Chondroadherin expression changes in skeletal development

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Chondroadherin is a cartilage protein with cell binding properties. The expression of chondroadherin was studied in rat tissues and during postnatal femoral head development. For design of oligonucleotide probes and primers a 1664 bp, full length, rat chondroadherin cDNA was isolated from a rat chondrosarcoma library and sequenced. Northern blot analysis showed chondroadherin mRNA to be present in femoral head and rib cartilage, as well as in tendon. More sensitive reverse-transcriptase PCR additionally identified the mRNA in calvaria, long bone and bone marrow. Localization of chondroadherin by immunocytochemistry in the developing femoral head from postnatal day 14 to day 60 showed presence of the protein in cartilaginous regions. With increasing age a very distinct localization of chondroadherin was seen in the territorial matrix around late proliferative cells in the growth plate as well as in the developing articular cartilage in the maturing femoral head. Localization of chondroadherin mRNA by *in situ* hybridization was in agreement with immunocytochemistry with strong hybridization signals in late proliferative cells in the growth plate. In the articular cartilage the expression was restricted to cells in the lower regions. A three-fold increase of cartilage chondroadherin content in the growing femoral head was demonstrated by Western blot analysis. The high expression of this cell binding protein in a dynamic region of cartilage suggests an important role for chondroadherin in the regulation of chondrocyte growth and proliferation.

INTRODUCTION

Chondroadherin is a non-collagenous extracellular matrix protein which was first isolated and purified from bovine cartilage [1]. The determination of the primary structure of the protein identified it as a member of the leucine-rich repeat (LRR) family of proteins [2,3]. Chondroadherin has been shown to promote attachment of chondrocytes and fibroblasts efficiently [4], mediated via the integrin $\alpha_2\beta_1$ [5]. More recently, a preparation of the protein from bovine bone was reported to promote attachment of osteoblasts [6]. Further clues as to the function of the protein are now presented in studies of the expression of the protein during tissue differentiation and remodelling.

As a model to study the expression of cartilage macromolecules in a dynamic situation with marked tissue changes, we have utilized the normal, postnatal development of the rat femoral head, which includes the formation of a secondary ossification centre and an articular cartilage [7,8]. The growth cartilage is characterized by distinct tissue compartments with cells showing different activities, the resting, proliferative, hypertrophic and calcified zones [9]. The hypertrophy of the chondrocytes and initiation of calcification occur in the secondary ossification centre of the rat femoral head at discrete time points, i.e. at 18 and 21 days *post partum*, respectively [8,10]. At the same time the superficial parts develop into a functional articular cartilage and remain uncalcified. Why some parts of primary cartilage-skeleton become calcified and develop into bone while others remain non-calcified is not known, but the process must be tightly regulated at the molecular level. Thus, a number of non-collagenous cartilage matrix proteins are likely to have different roles in the endochondral ossification process [11]. In accordance, it has been shown that the abundance of several matrix constituents changes during the calcification process as shown in studies of extractable macromolecules such as the highly negatively charged proteoglycans [7] and cartilage oligomeric matrix protein (COMP) [8].

In the present study we determined the primary structure of rat chondroadherin. Expression of chondroadherin mRNA in various rat tissues was studied by Northern blot analysis and reverse-transcriptase PCR (RT-PCR) as well as by *in situ* hybridization. In parallel, the localization of the chondroadherin protein was investigated by immunostaining of the maturing femoral head of Wistar rats from day 14 to day 60. The findings from histology were corroborated in studies of altered quantities of the protein in the maturing femoral head.

MATERIALS AND METHODS

Cloning and sequence analysis of cDNA

A rat chondrosarcoma cDNA library [12] was screened with a cDNA probe from a bovine chondroadherin clone [2]. The cDNA sizes were estimated by electrophoresis on a 1% (w/v) agarose gel after digestion with restriction enzyme *Eco*RI. A full length, 1.6 kbp clone was isolated and purified with a Plasmid Midi Kit (QIAGEN. Inc.). DNA sequencing was performed in full on both strands by standard double-strand dideoxy termination sequencing using T3, T7 and synthetic internal specific primers.

Analysis of RNA

Total RNA from various rat tissues and rat chondrosarcoma cells was prepared by single-step RNA isolation according to

Abbreviations used: COMP, cartilage oligomeric matrix protein; ECM, extracellular matrix; GAG, glycosaminoglycan; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LRR, leucine rich repeat; PTHrP, parathyroid hormone related protein; RT-PCR, reverse-transcriptase PCR; SSC, sodium chloride and sodium citrate; SSPE, sodium chloride, sodium phosphate, and EDTA; DAB, diaminobenzidine tetrahydrochloride.

The nucleotide sequence data reported have been submitted to GenBank under the accession number AF004953.

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Chomczynski and Sacchi [13]. 10 μg of total RNA from various rat tissues were separated on 1% agarose/2.2 M formaldehyde gel and transferred to a nylon membrane (Hybond™-N, Amersham) by capillary transfer. Probes were labelled with a [^{32}P]dCTP by using a random primed DNA labelling kit (Boehringer Mannheim, Germany). After ultraviolet light crosslinking the filter was prehybridized with a prehybridization solution (5 × SSPE, 5 × Denhardt's solution, 0.2% SDS, 10% dextran sulphate, 50% formamide and 0.1% denatured salmon sperm DNA) for 2 h, then the ^{32}P-labelled cDNA probe was added to the prehybridization solution at 10^6 c.p.m./ml and incubated at 42 °C overnight. The filters were washed twice with 2 × SSPE containing 0.1% SDS and with 1 × SSPE, followed by final washing once with 0.1 × SSPE, at 42 °C for 15 min. The filter was exposed to Cronex® 4 medical X-ray film (DuPont, U.S.A.) at −70 °C overnight and developed.

