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REVIEW ARTICLE

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Syndecan-3: a cell-surface heparan sulfate proteoglycan important for chondrocyte proliferation and function during limb skeletogenesis

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Abstract Syndecans are single-pass integral membrane components that serve as co-receptors for growth factors and cytokines and can elicit signal transduction via their cytoplasmic tails. We review here previous studies from our groups on syndecan-3 biology and function in the growth plates of developing long bones in chick and mouse embryos. Gain- and loss-of-function data indicate that syndecan-3 has important roles in restricting mitotic activity to the proliferative zone of growth plate and may do so in close cooperation and interaction with the signaling molecule Indian hedgehog (IHH). Biochemical and protein-modeling data suggest a dimeric/oligomeric syndecan-3 configuration on the chondrocyte's cell surface. Analyses of embryos misexpressing syndecan-3 or lacking IHH provide further clues on syndecan-3/IHH interdependence and interrelationships. The data and the conclusions reached provide insights into mechanisms fine-tuning chondrocyte proliferation, maturation, and function in the developing and growing skeleton and into how abnormalities in these fundamental mechanisms may subtend human congenital pathologies, including osteochondromas in hereditary multiple exostoses syndrome.

Key words Syndecan-3 · Chondrocyte proliferation · Indian hedgehog · Growth plate · Chondrocyte maturation · Limb skeletogenesis

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Introduction

The heparan sulfate proteoglycan family comprises macromolecules with exquisite structural and functional diversity [1–5]. Current family members include syndecan-1 through -4, which are single-pass integral membrane components; glypican-1 through -6, which are associated with cell surface via a glycosyl phosphatidyl inositol linkage; and pericellular/extracellular matrix proteoglycans such as perlecan, agrin, and the hybrid proteoglycan-collagen type XVIII. These macromolecules regulate a number of important processes, including cell proliferation, cell differentiation, cell migration, cell–cell interactions, and tissue and organ morphogenesis.

Syndecans are particularly interesting molecules. They serve as receptors and co-receptors of important cytokines, growth factors, and extracellular matrix components and can participate in pathologies when their expression, structure, properties, or distribution are altered [6,7]. Their membrane-intercalated structure allows them to directly connect the pericellular milieu with the cellular interior [8]. Molecular genetics has provided a full description of nature and primary structure of their core proteins [1], and biochemical analyses have provided details on structural-functional relationships along the heparan sulfate chains [9,10]. Syndecan core proteins are encoded by different genes and display distinct ectodomains, but share homologous transmembrane and cytoplasmic domains, except for a short variable region in the middle of the cytoplasmic domain. For instance, syndecan-4 has a relatively short ectodomain of about 140 amino acids bearing two heparan sulfate chains and capable also of protein–protein interactions, and its intracellular domain is involved in signal transduction through interaction/activation of protein kinase C [11]. Syndecan-3 instead has a rather long ectodomain of

more than 320 amino acids with typical heparan sulfate attachment sites toward the N-terminus plus an additional three sites near the transmembrane domain; its cytoplasmic domain has potentials for signal transduction and can interact with tyrosine kinases c-Src and c-Fys, actin-binding proteins and F-actin, and other important components [6].

Functional studies

Bernfield and coworkers carried out seminal work in syndecan biology with their original studies on syndecan-1, the founding member of the family, and described strong evidence for syndecan-1 roles in cell–extracellular matrix interactions, basal lamina organization, and tissue and organ morphogenesis [1]. Carey and collaborators were among the first to focus on syndecan-3 as an important effector of biological processes via heparan sulfate–protein interactions as well as protein–protein interactions [12,13]. Kosher and coworkers cloned syndecan-3 in the chick and studied its role in limb development [14]. They found that syndecan-3 is expressed in the progress zone in early limbs that lies beneath the apical ectodermal ridge and is constituted by rapidly proliferating mesenchymal and skeletal progenitor cells. These workers microinjected polyclonal syndecan-3 neutralizing antibodies into the progress zone of early chick embryos in ovo and found that the resulting interference with syndecan-3 function inhibited local cell proliferation and resulted in undergrowth and severe skeletal abnormalities [15]. These studies represented one of the first hints that one of the roles of syndecan-3 is to regulate proliferation of skeletal cells. This finding is in line with previous and recent evidence that experimental digestion of heparan sulfate chains from the cell surface or action by endogenous sulfatases can decrease the responses of many cell types to growth factors [2,16–19]. Conversely, exogenous heparin or heparan sulfate chains enhance mitotic responses of cells. What remains less clear, however, is exactly how heparan sulfate proteoglycans including syndecan-3 mediate the mitogenic responses. Several growth factors, including fibroblast growth factors (FGFs), or hedgehog proteins contain a heparan/heparin-binding domain; thus, it is possible that heparan sulfate proteoglycans bind the factors and "present/deliver" them to the respective receptors, which in that case are FGFRs and *Patched*, respectively [2,6,16,17]. These and related studies inspired us to investigate the biology of syndecan-3 further, specifically in chondrocyte development and function. Before reviewing our progress in this area, we briefly summarize the major steps of skeletogenesis involving chondrocytes.

Chondrocytes and skeletogenesis

Chondrocytes are the direct descendants of mesenchymal and ectomesenchymal cell condensations emerging at specific times and sites within the primordia of limbs, trunk, and head in the early embryo. Upon their differentiation from the condensed cells, the chondrocytes begin to assemble the cartilaginous blueprint and framework of the future skeleton; this critical but still poorly understood process allows the chondrocytes to provide configuration, structure, and organization of each skeletal element, be it a femur or a vertebra [20]. Within each skeletal anlage, the chondrocytes become organized in growth plates that contain reserve, proliferative, prehypertrophic, hypertrophic, and mineralizing zones of chondrocyte maturation [21,22]. This stepwise maturation process is required for transition from the cartilaginous skeleton to the definitive osseus skeleton. In a developing long bone, for example, ossification starts in association with the prehypertrophic zone with formation of an intramembranous bone collar around the incipient diaphysis. This step is followed by onset of endochondral ossification within the diaphysis, during which hypertrophic cartilage is invaded by populations of progenitor cells that give rise to bone and marrow. The chondrocyte maturation and ossification processes continue to attract much interest, given their fundamental nature in normal development and growth [23–25], and their vulnerability to congenital defects that can be as serious and devastating as achondroplasia or fibrodysplasia ossificans progressiva [26,27]. Thus, there have been important advances in understanding the chondrocyte maturation and ossification processes and their pathologies, but we are far from having a detailed and mechanistic understanding of them.

