Decorin-transforming Growth Factor-β Interaction Regulates Matrix Organization and Mechanical Characteristics of Three-dimensional Collagen Matrices*

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The small leucine-rich proteoglycan decorin has been demonstrated to be a key regulator of collagen fibrillogenesis; decorin deficiencies lead to irregularly shaped collagen fibrils and weakened material behavior in postnatal murine connective tissues. In an in vitro investigation of the contributions of decorin to tissue organization and material behavior, model tissues were engineered by seeding embryonic fibroblasts, harvested from 12.5–13.5 days gestational aged decorin null (Dcn−/−) or wild-type mice, within type I collagen gels. The resulting three-dimensional collagen matrices were cultured for 4 weeks under static tension. The collagen matrices seeded with Dcn−/− cells exhibited greater contraction, cell density, ultimate tensile strength, and elastic modulus than those seeded with wild-type cells. Ultrastructurally, the matrices seeded with Dcn−/− cells contained a greater density of collagen. The decorin-null tissues contained more biglycan than control tissues, suggesting that this related proteoglycan compensated for the absence of decorin. The effect of transforming growth factor-β (TGF-β), which is normally sequestered by decorin, was also investigated in this study. The addition of TGF-β1 to the matrices seeded with wild-type cells improved their contraction and mechanical strength, whereas blocking TGF-β1 in the Dcn−/− cell-seeded matrices significantly reduced the collagen gel contraction. These results indicate that the inhibitory interaction between decorin and TGF-β1 significantly influenced the matrix organization and material behavior of these in vitro model tissues.

Decorin, a member of the small leucine-rich proteoglycan family (1), regulates a myriad of functions in the extracellular matrix including collagen fibrillogenesis (2), collagen degradation (3), cell growth (4–6), and extracellular signaling (7, 8). Decorin consists of a core protein of ~40 kDa attached to a single chondroitin/dermatan sulfate glycosaminoglycan (GAG)2 chain (9, 10). Decorin’s regulation of collagen fibrillogenesis is putatively facilitated through binding of type I collagen molecules to the inner leucine-rich region of decorin core proteins (11). Decorin also binds to other collagens including types II, III, VI, and XIV, thereby affecting a variety of extracellular matrix components (12).

Decorin also participates in many important intracellular and extracellular signaling processes, including ligation of the epidermal growth factor receptor (5, 7, 13, 14), which up-regulates cyclin-dependent kinase inhibitor p21 and ultimately arrests cells in the G1 phase of the cell cycle. In addition, decorin has been shown to bind and inhibit all three mammalian isoforms of transforming growth factor-β (TGF-β1, -β2, -β3) (12, 15, 16), even when bound to collagen. As with collagen, this binding takes place via the protein core and not the GAG chains (5, 17). Conversely, the degradation of decorin by matrix metalloproteases, such as during tissue repair processes, releases the bound TGF-β (18, 19). Thus, whether binding to TGF-β or the epidermal growth factor receptor, decorin has multiple mechanisms for the inhibition of cell proliferation. Correspondingly, decorin has been widely reported to inhibit the growth rates of various cell types (4, 5, 20), whether it is endogenously produced or added exogenously to cell cultures. Even in vivo, tissues such as the periodontal ligaments from decorin-deficient mice have been found to contain twice the fibroblast density of comparable tissues from wild-type mice (20). The involvement of free TGF-β in this process is indicated by findings that decorin-deficient mice show increased ligation of TGF-β to the receptors TGF-βRI and -βRII (21).

Many significant advances in determining the functional roles of decorin have been made possible through the decorin knock-out (Dcn−/−) mouse model (22). The Dcn−/− mouse has also served as a useful model for understanding inherited disorders such as osteogenesis imperfecta, infantile progeria, and Ehlers-Danlos syndrome, all of which have reported deficiencies in decorin expression (23). One of the most distinctive phenotypic characteristics of the Dcn−/− mice, compared with wild-type mice, is that their connective tissues (i.e. skin, tendons) demonstrate reduced material strength (2) and contain loosely packed collagen fibrils with highly irregular diameters.

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The abbreviations used are: GAG, glycosaminoglycan; TGF-β, transforming growth factor-β; PCNA, proliferating cell nuclear antigen; PBS, phosphate-buffered saline; SMA, smooth muscle α-actin.

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due to abnormal lateral fusion (22, 24). When treated with exogenous decorin, dermal Dcn−/− fibroblasts grown in three-dimensional culture recovered their wild-type phenotype and generated collagen fibrils with more uniform diameters (25).

Although investigations of the Dcn−/− mouse have illuminated the role of decorin in collagen fibrillogenesis, the complexity of the animal model, with its in vivo physiological regulatory and compensatory mechanisms, make it difficult to quantify the actual influence of decorin on tissue mechanics. In an attempt to address the role of decorin without complications due to in vivo compensation, three-dimensional collagen gels seeded with Dcn−/− cells were developed primarily to improve our understanding of the role of decorin in the composition and organization of collagen matrices. Collagen gel matrices are well characterized for investigating fibroblast behavior and provide a more biomimetic three-dimensional environment to cells than do two-dimensional surfaces (26–28). It has been shown that fibroblasts seeded in collagen gels migrate within the gel using α5β1 and other integrins (26–28). Upon binding of these integrins to extracellular matrix components, a cascade of signals is initiated to trigger motor proteins to power cell movement (26). The cells then bind and move collagen fibers and organize them in the direction of tension. Furthermore, even though cells tend to synthesize less collagen when they are seeded within collagen gels than when in two-dimensional culture, it has been demonstrated that the majority of the synthesized collagen is incorporated into the developing tissue, in the form of fibrils, as opposed to being lost into the media (29).

