Tyrosine kinase-type receptor ErbB4 in chondrocytes: interaction with connective tissue growth factor and distribution in cartilage

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Abstract **In order to identify receptor molecules that participate in the growth and differentiation of chondrocytes, we cloned a number of cDNA fragments from HCS-2/8 chondrocytic cells, by using tyrosine kinase-specific primers for amplification. The mRNA expression of one such receptor, ErbB4, was increased by connective tissue growth factor/hypertrophic chondrocyte-specific gene product (CTGF/Hcs24), which promotes all stages of the endochondral ossification in vitro. ErbB4 expression was observed through all stages of chondrocytic differentiation in vitro, corresponding to the wide distribution of CTGF/Hcs24 target cells. Furthermore, positive signals for erbB4 mRNA were detectable throughout most populations of chondrocytes, in growth and articular cartilage in vivo. These results demonstrate for the first time that ErbB4 is expressed in chondrocytes and may play some roles in chondrocytic growth and differentiation along with CTGF/Hcs24.**

Key words: Connective tissue growth factor; Receptor; Chondrocyte; Cartilage; ErbB4

1. Introduction

Endochondral ossification is a complex biological process that determines the growth and ossification of longitudinal bones, which tasks are accomplished by highly organized layers of chondrocytes. This process is under the control of a vast number of growth factors and cytokines. Previously, we cloned cDNA fragments preferentially expressed in a human chondrosarcoma-derived chondrocytic cell line, HCS-2/8 [1,2], by use of the differential display-polymerase chain reaction (PCR) [3]. One of these cDNA fragments was identical to a fragment of connective tissue growth factor/hypertrophic chondrocyte-specific 24 (CTGF/Hcs24) gene [4]. CTGF/Hcs24 is a cysteine-rich, heparin-binding protein whose gene belongs to the immediate early gene family known as CCN family that is composed of cyr61, ctg, nov [5–8], ctgf-3/wisp-2/rcop-1 [9,10], wisp-1/elm-1 [11], and wisp-3 [12]. CTGF/Hcs24 was shown to be mitogenic for fibroblasts in vitro and its gene expression in human fibroblasts was induced by transforming growth factor (TGF)-β [13]. Because of these characteristics, it has been implicated in human atherosclerosis and fibrotic disorders such as systemic scleroderma [14]. However, surprisingly, we found that recombinant CTGF/Hcs24 (rCTGF/Hcs24) promoted the growth and differentiation of chondrocytes at all stages in vitro and that its expression was highest in hypertrophic chondrocytes of growth cartilage among various normal tissues in vivo [15]. CTGF/Hcs24 expression in chondrocytes was induced by the addition of TGF-β or bone morphogenetic protein-2 [3]. These findings strongly suggest that CTGF/Hcs24 may play important roles in endochondral ossification.

In the earliest studies, binding of partially purified CTGF/Hcs24 to the platelet-derived growth factor (PDGF) type B receptor was suggested [4], but neither direct binding between the two, nor intracellular signal transduction was described. Based on the data that the mRNA expression of both CTGF/Hcs24 and PDGF type A receptor was upregulated in scleroderma fibroblasts, it was also suggested that CTGF/Hcs24 and PDGF type A receptors may have some interactions [14]. However, PDGF displays no such effects on chondrocytes as caused by CTGF/Hcs24, and thus the significance of any CTGF/Hcs24–PDGF receptor interaction would be questionable. Another study indicated that CTGF/Hcs24 and Cyr61 directly bound to integrin $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ on fibroblasts [16]. Cyr61 also induced adhesion of human fibroblasts mediated by integrin $\alpha_6\beta_1$ interaction with cell-surface heparan sulfate proteoglycans, and activated intracellular signaling molecules [17]. Most recently, the low density lipoprotein receptor/α_2 macroglobulin receptor was shown to be a specific receptor of CTGF/Hcs24 [18], but its function remains to be clarified.

In our previous study, we demonstrated specific receptors for CTGF/Hcs24 on HCS-2/8 cells [19] and found them to be associated with protein kinase activity (Nishida et al., manuscript in preparation). In this study, we cloned a number of tyrosine kinase-type DNA fragments from HCS-2/8 cells by using tyrosine kinase-specific primers. We also investigated the interaction between CTGF/Hcs24 and one of its putative receptors, ErbB4. This is the first report to show that ErbB4,

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a neuregulin (NRG) receptor, is expressed in both growth and articular cartilage in vivo.

