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ephrinB2 and EphB4 are Expressed on the Chondrocyte during Fracture Healing

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Summary. **Vascular formation is an essential process for bone regeneration, and several molecules important for its attainment have been discovered. ephrinB2, a trans-membrane protein, is one of the ligands of EphB4, a receptor of the ephrin family. These molecules have tyrosine kinase activities and regulate endothelial proliferation and differentiation in vascular formation in addition to axon guidance in nerve development. Recently, ephrinB2 mRNA expression was identified in the articular cartilage of chick embryos. These data let us hypothesize that ephrinB2-EphB4 interaction may be involved in mammalian cartilage and bone formation. We investigated these expressions of mRNA of these molecules during fracture healing, and the result of *in situ* hybridization showed that both ephrinB2 and EphB4 were co-localized in the cartilage, especially in the proliferating zone during the reparative phase. The results of immuno-histochemistry indicated that proteins of ephrinB2 and EphB4 were seen in the chondrocyte. On the other hand, *in vitro*, we used ATDC5 to investigate the expression of ephrinB2 and EphB4 in the chondrification, and those expressions reached their peak during the proliferating phase. This suggests that ephrinB2 expresses in the arterial endothelial cell, EphB4 expresses in the venous endothelial cell, and together form the boundary in the capillaries. These results indicated that ephrinB2 and EphB4 are involved in proliferation of the chondrocyte and that they have other functions such as the boundary formation between vascular and non vascular areas, for example, between bone and cartilage.**

Key words — ephrinB2, EphB4, fracture healing, mouse rib fracture, ATDC5.

INTRODUCTION

Ephrins and Eph families are ligands and receptors with tyrosine kinase activities. ELF-2, mouse ephrinB2, was cloned and sequence analyzed in 1995 as a new member of the Eph ligand family. *In situ* hybridization of mouse embryos has show that the expression is a segmental pattern in the hindbrain region and the segmenting mesoderm, suggesting roles for ELF-2 in patterning these regions of the embryo[1]. MDK2, mouse EphB4, was cloned in 1995 as a novel receptor tyrosine kinase of the eck/eph family. *In situ* hybridization and Northern blot analyses of mouse embryos indicated its abundant expression in various organs during embryonic development, and these data suggest an important role in gestational growth and differentiation[2]. Otherwise, EphB4 promotes tumor growth by stimulating angiogenesis through ephrinB2[3]. Soluble EphB4 inhibits tumor growth and angiogenesis[4]. The over-expression of ephrinB2 suppressed tumor growth and vascular function in this *in vivo* colon cancer model[5]. Thus various function analyses of ephrinB2 and EphB4 proceeded and these molecules were thought to be concerned with neural and vascular development and tumor growth. The aim of this study was to analyze the distribution and quantification of ephrinB2 and EphB4 mRNAs and proteins in bone regeneration during skeletal development to analyze the mice fracture model and investigate these expressions during chondrogenesis to analyze the chondrification of the ATDC5.

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Abbreviations– H&E, hematoxylin-eosin; ITS-X, Insulin-Transferrin-Selenium-X Supplement; TRAP, tartrate-resistant acid phosphatase.

MATERIALS AND METHODS

Animals and tissue preparation

A total of 54 male ICR mice (5-6 weeks of age, weighing 28 ± 3 g; Nihon Charles River, Tokyo), was anesthetized with 50 mg/kg of ketamine hydrochloride (Sankyo, Tokyo) and 10 mg/kg of xylazine hydrochloride (Bayer, Tokyo). A transverse fracture was made on the right eighth rib of each mouse using scissors as previously described[6,7,8]. For sham surgery, the left eighth rib was exposed, but not cut. These procedures were reviewed and approved by the animal care and use committee of Niigata University. After the Surgery, groups of six mice each were killed on days 1, 3, 5, 7, 10, 14, 18, 21 and 28. A total of 27 mice was perfused through the left ventricle with 4%PFA under deep anesthesia. The fracture sites together with the surrounding soft tissues (4 × 4 mm) were harvested, and half of them were immersed in the ISOGEN reagent (Nippon Gene Co.Ltd., Tokyo) for the extraction of total RNAs. The remaining half was fixed with 4% paraformaldehyde in a 0.1 mol/L phosphate buffer, decalcified in a 0.5 mol/L ethylene-diamine tetra acetic acid (EDTA); pH7.5) solution, and embedded in paraffin wax. Serial sections (5-μm-thick) were made and mounted on 3-amino-propyl-triethoxysilane-coated glass slides and stored at 4°C for the following examination.

Histology

The sections were stained with hematoxylin-eosin (H&E). To detect the cartilage matrix, toluidine blue-O staining was carried out. To detect osteoclasts, tartrate-resistant acid phosphatase (TRAP) staining was performed according to the modified Burstone method [9].

Reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative PCR

Total RNAs were extracted from the samples immersed in ISOGEN according to the manufacturers' instruction and reverse-transcribed using random primers (Promega, Wisconsin, USA) and M-MLV reverse transcriptase (Invitrogen, California, USA). PCR was performed with the following primers: ephrinB2 (accession number U30244, amplified fragments 411bp and 243bp), 5'-aggaatcacggtccaacaaga-3' as the ephrinB2 sense primer, 5'-atagtccccgctgaccttct-3' as the anti-sense primer and 5'-gtctcctgcggtacttgagc-3' as the nested primer; EphB4 (accession number XM109433, amplified fragments 499bp and 243bp), 5'-agcgctctggacaagatgat-3' as the sense primer, 5'-ttccccaaatcctgtgtctc-3' as the anti-sense primer, and 5'-tgactccaattcggagaagg-3' as the nested primer. Each PCR product was separated on agarose gels by electrophoration and visualized with UV light. After checking each expression, the cDNA samples of the sham Surgery and fracture groups were subjected to quantitative PCRs using a LightCycler (Roche Diagostics, Mannhime, Germany) for ephrinB2, EphB4, and GAPDH mRNAs. The PCR program was as follows: dissociation of the Taq antibody for 10 min at 95 °C, then 40 cycles of denaturation for 0 sec at 95 °C, annealing for 10 sec at 60 °C, and extension for 13 sec at 72 °C. To construct melting curves of the PCR products, an additional protocol after the completion of 40 cycles of PCR consisted of 10 sec at 60 °C and 0 sec at 96 °C. The transition from 60 to 96 °C was effected in 0.2 °C steps with continuous fluorescence monitoring, with the plotted loss of fluorescence versus temperature. The data was analyzed using LightCycler analysis software according to the manufacture's instruction. The quantity was calculated by the following formula:

fracture [ephrinB2 (or EphB4) / fracture GAPDH] / [sham ephrinB2 (or EphB4) / sham GAPGD]

to standardize and remove the effect of any growth.

Preparation of probes for mRNA *in situ* hybridization

Digoxigenin-labeled single-strand RNA probes were prepared using the DIG RNA labeling kit (Boehringer Mannheim GmbH, Biochemica, Manheim, Germany) according to the manufacturer's instructions. Fragments of mouse ephrinB2 cDNA (0.4 kb) and fragments of mouse EphB4 cDNA (0.4 kb) were obtained by RT-PCR and subcloned into Bluescript SK (+) plasmids. These plasmids were linearized by digestion with BamHI and EcoRI and transcribed with T7 and T3 RNA polymerases for antisense and sense probes, respectively.

In situ hybridization

In situ hybridization was performed as previously described, with minor modifications. The sections were digested by 5 μg/ml of protainase K solution (Boehringer Mannheim GmbH, Manheim, Germany) at 37 °C for 10 min. They were hybridized with 0.5 μg/ml of the probes in a hybridization buffer at 50 °C for 16 h. After washing and RNaseA (Boehringer Mannheim GmbH, Manheim, Germany) treatment (10 μg/ml) at 37 °C for 30 min, the signals were detected using the DIG detection kit (Boehringer Mannheim