cDNA synthesis and RT-PCR

Total RNA used for synthesis of cDNA from various rat tissues and rat chondrosarcoma cells was prepared as mentioned above. Samples were reverse transcribed into single stranded cDNA by using Superscript™II (Gibco BRL, Life Technologies) according to the manufacturer's instructions. Immediately before cDNA synthesis the RNA samples were heated to 70 °C for 10 min with oligo(dT) primer and quickly cooled on ice. Then first strand buffer, dithiothreitol, dNTP mixture and Superscript™II were added. Samples were incubated, first at 42 °C for 50 min and then at 70 °C for 15 min. The cDNAs were amplified using 1 μM of a 21-mer upstream primer I (CGCTGGCTCTACCTGTCAGAA), located at position 568 and 1 μM of a 20-mer downstream primer II (GACCTCTTGGTGGGGGATTT), located at position 1087 of the cDNA sequence (see Figure 1). Amplification of cDNA was carried out in a thermocycler using Taq DNA polymerase and buffer supplied with the enzyme (Gibco BRL, Life Technologies) at 1 mM $MgCl_2$. The PCR was initiated by a hot start followed by 30 cycles at 94 °C, for 30 s; 58 °C, for 30 s; 72 °C, for 2 min. 2% of the products from the first amplification were re-amplified with a pair of nested primers: 1 μM of a 21-mer upstream primer III (CTTTGGCAGATACCTGGAGAC), located at position 771 and 1 μM of a 20-mer downstream primer IV (CTTGAACTTGGCTGGTGAAG), located at position at 1028 of cDNA sequence (see Figure 1). The PCR was performed as above by 25 cycles at 94 °C, for 30 s; 62 °C, for 30 s; 72 °C, for 2 min. The identity of the PCR products was verified by sizing 10% of the products by 1.5% agarose gel electrophoresis and by digestion with restriction enzyme *Afl*III (Boehringer Mannheim, Germany), which cleaves at a unique site at position 861 of the nucleotide sequence.

Control PCR amplification was performed with a pair of 21-mer primers designed from rat collagen αI (position 970 and 1459) to verify that chondroadherin negative samples contained intact, amplifiable cDNA. All samples contained amplifiable collagen cDNA (data not shown).

Analysis of protein

Whole femoral heads, from each age studied, were homogenized by the use of a Polytron and extracted overnight at 4 °C with 10 ml of 4 M guanidinium chloride, 0.1 M ϵ-aminocaproic acid, 5 mM benzamidine hydrochloride, 0.05 M sodium acetate and 5 mM *N*-ethylmaleimide, pH 5.8, containing 10 mM EDTA per g of tissue. Macromolecules in extracts were precipitated with 10 volumes of 95% ethanol containing 50 mM sodium acetate, washed once with 70% ethanol and finally dissolved in non-reducing electrophoresis reagent. Samples from each age group corresponding to 2, 1 and 0.5 mg tissue respectively were separated on SDS 4–16% polyacrylamide gel and proteins were transferred to a nitrocellulose membrane [14]. The membrane was blocked with 3% dried milk and incubated with the same antiserum as used in immunocytochemistry (1:500 dilution in 0.01 Tris buffer, pH 7.4). Bound antibodies were detected after incubation with secondary antibodies conjugated with horseradish peroxidase by chemiluminescence using the ECL system (Amersham, Life Science). Bands corresponding to 1 mg tissue samples were chosen for semiquantitative analyses since the density of these bands were within the linear range of the film sensitivity. The bands detected were quantified by scanning densitometry with the Gel-Pro Analyser program (Ver. 2.0, Media Cybernetics, MD, U.S.A.).

Quantification of glycosaminoglycan

The same extracts as used for quantification of chondroadherin were directly precipitated with Alcian Blue for glycosaminoglycan quantification [15].

Preparation of tissue sections

Femoral heads from rats of the Wistar strain aged 14–60 days were used in the present study. The femoral heads were dissected free from surrounding tissues, immediately frozen in liquid nitrogen and embedded in Tissue-Tek O.C.T. compound (Miles Scientific Co., Naperville, IL, U.S.A.). Embedded tissues were cut into 6 μm sections in a cryostat at −25 °C. Sections were mounted on gelatin-coated glass coverslips. Toluidine Blue and von Kossa's staining were used to provide a general background and discern calcification as previously described [8,16].

Immunocytochemistry

For immunocytochemistry, an antiserum recognizing a C-terminal peptide (KRSKKAGRH) of rat/bovine chondroadherin raised in rabbits was used. This antiserum has been shown to react with chondroadherin only in crude cartilage extracts. An antiserum against pig collagen X was a kind gift from Dr Rucklidge, Aberdeen, U.K. The ABC avidin–biotin–peroxidase complex method was used for staining as previously described [8]. Sections for immunocytochemistry were air-dried at room temperature, fixed in acetone, then stored at −20 °C until stained. After rinsing with PBS the sections were pre-treated with 0.5% hydrogen peroxide at room temperature for 20 min to inhibit endogenous peroxidase. Subsequently digestion with chondroitinase ABC (Sigma), 40 munits/ml in 0.05 M Tris, 0.15 M sodium chloride containing 0.1% (w/v) BSA, pH 7.4, was used to increase penetration of antibodies. Sections were treated with 1.5% (v/v) normal goat serum for 30 min at room temperature prior to incubation with primary antisera. Excess serum was gently blotted off and the sections were incubated with primary antiserum (1:500 in PBS containing 0.25% BSA) at 4 °C overnight. After thorough rinsing the sections were consecutively incubated with an affinity-purified, biotinylated goat anti-rabbit immunoglobulin antibody (1:200 in PBS), and with avidin–biotin–peroxidase complex for 30 min, at room temperature. After rinsing the sections were developed with freshly prepared 0.06% diaminobenzidine tetrahydrochloride (DAB) and finally sealed with Permount (Fisher Scientific Company, Fair Lawn, NJ, U.S.A.). They were observed and photographed with a Nikon Microphot-FAX microscope. Sections were also stained omitting pre-treatment with enzyme.

