Invited Review

Molecular signaling in bone fracture healing and distraction osteogenesis

Z. Liu[1,2], F.P. Luyten[1], J. Lammens[2] and J. Dequeker[1]
Departments of [1]Rheumatology and [2]Orthopaedic Surgery, U. Z. Leuven, Pellenberg, Belgium

Summary. The process of fracture healing has been described in detail in many histological studies. Recent work has focused on the mechanisms by which growth and differentiation factors regulate the fracture healing process. Rapid progress in skeletal cellular and molecular biology has led to the identification of many signaling molecules associated with the formation of skeletal tissues, including members of the transforming growth factor-ß (TGF-ß) superfamily and the insulin-like growth factor (IGF) family. Increasing evidence indicates that they are critical regulators of cellular proliferation, differentiation, extracellular matrix biosynthesis and mineralization. Limb lengthening procedure (distraction osteogenesis) is a relevant model to investigate the *in vivo* correlation between mechanical stimulation and biological responses as the callus is stretched by a proper rate and rhythm of mechanical strain. This model also provides additional insights into the molecular and cellular events during bone fracture repair. TGF-ß1 was significantly increased in both the distracted callus and the fracture callus. The increased level of TGF-ß1, together with a low concentration of calcium and an enhanced level of collagen synthesis, was maintained in the distracted callus as long as mechanical strain was applied. Less mineralization is also associated with a low level of osteocalcin production. These observations provide further insights into the molecular basis for the cellular events during distraction osteogenesis.

Key words: Growth factors, fracture healing, Distraction osteogenesis, Mechanical strain

Introduction

It is common knowledge that bone tissue has a substantial capacity for regeneration and repair in response to injury. Bone healing is in some way very

similar to the healing of soft tissues as they both require the cellular regulation of chemotaxis, proliferation, differentiation, extracellular matrix synthesis and formation of new tissue at the site of injury. In contrast to the frequent scar formation during soft tissue healing, bone heals by regeneration of the normal osseous anatomy without formation of scar tissue. The cellular events associated with the repair of bone injury involve, in addition to proliferation of progenitor cells and the influx of inflammatory cells also found in soft tissue wounds, the formation of bone by means of intra-membranous and endochondral ossification. Cells participating in this process include platelets, monocytes, mesenchymal cells, fibroblasts, endothelial cells, chondrocytes, osteoblasts, and osteoclasts (Erlebacher et al., 1995). Among these types of cells, osteoblasts play a critical role in this process, because they are responsible for the synthesis and mineralization of the bone matrix (Rodan and Noda, 1991; Aubin et al., 1992; Gehron Robey et al., 1992; Rodan, 1992).

Limb lengthening procedures (distraction osteo-genesis) consist mainly of an osteotomy of the shortened bone and a subsequent daily distraction of the newly formed callus leading to new bone formation. The clinical importance of the treatment is to fill large bone defects caused by infection, accidental injury, tumor resection, or congenital deformation, and to retain the original bone length. With adequate blood supply, rigid fixation, and proper rate and rhythm of distraction, a large amount of new bone rapidly develops within the distraction gap. This biological phenomenon appears to contradict the dogma that a fracture subjected to stretching forces may become a risk for a nonunion. However, a large number of studies have shown that bone formation activity is markedly increased during distraction osteogenesis (Monticelli et al., 1981; Alho et al., 1982; Paley, 1988; Ilizarov, 1989; Aronson, 1991; Peltonen et al., 1992). This increased activity has been attributed to the "tensioning" stimulatory effect on blood flow and bone forming cells, which results in rapid regeneration, bone formation and consolidation. To further understand this phenomenon, it is of importance, using modern tools of molecular biology, to analyze the

Table 1. Morphogens, as well as their receptors and transducer proteins, are expressed in fracture healing.

	IMMEDIATE INJURY RESPONSE	INTRAMEMBRANOUS OSSIFICATION	CHONDROGENESIS AND ENDOCHONDRAL OSSIFICATION
TGF-ß1	TGF.ß1 protein is extracellularly present[1] in hematoma. TGF-ß1 mRNA is weakly expressed[2] in proliferating mesenchymal cells and endothelial cells of vessels.	TGF-ß1 mRNA is strongly expressed in proliferating osteoblasts.	TGF-ß1 mRNA is strongly expressed in proliferating chondrocytes, not in hypertrophic chondrocytes.
BMP-2/4	BMP-2/4 proteins are strongly present in undifferentiated mesenchymal cells.	BMP-2/4 proteins are strongly present in proliferating osteoblasts.	BMP-2/4 proteins are strongly present in proliferating chondrocytes, weakly in mature and hypertrophic chondrocytes, and strongly in osteoblasts near to endochondral ossification front.
OP-1 (BMP-7)	OP-1 protein is strongly present in undifferentiated mesenchymal cells.	OP-1 protein is strongly present in proliferating osteoblasts.	OP-1 protein is strongly present in proliferating chondrocytes, and weakly in mature chondrocytes.
IGF-1	IGF-1 mRNA is not expressed.	IGF-1 mRNA is expressed in osteoblasts.	IGF-1 is expressed and present in prehypertrophic chondroblasts and osteoblasts.
IGF-II	IGF-II mRNA is expressed in endothelial cells of vessels in granulation tissue.	IGF-II mRNA is expressed in osteoblasts.	IGF-II is expressed and present in prehypertrophic chondrocytes, osteoblasts, and some osteoclasts.
SMAD2, 3, 4	mRNA for Smad2, 3 and 4 is not expressed. Smad2 protein is not present.	Smad2 protein is not present.	mRNA for Smad2, 3 and 4 is up-regulated. Smad2 protein is present in chondroblasts and chondrocytes.
BMPR I/II	BMR-I/II receptors are strongly present on undifferentiated mesenchymal cells.	BMPR-I/II receptors are strongly present on osteoblasts.	BMPR-I/II receptors are strongly present on proliferating chondrocytes, weakly on hypertrophic chondrocytes, and strongly on osteoblasts near to ossification front.

[1]: be present: determined by immunostaining; [2]: be expressed: determined by northern analysis or in situ hybridization.

molecular mechanisms associated with this process.