In this study, Dcn−/− cell-seeded collagen gels were grown for 4 weeks under tension to quantify the influence of decorin on cell proliferation, collagen fibril organization, and tensile strength. Because Dcn−/− and wild-type cells have different capacities for modulating TGF-β activity, the influence of TGF-β1 on the above-mentioned parameters was also assessed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Embryonic fibroblasts were isolated from euthanized Dcn−/− or wild-type Balb/c mouse embryos (12.5 to 13.5 gestational days old) using an established protocol (www.molgen.mpg.de/~rodent/MEF_protocol.pdf, date accessed: 5/21/04). Briefly, the embryo bodies were separated from the heads under sterile conditions, the bodies were finely minced, and the minced tissues were placed in an incubated shaker (37 °C, 150 rpm) for 20–30 min within a trypsin/EDTA solution (1–2 ml per embryo) containing DNase (2 mg) and collagenease III (1 mg/ml of trypsin/EDTA) to dissolve the tissue. The resulting cell suspension was then centrifuged, plated in T-25 tissue culture flasks (1 embryo per flask), and supplemented with Dulbecco’s modified Eagle’s medium (high glucose; Mediatech, Inc., Herndon, VA), 10% fetal bovine serum (Hyclone, Logan, UT), 1% antibiotic/antimycotic/antifungal solution (Mediatech, Inc.), and 1% L-glutamine (Mediatech, Inc.). The cells were cultured in an incubator (37 °C, 5% CO2, 95% humidity) with changes of medium every 48 h. After the adherent fibroblasts become confluent (1–2 days), they were passaged and grown in tissue culture flasks according to standard procedures. Cells were harvested from 3 separate sets of embryos obtained over several months. Most of the following experiments were performed using cells from at least two of these primary cell cultures.

**Gel Preparation**—The collagen gel matrices were prepared using acid-soluble type I collagen (from rat tail tendon, BD Biosciences) using an established protocol (30). The collagen matrices contained a final collagen concentration of 2 mg/ml and final cell concentrations of either 1 or 2 × 106 cells/ml. The collagen/cell mixture was poured into uniaxially oriented wells (40 × 5 × 5 mm) within custom-designed silicone rubber molds (Fig. 1). Micro-porous polyurethane foam holders (~3 mm diameter, from Sawbones Worldwide, Vashon, WA) were used to anchor the gel matrices at the ends of each well. The collagen/cell mixture solidified in 1–2 h. The gels were cultured in the tissue culture incubator for 4 weeks, and the medium was changed every 48 h. A group of the collagen gels was supplemented with 1 ng/ml of recombinant human TGF-β1 (R&D Systems) added to the culture medium. A subset of gels containing wild-type cells were also treated with 5 and 10 ng/ml of TGF-β1. The TGF-β1-supplemented gels were compared with gels containing wild-type and Dcn−/− cells cultured with regular media. In addition, because the gels seeded with wild-type cells at the 1 × 106 cells/ml concentration had very delayed contraction, contraction in those gels was initiated by supplementing with recombinant human TGF-β1 (only on day 2).

To verify the influence of TGF-β1 on the collagen gels seeded with the Dcn−/− cells, a group of gels containing Dcn−/− cells were cultured in media supplemented with the TGF-β receptor kinase inhibitor SB431542 (Sigma). The concentration of SB431542 was optimized to be 10 μM and was prepared from 20 mM stock solutions of SB431542 dissolved in dimethyl sulfoxide (Me2SO). The control gels for this group were cultured with media containing Me2SO in the same proportions (5 μl for 10 ml of media) as the experimental samples.
Western Blot—Western blotting was used to verify that the wild-type cells were synthesizing decorin. After Dcn$^{-/-}$ and wild-type cells were cultured in T-75 flasks for 5 days, equivalent amount of conditioned media were collected and mixed with ion exchange beads (Q-Sepharose, Amersham Biosciences) as previously described (30, 31). The beads were then rinsed with 40 column volumes of 7 M urea buffer (2 mM EDTA, 0.05 M Tris, 0.5% Triton X-100, pH 7.5) containing 0.25 M NaCl. The purified proteoglycans were eluted with 4 column volumes of 7 M urea buffer containing 3 M NaCl, ethanol precipitated, treated with chondroitinase ABC containing 0.1% bovine serum albumin, separated using SDS-PAGE, and transferred to nitrocellulose. The nitrocellulose membrane was treated with rabbit anti-mouse decorin primary antibodies (LF113 diluted 1:6000, courtesy of Dr. Larry Fisher, NIDCR, National Institutes of Health (32)) and goat anti-rabbit horseradish peroxidase-linked secondary antibodies (1:20000 dilution, Amersham Biosciences). The bands for decorin were detected using chemiluminescent exposure to radiographic film.

