Collagenous Tissue Engineered Constructs to Investigate the Role of Decorin on Collagen Fibrillogenesis and Tissue Mechanics

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Abstract
Collagenous Tissue Engineered Constructs to Investigate the Role of Decorin on Collagen Fibrillogenesis and Tissue Mechanics
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Decorin, a small leucine rich proteoglycan, has been reported to control collagen fibrillogenesis, thereby influencing the tensile properties of collagenous tissues. In this research work, decorin deficient cells (Dcn⁻反感) were combined with tissue-engineered collagen gels to study the contribution of decorin to cell proliferation, collagen fibrillogenesis, and tensile strength, as well as the interaction between decorin and transforming growth factor (TGF)-beta. This project was driven by the hypothesis that decorin deficient cells grown in tissue-engineered constructs would show significant biomechanical influence over the tensile behavior of collagenous tissues by controlling collagen fibrillogenesis.

Since decorin inhibits cell adhesion and cell migration on various matrix molecules, such as collagen and fibronectin, the adhesive characteristics of Dcn⁻反感 cells were first investigated to understand how the absence of decorin would influence cell organization in the collagen gels. Since β1 integrins play an integral role in cell-mediated adhesion, the contributions of α2 and β1 subunits of integrin were also investigated. In this study, Dcn⁻反感 cells showed significantly higher adhesion to both collagen and fibronectin substrates. For both collagen and fibronectin substrates, blocking either α2 or β1 integrin subunits blocked cell adhesion differently for the wild-type and Dcn⁻反感 cells, suggesting that distinct mechanisms of adhesion are utilized by these cell types. Finally, Dcn⁻反感 cells showed greater migration on the collagen substrate compared to wild-type controls.
To further determine how decorin participates in matrix organization, collagen gels containing the Dcn−/− cells and wild-type control cells were grown under 2 different mechanical conditions – static tension and dynamic tension. The static tension collagen gels seeded with Dcn−/− cells showed greater gel contraction, matrix organization, ultimate tensile strength and elastic modulus than those seeded with wild-type cells. Moreover, addition of TGF-beta to the wild-type cell-seeded gels made them similar to Dcn−/− cell-seeded gels. Conversely, when the collagen gels containing Dcn−/− cells were treated with a TGF-beta receptor kinase inhibitor, they demonstrated reduced contraction. These results indicate that the inhibitory interaction between decorin and TGF-beta significantly influenced the matrix organization and material behavior of these in vitro model tissues.

The dynamic gels were grown with the above cell types in a Flexcell Tissue Train™ culture system under cyclic 5% uniaxial strain at 0.1 Hz, while the static gels were cultured under static tension. Interestingly, it was found that some measured outcomes such as collagen fibril density, PG density, maximum load and stiffness were altered with mechanical stimulation regardless of the cell type used. On the other hand, unique outcomes regarding cell density, collagen fibril diameter, and biglycan expression were observed in response to cyclic strain in the Dcn−/− cell-seeded gels only. These results led us to conclude that decorin-mediated tissue organization is heavily dependent upon tissue type and the amount of strain imparted on the tissue.
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## Common Abbreviations

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<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>Dcn&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Decorin knockout</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan sulfate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>HS</td>
<td>Heparan sulfate</td>
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<tr>
<td>HA</td>
<td>Hyaluronan</td>
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<tr>
<td>KS</td>
<td>Keratan sulfate</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycan</td>
</tr>
<tr>
<td>SLRP</td>
<td>Small leucine rich proteoglycan</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
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CHAPTER 1

Thesis Overview

Decorin, a small leucine-rich proteoglycan, participates in a myriad of functions in the extracellular matrix (ECM) including collagen fibrillogenesis [2] and degradation [3], growth factor modulation [4], cell proliferation [5-7] and cell migration [8]. Excess amounts of decorin have also been reported in various pathological conditions such as myocardial infarction [9, 10], arthritic joints [11], tumor stroma [12], and angiogenesis [13], while the absence of decorin has been associated with diseases such as osteogenesis imperfecta, infantile progeria, and Ehlers-Danlos syndrome [14]. In spite of the diverse biological roles of this molecule, very little research has been performed to elucidate the contributions of decorin to the organization of the ECM.

Previously, the role of decorin in collagen fibrillogenesis has been primarily studied using the decorin knockout mouse model, in which the absence of decorin resulted in irregular collagen fibril diameters and distributions [15]. However, the complexity of in vivo systems makes it difficult to ascertain the exact mechanism that was triggered by the absence of this molecule. In this research work, a less complex approach was taken by using decorin deficient cells to develop tissue engineered collagen constructs. This in vitro model then allowed us to examine different experimental conditions to obtain a better understanding of native tissue mechanics.

The central hypothesis of this project was that decorin deficient cells grown in tissue-engineered constructs would exert significant influence over the mechanical properties of collagenous tissues. In order to investigate the effects of decorin on collagen fibril formation,
tissue architecture, and tissue mechanics, the following Specific Aims were proposed and investigated:

1. Determine the contributions of decorin to cell phenotype, cell adhesion and cell migration in 2-D cell culture.
2. Determine the contributions of decorin to collagenous tissue organization and mechanics and interactions between decorin and transforming growth factor β (TGF-β) using 3-D tissue engineered constructs cultured under static tension.
3. Determine the effects of decorin, TGF-β, and dynamic mechanical stimulation on the tissue organization and strength-associated characteristics of tissue engineered constructs.

This thesis begins with an introduction to the major components of the ECM, with particular emphasis on decorin and the various external factors that were investigated in this research work. Chapter 3 provides a review of the current research and available tools for studying proteoglycans and presents ideas regarding the importance of utilizing tissue-specific proteoglycan composition in tissue engineering. Chapter 4 presents results from Aim 1, where the properties of adhesion and migration were characterized for both cell types in 2-D cultures. The contributions of α2 and β1 integrin subunits to cell adhesion were also determined in this Aim. The surface behavior of these cells in this 2-D study provided insight regarding how these cells organized the 3-D collagen matrices. Chapter 5 begins the investigation into collagen constructs and presents a set of studies used to optimize the collagen constructs prior to conducting Aim 2. The research described in Chapter 6 then
utilized the results obtained in Chapter 5 to study the effects of decorin on various characteristics of the collagen constructs including collagen synthesis and organization, material parameters, cell proliferation, and gel contraction. The influence of unbound TGF-β within the decorin deficient cell-seeded gels is also described in this chapter. Chapter 7 presents research that investigated whether the exogenous addition of decorin could reverse the behavior of the collagen constructs containing decorin deficient cells. Chapter 8 presents research that utilized a more physiological condition, cyclic strain, in which to grow the collagen constructs and to determine if this mechanical conditioning influenced the outcomes measured in Aim 2. Finally, Chapter 9 presents the conclusions of this thesis and future directions for this research area.
CHAPTER 2

Background

2.1 Extracellular matrix

The extracellular matrix (ECM) creates an intricate mesh for cell attachment and organization. The cells secrete the ECM as well as organize the matrix [16]. The ECM maintains the environment for the growth, development, and separation, of tissues. For example, the ECM has been shown to promote cell migration and neurite growth cone movement towards its target cell [17]. In addition, the ECM provides tissues with mechanical strength, rigidity and elasticity. The ECM consists of a wide variety of molecules, mainly proteins and polysaccharides, which regulate cell attachment and function. Major components of the ECM include collagen, elastin, proteoglycans (PGs), glycosaminoglycans (GAGs), and glycoproteins. These ECM molecules participate in cell growth, proliferation, and differentiation. They are also involved in intracellular signaling and growth factor binding. Tissues can have different morphology based on their relative ECM composition and organization, thereby giving diversity to connective tissues and organs. For example, the matrix can be calcified in bones, transparent in the cornea, or ropelike in tendon. The ECM molecules are constantly degraded and renewed to maintain a proper cellular environment. The ECM components of particular relevance to this research are discussed in the following sections.
2.1.1 Collagen

Collagen is the most abundant protein of the ECM. The main roles of collagen are to maintain the structural integrity of the ECM and resist stretching forces. Collagen also promotes cell organization and attachment to the ECM. Collagen fibers appear as crimped structures when viewed by light microscopes. The extents of fiber crimp as well as collagen fibril diameter and organization have been reported to influence the mechanical function of tissues [18, 19].

Collagen has a hierarchical structure (Figure 2-1), in which the first level is the formation of the collagen molecule. The process of collagen molecule assembly starts with the secretion of procollagen molecules from the Golgi apparatus [16]. After secretion from the cell, the procollagen molecules are processed into collagen via proteolytic removal of the C- and N-propeptide portions of the molecule. The collagen molecule consists of a triple-helix, which dictates its structural and chemical properties. The triple helix consists of three \( \alpha \) chains made up of amino acid sequences twisted in a left-handed helical structure known as polyproline type II helix [20]. The twisted \( \alpha \)-chains provide a structural rigid rod-like structure to collagen [17]. The three chains are in turn wound around each other in a right-handed superhelix. The triple helical conformation is stabilized by an extensive network of hydrogen bonding between water molecules that surround the triple helix and the amino acids. The rotation angle in the right-handed superhelix is 30° for every amino acid triplet, which makes every third amino acid lie at the center of the triple helix [21]. Proper folding of the \( \alpha \)-chains requires that glycine (Gly) be present as every third amino acid residue [17]; therefore, the typical sequence of amino acids for the collagen molecule is Gly-X-Y, where
X, Y can be any amino acid. X is often proline and Y is hydroxyproline, a post-translational modification of proline.

![Collagen molecule organization](image)

Figure 2-1: Demonstration of the organization of collagen molecule; A) triple helical organization of a collagen molecule; B) & C) Quarter-stagger model of collagen molecules aggregation, D: D-period; D) Hierarchical structure of collagenous tissue. [22]

The presence of a large number of hydroxyproline residues improves the stability of the helical structure by the formation of additional hydrogen bonds. Even though other amino
acids can be present in the Gly-X-Y sequence, they are not found to follow a repeating pattern as notable as that of proline and hydroxyproline.

