Synergistic effect of IGF-I and TGF-ß1 on fracture healing in rats

Single versus combined application of IGF-I and TGF-ß1

Gerhard Schmidmaier, Britt Wildemann, Tobias Gäbelein, Joanna Heeger, Frank Kandziora, Norbert P Haas and Michael Raschke

Department of Trauma and Reconstructive Surgery, Charité, Humboldt-University of Berlin, Germany
Correspondence: gerhard.schmidmaier@charite.de
Submitted 02-04-10. Accepted 02-11-16

ABSTRACT **During the last few decades, knowledge about growth factors and their function has increased. However, little is known about the interaction of these factors during bone growth and fracture healing. In vitro studies have shown a higher rate of cell proliferation and cell metabolism after the use of IGF-I and TGF-ß1 in combination, as compared to the single use of these factors. The purpose of this study was to investigate a possible synergistic effect of these growth factors in vivo, using a fracture model.**

A midshaft fracture of rat tibia (n = 84) was intramedullary stabilized with poly(D,L-lactide)-coated or uncoated titanium K-wires. The growth factors IGF-I and TGF-ß1, singly or in combination, were incorporated in the coating and continuously released during fracture healing. 28 days after fracture, we performed mechanical tests and histomorphological analyses.

We found a greater stimulating effect of IGF-I on fracture healing than of TGF-ß1. The combined application of both growth factors resulted in a significantly higher maximum load and torsional stiffness than the use of only one of them. The histomorphometric analyses showed an increase in remodeling of the fracture callus in this group with less cartilaginous and more mineralized tissue than in the other groups. Both growth factors seem to have a synergistic effect on fracture healing in this model.

Increasing evidence indicates that growth factors, such as insulin-like growth factor (IGF) or transforming growth factor-beta (TGF-ß), are important regulators of cellular proliferation, differentiation, extracellular matrix synthesis and mineralization (Mohan and Baylink 1991, Linkhart et al. 1996, Trippel et al. 1996, Lind 1998). TGF-ß1 is expressed at high levels in osteoblasts during bone development (Kim and Ballock 1993) and healing (Joyce et al. 1990a, Steinbrech et al. 2000, Tatsuyama et al. 2000). It affects cell proliferation and phenotypic gene expression in vitro (Hock et al. 1990, Centrella et al. 1991) and stimulates bone healing in vivo (Gombotz et al. 1994, Fujimoto et al. 1999). IGF-I stimulates the replication of osteoblasts and the synthesis of bone matrix in vitro (Canalis 1980, Hock et al. 1988) and healing of bone defects in vivo (Thaller et al. 1993a).

The use of these factors individually or in combination stimulates cell proliferation and cell metabolism. A synergistic effect of IGF-I and TGF-ß1 on cell proliferation, matrix synthesis and differentiation was seen in chondrocyte cell culture (Tsukazaki et al. 1994, Matsumura et al. 2000). Similar results have been reported concerning the effects on proliferation and differentiation of osteoblasts (Kasperk et al. 1990).

We studied the effects of the growth factors IGF-I and TGF-ß1 on fracture healing when applied locally alone or together. The tibiae of rats were fractured in a standardized manner and the progress of healing was evaluated radigraphically. After 28 days, mechanical torsional testing and histomorphometric analyses were done.

Animals and methods

Animals and fracture model

A closed midshaft fracture of the right tibia of 5-month-old female Sprague-Dawley rats (Harlan-Winkelmann, Borchen, Germany) (n = 84) was produced, using a special fracture device, after sedating the animals with isoflurane and intraperitoneal anesthesia with a mixture of ketaminehydrochloride (100 mg/mL) (80 mg/kg body weight) and xylazine 2% (12 mg/kg body weight), as described elsewhere (Schmidmaier et al. 2001a). After closed reposition, the tibiae were intramedullary stabilized with coated or uncoated titanium Kirschner-wires (1.0 mm diameter, 3.0 cm length). All experiments were approved by the Ethics Committee on Animal Experiments.

