

Hepatocyte Growth Factor/Scatter Factor Modulates Cell Motility, Proliferation, and Proteoglycan Synthesis of Chondrocytes

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Abstract. Hepatocyte growth factor/scatter factor (HGF/SF) is a multifunctional growth factor that promotes proliferation, motility, and morphogenesis in epithelial cells. Recently the HGF receptor, c-met proto-oncogene product, has been shown to be expressed in developing limb buds (Sonnenberg, E., D. Meyer, M. Weidner, and C. Birchmeier. 1993. *J. Cell Biol.* 123: 223–235), suggesting that some populations of mesenchymal cells in limb buds respond to HGF/SF. To test the possibility that HGF/SF is involved in regulation of cartilage development, we isolated chondrocytes from knee joints and costal cartilages of 23-d embryonic and 4-wk-old rabbits, and analyzed the effects of HGF/SF on migration and proliferation of these cells. We found that HGF/SF stimulated migration of cultured articular chondrocytes but did not scatter limb mesenchymal fibroblasts or synovial fibroblasts in culture. HGF/SF

also stimulated proliferation of chondrocytes; a maximum three-fold stimulation in DNA synthesis was observed at the concentration of 3 ng/ml of HGF/SF. Moreover, HGF/SF had the ability to enhance proteoglycan synthesis in chondrocytes. The responsiveness of chondrocytes to HGF/SF was also supported by the observation that they expressed the HGF/SF receptor. Addition of the neutralizing antibody to rat HGF/SF affected neither DNA synthesis nor proteoglycan synthesis in rat chondrocytes, suggesting a paracrine mechanism of action of HGF/SF on these cells. In situ hybridization analysis showed that HGF/SF mRNA was restrictively expressed in the areas of future joint regions in developing limb buds and in the intercostal spaces of developing costal cartilages. These findings suggest that HGF/SF plays important roles in cartilage development through its multiple activities.

HEPATOCYTE growth factor, also known as scatter factor (HGF/SF)¹, has been identified, purified, and shown to act as a potent mitogen for primary cultured hepatocytes, and its gene has been cloned (Nakamura et al., 1986, 1987, 1989; Tashiro et al., 1990; Miyazawa et al., 1989). Subsequent studies have revealed that HGF/SF stimulates proliferation of epithelial cells, such as renal tubular epithelial cells (Igawa et al., 1991), keratinocytes, and melanocytes (Rubin et al., 1991; Matsumoto et al., 1991), promotes motility of keratinocytes and canine kidney epithelial cells (MDCK cells) (Stoker et al., 1987;

Matsumoto et al., 1991), and induces the tube formation of MDCK cells (Montesano et al., 1991) and lumen formation of mammary cells (Tsarfaty et al., 1992). Thus HGF/SF is a multipotential factor which acts on epithelial cells as a mitogen, motogen, and morphogenetic factor.

In addition to epithelial cells, mesenchymal cells have been found to be targets of HGF/SF. Two independent studies have demonstrated that HGF/SF stimulates DNA synthesis of hematopoietic cells (Kmieciak et al., 1992; Mizuno et al., 1993). HGF/SF also promotes vascular endothelial cell migration, proliferation, and capillary-like tube formation (Grant et al., 1993). Further, Sonnenberg et al. (1993) have found by in situ hybridization that HGF/SF receptor gene is expressed in developing limb buds, suggesting that HGF/SF may also have roles in the development of mesenchymal cells.

In the limb buds, cartilage formation is proceeded by condensation of mesenchymal cells and differentiation of the condensed cells into chondrocytes, and cartilage is subsequently replaced by bone (Horton, 1990). This series of

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1. *Abbreviations used in this paper:* HGF/SF, hepatocyte growth factor/scatter factor; IGF-I, insulin-like growth factor-I; IGF-II, insulin-like growth factor-II; PTH, parathyroid hormone; TGF- β , transforming growth factor β ; ZPA, zone of polarizing activity; AER, apical ectodermal ridge; p.c., postcoitus.

processes, collectively known as endochondral ossification, is responsible for the formation of skeletal structures in limb buds. Many growth factors, including fibroblast growth factors (FGFs) (Frenz et al., 1993), bone morphogenetic proteins (BMPs) (Lyons et al., 1990), and insulin-like growth factors (Streck et al., 1992) have been shown to be expressed in developing limb buds. Expression of these growth factors is temporally and spatially specific, and they modulate chondrogenesis and proliferation and/or differentiation of chondrocytes in vitro (Tabin, 1991; Francis et al., 1993; Frenz et al., 1993; Iwamoto et al., 1989a). Therefore an interplay among these factors is thought to regulate skeletogenesis in developing limb buds.

In this study, we have investigated the possibility that HGF/SF is also a regulator of skeletogenesis. We found that chondrocytes expressed the HGF/SF receptor and the HGF/SF stimulated the motility, proliferation, and proteoglycan synthesis in chondrocytes. Furthermore, in situ hybridization analysis revealed that HGF/SF mRNA was restricted to the areas of the future joint regions in developing limb buds and to intercostal spaces near the tips of developing costal cartilages. These findings suggest the implication of HGF/SF in cartilage development by its multiple stimulatory actions on motility, proliferation and maturation of chondrocytes.

Materials and Methods

Materials

Human and rat recombinant HGF/SF were purified from the conditioned medium of CHO cells transfected with an expression vector containing HGF/SF cDNA as described previously (Seki et al., 1991; Tashiro et al., 1990). We used human recombinant HGF/SF in all the experiments unless specified. A bovine active parathyroid hormone fragment (synthetic PTH [1-34]) was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Transforming growth factor- β (TGF- β), insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) were purchased from Cosmo Bio. Co. LTD. (Tokyo, Japan). A polyclonal antibody to HGF/SF was raised against rat recombinant HGF/SF.

