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Influence of Cyclic Strain and Decorin Deficiency on 3-D Cellularized Collagen Matrices

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Abstract

Cyclic strain evokes the expression of the small leucine-rich proteoglycans decorin and biglycan in 2-D cultures and native tissues. However, strain dependent expression of these proteoglycans has not been demonstrated in engineered tissues. We hypothesized that the absence of decorin may compromise the effect of cyclic strain on the development of engineered tissues. Thus, we investigated the contribution of decorin to tissue organization in cyclically-strained collagen gels relative to statically-cultured controls. Decorin null (*Dcn*^{-/-}) and wild-type murine embryonic fibroblasts were seeded within collagen gels and mechanically conditioned using a Flexcell[®] Tissue Train[®] culture system. After eight days, the cyclically-strained samples demonstrated greater collagen fibril density, proteoglycan content, and material strength for both cell types. On the other hand, increases in cell density, collagen fibril diameter, and biglycan expression were observed only in the cyclically-strained gels seeded with *Dcn*^{-/-} cells. Although cyclic strain caused an elevation in proteoglycan expression regardless of cell type, the type of proteoglycan differed between groups: the *Dcn*^{-/-} cell-seeded gels produced an excess of biglycan not found in the wild-type controls. These results suggest that decorin-mediated tissue organization is strongly dependent upon tissue type and mechanical environment.

1. Introduction

Decorin is a small-leucine rich proteoglycan (SLRP) that “decorates” collagen fibrils and mediates collagen fibrillogenesis in native and engineered tissues [1]. Decorin consists of a core protein of approximately 40 kDa and a single glycosaminoglycan (GAG) chain of repeating chondroitin/dermatan sulfate disaccharides [2,3]. Decorin binds to a variety of collagens, including types I, II, III, VI, and XIV [4,5], via attachment sites on its protein core. In addition, the GAG chains from the collagen-bound decorin extend out from the fibril surface and connect to neighboring fibrils, thereby forming interfibrillar bridges [6]. Decorin regulates collagen fibril diameter [7] and promotes tighter collagen fibril packing within the tissue. For

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this reason, decorin is believed to improve the mechanical integrity and strength of connective tissues.

Decorin is likely to be involved in the organization of cyclically strained engineered tissues, due to its altered expression in mechanically stimulated 2-D cultures and native tissues, as well as its interactions with other components of the extracellular matrix [8]. The application of mechanical stimulation to 3-D cultures (whether these are engineered tissues intended for regenerative medicine or simpler models intended for mechanobiology studies) is intended to mimic physiological conditions since most native tissues bear varying levels of strain, which significantly regulates their matrix composition and microstructure. Indeed, various engineered tissues such as heart valves, vascular tissues, and cartilage are commonly grown under physiologically-mimicking mechanical conditions in an attempt to achieve native tissue-like properties [9–11]. Similarly, collagenous matrices that have been grown under mechanical stimulation demonstrate improvements in cell alignment and collagen fibrillar orientation and packing [12]. In addition, several studies have shown that 2-D cell cultures and native tissues under cyclic strain will increase the synthesis of different matrix molecules, including type I collagen and small proteoglycans (PGs) such as decorin and biglycan [8,13,14]. Interestingly, a few studies have reported that mechanical stimulation can produce opposite trends in the expression of these PGs, i.e., reduced expression of decorin but increased expression of the related SLRP biglycan was observed in mechanically stimulated 2-D cultures and native tissues [8,15]. These few reports, however, have not presented conclusive data about the influence of mechanical stimulation on the expression and function of decorin. Similarly, even though increased total GAG expression has been reported in mechanically stimulated 3-D engineered tissues [8], alteration in specific PG expression and their contribution towards engineered tissue organization and material behavior has not been extensively investigated.

