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Influence of cyclic strain and decorin deficiency on 3D cellularized collagen matrices

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ABSTRACT

Cyclic strain evokes the expression of the small leucine-rich proteoglycans decorin and biglycan in 2D 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 8 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 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 2D cultures and native tissues, as well as its interactions with other components of the extracellular matrix [8]. The application of mechanical stimulation to 3D 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 2D 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 2D 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 3D engineered tissues [8], alteration in

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specific PG expression and the resulting 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 cultures were 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/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% CO_2, 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 were grown in customized 6-well culture plates that have elastomeric culture surfaces and nylon mesh anchors on 2 sides of each well. First, the culture plates were 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 μl of the collagen–cell mixture (Fig. 1a). The 3D 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 h 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. Fig. 1c shows mature collagen gels in phosphate buffered saline (PBS) at day 8.

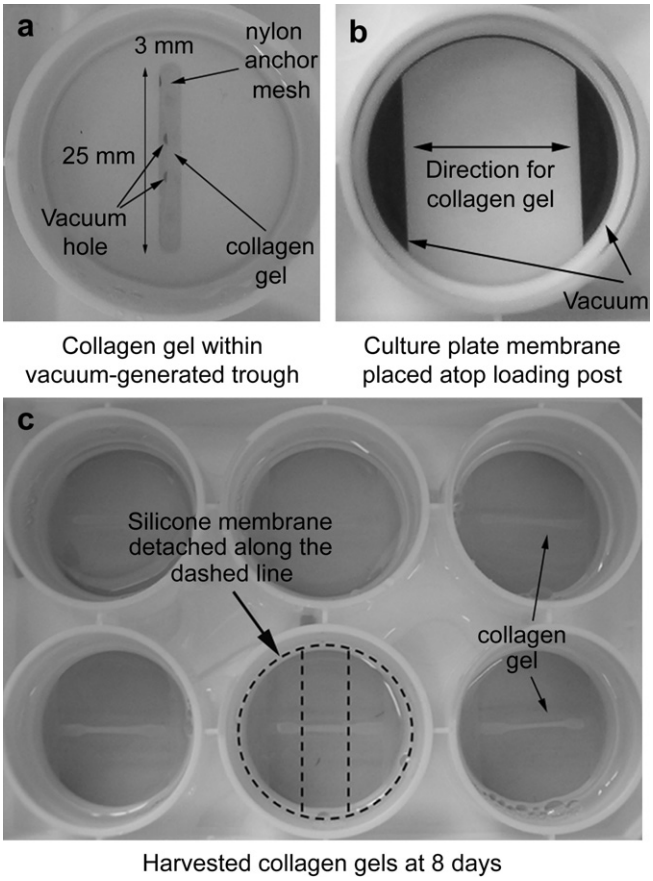

Fig. 1. Dynamic culture of collagen gels (a) vacuum-generated trough used to prepare the collagen gels; (b) solid loading post used to apply strain to collagen gels; (c) collagen gels after 8 days of culture. The dashed line in (c) shows how the collagen gels along with the silicone membrane were detached for mechanical testing.

2.2. Electron microscopy and image analysis

After 8 days, 1 collagen gel designated for electron microscopy from each group was 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 h, the gels were then trimmed to approximately 1 mm^3 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 $MgCl_2$ [19]. The samples were then immersed in 0.5% Na_2WO_4 in acetate buffer for 1 h and then overnight in 0.5% Na_2WO_4 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 10,000× and 3 images at 30,000× 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 (10,000×), the average collagen fibril diameter, and the average length of the GAG chains on the SLRPs bound to the collagen fibrils (30,000×). 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 30,000× images were counted and normalized per nanometer of fibril length. The resulting PG "density" was averaged from the 21 total measurements.