Cells and Cell Culture

Chondrocytes were isolated from 23-d embryonic and 4-wk-old postnatal New Zealand white rabbits and 4-wk-old Wistar rats as described previously (Kato et al., 1987). Articular chondrocytes and costal chondrocytes were isolated from femoral articular cartilage of knee joints and the hyaline cartilage of ribs. Synovial fibroblasts were isolated from synovial tissue of the knee joints. Minced synovial tissue fragments were cultured in DME containing 10% FBS (medium A) for 10 d, and overgrown cells were collected by trypsinization. Embryonic mesenchymal cells were isolated from the limb bud muscle tissue of 20-d rat embryos as described previously (Thompson et al., 1985). The cells were fed every other day.

DNA Synthesis

The rate of DNA synthesis was estimated by measuring the incorporation of [3 H]thymidine ([6- 3 H]thymidine; Amersham Corp., Arlington Heights, IL, S.A. 20 Ci/mmol) into 5% TCA-insoluble cell precipitates. Cells were seeded at a density of 1.5×10^4 cells/6-mm well in a 96-well plates and grown until confluence. For growth arrest, the cells were preincubated with 0.1 ml of DME containing 0.3% FBS for 24 h, after which various concentrations of HGF/SF were then added to the medium and the incubation continued for a further 24 h. 1 μ Ci/ml of [3 H]thymidine was added 3 h before the end of incubation. After labeling, the cultures were washed three times with ice-cold PBS, twice with 5% TCA containing 3 mM thymidine and once with ethanol/diethyl ether (3:1). The residues in the wells were solubilized in 100 μ l of 0.1 N NaOH, and transferred into scintillation vials. The solution was neutralized by adding 1 N HCl, and radioactivity was measured by scintillation counter (Rack-beta; Pharmacia LKB, Uppsala, Sweden).

Proteoglycan Synthesis

Chondrocytes were seeded at a density of 1.5×10^4 cells/6-mm well and maintained in 0.1 ml of medium A. When the cells became confluent they were preincubated for 24 h in 0.1 ml DME supplemented with 0.3% FBS. They were then incubated for 24 h in 0.1 ml of DME supplemented with 0.3% FBS and various concentrations of HGF/SF. 1 μ Ci/ml of [35 S]sulfate was added 20 h before the end of incubation. Proteoglycan synthesis was determined by measuring incorporation of [35 S]sulfate into materials precipitated with cetylpyridinium chloride after protease digestion (Kato et al., 1980). The parallel cultures which received the identical treatment were used for determination of DNA contents (Koike et al., 1990). The incorporation of [35 S]sulfate into glycosaminoglycans was standardized on the basis of μ gDNA. The data were statistically assessed by student's *t*-test.

Total RNA Preparation and Reverse-transcribed PCR

Total RNA was prepared by the method of Smale and Sasse (1992) with minor modifications. Freshly isolated cartilage fragments (0.1 g wet weight) or confluent cultures of chondrocytes (90 mm dish \times 2) were quickly homogenized in 2 ml of 4 M guanidine thiocyanate, 0.1 M Tris-HCl (pH 7.5) and 1% 2-mercaptoethanol (4 M GITC solution). The homogenate was mixed with 100 μ l of 10% sodium lauryl sarcosine, and spun for 5 min using a microcentrifuge. 2 ml of supernatant was overlaid on an equal volume of 1.6 g density gradient of cesium trifluoroacetate, 1 mM EDTA (pH 8.0) in a Beckman polyallomer centrifuge tube (13 \times 51 mm). The samples were then centrifuged at 35,000 rpm (147,000 *g*) for 20 h at 18°C. After removal of the supernatant by aspiration, the precipitate was dissolved in 200 μ l 4 M GITC solution and then extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The extracts were mixed with 20 μ l of 3 M sodium acetate (pH 4.8) and precipitated with 2 vol (440 μ l) of ethanol. The pellet was dissolved in 50–100 μ l DEPC-treated water.

First strand cDNA synthesis from 0.5 μ g total RNA was performed using Superscript reverse transcriptase (GIBCO BRL, Gaithersburg, MD) and downstream antisense primers. Subsequent amplification was carried out for 35 or 40 cycles under the following conditions: 94°C for 30, 58°C for 1 min, and 72°C for 1.5 min. Primer sequences for PCR amplification were as follows: 5'-CAGT(A/G)ATGATCTCAATGGGCAAT-3' and 5'-AATGCCCTTCTCTATGACTTC-3' for rat (Park et al., 1987) and mouse (Chan et al., 1988) c-met; respectively, generating a 725-bp fragment. The amplified products were analyzed by 1.5% agarose gel electrophoresis, transferred to the nylon membrane and then hybridized with 32 P-labeled nick translated rat c-met cDNA probe (Honda, S., and T. Nakamura, manuscript in preparation) to verify the amplified products are c-met as described previously (Delidow et al., 1993).

In Situ Hybridization

A 1.4-kb EcoRI fragment of a rat HGF/SF cDNA (RBC1 clone) (Tashiro et al., 1990) was subcloned into the pGEM7 vector to synthesize both antisense and sense run-off transcripts labeled with [α - 35 S]-UTP (400 Ci/mmol; Amersham Corp.). The labeled transcripts were alkaline hydrolyzed to 50–150 nucleotides for use as riboprobes.

In situ hybridization was performed as described previously (Noji et al., 1990). Briefly, samples were fixed in 4% paraformaldehyde in phosphate-buffered saline, dehydrated in ethanol, cleared with toluene, and embedded in paraffin. Sections 5- μ m thick were cut and then mounted on poly-L-lysine-coated glass slides. The sections were deparaffinized, treated with glycine and acetic anhydride, and hybridized with probes at 50°C for 16 h. After hybridization, the slides were washed with 2 \times SSC at 50°C for 1 h, treated with RNase A (20 μ g/ml) at 37°C for 30 min, and then washed twice with 0.1 \times SSC at 50°C for 1 h. The slides were dipped into emulsion (1:1 dilution of Kodak NBT-2) and exposed for 2 wk, after which they were developed with Kodak D-19, fixed, and stained with hematoxylin and eosin.

