Title: Disruption of endogenous perlecan function improves differentiation of rat articular chondrocytes *in vitro*

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ABSTRACT

Heparan sulfate (HS) and heparan sulfate proteoglycans (HSPG) are necessary for normal cartilage development and chondrocyte differentiation. However, recent studies demonstrated that HSPG accelerate dedifferentiation and catabolism in chondrocytes from degenerative cartilage. In this study, we investigated the inhibitory effect of HSPG on chondrocyte differentiation *in vitro*. Rat articular chondrocytes were cultured at low (0.3×10^4 cells/cm^2) and high (1.5×10^5 cells/cm^2) density in the presence or absence of heparitinase I, an HS degrading enzyme. Cells cultured at low density dedifferentiated and exhibited an elongated morphology, and treatment with heparitinase I precluded cell elongation. Conversely, populations of chondrocytes cultured at high density exhibited either a dedifferentiated or differentiated phenotype. Glycosaminoglycan accumulation increased in heparitinase I-treated cells. To determine the function of perlecan, an important HSPG for cartilage development, in chondrocyte differentiation, rat chondrocyte cultures were exposed to an anti-perlecan antiserum to inhibit perlecan function. Western blotting analysis indicated that preventing perlecan activity increased type II collagen synthesis. Our results suggest that HSPG are negative regulators of chondrocyte differentiation *in vitro* and that perlecan contributes to chondrocyte dedifferentiation *in vitro*.

Keywords: culture, extracellular matrix, HS, HSPG, perlecan

INTRODUCTION

Chondrocyte differentiation is regulated by various extracellular signaling molecules, such as morphogens and growth factors (GF) that act in developing and adult cartilage (Ellman et al. 2008; Minina et al. 2001). During cartilage degeneration or *in vitro* expansion, these signaling factors are abnormally activated, resulting in a catabolic state and a dedifferentiated phenotype (Im et al. 2007; Yuasa et al. 2008). During adulthood, articular cartilage exhibits a limited repair potential, such that this tissue is prone to severe degeneration and osteoarthritis (OA) in response to trauma. Although chondrocytes are capable of regenerative responses, such as increased proliferation and collagen expression, as OA progresses these abilities are unable to prevent cartilage degeneration. Chondrocytes from osteoarthritic cartilage exhibit a catabolic state and dedifferentiated phenotype, elongated fibroblastic morphology, a reduced collagen type II to type I expression ratio and expression of proteoglycan (PG), and increased expression of collagen-degrading enzymes (Tesche & Miosge 2005). Similar phenotypic changes occur in chondrocytes expanded *in vitro* (Tesche & Miosge 2005). Autologous chondrocyte implantation is a possible treatment for degenerative cartilage diseases, but requires *in vitro* expansion of chondrocytes (Batty et al. 2011). However, the expansion procedure might result in fibrocartilaginous tissue formation that is inadequate to restore articular cartilage function. The ability of various factors, such as bone morphogenetic proteins (BMP) and fibroblast growth

factors (FGF), to induce a differentiated and anabolic phenotype in chondrocytes *in vitro* and *in vivo*, was investigated (Gouttenoire et al. 2010; Moore et al. 2005). However, despite these efforts, induction of a differentiated and anabolic articular chondrocyte phenotype remains difficult to attain.

During cartilage development, the distribution and function of extracellular signaling molecules are regulated by heparan sulfate proteoglycans (HSPG) (Koziel et al. 2004; Grobe et al. 2005). HSPG are composed of a core protein that is bound mainly to heparan sulfate (HS) chains. The core protein determines the distribution of HS, which interacts with heparin-binding molecules, such as FGF, BMP, and Wnts (Whitelock et al. 2008; Manon-Jensen et al. 2010). Thus, HSPGs modulate the presentation of heparin-binding molecules to their cognate receptors.

The importance of HSPG function in cartilage formation is documented by studies of mutations in exostosin-1 (EXT1), an enzyme responsible for HS synthesis (Nadanaka & Kitagawa 2008). Mice carrying an *Ext1* hypomorphic allele exhibit a delay in chondrocyte hypertrophy and skeletal growth retardation (Koziel et al. 2004). Acordingly, conditional knockout of *Ext1* in limb buds during early embryonic development impairs mesenchymal cell condensation and cartilaginous primordium formation (Matsumoto et al. 2010). Perlecan is a multifunctional HSPG that is essential for the normal development of various organs and is critical for cartilage development (Knox & Whitelock 2006). Disruption of the perlecan gene causes dwarfism and dyssegmental dysplasia in mice (Arikawa-Hirasawa et al. 1999). During

cartilage development, the amount of perlecan in the stromal extracellular matrix (ECM) increases (Melrose et al. 2006). In addition, perlecan might regulate the distribution and presentation of Indian hedgehog (Ihh) and BMP (Melrose et al. 2008). Furthermore, multiple *in vitro* studies have demonstrated that perlecan promotes BMP-stimulated chondrogenic differentiation (Gomes et al. 2003, 2006; Yang et al. 2006). In addition, cell membrane-associated HSPG, such as syndecans, contribute to cartilage development by regulating the distribution of Ihh (Shimo et al. 2004). Mice harboring individual *syndecan-2, -3, or -4* null alleles do not exhibit cartilage defects, probably because of genetic compensation from the other syndecan genes (Bertrand et al. 2013; Pap & Bertrand 2013).

Although HSPG are important for normal cartilage development, recent studies suggest that HSPG inhibit cartilage repair in degenerative disease of the joint. In a mouse model of OA, ablation of *syndecan-4* delayed cartilage degeneration (Echtermeyer et al. 2009). Mice null for *endosulfatase-1*, which encodes for a protein that impairs HS function by removing sulfate groups from HS chains, exhibit more pronounced cartilage degeneration under conditions of OA induction (Otsuki et al. 2010). These studies suggest that HSPG primarily have negative effects on cartilage repair by dedifferentiated and catabolic chondrocytes. Perlecan expression may increase in cartilage from OA patients (Tesche & Miosge 2004), raising the possibility that perlecan contributes to the progress of dedifferentiation and tocatabolic responses in cartilage.

In this study, we cultured chondrocytes in the absence of inducers of

chondrocyte differentiation and examined the role of HSPG in chondrocyte differentiation by disrupting the function of HS and perlecan.

MATERIALS AND METHODS

Chondrocyte culture

Rat articular chondrocytes were cultured as described by Gosset et al. (2008), with some modifications. Neonatal Wistar rats were euthanized with an overdose of sodium pentobarbital (Dainippon Sumitomo Pharma, Osaka, Japan), in accordance with the guidelines of the ethics committee of Hokkaido University (Japan), and articular cartilage was obtained from knee joints. The cartilage was treated with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 0.1% collagenase (Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 30 min, and the surface of the cartilage was washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.4). After treatment with 0.02% collagenase for 12 h, the cartilage was disrupted mechanically by pipetting. Dissociated chondrocytes were filtered through nylon meshes with 40-μm pores (BD Biosciences, Franklin Lakes, NJ, USA). Subsequently, cells were cultured in DMEM/F-12 (Sigma) containing 5% fetal bovine serum (FBS; Cell Culture Technologies, Gravesano, Switzerland), 164 μg/mL L-ascorbic acid phosphate magnesium salt n-hydrate (Wako), 63.3 μg/mL penicillin G potassium (Nacalai Tesque, Kyoto, Japan), and 100 μg/mL streptomycin sulfate (Nacalai Tesque). The cells were cultured at a low density (0.3×10^4 cells/cm^2) to accelerate dedifferentiation, or at a high density (1.5×10^5 cells/cm^2) to induce differentiation.