Bone fracture healing is believed to be regulated by systemic and local factors. Recent studies have demonstrated that growth factors are central regulators of cellular proliferation, differentiation, and extracellular matrix biosynthesis and mineralization during this process. This paper is a brief overview of recent progress from studies which demonstrated the expression of local growth factors associated with cellular events in both *in vitro* and *in vivo* models. Although bone forming cells synthesize and release many growth factors and cytokines such as platelet-derived growth factor (PDGF) and fibroblast growth factors (FGFs), we will focus on transforming growth factor beta (TGF-ß), bone morphogenetic proteins (BMPs) and insulin-like growth factors (IGFs) because there is growing evidence that these growth factors function in the regulation of bone formation and their actions on bone formation have been extensively investigated.

Growth factors are present during fracture healing

The bone fracture healing process has been histologically divided into four distinct stages: (a) hematoma and immediate injury response; (b) primary intramembranous bone formation; (c) chondrogenesis; and (d) endochondral ossification. Each stage is characterized by specific cellular and matrix markers (Bolander, 1992). Cellular events involved in this process include chemotaxis of mesenchymal precursors

and proliferation of committed bone-forming cells occurring in the early stage of differentiation at the beginning of the fracture healing process (Iwaki et al., 1997). Cells subsequently undergo differentiation and synthesize distinct extracellular matrix, leading to intramembranous and endochondral ossification with formation and mineralization of a fracture callus. Finally, most cells are removed from the fracture site via apoptosis (Landry et al., 1997). Cell populations associated with these events mainly arise from mesenchymal precursors present in the bone marrow, including the endosteum, and the periosteum. These precursor cells can undergo osteogenic, chondrogenic, myogenic, tendogenic, ligamentagenic, and adipogenic differentiation forming a variety of mesenchymal tissues, including cartilage and bone (Bruder et al., 1994). In case of a fracture, the precursor cells enter a lineage following their commitment to particular chondrogenic and osteogenic pathways.

The above mentioned cellular events are induced and controlled by specific molecular signals, including TGF-ßs, BMPs and IGFs. Growth factor regulation may function via one or all of three pathways: endocrine, paracrine, and autocrine (Joyce et al., 1991). In addition, specific receptors and cytoplasmic signal transducers, e.g. recently discovered members of the SMAD family, are crucially involved (Heldin et al., 1997; Baker and Harland, 1997). Recent studies demonstrated that these growth factors, as well as their receptors and signal transducer proteins, are present in the fracture callus

TGF-ß

A number of *in vitro* studies have shown that TGF-ß has a variety of effects on osteoblast-like cells, depending on the source of cells, culture conditions and other parameters. Judging from these *in vitro* studies, however, the primary function of TGF-ß appears to be the stimulation of cell proliferation and matrix bio-synthesis (Boyan et al., 1994; Centrella et al., 1994; Harris et al., 1994; Ballock et al., 1997). This is supported by a recent study on the temporal expression pattern of TGF-ß in a fetal rat calvarial-derived osteo-blast model (Owen et al., 1990). In this model, TGF-ß and collagen type I were co-expressed during the proliferation period of osteoblast growth, then gradually down-regulated with collagen mRNA being maintained at a low level during osteoblast maturation. Several lines of evidence indicated that TGF-ß has an inhibitory effect on osteoblast differentiation (Talley Ronsholdt et al., 1995; Cheifetz et al., 1996). On the other hand, TGF-ß1 has been shown to stimulate chondrogenic differentiation in periosteal mesenchymal cell cultures (Izumi et al., 1992; Iwasaki et al., 1993, 1995; Miura et al., 1994) and osteoblast-like cell cultures (Basic et al., 1996). In the *in vivo* studies, both TGF-ß1 and -ß2 have been reported to stimulate bone formation by inducing differentiation of periosteal mesenchymal cells into chondroblasts and osteoblasts (Noda and Camilliere, 1989; Joyce et al., 1990b). Noteworthily, TGF-ßs dose-dependently increased the ratio of cartilage formation to intramembranous bone formation and the endochondral ossification just occurred after discontinuing daily injection of TGF-ßs (Joyce et al., 1990b). Interestingly, TGF-ß1 with a collagenous carrier matrix was reported to promote endochondral bone formation in extraskeletal sites in adult baboons (Ripamonti et al., 1997).

Using Northern analysis, in situ hybridization and immunohistochemistry, it is possible to investigate the expression and presence of individual growth factors within the fracture site during fracture repair. Joyce et al. (1990a) have demonstrated that extracellular TGF-ß1 was immunohistochemically localized in the hematoma as early as 24 hr post fracture and mRNA for TGF-ß1 was up-regulated during chondrogenesis and endo-chondral ossification. Andrew et al. (1993a) have shown that TGF-ß1 mRNA was detected by in situ hybriza-tion in proliferating mesenchymal cells in granulation tissue at the early stage of fracture repair, and in proliferating plump polygonal osteoblasts and small chondrocytes during the matrix formation stage. Consistently, Si et al. (1997) have also reported that TGF-ß1 mRNA was higher in chondrocytes and active differentiated osteoblasts during the stages of chondro-genesis and endochondral ossification in the rabbit mandible fracture healing model. Recently, Lammens et al. (1998) have shown that in a dog tibia fracture healing model, the level of TGF-ß1 protein in callus extracts was increased within the first month, peaked by 5 weeks after fracture, and then gradually decreased down to a basal level by 13 weeks post fracture. Meanwhile, an approximate similar synthesis pattern of collagen was documented in the same model.

TGF-ß receptors have been identified and characterized in osteoblasts (Kells et al., 1992; Takeuchi et al., 1995). Receptors (ActRI, II, IIB) for activin, a TGF-ß superfamily member, have been reported to be expressed by osteoblasts during fracture healing (Shuto et al., 1997). The Smad 2, 3 and 4 are intracellular signal transducer proteins in the TGF-ß/activin signaling transductive pathway (Heldin et al., 1997). Recently, the expression of Smad 2, 3, and 4 was documented in a rat fracture healing model (Matsui et al., 1998). Analysis of the Smad expression indicates that TGF-ß mediated signaling is restricted to a region of the chondroblast proliferation and differentiation during the stages of chondrogenesis and endochondral ossification.

