

Expression of Neurotrophins and Their Receptors Tropomyosin-related kinases (Trk) under Tension-stress during Distraction Osteogenesis

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The localization and expression of neurotrophins and their receptors during distraction osteogenesis was investigated in 72 male rat femurs (11 weeks old) to further clarify the concurrence of cellular and molecular events of new bone formation. After osteotomy, a 7-day lag phase was followed by distraction at the rate of 0.25 mm/12 h for 21 days (distraction phase), and a 7-day consolidation phase. The localization of neurotrophins (NGF, BDNF and NT-3) and their receptors tropomyosin-related kinases (TRKA, TRKB and TRKC) by immunostaining showed positive staining in bone forming cells in each stage, although the presence and staining intensity varied by cell type and phase. The expressions of NGF, BDNF and NT-3 by real-time polymerase chain reaction (real-time PCR) showed that the peak of the mRNA expression of NGF occurred 10 days after distraction. NT-3 increased during bone extension, but decreased when distraction stopped. In contrast, BDNF continued to increase gradually throughout the distraction and consolidation phases. These findings suggest that neurotrophins and their receptors may play different roles in endochondral and intramembranous ossification in distraction osteogenesis. The tension stress caused by distraction may stimulate the expression of neurotrophins and their receptors, and promote osteogenesis.

Key words: neurotrophin, Trk, distraction osteogenesis, mechanical stress

The neurotrophins (NTs), a family of secreted proteins, have diverse important functions in the normal development of the central and peripheral nervous systems [1]. NTs are best known for their roles in promoting the differentiation and survival of various types of neurons [2]. The 3 best-known NTs, nerve growth factor (NGF), brain-derived neu-

rotrophic factor (BDNF), and neurotrophin-3 (NT-3) are selectively recognized by 3 tropomyosin-related kinases (Trk) TRKA, TRKB, and TRKC, respectively [3]. The NTs and their receptors are also produced by a growing list of nonneural cells [4-7]. The results of *in vitro* analyses of the mRNA expression of NTs and TRK receptors in osteoblastic cell lines suggest that the neurotrophins may play a role in the regulation of bone formation [8-10]. In the osteoblast cell line MC3T3-E1, NT-3 promoted the proliferation of cells via TRKC [8]. Asaumi *et al.*

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made an *in vivo* mouse rib fracture model to observe the mRNA expression after fracture, and reported that the expression of the mRNA of 2 of the NTs was increased during the fracture healing process (8-day observation period), and that localization of NTs and their receptors were observed in bone forming cells (20-day observation period) [11]. Their results also suggested that NTs and their receptors may play a role during osteogenesis.

One of the problems in advancing the understanding of the pathways and mechanisms by which NTs function in non-neuronal cells has been the lack of a suitable *in vivo* model. The authors of the current study contend that such a model already exists, although it is little known outside the periodontal and orthopaedic fields. Distraction osteogenesis (DO) applies tension stress to lengthen bone by means of callus distraction, a process histologically similar to that of fracture healing, but with a rate of osteogenesis greater than that in embryonic development [12]. Sato *et al.* reported that mechanical tension-stress modulates cell shape and phenotype, and stimulates the expression of the mRNA of bone matrix proteins [13]. Recent studies have reported that mechanical tension-stress influences the genetic expression of bone morphogenetic protein (BMP), insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF) and transforming growth factor β (TGF β) [14, 15]. DO models extend the periods of callus building and bone formation, provide larger sampling areas for visualization, and allow evaluation of the concurrence of cellular and molecular events. To the best of our knowledge, the current study is the first report of a rat femur distraction model to clarify the expression and localization of NTs and their receptors under the mechanical stress of DO.

Materials and Methods

Experimental model for DO. The experimental protocols complied with animal welfare regulations approved by the Laboratory Animal Center for Biochemical Research, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Science. Seventy-two male Wistar rats (11 weeks old) were studied. The rats were anesthetized by intramuscular administration of ketamine before a longitudinal skin incision was made on the lateral

aspect of the right femur, and the fascia was cut longitudinally. After the muscles were separated, the right femur was exposed. Four 1.5-mm half pins were inserted into the axis of the femoral shaft, and clamped to a monolateral external fixator (Hoffman Mini Lengthening System; Stryker, Plan-les-Ouates, Switzerland) [16]. A transverse osteotomy was gently made between the second and third half-pins. The muscles were returned to their normal position, and the skin incision sutured. All of the rats were maintained in cages with free access to food and water. For the distracted group, there was a lag phase of 7 days after osteotomy, a distraction phase of 21 days during which gradual distraction was at a rate of 0.25 mm /12 h, and a consolidation phase of 7 days during which the external fixator remained in place without distraction. Rats in the nondistracted group underwent osteotomy and external fixation without distraction. Groups of 6 rats were euthanized at various stages for histologic examination and extraction of RNA (Table 1). At each stage after osteotomy, 1 rat from each group was euthanized by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). At each of the 4 time points listed in Table 1, material from the one animal that had been euthanized was used for immunohistochemical study.

Tissue preparation. Samples from 24 rats were obtained at the 4 time points (Table 1). The rats were perfused with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The distracted femurs were dissected with the surrounding soft tissues and the external fixator still in place, and fixed overnight in the same solution. The samples were then dehydrated with an ethanol series, decalcified with 20% ethylene-diamine tetraacetate (EDTA), and embedded in paraffin according to the standard procedure. Longitudinal semi-thin sections of 6.0- μ m thicknesses were cut on a microtome, mounted on slide glasses, and stored at 4 °C until being stained with Safranin O fast-green and then immunostained.