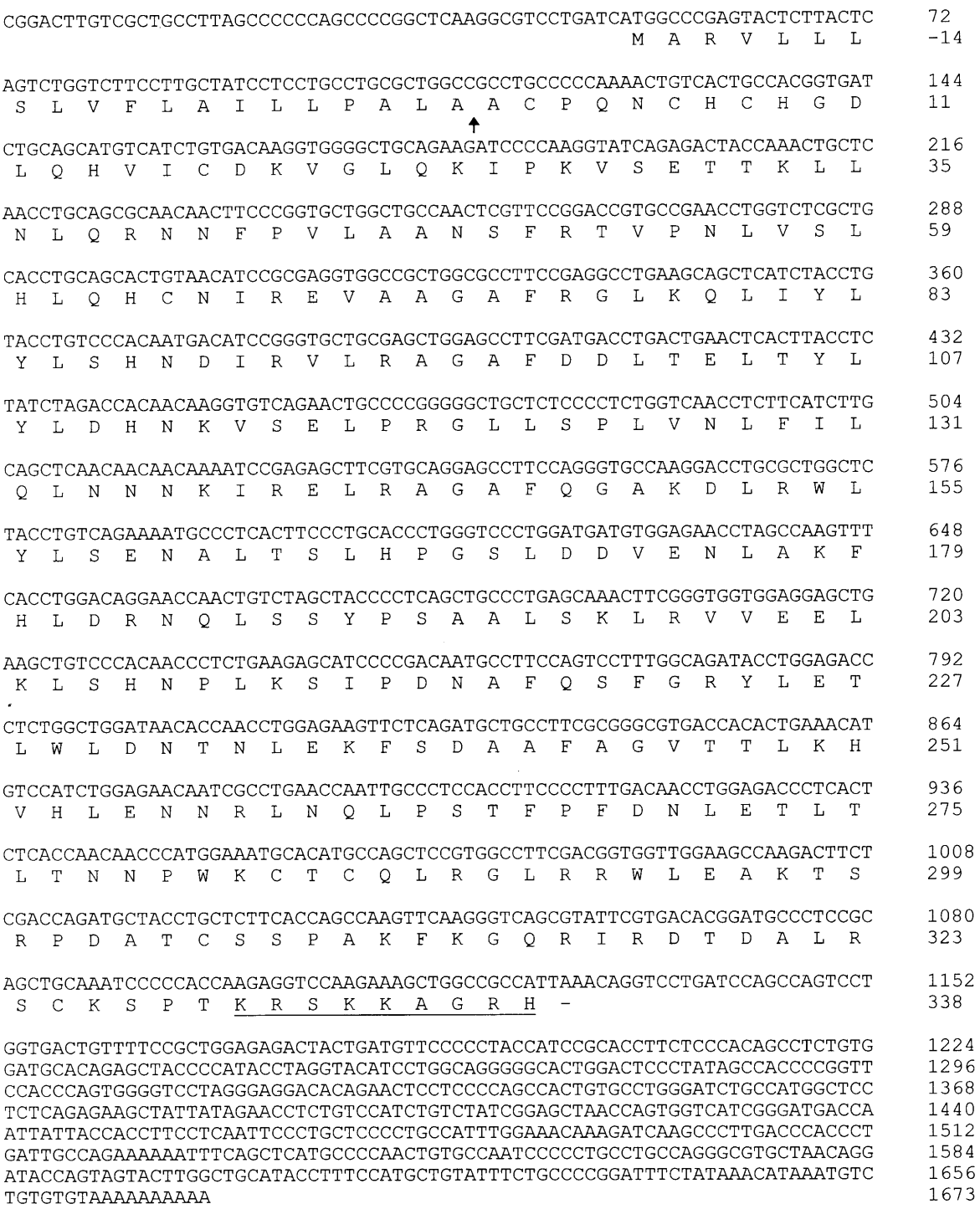

Figure 1 Nucleotide and deduced protein sequence for rat chondroadherin cDNA

A potential signal peptide cleavage site is indicated by an arrow. Underlined is the C-terminal peptide used to generate the rabbit antiserum used in this study.

For immunostaining with antiserum against collagen X, sections were demineralized with EDTA as previously described [8], and then pre-digested with chondroitinase ABC as above plus hyaluronidase (Sigma, 1 mg/ml) followed by staining with antiserum (1:200 in PBS) containing 0.25% BSA as mentioned above. Other sections were stained without treatment with EDTA. Routine control experiments for checking antiserum performance were done by replacing the specific antibody with preimmune serum or antiserum dilution buffer.

In situ hybridization

For *in situ* hybridization, a 50-mer rat oligonucleotide probe corresponding to nucleotides 421–372 of rat chondroadherin

cDNA was used. The sequence of this chondroadherin antisense probe was 5′-GGTCAGTCAGGTCATCGAAGGCTCCAGC-TCGCAGCACCCGGATGTCATTG-3′. The probe was labelled with [α-^{35}S]dATP (Amersham, Solna, Sweden) using terminal deoxynucleotidyl transferase (Gibco BRL, Life Technologies) as previously described [8]. Negative control sections were obtained by treatment with RNase A prior to hybridization. Initially, antisense and sense RNA probes, covering base 240–430, were tested giving a hybridization pattern in cartilage essentially identical to the results presented with the oligonucleotide probe. However, unacceptably high background was obtained with the RNA probes in calcified regions precluding their further use.

