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Ribozyme-Mediated Perlecan Knockdown Impairs Chondrogenic Differentiation of C3H10T1/2 Fibroblasts

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Abstract

Perlecan (Pln) is an abundant heparan sulfate (HS) proteoglycan in the pericellular matrix of developing cartilage, and its absence dramatically disrupts endochondral bone formation. This study examined two previously unexamined aspects of the function of Pln in mesenchymal chondrogenesis *in vitro*. Using the well established high density micromass model of chondrogenic differentiation, we first examined the requirement for endogenous Pln synthesis and secretion through the use of Pln-targeted ribozymes in murine C3H10T1/2 embryonic fibroblasts. Second, we examined the ability of the unique N-terminal, HS-bearing Pln domain I (PlnDI) to synergize with exogenous bone morphogenetic protein-2 (BMP-2) to support later stage chondrogenic maturation of cellular condensations. The results provide clear evidence that the function of Pln in late stage chondrogenesis requires Pln biosynthesis and secretion, because 60-70% reductions in Pln greatly diminish chondrogenic marker expression in micromass culture. Additionally, these data support the idea that while early chondrocyte differentiation can be supported by exogenous HS-decorated PlnDI, efficient late stage PlnDI supported chondrogenesis requires both BMP-2 and Pln biosynthesis.

Keywords

perlecan; chondrogenesis; BMP-2; cartilage; heparan sulfate proteoglycan

Introduction

Patterning in developing tissues including cartilage requires heparan sulfate proteoglycans (HSPGs), key components of the extracellular matrix (ECM). Heparan sulfate (HS) influences the local activities of bound growth factors such as fibroblast growth factor-2 (FGF-2) through localization and storage (Rifkin and Moscatelli, 1989; Vlodavsky et al, 1996), by potentiation of long term bioavailability (Ruoslahti and Yamaguchi, 1991; Pantoliano et al, 1994), and by presentation to cell surface receptors (Yayon et al, 1991; Rapraeger et al, 1991; Ornitz and Leder, 1992; Spivak-Kroizman et al, 1994; Moy et al, 1997). Bone morphogenetic proteins (BMPs) regulate the formation and growth of cartilage (Minina et al, 2001; Minina et al, 2002) and belong to the transforming growth factor- β (TGF- β) superfamily of secreted proteins (Hogan, 1996; Kingsley, 1994; Storm et al, 1994). Because BMPs are heparin binding proteins whose activities are enhanced both by heparin and HS (Takada et al, 2003), the current understanding is that the bioavailability of these molecules is determined by binding and release from HSPGs in the surrounding ECM. Perlecan (Pln) is the major HSPG in developing cartilage ECM where it can support BMP signaling. Perlecan expression in the elongating growth plate

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follows that of collagen type II and precedes that of collagen type X in hypertrophic (stage II) chondrocytes localized in the columnar, calcifying cartilage (Farach-Carson et al, 2005).

Pln is comprised of a 400-kDa core protein, decorated with 2-3 glycosaminoglycan (GAG) side chains at its N-terminus and sometimes one at its C-terminus (Hassell et al, 1980; Ledbetter et al, 1985; Ledbetter et al, 1987). The greatest deposition of Pln during embryonic development occurs in cartilage (SundarRaj et al, 1995; French et al, 1999). Strong expression is observed in the pericellular space and in the territorial matrix between prehypertrophic and hypertrophic regions in endochondral bone. Thus, Pln is a marker of chondrocyte differentiation and maturation. Pln's essential role in normal cartilage and bone formation is demonstrated by observations of severe cartilage and bone abnormalities in mice deficient in Pln expression (Arikawa-Hirasawa et al, 1999; Costell et al, 1999) and in humans harboring mutations in the Pln gene (Arikawa-Hirasawa et al, 2001; Arikawa-Hirasawa et al, 2002; Arikawa-Hirasawa et al, 2001; Aberfeld et al, 1965).

In the present study, we the multipotential embryonic (Taylor employed murine fibroblast cell line, C3H10T1/2 and Jones, 1979), as a convenient model to investigate the requirement for active Pln secretion during chondrogenic differentiation *in vitro* (Denker et al, 1999; Haas and Tuan, 1999; Fischer et al, 2002; Seghatoleslami and Tuan, 2002; Seghatoleslami et al, 2003; Modarresi et al, 2005). Our laboratory previously reported that Pln promotes *in vitro* aggregation, condensation and early chondrogenic differentiation events in this cell line (French et al, 1999). The condensations resemble those found in condensing mesenchyme *in vivo* insofar as they display reduced collagen type I expression and increased expression of various chondrocyte markers including aggrecan, collagen type II, link protein, and Pln itself (French et al, 1999; French et al, 2002; Gomes et al, 2003). Subsequent investigation used recombinant fragments representing all five domains of Pln to demonstrate that the GAG-bearing domain I (PlnDI), but not other domains of Pln, support chondrogenic activity *in vitro* when added exogenously (French et al, 2002). C3H10T1/2 cells plated on intact Pln or on PlnDI express markers of immature chondrocytes; however, even after long term culture these cellular condensations do not express markers of chondrocyte hypertrophy/maturation, such as collagen type X, except in the presence of BMP-2 (Gomes et al, 2003).

