Expression of the Heparan Sulfate Proteoglycan, Perlecan, during Mouse Embryogenesis and Perlecan Chondrogenic Activity In Vitro

M.M. French,* S.E. Smith,* K. Akanbi,‡ T. Sanford,§ J. Hecht,§ M.C. Farach-Carson,‡ and D.D. Carson*

*Department of Biochemistry and Molecular Biology, University of Texas, M.D. Anderson Cancer Center, Houston, Texas 77030; ‡Department of Basic Science, University of Texas, Dental Branch, Houston, Texas 77030; and §Department of Pediatrics, University of Texas Medical School, Houston, Texas 77030

Abstract. Expression of the basement membrane heparan sulfate proteoglycan (HSPG), perlecan (Pln), mRNA, and protein has been examined during murine development. Both Pln mRNA and protein are highly expressed in cartilaginous regions of developing mouse embryos, but not in areas of membranous bone formation. Initially detected at low levels in precartilaginous areas of d 12.5 embryos, Pln protein accumulates in these regions through d 15.5 at which time high levels are detected in the cartilage primordia. Laminin and collagen type IV, other basal lamina proteins commonly found colocalized with Pln, are absent from the cartilage primordia. Accumulation of Pln mRNA, detected by in situ hybridization, was increased in d 14.5 embryos. Cartilage primordia expression decreased to levels similar to that of the surrounding tissue at d 15.5. Pln accumulation in developing cartilage is preceded by that of collagen type II. To gain insight into Pln function in chondrogenesis, an assay was developed to assess the potential inductive activity of Pln using multipotential 10T1/2 murine embryonic fibroblast cells. Culture on Pln, but not on a variety of other matrices, stimulated extensive formation of dense nodules reminiscent of embryonic cartilaginous condensations. These nodules stained intensely with Alcian blue and collagen type II antibodies. mRNA encoding chondrocyte markers including collagen type II, aggrecan, and Pln was elevated in 10T1/2 cells cultured on Pln. Human chondrocytes that otherwise rapidly dedifferentiate during in vitro culture also formed nodules and expressed high levels of chondrocytic marker proteins when cultured on Pln. Collectively, these studies demonstrate that Pln is not only a marker of chondrogenesis, but also strongly potentiates chondrogenic differentiation in vitro.

Key words: heparan sulfate proteoglycan • perlecan • chondrogenesis

CARTILAGE serves as a multifunctional tissue in the vertebrate body. It is found in adult structures, providing a flexible support in the nose, trachea, spine, and rib tips. Found also in the developing embryo, cartilage provides a framework for endochondral bone formation. During embryonic development, subsets of mesenchymal cells generate hypertrophic chondrocytes through a stepwise progression of chondrogenesis. This process begins with mesenchymal cell condensation. As condensed cells further differentiate into chondroblasts, collagen expression transitions from type I to type II (Castagnola et al., 1988). Further maturation into hypertrophic chondrocytes is marked by the expression of aggrecan and collagen type X (Kirsch and von der Mark, 1990; Elima et al., 1993). In endochondral bone formation, ossification of cartilage follows. In the search for chondrogenic factors, a variety of growth factors have been examined. Basic FGF (bFGF),[1] TGF-β1, and its family members, BMP-2 and -4, are the most commonly used growth factors for in vitro assays of chondrocyte differentiation. BMP-2 is of particular interest in chondrogenesis due to its ability to induce bone in vitro via an endochondral pathway (Katagiri et al., 1990; Wang et al., 1990; Wozney et al., 1990) and osteoblastic maturation in vitro (Yamaguchi et al., 1991). During development, BMP-2 mRNA is expressed in the dorsal condensing mesenchyme and later in the periosteum and osteogenic zone. TGF-β1 is also de-

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1. *Abbreviations used in this paper:* bFGF, basic FGF; CS, chondroitin sulfate; GAG, glycosaminoglycan; HP, heparin; HS, heparan sulfate; Pln, perlecan.

tected in the periosteum as well as in other bone cells and tissues (Lehnert et al., 1988). In vitro assays demonstrated that TGF-β1 can stimulate the formation of chondrocytes (Rosen et al., 1988; Frenz et al., 1991; Denker et al., 1995; Basic et al., 1996). BMP-4, another TGF-β family member, is a mesodermal inducing factor in *Xenopus*, and is required for the formation of mesoderm in mice (Winnier et al., 1995). As a promoter of mesodermal induction, BMP-4 might stimulate the condensation of mesenchyme, initiating the cascade of chondrogenesis. Along with these TGF-β superfamily members, bFGF is also thought to induce differentiation in mesodermal cells (Smith, 1993).

Cartilage is known to contain a variety of proteoglycans primarily carrying glycosaminoglycan chains other than heparan sulfate (HS). Aggrecan, decorin, biglycan, and fibromodulin are well studied chondroitin sulfate (CS), keratan sulfate, and dermatan sulfate proteoglycans, respectively, found in chondrocyte matrix (Roughley and Lee, 1994). Recently, the HS proteoglycan perlecan (Pln) has been identified as a component of chondrocyte matrix (Iozzo et al., 1994; SundarRaj et al., 1995; Handler et al., 1997). Syndecan-2, a cell surface HS proteoglycan, also has been identified in chondrocytes (David et al., 1993). Both Pln and syndecan-2 carry HS chains in cartilage (David et al., 1993; SundarRaj et al., 1995).

Previous studies with cultures of chick limb bud mesenchymal cells proposed a role for HS in formation and growth of chondrogenic regions. When these cells were cultured in the presence of soluble HS or heparin (HP), the numbers of aggregates and ^{35}S-sulfate incorporation increased. This effect was dependent on HS chain structure, size, and charge. CS, lacking L-iduronic acid, and less sulfated than HS derivatives, had little activity in this assay. The presence of HS or HP was proposed to promote the growth of the aggregates through assisting in formation of tighter aggregates, thereby detaching the cells from matrix molecules that would maintain cells in a less differentiated state (SanAntonio et al., 1987).

In this study, the pattern of Pln protein and mRNA expression during murine development was examined. High levels of Pln protein were detected in developing cartilage and compared with cartilage markers such as collagen type II and Alcian blue staining, an index of proteoglycan accumulation. Because of their multipotential nature, the mouse embryonic fibroblast cell line 10T1/2 has been used extensively in assays to study the developmental progression of chondrogenesis and osteogenesis in vitro (Taylor and Jones, 1979; Konieczny and Emerson, 1984; Ahrens et al., 1993; Gazit et al., 1993; Wang et al., 1993; Atkinson et al., 1997). Assays using this cell line, as well as human chondrocytes, demonstrate that Pln can both stimulate and maintain chondrogenic differentiation in vitro, suggesting a similar potentiating role in vivo.

Materials and Methods

Materials

CF-1 mice were obtained from Sasco. Laminin, rabbit anti–mouse laminin, fibronectin, collagen type IV (Col IV), rabbit anti–mouse collagen type IV, Matrigel, Pln, human recombinant bFGF, TGF-β1, and rabbit antibody to human bFGF, which does not cross-react with mouse bFGF, were all obtained from Becton Dickinson Labware. Rat mAb against

mouse Pln and mouse mAb to a Col II peptide sequence, recognizing both mouse and human epitopes, were purchased from Chemicon International, Inc. Rabbit polyclonal antibody against rat aggrecan, recognizing mouse and human aggrecan, was provided by Dr. Kurt Doege (Shriner's Hospital for Children Portland Unit). Mouse mAb to chicken Col II (antibody II-II6B3 used at 1:100 dilution, which recognizes mouse collagen type II) was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine (Baltimore, MD), and the Department of Biological Sciences, University of Iowa (Iowa City, IA) under contract N01-HD-6-2915 from the NICHD. HP, HP-BSA, bovine kidney HS (BK-HS), bovine intestinal mucosa HS (BIM-HS), CS, hyaluronidase, heparin sulfate proteoglycan, ascorbic acid, sodium pyruvate, and sodium citrate were obtained from Sigma Chemical Co. Species-specific, fluorescein-conjugated secondary antibodies were purchased from Amersham Corp. The mouse Pln cDNA (clone 5) was the generous gift of Dr. John Hassell (Shriners' Hospital, Tampa Bay, FL). BMP-2 and -4 and Balb-c 3T3 cells were the generous gifts of Drs. Randy Johnson and Benoit de Crombrugghe (University of Texas, M.D. Anderson Cancer Center, Houston, TX), respectively.

Immunofluorescent Detection of Extracellular Matrix Components

Under the I.A.U.C.C. approved guidelines for animal use, CF-1 female mice were subjected to superovulation by intraperitoneal injection of 5 IU pregnant mare serum gonadotropin followed by 5 IU human chorionic gonadotropin (hCG) 48 h later. After hCG injection, females were caged with stud males overnight. Females were inspected the next morning for vaginal plugs, indicating d 0.5 of pregnancy at noon. Uteri and embryos were collected on various days of pregnancy. The tissue was snap frozen in isopentane chilled by dry ice and stored at −70°C. 8-μm sections cut on a Reichert-Jung cryostat were allowed to air dry briefly and stored at −70°C until they were processed. Immunostaining was carried out as described previously (Carson et al., 1993). Sections were not decalcified before staining. In brief, sections or wells were fixed in 100% methanol for 10 min at room temperature, washed with Dulbecco's PBS without magnesium or calcium (D-PBS) twice for 5 min each, incubated with the primary antibody for 1 h at 37°C, washed with D-PBS three times for 5 min each, incubated with the secondary antibody for 45 min at 37°C, washed with D-PBS three times for 10 min, and mounted. Samples were stored in the dark at −20°C until they were photographed on a Leitz microscope equipped for epifluorescence. Some sections were pretreated with hyaluronidase to ensure no epitopes were masked. Frozen sections were washed in PBS three times for 4 min each before treatment with hyaluronidase (4 mg/ml in PBS, pH 5) at 37°C for 30 min. After treatment, sections were washed for 4 min in PBS and the standard staining procedure described above was followed, starting with fixation in methanol. Detection of HS chains in the tissue was performed as described previously (Carson et al., 1993). In brief, methanol fixed sections were rehydrated in 0.15 M NaCl, 20 mM EDTA, and 10 mM Tris, pH 8 (TEN), and incubated with human recombinant bFGF (0.05 μg/ml in TEN) for 2 h at 37°C in a humid chamber. Sections were then washed with TEN three times for 5 min each at room temperature, incubated with rabbit anti–human bFGF for 1 h at 37°C, washed again as before, incubated with FITC-conjugated donkey anti–rabbit antibody for 40 min at 37°C, washed three times for 10 min each, and mounted in glycerol/PBS (9:1, vol/vol) buffered to pH 8 with 0.5 M sodium carbonate buffer, pH 9, and containing 0.1% (wt/vol) *p*-phenylenediamine.