Immunostaining. The sections were deparaffinized in xylene and rehydrated in a graded series of alcohol (100% to 50%), then pretreated for antigen retrieval by 0.1% trypsin in distilled water. The sections were then immersed in 0.3% methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase, and rinsed in 0.05 M of Tris-

buffered saline (TBS: pH 7.6, for 5 min, 3 times) before immersion in blocking solution (Funakoshi, Tokyo, Japan) for 30 min at room temperature.

Antibodies against TRKA, NGF, TRKB, BDNF, TRKC and NT-3 (Santa Cruz Technologies, Santa Cruz, CA, USA) were used as primary antibodies. The sections were incubated for 1 h with a 1:150 dilution of primary antibodies at room temperature. After rinsing in TBS, the sections were sequentially incubated with diluted biotinylated secondary antibody (goat anti-rabbit IgG) solution for 30 min. The slides were washed for 5 min in TBS 3 times, and then incubated with ABC reagent (Vectastain Elite ABC Kit, Vector Labs, Burlingame, CA, USA) for 30 min. The reaction was visualized by immersion in diaminobenzidine hydrochloride and the substrate, 0.6 % hydrogen peroxide, for 1–2 min. The sections were finally counterstained with Mayer’s hematoxylin to visualize the nuclei, then rinsed in tap water for 10 min, dehydrated in a graded series of alcohol (70 % to absolute methanol), rinsed in xylene, and mounted [17, 18].

Sections for negative control were reacted with normal rat serum or with the secondary antibody alone. All the control sections were negative. Positive staining controls were included for each antibody, and, where present in the specimens, internal staining controls were also checked for appropriate reactions with each antibody.

RNA extraction. A total of 48 rats were euthanized for the preparation of samples for real-

time PCR (Table 1). The lengthened segments of femur were dissected from each sample, snap frozen in liquid nitrogen, and shattered by vigorous hammering. Total RNA was isolated from the shattered samples by ISOGEN Reagent (Nippon Gene, Tokyo, Japan), which was used according to the manufacturer’s instructions.

Real-time PCR. Total RNA (1 µg) was reverse-transcribed to cDNA by using oligo d (T) with reverse transcriptase (ReverTra Ace, Toyobo, Osaka, Japan). Then the cDNAs of NGF, BDNF and NT-3 were amplified with LA-Taq DNA polymerase (Takara, Tokyo, Japan), and the cDNAs of GAPDH were amplified as a control with the same enzyme. Primers for NGF, BDNF and NT-3 were from Search-LC (Heidelberg, Germany). The primer sequences of NGF, BDNF, NT-3 and GAPDH appear in Table 2. The amplification procedure for NGF cDNA under this protocol consisted of denaturation at 95 °C for 10 min and 40 cycles of amplification reactions at 95 °C for 10 sec, 60 °C for 10 sec and 72 °C for 6 sec, followed by melting curve analysis and cooling process. The melting curve analysis was initiated at 70 °C with elevation of the temperature up to 95 °C at a heating rate of 0.1 °C/s. The conditions were identical for BDNF and NT-3. For GAPDH, the conditions were the same, except that the cDNA was amplified for 30 cycles.

Real-time quantitative PCR reactions were performed on a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) using a

Table 1 Time course and number of rats used in this study

		Days after osteotomy				
		0	7	18	28	35
		← Lag phase →		Distraction phase (0.25 mm/12 h)		← Consolidation phase →
		Osteotomy	Beginning of distraction		End of distraction	
Histologic examination			n = 6			
RNA extraction	n = 6		n = 6			
		Distracted group		(n)	(n)	(n)
		Histologic examination		6	6	6
		RNA extraction		6	6	6
		Nondistracted group		(n)	(n)	(n)
		RNA extraction		6	6	6

LightCycler FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Mannheim, Germany) as recommended by the manufacturer.

Radiographic study. Antero-posterior and lateral view radiographs of the lengthened femur were taken at specific time points. At Day 7, radiographs were taken of both groups before distraction. Thereafter, only the distracted group was studied.

Statistical analysis. The results of 4 real-time PCR runs were expressed as the mean \pm SD for each PCR product. The nonpaired t-test was used for statistical comparisons between the distracted and nondistracted groups (StatView software for Macintosh, version 5.0, SAS Institute Inc., Cary, NC, USA).

Results

All 72 rats survived the operation and tolerated distraction well, and the external fixation devices caused no additional injuries even though several rats were kept in each cage. The femoral lengthening was successful at the times and rates described above with reproducible and homogenous results.

Radiographic findings. By day 7, a callus had begun to form around the cut ends of bone at the osteotomy site in both groups before distraction. At Days 18 and 28, the proximal and distal calluses had lengthened progressively, but were separated by a radiolucent area. By Day 35, the bony calluses were longer, and the radiolucent zone was smaller and partially fused (data not shown).

Histologic features of distraction osteogenesis.

(1) Lag phase. Cortical bone at the osteotomy

site was surrounded by a soft callus of regenerating cartilage and new bone. Areas of cartilage formation within the callus were confirmed by Safranin O fast-green staining. The mesenchyme contained cells morphologically consistent with fibroblasts (fibroblast-like cells). Typical endochondral ossification was observed (Fig. 1A, 2A). The histological events were consistent with previous reports of the lag phase and fracture healing [11, 13, 15, 16].