Because decorin sequesters transforming growth factor beta (TGF- β), we recently investigated the maturation of collagen gels grown from decorin deficient cells. In the absence of decorin, unbound TGF- β improved the contraction, organization, and material behavior of the collagen matrix in decorin knockout ($Dcn^{-/-}$) cell-seeded collagen gels grown under static tension [16]. This same influence was not apparent in the control gels grown with wild-type cells, in which the endogenously produced TGF- β was sequestered by the cell secreted decorin, but the exogenous addition of TGF- β made the characteristics of the control gels similar to those of the $Dcn^{-/-}$ cell-seeded gels. Given that expression of collagen, PGs, and TGF- β is mechanosensitive [8,13,14,17], it is unclear how mechanical stimulation would influence the organization of collagen matrices in the absence of decorin. Therefore, in this paper, collagen matrices containing embryonic fibroblasts from $Dcn^{-/-}$ and wild-type control mouse embryos were used as a platform to investigate the effects of the presence or absence of decorin and cyclic mechanical strain on matrix organization and material behavior. These cell-seeded collagen matrices were not intended for use in a regenerative medicine capacity, but rather to examine the general mechanobiological effects of decorin.

2. Materials and methods

2.1. Cell isolation and collagen gel preparation

Primary cell culture was isolated from euthanized 12.5 to 14.5 gestational day old wild-type or $Dcn^{-/-}$ mouse embryos, using established protocol for feeder cells [16]. Briefly, the bodies of the embryos were finely minced under sterile condition. The minced tissues were then digested using trypsin-EDTA solution (1–2 ml per embryo) containing DNAase (2 mg) and collagenase III (1 mg per ml of Trypsin-EDTA) in an incubated shaker. The resulting cell suspension was then centrifuged and the resulting pellet of cells was cultured in T-25 tissue culture flasks (1 embryo per flask). These embryonic fibroblasts were cultured with medium (high glucose Dulbecco's Modified Eagle Medium (DMEM), Mediatech, Inc., Herndon, VA)

containing 10% Fetal Bovine Serum (Hyclone, Logan, UT), 1% antibiotic/antimycotic/antifungal solution (Mediatech, Inc.), and 1% L-glutamine (Mediatech, Inc.). Several passages of cells were grown in an incubated, humidified environment (37°C, 5% CO₂, 95% humidity). Cells from passages P4-P6 were used for preparing the collagen gels [16].

The collagen gels were prepared with acid-soluble rat tail collagen type I (BD Biosciences, Franklin Lakes, NJ) at a collagen concentration of 2 mg/ml and cell concentration of 1×10^6 cells/ml, as previously described [16]. The collagen gels were formed in the incubator using the Flexcell® Tissue Train® culture system, which has been previously described by Garvin et al. [12]. The gels are grown in customized 6-well culture plates that have elastomeric culture surfaces and nylon mesh anchors on two sides of each well. First, the culture plates are placed on top of a set of posts, each containing a cylindrically shaped trough (Fig. 1a), and a constant vacuum was applied through the vertical holes within the post to deform the elastomeric membrane into this trough at the center of the well. The trough was filled with approximately 200 μ L of the collagen-cell mixture (Fig. 1a). The 3-D collagen gels were then placed in incubator and vacuum was maintained for 1 day to retain the trough. The gels solidified within an hour in the incubator and were anchored by the nylon mesh anchors at the ends of the trough. Culture medium (3–4 ml) was added to the wells containing the collagen gels 3–4 hours after preparation. After 1 day, the culture plates containing the collagen gels to be grown under static tension (0% strain) were removed from the Flexcell system and cultured for another 7 days in the incubator. At the same time, the culture plates containing the collagen gels to be grown under cyclic strain were removed from the post with the trough and placed on top of solid “loading” posts (shaped as rectangles with curved ends, Fig. 1b). After the culture plates were placed on the loading posts, vacuum was applied to the system, causing the anchor regions of the elastomeric membrane (the only region not supported by the loading posts, Fig. 1b) to be stretched downwards. This deformation of the membrane and anchors applied uniaxial strain to the gels. The collagen gels were subjected to 5% uniaxial tensile strain at 0.25 Hz for another 7 days; these strain conditions and the duration of culture had been previously optimized to achieve intact collagen gels that were suitable for mechanical testing. Regardless, a small percent of the gels broke during culture; any broken gels were discarded and not used in any further analyses. Approximately 40 intact gels for each group were obtained in this study, from which approximately 20 were used for mechanical testing and the rest were used for biochemical assays. Figure 1c shows mature collagen gels in phosphate buffered saline (PBS) at day 8.

