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Heparan Sulfate-Dependent Signaling of Fibroblast Growth Factor 18 by Chondrocyte-Derived Perlecan[†]

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ABSTRACT: Perlecan is a large multidomain proteoglycan that is essential for normal cartilage development. In this study, perlecan was localized in the pericellular matrix of hypertrophic chondrocytes in developing human cartilage rudiments. Perlecan immunopurified from medium conditioned by cultured human fetal chondrocytes was found to be substituted with heparan sulfate (HS), chondroitin sulfate (CS), and keratan sulfate (KS). Ligand and carbohydrate engagement (LACE) assays demonstrated that immunopurified chondrocyte-derived perlecan formed HS-dependent ternary complexes with fibroblast growth factor (FGF) 2 and either FGF receptors (FGFRs) 1 or 3; however, these complexes were not biologically active in the BaF32 cell system. Chondrocyte-derived perlecan also formed HS-dependent ternary complexes with FGF18 and FGFR3. The proliferation of BaF32 cells expressing FGFR3 was promoted by chondrocyte-derived perlecan in the presence of FGF18, and this activity was reduced by digestion of the HS with either heparinase III or mammalian heparanase. These data suggest that FGF2 and -18 bind to discrete structures on the HS chains attached to chondrocyte-derived perlecan which modulate the growth factor activities. The presence and activity of mammalian heparanase may be important in the turnover of HS and subsequent signaling required for the establishment and maintenance of functional osteo-chondral junctions in long bone growth.

Cartilage provides the framework for endochondral bone formation via a process in embryonic development known as chondrogenesis. The growth of long bone begins with condensation of the mesenchymal stem cells followed by proliferation, which causes the anlage to expand in length and increase in width following the differentiation of the chondroblastic mesenchymal cells into chondrocytes. This process results in chondrocytes being arranged in morphologically distinct zones, including resting, proliferating, prehypertrophic, and hypertrophic (*1*). These zones reflect the function of the chondrocytes as they are exposed to a plethora of signaling molecules, some of which interact with their surrounding matrix (*2*).

Perlecan, the major heparan sulfate (HS)[1] proteoglycan of basement membranes (*3*, *4*), has also been isolated from articular cartilage (*5*) and localized to chondrocytes in the hypertrophic regions of articular cartilage as well as to the growth plate of developing long bones, where it was shown to surround the cells and form a "basement membrane" of the chondron (*6*−*9*). While there is no direct evidence that chondrocyte-derived perlecan HS is involved in chondrogenesis, cartilage-derived perlecan has previously been shown to bind a number of growth factors involved in bone development, including those of the FGF family. Specifically, it has been shown to bind and regulate the activities of FGF1 (*10*), FGF2 (*11*−*14*), and FGF9 (*10*) by an HS-mediated mechanism and more recently FGF18 by binding to the protein core (*15*). FGF2 and FGF18 are involved in cartilage growth and maturation, where they have been implicated in the development of functional cartilage and bone tissue (*16*−*19*). FGF2 may provide an antagonistic signal to FGF18 as FGF2 has been shown to decrease the level of hypertrophic chondrocytes in cartilage tissues (*20*) and mice null for FGF2 have decreased bone mass (*21*). Mice overexpressing FGF2 exhibited a phenotype characterized by chondrodysplasia, where FGF2 is antiproliferative in the growth plate and there is an increased level of apoptosis in the growth plate chondrocytes, leading to dwarfism (*22*). Mice lacking FGF18 display expanded zones of proliferating and hypertrophic chondrocytes as well as an increased level of chondrocyte proliferation (*2*). Mice null for

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[1] Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); BSA, bovine serum albumin; CS, chondroitin sulfate; DPBS, Dulbecco's phosphate-buffered saline; DPBST, Dulbecco's phosphate-buffered saline with 0.1% (w/v) Tween 20; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; HS, heparan sulfate; KS, keratan sulfate; LACE, ligand and carbohydrate engagement assay; PVDF, polyvinylidene difluoride; RT, room temperature; SA-FITC, streptavidin-fluorescein; SA-HRP, streptatividin-horseradish peroxidase; TBS, 50 mM Tris-HCl and 0.15 M NaCl (pH 7.6); TBST, 50 mM Tris-HCl, 0.15 M NaCl, and 0.05% (w/v) Tween 20 (pH 7.6); TBST-WB, 20 mM Tris base, 136 mM NaCl, and 0.1% (w/v) Tween 20 (pH 7.6).

FGF18 have skeletal structures similar to those of animals null for FGFR3 characterized by elongated long bones (*16*, *23*, *24*). Interestingly, humans that have an activating mutation in FGFR3 also are shorter in stature, which supports the animal model data and suggests that it is a result of premature terminal differentiation of the chondrocytes (*25*, *26*). Together, these data suggest that perlecan, FGF18, and FGFR3 may affect the same signaling pathways that control the chondrocyte phenotype, resulting in the modulation of the tissue transition zone between cartilage and bone. In this study, we have investigated the HS chains of perlecan isolated from chondrocytes using structural analytical techniques as well as binding assays and the BaF32 cell system to investigate FGF2 and FGF18 complex formation and signaling.

EXPERIMENTAL PROCEDURES

Antibodies, Enzymes, and Reagents. The monoclonal antibodies against perlecan domains I (CSI-076) and IV (A7L6) and the polyclonal anti-FGF2 antibody were from Abcam (Cambridge, MA), while polyclonal anti-FGF18 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against HS (10E4) and heparinase III-generated HS stubs (3G10) were purchased from Seikagaku Corp. (Tokyo, Japan). Antibodies reactive for chondroitinase ABC generated unsulfated (1B5), 4-sulfated (2B6), 6-sulfated (3B3) CS stubs, and KS (5D4) were provided by B. Caterson (Cardiff University, Cardiff, Wales, U.K.). Biotinylated anti-mouse, anti-rabbit, or anti-goat/sheep whole immunoglobulin (Ig) secondary antibodies, streptavidin-horseradish peroxidase (SA-HRP), and streptavidin-fluorescein (SA-FITC) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, U.K.). Biotinylated anti-rat Ig secondary antibodies were purchased from Dako (Glostrup, Denmark). HRP-conjugated anti-mouse Ig raised in sheep and HRP-conjugated anti-human Fc/Fab secondary antibodies were purchased from Millipore (Billerica, MA). Endoglycosidase enzymes, chondroitinase ABC, and heparinase III (EC 4.2.2.8) were purchased from Seikagaku Corp. Mammalian heparanase was obtained from human platelets as described previously (*27*). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise.