Detection of Pln mRNA by In Situ Hybridization

Embryos were isolated on either d 14.5 or d 15.5 of pregnancy and frozen as described above. In situ hybridization was performed as described previously (Smith et al., 1997). The Pln probe was prepared as described previously (Smith et al., 1997). In brief, linearized Pln cDNA clone 5 was used in an in vitro transcription kit from Ambion Inc. Probes were purified using phenol/chloroform extraction and ethanol precipitation. Hydrolysis to reduce probe size was performed and afterwards probes were resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10 mM dithiothreitol and stored at −70°C until use.

10-μm frozen sections were prepared as described above and used in in situ hybridization as described (De et al., 1989; McMaster et al., 1992). Sections were not decalcified before use. In brief, sections were fixed in 4% (wt/vol) paraformaldehyde for 15 min after thawing. Each sample was
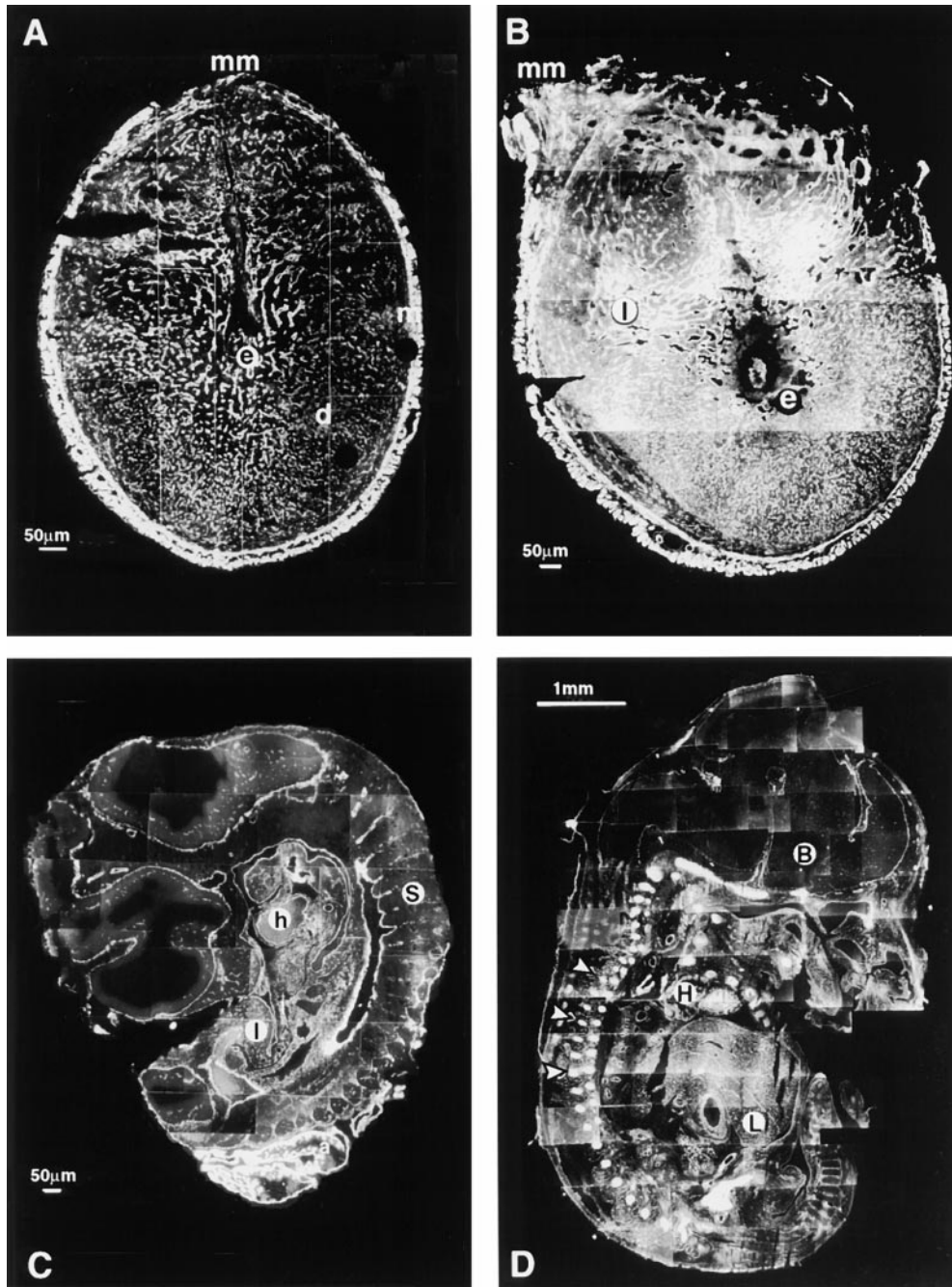
Figure 1. Detection of Pln in uteri and embryos by indirect immunofluorescence. Frozen sections of implantation sites of d 7.5 (A) and d 8.5 (B) mouse uteri, and d 11.5 (C) and d 15.5 (D) mouse embryos were probed with a mAb to Pln core protein. Pln is detected in the muscular and vascular elements of the uterus and in many extracellular matrices of the embryos. Arrowheads in the d 15.5 embryo indicate the cartilage primordia, which display high levels of Pln core protein. (A and B) d, decidua; m, myometrium; mm, mesometrial aspect of uterus; l, lacunae; e, embryo. (C and D) a, amnion; h, H, heart; s, somites; B, brain; l, L, liver. Magnification is indicated on the figure.

incubated with 2×10^7 cpm/ml ^{35}S-labeled riboprobe after prehybridization and covered with a siliconized coverslip. After hybridization at 45°C for 4 h, sections were digested with RNase A (20 µg/ml) at 37°C for 15 min. After 2 wk of autoradiography with Kodak NTB-2 emulsion, hybridized probe was visualized after development. Sections were counterstained with hematoxylin and eosin.

Culture of 10T1/2 Cells or Human Chondrocytes on Various Matrix Components

Tissue culture dishes of either 4 wells (Nunc) or 24 wells (Corning) were coated with 5 µg each of the following matrix components: Pln (9 nmol/well), HP (330 nmol/well), HP-BSA (HP chains covalently linked to BSA, 41 nmol/well), BSA (75 nmol/well), bovine intestinal mucosa HS (660 nmol/well), collagen type IV (16 nmol/well), laminin (5 nmol/well), fibronectin (11 nmol/well), or Matrigel. For coating wells, 5 µg of the matrix component was added to the well followed by D-PBS to attain a total volume of 200 µl. The area of wells in either the 4- or 24-well dishes is 1.76 cm² (4-well plates from Nunc, Nunclon, Cat. No. 176740; 24-well plates from Costar Corp., Cat. No. 3524). Matrigel was applied undiluted to the well as a thin layer and excess was removed immediately. The plate was allowed to dry overnight at 37°C. In all cases, on the following day wells were washed twice with D-PBS before adding cells. Coating efficiency was determined by including ^{125}I-iodine labeled Pln at the time of coating and counting material rinsed from the wells. Bound Pln gave a coating density of 1.5 µg Pln/cm².