Flow Cytometry—Because collagen gel contraction is influenced by cell contractility and cell-matrix adhesion, flow cytometry was used to detect any difference in the abundance of smooth muscle α-actin (SMA) (33) and α2β1 integrins (34) between the wild-type and Dcn$^{-/-}$ cells. The cells were trypsinized, and $2 \times 10^5$ cells for both cell types were washed with phosphate-buffered saline (PBS), and fixed with 100 μl of fixation buffer (1% paraformaldehyde in PBS at pH 7.5). Following fixation, flow cytometry buffer (1% bovine serum albumin, 0.1% sodium azide in PBS; Fisher Chemical) was added to each sample and the cells were centrifuged (5 min, 1500 × g). The pellet cells were permeabilized in 100 μl of 0.1% Triton X-100 buffer (PerkinElmer Life Sciences) and then incubated in 100 μl of buffer containing a 1:100 dilution of primary antibody (monoclonal mouse anti-human SMA with anti-mouse cross-reactivity, DakoCytomation, Denmark; rabbit anti-mouse integrin A2 from integrin β1 antibody kit, Chemicon International, Temecula, CA), although the controls were kept in buffer at 0 °C. Each sample was treated with appropriate secondary antibody (SMA: fluorescein-conjugated goat anti-mouse IgG, Jackson ImmunoResearch, West Grove, PA; integrin: fluorescein goat anti-rabbit IgG, Vector Lab, Burlingame, CA). Following incubation, the samples were suspended in 2 ml of buffer. Each sample was analyzed by flow cytometry using a FACScan device running BD Cell Quest Pro software (BD Biosciences) to collect median forward scatter, side scatter, and fluorescence light data.

Decorin-TGF-β Interaction within Collagen Matrices

Mechanical Testing—Mechanical testing was performed using an ELF 3200 uniaxial tensile tester with a 250-g load cell (EnduraTec, Minnetonka, MN); the resolution of the load cell was within 0.02%. After 4 weeks, the gels were separated from the anchors and kept immersed in PBS until mechanical testing was performed. For the load-elongation test, the gels were clamped within two-piece test grips, and kept hydrated by misting with PBS throughout the duration of the test (~1 min). All gels were preconditioned for 3 cycles (1 mm displacement at 1 Hz) and then stretched to 6 mm displacement at a strain rate of 6 mm/s while load and displacement test data were recorded. Most, but not all, of the gels ruptured prior to 6 mm of displacement and the maximum load (failure load or load at 6 mm) was recorded.

Stress was calculated by dividing the load data by the cross-sectional area of the gel. Gels contracted uniformly throughout, resulting in a cylindrical shape, thus it was assumed that the gel matrices were circular in cross-section making the area a function of the contracted gel diameter. Correspondingly, strength was calculated as the maximum load divided by the cross-sectional area. Strain was calculated by dividing the change in length (displacement) by the original grip to grip starting length. Stiffness and elastic modulus were calculated by determining the post-transition slopes of the load displacement and stress-strain curves, respectively.

Electron Microscopy—Mature collagen gels at 4 weeks were harvested and fixed with phosphate buffer solution containing 1% paraformaldehyde and 0.1% glutaraldehyde (both from Electron Microscopy Sciences, Hatfield, PA). The fixed gels were then processed as described by Liao and Vesely (35). Briefly, the collagen fibrils were stained with 1% uranyl acetate in maleate buffer (Electron Microscopy Sciences). The samples were then 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). The resulting images were analyzed using ImageJ software (NIH) to calculate the percentage area fraction of the collagen fibrils and the average collagen fibril diameter.

Water Content—Hydration of the mature collagen gel matrices samples was calculated from wet and dry weights. Briefly, the gels developed from both cell types were harvested after 4 weeks and their wet weight was measured. The samples were then lyophilized and the dry weight was measured. The percent water content was calculated by normalizing the difference between the wet and dry weight of the samples to the wet weight.

GAG Content—Fluorophore-assisted carbohydrate electrophoresis was used to determine the amount of GAGs secreted by the cells and retained within the mature collagen gels for both cell types ($n = 2$ for each cell type and cell concentration) (31). Briefly, the harvested gels were digested in proteinase-K solution (10 mg/ml in ddH$_2$O, Invitrogen) and vacuum concentrated. The samples were then treated with chondroitinases ABC and ACII (3 milliunits each, Associates of Cape Cod, Fallmouth, MA) to cleave the GAG chains into disaccharides, and fluorophore tagged with 2-aminoacridone HCl (Molecular Probes, Eugene, OR). The samples were then electrophoresed on a 20% acrylamide gel, and the gel bands were imaged and
Decin-TGF-β Interaction within Collagen Matrices

analyzed to quantify the various sulfated and unsulfated disaccharides.

Growth Study—A DNA assay was used to quantify the cell density in the mature collagen gels (36). Briefly, the mature gels were harvested at 4 weeks, lyophilized, then dissolved in proteinase-K solution (10 mg/ml in ddH2O, Invitrogen). The samples were sonicated for 4 min to rupture the cell membranes and facilitate release of DNA. After the samples were fluorotagged with Hoechst 33258 dye (Sigma), fluorescence emission was measured at 458 nm and compared with a standard curve generated from double-stranded calf thymus DNA (Sigma).