Results

HGF/SF Has Motogenic and Mitogenic Activities on Chondrocytes, But Not on Other Mesenchymal Cells

First we investigated whether mesenchymal cells in limb buds respond to HGF/SF. Since limb buds consist of various kinds of cells such as myogenic cells, chondrogenic

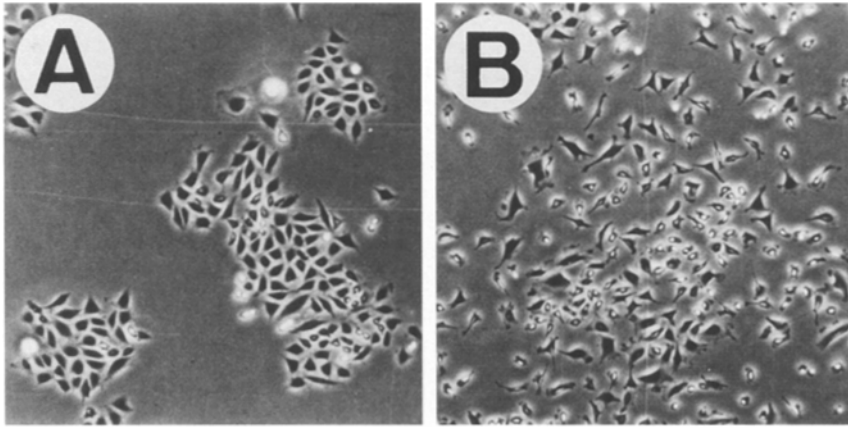


Figure 1. Stimulation of chondrocyte scattering by HGF/SF. Rabbit articular chondrocytes were seeded at a density of 3×10^3 cells/16-mm well, maintained in medium A for 2 d, and then treated with HGF/SF for 2 d. Phase contrast micrographs were taken at the end of incubation. (A) Control; (B) HGF/SF (3 ng/ml).

cells and fibroblasts, a target cell population of HGF/SF is difficult to determine using whole limb bud cell cultures. To clarify the effects of HGF/SF on homogeneous cells which express distinct phenotypes, we isolated chondrocytes from knee articular cartilage and costal cartilage, synovial cells from knee joints, and fibroblasts overgrown from limb muscle tissues.

To study the effects of HGF/SF on cell motility, the cultures were treated with HGF/SF. Fig. 1 showed the scattering action of HGF/SF on articular chondrocytes. In

control cultures, the chondrocytes exhibited a polygonal morphology and formed clusters (Fig. 1 A). In contrast the cells scattered and formed no clusters when treated with HGF/SF (Fig. 1 B). The factor failed to scatter fibroblasts and synovial cells (data not shown). Thus HGF/SF selectively acts on articular chondrocytes as a scatter factor.

To examine the effects of HGF/SF on proliferation of chondrocytes, confluent cultures were serum-starved for 24 h, and treated with various doses of HGF/SF. A dose-dependent increase in [3 H]thymidine incorporation into DNA was observed in chondrocytes isolated from 4-wk-old rabbits and 23-d rabbit embryos (Fig. 2 A). A maximum threefold stimulation in DNA synthesis occurred in the presence of 1 ng/ml HGF/SF, while fibroblasts showed no response (Fig. 2 A). HGF/SF also promoted cell proliferation of chondrocytes. Low-density cultures of articular chondrocytes maintained in DME containing 10% FBS were treated with 10 ng/ml of HGF/SF for 48 h, and cell numbers were determined at the end of this incubation period. At this dose, HGF/SF increased cell number about 1.8-fold (Fig. 2 B).

Cartilage formation requires not only proliferation, but also matrix production by chondrocytes. HGF/SF stimulated [35 S]sulfate incorporation into macromolecules (glycosaminoglycans) produced by chondrocytes in a dose-dependent manner (Fig. 3), with a maximal stimulation at 1 ng/ml, indicating that HGF/SF modulates proteoglycan synthesis in chondrocytes. This effect was weaker than those of TGF β (Inoue et al., 1989) and PTH (Koike et al., 1990) but comparable with those of IGF-I and IGF-II (Fig. 3) (Kato et al., 1980). The stimulation of proteoglycan synthesis by HGF/SF was transient. Long-term treatment (1 wk) with HGF/SF did not increase the total content of uronic acid which represents the amount of proteoglycans in culture (data not shown).

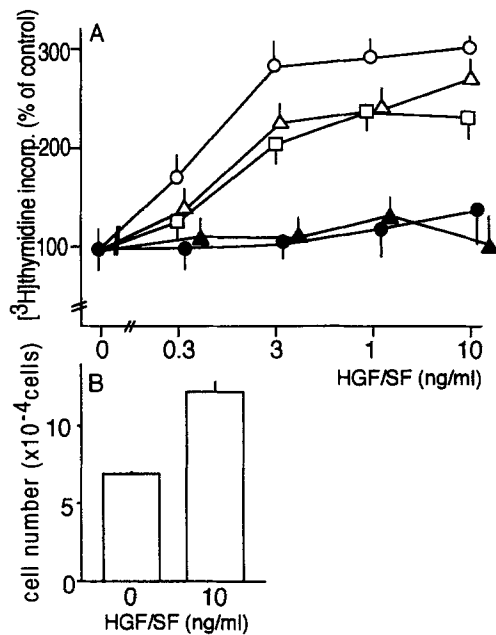


Figure 2. The effect of HGF/SF on proliferation of cultured chondrocytes. (A) Confluent cultures were treated with increasing concentrations of HGF/SF, and labeled with [3 H]thymidine as described in Materials and Methods. Values are indicated % of control and means of three cultures \pm standard deviation. The same tendency was observed in two independent experiments. (○) Articular chondrocytes isolated from 4-wk-old rabbits. (□) Chondrocytes isolated from costal cartilage. (Δ) Chondrocytes isolated from 23-d rabbit embryos. (●) Rat embryonic mesenchymal cells. (▲) Rabbit synovial fibroblasts. (B) Rabbit articular chondrocytes were seeded at a density of 1×10^4 cells/16-mm well and then treated with 10 ng/ml HGF/SF. Cell numbers were counted 2 d after the treatment.

