Published in final edited form as:
Tissue Eng. 2006 July ; 12(7): 2009–2024.

Chondrogenic Differentiation on Perlecan Domain I, Collagen II and Bone Morphogenetic Protein 2-Based Matrices

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Abstract

Extracellular matrix (ECM) molecules in cartilage, cooperate with growth factors to regulate chondrogenic differentiation and cartilage development. Domain I of perlecan (Pln) bears heparan sulfate chains that bind and release heparin binding growth factors (HBGFs). Our hypothesis was that Pln domain I (PlnDI) might be complexed with collagen II (P-C) fibrils to improve binding of bone morphogenetic protein-2 (BMP-2) and better support chondrogenesis and cartilage-like tissue formation *in vitro*. Our results showed that P-C fibrils bound more BMP-2 than collagen II fibrils alone, and better sustained BMP-2 release. Polylactic acid (PLA)-based scaffolds coated with P-C fibrils immobilized more BMP-2 than either PLA scaffolds or PLA scaffolds coated with collagen II fibrils alone. Multipotential mouse embryonic mesenchymal cells, C3H10T1/2, were cultured on two-dimensional P-C fibrils or three dimensional P-C/BMP-2-coasted (P-C-B) PLA scaffolds. Chondrogenic differentiation was indexed by glycosaminoglycan (GAG) production, and expression of the pro-chondrogenic transcription factor, Sox9, as well as cartilaginous ECM proteins, collagen II and aggrecan. Immunostaining for aggrecan, perlecan, tenascin and collagen X revealed that both C3H10T1/2 cells and primary mouse embryonic fibroblasts cultured on P-C-B fibrils showed the highest expression of chondrogenic markers among all treatment groups. Safranin O-Fast Green staining indicated that cartilage-like tissue was formed in the P-C-B scaffolds, while no obvious cartilage-like tissue formed in other scaffolds. We have concluded that P-C fibrils provide an improved biomimetic material for the binding and retention of BMP-2 and support chondrogenenic differentiation.

Keywords

Chondrogenesis; Perlecan; Bone Morphogenetic Protein-2 (BMP-2); Collagen II; Mesenchymal Cells; Tissue Engineering

Introduction

Cartilage is an avascular deformable tissue consisting of sparsely embedded chondrocytes in a specialized extracellular matrix (ECM). This ECM has dense collagen and proteoglycan networks that determine mechanical and functional properties of the tissue (1-3). The primary collagen component in cartilage is collagen II that interacts with the quantitatively minor collagens IX and XI to form heterotypic fibrils (1,2). Proteoglycan interactions with collagen fibrils and growth factors have been implicated in the regulation of ECM assembly and growth factor functions (2-4).

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Perlecan (Pln) is a heparan sulfate proteoglycan (HSPG) with a protein core of approximately 400 kDa and consists of five distinct domains(5). Pln domain I (PlnDI) is a 22 kDa protein core that contains three ser-asp-gly (SDG) motifs that serve as glycosaminoglycan (GAG) attachment sites decorated with two to three heparan sulfate (HS) chains and one chondroitin sulfate (CS) chain (5-8) of heterogeneous size. Through GAG chains attached to PlnDI, Pln functions as a ligand reservoir for storage and protection of heparin-binding growth factors (HBGFs) including fibroblast growth factor-2 (FGF-2) (7,8), vascular endothelial growth factor (VEGF) (9) and transforming growth factor β/bone morphogenetic proteins (TGF-β/BMPs) (6,10,11). Binding to GAG chains enhances the biological activities of these HBGFs (6,7,9-11). Thus, Pln and its GAG chains have a wide range of biological functions in cellular growth (7,8), angiogenesis (9), development (3,4,6,12) and tissue regeneration (13). During skeletal development, Pln is found in cartilage anlage after the expression of collagen II and aggrecan and is maintained as the major HSPG of adult cartilage (4,6,14,15). Pln null mice exhibit disorganized growth plates, severe cartilage defects, and skeletal abnormalities (16-18). Recently, several studies have demonstrated that Pln is crucial in chondrogenesis (3, 4,6,14,19). These actions may occur in concert with growth factors (4,9,11), such as BMP-2 and TGF-β1 (6,20,21), or growth factor binding proteins, such as the BMP binding polypeptide, noggin (6,22). In addition, Pln can maintain cartilage integrity and protect cartilage ECM from degradation (2,17). The murine mesenchymal stem cell line, C3H10T1/2, plated on surfaces coated with either intact Pln or recombinant PlnDI attach and aggregate into dense cell condensations that express chondrogenic markers including collagen II, aggrecan and link protein (4,14,19,20).

Collagen II fibrils support specific binding of a number of proteoglycans including fibromodulin (23,24), biglycan (25) and aggrecan (25,26). Both proteoglycan core proteins and their GAG chains mediate interactions with collagen II fibrils and modulate tensile strength of the ECM (25,27-29). In addition to its biomechanical functions, collagen II also plays a role in induction of chondrogenesis (1,3,16,30). Type IIA procollagen, but not type IIB collagen, binds BMP-2 and TGF-β1 (30). Other data suggest that interaction of BMP-2 with procollagen II is site-specific, and that the high-affinity binding site is located in the D-period of the collagen triple helix (31). Based on these properties, collagen II has been used to prepare or modify scaffolds in cartilage engineering applications (32-36). Collagen II can support chondrocyte infiltration and attachment (32,37,38) and maintains chondrocyte morphology and phenotype (33,34,39,40). Therefore, collagen II is an ideal candidate substrate to facilitate chondrogenesis and to use in cartilage tissue engineering.

During cartilage development, BMP-2 enhances recruitment of mesenchymal precursors to cartilage condensations, modulates expansion of condensation size and initiates BMP-dependent signaling cascades in mesenchymal progenitor cells for induction of chondrogenic differentiation (6,41-43). Multipotential precursor cells, such as C3H10T1/2 cells, cultured at high density initiate chondrogenesis following BMP-2 treatment (43-47). Because BMP-2 functions are enhanced by HS (4,6,10,11), and collagen II can bind GAG chains attached to proteoglycans (27-29), we hypothesized that PlnDI would bind both BMP-2 and collagen II fibrils via its GAG chains in a self-assembly process. Thus, PlnDI offered a novel, potential tool to enhance BMP-2 binding and function on scaffolds. In this investigation, we examined the interaction of BMP-2 with different substrates, including collagen II fibrils complexed with PlnDI, and the ability of these substrates to sustain BMP-2 release. In addition, we examined chondrogenic differentiation of C3H10T1/2 cells and primary mouse embryonic fibroblasts plated on these substrates, or seeded in scaffolds modified with the substrates. Collectively, these findings suggest that PlnDI improves substrate BMP-2 immobilization onto scaffolds or fibrils and promotes chondrogenic differentiation.

Materials and Methods:

Materials

Heparinases I, II and III, chondroitinase ABC, testicular hyaluronidase, heparan sulfate (HS), chondroitin sulfate (CS), bovine serum albumin (BSA), Tween 20, D-(+)-glucose and collagen II from bovine tracheal cartilage (C1188) were obtained from Sigma-Aldrich, (St. Louis, MO, USA). Recombinant human BMP-2 (rhBMP-2, 355-BM-010) and mouse monoclonal anti-human BMP-2 antibody (IgG2B, MAB3351) were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Rat anti-heparan sulfate proteoglycan monoclonal antibody (directed against perlecan domain IV, MAB1948) and rabbit anti-aggrecan polyclonal antibody (AB1031) were purchased from Chemicon International Inc. (Temecula, CA). Rabbit anti-chicken tenascin polyclonal antibody was a generous gift from Drs. R. Chiquet-Ehrismann and T. Sakakura (Friedrich Miescher Institute, Switzerland). Rabbit anti-mouse collagen X polyclonal antibody (NC2 #90) a generous gift from Dr. G. Lunstrum, (Shriners Hospital for Children, City, State). Rhodamine Red™ -X-conjugated affiniPure goat anti-rat IgG, sheep anti-mouse IgG conjugated HRP and normal rabbit serum were purchased from Jackson ImmuoResearch Laboratories, Inc. (West Grove, PA) Alexa fluor ®488 was obtained from Molecular Probes, Inc. (Eugene, OR) Neutr-Avidin horseradish peroxidase conjugated (NeutrAvidin™-HRP), 3,3′,5,5′tetramethylbenzidine (TMB, 1-Step™ Ultra TMBELISA), blocking buffer (SuperBlock™ Blocking Buffer) and chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate) were purchased from Pierce Biotechology, Inc. (Rockford, IL, USA). Polylactic acid (PLA) scaffolds were obtained from BD Biosicences (BD™ Three Dimensional OPLA® scaffolds).

rhBMP-2 to PlnDI Binding

A dot blotting format was employed to determine if PlnDI was functionally active in binding rhBMP-2 (7). PlnDI (12 μg) was digested with heparinases I, II, and III in PBS containing 1mM Ca^{2+} and Mg^{2+} for 4 h at 37° C. Digested and undigested PlnDI (3 μg) were blotted onto nitrocellulose, and subsequently blocked with 5% (w/v) fat-free milk powder in blocking buffer (SuperBlock™, Pierce Biotechnology, Inc.) for 1 h at room temperature. After washing with blocking buffer, 100 ng of rhBMP-2 was added to each well of the blotting apparatus and incubated for 4 h at room temperature. The membrane then was removed from the blotting apparatus, and blocked with 3% (w/v) BSA in blocking buffer for 1 h at 4°C, prior to incubation in 2.0 μg/ml of monoclonal mouse anti-human BMP-2 antibody in block buffer overnight at 4°C. After washing five times at room temperature with 0.05% (v/v) Tween 20 in PBS (PBS-T), the membrane was incubated with sheep anti-mouse IgG conjugated HRP (1:200,000) in blocking buffer for 1 h at room temperature. Following this incubation the membrane was washed again in PBS-T. The bound antibody was detected via enhanced chemiluminescence. The binding of rhBMP-2 to PlnDI was evaluated by densitometry and expressed as individual density values (IDV).