Hematoxylin & Eosin Staining (H&E)—The H&E stain was used as an additional measure to verify cell density in the collagen gel samples. Mature collagen gel matrices were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5-μm sections (37). Multiple sections were prepared from 4 samples for each cell type and cell concentration. Each slide was deparaffinized, hydrated to water, and stained with filtered hematoxylin 2 (Richard-Allen Scientific, Kalamazoo, MI) for 5 min and eosin Y (Richard-Allen Scientific) for 1 min. Finally, the slides were dehydrated and coverslipped. Images of the H&E-stained sections were then captured (ImagePro Express, Media Cybernetics, Bethesda, MD) and cell numbers were counted within a defined region of interest to determine the cell density.

Immunohistochemistry—Tissue sections on slides were processed and hydrated to water in the same manner as described for H&E and then immunohistochemically stained for either biglycan or proliferating cell nuclear antigen (PCNA) (n = 4 for each group). For biglycan, the slides were treated with 200 milliunits/ml of chondroitinase ABC (in 0.05 M Tris, 0.06 M sodium acetate, and 0.05 M NaCl), followed by blocking with 10% goat serum buffer. Sections were incubated with anti-mouse biglycan primary antibodies raised in rabbit (LF159 diluted 1:1000, courtesy of Dr. Larry Fisher) (32) and biotinylated anti-rabbit IgG secondary antibodies (1:500, Jackson ImmunoResearch). Positive staining was demonstrated via an avidin-binding complex and a chromogen reaction (Elite ABC and DAB kits, Vector Laboratories).

For PCNA, no chondroitinase ABC digest was required. The slides were incubated with rabbit polyclonal anti-mouse PCNA (diluted 1:500, Abcam, Cambridge, MA) for 1 h, and treated in the same procedure as described above.

Statistical Analysis—Replicate analyses were averaged to obtain mean values for each sample. Data were presented as mean ± S.D. Statistical evaluations (1, 2, and 3 factor analysis of variances) 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 α = 0.05.

RESULTS

Western Blot Verified Decorin Synthesis by Wild-type Cells—Because decorin expression in the murine dermis has been reported to appear at 14.5 days postconception (38), Western blotting was performed to verify that the wild-type cells were actually synthesizing decorin. Conditioned medium from cultures of the wild-type cells showed a positive band for decorin that was absent in the medium from the Dcn−/− cultures (Fig. 2).

Contraction Analysis as a Measure of Matrix Organization for the Collagen Gels—To determine differences in the cell-mediated remodeling of the collagen gels, gel contraction was analyzed by comparing the dimensions of the contracted collagen gel with the initial gel dimensions. The collagen gels populated with Dcn−/− cells showed greater contraction than wild-type cells during the first 2 days (p < 0.001, Fig. 3a) and throughout 2 weeks (p < 0.001,
Contraction and % water content for collagen gels grown with or without TGF-β1 at 4 weeks

|                  | 1 × 10⁶ Cells/ml | 2 × 10⁶ Cells/ml |
|------------------|------------------|------------------|
|                  | No TGF-β1 | TGF-β1      | No TGF-β1 | TGF-β1      |
| Contraction (at 4 weeks)* | Wild-type | 75.1 ± 3.2 | 84.2 ± 1.6 | 77.4 ± 6.4 | 85.2 ± 1.5 |
|                  | Dcn⁻/⁻  | 87.6 ± 3.2 | 85.7 ± 3.0 | 92.2 ± 0.7 | 88.1 ± 2.1 |
| Water content (%) | Wild-type | 93.7 ± 1.8 | 92.6 ± 0.2 | 94.6 ± 1.2 | 91.1 ± 1.0 |
|                  | Dcn⁻/⁻  | 91.5 ± 2.1 | 89.6 ± 0.9 | 89.1 ± 3.1 | 90.0 ± 1.5 |

*p < 0.001 for cell type, cell density, and TGF-β1 effects.

Role of TGF-β in Contraction of Collagen Gels—At 2 weeks, TGF-β1 improved the amount and onset of contraction for the 1 × 10⁶ cells/ml collagen gels containing wild-type cells, but did not significantly alter contraction for 2 × 10⁶ cells/ml gels (p < 0.001 for cell number and TGF-β1 interaction, Fig. 4); TGF-β1 treatment also did not alter contraction for the gels containing Dcn⁻/⁻ cells. At 4 weeks, however, TGF-β1 treatment resulted in a trend of reduced contraction for the Dcn⁻/⁻ cell-seeded gels, whereas an increased contraction was observed for the gels seeded with wild-type cells (Table 1, p < 0.001 for cell type, cell density, and TGF-β1 effects) when compared with gels treated with regular media. The TGF-β1-related increase in contraction for the gels containing wild-type cells was greater for those containing the lower initial cell concentration.

To further verify that the differences observed in collagen gels seeded with Dcn⁻/⁻ cells were influenced by TGF-β1, gels seeded with wild-type cells (1 × 10⁶ cells/ml) were treated with 5 and 10 ng/ml of TGF-β1. Both TGF-β1 concentrations resulted in early contraction comparable with the gels seeded with Dcn⁻/⁻ cells (Fig. 5a). On the other hand, when the Dcn⁻/⁻ cell-seeded gels were treated with a TGF-β receptor kinase inhibitor, a reduction in the initial rate of contraction as well as the overall amount of contraction was observed (p = 0.001, Fig. 5, b–d).