2.2. Electron microscopy and image analysis

After 8 days, one collagen gel designated for electron microscopy from each group were rinsed in PBS, and fixed in phosphate buffer solution containing 1% paraformaldehyde and 0.1% glutaraldehyde (both from Electron Microscopy Sciences, Hatfield, PA) while still attached to the mesh anchors. After fixation for at least 2 hours, the gels were then trimmed to approximately 1 mm³ sections and processed to stain for collagen fibrils and proteoglycans [16,18]. Briefly, to visualize proteoglycans, the gels were stained overnight with 1% cupromeronic blue (Sigma) in 0.2 M acetate buffer (pH 5.6, Electron Microscopy Sciences) containing 0.3 M MgCl₂ [19]. The samples were then immersed in 0.5% Na₂WO₄ in acetate buffer for 1 hour and then overnight in 0.5% Na₂WO₄ in 30% ethanol. The collagen fibrils were visualized by staining with 1% uranyl acetate in maleate buffer (Electron Microscopy Sciences). After staining, the samples were gradually dehydrated and embedded according to standard procedures and longitudinal sections of the samples were imaged with transmission electron microscopy (TEM, JEM 1010, JEOL, Tokyo, Japan). Five images at 10,000X and 3 images at 30,000X were obtained from each longitudinal section.

The resulting images were analyzed using Image J software (NIH) to calculate the percentage area fraction of the collagen fibrils (10,000X), the average collagen fibril diameter, and the average length of the GAG chains on the SLRPs bound to the collagen fibrils (30,000X). To measure the area fraction, each image had its background subtracted to reduce noise. The resulting image was then converted to binary. The number of saturated pixels, representing the area fraction of collagen fibrils, was measured over a defined threshold level that was optimized to measure only the collagen fibrils and not any background noise using the 'outline' analysis tool in ImageJ. The fibril diameters and lengths of the attached GAG chains were measured from collagen fibrils selected from the images using the 'straight line selection' tool, and in order to remove the potential for bias, only fibrils crossing straight lines drawn through the image were selected for analysis. In addition, the number of PGs attached to 7 randomly selected collagen fibrils in each of the three 30,000X images were counted and normalized per nm of fibril length. The resulting PG "density" was averaged from the 21 total measurements.

2.3. Mechanical testing

After 8 days, the culture plates containing intact collagen gels designated for mechanical testing were digitally photographed. The collagen gel diameter was then measured using Image-Pro Express software (Media Cybernetics, San Diego, CA) [16] using the dimensions of the trough (3 mm) to calibrate the image. The final collagen gels were cylindrical in shape with circular cross-sections, making the area a function of the measured gel diameter.

The collagen gels cultured under both static tension and cyclic strain conditions were mechanically tested using an ELF 3200 uniaxial tensile tester (EnduraTec, Minnetonka, MN) [16] with a 250 gram load cell. First, the silicone elastomer membranes with the attached, intact collagen gels were separated from the edge of the well of the culture plates using a scalpel. The central region of the silicone membrane between the anchors was then carefully trimmed away without detaching the collagen gels from the anchors (region indicated between the parallel dotted lines in Fig. 1c). Any gels that detached from the anchor meshes during this trimming process were not used for the mechanical tests, as they tended to slip from the grips. The collagen gels (with each end attached to the anchor mesh and a piece of silicone membrane) were then tightly gripped at each end. The grips were inserted into the tensile tester and the samples were stretched to failure at a strain rate of 10 mm/s while load and displacement data were being continuously recorded. The load and displacement data were then exported, converted to stress and strain, and analyzed to determine the ultimate strength and tensile elastic modulus of the collagen gels. Stress was calculated by dividing load by cross-sectional area using the previously measured gel diameter, assuming a uniform cross-sectional area of the gels. Strain was determined by dividing the change in displacement by the initial grip-to-grip distance (original length of the stretched tissue). The stiffness and elastic modulus was determined from the post-transition slope of the load-displacement and stress-strain curve respectively (Fig. 2). The strength was calculated as the stress value corresponding to the failure load. Material parameters from the different experimental groups were compared after normalizing to the mean values from the static tension wild type group. Sample size was 13 to 18 gels for each group.