Isolation of Human Fetal Chondrocytes. Human fetal feet were obtained with informed consent under ethical approval of the North Sydney and Central Coast Area Health Authority Human Research and Ethics Committee, while human fetal chondrocytes from the knee were harvested in accordance with institutional approval from the Human Research Ethics Committee of The University of New South Wales. The cartilage tissue used had no signs of vascularization and was cleaned and diced into 1 mm × 3 mm pieces before it was digested with sterile 0.1% (w/v) Pronase (from *Streptomyces griseus*, Roche) in DMEM on a rocker at 37 °C for 90 min followed by centrifugation at 1000 rpm for 3 min. A second digestion with 0.5% (w/v) collagenase in DMEM on a rocker at 37 °C for 20 h was subsequently performed, and cells were separated using a sterile 70 μm cell sieve (BD Biosciences, San Jose, CA) followed by centrifugation at 1000 rpm for 3 min. Chondrocytes were cultured in DMEM containing 50 μg/mL L-ascorbic acid, 10% (v/v) fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), and a 1% (v/v) penicillin/streptomycin mixture. Conditioned medium was collected every 3 days and stored at −20 °C until it was required.

Immunohistochemistry. Human fetal feet (12−14 weeks old) were obtained fixed and paraffin embedded as described previously (*28*). Sections were treated twice, 5 min each, with xylene to remove paraffin, and the slides were immersed in a series of ethanol solutions for 3 min each [twice at 100% (v/v), once at 95% (v/v), and once at 70% (v/v)] followed by several exchanges of water. Antigen epitopes were retrieved by immersion of the slides in 0.01 M sodium citrate (pH 6) followed by heat treatment in a decloaking chamber (Applied Medical) at 121 °C for 4 min. Slides were then rinsed with deionized water followed by blocking with 3% (v/v) H_2O_2 for 10 min. Additionally, some slides were treated with chondroitinase ABC (0.05 unit/mL) or heparinase III (0.01 unit/mL) in Dulbecco's phosphate-buffered saline (DPBS, pH 7.2) for 3 h at 37 °C. The slides were washed with 50 mM Tris-HCl, 0.15 M NaCl, and 0.05% (w/v) Tween 20 (pH 7.6) (TBST) and then blocked with 1% (w/v) bovine serum albumin (BSA) in TBST for 1 h at room temperature (RT). The slides were incubated with primary antibodies diluted in 1% (w/v) BSA in TBST at 4 °C for 16 h. Primary antibodies used included A7L6 (4 μg/mL), 10E4 (4 μg/mL), 5D4 (1:1000), CS56 (1:500), 1B5 (1:500), 2B6 (1:500), 3B3 (1:500), FGF2 (4 μg/mL), FGF18 (4 μg/mL), FGFR1 (4 μg/mL), FGFR3 (4 μg/mL), and type X collagen (4 μg/mL). Slides were then washed twice with TBST before being incubated with the appropriate biotinylated secondary antibodies (1:500) for 1 h at RT. The slides were washed twice with TBST and then incubated for 30 min with streptavidin-HRP (1:250) and rinsed four times with TBST before being developed with NovaRED chromogen stain (Vector Laboratories, Burlingame, CA). The slides were counterstained with hemotoxylin (Vector Laboratories) for 3 min and then rinsed with deionized water before being imaged using light microscopy.

Immunocytochemistry. Chondrocytes (passages 2−4) were cultured to confluence on microscope slides (Ultrafrost, Lomb Scientific, Taren Point, NSW, Australia), fixed with ice-cold acetone for 3 min, and rinsed with 50 mM Tris-HCl and 0.15 M NaCl (pH 7.6) (TBS). Selected slides were treated with chondroitinase ABC (0.05 unit/mL) or heparinase III (0.01 unit/mL) in PBS (pH 7.2) for 3 h at 37 °C. Slides were then blocked with 0.1% (w/v) casein in DPBS for 1 h at RT followed incubation with the primary antibodies at a final concentration of 2 μg/mL for 16 h at 4 °C. Primary antibodies used included CSI-076, 10E4, CS56, 2B6, 3B3, FGF2, FGF18, FGFR1, FGFR3, and type X collagen. Slides were rinsed twice with TBST and incubated with the appropriate biotinylated secondary antibodies (1:500) for 1 h at RT before being rinsed twice with TBST and incubated with SA-FITC (1:250) for 30 min at RT followed by four washes with TBST. The slides were then counterstained with 1 μg/mL 4′,6-diamidino-2-phenylindole, dilactate (DAPI) (Invitrogen) in DPBS for 10 min in the dark and rinsed four times with the deionized water before being imaged using fluorescence microscopy.

Immunopurification of Perlecan. Perlecan was isolated from the conditioned medium produced by cultured human fetal chondrocytes and human coronary artery endothelial cells (HCEC) by anion exchange and monoclonal antibody affinity chromatography, as described previously (*29*, *30*).

Western Blot Analysis. Purified perlecan samples (10 μg/lane) were treated with heparinase III (0.01 unit/mL) in DPBS (pH 7.2) at 37 °C for 16 h and electrophoresed through 3−8% Tris-Acetate NuPAGE SDS−PAGE gels (Invitrogen) for 1 h at 200 V in Tris-tricine buffer [50 mM tricine, 50 mM Tris base, and

0.1% (w/v) SDS (pH 8.3)]. Molecular weight markers (HiMark, Invitrogen) were electrophoresed on each gel. Samples were then transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) using transfer buffer [5 mM bicine, 5 mM Bis Tris, 0.2 mM EDTA, 0.005% SDS, and 10% (v/v) methanol (pH 7.2)] in a semidry blotter (Invitrogen) at 300 mA and 20 V for 1 h. The membrane was blocked with 1% (w/v) BSA in 20 mM Tris base, 136 mM NaCl, and 0.1% (w/v) Tween 20 (pH 7.6) (TBST-WB) for 2 h at RT followed by incubation with the perlecan domain IV antibody (A7L6, 0.1 μg/mL) diluted in 1% (w/v) BSA in TBST-WB for 16 h at 4 °C. Membranes were subsequently rinsed with TBST-WB, incubated with HRP-conjugated antibodies [1:50000 dilution in 1% (w/v) BSA in TBST-WB] for 45 min at RT, and rinsed with TBST-WB, 20 mM Tris base, and 136 mM NaCl (pH 7.6) before being imaged using chemiluminescent reagents (Femto reagent kit, Pierce Biotechnology, Rockford, IL) and X-ray film (Australian Imaging Distributors, North Ryde, NSW, Australia).