Previous studies have not determined if Pln biosynthesis and secretion is required for cartilage formation *in vitro*. Therefore, we employed a ribozyme knockdown strategy to determine if Pln synthesis is required for chondrogenic differentiation of C3H10T1/2 cells *in vitro* in the presence of BMP-2. We also investigated the possibility that recombinant PlnDI could replace intact Pln to support BMP-2 induced hypertrophic maturation of C3H10T1/2 nodules.

Materials and Methods:

Antibodies, Growth factors, and Proteins

The expression construct and antibodies specific for PlnDI (pCEP-Pu/PGI) were provided by the late Dr. Rupert Timpl (Max-Planck-Institut for Biochemistry, Martinsried Germany). Rabbit antibodies against mouse Pln were supplied by Dr. John Hassell (Shriners Hospital for Children, Tampa, FL). Rabbit antibody against mouse type X collagen (PXNC1-88) was provided by Dr. Greg Lunstrum (Shriners Children's Hospital, Portland, OR). Rabbit antibody against mouse type II collagen was purchased from Biodesign International (catalog no. T40025R; Biodesign, Kennebunk, ME). Peanut Agglutinin (PN)A Alexa Fluor 488 conjugate, which binds cell surface disaccharides, was purchased from Molecular Probes (Eugene, OR). Species specific Texas Red or Alexa Fluor 546 conjugated secondary antibodies were purchased from Amersham Corp. (Arlington Heights, IL) or Molecular Probes, respectively. rhBMP-2 (BMP_2) was purchased from R&D Systems (catalog no. 355-BM, Minneapolis,

MN USA). Recombinant PlnDI was prepared and characterized as described previously (Costell et al, 1997; Yang et al, 2005).

Creation of stable transfectants expressing ribozymes targeting Pln.

Perlecan Ribozyme. A hammerhead ribozyme targeting Pln mRNA was designed and tested according to previously published procedures (Montgomery and Dietz, 1997; Liu et al, 2000, Liu et al, 2002). In brief, the ribozyme contains a 24-nucleotide (nt) hammerhead domain flanked by two short complementary arms to the targeted region of Pln mRNA. This ribozyme is targeted to GUC at nt 396 (from the start AUG) of Pln mRNA, and thus named ribozyme 396. The cleavage site corresponds to a region of domain I near the center of the sperm protein, enterokinase and agrin (SEA) module (Wreschner et al, 2002). Ribozyme 396 was cloned into a U1 snRNA cassette used previously (Liu et al, 2000; Miller et al, 2003). The vector housing the U1 snRNA cassette includes a Zeocin (Invitrogen, Carlsbad, CA) resistance gene for selection. A control ribozyme, comprised of the scrambled PlnDI sequence, also was synthesized and ligated into the same expression vector. The active ribozyme 396 also has been stably transfected into human prostate cancer cells where it reduced perlecan expression by 80% (Savoré *et al.* in press).

Generation of cell lines stably transfected with Pln ribozyme. C3H10T1/2 cells ($\sim 5 \times 10^4$) were seeded into 24 well plates one day prior to transfection (Lipofectamine Plus, Invitrogen), according to the manufacturer's guidelines. Transfection was performed in the presence of linearized Pln ribozyme (2.8 μ g) or control vector constructs. Transfectants were carried in DMEM/F-12 medium (Gibco Life Sciences, Rockville, MD USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin, 100 μ g/ml of streptomycin sulfate, and the selection antibiotic Zeocin (100 μ g/ml). Individual foci of Zeocin resistant cells were present after 2 weeks of culture. Foci were isolated and expanded in the presence of Zeocin for verification of integration and expression of the ribozyme constructs. Stable transfectants were subsequently screened and selected for reduced Pln secretion by dot blot analysis of enriched conditioned medium collected in the absence of Zeocin (see below).

Enrichment and partial purification of Pln from conditioned medium. The partial purification of Pln from conditioned media was performed using anion exchange in a microfuge tube to determine if stable transfectants secreted reduced levels of Pln. First, 2-(diethyl amino) ethanol (DEAE) Sepharose CL-6B beads (10 ml) were equilibrated in 40 ml of equilibration buffer (0.05M Tris/HCl [pH 7.5], 2M urea, 0.25 M sodium chloride [NaCl], 2.5 mM ethylenediamine tetra-acetic acid [EDTA], 0.5 mM phenylmethylsulfonyl fluoride [PMSF] and 0.5 mM benzamidine). Post equilibration, the buffer was removed leaving a 10 ml slurry of beads. Then to each ml of conditioned medium (72 hrs, from confluent cultures), 0.1 ml of equilibrated beads were added, the samples were gently rocked on a nutator (3D orbital shaker) overnight at 4°C. Beads were pelleted by centrifugation at 8000 rpm for 30 sec, and resuspended in 0.2 ml of equilibration buffer, briefly centrifuged, and the supernatant discarded. To elute bound proteins, pellets were resuspended in 0.2 ml of elution buffer (0.05M Tris/HCl [pH 7.5], 2M Urea, 1.0 M NaCl, 2.5 mM EDTA, 0.5 mM PMSF and 0.5 mM benzamidine), and rocked on a nutator for 30 min at 4°C. The samples were pelleted by centrifugation, supernatant collected and diluted with 0.8 ml of phosphate buffer saline (PBS) to decrease the salt concentration.