Preparation of microplate coating with collagen II fibrils

Freeze-dried collagen II extracted from bovine tracheal cartilage was dissolved at 4 mg/ml in 0.5 M acetic acid for 48 h at 4°C to make collagen II dispersions (collagen II monomers). Collagen II fibrils were formed by dialyzing 2.5 ml of collagen II acid dispersion against 1 L of PBS (pH 7.4) for 48 h at room temperature, and then incubating for 24 h at 37°C as described previously (23). *In vitro* fibril formation was monitored by the increase in absorbance at 400 nm (24,25). The collagen II fibril preparation then was diluted with PBS to 1.0 mg/ml and stored at 4°C. Denatured collagen II fibrils were obtained by heating collagen II fibril preparations at 60°C for 30 min as previously described (23). To immobilize collagen II into plastic plates, each well of 96-well microplates was incubated with 10 μg of collagen II fibrils, or denatured collagen II fibril suspension or acid dispersion (collagen II monomer) in 100 μl

for 24 h at 37°C. Control wells were coated with 100 μl of 100 μg/ml BSA solution in PBS. After rinsing with PBS, the coated 96-well plates were stored at 4°C for future use. **Collagen II coating efficiency was determined by measuring hydroxyproline content of the coated well surfaces (48). All collagen forms used gave similar coating efficiencies ($^{+/-}$ 5%).**

PlnDI binding to collagen II fibrils

To determine if PlnDI bound to collagen II fibrils, a solid-phase binding assay was performed essentially as described previously (23-25). Briefly, PlnDI was biotinylated with Sulfo-NHS-LC-Biotin using EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit (Pierce Biotechology, Inc, Rockford, IL, USA), according to the manufacturer';s instructions. The association of biotinylated PlnDI with collagen II fibrils immobilized in microplates was determined by binding of NeutrAvidin conjugated horseradish peroxidase (NA-HRP). After blocking with 3% (w/v) BSA in PBS, 100 μl of biotinylated PlnDI in blocking buffer was added at increasing concentrations (0-600 μg/ml) to each well of a 96-well microplate and incubated for 2 hr at room temperature. After washing three times with PBS, the bound biotinylated PlnDI was incubated with NA-HRP (0.1 μg/ml) in 100 μl blocking buffer for 30 min at room temperature. The wells finally were incubated with 200 μl of TMB solution followed by washing with PBS. The reaction was stopped with 500 μ l of 2M sulfuric acid. The optical density was measured at 450 nm. The same assay was used to assess interactions of biotinylated PlnDI with denatured collagen fibrils and collagen II monomers.

The specific binding of biotinylated PlnDI to collagen II fibrils was evaluated further by competitive binding of unlabeled PlnDI. In the assay, 3 μg of biotinylated PlnDI was added to collagen II fibril-coated wells in the presence of increasing molar ratios of unlabelled PlnDI/ biotinylated PlnDI (from 0 to 40). The association of biotinylated PlnDI with collagen II fibrils was measured as described above.

To investigate to what extent the protein and GAG constituents of PlnDI mediated interactions with collagen II fibrils, biotinylated PlnDI (3 μg), digested or undigested with heparinases I, II and III and chondroitinase ABC, was added into each well of collagen II fibril-coated microplates. HS (25 μg/well) or CS (25 μg/well) were used to compete for biotinylated PlnDI binding (6 μg/well) to collagen II fibrils. Binding characteristics of biotinylated PlnDI, following digestion of HS or CS or in competition with HS or CS, were evaluated as described above.

Binding of rhBMP-2 to P-C fibrils

After immobilizing collagen II fibrils into 96-well microplates and blocking with 3% (w/v) BSA in PBS, PlnDI (3 μg/well), undigested or digested with heparinases I, II and III or chondroitinase ABC, was incubated with the collagen II fibrils resulting in the following substrates: PlnDI-collagen II fibrils (P-C fibrils), heparinases I, II and III-digested P-C fibrils and chondroitinase ABC-digested P-C fibrils. Surfaces coated with collagen II fibrils alone or BSA (BSA) served as controls. Solid-phase binding assays were employed to assess rhBMP-2 binding. In this experiment, rhBMP-2 (50 ng) in blocking buffer was added to each well and incubated for 2 h at room temperature. After washing three times with PBS, anti-human BMP-2 antibody conjugated to HRP and colorimetric reagents of the BMP-2 Quantikine ELISA Kit (R&D System, Inc. Minneapolis, MN) were used to identify the rhBMP-2 associated with these substrates, according to manufacturer';s instructions.

Quantification of rhBMP-2 release

The release kinetics of rhBMP-2 from P-C fibrils or collagen II fibrils, were measured using a sandwich ELISA. In 4-well plates (Nalge-Nunc International; Rochester, NY), collagen II fibrils (100 μg in 300 μl of PBS) were added to each well and then incubated with either PBS

or PlnDI (9 μg in 300 μl of PBS/well) to form substrates of collagen II fibrils alone or P-C fibrils, as described above. The coated 4-well plates were sterilized under UV irradiation in a standard tissue culture hood for 2 h. After washing with PBS, 200 ng of rhBMP-2 in 300 μl of release buffer (DMEM containing 1% (w/v) BSA, 100 U/ml penicillin and 100 μg/ml streptomycin) was added into each well, and incubated with the substrates for 2 h at 37°C. rhBMP-2 in the release buffer was determined in 0.8 ml collected at day 0. Next, release buffer (0.8 ml) was added into each well after which it was retrieved at 1, 3, 6, 12 days, and stored at -40° C. The content of rhBMP-2 in the release buffer was determined with a sandwich ELISA assay kit (Quantikine BMP-2 ELISA, R&D Systems, Inc, Minneapolis, MN), according to the manufacturer';s instructions. The content of rhBMP-2 associated with each substrate, and the percent of rhBMP-2 released from the substrates were calculated.

High density micromass cultures

The multipotential mouse embryonic fibroblast stem cell line, C3H10T1/2, was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in DMEM/F12 containing 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C in a humidified atmosphere of air: CO_2, 95:5 (v/v). High density micromass culture of C3H10T1/2 was employed as described previously (46,49). P-C fibrils and collagen II fibrils only substrates were pre-coated on 4-well plates as described above, and then incubated with rhBMP-2 (200 ng/well) in 300 μl of DMEM containing 5% (v/v) FBS for two h at room temperature to form P-C-B fibrils and C-B fibrils. After washing with PBS two times and sterilizing with UV irradiation for 30 min, the 4-well plates loaded with different substrates were air-dried in a laminar-flow hood, and then C3H10T1/2 cells were spot-seeded as 10 μl drops containing 1×10^5 cells, in the center of each well. After cells had attached for 1-2 h at 37°C, 0.8ml of chondrogenic differentiation medium (CMRL-1066 containing 15% (v/v) FBS, ascorbic acid (50 μg/ml), citrate (50 μg/ml), pyruvate (50 μg), 100 U/ml penicillin and 100 μg/ml streptomycin [14, 19, 20]), was added to each well. The medium was changed every 2 days. Cultures were maintained at 37°C in a humidified atmosphere of air: CO_2, 95:5 (v/v) until harvest.

Alcian blue staining for micromass culture

To observe chondrogenic differentiation of C3H10T1/2 cells on different substrates, Alcian blue staining was performed as described previously (21,46,49,50). Briefly, after 6 days of micromass culture, cells were rinsed with PBS, fixed with 10% (v/v) formalin containing 0.5% (w/v) cetylpyridinium chloride (CPC) for 10 min at room temperature, briefly rinsed with 3% (v/v) glacial acetic acid (pH 1.0) and then incubated in 1 ml of 0.5% (w/v) Alcian blue 8GX (Sigma) in 3% (v/v) glacial acetic acid (pH 1.0) overnight at room temperature.

Collagen II, aggrecan and SOX 9 mRNA expression

RNA was extracted from C3H10T1/2 cell micromass cultures at day 6. Each sample was comprised of four micromass cultures collected in cell lysis buffer from the RNeasy Mini Kit (QIAGEN; Valencia, CA), and passed through a Qiashredder homogenizer (QIAGEN) and Qiashredder spin column according to the manufacturer';s protocol. Isolated RNA was treated using the DNA-free Kit (Ambion, Austin, TX) and quantified spectrophotometrically. cDNA was generated from RNA using random hexamers and RNase inhibitor from GeneAmp RNA PCR Core kit (Applied Biosystems, Forster City, CA), and reverse transcriptase, dNTPs and RT buffer from the Omniscript RT Kit (QIAGEN) according to the manufacturer';s protocol. mRNA levels were determined using real-time quantitative PCR, performed using SYBR Green PCR Master Mix (Applied Biosystems, Warrington WA1 4SR, UK). PCR reactions were performed and monitored using ABI Prism 7700 Sequence Detection System (AB Applied Biosystems, Foster City, CA) with a two step cycling protocol (annealing and

elongation at 60°C, and denaturation at 94°C). The levels of expression of mRNA were calculated with the comparative threshold cycle (Ct) method with $2^{-\Delta\Delta Ct}$ formula (User Bulletin No.2, ABI Prism 7700 Sequence Detection System). The Ct value of each target sequence was subtracted from the Ct value of β-actin, to derive Δct. The calculation of ΔΔCt involved subtraction of the ΔCt value of C3H10T1/2 cells cultured on uncoated plates. The validation experiment demonstrated that the amplifying efficiency of the targets (collagen II, aggrecan and sox9) and reference (β-actin) were approximately equal (slope difference <0.1). Each sample was assessed in triplicate. Specificity of primers was verified by dissociation of amplicons. The primer pairs used for PCR reactions are listed in table 1.