Collagen molecules usually aggregate into fibrils. The self-assembly of triple-helix collagen molecules into microfibrils requires the formation of “nuclei”, a group of molecules in supersaturated form and of a stable size to be considered as a separate phase [23]. Aggregation of the nuclei forms the visible crystalline structure under an electron microscope [24]. Further lateral and end-to-end aggregation of the microfibrils (Figure 2-2) causes the formation of the collagen fibril with a visible D-period. The D-period is the characteristic cross-striations every 67 nm on the collagen fibrils, according to the “quarter-stagger” model [16]. For example, for tendinous tissues, the fibril diameters range from 20-180 nm [25].

Figure 2-2: The lateral and end-to-end aggregation of collagen molecules. [26]

After the fibrils have formed in the extracellular space, they are stabilized by the covalent cross-links between the lysine residues of the constituent adjacent collagen molecules [16]. If this cross-linking is inhibited, the strength of the collagen fibrils is greatly reduced, and collagenous tissues such as skin, tendons, and blood vessels become fragile and tend to tear. The degree of cross-linking varies depending on the requirement of mechanical
strength for the tissue. The stabilized fibrils bundle to form collagen fibers with diameters in the range of 0.2 to 12 μm [27]. The mechanical strength of the tissue also depends on the orientation of the collagen fibers [28].

When mechanical load is applied to tissues, the collagen molecules, fibrils, and fibers deform and eventually fail by a process called defibrillation [29]. Collagen molecules bear tissue strain via molecular deformation. Even though the exact mechanism of this mechanical energy transfer is unknown, it is thought that up to ~2% of tissue strain is carried by molecular stretching, and collagen fibrils can elongate up to 4% by molecular slippage [29]. However, up to 40% of the tissue strain is attributed to fibrils, and the mechanical characteristics such as extensibility and stiffness of collagenous tissues can be attributed to fiber crimping and to fibril diameters and packing [19, 29]. Collagen fiber uncrimping is primarily responsible for the extensibility of collagen fibrils. When mechanical load is applied to collagenous tissues, the initial load causes uncrimping of the collagen fibers [30]. The process begins as the crimped fibers start to straighten out under relatively small tensile loads. Initially, there is small resistance to tension as the fibers lengthen, but as elongation progresses, a larger number of fibers become taut. Eventually after all of the fibers are straightened out, the fibrils start bearing the load.

Collagen fibril diameters and packing contribute to tissue mechanics. Lim et al. attributed greater extensibility of collagenous tissues, such as chordae, to decreased fibril density [18]. In his study, he observed that fibril diameters in larger chordae (tendon-like structures of heart valves) (51.6 ± 0.2 nm) were smaller than that in smaller chordae (54.5 ± 0.2 nm), and that the larger chordae were more extensible. Larger fibrils will have more cross-linking, so they are stiffer than smaller fibrils. However, a reverse relationship is
observed between fibril diameter and density [19]. Fibrils with uniformly large diameter or a wide range of diameters cannot pack as tightly as fibrils with uniformly small diameter. This loose packing will reduce the load bearing cross-sectional area. Therefore, collagenous tissues are generally weaker and more extensible when they contain larger fibril diameters or wide range of diameters with loose packing.

2.1.2 Elastin

Elastin is responsible for the elastic recoil of load bearing tissues between loading cycles [31]. Elastic fiber sheaths that wrap around collagen fibers within tissues or tissue engineered constructs are thought to restore the native wavy (crimping) pattern of collagen fibers upon relaxation of the tissue [28]. Elastic fibers are composed of a central amorphous core of the protein elastin surrounded by microfibrillar proteins [20]. Cross-sections of elastic fibers have shown the presence of PGs within the elastin core [28]. Thin, parallel elastic fibers have been observed parallel to collagen fibers in uniaxial tissue-engineered (TE) constructs, similar to native chordae tendineae [31]. A high abundance of elastic fibers in engineered tissues are thought to reduce creep through elastic recoil of the crimp configuration of collagen fibers [26, 32].

2.1.3 Glycosaminoglycans

GAGs are linear polysaccharide chains. Each GAG polymer chain consists of repeating disaccharide units: a hexosamine in addition to a carboxylate and/or a sulfate ester [20]. After the GAGs are polymerized onto proteins giving rise to the “parent polymers,” enzymatic modifications of the sugar molecules give rise to unique GAG composition [33].
There are 4 main classes of GAGs: (1) hyaluronan, (2) chondroitin sulfate and dermatan sulfate, (3) heparan sulfate, and (4) keratan sulfate.

The disaccharide units in GAGs are composed of either of the two sugars N-acetyl glucosamine or N-acetyl galactosamine and a uronic acid such as glucuronic or iduronic acid [16]. The amino sugars are usually sulfated at 2, 4 or 6 positions and the uronic acids are usually sulfated at 2 position [16]. Other possible modifications to the sugar chain include addition of O-sulfate groups, replacement of the N-acetyl glucosamine groups by N-sulfate groups, or an isomerization of D-glucuronic acid into L-iduronic acid [17]. Each disaccharide in a GAG chain may contain any, all, or none of these modifications.

The GAG chains are highly negatively charged since they carry sulfate or carboxyl groups on most of their sugars [16]. In fact, GAGs are the most anionic molecules produced by animal cells. Due to their high charges, the sugar chains are too stiff to fold and are heavily hydrophilic. Thus, GAGs tend to form an extended conformation, occupy large volumes relative to their masses, and form gels even when present in low amounts. This porous gel formation is important for connective tissues since the gels fill the extracellular space and provide mechanical support to the tissue. The GAGs’ negative charge also attracts a variety of cations, mainly \( \text{Na}^+ \), which are also osmotically active. Thus, GAGs cause a large amount of water molecules to be locally bound to the matrix. This swelling pressure caused by the water molecules can be beneficial for specific tissues, such as cartilage, as they help withstand compressive forces.
2.1.4 Proteoglycans

PGs consist of a protein core and one or more GAG chains that are attached to the protein core via serine residues [16, 17]. A link tetrasaccharide is first attached to the serine residue, and the GAG chain is then synthesized at the link chain (Figure 2-3) [16]. PGs can contain up to 90% carbohydrate mainly in the form of long unbranched GAG chains that range from 30-90 disaccharides long. The GAG chains of the PG bind to proteins present on the cell surface, proteins secreted by cells, or different ECM proteins [17]. PGs are present within intracellular vesicles, on the cell surface, and in the ECM. PGs vary in type of GAGs, number and lengths of GAG chains, modifications in the repeating patterns of the disaccharides [16], and core protein structure [20]. For example, the protein core may contain polypeptide chains within the range of 10 Da to 400,000 Da [17]. For this reason, PGs can be very large, such as aggrecan, or they can be small, such as decorin. Despite this variability, all PGs are classified as a single type of ECM because of their GAG chains. The GAG chains control the structure and molecular interactions of the PG, giving them similar properties during separation from other biological molecules.

![Figure 2-3: General structure of proteoglycans. [16]](image)

PGs not only have a wide range of variations in their structure, but they also have many biophysical and biochemical functions in the ECM [17]. In addition to performing a space-filling role, the GAG chains of the PG form gels of varying pore sizes and charge
density and thereby regulate transport of different molecules and cells. PGs participate in intracellular signaling by acting as a receptor on the cell surface to bind to signaling molecules, such as growth factors, and proteins. This binding of PGs to proteins immobilizes, blocks, or delays release of proteins, such as proteolytic enzymes and proteases. Another major function of PGs is to influence fibrillogenesis of collagen fibers within the ECM.

**Small leucine rich proteoglycans (SLRPs)**

SLRPs are a subgroup of PGs that participate in collagen fibrillogenesis, growth factor modulation, and regulation of cellular growth [2]. SLRPs contain core proteins of approximately 30-50 kDa and one or more chondroitin sulfate, dermatan sulfate or keratan sulfate GAG chains [5]. The core proteins of SLRPs are rich in the amino acids leucine and asparagines [20]. The C-terminal of the core protein contains 9-12 leucine rich repeats that exhibit collagen binding properties [34]. The N-terminal shows variation in glycosylation and amino acid sequence; this terminal participates in cell-cell, cell-matrix and protein-protein interactions.

SLRPs can be divided into 3 major classes based on amino acid sequences [35]. Class I consists of decorin and biglycan, class II consists of fibromodulin, lumican, and, keratocan, and class III consists of epiphycan and osteoglycin. Both decorin (90-140 kDa) and biglycan (150-240 kDa) contain chondroitin sulfate or dermatan sulfate chains. Fibromodulin (60 Kda) and other class II SLRPs, on the other hand, contain keratan sulfate chains attached through N-linked oligosaccharides [20].

The core proteins of SLRPs are involved in the collagen binding, although fibrillogenesis is improved when GAG chains are present [36]. The GAG chains, on the other
hand, are involved in the SLRP to SLRP binding. The SLRPs bridge adjacent collagen fibrils by binding to the D-period. This binding usually involves 2 SLRPs connecting the collagen fibrils though mutual interaction via their GAG chains mediated by accessory proteins. Since the binding force between the GAG chains is stronger than the core protein-collagen fibril binding force, when the fibrils detach, one of the SLRPs remain attached to the fibril. The detached SLRP might drag the other SLRP bound to it, which then binds via the protein head to a neighboring D-period on the fibril (marked 2 in Figure 2-4). However, if only one SLRP is attached to the fibril, the GAG chain might remain hanging (marked 1 in Figure 2-4). In addition, it has been proposed that SLRPs control the fibril size or diameter and interfibrillar separation and also maintain the mechanical integrity of the aligned collagen fibrils [19].

