2

Fracture Repair

Charles Sfeir, DDS, PhD, Lawrence Ho, Bruce A. Doll, DDS, PhD, Kodi Azari, MD, and Jeffrey O. Hollinger, DDS, PhD

INTRODUCTION

The economic impact of musculoskeletal conditions in the United States represents $126 billion. Bone fracture repairs are among the most commonly performed orthopedic procedures; about 6.8 million come to medical attention each year in the United States *(1)*. Advances through research and enhanced understanding of fracture repair have enabled orthopedic surgeons to provide patients with many treatment options and improved outcome. In this chapter we will review the current knowledge of fracture from both chronological and molecular biology aspects; we will then address bone healing in elderly patients and the different technologies used to enhance fracture repair.

Bone fracture healing is a very remarkable process because, unlike soft tissue healing, which leads to scar formation, the end result of normal healing is the regeneration of the anatomy of the bone and complete return to function. In general, fracture healing is completed by 6–8 wk after the initial injury. Fracture healing can be divided into two major categories: primary (direct, cortical) bone healing and secondary (indirect, spontaneous) bone healing, with the latter being discussed first because it is more common. Both of these are very complex processes that involve the coordination of a sequence of many biological events. With the recent advances made in molecular biology, the identification of various signaling molecules during specific phases of the healing process has been made possible.

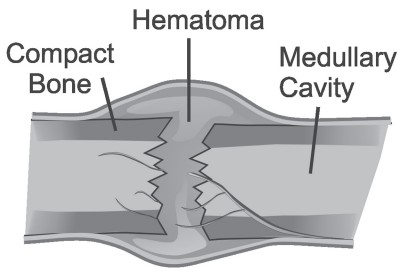
SECONDARY BONE HEALING

Secondary fracture healing is characterized by spontaneous fracture healing in the absence of rigid fixation of the fracture site, and it is the more common method of bone healing as mentioned above. The complete process has been described as having three to five phases *(2–8)*. The biology of bone fracture repair is an organized pattern for repair and perhaps is best elucidated when viewed in histological sections *(2,9)*. Fracture repair can be easily divided into three phases, each characterized by the presence of different cellular features and extracellular matrix components. In temporal order, the events reflect an inflammatory phase; a reparative phase that includes intramembranous ossification; chondrogenesis, and endochondral ossification, and a remodeling phase *(2,10)*. The phases of secondary bone fracture repair are illustrated in **Fig. 1**. It is important to note that these three phases overlap one another and in effect form a continuous healing process.

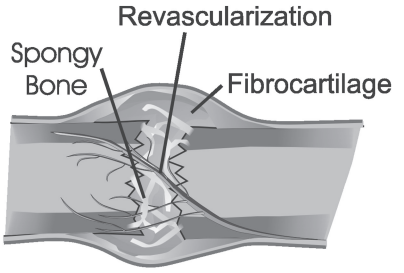
Inflammatory Phase

An injury that fractures bone damages not only the cells, blood vessels, and bone matrix, but also the surrounding soft tissues, including muscles and nerves *(11)*. Immediately following the injury, an inflammatory response is elicited, which peaks in 48 h and disappears almost completely by 1 wk postfracture. This inflammatory reaction helps to immobilize the fracture in two ways: pain causes the individual

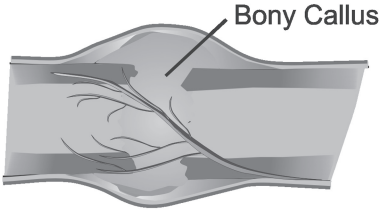
From: *Bone Regeneration and Repair: Biology and Clinical Applications*
Edited by: J. R. Lieberman and G. E. Friedlaender © Humana Press Inc., Totowa, NJ



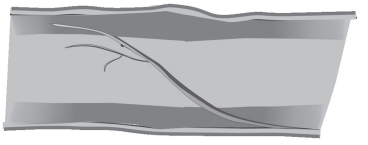
Inflammatory Phase- Vascular endothelial damage results in hematoma formation, complement cascade, and clotting cascade leading to the accumulation of PMNs, lymphocytes, platelets, blood monocytes, macrophages, neutrophils, osteoclasts, and undifferentiated cells. The cells express various genes including TGF-β, FGF-I, FGF-II, PDGF, IGF-I, IGF-II, BMP-2,-4,-7, osteonectin, BMPR I/II, IL-1, IL-6, and GMCSF.



Reparative Phase (Fibrocartilage Callus Formation)- Fibrous tissue and new cartilage are beginning to form and revascularization is taking place. The appearance of macrophages, chondroblasts, chondrocytes, osteoclasts, fibroblasts, and endothelial cells is seen along with the expression of TGF-β, FGF-1, FGF-2, PDGF, IGF-I, IGF-II, BMP-2,-4,-7, osteonectin, fibronectin, BMPR I/II, smads 2,3,4, IL-1, IL-6, and collagens (II,III,IV,V,VI,IX,X).



Reparative Phase (Bony Callus Formation)- Intramembranous and endochondral ossification is taking place to lay down new woven bone. The appearance of macrophages, chondroblasts, chondrocytes, osteoblasts, osteoclasts, and endothelial cells is seen along with the expression of TGF-β, FGF-1, FGF-2, PDGF, IGF-I, IGF-II, BMP-2,-4,-7, osteonectin, osteopontin, osteocalcin, BMPR I/II, smads 2,3,4, IL-1, IL-6, and collagens (I, II, III, IV, V, VI, IX, X).



Remodeling Phase- Replacement of woven bone by lamellar bone and the resorption of excess callus. Gradual modification of the fracture region leads to restoration of normal bone architecture.

Fig. 1. Schematic representation of the three stages of fracture repair.

to protect the injury, and swelling hydrostatically keeps the fracture from moving *(3)*. At the injured site, vascular endothelial damage results in the activation of the complement cascade, platelet aggregation, and release of its α-granule contents. This platelet degranulation releases growth factors and triggers chemotactic signals. The conductors of the clotting cascade are the platelets, which have the duty of hemostasis and mediator signaling through the elaboration of chemoattractant growth factors. Polymorphonuclear leukocytes (PMNs), lymphocytes, blood monocytes, and tissue macrophages are attracted to the wound site and are activated to release cytokines that can stimulate angiogenesis *(12)*. The early fracture milieu is characteristically a hypoxic and acidic environment, which is optimal for the activities of PMNs and tissue macrophages *(13)*. The extravasated blood collection will clot. Hematoma accumulates within the medullary canal between the fracture ends and beneath elevated periosteum and muscle. Its formation serves as a hemostatic plug to limit further hemorrhage as well as becoming a fibrin network that provides pathways for cellular migration *(3,11,14,15)*. Recent evidence also suggests that the hematoma serves as a source of signaling molecules that initiate cellular events essential to fracture healing *(10)*. This whole process creates a reparative granuloma and is referred to as an external callus *(10)*.

Reparative Phase

The reparative phase occurs within the first few days, before the inflammatory phase subsides, and lasts for several weeks. The result of this phase will be the development of a reparative callus tissue in and around the fracture site, which will eventually be replaced by bone. The role of the callus is to enhance mechanical stability of the site by supporting it laterally. Osteocytes located at the fracture ends become deficient in nutrients and die, which is observed by the presence of empty lacunae extending for some distance away from the fracture *(5)*. Damaged periosteum and marrow as well as other surrounding soft tissues may also contribute necrotic tissue to the facture site *(3)*. While these tissues are being resorbed, pluripotent mesenchymal cells begin to form other cells such as fibroblasts, chondroblasts, and osteoblasts. These cells may originate in injured tissues, while others migrate to the site with the blood vessels. During this phase, the callus can be comprised of fibrous connective tissue, blood vessels, cartilage, woven bone, and osteoid. As repair progresses, the pH gradually becomes neutral and then slightly alkaline, which is optimal for alkaline phosphatase activity and its role in the mineralization of the callus *(11)*. It has been shown that the earliest bone forms from the cells in the cambium layer of the periosteum *(16)*. The composition of repair tissue and rate of repair may differ depending on where the fracture occurs in bone, the extent of soft tissue damage, and mechanical stability of the fracture site *(11)*. A closer look at the reparative phase focuses on intramembranous ossification, chondrogenesis, and endochondral ossification.

Intramembranous ossification begins within the first few days of fracture, but the proliferative activities appear to stop before 2 wk after the fracture. Histological evidence first shows osteoblast activity in the woven bone opposed to the cortex within a few millimeters from the fracture site *(7)*. Bone formation in this area occurs by the differentiation of osteoblasts directly from precursor cells, without the formation of cartilage as an intermediate step. The region of this type of bone formation occurring in the external callus is often referred to as the hard callus *(10)*.

While intramembranous ossification is taking place, chondrogenesis occurs in the periphery of the callus, where lower oxygen tension is present *(5)*. Mesenchymal or undifferentiated cells from the periosteum and adjacent external soft tissues are also seen in the granulation tissue over the fracture site *(7)*. These cells become larger, start to take on the appearance of cartilage, and begin to synthesize an avascular basophilic matrix much like what is seen in the proliferating zone of the growth plate. This region of fibrous tissue and new cartilage is referred to as the soft callus, and eventually the cartilage will replace all fibrous tissue *(10)*.

By the middle of the second week during fracture healing, there is abundant cartilage overlying the fracture site and calcification begins by the process of endochondral ossification *(7)*. This process is much like the one observed in the growth plate. Hypertrophic chondrocytes first secrete neutral proteoglycanases that degrade glycosaminoglycans, because high levels of glycosaminoglycans are shown to inhibit mineralization *(17)*. Then, these cells and later osteoblasts release membrane-derived vesicles that contain calcium phosphate complexes into the matrix *(18)*. They also carry neutral proteases and alkaline phosphatase enzymes that degrade the proteoglycan-rich matrix and hydrolyze high-energy phosphate esters in order to provide phosphate ions for precipitation with calcium *(11)*. As the mineralization process proceeds, the callus calcifies becoming more rigid and the fracture site is considered internally immobilized *(3)*. Capillaries from adjacent bone invade the calcified cartilage, increasing the oxygen tension. This is followed by invasion of osteoblasts, which form primary spongiosa consisting of both cartilage and woven bone *(10)*. Eventually the callus is composed of just-woven bone, which connects the two fracture ends, and the remodeling process begins.

Remodeling Phase

The remodeling phase is the final phase in fracture healing and begins with the replacement of woven bone by lamellar bone and the resorption of excess callus *(11,13)*. Although this phase represents the normal remodeling activity of bone, it may be accelerated in the fracture site for several years

(19). Remodeling of fracture repair after all woven bone is replaced consists of osteoclastic resorption of poorly located trabeculae and formation of new bone along lines of stress *(20)*. The result of the remodeling phase is a gradual modification of the fracture region under the influence of mechanical loads until optimal stability is achieved, where the bone cortex is typically similar to the architecture it had before the fracture occurred *(3)*.

PRIMARY BONE HEALING

Primary bone healing requires rigid stabilization with or without compression of the bone ends. Unlike secondary bone healing, this rigid stabilization suppresses the formation of a callus in either cancellous or cortical bone *(21–29)*. Because most fractures occurring worldwide either are untreated or are treated in a way that results in some degree of motion (sling or cast immobilization, external or intramedullary fixation), primary healing is rare *(7)*. Although some have considered this type of healing to be a goal of fracture repair, in many ways it is not shown to be advantageous over secondary bone healing *(30,31)*. The intermediate stages are weak, and it does not occur in an anaerobic environment *(3)*. Primary bone healing can be divided further into gap healing and contact healing, both of which are able to achieve bone union without external callus formation and any fibrous tissue or cartilage formation within the fracture gap.

Gap Healing

Gap healing occurs in two stages, starting with initial bone filling and followed by bone remodeling. In the first stage of gap healing, the width of the gap is filled by direct bone formation. An initial scaffold of woven bone is laid down, followed by formation of parallel-fibered and/or lamellar bone as support *(28,29)*. The orientation of the new bone formed in this first stage is transverse to that of the original lamellar bone orientation. There are no connective tissues or fibrocartilage within this gap preceding the production of bone. In the second stage of gap healing, which happens after several weeks, longitudinal haversian remodeling reconstructs the necrotic fracture ends and the newly formed bone such that the fracture site is replaced with osteons of the original orientation *(32)*. The end result of normal gap healing is the return of the bone structure to the way it was before the fracture.