These studies revealed that TGF-ß arrives at the fracture site by the blood stream, most likely released by platelets, and is subsequently synthesized by bone forming cells within the callus throughout the fracture repair process. It may also be speculated that TGF-ß, along with other growth factors such as PDGF and FGFs, plays an important role to unlock cellular proliferation in mesenchymal cells, and together with other factors such as BMPs, induces differentiation of periosteal mesenchymal cells into chondroblasts and osteoblasts at the early stage of fracture repair. During the stages of chondrogenesis and endochondral ossification, TGF-ß stimulates the proliferation and synthetic activity of chondrocytes and osteoblasts, increasing the fracture callus size (Andrew et al., 1993a). However, TGF-ß signaling appears not to be required during the later phases of mineralization and remodeling (Le et al., 1998).

BMPs

The BMPs are a group of related proteins originally isolated and characterized from bovine bone matrix by their ability to ectopically induce endochondral bone formation, and form a unique subfamily within the TGF-ß superfamily. By molecular cloning, six related members of this family were originally identified and are termed BMP-2 through BMP-7 (also called osteogenic protein 1, OP-1). To date, the BMP-family has been continuously expanding (Reddi, 1997). Since it was first described by Urist (1965), the regulatory effects of BMPs on bone and cartilage formation have been extensively investigated in both *in vitro* and *in vivo* studies, using heterotopic/orthotopic bone formation, fracture healing, and embryogenesis. Findings from developmental biology and cell culture studies suggest that BMPs are critically involved in the osteoblastic and chondroblastic differentiation. BMPs were reported to induce cartilage and bone formation when injected

subcutaneously, intramuscularly, or periosteally into adult rats or other species (Reddi, 1997). BMPs are expressed in mouse embryos at sites of cartilage and bone formation (Hogan, 1996). BMPs were also shown to suppress myogenic expression and to induce chondrogenic or osteogenic expression both in multipotent and committed cell lines. Furthermore, the inductive effect of BMPs on osteoblast differentiation is strongly supported by a recent study which demonstrated that BMP-7 induced expression of Osf2/Cbfa1, a transcription factor associated with early osteoblast differentiation, in cells where it is not normally expressed, prior to the expression of any other osteoblast marker genes (Ducy et al., 1997).

The temporal and spatial expression patterns of some BMPs and their receptors, BMP type I and type II receptors (BMPR I and II), have been defined during fracture healing process in several *in vivo* models. Nakase et al. (1994) reported that mRNA for BMP-4 was induced very rapidly in less differentiated osteo-progenitors of the proliferating periosteum near the fracture site in the early stage of mouse fracture repair before new cartilage and bone formation, and decreased to an undetectable level on day 5 after fracture. The BMP-4-positive cells did not express bone Gla protein mRNA, which is a marker of more mature osteoblasts. Recently, Si et al. (1997) have demonstrated that BMP-2 mRNA was increased in undifferentiated mesenchymal cells, differentiating osteoblasts and chondroblasts at the stage of intramembranous bone formation and early chondrogenesis. Consistently, Onishi et al. (1998) have also observed that BMP-2/-4 and OP-1 were dramatically induced in the proliferating periosteum at the early stage after fracture by immunostaining. BMP-2/-4 and OP-1 were detected in various types of chondro-cytes; strongly in fibroblast-like spindle cells and proliferating chondrocytes. In the meantime, BMPR I and II receptors are expressed by various types of cells within the healing callus throughout the fracture repair process (Ishidou et al., 1995). These findings, taken together with the abundance of *in vitro* and *in vivo* data on chondrogenic and osteogenic activities, strongly suggest that BMP signaling is associated with the differentiation of mesenchymal cells into the chondro-blastic and osteoblastic lineage during fracture healing.

IGFs

IGFs have been reported to have a mitogenic activity for cells of the osteoblastic lineage and to stimulate osteoblast differentiated functions such as type I collagen and osteocalcin synthesis *in vitro* (Canalis et al., 1993; Dequeker et al., 1993; Mundy, 1993; Rosen et al., 1994; Mohan et al., 1995). Significant expression levels of IGFs were found in cells of developing peri-osteum, growth plate, and ectopic bone formation (Edwall et al., 1992; Prisell et al., 1993; Sandberg et al., 1993). These observations suggest that IGFs are involved in cartilage and bone formation and may play a

role in fracture healing. In the past few years, several *in vivo* studies have shown that IGFs are expressed in fracture healing callus by immunohistochemistry and in situ hybridization. Bourque et al. (1993) have reported that IGF-I protein was present in young chondroblasts at the edge of the cartilage during the chondrogenesis stage of the fracture healing. Edwall et al. (1992) have shown that the level of IGF-I mRNA in callus peaked on day 8 post fracture. IGF-I immunostaining was found in cartilaginous cells, osteoblasts, and myocytes in the fracture site, respectively 6 and 8 days after fracture. Andrew et al. (1993b) also reported that mRNA expression for both IGF-I and II was detected in osteo-blasts and non-hypertrophic chondrocytes at the stage of matrix formation and remodeling. Recently, Lammens et al. (1998) have demonstrated that IGF-I protein in callus extracts was increased at 13 weeks after fracture compared to normal bone. Interestingly, IGFs have been reported to increase osteoclast formation from mouse osteoclast precursors (Mochizuki et al., 1992; Hill et al., 1995). Andrew et al. (1993b) have also observed that IGF-II mRNA was detected in some osteoclasts in a fracture healing model. IGF-II- expressing osteoclasts were adjacent to osteoblasts that also expressed IGF-II, whereas most other osteoblasts at the stage of bone remodeling were negative for IGF-II. These findings suggest that IGFs are not only involved in proliferation and differentiation of osteoblastic and chondroblastic lineages, contributing to bone formation, but may also modulate osteoclast function, leading to bone remodeling during fracture repair.