Figure 2-4: TEM image showing detachment of SLRPs during tearing of fibrils; 1: SLRP remains attached at one end; 2: Detached SLRP gets attracted to a site at a neighboring D-period. [19]
It is not clearly understood how SLRPs transmit forces along the collagen fibrils. According to the shear lag theory, the shear force between fibrils is transmitted throughout the entire fibril length as well as from fibril to fibril by the interstitial PGs (Figure 2-5) [37]. The transmission of forces results in interfibrillar slippage revealed through the skewing of the interconnecting GAG chains. Even though the binding force between GAG chains is less than the PG core-fibril binding, the large number of PGs present between fibrils facilitates the proper load transfer between fibrils.

![Model of decorin-collagen fibril interaction.](image)

**Figure 2-5: Model of decorin-collagen fibril interaction.** [19]

SLRPs can have compensatory or non-compensatory functions. Decorin and fibromodulin, among other SLRPs, are known to regulate collagen organization and fibrillogenesis in the matrix [20]. Exogenous decorin and fibromodulin have been found to inhibit collagen fibrillogenesis in vitro [20, 38]. However, based on histology and immunohistochemistry, decorin has been found to bind to a specific site on the collagen fibril, which is different from the binding site of fibromodulin [20]. Unlike decorin and
fibromodulin, KS PGs lumican and fibromodulin reportedly bind to the same region of type I collagen [34]. Fibromodulin knockout mice reportedly have increased lumican deposition indicating that SLRPs can compensate for one another. In the cornea, lumican is thought to control the thickness of the collagen fibrils since lumican knockout mice show opaque corneas with abnormally thick collagen fibrils, indicating the role of lumican on corneal transparency. Even though lumican and Dcn\(^{+}\) mice show similar behavior with regard to collagen fibril organization and diameter, this corneal opaqueness is observed only in lumican knockout mice indicating non-compensating behavior between lumican and decorin [38].

While decorin and fibromodulin perform similar functions regarding collagen fibrillogenesis, decorin and biglycan are located and expressed in different regions in developing tissues [20]. While decorin is located on collagen fibers, biglycan is located on cell surfaces and pericellular matrices. Thus, decorin is thought to be involved in collagen fibrillogenesis, whereas biglycan is believed to be involved in tissue development. For this project, we investigated the role of the SLRP decorin on collagen fiber assembly, tissue organization, and mechanical strength.

2.2 Role of decorin in ECM

Decorin is a SLRP that contains a single GAG chain of chondroitin/dermatan sulfate [16]. Decorin consists of 3 main domains (Figure 2-6): (a) the N-terminal containing a dermatan/chondroitin sulfate side chain and repeating pattern of cysteine residues, (b) the central region of leucine-rich repeats, and (c) the cysteine rich C-terminal region [2, 5]. The cysteine rich N- and C-terminus tend to form disulfide-bonded loops [5]. Decorin binds to
different types of collagen including type I, II, III, and VI in addition to interacting with a variety of growth factors and their receptors such as TGF-β [2], vascular endothelial growth factor [39], insulin-like growth factor receptor [40] and epidermal growth factor receptor [6, 41-43]. However, Decorin and TGF-β interaction has been most widely studied. Decorin acts as a reservoir for TGF-β and prevents TGF-β from binding to their receptors [44] and activating the Smad pathways [45, 46]. The decorin-mediated regulation of cellular phenotype, interaction with TGF-β, and collagen fibrillogenesis was studied in this project. Even though decorin influence both cellular phenotype and collagen fibrillogenesis, the mechanisms utilized for these two processes are not believed to be interlinked.

![Figure 2-6: The 3 main domains of SLRP decorin; 1) The N-terminal, 2) The central leucine-rich repeats, 3) The C-terminal. Adapted from [5]](image)

2.2.1 Effect of decorin on cellular phenotype

Decorin influences the growth rate of certain cell types. For example, decorin has been observed to inhibit the growth rate of Chinese hamster ovary cells [5, 6, 20]. Decorin ligates the epidermal growth factor receptor, which then upregulates cyclin-dependent kinase p21 and arrests cells in the G1 phase of cell cycle [6, 41-43]. Even though the growth rate inhibition primarily occurs via the epidermal growth factor receptor, an interaction between decorin and TGF-β is considered to influence this growth control [4]. Because TGF-β is
crucial for matrix synthesis and proliferation, the limited growth is due to the presence of decorin since decorin is a functional antagonist to TGF-β. In a study with decorin deficient mice, the mean number of fibroblasts in the periodontal ligament was 1.9 fold higher than normal, which supports the role of decorin on cell growth [47]. In the same study, the addition of exogenous decorin was shown to inhibit cell growth in a dose-dependent manner.

2.2.2 Decorin-collagen interaction

The interaction between decorin and collagen is reported to influence the mechanical behavior of the fibrillar collagen [29]. Although the magnitude of the strain for fibrillar deformation of collagen depends on the tissue type, lateral interactions between collagen fibrils reportedly influence its mechanical behavior. Decorin is thought to influence fibril diameter by inhibiting lateral fusion of fibrils. The GAG chains from collagen-bound decorin extend out from the fibril surface and connect to neighboring fibrils forming interfibrillar bridges [48] and thus improve the mechanical integrity of connective tissues. In fact, even small amounts of decorin increase ultimate tensile strength, load and strain at failure of collagen fibers [29].

Many studies have looked at the binding sites of collagen and decorin and reported that decorin binds to a specific core protein binding site near the D-period of collagen type I [17, 20]. The 17-amino acid peptides at the N-terminal and the GAG chain are apparently not required for this decorin-collagen binding [49]. This leucine-rich core of decorin tends to form arch-shaped solenoid-like structures that can fit on a single collagen triple helix (Figure 2-7) [2, 35]. Further studies have shown that there are at least 2 collagen binding domains in
the decorin core protein and neither the N-terminal nor the central leucine rich repeats of the core protein can fully bind to fibrillar collagen alone [49].

Figure 2-7: The arch-shaped solenoid formed by decorin core protein. [50]

The role of decorin on collagen fibrillogenesis has been supported in studies of knock out mice. The skin of decorin knockout (Dcn\textsuperscript{−/−}) mice shows extreme fragility. This weakness is absent in heterozygous mice [2], indicating that both alleles need to be absent for fragile phenotype to show. Under an electron microscope, the Dcn\textsuperscript{−/−} mice showed collagen fibrils of highly irregular diameters and abnormal fibril organization in skin, tendon, and other tissues (Figure 2-8) [2, 47]. Fibrils of both extremities of diameter, very thick (660 nm) [2] and very thin (40-60 nm) [2], are present in larger numbers in Dcn\textsuperscript{−/−} mice tendons. In contrast, the wild type mice tendons have a more regular distribution of collagen fibrils (average 140 nm) [48]. Similar trends were observed in skin, even though the mean diameters are similar (116 nm and 119 nm, respectively). The abnormality in the collagen fibril diameter of Dcn\textsuperscript{−/−} mice is thought to be due to lateral fusion of thick and thin collagen fibrils. In addition, the Dcn\textsuperscript{−/−} fibrils are very loosely and irregularly packed with irregular outline, compared to the wild-
type fibrils, showing uncontrolled lateral fusion of fibrils. Since decorin slows down the rate and degree of collagen fibrillogenesis in vitro, it is thought that this increased time promotes uniform fibril formation. This irregular fibril organization reduced the tensile strength of the Dcn\textsuperscript{-/-} mice skin, i.e., their skin was fragile, had less ductility, and could not resist stretching [2]. Similarly, when dermal fibroblasts from decorin knockout mice (Dcn\textsuperscript{-/-}) were grown in static 3-D culture, they were able to recover for the decorin deficiency and form more uniform fibril diameters after decorin was exogenously supplied in the culture medium [51]. The Dcn\textsuperscript{-/-} mice also show increased collagen degradation [2], since the presence of decorin increases the energy required to dissociate collagen fibrils and thus stabilizes them [38].

![Collagen fibril diameter distribution in skin](image_url)

**Figure 2-8:** Collagen fibril diameter distribution in skin; top: wide variability in diameter size in decorin deficient mouse; bottom: more uniform diameters in wild type mouse. [15]
2.3 Tissue engineering

2.3.1 Need for tissue engineering

Even though advances in medical technology have significantly improved healthcare, the failure of tissues and organs remains a major problem, incurring tremendous cost to the healthcare system every year. Approximately half of total annual healthcare expenditures are attributed to tissue and organ failure [52]. The common practice for organ or tissue replacement is to transplant donated organs from another individual, surgically repair the organ or use mechanical devices. However, the patient has to live with a reduced quality of life with all these procedures. The number of donors for organs is also very limited. Therefore, even though these options effectively save lives, a better option would be to regenerate or repair the patient’s own tissues or organs.

Tissue engineering is the emerging field aimed towards growing tissues or organs in vitro using biomaterials, biological cells, and biotechnology. Tissue engineering would be more effective compared to other transplantation techniques since the tissue could be easily generated based on patient need. Since the tissues or organs are regenerated from the patient’s own cells, there would also be a reduced risk of immune response and disease transmission.

In addition to its application in medical field, tissue engineering provides the opportunity to study structure-function relationships of biological systems in vitro by mimicking internal tissue structure. This research work investigated the interaction between collagen and decorin by using TE constructs that mimic native tissues in uniaxial tension.
2.3.2  Tissue engineered collagen scaffolds

In order to mimic native tissues in vitro, the cells need to be seeded in the proper kind of scaffold. Since we are interested in decorin-collagen interaction, the logical choice is to use collagen as the scaffold. In addition, collagen gels are a well-characterized scaffold for investigations of fibroblasts because collagen is the most abundant protein in our body and the gels provide a more favorable 3-D environment for cells than do 2-D surfaces [53-55]. Fibroblasts seeded in collagen gels migrate within the gel using α2β1 and other integrins [53-55]. The integrin receptors bind to ECM components and then initiate a cascade of signals that trigger motor proteins to power cell movement [53]. The cells then organize the collagen fibers in the directions of tension. In addition, even though cells tend to synthesize less collagen in collagen gels than they do in 2-D culture, the majority of the synthesized collagen has been shown to be incorporated into the developing tissue as fibrils as opposed to being lost into the media [56]. The collagen gels are prepared by mixing a neutralized solution of acid extracted collagen and cells in serum-containing medium. The collagen polymerizes when the temperature is brought up to 37°C, forming a network of fine fibrils [28, 57]. The cells seeded within the gel align the collagen fibrils and compact the gel. The degree of compaction depends on the cell number, cell type, and collagen concentration used. The gels can be anchored to provide tension or grown as free-floating gels.