Syndecan-3 and chondrocyte proliferation: in vitro studies

What regulates chondrocyte proliferation in a developing skeletal element? How and why is it topographically localized and restricted to the proliferative zone? What maintains proliferation low in the reserve zone and what shuts off proliferation as the chondrocytes move from the proliferative zone to the prehypertrophic zone of the growth plate? What regulates the different rates of chondrocyte proliferation seen in different growth plates within the same developing organism and even within a single skeletal element, such as in the proximal and distal growth plates located at each epiphyseal end of a developing long bone? How is chondrocyte proliferation re-activated in pathologies such as osteoarthritis and what is its significance and implications? These are among the many questions for which there are at best only partial answers at the moment [28,29]. For example, several studies have pointed to the roles of growth hormone, insulin growth factors, and parathyroid hormone-related protein as positive regulators of chondrocyte proliferation in the growth plate and fibroblast growth factor receptors and their ligands as negative regulators [30–33]. However, it is not clear exactly how those circulating/local hormones regulate chondrocyte proliferation, particularly considering the different mitotic rates seen

in different growth plates within a given organism or even within a single skeletal element.

In our studies, we focused on syndecan-3 inspired by our initial results in developing chick embryo long bone anlagen providing the following realization, namely that syndecan-3 gene expression was confined and restricted precisely to the proliferative zone of each growth plate examined, regardless of stage of embryogenesis or site within the embryonic skeleton [34]. Syndecan-3 expression was preceded by a mitotically semiquiescent articular cap area expressing the matrix molecule tenascin-C, and was followed by the prehypertrophic zone expressing the secreted factor Indian hedgehog (IHH) (see following). These initial results led to several new questions but, most importantly, what is the function(s) of each of these molecules and of syndecan-3 in particular?

Because of its expression in the proliferative zone, we simply hypothesized that syndecan-3 must have a role in proliferation. To test this possibility, we isolated resting immature chondrocytes and maturing chondrocytes from caudal and cephalic portions of chick embryo sterna, a popular source of chondrocytes for experimental studies [35]. Cells were reared in standard monolayer culture for a few days during which the immature cells started to proliferate actively, and the maturing cells continued their proliferation. Subconfluent cultures were switched for 24h to medium containing 0.5% serum to reduce proliferation to a minimum, and were then exposed to an exogenous growth factor (specifically, FGF-2]. Chondrocyte proliferation was readily and quantitatively stimulated by FGF-2 as indicated by tritiated thymidine incorporation (Fig. 1A, closed circles), but this response was counteracted by concurrent exposure to heparinase-I (not shown), indicating that heparan sulfate chains were involved in the mitotic response.

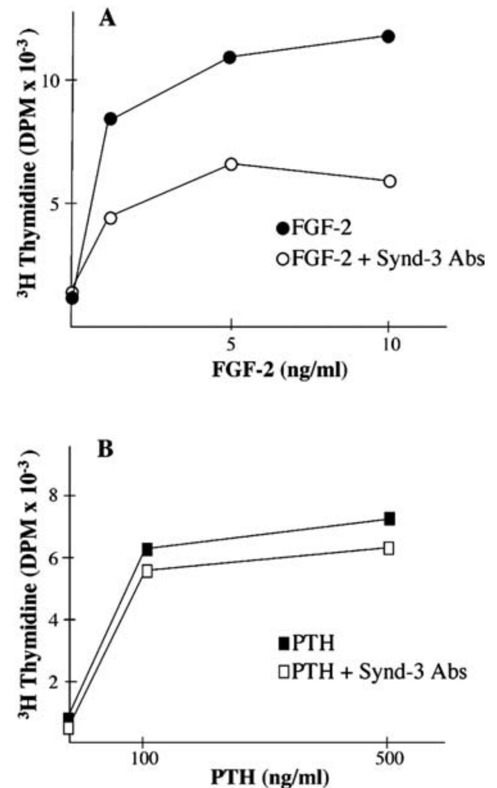

Fig. 1. Mitotic responses of chondrocytes. Subconfluent caudal sternal chondrocyte cultures were treated with indicated doses of **A** fibroblast growth factor (*FGF*)*-2* or **B** parathyroid hormone (*PTH*) for 24h in the absence or presence of 10μg/ml monospecific syndecan-3 antibodies. Cultures were pulse-labeled with [^{3}H]thymidine during the last 4h of treatment, and radioactivity was determined by scintillation counting. Data are averages of three separate experiments

To specifically test the syndecan-3 role, we raised polyclonal antibodies to syndecan-3 core protein similar to those used in the limb interference studies by Dealy et al. [15]. To maximize the probability that the antibodies would have neutralizing activity, we selected as antigen a large segment of the ectodomain (amino acids 215–313) previously proposed to have important functional roles, including protein–protein interactions [13]. The recombinant segment was used to raise rabbit polyclonal antibodies and also to purify them by affinity chromatography. The resulting monospecific antibodies indeed counteracted the stimulation of chondrocyte proliferation elicited by FGF-2 (Fig. 1A, open circles), but had no obvious effect on proliferation triggered by growth factors such as parathyroid hormone [36] that do not interact with, and require, heparan sulfate proteoglycans for action (Fig. 1B).

Syndecan-3 and chondrocyte proliferation and function: in vivo studies

To seek evidence for similar syndecan-3 roles in vivo, we misexpressed syndecan-3 in the developing limb of early chick embryos [37]. Syndecan-3 encoding RCAS viral particles were microinjected in the vicinity of the incipient cartilaginous humerus, radius, and/or ulna in day 4–5 chick embryos in ovo; contralateral wing buds were not injected and served as controls. Additional controls consisted of companion wing buds injected with insert-less viral particles. Embryos were reincubated for 3–6 days, injected with 5-bromodeoxyuridine (BrdU) 2h before harvesting, and processed for detection of incorporated BrdU and syndecan-3 gene expression. Figure 2A–D show the results obtained with day 7.5 embryos. In control embryos, syndecan-3 gene expression and proliferating chondrocytes exhibited predictable patterns and location at that stage [34,38,39] and were both restricted to the epiphyseal ends of radius and ulna (Fig. 2A,C, arrows). In contrast, in virally infected specimens syndecan-3 expression was quite strong and widespread (Fig. 2B) and proliferating chondrocytes were present throughout the anlagen, including the diaphyseal portion (Fig. 2D, arrowheads). Proliferation patterns were essentially unaffected in surrounding perichondrial and mesenchymal tissues (Fig. 2C,D).