Murine 10T1/2 cells obtained from ATCC were maintained as subconfluent cultures in tissue culture flasks with Dulbecco's modified Eagle's media/Ham's F12 (1:1) media containing 100 mg/ml streptomycin sulfate, 100 U/ml penicillin, 1.2 g/liter NaHCO₃, and 15 mM Hepes (pH 7.2) supplemented with 10% (vol/vol) heat-inactivated FBS. For matrix assays, cells were detached with trypsin/EDTA (Irvine Scientific) and plated on matrix-coated wells in CMRL-1066 media supplemented with antibiotics and either high (15% [vol/vol]) or low (2.5% [vol/vol]) FBS for prelimi-
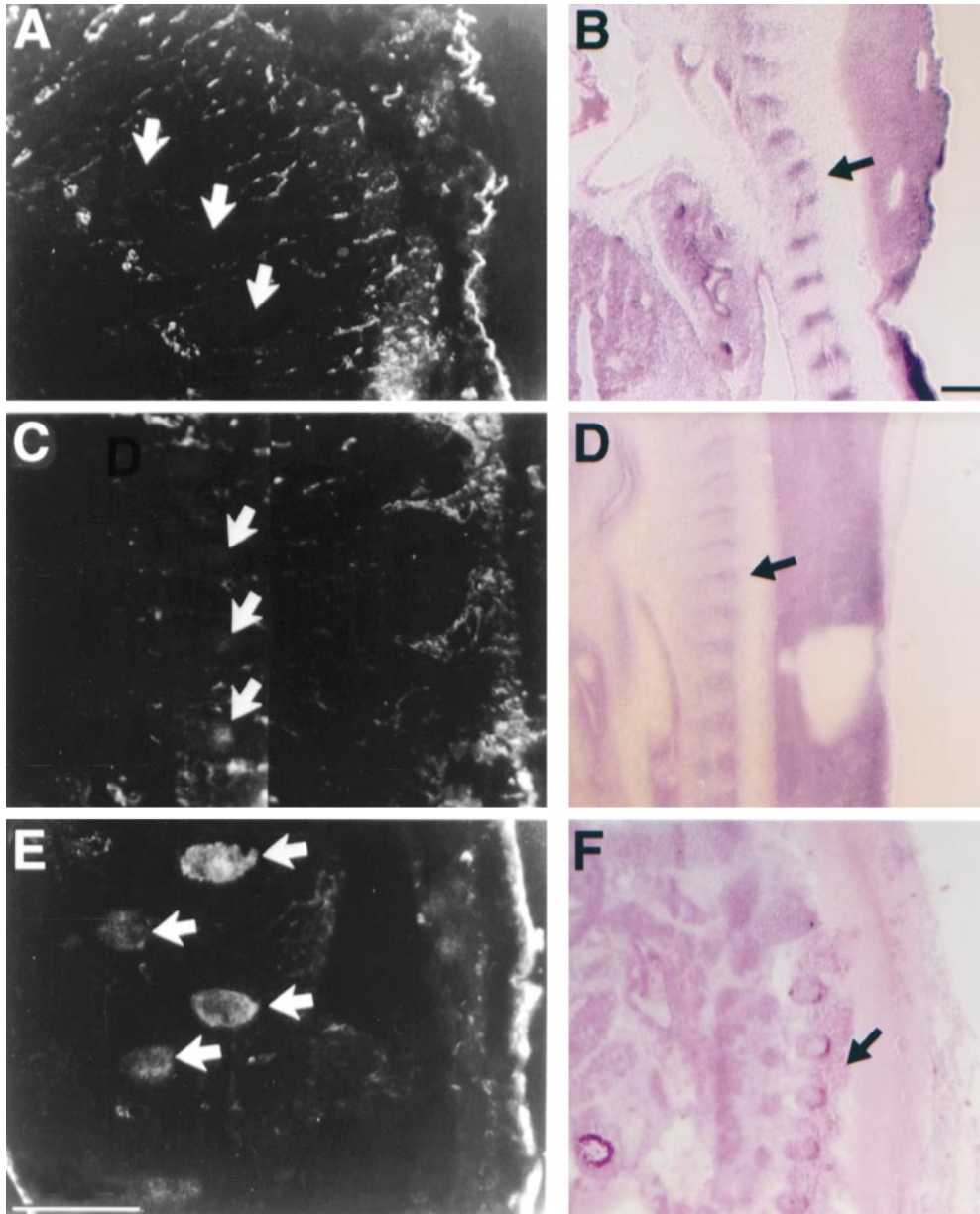
Figure 2. Pln accumulation in cartilage primordia. Longitudinal, frozen sections from embryos at d 12.5 (A and B), d 13.5 (C and D), and d 14.5 (E and F) were probed with a mAb to Pln (A, C, and E) or similar regions were stained with hematoxylin and eosin (B, D, and F). Regions of developing vertebrae are shown. Pln protein levels were low in the cartilage primordia (arrows) at d 12.5, but accumulated as development progressed through d 13.5 and d 14.5. In contrast, consistent, discrete expression of Pln is noted in surrounding tissues. Bars, 200 μm.

nary experiments. In later experiments, the media was supplemented with 15% (vol/vol) FBS, pyruvate (50 μg/mg), citrate (50 μg/ml), and ascorbic acid (50 μg/ml). Plating density was 2×10^5 cells per well (114,600 cells per cm^2). Media was added to bring the total volume in a well to 500 μl. Media was changed every other day during assays. For growth factor assays, growth factors were added at 1 ng/ml (bFGF, BMP-2 at 0.055 nM, TGF-β1 at 0.2 nM, and BMP-4 at 0.063 nM) to CMRL-1066 supplemented with 15% (vol/vol) FBS, citrate, ascorbate, and pyruvate. Medium was changed every other day and a fresh aliquot of growth factor added.

Human costochondral cartilage samples were acquired from pectus excavatum surgeries after appropriate consent was obtained and with institutional IRB approval. Cartilage samples were minced and digested in collagenase, 2 mg/ml, overnight at 37°C. The chondrocytes were plated and grown in DMEM with 15% FBS. All chondrocytes were expanded in monolayer cultures, and used at passage 3.

Formation of aggregates was assessed by visual examination using a microscope. Cells that had drawn together into dense, multi-layered regions reminiscent of condensing mesenchyme of developing cartilage, leaving areas of the well bare, were scored as aggregate positive. Also, cells in these regions were highly rounded compared with the fibroblastic morphology typical of 10T1/2 cells. To determine whether expression of chondrocyte markers was uniform throughout the aggregate mass, aggregates formed on Pln were fixed in paraformaldehyde, embedded in cryoprotectant, and sectioned. Alcian blue staining was performed before embedding. Sections were processed for immunostaining as described for tissue sections. Cells grown on plastic were similarly treated and scraped off the plate before embedding.

Enzymatic Digestion of Glycosaminoglycan (GAG) Chains

Wells were coated with Pln, as described above. Before plating of 10T1/2 cells, wells were subjected to digestion with chondroitinase ABC (Sigma Chemical Co.) or a mix of heparinases I, II, and III (Sigma Chemical Co.). To each well, 0.25 ml of enzyme mix containing 1 U/ml enzymes, 0.5 mM Mg^{2+}/Ca^{2+}, a protease inhibitor mixture (PIMS I described in Pimental et al., 1996) in D-PBS was added. Plates were incubated at 37°C for 4 h. After digestion, wells were rinsed with D-PBS before plating cells. As a control, 0.5 ml of CS or HP at 1 mg/ml was treated with enzyme or with buffer lacking enzyme for the same time. After digestion of control solutions, 1/10 volume 10% (wt/vol) cetylpyridinium chloride was added to precipitate and visualize GAGs to confirm enzyme activity. A successful digest resulted in at least 50% reduction of precipitated GAGs in the digested control compared with undigested. To determine if digestion re-
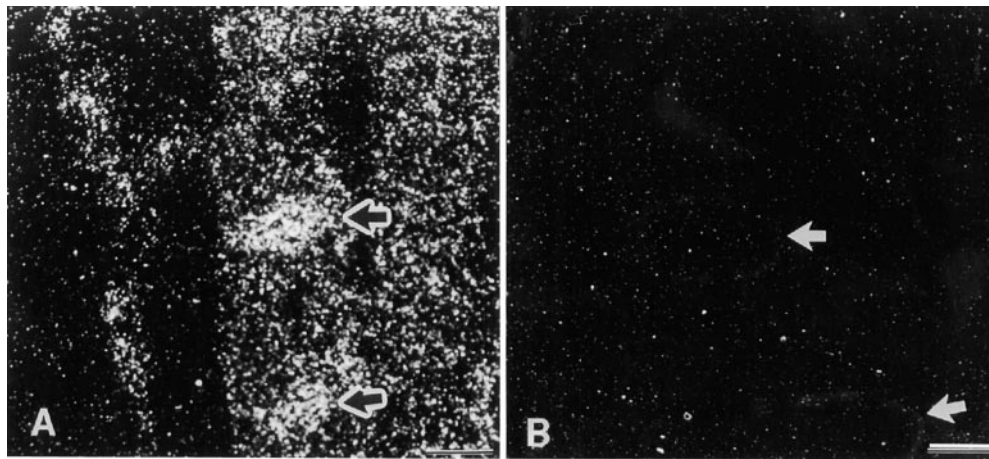
Figure 3. Pln mRNA expression in d 14.5 embryos. In situ hybridization was performed with Pln antisense (A) and sense (B) riboprobes. A region of the embryo including the cartilage primordia (arrows) is shown. The expression of Pln mRNA is higher in the discs than in the surrounding tissue (A). This expression is specific as determined by absence of signal with the sense control (B). Refer to Fig. 2 E for histological reference. Bar, 100 μm.

moved Pln from wells, digested and undigested Pln wells were subjected to an ELISA binding assay. No loss of Pln protein core through digestion was detected by this assay.

Identification of Chondrocytic Phenotype by Alcian Blue Staining

10T1/2 cells were cultured for 10 d on various matrices, washed twice with D-PBS, and fixed for 15 min in 10% (wt/vol) paraformaldehyde in PBS. After fixation, cells were washed twice with ultrapure water before Alcian blue (1% [wt/vol]) in 0.1 N HCl was added to the cells for 30 min. Cells were washed twice with 0.1 N HCl and allowed to dry. Photography was performed on a Nikon inverted microscope using bright-field conditions.

RNA Extraction

After culture for various times on different matrices, RNA was isolated from ~4 × 10^5 10T1/2 cells per treatment using the method of Chomczynski and Sacchi (1987). After determining the concentration of RNA for each sample, reverse transcription and polymerase chain reactions (RT-PCR) were performed.