2. Materials and methods

2.1. Cell culture

Four human cell lines were used in this study. A chondrosarcoma-derived chondrocytic cell line (HCS-2/8), a squamous cell carcinoma cell line (A431), a breast cancer cell line (MDA231) and a fibrosarcoma cell line (HT1080) were cultured in Dulbecco's modified minimum essential medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with L-glutamine (2 mM) and 10% fetal bovine serum (FBS). CTGF/Hcs24 stimulation of confluent HCS-2/8 cell cultures was performed with a rCTGF/Hcs24 at a concentration of 50 ng/ml. A murine cell line, ATDC5 cells were cultured in DMEM/Ham's F-12 (1:1) hybrid medium (ICN Biomedicals, Inc., Costa Mesa, CA, USA) supplemented with 5% FBS (ICN Biomedicals, Inc., Costa Mesa, CA, USA), 10 μg/ml human transferrin (Roche Molecular Biochemicals, Indianapolis, IN, USA), and 3×10^{-8} M sodium selenite (Sigma, St. Louis, MO, USA). For induction of chondrogenesis, ATDC5 cells were plated at a density of 6×10^4 cells/well in 6-well plates and cultured for 22 days in the above medium supplemented with 10 μg/ml of human recombinant insulin (Wako Pure Chemical, Osaka, Japan). All of the cells were cultured in a 5% CO_2 atmosphere at 37°C.

2.2. Cloning of cDNA fragments of tyrosine kinase-type receptors

Total RNA was isolated from confluent cultures of HCS-2/8 cells according to the method of Chomczynski et al. [20]. The RNA was treated with DNase I at 37°C for 30 min and was then reverse transcribed to cDNA by using an oligo dT_{16} primer with AMV-derived reverse transcriptase for 30 min at 42°C. Next, the cDNA was amplified with Taq Gold polymerase (Applied Biosystems/Perkin Elmer, Branchburg, NJ, USA) by using the following oligonucleotide primers for the conserved segments of the kinase domains of tyrosine kinase-type receptors: sense, 5′-GGAATTCCATCGIGATTTIGCIGCICG-3′; antisense, 5′-CCTCGAGATICCGTAGGTCCAIACGTC-3′. The amplification conditions were as follow: 95°C (10 min) for one cycle, then 95°C (30 s)–50°C (30 s)–72°C (30 s) for 40 cycles, and 72°C (7 min) for one cycle. The PCR products were separated by agarose gel electrophoresis. Next, these separated bands were excised from gels, and subcloned into pCR-script plasmid vector (Stratagene, La Jolla, CA, USA). Then, nucleotide sequences of these clones were determined by an automated DNA sequencing system (Applied Biosystems/Perkin Elmer), and the sequence homology was analyzed, by using the DDBJ homology search system BLAST.

2.3. Reverse transcriptase-mediated PCR (RT-PCR) analysis

From confluent HCS-2/8 cells, total RNA was isolated 5, 15, 30, 60, and 120 min after the addition of rCTGF/Hcs24 to the cultures, according to the method of Chomczynski et al. [20]. From ATDC5 cells, total RNA was isolated 2, 5, 8, 14, and 19 days after confluence. Isolation of total RNA was performed under standard tissue culture conditions in the cases of A431, MDA231 and HT1080 cells. The isolated total RNA (0.5 μg) was treated with 20 U of DNase I at 37°C for 30 min and was then reverse-transcribed to cDNA by using oligo dT_{16} primers with AMV-derived reverse transcriptase for 30 min at 42°C. Thereafter, the cDNA was amplified with Taq Gold polymerase (Applied Biosystems/Perkin Elmer), using the following pairs of oligonucleotide primers for erbB4 mRNA: forward (erbB4-sense), 5′-GACGAATTCCNATHAARTGGATGGC-3′; reverse (erbB4-antisense), 5′-CTGCTGTCAGCATCGATCAT-3′. The amplification conditions were as follow: 95°C (10 min) for one cycle then 95°C (30 s)–50°C (30 s)–72°C (30 s) for 45 cycles, and 72°C (7 min) for one cycle. RT-PCR analyses for CTGF/Hcs24, type X collagen, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH: control) mRNAs were performed as described previously [15]. The PCR products were analyzed by agarose gel electrophoresis.