(2) Distraction phase. At Day 18, the calluses around the bony ends had thickened and elongated. The calluses were connected by a vascularized fibrous zone. Fibroblast-like cells were no longer visible in the mesenchyme. Bone formation was observed at the sides of the fibrous zone. Osteoblast cells were seen in cartilage around newly-formed bone (Fig. 2).

At Day 28, the area of trabecular bone was increased and the cartilage area decreased. Pre-osteoblasts, osteoblasts, and fibroblast-like cells in the mesenchyme were arranged by stage of differentiation along the tension vector. The cells in the fibrous zone had apparently converted into bone-forming cells in a manner consistent with intramembranous ossification (Fig. 3).

(3) Consolidation phase. Fusion across the fibrous zone was partial by Day 35. Intramembranous ossification continued in the middle of the zone (Fig. 3A, 4A). In general, the histological features of this phase were consistent with those of the remodeling phase of fracture healing [11]. Although the proximal and distal new bone had fused partially in the middle of the fibrous interzone, the fusion was not yet complete, and intramembranous ossification was mainly seen there [16].

Immunostaining. A semi-quantitative evaluation of the localization of NTs and their receptors in lengthened femur is shown in Table 3 and 4. The frequency levels of the NT and receptor positive cells were divided into 4 groups: - : 0%, \pm : \sim 30%, + : \sim 60%, ++ : \sim 100%, based on the samples of 24 rats.

(1) Lag phase. At Day 7, positive staining for NTs and their receptors were observed in the cells participating in bone-forming areas. At sites of endochondral ossification, moderate positive staining for 2 of the NTs and their receptors were observed in the hypertrophic chondrocytes and osteoblasts.

Table 2 Sequences of the primers for Real-time PCR

	Sequences (5'-3')	Products size (bp)
GAPDH	(S) ACCACAGTCCATGCCATCAC	450
	(AS) TCCACCACCCTGTTGCTGTA	
NGF	(S) CCCCGAATCCTGTAGAGA	149
	(AS) CACGCAAGGCTGTATCTAT	
BDNF	(S) CAAAAGGCCAACTGAAGC	169
	(AS) CGCCAGCCAATTCTCTTT	
NT-3	(S) AACGAGGTGTAAAGAAGC	155
	(AS) TGCTATTTCGTATCCAGC	

(AS), Antisense primer; (S), Sense primer.

BDNF did not stain in the hypertrophic chondrocytes, but its cognate receptor TrkB showed moderate staining. In the fibroblast-like cells in the mesenchyme, no staining was observed for any of the NTs or the cognate receptors (Fig. 1B-G).

(2) Distraction phase. At Day 18, positive staining for NTs and their receptors were observed at sites of endochondral ossification (Fig. 2B-G). In the hypertrophic chondrocytes, NGF and TRKA showed strong positive staining, and BDNF, NT-3, TRKB

and TRKC showed moderate positive staining. In the osteoblasts, NGF and TRKA showed strong positive staining, and BDNF, NT-3, TRKB and TRKC showed moderate positive staining. In the fibroblast-like cells in the mesenchyme, NGF showed moderate positive staining, and NT-3, TRKA and TRKC showed weak positive staining. BDNF and TRKB showed no staining.

At Day 28, positive staining for all 3 of the NTs and their receptors were observed in sites of intra-