Analysis of Gene Expression by RT-PCR

PCR primers for various chondrocyte markers were acquired from GIBCO-BRL. Collagen type I α2: forward 5′ GAACGGTCCACGAT-TGCATG 3′, reverse 5′ GGCATGTTGCTAGGCACGAAG 3′; collagen type II α1: forward 5′ CACACTGGTAAGTGGGGCAAGACCG 3′, reverse 5′ GGATTGTGTTGTTTCAGGGTTCGGG 3′ (Shukunami et al., 1996); aggrecan: forward 5′ CTACGACGCCATCTGCTACA 3′, reverse 5′ ACGAGGTCCTCACTGGTGAA 3′ (Doege et al., 1987); Pln: forward 5′ CCTACGATGGCCTTTCCCT 3′, reverse 5′ TTGGCACT-TGCATCCTCCA 3′ (Smith et al., 1997). First-strand cDNA was synthesized with total RNA extracted from cultured cells by random hexamer priming using an RNA PCR kit (Perkin Elmer). In brief, purified total RNA (840 ng) was incubated at 42°C for 60 min with a mixture of 1 U of RNase inhibitor, 2.5 U of MuLV reverse transcriptase, 2.5 μM of random hexamers, 5 mM MgCl$_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1 mM each of dGTP, dATP, dTTP, and dCTP in a volume of 20 μl.

Aliquots of 1/10 (2 μl) of the cDNA were used to amplify coding sequences from type I and type II collagen, Pln, and aggrecan. The PCR conditions for collagen types I and II were 94°C for 30 s, 60°C for 1 min, 72° for 1 min for 25 cycles, and final extension at 72°C for 5 min. The PCR conditions for Pln and aggrecan were 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min for 25 cycles, and 94°C for 30 s, 68°C for 30 s, 72°C for 1.5 min for 25 cycles, respectively. The products were separated by electrophoresis through 4% Nusieve 3:1 agarose gels (FMC Bio Products) along with molecular weight markers and detected by ethidium bromide. PCR products were subsequently sequenced and determined to be specific for either Col I or Col II.

Results

Pln Expression in Development

The expression pattern of Pln protein at various stages of postimplantation development was analyzed by immunohistochemistry (Fig. 1). During early postimplantation stages, Pln was consistently detected in muscular elements such as the uterine myometrium and the developing heart in the embryo. In the d 7.5 uterus (Fig. 1 A) Pln was expressed in basal lamina of vascular elements, basement membrane of the epithelial cells of the residual lumen, in the amnion surrounding the embryo, and the embryo itself. At d 8.5, Pln protein was detected surrounding vascular elements in the decidua of the uterus and had accumulated to high levels in the developing lacunae (Fig. 1 B) as well as in the extracellular matrix of the decidua itself. At d 8.5, staining of the embryo persisted while extraembryonic tissue was negative. At later stages of development Pln protein is detected in basal lamina throughout the embryo (Fig. 1, C and D). Staining within the liver is seen, again defining vascular elements. The amnion (bottommost region, Fig. 1 C), consisting of two distinct layers separated by a basal lamina, stains intensely with anti-Pln. Muscular tissue such as the tongue and heart are positive, as are regions of the brain such as the choroid plexus. Notably, by d 15.5 of development, Pln staining was particularly intense in developing cartilage including the ribs and the cartilage primordia (Fig. 1 D). Membranous bone regions were negative for Pln staining.

To further examine the temporal expression of Pln in developing cartilage primordia, longitudinal sections containing dorsal regions from d 12.5, d 13.5, and d 14.5 were stained with Pln antibody (Fig. 2). While at d 12.5 (Fig. 2 A) Pln is essentially undetectable in the condensing intervertebral disc, by d 13.5 (Fig. 2 B) an initial accumulation of Pln was detectable. By d 14.5 (Fig. 2 C), Pln signal was clear and discretely localized within the discs, as well as in basal lamina of surrounding tissues. In situ hybridization confirmed that Pln mRNA accumulation also occurred in developing cartilage primordia (Fig. 3). At d 14.5, the level of Pln mRNA in cartilage primordia was higher than the surrounding tissue (Fig. 3 A). Sense cRNA probes
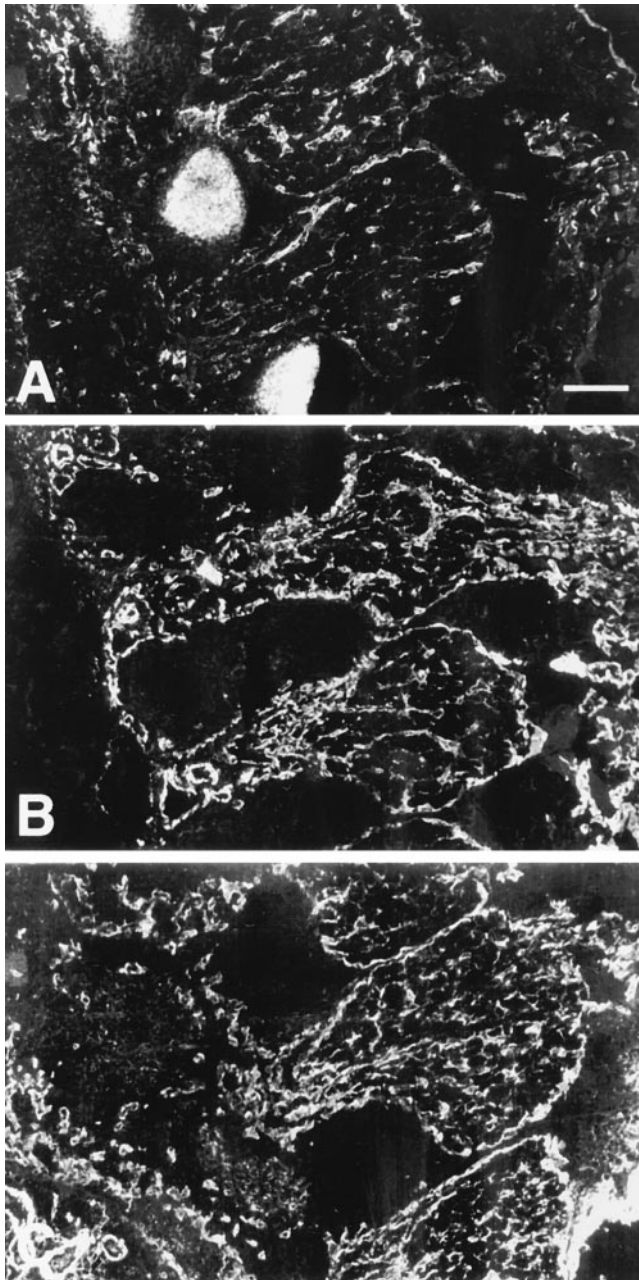
Figure 4. Laminin and collagen type IV are not expressed in cartilage primordia. Frozen sections of d 15.5 embryos were probed with antibodies to the basal lamina components Pln (A), collagen type IV (B), or laminin (C) by indirect immunofluorescence. While discrete sites of surrounding tissue react consistently with all three antibodies, only Pln is expressed in cartilaginous regions. Bar, 100 μm.

demonstrated negligible background hybridization signal (Fig. 3 B).

Other Basal Lamina Components Are Not Expressed by Cartilage Primordia

After examining Pln expression in cartilage primordia, immunohistochemistry was used to determine if other basal lamina proteins, namely collagen type IV and laminin, also

accumulated in these regions. As shown in Fig. 4, in the d 15.5 embryo, Pln protein levels were high (Fig. 4 A), while neither collagen type IV (Fig. 4 B) nor laminin (Fig. 4 C) accumulated in the discs, although these proteins were readily detectable in the surrounding tissue. Predigestion of sections with hyaluronidase had no effect on Pln staining and did not reveal collagen type IV or laminin staining (data not shown). Thus, these observations indicated that general accumulation of basal lamina components did not occur in developing cartilage. Immunostaining with antibodies to collagen type II demonstrated that accumulation of this early cartilage marker protein preceded that of Pln. As shown in Fig. 5, at d 11.5 of development cartilaginous condensations are moderately positive for collagen type II (Fig. 5 A), but negative for Pln (Fig. 5 B). By d 12.5, a similar region is strongly positive for collagen type II (Fig. 5 C) accompanied by low levels of Pln (Fig. 5 D). These observations suggested that while Pln expression is a component of the program of ongoing chondrogenesis, it is not an initial marker of this process.

At d 15.5 Pln accumulation persisted in the cartilage primordia regions (Fig. 6 A). As expected, these same structures strongly stained with Alcian blue (Fig. 6 B), consistent with the cartilaginous nature of this tissue. These structures also stained positively for HS using a bFGF-dependent HS detection system (Fig. 6 C). Staining by bFGF was completely blocked by inclusion of soluble HP (Fig. 6 D), thus indicating that HS chains rather than FGF protein receptors were the major ligands detected in the tissue. In contrast to the pattern observed at d 14.5, in situ hybridization performed at d 15.5 did not show a preferential accumulation of Pln mRNA in cartilage primordia relative to the surrounding tissue (Fig. 6 E, arrowheads). This finding indicated that persistent, high-level Pln mRNA expression was not required for persistent, high-level Pln protein retention in developing cartilage.