Preparation of ECM modified-PLA scaffolds and PlnDI/collagen II fibril-PLA scaffolds

Collagen II fibril-PLA scaffolds were prepared by coating collagen II fibrils on PLA sponges as described previously (35,51) with some modification. The PLA sponges (average pore size: 100-200 μm, hydration capacity: 30 μl, diameter: 4.2-5.2 mm, height: 3.9-4.5 mm, volume: 0.039 cm^3) were immersed in collagen II fibril solution (1.0 mg/ml in PBS) containing D-(+)-glucose (9 mM) and submitted to constant rotary agitation overnight at 4°C. The collagen II fibril-containing PLA sponges then were frozen at -80°C for 24 h, and subsequently lyophilized for an additional 24 h. The lyophilized collagen II fibril-PLA scaffolds were UV cross-linked as described previously using a UV crosslink chamber (Stratalinker 2400™, Stratagene Cloning Systems, La Jolla, CA, USA). To further fabricate P-C fibril-PLA scaffolds, collagen II fibril-PLA scaffolds were incubated with PlnDI (30 μg/ml) with constant rotary agitation for 2 h at room temperature. The structure of the scaffolds was observed employing scanning electron microscope (SEM).

rhBMP-2 binding to scaffolds

To investigate the binding of rhBMP-2 to various scaffolds, an ELISA was employed. After blocking with 3% (w/v) BSA in PBS, PLA, collagen II-PLA or P-CPLA scaffolds were incubated with rhBMP-2 (200 ng/ml) with constant rotary agitation for 2 h at room temperature, and then washed 3 times with PBS-T on shaker at room temperature to remove unbound rhBMP-2. rhBMP-2 binding to scaffolds was measured with the BMP-2 Quantikine ELISA Kit (R&D System, Inc. Minneapolis, MN) according to the manufacturer';s instructions. Each scaffold was further reacted with 3 ml of ELISA kit color reagent and then dried with a Kaydry wiper (Kimberly-Clark, Co., Roswell, GA, USA) to stop the reaction and immediately photographed. In addition, after addition of stop buffer 200 μl of the reactant solution was transferred to wells of 96-well plates for absorbance measurement at 450 nm.

Seeding scaffolds with cells and 3D tissue culture

Scaffolds, [PLA (PLA), collagen II-PLA and P-C PLA, were incubated with rhBMP-2 (200 ng/ml in PBS) for 2 h. These coated scaffolds were immersed in 20 ml of CMRL-1066 medium containing 10% (v/v) FBS, and then briefly dabbed with a sterile gauze to remove excess medium. To form cell-scaffold constructs, dynamic seeding was used to load C3H10T1/2 cells onto scaffolds, according to the manufacturer';s instruction (BD™ Three Dimensional OPLA® Scaffold, Guidelines for Use, BD Biosciences) as reported previously (52). Cell-scaffold constructs on PLA, collagen II-PLA and P-C PLA served as controls. Three scaffolds of each type were placed into 50 ml conical tubes (BD Falcon Conical Centrifuge Tubes) and then incubated in 1 ml of C3H10T1/2 cells suspension (2×10^7 cells/ml) in CMRL-1066 medium containing 15% (v/v) FBS. The tubes were placed on an orbital shaker (Lab-Line Instruments. Inc. Melrose Park, IL) and rotary agitated in an incubator at 37°C in a humidified atmosphere consisting of air: CO_2, 95/5 (v/v) at 250 rpm for 3 h. To maintain appropriate pH during extended incubation times, 4 ml of fresh CMRL-1066 medium containing 10% (v/v) FBS was added to each tube, and then the tubes were agitated for additional 12 h under the same

conditions. After gently washing with CMRL-1066 media to remove non-adherent cells, the cell-seeded scaffolds were transferred into 25 cm^2 cell culture flasks (Corning Incorporated, Corning, NY) and incubated in 8 ml of CMRL-1066 media, containing 15% (v/v) FBS, ascorbic acid (50 μg/ml), citrate (50 μg/ml), pyruvate (50 μg), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C in a humidified atmosphere of air: CO_2, 95:5 (v/v). Finally, dynamic culture was performed by placing the cell culture flask, fixed in a specially designed stand with an up-standing position, on the orbital shaker at 200 rpm in the incubator. The media was changed every 3 days. After 21 days of culture, cell-scaffold constructs were harvested for morphological analysis. Mouse embryonic fibroblasts (MEFs) were isolated from day 14 post coitum embryos of ICR mice using established methods (43). Differentiation experiments were carried out using cells between passage 3 and 4. Constructs of MEFs-scaffold were formed and cultured with same method used for C3H10T1/2 cells-scaffold constructs. As noted above, in each experiment three separate scaffolds were prepared in each test group and each experiment performed three times with similar results.

Histological and Immunohistochemical analysis

The cell-scaffold constructs were rinsed with PBS, fixed for 2 hr in 10% (w/v) formalin, dehydrated through a graded series of ethanol and, embedded in paraffin. Thick sections (10 μm) were cut through the center of scaffolds for Safranin O/Fast Green and von Kossa staining. For cryosectioning, the cell-scaffold constructs were embedded in O.C.T. (Sakura Finetek, Torrance, CA) frozen on dry ice. Sections of 30 μm thickness were cut through the center of cell-scaffold constructs for alkaline phosphatase (ALP), Oil Red staining and immunohistochemical analysis. The staining procedures of Safranin O/Fast Green, von Kossa, ALP and Oil-Red were performed according to standard histological protocols (33,43,53-55). For immunohistochemical analysis, crysections were fixed with 4% (w/v) paraformaldehyde in PBS for 30 min at room temperature followed by digestion with chondroitinase ABC (2.5 U/ml) for aggrecan staining, or with 0.25% (w/v) testicular hyaluronidase for Pln, tenascin and collagen X staining, for 1 h at 37°C. The specimens were blocked with DAKO® serum-free protein block (DAKO Co., Carpinteria, CA), and incubated with primary antibodies against aggrecan (rabbit anti-aggrecan polyclonal antibody, 1:50), perlecan (rat anti-perlecan domain IV monoclonal antibody, 1:60), tenascin (rabbit anti-tenascin polyclonal antibody, 1:100) or collagen X (rabbit anti-collagen X, 1:200), respectively, for 1 h at 37°C. After rinsing with PBS, sections then were incubated with secondary antibodies of Alexa fluor ®488 goat anti-rabbit (1:500) for aggrecan detection, Rhodamine Red™ -X-conjugated affiniPure goat anti-rat IgG (1:100) for perlecan detection or Alexa Fluor 568 goat anti-rabbit (1:50) for tenascin and collagen X detection for 1 h at 37°C. Sections then were rinsed three times with PBS, placed under glass coverslips and observed and photographed using confocal microscopy.

Statistical analysis

Unless otherwise stated, all values are expressed as means ± standard deviations (SD) and one-way ANOVA. All studies were assayed using samples from separate determinations in triplicate. Statistical significance was determined by a Tukey-Kramer multiple comparisons test; p values <0.01 were considered significant.

Results

rhBMP-2 Binding to PlnDI

A photograph of a representative dot blot depicting rhBMP-2 binding to PlnDI is shown in fig. 1A, B and demonstrates the heparan sulfate dependence, i.e., heparinase sensitivity, of the interaction. The densitometric quantitation of these data are summarized in fig. 1C. Together, these data demonstrate that PlnDI binds rhBMP-2 robustly compared to negative controls (BMP-2 + PBS and BMP-2 + heparinase) ($P<0.001$). In addition, HS chains attached in PlnDI

are largely responsible for rhBMP-2 since heparinase treatment greatly reduced the binding of rhBMP-2 ($P < 0.001$).

Binding of PlnDI to collagen II fibrils

The solid-phase assays provided a simple, quantitative assay for detection of protein binding to collagen II. Initially, immobilized collagen II fibrils were incubated with soluble, biotinylated PlnDI to determine if PlnDI could bind to collagen II fibrils. Biotinylated PlnDI interacted with collagen II fibrils in a saturable manner, as expected for specific binding. In contrast, biotinylated PlnDI bound poorly to BSA-coated surfaces and represented a nonspecific binding control ($P<0.001$, fig. 2). In addition, biotinylated PlnDI binding to collagen II fibrils saturated at concentrations of approximately 10-20 μg protein/ml, i.e., approximately 45-900 nM with half-saturation occurring at approximately 2.5 μg protein/ml, i.e., approximately 110 nM (fig. 2A). As an additional specificity control, unlabeled PlnDI was used to compete for the biotinylated PlnDI binding. Biotinylated PlnDI binding to collagen II fibrils was blocked >80% in a dose-dependent fashion by unlabeled PlnDI (fig. 2B), suggesting that most binding was due to interactions with PlnDI and not biotin.

Next, collagen II fibril-coated plates were incubated with biotinylated PlnDI that had been predigested with heparinases I, II and III or chondroitinase ABC. Binding was reduced significantly by predigestion with either heparinase or chondroitinase with maximal inhibition observed with combined predigestion ($P<0.001$; fig. 3A). Similar results were obtained in another type of experiment in which excess soluble HS or CS was used to compete for binding sites in collagen II fibrils ($P<0.001$; fig. 3B). These results demonstrated that both HS and CS of PlnDI contribute to binding to collagen II fibrils.