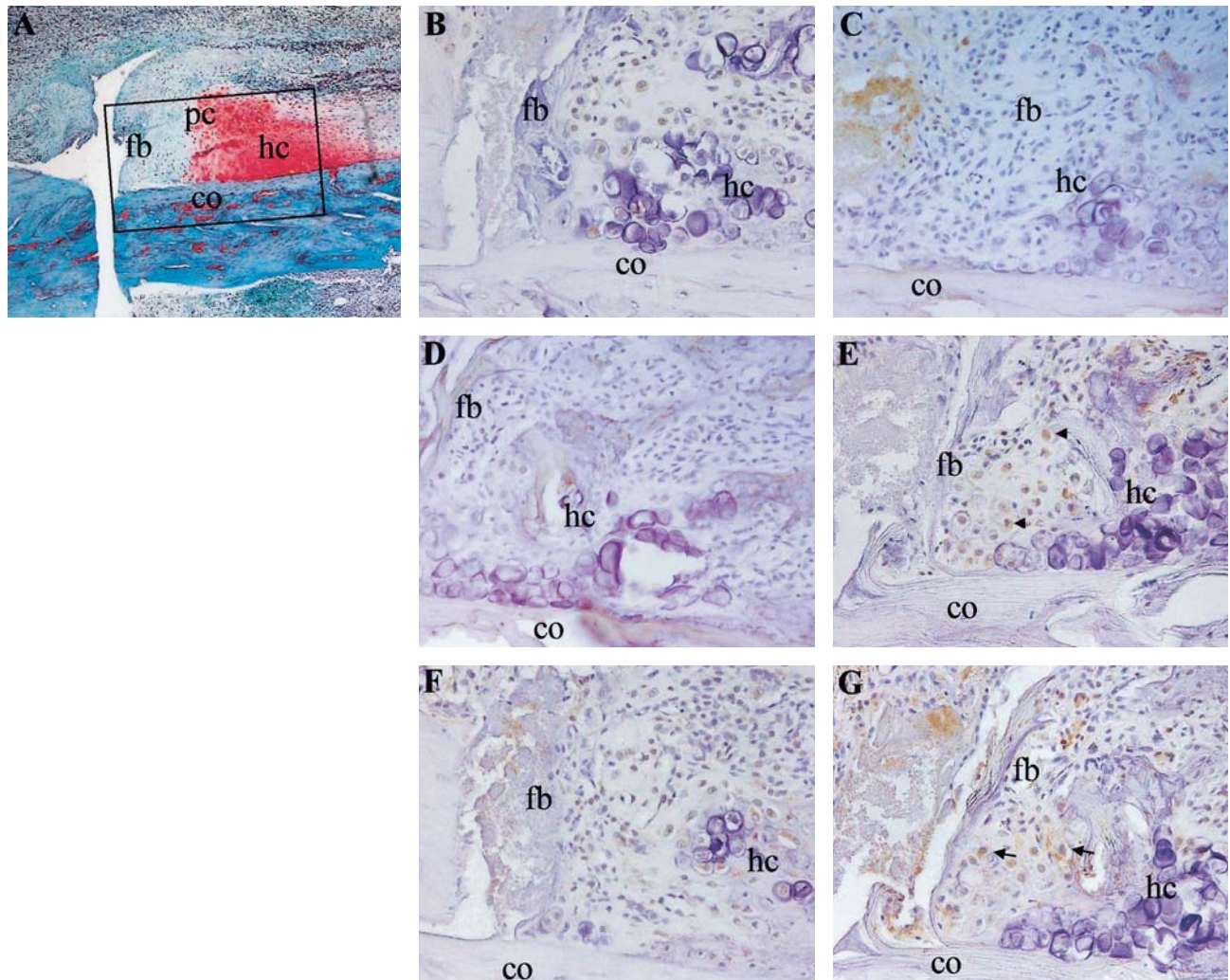


Fig. 1 Light microscope observation of osteotomy site in rat femur on Day 7 before distraction. Safranin O fast-green staining (A), positive immunohistochemical staining for TRKA (B), NGF (C), TRKB (D), BDNF (E), TRKC (F), and NT-3 (G) (Enlargement of inset in A). Cortical bone (co), fibroblast-like cells in mesenchyme (fb), hypertrophic chondrocyte zone (hc), and proliferating chondrocytes (pc). Arrowheads indicate BDNF and arrows indicate NT-3 in hypertrophic chondrocytes. A \times 75, B-G \times 250.

membranous ossification (Fig. 3B-G). NGF and TRKA showed moderate positive staining, while BDNF, NT-3, TRKB and TRKC showed strong positive staining in the osteoblasts and fibroblast-like cells.

(3) Consolidation phase. At Day 35, the 3 NTs and their receptors continued to be localized in areas of bone formation. In sites of intramembranous ossification, BDNF, TRKB and TRKC showed strong

positive staining in osteoblasts and fibroblast-like cells. NGF and TRKA showed weak positive staining, and NT-3 showed moderate positive staining in fibroblast-like cells in the mesenchyme. NGF, NT-3 and TRKA showed moderate positive staining in the osteoblasts (Fig. 4B-G).

Similar results were obtained for each distracted group of 6 rats (Table 3 and 4).

(4) Detection of NGF mRNA by real-time PCR.