To determine whether these changes had additional consequences on long bone development, embryos were examined at later stages by anatomy, histochemistry, and in situ

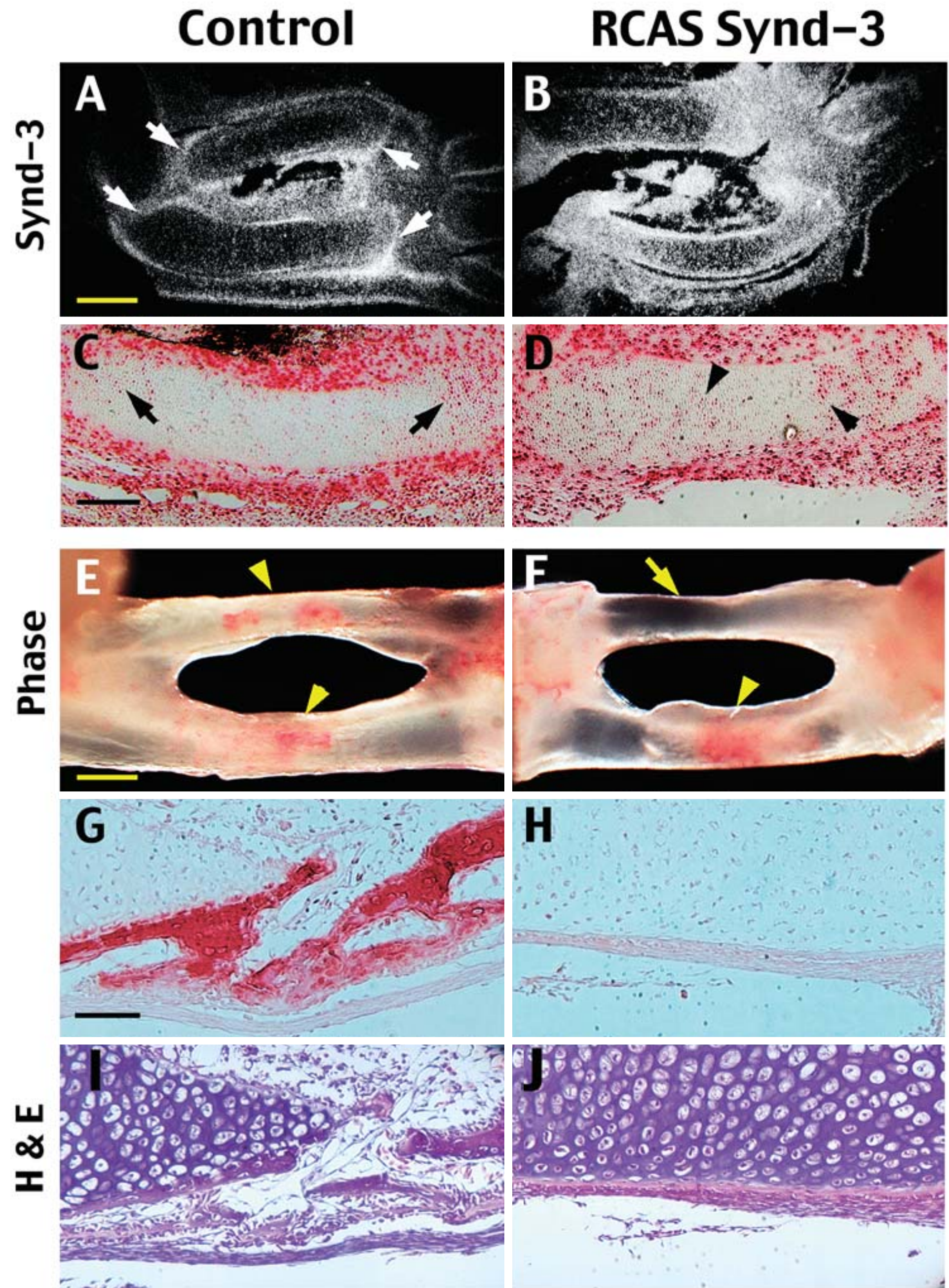

Fig. 2. Skeletal consequences of syndecan-3 (*Synd-3*) misexpression in developing chick limbs. Syndecan-3-encoding RCAS viral particles were microinjected in the vicinity of radius and ulna in day 4–5 chick embryos in ovo, and eggs were reincubated and sacrificed at later time points. Proliferation was monitored by injection of 5-bromo-deoxyuridine (*BrdU*) 2 h before harvesting. **A–D** Longitudinal sections from control (**A** and **C**) and syndecan-3-misexpressing (**B** and **D**) day 7.5 chick embryos processed for in situ hybridization analysis of syndecan-3 gene expression (**A,B**) and BrdU-labeled proliferating cells (**C,D**). **E–J** Anatomic and histological analyses of day 10 embryos. Note the entirely cartilaginous syndecan-3-misexpressing radius (*arrow* in **F**) and the ossifying radius in control (*arrowheads* in **E**). Note alizarin red-staining bone matrix and invading bone and marrow cells in control (**G** and **I**) and their absence in syndecan-3 misexpressing tissue (**H** and **J**). See text for further details. *Bars* **A,B** 150 μm; **C,D** 75 μm; **E,F** 250 μm; **G–J** 35 μm

hybridization [37]. Results from day 10 embryos are shown in Fig. 2E-2J. Control uninfected radius and ulna had the expected normal appearance and a diaphysis that was rich in blood vessels (Fig. 2E, arrowheads) and displayed alizarin red-staining bone matrix and endochondral bone and marrow (Fig. 2G,I). In contrast, the virally infected syndecan-3 misexpressing radius was entirely cartilaginous (Fig. 2F, arrow), and lacked alizarin red-staining bone matrix, endochondral bone, bone collar, and marrow (Fig. 2H,J). The adjacent ulna that had remained uninfected displayed features similar to control (Fig. 2F, arrowhead), indicating that there were no side effects or systemic changes.