In Vitro Assays of Chondrogenic Differentiation

Given the dramatic accumulation of Pln in developing cartilage, it was considered that Pln might potentiate the chondrogenic process. Initially, 10T1/2 cells were plated on a variety of matrices and visually inspected for morphological changes typical of chondrogenesis, i.e., aggregate formation. When plated on tissue culture plastic (Fig. 7 A), 10T1/2 cells maintained their fibroblastic phenotype. If plated on Pln, cells became rounded and aggregated into nodules that stained positively with Alcian blue (Fig. 7, B and D). A scoring index was developed to describe different behaviors observed during in vitro culture and is described in Table I. Of a variety of extracellular matrix components studied, only Pln and, to a lesser extent, the Pln-containing complexes in Matrigel were able to induce nodules (Table I). While heparinase digestion severely reduced Pln's activity in this regard, HP, HS, or HP-BSA displayed very little activity, indicating that both the protein and HS chains of Pln are required. 10T1/2 cells plated on a variety of other matrices or tissue culture plastic maintained a fibroblastic phenotype. Some Pln preparations from sources other than Becton Dickinson did not display this activity, presumably due to differences in HS chain composition (see below). Various potential chon-
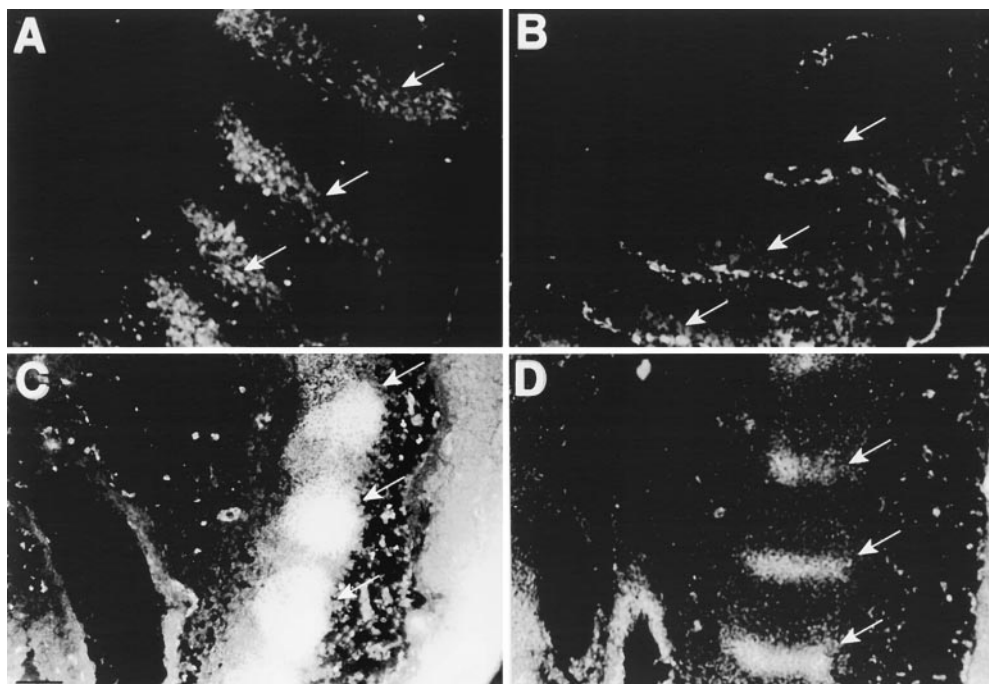
Figure 5. Collagen II expression precedes that of Pln in developing embryos. Sections from d 11.5 (A and B) and d 12.5 (C and D) were probed with antibodies to collagen type II (II6B3) (A and C) and Pln (B and D). At d 11.5, collagen type II is detectable in the spinal region while Pln is undetectable. By d 12.5, collagen type II is intense and Pln is beginning to accumulate. Bar, 100 μm.

drogenic growth factors, including BMP-2, BMP-4, bFGF, and TGF-β1, were added to the culture medium of cells plated on inactive Pln preparations and failed to induce aggregate formation (Table II). Moreover, extraction of Pln-coated surfaces with high salt, a procedure expected to remove HS-bound growth factors, had no effect on aggregate formation (Table I). Thus, contamination of "active" Pln preparations with these growth factors did not account for its chondrogenic activity. Interestingly, Balb-c 3T3 cells, plated on active Pln, form aggregates, but many of these aggregates remained Alcian blue negative. Thus, while other embryonic fibroblastic cell lines respond to Pln, there appear to be differences in the degree of this response (see below). 10T1/2 cell aggregates also accumulated large amounts of collagen type II (Fig. 7 F) as well as aggrecan (data not shown). In contrast, 10T1/2 cells cultured on tissue culture plastic failed to accumulate collagen type II (Fig. 7 E). Aggregates sectioned and probed for chondrocyte markers were positive for matrix molecules such as collagen type II and aggrecan (Fig. 8, C and D). Alcian blue staining was also present in the aggregate (Fig. 8 A). Hematoxylin and eosin staining show distinctive nonfibroblastic morphology when cultured on Pln (Fig. 8 B) compared with 10T1/2 cells cultured on plastic (Fig. 8 F). Cells cultured on plastic again express little collagen type II (Fig. 8 E). RT-PCR analyses of RNA isolated from 10T1/2 cells cultured on Pln also demonstrated increased expression of collagen type II mRNA (Fig. 9).

Plating of 10T1/2 cells on Pln digested with heparinase, to remove HS chains, resulted in delayed and incomplete aggregation into nodules (Fig. 10 D). In contrast, nodule formation was both rapid and complete on chondroitinase-digested Pln (Fig. 10 C) and was indistinguishable from undigested Pln (Fig. 10 B). ELISA demonstrated that neither heparinase nor chondroitinase digestion reduced the amount of Pln protein core bound to the tissue culture surfaces (data not shown). The kinetics and extent of differentiation on Pln is described in Fig. 11. 10T1/2 cells efficiently aggregated within 1 d of culture on Pln and became uniformly Alcian blue positive within 4 d. Chondroitinase digestion had no effect on these parameters while heparitinase digestion severely and persistently retarded all aspects of this process. Collectively, these in vitro studies indicated that while the protein core of Pln participates in promotion of chondrogenesis, the HS chains also are required for maximal activity. Furthermore, different embryonic fibroblast cell lines display different degrees of Pln responsiveness.

To examine the behavior of stable chondrocytes on Pln, human chondrocytes were plated on tissue culture plastic or Pln and examined for the expression of aggrecan or collagen type II. On plastic, human chondrocytes appeared fibroblastic (Fig. 12, B and G) and expressed lower levels of aggrecan (Fig. 12 A). In contrast, chondrocytes on Pln formed aggregates (Fig. 12, D and H) maintaining their rounded morphology, and accumulated high levels of aggrecan (Fig. 12 C). Similar to murine 10T1/2 cells, human chondrocytes cultured on Pln also accumulated high levels of collagen type II (Fig. 12 F). When human fibroblasts are cultured on Pln, they fail to attach (Fig. 12 E).

Discussion

As a component of extracellular matrix and basal lamina, Pln is found in various embryonic and adult tissues. The major cartilage proteoglycans are of the chondroitin/dermatan sulfate variety; however, expression of Pln has been reported recently (Iozzo et al., 1994; SundarRaj et al., 1995; Handler et al., 1997). While the Pln core protein has the ability to carry CS chains, it has been demonstrated that Pln in cartilage also carries HS chains (SundarRaj et al., 1995). Consistent with these findings, we detected both Pln protein and mRNA as well as HS chains in developing mouse cartilage. One description of Pln expression
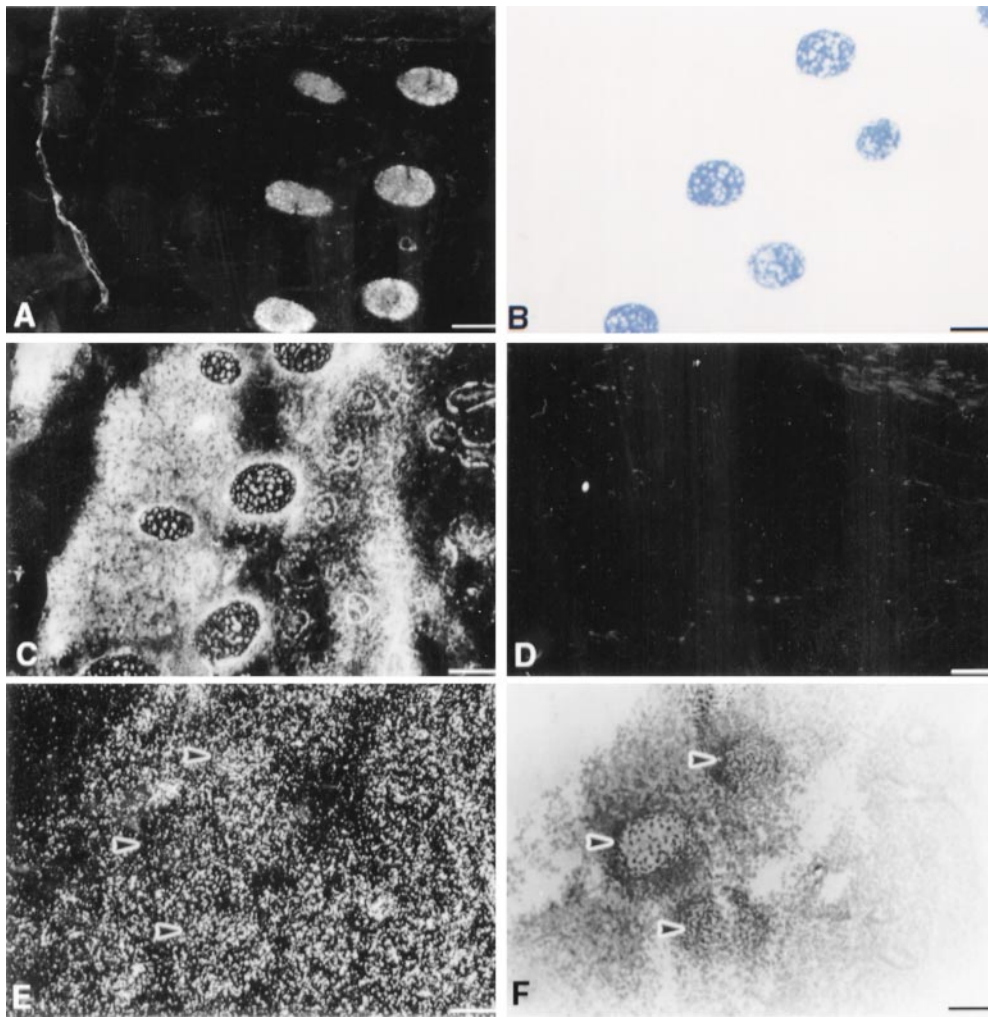
Figure 6. Analysis of cartilage primordia of d 15.5 embryo. (A) Pln expression, determined by immunostaining, is high in the cartilage primordia. To define characteristics of these discs, embryos were stained with Alcian blue (B). The positive Alcian blue staining is indicative of proteoglycan accumulation characteristic of cartilaginous tissue. The surrounding tissue is negative for Alcian blue staining. (C) Deposition of HS in cartilage primordia was determined by staining with bFGF as described in Materials and Methods. HS is seen in the discs and the chondrocytes as well as in the surrounding tissue. The specificity of staining for HS is demonstrated by competition with HP included during the staining procedure (D). By this stage of development, no preferential accumulation of Pln mRNA in developing cartilage was apparent by in situ hybridization with Pln antisense probe (E). Sense probe signal was similar to that seen in Fig. 5 (data not shown). Bright-field photography (F) of the same site confirms the location of the discs (arrowheads). Bars, 100 μm.