ELISA Analysis. Immunopurified chondrocyte-derived perlecan (5 μg/mL) was adsorbed onto wells of a 96-well ELISA plate (Greiner Bio One, GmbH, Frickenhausen, Baden-Württemberg, Germany) for 2 h at RT, rinsed twice with DPBS, and blocked with 0.1% (w/v) casein in DPBS for 1 h at RT. Wells were rinsed twice with DPBS with 0.1% (w/v) Tween 20 (DPBST) and incubated with antibodies against perlecan domain IV (mAb A7L6, 2 μg/mL), HS (mAb 10E4, 2 μg/mL), CS (mAb CS56, 1:500), and KS (mAb 5D4, 1:500) diluted in 0.1% (w/v) casein in DPBST. Wells were then rinsed twice with DPBST followed by incubation with biotinylated secondary antibodies diluted in 0.1% (w/v) casein DPBST for 1 h at RT followed by two rinses with DPBST. Wells were then incubated with SA-HRP (1:500) for 30 min at RT and rinsed four times with DPBST. Binding of the antibodies to the samples was detected using the colorimetric substrate 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and the absorbance was measured at 405 nm.

Ligand and Carbohydrate Engagement Assay (*LACE*). Perlecan (5 μg/mL) was adsorbed onto 96-well high-binding ELISA plates for 16 h at 4 °C. Wells were washed with DPBS, and selected wells were digested with endoglycosidase enzymes, chondroitinase ABC (0.05 unit/mL), heparinase III (0.01 unit/mL), or mammalian heparanase (2 ng/mL) in 0.01% (w/v) BSA with DPBS (pH 7.2) for 3 h at 37 °C. Mammalian heparanase was found to degrade perlecan HS at pH 7.2 and inhibit the formation of ternary complexes with FGFs and their receptors. Wells were blocked with 3% (w/v) BSA in DPBS for 1 h at RT and washed twice with DPBST. Either recombinant human FGF2 or FGF18 (5 nM) (Invitrogen) and either soluble recombinant human FGFR1c or -3c−human IgG chimeric proteins (5 nM) (R&D Systems, Minneapolis, MN) were incubated in PBST for 10 min before being transferred into each well and incubated for 1 h at 37 °C. Controls were incubated with FGFR1c or -3c only. Wells were then washed twice with DPBST and incubated with HRP-conjugated anti-human IgG secondary antibody (1:1000) in DPBST for 1 h at 37 °C. Wells were washed four times with DPBST before the addition of ABTS, and the absorbance was measured at 405 nm.

BaF32 Cell Proliferation Assays. BaF32 cells are from an IL-3-dependent and HS proteoglycan deficient myeloid B cell line that has been stably transfected with either FGFR1c or FGFR3c (*31*, *32*). BaF32 cells represent a model system developed to identify HS and heparin structures that interact with FGFs and their receptors. The readout of this assay is cell proliferation which indicated the formation of ternary complexes on the cell surface. BaF32 cells were maintained in RPMI 1640 medium containing 10% (v/v) FBS, 10% (v/v) WEHI-3BD conditioned medium, and a 1% (v/v) penicillin/streptomycin mixture. WEHI-3BD cells were maintained in RPMI 1640 medium supplemented with 2 g/L sodium bicarbonate, 10% (v/v) FBS, and a 1% (v/v) penicillin/streptomycin mixture, and the conditioned medium was collected three times per week and stored at −20 °C until it was required. For the mitogenic assays, the BaF32 cells were transferred into IL-3 depleted medium for 24 h prior to experimentation and seeded into 96-well plates at a density of 2×10^4 cells/well in the presence of chondrocyte-derived perlecan (40 μg/mL) and either FGF2 (0.03 nM) or FGF18 (9 nM). Heparin (30 nM) with either FGF2 (0.03 nM) or FGF18 (9 nM) was used as a positive control for the assay, while cells in the presence of medium only were used as a negative control. To investigate the role of the glycosaminoglycan chains attached to chondrocyte-derived perelcan, endoglycosidase digestions were also performed in situ with chondroitinase ABC (0.05 unit/mL), heparinase III (0.01 unit/mL), or mammalian heparanase (2 ng/mL) at pH 7.2 and 37 °C for 16 h prior to commencement of the cell assay. Cells were incubated for 96 h in 5% CO_2 at 37 °C, and the number of cells present was assessed using the MTS assay. The MTS reagent (Promega, Madison, WI) was added to the cell cultures 6 h prior to measurement of the absorbance at 490 nm.

Statistical Analysis. A student's t test (two samples, two-tailed distribution assuming equal variance) was used to compare statistical significance. $p < 0.05$ results were considered significant. Experiments were performed in triplicate, and experiments were repeated.

RESULTS

Immunolocalization of Perlecan, Glycosaminoglycans, FGFs, and FGFRs in the Developing Human Anlagen. The presence of perlecan was detected in the middle zone of developing cartilage rudiments in human feet (marked by asterisks in Figure 1A) and in the developing growth plates located in either end of the metatarsal [filled arrow, proximal end; empty arrow, distal end (Figure 1A)]. When the region identified by the filled arrow in Figure 1A was examined under higher power, it was noted that the perlecan immunoreactivity was localized to the pericellular matrix, which is in close association with the cell and lacunae membranes (Figure 1B). Chondrocytes in this same region stained positively for type X collagen (Figure 1C), indicating that these cells were hypertrophic in nature. Regions of the developing foot that had significant immunoreactivity for type X collagen also demonstrated the presence of HS stubs (Figure 1D) generated after digestion with heparinase III, native CS (Figure 1E), and 4-sulfated (Figure 1G) and 6-sulfated (Figure 1H) CS stubs generated after digestion with chondroitinase ABC. In contrast, the same regions showed no reactivity toward the antibody that recognized the unsulfated CS stubs (Figure 1F). Interestingly, the presence of the HS stubs (Figure 1D) and 4-sulfated CS stubs (Figure 1G) was confined to the pericellular matrix, whereas the 6-sulfated CS stubs (Figure 1H) were also detected in the interterritorial matrix (Figure 1H), which was most likely attached to aggrecan. Chondrocytes in regions of type X collagen staining also stained for the presence of FGF2 (Figure 1I), FGF18 (Figure 1L), FGFR1 (Figure 1J), and FGFR3 (Figure 1K). FGF18 was also detected in the tissue between the developing cartilage joints (Figure 1M)