2.4. In situ hybridization and histological analyses

ICR mice (3 weeks old) were used for this study. The knee and temporomandibular joints of the animals were removed and immediately immersed in 4% paraformaldehyde and kept in it for 24 h. Next, the specimens were decalcified with 10% ethylene diamine tetraacetic acid for 2 weeks, embedded in paraffin, and cut into 4-μm sections. The knee sections were hybridized with digoxigenin-labeled anti-sense riboprobes for the coding region of the extracellular domain of erbB4 and the entire coding region of ctgf, as described previously [3]. Labeled sense riboprobes were utilized for negative control experiments to ensure the signal specificity. The same sections were also subjected to nuclear staining with methylgreen.

3. Results

3.1. Screening and identification of the tyrosine kinase-type receptor cDNA fragments expressed in HCS-2/8 cells

The conserved tyrosine kinase domain-encoding cDNA

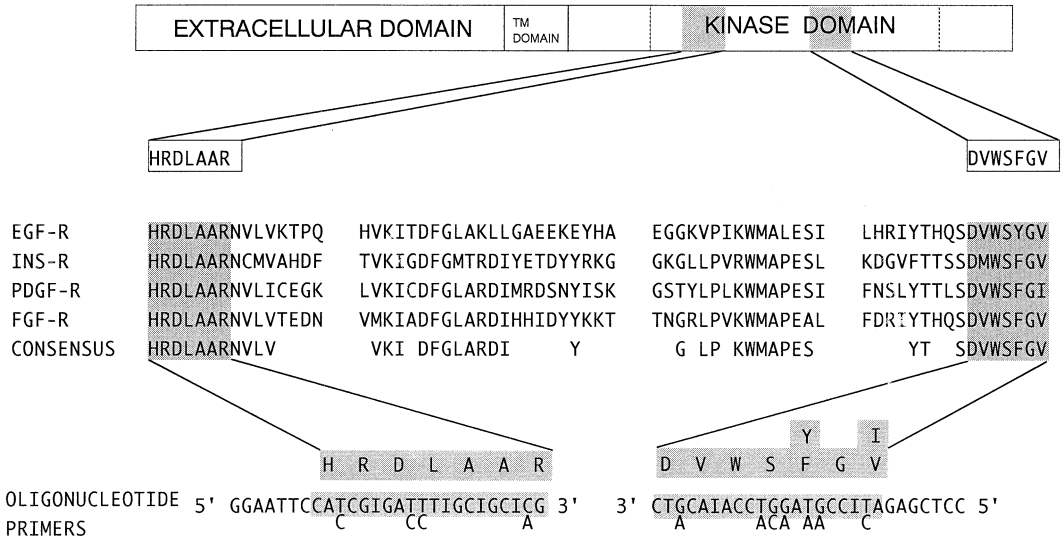

Fig. 1. Conserved amino acid sequences and their corresponding cDNA sequences among the kinase domains of a variety of tyrosine kinase-type receptors. The two sequences, one downstream and one upstream, utilized for designing oligonucleotide primers are shown in single-letter codes. The resultant primers contained several deoxyinosines instead of mixed deoxyribonucleotides, as shown at the bottom. Abbreviations: EGF-R, epidermal growth factor receptor; INS-R, insulin receptor; PDGF-R, platelet-derived growth factor receptor; FGF-R, fibroblast growth factor receptor.

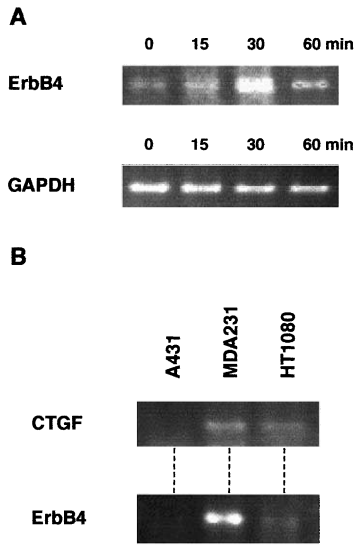

Fig. 2. A: Induction of erbB4 gene expression by rCTGF/Hcs24 in HCS-2/8 cells. Total RNA was sampled at each time point after the addition of rCTGF/Hcs24 (50 ng/ml) and analyzed by RT-PCR. GAPDH gene expression was analyzed and displayed as an internal control. Similar results were obtained with rabbit primary growth cartilage cells (data not shown). B: Concomitant expression of ctgf and erbB4 among human tumor-derived cell lines other than chondrocytic HCS-2/8. A431, MDA231, and HT1080 originated from human squamous cell carcinoma, breast cancer, and fibrosarcoma, respectively.