Dot blot analysis of partially purified Pln. Dot blot analysis was performed to quantitate the level of Pln secreted by stable transfectants as follows. Partially purified Pln samples (in triplicate), were generated as above, and diluted to form a 6.2 % (v/v) solution in PBS. Samples and Pln standards were applied to the blotting apparatus and allowed to bind the nitrocellulose membrane by gravity flow at room temperature. The blot was removed from the apparatus, briefly air dried and rehydrated in PBS prior to blocking in 25 ml of PBS containing 3% (w/v) BSA and 0.05% (v/v) Tween 20, and subjected to constant rotary agitation for 4 hrs at 4°C.

The Pln polyclonal antibody was added to the blocking solution (1:5000 dilution), and the blot incubated with constant rotary agitation overnight at 4°C. The blot then was rinsed three times with PBS (50 ml) containing 0.05% (v/v) Tween 20 and incubated with the secondary antibody (donkey anti-rabbit-HRP, Jackson Immuno. Cat #711-035-152, 1:400,000 dilution) in PBS (25 ml) containing 0.05% (v/v) Tween-20. After incubating 40 min at room temperature with constant rotary agitation, the blot was rinsed and the bound secondary antibody was detected and visualized by enhanced chemiluminescence (Amersham Corp.) and brief film exposure. Blots subsequently were scanned by densitometry and each individual value expressed relative to cell number. A standard curve, employing mouse specific HSPG purchased from Sigma (St. Louis, Mo), was used to quantify Pln secretion and the data expressed as micrograms of Pln secreted per 10⁶ cells, collected after 3 days of culture.

Growth analysis of stable transfectants. For growth analysis, stably transfected cells were maintained in DMEM/F-12 medium (Gibco Life Sciences, Rockville, MD USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin, 100 µg/ml of streptomycin sulfate, and in the presence of Zeocin (100 µg/ml) until 50% confluent. Zeocin free medium then was used until the cultures became 90% confluent. After passage, the cells were seeded into six well plates (in triplicate) at 10,000 cells per well, and carried in Zeocin free medium. To determine cell number, cells were trypsinized and counted using a hemocytometer every two days for two weeks. Data are expressed as mean cell number per well +/- the standard deviation of triplicate determinations in each case.

Response of knockdown clones to BMP-2. Cells were seeded (in triplicate) into eight well “permanox” chambered cover slides (Nalge Nunc International Corp., Naperville, IL) at 92,000 cells/well, and carried in 0.25 ml of CMRL-1066 medium supplemented with 15% (v/v) heat-inactivated FBS, 50 µg/ml ascorbic acid, 50 µg/ml citrate, 50 µg/ml pyruvate, 100 U/ml of penicillin, and 100 µg/ml of streptomycin sulfate. After two days, the treatment group was supplemented with 100 ng/ml of BMP-2. On days 3, 5, 7 and 9, cells were trypsinized and counted using a hemocytometer to determine cell number.

High density micromass cultures. The micromass culture technique was modified from Denker et al., 1995 and 1999. Cultures were prepared from a 10 µl drop of medium (CMRL-1066, supplemented with 15% [v/v] FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin sulfate, ascorbic acid [50 µg/ml], citric acid [50 µg/ml], and pyruvate [50 µg/ml]) containing 200,000 cells, placed in the center of uncoated borosilicate chambered coverglass (0.9 cm²/well, Nalge Nunc). The chambered coverglasses were placed in humidified box and left for two hrs in a tissue culture incubator set at 37°C in a humidified atmosphere of air:CO₂, 95:5 (v/v). Following this incubation, one ml of medium was added with care to avoid disturbing the cell mass. BMP-2 (100 ng/ml) was added to half of the micromass cultures on day two of culture. Thereafter, the medium was changed every 3rd day and supplemented with or without BMP-2, as on day 1. On day twelve (day ten of BMP-2 treatment), the cultures were harvested and analyzed for the presence of chondrogenic markers. All cultures were performed in triplicate and repeated at least twice.

PlnDI supported condensations. Cellular condensations were created as previously described (French et al, 1999), with some modification. To facilitate confocal microscopic analysis of cell condensations, permanox chambered slides (8 well, no. 177445; Nalge-Nunc, Naperville, IL) were used. Briefly, for coating wells, 2.5 µg of PlnDI was added to the well in a final volume of 100 µl in Dulbecco's phosphate buffered saline (D-PBS) and then incubated overnight at 37° with lids askew. On the following day, the dry wells were rinsed twice with D-PBS before addition of cells. Cells were added to wells (9.2 × 10⁴ cells/well), in CMRL-1066 medium. The CMRL-1066 medium (Gibco Life Sciences, Rockville, MD USA) was supplemented with 15% (v/v) heat-inactivated FBS, 100 U/ml of penicillin, 100 µg/ml of

streptomycin sulfate, ascorbic acid (50 µg/ml), citrate (50 µg/ml), and pyruvate (50 µg/ml). Media were changed every 48 hrs throughout the experiment. Formation of condensations was assessed by visual inspection using light microscopy. Cells that had self-assembled into dense, multilayered cellular condensations reminiscent of condensing mesenchyme in developing cartilage were scored as positive described as previously (French et al, 1999). In addition, these condensations were positive for PNA-binding and expressed collagen type II mRNA and protein. BMP-2 media supplementation (100 ng/ml) was initiated on experimental day three, corresponding to two days after condensation formation, and BMP-2 was maintained with each medium change throughout the experiment. In all chondrogenic differentiation experiments, cells were seeded in triplicate for each condition and each experiment was repeated a minimum of three times.