2.3.3  Cell alignment and cell mediated contraction in scaffold

The traction forces exerted by cells are clearly observed in collagen-based scaffolds. These traction forces vary according to cell type. Fibroblasts, which are thought to provide tension during wound contraction, provide the strongest traction force for collagen gel
contraction [29], though other cells can contract collagen gels as well. The exact mechanism of why and how cells exert traction forces and why cells align themselves along the axis of mechanical constrains is not clearly understood [28]. It has been proposed that the tensional forces generated by fibroblasts are due to a balance between actin-myosin motor elements in microfilaments and compressive loading onto the microtubular element that maintain cell shape [58]. Fibroblasts differentiate into myofibroblasts, which in turn organize the actin cytoskeleton [59]. It has also been shown that the tensional force between fibroblasts and collagen fibrils is mediated by members of the β1 integrin family, in particular by α2β1 integrin [60]. Another explanation for this traction force is believed to be due to cell locomotion or migration [28]. According to this theory, as fibroblasts move and organize themselves within the gel, they bundle and orient collagen fibrils and provide the traction force for gel contraction. Cells align the nearby collagen fibrils first, and the traction force is then propagated throughout the interconnected collagen network. This traction force exerted by the cells is manifested by the macroscopic gel compaction [1]. Figure 2-9 shows one of the most widely accepted biomechanical feedback mechanisms [1]. The cell alignment and collagen synthesis is significantly different in free floating vs. anchored gels [54, 61]. Even though cells align themselves along collagen fibrils, orientation is random in free-floating gels [1]. In anchored gels, the collagen fibrils are highly aligned along the axis of strain, and the cells migrate and orient themselves around the fibrils.
Cell contractility can be influenced by external biological factors such as growth factors [28, 60, 62-68]. The activity of serum in regular media can be enhanced or replaced by different growth factors. Both TGF-β [65] and platelet derived growth factor [64] have been shown to stimulate gel contraction, whereas fibroblast growth factor inhibits gel contraction [62, 66]. TGF-β causes fibroblasts to differentiate into myofibroblasts, which are more contractile [69], by inducing expression of smooth muscle α-actin (SMαA) [59, 60]. Myofibroblasts are responsible for wound contraction and exhibit actin stress fibers along the axis of cell alignment. Other factors that influence construct compaction include cell type, cell passage, cell-seeding density, and cell-synthesized collagen [28, 70].

Figure 2-9: Self-organizing biomechanical feedback mechanism in tissue-engineered constructs. Adapted from [1]
2.3.4 *Effect of mechanical stimulation on collagen and decorin*

Mechanical stimulation of tissue-engineered constructs tends to stimulate the production of collagen. Tensile stretching increases cellular collagen deposition as well as fibrillar alignment and packing, possibly due to greater cross-linking between amino acid residues within or between adjacent fibrils [29]. Cyclic mechanical strain has been found to increase collagen synthesis two to four fold in a variety of cell types [71, 72]. Mechanical stimulation also increases cell and collagen fiber alignment [73]. Cyclic strain significantly improves ultimate tensile strength and elastic modulus of collagen gels when cultured for several weeks. In a study comparing scaffolds derived from collagen, fibrin, and a mixture of collagen-fibrin (1:1), each of the cell seeded gels under mechanical stimulation showed higher compaction, elastic modulus, ultimate tensile stress and toughness compared to static gels [74], possibly due to increased collagen deposition and alignment [75]. In addition, even though the collagen-fibrin gel in that study showed less compaction similar to fibrin gels, it had the highest linear modulus, ultimate tensile strength and toughness. This indicates that the combination of fibrin and collagen improves mechanical behavior more than collagen alone.

For the collagen gels grown under static loading or no loading, the addition of exogenous decorin has been shown to improve tensile strength and failure load of collagen fibers. Decorin binds adjacent collagen fibers and creates a meshwork, thereby improving mechanical strength of collagen by aligning and stabilizing collagen molecules and fibrils [29]. However, while stretching of acellular collagen fibers improves its mechanical behavior, it has been shown to reduce the level of decorin present in the collagen fibers [29, 76]. The loss of decorin has been proposed to act as a regulatory signal for increased collagen
production by cells [29]. It is possible that stretching breaks the link between collagen and decorin, as previously discussed by the shear lag theory, leading to its diffusion out of the tissue. The released decorin, in response to large mechanical load, can then stimulate cells to produce more collagen to bear increased load. Since mechanical stimulation the causes the formation of larger collagen fibril diameters that are packed densely, it has also been proposed that the mechanical stretching-induced loss of existing decorin may allow the lateral fusion of collagen fibers. Since a positive correlation is observed between mean fibril diameter and maximum load and stiffness [48], this stretch-induced loss could improve the mechanical strength of collagen.
CHAPTER 3

Importance of Tissue-Specific Proteoglycan Composition in Tissue Engineering

This chapter addresses various methods of integrating proteoglycans (PGs) into the design of engineered tissues and provides insight for designing tissue-engineered disease models that leverage current knowledge of PG biology. Even though PGs show immense possibilities in tissue engineering applications, they have seldom been utilized to their full potential. The most common tissue engineering application of PGs has been in scaffolds (i.e., matrigels and collagen-chondroitin sulfate matrices), where PGs or their glycosaminoglycan (GAG) chains are incorporated into the scaffold to promote cell growth, tissue remodeling, and intracellular signaling. In addition, many studies have reported the total amount of PGs synthesized within engineered tissues, but have not delineated which specific PGs or GAG classes are involved in engineered tissue development. In native tissues, various PGs are dynamically and differentially regulated to achieve specific biophysical and biological functions, such as compressibility and transparency. Therefore, the targeted modulation of specific PGs (via exogenous addition, endogenous stimulation with growth factors, or mechanical stimulation) may help engineered tissues to achieve native tissue properties. The PG composition of engineered tissues could also be modified to achieve disease models in vitro and thus provide a way to study the effect of external agents on PG-related disease mechanisms.

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3.1 Introduction

Tissue engineering is the active and promising area of research aimed towards designing tissues or organs in vitro using various combinations of cells, biomaterials, and techniques that enhance cell/matrix differentiation. This field also provides the opportunity to study structure-function relationships of biological systems in vitro by mimicking tissue structure. Many research groups have harnessed advances in tissue engineering to study the structure and function of the extracellular matrix (ECM) [29, 77]. However, proteoglycans, a major component of the ECM, have been given less attention than the fibrillar components of the ECM (such as collagen, elastin, and fibronectin) regarding their potential applications in tissue engineering.

Proteoglycans (PGs) consist of one or more glycosaminoglycan (GAG) chains that are attached, via a link tetrasaccharide [16], to serine residues within a core protein [33]. GAGs are long chains of repeating disaccharide units that are variably sulfated (Figure 3-1a-b). There are four main classes of GAGs: hyaluronan (HA), chondroitin sulfate (CS) and dermatan sulfate (DS), heparan sulfate (HS), and keratan sulfate (KS), among which only HA does not covalently attach to a PG core protein via a link tetrasaccharide. PGs as a group exhibit great structural diversity because each type of PG may contain different kinds of GAGs, different numbers and lengths of GAG chains, modifications in the repeating patterns of the disaccharides by a complex pattern of sulfate groups [16], and different core protein structure [78]. PGs can be present in monomeric form or can form aggregates by complexing with HA [79]. Figure 3-1c-d shows a simplified hierarchical organization for the large interstitial PG aggrecan and an aggrecan aggregate.
Both the core protein and the GAG chains of PGs play key roles in tissue remodeling, intracellular signaling, uptake of proteins, cell migration, and many other crucial functions in native tissues [16, 17]. PGs participate in intracellular signaling by acting as receptors on the
cell surface to bind to signaling molecules, such as growth factors, and other proteins [17]. This PG binding can immobilize, block, or delay the release of proteins, such as proteolytic enzymes and proteases, that are involved in cell migration and tissue remodeling [17]. The GAG chains of the PG bind to proteins present on the cell surface as well as to many soluble and ECM proteins [17]; there are numerous reports, relevant to both native and engineered tissues, of PGs influencing the production of other ECM. For example, PGs and their GAG chains have been reported to influence elastic fiber assembly [81, 82]. The elastin binding protein, a molecular chaperone that protects the elastin precursor molecule tropoelastin from self-aggregation and premature degradation, undergoes a conformational change in the presence of excess CS or DS GAGs. This change leads to the release of tropoelastin from the elastin binding protein, which is then degraded, and consequently results in impaired elastogenesis [82]. The small leucine-rich family of PGs and their GAG chains bind and organize collagen fibrils [79]; this important function will be discussed in more detail toward the end of this review. Furthermore, many GAG-rich PGs, particularly the hyalectins, will aggregate with HA [79]; this local accumulation of negatively charged GAGs creates an osmotic imbalance that draws in large amount of water [83], thus creating stiff supramolecular assemblies that can influence the mechanical characteristics of connective tissues [79]. PGs likely have similar contributions to tissue-engineered (TE) scaffolds and they can be exogenously added or their synthesis stimulated to impart native tissue-like properties.

PGs are generally categorized according to whether they are located in the extracellular space or on the cell surface. The PGs located in the extracellular space consist of three groups [79]: (1) basement membrane PGs, (2) hyalectins, and (3) small leucine rich
PGs (SLRPs). Basement membrane contains three PGs: perlecan, agrin, and bamacan. Perlecan is the most characterized basement membrane PG; it contains HS or CS GAG chains and participates in cellular lipid uptake and metabolism, cell adhesion and growth, and growth factor regulation [79]. Agrin, which contains HS chains, is the major and most characterized PG of neuromuscular junctions and causes the aggregation of acetylcholine receptors via signaling pathways that involve muscle-specific tyrosine kinase receptors [84]. The importance of agrin in acetylcholine receptor aggregation has been underscored by mutant mice studies, in which exons associated with acetylcholine receptor clustering of agrin were spliced causing the mice to die perinatally and preventing the formation of acetylcholine receptors associated with motor neurons [79].