Gene expression patterns in day 10 control uninfected elements were typical (Fig. 3). Syndecan-3 transcripts were abundant and limited to the proliferative zone of growth plate (Fig. 3A, arrow) lying below the articular cap (Fig. 3A, *ac*). Indian hedgehog (IHH) transcripts were equally prominent and restricted to the prehypertrophic zone (Fig. 3B, arrow), and type X collagen and osteopontin RNAs were seen primarily in diaphyseal hypertrophic and posthypertrophic zones, respectively (Fig. 3C,D, arrows). In syndecan-3 misexpressing element, however, syndecan-3 transcripts were very abundant and present in epiphysis, metaphysis, and diaphysis, and represented endogenous

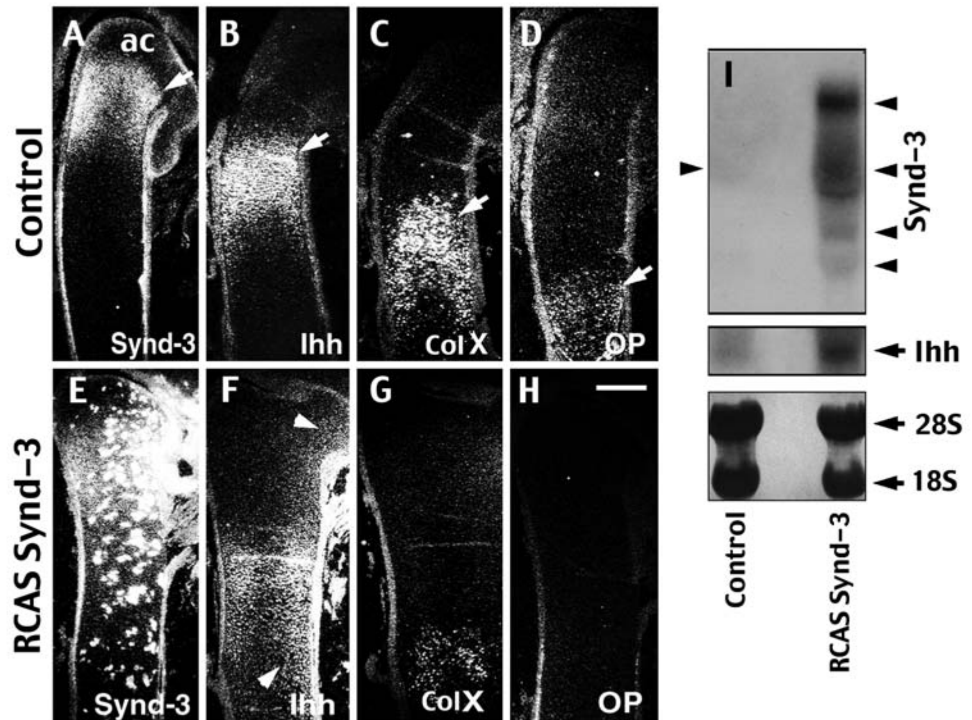

Fig. 3. Gene expression in control and syndecan-3-misexpressing tibia and cultured chondrocytes. **A–D** and **E–H** Longitudinal sections from day 10 control (**A–D**) and syndecan-3-misexpressing wings (**E–H**) were processed for expression analysis by in situ hybridization of syndecan-3 (*Synd-3*); Indian hedgehog (*IHH*); type X collagen (*ColX*); and osteopontin (*OP*). **I** Northern blot analysis of syndecan-3 and IHH expression in control and syndecan-3-overexpressing (RCAS Synd-3] cultures. Note the single faint main syndecan-3 transcript in control lane (*single arrowhead on left*), and the multiple abundant transcripts in overexpressing cells that reflect cellular and virally encoded transcripts of varying sizes as expected. *Bar* **A–D**, **E–H** 150 μm

and virally encoded transcripts (Fig. 3E). Interestingly, IHH gene expression had been affected as well, and IHH transcripts were similarly abundant and widespread and were present even in epiphysis and diaphysis (Fig. 3F, arrowheads), clearly overlapping the misexpression patterns of syndecan-3. Type X collagen expression was severely reduced (Fig. 3G) and osteopontin RNAs were essentially undetectable (Fig. 3H).

To verify that the sharp and unexpected increase in IHH gene expression was directly linked to syndecan-3 misexpression, we determined whether IHH gene expression would increase in chondrocytes in culture overexpressing syndecan-3 (see below for further details). Northern blot analysis indeed revealed that syndecan-3-overexpressing cultures (RCAS Synd-3) contained much higher levels of IHH transcripts (Fig. 3I) than companion control RCAS cultures, which exhibited expected low levels of both IHH and syndecan-3 RNAs (Fig. 3I) (Shimo et al., in preparation).

Clearly, normal syndecan-3 expression patterns are important for chondrocyte proliferation as well as maturation, and syndean-3 misexpression is incompatible with normal progression of maturation and ossification and normal expression of IHH.

Endogenous mitogens

As already mentioned, the nature of the endogenous mitogens regulating mitotic activity in the proliferative zone remains one of the least understood aspects of growth plate biology. Genetic evidence from in vivo studies had pointed to the possibility that IHH produced in prehypertrophic zone may be one such endogenous factor [40–42]. This possibility was particularly attractive because it would imply that the mitotically quiescent prehypertrophic chondrocytes regulate proliferation in the preceding zone of the growth plate [24,40]. It would also relate well to the close coordination of syndecan-3 and IHH gene expression patterns and interrelated functions suggested by our data reviewed above. Thus, we carried out experiments in vitro to test directly whether IHH is a mitogen for chondrocytes and whether syndecan-3 is involved in its action [37]. Immature and maturing sternal chondrocytes were reared in monolayer culture as above; one-half of the cultures were infected with retroviral particles encoding full-length chick syndecan-3 and the remainder were infected with insert-less control virus. Immunoblot analysis showed that syndecan-3 levels had increased two- to threefold in day 10 cultures expressing the syndecan-3 transgene. Control and syndecan-3-overexpressing cultures were then treated with increasing amounts of recombinant N-terminal half of IHH (rIHH-N) for 24 h, and proliferation rates were monitored by BrdU incorporation. This analysis revealed that rIHH-N stimulated proliferation 20%–40% compared to control cultures. Stimulation was much more conspicuous in cultures overexpressing syndecan-3, and was counteracted in all cases by treatment with heparinase I (not shown) or neutralizing antibodies to syndecan-3 (Fig. 4). It is interesting to note that both heparinase I and antibody treatments reduced baseline proliferation rates in the absence of exogenous rIHH-N, indicating that they were also interfering with endogenous growth factors including IHH.