2.4. Cell density

Cell density within the collagen gels cultured under static tension or cyclic strain was measured using a DNA assay [16]. Briefly, the collagen gels were harvested at 8 days and lyophilized overnight. The dried samples were weighed, re-hydrated in 1 ml of 100 mM ammonium acetate, then digested by adding a 100 μ l aliquot of Proteinase-K solution (10 mg/ml in ddH₂O, Invitrogen, Carlsbad, CA) and incubating at 60°C for 2 hours. The solubilized samples were sonicated for 4 minutes to rupture the cell membranes and release the DNA, then fluorotagged with Hoechst 33258 dye (Sigma, St. Louis, MO). Fluorescence emission was measured at 458

nm and was compared with standards prepared from double stranded DNA from calf thymus (Sigma) to determine the mass of DNA present. The mass of DNA was normalized to the dry weight of each collagen gel. Sample size was 7 to 10 gels for each group.

2.5. Western blotting of proteoglycans

The culture medium was changed and collected from the Tissue Train culture plates every 2 days for gels grown under static or dynamic conditions and was pooled for each sample for the entire culture period. The media and the mature collagen gels were then analyzed to determine if there was any compensatory synthesis of biglycan, a SLRP closely related to decorin, in the collagen gels containing $Dcn^{-/-}$ cells.

The media samples were processed for western blotting as previously described [16,20,21]. Briefly, to isolate the PGs, 9 ml of media samples were mixed with Q-sepharose ion exchange beads (Amersham Biosciences, Uppsala, Sweden). The beads were then rinsed with 40 column volumes of 7 M urea buffer with 0.25 M NaCl and eluted with 4 column volumes of 7 M urea buffer with 3 M NaCl. The PGs were then precipitated from the urea by the addition of ethanol and the precipitate was digested with chondroitinase ABC containing 0.1% BSA. The samples were then separated in 10% Tris-HCl SDS-PAGE gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membrane. The membrane was then treated with anti-biglycan primary antibody (1:6000 dilution of LF159, courtesy of Dr. Larry Fisher [22]) and goat anti-rabbit horseradish peroxidase-linked secondary antibody (1:20000 dilution, Amersham Biosciences UK Limited, Buckinghamshire, UK). The bands for biglycan were then detected using chemiluminescent exposure to radiographic film. The optimal elution volume was determined to be 900 μ l, which resulted in a large biglycan band from the media samples collected from the dynamically cultured $Dcn^{-/-}$ cell-seeded gels. Sample size was 8 to 11 medium samples (each pooled across the entire culture duration) for each group.

The collagen gel samples were lyophilized overnight, weighed, then digested in extraction buffer (4 M guanidine HCl, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.05 M ammonium acetate, 0.01 M ethylenediamine tetraacetic acid (EDTA), 0.1 M 6-aminohexanoic acid, 0.08% benzamidinium HCl, 10 mM N-ethyl maleimide, 1 mM phenylmethylsulfonyl fluoride (PMSF); 1 ml per 25 mg tissue dry weight) [20,21]. After extraction, the supernatant was centrifuged at 13,000 rpm at 4°C for 30 minutes and dialyzed four times against 7 M urea buffer (containing 2 mM EDTA, 0.05M Tris, 0.5% Triton X-100, pH 7.5) to remove the guanidine. After dialysis, Q-sepharose beads (amount calculated based on initial tissue dry weight) were added to the extracted tissue volumes and then processed in a similar way as media samples. Since the collagen gels had very low dry weight, the entire extraction volume was used for western blotting, and the biglycan bands thus represented the amount of this PG per collagen gel sample. Sample size was 7 to 10 gels for each group. The bands detected for biglycan from the media and collagen gel samples were further analyzed to obtain integrated optical density (IOD) values using Gel Pro Analyzer (Media Cybernetics) software. Since at least 7 samples were run for each group, the samples had to be run into multiple gels and the bands were not normalized to any particular band. However, care was taken to distribute the wild-type and $Dcn^{-/-}$ samples in each gel and the exposure time to radiographic film were kept to be around 2 min to avoid experimental bias.

2.6. Statistical analysis

Replicate analyses were averaged to obtain mean values for each sample. Data were presented as mean and standard deviations. Statistical evaluations (2 factor ANOVAs) were performed using SigmaStat software (SPSS, Chicago, IL). When a significant difference was observed between groups, post-hoc testing was performed for subgroup comparisons. The level of significance was set at $\alpha=0.05$.