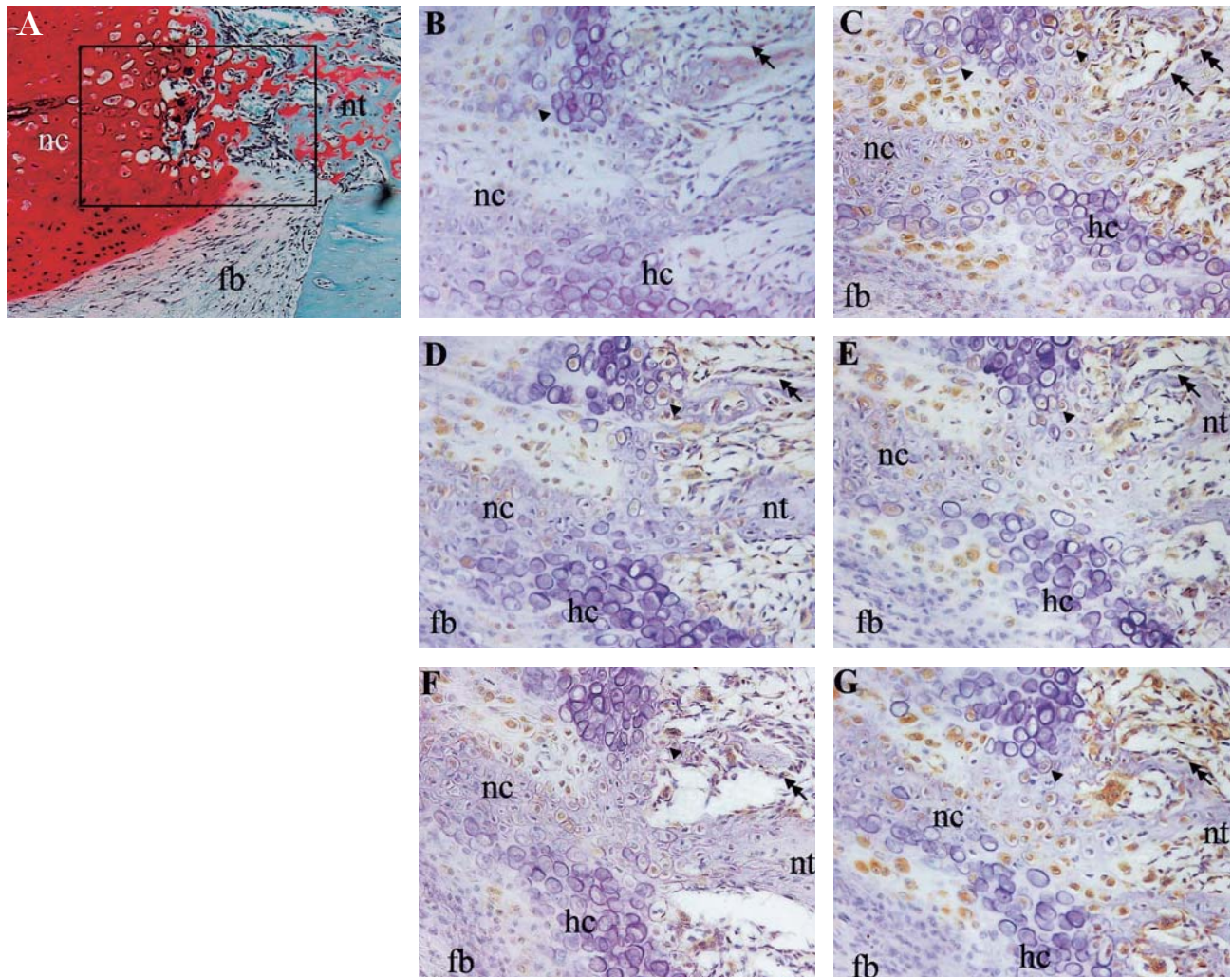


Fig. 2 Light microscope observation of distraction site in rat femur on Day 18. Safranin O fast-green staining (A), positive immunohistochemical staining for TRKA (B), NGF (C) especially strong in hypertrophic chondrocytes and osteoblasts, TRKB (D), BDNF (E), TRKC (F), and NT-3 (G) (Enlargement of inset in A). New trabecular bone (nt), new cartilage (nc), fibroblast-like cells in mesenchyme (fb), and hypertrophic chondrocyte zone (hc). Arrowheads indicate NTs and cognate receptors in hypertrophic chondrocytes, and the double-headed arrows indicate them in osteoblasts.

A \times 200, B-G \times 250.

The expression level of NGF mRNA in the lengthened femur was disclosed by real-time PCR with synthetic primers specific for NGF (Table 2). The PCR product sizes were consistent with those expected from their primer design. The expression of NGF mRNA was increased during the lag phase and early distraction phase. In the nondistracted group, after the expression of NGF mRNA showed its peak level at Day 18, it declined gradually to a lower level. However in the distracted group, the expression fur-

ther increased after the beginning of distraction until its peak level at Day 18, after which it decreased to almost the predistracted level (Fig. 5A).

(5) Detection of BDNF mRNA by real-time PCR. The expression level of BDNF mRNA in the lengthened femur was disclosed by real-time PCR with synthetic primers specific for BDNF. The PCR product sizes were consistent with those expected from their primer design. In the distracted group, the expression of BDNF mRNA increased gradually