during embryonic development has been reported (Handler et al., 1997), with similar results to those presented here. An examination of Pln expression throughout murine development demonstrated a relationship with chondrogenesis. During this process, Pln protein was found to accumulate in the cartilage primordia progenitor cells. This does not reflect a general expression of basal lamina components by the cells, since neither laminin nor collagen type IV was detected in these tissues. (In contrast, laminin has been found in human nasal cartilage [Sunder-Raj et al., 1995].) A hallmark of chondrocyte differentiation, collagen II expression, was apparent by d 12.5, a time when Pln was only faintly detected. Thus, these cells appear to be committed to initial aspects of the chondrocytic pathway before displaying high level Pln expression. It is possible that low levels of Pln accumulate before collagen type II and trigger further chondrocytic differentiation in vivo. In this regard, 10T1/2 cells and human chondrocytes also respond to culture on Pln by expressing high levels of Pln themselves (data not shown). Alternatively, Pln expression may be independent of collagen type II or Pln may promote more complete differentiation of cells already poised to undergo chondrogenesis. 10T1/2 cells re-

spond more rapidly and completely than Balb-c 3T3 cells. Similarly, human chondrocytes, but not human fibroblasts, can respond to Pln in vitro. It is not clear what factors, e.g., cell surface receptors, transcription factors, etc., predispose cells to Pln responsiveness.

Initial assays determined that 10T1/2 plated on Pln and, to a lesser extent, the Pln-containing matrix, Matrigel, formed aggregates and stained positively with Alcian blue. The ability of 10T1/2 cells to undergo chondrogenic differentiation in vitro may be enhanced by the chondrogenic transcription factor, SOX9 (Bell et al., 1997). In developing cartilage, SOX9 expression precedes that of Pln (Bell et al., 1997). Interestingly, 10T1/2 cells express much higher levels of SOX9 than Balb-c 3T3 cells (Lefebvre et al., 1997). This may account, in part, for the ability of 10T1/2, but not 3T3 cells to differentiate more completely in response to Pln; however, 3T3 cells stably transfected with SOX9 form aggregates, but fail to stain with Alcian blue as completely as 10T1/2 cells (data not shown). Collectively, these data suggest that while SOX9 may be necessary, it is not sufficient to promote chondrogenesis either alone or in combination with Pln.

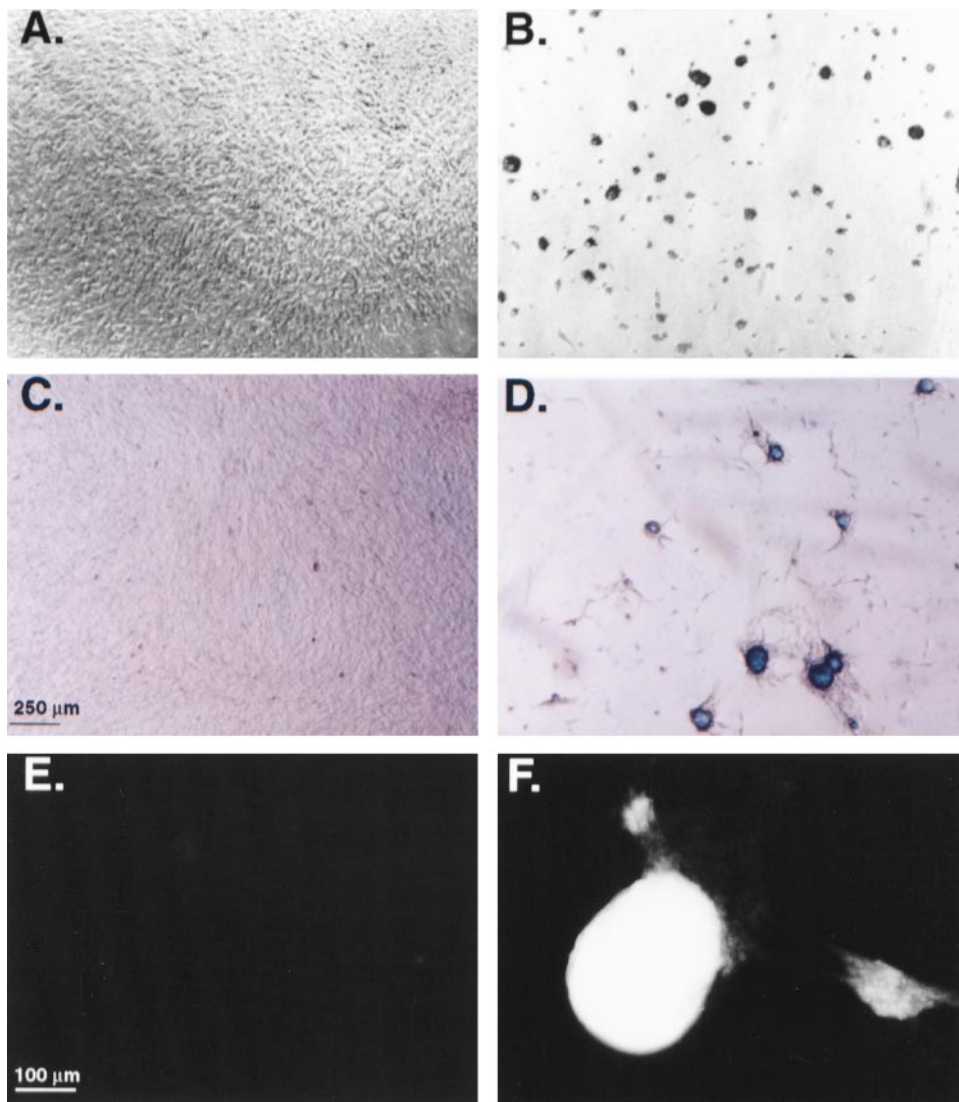As described previously, other extracellular matrix mol-

Figure 7. Detection of chondrocyte markers in 10T1/2 cells. After culture on tissue culture plastic (A, C, and E) or Pln (B, D, and F) for 9 d, 10T1/2 cells maintained a fibroblastic appearance on tissue culture plastic (A) but formed aggregates (B) on Pln reminiscent of mesenchymal condensation of developing cartilage. Only cells cultured on Pln stained positively with Alcian blue (compare C and D) and expressed collagen type II protein detected with II6B3 antibody (compare E and F). Magnification for A–D is indicated in C, and magnification for E and F is indicated in E.

ecules and glycosaminoglycans fail to induce differentiation. Heparinase-treated Pln displayed inductive ability, albeit at a greatly reduced rate compared with intact Pln. These experiments indicate that cooperation between the Pln core protein and its constituent HS chains occurs in the potentiation of chondrogenesis. To exclude action of potential contaminating growth factors, certain inactive Pln preps, obtained from either commercial sources or prepared in our laboratory, were supplemented with growth factors previously proposed as chondrogenic agents, i.e., bFGF, BMP-2, BMP-4, and TGF-β1. These potential contaminants failed to stimulate nodule formation on inactive Pln preparations. It was surprising that neither BMPs nor TGF-β1 stimulated chondrogenesis on surfaces coated with inactive Pln since these growth factors do so when 10T1/2 cells are cultured under other conditions (Katagiri et al., 1990; Denker et al., 1995; Atkinson et al., 1997). The assay developed here relies on the response of the cells to a matrix molecule presented in a solid phase, presumably similar to the state found in vivo. The features differentiating inactive Pln from the active preparations have not yet been clarified. However, it is clear that both the GAG chains and the protein core participate in this activity.

Damage to the protein core or HS chain alteration, e.g., heparanase digestion or undersulfation, might result in a lack of activity in the 10T1/2 assay. We have found that chondrogenic activity maps to particular recombinant Pln domains and requires glycosaminoglycans (French, M., M. Hook, R. Timpl, and D.D. Carson, unpublished observations). It is possible that, in the absence of appropriate glycosaminoglycan chains, portions of cell surface receptors or binding sites become occupied that require glycosaminoglycan interactions to transmit signals fully. FGF receptors require such interactions with HS chains in complexes with FGFs (Yayon et al., 1991; Aviezer et al., 1994; Kan et al., 1996). In the absence of appropriate glycosaminoglycan complexes at these sites, cells may become locked in a state preventing them from responding to other signals. In addition, the observation that the Pln that is active in solid phase assays is inactive in soluble form also indicates that the context of Pln presentation is an important aspect of this response. The receptors involved in Pln recognition by 10T1/2 cells must be identified to understand these responses in molecular detail.