3. Results

3.1. Nanostructure

The collagen gels were cultured in Flexcell® Tissue Train® culture system (Fig. 1) and after either static or dynamic culture for 8 days, were harvested and processed for TEM. Analysis of TEM images showed a statistically significant difference in collagen content, collagen fibril diameter, length and number of PGs between the mechanically stimulated collagen gels and the static gels. There was greater collagen content (i.e., area fraction of the TEM images) within the mechanically stimulated gels for both cell types, with more long fibrils visible in the longitudinal sections of the cyclically-strained collagen gels than in the static gels (Fig. 3a and 3b). The $Dcn^{-/-}$ cell-seeded gels also contained more collagen than those containing wild-type cells for both mechanical conditions (2-way ANOVA: $p < 0.05$ for cell type and $p < 0.005$ for mechanical condition). Figure 4a shows a representative TEM image in which the short linear electron-dense stains regularly spaced along the periphery of the collagen fibrils are the sulfated GAG chains of SLRPs [19]. Compared to collagen gels grown under static strain, the cyclically-strained collagen gels containing $Dcn^{-/-}$ cells demonstrated greater collagen fibril diameter, whereas those containing wild-type cells showed reduced fibril diameter (Fig. 4b). Two-way ANOVA (cell type and mechanical conditioning) showed statistically significant difference ($p < 0.001$) between the groups only for the combined effect of the above factors. To determine which interactions of the above factors had significant effects on the group comparisons, post-hoc Holm-Sidak pairwise comparison showed significant difference ($p < 0.05$) between all the interactions, in particular between the cell types under both static and dynamic mechanical conditions. Similarly, the collagen gels cultured under cyclic strain contained a higher density of PGs compared to the statically-cultured gels (Fig. 4c, $p < 0.01$ for cell type and $p < 0.001$ for mechanical condition). Furthermore, the GAG chains of these PGs were more elongated in the $Dcn^{-/-}$ cell-seeded collagen gels as compared to those with wild-types (Fig. 4d). Two-way ANOVA showed statistically significant difference in length of PGs for cell type ($p < 0.001$), mechanical condition ($p < 0.001$) and their combined effect ($p < 0.05$).

3.2. Material behavior

The collagen gels cultured under cyclic strain demonstrated higher maximum load ($p < 0.05$) and stiffness ($p < 0.01$) for both $Dcn^{-/-}$ and wild-type cell-seeded gels, whereas no difference was observed between the cell types under either mechanical condition (Fig. 5a and 5b). After the load and displacement data were converted to stress and strain, only the elastic modulus was significantly higher for the cyclic strain condition ($p < 0.005$), again for both cell types (Fig. 5c and 5d). When considering only the gels grown under the dynamic strain condition, the collagen gels containing $Dcn^{-/-}$ cells demonstrated a higher elastic modulus ($p < 0.02$) and a trend of greater strength ($p = 0.082$) as compared to those containing wild-type cells.

3.3. Cell density

The cell density in the collagen gels was dependent on both the cell type and the mechanical condition during culture. After 8 days, DNA content was greatest in the cyclically-strained collagen gels containing $Dcn^{-/-}$ cells (Fig. 6, $p < 0.004$ for the combined effect of cell type and mechanical condition). Given that the sample dry weights were comparable, these elevations in cell density reflect greater numbers of cells within the collagen gels. To further determine which interactions of the above factors had significant effect, post-hoc Holm-Sidak pairwise analysis showed significant difference only between cell type within dynamic condition ($p < 0.005$) and between $Dcn^{-/-}$ cell-seeded gels grown under static and cyclic strain ($p = 0.01$). There was no effect of mechanical culture condition on the DNA content of the wild-type cell-seeded gels.