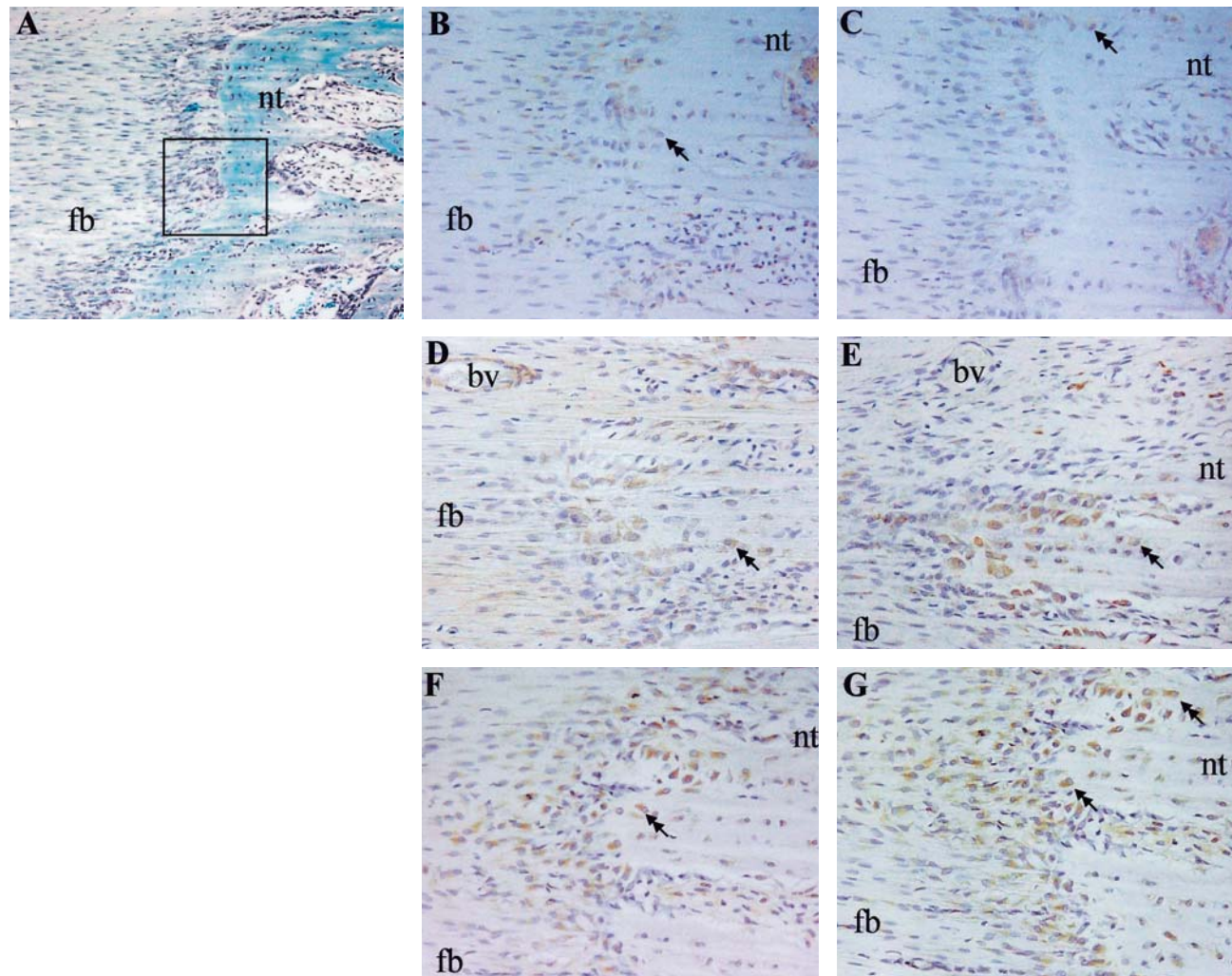


Fig. 3 Light microscope observation of distraction site in rat femur on Day 28. Safranin O fast-green staining (A), positive immunohistochemical staining for TRKA (B), NGF (C), TRKB (D), BDNF (E), TRKC (F), and NT-3 (G) especially strong in osteoblasts (Enlargement of inset in A). New trabecular bone (nt), fibroblast-like cells in mesenchyme (fb), and blood vessels (bv). Double-headed arrowheads indicate NTs and cognate receptors in osteoblasts. A \times 200, B-G \times 250.

until Day 18 and continued to increase until the end of the experiment. In the nondistracted group, after the expression of BDNF mRNA reached its peak level at Day 18, it declined gradually (Fig. 5B).

(6) Detection of NT-3 mRNA by real-time PCR. The expression level of NT-3 mRNA in the lengthened femur was analyzed by real-time PCR with synthetic primers specific for NT-3. The sizes of the PCR products were consistent with those expected from their primer design. The expression

of NT-3 mRNA increased during the lag phase and distraction phase. In the nondistracted group, after the expression of NT-3 mRNA showed its peak level at Day 7, it gradually decreased. However, in the distracted group, the expression showed its peak level at Day 28 after osteotomy (Fig. 5 C).