To determine if the physical form of collagen II impacted PlnDI binding, 96-well microplates were coated with natural collagen II fibrils, heat-denatured fibrils and collagen II monomers. The results demonstrated that significantly more biotinylated PlnDI bound to native fibrils than either denatured fibrils or monomers ($P<0.001$; fig. 4). Collagen II monomers bound significantly more PlnDI than denatured fibrils ($P<0.01$).

rhBMP-2 binding to P-C fibrils

We next set out to determine if rhBMP-2 could bind to P-C fibrils using a solid-phase binding assay. P-C fibril complexes bound significantly more rhBMP-2 than collagen II fibrils alone ($P<0.001$; fig. 5). Digestion of PlnDI with either heparinase (DH-P-C) or chondroitinase (DC-P-C) significantly reduced rhBMP-2 binding to P-C fibril complexes, although the residual binding was still significantly greater than to collagen fibrils alone ($P<0.001$). Thus, as was the case for PlnDI binding to collagen II fibrils, both HS and CS GAG chains contributed greatly to binding rhBMP-2 to P-C fibril complexes.

rhBMP-2 release kinetics

rhBMP-2 release from P-C fibril complexes and collagen II fibrils alone was evaluated *in vitro* by incubation of these substrates in a physiological buffer for up to 12 days (fig. 6). rhBMP-2 release was quantified using a sandwich ELISA. P-C fibril complexes initially bound 112 ng ± 4 of rhBMP-2 (day 0) in contrast with collagen II fibrils alone that bound only 49 ng ± 3 of rhBMP-2 (day 0). After 3 days, P-C fibrils retained 103ng ± 4 of rhBMP-2 (fig. 6A), releasing only 7.3% ± 3.4% of initially bound rhBMP-2. In contrast, at the same time collagen II fibrils alone retained 26ng ± 6 of rhBMP-2 releasing 47.7% ± 4.9% of initially bound rhBMP-2 (fig. 6B). After 12 days of incubation, P-C fibril complexes retained 72 ng ± 5 rhBMP-2 (fig. 6A) releasing 41.5% ± 5.7% of initially bound rhBMP-2. At this time point, collagen II fibrils alone retained very little, i.e., 13 ng ± 3, rhBMP-2 releasing 71.3% ± 3.7% of initially bound rhBMP-2 (fig. 6B). These findings demonstrated that P-C fibril complexes

not only immobilized significantly more rhBMP-2, but also retained the HBGF well during extended incubation in physiological buffer.

C3HT101/2 chondrogenic differentiation

C3H10T1/2 cells were placed in micromass cultures on collagen II fibrils (C), P-C fibril complexes, collagen II fibrils with bound rhBMP-2 (C-B fibrils) or P-C-B fibrils. After 6 days of culture, these cultures were stained with Alcian blue as an index of chondrogenic differentiation, i.e., GAG accumulation (fig. 7). Micromass cultures displayed positive staining when plated on both P-C-B and C-B fibrils, and negative staining when plated without BMP-2; however, Alcian blue staining of micromass cultures on P-C-B fibrils was much more robust than on C-B fibrils in the absence of PlnDI.

Chondrogenic differentiation also was evaluated by examining chondrocyte-marker gene expression by real time PCR (fig. 8). Collagen II, aggrecan and sox9 mRNA content were normalized to β-actin mRNA in each sample. Expression of all three marker mRNAs was highest when micromass cultures were plated on P-C-B fibrils ($P<0.001$); however, marker mRNA expression was higher for micromass cultures plated on C-B fibrils than on either other matrix in the absence of BMP-2 ($P<0.01$). No significant difference in marker mRNA expression was found between collagen II fibrils with or without PlnDI without BMP-2 ($P>0.05$). These results demonstrated that all components of P-C-B fibril complex are required to support optimal C3H10T1/2 chondrogenic differentiation in high-density micromass culture, and that addition of BMP-2 is absolutely critical regardless of matrix.

Physical properties and binding capacity of different scaffolds

Scanning electron microscopy revealed that scaffolds of collagen II fibrils/PLA and P-C fibrils-PLA maintained the porous structures normally observed with uncoated PLA scaffolds [49; data not shown]. An ELISA-based assay was used to index rhBMP-2 binding to the various scaffolds. Figure 9 shows that P-C fibril-PLA scaffolds displayed the highest rhBMP-2 binding; however, collagen II fibril/PLA scaffolds also displayed binding significantly above that of PLA alone, albeit much lower than that of P-C fibril-PLA scaffolds.

Histochemical and immunohistochemical analysis

The morphology of cell-scaffold constructs was examined histologically with Safranin O/Fast Green staining, which stains negatively charged GAGs red and nuclei **dark purple/black.** Sections from P-C-B-PLA scaffolds were strongly positive for GAG compared with other constructs, and revealed round chondrocyte-like cells embedded in lacunae and surrounded by abundant ECM (fig. 10D). Sections from C-B-PLA scaffolds revealed fibroblast-like cells embedded in compacted ECM that thickly covered the exterior scaffold surface (fig. 10E). Cells seeded into BMP-2-PLA (fig. 9F), P-C-PLA (fig. 9A), collagen II-PLA (fig. 10B) and PLA alone (fig. 10C) scaffolds demonstrated no obvious cartilage-like tissue. Alkaline phosphatase staining for chondrocyte maturation (fig. 10G-I) showed weak staining in some regions of P-C-B-PLA scaffolds (fig. 10G), but none in the other constructs. Von Kossa staining of cell-scaffold constructs showed that no mineralized ECM was present in any of the scaffold constructs (fig.10J-L). Immunohistochemical staining for the cartilage ECM markers, aggrecan, Pln and tenascin, showed strong positive staining in P-C-B-PLA scaffolds (fig.11A, D and G) with weak to no signal in the other scaffolds. A thin layer of Pln was evident at the exterior surface of collagen II-BMP2-PLA and BMP-2-PLA scaffolds (fig. 11E and F). Staining for collagen X, a marker of late hypertrophic chondrocyte differentiaton only was found in isolated regions of P-C-B-PLA scaffolds (fig. 11J), but was virtually absent in the other scaffolds (fig. 11K and L).

For experiments with primary mouse embryonic fibroblasts, cells seeded on P-C-BPLA scaffolds also demonstrated cartilage-like tissue formation after 21 days of culture (fig. 12). Compared with cells cultured on C-B-PLA and B-PLA scaffolds, embryonic fibroblasts on P-C-B-PLA scaffolds displayed more GAG accumulation (as indexed by Alcian Blue staining; data not shown) and much higher chondrogenic marker expression (aggrecan, perlecan, tenascin). As with C3H10T1/2 cells, marginal expression of collagen X was observed on P-C-B scaffolds (fig. 12M). In addition, embryonic fibroblasts cultured on P-C-B scaffolds deposited more extracellular matrix and displayed morphological characteristics more similar to chondrocytes than under the other conditions (fig. 12, panels A-C).

Discussion

In developing biomimetic materials or scaffolds for tissue engineering, bioactive ECM molecules, such as collagen, fibronectin and laminin, have been used to improve biological activity of the scaffolds (51-53,56). These components facilitate cell attachment, proliferation, differentiation, and the differentiated functions of cells (7,34,53,56). Nonetheless, in the design strategies of biomimetic scaffolds, shorter polypeptide or peptide sequences often have advantages over the usually very large ECM proteins because of their superior bioavailability and stability properties and for mass production improved feasibility (53). In this regard, we have used a recombinant fragment of Pln, PlnDI, expressed by a transfected mammalian cell line and purified from conditioned medium. This fragment is substantially smaller than intact Pln (approximately 22 kDa versus 800 kDa core protein) is appropriately decorated with GAG chains, binds HBGFs well and promotes cell proliferation (7). Both intact Pln and PlnDI stimulate cartilage differentiation and promote the action of chondrogenic growth factors, such as BMP-2 and TGF-β1 (4,6,14,19,20). The HBGF, BMP-2, plays key roles during chondrogenesis and was used to induce chondrogenic differentiation of mesenchymal stem cells and subsequent cartilage-like tissue formation in high-density culture (43-46). Thus, we considered that combining BMP-2 with proteins derived from cartilage ECM such as collagen type II and Pln would promote chondrogenic differentiation of mesenchymal stem cells. As a first step, we established that rhBMP-2 bound immobilized PlnDI with high affinity and stability and was abolished by digestion of HS chains in PlnDI with heparinases I, II and III. The latter observation demonstrated that the interaction between BMP-2 and PlnDI is dependent on the HS attached on its core protein. This interaction is consistent with previous studies demonstrating that HS can regulate and enhance BMP-2 functions (10,11).

Collagen II is a fibril-forming collagen believed to be an effective substrate in engineering cartilage (32-36). Collagen II fibrils can interact with various proteoglycans that regulate collagen II fibril formation and ECM network assembly (25,27-29). Heat-denatured collagen II fibrils fail to interact with these proteoglycans (23,27), suggesting that the triple helical structure of native collagen II is crucial for these interactions; however, the characteristics of the interactions are not very clear. Some studies indicate that proteoglycan binding to collagen II fibrils is mediated by CS or HS (23,27-29), while other studies indicate a primary role for the proteoglycan core protein in these interactions (24-26). In the present studies, we demonstrated that the interaction between PlnDI and collagen II fibrils was dependent on both HS and CS. Moreover, the interaction was abolished by heat-denaturation of collagen II fibrils demonstrating a requirement for appropriate three-dimensional structure of the fibrils. The specificity of PlnDI binding to collagen II fibrils was verified by the demonstration of concentration-dependent and saturable binding and competition by unlabeled PlnDI, but not the unrelated protein, BSA. In addition, PlnDI bound to collagen II fibrils much better than to collagen II monomers. Thus, it appears that the fibrillar configuration of collagen II contributes to optimal PlnDI binding.

These data indicate that P-C fibril complexes can be readily formed as a basis to develop new substrates for growth factor binding and cell culture. This substrate has superior functions than collagen II fibrils alone since it binds more HBGFs than collagen II-only substrates, regardless of the collagen II form or scaffold used. In addition, P-C fibril complexes not only retained but also sustained BMP-2 release, better than collagen II fibrils alone. To further investigate the potential value in tissue engineering, P-C fibril complexes were used to coat PLA scaffolds as described by Chen (51). rhBMP-2 interactions with different scaffolds were evaluated by a modified ELISA. These studies again demonstrated that P-C fibril complexes improved PLA scaffolds function via improved bindning and retention of BMP-2.

To mimic events in chondrogenic differentiation (41-44) and chondrogenesis of mesenchymal cells *in vitro*, high density cell culture systems, including micromass or pellet cultures, have been used in combination with BMP-2 for both the C3H10T1/2 mesenchymal progenitor cell line (45,46) as well as primary cultures of mouse embryonic fibroblasts and bone marrow stromal cells (43,44). We also employed micromass cultures of C3H10T1/2 cells plated on different substrates on which rhBMP-2 was pre-loaded. Alcian blue staining showed that the micromass cultures plated on P-C-B fibrils appeared more differentiated, i.e., accumulated more GAG, than the micromass cultures on other substrates. To verify the differentiated state of C3H10T1/2 cells, expression of the chondrogenic marker genes, Sox9, aggrecan and collagen II, was evaluated by real time PCR. Consistent with the results of Alcian blue staining, we found that mRNA expression of all chondrogenic markers was most robust when micromass cultures were plated on P-C-B fibrils. This effect is apparently due to the ability of P-C fibrils to bind and retain more BMP-2 than other substrates.