Local application of growth factors

The growth factors, recombinant human IGF-I (R&D System, Wiesbaden, Germany) (5% w/w) and recombinant human TGF-ß1 (R&D Systems, Wiesbaden, Germany) (1% w/w), were locally applied using poly(D,L-lactide) (Boehringer, Ingelheim, Germany) coated titanium Kirschner-wires as the drug carrier. The properties of the PDLLA coating and the release characteristics of the incorporated growth factors have been described elsewhere (Schmidmaier et al. 2001b). Briefly, about 50% of these incorporated growth factors were released within the first 48 hours followed by a sustained release of a further 30% during the next 40 days. The 10-μm thin coating had a high mechanical stability on the metallic implant.

Following groups were studied: group I – uncoated implants; group II – implants coated with TGF-ß1 (1% w/w, 10 μg); group III – implants coated with IGF-I (5% w/w, 50 μg; group IV – implants coated with IGF-I (5% w/w, 50 μg) and TGF-ß1 (1% w/w, 10 μg).

Radiographic evaluation

Radiographs were taken throughout the observation period of 28 days in posteroanterior and lateral views. We used Microvision C Mammography films (Sterling Diagnostic, Newark, USA) and a Mobilett Plus X-ray unit (Siemens AG, Erlangen, Germany). The fracture callus on day 28 was described by two independent observers, using the following parameters: A) complete bridging (4 cortices are bridged), B) unilateral bridging (1–3 cortices are bridged), and C) no bridging (no cortex is bridged).

Body weight and body temperature

Rectal body temperature was measured and body weight determined with a precision scale during the experiment. Other signs of local or systemic infections were evaluated.

Mechanical testing

Randomly, 10 animals in each group were killed 28 days after the fracture and both tibiae were dissected free from soft tissue for torsional testing. The unfractured side served as the control and the data were expressed as a percentage of the unfractured tibia. After dissecting the bones and removing the K-wire, the proximal and distal ends were embedded into two moulds with bone cement (Beracryl, Troller, Fullenbach, Switzerland). Each mould was connected to a pivoted axis. A linear constant feet rate, derived from a material testing machine (Zwick 1455, Ulm, Germany), was loaded by a lever attached to one of the pivoted axes. The bone was preloaded by an axial force of 5 N and a constant linear propulsion (v = 2 mm/min) was applied by the testing machine. The translation of the material testing machine was transformed into a uniform torsional movement. The free axis was connected with a strain-gauge (F_{max} = 50 N, HBM-Germany) that determined the torsional force and transferred the data to a calculator.

Histomorphology and histomorphometric analysis

For the histological analyses, 10 fractured tibiae in each group were harvested 28 days after the fracture. The tibiae were fixed for 2 days in 10% normal buffered formaldehyde, followed by dehydration in increasing concentrations of ethanol, and embedded undecalcified in methylmethacrylate (Technovit 9100, Heraeus, Wehrheim, Germany). Longitudinal sections in a sagittal plane were cut at 5 μm, using a Leica SM 2500S (Bensheim, Germany) with a stainless steel knife at 40°. The following stains were used: a) von Kossa and b) a combination of safranin-orange and light green.

Histomorphometric parameters in the fracture callus were measured, using a Leica DM-RB (Bensheim, Germany) microscope and an image analysis system (KS 400, Zeiss, Göttingen, Germany). The following parameters were measured at a magnification of 1.6× and structural indices were calculated:

Diameter of the tibia at the fracture gap (Ti.Dm): including Ct.Wi and Ma.Dm (mm);

Bone density of the corticalis: mineralized area/cortical bone area: Md.Ar/Ct.B.Ar (%);

Callus area: Cl.Ar (mm^2);

Mineralized area of the Cl.Ar: Md.Ar/Cl.Ar (%);

Cartilage area of the Cl.Ar: Cg.Ar/Cl.Ar (%).

Ar: Area, B: Bone, Cg: Cartilage, Ct: Corticalis, Dm: Diameter, Ma: Marrow, Md: Mineralized, Wi: Width (Parfitt et al. 1987). Cl: Callus (abbreviation not included in Parfitt et al. 1987).

The tibia's diameter (baseline) was measured to determine the size of the region of interest (ROI). The callus was divided into its proximal and distal parts and a 1.5 length of the baseline was used to define the ROI in the proximal and distal callus halves. The total diameter of the callus was included in the ROI. To make a histomorphometric comparison between the four groups, we measured the mineralized volume of the cortices, the area of the whole calluses, the mineralized and cartilaginous volume of the calluses (Schmidmaier et al. 2002).