Discussion

More recent studies provide some insights into

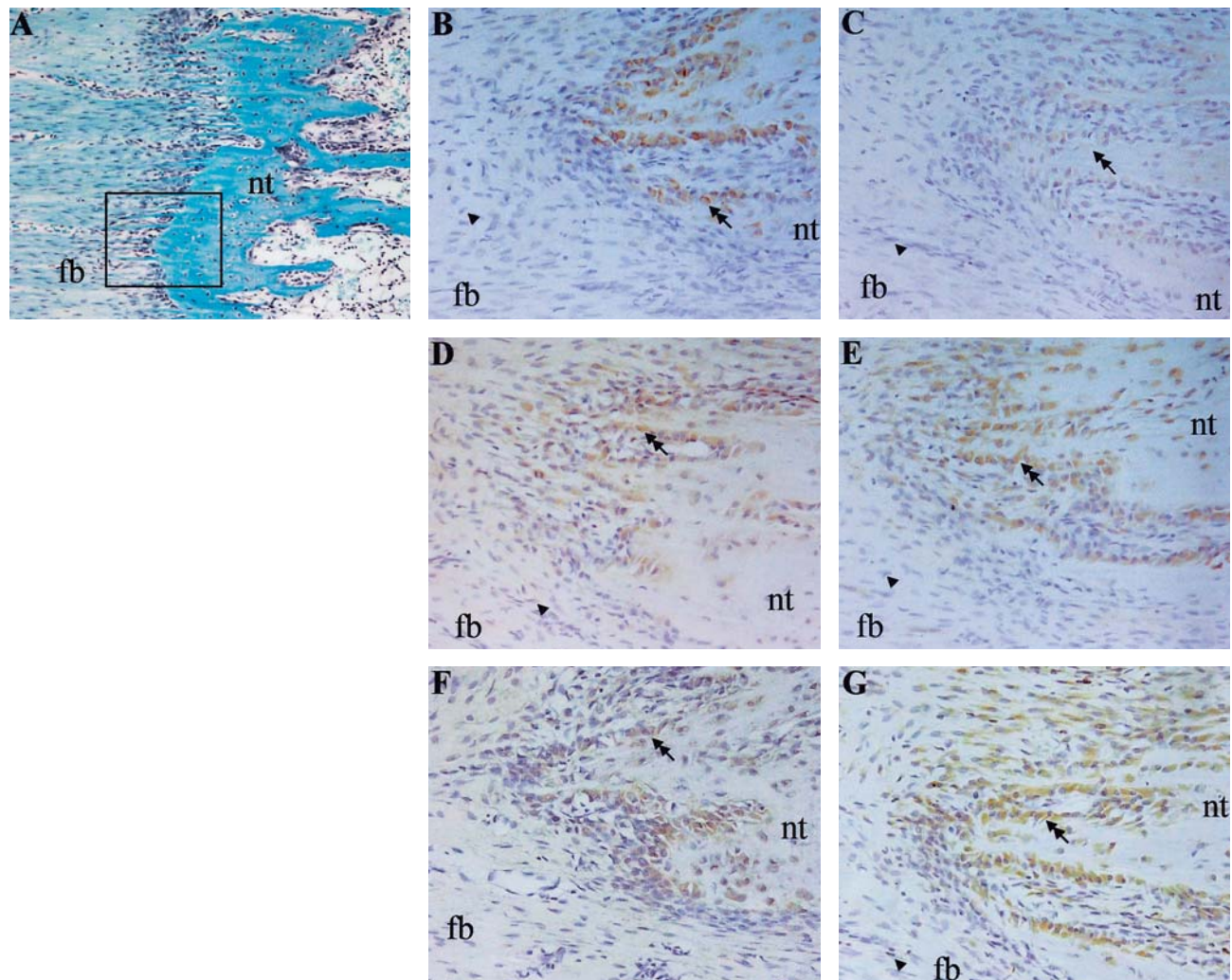


Fig. 4 Light microscope observation of distraction site in rat femur on Day 35. Safranin O fast-green staining (A), positive immunohistochemical staining for TRKA (B), NGF (C) especially strong in osteoblasts TRKB (D), BDNF (E), TRKC (F), and NT-3 (G) (Enlargement of inset in A). New trabecular bone (nt) and fibroblast-like cells in mesenchyme (fb). Arrowheads indicate NTs and cognate receptors in fibroblast-like cells, and double-headed arrows indicate them in the osteoblasts. A $\times 200$, B-G $\times 250$.

Table 3 Localization of neurotrophins and their receptors (TRK) at the distraction callus in endochondral ossification

	Days after osteotomy											
	7						18					
	NGF	BDNF	NT-3	TRKA	TRKB	TRKC	NGF	BDNF	NT-3	TRKA	TRKB	TRKC
Endochondral ossification												
Hypertrophic chondrocytes	+	-	+	+	+	+	++	+	+	++	+	+
Osteoblasts	+	+	+	+	+	+	++	+	+	++	+	+
Fibroblast-like cells in Mesenchyme	-	-	-	-	-	-	+	-	±	±	-	±

Positive cell labeled by anti neurotrophins and their receptors antibodies were expressed: -, no staining (0%); ±, weak staining (~30%); +, moderate staining (~60%); ++, strong staining (~100%).

Table 4 Localization of neurotrophins and their receptors (TRK) at the distraction callus in intramembranous ossification

	Days after osteotomy											
	28						35					
	NGF	BDNF	NT-3	TRKA	TRKB	TRKC	NGF	BDNF	NT-3	TRKA	TRKB	TRKC
Intramembranous ossification												
Fibroblast-like cells in Mesenchyme	+	++	++	+	++	++	±	++	+	±	++	++
Osteoblasts	+	++	++	+	++	++	+	++	+	+	++	++

Positive cell labeled by anti neurotrophins and their receptors antibodies were expressed: -, no staining (0%); ±, weak staining (~30%); +, moderate staining (~60%); ++, strong staining (~100%).