Greater Expression of α₂β₁, Integrin and SMA by Dcn⁻/⁻ Cells—Compared with wild-type cells, the Dcn⁻/⁻ cells showed higher median fluorescence intensities for both SMA and α₂β₁ integrin (2.3 versus 8.5 for SMA and 2.98 versus 7.3 for α₂β₁ integrin, for wild-type and Dcn⁻/⁻, respectively). The same trend was observed for median forward scatter (7.4 versus 10.2 for SMA and 8.3 versus 12 for α₂β₁ integrin, for wild-type and Dcn⁻/⁻, respectively) and side scatter (62.3 versus 78 for SMA and 66.7 versus 97.4 for α₂β₁ integrin, for wild-type and Dcn⁻/⁻, respectively) values. The higher forward and side scatter values indicate larger cell size and greater cell complexity of the Dcn⁻/⁻ cells.

Greater Mechanical Strength and Elastic Modulus for Collagen Gels Populated with Dcn⁻/⁻ Cells—To obtain material parameters of the collagen gels, the gels were stretched up to 6 mm, and the resulting load and displacement data were converted to stress-strain curves. Fig. 6a shows representative stress-strain curves (each showing raw data from one tensile testing measurement) used to obtain the material parameters of

Decerin-TGF-β Interaction within Collagen Matrices

FIGURE 4. Dcn⁻/⁻ cells contracted the collagen matrices more than wild-type cells throughout 2 weeks. The collagen gels were prepared with 2 cell concentrations (1 and 2 × 10⁶ cells/ml) and were digitally photographed every 2 days for the first 2 weeks, because most of the contraction occurred during this period. Three measurements of the diameter of each contracted gel were taken, which were then averaged and compared with the original diameter to obtain percentage contraction. Panels a and c show contraction for 1 and 2 × 10⁶ cell concentrations, respectively. Greater contraction was observed for the Dcn⁻/⁻ collagen gels for up to 2 weeks for both cell concentrations (n = 12, p < 0.001). The addition of TGF-β1 significantly improved contraction of the gels seeded with wild-type cells at the 1 × 10⁶ cells/ml density only (p = 0.016). Panels b and d show the rate of contraction over 2 weeks corresponding to the gels from panels a and c, respectively. The values represent mean ± S.D.
Decorin-TGF-β Interaction within Collagen Matrices

The collage gels from both cell types. Collagen gels containing Dcn−/− cells demonstrated greater stiffness, maximum load, elastic modulus, and strength compared with the gels containing the wild-type cells. This difference was most significant for strength and elastic modulus (Fig. 6, b and c, ~9.5-fold higher for both cell concentrations, p < 0.001) due to the higher contraction of the matrix by Dcn−/− cells, because elastic modulus and strength are normalized to a cross-sectional area. A trend of higher mechanical strength and elastic modulus (~1.5-fold, p = 0.08) were observed for the tissues containing the higher cell concentration for both cell types.

TGF-β1 influenced material parameters for both cell types (p < 0.005 for combined effects of cell type and TGF-β1), but this influence depended on the initial cell concentration used for preparing the gels (p < 0.05 for combined effects of initial cell density and TGF-β1). TGF-β1 improved both mechanical strength and elastic modulus for the gels seeded with wild-type cells, an effect that was greater for the lower cell concentration (p < 0.001, Fig. 6, b and c). In contrast, TGF-β1 did not significantly alter strength or modulus of the collagen gels seeded with Dcn−/− cells.

Ultrastructural Analysis of Collagen Content and Matrix Organization—TEM was used to observe the ultrastructure of the collagen gel matrices and quantify the influence of decorin on collagen content and collagen fibril diameter for both cell types. Despite identical culture conditions for all collagen gel matrices, the Dcn−/− cells within the mature gels appeared more elongated than the wild-type cells. These same collagen gels demonstrated more collagen fibrils in the Dcn−/− pericellular matrix, indicating the active involvement of these cells in organizing the existing and newly synthesized collagen fibrils (Fig. 7). The collagen gels containing Dcn−/− cells (both cell concentrations) also had greater collagen content, as measured by the area fractions of collagen fibrils, than those containing the wild-type cells (p < 0.001 for cell type and cell concentration, Fig. 8). TGF-β1 treatment resulted in greater area fractions of collagen fibrils for gels containing wild-type cells (at both initial cell densities) and for the gels containing the Dcn−/− cells (lower cell density only) when compared with untreated gels (p < 0.001). However, within the TGF-β1-treated group, the gels containing Dcn−/− cells had greater collagen area fractions than wild-type controls (p < 0.001, Fig. 8b). The fibrils from collagen gels containing Dcn−/− cells showed a trend of slightly higher average diameter (Fig. 8c); however, no significant difference was observed between collagen fibril diameters among cell types or cell concentrations, with or without TGF-β1.

Greater Hydration of Collagen Gels Populated with Wild-type Cells—The degree of hydration in collagen gels tends to be inversely proportional to contraction trends and is often associated with matrix organization (39). The percent water content was calculated by normalizing the difference of the wet weight and dry weight of the samples to the wet weight. The collagen gels with Dcn−/− cells had lower percent water content compared with the gels with wild-type cells at 4 weeks for both untreated (p = 0.001) and TGF-β1-treated conditions (Table 1, p < 0.001 for cell type and p = 0.05 for TGF-β1).