Growth factors are present during distraction osteogenesis

Distraction osteogenesis has to be considered as a particular form of fracture healing, as it differs from callus formation or osteonal (primary) bone healing. After a percutaneous osteotomy, the newly forming bone is subjected to a mechanical longitudinal strain, allowing for the formation of large amounts of callus. This callus appears to result mainly from intramembranous ossification (Lavrishcheva and Mikhailova, 1985; Aronsen and Harrison, 1987; Jazrawi et al., 1998; Delloye et al., 1990). Distraction osteogenesis can be dynamically divided into three stages: a) latent stage; b) distraction stage; and c) consolidation stage (Fig. 1). In the latent stage (normally one week after osteotomy), similar to fracture healing, the hematoma is replaced by granulation tissue and intramembranous bone formation occurs flanking the granulation tissue under the periosteum. During the distraction stage, with gradual and continuous distraction, the maturation of newly forming bone in the distraction gap appears different, some areas are more calcified while the newly regenerated tissue elsewhere is less dense with more cellular and vascular components (Costantino et al., 1990). Radiological examination has also shown that there are high and low radiodensity zones in the
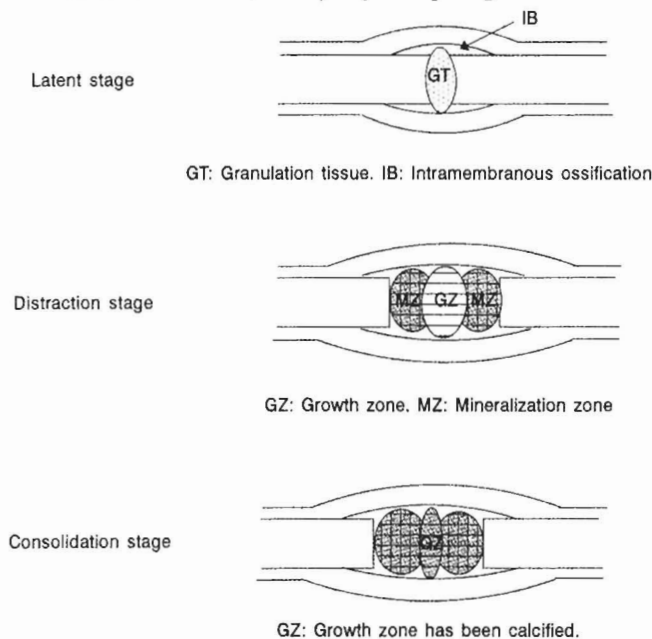
Table 2. Expression of morphogens, collagenous and non-collagenous proteins in distraction osteogenesis.

	LATENT	DISTRACTION	CONSOLIDATION
TGF-ß1	▲	▲▲	▲▲
BMP-2/4	▲	▲	▲▲
IGF-1	ND	NC	▲
Osteocalcin	ND	▼	▼
Collagen	ND	▲▲	▲▲

▲: inceased compared to normal bone; ▲▲: dramatically increased compared to normal bone; ▼: decreased compared to normal bone; ND: not determined; NC: no change compared to normal bone.

distraction gap. In combination with radiographic data, the distracted callus can be histologically divided into the growth zone and the mineralization zone. The growth zone is composed predominantly of longitudinal fibres and fibroblast-like cells (Kojimoto et al., 1988; Yasui et al., 1997). The majority of proliferating cells are present in the edge of the growth zone during distraction (Kojimoto et al., 1988; Aronson et al., 1997; Li et al., 1997). In the consolidation stage, growth zone is calcified, and in the meantime, newly formed bone is gradually remodeled into lamellar bone.

The mineralization was reported to begin from both sides adjacent to the original cortices in the callus during the distraction period (Delloye et al., 1990; Lammens et al., 1997). In the past several years, numerous *in vitro* studies have shown that proliferation and differentiation of bone forming cells (osteoblasts and chondrocytes) are subjected to regulation by mechanical strain (Meikle et al., 1979; Somjen et al., 1980; Hasegawa et al., 1985; Buckley et al., 1988; Burger et al., 1992; Harter et al., 1995; Stanford et al., 1995). By compiling these data and

IB

Latent stage

GT

GT: Granulation tissue. IB: Intramembranous ossification

Distraction stage

MZ GZ MZ

GZ: Growth zone. MZ: Mineralization zone

Consolidation stage

GZ

GZ: Growth zone has been calcified.

Fig. 1. Models for distraction osteogenesis.

correlating the magnitudes of strain, it appears that at high levels of strain, cells increase their proliferative activity. At lower levels of strain, the response of cells indicates a more differentiated state. As the magnitude of strain is heterogeneous across the distraction gap, osteoblasts that experience the greatest strain increase their proliferative activity and, on the other hand, osteoblasts that experience less strain respond with differentiation, which results in mineralization.

Although increasing evidence indicates that mechanical strain induces a large amount of newly forming bone in the distraction gap, there is no significant difference in the bone formation rate between distraction osteogenesis and fracture healing (Welch et al., 1998). In distraction osteogenesis, therefore, bone forms just as rapidly as it does during fracture repair, but as long as mechanical strain is applied. Thus, a number of questions concerning distraction osteogenesis have arisen: a) why can mechanical strain stimulate and maintain the process of osseous regeneration? and b) does mechanical strain induce any specific growth factor that is involved in the distraction cellular events? Over the past few years, it has been shown by *in vitro* and *in vivo* studies that mechanical strain induces the expression of some growth factors such as TGF-ßs, BMPs and IGF-I, which showed different expession patterns when compared to normal bone fracture repair (Table 2). Holbein et al. (1995) reported that cyclic stretching significantly increased the production of active TGF-ß and cellular proliferation in primary human osteoblast culture. The level of TGF-ß was increased in the sera from patients who had distraction between the third and fourth weeks during distraction. Lammens et al. (1998) have recently demonstrated that, in a canine tibia distraction model, the level of TGF-ß1 protein was increased in distracted callus as long as continuous traction was applied, whereas TGF-ß1 in osteotomized callus was first increased and gradually decreased to a basal level afterwards. Consistently, levels of TGF-ß1 in sera from dogs with a tibia distraction were increased immediately after application of distraction and then maintained at a high level during the whole experimental period. In addition, a constantly increased level of collagen synthesis and low concentration of calcium were observed in distracted callus during the distraction period. Recently, Tay et al. (1998) reported that mRNA for TGF-ß2 was detected by in situ hybridization in the growth zone of the distracted callus during the distraction period. These findings suggest that the mechanical strain stimulates and maintains callus formation by inducing TGF-ß production, possibly preventing differentiation and delaying mineralization.