HGF/SF Acts on Chondrocytes via a Paracrine Mechanism

In general, HGF/SF is produced by mesenchymal cells and acts on target cells via a paracrine mechanism. Since chondrocytes are mesenchymal cells, we examine whether chondrocytes produced HGF/SF and use it in an autocrine fashion or whether they respond to HGF/SF via a paracrine mechanism. We used a polyclonal antibody against rat HGF/SF to neutralize endogenous HGF/SF. Rat articular chondrocytes were treated with or without the anti-

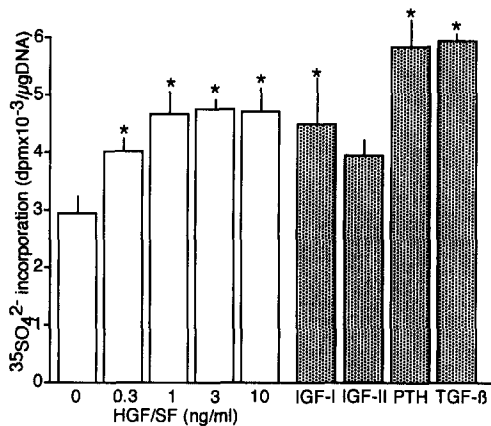


Figure 3. The effect of HGF/SF on proteoglycan synthesis by cultured chondrocytes. Confluent cultures of articular chondrocytes were treated with various factors and labeled with [³⁵S]sulfate as described in Materials and Methods. The concentrations of these factors were as follows: *IGF-I*, 100 ng/ml; *IGF-II*, 100 ng/ml; *PTH*, 10⁻⁷ M; *TGF-β*, 3 ng/ml. Histograms show the amount of [³⁵S]sulfate incorporation which represents synthetic activity of sulfated proteoglycans. Values are means of three cultures ± standard deviation. **P* < 0.05 vs. control.

body in the presence or absence of rat HGF/SF and then labeled with [³H]thymidine or [³⁵S]sulfate. The antibody alone affected neither DNA synthesis nor proteoglycan synthesis in control cultures (Fig. 4, *A* and *B*). However, the antibody completely blocked the effects of exogenously added HGF/SF (Fig. 4, *A* and *B*). These findings indicate that chondrocytes in culture do not produce enough HGF/SF to modulate their own functions.

Expression of the HGF/SF Receptor Gene (*c-met*) in Chondrocytes

Since the findings above showed that chondrocytes responded to HGF/SF, it was expected that chondrocytes express the HGF/SF receptor (*c-met*). Expression of *c-met* in chondrocytes in vivo and in vitro was examined by RT-

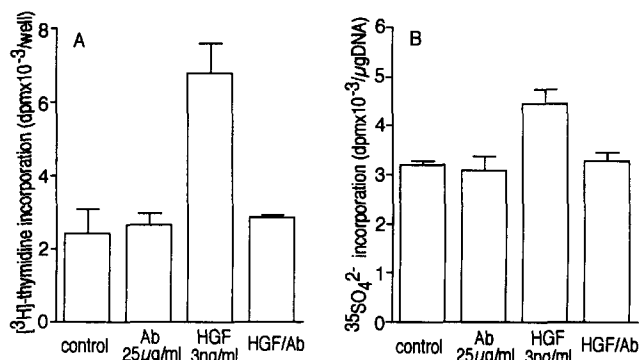


Figure 4. The effects of HGF/SF neutralizing antibody on DNA and proteoglycan synthesis by chondrocytes. Confluent cultures of rat articular chondrocytes were treated with or without 25 μg/ml of affinity-purified IgG fraction of an anti-rat-HGF/SF polyclonal antibody in the presence or absence of 3 ng/ml rat HGF/SF, and then labeled with either [³H]thymidine (*A*) or [³⁵S]sulfate (*B*). Values are means of three cultures ± standard deviation.

PCR. Articular and rib cartilages were dissected out from 4-week-old rats and total RNA was extracted as described in Materials and Methods. The extracted RNA was reverse transcribed, and amplified using *c-met* primers. We detected trace amounts of *c-met* expression in both articular and rib cartilages after 40 cycles of PCR amplification (Fig. 5). In contrast, significant expression of the *c-met* gene were detected in cultured chondrocytes after 35 cycles of PCR amplifications, indicating that chondrocytes expressed higher levels of *c-met* mRNA in culture in vitro than in vivo (Fig. 5).

Expression of HGF/SF mRNA in the Developing Limb

Our data raise the possibility that HGF/SF acts on chondrocytes in vivo. Since the paracrine mechanism of the action of HGF/SF on chondrocytes was indicated, we examined whether HGF/SF is produced in the developing limb buds and the costal regions where cartilage development proceeds actively.

In 10.5-d postcoitus (p.c.) embryos, diffuse expression of HGF/SF mRNA was detected around the proximal region of the limb (Fig. 6, *A-D*). At 11-d p.c., HGF/SF mRNA was detected over the entire proximal region (Fig. 6, *E-H*). At this stage, no cartilaginous condensation occurred in the limb. As cartilaginous condensation proceeded, ex-