GAG Synthesis in Collagen Gels Depended on Initial Cell Density for Both Cell Types—To determine whether there was a relation between hydration and GAG content, fluorophore-assisted carbohydrate electrophoresis was used to quantify the content of sulfated GAGs and hyaluronan in the mature gels for both cell types. When normalized to the final DNA content, total GAG content in the collagen gels seeded with either type of cell was dependent upon the initial seeding density and greater for the higher initial density (2 × 10^6 cells/ml), as shown in Table 2 (p < 0.05 for effect of cell density). The collagen gels seeded with wild-type cells showed a trend of more GAGs per cell than those seeded with Dcn−/− cells; however, this difference was significant only for sulfated GAGs (p = 0.035 for cell type). No significant difference in hyaluronan content was observed between the cell types.
Greater Cell Density for Collagen Gels Populated with Decorin-deficient Cells as Compared with Those with Wild-type Cells—A DNA assay was used to determine the final cell concentration within the collagen gels. At 4 weeks, the gels containing the \( \text{Dcn}^{-/-} \) cells had a greater cell density than the gels containing the wild-type cells (Fig. 9). This difference was more pronounced for the lower initial cell concentration (higher by 2.8-fold as compared with 1.2-fold for the higher initial cell concentration, \( p < 0.05 \) for cell type). TGF-\( \beta 1 \) influenced the final cell density for both cell types and cell concentrations (Fig. 9, \( p < 0.001 \) for cell type, cell concentration, and TGF-\( \beta 1 \) effects). However, even though all gels seeded with 1 \( \times 10^6 \) cells/ml showed about 2–3-fold increase in final cell density after TGF-\( \beta 1 \) treatment, gels seeded with 2 \( \times 10^6 \) cells/ml had different magnitude responses. Those final densities were 2.5-fold greater for the wild-type cell-seeded gels but 7-fold greater for the \( \text{Dcn}^{-/-} \) cell-seeded gels.

To verify these differences in cell density, the numbers of cells within an imaged region of interest were also measured for H&E-stained collagen gels. There was a significantly higher cell density in \( \text{Dcn}^{-/-} \) cell-seeded gels for both initial seeding densities when compared with wild-type cell-seeded gels (Fig. 9, \( b \) and \( c \), \( p < 0.001 \)). The cell nuclei also appeared more elongated in \( \text{Dcn}^{-/-} \) cell-seeded gels.

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To determine whether the cells measured using the H&E and DNA were alive or dead, cells expressing PCNA were examined using immunohistochemistry (Fig. 9d). PCNA was found to stain all nuclei within the histological sections of the collagen
Decorin-TGF-β Interaction within Collagen Matrices

FIGURE 8. Collagen gels populated with Dcn−/− cells had greater collagen content and organization than collagen gels containing wild-type cells. Panel a shows representative longitudinal section images taken using transmission electron microscopy; image magnification ×30,000. Three images were taken of each longitudinal section of collagen gels for both cell types and cell concentrations (1 and 2 × 10^6 cells/ml), with or without TGF-β1.

| TABLE 2 | GAG content within collagen gels at 4 weeks, as measured by fluorophore-assisted carbohydrate electrophoresis analysis |
|-----------------------------------------------|
| Wild-type | Dcn−/− | Wild-type | Dcn−/− |
| 1 × 10^6 Cells/ml | 2 × 10^6 Cells/ml | 1 × 10^6 Cells/ml | 2 × 10^6 Cells/ml |
| Total GAG (pmol/ng DNA) | 0.85 ± 0.16 | 0.66 ± 0.03 | 1.65 ± 0.38 | 1.22 ± 0.20 |
| Hyaluronan (pmol/ng DNA) | 0.14 ± 0.00 | 0.10 ± 0.02 | 0.40 ± 0.09 | 0.56 ± 0.15 |
| Sulfated GAGs (pmol/ng DNA) | 0.72 ± 0.16 | 0.56 ± 0.05 | 1.25 ± 0.29 | 0.66 ± 0.05 |

*p < 0.05 for cell type. Sulfated GAGs represent total amount measured for chondroitin 4-sulfate, 6-sulfate, 2,4-sulfate, and 2,6-sulfate. p = 0.058 for initial cell density. *p < 0.05 for cell type.

gel, indicating that the differences in cell density between the collagen gels derived from the different cell types could be attributed to live, proliferating cells.

Decorin-deficient Collagen Gels More Strongly Express Biglycan—It has been widely reported that when one small leucine-rich proteoglycan is knocked out, the synthesis of other small leucine-rich proteoglycans tends to be up-regulated to compensate for this deficiency (40, 41). To determine whether such compensation was occurring in these three-dimensional collagen gel matrices, immunohistochemistry was used to demonstrate the abundance of biglycan within the collagen gels. Overall, the collagen gels seeded with the Dcn−/− cells showed stronger staining for biglycan than did the gels seeded with the wild-type cells (Fig. 10).

DISCUSSION

The main findings of this study are that collagen gels seeded with Dcn−/− cells showed distinct behavior from those seeded with wild-type cells, resulting in increased gel contraction, matrix organization, ultimate tensile strength, and elastic modulus. Even though the mechanical behavior of the collagen gels containing the Dcn−/− cells contradicted the general trend reported for Dcn−/− tissues from adult mice (2, 20, 24), these data support some recent trends reported for Dcn−/− tendons (23) and immature tendons in particular (40). Moreover, TGF-β1 was shown to regulate many of the differences observed between the Dcn−/− and wild-type groups.