3.4. Biglycan expression

Western blotting was used to detect the SLRP biglycan in both the mature collagen gel samples and in the conditioned culture medium. Due to the small mass of the gel samples (~1 mg), blotting was performed using the entire mass of the gel and was not normalized to dry or wet weight, so the biglycan band represented the entire amount in the gel for each cell type and mechanical condition. The biglycan bands were either faint or absent for the medium and gel samples from all static cultures for both cell types, and were even faint for the dynamically cultured gels containing wild-type cells. A thick biglycan band, however, was observed for both medium and gel samples from the cyclically-strained gels containing $Dcn^{-/-}$ cells. Figure 7a shows a representative western blot image for the media samples; a similar trend (usually with thicker bands) was observed in the images of the gel samples. 2-way ANOVA analysis of the IOD values for both media and gel samples showed statistically significant difference for cell type and mechanical condition used (Fig 7b and 7c, $p < 0.001$). For the combined effect of the above factors, post-hoc Holm-Sidak pairwise comparison showed that no significant difference existed between gels grown with either cell types grown under static condition or between gels with wild-types cells grown under static or cyclic strain. In other words, significantly higher amounts of biglycan were retained within the gel and secreted into the medium only in the cultures of collagen gels containing $Dcn^{-/-}$ cells undergoing cyclic strain ($p < 0.001$).

4. Discussion

This work investigated how the presence or absence of decorin and cyclic tensile strain influenced the material, biochemical and biophysical parameters of collagen gels containing murine embryonic fibroblasts. Our results demonstrated that cyclic strain induced greater collagen fibril density, PG density, maximum load, and stiffness regardless of the cell type (wild-type or $Dcn^{-/-}$). In contrast, strain-dependent improvements in cell density, collagen fibril diameter, GAG chain length, and compensatory biglycan expression were observed only in the $Dcn^{-/-}$ cell-seeded gels.

Compared to the static gels, the gels grown under cyclic mechanical strain demonstrated greater stiffness and elastic modulus, and withstood a higher failure load. These improvements in material behavior with mechanical stimulation have been commonly reported for engineered tissues, and are attributed to improved collagen synthesis and organization [12,23–25]. For example, Pins et al. found that greater stretch of self-assembled collagen fibers resulted in both improved collagen fibril alignment as measured by TEM as well as higher tangential elastic moduli [25]. In addition, Garvin et al. found that the application of cyclic tensile stretch via the Flexcell system resulted in greater expression of collagen types III and XII, greater expression of prolyl hydroxylase (involved in collagen synthesis), and a measured elevation in ultimate tensile strength [12]. Correspondingly, the cyclically-strained gels also contained a greater density of collagen fibrils than found in the static gels. Alternatively, cyclic strain might directly influence the expression and activity of GAG synthesizing and polymerizing enzymes leading to elongation of the newly synthesized GAGs. This would then affect collagen assembly and fibril packing within the tissue. In addition, the cyclically strained collagen gels seeded with $Dcn^{-/-}$ cells contained the greatest final density of cells, as shown in our previous study [16]. It has been widely recognized that decorin can inhibit cell proliferation, both by sequestering TGF- β and by blocking the epidermal growth factor receptor [26–30].

Gels grown under the cyclic strain condition also demonstrated greater PG density, altered collagen fibril diameters, and longer GAG chains consistent with previous reports that mechanical stimulation of engineered [31] or native tissues [32] alters the production of PGs and total GAGs. To our knowledge, however, this is the first study to show the effects of cyclic strain on collagen fibril diameter, PG density, and GAG chain length in engineered tissues.

SLRPs have been demonstrated to regulate collagen fibrillogenesis and fibril packing [25] via their core protein [5] as well as their GAG chains [25]. Therefore, the measured increase in the number of PGs (SLRPs) bound to the collagen fibrils of the cyclically-strained gels, as well as the greater length of the GAG chains on these PGs, may also contribute to the formation of stiffer and stronger engineered tissues. In addition, the $Dcn^{-/-}$ cell-seeded gels contained a greater density of PGs with longer GAG chains likely due to the compensatory production of biglycan and possibly other SLRPs. The greater length of the GAG chains in the $Dcn^{-/-}$ cell-seeded gels might have been influenced by the unbound TGF- β , since TGF- β has been reported to lengthen GAG chains [33]. Although the TEM images demonstrated large quantities of SLRPs in all engineered tissues, our western blotting results lead us to speculate that these SLRPs were predominantly biglycan in the $Dcn^{-/-}$ cell-seeded gels and decorin in the wild-type cell-seeded gels. In fact, cyclic strain caused a dramatic elevation in the amount of biglycan in the $Dcn^{-/-}$ cell-seeded gels, but not in the wild-type controls. This greater amount of biglycan could also be attributed to the greater number of cells within the cyclically strained $Dcn^{-/-}$ cell-seeded gels. Biglycan, a SLRP highly homologous to decorin, contains 2 chondroitin/dermatan sulfate GAG chains and is thought to participate in tissue development [4]. Even though decorin and biglycan are often found in different regions of the extracellular matrix and respond differently to growth factors [4], biglycan competes with decorin for the same binding site on type I collagen fibrils [4]. Thus, compensatory behavior of biglycan has been reported in $Dcn^{-/-}$ cell-seeded collagen gels and native tissues [16,34,35].