Contact Healing

In contrast to gap healing, contact healing occurs where fragments are in direct apposition and osteons actually are able to grow across the fracture site, parallel to the long axis of the bone, without being preceded by the process of transverse bone formation between fracture ends *(23,26,28,29)*. Under these conditions, osteoclasts on one side of the fracture undergo a tunneling resorptive response, forming cutting cones that cross the fracture line. This resorptive cavity that develops allows the penetration of capillary loops and eventually the establishment of new haversian systems. These blood vessels are then accompanied by endothelial cells and osteoprogenitor cells for osteoblasts leading to the production of osteons across the fracture line *(7)*. The result of normal contact healing will also eventually lead to regeneration of the normal bone architecture.

The biology of bone fracture repair is a very complex process that leads to the regeneration of normal bone architecture. Primary bone healing occurs when there is rigid stabilization of the fracture site and the fracture callus is inhibited. Gap healing and contact healing are both considered to be primary bone healing processes. Secondary bone healing occurs when there is no rigid fixation of the fractured bone ends, which leads to the development of a fracture callus. This process is a little more complicated and consists of an inflammatory phase, a reparative phase, and a remodeling phase. Normal fracture repair is orchestrated through the expression of many different genes, which are turned on and off at very specific times throughout healing. Important gene expression includes TGF-β, FGF, PDGF, IGF, BMP, osteonectin, osteocalcin, osteopontin, fibronectin, BMPR, Smads, IL-1, IL-6, GMCSF, MCSF,

and various collagen isotypes. The well-regulated expression of these genes enables the cellular interactions to take place that are responsible for restoring bone morphology and function.

GENE EXPRESSION DURING FRACTURE REPAIR

As described above, the process of fracture repair can be divided into three distinct phases: inflammation, reparative, and remodeling. During these phases, interactions among many different cells via various growth factors, cytokines, receptors, and intermediate signaling molecules take place. With recent advances in molecular biology, the identification and characterization of many of these interactions can now be elucidated. Although several growth factors and extracellular matrix proteins are involved in the repair process, **Table 1** and the following section summarizes the most investigated ones. The temporal and spatial expression of these growth factors and extracellular matrix proteins during different phases of bone repair is described below.

Transforming Growth Factor-β (TGF-β)

TGF-β is produced in the fracture site by platelets, inflammatory cells (monocytes, macrophages), osteoblasts, osteoclasts, and chondrocytes *(10)*. It is extracellularly present in the hematoma (fracture site and periosteum) during the immediate injury response (within 24 h). In the inflammatory phase, the mRNA of TGF-β is weakly expressed in proliferating mesenchymal cells and endothelial cells. It is strongly expressed in proliferating osteoblasts during intramembranous ossification, and strongly expressed in proliferating chondrocytes, not hypertrophic chondrocytes, during the chondrogenesis and endochondral ossification phases *(33)*. It exists first as an inactive precursor peptide that is activated by the acidic conditions of the callus or proteases and becomes the most potent chemoattractant identified for macrophages *(34–37)*. TGF-β also has many other roles, including promoting angiogenesis, which is essential for orderly fracture repair *(10)*; stimulating bone formation by inducing differentiation of periosteal mesenchymal cells into chondroblasts and osteoblasts *(38–40)*; regulating cartilage matrix calcification; and stimulating osteoblast activity and intraosseus wound regeneration *(13, 41,42)*. Other actions include inhibiting osteoblast differentiation and mineralization *(43,44)*, inhibiting osteoclast activity and the formation of osteoclasts *(45)*, and also increasing the production of other bone and cartilage components such as types I, II, III, IV, VI, and X collagen, fibronectin, osteopontin, osteonectin, thrombospondin, proteoglycans, and alkaline phosphatase *(40,46,47)*.

Fibroblast Growth Factors (FGFs)

FGFs are produced by inflammatory cells, osteoblasts, and chondrocytes within the fracture callus. There are two forms of FGF, designated FGF-I and FGF-II. FGF-I is expressed in macrophages and periosteal cells in the inflammatory phase of fracture. It is then expressed in osteoblasts during intramembranous ossification, followed by maximum expression in immature chondrocytes during chondrogenesis. During endochondral ossification, FGF-I is expressed only in osteoblasts. FGF-II has similar expression throughout repair, without any peaks. It is present in macrophages during the inflammatory phase, in osteoblasts during intramembranous ossification, in chondrocytes during chondrogenesis, and in hypertrophic chondrocytes and osteoblasts during endochondral ossification *(10)*. FGFs promote blood vessel formation *(48)*, has autocrine, intracellular functions, and stimulates type 4 collagenase *(10)*. FGF-II also serves as a chemoattractant and mitogen for chondrocytes and regulates differentiation of growth plate chondrocytes *(49,50)*.

Platelet-Derived Growth Factors (PDGFs)

PDGFs are produced by platelets, monocytes, activated tissue macrophages, and endothelial cells in the fracture callus. After being weakly expressed in the inflammatory phase, PDGF expression rises and remains constant throughout repair *(10)*. PDGF has many roles including having receptor tyrosine

Table 1
Gene Expression during Fracture Repair

Gene expression	Function	Temporal and spatial expression
Transforming growth factor-β (TGF-β)	–Most potent chemoattractant for macrophages *(34–37)* –Promotes angiogenesis *(10)* –Induces differentiation of periosteal mesenchymal cells into chondroblasts and osteoblasts *(38–40)* –Regulates cartilage matrix calcification and stimulates osteoblast activity *(13,41,42)* –Increases production of types I, II, III, IV, VI, and X collagen, fibronectin, osteopontin, osteonectin, thrombospondin, proteoglycans, and alkaline phosphatase *(40,46,47)*	–Produced by platelets, inflammatory cells (monocytes, macrophages), osteoblasts, osteoclasts, mesenchymal cells, endothelial cells, and chondrocytes *(10,33)* –Weakly expressed in proliferating mesenchymal cells and endothelial cells in the inflammatory phase, strongly expressed in proliferating osteoblasts during intramembranous ossification, and strongly expressed in proliferating chondrocytes during chondrogenesis and endochondral ossification *(33)*
Fibroblast growth factor-I (FGF-I)	–Promotes blood vessel formation *(48)*, has autocrine, intracellular functions, and stimulates type 4 collagenase *(10)*	Expressed in macrophages and periosteal cells in inflammatory phase, in osteoblasts during intramembranous ossification, maximum expression occurs in immature chondrocytes during chondrogenesis, and it is expressed in osteoblasts during endochondral ossification *(10)*
Fibroblast growth factor-II (FGF-II)	–Promotes blood vessel formation *(48)*, has autocrine, intracellular functions, and stimulates type 4 collagenase *(10)* –A chemoattractant and mitogen for chondrocytes and regulates differentiation of growth plate chondrocytes *(49,50)*	Constant expression throughout repair in macrophages during the inflammatory phase, in osteoblasts during intramembranous ossification, in chondrocytes during chondrogenesis, and in hypertrophic chondrocytes and osteoblasts during endochondral ossification *(10)*
Platelet-derived growth factor (PDGF)	–Has receptor tyrosine kinase activity, stimulates mesenchymal cell proliferation, helps form cartilage and intramembranous bone, and initiates callus formation *(10)* –Potent mitogen for connective tissue cells, stimulates bone cell DNA and protein synthesis, and promotes resorption via prostaglandin synthesis *(51)* –Enables cells to respond to other biologic mediators, increases type I collagen *in vitro*, modulates blood flow *(13,52,53)* –Increases expression of *c-myc* and *c-fos* protooncogenes *(40,54)*	Constant expression in platelets, monocytes, activated tissue macrophages, and endothelial cells in the fracture callus after being weakly expressed in the inflammatory phase *(10)*
Insulin-like growth factor-I (IGF-I)	–Increases collagen synthesis and decreases collagen degradation *(40,62)* –Stimulates clonal expansion of chondrocytes in proliferative zone *(57)*	–In osteoblasts during the intramembranous ossification phase and present in prehypertrophic chondrocytes *(55)* –mRNA peaks at 8 d postfracture *(56)*

Table 1 (Continued)

Gene expression	Function	Temporal and spatial expression
Insulin-like growth factor-I (IGF-I) *(continued)*	–Stimulates replication of preosteoblastic cells *(51)* –Increases osteoclast formation from mouse osteoclast precursors *(59,60)*	–IGF-I in callus extracts increased at 13 wk after fracture *(58)*
Insulin-like growth factor-II (IGF-II)	–Increases collagen synthesis and decreases collagen degradation *(40,62)* –Increases osteoblast precursor cell proliferation during resorption *(37)* –Promotes cartilage matrix synthesis *(13)* –Modulates osteoclast function leading to bone remodeling *(33)*	–IGF-II mRNA is in fetal rat precartilaginous condensations, perichondrium, and proliferating chondrocytes *(61)* –IGF-II mRNA is detected in some osteoclasts next to osteoblasts that also expressed IGF-II, whereas most other osteoblasts in bone remodeling were negative for IGF-II *(55)*
Bone morphogenetic proteins (BMP-2, BMP-4, BMP-7)	–BMP-2 increases rat osteoblast IGF-I and IGF-II expression *(69)* –BMP-2 increases TGF-β and IL-6 expression in HOBIT cells *(70)* –BMP-4 stimulates TGF-β expression in monocytes *(71)* –BMP-4 binds to type IV collagen, type I collagen, and heparin *(74)*, and may explain in part the role of vasculogenesis and angiogenesis in fracture healing *(74,75)* –BMP-7 induces expression of Osf2/Cbfa1, a transcription factor associated with early osteoblast differentiation *(76)* –BMP-7 or osteogenic protein-1 (OP-1) *(72)*, increases IGF type 2 receptor expression *(73)*	–Produced by primitive mesenchymal and osteoprogenitor cells, fibroblasts, and proliferating chondrocytes *(66–68)* –Present in newly formed trabecular bone and multinucleated osteoclast-like cells *(68)* –Strongly present in undifferentiated mesenchymal cells during the inflammatory phase *(33,68)* –Strongly present in the proliferating osteoblasts in intramembranous ossification *(33,68)* –During chondrogenesis and enochondral ossification, BMP-2 and -4 are in proliferating chondrocytes, weakly in mature and hypertrophic chondrocytes, and strongly in osteoblasts near endochondral ossification front, BMP-7 is in proliferating chondrocytes and weakly in mature chondrocytes *(33,68)*
Osteonectin	–Most abundant noncollagenous organic component of bone and serves to bind calcium *(82)* –May regulate tissue morphogenesis *(7)*	–mRNA is found throughout the healing process *(83,84)* –Expression peaks in the soft callus on d 9 and a prolonged peak in expression in the hard callus observed from d 9 to d 15 *(85)* –In d 4–7, the osteonectin signal is found to be strongest in the osteoblastic cells where intramembranous ossification was occurring *(7)* –By d 10, osteonectin signal diminishes, is detected only at the endochondral ossification front, and only weakly in proliferative chondroctyes *(7,84)*
Osteocalcin	–Participates in regulation of hydroxyapatite crystal growth *(40)*	–Thought to be osteoblast-specific *(7)* –Osteocalcin was not detected in the soft callus but was in the hard callus, and initiation of osteocalcin occurred between d 9 and d 11, with peak expression at about d 15 *(85)*

(continued)

Table 1 (Continued)