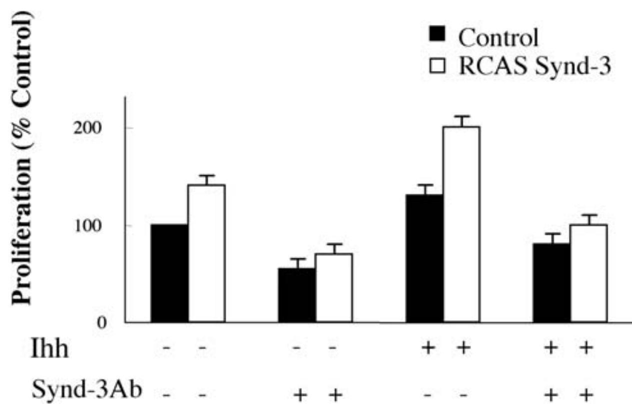

Fig. 4. Mitotic responses of chondrocytes to exogenous IHH. Semiconfluent chondrocyte cultures were grown in control condition in the absence of additives (−) or were treated (+) for 24 h with 1 μg/ml recombinant IHH in absence or presence of 10 μg/ml monospecific syndecan-3 antibodies. Proliferation was measured by BrdU incorporation during the last 4 h of culture, and value in control untreated cultures was set at 100%

Syndecan-3 organization on chondrocyte cell surface

The foregoing results support the notion that syndecan-3 has a positive role in proliferation together with IHH, but raised another question. How can antibodies directed against syndecan-3 core protein interfere with FGF-2 mitogenic responses? Does the core protein have a role? There is evidence that the core proteins of other proteoglycans can bind growth factors directly [43], but such evidence has never been reported for syndecan-3. Among other possibilities we considered was the following. Because the syndecan-3 neutralizing antibodies used above were against a large ectodomain segment proposed to have protein–protein interaction activity [13], we reasoned that perhaps syndecan-3 is present as a dimer or oligomer on the surface of chondrocytes and this configuration may have functional significance. To test this possibility, chick chondrocytes in monolayer were exposed to the reversible cross-linker dimethyl suberimidate for 3 h and the resulting cell homogenates were processed for electrophoresis and immunoblotting before and after treatment with a reducing agent. This approach did reveal that syndecan-3 molecules were in fact present as dimers/oligomers on the chondrocytes surface [39]. We used computer-assisted protein modeling to analyze the syndecan-3 ectodomain and identify possible motifs that could lead to protein–protein interactions. This analysis suggested that a portion of the ectodomain from amino acid 124 to 196 has a potentially extended stalk-like configuration consisting of several β-strand segments and reflecting its high threonine-serine-proline content. This structure would enable contiguous syndecan-3 molecules to form dimers/oligomers in which the β-strand segments would align and form stable parallel β-sheet structures. We have reported the resulting three-dimensional (3-D) model of avian syndecan-3 dimer in our earlier paper [39].

Mammalian growth plates

We have recently initiated new lines of experimentation to extend our work as reviewed here to mammalian systems and determine whether the regulation of chondrocyte behavior and function is similar in avian and mammalian growth plates. We have just completed a detailed analysis of the gene expression patterns of syndecan-1, -2, -3, and -4 in mouse embryo growth plates (Koyama et al., in preparation). Data obtained with wild-type E17.5 mouse embryo tibias indicated that as in chick, syndecan-3 was strongly expressed in the proliferative zone of growth plate (Fig. 5D, arrow) identifiable by expression of proliferation marker histone H4C (Fig. 5I, arrow). It is noteworthy that there were also a few proliferating chondrocytes at the epiphyseal end (likely representing articular cap/reserve zone) that did not express syndecan-3 strongly (Fig. 5I, arrowhead). Syndecan-3 as well as syndecan-1 and -2 were expressed in endochondral bone and perichondrium/periosteum (Fig. 5B–D, arrowheads) [44], whereas syndecan-4 was expressed at low levels throughout the anlage (Fig. 5E). Other gene markers had predictable expression, with collagen X strong in the hypertrophic zone (Fig. 5F) and osteopontin (OP) and vascular endothelial cell growth factor (VEGF) in posthypertrophic cells and intramembranous collar and endochondral bone (Fig. 5G,H).

To test whether the relationship between syndecan-3 and IHH suggested by our chick studies may be valid in the mouse as well, we analyzed syndecan gene expression patterns in IHH-null E17.5 mouse embryos (littermates of the above wild-type embryos) [45]. Interestingly, syndecan-3 gene expression was disregulated and characterized nearly the entire cartilaginous tibia (Fig. 5M). Equally interesting was the finding that expression of syndecan-2 and -4 was undetectable (Fig. 5L,N). Syndecan-1 expression was confined to an abnormal epiphyseal region that was undergoing ectopic ossification (Fig. 5K) as indicated by its histology (Fig. 5J) and ectopic expression of collagen X, osteopontin, and VEGF (Fig. 5O–Q). However, there was no obvious evidence for an intramembranous bone collar around the diaphysis. Last, the null tissue did not contain a typical proliferative zone (Fig. 5R), and the few proliferating chondrocytes present were limited to the most epiphyseal region (Fig. 5R, arrowhead) possibly representing remnants of articular cap or reserve zone.

Discussion

Our studies have examined the biology of syndecan-3 in the growth plate of developing skeletal elements [37–39,41]. The results provide evidence that this cell-surface component mediates the specific response of chondrocytes in vitro to such potent mitogens as FGF-2, having no apparent role in the modulation of mitotic activity in response to heparin-independent growth factors such as parathyroid hormone (PTH). Syndecan-3 also regulates chondrocyte prolifera-