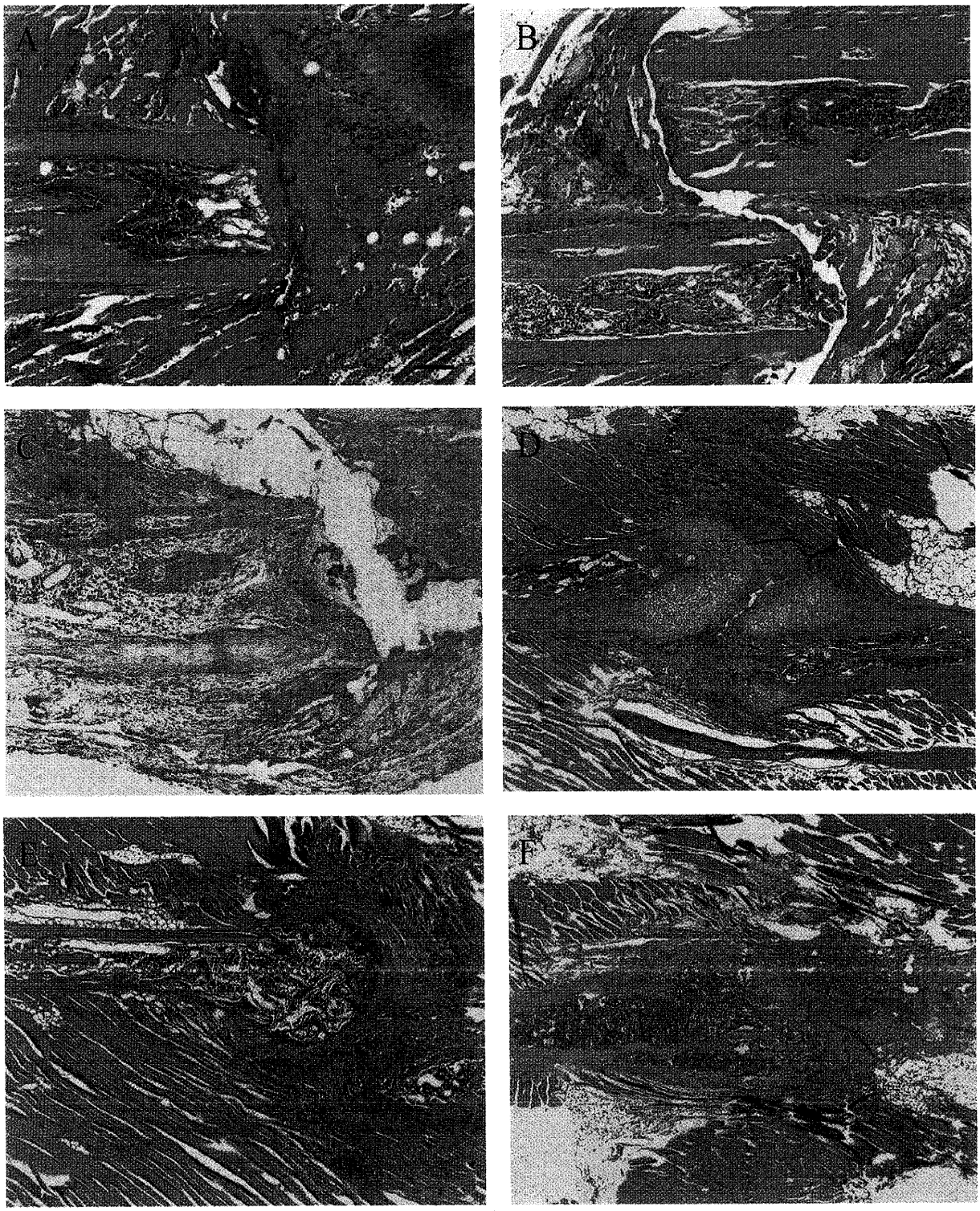

Fig 1. Histological features of fracture healing. **A.** Erythrocytes and fibroblasts accumulated beneath the periosteum and between the fracture stumps on day 3. The torn periosteum shows proliferation of the repair tissues. **B.** Woven bones and cartilage tissues are detected in the proliferating periosteal repair tissues on day 5. Woven bones are also observed in the intramedullary space of the fracture ends. **C.** The fracture stumps are lined by multinuclear osteoclast-like cells, exhibiting positive signals for TRAP staining and resorbing the matrix of the fracture ends. **D.** The fracture ends are partially bridged by a growing matrix, i.e. "callus", on day 7. The center of the callus contains a large amount of cartilage tissues. Newly formed bones are identified at the edges of the callus. **E.** The fracture ends are completely bridged by the callus in which cartilage is replaced by immature trabecular bones and bone marrow spaces on day 14. **F.** The cartilage tissues are completely replaced by newly formed trabecular bones and the callus unites the fracture ends by day 21. All rib fractures were healed without any non-unions in this study. *Bar*, 100 μm.

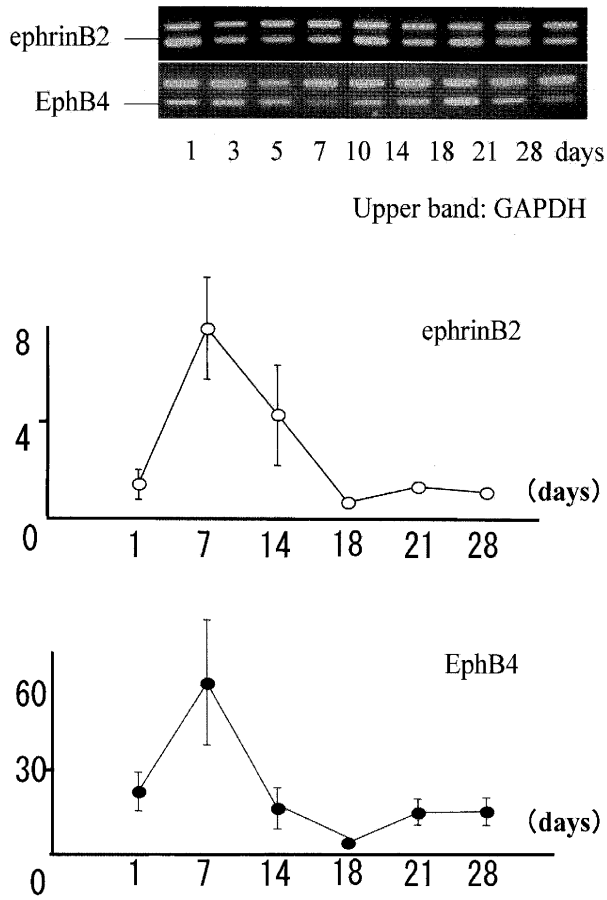

Fig 2. Reverse transcriptase polymerase chain reaction (RT-PCR). Both ephrinB2 and EphB4 mRNAs were detected during all periods of the fracture healing. The results of quantitative PCR using a LightCycler (Roche Diagostics, Mannhime, Germany) show that eprinB2 and EphB4 mRNAs peaked on day 7. The expression of ephrinB2 mRNA on day 7 was four times of that on day 1, and the expression of EphB4 mRNA on day 7 was double that on day 1. The expression of EphB4 mRNA was about 10 times that of ephrinB2 mRNA.

GmbH, Manheim, Germany) according to the manufacturer's instructions.

Immunohistochemistry

After the de-paraffinization and re-hydration, the sections were treated with hyallonidase at 37 °C for 30 min. Endogenous peroxidase activity was quenched by immersion with 0.3% hydrogen peroxide in methanol at 37 °C for 15 min. Anti–ephrinB2 and anti-EphB4, produced in gout (Sigma-Aldrich, St. Louis, MO, USA), were used as primary antibodies. The sections were incubated at 4 °C for 2 h with 1:100 dilution of the primary antibodies. Then they were treated with the peroxidase-conjugated rabbit anti-goat IgG antibody (DAKO cytomation, Glostrup, Denmark) at room temperature for 30 min. The peroxidase reactions were visualized with 3'-diaminbenzidine tetrahydrochloride-0.01% hydrogen peroxide. The sections were counterstained with hematoxylin to stain nuclei.