The hybridization solution contained 50 % (v/v) deionized formamide, 10 % w/v) dextran sulphate, 1 × Denhardt's solution (0.02 %, w/v, each of BSA, Ficoll and polyvinylpyrrolidone), 4 × SSC (sodium chloride and sodium citrate), denatured salmon sperm DNA (0.5 μg/μl) and yeast tRNA (0.25 μg/μl), 1 % (w/v) sodium *N*-lauroylsarcosinate and 20000 c.p.m. of ^{35}S-labelled oligonucleotide probe per μl. Dithiothreitol was added to 0.1 M to the hybridization solution directly prior to use.

Sections were directly stored in RNase-free boxes at −70 °C. The hybridization procedures used were similar to those used in a previous study [8] with minor modifications of the concentrations of probes and washing solution (SSC), as well as washing temperature. Briefly, slides were removed from the freezer, thawed and dried with a hair dryer. Slides were then post-fixed in 4 % (w/v) freshly prepared paraformaldehyde in PBS, acetylated with 0.25 % (v/v) acetic anhydride in 0.1 M triethanolamine buffer, and finally dehydrated in increasing concentrations of ethanol. Each section was hybridized with 10^6 c.p.m. of labelled antisense oligonucleotide probe in a humid chamber at 37 °C overnight. After hybridization, the sections were washed in 2 × SSC at 50 °C and then dehydrated in an ascending series of ethanol containing 0.3 M ammonium acetate. After dipping in Ilford K-5 photographic emulsion (Ilford, England), the sections were stored with desiccant at 4 °C for 2–3 weeks. The photoemulsion was developed, fixed and sections were counterstained with haematoxylin and finally mounted in Kaiser's medium (glycerol/gelatin; Merck, Darmstadt, Germany). The slides were examined and photographed with both bright- and dark-field illumination.

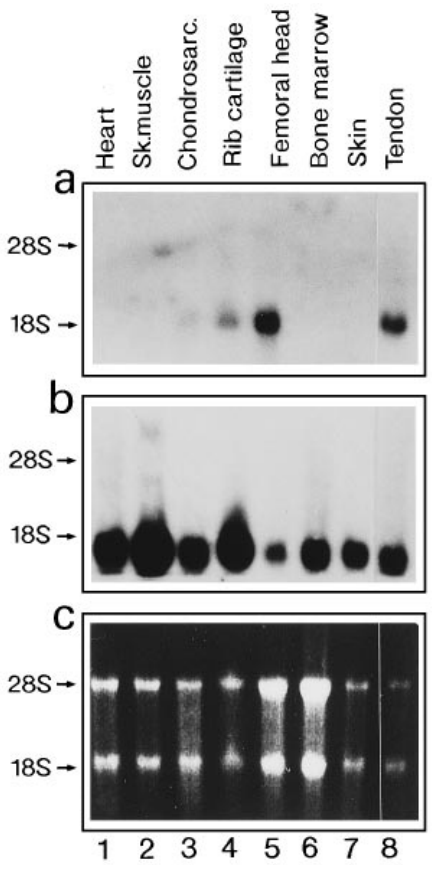

Figure 2 Northern blot analysis of various rat tissues and chondrosarcoma cells

10 μg each of total RNA from the rat tissues indicated was analysed by Northern blotting with the chondroadherin cDNA probe (**a**) and with the GAPDH cDNA probe (**b**) and by staining with ethidium bromide (**c**). Lanes: (1) heart; (2) skeletal muscle; (3) rat chondrosarcoma cells; (4) rib cartilage; (5) femoral head; (6) bone marrow; (7) skin, and (8) tendon.

RESULTS

Sequence of rat chondroadherin

The rat chondrosarcoma cDNA clone of 1651 bp (Figure 1) appears to encode the full length sequence of rat chondroadherin since the size of the cDNA correspond to the size of the mRNA, see below. No classical AATAAA polyadenylation signal is present making the assignment of the 3′ end uncertain. However, a potential alternative polyadenylation signal is an ATAAAC-ATAAA sequence beginning 20 bases upstream of the 3′ poly(A) stretch. The length of the 5′-untranslated region is similar to the length of the same region in bovine chondroadherin cDNA. Thus the rat clone most likely represents a full length cDNA. The translated sequence for rat chondroadherin is highly similar to bovine chondroadherin with 93 % identity at the amino acid level. The C-terminal region chosen for generation of the polyclonal rabbit antiserum is completely conserved.

Tissue distribution of rat chondroadherin RNA

Northern blot analysis was performed on total RNA prepared from various rat tissues and freshly isolated rat chondrosarcoma cells (Figure 2). A 1.6 kb chondroadherin mRNA was detected in the whole femoral head and rib cartilage preparations. A weak signal was also detected in the chondrosarcoma RNA preparation. Surprisingly, a strong hybridization signal for chondroadherin mRNA was found in rat tail tendon RNA preparations (Figure 2a). The quality and amounts of the isolated RNA was confirmed by hybridization with a rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe (Figure 2b) or by staining the separation gel with ethidium bromide (Figure 2c).

The results from Northern blot analysis were confirmed and further extended by using RT-PCR analysis. cDNAs from various tissues were amplified in a two-step procedure with nested primers. Agarose gel electrophoresis showed a predicted product with a size of about 277 bp in femoral head, rib cartilage, chondrosarcoma cells and tendon, but also in calvaria, bone and bone marrow RNA preparations (Figure 3). The identity of the PCR products was confirmed by digestion with the restriction enzyme *Afl*III and agarose electrophoresis (data not shown).