Statistics

All animals were randomized in a blinded manner for radiographic evaluation, histological or biomechanical investigation. The data were compared, using one-way ANOVA for independent samples. The radiographic score was analyzed, using the Chi^2-test. Both tests were checked by the Bonferroni correction. Interobserver variability in the radiographic evaluation was determined with kappa statistics. Statistical differences were set at a 95% confidence level. The values are given as mean (SD). SPSS (release 10.0; SPSS Inc. Chicago, Illinois) software was used in the statistical evaluation. To analyze a synergistic effect the differences from the control group were added. If they exceeded the sum, the effect was regarded as synergistic.

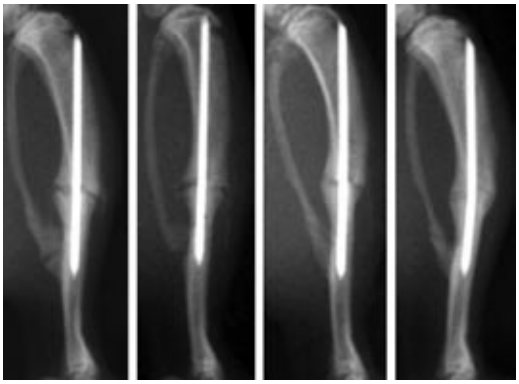

Figure 1. The right tibiae 28 days after fracture and intramedullary stabilization with coated and uncoated titanium K-wires. The gradual consolidation of the fracture in group IV (TGF-ß1 + IGF-I) is clearly seen, as compared to the other groups. Groups II and III treated with TGF-ß1 or IGF-I show better consolidation than control group I.

Results

4 animals were excluded from the study: 2 died during anesthesia and 2 because of a complicated tibial fracture on day 0.

Radiographic examinations

The radiographs of the uncoated group I showed less consolidation than the growth factor groups after 28 days (Figure 1). Only 3 animals in group I had unilateral or complete bridging (A+B) of the fracture ($p < 0.05$). The tibiae in group II (TGF-ß1) were bridged in 6 of 10 cases, the fractures in group III (IGF-I) in 7 of 10 and in the group with both IGF-I + TGF-ß1 (group IV), 9 of 10 animals showed unilateral or complete consolidation after 28 days (Table 1).

Mechanical testing

The maximum load and torsional stiffness (torsional stability) were higher ($p < 0.05$) in all the growth factor groups (II, III and IV) than in control group I. As compared to the unfractured contralateral tibia, control group I had a mean maximum load of 35 (10)% and a mean torsional stiffness of 54 (12)%. The IGF-I group III had greater ($p < 0.05$) torsional stability (mean maximum load of 100 (12)% and mean torsional stiffness of 98 (9.0)%) than control group I and TGF-ß1 group II (mean maximum load of 82 (7.5)% and mean torsional

Table 1. Radiographic analysis

Consolidation	Group I uncoated	Group II TGF-ß1	Group III IGF-I	Group IV IGF-I+TGF-ß1
A. Complete bridging (n)	1 [a]	3	4	5
B. Incomplete bridging (n)	2	3	3	4
C. No bridging (n)	7 [a]	4	3	1 [a]