Culture of the mouse chondrocyte-like cell, ATDC5

ATDC5 cells were cultured in Dulbecco's modified Eagle's and nutrient mixture F-12 media (D-MEM/F-12) (Invitrogen, California, USA) containing 5% fetal bovine serum (FBS: Invitrogen, California, USA) as a maintenance medium at a cell density of $6 \times 10^{5)}$ cell/well in a 6-multiwell plate (Corning, New York, NY, USA) at 37 °C in a humidified atmosphere of 5% CO2. When the cell reached confluence, 1% Insulin-Transferrin-Selenium-X Supplement (ITS-X) (Invitrogen, California, USA) was added twice/week for three weeks. The cells were harvested at one, two and three weeks to extract the total RNAs and the wells were stained with Safranin-O light green staining. Both methods were performed to three control groups and three ITS-X groups.

Safranin-O light green staining of ATDC5

At each time point, cells were rinsed with distilled water and stained with Safranin O (Wako, Osaka) for 4 min and rinsed in distilled water with one quick dip, then soaked in 5% phosphotungstic acid for one min, washed three times in distilled water, counterstained with 0.02% Light Green (Sigma-Aldrich, St. Louis, MO, USA) for one min, and washed until no green ran out[10].

Quantitative analysis of ephrinB2 and EphB4 during chondrogenesis

RT-PCR was performed at each time point with the primers for ephrinB2, EphB4, and LightCycler® (Roche Diagostics, Mannhime, Germany) were performed as described above. The data were analyzed using LightCycler analysis software according to the manufacture's instruction. The quantities were normalized to the expression of GAPDH mina and evaluated statistically by Fisher's Protected Least Significant Difference using statistical software (Stat View®, SAS Institute Inc., NC, USA). Aggrecan, type X collagen, sox9, as follows:

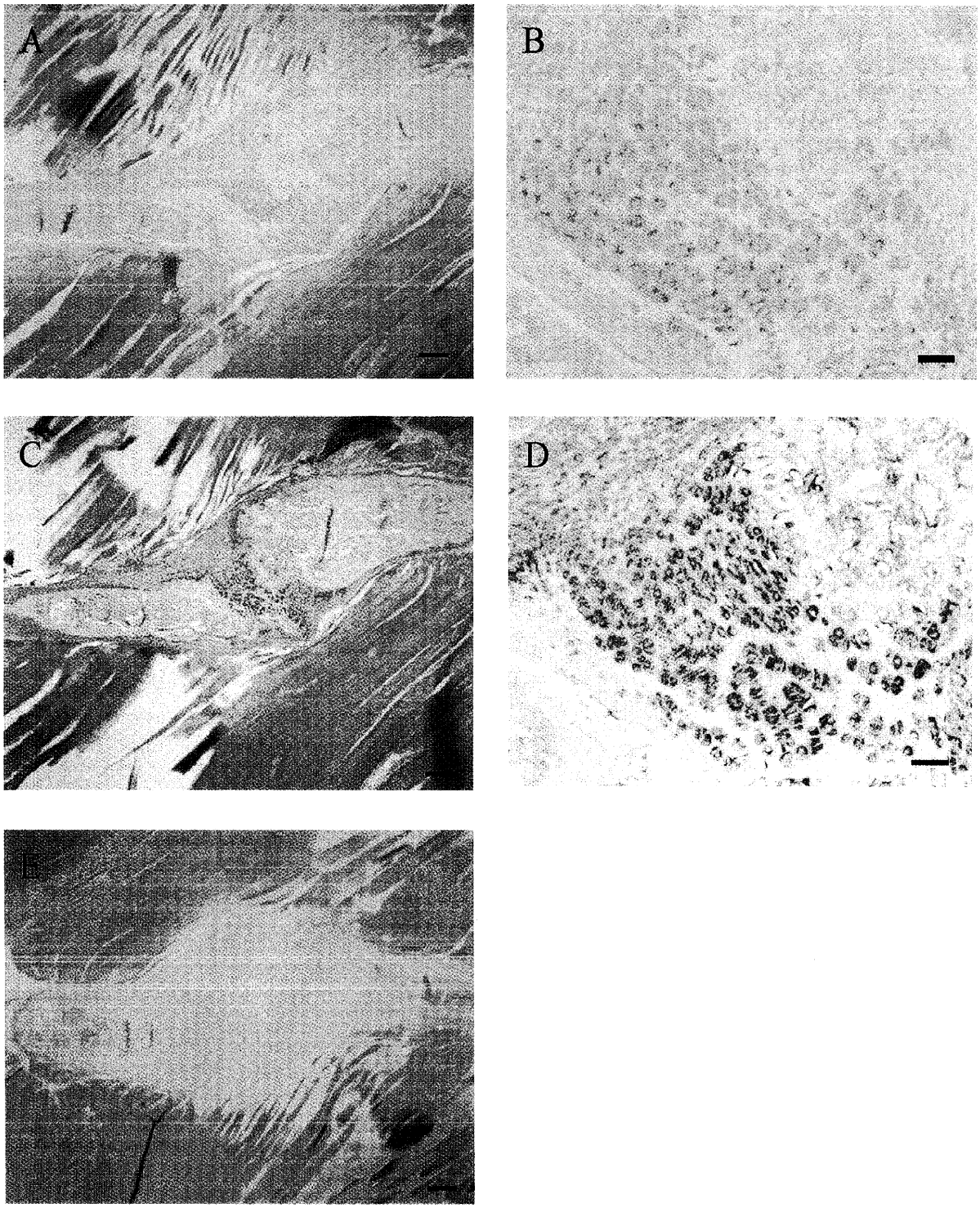

Fig 3. *In situ* hybridization of ephrinB2 and EphB4. **A.** Positive signals of ephrinB2 are seen on the periosteum and especially on the fracture site on day 5. **B.** In a high magnification of the fracture site, the signals can be seen on the chondrocytes, and the signals of the proliferating zone are stronger than that of the hypertrophic zone. **C.** The signal of the sense probe of ephrinB2 is not seen. **D.** In the serial section of the ephrinB2, where we performed *in situ* hybridization of EphB4, the signals are seen in the same area of ephrinB2. **E.** In a high magnification of fracture site, localization and intensity are the same as ephrinB2, but signals of EphB4 are stronger than those of ephrinB2. **A, C** and **E.** *Bar*, 100 μm; **B** and **D.** *Bar*, 50 μm.

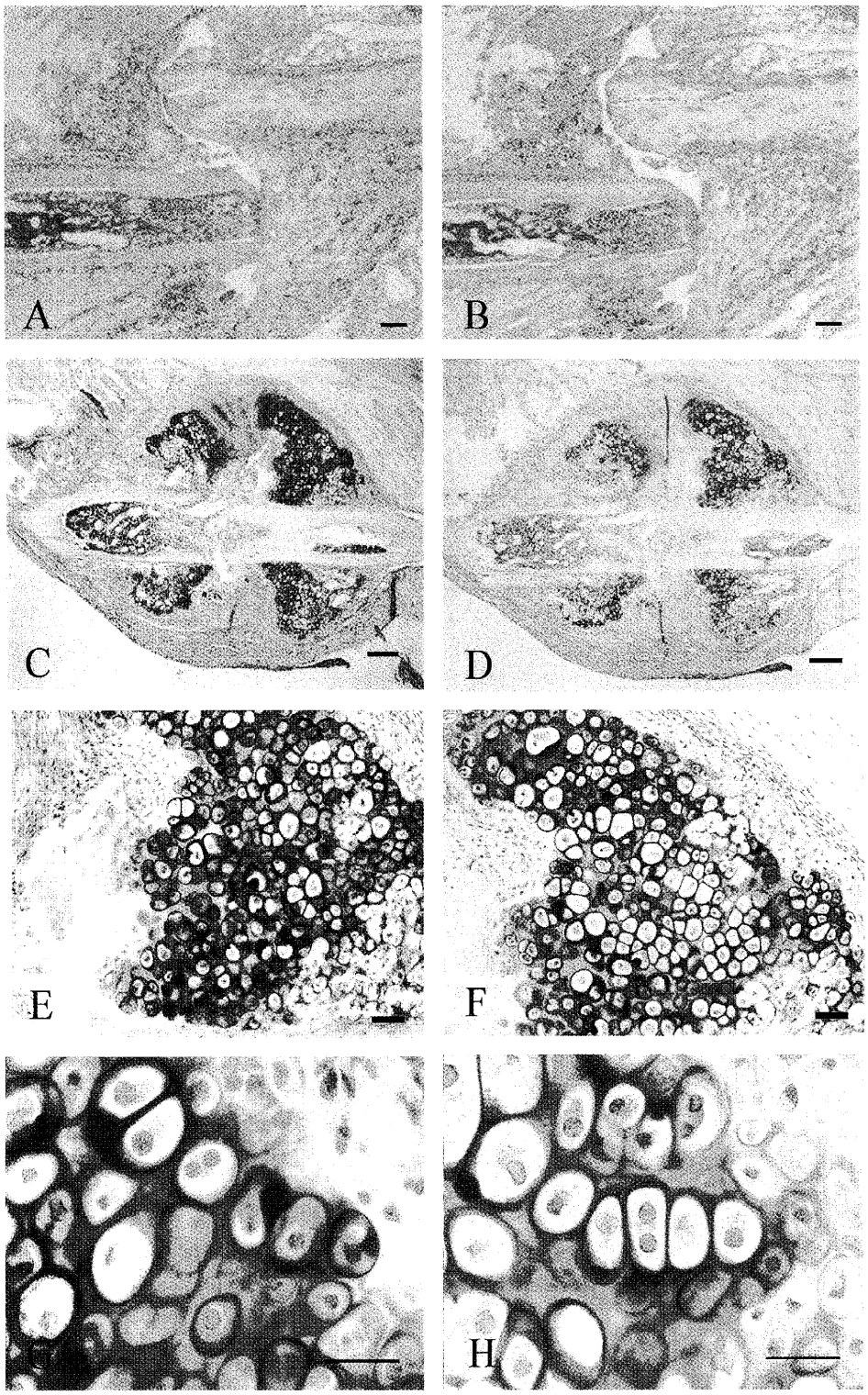

Fig 4. Immunohistochemistry of ephrinB2 and EphB4. **A.** Immunohistochemistry of ephrinB2 in the mouse rib fracture on day 3 shows proteins produced in the periosteum and around the fracture site. **B.** In the serial section of ephrinB2, EphB4 proteins are produced in the same area of ephrinB2. **C.** In the sections on day 10, the proteins can be seen around the fracture sites, especially in the callus. **E** and **G.** In a high magnification, the chondrocytes express ephrinB2. **D, F** and **H.** EphB4 proteins are produced in the same area. **A, B, C** and **D.** *Bar*, 100 μm; **E** and **F.** *Bar*, 50 μm; **G** and **H.** *Bar*, 10 μm.