Osteocalcin is a phenotypic marker of osteoblasts and is expressed during the mineralization stage of osteoblasts. The relationship between proliferation and phenotypic expression has been well characterized in the calvarial osteoblast differentiation model (Lian et al., 1991). In that model, subconfluent calvarial-derived

cells demonstrated a specific temporal sequence of initial proliferation and collagen synthesis associated with low expression of osteocalcin, followed later by an increase of alkaline phosphatase activity and osteocalcin gene expression when proliferation was decreased. The inverse relationship between proliferation and differentiation of osteoblasts has been observed in the cultures of osteoblast-like cells with strain (Stanford et al., 1995), and it has been demonstrated that proliferation was increased and the expression of osteocalcin was depressed in response to strain. Lammens et al. (1998) have also observed the inverse relationship in a canine tibia distraction model, which showed that TGF-ß1 was increased after distraction and then maintained at a high level together with low expression of osteocalcin compared with normal bone during the experimental period. Less mineralization (low concentration of calcium and low ash content) was associated with a suppressed expression of osteocalcin. These observations suggest that, under the stimulation by mechanical strain, increased TGF-ß levels stimulate proliferation of osteoprogenitors and biosynthesis of extracellular matrix. On the other hand, TGF-ß suppresses expression of osteocalcin by delaying differentiation of osteoblasts into the mineralization stage. Thus, a progressively increasing number of osteoprogenitors that are accumulated at the proliferation stage increases extracellular matrix synthesis and soft callus formation to fill the gap enlarged by continuous distraction.

Recently, the expression of BMP-2 and -4 has been demonstrated in distraction osteogenesis (Cho et al., 1998). Both BMP-2 and -4 showed delayed peak expression in distraction osteogenesis compared to fracture repair. BMP-2 expression was remarkably up-regulated during the consolidation phase. These findings strongly imply that BMP signals are closely associated with mineralization and bone remodeling of distracted callus.

The expression of IGF-I has also been documented in the distraction osteogenesis process. Schumacher et al. (1996) have shown that periosteal IGF-I was increased during bone distraction and returned to a basal level after bone distraction in a rabbit tibia distraction model. Lammens et al. (1998) have reported that IGF-I was increased in serum during the distraction period, and was followed by an increased skeletal IGF-I both in distracted callus and normal bone after distraction. These findings suggest that the mechanical strain also induces IGF-I production in cells of distracted periosteum, which may lead to bone formation. A late increase in IGF-I in distracted callus may be induced by increased BMP-2 levels (Canalis and Gabbitas, 1994) and is probably associated with bone remodeling, since secondary bone remodeling was reported to be significantly increased in distraction osteogenesis (Welch et al., 1998). In addition, other stretched tissues, including muscle, skin, vessels, and nerves, might contribute to an increased systemic IGF-I concentration, which results in an increased storage of IGF-I in the bone. However, the correlation

between mechanical stimulation and IGFs expression in distraction osteogenesis remains at present unkown due to the limited data.

A hypothesis and reminiscence

Findings from histological examination suggest that mechanical strain stimulates and maintains the process of osseous regeneration most probably by maintaining the proliferative state of osteoprogenitors in the growth zone. Thus, the biological function of the growth zone in distraction osteogenesis appears in some way very similar to the "progress zone" (PZ) in vertebrate limb development. Indeed, the maintenance of the PZ is critical for outgrowth of limb bud (Johnson and Tabin, 1997). FGFs, as well as other related molecular signals such as WNTs, have been demonstrated to play important roles in this context (Johnson and Tabin, 1997; Kengaku et al., 1998). Moreover, chondrogenesis was reported to be inhibited by FGF-2 (Kato and Iwamoto, 1990), Wnt-1 (Rudnicki and Brown, 1997), or mechanical strain (Jazrawi et al., 1998). These observations may provide a clue that these molecular signals might be additional players in distraction osteogenesis. Recently, Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) have been shown to be involved in endochondral bone formation in both embryonic development and fracture healing (Vortkamp et al., 1998). These data further corroborate the hypothesis that bone formation in these processes, including distraction osteogenesis, is most likely controlled by similar molecular mechanisms.

Conclusion

Fracture healing is a complex tissue regeneration and repair process which involves chemotaxis of mesenchymal cells, proliferation and differentiation of committed bone-forming cells, extracellular matrix synthesis and callus formation, maturation and remodeling. Current data suggest that these cellular events are precisely controlled and regulated by specific morphogens, while such molecular signals are also implicated in bone formation during embryonic development. The secreted signals exert their functions via a cascade of molecular events which consist of biosynthesis, secretion, activation, transportation, signal transduction and gene expression. Indeed, these molecular events are believed to be influenced by many factors, including blood supply, mechanical stimuli, extracellular and intracellular microenvironment, etc.

Recent studies have demonstrated that a number of morphogens are involved in fracture repair, including TGF-ß, BMPs and IGFs. Additional data are required to identify the specific functions of these morphogens in the bone healing process. TGF-ß most probably functions as a regulator to stimulate proliferation of bone forming cells and extracellular matrix synthesis. BMPs may stimulate differentiation of mesenchymal cells into

osteoblastic and chondroblastic lineage, leading to intramembranous bone formation and endochondral ossification in fracture callus. IGFs seem not only to regulate proliferation and differentiation of bone forming cells, contributing to bone formation, but also to regulate osteoclast function, participating in bone remodeling. The mechanical strain can stimulate and maintain the process of osseous regeneration by inducing specific morphogen expression, synthesis and processing.

References

Alho A., Bang G., Karaharju E. and Armond I. (1982). Filling of a bone defect during experimental osteotaxis distraction. Acta Orthop. Scand. 53, 29-34.

Andrew J.G., Hoyland J., Andrew S.M., Freemont A.J. and Marsh D. (1993a). Demonstration of TGF-beta 1 mRNA by in situ hybridization in normal human fracture healing. Calcif. Tissue Int. 52, 74-78.

Andrew J.G., Hoyland J., Freemont A.J. and Marsh D. (1993b). Insulin like growth factor gene expression in human fracture callus. Calcif. Tissue Int. 53, 97-102.

Aronsen J. and Harrison B. (1987). Mechanical induction of osteogenesis by distraction of a metaphyseal osteotomy in long bones. Orthop. Trans. 12, 180-187.

Aronson J. (1991). The biology of distraction osteogenesis. In operative principles of Ilizarov. Bianchi Maiocchi A. and Aronsen J. (eds). Williams and Wilkins. Baltimore MD, USA. pp 42-52.