Normal human articular chondrocytes (NHAC) were purchased from Lonza (Basel, Switzerland). Cells were fully dedifferentiated by extensive expansion in DMEM containing 10% FBS, 10 ng/mL FGF-2 (BD biosciences), 63.3 μg/mL penicillin G potassium, and 100 μg/mL streptomycin sulfate. The redifferentiation of NHAC was induced by seeding on cell culture inserts (0.4-μm pore size; BD Biosciences) and co-culturing with rat primary chondrocytes previously seeded on 6-well cell culture plates (Thermofisher Scientific, Waltham, MA, USA), in chondrogenic differentiation medium (CDM) for 14 days. DMEM-high glucose containing 0.1% bovine serum albumin (BSA; Wako), 82 μg/mL L-ascorbic acid phosphate magnesium salt n-hydrate, 10 μg/mL insulin (Sigma), 10 μg/mL holo-transferrin (Sigma), 30 nM sodium selenite (Wako), and 10 ng/mL TGF-β1 (Wako) was used as CDM. The co-cultured cells were examined for perlecan mRNA expression and compared to monocultures in CDM. Co-cultures of rat primary chondrocytes and NHACs were routinely conducted at 37°C in a humidified atmosphere containing 5% CO_2. The media was replaced every other day.

Disruption of endogenous HS function

The function of HS chains expressed by chondrocytes was disrupted by addition of 1 mU/mL heparitinase I (Seikagaku, Tokyo, Japan), an HS degrading enzyme, and 100 μg/mL sodium heparin (Nacalai) to the culture medium. At this concentration, heparin interacts with various GF and cytokines leading to the disruption of HS function (Irie et al. 2003; De Wit et

al. 2005; Belleudi et al. 2007; Santiago et al. 2012).

Blocking of endogenous perlecan function and supplementation of exogenous perlecan

Perlecan and anti-perlecan antiserum (anti-Pln) were produced as described previously (Nakamura et al. 2014). Blocking of perlecan function was carried out by adding 1/500 volume of anti-Pln to the culture medium every day. Pre-immune serum (PIS) was added as a control. Purified perlecan was dissolved in PBS and added to the culture medium at 5 μg/mL. PBS was added to the medium as a control.

Morphological analysis of chondrocyte differentiation

For morphological evaluation of chondrocyte differentiation, the roundness of chondrocytes was measured as described by Kino-Oka et al. (2009). Chondrocytes seeded on 96-well plates (Thermo Fisher Scientific) at a density of 0.3×10^4 cells/cm^2 were cultured for 2 days, fixed with 10% formalin, and stained with 1% toluidine blue solution for 1 h. Images of cells in each well were acquired with an EVOS fl AMF-4302 microscope (Advanced Microscopy Group, Bothell, WA, USA) equipped with a ×20 objective. The roundness of an individual cell (Rc) was defined by the following equation:

$$Rc = \frac{2(\pi Ac)^{1/2}}{lc}$$

where the area (Ac) and peripheral length (lc) of each cell were evaluated with ImageJ image analysis software (downloaded from the National

Institutes of Health, Bethesda, MD, USA; available at http://rsb.info.nih.gov/ij). The *Rc* values of more than 100 cells in each well were evaluated. Cells exhibiting an $Rc > 0.9$ were defined as round-shaped, and the frequency of round-shaped cells was calculated.

Determination of glycosaminoglycan content

Chondrocytes seeded at a density of 1.5×10^5 cells/cm^2 on 96-well plates were cultured for 4 days. The cell/ECM layers were collected in sulfuric acid containing 0.95% boric acid, and the uronic acid content was determined by the carbazole reaction method of Bitter and Muir (1962). The glycosaminoglycan (GAG) content was estimated by doubling the uronic acid content.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

NHAC were lysed in Isogen (Wako), and total RNA was isolated according to the manufacturer's instruction. First strand cDNA was synthesized from 1 μg of total RNA using an oligo d(T)18 primer (New England BioLabs, Ipswich, MA, USA) and reverse transcriptase (M-MLV, RNase H+; Wako). For PCR amplification, FastStart Taq DNA Polymerase (Roche Diagnostics, Basel, Switzerland) was used according to the manufacturer's instruction. Specific primers were designed as follows: 5′-CGATGGATTCCAGTTCGAGTA-3′ and 5′-GTTTACACGAAGCAGGCAGG-3′ for type I collagen (*COL1A1*); 5′-GGAGACTACTGGATTGA-3′ and 5′-GCGTGAGGTCTTCTGTG-3′ for type II collagen (*COL2A1*); 5′-TCCAGGTAAAGTTGGTCCTCCG-3′ and

5′-AAGAAATCCGCACTCCCTTCC-3′ for type IX collagen (*COL9A2*); 5′-GGTCCAGGTGGAAAACGAAAC-3′ and 5′-CTCTCACTTCTATCGAGGGGT-3′ for type XI collagen (*COL11A1*); 5′-TGGACACATTCGTACCTTTCTGA-3′ and 5′-ACTCCGACTCCAGCGTGTCT-3′ for perlecan (*HSPG2*); and 5′-GGTGAAGGTCGGTGTCAACGGATT-3′ and 5′-GATGCCAAAGTTGTCATGGATGACC-3′ for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The resulting PCR products were electrophoresed on 2% agarose gels and stained with 0.5 μg/mL ethidium bromide (Life Technologies, Carlsbad, CA, USA).

Immunofluorescence and Alcian blue staining

Chondrocytes seeded at a density of 0.3×10^4 or 1.5×10^5 cells/cm^2 were cultured for 4 days and fixed with 10% formalin. The cells were incubated with the following primary antibodies at 4°C over night: rabbit anti-rat collagen type I (×100 dilution), rabbit anti-mouse collagen type II (×100 dilution) (all purchased from LSL, Tokyo, Japan), and anti-Pln (×80 dilution). Subsequently, cells were then stained fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G (×200 dilution; MP Biomedicals, Solon, OH, USA) and 4',6-diamidino-2-phenylindole (DAPI; ×500 dilution; Dojindo Laboratories, Kumamoto, Japan) at 37°C for 30 min. Fluorescent images were acquired using an inverted fluorescence microscope (EVOS xl core AMEX-1200; Advanced Microscopy Group). Because differentiated chondrocytes were embedded in a dense GAG matrix, which precludes

reaction of antibodies with pericellular ECM components, cells cultured at high density were treated with PBS containing 1% hyaluronidase at 37°C for 30 min before exposure to primary antibodies. Following formalin fixation, cells were stained with 1% Alcian blue dissolved in 3% acetic acid and observed with an EVOS fl AMF-4302 microscope.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Chondrocytes seeded at a density of 1.5×10^5 cells/cm^2 on 12-well plates were cultured for 6 days. The layer of ECM and chondrocytes was collected in a 0.5 N NaOH solution, and protein concentration was measured using the Biuret method (Gorall et al. 1949). Samples containing equal amounts of protein were subjected to SDS-PAGE with 5% polyacrylamide gels. For western blotting analysis, separated proteins were blotted onto nitrocellulose membranes, which were rinsed with PBS containing 0.5% Nonidet P-40 and then with PBS containing 0.05% Tween 20 (T-PBS; pH 7.4). Membranes were blocked with T-PBS containing 2% casein (Wako) and 1% BSA and then incubated with primary antibodies. The membranes were washed with T-PBS three times and exposed to horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, IgA, and IgM (MP Biomedicals). HRP was detected with 3,3′-diaminobenzidine (Dojindo Laboratories) and hydrogen peroxide (Wako). For detection of high molecular weight proteoglycans, such as aggrecan and hyaluronan, the gels were stained with Coomassie brilliant blue and then with Alcian blue. Collagens secreted into the medium were extracted by

adding 1/3 volume of saturated ammonium sulfate solution and were analyzed by western blotting.

Statistical analysis

Data are shown as means ± SD. Student's *t*-test was applied to determine the statistical significance of differences between groups, and $p < 0.05$ was considered significant.

RESULTS

Disruption of HS prevents chondrocyte dedifferentiation

To determine whether HSPG inhibited chondrocyte differentiation *in vitro*, HS function was disrupted by exposing cell cultures to heparitinase I and a high concentration of heparin. Rat primary chondrocytes cultured at a low density in control medium for 2 days began to dedifferentiate (Figs. 1a, 2a). Some cells retained a spherical, differentiated morphology and were intensely stained with toluidine blue. Conversely, other cells showed a dedifferentiated morphology, characterized by a flattened appearance, and faint toluidine blue staining. Exposure of chondrocyte cultures to 1 mU/mL heparitinase I or 100 μg/mL sodium heparin for 2 days increased the number of spherical cells (Figs. 1b, 2b) and the frequency of round-shaped cells (Figs. 1c, 2c).