Alcian blue GAG labeling. Micromass cultures (in triplicate) were fixed for 15 min at room temperature with 10% (w/v) neutral buffered formalin (Sigma), containing 0.5% (w/v) cetylpyridinium chloride, rinsed once with PBS, then twice with 3% (v/v) glacial acetic acid (pH 1.0), prior to a 4 hr room temperature incubation with 0.5% (w/v) Alcian Blue 8GX (Lev and Spicer, 1964) in 3% (v/v) glacial acetic acid (pH 1.0). Post staining, cells were rinsed twice with 3% (v/v) glacial acetic acid (pH 1.0), then twice with 3% (v/v) glacial acetic acid (pH 2.5), air dried, and finally photographed with a digital camera (Coolpix 990, Nikon Japan) attached to a phase contrast microscope (Nikon, Japan).

Oil Red O lipid labeling. Triplicate cultures were fixed for 15 min at room temperature with 10% (v/v) paraformaldehyde (Electron Microscopy Sciences, Ft Washington, PA USA). During fixation, a working solution of Oil Red O (Lillie and Ashburn, 1943, Lillie, 1944) was prepared by diluting 6 ml of a stock solution, containing 0.5 g Oil Red O (C.I. 26125) in 100 ml isopropyl alcohol, into 4 ml of distilled water. The solution was allowed to stand for 15 min, then filtered using #1 Whatman filter paper immediately prior to use. Post fixation, the cells were rinsed twice with distilled water, then stained for 15 min with the working solution. The stained cells were rinsed four times with distilled water, air dried, and photographed with a digital camera attached to a phase contrast microscope under 10X optics.

Immunofluorescent detection of ECM components. After 3, 6, 9 or 12 days in culture, cell condensations and monolayers were rinsed twice with Ca²⁺/Mg²⁺ free D-PBS. The specimens were fixed, washed three times for 5 min each at room temperature with D-PBS, and incubated with the primary antibody for 1 hr at 37° in a humidified chamber. After three washes for 5 min each at room temperature in D-PBS, cell condensations and monolayers were incubated with the secondary antibody for 45 min at 37° in a humidified chamber and finally washed three times for 5 min each at room temperature with D-PBS and mounted. For Pln detection, cell condensations and monolayers were fixed in 100% methanol for 10 min at room temperature; however, for types II and X collagen detection, samples were fixed for 30 min on ice in ethanol:acetic acid, 95:5 (v/v). To promote antibody penetration, cell condensations and monolayers were incubated with 0.02% (w/v) type IV-S testicular hyaluronidase (H3884; Sigma, St. Louis, MO) for 30 min at room temperature and rinsed three times with PBS before primary antibody incubation. For PNA staining, the condensations were fixed in freshly prepared electron microscopy grade 4% (w/v) paraformaldehyde (15710, Electron Microscopy Sciences) in PBS for 20 min at room temperature. After three washes for 5 min each at room temperature in D-PBS, the condensations were incubated with PNA-Alexa 488 (100 µg/ml), for 45 min at room temperature. All condensations were washed three times in D-PBS for 5 min each at room temperature prior to mounting in Vectashield mounting medium for fluorescence with DAPI (H-1200, Vector Laboratories, Inc., Burlingame, CA USA).

Results

Analysis of Pln knockdown clones. Four independently isolated clones were selected for subsequent analysis: two served as controls and two others as test/knockdown clones. Control ribozyme clones 14 and 16 (CR14 and CR16), stably transfected with the scrambled annealing arms, were selected because they secreted levels of Pln similar to the parental, C3H10T1/2 cell line (fig. 1). Pln knockdown clones 12 and 18 (PR12 and PR18), stably transfected with active Pln specific ribozymes, were selected for their reduced levels of Pln secretion (~70 and 60%, respectively), relative to the parental cell line (fig. 1).

Proliferative response of knockdown clones +/- BMP-2. A growth curve analysis of stably transfected cells determined the effects of reduced Pln expression on cell proliferation. As shown in figure 2A, the basal growth characteristics of these cells are nearly identical. To determine the effect of BMP-2 treatment on Pln knockdown clone proliferation, cells were treated for 7 days, beginning on day two of culture. As shown in figure 2B, all clones responded similarly to BMP-2, and displayed a modest (20-25%) increase in proliferation. Similar results were obtained during shorter (3-5 days) BMP-2 treatment periods (data not shown). Collectively, these results suggest that cell proliferation was not impacted by a reduction in Pln expression.