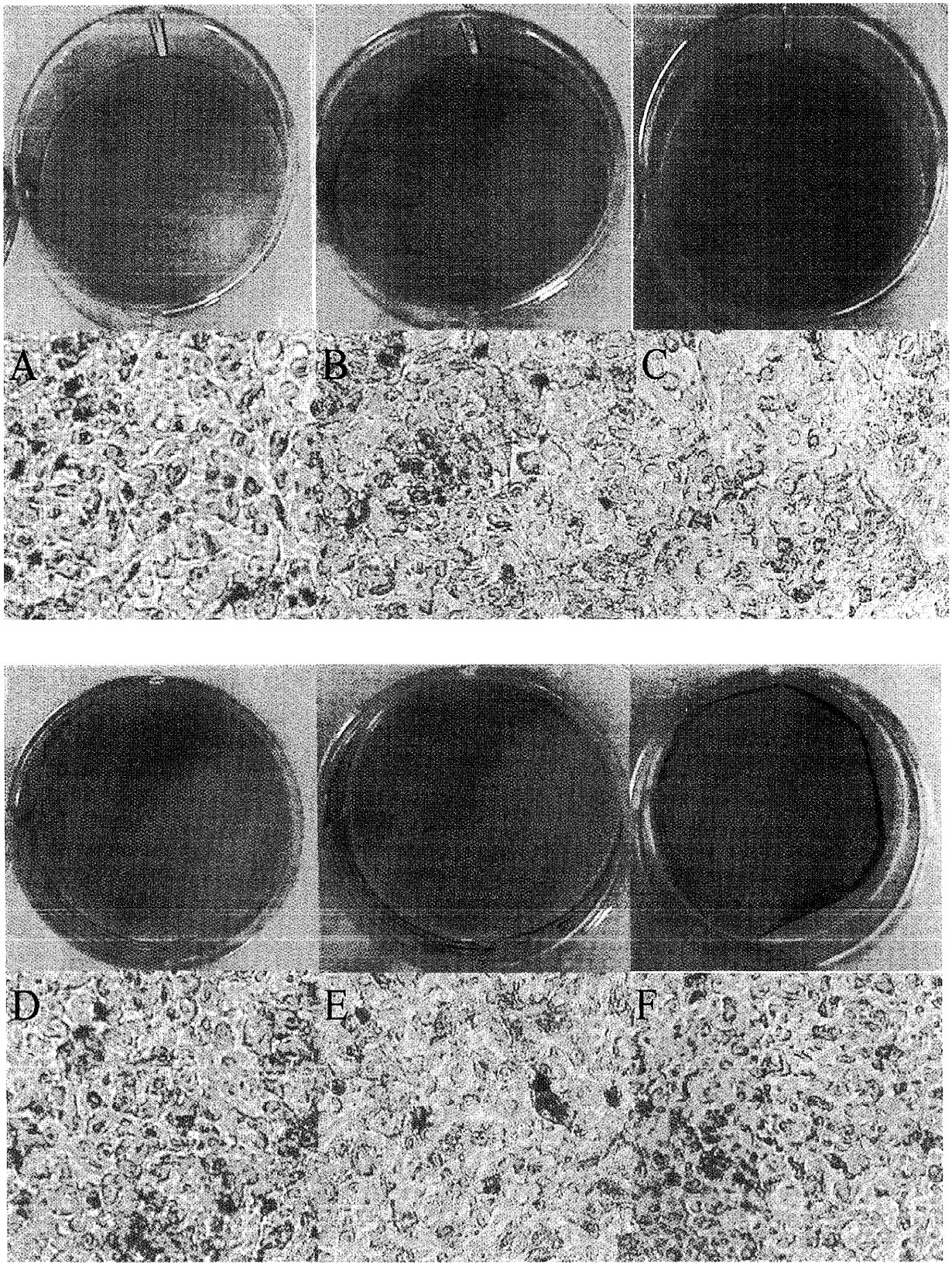

Fig 5. ATDC5 culture stain legend. **A** and **B.** Neither cell after one week in the maintenance medium nor in the medium with ITS-X added are stained by Safranin-O. **C** and **D.** After two weeks, the cells in the maintenance medium are stained slightly red, and in the cells in the medium with ITS-X added, the cartilage nodule is stained red. **E** and **F.** After three weeks, cells in the maintenance medium are stained as at two weeks, and in cells in the medium with ITS-X added, the cartilage nodule is stained more thickly red.