After 2 days of culture at a high density in control medium, chondrocytes exhibited either an elongated or spherical appearance, and number of cells presenting either morphologies increased after 4 days (Figs. 3a, c, 4a, c). These observations suggest that dedifferentiation and differentiation were occurring in the culture system. Cultures treated with heparitinase I or heparin exhibited a higher number of spherical cells (Figs. 3b, d, 4b, d) and higher levels of GAG accumulation than control cultures (Figs. 3e, 4e). SDS-PAGE analysis revealed that high molecular weight PG increased in the cell/ECM layer of heparin-treated cells (Fig. 4f).

Perlecan expression by dedifferentiated chondrocytes

Perlecan is an essential HSPG for cartilage development that is upregulated in cartilage from OA patients (Tesche & Miosge 2004). Therefore, we examined whether perlecan is upregulated during chondrocyte dedifferentiation *in vitro*. NHAC fully dedifferentiated by *in vitro* expansion were induced to differentiate by co-culture with rat primary chondrocytes. Type II, IX, and XI collagen mRNA levels were higher in co-cultured NHAC than in cultures of NHAC without other cells (Fig. 5). Conversely type I collagen mRNA expression was not different between co-cultures and monocultures (Fig. 5). These results indicate that co-culture of NHAC with rat primary chondrocytes induced NHAC redifferentiation. Perlecan mRNA expression was low in the co-cultured NHAC, indicating that dedifferentiation increases perlecan mRNA expression in chondrocytes.

The distribution of perlecan in dedifferentiated and differentiated rat chondrocytes was examined by immunofluorescence. Cells cultured at a low density for 4 days showed extensive dedifferentiation (Fig. 6a). Morphology was flattened and elongated, and Alcian blue staining was not observed in the ECM, although it was present in the nucleus. Type II collagen staining was faint (Fig. 6c), but type I collagen staining revealed the presence of an extensive extracellular network (Fig. 6e). In dedifferentiated cells, perlecan staining indicated the presence of a fine extracellular network (Fig. 6g). Primary high-density chondrocytes cultures presented spherical cells intensely stained with Alcian blue and elongated cells exhibiting faint Alcian blue staining (Fig. 6b). Without pre-treatment with hyaluronidase, spherical

cells exhibited faint type II collagen and perlecan staining, whereas staining for type I collagen and perlecan revealed the presence of an extracellular network surrounding the elongated cells (Supplemental data Fig. S1). Staining for type II collagen in hyaluronidase-treated cells was intense around spherical cells (Fig. 6d). Type I collagen staining exposed a complex network in the proximity of elongated cells, but was not observed in the surroundings of spherical cells (Fig. 6f). Similar to findings in rat normal cartilage (Melrose et al. 2006), perlecan was detected around the spherical, differentiated cells, whereas it formed a network connecting elongated cells (Fig. 6h).

Suppression of perlecan function prevents chondrocyte dedifferentiation

To determine whether perlecan is involved in the dedifferentiation of chondrocytes, perlecan function was suppressed with anti-Pln. Rat primary chondrocytes cultured at a low density in the presence of anti-Pln for 2 days appeared less elongated than cells cultured with PIS (Fig. 7a, b). The frequency of round-shaped cells was higher in the context of perlecan inhibition than under basal conditions (Fig. 7c). Perlecan inhibition had no effect on cellular morphology in chondrocytes cultured at a high density for 4 days (Fig. 8a, b). However, perlecan-blocking insignificantly increased GAG accumulation by chondrocytes cultured at a high density (Fig. 8c). The addition of bovine kidney perlecan to the culture medium did not decrease GAG accumulation by chondrocytes (data not shown), probably because the amount of endogenous perlecan expressed by chondrocytes was sufficient.

Although anti-Pln, unlike heparitinase I and heparin, did not have statistically significant effects on frequency of round-shaped cells and GAG accumulation, the possibility that perlecan inhibits chondrocyte differentiation *in vitro* cannot be excluded. After 6 days of culture, the number of spherical cells was higher in the context of perlecan inhibition than under basal conditions (Fig. 9a, b). Type I and II collagen expression was analyzed by western blotting (Fig. 9c, d). Type I collagen bands of similar sizes and intensities were observed in media and cell/ECM layers from cultures exposed to anti-Pln and from control cultures. More than 5 bands for collagen type II were detected in the media and in the cell/ECM layer samples from control and experimental cultures. In accordance with a report by Aubert-Foucher (2014), the bands between 116 and 200 kDa were identified as the α1 chains of procollagen type IIA/D [Pro-α1(II)A/D] and IIB [Pro-α1(II)B], the C-terminal-processed forms of Pro-α1(II)A/D [P_N-α1(II)A/D] and Pro-α1(II)B [P_N-α1(II)B], the N-terminal-processed form of Pro-α1(II)A/B/D [P_C-α1(II)], and the α1 chain of mature collagen type IIA/B/D [α1(II)]. The levels of the type II collagen precursors [Pro-α1(II)A/D and P_N-α1(II)B] in the media were higher in the presence than in the absence of anti-Pln. Levels of precursor and mature type II collagen levels were higher in the cell/ECM layer of cultures in the context of perlecan inhibition than under basal conditions. These findings suggest that perlecan inhibition increases both *de novo* and net type II collagen synthesis. In addition, an increase in the levels of the procollagen type IIB isoform, which is predominantly expressed in mature chondrocytes (Gouttenoire et al. 2004),

1 was observed in the presence of anti-Pln. Finally, Alcian blue staining
2 revealed higher levels of high molecular weight PG in the cell/ECM layer in
3 the context of perlecan inhibition than under basal conditions (Fig. 9d).
4

DISCUSSION

Although perlecan and other HSPG are important for cartilage development, our data indicate that these molecules inhibit chondrocyte differentiation *in vitro*.

Disruption of HS chains and perlecan function either prevented dedifferentiation or promoted differentiation of chondrocytes either cultured at a low or a high density, respectively. Previous studies have reported that endogenous perlecan promotes the chondrogenic effects of BMP-2 on the C3H10T1/2 mesenchymal cell line (Yang et al. 2006). In contrast, our results suggest that endogenous HSPG preferentially mediate extracellular signaling events that inhibit chondrocyte differentiation in the absence of strong chondrogenic factors, such as BMP. Because HS chains of perlecan and other HSPG interact with various molecules (Knox & Whitelock 2006), the identity of the factors interacting with HSPG in cultured chondrocytes is elusive. Dedifferentiated chondrocytes express low levels of chondrogenic BMP-2 and BMP-4, and exhibit activation of canonical Wnt signaling, which promotes dedifferentiation (Yuasa et al. 2008; Cheng et al. 2012). Consequently, these observations suggest that changes in the expression pattern of HS-binding molecules during chondrocyte dedifferentiation contribute to further advance this process.

The mechanisms whereby HSPG disrupted chondrocyte differentiation under high density culture conditions are not apparent, since these culture conditions are commonly chondrogenic. One possible explanation is that high

density PG aggregates define microenvironments that either favor differentiation or dedifferentiation. We showed that chondrocytes cultured at a high density contained both dedifferentiated and differentiated cell populations. The differentiated population was observed in association with high-density PG aggregates, which prevented infiltration of the antibodies used for immunofluorescent staining. Therefore, PG aggregation might prevent contact with factors that promote dedifferentiation, such as selected Wnt molecules (Yuasa et al. 2008) secreted by dedifferentiated cells. Moreover, because perlecan contains negatively charged GAG chains (Knox & Whitelock 2006), we speculate that high-density PG aggregates repel perlecan expressed by dedifferentiated chondrocytes. Negatively charged molecules do not readily penetrate the cartilage ECM (Bansal et al. 2011). Therefore, it is probable that perlecan expressed by dedifferentiated chondrocytes preferentially mediates reactions occurring in proximity of these cells.

Therapeutic antibodies are utilized for the treatment of various diseases. In 2011, the global market for these drugs was reported to be valued about 44.6 billion dollars (BCC research 2012). Intravenous injection of Tanezumab, an antibody against nerve growth factor, improved pain of OA in a phase III clinical trial (Spierings et al. 2013). Moreover, an antibody against syndecan-4 has been reported to prevent OA progression in the mouse knee. Our results and recent reports demonstrating that HSPG contribute to chondrocyte dedifferentiation suggest that antibodies against perlecan or HSPG are candidate drugs to prevent or correct cartilage degeneration.