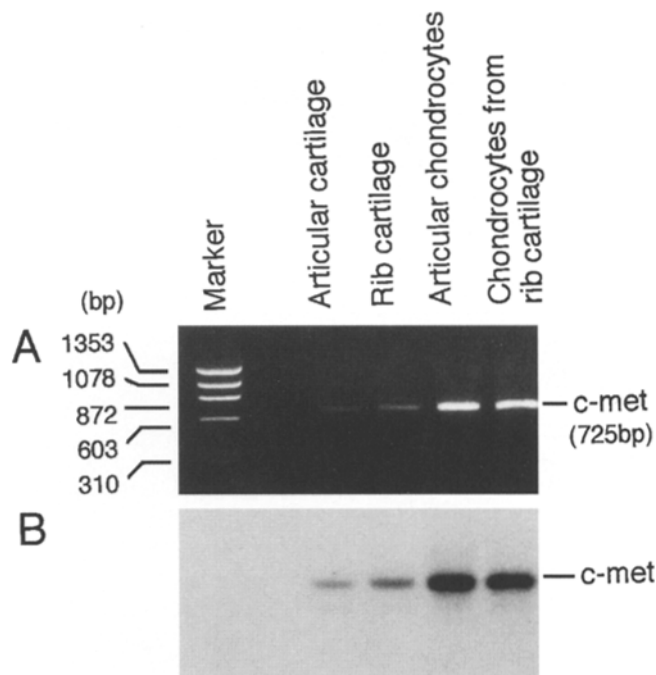


Figure 5. Expression of *c-met* in chondrocytes in vitro and in vivo. Total RNA was prepared from articular and rib cartilages from cartilages of 4-wk-old Wistar rats and cultured chondrocytes isolated from these cartilages. 0.5 μg of the total RNA from each samples were reverse-transcribed, amplified 35 cycles (cultured chondrocytes) or 40 cycles (cartilages), and analyzed by 1.5% agarose gel electrophoresis (*A*). Amplified products were transferred to the nylon membrane and then hybridized with [³²P]-labeled rat *c-met* cDNA probe to verify the amplified products are *c-met* (*B*).

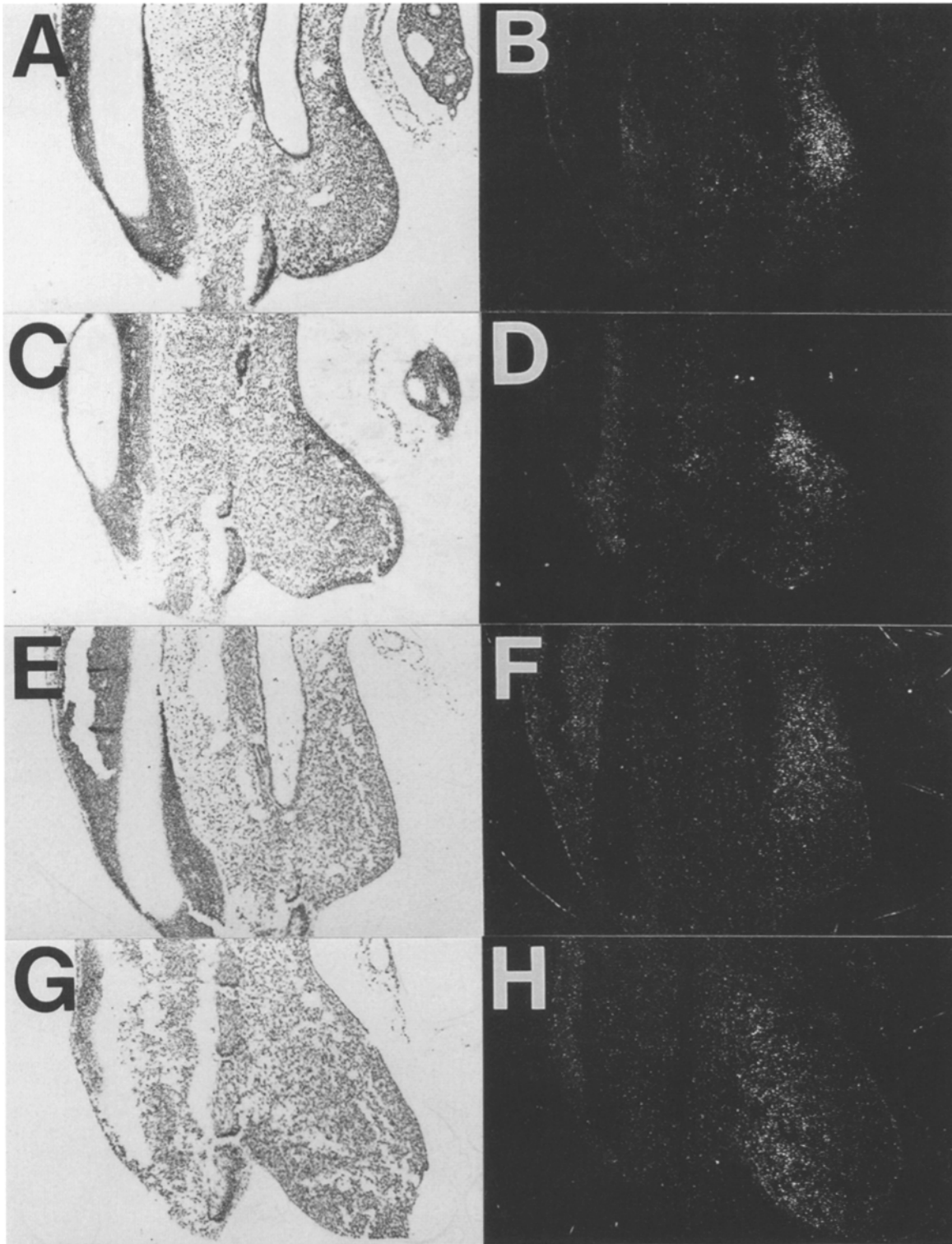


Figure 6. Localization of the HGF/SF mRNA in limb buds at the early developmental stages. Sagittal sections of the hind limb of various ages of mouse embryos were hybridized with a ^{35}S -labeled rat HGF/SF riboprobe. Bright-field (*left*) and corresponding dark-field (*right*) photomicrographs were taken after in situ hybridization, autoradiography, and staining. (*A–D*): sections of 10.5-d p.c. embryo. (*E–H*): sections of 11-d p.c. embryo.

pression of the HGF/SF gene became more restricted (Fig. 7). When stylopodial, zeugopodial, and autopodial elements were formed, expression of the HGF/SF gene was observed in the future ankle and knee joint regions (Fig. 7, *A–D*). At later stages (13-d p.c.), HGF/SF transcripts were

restricted to mesenchymal cells adjacent to cartilaginous condensations at the ankle and knee regions (Fig. 7, *E–J*). In the forelimb, we also observed expression of HGF/SF gene in the future wrist and elbow joint regions (data not shown). At 16-d p.c., tibia and tarsal bones were clearly