Hyalectins, the second group, are large PGs with a tridomain structure, in which the C-terminal region binds to cell surface lectins, the central region binds to the GAG chains, and the N-terminal region binds to HA to form PG aggregates [79]. The binding of HA to hyalectins is mediated by link proteins. Hyalectins, which include the PGs versican, aggrecan, neurocan, and brevican, thereby provide a bridge between the cell surface and the ECM. Versican, the largest member of this chondroitin sulfate PG (CSPG) family, controls fibroblasts’ attachment to basement membrane glycoproteins, such as laminin, as well as cellular growth via epidermal growth factor (EGF)-like regions in the N-terminal domain of the core protein [85]. Aggrecan, the main PG of cartilaginous tissues, helps bear compressive loads by its highly hydrated nature imparted by numerous sulfated GAG chains [79].

SLRPs, the third group, include decorin, biglycan, lumican, fibromodulin and several other small PGs that participate in collagen fibrillogenesis, growth factor modulation, and
regulation of cellular growth [2]. The core proteins of SLRPs, which are involved in collagen binding, are rich in the amino acids asparagine and leucine [78].

Cell surface PGs include syndecans, glypicans, thrombomodulin, and CD44 [85, 86]. These PGs bind to a variety of ECM molecules and ligands. Syndecans contain HS and/or CS chains and bind to collagen types I, III, V, fibronectin, thrombospondin, and other ECM molecules to form a stable pericellular matrix and participate in intracellular signaling. Glypicans contain HS chains and facilitate the signaling and transport of growth factors [86] such as fibroblast growth factor (FGF) via binding to the GAG chain [87]. Thrombomodulin participates in the anticoagulatory system, whereas the HA-binding PG CD44 contributes to cell adhesion and recognition, especially in immunity [85]. Table 3-1 summarizes commonly studied PGs and their functions of relevance to tissue engineering. More comprehensive explanations of PG functions and biology can be found in excellent reviews by Iozzo et al. [79], Hardingham et al. [85], and Fransson et al. [86].

Even though the quantities of PGs and GAGs are often assessed in tissue engineering research, these molecules are predominantly treated as a single group. Specific PGs, however, have been observed to be present in varying abundance in different native tissues, suggesting their roles in tissue-specific behavior [88]. Due to the immense diversity of functions of PGs, this paper will review some of the roles and applications of PGs and GAGs that pertain to tissue engineering, address ways to engineer TE constructs that contain specific PGs and GAGs and thus better mimic native tissues, and then highlight some beneficial aspects of the SLRPs that offer great potential to this developing field.
### Table 3-1: Commonly studied PGs, their GAG chains, and primary functions

<table>
<thead>
<tr>
<th>PG</th>
<th>Group/location</th>
<th>GAG chain(s)</th>
<th>Primary functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perlecana[79]</td>
<td>Basement membrane PGs</td>
<td>HS or CS</td>
<td>Cellular lipid uptake and metabolism, cell adhesion and growth</td>
</tr>
<tr>
<td>Agrin[79]</td>
<td>Basement membrane PGs</td>
<td>HS</td>
<td>Aggregation of acetylcholine receptors</td>
</tr>
<tr>
<td>Versican[79]</td>
<td>Hyalectins</td>
<td>CS or DS</td>
<td>Attachment to basement membrane proteins, cell proliferation, cell migration; withstand compressive loading</td>
</tr>
<tr>
<td>Aggreccan[79]</td>
<td>Hyalectins</td>
<td>CS and KS</td>
<td>Withstand compressive loading</td>
</tr>
<tr>
<td>Decorin*, Biglycan*, Lumican†, Fibromodulin†[79]</td>
<td>SLRPs</td>
<td>*CS or DS †KS</td>
<td>Collagen fibrillogenesis, growth factor modulation</td>
</tr>
<tr>
<td>Syndecans[85, 86]</td>
<td>Cell surface PGs</td>
<td>HS or CS</td>
<td>Bind to collagen I, III, V, fibronectin, thrombospondin</td>
</tr>
<tr>
<td>Glypicans[85, 86]</td>
<td>Cell surface PGs</td>
<td>HS</td>
<td>Signaling and transport of growth factors</td>
</tr>
</tbody>
</table>

### 3.2 Assessment of PGs in tissue-engineered constructs

Despite the ubiquitous distribution and diverse functions of PGs, the characteristics of individual PGs have rarely been studied in tissue engineering. In the limited reports that do address PGs, most have focused on determining the total amount of GAGs and seldom have investigated the specific PGs synthesized within TE constructs or secreted into the surrounding culture medium. The most common technique reported for determining total sulfated GAGs and PGs in TE constructs as well as native tissues is by radiolabeling [76, 89-91], such as with $^{35}$S, or through commercially available assays such as the Blyscan assay (Biocolor Ltd., Belfast, Northern Ireland) for sulfated GAGs. The Blyscan assay uses 1,9-dimethylmethylenedi blue dye to label sulfated PGs and/or the protein free sulfated GAGs.
chains. Western blotting has also been used to detect the core protein of specific PGs in TE constructs [92, 93]. A newer and less widely used technique is fluorophore-assisted carbohydrate electrophoresis (FACE) [92]. To perform FACE, briefly, the samples are digested with GAG-specific enzymes such as chondroitinase or heparinase to cleave the GAG chains into disaccharides, stoichiometrically labeled with a fluorophore tagged, and then electrophoresed on a monosaccharide gel. The gel bands can then be imaged and analyzed to quantify the variously sulfated and unsulfated disaccharides. Some, but not all, of these techniques have been applied to the field of tissue engineering.

Various microscopic and histological modalities can be used for qualitative analysis of the GAGs and PGs synthesized within TE constructs. Electron microscopy can be used to examine the association between PGs and other ECM components, such as the collagen fibrils in the TE scaffolds. To our knowledge, only one group has performed such an analysis. Rothenburger et al. demonstrated three different sizes of PGs in collagen scaffolds seeded with valvular interstitial cells, similar to that found in native valves [94, 95]. Using cupromeronic blue staining for transmission electron microscopy, they observed that small sized (65 nm) PGs, such as decorin, were aligned along the 60 nm collagen type I/III fibrils and that large sized PGs (180 nm), presumably versican, were located outside the collagen bundles in an amorphous ECM matrix. Heparan sulfate PGs (HSPGs), such as the syndecan family and perlecan, were present at the cell surface. Their study demonstrated that PGs organize in tissue-engineered scaffold in a similar fashion as in native tissues and provided one of the most thorough PG investigations to date in the field of tissue engineering.
3.3 PGS or their GAGS grafted in scaffolds

Tissue morphology varies due to the differential organization and composition of ECM components, including the PGs. To replicate this diversity in engineered tissues, PGs or their GAG chains have frequently been grafted to TE scaffolds. Most notably, CS GAGs have been used to create collagen-GAG scaffolds; likewise, the PG perlecan is a component of the matrigel scaffold. In addition, the GAG HA, as well as HA derivatives, have been widely used as scaffolds [83], either alone [96-98] or in combination with other proteins or polymers [99-103], such as fibrin, collagen, and chitosan. Since HA scaffolds and their applications in TE have recently been reviewed [83], this review will be limited to scaffolds that contain PGs or sulfated GAGs. Table 3-2 summarizes the scaffolds discussed in this section and their main applications relevant to tissue engineering.

Table 3-2: Advantages and main applications of collagen-GAG and matrigel scaffolds in tissue engineering

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>PGs or GAGs incorporated</th>
<th>Advantages</th>
<th>Applications</th>
</tr>
</thead>
</table>
| Collagen-GAG | Primarily CS             | • Better tissue regeneration  
• Induce higher PG synthesis  
• Specific PGs can be grafted to achieve tissue-specific behavior | • Nerve regeneration[104-108]  
• Artificial cornea[109, 110]  
• Artificial skin [111, 112][113]  
• Bone[114]  
• Cardiac and vascular tissue[115]  
• Fat grafting[116]  
• Liver-like tissue[117] |
| Matrigel    | Perlecan                 | • Derived from basement membrane, hence appropriate model for tumor cell invasion and cell migration studies |                                                  |
3.3.1 Collagen-GAG scaffolds

Collagen-GAG scaffolds have been extensively used for studying the function of PGs in TE scaffolds. Collagen-GAG scaffolds are convenient for TE applications because they can be sterilized by both heat and chemical procedures and can be manufactured with a variety of pore structures and a large range of degradation rates [118]. The collagen-GAG scaffold is fabricated using a freeze-drying process in which a collagen/GAG suspension is solidified, leaving the collagen-GAG to co-precipitate between growing ice crystals [118]. The range of pore sizes developed during this freezing process is biologically important because the pores need to be large enough to allow cell migration within the scaffold, and each cell type requires an optimal pore size. To prevent the elution of the GAG chains after implantation within the body (due to the neutral pH), the GAG chains are grafted to the scaffold through exposure to high temperature [119], which also sterilizes the scaffold [118, 119].

The incorporation of GAG into a collagen scaffold has been found to improve tissue growth and regeneration over the use of collagen alone [77, 118]. However, the role of the GAG component, most frequently chondroitin-6-sulfate, in the collagen-GAG scaffold is not completely understood. Although the GAGs are recognized to be water-binding [120, 121] and indirectly participate in collagen fibril organization [120], the presence of GAGs has not been found to alter the morphology, in vitro degradation [120], or mean tensile strength [121] of native unseeded collagen-GAG matrices. The GAG component, however, has been observed to delay matrix degradation in vivo [104], which indicates some possible interaction between cells and the GAGs. The collagen-GAG combination also induces more native tissue-like ECM composition in the engineered tissue than would collagen alone, possibly
through interactions with growth factors [77], which explains its attractiveness in regenerative tissue engineering. Furthermore, the collagen-GAG scaffolds induce cells to retain more PG aggregates within the scaffold (60% of the total newly synthesized PGs), similar to native tissues, compared to monolayer cell cultures, in which only 40% of the total newly synthesized PGs were retained within the cell layer [122]. These synthesized PGs were also larger (i.e., had more GAG chains) than those produced by cells in monolayer culture; because these larger PGs can entrap greater volumes of water, their presence likely contributes to the compressive and viscoelastic mechanical properties of the TE constructs.