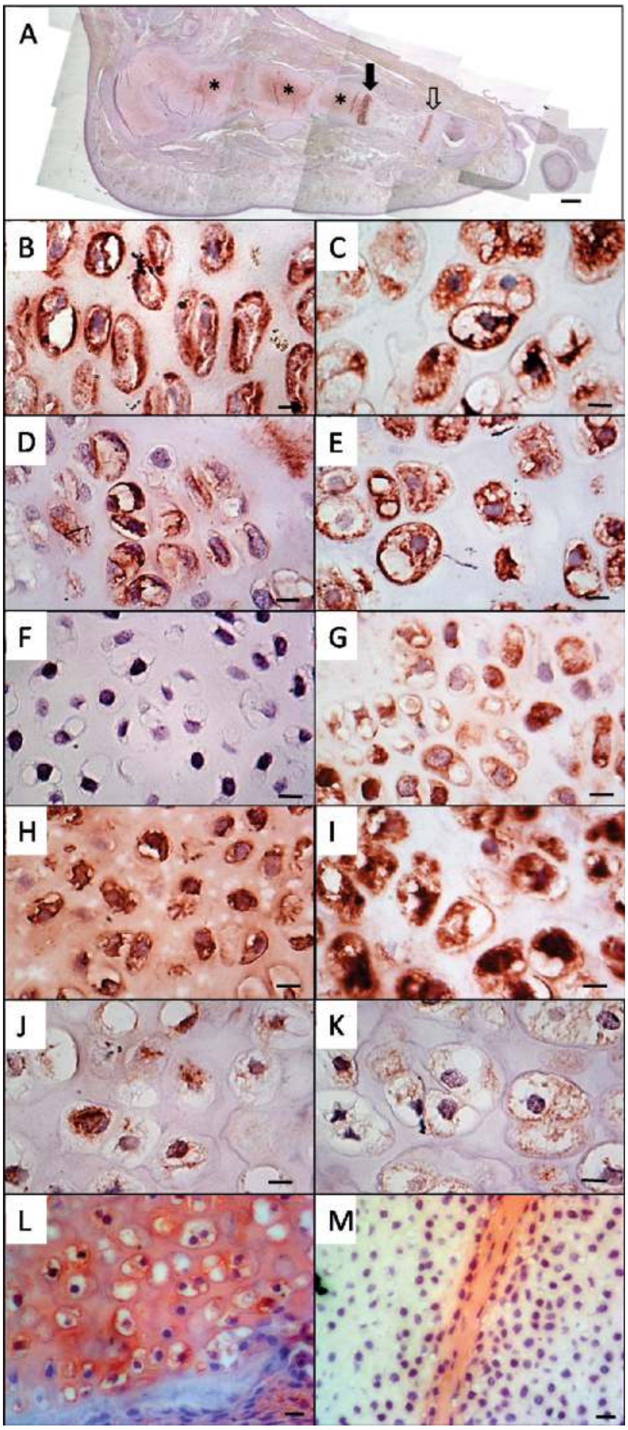

FIGURE 1: Immunolocalization of perlecan, type X collagen, GAGs, FGFs, and FGF receptors 1 and 3 in developing human cartilage rudiments. The sections were probed for the presence of perlecan (marked by asterisks and arrows in panel A, mAb A7L6). A region was selected from panel A (marked by the filled arrow at 25× magnification) and viewed at a higher (1000×) magnification (B, mAb A7L6). Similar regions were also investigated at the higher magnification for type X collagen (C), as well as the presence of HS stubs generated by heparinase III enzyme digestion (D, mAb 3G10) and native CS (E, mAb CS56), while CS stubs were generated by chondroitinase ABC digestion and probed for the presence of unsulfated CS stubs (F, mAb 1B5), 4-sulfated CS stubs (G, mAb 2B6), and 6-sulfated CS stubs (H, mAb 3B3). The sections were probed for the presence of FGF2 (I), FGFR1 (J), FGFR3 (K), and FGF18 (L and M). The nuclei were counterstained with hemotoxylin (the scale bar is 500 μm in panel A, 10 μm in panels B−K, and 20 μm in panels L and M).

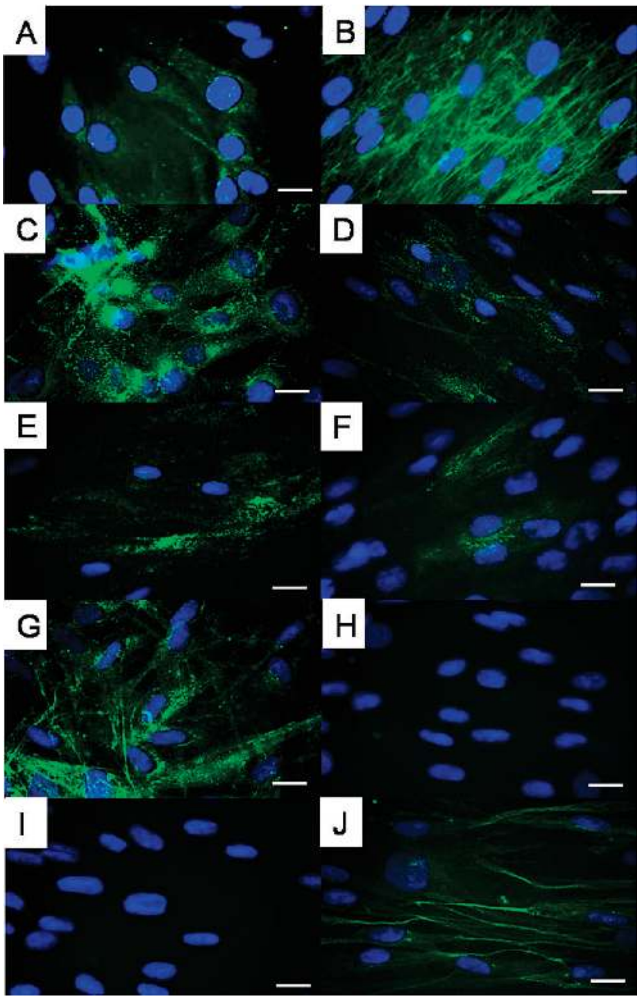

FIGURE 2: Expression of perlecan, glycosaminoglycans, FGFs, FGF receptors, and type X collagen in cultured human fetal chondrocytes. Human fetal chondrocytes were cultured on glass slides and probed for the presence of type X collagen (A), perlecan (B, mAb CSI-076), HS (C, mAb 10E4), native CS (D, mAb CS56), 4-sulfated CS stubs (E, mAb 2B6), 6-sulfated CS stubs (F, mAb 3B3), FGF2 (G), FGF18 (H), FGFR1 (I), or FGFR3 (J). Specific staining was shown by FITC (green), while the nuclei were counterstained with DAPI (blue) at 500× magnification (the scale bar is 20 μm).

and in the perichondrium surrounding the cartilage (data not shown).