Although the greater SLRP density found in the dynamically cultured gels was consistent with their material behavior, the trend observed for the mean collagen fibril diameter was unexpected. We observed that cyclic strain caused a significant reduction in fibril diameter for wild-type cell-seeded gels, whereas the opposite trend was observed for the fibrils in the $Dcn^{-/-}$ cell-seeded gels. These results suggest that the biglycan and decorin, the predominant SLRPs in the $Dcn^{-/-}$ and wild-type cell-seeded gels, respectively, regulate collagen fibrillogenesis differently in the presence of mechanical stimulation. To our knowledge, this is the first study to determine changes in fibril diameter in cyclically-strained cell-seeded collagen gels, although Pins et al. have studied changes in acellular self-assembled collagen fibers in response to static strain [25].

Our research has important implications for tissue engineering and for reports describing unexpected relationships between decorin and the material behavior of native tissues. Decorin deficiency was reported to cause irregularities in the collagen fibril diameter and distribution within adult mouse tissues such as skin and tendon, which in turn reduced their material strength [36]. Thus, it was believed that absence of decorin would lead to weaker tissues and that the opposite trend could be expected if decorin is overexpressed. However, either superior or similar material parameters have recently been reported for tendons from decorin deficient adult [37] and postnatal mice [34] as well as decorin deficient engineered gels [16], compared to wild-type controls. In addition, greater than normal decorin expression has been reported in prolapsed mitral valve [38] in which the tissue is weakened [39], and our lab has histochemically demonstrated that excess decorin is present valves that were exposed to altered shear stress [40]. These unexpected results, taken together with the observations from this study, lead us to postulate that decorin-mediated tissue organization is heavily dependent upon tissue type and the amount of strain experienced by the tissue. The causative relationships between altered material behavior, levels of decorin expression, and biglycan compensation, will be important topics to address in future investigations.

This study had a number of limitations. First, the TEM images in this study were obtained from only one gel for each experimental group, although these gels were randomly selected and expected to be representative of their respective groups. In addition, some of the GAG chain lengths measured from the TEM sections might have been out of plane. This experimental

error, however, was expected to affect the different TEM images consistently and should not influence the trend reported in this study. Also, the rat tail collagen type I that was used to prepare the collagen gels also contained some decorin impurity, as measured using western blotting. However, since both decorin deficient and control gels were treated under the same experimental conditions, the differences observed were believed to be due to the absence of endogenous decorin. Finally, our approach did not permit us to distinguish any new synthesized collagen secreted by the murine cells from the rat tail collagen used in preparing the gels. It was expected, however, that the amount of collagen secreted by the cells over 8 days would have been only a minor fraction of the total collagen within the final gel. Regardless, our method did permit our analysis of the cell-mediated reorganization of the collagen fibrils within the matrix.

It should be noted that, similar to our previous study on statically-cultured collagen gels [16], free cell-secreted TGF- β is also expected to have influenced some of the differences observed between the Dcn^{-/-} and wild-type cell-seeded cyclically strained gels in this study. In fact, TGF- β had been reported to upregulate the expression of chondroitin/dermatan sulfate PGs [41] and elongate their GAG chains [33] in static 2-D cultures. Unlike our previous study, however, in which the endogenous TGF- β had a significant influence on the static tension gels [16], in this study the differences between the static tension gels were less pronounced and the endogenously produced free TGF- β did not appear to influence the statically-cultured collagen gels. This conclusion was based on our observations that the exogenous addition of 1 ng/ml TGF- β to the static tension wild-type gels caused them to contract far more than the Dcn^{-/-} gels (data not shown). However, because cyclic strain is reported to upregulate the synthesis of TGF- β [17], we believe that the differences reported in this study were caused by the interactions between decorin, TGF- β , and cyclic strain, but not due to free TGF- β alone.