Even though collagen-GAG scaffolds primarily incorporate GAG chains as opposed to PGs, the scaffold properties do not change significantly if PGs are used instead. For example, when the CS in the scaffold was replaced by DS, the PG decorin, or the PG aggregcan, no difference in the resulting mechanical behavior of the scaffold could be observed [104]. However, incorporation of decorin and DS delayed contraction of the scaffold compared to incorporated CS or aggregcan. Although varying the GAG component within the scaffold may affect its biophysical or biological function, these different collagen-GAG scaffolds echo the diversity of native tissues [121]. For example, DS might be appropriate to include in a TE skin scaffold, whereas aggregcan incorporation would be suitable for cartilage. The influence of GAG components on scaffold properties were also supported by Moghe et al. in a study where hepatocytes were cultured in a variety of collagen I, heparan sulfate, and matrigel combinations [123]. The authors observed that heparan sulfate component of the collagen I-heparan sulfate sandwich induced expression of EGF receptors leading to basal polarization of the hepatocytes. In addition, they also reported that while a collagen I-heparan sulfate sandwich exhibited cell surface markers similar to
adult liver, hepatocyte attachment, polarity, morphology, connexin and albumin expression, among other characteristics, varied based on the ECM composition used.

Various collagen-GAG scaffolds have been successfully used in tissue-engineered applications ranging from nerve regeneration to artificial skin [112]. Collagen-GAG scaffolds have also been seeded with dental cells derived from enamel, dentin, and cementum [124], knee meniscus cells [112] and oral mucosal cells [125]. Silicone tubes filled with collagen-GAG matrix are the most common current applications for nerve regeneration [104]. This silicone tube-collagen-GAG combination has shown to induce significantly greater number of axons per nerve, number of large diameter axons, mean fiber diameter and action potential conduction velocity when compared to unfilled tubes, in which regeneration was poor [108]. More recently, biodegradable collagen tubes filled with collagen-GAG matrix have been investigated in rat models [104-108]; these conditions reportedly regenerated a large number of axons, similar to autograft controls. Collagen-GAG scaffolds are widely used as artificial skin since these natural scaffolds produce less hypertrophic scarring than do control grafts (autografts, allografts, xenografts and a synthetic dressing) [112]. An unseeded collagen-GAG scaffold affixed to a disposable silicone sheet (INTEGRA, Ethicon, Inc., Somerville, NJ) was first FDA approved as an artificial skin for burn treatment in 1996 [111] and has an acceptance of 80% compared to autografts. The INTEGRA dermal matrix has also been cultured in a semi-automated perfusion culture system and has shown improved cell growth and differentiation in cell layers compared to regular culture [111]. However, cultured skin substitutes of collagen-GAG scaffolds preseeded with autologous fibroblasts and keratinocytes showed a clinical success rate of
only 50% (which the authors attributed to protease activity in the wound area), and therefore these cell seeded grafts have not been accepted as skin replacements [113].

Collagen-GAG scaffolds have also recently been used as substrates for osteogenic and chondrogenic tissue development [114]. The authors suggested that collagen-GAG scaffolds are very appropriate for bone tissue engineering, since osteoblasts seeded in collagen-GAG scaffolds show greater adhesion and proliferation and express more markers for osteoblast function, such as alkaline phosphatase, when compared to other scaffolds used for bone such as polystyrene, titanium, and poly-lactic or poly-glycolic acid. In this study, the adult mesenchymal stem cells seeded in collagen-GAG scaffolds, when treated with osteogenic factors, such as dexamethasone, ascorbic acid, and β-glycerophosphate, showed cellular migration, matrix mineralization, production of collagen I and osteocalcin similar to poly(ethylene glycol)-based and agarose-based hydrogels by 21 days.

A variation of traditional collagen-GAG scaffolds has been utilized in artificial corneas, where keratan sulfate (KS) and lumican, a KS SLRP, have been used to improve corneal transparency.[109] The transparency of the cornea is attributed to the structure of corneal stroma, a specialized tissue composed of multiple lamellae of parallel, highly regular collagen fibrils. The stroma also contains GAGs and PGs including lumican, which binds to the collagen fibrils. In a study by Doillon et al., addition of type A CS (chondroitin 4-sulfate, found in humans in cartilage, bone, cornea, skin and arterial wall) [126] and type C CS (chondroitin-6-sulfate, found primarily in fish and shark cartilage) [126] to collagen-GAG scaffolds improved transparency of the TE cornea; maximum transparency was observed for type C CS [110] indicating a role for GAG fine structure.
3.3.2 Matrigel scaffolds

Matrigel, a unique PG-containing scaffold, has also been widely studied and reviewed for tissue engineering applications [127, 128]. Matrigel is a soluble basement membrane extract derived from Engelbreth-Holm-Swarm tumor cells [127-130]. The main components of matrigel include laminin, fibrin, nidogen, entactin, collagen IV, perlecan, and growth factors such as transforming growth factor (TGF)-β, EGF, insulin like growth factor 1 (IGF-1), platelet derived growth factor (PDGF), and nerve growth factor [128]. Since matrigel consists of basement membrane components, one primary application of this scaffold has been to study tumor cell migration and invasion of the basement membrane [127, 129, 131, 132]. Other major applications of matrigel include angiogenesis models [127, 133, 134] as well as cell proliferation, differentiation, and migration studies [127, 128, 135, 136]. Unlike the collagen-GAG scaffold, to our knowledge, the groups studying matrigel for tissue engineering have neither studied the PGs synthesized in the scaffold, the contribution of the PG component present in the matrigel, nor have applied external PGs to change the scaffold properties. Nevertheless, matrigel is commonly used for tissue engineering applications, albeit with a primary focus on engineering cardiac and vascular tissues [115, 137, 138]. For example, Abilez et al. demonstrated that combining matrigel and shear stress promoted the differentiation of cells into layers similar to native blood vessels [115]. Similarly, Cassell et al. reported matrigel to be better than fibrin, but not poly-D,L-lactic-co-glycolic acid (PLGA), for generating vascularized and transplantable tissue [137]. Another application of matrigel in tissue engineering has been for adipogenesis, where Kelly et al. demonstrated that besides encouraging neovascularization, matrigel can induce adipose cell generation when the scaffold is in contact with surrounding adipose tissue [116]. Nahmias et al. used matrigel
to establish endothelial vascular structures and then leveraged their interactions with mature hepatocytes to direct and support liver sinusoid formation, which could then be used to study liver toxicity, ischemia, and fibrosis [117]. However, one shortcoming of matrigel in tissue engineering is that since it is derived from mouse tumor, it may not be acceptable for human use [137].

Compared to the other major components of matrigel, the function of the heparan sulfate PG perlecan is less studied, even though perlecan is thought to play key roles in blood vessel growth and structural integrity [139]. The C terminus of perlecan in particular has been reported to inhibit endothelial cell migration, collagen-induced endothelial tube formation, and blood vessel growth [139]; therefore, it has been suggested that the presence of perlecan might indirectly retard tumor growth in vivo. In addition, perlecan is thought to participate in the control of tumor cell growth and metastatic behavior by its association with different growth factors, which bind to HS, such as FGF [140]; nevertheless, the degree of tumor growth and invasion depends on the location of perlecan and the cell type since perlecan can act both as an adhesive and antiadhesive protein [79]. Similarly, perlecan could be utilized in tissue engineering by harnessing its interactions with growth factors and cell surface receptors [128].

3.4 Factors that influence PG production

Obtaining tissue-specific PG production will be critical for successful tissue engineering since PGs influence the mechanical and structural characteristics of native tissues as well as TE constructs. Variability in PG production and accumulation in tissues could lead to different mechanical and physical behavior, i.e., compressibility [88],
extensibility [92], and transparency [109]. However, engineered tissues have demonstrated difficulties achieving the same PG levels as found in native tissues [141, 142] possibly due to an inability to retain PGs or due to lower PG productions by the seeded cells. Therefore, targeted stimulation to achieve specific PG production might be desirable to attain native tissue characteristics in the engineered scaffold. For example, high PG production is important for tissues such as nucleus pulposus, the central portion of intervertebral disk, and hyaline cartilage [88]. Nucleus pulposus cells reportedly synthesize more PGs than collagen, resulting in a GAG to hydroxyproline ratio more than five-fold of that found in cartilage. Therefore, a tissue engineered nucleus pulposus should contain high ratio of PG (especially aggrecan) to collagen in the ECM to resist compression, whereas a hyaline cartilage should contain more collagen than PG to mimic its native mechanical characteristics. Obtaining tissue-specific PG production should be a future goal in the field of tissue engineering. Table 3-3 summarizes a variety of stimuli and their reported effects on PG and GAG synthesis.
### Table 3-3: Various external factors differentially stimulate PG and GAG synthesis in tissue engineered constructs