Gene expression	Function	Temporal and spatial expression
Osteopontin	–Interacts with CD-44, which is a cell-surface glycoprotein that binds hyaluronic acid, type I collagen, and fibronectin *(88)* –Mediates cell–cell interaction in bone repair and remodeling *(7)* –Helps anchor osteoclasts to bone through vitronectin receptors *(91)*	Detected in osteocytes and osteoprogenitor cells in the subperiosteal hard callus, and by d 7 after fracture it is found in the junction between the hard and soft callus *(7,89,90)*
Fibronectin	–Helps in adhesion and cell migration *(7)* –Plays an important role in the establishment of provisional fibers in cartilaginous matrices *(7)*	–Produced by fibroblasts, osteoblasts, and chondrocytes and is detected in the hematoma within the first 3 d after fracture and in the fibrous portions of the provisional matrices *(7)* –Low levels of fibronectin mRNA in intact bone and marked expression in the soft callus within 3 d after fracture that reaches peak level at d 14 *(92)*
Bone morphogenetic protein receptors (BMPR-I, -II)	–Findings suggest an association of the receptors with the differentiation of mesenchymal cells into chondroblastic and osteoblastic lineages *(33)*	–Strongly present in undifferentiated mesenchymal cells during the inflammatory phase, in proliferating osteoblasts during intramembranous ossification, and are found in proliferating chondrocytes, weakly in mature and hypertrophic chondrocytes, and strongly in osteoblasts near the endochondral ossification front during chondrogenesis and endochondral ossification *(33,93)*
smads (2, 3, 4)	–Components of the intracellular signaling cascade that starts with BMPs *(94,95)* –smad 2 and smad 3 help to mediate TGF-β signaling *(94)* –smad 4 forms a heterodimeric complex with other pathway restricted smads and translocates into the nucleus to modulate important BMP response genes *(96)*	–In the inflammatory phase, the mRNA for smads 2, 3, 4 are not expressed, and in chondrogenesis and endochondral ossification, the mRNA for smads 2, 3, 4 are upregulated and the smad 2 protein is present in chondroblasts and chondrocytes *(33)*
Interleukin-1 (IL-1)	–Induces the secretion of IL-6, GMCSF, and MCSF *(98)* –May stimulate activities of neutral proteases to selectively degrade callus tissue *(17,99)* –May increase fibroblastic collagen synthesis, collagen cross-linking, and stimulate angiogenesis *(98,100–103)*	–Produced by macrophages and is expressed at low constitutive levels throughout fracture healing but can be induced to high activities in the early inflammatory phase (d 3) *(97)*
Interleukin-6 (IL-6)	–Very sensitive to IL-1 stimulation *(106)* –May be a stimulator of bone resorption *(107–109)*	–Produced by osteoblasts during fracture repair *(104,105)* –Shows a high constitutive activity early in the healing process *(97)*
Granulocyte-macrophage colony-stimulating factor (GMCSF)	–May stimulate formation of osteoclasts, increase the proliferation of T-lymphocytes, and stimulate cytokine secretion *(102,111–114)*	–Produced by T-lymphocytes during the fracture healing process and is expressed at early time points after fracture *(97)*

Table 1 (Continued)

Gene expression	Function	Temporal and spatial expression
Granulocyte-macrophage colony-stimulating factor (GMCSF) *(continued)*	–Associated with increased fibroblast migration and collagen synthesis *(115–117)* –Associated with the proliferation and differentiation of granulocytic and monocyte/macrophage lineages *(118)* –May suppress the expression of receptors for other cytokines in different cell types *(97,111)*	–May be produced from osteoblasts *(102,111–114)*
Macrophage colony-stimulating factor (MCSF)	–An important growth factor for development of macrophage colonies by hematopoietic tissues *(121)*	–Lack of expression in the fracture callus may be due to complex interactions between immune, hematopoietic and musculoskeletal systems not yet understood *(97)* –Constitutive secretion by osteoblast-like cells in culture is observed *(119,120)*
Collagens (types I, II, III, IV, V, VI, IX, X, XI)	–Type I collagen aids in developing cross-linkages which produce collagen fibrils that mature to collagen fibers, creating regions allowing for the deposition and growth of hydroxy-apatite crystals *(13)* –Aberrations in type III collagen production may lead to delayed union or nonunion *(124)* –Type IV (and types I and X) may aid in converting mesenchymal lineage cells into osteoblasts *(128)* –Type V and XI may regulate the growth and orientation of type I and type II collagen in cartilaginous and noncartilagenous tissues *(129,130)* –Type V collagen has been associated with blood vessels in granulation tissue *(124)* –Type IX may mediate interactions between collagen fibrils and proteo-glycans in cartilage *(40,132)* –Type X collagen may play a role in the mineralization of cartilage *(40)*	–Type I is associated with bone, type II with cartilage, types III and V with granulation tissue, types IV and VI with the endothelial matrix, and type X with hypertrophic cartilage *(123)* –Mechanically stable fractures have predominately type I collagen along with types II and V *(124)* –Mechanically unstable fractures are characterized by initial production of types III and V collagen which is replaced by types II and IX collagen and very little type I collagen *(122)* –Type II collagen mRNA is detectable as early as d 5 postfracture in cells that have chondrocytic phenotype, has a peak expression approximately 9 d after fracture in the mouse and rat, and by d 14 after fracture the expression of mRNA for type II chain becomes absent *(7,85,125,126)* –Type III collagen mRNA increases rapidly during the first week of fracture healing *(127)* –Type V collagen is expressed through out healing process with the highest accumulation of type V collagen in the subperiosteal callus *(89)* –Expression of type IX collagen and aggrecan coincides with expression of type II collagen *(40,132)* –Expression of type X collagen occurs later than that of other cartilage specific genes *(40)*

kinase activity, stimulating mesenchymal cell proliferation, initiating fracture repair, helping to form cartilage and intramembranous bone, and initiating callus formation *(10)*. They are released from the α-granules of platelets and become potent mitogens for connective tissue cells, stimulate bone cell DNA and protein synthesis, and promote resorption via prostaglandin synthesis *(51)*. PDGF also serves as a competence factor that enables cells to respond to other biological mediators; increase type I collagen *in vitro*; modulate blood flow, which has a positive impact on wound healing *(13,52,53)*; and are shown to increase expression of *c-myc* and *c-fos* protooncogenes, which encode nuclear proteins involved in regulating cell proliferation, growth, and differentiation *(40,54)*.

Insulin-Like Growth Factors (IGFs)

IGFs are also often referred to as somatomedins or sulfation factors. IGF expression is high in cells of the developing periosteum and growth plate, healing fracture callus tissue, and developing ectopic bone tissue induced by DBM *(40,47,55,56)*. IGFs produced by bone cells not only act as autocrine and paracrine regulators, but also become incorporated into bone matrix and are later released during resorption, which increases osteoblast precursor cell proliferation *(37)*. IGFs may also become secreted by chondrocytes and respond in an autocrine manner to promote cartilage matrix synthesis *(13)*. However, IGFs may not only contribute to bone formation, they may modulate osteoclast function, leading to bone remodeling during fracture repair *(33)*.

IGF-I mRNA is not expressed in the inflammatory phase of repair. However, mRNA expression is seen in osteoblasts during the intramembranous ossification phase and are also present in prehypertrophic chondrocytes *(55)*. Actually, the level of mRNA peaks at 8 d postfracture *(56)*. IGF-I may stimulate clonal expansion of chondrocytes in proliferative zone through an autocrine mechanism, much like in the chondrogenesis stage of fracture repair *(57)*. IGF-I also stimulates replication of preosteoblastic cells and induces collagen production by differentiated osteoblasts *(51)*. It should be noted that IGF-I in callus extracts increased at 13 wk after fracture *(58)*, and has been shown to increase osteoclast formation from mouse osteoclast precursors, which suggests some involvement during remodeling *(59,60)*. In addition, IGF-II mRNA is observed in fetal rat precartilaginous condensations, perichondrium, and proliferating chondrocytes *(61)*. IGF-II mRNA is detected in some osteoclasts in the fracture healing model next to osteoblasts that also expressed IGF-II, whereas most other osteoblasts in bone remodeling were negative for IGF-II *(55)*. IGF-I and IGF-II have been observed to increase collagen synthesis and decrease collagen degradation *(40,62)*.

Bone Morphogenetic Proteins (BMPs)

BMPs are members of the TGF-β superfamily and were discovered as the noncollagenous and water-soluble substances in bone matrix that have osteoinductive activity *(63–65)*. In general, recent studies reveal increased presentation of BMP-2, -4, and -7 in the primitive mesenchymal and osteoprogenitor cells, fibroblasts, and proliferating chondrocytes present at the fracture site *(66–68)*. In a rat model, mesenchymal cells that had migrated into the fracture gap and had begun to proliferate showed increased statement of BMP-2 and -4 *(66)*. In a similar rat fracture healing model, it was confirmed that BMP-2, -4, and -7 were present in newly formed trabecular bone and multinucleated osteoclast-like cells *(68)*. More specifically, when the expression is broken down into the phases of healing, BMP-2, -4, and -7 are strongly present in undifferentiated mesenchymal cells during the inflammatory phase. During intramembranous ossification, these BMPs are strongly present in the proliferating osteoblasts. In chondrogenesis and endochondral ossification, BMP-2 and -4 are found in proliferating chondrocytes, weakly in mature and hypertrophic chondrocytes, and strongly in osteoblasts near endochondral ossification front. In these later stages of healing, BMP-7 is found in proliferating chondrocytes and weakly in mature chondrocytes *(33,68)*.

BMPs affect expression of other growth factors that may function to mediate the effects of BMPs on bone formation *(37)*. BMP-2 increased rat osteoblast IGF-I and IGF-II expression *(69)*, and increased

TGF-β and IL-6 expression in HOBIT cells *(70)*. BMP-4 stimulated TGF-β expression in monocytes *(71)*. BMP-7 or osteogenic protein-1 (OP-1) *(72)* is shown to increase IGF type 2 receptor expression *(73)*.

BMPs also have other roles in fracture repair. BMP-4 binds to type IV collagen, type I collagen, and heparin *(74)*. The interaction of BMP-4 with type IV collagen and heparin may explain in part the role of vasculogenesis and angiogenesis in bone development such as in fracture healing *(74,75)*. BMP-7 also stimulates normal human osteoblast proliferation by inducing expression of Osf2/Cbfa1, a transcription factor associated with early osteoblast differentiation *(76)*. It should be noted that although they were identified and named because of their osteoinductive activity *(77,78)*, the BMPs play many diverse roles during embryonic and postembryonic development as signaling molecules in a wide range of tissues *(79,80)*. In conclusion, a number of findings suggest that BMP-2, -4, and -7 work to promote fracture healing and bone regeneration *(81)*.

Osteonectin is one of many extracellular matrix proteins involved with bone repair and regeneration. In fact, osteonectin is the most abundant noncollagenous organic component of bone and serves to bind calcium *(82)*. Osteonectin mRNA is found throughout the healing process *(83,84)*. Its expression peaks in the soft callus on d 9, and a prolonged peak in expression in the hard callus is observed from d 9 to d 15 *(85)*. During d 4–7, the osteonectin signal is found to be strongest in osteoblastic cells where intramembranous ossification was occurring *(7)*. By d 10, this signal diminished and the signal was detected only at the endochondral ossification front. No osteonectin was detected in hypertrophic chondrocytes and only weakly in proliferative chondroctyes *(7,84)*. Incidentally, type I and V collagen followed similar expression patterns, which suggests that osteonectin may regulate tissue morphogenesis *(7)*.

Osteocalcin, an osteoblast-specific protein, contains three γ-carboxyglutamic acid residues, which provide it with calcium-binding properties. Osteocalcin has been suggested to participate in regulation of hydroxyapatite crystal growth *(40)*, and may possess other functions, as it is also expressed in human fetal tissues *(86)*. In one study, osteocalcin was not detected in the soft callus but was detected in the hard callus. Initiation of osteocalcin occurred between d 9 and d 11, and peak expression was at about d 15 *(85)*. Osteocalcin levels in plasma depend on the formation of new bone, and the concentration may be an indicator of the activity of osteoblasts *(87)*.

Osteopontin, an extracellular matrix protein known to be important in cellular attachment, interacts with CD-44, which is a cell-surface glycoprotein that binds hyaluronic acid, type I collagen, and fibronectin *(88)*. *In situ* studies have shown that this protein is detected in osteocytes and osteoprogenitor cells in subperiosteal hard callus; however, little is seen in cuboid osteoblasts and by d 7 after fracture. Osteopontin is found in the junction between the hard and soft callus *(7,89,90)*. The coexistence of CD-44 and osteopontin in osteocytes and osteoclasts implies the presence of an osteopontin/CD-44 mediated cell–cell interaction in bone repair *(7)*. Another theory suggests that osteopontin helps anchor osteoclasts to bone through vitronectin receptors, helping in the resorption process *(91)*.