Relationship between Cell Density, Collagen Density, Contraction, and Material Behavior—The increased contraction of the collagen gels by the Dcn−/− cells illuminates the role of the proteoglycan decorin in extracellular matrix organization. Even in the absence of cells, decorin can slow the rate of fibrillogenesis in vitro, putatively by binding to collagen fibril surfaces and inhibiting their lateral growth; this mediation facilitates the development of uniform spacing between collagen fibrils (2,

treatment. The images were analyzed to calculate the percentage area fraction of collagen and the diameter of the collagen fibrils. Panel b shows the area fraction for collagen fibrils calculated from the transmission electron microscopy images, indicating higher collagen content in the gels containing the Dcn−/− cells (p < 0.001). The addition of TGF-β1 increased the percentage area fraction for both cell types, although the improvement was greater for wild-type cells. Panel c shows collagen fibril diameter measured from the transmission electron microscopy images for all of the groups. The graph legend is the same as in panel b. The values represent mean ± S.D.
Delayed contraction of collagen matrices has also been reported for acellular collagen-GAG scaffolds in which the GAG component was replaced by decorin (43), and for collagen gels seeded with either cells transfected to overexpress decorin or control cells in the presence of exogenously added decorin (44). Interestingly, one conflicting study observed greater collagen gel contraction by decorin overexpressing cells (created through retroviral transfection), but this trend was not supported when recombinant decorin was added to the same untransfected cell line (45). Based on the majority of the reported interactions of decorin with collagen, these Dcn<sup>−/−</sup> cell-seeded collagen gels were expected to show accelerated contraction and poor collagen fibril organization. Whereas the latter result was not demonstrated by TEM, there was indeed significantly greater contraction by the collagen gels seeded with Dcn<sup>−/−</sup> cells.

The greater number of cells within the Dcn<sup>−/−</sup> cell-seeded collagen gels might also have influenced several other gel characteristics, such as collagen density and material parameters. Because decorin inhibits cell growth (4, 5, 20), the decorin-deficient collagen gels had higher cell densities, similar to what has been shown in periodontal tissues from adult Dcn<sup>−/−</sup> mice (20). Taken together, the improved cell density and greater contraction are likely responsible for the greater collagen density observed from the TEM images of Dcn<sup>−/−</sup> cell-seeded gels. Additionally, the high collagen density and low cross-sectional area of these gels contributed to their superior mechanical strength and elastic modulus as compared with the gels containing wild-type cells. Another influential factor could be the greater abundance of biglycan observed in the collagen gels containing Dcn<sup>−/−</sup> cells. Biglycan is similarly believed to be involved with tissue development (46), and has

![Image](https://example.com/image.png)
been shown to be up-regulated during the growth of murine Den-/- tendons (40). The excess biglycan in these collagen gel matrices containing Den-/- cells may have compensated for the absent decorin in regulating collagen fibrillogenesis.

Comparison of Three-dimensional Collagen Gels with Adult and Juvenile Native Tissues—These Den-/- cell-seeded gels behaved differently than originally expected based on reported characteristics of knock-out mouse tissues. Den-/- tissues from postnatal knock-out mice have been characterized as having larger collagen fibril diameters, irregular fibril outlines, and loose fibril packing, leading to lower mechanical strength (22); in contrast, the decorin-deficient engineered tissues were actually much stronger than the wild-type controls. However, our results are in agreement with data recently reported for decorin-deficient tendons. Robinson et al. (23) measured a higher elastic modulus for Den-/- tissues for both patellar and flexor digitorum longus tendons. In another study, Zhang et al. (40) investigated the mechanical characteristics of Den-/- flexor digitorum longus tendons at different postnatal days. They observed that mature Den-/- tendons (postnatal 150) had significantly lower strength and stiffness, but at the end of the fibril growth phase (postnatal day 60), there was no significant difference between Den-/- and wild-type tendons. In fact, their data at postnatal day 60 showed a trend of higher stiffness, maximum stress, and elastic modulus for the Den-/- tendons. Because collagen gels in this study were grown for 4 weeks and the cells used in the collagen gels were embryonic fibroblasts, the tissues created here could be considered as a model of a developing tissue. Hence, it could be expected that the mechanical characteristics of our engineered tendons would be more similar to postnatal day 60 than to the mature tendons. It may also be that the effect of decorin on collagen fibrillogenesis requires a longer culture period to become evident and that if our gels were grown longer, the effect of decorin on fibrillogenesis would become more obvious.

Role of TGF-β1 on Maturation of Three-dimensional Collagen Gels—To determine whether the significant differences in collagen gel contraction between the wild-type and Den-/- cells were present in the early stages of gel formation, the hourly contraction was measured for several hours over a 2-day period. It was observed that the Den-/- cells initiated contraction of the matrix several hours before the wild-type cells. To understand these early contractile differences, the role of TGF-β1 was investigated.