5. Conclusion

In this study, we have demonstrated that the culture conditions and mechanical stimuli applied to collagen matrices seeded with murine embryonic fibroblasts play a critical role in modulating the synthesis, assembly, and organization of extracellular matrix components. Cyclically-strained samples demonstrated greater collagen fibril density, PG density, GAG chain length, and stiffness regardless of cell type. The application of strain to decorin deficient collagen matrices specifically increased cell density, collagen fibril diameter, and biglycan synthesis, possibly to compensate for the missing decorin. This work contributes to the small but growing body of knowledge regarding the expression and function of specific PGs in response to strain. Ultimately, an improved knowledge of decorin, other extracellular matrix components, their interactions, and feedback via cell-matrix interactions, will be necessary for successful creation of engineered tissues that mimic native tissues.

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Fig. 1.

Dynamic culture of collagen gels: (a) Vacuum-generated trough used to prepare the collagen gels; (b) Solid loading post used to apply strain to collagen gels; (c) Collagen gels after 8 days of culture. The dashed line in part (c) shows how the collagen gels along with the silicone membrane were detached for mechanical testing.

Fig. 2. Representative plots for: (a) Load-displacement; (b) Stress-strain curves. The load-displacement plots were used to calculate maximum load and stiffness, while the stress-strain plots were used to calculate strength and elastic modulus for the collagen gels.

Fig. 3.

Collagen fibril density in statically and dynamically cultured collagen gels containing either *Dcn*^{-/-} or wild-type cells grown for 8 days: (a) TEM images of longitudinal sections of collagen gels; (b) Area fraction for collagen fibrils as measured from the TEM images. * indicates $p < 0.05$ between static and dynamic condition for the same cell type and ^ indicates $p < 0.05$ between the cell-types for the same culture condition. Scale bar in part (a) is 2 microns.

Fig. 4.

Collagen fibril diameter, PG density, and length of GAG chains in statically and dynamically cultured collagen gels containing either *Dcn*^{-/-} or wild-type cells at 8 days: (a) TEM image showing collagen fibrils (long, thick dark gray lines) and the GAG chains of PGs bound to the fibrils (short black lines); (b) Collagen fibril diameter; (c) PG density; (d) Length of GAG chains. * indicates p < 0.05 between static and dynamic condition for the same cell type and ^ indicates p < 0.05 between the cell-types for the same culture condition. Scale bar in part (a) is 500 nm.

Fig. 5.

Normalized material parameters for statically and dynamically cultured collagen gels containing either *Dcn*^{-/-} or wild-type cells at 8 days: (a) Load normalized to static tension wild-type (8.67 ± 1.74 g); (b) Stiffness normalized to static tension wild-type (3.18 ± 0.79 g); (c) Strength normalized to static tension wild-type (104.34 ± 37.26 kPa); and (d) Elastic Modulus normalized to static tension wild-type (429.50 ± 189.46 kPa). * indicates $p < 0.05$ between static and dynamic condition for the same cell type and ^ indicates $p < 0.05$ between the cell-types for the same culture condition. Values represent normalized mean $\pm$ SD ($n=13$ to 18 gels for each group).

Fig. 6. Final DNA content for statically and dynamically cultured collagen gels containing either *Dcn*^{-/-} or wild-type cells at 8 days. * indicates p<0.05 between static and dynamic condition for the same cell type and ^ indicates p<0.05 between the cell-types for the same culture condition. Values represent mean ± SD (n=7 to 10 gels for each group).

Fig. 7.

Amount of biglycan synthesized by Dcn^{-/-} and wild-type cells under static and dynamic culture conditions: (a) Image of Western blot membrane containing conditioned culture medium samples; (b) Biglycan secreted into the culture medium; and (c) Biglycan retained within the collagen gels. * indicates $p < 0.05$ between static and dynamic condition for the same cell type and ^ indicates $p < 0.05$ between the cell-types for the same culture condition. BSA: bovine serum albumin. Values represent mean $\pm$ SD (n=7 to 11 gels and media samples for each group).