Immunolocalization of Type X Collagen, Perlecan, Glycosaminoglycans, FGFs, and FGFRs in Cultured Human Fetal Chondrocytes. When the cells were isolated from developing cartilage rudiment tissue and cultured, some of the cells were found to express type X collagen, supporting the idea that some of the cells were hypertrophic (Figure 2A). Most cells expressed and secreted perlecan into the pericellular/extracellular space as microfibrillar structures (Figure 2B). HS was detected with a punctate and granular staining pattern (Figure 2C). Staining for CS showed that some cells exhibited intracellular perinuclear staining, such as that seen for the native CS (Figure 2D) and the 4- and 6-sulfated CS stubs (panels E and F of Figure 2, respectively). FGF2 was detected intracellularly and extracellularly where it appeared to be localized in microfibrillar structures (Figure 2G). FGF18 (Figure 2H) and FGFR1 (Figure 2I) did not show any significant staining as compared to a no primary control (data not shown); however, some of the cells exhibited positive staining for FGFR3 (Figure 2J).

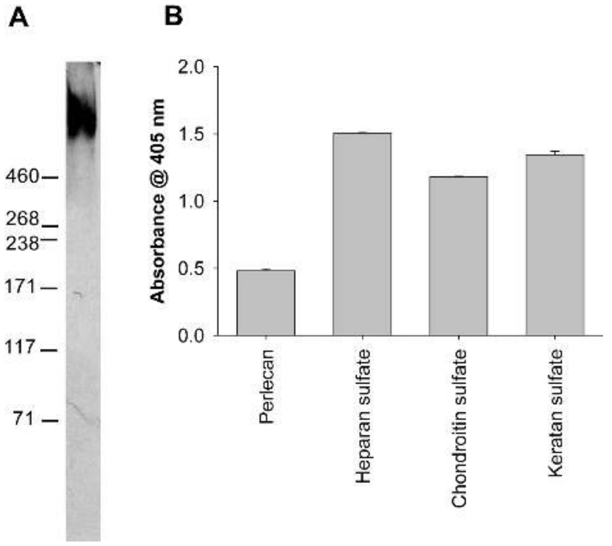

FIGURE 3: Characterization of immunopurified chondrocyte-derived perlecan. Immunopurified chondrocyte-derived perlecan was electrophoresed through 3 to 8% SDS−PAGE gels, electroblotted onto a PVDF membrane, and probed with an anti-perlecan antibody (domain IV, mAb A7L6) (A). Immunopurified chondrocyte-derived perlecan was coated onto 96-well plates (B) and probed with antibodies against perlecan (domain IV, mAb A7L6), heparan sulfate (HS, mAb 10E4), chondroitin sulfate (CS, mAb CS56), or keratan sulfate (KS, mAb 5D4). Data are means ± the standard deviation ($n = 3$).

Biochemical Characterization of Chondrocyte-Derived Perlecan. Immunopurified chondrocyte-derived perlecan isolated from cells in culture was characterized with respect to its molecular mass and glycosaminoglycan composition. Chondrocyte-derived perlecan was found to have a molecular mass in excess of 600 kDa when probed with a perlecan protein core antibody against domain IV (Figure 3A). Immunopurified chondrocyte-derived perlecan was probed with antibodies against HS, CS, and KS by an ELISA which confirmed that this material contained all three glycosaminoglycan types (Figure 3B). The substructure of the CS chains was further analyzed and shown to contain both 4- and 6-sulfated CS stub structures (data not shown).

Ternary Complexes Formed among Perlecan, Growth Factors, and Receptors. Chondrocyte-derived perlecan formed ternary complexes with FGF2 and either FGFR1 (Figure 4a) or FGFR3 (Figure 4b). Digestion of chondrocyte-derived perlecan with either heparinase III or mammalian heparanase inhibited the ability of perlecan to form complexes. Chondrocyte-derived perlecan formed HS-dependent ternary complexes with FGF18 and FGFR3 (Figure 4d), but not with FGF18 and FGFR1 (Figure 4c). In contrast, endothelial-derived perlecan supported the formation of HS-dependent complexes between FGF18 and either FGFR1 or FGFR3 (Figure 4c,d). The complexes formed between chondrocyte-derived perlecan, FGF18, and FGFR3 were also sensitive to both heparinase III and mammalian heparanase digestion; however, when endothelial-derived perlecan was used to form these complexes, it was sensitive only to heparinase III digestion (Figure 4d). These contrasting results between chondrocyte- and endothelial-derived perlecan suggest that it is not simply a difference in the amount of HS that elicits the different responses but is suggestive of significant differences in the structure of the HS.

The binding of FGFR3 to perlecan in the absence of FGF18 was assessed to ensure that both FGF18 and FGFR3 needed to be present to form a complex (Figure 5). FGFR3, itself, did not bind to either chondrocyte- or endothelial-derived perlecan in either the presence or absence of HS. This supports the finding that perlecan HS, FGF18, and FGFR3 need to be present to form a complex.

FGF Growth Promoting Activities of Perlecan. The activities of the ternary complexes formed between perlecan, growth factors, and their cognate receptors were tested using the BaF32 cell system expressing either FGFR1 (Figure 6A,C) or FGFR3 (Figure 6B,D). Commercially available heparin and either FGF2 or FGF18 were used as positive controls for the assay, while cells in the presence of medium only were used as a negative control. Proliferation of the FGFR1-expressing BaF32 cells in the presence of chondrocyte-derived perlecan and FGF2 was found to support increased but not statistically significantly ($p < 0.05$) greater proliferation compared to that of the medium only control (Figure 6A), suggesting that chondrocyte-derived perlecan elicited only weak activity in the presence of FGF2. Chondrocyte-derived perlecan did not support the proliferation of FGFR1-expressing BaF32 cells in the presence of FGF18 (Figure 6A). Digestion of chondrocyte-derived perlecan with endoglycosidase enzymes did not significantly ($p < 0.05$) change the level of proliferation of FGFR1-expressing BaF32 cells in the presence of FGF2 compared to that of undigested chondrocyte-derived perlecan (Figure 6C). Both endothelial-derived perlecan and heparin supported the proliferation of FGFR1-expressing BaF32 cells in the presence of FGF2, but not in the presence of FGF18 (Figure 6A).