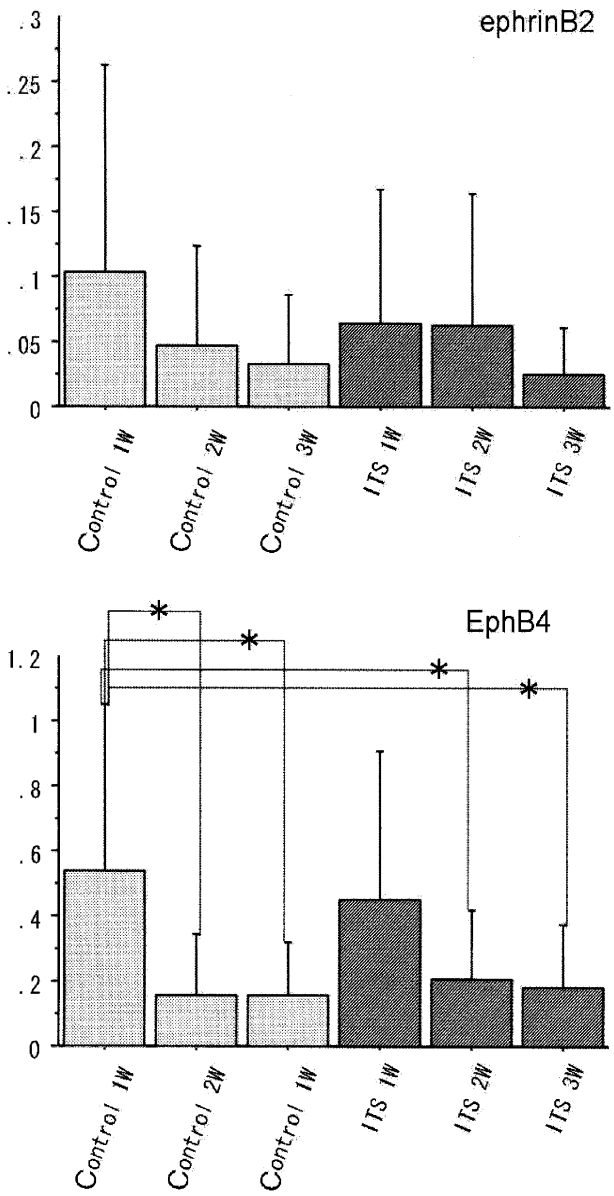

Fig 6. Quantitative RT-PCR of ATDC5 legend. Both ephrinB2 and EphB4 are expressed during the three weeks, and quantitative analysis with LightCycler (Roche Diagostics, Mannhime, Germany) shows that the expression of eprinB2 and EphB4 mRNAs peaked on day 7. The expression of EphB4 mRNA is about five times higher than that of ephrinB2 mRNA.

5'- TTTGAGGAGGCACAGCAGG -3' for the aggrecan sense primer; 5'- GGGCTCACAATGGGGTATC -3' for the aggrecan anti-sense primer; 5'- ACTCACGTTTGGGTAGGCC -3' for the type X sense primer; 5'- TCAGAGGAGTAGAGGCCGT -3' for the type X anti-sense primer; 5'- ATGACCGACGAGCAGGAGAA -3' for the sox9 sense primer, and 5'- TGCACGTCGGTTTTGGGAGT -3' for the sox9 anti-sense primer. The PCR protocol was the same as described above.

RESULTS

Histological features of fracture healing

H&E staining is shown in Fig. 1. We observed a hematoma between the fracture fragments and the proliferation of fibroblasts around the fracture sites on days 1 and 3. On days 5 and 7, endochondral and intramembranous ossifications were identified around the fracture sites, with a fracture callus in which hypertrophic chondrocytes and new woven bones were observed. On day 10, a bridging callus was detected between the fracture stumps, and trabecular bone formation was seen in the marrow spaces. On day 14, the trabeculae bones were newly formed trabeculae. Bone remodeling, replaced with bone marrow, was also identified in the callus on day 18 (Fig. 1).

RT-PCR and quantitative PCR for ephrinB2 and EphB4 mRNAs

ephrinB2 and EphB4 mRNAs were expressed during all phases (Fig. 2). The quantitative PCR showed that the expression of eprinB2 and EphB4 mRNAs peaked on day 7. The expression of ephrinB2 and EphB4 on day 7 was four and two times higher than those on day 1, respectively. The expression of EphB4 mRNA was about 10 times higher than that of ephrinB2 mRNA (Fig. 2).

In situ hybridization

The distributions of ephrinB2 and EphB4 mRNAs were similar in the fracture sites. On day 1, the expression of both mRNAs was observed in the periosteum with faint signals in the bone marrow (Fig. 3). On days 3, 5, and 7, we identified the expression in the cartilage of the fracture callus from the proliferating zone to the hypertrophic zone as well as in the periosteum and the bone marrow. The expression was intense in the prehypertrophic chondrocytes. The hypertrophic chondrocytes and cells of the granulation tissue around the fracture sites also expressed faintly positive signals for ephrinB2 and EphB4 mRNAs (Fig. 3). We did not detect any signals in the fractures with the sense probes. Triplicated studies showed the same results.

Immunohistochemistry

The immunohistochemical localization of ephrinB2 and EphB4 in the fracture sites was similar to those of the mRNA expression of eprinB2 and EphB4 (Fig. 4). On day 1, faintly positive signals for ephrinB2 were detected around the fracture sites. Fibroblasts around fracture sites and vascular endothelial cells showed positive signals on day 3. Intense signals were identified in the proliferating chondrocytes, with faint signals in the hypertrophic chondrocytes on days 5 and 7 (data not shown). On day 10, the hypertrophic chondrocytes exhibited a positive signal, as did the proliferating chondrocytes. The distribution of the positive signals for EphB4 was almost same as those of ephrinB2. The same results were obtained in the other two mice in each group.