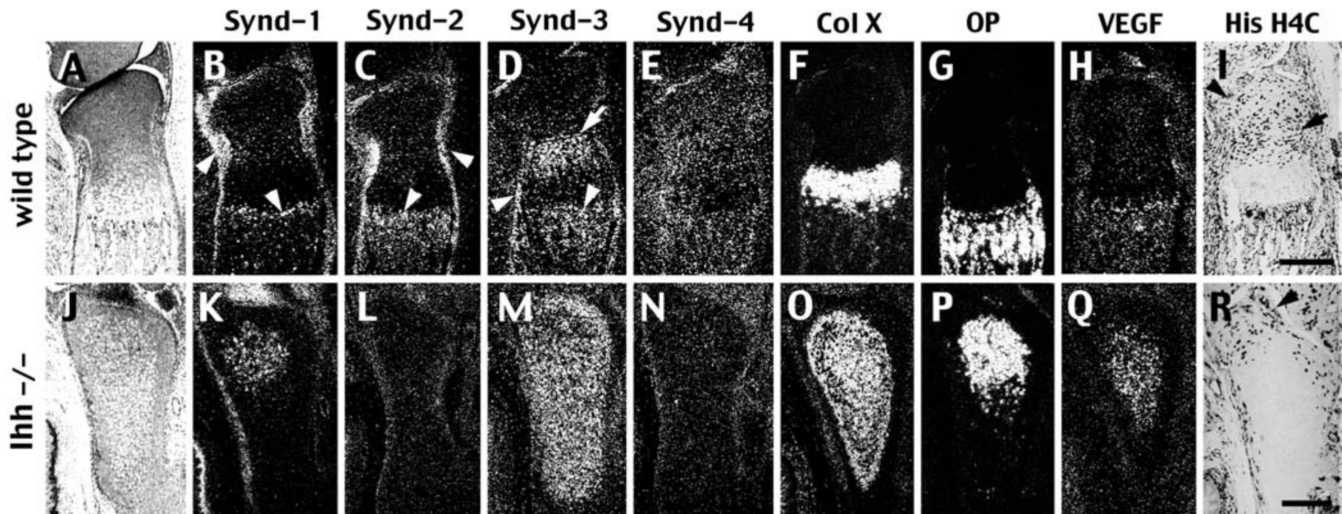

Fig. 5. In situ hybridization analysis of gene expression and chondrocyte proliferation in E17.5 wild type (**A–I**) and IHH-null (**J–R**) mouse embryo tibias. Longitudinal sections were hybridized with radiolabeled riboprobes encoding indicated genes. Cell proliferation in **I** and **R** was revealed by hybridization with probe encoding histone H4C, which is expressed by proliferating cells. See text for details. *Bars* **A–I** 80 μm; **J–R** 60 μm

tion in vivo, as indicated by the widespread proliferative behavior seen in developing chick limb skeletal elements misexpressing syndecan-3. In addition, our studies identify IHH as a likely endogenous syndecan-3-binding mitogen. This is in line with the proven ability of hedgehog proteins to act as growth factors for several cell types and the ability of IHH to diffuse away from its site of synthesis in prehypertrophic zone into surrounding zones and perichondrial tissue [46,47]. Last but not least, our data show that syndecan-3 expression in the proliferative zone is shared by both avian and mammalian growth plates, indicating that syndecan-3 function is conserved; the possible importance of syndecan-3 and syndecans in general in mouse embryo growth plate biology is reaffirmed by the chaotic patterns of syndecan gene expression and abnormal chondrocyte behavior seen in IHH-null mouse embryo growth plates. It is thus proper to conclude that syndecan-3 is an important player in regulating proliferation as well as maturation in the growth plate. Syndecan-3 would exert its roles by virtue of its restricted patterns of gene expression, its receptor/coreceptor properties, and its ability to bind endogenous ligands including IHH. Mitotic activity occurring outside the proliferative zone, such as that characterizing most epiphyseal articular/reserve chondrocytes (Fig. 5I), could thus be independent of syndecan-3/IHH action and may reflect action by other factors, such as PTH-related protein (PTHrP).

Syndecans and syndecan-3 in particular owe their biological properties to their heparan sulfate chains and their core proteins. Heparan sulfates bear an extraordinary degree of biological sophistication and specificity embedded into their saccharide sequence and modifications [9,10], and the structural diversity of syndecan core proteins speaks volumes to the fact that each must have specific properties and action. At present, however, we remain largely ignorant about the specific sequence and structure of heparan sulfate chains in growth plate syndecans and whether the chains present on syndecan-3 in the proliferative zone are identical or different from those present in other syndecans expressed at neighboring sites. Likewise, not much is known about whether syndecan-3 is involved in signal transduction in chondrocytes and whether this occurs via proteins such as src or the actin cytoskeleton. Thus, we regard our data on the presence of syndecan-3 dimers/oligomers on the chondrocyte cell surface as an important step in clarifying the biology of this molecule. Carey, Bernfield, and coworkers had postulated that syndecan-3 molecules are present on the cell surface as dimers, but direct evidence was lacking [6,13]. Such configuration would have many advantages. It could further increase syndecan-3 ability to bind and concentrate growth factors, increasing their local density and probability of encounter and interactions with the signaling receptors. It would also offer syndecan-3 and syndecans in general another level of control, as the molecules could shift from monomers to dimers/oligomers and back to monomers as needed. Syndecan dimerization is postulated to be required for signal transduction [1,6], a process reminiscent of that required for receptors such as FGFRs or bone morphogenetic factor receptors (BMPRs). Thus, it is conceivable that function of monomeric syndecan-3 molecule may be exerted for the most part by its heparan sulfate chains, whereas function of dimeric/oligomeric syndecan-3 molecules would involve also their core proteins and elicit signal transduction.

Since its identification about 10 years ago, IHH has attracted a considerable amount of attention. IHH and its other family members owe their popularity to the fact that they have fundamental roles in development. Several

groups including ours first reported that IHH is expressed in the prehypertrophic zone of the growth plate [40,48,49]. We went on to show that IHH expression coincides with formation of the intramembranous bone collar around the incipient diaphysis of developing long bones, and that IHH induce osteogenic cell differentiation [49,50]. These findings led us to propose that a key function of IHH in the growth plate is to induce and regulate osteogenesis. Others went on to show that IHH may have another role, that is, to limit the rates of chondrocyte maturation and hypertrophy in cooperation with peri-articular-derived PTHrP [40,51]. Genetic studies provided firm evidence for both roles as well as evidence that IHH has yet one more role in growth plate, that is, a role in chondrocyte proliferation [42]. What has remained unclear is whether IHH action in proliferation is direct and how it occurs. Our studies [37] reviewed here provide evidence for a direct role of IHH in chondrocyte proliferation, and can also account for the topographical restriction of proliferation in the growth plate. Proliferation would be limited to the zone where syndecan-3 is expressed and where an effective IHH–syndecan-3 interactions occurs. The delicate balance between, and the mutual interdependence of, syndecan-3 and IHH are reaffirmed by our new data on syndecan-3 gene expression in wild-type and IHH-null mouse embryos. The disregulated and widespread expression of syndecan-3 throughout much of the IHH-null cartilaginous long bone anlagen, combined with absence of proliferative growth plate chondrocytes, strongly indicates that a normal syndecan-3/IHH loop is important for normal proliferation and normal progression and completion of chondrocyte maturation. The broad and deregulated expression of syndecan-3 could thus be viewed as a futile attempt to restore/promote chondrocyte proliferation in the absence of endogenous IHH mitogenic action.