n 10 in each group and at each time
[a] $p < 0.05$ (Chi^2) in all groups

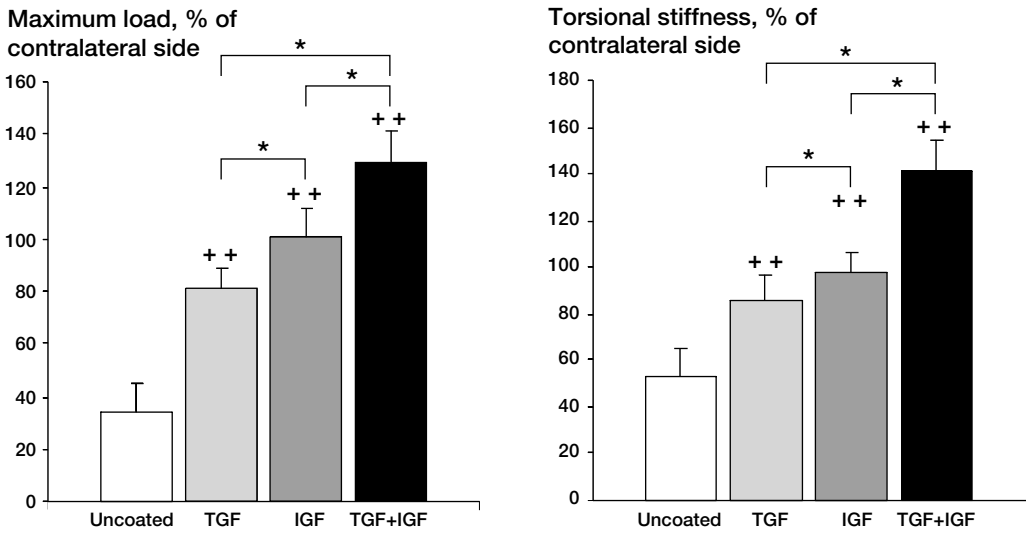

Figure 2. Mechanical torsional testing 28 days after fracture, as compared to the contralateral unfractured tibiae (in %). The highest maximal load (left) and torsional stiffness (right) were found in group IV (IGF-I + TGF-ß1) followed by group III (IGF-I) and then group II (TGF-ß1). The findings in all experimental groups were higher than in control group I (++, $p < 0.05$), *$p < 0.05$.

stiffness of 86 (11)%). The greatest ($p < 0.05$) torsional stability was measured in group IV with the combined application of IGF-I and TGF-ß1, a mean maximum load of 129 (12)% and mean torsional stiffness of 141 (14)% (Figure 2). The enhancement of the torsional stiffness in group IV exceeded that in groups II and III together.

Histomorphology and histomorphometric analyses

The calluses of all groups were composed of fibroblasts, cartilage cells and newly-formed trabecular bone (Figure 3). 28 days after fracture, healing was in the phase of enchondral ossification. All growth factor groups showed more callus remodeling than the control group. The control parameters, like the diameter of the tibiae (baseline) and the mineralized area of the cortices, showed no significant differences between the groups. A slightly larger callus area (Cl.Ar) was found in group IV than in the other groups. Group IV, which had been treated with both IGF-I and TGF-ß1, showed significantly more mineralized tissue and less cartilage in the callus than the other groups. In the control group, more cartilage was measured than in all other groups (Table 2).

Body weight and body temperature

No statistically significant differences were found between the groups during the experiment in mean body temperature and body weight (data not shown). None of the animals examined showed any signs of local or systemic infections during the experiment.

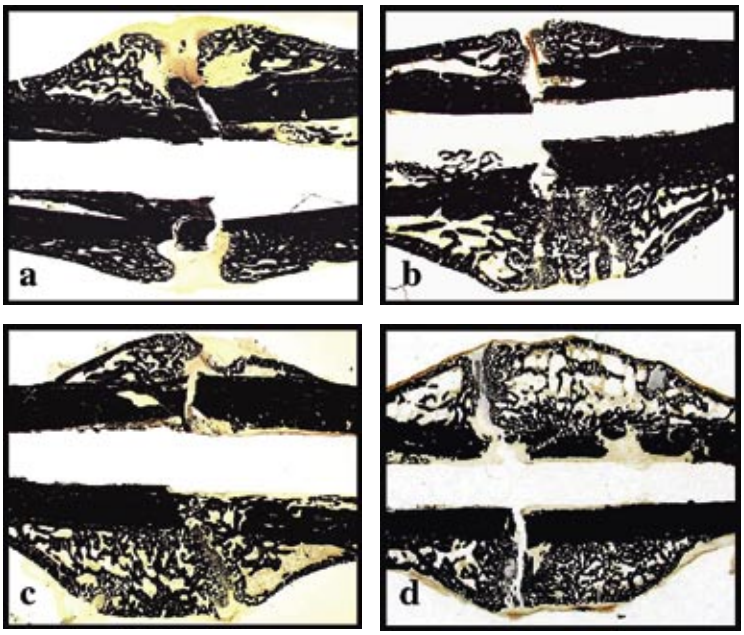

Figure 3. Histological sagittal sections of tibiae 28 days after fracture stained with von Kossa. Note the percentages of mineralized tissue stained black. a) uncoated implant, b) TGF-ß1, c) IGF-I, d) IGF-I & TGF-ß1. Progressed remodeling of the callus can be seen in the growth factor groups compared to the control group. In control group I, which was not treated with growth factors, much more soft tissue and cartilage were visible in the callus than in the groups treated with growth factors.