Aronson J., Shen X.C., Gao G.G., Miller F., Quattlebaum T., Skinner R.A., Badger T.M. and Lumpkin C.K. Jr. (1997). Sustained proliferation accompanies distraction osteogenesis in the rat. J. Orthop. Res. 15, 563-569.

Aubin J.E., Turksen K. and Heersche J.M.M. (1992). Osteoblastic cell lineage. In: Cellular and molecular biology of bone. Noda M. (ed). Academic Press. San Diego, CA, USA. pp 1-46.

Baker J.C. and Harland R.M. (1997). From receptor to nucleus: the Smad pathway. Curr. Opin. Genet. Dev. 7, 467-473.

Ballock R.T., Heydemann A., Izumi T. and Reddi A.H. (1997). Regulation of the expression of the type-II collagen gene in periosteum-derived cells by three members of the transforming growth factor-beta superfamily. J. Orthop. Res. 15, 463-467.

Basic N., Basic V., Bulic K., Grgic M., Kleinman H.K., Luyten F.P. and Vukicevic S. (1996). TGF-beta and basement membrane matrigel stimulate the chondrogenic phenotype in osteoblastic cells derived from fetal rat calvaria. J. Bone Miner. Res. 11, 384-391.

Bolander M.E. (1992). Regulation of fracture repair by growth factors. Proc. Soc. Exp. Biol. Med. 200, 165-170.

Bourque W.T., Gross M. and Hall B.K. (1993). Expression of four growth factors during fracture repair. Int. J. Dev. Biol. 37, 573-579.

Boyan B.D., Schwartz Z., Park Snyder S., Dean D.D., Yang F., Twardzik D. and Bonewald L.F. (1994). Latent transforming growth factor-beta is produced by chondrocytes and activated by extracellular matrix vesicles upon exposure to 1,25-(OH)2D3. J. Biol. Chem. 269, 28374-28381.

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, 283-294.

Buckley M.J., Banes A.J., Levin L.G., Sumpio B.E. Sato M., Jordan R., Gilbert J., Link G.W. and Tran Son Tay R. (1988). Osteoblasts increase their rate of division and align in response to cyclic,

mechanical tension in vitro. Bone Miner. 4, 225-236.

Burger E.H., Klein Nulend J. and Veldhuijzen J.P. (1992). Mechanical stress and osteogenesis in vitro. J. Bone Miner. Res. 7 (Suppl. 2), S397-401.

Canalis E., Pash J. and Varghese S. (1993). Skeletal growth factors. Crit. Rev. Eukaryot. Gene Expr. 3, 155-166.

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, 1999-2005.

Centrella M., Horowitz M.C., Wozney J.M. and McCarthy T.L. (1994). Transforming growth factor-beta gene family members and bone. Endocr. Rev. 15, 27-39.

Cheifetz S., Li I.W., McCulloch C.A., Sampath K. and Sodek J. (1996). Influence of osteogenic protein-1 (OP-1; BMP-7) and transforming growth factor-beta 1 on bone formation in vitro. Conn. Tissue Res. 35, 71-78.

Cho T.J., Choi I.H. and Chung C.Y. (1998). BMP-2 and BMP-4 mRNA expression during distraction osteogenesis in rat tibia. 44th Annual Meeting for Orthopaedic Research Society of USA, Transactions 23, 891 (Abstract)

Costantino P.D., Shybut G., Friedman C.D., Pelzer H.J., Masini M., Shindo M.L. and Sisson G.A. Sr. (1990). Segmental mandibular regeneration by distraction osteogenesis. An experimental study. Arch. Otolaryngol. Head. Neck Surg. 116, 535-545.

Delloye C., Delefortrie G., Coutelier L. and Vincent A. (1990). Bone regenerate formation in cortical bone during distraction lengthening. An experimental study. Clin. Orthop. 250, 34-42.

Dequeker J., Mohan S., Finkelman R.D., Aerssens J. and Baylink D.J. (1993). Generalized osteoarthritis associated with increased insulin-like growth factor types I and II and transforming growth factor beta in cortical bone from the iliac crest. Possible mechanism of increased bone density and protection against osteoporosis. Arthritis Rheum. 36, 1702-1708.

Ducy P., Zhang R., Geoffroy V., Ridall A.L. and Karsenty G. (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89, 747-754.

Edwall D., Prisell P.T., Levinovitz A., Jennische E. and Norstedt G. (1992). Expression of insulin-like growth factor I messenger ribonucleic acid in regenerating bone after fracture: influence of indomethacin. J. Bone Miner. Res. 7, 207-213.

Erlebacher A., Filvaroff E.H., Gitelman S.E. and Derynck R. (1995). Toward a molecular understanding of skeletal development. Cell 80, 371-378.

Gehron-Robey P., Bianco P. and Termine J.D. (1992). The cellular biology and molecular biochemistry of bone formation. In: disorders of bone and mineral metabolism. Coe F.L. and Flavus M.J. (eds). Raven Press. New York, NY, USA. pp 241-269.

Harris S.E., Bonewald L.F., Harris M.A., Sabatini M., Dallas S., Feng J.Q., Ghosh Choudhury N., Wozney J. and Mundy G.R. (1994). Effects of transforming growth factor beta on bone nodule formation and expression of bone morphogenetic protein 2, osteocalcin, osteopontin, alkaline phosphatase, and type I collagen mRNA in long-term cultures of fetal rat calvarial osteoblasts. J. Bone Miner. Res. 9, 855-863.

Harter L.V., Hruska K.A., and Duncan R.L. (1995). Human osteoblast-like cells respond to mechanical strain with increased bone matrix protein production independent of hormonal regulation. Endocrinology 136, 528-535.

Hasegawa S., Sato S., Saito S., Suzuki Y. and Brunette D.M. (1985). Mechanical stretching increases the number of cultured bone cells synthesizing DNA and alters their pattern of protein synthesis. Calcif. Tissue Int. 37, 431-436.

Heldin C.H., Miyazono K. and ten Dijke P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 390, 465-471.

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, 124-131.

Hogan B.L. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev. 10, 1580-1594.