Chondrogenic differentiation of knockdown clones. C3H10T1/2 fibroblasts were cultured in high density micromass (in triplicate), on borosilicate coverglass to determine the consequences of reduced Pln expression on chondrogenic differentiation, initially indexed by Alcian blue staining, a marker of GAG accumulation. In the absence of BMP-2, cells stably transfected with the control ribozyme (clones CR14 and CR16) demonstrated weakly positive Alcian blue staining (fig. 3; only CR16 is shown) localized to the center of the micromass. As expected, supplementation with BMP-2 enhanced Alcian blue staining. In contrast, micromass cultures of both Pln knockdown clones (PR12 and PR18) demonstrated barely detectable Alcian blue staining in the absence of BMP-2. Compared to treated controls (CR14 and CR16) Alcian blue labeling was reduced in both Pln knockdown clones (PR12 and PR18). Moreover, BMP-2 treatment of these cultures only modestly increased Alcian blue staining compared to controls. Interestingly, we noted that the treated PR12 culture stained weakly at the periphery, and the PR18 at the center of the culture, a finding that we suspect is due to the migration of PR12 cells away from the micromass center. The morphology of PR12 cells at the periphery of the micromass culture suggests that these cells are not chondrocytes, but fibroblasts.

As an additional test of chondrogenic differentiation, indirect immunostaining with antibodies directed against early (aggrecan and collagen type II), and late stage (collagen type X) chondrogenic markers was performed. Aggrecan immunolabeling was not detected in untreated micromass cultures; however, aggrecan immunolabeling was detected in BMP-2 treated micromass control cultures (compare fig. 4A2, B2 and C2 with fig. 4A1). The intensity of aggrecan immunolabeling in Pln knockdown clones was weak in the center of the micromass (Figure 4, B1 and C1); however, it was noted that a few cells at the periphery of the micromass expressed aggrecan at levels comparable to controls, a pattern similar to that observed with Alcian blue staining (not shown). Collagen type II immunolabeling was detected in all micromass cultures, regardless of BMP-2 treatment (fig. 4 panels D1-F2). In BMP-2 treated micromass cultures, collagen type II labeling intensity was greatest in controls followed by PR18 and finally PR12 (fig. 4 panel D1 vs E1 and F1, respectively). Moreover, BMP-2 treatment of micromass cultures only enhanced expression of collagen type II in control cultures (fig. 4 panel D1 vs D2). If anything, BMP-2 treatment of knockdown clones may have decreased expression of collagen type II, but the effect was modest. Collagen type X immunolabeling was not detected in BMP-2 treated or untreated cultures (not shown).

Adipogenic differentiation of knockdown clones. Microscopic examination of micromass cultures revealed intracellular droplets that had accumulated in cells of BMP-2 treated, Pln knockdown clones. To determine if this corresponded to lipid accumulation, we employed Oil Red O labeling. The accumulation of red, lipid filled droplets in high density cultures of Pln knockdown clones treated with BMP-2 demonstrated that these structures accumulated to a greater extent in Pln knockdown clones, PR12 and PR18 (fig. 5 C and D) than in control clones, CR14 and CR16. This behavior was most noticeable in clone PR12 cultures, and suggested that loss of Pln influenced cell fate in C3H10T1/2 cells.

Analysis of chondrogenic markers in PlnDI supported condensations. To determine if exogenous PlnDI could support late stage chondrogenic differentiation driven by BMP-2, we cultured C3H10T1/2 cells on surfaces coated with or without PlnDI. Following one day of BMP-2 treatment, the signal intensity for the early chondrogenic markers, PNA binding and collagen type II were elevated in condensations treated with BMP-2 relative to untreated condensations (green signal, fig. 6. A vs B, and red signal, C vs D, respectively). In contrast, the signal intensity for Pln was not different in BMP-2 treated condensations compared to untreated condensations at this time point (red signal, fig. 6; Day 3, panel E vs F).

By day 6 of culture, (day 4 of BMP-2 supplementation), the intensity of PNA labeling in BMP-2 treated condensations was slightly reduced compared to BMP-2 treated condensations from day 3 cultures (green signal, fig. 6, day 6 vs day 3, panel A); however, under these conditions, obvious differences in PNA signal intensity between BMP-2 treated and untreated condensations were observed (green signal, fig. 6, day 6, panel A vs B). The signal intensities for both collagen type II and Pln remained elevated and unchanged in BMP-2 treated condensations compared to treated condensations in day 3 cultures. Interestingly, the signal intensity for collagen type II in untreated condensations was elevated at day 6 compared to untreated condensations on day 3 (red signal, fig. 6, day 6 vs day 3, panel D). In a manner similar to condensations supported by intact Pln and treated with BMP-2, the signal intensity for collagen type II and Pln in PlnDI-supported condensations treated with BMP-2 also was enhanced compared to signals in untreated condensations (red signal, fig. 6, day 6, panels C vs D, and E vs F). The signal intensity for Pln in day 6 untreated condensations had declined compared to that of day 3 condensations (red signal, fig. 6, day 6 vs day 3, panel F) suggesting that a differentiated state was not maintained in the absence of BMP-2.