fragments were amplified from total RNA isolated from HCS-2/8 cells by RT-PCR utilizing two primers comprising several deoxyinosines for ambiguous nucleotide sequences (Fig. 1). The amplified product was then subcloned into the pCR-script vector, by using a commercially available kit (Stratagene). Among a number of colonies obtained, 398 clones were subjected to the initial screening to verify the insertion of the cDNA fragment. As a result, 181 clones were forwarded for nucleotide sequencing after purification through an ion-exchange column (Qiagen, Hilden, Germany). Identity of the obtained nucleotide sequences was determined by using the DDBJ homology search system BLAST. Consequently, 29 clones were identified as fragments of the macrophage colony-stimulating factor receptor (M-CSFR) gene, nine clones as portions of the human EPH-like receptor protein kinase 8 (HEK8) gene, and one clone was found to be a fragment of the NRG receptor (ErbB4) gene [21].

3.2. Upregulation of erbB4 gene expression by CTGF

Next, we evaluated the interaction of these tyrosine kinase-type receptors with CTGF/Hcs24 by adding exogenous rCTGF/Hcs24 [15] to HCS-2/8 cell cultures. In previous reports, 50 ng/ml of rCTGF/Hcs24 was effective enough to display biological function on chondrocytic cells. Therefore, HCS-2/8 cells were treated with this fixed dose of rCTGF/Hcs24, and total RNA was harvested following a time course. A pair of erbB4 primers that recognized the erbB4 mRNA of multiple mammalian species was prepared. RT-PCR analysis with these primers clearly showed rapid induction of erbB4 gene expression in HCS-2/8 cells upon CTGF/Hcs24 stimulation (Fig. 2A). Similar results were obtained from another series of experiments with rabbit primary growth cartilage chondrocytes (data not shown). The cooperative expression of erbB4 and ctgf genes was confirmed by analyzing their gene expression patterns in other non-chondrocytic cell lines (Fig. 2B). A squamous cell carcinoma cell line, A431, showed no detectable gene expression of either ctgf, or erbB4, whereas a CTGF/Hcs24-producing breast cancer cell line, MDA231, displayed a significant level of erbB4 mRNA expression. Under the same experimental conditions, ctgf gene expression was marginally detectable in the fibrosarcoma cell line HT1080, which also gave a faint signal for erbB4 mRNA. As such, the data indicated that the gene expression of the tyrosine kinase-type receptor erbB4 was significantly upregulated by CTGF/Hcs24, which action may represent the upregulation of receptors by their specific ligands. However, no upregulation was observed in the case of HEK8 by CTGF/

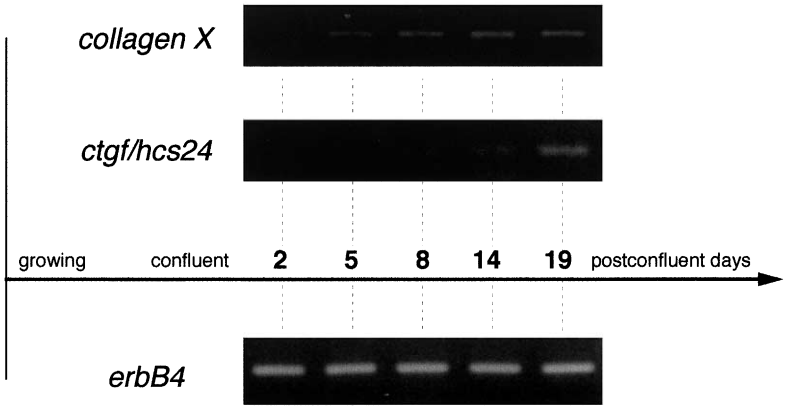

Fig. 3. Comparative analysis of the expression time course of type X collagen, CTGF/Hcs24 and ErbB4 genes during chondrocytic differentiation. After reaching confluence, ATDC5 cells were maintained under differentiation-inducing cell culture conditions as described in Materials and Methods. Total RNA was isolated at the indicated postconfluent days. ErbB4 gene expression was observed regardless of the differentiation stage. These analyses were repeated at least twice with comparable results, and a set of typical data are displayed here.