Holbein O., Neidlinger Wilke C., Suger G., Kinzl L. and Claes L. (1995). Ilizarov callus distraction produces systemic bone cell mitogens. J. Orthop. Res. 13, 629-638.

Ilizarov G.A. (1989). The tension-stress effect on the genesis and growth of tissues. Part I. The influence of stability of fixation and soft-tissue preservation. Clin. Orthop. 238, 249-281.

Ishidou Y., Kitajima I., Obama H., Maruyama I., Murata F., Imamura T., Yamada N., ten Dijke P., Miyazono K. and Sakou T. (1995). Enhanced expression of type I receptors for bone morphogenetic proteins during bone formation. J. Bone Miner. Res. 10, 1651-1659.

Iwaki A., Jingushi S., Oda Y., Izumi T., Shida J.I., Tsuneyoshi M. and Sugioka Y. (1997). Localization and quantification of proliferating cells during rat fracture repair: detection of proliferating cell nuclear antigen by immunohistochemistry. J. Bone Miner. Res. 12, 96-102.

Iwasaki M., Nakata K., Nakahara H., Nakase T., Kimura T., Kimata K., Caplan A.I. and Ono K. (1993). Transforming growth factor-beta 1 stimulates chondrogenesis and inhibits osteogenesis in high density culture of periosteum-derived cells. Endocrinology 132, 1603-1608.

Iwasaki M., Nakahara H., Nakata K., Nakase T., Kimura T. and Ono K. (1995). Regulation of proliferation and osteochondrogenic differentiation of periosteum-derived cells by transforming growth factor-beta and basic fibroblast growth factor. J. Bone Joint Surg. Am. 77, 543-554.

Izumi T., Scully S.P., Heydemann A. and Bolander M.E. (1992). Transforming growth factor beta 1 stimulates type II collagen expression in cultured periosteum-derived cells. J. Bone Miner. Res. 7, 115-121.

Jazrawi L.M. Majeska R.J., Klein M.L., Kagel E., Stromberg L. and Einhorn T.A. (1998). Bone and cartilage formation in an experimental model of distraction osteogenesis. J. Orthop. Trauma. 12, 111-116.

Johnson R.L. and Tabin C.J. (1997). Molecular models for vertebrate limb development. Cell 90, 979-990.

Joyce M.E., Jingushi S. and Bolander M.E. (1990a). Transforming growth factor-beta in the regulation of fracture repair. Orthop. Clin. North Am. 21, 199-209.

Joyce M.E., Roberts A.B., Sporn M.B. and Bolander M.E. (1990b). Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. J. Cell Biol. 110, 2195-2207.

Joyce M.E., Jingushi S., Scully S.P. and Bolander M.E. (1991). Role of growth factors in fracture healing. In: Clinical and experimental approaches to dermal and epidermal repair: normal and chronic wounds. Wiley Liss Inc. New York, NY, USA. pp 391-416.

Kato Y. and Iwamoto M. (1990). Fibroblast growth factor is an inhibitor of chondrocyte terminal differentiation. J. Biol. Chem. 265, 5903-5909.

Kells A.F., Schwartz H.S., Bascom C.C. and Hoover R.L. (1992).

Identification and analysis of transforming growth factor beta receptors on primary osteoblast-enriched cultures derived from adult human bone. Connect. Tissue Res. 27, 197-209.

Kengaku M., Capdevila J., Rodriguez-Esteban C., De La Pena J., Johnson R., Belmonte J. and Tabin C. (1998). Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. Science 280, 1274-1277.

Kojimoto H., Yasui N., Goto T., Matsuda S. and Shimomura Y. (1988). Bone lengthening in rabbits by callus distraction. The role of periosteum and endosteum. J. Bone Joint Surg. Br. 70, 543-549.

Lammens J., Aerssens J., Nijs J., Mokassa L., Dequeker J., Bouillon R. and Fabry G. (1997). Biochemical and density assessment of the new bone in late remodeling after callus distraction. J. Orthop. Res. 15, 391-397.

Lammens J., Liu Z., Aerssens J., Dequeker J. and Fabry G. (1998). Distraction bone healing versus osteotomy healing: a comparative biochemical analysis. J. Bone Miner. Res. 13, 279-286.

Landry P., Sadasivan K., Marino A. and Albright J. (1997). Apoptosis is coordinately regulated with osteoblast formation during bone healing. Tissue Cell 29, 413-419.

Lavrishcheva G.I. and Mikhailova L.N. (1985). Histogenesis of skeletogenic tissue in bone regeneration during distraction. Bull. Eksp. Biol. Med. 99, 198-201.

Le A.X., Iwasaki M., Filvaroff E.H., Heimann M.R., Lotz J.C., Derynck R.M. and Helms J.A. (1998). Interrupture of TGF-ß signaling in osteoblasts does not impair frarture healing. 44th Annual Meeting for Orthopaedic Research Society of USA. Transactions 23, 231 (Abstract)

Li G., Simpson A.H., Kenwright J. and Triffitt J.T. (1997). Assessment of cell proliferation in regenerating bone during distraction osteogenesis at different distraction rates. J. Orthop. Res. 15, 765-772.

Lian J.B., Stein G.S., Bortell R. and Owen T.A. (1991). Phenotype suppression: a postulated molecular mechanism for mediating the relationship of proliferation and differentiation by Fos/Jun interactions at AP-1 sites in steroid responsive promoter elements of tissue-specific genes. J. Cell Biochem. 45, 9-14.

Matsui N., Shuto N., Sarkar G., Bronk J., Mizuno K. and Bolander M.E. (1998). Expression of Smad2, 3 and 4 in fracture callus and growth plate during chondrogenesis. 44th Annual Meeting for Orthopaedic Research Society of USA. Transactions 23, 56 (Abstract)

Meikle M.C., Reynolds J.J., Sellers A. and Dingle J.T. (1979). Rabbit cranial sutures in vitro: a new experimental model for studying the response of fibrous joints to mechanical stress. Calcif. Tissue Int. 28, 137-144.

Miura Y., Fitzsimmons J.S., Commisso C.N., Gallay S.H. and O'Driscoll S.W. (1994). Enhancement of periosteal chondrogenesis in vitro. Dose-response for transforming growth factor-beta 1 (TGF-beta 1). Clin. Orthop. 271-280.