Because decorin sequesters TGF-β, Den-/- cell cultures and tissues will contain unbound TGF-β that is therefore available to stimulate a number of effects on cells in vivo and in vitro. The application or enhanced expression of TGF-β has been linked to increased cell proliferation and migration (47, 48), increased collagen synthesis (49, 50), and suppressed collagenase activity (51, 52) by fibroblasts cultured from various tissues. TGF-β causes fibroblasts to differentiate into myofibroblasts, resulting in their greater expression of SMA (33, 53) and α₁β₁ integrins (54), which as shown here would cause greater contraction of the collagen matrix. Similarly, many of the effects of TGF-β, such as cell migration (55, 56), hyaluronan synthesis (56, 57), proteoglycan synthesis (58), and cell proliferation have been shown to be dependent upon cell density, that is, greater response is observed for lower cell density. The cell density-dependent behavior of TGF-β1 has been attributed to either improved binding of TGF-β by the fibroblasts (59) or increased expression of TGF-β1 receptors (60).

For the collagen gels grown from the wild-type cells, the influence of TGF-β1 was strongly dependent upon cell density. In these cultures, the addition of TGF-β1 increased the contraction, collagen density, strength, elastic modulus, and final cell density of collagen gels of both initial seeding densities. Overall, the addition of TGF-β1 to the wild-type cell-seeded gels made them behave more like the untreated Den-/- cell-seeded gels. The gels containing the lower initial cell densities, however, were more profoundly changed than were those generated from the higher cell density. In general, this improvement in material parameters was expected because TGF-β1 treatment has been reported to increase the mechanical strength and modulus of tissues regenerated from patellar tendon (61) or collagen gels prepared with smooth muscle cells (62). Because these wild-type cells are capable of synthesizing decorin, which sequesters TGF-β1 (12, 15), it is likely that the TGF-β1 present within the regular culture conditions (from serum and endogenous production) is partially bound by decorin. As a result, fewer of the wild-type cellular TGF-β receptors would be ligated, leaving them free to bind to the TGF-β1 that was added in these experiments. Because the same dose of TGF-β1 was added to both the lower and higher cell density-based gels, this dose would be expected to have a stronger effect with gels derived from the lower cell density (and fewer TGF-β receptors to saturate) and conversely a reduced effect on gels derived from the higher cell density.

The collagen gels grown from the Den-/- cells were less influenced by the addition of TGF-β1, although there were some notable effects of initial cell density. Adding TGF-β1 had a greater effect on the collagen density of the lower cell density collagen gels, but ultimately there was no difference in collagen density between the two groups (low and high cell density) of TGF-β1-treated collagen gels. The contraction and mechanical properties were not significantly affected by the TGF-β1. In contrast, the addition of TGF-β1 had a greater influence on the final cell concentration of the collagen gels derived from the higher initial cell density as opposed to those derived from the lower cell density. Given that these cells could not synthesize decorin, which would therefore not be able to sequester TGF-β, the amounts of TGF-β normally present within the cell culture would be free to act upon the cells through ligation of the TGF-β receptors. Over time, the collagen gels containing the lower density of Den-/- cells would be more likely to have maximal saturation of TGF-β receptors, whereas there may be unsaturated TGF-β receptors present within the collagen gels containing the high density of Den-/- cells. With addition of excess TGF-β1, those cells within the latter set of gels (higher initial cell density) could be stimulated to proliferate, which would correspond to the profound increase in cell density upon stimulation of the Den-/- cells with TGF-β1.

Because the contraction kinetics were similar for both cell types from 4 days onwards, the role of free TGF-β in the early contraction of the collagen gels was further demonstrated by
dose dependence and inhibition studies. When the gels containing wild-type cells were treated with 5 and 10 ng/ml of TGF-β1, the early contraction of these gels increased in a dose-dependent manner, with the 5 ng/ml stimulating contraction that matched that of the gels containing Dcn+/− cells and the 10 ng/ml stimulating even higher contraction. Conversely, when the collagen gels containing Dcn+/− cells were treated with a TGF-β receptor kinase inhibitor, the activity of the free TGF-β1 was blocked and the gels demonstrated reduced contraction. Overall, in addition to the initial cell density, the availability of free TGF-β1 was the main factor that appeared to govern the early contraction of the gels.

Limitations of Current Research—The addition of a single dose of TGF-β1 at day 2 to the gels seeded with the lower density of wild-type cells might have influenced other extracellular matrix components in addition to increasing the contraction of the collagen gels. However, because similar results were observed for the higher cell density-based wild-type gels (grown without TGF-β), this one-time addition of TGF-β likely did not alter the parameters measured in this study. Indeed, the results of an unanchored collagen gel study indicated that a sustained application of TGF-β was needed to alter these outcomes (results not shown); similar findings have also been reported by Walsh et al. (63). Furthermore, because collagen gels demonstrate viscoelastic behavior, as reported by Wagenseil et al. (64), the material parameters reported in this paper were dependent on the stretch rate and preconditioning cycles used. It would be of interest in the future to investigate their differential behavior in response to time dependent materials testing conditions.

Conclusions—This study demonstrated that in the absence of decorin, free TGF-β greatly influenced the contraction, final cell density, and mechanical strength of three-dimensional cell-seeded collagen gel matrices, resulting in unexpected behavior of the collagen gels seeded with Dcn−/− cells. This study sheds new light on the differences between engineered and native tissues and important caveats about the use of embryonic fibroblasts. Overall, the use of a three-dimensional engineered tissue approach to investigate the biomechanical contributions of decorin should continue to reveal more about the diverse functionalities of this fascinating molecule.

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Decerin-TGF-β Interaction within Collagen Matrices

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