The challenges that remain ahead are many and complex. In addition to those mentioned above, work will have to be directed toward understanding how syndecan-3 expression becomes restricted to the proliferative zone and how syndecan-3 mediates IHH action in vivo. We should mention that syndecan-3-null mice have been described to be relatively normal, but no detailed analyses of their developing skeletons were reported [52]. On the other hand, the severe skeletal phenotype of mice lacking perlecan has been documented, and the defects include a disorganized growth plate with aberrant IHH gene expression [53]. Thus, it would be interesting to examine the syndecan-3-null mice in greater detail and to study also the perlecan-null mice for possible aberrant expression of syndecans and syndecan-3 in particular. Furthermore, we show here that syndecan-1 and syndecan-2 are preferentially expressed in perichondrium/periosteum and endochondral bone, leading to the possibility that these molecules may have roles in osteogenesis via their site-specific interactions with local potent cytokines including BMPs and IHH itself. In this context, it is important to point out also studies indicating that defects in heparan sulfate synthesis due to mutations in glycosyltransferase EXT1 and EXT2 genes cause human skeletal pathologies such as osteochondromas in hereditary multiple exostoses syndrome [54–56]. These tumors form in association with the growth plate and are likely to be caused by unhindered diffusion and leakage of IHH into surrounding perichondrial tissues, where it would act as an inducer of ectopic chondrogenesis and osteogenesis [57] and tumor formation.

In sum, ongoing and future studies promise to shed further light on mechanisms by which syndecan-3 and other heparan sulfate proteoglycans bring about normal chondrocyte proliferation and maturation process in growth plates and normal cartilage-to-bone transition and on how abnormalities in these fundamental pathways may subtend human pathologies spanning from growth retardation to musculoskeletal neoplasias.

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References

1. Bernfield M, Kokenyesi R, Kato M, Hinkes MT, Spring J, Gallo RL, Lose EJ (1992) Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. Annu Rev Cell Biol 8:365–393
2. Carey DJ (1997) Syndecans: multifunctional cell surface coreceptors. Biochem J 327:1–16
3. Iozzo RV (2001) Heparan sulfate proteoglycans: intricate molecules with intriguing functions. J Clin Invest 108:165–167
4. De Cat B, David G (2001) Developmental roles of the glypicans. Semin Cell Dev Biol 12:117–125
5. Filmus J (2001) Glypicans in growth control and cancer. Glycobiology 11:19R–23R
6. Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M (1999) Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 68:729–777
7. Reiland J, Sanderson RD, Waguespack M, Barker SA, Long R, Carson DD, Marchetti D (2004) Heparanase degrades syndecan-1 and perlecan heparan sulfate: functional implications for tumor cell invasion. J Biol Chem 279:8047–8055
8. Woods A (2001) Syndecans: transmembrane modulators of adhesion and matrix assembly. J Clin Invest 107:935–941
9. Vives RR, Pye DA, Salmivirta M, Hopwood JJ, Lindahl U, Gallagher JT (1999) Sequence analysis of heparan sulphate and heparin oligosaccharides. Biochem J 339:767–773
10. Nakato H, Kimata K (2002) Heparan sulfate fine structure and specificity of proteoglycan functions. Biochim Biophys Acta 1573:312–318
11. Oh E-S, Woods A, Couchman JR (1997) Multimerization of the cytoplasmic domain of syndecan-4 is required for its ability to activate protein kinase C. J Biol Chem 272:11805–11811
12. Chernousov MA, Carey DJ (1993) *N*-Syndecan (syndecan-3) from neonatal rat brain binds basic fibroblast growth factor. J Biol Chem 268:16810–16814
13. Asundi VK, Carey DJ (1995) Self-association of *N*-syndecan (syndecan-3) core protein is mediated by a novel structural motif in the transmembrane domain and ectodomain flanking region. J Biol Chem 270:26404–26410
14. Gould SE, Upholt WB, Kosher RA (1995) Characterization of chicken syndecan-3 as a heparan sulfate proteoglycan and its expression during embryogenesis. Dev Biol 168:438–451
15. Dealy CN, Seghatoleslami MR, Ferrari D, Kosher RA (1997) FGF-stimulated outgrowth and proliferation of limb mesoderm is dependent on syndecan-3. Dev Biol 184:343–350
16. Yayon A, Klagsbrun M, Esko JD, Leder P, Ornitz DM (1991) Cell surface, heparin-like molecules are required for binding to basic