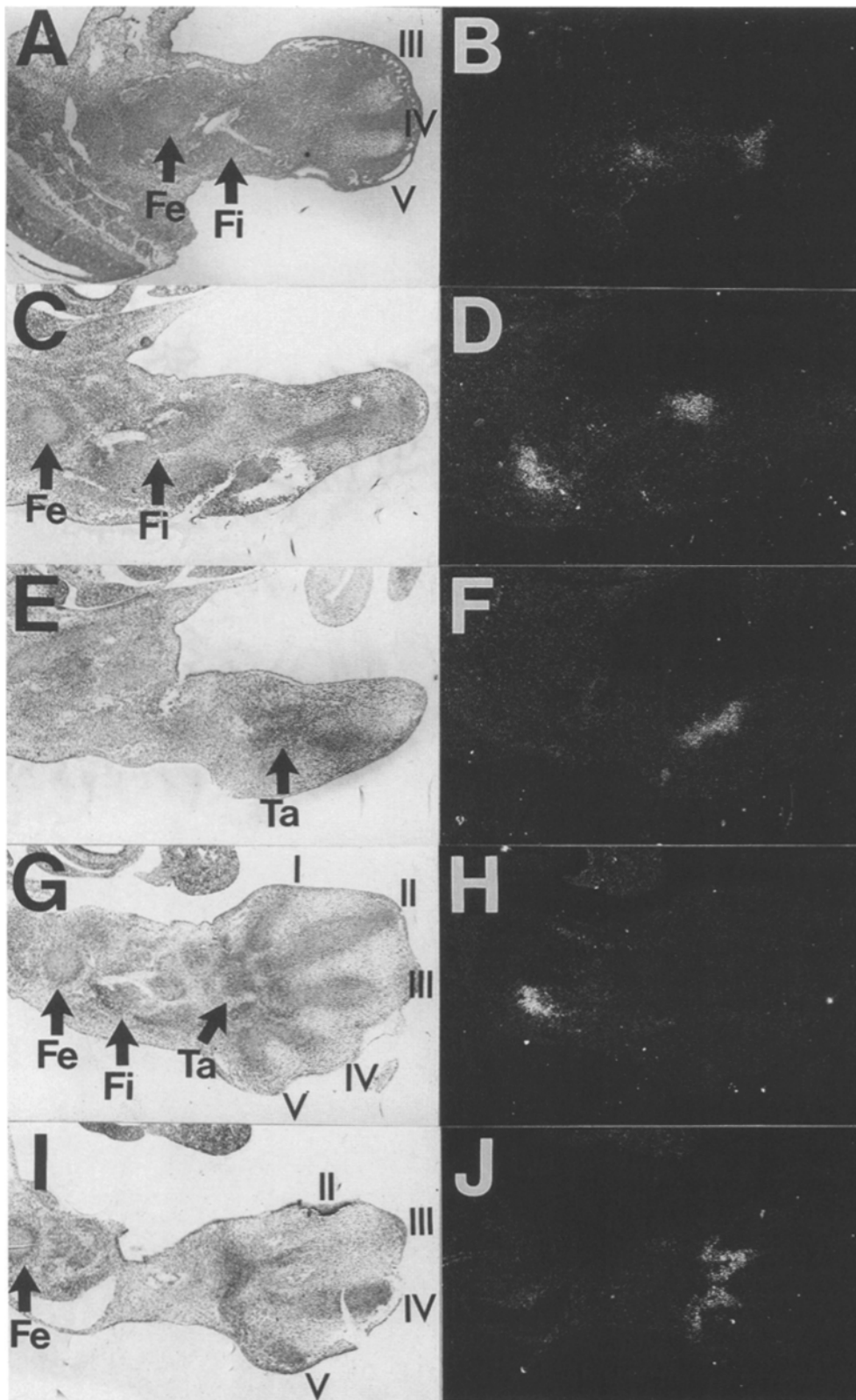


Figure 7. Localization of the HGF/SF mRNA in limb buds at the digit-forming stages. Sagittal sections of the hind limbs of various ages of mouse embryos were hybridized with ^{35}S -labeled rat HGF/SF riboprobe. Bright-field (left) and corresponding dark-field (right) photomicrographs were taken after in situ hybridization, autoradiography, and staining. **A** and **B** Sections of 12.5-d p.c. embryo. (**C-F**) Sections of 13-d p.c. embryo. (**G-J**) Sections of 14-d p.c. embryo. *Fe*, femur; *Fi*, fibula; *Ta*, tarsal bone; *I*, *II*, *III*, *IV*, and *V*, digit number.

separated, while joint formation between tarsal and digit bones was still at early stages (Fig. 8 *A*). In ankle joints, slight expression of HGF/SF gene was detected in the perichondrium of the distal surface of tibia (Fig. 8, *A* and *B*). In contrast, stronger expression of the gene was detected in the future joint space between tarsal and digit bones. Fig. 9 summarizes the expression pattern of HGF/SF gene

in developing limb buds. Throughout the experiment, no significant signals for HGF/SF mRNA were detected in the bifurcation between tibia and fibula or ulna and radius, or in the interdigit spaces.

The HGF/SF gene was also expressed in the intercostal mesenchyme around the tips of elongated precartilaginous condensations of ribs (Fig. 8, *C-F*). Expression of HGF/SF

gene was observed throughout cartilage formation. After the completion of rib cartilage formation, expression of HGF/SF gene decreased and eventually disappeared (data not shown). No positive hybridization signal was detected in the precartilaginous condensations or cartilage itself (Fig. 8, C-F).

Discussion

It is generally accepted that mesenchymal cells are a source of HGF/SF, but they are not the target cells. The present study, however, demonstrates that chondrocytes express the HGF/SF receptor and have the ability to respond to this factor. Gherardini and Stoker (1991) have reported that HGF/SF induces the tube formation of endothelial cells in three dimensional culture. Kmiecik et al. (1992) and Mizuno et al. (1993) have reported that HGF/SF stimulates DNA synthesis of hematopoietic cells, which

are also of mesenchymal origin. These findings indicate that HGF/SF acts on a variety of cells of both epithelial and mesenchymal origin.