<table>
<thead>
<tr>
<th>External Stimuli</th>
<th>Cell/tissue type</th>
<th>Effect on PG/GAG synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth factors and cytokines:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β, bFGF, and IGF-1[143]</td>
<td>Annulus fibrosus and nucleus pulposus</td>
<td>↑ total PG</td>
</tr>
<tr>
<td>TGF-β and growth factors in FBS[109]</td>
<td>Keratocytes</td>
<td>↓ KSPGs, ↑ DSPGs</td>
</tr>
<tr>
<td>PDGF and TGF-β[144]</td>
<td>Chondrocyte</td>
<td>↓ aggrecan, ↑ total PG</td>
</tr>
<tr>
<td>EGF, FGF, PDGF-AB, IGF-1[145]</td>
<td>Smooth muscle cells</td>
<td>↑ GAGs (DS, HS)</td>
</tr>
<tr>
<td>Interleukin-4[146]</td>
<td>Gingival fibroblasts</td>
<td>↑ CSPG in cell layer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ HSPG in media</td>
</tr>
<tr>
<td><strong>Antibiotics:</strong></td>
<td></td>
<td></td>
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<tr>
<td>Staurosporine[147]</td>
<td>Chondrocyte</td>
<td>↑ GAG</td>
</tr>
<tr>
<td><strong>Mechanical stimulation:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High shear vs. low shear[91]</td>
<td>Endothelial cells</td>
<td>↑↑ (high) and ↑ (low) total PG</td>
</tr>
<tr>
<td>Cyclic pressure and high amplitude[148]</td>
<td>Chondrocyte</td>
<td>↑ total PG</td>
</tr>
<tr>
<td>Dynamic culture[110]</td>
<td>Human dermal fibroblasts</td>
<td>↓ GAG</td>
</tr>
<tr>
<td>Tensile stretching[76]</td>
<td>Arterial smooth muscle cells</td>
<td>↑ biglycan, ↓ decorin</td>
</tr>
</tbody>
</table>

3.4.1 **Growth factors and cytokines**

Increased PG and GAG synthesis can be achieved using chemical stimuli such as growth factors and cytokines, which are often used in tissue engineering. However, their influence depends on the type of cultured cells or the tissue being engineered, as different cells tend to respond to the stimulating factors in distinct ways. For example, growth factors such as TGF-β, basic fibroblast growth factor (bFGF) and IGF-1 all increased PG production for annulus fibrosus and nucleus pulposus cells seeded within a 3-D scaffold [143], with the maximum PG production observed for TGF-β. Depending on the cell type, the constructs treated with TGF-β contained two to five times as many PGs as those treated with fetal bovine serum (FBS) alone. This effect was not only due to increased cell density, but also to
increased PG production by the cells. PG accumulation was also significantly greater when bFGF and TGF-β were used in combination in the same study. The retention of specific PGs, such as aggrecan, in the scaffold can be improved by adding the GAG hyaluronan to promote PG aggregate formation [88]. This retention of aggrecan is significant for the design of engineered tissues such as nucleus pulposus since aggrecan must be present in a large amount to resist compression [88].

Growth factors such as TGF-β can also stimulate or inhibit specific PG production in cell cultures [109]. In monolayer culture, keratocytes that are involved in the wound healing response in the cornea secreted normal PGs in serum free medium [109]. However, when the same cells were treated with TGF-β and/or with FBS (which contain many growth factors), they reduced their production of keratan sulfate PGs (KSPGs), such as lumican, similar to wound healing and inflammatory responses in vivo. Dermatan sulfate PGs (DSPGs) responded to growth factors in the opposite manner as KSPGs in this study [109]. Biglycan, a DSPG, while not present in normal cornea, appeared within 24 hours of treatment with growth factors and increased during the period of treatment. On the other hand, chondrocyte cells treated with human platelet supernatant, which contains both PDGF and TGF-β, showed a reduced transcription rate for aggrecan and increased collagen I synthesis similar to wound healing chambers [144]. The treated chondrocytes, however, showed increased cell proliferation and overall PG synthesis. Smooth muscle cells also showed increased GAG synthesis (i.e., DS, HS) for growth factors including EGF, FGF, PDGF-AB, and IGF-1 regardless of their effect on cell proliferation [145]. In a similar manner, since TGF-β is a functional antagonist to the DSPG decorin [5, 6], it could be used to inhibit decorin synthesis
in tissue surrogates. Therefore, depending on the tissue-specific need, specific PG synthesis might be stimulated in the TE constructs using certain growth factors.

Cytokines, similar to growth factors, can also stimulate or inhibit specific PG production in tissue surrogates. In a study on cultured gingival fibroblasts, interleukin-4 stimulated the synthesis of CSPG in the cell layer and increased the secretion of HSPG into the media, suggesting that interleukin-4 might reduce retention of HSPGs syndecan and perlecain in the matrix [146]. Similarly, interleukin-4 might be applied to TE constructs if a higher ratio of CSPGs to HSPGs is desired for certain tissue types.

In addition to growth factors and cytokines, external factors such as Staurosporine, an antibiotic known to inhibit protein kinase C and disrupt cytoskeletal structure, has been shown to increase GAG production [147]. In fact, for chondrocyte cells grown in a collagen-GAG matrix, Staurosporine has been reported to induce a 10 fold increase in GAG production by 15 days when compared to the untreated group [147]. In the same study, Staurosporine also markedly reduced contraction of the collagen matrix; reduced contraction is beneficial for treating cartilage defects. Since GAGs function as space fillers, the increased GAG production might have contributed to the reduced contraction. Nevertheless, since this antibiotic is a protein kinase C inhibitor, it can only be used \textit{in vitro} for experimental TE purposes, but not for \textit{in vivo} applications [147].

\subsection{Mechanical stimulation}

Mechanical stimulation has likewise been shown to stimulate differential PG production and accumulation in cell cultures, native tissues, and in engineered tissues [91][148][149]. As with chemical modulation, the amount of PGs produced reportedly
depends on the type and magnitude of mechanical stimulation applied to the cells or tissues. For example, application of low shear vs. high shear to endothelial cells caused quantifiable difference in PGs, primarily the level of PG production, in the isolated medium [91]. However, shear flow duration of at least 24 hours was required to see any changes in PG production. In the same study, higher shear significantly increased the level of PG compared to low shear, while both were higher than static culture. In addition, the ratio of high molecular weight PGs to low molecular weight PGs was higher for low shear treated material than high shear treated materials. Since in this study, the high molecular weight PG fraction was more effective in preventing FGF-2 mediated vascular smooth muscle cell binding and growth, shear modulated PG production by endothelial cells might influence other PG-mediated vascular activity, such as that of heparin binding growth factors. Similarly, chondrocyte cells seeded in agarose gels, when subjected to intermittent mechanical stimulation, showed increased total PG synthesis [148]. The authors demonstrated that even very short periods of intermittent stimulation (6 minutes every second day) stimulated matrix production. With stimulation for 4 weeks, PG accumulation in the tissue surrogate was 30% higher compared to unstimulated controls. In the same study, when cyclic hydrostatic pressure was applied to chondrocytes grown in monolayers, PG synthesis was increased after 1-4 hours of stimulation [148]. The amplitude of the mechanical stimulation also appeared to influence ECM production, in that higher amplitudes were favored for PG synthesis while low amplitudes were preferred for collagen production. In addition, matrix synthesis was higher when more ECM was present in the agarose gels; this might be a possible reason for higher ECM accumulation in gels stimulated for 4 weeks as compared to 2 weeks.
Some controversy remains in the existing literature regarding mechanical stimulation and PG synthesis. It has been observed that mechanically stimulated cells, while demonstrating a higher initial PG synthesis, produced less PG in the long run than statically cultured cells. In a study on human dermal fibroblasts cells seeded on porous poly(ethylene glycol)-terephthalate/poly(butylene terephthalate) copolymer scaffold, the cells subjected to shear stress in 3-D culture produced significantly higher amount of total GAGs when compared to statically cultured cells [149]. However, after a few days, statically cultured cells produced higher amount of GAG than dynamic culture suggesting that medium flow did not favor GAG deposition on the scaffold. The authors proposed that the shear stress applied to the fibroblasts in this dynamic culture condition played a role in the production and degradation of ECM components involved in controlling tissue integrity and hence caused the reduction in GAG content. There may be an alternative explanation that the authors did not consider, that of the differential temporal synthesis of distinct PGs and GAGs. Even though mechanical stimulation improves the strength of engineered tissues by stimulating higher collagen synthesis and a more uniform alignment of collagen fibers in mechanically stimulated tissues and constructs [29, 71-73], SLRPs are thought to contribute to this higher strength by controlling collagen fibril organization and development. Therefore, the level of SLRPs is expected to increase with mechanical stimulation. In a conflicting study, however, the level of decorin was actually lower in mechanically stimulated tissues [29, 76]. In fact, the various SLRPs may behave differently: biglycan has been shown to increase in response to mechanical stimulation, while the level of decorin decreases [29, 76]. The loss of decorin has been proposed to act as a regulatory signal for increased collagen production by cells [29]. Since mechanical stimulation causes larger collagen fibril diameters with dense fibril
packing to form, thereby increasing effective load-bearing area, it has also been proposed that the mechanical stretching-induced loss of existing decorin may allow the lateral fusion of collagen fibers. Given that a positive correlation is observed between mean fibril diameter and maximum load and stiffness [48], this fusion would improve the mechanical strength of collagen.

Overall, recreating the functionality of native ECM is an extremely important goal for successful tissue engineering, and growth factors, cytokines, and mechanical stimulation are some of the possible ways of stimulating or controlling specific PG synthesis in engineered tissues.

3.5 The potential of SLRPs and other PGS in future engineered tissue design

PG synthesis can be modified or enhanced not only to achieve native tissue composition, but also to obtain additional desirable qualities in the TE surrogates. For example, DSPG SLRPs, such as decorin and biglycan, have been found to demonstrate antithrombotic properties by forming a DS-heparin cofactor II complex that inactivates thrombin [150-154]. The DS-heparin cofactor II complex can inhibit both fibrin clot bound thrombin as well as surface bound thrombin, unlike heparin, which can only inhibit fibrin clot bound thrombin [150, 153, 154]. This antithrombotic quality of DSPGs could be utilized in TE surrogates designed for replacing tissues in the circulatory system. In fact, the SLRP decorin is widely used in cancer research due to decorin’s inhibitory effect on cell growth [155-157]; however, the research so far is only limited to 2-D cell culture studies and does not yet appear to have been applied to tissue engineering.
PG synthesis can be altered in different diseased conditions, which relate to the importance of appropriate PG function in engineered tissues. For example, a transient expression of lumican is observed in chronic pancreatitis-like lesions causing fibrosis of the pancreas [158]. Likewise, overexpression of biglycan, decorin and fibromodulin occurs in experimental osteoarthritic cartilages [159]. Experimental injury to the annulus fibrosus, the outer portion of the intervertebral disc, stimulated expression of biglycan and decorin [160]. Since these PGs are involved in type I/II collagen fibrillogenesis, the authors suggested that the overexpression of these PGs might explain why annular lesions in the avascular inner annulus do not heal. In addition, the levels of different PG classes are altered in heart valve diseases and are believed to be partly responsible for the disorganized collagen fibers and valvular dysfunction [92].