Synthetic PLA scaffolds are easily processed into desired shapes, pore size and microstructure, and are mechanically strong, compared with collagen scaffolds (51). Nonetheless, PLA scaffolds lack cell recognition signals, and their hydrophobic properties hinder uniform cell seeding in three dimensions (51,53,56). Therefore, synthetic scaffolds have been combined with bioactive molecules from ECM to improve their utility for tissue engineering. Surface modification of biomaterials with bioactive molecules is one method to make biomimetic materials and scaffolds (53). The finding that P-C fibril complexes effectively bind and retain BMP-2 suggested that these complexes are useful to coat and improve function of PLA scaffolds. Histological analysis revealed that cartilage-like tissue formed in P-C-B-PLA with abundant GAG accumulation as shown by Safranin O-Fast Green staining. In contrast, we did not find cartilage-like tissue in other scaffolds tested, and noted that fibroblastic and adipocyte-like cells mainly appeared in other scaffold constructs. The finding was confirmed by immunohistochemical analysis for expression of chondrogenic matrix components. Both aggrecan and Pln itself were used as additional markers of chondrogenesis (3-5,12). During cartilage development, tenascin appears in mesenchymal cell condensations preceding chondrocyte differentiation while in adult cartilage, tenascin is abundantly expressed in articular cartilage and tracheal rings, but not mature bone matrix (16,57,58). Therefore, tenascin was used as another marker of chondrogenic differentiation. Cell lines may adapt or mutate during extended passaging in cell culture. Therefore, their responses are not necessarily reflective of responses of cells in tissues or primary cell cultures. To address this concern, we utilized primary cultures of mouse embryonic fibroblasts that possess stem cell qualities (43). Our results demonstrated that these primary cell cultures behaved very similarly to the C3H10T1/2 cell line. This suggests that this approach can be used to generate cartilage-like tissue implants from primary cultures obtained from patients. Collectively, our observations indicate that scaffolds coated with P-C fibril complexes facilitate chondrogenic differentiation of mesenchymal progenitors in the presence of BMP-2 and are much superior to PLA scaffolds alone or coated with other combinations of PlnDI, collagen II fibrils and BMP-2.

Chondrogenic differentiation of mesenchymal progenitor cells *in vitro* requires the complex influences of growth factors including BMPs and TGF-βs as well as cell-cell and cell-matrix interactions (41,45,50). In response to BMP-2, C3H10T1/2 cells undergo both chondrogenesis and osteogenesis (45). In our study, we observed little or no mineralized matrix under any condition tested; however, we detected modest alkaline phosphatase and collagen X expression in P-C-B-PLA scaffolds at regions closed to the exterior surface, demonstrating that some chondrocytes in this area underwent hypertrophic differentiation. Uniform chondrogenic differentiation is preferred for fabricating permanent cartilage; however, considering that the generation of a functional osteochondral junction is desirable for articular cartilage resurfacing, the finding that hypertrophic chondrocytes occur at the scaffold periphery is interesting and may even prove advantageous for proper tissue integration (54). These observations are in marked contrast to studies using PlnDI in combination with collagen type I and FGF-2 which effectively drives osteoblastic, rather than chondrocytic, differentiation (7). Therefore, both the growth factors used and the matrix components of the scaffold appear to be strong influences on cell fate in tissue engineering applications.

In summary, by virtue of their ability to bind and retain key growth factors, PlnDI containing substrates have excellent potential in the development of biomimetic scaffolds for tissue regeneration, repair, and replacement. In addition to BMP-2, other HBGFs, such as FGF-2, VEGF, PDGF and HB-EGF, might be complexed with PlnDI coated scaffolds in conjuction with an collagen type I or type II to promote chondrogenesis, osteogenesis, vasculogenesis and other aspects of tissue morphogenesis.

Acknowledgments:

We appreciate the many helpful discussions and comments of Ms. JoAnne Julian, Dr. Kirk Czymmek, Dr. Ying Shao, Dr. Catherine Kirn-Safran and Mr. Rick Focht. We also acknowledge the excellent secretarial support of Ms. Sharron Kingston. This work was supported by NIH R01 DE13542 (to D.D.C. and M.C.F-C.), NIH P20-PR16458 (to M.C.F.C. and R.R.G.) and NRSA F32 AG20078 (to R.R.G.).

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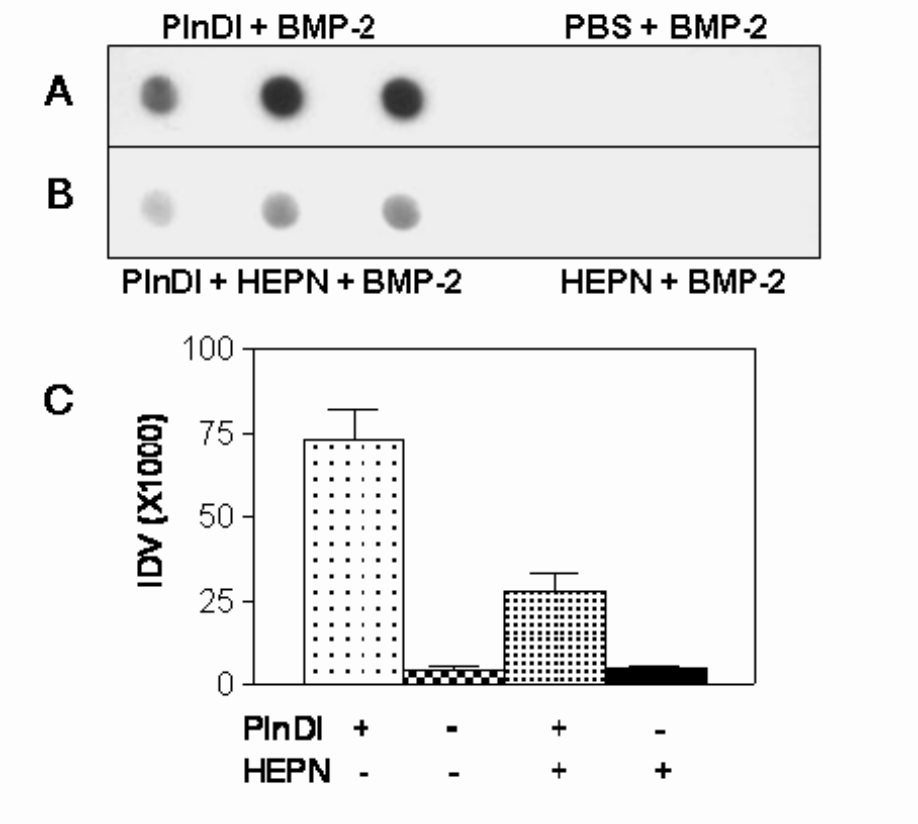

Fig. 1.

BMP-2 binding to perlecan domain I (PlnDI). In rows A & B, PlnDI or PBS vehicle was immobilized on nitrocellulose. In row B, PlnDI or PBS also were digested with heparitinases I, II and III (HEPN) then immobilized on nitrocellulose. All wells subsequently were incubated with BMP-2 and bound BMP-2 detected. Panel C summarizes densitometric measurements performed on the dot blots above. From left to right in the bar graph: detection of binding of BMP-2 to PlnDI, PBS, PlnDI digested with HEPN and HEPN alone. Assays were performed in triplicate. Each bar indicates the mean ± SD.

Fig.2.

PlnDI binding to collagen II fibrils. A) Wells of 96-well microplates were coated with collagen II fibrils (●) or BSA (o), followed by incubation with 100 μl of biotinylated PlnDI at the indicated concentrations. **B)** Non biotinylated PlnDI was used to compete for biotinylated PlnDI binding to collagen II fibrils at increasing molar ratios of PlnDI/biotinylated PlnDI. Assays were performed in triplicate. Values given are the mean± SD in each case.

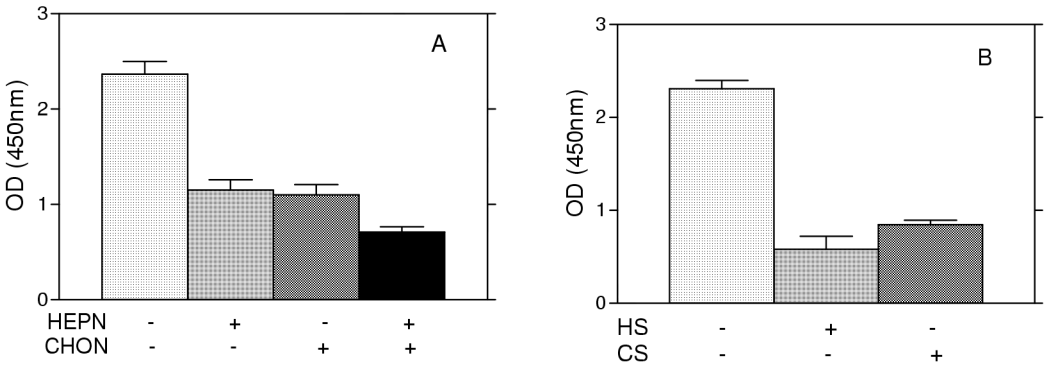

Fig.3.

PlnDI binding to collagen II fibrils is HS and CS dependent. A) Binding of biotinylated PlnDI, digested or undigested with heparitinases I, II and III (HEPN) and/or chondroitinase ABC (CHON) as indicated on the figure, to collagen II fibrils coated on polyethylene wells was determined. **B)** Biotinylated PlnDI was mixed with either HS (250 μg/ml) or CS (250 μg/ml), and then incubated with collagen II fibrils coated on polyethylene wells. Binding was determined. All assays were performed in triplicate and results of a representative experiment are shown. Each bar indicates the mean ± SD.