Autologous chondrocyte implantation requires *in vitro* preparation of well-differentiated chondrocytes, so that disruption of endogenous perlecan/HSPG function in culture may provide a suitable cell source for this procedure.

Although it remains unclear whether OA induces perlecan or other HSPG in any jointsother than knee, endosulfatase-1 and -2 prevented knee and temporomandibular OA progression (Otsuki et al. 2010). Therefore, disruption of HSPG function might be effective for treatment of OA irrespective of joint location.

In conclusion, perlecan inhibits chondrocyte differentiation in a dedifferentiative environment. Inhibiting the function of perlecan and/or other HSPG may promote chondrocyte differentiation.

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Figure 1 Heparitinase treatment prevents chondrocyte dedifferentiation. Rat primary chondrocytes seeded at a density of 0.3×10^4 cells/cm^2 were cultured in the presence or absence of 1 mU/mL heparitinase I for 2 days and stained with toluidine blue. Representative images of control (a) and heparitinase-treated (b) cells. Bars, 50 μm. Frequency of round-shaped cells (c). Data represent means ± SD (n = 3). *, $P < 0.05$.

Figure 2 Heparin treatment prevents chondrocyte dedifferentiation. Rat primary chondrocytes seeded at a density of 0.3×10^4 cells/cm^2 were cultured in the presence or absence of 100 μg/mL heparin for 2 days and stained with toluidine blue. Representative images of control (a) and heparin-treated (b) cells. Bars, 50 μm. Frequency of round-shaped cells (c). Data represent means ± SD (n = 3). *, $P < 0.05$.

Figure 3 Heparitinase treatment increases GAG accumulation in chondrocytes. Rat primary chondrocytes seeded at a density of 1.5×10^5 cells/cm^2 were cultured in the presence or absence of 1 mU/mL heparitinase I for 4 days. Representative phase contrast microscopy images of control (a, c) and heparitinase-treated (b, d) cells. Bars, 50 μm. GAG accumulation in the cell/ECM layers was assessed using the carbazole reaction method (e). Data represent means ± SD (n = 3). *, $P < 0.05$.

Figure 4 Heparin treatment increases GAG accumulation in chondrocytes. Rat primary chondrocytes seeded at a density of 1.5×10^5 cells/cm^2 were

cultured in the presence or absence of 100 μg/mL heparin for 4 days. Representative phase contrast microscopy images of control (a, c) and heparin-treated (b, d) cells. Bars, 50 μm. GAG accumulation in the cell/ECM layers was assessed using the carbazole reaction method (e). Data represent means ± SD (n = 3). *, $P < 0.05$. The accumulation of high molecular weight PG in the cell/ECM layers of control (lane 1), and heparin-treated (lane 2) cells was examined by SDS-PAGE (f). High molecular weight PG stained with Alcian blue are indicated by an arrow.

Figure 5 Perlecan mRNA expression increases in dedifferentiated NHAC. NHAC were cultured independently (lane 1) or co-cultured with rat primary chondrocytes (lane 2) for 14 days, and the mRNA levels of collagen type II (*COL2A1*), type IX (*COL9A2*), type XI (*COL11A1*), type I (*COL1A1*), and perlecan (*HSPG2*) were examined by RT-PCR. *GAPDH* expression was used as an internal control.

Figure 6 Distribution of perlecan in dedifferentiated and differentiated chondrocytes. Rat primary chondrocytes seeded at a density of 1.2×10^4 cells/cm^2 (low density; a, c, e, g) and 1.5×10^5 cells/cm^2 (high density; b, d, f, h) were cultured for 4 days. GAG was stained with Alcian blue (a, b). Collagen type II (c, d), collagen type I (e, f), and perlecan (g, h) were stained with specific antibodies. Bars, 50 μm.

Figure 7 The effect of perlecan inhibition on dedifferentiating chondrocytes

morphology. Rat primary chondrocytes seeded at a density of 0.3×10^4 cells/cm^2 on 96-well plates were cultured with PIS or anti-Pln and stained with toluidine blue. Representative images of PIS-treated (a) and anti-Pln-treated (b) cells. Bars, 50 μm. Frequency of round-shaped cells (c). Data in c represent means ± SD (n = 3).

Figure 8 The effect of perlecan inhibition on GAG accumulation in differentiating chondrocytes. Rat primary chondrocytes seeded at a density of 1.5×10^5 cells/cm^2 were cultured for 4 days, and perlecan was blocked in the last 2 days. Representative phase contrast microscopy images of cells cultured with PIS (a) and anti-Pln (b). Bars, 50 μm. GAG accumulation in the cell/ECM layers was assessed using the carbazole reaction method (c). Data represent means ± SD (n = 3).

Figure 9 Perlecan inhibition increased expression of cartilaginous ECM by chondrocytes. Rat primary chondrocytes seeded at a density of 1.5×10^5 cells/cm^2 were cultured for 6 days, and perlecan was blocked in the final 4 days of culture. Representative phase contrast microscopy images of cells treated with PIS (a) or cultured under conditions of perlecan function inhibition (b); arrows indicate flattened cells. Bars, 50 μm. The expression of cartilaginous ECM components in cells treated with PIS (lane 1) or cultured under conditions of perlecan function inhibition (lane 2) was examined by SDS-PAGE and western blotting (c, d). Levels of collagen type I (Col I) and type II (Col II) in the media between 5 and 6 days of culture (c), and in the

cell/ECM layers (d) were examined by western blotting. GAG accumulation in the cell/ECM layers was examined by staining SDS-PAGE gels with Alcian blue (AB) (d). An arrow indicates high molecular weight PG stained with Alcian blue.

Fig1

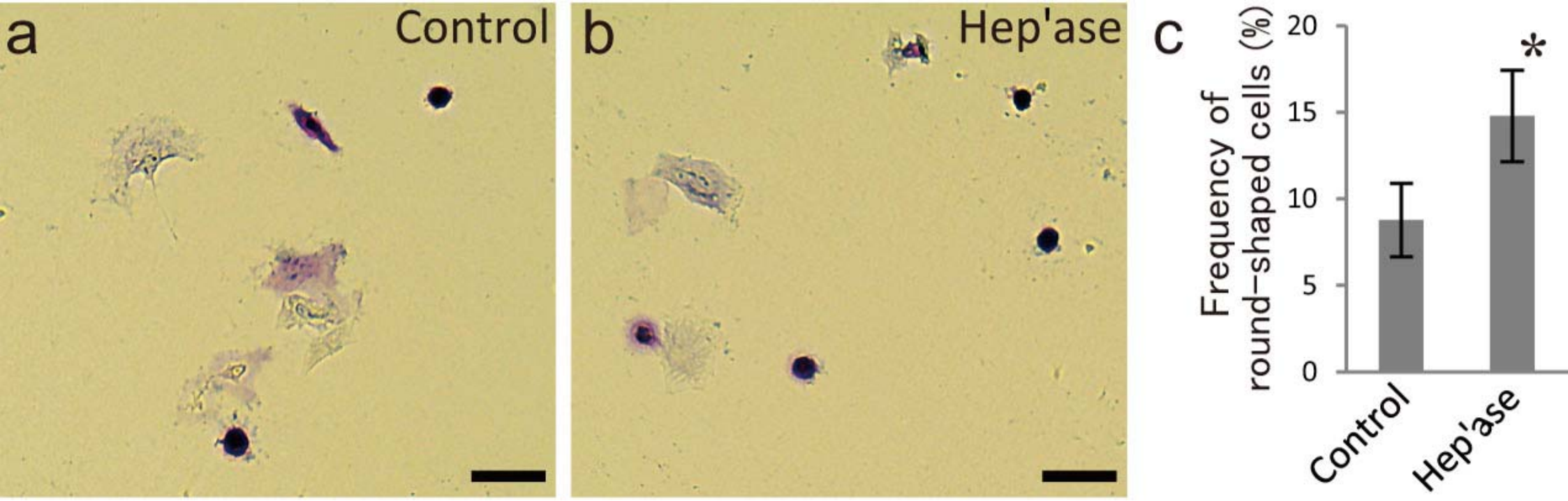

a
Control
b
Hep'ase
c
Frequency of round-shaped cells (%)
20
15
10
5
0
*
Control
Hep'ase