HGF/SF stimulated both proliferation and proteoglycan synthesis in chondrocytes. The potency of HGF/SF as a mitogen in chondrocytes was weaker than that of FGF, which is the most potent growth factor for these cells (Kato et al., 1987). However, under the same culture conditions, HGF/SF also stimulated proteoglycan synthesis by chondrocytes, while FGF did not. Further, HGF/SF functions as a mitogen for chondrocytes. No growth factors have been previously identified which modulate migration, proliferation, and proteoglycan synthesis of chondrocytes. Thus HGF/SF is an unique multifunctional factor for chondrocytes.

Our in situ hybridization analysis revealed that HGF/SF gene was expressed in embryonic sites where chondrogenesis is actively taking place. In the limb bud, we found a re-

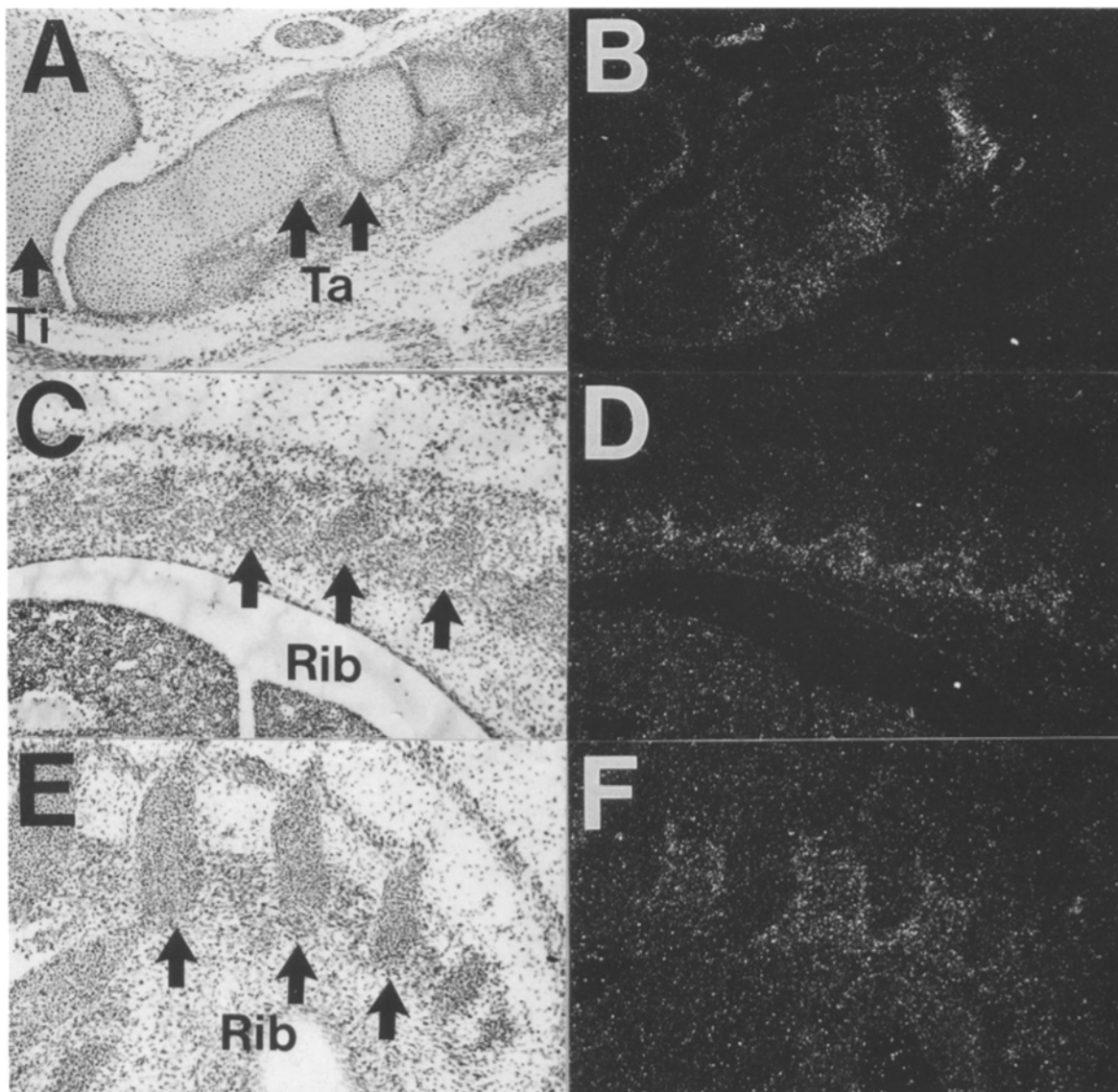


Figure 8. Expression of the HGF/SF mRNA in the developing limb and thorax. Transverse section of hind limb of 16-d p.c. mouse embryo (A and B) and sagittal sections of the thorax of 13-d p.c. (C and D) and 14-d p.c. (E and F) mouse embryos were hybridized with a rat HGF/SF riboprobe. Bright-field (left) and corresponding dark-field (right) photomicrographs were taken after in situ hybridization, autoradiography, and staining. Ta, tarsal bones; Rib, precartilaginous condensation of rib cartilage.