Chondrocyte-derived perlecan supported the proliferation of the FGFR3-expressing BaF32 cells in the presence of FGF18, the level of which was significantly ($p < 0.05$) greater than that of the negative control and equal to that of the positive heparin control (Figure 6B). Digestion of chondrocyte-derived perlecan with either heparinase III or mammalian heparanase significantly ($p < 0.05$) reduced the level of proliferation compared to that of the undigested chondrocyte-derived perlecan (Figure 6D). Interestingly, digestion with mammalian heparanase significantly reduced the amount of proliferation to a level that was statistically similar to that achieved in the medium only negative control (Figure 6D). Chondrocyte-derived perlecan did not promote the proliferation of FGFR3-expressing BaF32 cells in the presence of FGF2 above the medium only control (Figure 6B). In contrast, endothelial-derived perlecan promoted the proliferation of FGFR3-expressing BaF32 cells in the presence of either FGF2 or FGF18 (Figure 6B).

DISCUSSION

This work shows that the HS decorating chondrocyte-derived perlecan protein core mediates the binding of FGF18 and the subsequent activation of FGFR3. It also shows that digestion of the HS decorating chondrocyte-derived perlecan can inhibit this signaling, supporting the hypothesis that FGF18 signaling in developing cartilage tissues is a result of the concerted actions and local concentrations of perlecan and the enzyme that degrades its HS.

The localization of perlecan to the pericellular matrix of chondrocytes in the developing cartilage rudiments supports the hypothesis that perlecan performs an important role in the development of these tissues (*10*, *28*, *33*, *34*). The staining pattern for the immunolocalization of FGF2 and perlecan was similar in some regions of the developing cartilage, supporting the idea that perlecan is a mechanotransductor by binding FGF2, which

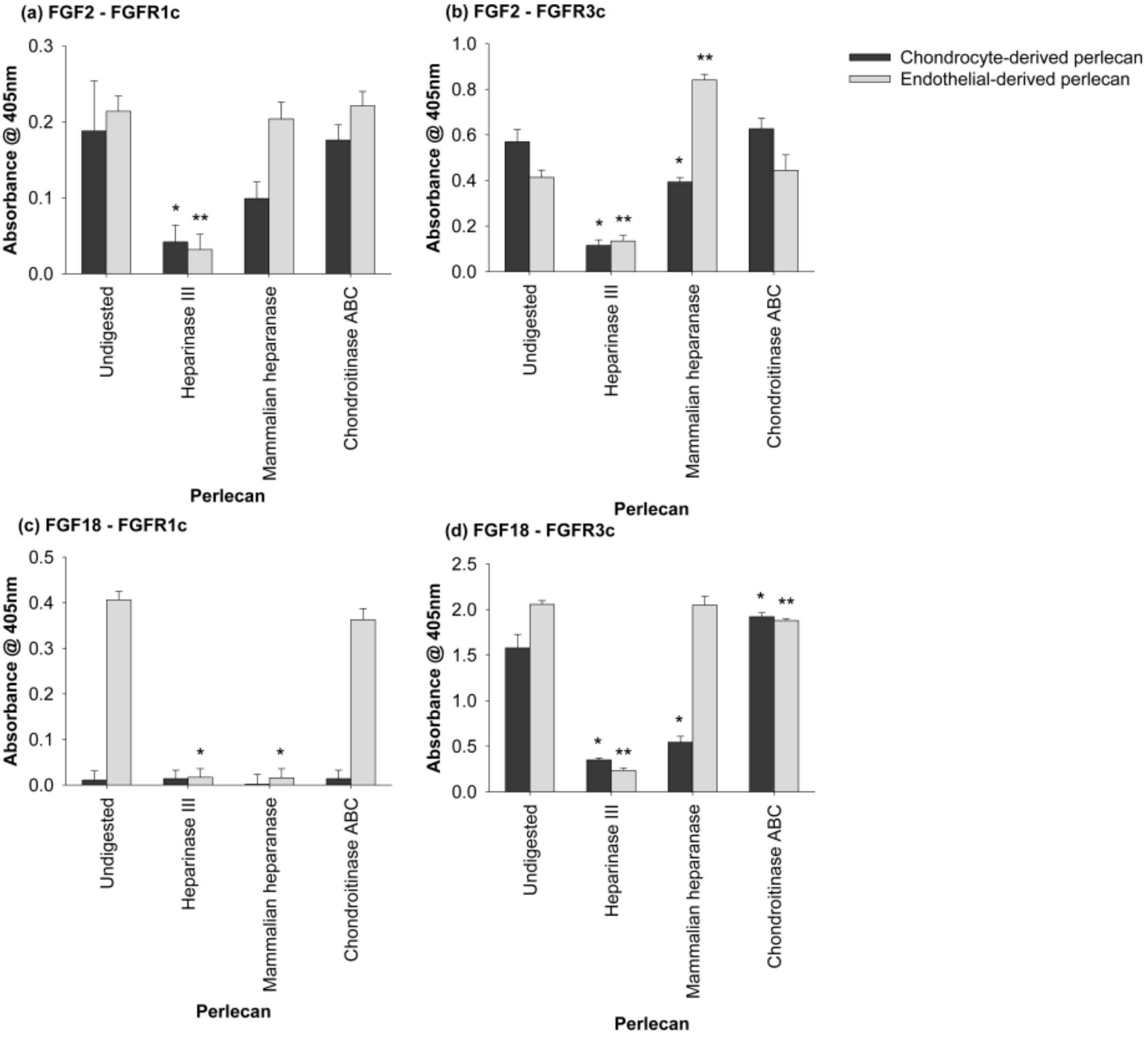

FIGURE 4: Ternary complexes formed among perlecan, either FGF2 or FGF18, and either FGF receptor type 1 or 3. Perlecans were adsorbed onto wells of a 96-well plate and were either undigested or digested in situ with heparinase III, mammalian heparanase, or chondroitinase ABC before the LACE assay was performed. Measurements were corrected for absorbance measurements detected in the absence of FGF and are presented as means ± the standard deviation ($n = 3$). Significant differences were analyzed using a Student's t test: (*) $p < 0.05$ compared to undigested chondrocyte-derived perlecan and (**) $p < 0.05$ compared to the undigested endothelial-derived perlecan.

results in chondrocytes being able to receive proliferative signals in response to mechanical stimuli (*14*). FGF2, FGF18, FGFR1, and FGFR3 were immunolocalized to similar regions of the cartilage tissue, which contained hypertrophic chondrocytes as identified by their size and the presence of type X collagen, supporting hypotheses that these factors may be involved in the regulation of chondrocyte proliferation and terminal differentiation prior to apoptosis (*18*, *35*−*39*). FGF18 was detected in the developing cartilage tissue between the developing cartilage joints and the perichondrium surrounding the cartilage rudiment as reported previously using mRNA in situ hybridization (*2*, *18*). FGF18 could not, however, be detected in the in vitro chondrocyte cultures, suggesting that FGF18 was not produced by these cells and may act as a paracrine signaling factor.