By day 12 of culture, i.e., day 10 of BMP-2 treatment, PNA binding sites were not detectable in condensations from either culture condition (data not shown). Signal intensities for collagen type II and Pln were reduced in condensations from both culture conditions relative to condensations from day 6 cultures (fig. 6, day 12 vs. day 6, panels A vs. C and B vs. D for collagen type II; panels C vs. E and D vs. F for Pln); however, the signals for collagen type II and Pln remained elevated in day 12 condensations treated with BMP-2 relative to untreated (fig. 6, day 12, panels A vs. B for collagen type II; panels C vs. D for Pln). Similar to condensations supported with intact Pln, condensations supported with PlnDI and treated with BMP-2 also expressed collagen type X. This expression first was detected at low levels in condensations in 9 day cultures. By day 12 of culture, the signal intensity for collagen type X was elevated and localized to both intra- and extracellular/pericellular compartments (fig. 6 day 12, panel E). In contrast, collagen type X was not detectable in condensations in the absence of BMP-2.

To determine if exogenous PlnDI could rescue the chondrogenic phenotype of Pln knockdown clones, cells were seeded on tissue culture surfaces coated with PlnDI, and treated with BMP-2. Following 12 days of culture [10 days of BMP-2 treatment] only one cell in all condensations derived with Pln knockdown clones was immunopositive for collagen type X relative to control

parental cells, indicating that culture on a PlnDI substrate could not efficiently rescue differentiation in the absence of Pln biosynthesis.

Discussion

Territorial boundaries and cell adhesion in cartilage are established by ECM and cell surface adhesion molecules including fibronectin, tenascin, N-cadherin, N-CAM, and syndecan (Haas and Tuan, 1999; Chimal-Monroy and Diaz de Leon, 1999; Travella et al, 1994; Oberlender and Tuan, 1994; Oberlender and Tuan, 1994; Delise and Tuan, 2002). Cartilage also is rich in Pln, a large essential HSPG involved in sequestration and delivery of heparin binding growth factors such as FGF-2 and BMP-2. In this study, we examined two critical aspects of Pln function in the chondrogenic differentiation of C3H10T1/2 embryonic fibroblasts *in vitro*: 1) the requirement for endogenous Pln synthesis and secretion and; 2) the ability of PlnDI to synergize with exogenous BMP-2 to support later stage chondrogenic maturation. Employing two different models to trigger *in vitro* chondrogenesis of C3H10T1/2 cells, i.e., micromass culture and culture on PlnDI coated surfaces, and a ribozyme knockdown approach, we demonstrated that even a partial, i.e., 60-70%, reduction in endogenous Pln secretion severely impaired chondrogenic differentiation, even when exogenous BMP-2 or a PlnDI substrate was provided.

The defect we observed in chondrogenic differentiation is unlikely to be due to impaired cell proliferation for several reasons. First, mesenchymal condensation does not rely on proliferation to support chondrogenic differentiation (DeLise et al, 2000), and cell proliferation within high density micromass cultures occurs predominately at the periphery (Mello and Tuan, 1999). Second, we found that the rate of proliferation of knockdown clones, as well as their response to BMP-2, was not different from the parental line in monolayer culture. On the other hand, Pln knockdown cells demonstrate reduced BMP-2 responsiveness in micromass cultures. Differentiation of C3H10T1/2 cells under these conditions is affected by the initial plating density and by BMP-2 dose (Wang et al, 1993; Denker et al, 1999). Sulfated polysaccharides, including HS and heparin, directly interact with and enhance the activity of a number of growth factors, including BMPs (Yayon et al, 1991; Rapraeger et al, 1991; Aviezer et al, 1994; Ohkawara et al, 2002; Irie et al, 2003). With regard to PlnDI, we recently observed that both BMP-2 and FGF-2 bind directly to PlnDI in a HS dependent manner (Yang et al, 2005). Thus, it is intriguing to speculate that BMP-2 interactions with HS chains of Pln are required to coordinate chondrogenic differentiation. While outside the scope of these experiments, it also is likely that loss of Pln influences responses to FGF-2. Interestingly, Pln knockdown cells poorly expressed collagen type X in response to BMP-2 even when they were cultured on surfaces coated with PlnDI, indicating either that PlnDI supplied exogenously is not presented in the proper context or that other domains of Pln are needed to support late stage differentiation. The latter notion is consistent with observations of mouse mutants in which domain I of Pln was selectively deleted and which displayed grossly normal cartilage development (Rossi et al, 2003). We found that neither Pln nor PlnDI added in solution induces chondrogenic differentiation in high density cultures *in vitro* (French et al, 1999 and Gomes et al, unpublished observations). Thus, deposition of Pln into an organized territorial matrix must be necessary to support chondrogenic differentiation. Additional support that Pln knockdown cells have an attenuated differentiation response to BMP-2 stems from our observations of adipogenic differentiation in BMP-2 treated Pln knockdown clones. While it is well documented that low doses of BMP-2 stimulate adipogenic differentiation of C3H10T1/2 cells (Wang et al, 1993; Ahrens et al, 1993; Taylor and Jones, 1979; Date et al, 2004), a role for Pln in this process has not been reported. Because the activity of HS binding growth factors can be modulated by Pln through its associated HS chains, low levels of Pln expression may lower BMP-2 activity and favor adipogenic differentiation.