Rinsing of Pln-coated surfaces with high salt concentrations, a treatment that would be expected to release HS-

Table I. Effects of ECM Components on 10T1/2 Aggregate Formation

Plating material	Result	Plating material	Result
ECM components		Glycosaminoglycans	
Laminin	0	HP	0
Collagen type IV	0	Bovine kidney HS	0
Laminin + collagen type IV	0	Bovine intestinal mucosa HS	0
Fibronectin	0	CS	0
Pln	3	HP–BSA	1
Pln + heparinase	1	Other	
Pln + chondroitinase ABC	3		
Pln + NaCl wash	3	BSA	0
Matrigel	1	PBS	0

10T1/2 cells were cultured on surfaces coated with the indicated ECM components. Differentiation was scored on the basis of the formation of cell aggregates and Alcian blue staining as shown below. 0 = no aggregates formed; 1 = inefficient cell recruitment into aggregates; 2 = efficient recruitment of all cells with nonuniform or incomplete aggregate staining with Alcian blue; 3 = efficient recruitment of cells into aggregates, uniform aggregate staining with Alcian blue.
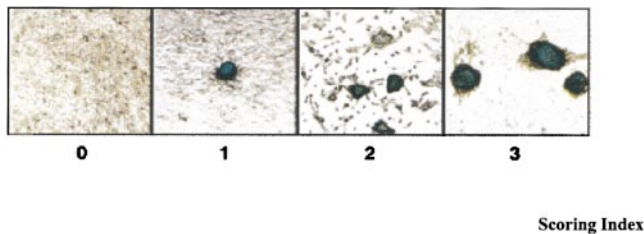

Scoring Index

Table II. Growth Factors Do Not Restore 10T1/2 Aggregate Formation on Inactive Pln Preparations

Growth factor	Plating material	Result
bFGF	PBS	0
1 ng/ml	I-Pln	0
BMP-2	PBS	0
1 ng/ml	I-Pln	0
BMP-4	PBS	0
1 ng/ml	I-Pln	0
BMP-2 + -4	PBS	0
1 ng/ml	I-Pln	0
TGF-β	PBS	0
1 ng/ml	I-Pln	0

Growth factors were added to a Pln preparation lacking aggregate-inducing activity (I-Pln). Growth factors were scored using the scoring index described in Table I.

bound growth factors (McCaffrey et al., 1992) had no effect on this inductive activity. From these assays, inductive activity appears to be dependent in part on both the Pln core protein and the HS chains. This may account for the lack of inductive activity in some Pln preparations. If cells require interaction with both the HS chains and the core protein to elicit a full response, one or the other interaction might act to block stimulation from another source. Thus, contact with the core protein of inactive Pln primes
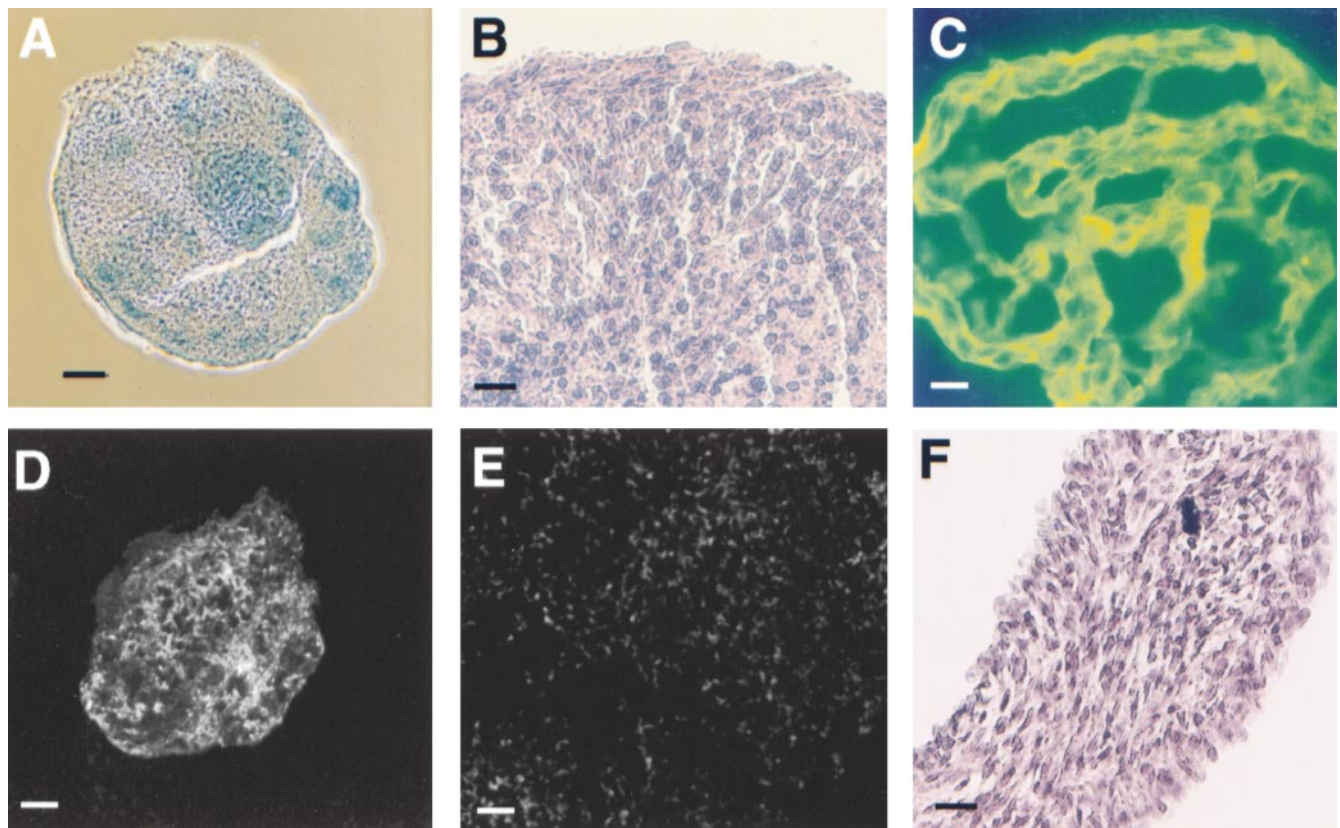

Figure 8. Internal expression of chondrocyte markers by 10T1/2 aggregates. 10T1/2 cells were cultured for 7 d on Pln (A–D) or plastic (E and F). Aggregates from cultures on Pln or scraped cells from cultures on plastic were sectioned and probed for different chondrocyte markers. Alcian blue staining (A) is present in regions throughout the aggregate. Hematoxylin and eosin staining of aggregates (B) demonstrates a nonfibroblastic morphology compared with a culture from tissue culture plastic (F). Collagen type II detected with rabbit anti–mouse antibody from Chemicon (C) and aggrecan (D) are also present within the aggregate. Cells cultured on plastic fail to express similar levels of collagen type II (E). Bars, A, 100 μm; B and F, 250 μm; C, 800 μm; D and E, 200 μm.
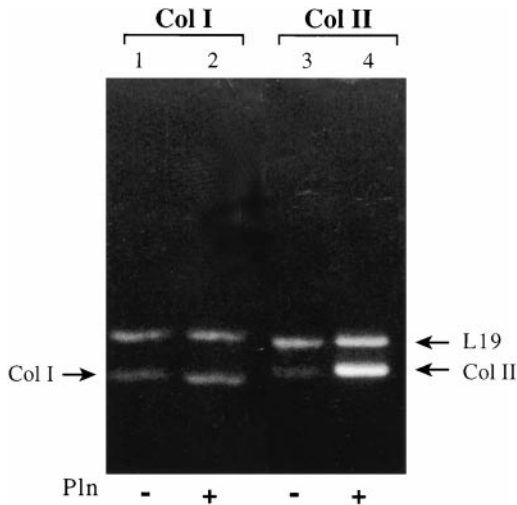
Figure 9. Analysis of chondrocyte marker expression by RT-PCR. RNA from 10T1/2 cells cultured on tissue culture plastic (lanes 1 and 3) or Pln (lanes 2 and 4) for 9 d was reverse transcribed and products for collagen types I (Col I) and II (Col II) amplified. As a positive control for the assay, product for the ribosomal protein L19 was also amplified. Cells cultured on plastic or Pln express similar levels of collagen type I mRNA; however, cells cultured on Pln express much higher levels of mRNA for the chondrocyte marker, collagen type II.

Figure 11. Kinetics of cell aggregation on Pln. Cells cultured on Pln for the indicated number of days were inspected for both recruitment of cells into aggregates and staining with Alcian blue using the scoring index described in Table I. Balb-c 3T3 cells aggregate, but not all aggregates stain with Alcian blue. 10T1/2 cells rapidly form aggregates that all stain with Alcian blue and this process is unaffected by chondroitinase treatment of the Pln. In contrast, heparinase digestion of Pln reduces the rate and the extent of the response by the cells.

the 10T1/2 cells to respond to stimulation from a linked HS chain. In the absence of the chain, the cell may be unable to respond to TGF-β or BMP-4 signals. Variations in isolation protocols or the actions of tissue heparanases during isolation could account for a depletion of specific classes of HS structures in these preparations. A preference for more highly negative molecules containing L-iduronic acid by differentiating chondrocytes in micromass culture has been demonstrated by SanAntonio et al.
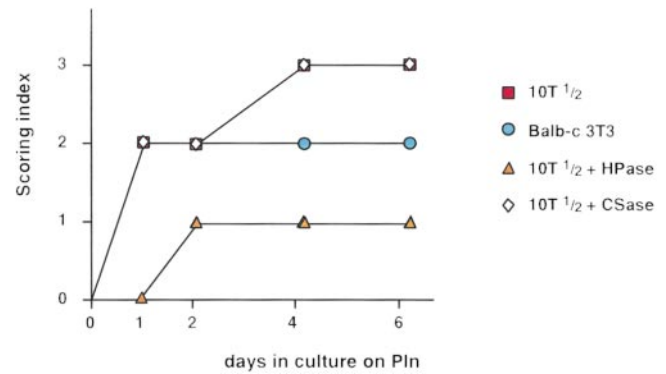
(1987). If larger and more negatively charged HS chains were selected against in the isolation process, or were degraded by tissue heparanases during isolation, the stimulatory effect of Pln might be lost or reduced. Another possibility is the large Pln core protein may become partially denatured or otherwise modified at subtle, yet critical, sites during isolation.