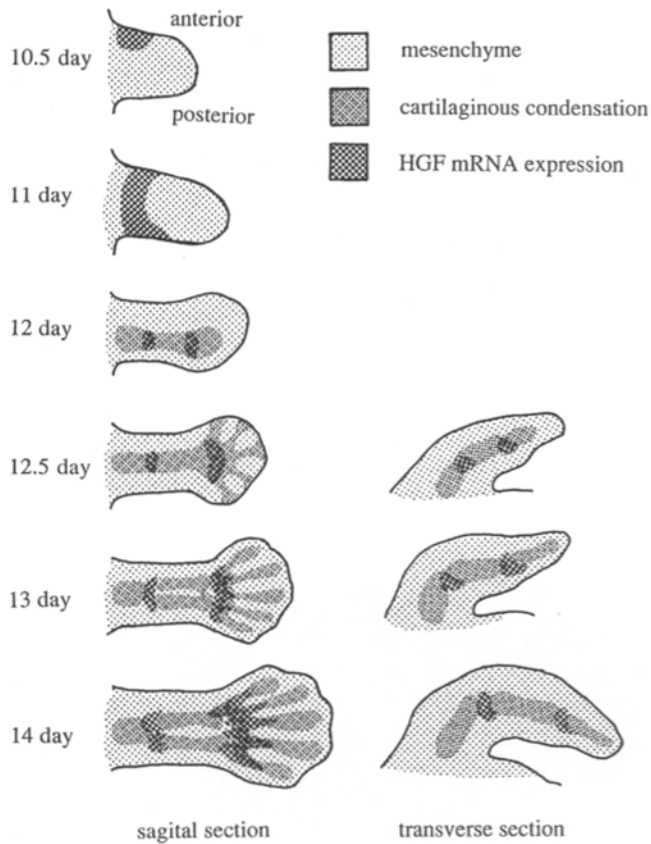


Figure 9. Expression of the HGF/SF gene in developing limb buds. Schematic drawings show the areas of HGF/SF gene expression during limb development. The drawings were based on the results of observations of serial sagittal sections from two independent experiments. HGF/SF gene was first expressed in antero-proximal portion of limb buds, and spread in entire proximal region. As cartilaginous condensations proceeded, expression of the gene became restricted in the future joint regions.

stricted expression pattern of the HGF/SF gene to areas of future joint regions, such as wrist, ankle, elbow, and knee region. In the developing ribs, expression of HGF/SF gene was detectable in the intercostal spaces near the tips of developing costal cartilages. In contrast to HGF/SF, we failed to detect the expression of the HGF/SF receptor, c-met, *in vivo* by *in situ* hybridization. Only RT-PCR revealed expression of the receptor in the costal cartilages. We conclude that chondrocytes express the HGF/SF receptors, although the expression level is probably quite low. Taken together with the finding that HGF/SF modulates the function of chondrocytes *in vitro*, this factor is likely to be involved in proliferation and differentiation of chondrocytes *in vivo*. The target cells for HGF/SF could not be determined in the limb buds, thereby the role of HGF/SF in the limb buds is still unclear. However, it is possible that HGF/SF is implicated in the control of chondrogenesis. In addition, HGF/SF may be an inducer of chondrogenesis, because the HGF/SF gene is expressed in limb buds before mesenchymal condensation for chondrogenesis.

HGF/SF has strong morphogenetic activities on various cell types (Matsumoto and Nakamura, 1992b) and its expression has been observed in sites where tissue organiza-

tion actively occurs (Sonnenberg et al., 1993). This indicates that the factor may regulate morphogenesis of various organs. We find that HGF/SF gene is expressed in limb buds at the fairly early stage (11-d p.c.). At this stage cartilaginous condensation has not occurred yet. The finding that HGF/SF is present in limb buds before chondrocytes appear suggests that this factor may also have a regulatory role on pattern formation of limb, in addition to its control of cartilage development. The complex architecture of the limb cartilage patterns was formed by the sequential segmentation and branching processes of precartilaginous condensation. These processes seem to be regulated by interaction of diffusible factors from the zone of polarizing activity (ZPA) and the apical ectodermal ridge (AER) (Saunders et al., 1976; Hinchliffe and Gumpel-Pinot, 1981). Yonei et al. (1993) have reported that transplantation of MRC-5 cells, producers of HGF/SF (Stoker et al., 1987), into anterior-proximal region of chick limb buds caused duplication of digit formation. Since ZPA is essential for the polarization of anterior-posterior axis of the limb, their results raised the possibility that HGF/SF may be a part of ZPA factors. However, at early stages of limb development, we found that HGF/SF gene is not expressed around ZPA region. Our results suggest that HGF/SF does not mediate a signal from ZPA and that MRC-5 may produce another regulatory molecules. However, HGF/SF might indirectly affect proximal-distal growth of limb by acting on AER.

We found that the cultured chondrocytes express considerable amounts of c-met mRNA whereas expression of c-met mRNA in cartilage tissue was low. In cartilage, the proliferation and other metabolic activities of chondrocytes are strictly regulated by the mechanisms such as cell-extracellular matrix and cell-cell interactions, and the proliferative activity of chondrocytes is extremely low except in the proliferating zone of growth plate cartilages. However, once chondrocytes are liberated from their surrounding extracellular matrix by enzyme digestion and cultured *in vitro*, the cells duplicate actively (Iwamoto et al., 1989b). There may be a correlation between the expression of the HGF/SF receptor on chondrocytes and their proliferative activity. In a preliminary experiment, we observed up-regulation of expression of c-met and HGF/SF mRNAs in experimentally injured articular cartilage (data not shown). Articular cartilage of osteoarthritis often exhibit mitotic profiles of chondrocytes as well as degradation of cartilage matrix. The mitotic profiles are thought to be the results from recovery of the injured cells and release from the regulation by the extracellular matrix. We assume that HGF/SF might be also involved in the repair of the injured cartilage by promoting proliferation of chondrocytes. It is noteworthy that 5' regulatory elements of the HGF/SF gene contain the response elements for IL-1 and IL-6 (Miyazawa et al., 1991), which are closely related with joint diseases such as rheumatoid arthritis and osteoarthritis (Duff, 1989; Lipsky et al., 1989; Venn et al., 1993). Indeed IL-1 induces expression of HGF/SF in stromal cells *in vitro* (Matsumoto et al., 1992a). In conclusion, HGF/SF may regulate cartilage development both physiologically and pathologically through the multiple actions on chondrocytes, including stimulation of cell motility, proliferation and proteoglycan synthesis.

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