Table 2. Histomorphometric analysis. Mean (SD)

	Group I uncoated	Group II TGF-ß1	Group III IGF-I	Group IV IGF-I+TGF-ß1
Tibia diameter (mm)	2.6 (0.3)	2.6 (0.2)	2.6 (0.2)	2.6 (0.2)
Mineralized area/cortical area (%)	93 (1.9)	92 (1.2)	92 (1.0)	91 (1.9)
Callus area (mm^2)	18 (5.8)	19 (3.3)	19 (4.3)	21 (5.0)
Mineralized area/callus area (%)	51 (4.0)	52 (3.6)	50 (4.1)	58 (3.8) [a]
Cartilage area/callus area (%)	14 (1.4) [a]	10 (2.1)	9 (3.0)	7 (1.5) [a]

n 10 in each group
[a] Significant difference from all groups, $p < 0.05$, ANOVA

Discussion

Various authors have reported that growth factors modify the actions of other growth factors and possible interactions may have a synergistic effect on fracture healing (Tsukazaki et al. 1994, Matsumura et al. 2000). The aim of our study was to determine the effect of IGF-I and TGF-ß1 on fracture healing in a rat model and to compare these results with the application of both growth factors.

IGF-I (50 μg) and TGF-ß1 (10 μg) were incorporated in a PDLLA coating of implants to obtain an effective local level of the growth factors on the fracture side for a long period (Schmidmaier et al. 2001b). The concentration was selected because of previous studies on the dose-response ratio of this factor-combination on spinal fusion, using the same method of local application (Kandziora et al. 2002). The results of the present study indicate that both growth factors, IGF-I and TGF-ß1, have a partly synergistic effect on fracture healing, when combined in a rat model. The latter combination had greater than additive effects on torsional stiffness and the mineralization of the periosteal callus than single application of these growth factors. The fractures treated with IGF-I showed a higher mechanical stability than the fractures treated with TGF-ß1. The combination of both factors enhanced the torsional stability of the fractured tibiae, as compared to the single applications and the control group. The radiographic and histomorphometric analyses showed no marked differences

between groups II (TGF-ß1) and III (IGF-I). However, group IV, which had been treated with both IGF-I and TGF-ß1, showed a continuous increase in callus remodeling with less cartilage and more mineralized tissue in the callus than in the other groups. Therefore, both growth factors from the PDLLA coating seem to interact, and accelerate the effects on bone metabolism and healing.

We found no significant differences between the groups in mean body temperature and body weight. This accords with studies reporting no systemic or local effects due to the local application of growth factors (Schmidmaier et al. 2001a).

IGF-I or TGF-ß1 alone improve matrix formation and bone remodeling by direct and indirect mechanisms (Hock et al. 1988, Joyce et al. 1990b, Mohan and Baylink 1991, Beck et al. 1993, Thaller et al. 1993b, Lind 1998, Fujimoto et al. 1999). An increase in callus formation and greater bending strength have been noted after injecting TGF-ß (Nielsen et al. 1994) or incorporating TGF-ß in tricalcium-phosphate or hydroxyapatite in canine models (Lind 1998). Bone growth was also stimulated by daily injections of 5 μg IGF-I (Isgaard et al. 1986). Continuous infusion of IGF-I into an artery in the right hind limb of rats for up to 14 days induced cortical and trabecular bone formation (Spencer et al. 1991). A critical-size calvarial defect in rats healed better after treatment with IGF, using an osmotic infusion pump (Thaller et al. 1993a). However, until now, no study has compared the effects of single and combined application of IGF-I and TGF-ß1 on fracture healing.