Fibronectin is a protein that helps in adhesion and cell migration, making it important in the repair process. In the fracture callus, this protein is produced by fibroblasts, osteoblasts, and chondrocytes. It is detected in the hematoma within the first 3 d after fracture and in the fibrous portions of the provisional matrices and less in the cartilage matrix *(7)*. There was no evidence of this protein in the periosteum, in osteoblasts, or osteocytes of periosteal woven bone using *in situ* hybridization. Northern hybridization showed low levels of fibronectin mRNA in intact bone and marked expression in the soft callus within 3 d after fracture, reaching a peak level at d 14 *(92)*. Because fibronectin production appears to be greatest in the earlier stages of repair, it is thought that it plays an important role in the establishment of provisional fibers in cartilaginous matrices *(7)*.

Bone Morphogenetic Protein Receptors (BMPRs)

The receptors for BMPs are strongly present in undifferentiated mesenchymal cells during the inflammatory phase. Then, they are strongly present in proliferating osteoblasts of intramembranous ossifica-

tion. BMPR I/II are found in proliferating chondrocytes, weakly in mature and hypertrophic chondrocytes, and strongly in osteoblasts near the endochondral ossification front during chondrogenesis and endochondral ossification *(93)*. The association of these receptors with the differentiation of mesenchymal cells into chondroblastic and osteoblastic lineages has been suggested *(33)*.

Smads are essential components of the complex intracellular signaling cascade that starts with BMPs *(94,95)*. During the inflammatory phase, the mRNA for smads 2, 3, 4 are not expressed, and smad 2 protein is not present. During the intramembranous ossification phase, smad 2 is still not present yet. In chondrogenesis and endochondral ossification, the mRNA for smads 2, 3, 4 are upregulated and the smad 2 protein is present in chondroblasts and chondrocytes *(33)*. Smad 2 and smad 3 help to mediate TGF-β signaling *(94)*. Smad 4 forms a heterodimeric complex with other pathway-restricted smads and translocates into the nucleus in order to modulate important BMP response genes *(96)*.

Interleukin-1 (IL-1)

IL-1 is an important cytokine produced by macrophages and is expressed at low constitutive levels throughout fracture healing but can be induced to high activities in the early inflammatory phase (d 3) *(97)*. It induces the secretion of IL-6, GMCSF, and MCSF, which means that the early expression of IL-1 may indicate a triggering mechanism that initiates a cascade of events that regulate repair and remodeling *(98)*. IL-1 may stimulate activities of neutral proteases to selectively degrade callus tissue *(17,99)*. The action of macrophages, which include increasing fibroblastic collagen synthesis, increasing collagen crosslinking, stimulating angiogenesis, and improving wound breaking strength, may also be attributed to IL-1 production *(98,100–103)*.

Interleukin-6 (IL-6) is an important cytokine that is produced by osteoblasts during fracture repair *(104,105)*. It is very sensitive to IL-1 stimulation *(106)*, and shows a high constitutive activity early in the healing process *(97)*. Several lines of evidence suggest that it is a stimulator of bone resorption *(107–109)*.

Granulocyte-Macrophage Colony-Stimulating Factor (GMCSF)

T-lymphocytes have been identified morphologically in fracture calluses and may be a part of the healing process *(110)*. GMCSF is produced by T-lymphocytes during the fracture healing process and is expressed at early time points after fracture but then gradually declines *(97)*. It is also suggested that GMCSF may be produced from osteoblasts to stimulate formation of osteoclasts, increases the proliferation of T-lymphocytes, and stimulates cytokine secretion *(102,111–114)*. This cytokine activity has been associated with increased fibroblast migration and collagen synthesis *(115–117)*, and the proliferation and differentiation of granulocytic and monocyte/macrophage lineages *(118)*. GMCSF may also suppress the expression of receptors for other cytokines in different cell types *(97,111)*.

Macrophage Colony-Stimulating Factor (MCSF) was not detected in the fracture callus according to one study *(97)*; however, constitutive secretion by osteoblast-like cells in culture is observed *(119, 120)*. It has been shown to be an important growth factor for development of macrophage colonies by hematopoietic tissues *(121)*. The lack of expression in the fracture callus may be due to the complex interactions among immune, hematopoietic, and musculoskeletal systems as a result of injury, which are not yet understood *(97)*.

Collagens

The overall quantity and type of collagen influences callus formation and fracture healing and the expression of these extracellular matrix proteins has also been documented *(122)*. There are at least 18 isotypes of collagens: type I is associated with bone, type II with cartilage, types III and V with granulation tissue, types IV and VI with the endothelial matrix, and type X with hypertrophic cartilage *(123)*. Mechanically stable fractures have predominately type I collagen, along with types II and V *(124)*. Mechanically unstable fractures are characterized by initial production of types III and V collagen, which is replaced by types II and IX collagen and very little type I collagen *(122)*.

Type I collagen, which is the main collagen type in bone, aids in developing cross-linkages. These linkages produce collagen fibrils that mature to collagen fibers, creating regions allowing for the deposition and growth of hydroxyapatite crystals about 10 d postfracture *(13)*. Type II collagen is a major structural protein of cartilage and has a peak expression approx 9 d after fracture in the mouse and rat. Pro-α-2 collagen mRNA is seen in the proliferative chondrocytes. By d 14 after fracture, expression of mRNA for type II collagen becomes absent. Almost all chondrocytes are hypertrophied, and there is no expression of type 2 procollagen chain. Type II mRNA is detectable as early as d 5 postfracture *(7,85,125,126)*. Type III collagen mRNA increases rapidly during the first week of fracture healing *(127)*, particularly in bone, and aberrations in its production may lead to delayed union or nonunion *(124)*. Type IV (and types I and X) may aid in converting mesenchymal lineage cells into osteoblasts *(128)*. Types V and XI have a closely related structures it has been suggested that they regulate the growth and orientation of type I and type II collagen in cartilaginous and noncartilagenous tissues *(129,130)*. Type V collagen is expressed in both soft and hard callus throughout the healing process. The highest accumulation of type V collagen was detected in the subperiosteal callus, where intramembranous ossification was taking place *(89)*. Type V collagen has also been associated with blood vessels in granulation tissue *(124)*. Type XI collagen is found in cartilage and is a minor component of collagen fibrils, but expression of this collagen type is not restricted to cartilage *(40,131)*. The expression of type IX collagen and aggrecan coincides with expression of type II. Type IX collagen is seen in cartilage and may mediate interactions between collagen fibrils and proteoglycans *(40,132)*. The expression of type X collagen, a marker for hypertrophic chondrocytes during endochondral ossification, occurs later than that of other cartilage-specific genes and may play a role in the mineralization of cartilage *(40)*.

As our understanding of bone repair at a molecular level increases, we will be able to engineer comprehensive bone regenerative therapies. This knowledge will guide us to better design delivery systems that are biology driven; for example, if multiple growth factors are being delivered to a fractured bone site, one might imagine that different growth factors could be released at different times to optimize the healing cascade. Another area of research that will also influence our therapy design is the bone healing related to age; research indicates that bone repair is different between young and elderly patients. This topic is discussed in the following section.

FRACTURE HEALING IN THE ELDERLY

It has been established that bone formation during bone remodelling and fracture healing in the elderly patient appears to be reduced. Causes include a reduced number of recruited osteoblast precursors, a decline in proliferative activity of osteogenic precursor cells, and a reduced maturation of osteoblast precursors. Advanced age-related changes occur in the bone mineral, bone matrix *(133)*, and osteogenic cells *(134,135)*. Common clinical experience indicates that fractures heal faster in children than in adults *(136)*. Mechanisms causing these alterations are unclear. The observations have been attributed to slow wound healing, reflecting a general functional decline in the homeostatic mechanisms during aging and senescence. Furthermore, differences in fracture healing in the elderly population can be caused by local or systemic changes in hormonal and growth factor secretion and altered receptor levels, or changes in the extracellular matrix composition.

Several publications deal with the delicate relationship between bone resorption and bone formation and its imbalances, leading to osteopenia and osteoporosis. Presently, less information is obtainable as to similarities and changes in the process of fracture healing in the elderly patient in comparison to the physiological process of bone healing in children and young adults. In addition, the data obtained in animal fracture healing models (rat, rabbit) are difficult to transfer to the human physiological fracture repair process in the elderly patient.

General cellular and biochemical processes of fracture repair in the elderly, healthy (nonosteoporotic) patient receive less focus. Demographic changes and with an overaging population, steadily increas-

ing fracture numbers in the elderly population will mandate more emphasis as a means to enhance the process.

In vitro evidence of age-related changes in cell behavior indicate a reduced proliferative capacity. Christiansen et al. have demonstrated that serially passaged cultures of human trabecular osteoblasts exhibit limited proliferative activity and undergo cellular aging. They reported a number of changes during serial passaging of human trabecular osteoblasts, which include alterations in morphology and cytoskeleton organization; an increase in cell size and higher levels of senescence-associated β-galactosidase activity. They studied changes of topoisomerase I levels during cellular aging of human trabecular osteoblasts. They reported an age-related progressive and significant decline in steady-state mRNA levels of this gene in human bone cells undergoing cellular aging *in vitro (137)*. Taken together, these observations facilitate a further understanding of reduced osteoblast functions during cellular aging. These results concur with previous former findings of a correlation between donor age and the impairment of osteoblastic functions such as production of Col I, OC, and other extracellular matrix components in *in vitro* culture of human mature osteoblasts *(138–140)*.

Martinez et al. examined the cell proliferation rate and the secretion of C-terminal type I procollagen and alkaline phosphatase (ALP). They noted a lower proliferation rate and osteocalcin secretion in osteoblastic cells from the older donors than in those from younger subjects. They also found significant differences of these parameters in relation to the skeletal site of origin *(141)*. Theoretical basis of these experiments and their importance for the understanding of the process of bone aging and bone healing in the elderly patient is the consideration as a useful tool for evaluating osteoblastic alterations associated with bone pathology and aging *(142)*. Other groups have shown that human bone-derived cells show a dramatic decrease in their proliferative capacity with donor age. Studying the gender and age-related changes in iliac crest cortical bone and serum osteocalcin in humans subjects, Vanderschueren et al. *(143)* also detected a significant age-related decline of bone and serum osteocalcin content with age in vivo. Furthermore, a parallel decrease in age-matched groups revealed a generally higher concentration of bone and serum osteocalcin in men.

With advancing age, the membrane-like arrangement of the osteogenic cells in the periosteum is lost, leaving a reduced number of precursor cells to draw from *(134)*. These electron microscopy-based results were confirmed by an organ culture model investigating the relationship between chondrogenic potential of periosteum and aging. In this model, periosteal explants from the medial tibiae of rabbits (age range between 2 wk and 2 yr) were cultured in agarose suspension conditions conductive for chondrogenesis. A significant decline of chondrogenic potential of periosteum with increasing age was apparent. Furthermore, a significant decrease of proliferative activity was found by ^{3}H-thymidin incorporation *(144)*.

Enhancing Fracture Healing

The goal is to accelerate or to assure the healing of a fracture, which is likely not able to heal without invasive or noninvasive intervention. Several methods could be used to enhance bone fracture healing. The approaches could be biological or mechanical and biophysical enhancement *(145–147)*. In this section we will focus on the biological approaches.

The local methods for fracture enhancements involve the use of biological bone grafts, synthetic grafts, and delivery of growth factors. The autologous cancellous bone graft is considered the gold standard and has been extensively used in orthopedics. This type of grafting material will provide some living bone-producing cells, inductive growth factors, and hydroxyapatite mineral. The disadvantages are morbidity at the donor site, scarring and risk of infection, and most often the graft volume needed is greater than what is available. Thus, the need for alternative graft material has been sought, but none yet provide all the qualities of autologous cancellous bone. Different categories of grafting materials are available and are summarized in **Table 2**.