Mochizuki H., Hakeda Y., Wakatsuki N., Usui N., Akashi S., Sato T., Tanaka K., and Kumegawa M. (1992). Insulin-like growth factor-I supports formation and activation of osteoclasts. Endocrinology 131, 1075-1080.

Mohan S., Nakao Y., Honda Y., Landale E., Leser U., Dony C., Lang K. and Baylink D.J. (1995). Studies on the mechanisms by which insulin-like growth factor (IGF) binding protein-4 (IGFBP-4) and IGFBP-5 modulate IGF actions in bone cells. J. Biol. Chem. 270 20424-20431.

Monticelli G., Spinelli R. and Ronucci E. (1981). Distraction

epiphysiolysis as a method of limb lengthening. Clin. Orthop. 154, 262-273.

Mundy G.R. (1993). Factors which stimulate bone growth *in vivo*. Growth Regul. 3, 124-128.

Nakase T., Nomura S., Yoshikawa H., Hashimoto J., Hirota S., Kitamura Y., Oikawa S., Ono K. and Takaoka K. (1994). Transient and localized expression of bone morphogenetic protein 4 messenger RNA during fracture healing. J. Bone Miner. Res. 9, 651-659.

Noda M. and Camilliere J.J. (1989). *in vivo* stimulation of bone formation by transforming growth factor-beta. Endocrinology 124, 2991-2994.

Onishi T., Ishidou Y., Nagamine T., Yonemori K. Yone K., Murata F., Imamura T., Kato M., Sampath K., Dijke P., Miyazono K. and Sakou T. (1998). Distinct and overlapping patterns of localization of BMP family members and a BMP type II receptor during fracture healing in rats. Bone 22, 605-612.

Owen T.A., Aronow M., Shalhoub V., Barone L.M., Wilming L., Tassinari M.S., Kennedy M.B., Pockwinse S., Lian J.B. and Stein G.S. (1990). Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J. Cell Physiol. 143, 420-430.

Paley D. (1988). Current techniques of limb lengthening. J. Pediatr. Orthop. 8, 73-92.

Peltonen J., Kahri A., Lindberg L.A., Heikkila P.S., Karaharju E.O., and Aalto K.A. (1992). Bone formation after distraction osteotomy of the radius in sheep. Acta. Orthop. Scand. 63, 599-603.

Prisell P.T., Edwall D., Lindblad J.B., Levinovitz A. and Norstedt G. (1993). Expression of insulin-like growth factors during bone induction in rat. Calcif. Tissue Int. 53, 201-205.

Reddi A.H. (1997). Bone morphogenetic proteins: an unconventional approach to isolation of first mammalian morphogens. Cytokine. Growth Factor. Rev. 8, 11-20.

Ripamonti U., Duneas N., Van Den Heever B., Bosch C. and Crooks J. (1997). Recombinant transforming growth factor-beta1 induces endochondral bone in the baboon and synergizes with recombinant osteogenic protein-1 (bone morphogenetic protein-7) to initiate rapid bone formation. J. Bone Miner. Res. 12, 1584-1595.

Rodan G.A. (1992). Introduction to bone biology. Bone 13 (Suppl. 1), S3-6.

Rodan G.A. and Noda M. (1991). Gene expression in osteoblastic cells. Crit. Rev. Eukaryot. Gene Expr. 1, 85-98.

Rosen C.J., Donahue L.R. and Hunter S.J. (1994). Insulin-like growth factors and bone: the osteoporosis connection. Proc. Soc. Exp. Biol. Med. 206, 83-102.

Rudnicki J.A. and Brown A.M. (1997). Inhibition of chondrogenesis by Wnt gene expression *in vivo* and in vitro. Dev. Biol. 185, 104-118.

Sandberg M.M., Aro H.T. and Vuorio E.I. (1993). Gene expression during bone repair. Clin. Orthop. 289, 292-312.

Schumacher B., Albrechtsen J., Keller J., Flyvbjerg A. and Hvid I. (1996). Periosteal insulin-like growth factor I and bone formation. Changes during tibial lengthening in rabbits. Acta Orthop. Scand. 67, 237-241.

Shuto T., Sarkar G., Bronk J.T., Matsui N. and Bolander M.E. (1997). Osteoblasts express types I and II activin receptors during early intramembranous and endochondral bone formation. J. Bone Miner. Res. 12, 403-411.

Si X., Jin Y., Yang L., Tipoe G.L. and White F.H. (1997). Expression of BMP-2 and TGF-beta 1 mRNA during healing of the rabbit mandible. Eur. J. Oral Sci. 105, 325-330.

Somjen D., Binderman I., Berger E. and Harell A. (1980). Bone remodelling induced by physical stress is prostaglandin E2 mediated. Biochim. Biophys. Acta 627, 91-100.

Stanford C.M., Morcuende J.A. and Brand R.A. (1995). Proliferative and phenotypic responses of bone-like cells to mechanical deformation. J. Orthop. Res. 13, 664-670.

Takeuchi Y., Fukumoto S. and Matsumoto T. (1995). Relationship between actions of transforming growth factor (TGF)-beta and cell surface expression of its receptors in clonal osteoblastic cells. J. Cell Physiol. 162, 315-321.

Talley Ronsholdt D.J., 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.

Tay B.K., Le A.X., Gould S.E. and Helms J.A. (1998). Molecular and biochemical characterization of distraction osteogenesis: a mouse model. 44th Annual Meeting for Orthopaedic Research society of USA. Transactions 23, 601(Abstract)

Urist M.R. (1965). Bone: formation by autoinduction. Science 150, 893-899.

Vortkamp A., Pathi S., Peretti G.M., Caruso E.M., Zaleske D.J. and Tabin C.J. (1998). Recapitulation of signals regulating embryonic bone formation during postnatal growth and in fracture repair. Mech. Dev. 71, 65-76.

Welch R.D., Birch J.G., Makarov M.R. and Samchukov M.L. (1998). Histomorphometry of distraction osteogenesis in a caprine tibia lengthening model. J. Bone Miner. Res. 13, 1-9.

Yasui N., Sato M., Ochi T., Kimura T., Kawahata H., Kitamura Y. and Nomura S. (1997). Three modes of ossification during distraction osteogenesis in the rat. J. Bone Joint Surg. Br. 79, 824-830.