Glycosaminoglycan characterization of the chondrocyte-derived perlecan suggested that it was secreted as a full-length molecule substituted with HS, KS, and CS. This is in agreement with previous reports describing perlecan isolated from human and bovine cartilage tissues as a proteoglycan decorated with HS, CS, or both (*7*, *10*). However, the precise location, structure, and role of the glycosaminoglycans remain to be determined.

FGF2 has been shown to bind to perlecan derived from fetal bovine growth plates in an HS-dependent manner (*13*), while the role of FGF18 has also been shown to be important in cartilage development (*18*). Thus, in this study, we were interested in determining whether the HS on the chondrocyte-derived perlecan was able to interact with FGF2 and FGF18 to form ternary complexes with either FGFR1 or FGFR3 in vitro. The HS only endothelial-derived perlecan was used as a control to compare with chondrocyte-derived perlecan in the LACE assays. Chondrocyte-derived perlecan formed HS-dependent ternary complexes with FGF2 and FGFR1 or FGFR3, which were sensitive to both heparinase III and mammalian heparanase digestion. In contrast, endothelial cell-derived perlecan was only sensitive to heparinase III digestion, indicating differences in the structure of HS produced by the two cell types. Chondrocyte-derived perlecan also formed HS-dependent ternary complexes with FGF18 and FGFR3, while complexes were not formed between FGF18 and FGFR1, suggesting that the HS motif on chondrocyte-derived perlecan may be specific for certain complexes. Previous studies have shown that FGF18 binds to FGFR3 with a higher specificity than to other FGFRs (*18*)

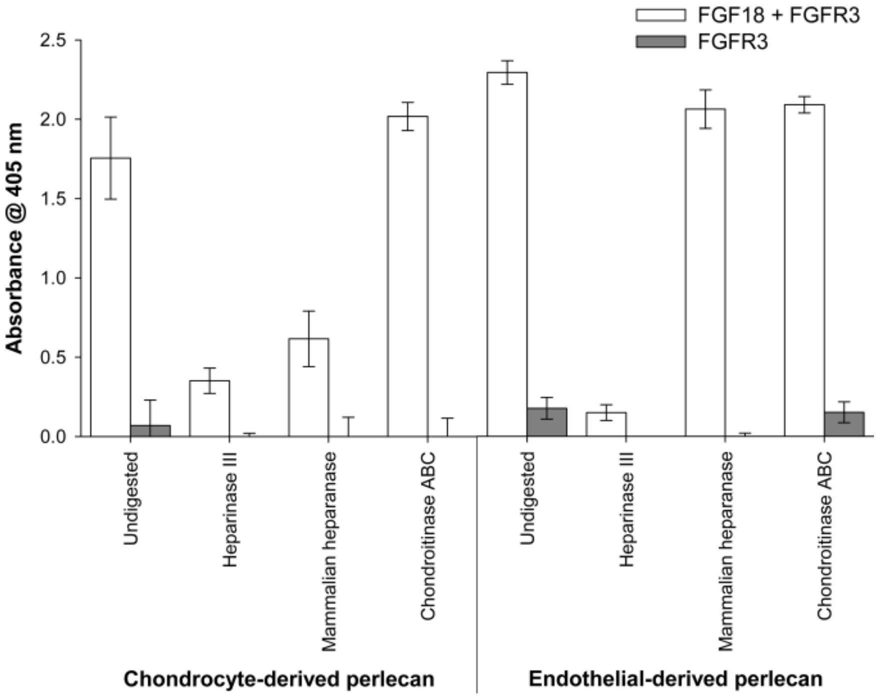

FIGURE 5: Complexes formed among perlecan, FGF18, and FGFR3 or between perlecan and FGFR3. Perlecans were adsorbed onto wells of a 96-well plate and were either undigested or digested in situ with heparinase III, mammalian heparanase, or chondroitinase ABC before a LACE assay was performed. Measurements were corrected for absorbance measurements detected in the absence of perlecan and are presented as means ± the standard deviation ($n = 3$).

and that FGF18 has a greater receptor selectivity than FGF2 (*39*, *40*). FGF18 has also been shown to bind to the protein core of perlecan (*15*), which may explain the residual binding demonstrated among heparinase III-treated perlecan, FGF18, and FGFR3. However, the complexes formed between FGF18, FGFR3, and perlecan are predominantly formed via interactions with the HS chains, and FGFR3 alone does not bind directly to the HS chains as there was no significant binding in the absence of FGF18.

We were interested in determining whether chondrocyte-derived perlecan could promote signaling of FGF2 and FGF18 and whether digestion by mammalian heparanase affected the biological activity that was determined by the proliferation of FGFR1- and FGFR3-expressing BaF32 cells in situ. The BaF32 cell assays indicate signaling of the complexes formed between perlecan, growth factors, and growth factor receptors through their proliferation. Chondrocyte-derived perlecan did not stimulate the statistically significant ($p < 0.05$) proliferation of FGFR1-expressing BaF32 cells in the presence of FGF2 or -18 above the medium only control and hence did not provide a biologically active signal. These data are in agreement with previous studies using perlecan isolated from bovine growth plates (*13*). The LACE assay demonstrated that chondrocyte-derived perlecan did not form ternary complexes with FGF18 and FGFR1; however, chondrocyte-derived perlecan was able to form ternary complexes with FGF2 and FGFR1. It has been shown previously that perlecan can sequester FGF2 away from its high-affinity receptor, preventing its activation (*12*, *13*) and degradation (*41*). The LACE assay data together with the BaF32 cell-based assay data presented here show that while chondrocyte-derived perlecan can form ternary complexes with FGF2 and FGFR1, it was not able to signal to cells, suggesting that the complexes are in an inactive state. This novel phenomenon was also observed for complexes formed between chondrocyte-derived perlecan, FGF2, and FGFR3. The ability of chondrocyte-derived perlecan to form biologically inactive ternary complexes is likely due to the structure of its HS. The importance of HS structure with respect to biological activity has been reported previously in both the endothelial and neuroepithelial systems. Different sources of endothelial-derived perlecan have been reported to provide differential regulation of FGF2-mediated cell signaling (*42*), while neuroepithelial cells at different stages of development have structurally distinct forms of HS (*43*) that selectively interact with specific FGF−FGFR complexes to regulate activation (*44*).