A likely explanation for the observation that PlnDI knockout mice (Rossi et al, 2003) develop apparently normal cartilage despite the clear importance of HS in cartilage development is that other cartilage ECM and cell surface molecules such as aggrecan, syndecan or even domain V of Pln are substituted with HS to compensate for the absence of PlnDI HS (Govindraj et al, 2002). In any event, the severe cartilage phenotype observed with Pln nulls *versus* the normal cartilage phenotype observed with PlnDI null mice suggests that other Pln domains serve critical role(s) in normal cartilage development. Domains II-V of Pln interact with a variety of cell surface and ECM molecules (Battaglia et al, 1992; Brown et al, 1997; Sasaki et al, 1998; Gohring et al, 1998; Peng et al, 1999; Talts et al, 1999; Mongiat et al, 2003; Miosge et al, 2003) and also may be important for normal secretion and matrix assembly. Thus, there are many ways in which the lack of Pln domains II-V could lead to impaired interactions that would compromise normal cartilage development.

A second model culture system was used to investigate the ability of BMP-2 to drive chondrogenic maturation on PlnDI coated surfaces. We used collagen type II and PNA binding as markers of early chondrogenic differentiation. PNA is a lectin that selectively binds cell surface disaccharides on condensing chondroprogenitor cells, and precedes the deposition of a cartilaginous ECM (Zimmermann and Thies, 1984; Aulthouse and Solursh, 1987; Milaire, 1991). Collagen type II provided a matrix marker of early chondrocyte differentiation. PlnDI supported condensations expressed high levels of PNA binding sites, and collagen type II after 24 hr of BMP-2 treatment. In the absence of BMP-2, condensations displayed little or no PNA binding sites or collagen type II. Under these conditions, collagen type II expression was not detected until day six of culture. In contrast, BMP-2 treatment both accelerated and greatly enhanced overall expression of both markers. The ability of BMP-2 to alter the timing of chondrogenic differentiation in condensations was further demonstrated by the early appearance of collagen type X. Collagen type X was first detected in BMP-2 treated condensations at day 9 of culture (data not shown), but not until day 14 for BMP-2 treated C3H10T1/2 embryonic fibroblasts cultured in monolayer, on plastic. Together, these data suggest the onset of chondrocyte differentiation in PlnDI-supported condensations occurs earlier in the presence of BMP-2. Moreover, the observation that BMP-2 accelerates and enhances chondrogenic maturation of PlnDI supported condensations is consistent with our previous observations employing intact Pln (Gomes et al, 2003). Interestingly, in spite of differences that exist between the models used, our observations generally agree with a recent report by Seghatoleslami et al. (2003) indicating that BMP-2 treatment of C3H102T1/2 cell condensations advances the signaling program involved in chondrogenesis. Under normal conditions, we thus propose that HS-bearing Pln organized in the territorial matrix of cartilage or produced by cultured chondrocytes increases BMP-2 activity and favors chondrogenesis over adipogenesis.

In summary, we have demonstrated using a knockdown approach that normal levels of Pln secretion are critical for the chondrogenic differentiation of high density micromass cultures in the presence of BMP-2. While BMP-2 treatment of murine C3H10T1/2 embryonic fibroblasts supports chondrogenesis, as has been elegantly demonstrated by other investigators (Wang et al, 1993; Denker et al, 1999; Haas and Tuan, 1999), the supporting role played by Pln during BMP-2 stimulated chondrogenic differentiation has not been appreciated. These data are consistent with *in vivo* observations and demonstrate a direct role for Pln in the differentiation of mesenchymal stem cell lines into chondrocytes. The requirement for Pln biosynthesis is most likely to reflect complex associations with other ECM components that cannot be recapitulated simply by addition of exogenous Pln or PlnDI either in soluble form or as a simple surface coating. In contrast, PlnDI coated surfaces can be used to initiate early chondrogenesis in precursor cells then able to produce and assemble their own Pln-containing territorial matrix.

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Figure 1.

Quantification of Pln secretion. Dot blot analysis of Pln secretion by C3H10T1/2 murine embryonic fibroblasts stably transfected with ribozymes specific for Pln. Conditioned medium was blotted onto nitrocellulose and probed with Pln specific antibodies. Following exposure to film, blots were scanned by densitometry, and individual density values were expressed relative to cell number and averaged. A standard curve employing known amounts of Pln was used to quantify the amount of Pln bound. C3H10T1/2, parental cell line; CR14 and CR16 C3H10T1/2 cell clones stably transfected with the control ribozyme; PR12 and PR18 C3H10T1/2 cell clones stably transfected with Pln ribozymes. The secretion of Pln is reduced ~70 and 60 % in knockdown clones PR12 and PR18, respectively. These data represent the mean $\pm$ S.D. of triplicate determinations in each case (* $P < 0.01$).