Table 2
Alternative Grafts Used to Enhance Fracture Healing

Absorbable	Nonabsorbable
Natural	Synthetic polymers
• Allogeneic bone	• Polytetrafluoroethlene
• Collagen	• Synthetic composite
• Collagen-GAG	• Bioactive glasses
• Fibrin	• Calcium-based ceramic grafts
• Hyaluranic acid	Hydroxyapatite
Natural mineral	Composite
• Hydroxyapatite	• Calcium-collagen composite
• Xenogeneic derivatives (anorganic bone)	
Synthetic	
• Polylactic acid	
• Polyglycolic acid	
• Tri-calcium phosphate	
• Calcium sulfate	
Cellular grafts	
• Autogenous bone marrow grafts	
• Autogenous bone grafts	

In addition to grafts, bone marrow has been shown to contain a population of mesenchymal stem cells that are capable of differentiating into osteoblasts and form bone as well as other connective tissues. Connolly et al. reported that injectable bone marrow cells could stimulate osteogenic repair. They developed techniques for clinical application by harvesting autologous bone marrow, centrifuging, and concentrating the osteogenic marrow prior to implantation. Garg et al. *(148)* also reported the successful use of autogenous bone marrow as an osteogenic graft. Seventeen of the 20 ununited long bone fractures healed according to clinical and radiographic criteria.

Extensive research has been carried out and in progress aimed at isolating, purifying and expanding marrow-derived mesenchymal cells *(149–152)*. Once these cells are isolated, they may be expanded (not differentiated) in a specialized medium and ultimately yield a source of cells that are highly osteogenic. These cells could then be delivered to enhance bone repair *(150,153,154)*.

Other attempts to enhance bone healing are the use of osteoinductive factors such as recombinant growth factors. This osteoinductive therapy induces mitogenesis of undifferentiated perivascular mesenchymal cells and leads to the formation of osteoprogenitor cells with the capacity to form bone. Several growth factors are potentially beneficial for bone and cartilage healing, such as TGF-β, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and the BMPs. Since these factors have been shown to be produced during fracture repair and to participate in the regulation of the healing process, it was logical to administer some of these factors exogenously at the site of injury. Extensive research has been carried to enhance bone healing in different animal models; we summarize these advances in **Table 3**.

Although there is increasing evidence supporting the use of growth factors to enhance fracture healing, the clinical data have been hindered by the selection of optimal carrier and dosage. Only three peer-reviewed clinical studies using rhBMP have been published *(183–185)*, and BMP doses suggesting efficacy ranged from 1.7 to 3.4 mg. These results mute clinical enthusiasm. To overcome difficulties using growth factors, alternatives have been investigated. Such alternatives are gene therapy for fracture healing.

Table 3
Growth Factors and Delivery Systems Used in Different Animal Models to Enhance Bone Healing

Growth	Carrier	Animal	Tissue regenerated	References
TGF-β1	Gelatin	Rabbit	Skull bone	*(155)*
	PLGA	Rat	Skull bone	*(156)*
	Collagen	Mouse	Dermis	*(157)*
FGF-1	Demineralized bone matrix (DBM)	Rabbit	Long bone	*(158)*
FGF-2	Alginate	Mouse	Angiogenesis	*(159)*
FGF-2	Agarose/heparin	Mouse, pig	Angiogenesis	*(160,161)*
FGF-2	Gelatin	Mouse	Angiogenesis	*(162)*
FGF-2	Gelatin	Rabbit, monkey	Skull bone	*(162,163)*
FGF-2	Fibrin gel	Rat	Long bone	*(164)*
FGF-2	Collagen minipellet	Rabbit	Long bone	*(165)*
FGF-2	Collagen	Mouse	Cartilage	*(166)*
RhBMP2	PLA	Dog	Maxilla	*(167)*
BMP	PLA	Dog	Long bone	*(168)*
rhBMP2	PLA (porous)	Dog	Vertebrae	*(169)*
rhBMP2	PLA-coating gelatin sponge	Dog	Long bone, maxilla	*(170)*
rhBMP7	Collagen	Dog	Vertebrae	*(171)*
rhBMP7	Collagen	Monkey	Long bone	*(171)*
rhBMP2	Porous HA	Monkey	Skull	*(172)*
rhBMP2	PLA/PGA	Rabbit	Long bone	*(173)*
rhBMP2	Porous HA	Rabbit	Skull	*(174)*
rhBMP2	PLA	Rabbit	Long bone	*(175)*
rhBMP2	Injection into intervertebral disk	Rabbit	Vertebrae	*(176)*
rhBMP2	Gelatin	Rabbit	Skull	*(177)*
rhBMP2	PLGA	Rat	Long bone	*(178)*
rhBMP2	PLA	Rat	Skull bone	*(179)*
rhBMP2	Collagen sponge	Rat	Skull	*(173)*
rhBMP2	PLA-PEG copolymer	Rat	Long bone	*(180)*
rhBMP2	Inactive bone matrix	Sheep	Long bone	*(181)*
rhBMP2	PLGA	Sheep	Long bone	*(182)*

Fracture Enhancement via Gene Therapy

Gene-based delivery systems offer the potential to deliver and produce proteins locally at therapeutic levels and in a sustained fashion within the fracture site. To transfer genes into a cell, two main choices have to be made. The first is to determine the gene delivery vehicle, known as the vector. The second is to determine if the genes should be introduced into the cell in vivo or ex vivo.

To introduce exogenous DNA into the cell and more specifically into the nucleus where the transcriptional machinery resides, vectors must be used. These vectors could be viral or nonviral. Each system has its advantages and disadvantages. Naked DNA delivery is usually achieved by direct local injection; more recently, combining the DNA with cationic liposomes or other transfecting agents or a biodegradable polymer improved the transfection efficiency. Although transfection efficiency in general was lower than with viral vectors, gene expression from delivered plasmid DNA was sufficient to promote osteogenesis *(186,187)* and angiogenesis *(188–190)*. The main advantages of plasmid DNA are cost, safety, transient expression, and less antigenicity than viral vectors.

Viral vectors have been developed from various viruses. The most widely used viruses are derived from retroviruses, adenoviruses, adeno-associated, and herpes simplex viruses. **Table 4** summarizes the clinical research conducted so far in orthopedics using these various viruses.

With continuing advances in gene technology, gene therapy will likely become increasingly important in healing both acute and chronic wounds. As our understanding of the physiology of bone fracture

Table 4
Summary of Gene Therapy to Bone

Virus type/gene delivered	Tissue targeted	References
Retroviral		
• lacZ marker gene, hBMP-7	Periosteal cells/rabbit femoral osteochondral defects	*(191)*
• Collagen alpha 1	*In vitro* expression in bone marrow stromal cells	*(192)*
• LacZ marker gene	Human osteoprogenitors bone marrow fibroblast were transduced with retrovirus-LacZ and implanted in calvariae of SCID mouse	*(193)*
• BMP-2 and BMP-4	Ectopical expression in developing chick limbs	*(194)*
Adenoviruses		
• LacZ	Rabbit femur (diaphysis)	*(195)*
• BMP-2	Rabbit femur	*(196)*
• FGF		
• BMP-7	Adeno-CMV-BMP-7 virus particles mixed with bovine bone-derived collagen carrier and was implanted into mouse muscle and dermal pouches	*(197)*
• BMP-7	*Ex vivo* transduction of human gingival fibroblasts or rat dermal fibroblasts. The transduced cells were then implanted in critical size skeletal defects in rat calvariae	*(198)*
• LacZ	Rat mandibular osteotomy model,	*(199)*
• BMP-9	Injection of 7.5×10^8 pfu of a BMP-9 adenoviral vector in the lumbar paraspinal musculature.	*(200)*
• Human TGF-β1	Rabbit lumbar intervertebral disks	*(201)*
• BMP-2	Athymic nude rats were injected with Ad-BMP-2 in the thigh musculature	*(202,203)*
• LacZ	Direct injection into the temporomandibular joints of Hartley guinea pigs	*(204)*
• BMP-2	Intramuscular direct injection	*(205)*
Adeno-associated viruses (AAVs)		
• Murine IL-4	Synovial tissues	*(206)*
• To the best of our knowledge, no AAV vectors have been used to enhance bone fracture repair. The difficulty in preparing and purifying this viral vector in large quantities remains a major obstacle for evaluating AAV vectors in clinical trials. Recently, methods for producing a high titer *(207)* and purification *(208)* were published. These advances will allow further studies using AAV vectors.		
Herpex simplex virus type 1 (HSV-1)		
• Has not been used in bone fracture healing models. The HSV-1 amplicon vector is a very promising genetic vehicle for *in vivo* gene delivery. The HSV-1 amplicon vectors consists of a plasmid containing a transgene(s) and the HSV-1 origin of DNA replication and packaging sequence, packaged in a HSV-1 virion free of HSV-1 helper virus.		

repair and the role of the various repair regulators at the molecular level increases, this will ultimately accelerate the progress of gene therapy. In addition, the transfection efficiency and the safety of the delivery systems is expected to improve, providing a therapy with fewer hurdles to overcome in order to become an accepted therapy.

In summary, newly developed comprehensive therapies based on biological understanding, using either recombinant proteins or their genes, will enhance bone regeneration. The challenging task of tissue engineering bone is being tackled by many multidisciplinary research groups involving engineers, biologists, and polymer chemists. This effort should yield optimization of current therapies or the development of therapies that will enhance clinical treatment outcomes.