Chondrocyte-derived perlecan stimulated the proliferation of FGFR3-expressing BaF32 cells in the presence of FGF18, and this activity was modulated by either heparinase III or mammalian heparanase. This suggests a role for mammalian heparanase in vivo in the turnover and processing of the FGF18−FGFR3 signal in hypertrophic chondrocytes in cleaving the HS attached to perlecan that promotes the formation of biologically active complexes. Consistent with this, FGF18 has been known to signal via FGFR3 to promote chondrogenesis (*36*) while heparanase mRNA was recently localized at the chondro-osseus junction of murine growth plates (*45*, *46*), which further supports the hypothesis that mammalian heparanase plays an important role in the turnover of HS attached to chondrocyte-derived perlecan in cartilage development.

In conclusion, this work demonstrates that chondrocyte-derived perlecan HS was capable of binding and supporting the biological activity of FGF18 through the formation of ternary complexes with FGFR3. Chondrocyte-derived perlecan was also capable of binding FGF2 through the formation of ternary complexes with FGFR1 or FGFR3; however, they did not form biologically active complexes. The inhibition of FGF18 activity by mammalian heparanase leads to subsequent events of terminal differentiation of hypertrophic chondrocytes and apoptosis which

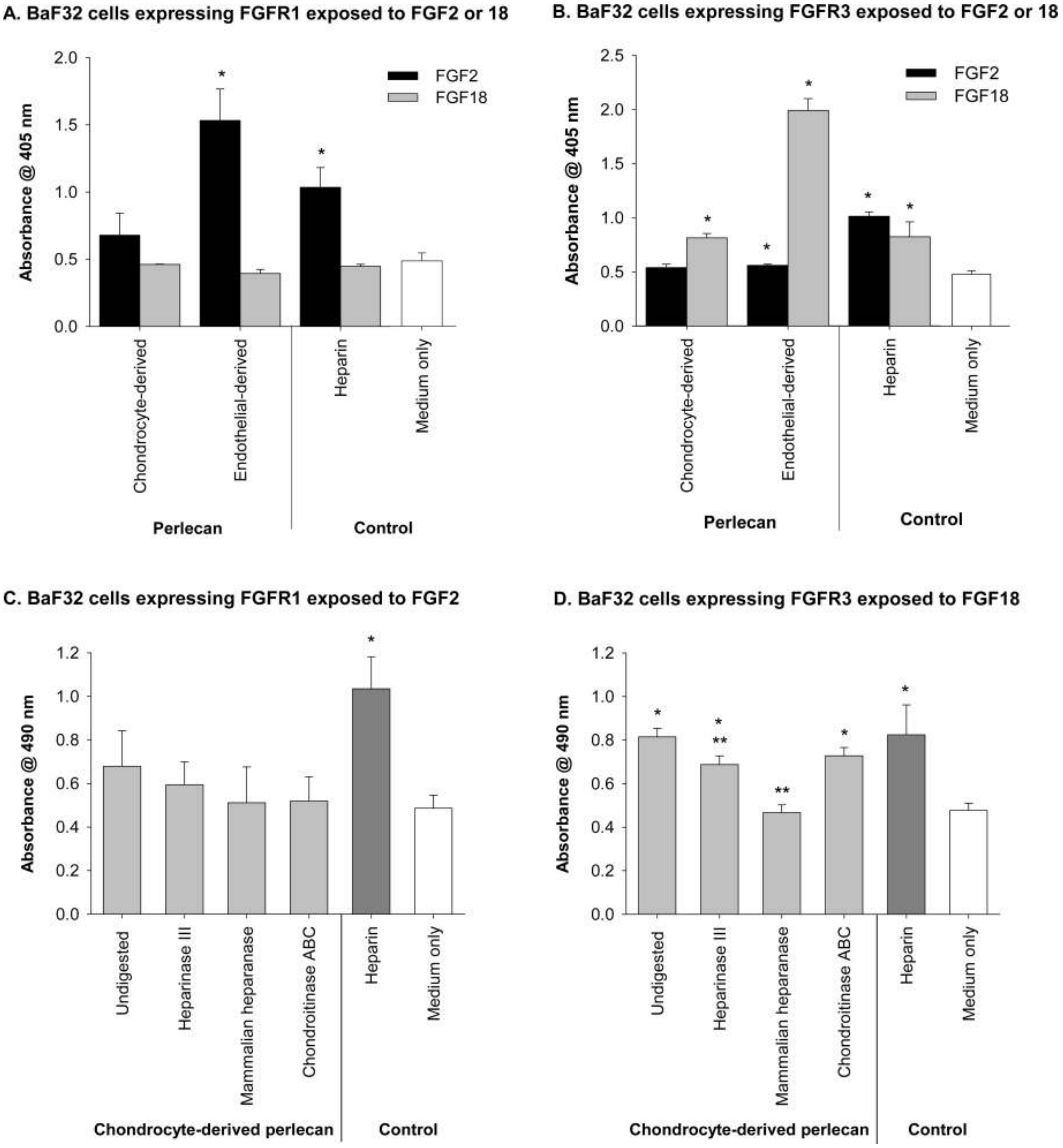

FIGURE 6: Proliferation of BaF32 cells expressing either FGFR1 or FGFR3 in the presence of chondrocyte- or endothelial-derived perlecan with either FGF2 or FGF18. FGFR1-expressing (A) or FGFR3-expressing (B) BaF32 cells were incubated with chondrocyte- or endothelial-derived perlecan in the presence of either FGF2 (black bars) or FGF18 (gray bars) and compared to cells with heparin in the presence of either FGF2 or FGF18. Medium only was used as a negative control (white bars). Chondrocyte-derived perlecan was either left undigested or digested with heparinase III, mammalian heparanase, or chondroitinase ABC in situ prior to incubation with either FGFR1-expressing BaF32 cells in the presence of FGF2 (C) or FGFR3-expressing BaF32 cells in the presence of FGF18 (D). Data are presented as means ± the standard deviation ($n = 3$). Significant differences were analyzed using a Student's *t* test: (*) $p < 0.05$ compared to medium only and (**) $p < 0.05$ for the endoglycosidase-digested perlecan samples compared to the undigested perlecan.

itself is a forerunner of the development of bone. These events are controlled by the synthesis and turnover of the growth factors, their receptors, and HS, which all act in concert to produce functional components of the skeletal system with the correct architecture.

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