Routine histology

Routine histological examinations were carried out at different stages of endochondral bone formation and articular cartilage differentiation in the rat femoral head. Changes in chondrocyte

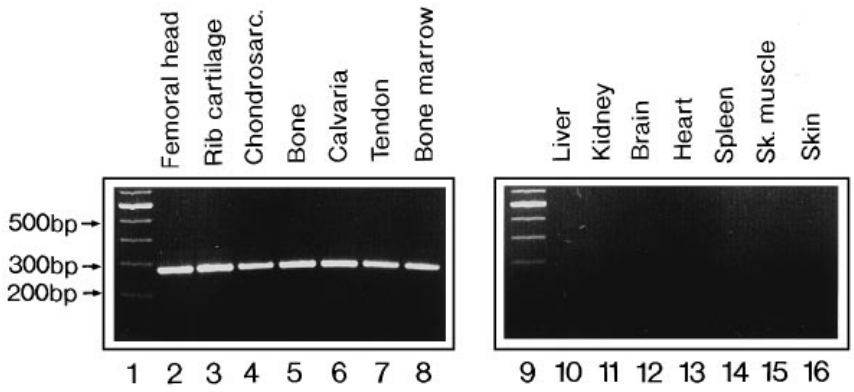

Figure 3 RT-PCR analysis of various rat tissues

10% (5 μl) of RT-PCR products with nested primers from the rat tissues indicated were electrophoresed on a 1.5% agarose gel. Lanes: (1) molecular-mass marker; (2) femoral head; (3) rib cartilage; (4) chondrosarcoma cell; (5) bone; (6) calvaria; (7) tendon; (8) bone marrow; (9) molecular-mass marker; (10) liver; (11) kidney; (12) brain; (13) heart; (14) spleen; (15) skeletal muscle, and (16) skin.

morphology typical for hypertrophy on day 18 in the secondary ossification centre and appearance of mineralization on day 21 as well as ossification around day 45 were similar to those described previously [8,10].

Localization and expression of chondroadherin during femoral head maturation

An anti-peptide antiserum raised against the C-terminal amino acids of rat/bovine chondroadherin was used for immunocytochemistry. The anti-peptide antiserum is highly specific and recognizes only chondroadherin on protein blots of crude cartilage extracts (see Figure 6 below). Another polyclonal rabbit antiserum raised against isolated bovine chondroadherin, which works well in ELISA assays on bovine tissue extracts [1], did not stain rat tissue sections. Immunocytochemistry with rat femoral heads of different ages showed a general distribution of chondroadherin to all cartilaginous regions. The strongest immunoreactivity was at all ages detected in the growth cartilage region (Figures 4A, 4C, 4E and 5A).

At the youngest age examined, day 14, chondroadherin was readily detectable in the cartilage, with clear staining in the growth plate, but with little more than background staining in the bulk of the cartilage (Figure 4A). The more superficial parts of the femoral head corresponding to the developing articular cartilage layer, however, showed some weak immunostaining (Figure 4A). At this stage, mRNA for chondroadherin was only detected by the use of *in situ* hybridization at the lower level in growth cartilage region (Figure 4B). No mRNA was detected in the underlying bone trabeculae.

At the time of onset of calcification, day 20, the staining for chondroadherin decreased somewhat in the centre portion of the femoral head when the cells had entered hypertrophy (Figure 4C). At this stage a larger number of chondrocytes in the primary cartilage growth zone expressed detectable levels of chondroadherin mRNA (Figure 4D). Only low expression of chondroadherin mRNA was seen at other sites. With increasing age, the area of mineralization expanded and concomitantly the presence of chondroadherin at this tissue compartment decreased (Figure 4C and 4E). At day 33, chondroadherin was abundant in a narrow zone in the growth cartilage region as well as in the region corresponding to the developing articular cartilage (Figure 4E). At this stage chondroadherin mRNA levels appeared to be substantially increased in both the growth cartilage region and in forming articular cartilage as seen by *in situ* hybridization (Figure 4F), consistent with results from immunostaining. There was neither detectable immunostaining nor hybridization for mRNA in the now calcified secondary ossification zone.

The intensity of chondroadherin staining in the articular cartilage increased further at day 60, both with regard to immunostaining and *in situ* hybridization (Figure 5A and 5D), and remained very intense in a narrow zone of the primary growth cartilage region. Chondroadherin is mainly present in the territorial matrix of the deeper parts of the articular cartilage but there is some weaker staining in the interterritorial matrix (Figure 5B). This corresponds to sites with marked hybridization for chondroadherin mRNA (Figure 5D and 5E). Abundant protein was seen in the growth cartilage plate around the lower region of proliferative chondrocytes (Figure 5A and 5C). Moreover, strong mRNA hybridization signals were detected in the late proliferative chondrocytes above the hypertrophic chondrocytes in the primary growth cartilage region (Figure 5F).

Immunostaining for collagen X was used as a reference for hypertrophic chondrocytes and showed localization below the zone of most intense chondroadherin staining. Collagen X was localized interterritorially in the lower part of the cartilage growth region with staining extending into cartilage spiculae between the bone trabeculae (Figure 5G and 5I). Collagen X was also detectable in the secondary ossification centre in a region close to articular cartilage (Figure 5H). In general, sections not pre-digested with enzymes showed a similar but weaker staining pattern. The controls for antibody specificity, with the preimmune rabbit serum, showed no immunostaining either in cartilage or in bone (Figure 4G). There were no detectable hybridization signals in control sections for *in situ* hybridization after pretreatment with RNase (Figure 4H).