A. **Figure 2.**

Growth responses of Pln knockdown clones. A. Growth curves for murine C3H10T1/2 embryonic fibroblasts stably transfected with control ribozymes or those specific to Pln were performed as described in “Materials and Methods”. Cell numbers were determined by hemocytometer counting in triplicate cultures at each time point. The data represents the average cell density for control clone 16 (CR16), and Pln knockdown clones PR12 and PR18, during 14 days of culture. All clones share similar growth patterns. **B.** BMP-2 growth responses of Pln knockdown clones were determined as described in “Materials and Methods”. The bars represent the mean ($\pm$ standard deviation) cell density of triplicate determinations of BMP-2 (100 ng/ml) treated (white bars) and untreated (black bars) clones determined on day nine of culture. All cells responded to BMP-2 supplementation to a similar extent, and similar effects were observed at earlier culture times.

Figure 3.

Alcian blue staining of high density micromass cultures. Alcian blue staining of micromass cultures was performed following ten days of culture with and without BMP-2 (100 ng/ml), as described in “Materials and Methods”. Micromass cultures containing cells from the ribozyme control clone, CR16 demonstrate strong Alcian blue labeling (panel A) following BMP-2 treatment. In contrast, Pln knockdown clones (PR12 and PR18) stain weakly for Alcian blue, and demonstrate a different labeling distribution relative to control (panels C and E vs. A). Alcian blue staining in control clones is intense and localized to the entire micromass; however, knockdown clones demonstrate weak or no labeling in the center and the majority of the staining on clone PR12 is at the periphery, surrounding the micromass. Data presented are representative images collected from triplicate cultures

Figure 4.

Indirect immunohistochemical analysis of micromass cultures treated with BMP-2.

Micromass cultures were prepared with and without BMP-2 (100 ng/ml) treatment for ten days, and immunostained as described in “Materials and Methods”. Panels A1, B1, C1, D1, E1, and F1 are from high density micromass cultures in the presence of BMP-2, while panels A2, B2, C2, D2, E2, and F2 are culture in the absence. The top six panels (A1-C2) are from cultures stained with antibodies specific to aggrecan, and the bottom six panels (D1-F2) are from cultures stained with antibodies to collagen type II and counter stained with the nuclear dye Syto13. Note that the signal intensity for aggrecan and collagen type II in the control clone (CR16) is enhanced relative to untreated cultures (A1 vs. A2 and D1 vs. D2). Aggrecan and collagen type II signal intensities in the control clone (CR16) treated with BMP-2 also are elevated above BMP-2 treated knockdown clones PR18 and PR12 (A1 vs. B1 and C1, and D1 vs. E1 and F1). All images are optical sections, captured with a laser scanning confocal microscope under identical settings. (magnification = 20X).

Figure 5.

Oil Red O staining of high density micromass cultures. Monolayer cultures were maintained for ten days in the presence of BMP-2 (100 ng/ml) prior to staining with Oil Red O as described in “Materials and Methods”. Panels A and B represent control clones CR14 and CR16, respectively, and are negative for Oil Red O. Panels C and D, represent clones PR18 and PR12, respectively, and are positive for Oil Red O. Major areas of lipid droplet accumulation are denoted by black arrows. Clone PR12 has a greater abundance of lipid filled cells than clone PR18. All images were captured under the same magnification as indicated by the bar in panel D (bar= 50 μ m). Data presented are representative images collected from triplicate cultures.

Figure 6.

Immunohistochemical analysis of chondrogenic markers in C3H10T1/2 cell

condensations formed on PlnDI and treated with BMP-2. A. On days 3, 6, and 12 of culture, both BMP-2 treated and untreated PlnDI-stimulated nodules were collected and labeled with PNA, or antibodies specific to collagen type II, collagen type X, or Pln as described in “Materials and Methods”. In day 3 condensations treated with BMP-2 the signal intensities for PNA (green) and collagen type II (red) are enhanced relative to untreated condensations (panels A vs. B, and C vs. D, respectively). The intensity of Pln labeling (red signal) in treated and untreated condensations is similar (panels E vs. F). Relative to day 3 condensations, the signal for PNA binding in day 6 condensations is reduced (day 6, panel A and B). The signal intensity for collagen type II in untreated condensations is elevated (day 3 D vs. day 6 C), but not different from treated day 6 condensations (day 6 panel C vs. D). The signal intensity for Pln is similar in treated (day 6 E vs. day 3 E), but reduced in untreated condensations (day 6 F vs. day 3 F and day 6 E). In day 12 condensations the signal intensities for collagen type II and Pln are reduced relative to condensations from day 3 and 6 cultures. BMP-2 treated condensations maintain a higher signal intensity for collagen type II and Pln relative to untreated condensations (C vs. D; and E vs. F). Day 12 condensations treated with BMP-2 express high levels of collagen type X (red signal, A). Untreated condensations do not express collagen type X (B). **B.** Representative images of collagen type X expression (red signal) in condensations from control and Pln knockdown clones following 10 days of BMP-2 treatment. In all panels the blue signal represents DAPI labeling of cell nuclei. All images were captured under

identical conditions, employing the same magnification as indicated by the bar in panel D (bar in 6A = 50 μm , and in 6B = 100 μm).