Culture of ATDC5

The cells were almost confluent after one week. Neither cells in maintenance medium nor in the medium with ITS-X added were stained by Safranin-O. After two weeks, the cells reached confluence, and differentiation was seen; the cells in the maintenance medium were partially stained slightly red, and in the cells in medium with ITS-X added and the cartilage nodules were stained red. After three weeks, cells in the maintenance medium were unchanged, and in the cells in medium with ITS-X added, the cartilage nodules were stained more thickly red (Fig. 5).

RT-PCR and quantitative analyses of ephrinB2, EphB4 in ATDC5

Both ephrinB2 and EphB4 were expressed during the three weeks, and quantitative analysis with LightCycler (Roche Diagostics, Mannhime, Germany) showed that the expression of eprinB2 and EphB4 mRNAs peaked on day 7. The expression of EphB4 mRNA was about five times higher than that of ephrinB2 mRNA (Fig. 6).

DISCUSSION

Ephrin and Eph families are respectively ligands and receptors with tyrosine kinase activities. These have been identified in several tissues - such as nerve, vascular, and tumor tissues. Little is known about the expression of these molecules during the healing process after injuries. In other animal trauma models such as a rat brain trauma model in which a subdural hematoma was created, most Eph kinase mRNA was not regulated during the recovery[11]; however, EphB4 mRNA and ephrins were not investigated. This is the first report to investigate the mRNA expression and protein production of ephrinB2 and EphB4 in the trauma model.

The co-localization of ephrin and Eph proteins was observed in the sprouting of the vascular formation during vascular development. *In vitro* studies, using a OP9 stromal cell transfected ephrinB2 or EphB4 cDNA and a para-aortic splanchnopleura co-culture system, found that the stromal cells expressing ephrinB2 promoted vascular network formation and ephrinB2+ endothelial cell proliferation and also induced the recruitment and proliferation of alpha-smooth muscle actin-positive cells. Stromal cells expressing EphB4 inhibited vascular network formation, ephrinB2+ endothelial cell proliferation, and arpha smooth muscle actin-positive cell recruitment and proliferation[12]. Using a 32D cell, a hematopoietic cell line that has a requirement of IL-3 or 5% WEHI-condition medium for growth, and transfected EphB4, these 32D EphB4 cells exhibited a higher proliferation rate than did control cells[13]. The stimulation of EphB4 receptors promotes micro-vascular endothelial cell migration and proliferation[14]. *In vivo* studies, mice lacking ephrinB2 at 9.5 embryonal day exhibited a pale yolk sac and a primitive vasculature with a poorly organized network, confirming that ephrinB2 has an essential role in early vascular development[15]. Therefore, ephrin-Eph coupling is essential for regulating endothelial proliferation (sprouting) in vascular formation.

In our study, mRNA expression and the protein production of ephrinB2 and EphB4 were identified on the chondrocytes in the fracture callus. ephrinB2 and EphB4 were identified especially in the proliferating chondrocyte and not in the hypertrophic chondrocyte, which was the terminal differentiated chondrocyte. Quantitative RT-PCR demonstrated that the mRNA expression of ephrinB2 and EphB4 peaked in the early proliferation stage of the fracture callus. In the experiment on ATDC5 chondrification, quantitative RT-PCR demonstrated that the mRNA of ephrinB2 and EphB4 peaked in the proliferation phase. From these data, we speculate that the ephrinB2-EphB4 coupling during fracture healing seemed to play same role as the sprouting in vascular formation to regulate cell proliferation.

ephrinB2 knockout mice show that ephrinB2 and EphB4 mRNAs are also expressed in the arterial and venous endothelial cells, respectively[16]. In an experiment with the pharmacological inhibition of ERK1/2 of HUVECs, ephrinB2-Fc suppressed VEGF-induced proliferation and migration and Angl-induced migration in venous endothelial cells[17]. These results

indicated that ephrin-Eph coupling plays a key role in "boundary formation" between the artery and vein in vascular formation[18]. Otherwise, in neural development, using Zebrafish, Val controls eph expression as well as the interactions between EphB4a and ephrinB2a mediate cell sorting and "boundary formation" in the segmenting caudal hindbrain[19].

Recently, eprinB2 was identified in the epiphyseal cartilage of long bone[20], and its distribution was similar to that of chondromodulin-I, a potent vascular inhibitory factor, as well as chondrogenic growth factor[21,22]. In our study, not only ephrinB2 but also EphB4 were both identified on chondrocytes during fracture healing. Cartilage is a non-vascular matrix in skeletal development and bone repair. Ephrins and Eph families are molecules initially identified in endothelial development. Taken together, these findings show that ephrin-Eph coupling may contribute to certain relationships, such as a boundary formation, between non-vascular cartilage and newly formed vessels in fracture healing.

In conclusion, eprinB2 and EphB4 may play a key role in chondrogenesis during fracture healing, such as promoting the angiogenesis around fracture sites while cooperating with VEGF, or probably control the boundary formation between newly formed cartilage and newly formed bone around the fracture site. This comprises the first report on ephrin-Eph coupling in bone regeneration, and future studies are awaited.

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