Chondroadherin and glycosaminoglycan tissue content

To confirm that there was an actual increase in chondroadherin protein content during femoral head development as indicated by immunostaining and *in situ* hybridization, we performed semiquantitative determination of the molecule in extracts of whole femoral heads (Figure 6 and Table 1). Only one major band corresponding to the expected size of about 36 kDa was

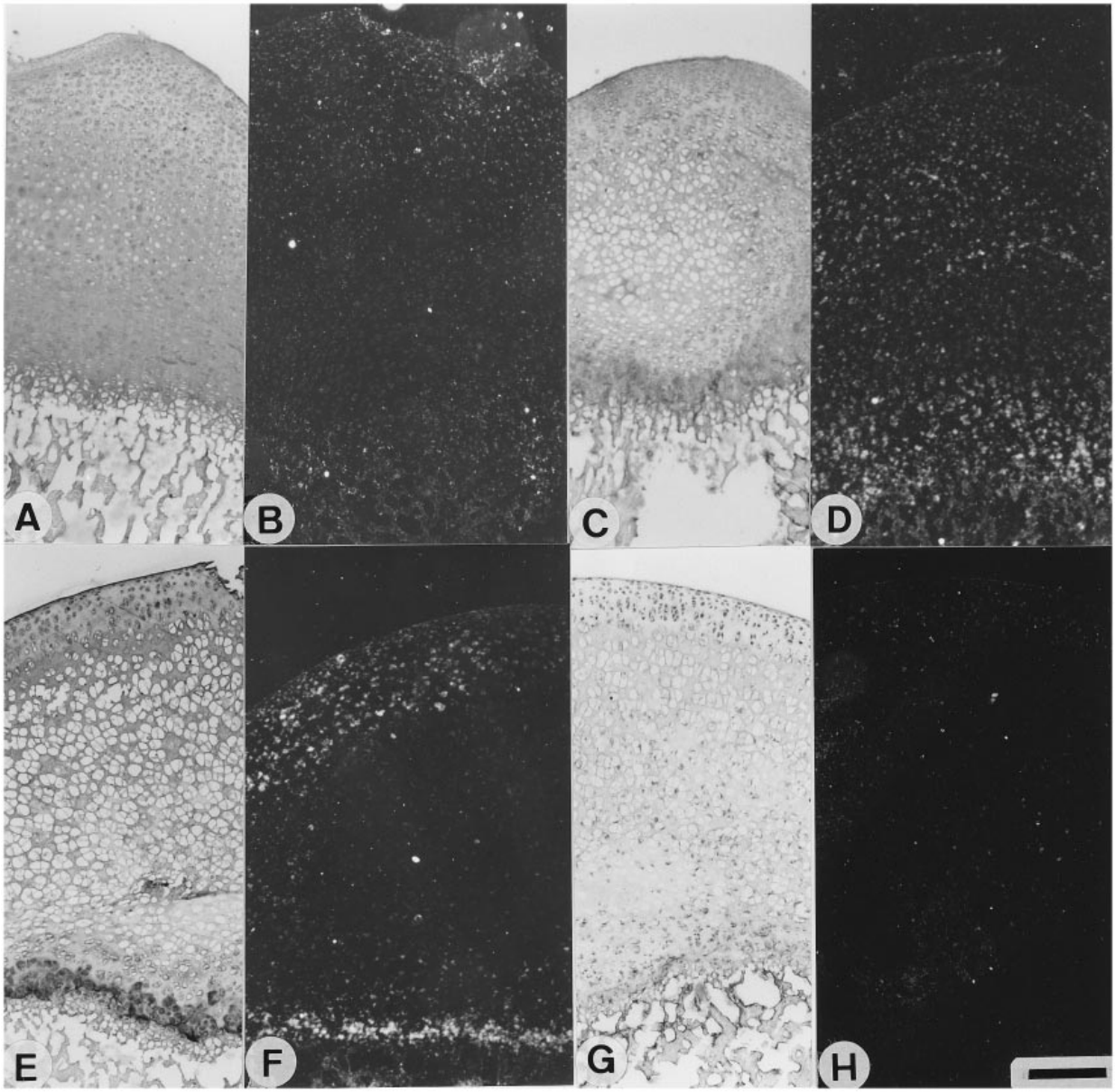

Figure 4 Sections of day 14 (A and B), day 20 (C and D) and day 33 (E to H) rat femoral heads: immunostaining for chondroadherin (bright-field illumination) and *in situ* hybridization with the ^{35}S-labelled chondroadherin oligonucleotide probe (dark-field illumination)

(**A**) Section stained with antiserum against chondroadherin showing moderate staining in the cartilage growth region and very weak staining in the rest of the immature femoral head cartilage. (**B**) Detectable hybridization to chondroadherin mRNA only in chondrocytes in the lower part of the growth cartilage zone. (**C**) Increased immunostaining for chondroadherin in the primary growth cartilage zone and weak staining of the immature femoral head cartilage. (**D**) Moderate expression of mRNA for chondroadherin detected in chondrocytes of the primary growth cartilage region. (**E**) Increased staining for chondroadherin in a narrow zone of the primary growth cartilage region and initial, increased staining in the forming articular cartilage layer. Also, further reduction of chondroadherin immunostaining in the region of the secondary ossification centre compared with (**A**) and (**C**). (**F**) Strong hybridization in proliferative chondrocytes of the primary cartilage growth region and increased hybridization now restricted to the lower articular cartilage chondrocytes. (**G**) Control section stained with preimmuno-serum showing no positive staining in cartilage or bone. (**H**) Control section pre-treated with RNase A showing no hybridization signal in cartilage or bone. The scale bar (**A**–**H** same magnification) is 300 μm.

Figure 5 Sections of day 60 femoral head: immunostaining for chondroadherin (A–C) or for collagen X (G–I), and *in situ* hybridization with the ^{35}S-labelled chondroadherin oligonucleotide probe (D–F)

(**A**) Intense immunostaining for chondroadherin in the growth cartilage region and moderate staining in articular cartilage layer. (**B**) High magnification of articular cartilage clearly showing chondroadherin localized to the territorial matrix as well in the interterritorial matrix. (**C**) High magnification of cartilage growth plate showing chondroadherin localized territorially to proliferative cell and upper hypertrophic chondrocytes in the growth cartilage region. (**D**) Hybridization signals in proliferative chondrocytes in the articular cartilage and in the growth cartilage region. (**E**) High magnification of articular cartilage showing major expression of chondroadherin in chondrocytes of the deep articular cartilage layer. (**F**) High magnification of cartilage growth region showing proliferative and upper hypertrophic chondrocytes to express high levels of mRNA for chondroadherin. (**G**) Immunostaining for collagen X localized to the matrix of the lower growth cartilage region and secondary ossification centre as well as extending into trabecular bone. (**H**) High magnification of articular cartilage showing the presence of collagen X in the area of secondary ossification centre close to the articular cartilage. (**I**) High magnification of the growth cartilage zone showing localization of collagen X in the lower hypertrophic chondrocytes. The scale bar (first column, **A**, **D** and **G**) is 300 μm; scale bars (second and third columns, **B**, **C**, **E**, **F**, **H** and **I**) are 40 μm.