Fig2

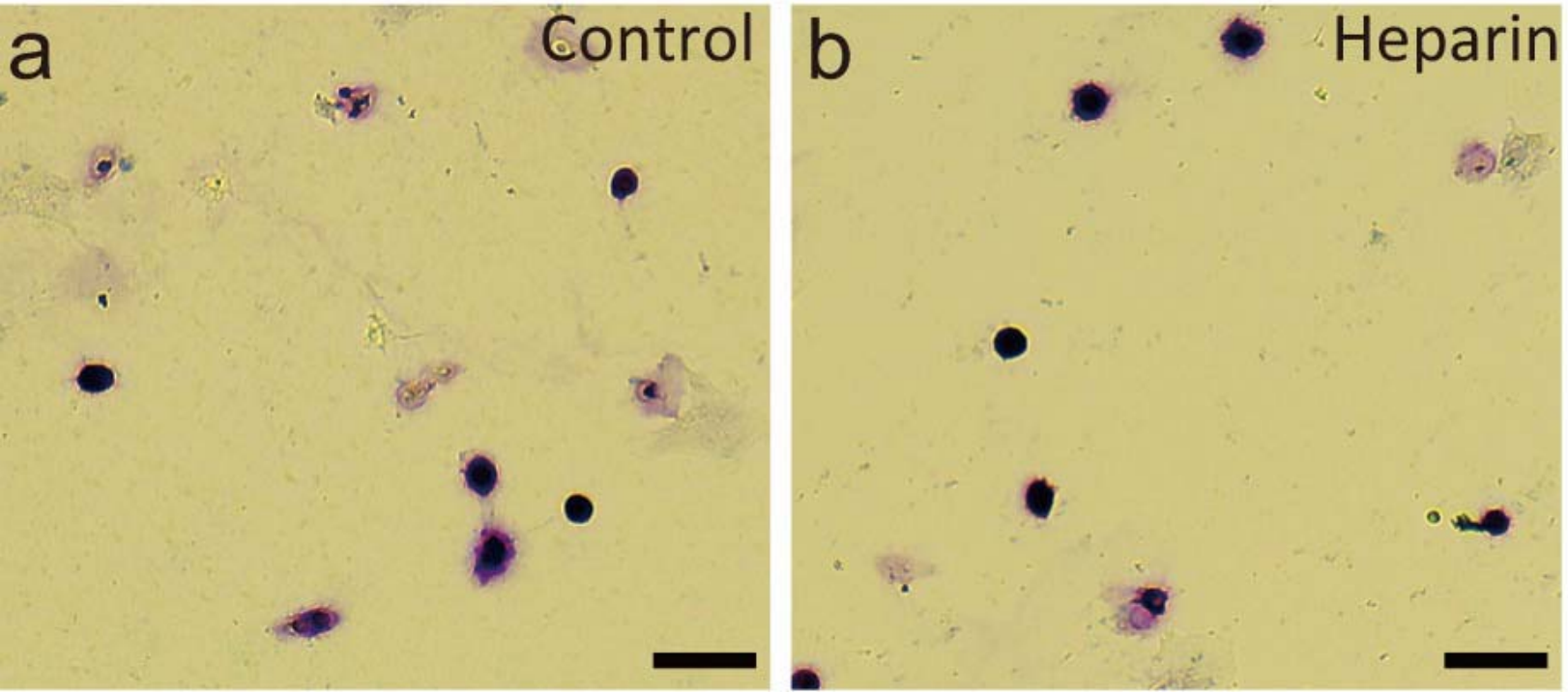
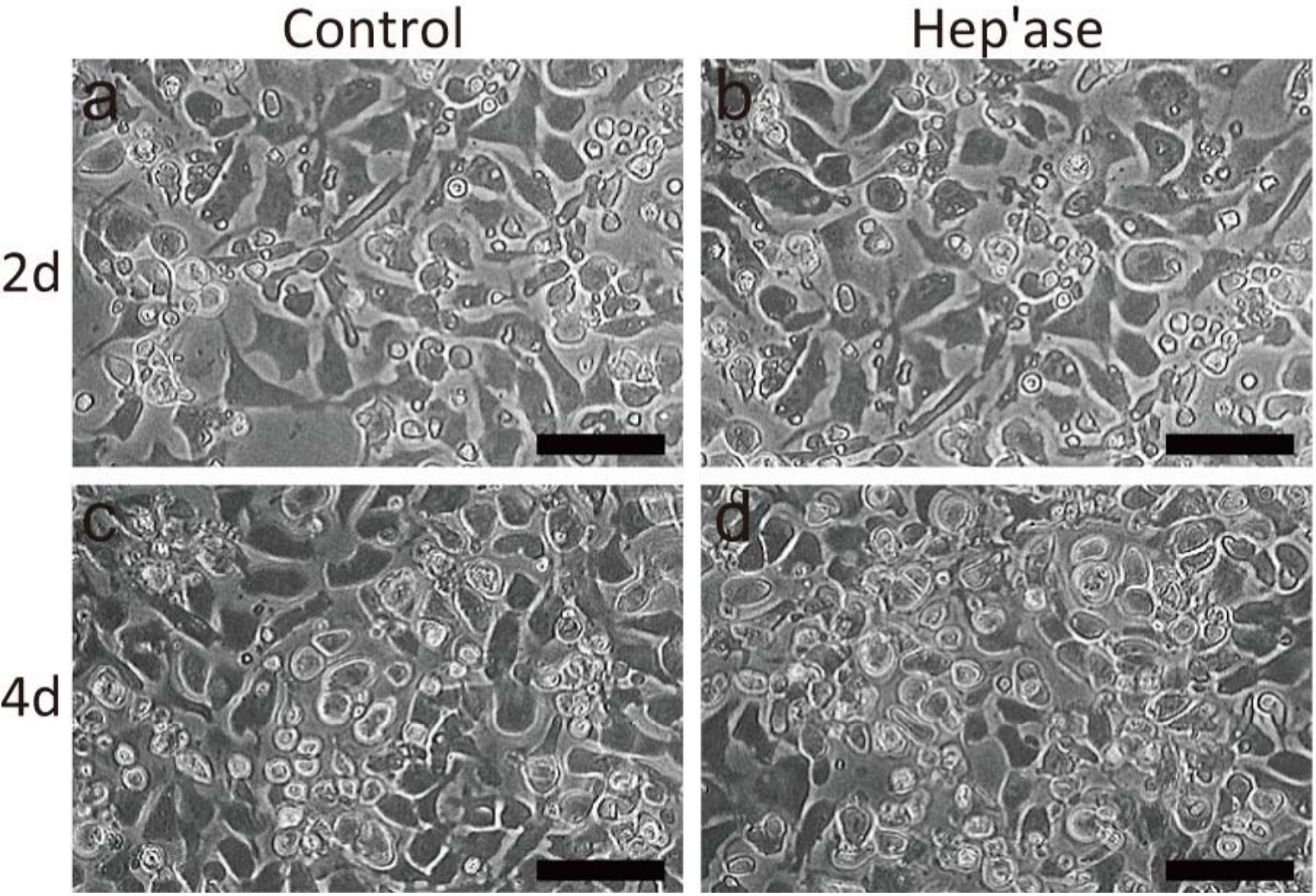

a
Control
b
Heparin
c
Frequency of round-shaped cells (%)
80
60
40
20
0
*
Control
Heparin