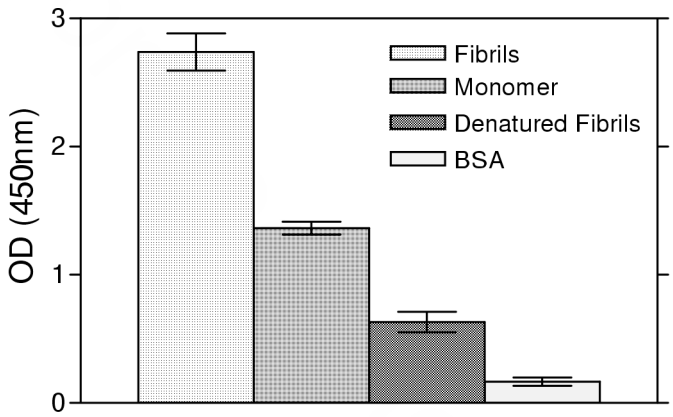

Fig.4.

PlnDI binding to different forms of collagen II. Binding of biotinylated PlnDI to collagen II fibrils, collagen II monomers, heat-denatured collagen II fibrils or BSA was determined. Each assay was performed in triplicate and the results of a representative experiment are shown. Each bar represents the mean ± SD.

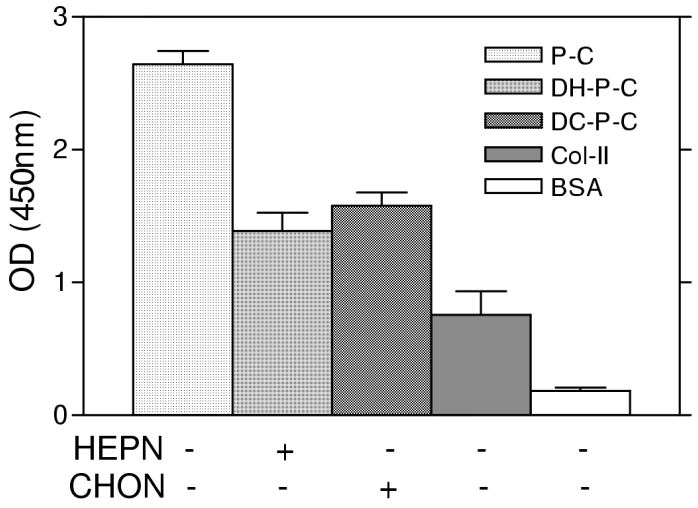

Fig.5.

BMP-2 binding to PlnDI associated with collagen II fibrils. Binding of BMP-2 to PlnDI/collagen II fibril complexes (P-C), heparitinase-digested PlnDI digested/collagen II fibril complexes (DH-P-C), chondroitinase ABC-digested PlnDI/collagen II fibril complexes (DC-P-C), was evaluated with a solid phase binding assay. Coating with collagen II alone (Coll-II) or BSA were used as controls. Each bar indicates the mean ± SD of triplicate determinations from a representative experiment.

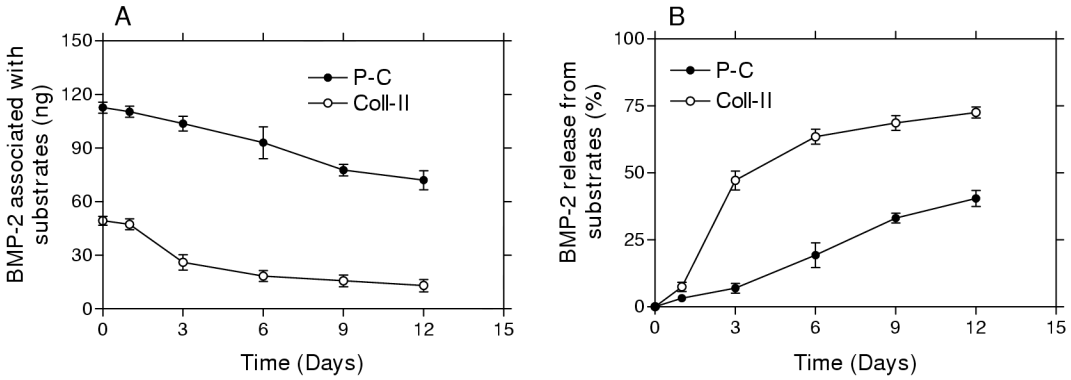

Fig.6.

BMP-2 release from PlnDI/collagen II fibril complexes and collagen II fibrils. Complexes of PlnDI/collagen II fibrils (PlnDI/Coll-II) (●) and collagen II fibrils alone (Coll-II) (o) were pre-coated on surfaces and subsequently incubated with BMP-2. Released BMP-2 was determined as the indicated time by ELISA. **A)** The amount of BMP-2 bound was calculated by subtracting the amount of BMP-2 released from the amount determined to be bound at time zero. **B)** BMP-2 release was calculated as the percentage of BMP-2 released at the indicated time relative to the amount bound to the scaffold at time zero. All points reflect the means ± SD of triplicate determinations in each case.

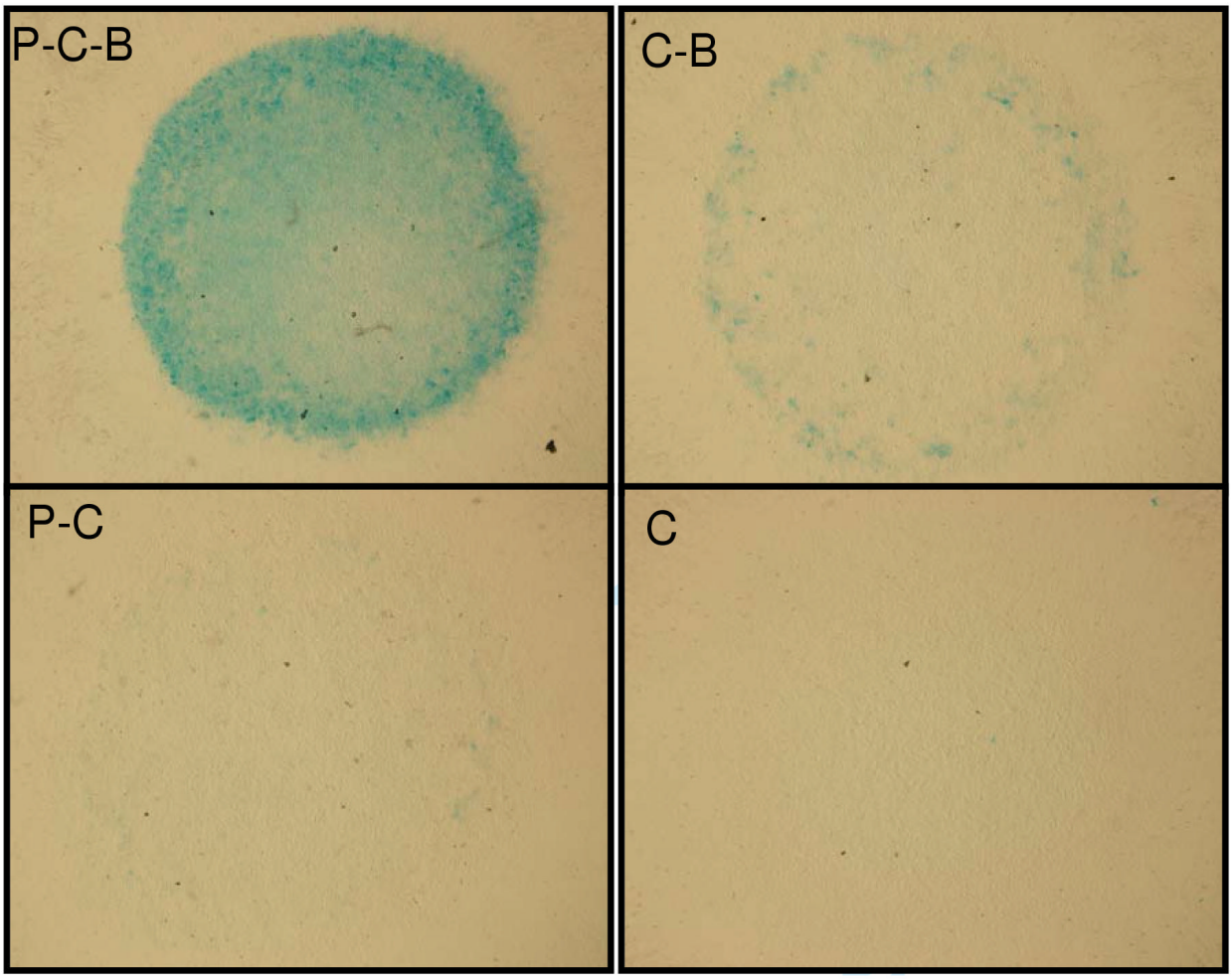

Fig.7.

Alcian Blue staining of micromass cultures of C3HT1/2 cells on different substrates. High density micromass cultures of C3H10T1/2 cells (1×10^5/10ul) were plated on the indicated substrates for 6 days followed by Alcian Blue staining. The substrates used were PlnDI-collagen II fibril-BMP2 complexes (P-C-B), collagen II fibril-BMP-2 complexes (C-B), PlnDI-collagen II fibril complexes (P-C) and collagen II fibrils alone (C).