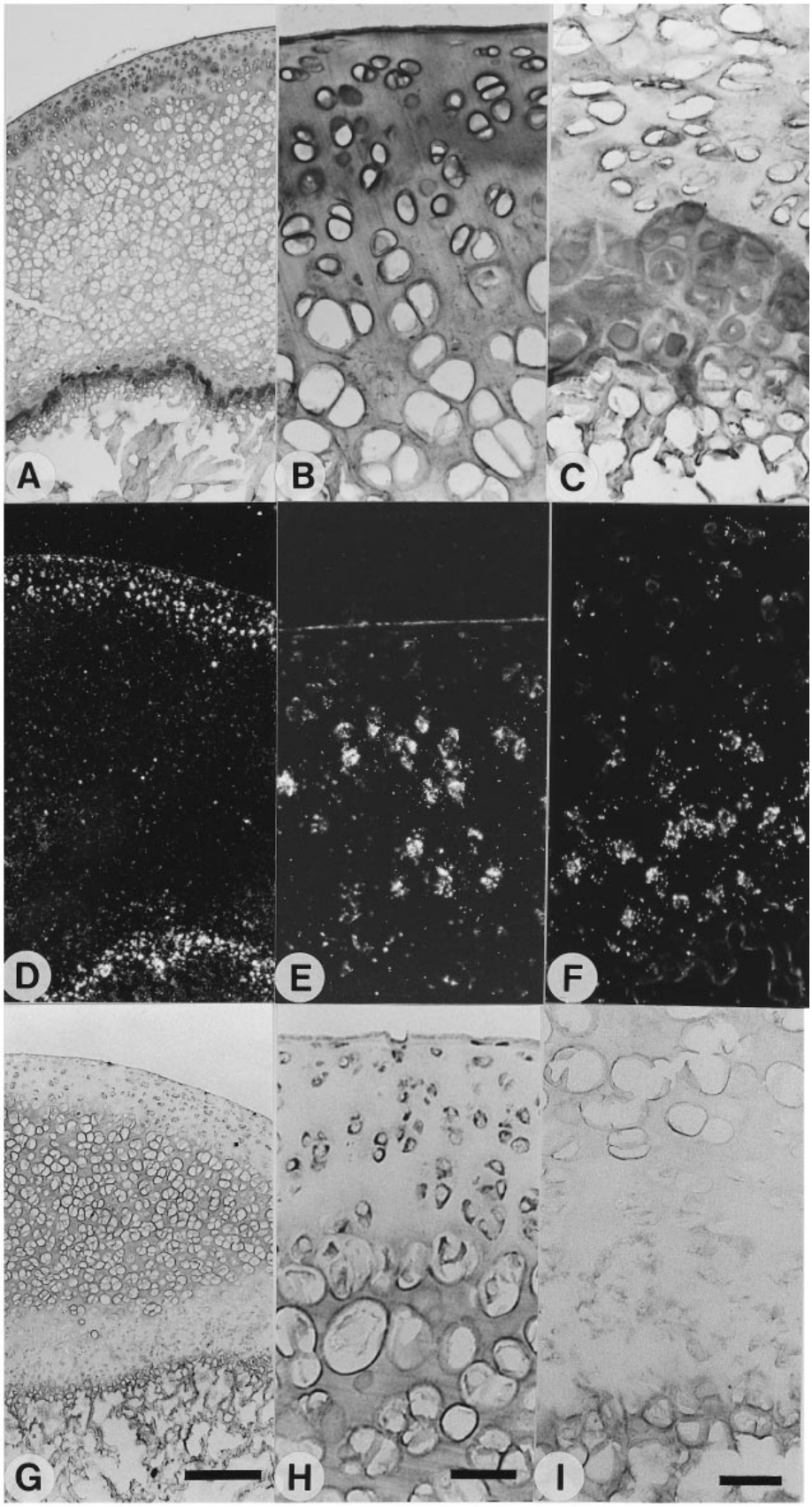

Figure 5 **For legend see opposite page.**

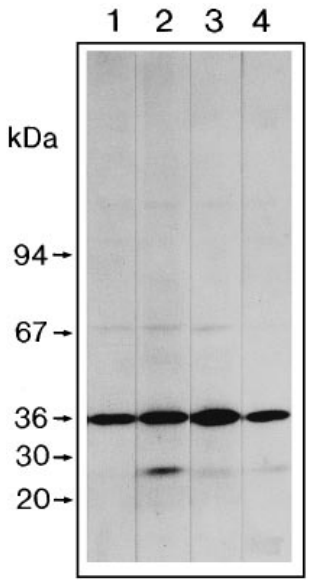

Figure 6 Western blot analysis of whole femoral heads of various ages

Samples of extracts of whole femoral heads corresponding to 1 mg of tissue were separated on SDS/4–16% PAGE gel and transferred to nitrocellulose membrane for immunoblotting. Chondroadherin was detected by chemiluminescent detection with the same antiserum as used for immunocytochemistry. Lanes: (1) day 14; (2) day 20; (3) day 33, and (4) day 60.