Fig3


Control
Hep'ase
a
b
c
d
2d
4d

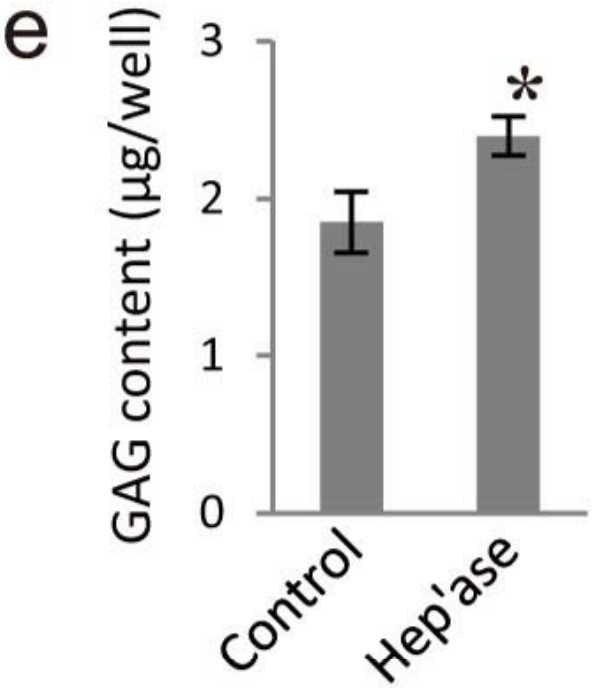
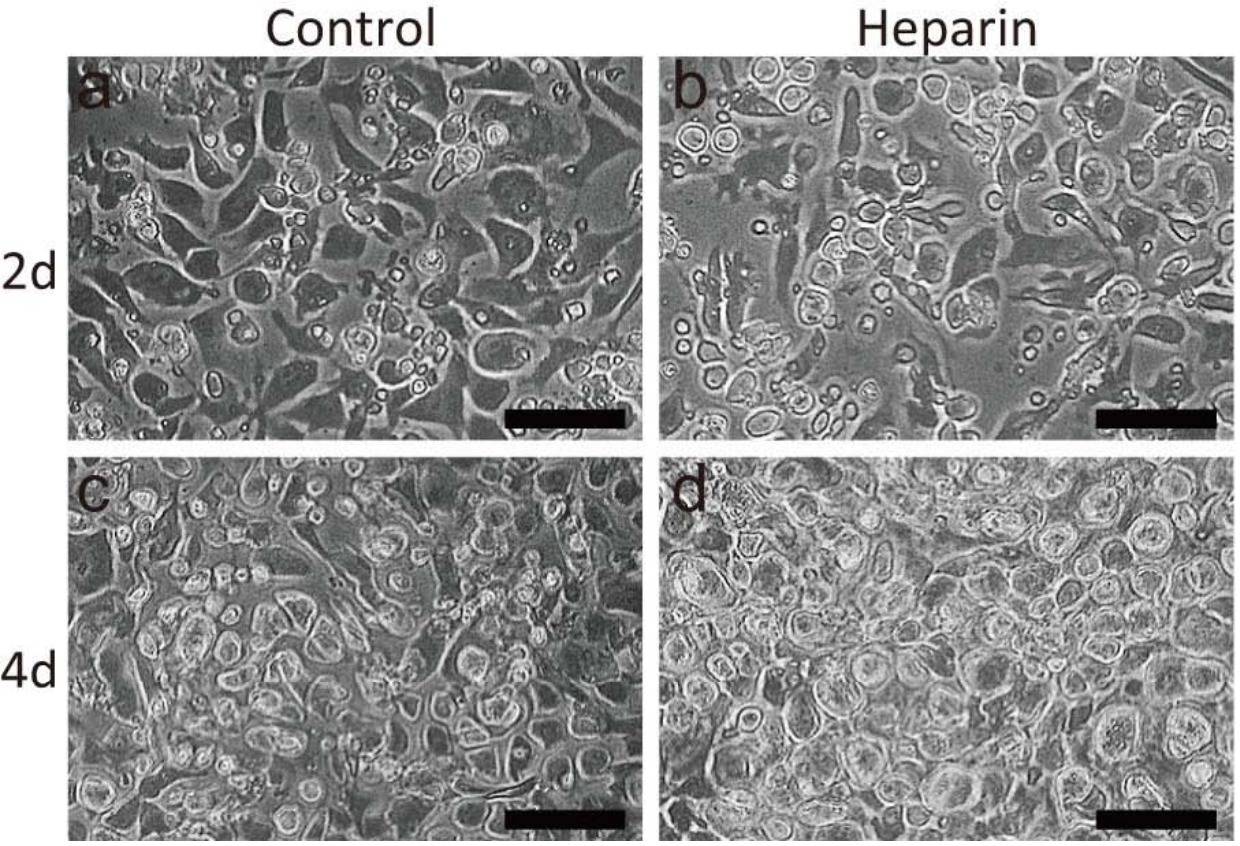
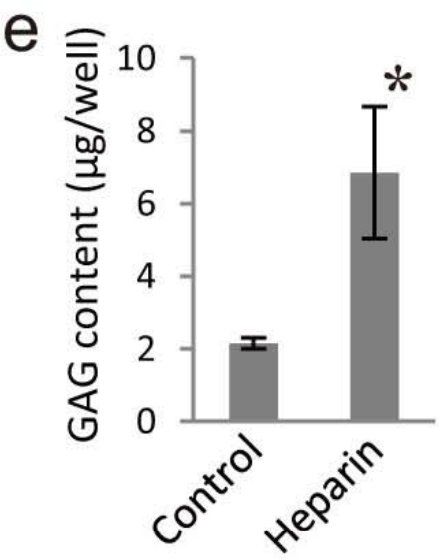

e
GAG content (μg/well)
3
2
1
0
*
Control
Hep'ase

Fig4


Control
Heparin
a
b
c
d
2d
4d


e
GAG content (μg/well)
10
8
6
4
2
0
*
Control
Heparin

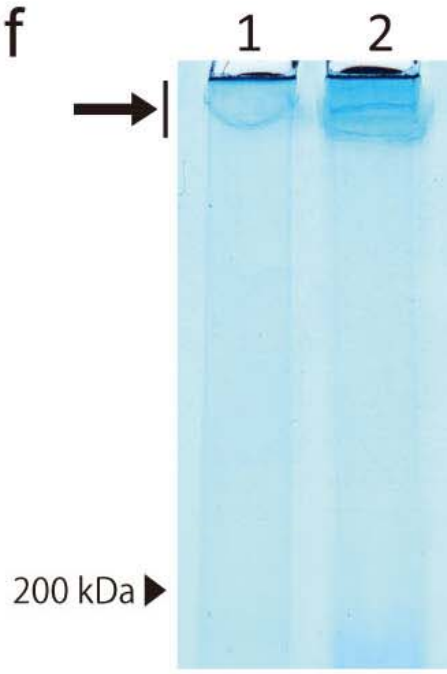
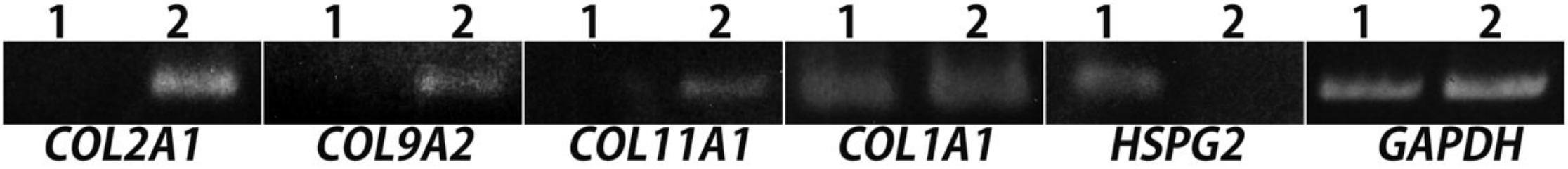