REFERENCES

1. Praemer, A, Furner, S., et al. (1992) Musculoskeletal conditions in the United States. American Academy of Orthopaedic Surgeons, Park Ridge, IL.
2. Cruess, R. L. and Dumont, J. (1975) Fracture healing. *Can. J. Surg.* **18(5),** 403–413.
3. Madison, M. and Martin, R. B. (1993) Fracture healing, in *Operative Orthopaedics* (Chapman, M. W., ed.), Lippincott, Philadelphia, pp. 221–228.
4. Frost, H. M. (1989) The biology of fracture healing. An overview for clinicians. Part I. *Clin. Orthop.* **248,** 283–293.
5. McKibbin, B. (1978) The biology of fracture healing in long bones. *J. Bone Joint Surg. Br.* **60B(2),** 150–162.
6. White, A. A. I., Punjabi, M. M., and Southwick, W. O. (1977) The four biomechanical stages of fracture healing. *J. Bone Joint Surg.* **59A,** 188.
7. Thomas, T. A. (1998) The cell and molecular biology of fracture healing. *Clinical Orthopaedics & Related Research. Fracture Healing Enhancement* **1(355 Suppl),** S7–S21.
8. Marsh, D. R. and Li, G. (1999) The biology of fracture healing: optimising outcome. *Br. Med. Bull.* **55(4),** 856–869.
9. Nemeth, G. G., Bolander, M. E., and Martin, G. R. (1988) Growth factors and their role in wound and fracture healing. *Prog. Clin. Biol. Res.* **266,** 1–17.
10. Bolander, M. E. (1992) Regulation of fracture repair by growth factors. *Proc. Soc. Exp. Biol. Med.* **200(2),** 165–170.
11. Buckwalter, J. A., et al. (1996) Healing of the musculoskeletal tissues, in *Rockwood and Green's Fractures in Adults* (Rockwood, C. A., et al., ed.), Lippincott-Raven, Philadelphia, pp. 261–304.
12. Glowacki, J. (1998) Angiogenesis in fracture repair. *Clin. Orthop.* **355(Suppl),** S82–S89.
13. Hollinger, J. and Wong, M. E. (1996) The integrated processes of hard tissue regeneration with special emphasis on fracture healing. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endodont.* **82(6),** 594–606.
14. Grundnes, O. and Reikeras, O. (1993) The role of hematoma and periosteal sealing for fracture healing in rats. *Acta Orthop. Scand.* **64(1),** 47–49.
15. Grundnes, O. and Reikeras, O. (1993) The importance of the hematoma for fracture healing in rats. *Acta Orthop. Scand.* **64(3),** 340–342.
16. Tonna, E. A. and Cronkite, E. P. (1963) The periosteum: autoradiographic studies on cellular proliferation and transformation utilizing tritiated thymidine. *Clin. Orthop.* **30,** 218–233.
17. Einhorn, T. A., et al. (1989) Neutral protein-degrading enzymes in experimental fracture callus: a preliminary report. *J. Orthop. Res.* **7(6),** 792–805.
18. Brighton, C. T. and Hunt, R. M. (1986) Histochemical localization of calcium in the fracture callus with potassium pyroantimonate. Possible role of chondrocyte mitochondrial calcium in callus calcification. *J. Bone Joint Surg. Am.* **68(5),** 703–715.
19. Wendeberg, B. (1961) Mineral metabolism of fractures of the tibia in man studied with external counting of strontium 85. *Acta Orthop. Scand. Suppl.* **52,** 1–79.
20. Frost, H. M. (1980) Skeletal physiology and bone remodeling, in *Fundamental and Clinical Bone Physiology* (Urist, M. R. ed.), Lippincott, Philadelphia, pp. 208–241.
21. Allgower, M. and Spiegel, P. G. (1979) Internal fixation of fractures: evolution of concepts. *Clin. Orthop.* **138,** 26–29.
22. Muller, M. E., Allgower, M., and Willenegger, H. (1965) *Technique of internal fixation of fractures.* Springer-Verlag, New York.
23. Perren, S. M. (1979) Physical and biological aspects of fracture healing with special reference to internal fixation. *Clin. Orthop.* **138,** 175–196.
24. Perren, S. M. (1989) The biomechanics and biology of internal fixation using plates and nails. *Orthopedics* **12(1),** 21–34.
25. Perren, S. M., Cordey, J., and Gautier, E. (1987) Rigid internal fixation using plates: terminology, principle and early problems, in *Fracture Healing* (Lang, J. M., ed.), Churchill Livingstone, New York, pp. 139–151.
26. Perren, S. M., Huggler, A., and Russenberger, S. (1969) Cortical Bone Healing. *Acta Orthop. Scand. Suppl.* 125.
27. Schatzker, J., Waddell, J., and Stoll, J. E. (1989) The effects of motion on the healing of cancellous bone. *Clin. Orthop.* **245,** 282–287.
28. Schenk, R. (1987) Cytodynamics and histodynamics of primary bone repair, in *Fracture Healing* (Lang, J. M., ed.), Churchill Livingstone, New York, pp. 23–32.
29. Schenk, R. and Willenegger, H. (1967) Morphological findings in primary fracture healing: callus formation. *Simp. Biol. Hungar.* **7,** 75–80.
30. Rahn, B. A., et al. (1971) Primary bone healing. An experimental study in the rabbit. *J. Bone Joint Surg. Am.* **53(4),** 783–786.
31. Schenk, R. and Willenegger, H. (1963) Histologie der primaren Knochenheilung. *Arch. Klin. Chir.* **19,** 593.
32. DeLacure, M. D. (1994) Physiology of bone healing and bone grafts. *Otolaryngol. Clin. N. Am.* **27(5),** 859–874.
33. Liu, Z., et al. (1999) Molecular signaling in bone fracture healing and distraction osteogenesis. *Histol. Histopathol.* **14(2),** 587–595.

34. Bonewald, L. F. and Dallas, S. L. (1994) Role of active and latent transforming growth factor beta in bone formation. *J. Cell Biochem.* **55(3),** 350–357.
35. Dallas, S. L., et al. (1994) Characterization and autoregulation of latent transforming growth factor beta (TGF beta) complexes in osteoblast-like cell lines. Production of a latent complex lacking the latent TGF beta-binding protein. *J. Biol. Chem.* **269(9),** 6815–6821.
36. Miyazono, K., Ichijo, H., and Heldin, C. H. (1993) Transforming growth factor-beta: latent forms, binding proteins and receptors. *Growth Factors* **8(1),** 11–22.
37. Linkhart, T. A., Mohan, S., and Baylink, D. J. (1996) Growth factors for bone growth and repair: IGF, TGF beta and BMP. *Bone* **19(1 Suppl),** 1S–12S.
38. Joyce, M. E., et al. (1990) Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. *J. Cell Biol.* **110(6),** 2195–2207.
39. Noda, M. and Camilliere, J. J. (1989) *In vivo* stimulation of bone formation by transforming growth factor-beta. *Endocrinology* **124(6),** 2991–2994.
40. Sandberg, M. M., Aro, H. T., and Vuorio, E. I. (1993) Gene expression during bone repair. *Clin. Orthop.* **289,** 292–312.
41. Mundy, G. R. (1993) Cytokines and growth factors in the regulation of bone remodeling. *J. Bone Miner. Res.* **8(Suppl 2),** S505–S510.
42. Beck, L. S., et al. (1991) *In vivo* induction of bone by recombinant human transforming growth factor beta 1. *J. Bone Miner. Res.* **6(9),** 961–968.
43. Talley Ronsholdt, D., Lajiness, E., and Nagodawithana, K. (1995) Transforming growth factor-beta inhibition of mineralization by neonatal rat osteoblasts in monolayer and collagen gel culture. *In Vitro Cell Dev. Biol. Anim.* **31,** 274–282.
44. Cheifetz, S., et al. (1996) Influence of osteogenic protein-1 (OP-1;BMP-7) and transforming growth factor-beta 1 on bone formation in vitro. *Connect. Tissue Res.* **35(1–4),** 71–78.
45. Pfeilschifter, J., Seyedin, S. M., and Mundy, G. R. (1988) Transforming growth factor beta inhibits bone resorption in fetal rat long bone cultures. *J. Clin. Invest.* **82(2),** 680–685.
46. Massague, J. (1990) The transforming growth factor-beta family. *Annu. Rev. Cell Biol.* **6,** 597–641.
47. Prisell, P. T., et al. (1993) Expression of insulin-like growth factors during bone induction in rat. *Calcif. Tissue Int.* 1993. **53(3),** 201–205.
48. Eppley, B. L., et al. (1988) Enhancement of angiogenesis by bFGF in mandibular bone graft healing in the rabbit. *J. Oral. Maxillofac. Surg.* **46(5),** 391–398.
49. Trippel, S. B., et al. (1992) Interaction of basic fibroblast growth factor with bovine growth plate chondrocytes. *J. Orthop. Res.* **10(5),** 638–646.
50. Chintala, S. K., Miller, R. R., and McDevitt, C. A. (1994) Basic fibroblast growth factor binds to heparan sulfate in the extracellular matrix of rat growth plate chondrocytes. *Arch. Biochem. Biophys.* **310(1),** 180–186.
51. Canalis, E., McCarthy, T., and Centrella, M. (1988) Growth factors and the regulation of bone remodeling. *J. Clin. Invest.* **81(2),** 277–281.
52. Centrella, M., et al. (1992) Isoform-specific regulation of platelet-derived growth factor activity and binding in osteoblast-enriched cultures from fetal rat bone. *J. Clin. Invest.* **89(4),** 1076–1084.
53. Robson, M. C., et al. (1992) Platelet-derived growth factor BB for the treatment of chronic pressure ulcers. *Lancet* **339(8784),** 23–25.
54. Muller, R., et al. (1984) Induction of c-fos gene and protein by growth factors precedes activation of c-myc. *Nature* **312(5996),** 716–720.
55. Andrew, J., et al. (1993) Insuling like growth factor gene expression in human fracture callus. *Calcif. Tissue Int.* **53,** 97–102.
56. Edwall, D., et al. (1992) Expression of insulin-like growth factor I messenger ribonucleic acid in regenerating bone after fracture: influence of indomethacin. *J. Bone Miner. Re*s. **7(2),** 207–213.
57. Nilsson, A., et al. (1986) Regulation by growth hormone of number of chondrocytes containing IGF-I in rat growth plate. *Science* **233(4763),** 571–574.
58. Lammens, J., et al. (1998) Distraction bone healing versus osteotomy healing: a comparative biochemical analysis. *J. Bone Miner. Res.* **13(2),** 279–286.
59. Hill, P. A., Reynolds, J. J., and Meikle, M. C. (1995) Osteoblasts mediate insulin-like growth factor-I and -II stimulation of osteoclast formation and function. *Endocrinology* **136(1),** 124–131.
60. Mochizuki, H., et al. (1992) Insulin-like growth factor-I supports formation and activation of osteoclasts. *Endocrinology* **131(3),** 1075–1080.
61. Beck, F., et al. (1987) Histochemical localization of IGF-I and -II mRNA in the developing rat embryo. *Development* **101(1),** 175–184.
62. McCarthy, T. L., Centrella, M., and Canalis, E. (1989) Regulatory effects of insulin-like growth factors I and II on bone collagen synthesis in rat calvarial cultures. *Endocrinology* **124(1),** 301–309.
63. Urist, M. R. and Strates, B. S. (1971) Bone morphogenetic protein. *J. Dent. Res.* **50(6),** 1392–1406.

64. Urist, M. R., Iwata, H., and Strates, B. S. (1972) Bone morphogenetic protein and proteinase in the guinea pig. *Clin. Orthop.* **85,** 275–290.
65. Urist, M. R., et al. (1983) Human bone morphogenetic protein (hBMP). *Proc. Soc. Exp. Biol. Med.* **173(2),** 194–199.
66. Bostrom, M. P., et al. (1995) Immunolocalization and expression of bone morphogenetic proteins 2 and 4 in fracture healing. *J. Orthop. Res.* **13(3),** 357–367.
67. Nakase, T., et al. (1994) Transient and localized expression of bone morphogenetic protein 4 messenger RNA during fracture healing. *J. Bone Miner. Res.* **9(5),** 651–659.
68. Onishi, T., et al. (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(6),** 605–612.
69. Canalis, E. and Gabbitas, B. (1994) Bone morphogenetic protein 2 increases insulin-like growth factor I and II transcripts and polypeptide levels in bone cell cultures. *J. Bone Miner. Res.* **9(12),** 1999–2005.
70. Zheng, M. H., et al. (1994) Recombinant human bone morphogenetic protein-2 enhances expression of interleukin-6 and transforming growth factor-beta 1 genes in normal human osteoblast-like cells. *J. Cell Physiol.* **159(1),** 76–82.
71. Cunningham, N. S., Paralkar, V., and Reddi, A. H. (1992) Osteogenin and recombinant bone morphogenetic protein 2B are chemotactic for human monocytes and stimulate transforming growth factor beta 1 mRNA expression. *Proc. Natl. Acad. Sci. USA* **89(24),** 11740–11744.
72. Ozkaynak, E., et al. (1990) OP-1 cDNA encodes an osteogenic protein in the TGF-beta family. *EMBO J.* **9(7),** 2085–2093.
73. Knutsen, R., et al. (1995) Regulation of insulin-like growth factor system components by osteogenic protein-1 in human bone cells. *Endocrinology* **136(3),** 857–865.
74. Paralkar, V. M., et al. (1990) Interaction of osteogenin, a heparin binding bone morphogenetic protein, with type IV collagen. *J. Biol. Chem.* **265(28),** 17281–17284.
75. Wozney, J. M. (1992) The bone morphogenetic protein family and osteogenesis. *Mol. Reprod. Dev.* **32(2),** 160–167.
76. Ducy, P., et al. (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89(5),** 747–754.
77. Celeste, A. J., et al. (1990) Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone. *Proc. Natl. Acad. Sci. USA* **87(24),** 9843–9847.
78. Wozney, J. M. (1989) Bone morphogenetic proteins. *Prog. Growth Factor Res.* **1(4),** 267–280.
79. Hogan, B. L. (1996) Bone morphogenetic proteins in development. *Curr. Opin. Genet. Dev.* **6(4),** 432–438.
80. Hogan, B. L. (1996) Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10(13),** 1580–1594.
81. Sakou, T. (1998) Bone morphogenetic proteins: from basic studies to clinical approaches. *Bone* **22(6),** 591–603.
82. Bolander, M. E., et al. (1988) Osteonectin cDNA sequence reveals potential binding regions for calcium and hydroxyapatite and shows homologies with both a basement membrane protein (SPARC) and a serine proteinase inhibitor (ovomucoid). *Proc. Natl. Acad. Sci. USA* **85(9),** 2919–2923.
83. Hiltunen, A., et al. (1994) Retarded chondrogenesis in transgenic mice with a type II collagen defect results in fracture healing abnormalities. *Dev. Dyn.* **200(4),** 340–349.
84. Yamazaki, M., Majeska, R. J., and Moriya, H. (1997) Role of osteonectin during fracture healing. *Trans. Orthop. Res. Soc.* **22,** 254.
85. Jingushi, S., Joyce, M. E., and Bolander, M. E. (1992) Genetic expression of extracellular matrix proteins correlates with histologic changes during fracture repair. *J. Bone Miner. Res.* **7(9),** 1045–1055.
86. Metsaranta, M., et al. (1989) Localization of osteonectin expression in human fetal skeletal tissues by *in situ* hybridization. *Calcif. Tissue Int.* **45(3),** 146–152.
87. Price, P. A., Williamson, M. K., and Lothringer, J. W. (1981) Origin of the vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone. *J. Biol. Chem.* **256(24),** 12760–12766.
88. Nakamura, H., et al. (1995) Localization of CD44, the hyaluronate receptor, on the plasma membrane of osteocytes and osteoclasts in rat tibiae. *Cell Tissue Res.* **280(2),** 225–233.
89. Yamazaki, M., et al. (1997) Spatial and temporal expression of fibril-forming minor collagen genes (types V and XI) during fracture healing. *J. Orthop. Res.* **15(5),** 757–764.
90. Hirakawa, K., et al. (1994) Localization of the mRNA for bone matrix proteins during fracture healing as determined by *in situ* hybridization. *J. Bone Miner. Res.* **9(10),** 1551–1557.
91. Reinholt, F. P., et al. (1990) Osteopontin—a possible anchor of osteoclasts to bone. *Proc. Natl. Acad. Sci. USA* **87(12),** 4473–4475.
92. Bourghol, A., et al. (1996) Fibronectin expression during fracture healing in the rat. *Trans. Orthop. Res. Soc.* **21,** 618.
93. Ishidou, Y., et al. (1995) Enhanced expression of type I receptors for bone morphogenetic proteins during bone formation. *J. Bone Miner. Res.* **10(11),** 1651–1659.
94. Baker, J. C. and Harland, R. M. (1997) From receptor to nucleus: the Smad pathway. *Curr. Opin. Genet Dev.* **7(4),** 467–473.
95. Heldin, C. H., Miyazono, K., and Dijke, P. (1997) TGF[beta] signaling from cell membrane to nucleus through Smad proteins. *Nature* **390,** 465–471.