Hcs24 stimulation (data not shown). Also, no remarkable findings were obtained for any M-CSFR–CTGF/Hcs24 interaction.

3.3. Expression of erbB4 gene along with chondrocytic differentiation

According to our previous findings, ctgf expression was restricted to terminally differentiated hypertrophic stages, whereas its specific receptors were displayed in immature to mature chondrocytes, suggesting CTGF/Hcs24-independent induction of such receptors in cartilage. Thus, we analyzed the erbB4 gene expression following the course of in vitro differentiation of the murine chondrocytic ATDC5 cell line. Therefore, unlike the case of tumor cell lines in Fig. 2B, expression patterns of ctgf and erbB4 were not expected to be parallel in this experiment. Under these culture conditions, expression of type X collagen mRNA gradually increased along with time, representing the induction of differentiation. After day 8, late-phase expression of ctgf was strongly induced. These findings represent terminal differentiation of those cells towards hypertrophy. In contrast to such differential expression of the ctgf gene, the erbB4 gene appeared to be expressed constitutively until the onset of hypertrophy, which is consistent with the ubiquitous demonstration of CTGF/Hcs24 receptor(s) among chondrocytes in growth cartilage.

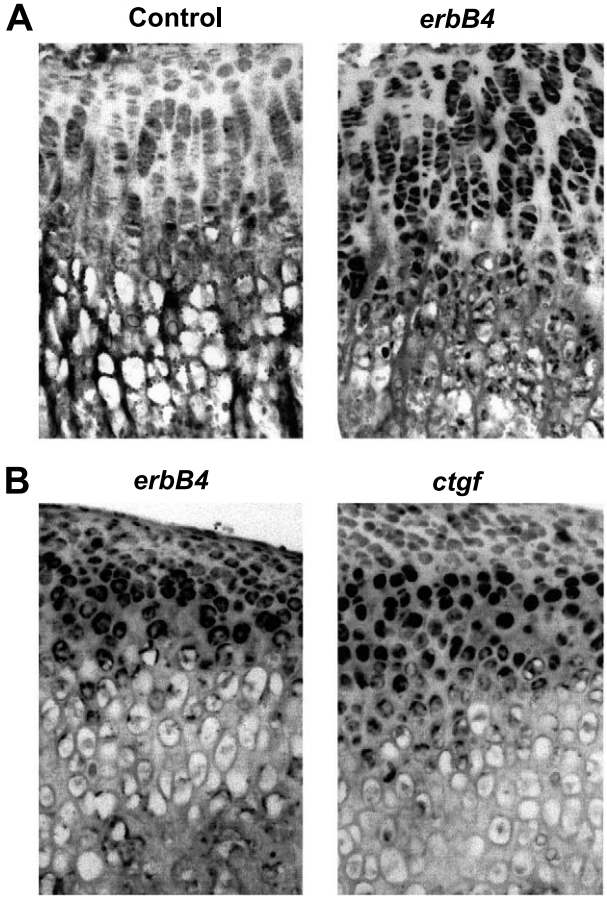

Fig. 4. Expression of erbB4 gene in cartilage in vivo. Normal mouse femur growth plate and temporomandibular joint were analyzed by in situ hybridization with specific probes against erbB4 and ctgf. Expression of the erbB4 gene is observed throughout all of the stages of differentiating chondrocytes in the growth plate (A: right panel). In articular cartilage (B: left panel) erbB4-positive cells are found sporadically near the joint surface, and more prominent erbB4 expression is observed among more differentiated chondrocytes inside. Similarly, the distribution of ctgf-positive cells is restricted to relatively differentiated articular chondrocyte layers (B: right panel). Control experiments with sense riboprobes for erbB4 (A: left panel) and ctgf (data not shown) gave no significant signals.