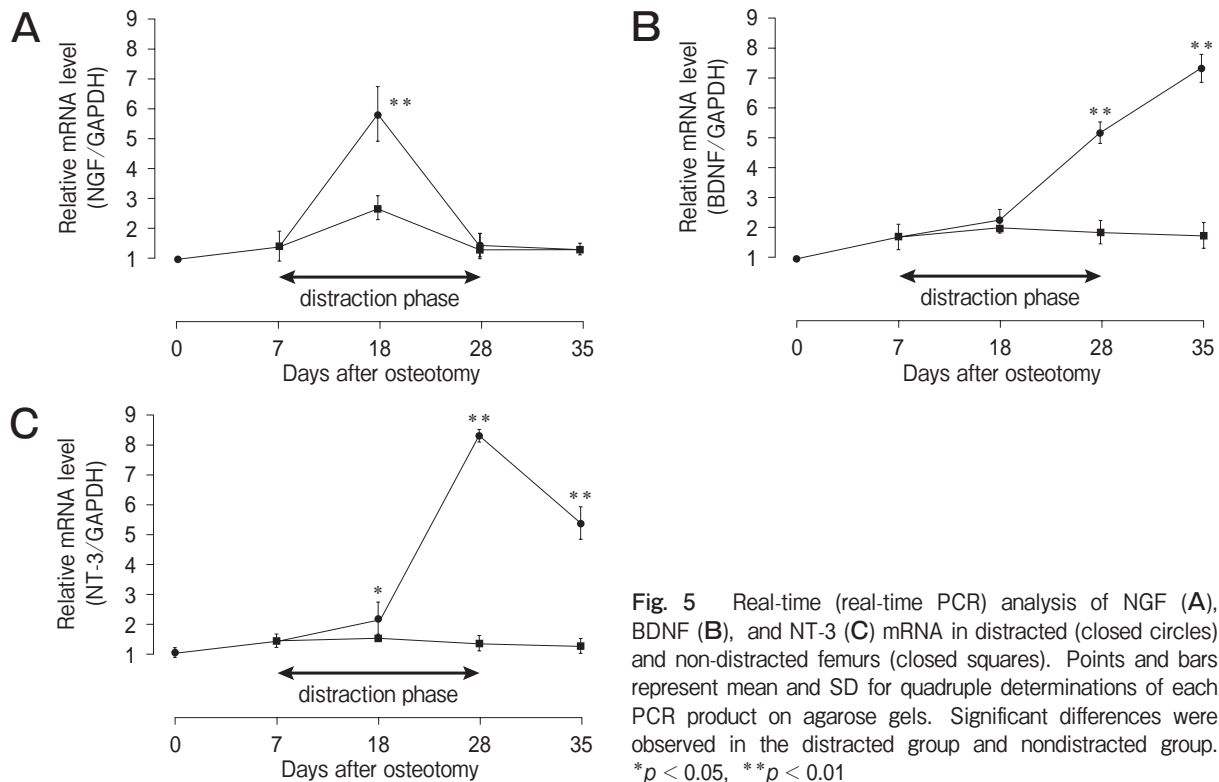


Fig. 5 Real-time (real-time PCR) analysis of NGF (A), BDNF (B), and NT-3 (C) mRNA in distracted (closed circles) and non-distracted femurs (closed squares). Points and bars represent mean and SD for quadruple determinations of each PCR product on agarose gels. Significant differences were observed in the distracted group and nondistracted group. * $p < 0.05$, ** $p < 0.01$

the cellular and molecular mechanisms of distraction osteogenesis (DO). It was reported that mechanical tension-stress modulated cell shape and phenotype, and stimulated the expression on the mRNA for BMPs [13]. Expression levels of mRNA of NTs during distraction in the current study were higher than in the nondistracted group. The variety of cell types expressing NTs and their receptors in DO is much greater than that detected in the fracture healing process in a mouse model [11]. Tang *et al.* reported that DO and the secondary Wallerian degeneration triggered the proliferation of Schwann cells, and that chronic stretching might effectively promote the synthesis of NGF and other NTs in Schwann cells [19]. These findings suggest that NGF may promote the development of nerve around bone tissue during DO. In our previous study of a mice rib fracture model, NTs and their receptors were involved in the regulation of bone formation as an autocrine and paracrine factor [11]. Taken together, these findings suggest that mechanical tension-stress may stimulate the expression of NTs and promote osteogenesis.

We used immunohistochemistry and real-time PCR in a rat femur distraction model to observe changes in NGF, BDNF, and NT-3 and their cognate receptors in bone during DO. Immunostaining demonstrated that the NTs and their receptors showed positive staining in almost all bone forming cells in both groups, but not equally. The presence and intensity of staining varied by cell type and phase. In the early stage of distraction, localization of NTs and their receptors was observed in chondrocytes and in osteoblasts in endochondral ossification. At the late stage of distraction and the consolidation phase, the localization of NTs and their receptors was observed in osteoblasts in intramembranous ossification. Localization in such cells suggests that NTs may promote endochondral ossification and intramembranous ossification in DO.

In our previous study of a mice rib fracture model, TRKB was not detected [11], but in the current rat femur distraction model, TRKB was revealed. Although the reason for the difference is unclear, BDNF in the mouse model may recognize TRKB isoforms that were deleted in their tyrosine kinase domains, or BDNF may use other receptors. The peak of NGF staining was observed at Day 18,

BDNF was at Day 35, and NT-3 was at Day 28. These results were consistent with the results of real-time PCR.

Real-time PCR demonstrated that the expression levels of mRNA of the NTs were increased during DO. At Day 7 (lag phase), the expressions of all NTs were slightly increased. These results were also consistent with the results of our previous study of a mouse rib fracture model [11]. The expression level of mRNA of NGF was higher in the distracted group with peak mRNA expression at Day 18, followed by a sharp decrease, possibly indicating a function quite different from that of BDNF. The expression levels of mRNA of NT-3 increased during distraction, reaching the peak level at Day 28, and decreased after distraction stopped. It is thought that the expression level was gradually promoted by the mechanical tension-stress. Concurrent with this pattern of expression were the observations of the fibroblast-like cells which were seen in the mesenchyme before distraction began, then seemed to disappear during distraction, and were once again visible after distraction stopped. Were they actually fibroblasts, or merely morphologically consistent in appearance? Since we did not specifically stain for fibroblasts, our results cannot answer these questions. Since they were aligned along the tension vector, it is reasonable to speculate that tension stress caused them to stretch, and in doing so altered their expression. Subsequent studies must resolve the identity of the fibroblast-like cells and their possible relationship with NT-3 and its cognate receptor. In contrast, the expression level of mRNA of BDNF continued to increase throughout the bone extension and consolidation phases. This phenomenon may be very important, because BDNF participates in regulating the development and remodeling of bony tissue [20] and stimulates the expression of bone-related proteins [21]. BDNF may be an attractive therapeutic factor for further study in bone formation. The expression of NT-3 and TRKC in osteoblast-like cells shown in immunostaining, and the high level expression of NT-3 mRNA by real-time PCR, suggest an autocrine loop function during DO *in vivo* [22]. NGF and TRKA might also contribute to DO in a similar autocrine manner. We conclude that the mechanical tension-stress of DO appears to stimulate the expression of NGF, BDNF, and NT-3, and to

promote osteogenesis.

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