On the other hand, the effects on bone cell and chondrocyte metabolism have been evaluated in several in vitro studies (Piche and Graves 1989, Pfeilschifter et al. 1990). The combined application of IGF-I and TGF-ß1 had a synergistic effect on cell proliferation, matrix synthesis and differentiation in chondrocyte cell culture (Tsukazaki et al. 1994, Matsumura et al. 2000). Similar findings have been noted concerning the effects of various growth factors on proliferation and differentiation of osteoblasts (Kasperk et al. 1990). These in vitro studies accord with our results showing a more than additive effect of the combined application of IGF-I and TGF-ß1 on fracture healing in vivo, as compared to the single application, concerning torsional stiffness and callus mineralization. Comparable results have also been obtained using growth factor combinations to stimulate wound healing in vivo. The combination of PDGF-2 and IGF-I or PDGF-2 and TGF-alpha had a synergistic effect on porcine skin wound healing (Lynch et al. 1989).

However, the chronology and interaction of the enormous number of growth factors involved in bone growth and fracture healing are still not understood. Further studies should concentrate on cellular processes during the various phases of fracture healing to obtain more information about the role and activity of different growth factors during healing.

This study was supported by Deutsche Forschungsgemeinschaft DFG (Schm 1436/1-1).

No competing interests declared.

Beck L, Amento E, Xu Y, Deguzman L, Lee W, Nguyen T, Gillet N. TGF-beta1 induces bone closure of skull defects - Temporal dynamics of bone formation in defects exposed to rhTGF-beta1. J Bone Miner Res 1993; 8 (6): 753-61.

Canalis E. Effect of insulin-like growth factor I on DNA and protein synthesis in cultured rat calvaria. J Clin Invest 1980; 66 (4): 709-19.

Centrella M, McCarthy T L, Canalis E. Transforming growth factor-beta and remodeling of bone. J Bone Joint Surg (Am) 1991; 73 (9): 1418-28.

Fujimoto R, Tanizawa T, Nishida S, Yamamoto N, Soshi S, Endo N, Takahashi H E. Local effects of transforming growth factor-beta1 on rat calvaria: changes depending on the dose and the injection site. J Bone Miner Metab 1999; 17 (1): 11-7.

Gombotz W R, Pankey S C, Bouchard L S, Phan D H, Puolakkainen P A. Stimulation of bone healing by transforming growth factor-beta 1 released from polymeric or ceramic implants. J Appl Biomater 1994; 5 (2): 141-50.

Hock J, Centrella M, Canalis E. Insulin-like growth factor I has independent effects on bone matrix formation and cell replication. Endocrinology 1988; 122 (1): 254-60.

Hock J, Canalis E, Centrella M. Transforming growth factor-beta stimulates bone matrix apposition and bone cell replication in cultured fetal rat calvariae. Endocrinology 1990; 126 (1): 421-6.

Isgaard J, Nilsson A, Lindahl A, Jansson J, Isaksson O. Effects of local administration of GH and IGF-I on longitudinal bone growth in rats. Am J Physiol 1986; 250 (4 Pt 1): 367-72.

Joyce M, Jingushi S, Bolander M. Transforming growth factor in the regulation of fracture repair. Orthop Clin North Am 1990a; 21 (1): 199-209.

Joyce M, Roberts A B, Sporn M, Bolander M E. Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. J Cell Biol 1990b; 110 (6): 2195-207.

Kandziora F, Pflugmacher R, Scholz M, Schäfer J, Schollmeier G, Schmidmaier G, Duda G, Raschke M, Haas N P. Dose-dependent effects of combined IGF-I and TGF-ß1 application in a sheep cervical spine fusion model. Eur Spine J 2002; 4 (in press).

Kasperk C H, Wergedal J E, Mohan S, Long D L, Lau K H, Baylink D J. Interactions of growth factors present in bone matrix with bone cells: effects on DNA synthesis and alkaline phosphatase. Growth Factors 1990; 3 (2): 147-58.

Kim S J, Ballock R T. Cellular and molecular biology of transforming growth factor ß. In: Cellular and Molecular Biology of Bone. Academic Press 1993; 3: 97-129.

Lind M. Growth factor stimulation of bone healing. Effects on osteoblasts, osteotomies, and implant fixation. Acta Orthop Scand (Suppl 283) 1998: 2-37.