3.4. Expression of erbB4 in growth and articular cartilage in vivo

Based on the results obtained from the in vitro experiments, we subsequently carried out in situ hybridization analysis of erbB4 gene expression and confirmed that chondrocytes in growth plate and articular cartilage did express erbB4 in vivo. Except in the late hypertrophic zones, erbB4 expression was observed in almost all of the differentiation stages of chondrocytes in the epiphysial growth plate cartilage, which is in sharp contrast to the restricted expression of ctgf in the hypertrophic zone (Fig. 4) [3]. These findings are consistent with the results in Fig. 3, demonstrating different expression patterns of ctgf and erbB4 along the course of chondrocytic differentiation. In articular cartilage, erbB4 and ctgf were expressed sporadically in chondrocytes near the joint surface and were highly expressed in the chondrocytes further down, which morphologically correspond to the proliferative and prehypertrophic chondrocytes. The negative control with each sense probe gave no significant staining.

4. Discussion

In seeking specific receptors for CTGF/Hcs24 on chondrocytes, we found that the erbB4 gene was expressed in HCS-2/8, a human chondrocytic cell line. Furthermore, we demonstrated that erbB4 gene was expressed in chondrocytes in growth and articular cartilage. There have been no previous reports describing the expression of the erbB4 gene in connective tissue. This is partly because ErbB4 [21], a NRG receptor, has been believed to be one of the neural tissue-specific factors. However, our study suggests another novel biological role of ErbB4, one in cartilaginous tissues.

The possible contribution of neurotrophic factors and their receptors in the growth and development of chondrocytes has been previously described in a few reports. Such findings are typically represented by the case of neurotrophins during the bone regeneration process. In a mouse longitudinal bone fracture model, neurotrophins such as nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 were specifically expressed in bone-forming cells, together with their specific receptors tropomycsin receptor kinase (TRK) A and TRKC, suggesting their active function in bone reconstruction [22]. Whereas the roles of these neural factors and their receptors in the bone regeneration process still remain to be clarified, their functions in neurons have been well-characterized; and it is known that they promote both differentiation and survival of neuronal cells. Interestingly, similar effects on neuronal tissues were conferred also by NRG, the ErbB4 ligand [23]. Considering the functional similarity between NRG and neurotrophins in neuronal tissues, it seems reasonable that NRG would contribute also to the formation and maintenance of connective tissues, which actions may be mediated by ErbB4. Therefore, roles of NRG/ErbB4 in endochondral ossification, articular cartilage maintenance, and fracture healing should be investigated in the near future.

Whether the ErbB4 ligand NRG is actually expressed in normal cartilage and fracture healing process, or not, is unclear. However, interestingly, CTGF/Hcs24 was recently found to be expressed during fracture healing as well as in normal cartilage [26]. While pursuing CTGF/Hcs24 receptors, we re-discovered ErbB4 as a chondrocytic receptor mediating certain signaling. To date, several cell-surface receptors have been reported to interact with CTGF/Hcs24, but none of them have been shown to transduce signals into the cells through a phosphorylation cascade. However, it was already proven that CTGF/Hcs24 is capable of activating a signal transduction cascade mediated by mitogen-activated protein kinases in chondrocytes [24]. ErbB4 is a tyrosine kinase-type receptor, and we found that erbB4 gene expression was upregulated by the CTGF/Hcs24 signal. Only with such indirect findings, we ought to avoid to conclude that ErbB4 is one of the specific receptors of CTGF/Hcs24. Thus, direct binding between ErbB4 and CTGF/Hcs24 molecules is currently being evaluated. Also, the mechanism by which erbB4 is expressed at earlier stages of chondrocyte differentiation (Fig. 3) is unclear. Nevertheless, it may be ascribed to the compensatory effect by the Cyr61 molecules which is structurally and functionally quite similar to CTGF/Hcs24. Of note, Cyr61 was reported to be expressed during early stages of chondrogenesis [25]. Therefore, comparative investigation of CTGF/Hcs24 and Cyr61 is of critical importance for comprehensive understanding of the function of these factors in cartilage. However, it is already clear that ErbB4 should play some biological role(s) in chondrocytes in vivo. Knocking out the erbB4 genes in mice and cartilage-oriented analysis of these animals may give us remarkable insights into the function of ErbB4 in cartilaginous tissues as well as in neuronal tissues.

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