Table 1 Analyses of growth and glycosaminoglycan contents in femoral heads of various ages

Whole femoral heads from the various ages were extracted and processed as described in the methods section. Chondroadherin (CHAD) content was quantitated by densitometry of immunoblots of samples corresponding to 1 mg tissue wet weight. Values are given as arbitrary unit density. Glycosaminoglycan (GAG) content in extracts was determined by Alcian Blue precipitation. The data are expressed in relation to wet weight and per femoral head. The ratio of CHAD/GAG gives an estimate of the relative increase of chondroadherin in cartilage. N, number of femoral heads used; FH, femoral head; CHAD, chondroadherin; GAG, glycosaminoglycan.

Age (days)	N	Average wet weight/FH (mg)	CHAD		GAG		CHAD/GAG per FH
			Density/mg	Density/FH	μg/mg	μg/FH	
14	20	5.6	108	609	31	174	3.5
20	10	9.6	172	1663	45	441	3.8
33	10	23.1	216	4450	21	505	8.8
60	16	32.4	158	5126	16	520	9.9

detected in extracts by Western blot analysis with the anti-peptide antisera, indicating the high specificity of the antiserum. Interestingly, from day 20 a minor band appeared with a size of about 25 kDa. This most likely represents a C-terminal domain degradation fragment of chondroadherin. The bands detected were quantified by scanning densitometry and data showed that the total amount of the protein in the femoral head increased with growth of rats, especially from day 20 to day 33. The content of chondroadherin per femoral head increased approx. eight-fold from 609 (density/femoral head) to 5126 (Table 1). At the same time the weight of the femoral heads increased approx. six-fold. Thus the content of chondroadherin increased more than could be accounted for by the growth of the femoral head, corroborating the findings from immunocytochemistry. However, the values represent an underestimate of chondroadherin content since the true cartilage content of the aged tissue is lower. This follows from the calcification of cartilage and bone formation. The content of chondroadherin should preferably be estimated in relation to true cartilage content. We therefore used the amount of extractable glycosaminoglycans as a measure for cartilage tissue. When the chondroadherin values were expressed in relation to glycosaminoglycan content, the results indicated an approximately three-fold increase of chondroadherin in the cartilage compartment. Again, the most prominent increase was seen between day 20 and 33, which is consistent with the findings by immunocytochemistry and *in situ* hybridization.

DISCUSSION

Chondroadherin is expressed by cartilaginous cells. This is supported by immunocytochemistry which shows that the protein is laid down where it is synthesized. The low level of mRNA, detected by RT-PCR in long bone and calvaria is interesting, and confirms previous reports that the protein is detected in bovine bone extracts by immunological methods [1,6]. This restricted tissue distribution for chondroadherin is unusual among LRR proteins of the ECM. Most of the LRR-containing proteoglycans show wide tissue distribution. Biglycan, decorin, fibromodulin and lumican are all expressed in a number of connective tissues [12,17,18].

In tendon, expression of mRNA for chondroadherin was detected by both Northern blot and RT-PCR, while the protein was not detected by immunostaining. Apparently the level of the protein is too low to be detectable by the antibodies in the rat tendon section. Another prominent cartilage protein, COMP, is also found in tendon [19]. It is particularly interesting that the two tissues have a number of proteins in common, also including collagen II and aggrecan [20]. Indeed, tendon contains fibrocartilaginous parts, where compression due to pressure from, e.g., bone creates a load situation similar to that in cartilage.

Chondroadherin mRNA was detected in bone marrow cells by RT-PCR. Support was obtained by *in situ* hybridization, in initial experiments using RNA probes, showing that some bone marrow cells express chondroadherin (data not shown). The character of these bone marrow cells could not be established from the *in situ* hybridization.

The change in chondroadherin localization and expression upon maturation of the femoral head is very pronounced. Similarly, collagen II expression is low in immature cartilage and increases upon maturation [21]. The rather restricted distribution of chondroadherin to the lower part of the proliferative zone is intriguing, suggesting a role in regulating cell division, particularly considering the apparent absence of cell spreading and growth of chondrocytes on chondroadherin coated surfaces [4].

Another protein showing a similar distribution of expression is Indian hedgehog, which is upstream in the signalling pathway of parathyroid hormone related protein (PTHrP), a factor apparently preventing the change to the hypertrophic phenotype of chondrocytes [22]. Whether the expression of chondroadherin is influenced by these factors is not known. To our knowledge no other extracellular matrix molecule studied thus far has shown a corresponding distinct localization around a portion of the proliferative chondrocytes of the cartilage growth plate. This very distinct localization indicates a major change in the properties of the territorial matrix immediately surrounding the cells.

No apparent immunoreactivity was seen in diaphyseal bone trabeculae, although mRNA expression was detected in bone by RT-PCR. In contrast remnants containing COMP persisted after

calcification of the matrix [8]. It appears that chondroadherin is highly expressed by late proliferative chondrocytes and that it is efficiently cleared from the cartilage upon calcification and ossification.

An interesting finding is the cleavage fragment of about 25 kDa in samples of growth cartilage from day 20 to day 60. This is consistent with the elimination of chondroadherin from the tissue, when cells undergo hypertrophy. The size of the fragment recognized by the antiserum reacting with the C-terminal part of the molecule, indicates a major cleavage site in the central region of chondroadherin. The mechanisms and the regulation of this cleavage need to be elucidated. Chondroadherin is a member of the LRR protein family. A number of these proteins have been shown to interact with matrix constituents like collagens [23–25]. While chondroadherin has been shown to bind the integrin $\alpha_2\beta_1$ on cells [5], thereby potentially mediating attachment and/or cell signalling, it is not clear whether the protein can bind to other matrix constituents. To further elucidate the functions of chondroadherin, its interactions will have to be further studied. Current data suggest a role in regulating cell growth and migration.

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