Linkhart T A, Mohan S, Baylink D J. Growth factors for bone growth and repair: IGF, TGF-beta and BMP. Bone (1 Suppl) 1996; 19: 1S-12S.

Lynch S E, Colvin R B, Antoniades H N. Growth factors in wound healing. Single and synergistic effects on partial thickness porcine skin wounds. J Clin Invest 1989; 84 (2): 640-6.

Matsumura T, Whelan M C, Li X Q, Trippel S B. Regulation by IGF-I and TGF-beta1 of Swarm-rat chondrosarcoma chondrocytes. J Orthop Res 2000; 18 (3): 351-5.

Mohan S, Baylink D J. Bone growth factors. Clin Orthop 1991; 263: 30-48.

Nielsen H M, Andreassen T T, Ledet T, Oxlund H. Local injection of TGF-beta increases the strength of tibial fractures in the rat. Acta Orthop Scand 1994; 65 (1): 37-41.

Parfitt A M, Drezner M K, Glorieux F H, Kanis J A, Malluche H, Meunier P J, Ott S M, Recker R R. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 1987; 2 (6): 595-610.

Pfeilschifter J, Oechsner M, Naumann A, Gronwald R, Minne H, Ziegler R. Stimulation of bone matrix apposition in vitro by local growth factors: a comparison between insulin-like growth factor I, platelet-derived growth factor and transforming growth factor beta. Endocrinology 1990; 127 (1): 69-75.

Piche J E, Graves D T. Study of the growth factor requirements of human bone-derived cells: a comparison with human fibroblasts. Bone 1989; 10 (2): 131-8.

Schmidmaier G, Wildemann B, Bail H, Lucke M, Fuchs T, Stemberger A, Flyvbjerg A, Haas N P, Raschke M. Local application of growth factors (insulin-like growth factor-1 and transforming growth factor-ß1) from a biodegradable poly(D,L-lactide) coating of osteosynthetic implants accelerates fracture healing in rats. Bone 2001a; 28 (4): 341-50.

Schmidmaier G, Wildemann B, Stemberger A, Haas N P, Raschke M. Biodegradable poly(D,L-lactide) coating of implants for continuous release of growth factors. J Biomed Mater Res 2001b; 58 (4): 449-55.

Schmidmaier G, Wildemann B, Cromme F, Kandziora F, Haas N P, Raschke M. BMP-2 coating of titanium implants increases biomechanical strength and accelerates bone remodeling in fracture treatment. Bone 2002; 30 (6): 816-22.

Spencer E M, Liu C C, Si E C, Howard G A. In vivo actions of insulin-like growth factor-I (IGF-I) on bone formation and resorption in rats. Bone 1991; 12 (1): 21-6.

Steinbrech D S, Mehrara B J, Rowe N M, Dudziak M E, Luchs J S, Saadeh P B, Gittes G K, Longaker M T. Gene expression of TGF-beta, TGF-beta receptor, and extracellular matrix proteins during membranous bone healing in rats. Plast Reconstr Surg 2000; 105 (6): 2028-38.

Tatsuyama K, Maezawa Y, Baba H, Imamura Y, Fukuda M. Expression of various growth factors for cell proliferation and cytodifferentiation during fracture repair of bone. Eur J Histochem 2000; 44 (3): 269-78.

Thaller S R, Dart A, Tesluk H. The effects of insulin-like growth factor-1 on critical-size calvarial defects in Sprague-Dawley rats. Ann Plast Surg 1993a; 31 (5): 429-33.

Thaller S R, Hoyt J, Tesluk H, Holmes R. The effect of insulin growth factor-1 on calvarial sutures in a Sprague-Dawley rat. J Craniofac Surg 1993b; 4 (1): 35-9.

Trippel S, Coutts R, Einhorn T, Mundy R, Rosenfeld R. Growth factors as therapeutic agents. J Bone Joint Surg (Am) 1996; 78 (8): 1272-86.

Tsukazaki T, Usa T, Matsumoto T, Enomoto H, Ohtsuru A, Namba H, Iwasaki K, Yamashita S. Effect of transforming growth factor-beta on the insulin-like growth factor-I autocrine/paracrine axis in cultured rat articular chondrocytes. Exp Cell Res 1994; 215 (1): 9-16.