96. Reddi, A. H. (1998) Initiation of fracture repair by bone morphogenetic proteins. *Clin. Orthop.* **355(Suppl),** S66–S72.
97. Einhorn, T. A., et al. (1995) The expression of cytokine activity by fracture callus. *J. Bone Miner. Res.* **10(8),** 1272–1281.
98. Beuscher, H. U., et al. (1992) Transition from interleukin 1 beta (IL-1 beta) to IL-1 alpha production during maturation of inflammatory macrophages *in vivo*. *J. Exp. Med.* **175(6),** 1793–1797.
99. Brown, C. C., Hembry, R. M., and Reynolds, J. J. (1989) Immunolocalization of metalloproteinases and their inhibitor in the rabbit growth plate. *J. Bone Joint Surg. Am.* **71(4),** 580–593.
100. Leibovich, S. J. and Ross, R. (1975) The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. *Am. J. Pathol.* **78(1),** 71–100.
101. Polverini, P. J., et al. (1977) Activated macrophages induce vascular proliferation. *Nature* **269(5631),** 804–806.
102. Zlotnik, A., Daine, B., and Smith, C. A. (1985) Activation of an interleukin-1-responsive T-cell lymphoma by fixed P388D1 macrophages and an antibody against the Ag:MHC T-cell receptor. *Cell. Immunol.* **94(2),** 447–453.
103. Browder, W., et al. (1988) Effect of enhanced macrophage function on early wound healing. *Surgery* **104(2),** 224–230.
104. Feyen, J., et al. (1989) Interleukin-6 is produced by bone and modulated by parathyroid hormone. *J. Bone Miner. Res.* **4,** 633–638.
105. Horowitz, M. C., et al. (1990) Il-6 secretion by osteoblasts. *Trans. Orthop. Res. Soc.* **15,** 379.
106. Helle, M., et al. (1988) Interleukin 6 is involved in interleukin 1-induced activities. *Eur. J. Immunol.* **18(6),** 957–959.
107. Lowik, C., et al. (1989) Parathyroid hormone (PTH) and PTH-like protein (PLP) stimulate interleukin-6 production by osteogenic cells: a possible role of interleukin-6 in osteoclastogenesis. *Biochem. Biophys. Res. Commun.* **162,** 1546–1552.
108. Roodman, G. (1992) Interleukin-6: An osteoptropic factor? *J. Bone Miner. Res.* **7,** 475–478.
109. Jilka, R. L., et al. (1992) Increased osteoclast development after estrogen loss: mediation by interleukin-6. *Science* **257(5066),** 88–91.
110. Hulth, A., et al. (1985) Appearance of T-cells and Ia-expressing cells in fracture healing in rats. *Int. J. Immunother.* **1,** 103–106.
111. Gliniak, B. C. and Rohrschneider, L. R. (1990) Expression of the M-CSF receptor is controlled posttranscriptionally by the dominant actions of GM-CSF or multi-CSF. *Cell* **63(5),** 1073–1083.
112. Horowitz, M. C., et al. (1989) Parathyroid hormone and lipopolysaccharide induce murine osteoblast-like cells to secrete a cytokine indistinguishable from granulocyte-macrophage colony-stimulating factor. *J. Clin. Invest.* **83(1),** 149–157.
113. Horowitz, M. C., et al. (1989) Osteotropic agents induce the differential secretion of granulocyte-macrophage colony-stimulating factor by the osteoblast cell line MC3T3-E1. *J. Bone Miner. Res.* **4,** 911–921.
114. Horowitz, M. C., Friedlaender, G. E., and Qian, H.-T. (1994) T cell activation and the immune response to bone allografts. *Trans. Orthop. Res. Soc.* **19,** 180.
115. Barbul, A., et al. (1986) Interleukin 2 enhances wound healing in rats. *J. Surg. Res.* **40(4),** 315–319.
116. Dinarello, C. A. and Mier, J. W. (1987) Lymphokines. *N. Engl. J. Med.* **317(15),** 940–945.
117. Turck, C. W., Dohlman, J. G., and Goetzl, E. J. (1987) Immunological mediators of wound healing and fibrosis. *J. Cell Physiol. Suppl.* **Suppl(5),** 89–93.
118. Nicola, N. A. (1989) Hemopoietic cell growth factors and their receptors. *Annu. Rev. Biochem.* **58,** 45–77.
119. Felix, R., Fleisch, H., and Elford, P. R. (1989) Bone-resorbing cytokines enhance release of macrophage colony-stimulating activity by the osteoblastic cell MC3T3-E1. *Calcif. Tissue Int.* **44(5),** 356–360.
120. Horowitz, M. C., et al. (1989) Functional and molecular changes in colony stimulating factor secretion by osteoblasts. *Connect Tissue Res.* **20(1–4),** 159–168.
121. Stanley, E. R., et al. (1976) Factors regulating macrophage production and growth: identity of colony-stimulating factor and macrophage growth factor. *J. Exp. Med.* **143(3),** 631–647.
122. Hayda, R. A., Brighton, C. T., and Esterhai, J. L. Jr. (1998) Pathophysiology of delayed healing. *Clin. Orthop.* **355(Suppl),** S31–S40.
123. Pan, W. and Einhorn, T. A. (1992) The biochemistry of fracture healing. *Curr. Orthop.* **6,** 207–213.
124. Page, M., Hogg, J., and Ashhurst, D. E. (1986) The effects of mechanical stability on the macromolecules of the connective tissue matrices produced during fracture healing. I. The collagens. *Histochem. J.* **18(5),** 251–265.
125. Hiltunen, A., Aro, H. T., and Vuorio, E. (1993) Regulation of extracellular matrix genes during fracture healing in mice. *Clin. Orthop.* **297,** 23–27.
126. Sandberg, M., et al. (1989) *In situ* localization of collagen production by chondrocytes and osteoblasts in fracture callus. *J. Bone Joint Surg. Am.* **71(1),** 69–77.
127. Multimaki, P., Aro, H., and Vuorio, E. (1987) Differential expression of fibrillar collagen genes during callus formation. *Biochem. Biophys. Res. Commun.* **142(2),** 536–541.
128. Paralkar, V. M., Vukicevic, S., and Reddi, A. H. (1991) Transforming growth factor beta type 1 binds to collagen IV of basement membrane matrix: implications for development. *Dev. Biol.* **143(2),** 303–308.
129. Birke, D., et al. (1990) Collagen fibrillogenesis *in vitro*: interaction of types I and V collagen regulates fibril diameter. *J. Cell Sci.* **95,** 649–657.

130. Mendler, M., et al. (1989) Cartilage contains mixed fibrils of collagen types II, IX, and XI. *J. Cell Biol.* **108(1),** 191–197.
131. Mayne, R. and Burgeson, R. E., eds. (1987) *Structure and Function of Collagen Types.* Academic Press, Orlando, FL.
132. Shimokomaki, M., et al. (1990) The structure and macromolecular organization of type IX collagen in cartilage. *Ann. NY Acad. Sci.* **580,** 1–7.
133. Tonna, E. A. (1958) Histochemical and antoradiograhic studies on the effects of aging of the mucopolysaccharides of the periosteum. *J. Biophys. Biochem. Cytol.* **6,** 171–175.
134. Tonna, E. A. (1978) Electron microscopic study of bone surface changes during aging. The loss of cellular control and biofeedback. *J. Gerontol.* **33(2),** 163–177.
135. Syftestad, G.T. and Urist, M.R. (1982) Bone aging. *Clin. Orthop.* **162,** 288–297.
136. Mears, D.C. (1999) Surgical treatment of acetabular fractures in elderly patients with osteoporotic bone. *J. Am. Acad. Orthop. Surg.* **7(2),** 128–141.
137. Christiansen, M., et al. (2000) CBFA1 and topoisomerase I mRNA levels decline during cellular aging of human trabecular osteoblasts. *J. Gerontol. A Biol. Sci. Med. Sci.* **55(4),** B194–B200.
138. Ankersen, L.(1994) Aging of humantravecular osteoblasts in culture. *Arch. Gerontol. Geriatr.* **4(Suppl),** 5–12.
139. Kassem, M., et al. (1997) Demonstration of cellular aging and senescence in serially passaged long-term cultures of human trabecular osteoblasts. *Osteopor. Int.* **7(6),** 514–524.
140. Chavassieux, P. M., et al. (1990) Influence of experimental conditions on osteoblast activity in human primary bone cell cultures. *J. Bone Miner. Res.* **5(4),** 337–343.
141. Martinez, M. E., et al. (1999) Influence of skeletal site of origin and donor age on osteoblastic cell growth and differentiation. *Calcif. Tissue Int.* **64(4),** 280–286.
142. Marie, P. J. (1995) Human endosteal osteoblastic cells: relationship with bone formation. *Calcif. Tissue Int.* **56(Suppl 1),** S13–S16.
143. Vanderschueren, D., et al. (1990) Sex- and age-related changes in bone and serum osteocalcin. *Calcif. Tissue Int.* **46(3),** 179–182.
144. O'Driscoll, S. W., et al. (2001) The chondrogenic potential of periosteum decreases with age. *J. Orthop. Res.* **19(1),** 95–103.
145. Evans, R. D., Foltz, D., and Foltz, K. (2001) Electrical stimulation with bone and wound healing. *Clin. Podiatr. Med. Surg.* **18(1),** 79–95, vi.
146. Spadaro, J. A. (1997) Mechanical and electrical interactions in bone remodeling. *Bioelectromagnetics* **18(3),** 193–202.
147. Goodman, S. and Aspenberg, P. (1993) Effects of mechanical stimulation on the differentiation of hard tissues. *Biomaterials* **14(8),** 563–569.
148. Garg, N. K., Gaur, S., and Sharma, S. (1993) Percutaneous autogenous bone marrow grafting in 20 cases of ununited fracture. *Acta Orthop. Scand.* **64(6),** 671–672.
149. Caplan, A. I. (1994) The mesengenic process. *Clin. Plast. Surg.* **21(3),** 429–435.
150. Bruder, S. P., Fink, D. J., and Caplan, A. I. (1994) Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. *J. Cell Biochem.* **56(3),** 283–294.
151. Goshima, J., Goldberg, V. M., and Caplan, A. I. (1991) The osteogenic potential of culture-expanded rat marrow mesenchymal cells assayed *in vivo* in calcium phosphate ceramic blocks. *Clin. Orthop.* **262,** 298–311.
152. Caplan, A. I. (1991) Mesenchymal stem cells. *J. Orthop. Res.* **9(5),** 641–650.
153. Goshima, J., Goldberg, V. M., and Caplan, A. I. (1991) The origin of bone formed in composite grafts of porous calcium phosphate ceramic loaded with marrow cells. *Clin. Orthop.* **269,** 274–283.
154. Dennis, J. E. and Caplan, A. I. (1993) Porous ceramic vehicles for rat-marrow-derived (Rattus norvegicus) osteogenic cell delivery: effects of pre-treatment with fibronectin or laminin. *J. Oral Implantol.* **19(2),** 106–115.
155. Hong, L., et al. (2000) Promoted bone healing at a rabbit skull gap between autologous bone fragment and the surrounding intact bone with biodegradable microspheres containing transforming growth factor-beta1. *Tissue Eng.* **6(4),** 331–340.
156. Gombotz, W. R., et al. (1993) Controlled release of TGF-beta 1 from a biodegradable matrix for bone regeneration. *J. Biomater. Sci. Polym. Ed.* **5(1–2),** 49–63.
157. Mustoe, T. A., et al. (1987) Accelerated healing of incisional wounds in rats induced by transforming growth factor-beta. *Science* **237(4820),** 1333–1336.
158. Mackenzie, D. J., et al. (2001) Recombinant human acidic fibroblast growth factor and fibrin carrier regenerates bone. *Plast. Reconstr. Surg.* **107(4),** 989–996.
159. Downs, E. C., et al. (1992) Calcium alginate beads as a slow-release system for delivering angiogenic molecules *in vivo* and *in vitro*. *J. Cell Physiol.* **152(2),** 422–429.
160. Edelman, E. R., et al. (1991) Controlled and modulated release of basic fibroblast growth factor. *Biomaterials* **12(7),** 619–626.
161. Harada, K., et al. (1994) Basic fibroblast growth factor improves myocardial function in chronically ischemic porcine hearts. *J. Clin. Invest.* **94(2),** 623–630.