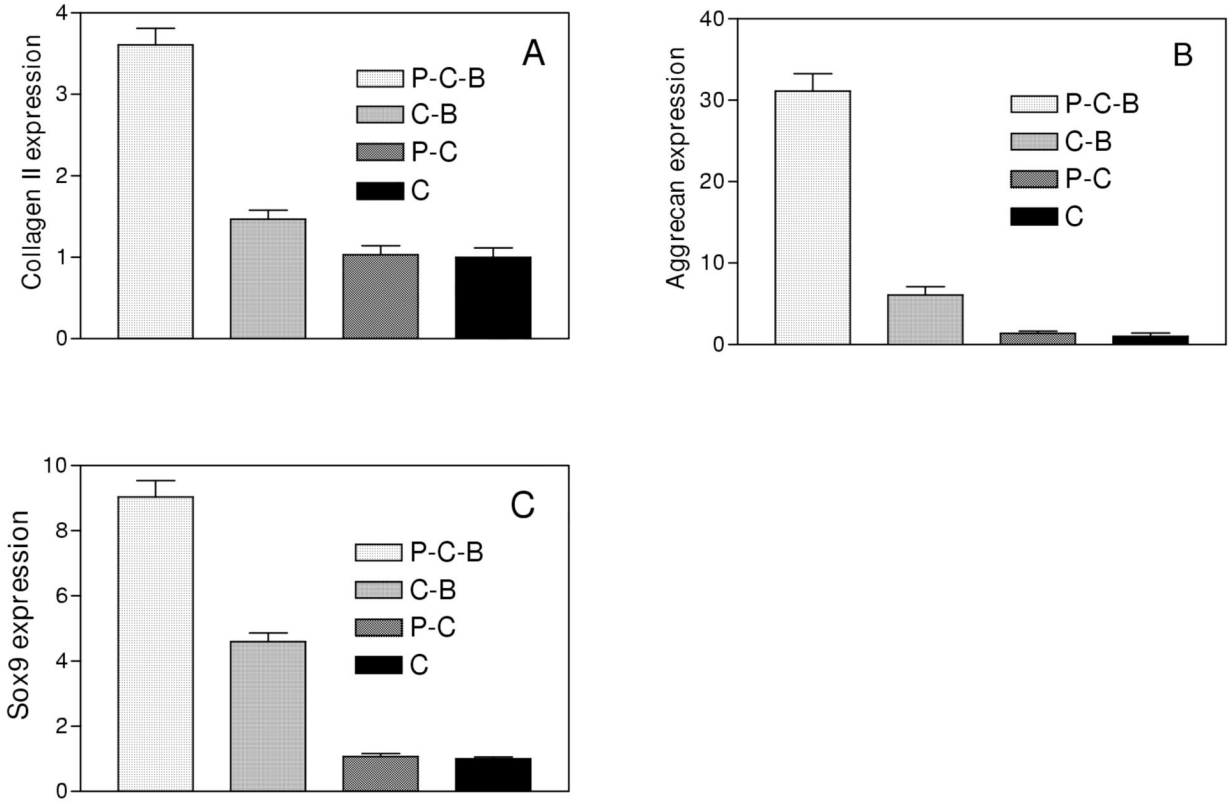

Fig. 8.

Chondrogenic differentiation marker mRNA expression by micromass cultures of C3H10T1/2 cells. Total RNA was extracted from micromass cultures of C3H10T1/2 cells cultured on different substrates (abbreviations same as described in legend to panel 7) after 6 days of culture and relative levels of expression of mRNA encoding collagen II (**A**), aggrecan (**B**) or sox9 (**C**) was evaluated by real-time RT-PCR. The values on the Y axes in each group represent arbitrary values relative to the ratio of target mRNA C_T to GAPDH C_T for each marker. Values represent means ± SD of triplicate determinations of separate RNA isolates in each case.

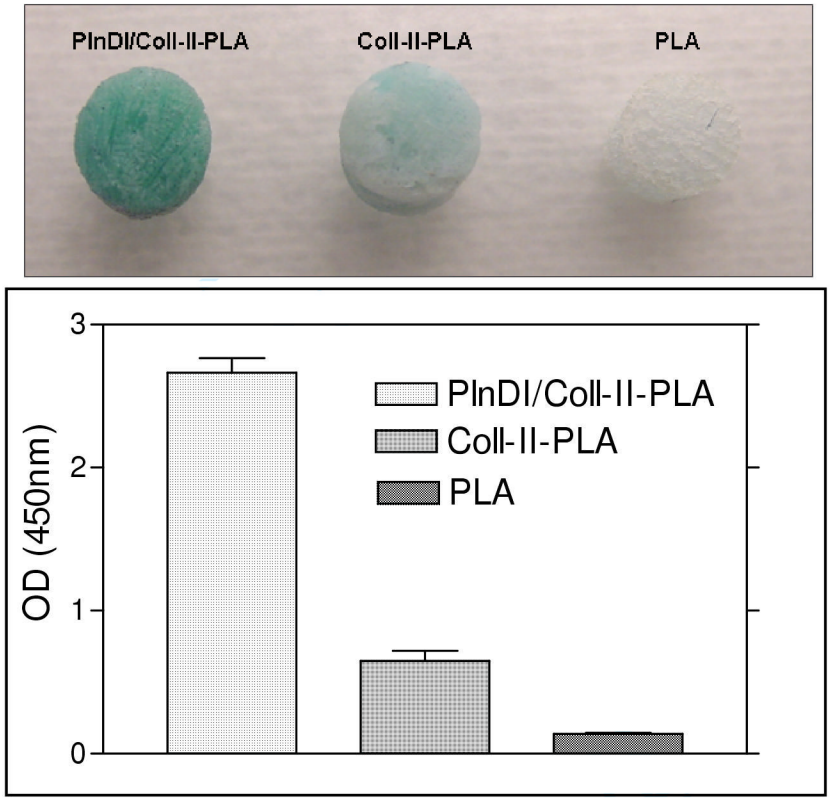

Fig.9.

BMP-2 binding to different three-dimensional scaffolds. An ELISA-based assay was used to determine BMP-2 binding to scaffolds. The scaffolds were constructed of PlnDI/collagen II fibrils-PLA scaffolds (PlnDI/Coll-II-PLA), collagen II fibrils-PLA (Coll-II-PLA) or PLA alone (PLA). The upper panel shows a photograph of scaffolds retaining the blue reaction product generated by the ELISA indicating BMP-2 retention. The lower bar graph shows the quantitation of dye in each scaffold following extraction and measurement of OD_{450} in the extracts. Each bar represents the mean + SD of triplicate determinations.

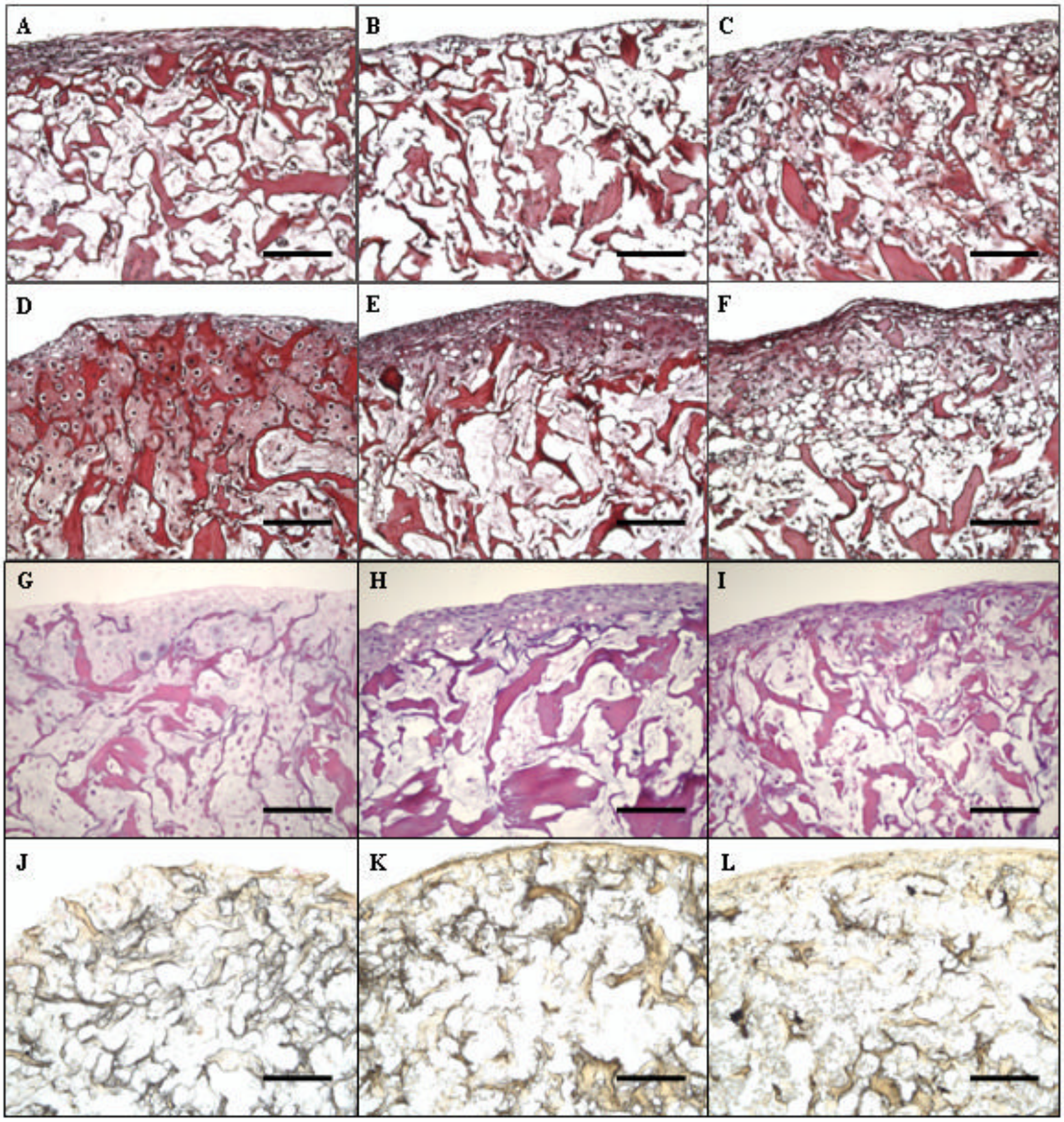

Fig. 10.