f
1
2
200 kDa

Fig5



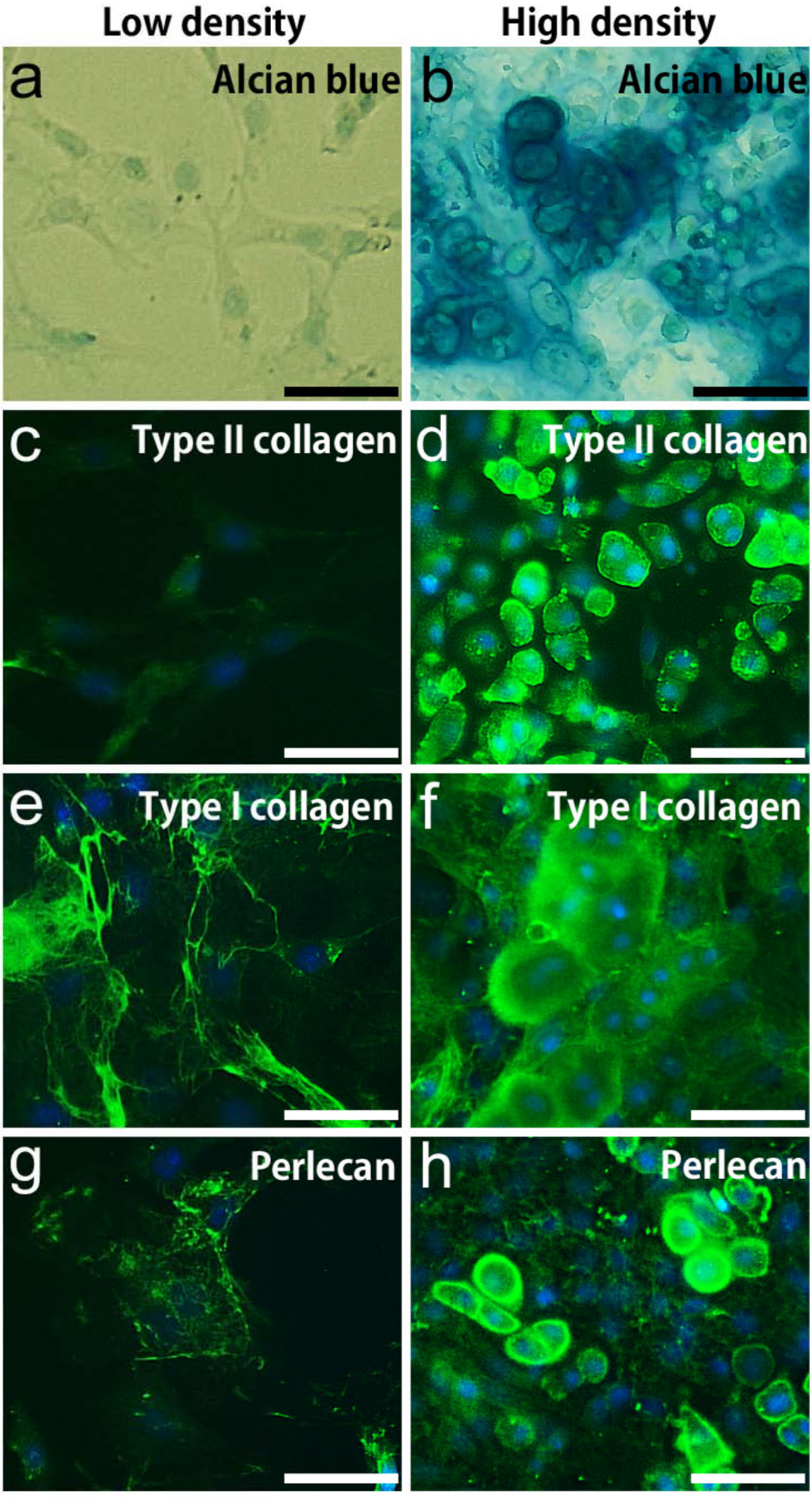
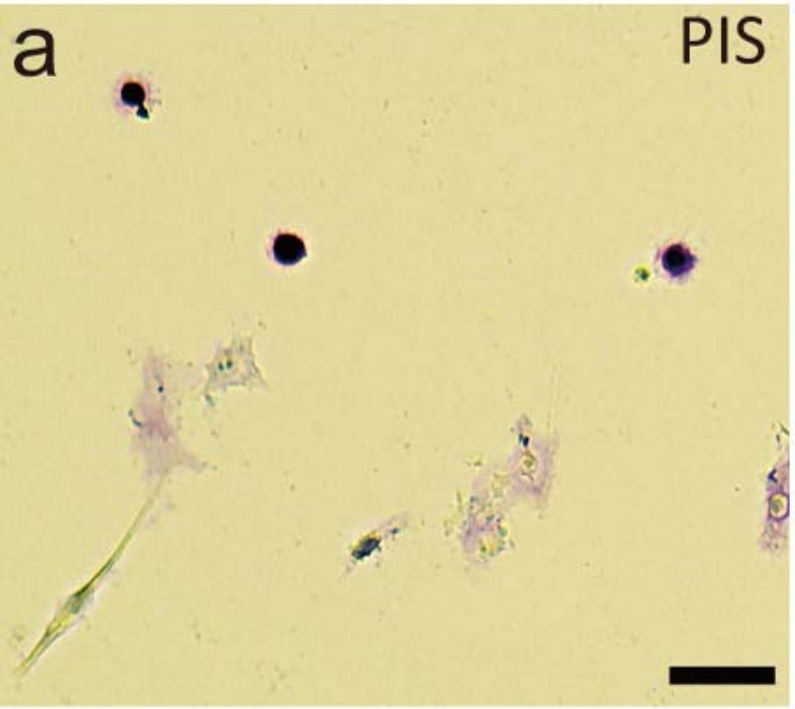
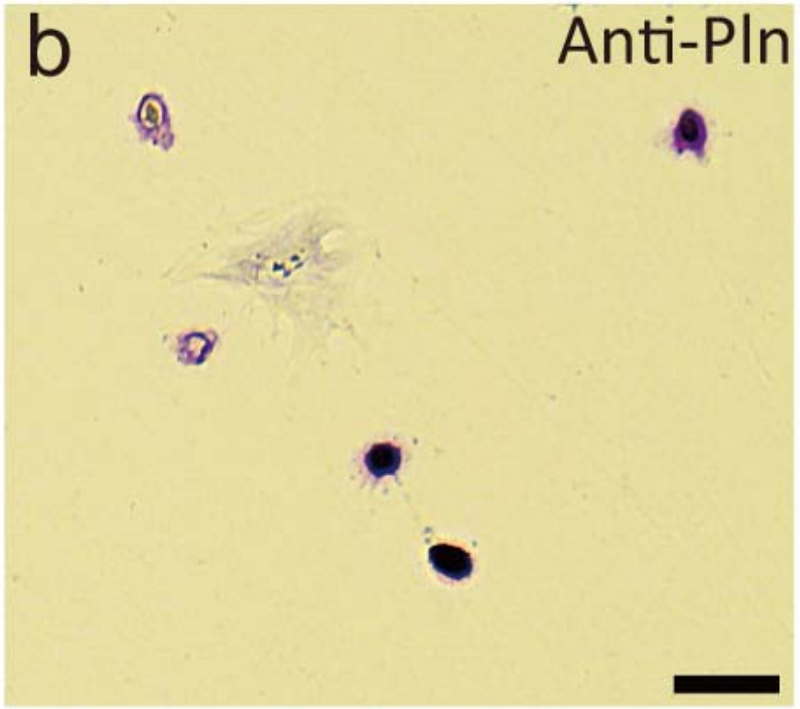
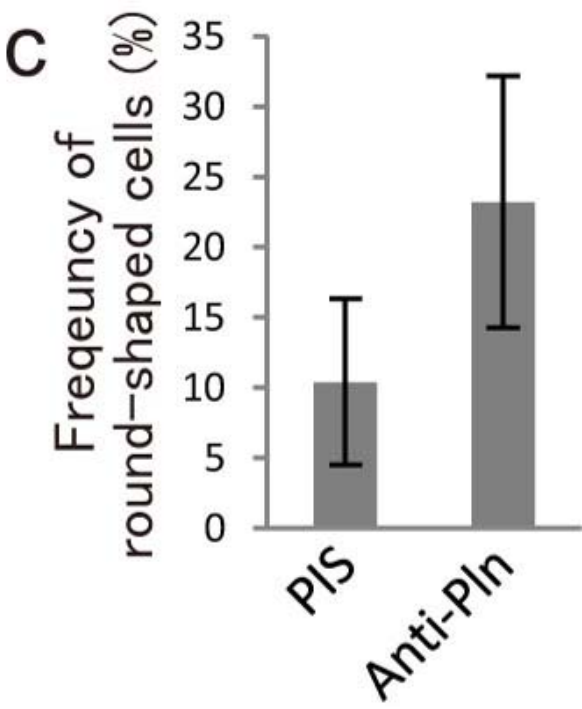

Low density
High density
a
Alcian blue
b
Alcian blue
c
Type II collagen
d
Type II collagen
e
Type I collagen
f
Type I collagen
g
Perlecan
h
Perlecan