A simple model for the action of Pln on chondrogenesis focuses on attachment of the cells to a matrix. If cell–matrix interactions are impaired, then the cells would remain more rounded and attach to each other rather than the matrix, forming aggregates. When cultures from chick limb buds were supplemented with soluble HS or HP, increased aggregate formation and sulfate incorporation,
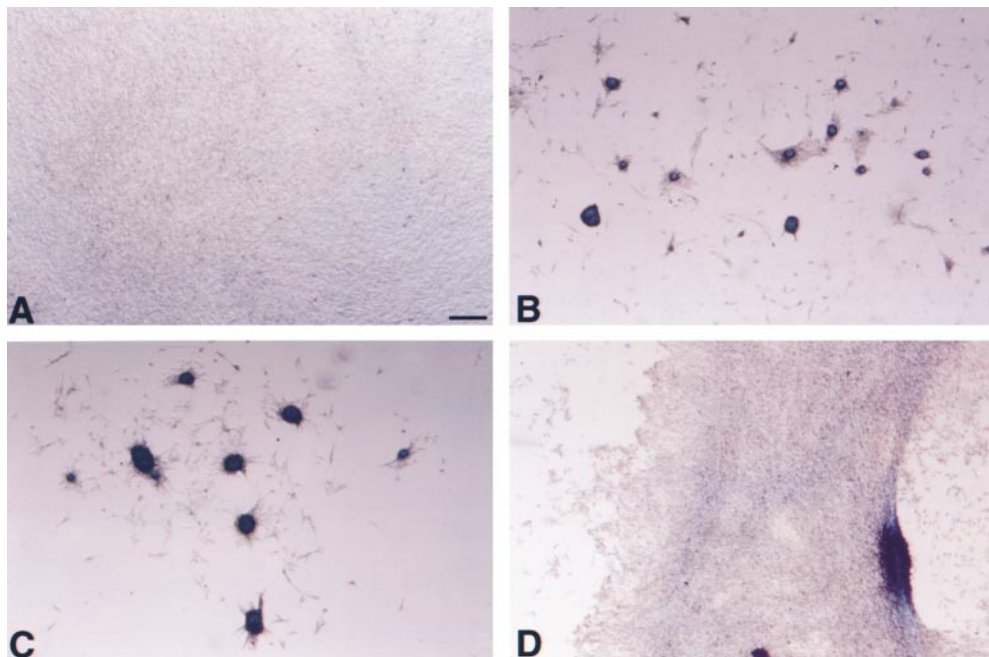

Figure 10. Induction of chondrogenic phenotype by enzymatically modified Pln. 10T1/2 cells were cultured on tissue culture plastic (A), Pln (B), Pln treated with chondroitinase (C), or Pln treated with heparinases (D) for 7 d. Both the cells on Pln and on chondroitinase-treated Pln rapidly formed aggregates that stain positively with Alcian blue. In contrast, 10T1/2 cells on heparinase-treated Pln form aggregates poorly, and fail to stain strongly with Alcian blue. Bar, 500 μm.
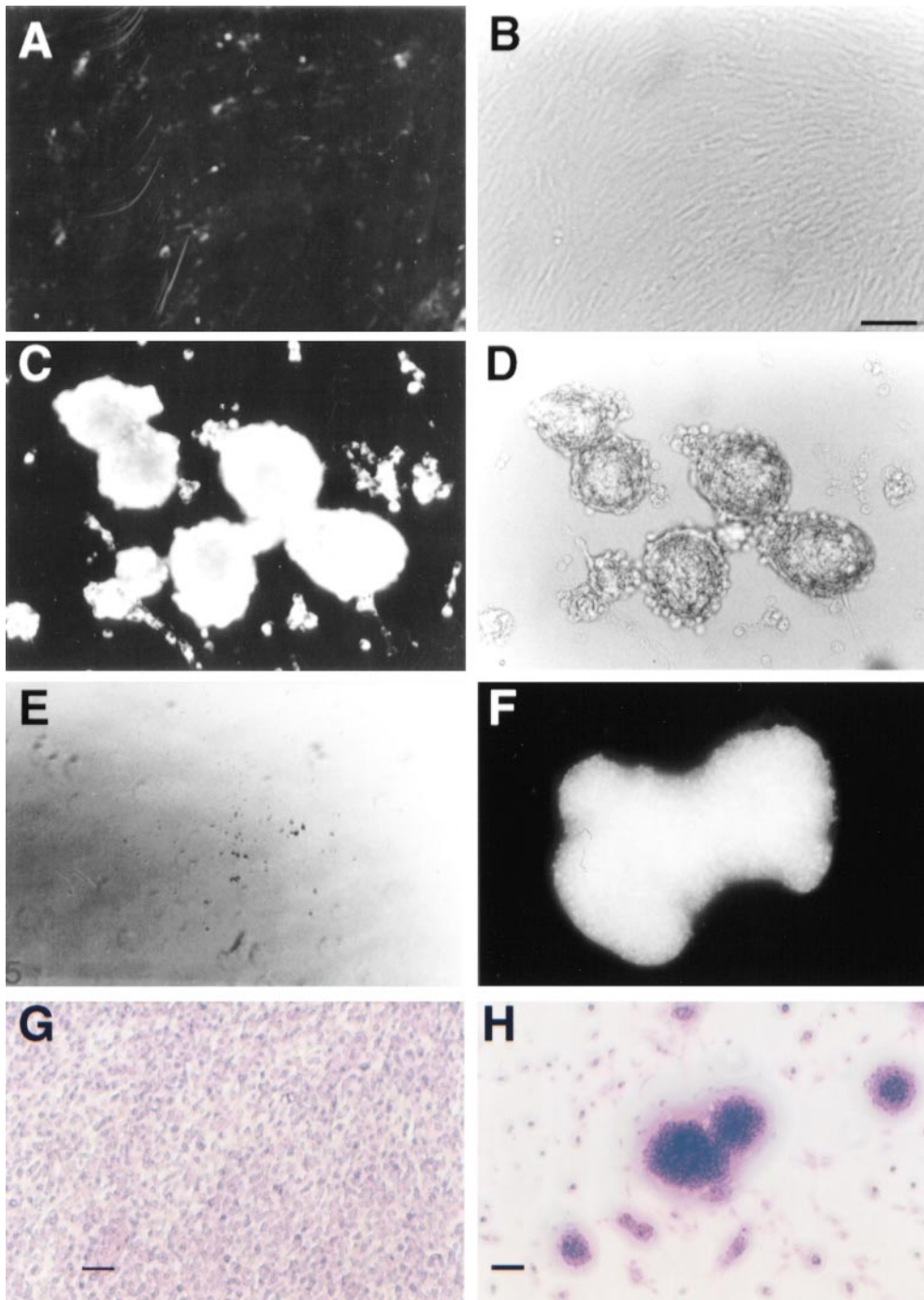
Figure 12. Maintenance of human chondrocytes by Pln. Human chondrocytes were cultured on either tissue culture plastic (A, B, and G) or Pln (C, D, and F) for 7 d and probed with antibodies recognizing aggrecan (C) or collagen type II detected by mouse mAb from Chemicon (F). On plastic, chondrocytes express low levels of aggrecan and appear fibroblastic. On Pln, aggrecan protein is discretely localized to specific cells and aggregates. The morphology, more rounded and aggregated, is typical of chondrocytes. Human fibroblasts do not adhere to Pln (E). Chondrocytes on plastic (G) and Pln (H) stained with hematoxylin and eosin. Bar, 100 μm. Magnification for A–F is indicated in B.

i.e., glycosaminoglycan synthesis, was observed (SanAntonio et al., 1987). In these studies, it was suggested that a possible effect of the HS/HP was to free the cells from their attachment to the matrix, allowing them to round up and promoting cell–cell interaction. The induction of chondrogenesis observed in the present studies is more complex. Induction of chondrogenesis in vitro appears to require some specific interaction and binding to Pln. 10T1/2 cells attach, but do not spread, on Pln. Also, Pln can maintain human chondrocytes in their differentiated form in vitro. In contrast, human fibroblasts do not attach to or differentiate on Pln-coated surfaces. It remains unclear which receptor systems trigger chondrogenesis in vitro.

Such systems appear to involve both HS- and Pln-recognizing events. Complexes containing integrin subunits α_v, β_3, and β_1 have been reported to bind Pln, although Pln lacking HS chains appeared to be more effectively recognized (Hayashi et al., 1992). These integrin subunits also have been detected in developing cartilage (Woods et al., 1994).

While several potential chondrogenic factors have been identified, none of these factors have been examined in the context of interactions with the extracellular matrix surrounding the cells in vivo. In this study, examination of temporal and spatial patterns of the extracellular matrix protein, Pln, supports a role for this protein in the program

of maturation or maintenance of chondrocytes. The combination of appropriate extracellular matrix molecules with various growth factors, secreted either by chondrocytes or surrounding cells, may prove to be an essential mix for development of cartilage in vivo.

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