Histological analysis of C3H10T1/2 cells seeded in different scaffolds. C3H10T1/2 cells were seeded and cultured dynamically for 21 days on each scaffold followed by Safranin O-fast green straining (**A-F**), alkaline phosphatase staining (pink, **G-I)** or von Kossa staining (**J-L**). The scaffolds were constructed of PlnDI-collagen II fibrils PLA (**A**), collagen II fibrils-PLA (**B**) or PLA (C), BMP-2-PlnDI-collagen II-PLA (**D, G, J**), BMP-2-collagen II fibrils-PLA (**E, H, K**) or BMP-2-PLA (**F, I, L**). **(Scale bar = 200μm).**

fibrils-PLA (E,H and K) or BMP-2-PLA (F,I and L). (Scale bar = 200μm).

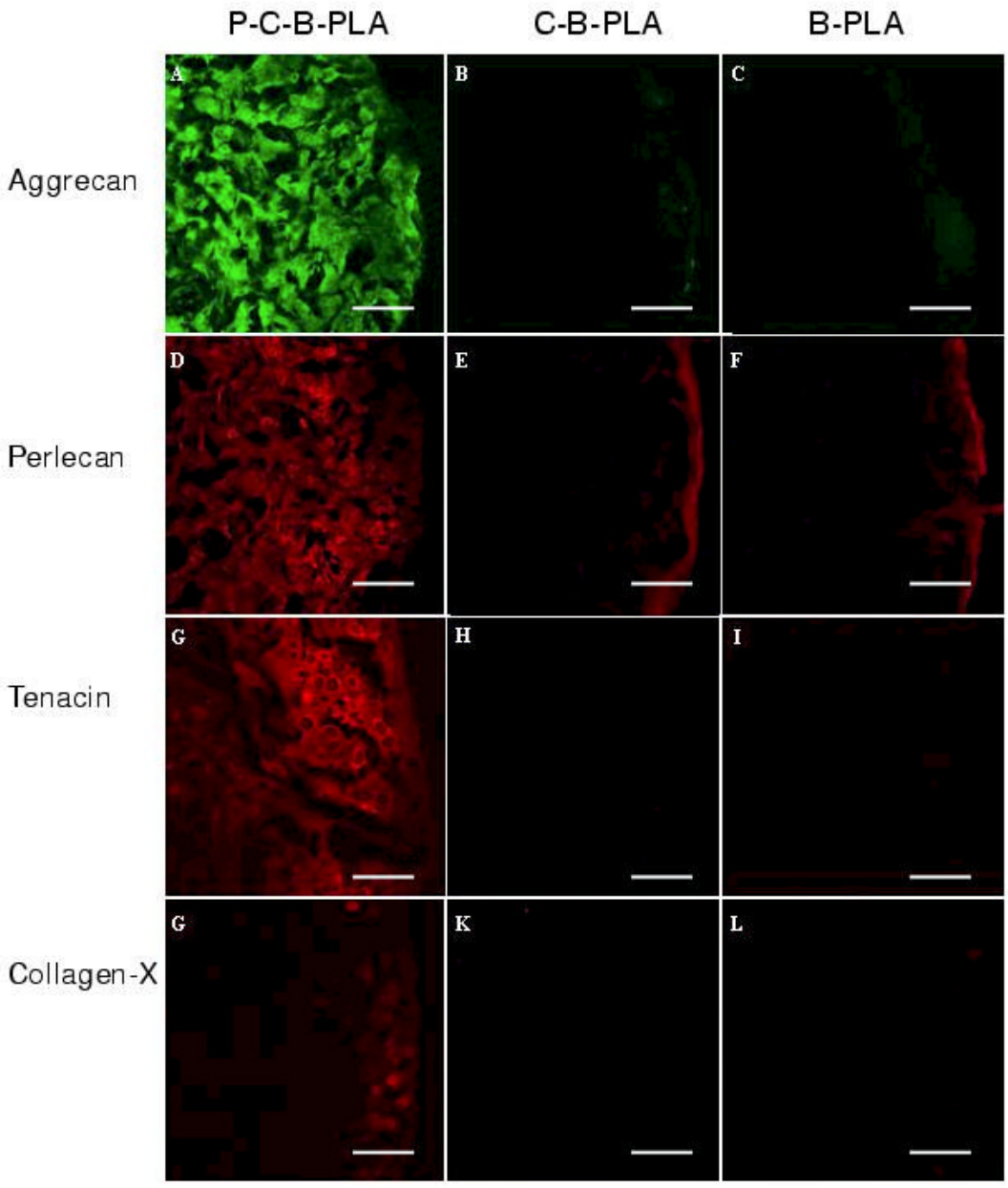

Fig. 11.

Immunohistochemical analysis of chondrogenic markers by C3H101/2 cells seeded in different scaffolds. C3H10T1/2 cells-scaffolds were seeded and cultured dynamically for 21 days in different scaffolds. The scaffolds used were PlnDI-collagen II fibrils-BMP-2-PLA (P-C-B-PLA, panels A, D, G and J), collagen II fibrils-BMP-2-PLA (C-B-PLA, panels B, E, H and K) and BMP-2-PLA (B-PLA, panels C, F, I and K). The sections of cell-scaffold constructs were stained for aggrecan (A-C), perlecan (D-F), tenacin (G-I) or collagen X (J-K). (Scale bar = 200μm).

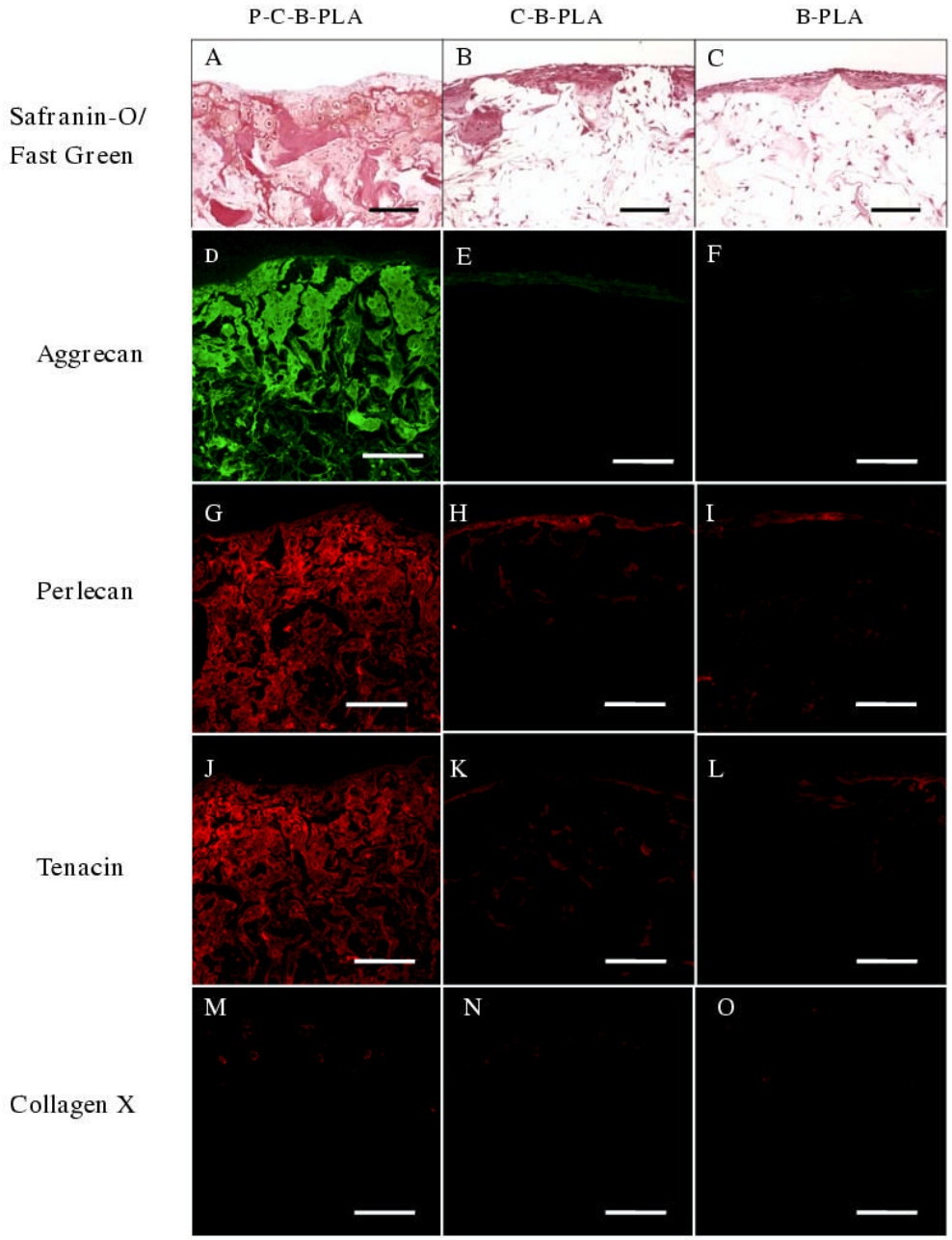

Fig. 12.

Histological and Immunohistochemical analysis of chondrogenic markers by mouse embryonic fibroblasts (MEFs) seeded in different scaffolds. MEFs were seeded and cultured dynamically for 21 days in the indicated scaffolds followed by sectioning and staining by Safranin O-fast green (A-C) or immunostaining for aggrecan (D-F), perlecan (domain IV) (G-I), tenacin (J-L) and collagen X (M-O). The abbreviation for the scaffolds in each column are the same as described in the legend to figure 11. (Scale bar = 200μm).

Table I:

Specific primers used for real time **PCR**

Genes	Forward	Reverse
β-actin	5′AAATCGTGCGTGACATCAAAGA3′	5′GCCATCTCCTGCTCGAAGTC3′
Collagen II	5′CTCATCCAGGGCTCCAATGA3′ 5′TCCTTCAGGGCAGTGTATGTGA3′	
Aggrecan	5′CAGGGTTCCCAGTGTTCAGT3′	5′CCAGAAGACTCTCCACTGCC3′
Sox9	5′GAGGCCACGGAACAGACTCA3′ 5′CAGCGCCTTGAAGATAGCATT3′	