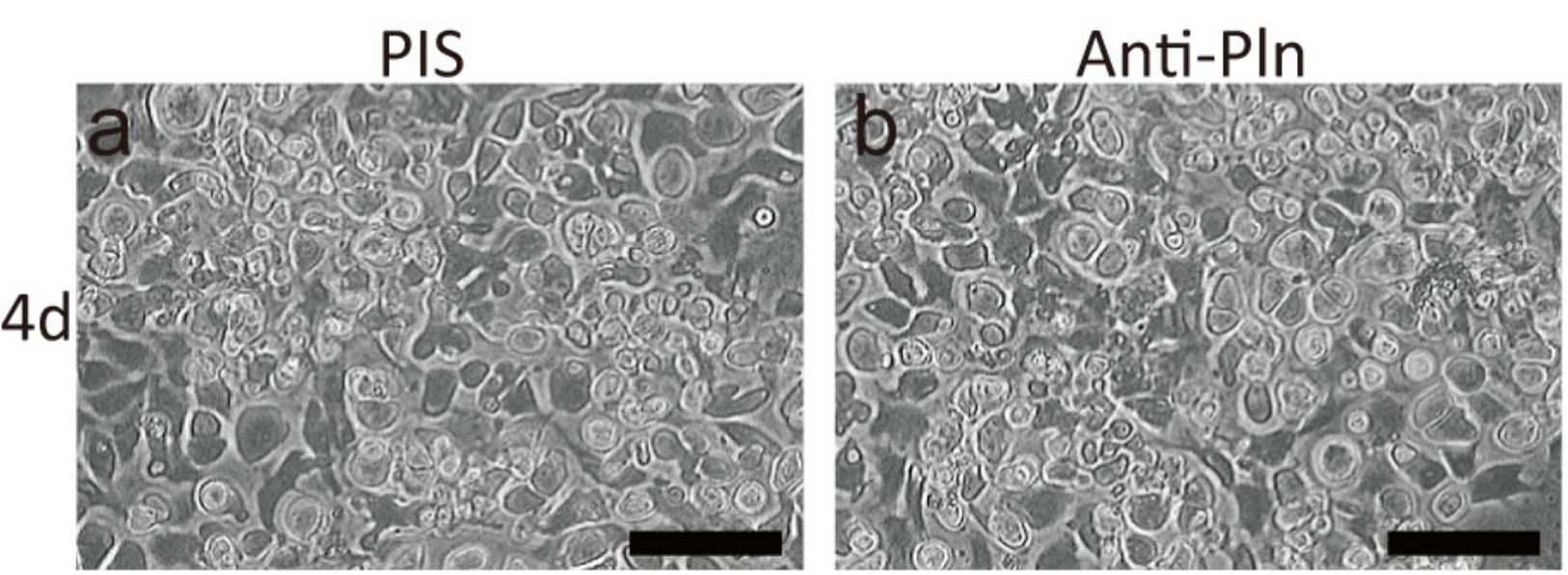
Fig6

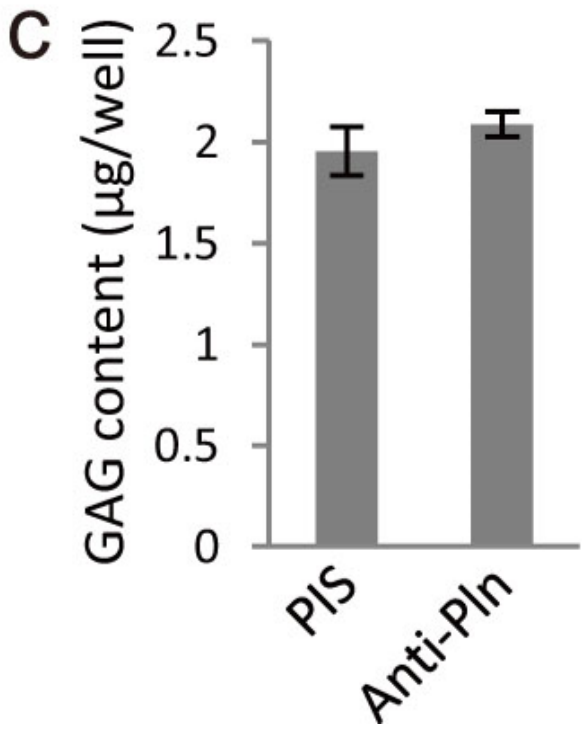
Fig7

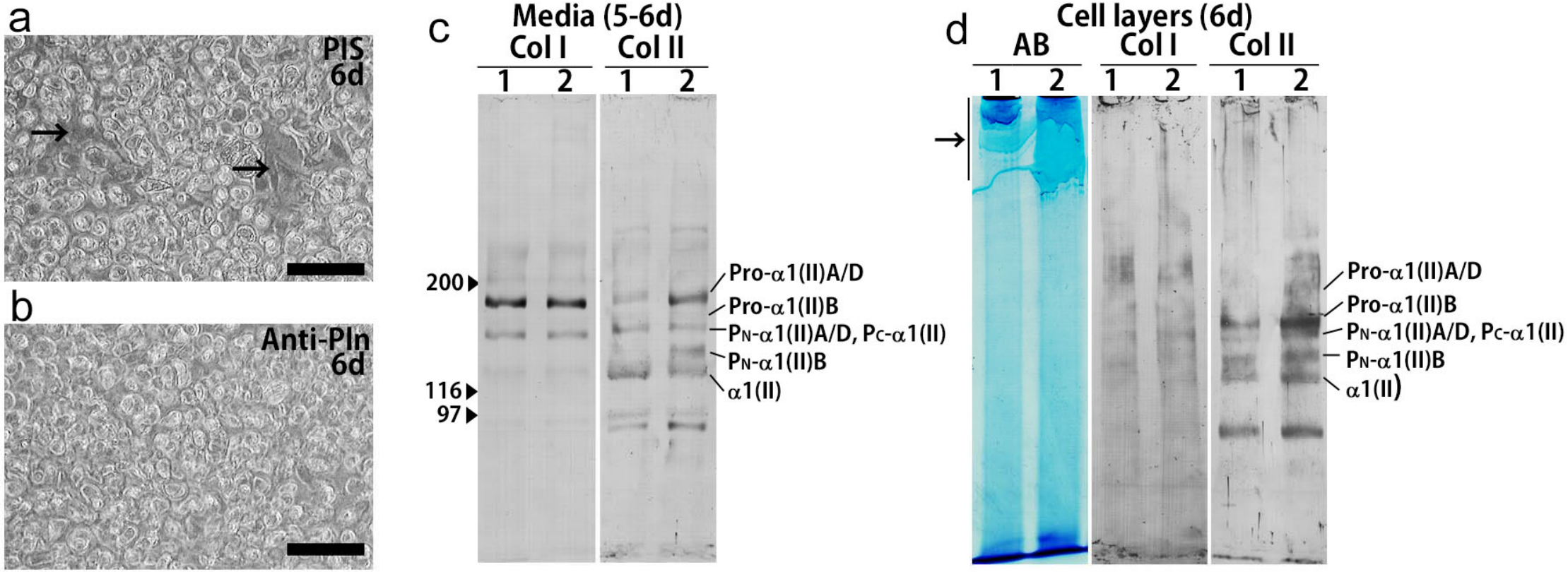

a
PIS


b
Anti-Pln


c
Freqeuncy of round-shaped cells (%)
35
30
25
20
15
10
5
0
PIS
Anti-Pln


PIS
Anti-Pln
a
b
4d


c
GAG content (μg/well)
2.5
2
1.5
1
0.5
0
PIS
Anti-Pln


a
PIS
6d
b
Anti-PIn
6d
c
Media (5-6d)
Col I
Col II
1
2
1
2
200
116
97
Pro-α1(II)A/D
Pro-α1(II)B
PN-α1(II)A/D, PC-α1(II)
PN-α1(II)B
α1(II)
d
Cell layers (6d)
AB
Col I
Col II
1
2
1
2
1
2
Pro-α1(II)A/D
Pro-α1(II)B
PN-α1(II)A/D, PC-α1(II)
PN-α1(II)B
α1(II)