162. Tabata, Y., et al. (1999) Skull bone regeneration in primates in response to basic fibroblast growth factor. *J. Neurosurg.* **91(5),** 851–856.
163. Tabata, Y., et al. (1998) Bone regeneration by basic fibroblast growth factor complexed with biodegradable hydrogels. *Biomaterials* **19(7–9),** 807–815.
164. Kawaguchi, H., et al. (1994) Stimulation of fracture repair by recombinant human basic fibroblast growth factor in normal and streptozotocin-diabetic rats. *Endocrinology* **135(2),** 774–781.
165. Inui, K., et al. (1998) Local application of basic fibroblast growth factor minipellet induces the healing of segmental bony defects in rabbits. *Calcif. Tissue Int.* **63(6),** 490–495.
166. Fujisato, T., et al. (1996) Effect of basic fibroblast growth factor on cartilage regeneration in chondrocyte-seeded collagen sponge scaffold. *Biomaterials* **17(2),** 155–162.
167. Hollinger, J. and Wong, M. E. (1996) The integrated processes of hard tissue regeneration with special emphasis on fracture healing. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endodont.* **82(6),** 594–606.
168. Heckman, J. D., et al. (1991) The use of bone morphogenetic protein in the treatment of non-union in a canine model. *J. Bone Joint Surg. Am.* **73(5),** 750–764.
169. Muschler, G. F., et al. (1994) Evaluation of human bone morphogenetic protein 2 in a canine spinal fusion model. *Clin. Orthop.* **308,** 229–240.
170. Miyamoto, S., et al. (1992) Evaluation of polylactic acid homopolymers as carriers for bone morphogenetic protein. *Clin. Orthop.* **278,** 274–285.
171. Cook, S. D., et al. (1994) *In vivo* evaluation of recombinant human osteogenic protein (rhOP-1) implants as a bone graft substitute for spinal fusions. *Spine* **19(15),** 1655–1663.
172. Ripamonti, U., et al. (1992) Osteogenin, a bone morphogenetic protein, adsorbed on porous hydroxyapatite substrata, induces rapid bone differentiation in calvarial defects of adult primates. *Plast. Reconstr. Surg.* **90(3),** 382–393.
173. Zellin, G. and Linde, A. (1997) Treatment of segmental defects in long bones using osteopromotive membranes and recombinant human bone morphogenetic protein-2. An experimental study in rabbits. *Scand. J. Plast. Reconstr. Surg. Hand. Surg.* **31(2),** 97–104.
174. Ono, I., et al. (1992) A study on bone induction in hydroxyapatite combined with bone morphogenetic protein. *Plast. Reconstr. Surg.* **90(5),** 870–879.
175. Zegzula, H. D., et al. (1997) Bone formation with use of rhBMP-2 (recombinant human bone morphogenetic protein-2). *J. Bone Joint Surg. Am.* **79(12),** 1778–1790.
176. Muschik, M., et al. (2000) Experimental anterior spine fusion using bovine bone morphogenetic protein: a study in rabbits. *J. Orthop. Sci.* **5(2),** 165–170.
177. Hong, L., et al. (1998) Comparison of bone regeneration in a rabbit skull defect by recombinant human BMP-2 incorporated in biodegradable hydrogel and in solution. *J. Biomater. Sci. Polymer. Ed.* **9(9),** 1001–1014.
178. Lee, S. C., et al. (1994) Healing of large segmental defects in rat femurs is aided by RhBMP-2 in PLGA matrix. *J. Biomed. Mater. Res.* **28(10),** 1149–1156.
179. Kenley, R., et al. (1994) Osseous regeneration in the rat calvarium using novel delivery systems for recombinant human bone morphogenetic protein-2 (rhBMP-2). *J. Biomed. Mater. Res.* **28(10),** 1139–1147.
180. Miyamoto, S., et al. (1993) Polylactic acid-polyethylene glycol block copolymer. A new biodegradable synthetic carrier for bone morphogenetic protein. *Clin. Orthop.* **294,** 333–343.
181. Gerhart, T. N., et al. (1993) Healing segmental femoral defects in sheep using recombinant human bone morphogenetic protein. *Clin. Orthop.* **293,** 317–326.
182. Kirker-Head, C. A., et al. (1998) Healing bone using recombinant human bone morphogenetic protein 2 and copolymer. *Clin. Orthop.* **349,** 205–217.
183. Boden, S. D., et al. (2000) The use of rhBMP-2 in interbody fusion cages. Definitive evidence of osteoinduction in humans: a preliminary report. *Spine* **25(3),** 376–381.
184. Boyne, P. J., et al. (1997) A feasibility study evaluating rhBMP-2/absorbable collagen sponge for maxillary sinus floor augmentation. *Int. J. Periodont. Restor. Dent.* **17(1),** 11–25.
185. Cochran, D. L., et al. (2000) Evaluation of recombinant human bone morphogenetic protein-2 in oral applications including the use of endosseous implants: 3-year results of a pilot study in humans. *J. Periodontol.* **71(8),** 1241–1257.
186. Fang, J., et al. (1996) Stimulation of new bone formation by direct transfer of osteogenic plasmid genes. *Proc. Natl. Acad. Sci. USA* **93(12),** 5753–5758.
187. Bonadio, J., et al. (1999) Localized, direct plasmid gene delivery *in vivo*: prolonged therapy results in reproducible tissue regeneration. *Nat. Med.* **5(7),** 753–759.
188. Baumgartner, I., et al. (1998) Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* **97(12),** 1114–1123.
189. Symes, J. F., et al. (1999) Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease. *Ann. Thorac. Surg.* **68(3),** 830–836; discussion 836–837.
190. Losordo, D. W., et al. (1998) Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation* **98(25),** 2800–2804.

191. Mason, J. M., et al. (1998) Expression of human bone morphogenic protein 7 in primary rabbit periosteal cells: potential utility in gene therapy for osteochondral repair. *Gene Ther.* **5(8),** 1098–1104.
192. Stover, M. L., et al. (2001) Bone-directed expression of Col1a1 promoter-driven self-inactivating retroviral vector in bone marrow cells and transgenic mice. *Mol. Ther.* **3(4),** 543–550.
193. Oreffo, R. O., Virdi, A. S., and Triffitt, J. T. (2001) Retroviral marking of human bone marrow fibroblasts: *in vitro* expansion and localization in calvarial sites after subcutaneous transplantation *in vivo*. *J. Cell Physiol.* **186(2),** 201–209.
194. Duprez, D., et al. (1996) Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. *Mech. Dev.* **57(2),** 145–157.
195. Baltzer, A. W., et al. (1999) A gene therapy approach to accelerating bone healing. Evaluation of gene expression in a New Zealand white rabbit model. *Knee Surg. Sports Traumatol. Arthrosc.* **7(3),** 197–202.
196. Baltzer, A. W., et al. (2000) Genetic enhancement of fracture repair: healing of an experimental segmental defect by adenoviral transfer of the BMP-2 gene. *Gene Ther.* **7(9),** 734–739.
197. Franceschi, R. T., et al. (2000) Gene therapy for bone formation: *in vitro* and *in vivo* osteogenic activity of an adenovirus expressing BMP7. *J. Cell Biochem.* **78(3),** 476–486.
198. Krebsbach, P. H., et al. (2000) Gene therapy-directed osteogenesis: BMP-7-transduced human fibroblasts form bone *in vivo*. *Hum. Gene Ther.* **11(8),** 1201–1210.
199. Spector, J. A., et al. (2000) Expression of adenovirally delivered gene products in healing osseous tissues. *Ann. Plast. Surg.* **44(5),** 522–528.
200. Helm, G. A., et al. (2000) Use of bone morphogenetic protein-9 gene therapy to induce spinal arthrodesis in the rodent. *J. Neurosurg.* **92(2 Suppl),** 191–196.
201. Nishida, K., et al. (1999) Modulation of the biologic activity of the rabbit intervertebral disc by gene therapy: an *in vivo* study of adenovirus-mediated transfer of the human transforming growth factor beta 1 encoding gene. *Spine* **24(23),** 2419–2425.
202. Alden, T. D., et al. (1999) *In vivo* endochondral bone formation using a bone morphogenetic protein 2 adenoviral vector. *Hum. Gene Ther.* **10(13),** 2245–2253.
203. Alden, T. D., et al. (1999) Percutaneous spinal fusion using bone morphogenetic protein-2 gene therapy. *J. Neurosurg.* **90(1 Suppl),** 109–114.
204. Kuboki, T., et al. (1999) Direct adenovirus-mediated gene delivery to the temporomandibular joint in guinea-pigs. *Arch. Oral. Biol.* **44(9),** 701–709.
205. Musgrave, D. S., et al. (1999) Adenovirus-mediated direct gene therapy with bone morphogenetic protein-2 produces bone. *Bone* **24(6),** 541–547.
206. Watanabe, S., et al. (2000) Adeno-associated virus mediates long-term gene transfer and delivery of chondroprotective IL-4 to murine synovium. *Mol. Ther.* **2(2),** 147–152.
207. Xiao, W., et al. (1999) Gene therapy vectors based on adeno-associated virus type 1. *J. Virol.* **73(5),** 3994–4003.
208. Gao, G., et al. (2000) Purification of recombinant adeno-associated virus vectors by column chromatography and its performance *in vivo*. *Hum. Gene Ther.* **11(15),** 2079–2091.