fibroblast growth factor to its high affinity receptor. Cell 64:841–848
17. Rapraeger AC (1995) In the clutches of proteoglycans: how does heparan sulfate regulate FGF binding? Chem Biol 2:645–649
18. Dhoot GK, Gustafsson MK, Ai X, Sun W, Standiford DM, Emerson CP (2001) Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. Science 293:1663–1666
19. Wang S, Ai X, Freeman SD, Pownall ME, Lu Q, Kessler DS, Emerson CP (2004) QSulf1, a heparan sulfate 6-*O*-endosulfatase, inhibits fibroblast growth factor signaling in mesoderm induction and angiogenesis. Proc Natl Acad Sci USA 101:4833–4838
20. Hinchliffe JR, Johnson DR (1980) The development of the vertebrate limb. Oxford University Press, New York, pp 72–83
21. Hinchliffe JR, Johnson DR (1983) Growth of cartilage. In: Hall BK (ed) Cartilage. Development, differentiation, and growth, vol 2. Academic Press, New York, pp 255–295
22. Hunziker EB (1994) Mechanism of longitudinal bone growth an its regulation by growth plate chondrocytes. Microsc Res Tech 28:505–519
23. Iwamoto M, Enomoto-Iwamoto M, Kurisu K (1999) Actions of hedgehog proteins on skeletal cells. Crit Rev Oral Biol Med 10:477–486
24. Pacifici M, Gentili C, Yin M, Iwamoto M, Enomoto-Iwamoto M, Abrams WR, Koyama E (2002) Indian hedgehog and retinoids orchestrate multiple growth plate functions in developing long bones: the growth plate as a highly interactive structure. In: Shapiro IM, Boyan B, Anderson HC (eds) The Growth Plate. IOS Press, Amsterdam, pp 1–17
25. Kronenberg HM (2003) Developmental regulation of the growth plate. Science 423:332–336
26. Zelzer E, Olsen BR (2003) The genetic basis of skeletal diseases. Nature (Lond) 423:343–348
27. Kaplan FS, Glaser DL, Hebela N, Shore EM (2004) Heterotopic ossification. J Am Acad Orthop Surg 12:116–125
28. Farnum CE, Wilsman NJ (1993) Determination of proliferative characteristics of growth plate chondrocytes by labeling with bromodeoxyuridine. Calcif Tissue Int 52:110–119
29. Wilsman NJ, Farnum CE, Green EM, Lieferman EM, Clayton MK (1996) Cell cycle analysis of proliferative zone chondrocytes in growth plates elongating at different rates. J Orthop Res 14:562–572
30. Liu JL, LeRoith D (1999) Insulin-like growth factor I is essential for postnatal growth in response to growth hormone. Endocrinology 140:5178–5184
31. Butler AA, LeRoith D (2001) Tissue-specific versus generalized gene targeting of the IGF1 and IFG1R genes and their roles in insulin-like growth factor physiology. Endocrinology 142:1685–1688
32. Kobayashi T, Chung U-I, Schipani E, Starbuck M, Karsenty G, Katagiri T, Goad DL, Lanske B, Kronenberg HM (2002) PTHrP and Indian hedgehog control differentiation of growth plate chondrocytes at multiple steps. Development (Camb) 129:2977–2986
33. Colvin JS, Bohne BA, Harding GW, McEwen DG, Ornitz DM (1996) Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. Nat Genet 12:390–397
34. Shimazu A, Nah H-D, Kirsch T, Koyama E, Leatherman JL, Golden EB, Kosher RA, Pacifici M (1996) Syndecan-3 and the control of chondrocyte proliferation during endochondral ossification. Exp Cell Res 229:126–136
35. Gibson GJ, Flint MH (1985) Type X collagen synthesis by chick sternal cartilage and its relationship to endochondral ossification. J Cell Biol 101:277–284
36. Koike T, Iwamoto M, Shimazu A, Nakashima K, Suzuki F, Kato Y (1990) Potent mitogenic effects of parathyroid hormone (PTH) on embryonic chick and rabbit chondrocytes. J Clin Invest 85:626–631
37. Shimo T, Gentili C, Iwamoto M, Wu C, Koyama E, Pacifici M (2004) Indian hedgehog and syndecan-3 coregulate chondrocyte proliferation and function during chick limb skeletogenesis. Dev Dyn 229:607–617
38. Koyama E, Leatherman JL, Shimazu A, Nah H-D, Pacifici M (1995) Syndecan-3, tenascin-C and the development of cartilaginous skeletal elements and joints in chick limbs. Dev Dyn 203:152–162
39. Kirsch T, Koyama E, Liu M, Golub EE, Pacifici M (2002) Syndecan-3 is a selective regulator of chondrocyte proliferation. J Biol Chem 277:42171–42177
40. Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ (1996) Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 273:613–622
41. Koyama E, Shimazu A, Leatherman JL, Golden EB, Nah H-D, Pacifici M (1996) Expression of syndecan-3 and tenascin-C: possible involvement in periosteum development. J Orthop Res 14: 403–412
42. Long F, Zhang XM, Karp S, Yang Y, McMahon AP (2001) Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. Development (Camb) 128:5099–5108
43. Mongiat M, Taylor K, Otto J, Aho S, Uitto J, Whitelock JM, Iozzo RV (2000) The core protein of the proteoglycan perlecan binds specifically to fibroblast growth factor-7. J Biol Chem 275:7095–7100
44. David G, Bai XM, vad der Schueren B, Marynen P, Cassiman J-J, van der Berghe H (1993) Spatial and temporal changes in the expression of fibroglycan (syndecan-2) during mouse embryonic development. Development (Camb) 119:841–854
45. St-Jacques B, Hammerschmidt M, McMahon AP (1999) Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev 13:2076–2086
46. Gritli-Linde A, Lewis P, McMahon AP, Linde A (2001) The whereabouts of a morphogen: direct evidence for short- and graded long-range activity of hedgehog signaling peptides. Dev Biol 236:364–386
47. Yin M, Gentili C, Koyama E, Zasloff M, Pacifici M (2002) Antiangiogenic treatment delays chondrocyte maturation and bone formation during limb skeletogenesis. J Bone Miner Res 17:56–65
48. Bitgood MJ, McMahon AP (1995) Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. Dev Biol 172:126–138
49. Koyama E, Leatherman JL, Noji S, Pacifici M (1996) Early chick limb cartilaginous elements possess polarizing activity and express *Hedgehog*-related morphogenitic factors. Dev Dyn 207:344–354
50. Nakamura T, Aikawa T, Enomoto-Iwamoto M, Iwamoto M, Higuchi Y, Pacifici M, Kinto N, Yamaguchi A, Noji S, Kurisu K, Matsuya T (1997) Induction of osteogenic differentiation by hedgehog proteins. Biochem Biophys Res Commun 237:465–469
51. Lanske B, Karaplis AC, Lee K, Lutz A, Vortkamp A, Pirro A, Karperien M, Defize LHK, Ho C, Mulligan R, Abou-Samra AB, Juppner H, Segre GV, Kronenberg HM (1996) PTH/PTHrp receptor in early development and Indian hedgehog-regulated bone growth. Science 273:663–666
52. Kaksonen M, Pavlov I, Voikar V, Lauri SE, Hienola A, Riekki R, Lakso M, Taira T, Rauvala H (2002) Syndecan-3-deficient mice exhibit enhanced LTP and impaired hippocampus-dependent memory. Mol Cell Neurosci 21:158–172
53. Arikawa-Hirasawa E, Watanabe H, Takami H, Hassell JR, Yamada Y (1999) Perlecan is essential for cartilage and cephalic development. Nat Genet 23:354–358
54. Hecht JT, Hogue D, Strong LC, Hansen MF, Blanton SH, Wagner H (1995) Hereditary multiple exostosis and chondrosarcoma: linkage to chromosome II and loss of heterozygosity for EXT-linked markers on chromosome II and 8. Am J Hum Genet 56:1125–1131
55. McCormick C, Duncan G, Goutsos KT, Tufaro F (2000) The putative tumor suppressors EXT1 and EXt2 form a stable complex that accumulates in the Golgi complex and catalyzes the synthesis of heparan sulfate. Proc Natl Acad Sci USA 97:668–673
56. Tiet TD, Alman BA (2003) Developmental pathways in musculoskeletal neoplasia: involvement of the Indian hedgehog-parathyroid hormone-related protein pathway. Pediatr Res 53: 539–543
57. Enomoto-Iwamoto M, Nakamura T, Aikawa T, Higuchi Y, Yuasa T, Yamaguchi A, Nohno T, Noji S, Matsuya T, Kurisu K, Koyama E, Pacifici M, Iwamoto M (2000) Hedgehog proteins stimulate chondrogenic cell differentiation and cartilage formation. J Bone Miner Res 15:1659–1668