Studies with knock out mice show the importance of PGs in maintaining normal function in our body. While decorin-deficient mice show resistance to Lyme disease [161], their skin shows fragility similar to Ehlers-Danlos syndrome [162]. Biglycan and fibromodulin knockout mice, on the other hand, have been valuable models for musculoskeletal diseases [162, 163]. Even though collagen fibril abnormalities are observed for all decorin, biglycan, and fibromodulin deficiencies, only biglycan and fibromodulin deficiency results in ectopic ossification in the tendons, lower bone mass, osteoarthritis, and impaired gait [162-166].

While knockout mouse models and 2-D cell culture studies have generated useful knowledge about the possible functions of PGs, tissue engineering should also provide a convenient way to mimic these diseased states in vitro and apply different external factors to achieve a better understanding of the disease mechanisms. Exogenously added PGs as well
as genetically engineered cells (that overproduce or are deficient in selected PGs or GAGs) can be appropriately used either to mimic diseased states *in vitro* or to determine potential treatments of diseases depending on the PG under study.

### 3.6 Conclusions

Even though some TE constructs are successfully being used in regenerative medicine, in most cases, their mechanical strength is several orders of magnitude lower than native tissue strength and need to be improved before can be used *in vivo*. A less widely recognized problem for TE constructs is their reduced ability to produce and retain PGs and GAGs, essential components of the extracellular matrix. PG production can be stimulated in TE constructs using growth factors and mechanical stimulation. To promote tissue organization, regeneration and growth factor binding, PGs have already been incorporated into collagen-GAG and matrigel scaffolds and have achieved ECM composition that is closer to more native tissues than those produced by scaffolds without PGs. PGs also show immense possibilities for using engineered tissues to model disease states, such as investigating how the SLRPs’ roles in collagen fibrillogenesis may be altered in connective tissue diseases. Overall, PGs have immense possibilities in the structural development and healing of engineered tissues and should be modulated to achieve native ECM-like composition or to study disease mechanisms.
CHAPTER 4

Enhanced Proliferation, Adhesion and Migration by Dcn" Cells on 2-D Collagen-Coated Surfaces

The proteoglycan decorin putatively inhibits cell adhesion and cell migration on various extracellular matrix substrates through interactions with β1 integrins. This chapter therefore discusses the adhesive, migration, and proliferative characteristics of decorin knockout (Dcn") murine embryonic fibroblasts compared to wild-type controls on collagen-coated, fibronectin-coated, and uncoated tissue culture plates. The Dcn" cells showed significantly greater proliferation than wild-type controls on all substrates. The Dcn" cells also showed significantly greater adhesion to both collagen and fibronectin; both cell types showed greater adhesion to collagen. For collagen, blocking either α2 or β1 integrin subunits significantly reduced adhesion for both cell types; however, the effect was stronger for the Dcn" cells, implying these cells bind to collagen via a decorin-integrin complex. For fibronectin, blocking both α2 and β1 integrin subunits slightly reduced adhesion for wild-type cells, but did not affect Dcn" cells. This result suggests that adhesion to fibronectin by wild-type and Dcn" cells is predominantly mediated by non-α2, non-β1 integrins, which also interact with decorin. Finally, compared to wild-type cells, Dcn" cells showed faster migration on both collagen-coated and uncoated substrates. This chapter also discusses the effects of decorin on the biology of various integrins that participate in cell proliferation, adhesion, and migration on various substrates.

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4.1 Introduction

Decorin, a member of the small leucine rich proteoglycan family, is an important regulator of collagen fibrillogenesis [2], collagen degradation [3], and matrix organization [167]. Decorin also modulates collagenase gene expression and the availability of growth factors in the extracellular matrix by binding of the decorin core protein to fibronectin [168, 169], collagen [170], and transforming growth factor beta (TGF-β) [4, 171]. Increased decorin expression has also been reported in healing wounds [172], infarcted myocardium [9, 10], tissues undergoing angiogenesis [13], tumor stroma [12], and arthritic joints [11]. The involvement of decorin in the above tissue remodeling and pathogenesis can be attributed in part to the inhibitory effect of decorin on cell adhesion [173, 174] as well as the ability of decorin to bind to TGF-β [4] and epidermal growth factor receptors [6, 41-43]. Due to both of these effects, the addition of decorin has been shown to influence the proliferation of many different cell types [5, 6, 47].

Cell adhesion to extracellular matrix substrates, predominantly fibronectin, collagen, vitronectin, fibrinogen, and laminin, regulates several aspects of normal cell behavior, such as proliferation, migration, gene expression, and protein synthesis [168, 175]. Cell adhesion to these matrix molecules occurs mainly through β1 integrins and triggers a variety of signaling pathways [175]. Interestingly, the negative influence of decorin on cell adhesion to these substrates has also been shown to be mediated via the β1 subunit of integrin [8, 174, 176]. It is not confirmed, however, whether this influence is mediated by the β1 subunit binding to the glycosaminoglycan (GAG) chain of decorin [8] or to the core protein [169, 177].
This influence of decorin on cell adhesion to matrix components likely also affects the development and maturation of engineered tissues. We recently reported that 3-D collagen gels seeded with decorin knockout (Dcn<sup>+/−</sup>) murine embryonic fibroblasts (MEF) showed greater matrix organization, cell proliferation, ultimate tensile strength, and elastic modulus than those seeded with wild-type control cells [178]. The mechanical behavior of the Dcn<sup>+/−</sup> cell-seeded gels was consistent with trends reported for native immature Dcn<sup>+/−</sup> tissues [179] but was different from the mechanical behavior of tissues from postnatal Dcn<sup>+/−</sup> mice [15]. Even though it was determined that TGF-β substantially influenced the above properties, we speculated that the observed characteristics of the developing 3-D collagen gels might also be attributed to the adhesive and migration characteristics of the Dcn<sup>+/−</sup> cells, which would result in their greater contraction of the collagen gels. We also found that the Dcn<sup>+/−</sup> cells expressed greater amounts of α<sub>2</sub>β<sub>1</sub> integrin than wild-type cells. Therefore, in this study we assessed the effect of decorin on the proliferative, adhesive, and migration behavior of Dcn<sup>+/−</sup> and wild-type MEFs grown on uncoated or collagen-coated tissue culture plastic, using the rationale that the surface-dependent cell behavior from these 2-D experiments would be consistent with observations from the 3-D tissue model. Since the Dcn<sup>+/−</sup> and wild-type cells were previously shown to express different amounts of α<sub>2</sub>β<sub>1</sub> integrins, the contribution of α<sub>2</sub> and β<sub>1</sub> integrin subunits to cell adhesion was also investigated. In addition, the cells’ adhesion to fibronectin was investigated to determine if the adhesive interactions between decorin and α<sub>2</sub>β<sub>1</sub> integrins were substrate-specific.
4.2 Experimental procedures

4.2.1 Cell culture

Embryonic fibroblasts were chosen as the cell type for this research work since we wanted a general cell type to study decorin and collagen interaction. Cells harvested from tissues such as skin or tendon would demonstrate tissue-specific behavior and the cell behavior would also be influenced by the stain experienced by the tissue. Even though embryonic fibroblasts have unique behavior and are not truly general for all tissue-type, they are believed to bear low non-uniform strains and since we investigated the influence of strain on matrix organization (Chapter 8), embryonic fibroblasts were considered suitable for this research. Embryonic fibroblasts also have been widely characterized and yield a large cell population from each harvest of embryos, which was found very suitable for this research.

Embryonic fibroblasts were isolated from euthanized Dcn<sup>−/−</sup> or wild-type mouse embryos (12.5 to 13.5 gestational days old) from Balbc background, as previously described [178]. The cells were maintained in an incubator (37°C, 5% CO₂, 95% humidity) and supplemented with culture medium containing high glucose Dulbecco's Modified Eagle Medium (DMEM, 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 medium was changed every 2 days and the cells were passaged upon confluence. Cells from passage numbers P5-P8 were used in this study.

4.2.2 Proliferation assays

The growth studies for the different types of cells were performed on uncoated and collagen coated tissue culture plastic. For the study using uncoated tissue culture plates, cells
were grown in 6-well plates at an initial seeding density of 1x10^5 cells/well (10.5x10^3 cells/cm^2) and the medium was changed every alternate day. The cells were harvested from triplicate wells every 2 days up to a total of 10 days. Cell numbers were measured using a hemacytometer (trypan blue exclusion).

For the study using collagen-coated plates, the cells were grown in 12-well plates coated with type I collagen (BD Biosciences). Cells were seeded at an initial density of 1.5x10^5 cells/well (3.9x10^4 cells/cm^2) and the medium changed every alternate day. Since the cells were grown on collagen, instead of trypsinizing the cells, an MTT assay (Sigma, St. Louis, MO) was performed according to the manufacturer’s instructions on the cells from triplicate wells every 2 days up to a total of 10 days. The higher cell density used in this study was required to achieve suitable absorbance values for the MTT assay. To perform the MTT assay, the culture media was first aspirated to remove any unadhered cells, then 1 ml of new media along with 100 μl of MTT reagent (5 mg/ml in sterile PBS, Sigma) was added to each well and the adherent cells were incubated at 37°C for 4 hours. After the incubation period, 1 ml of MTT solvent was added to each well and mixed by trituration until the formazan blue crystals dissolved. The absorbance of the resulting formazan blue product was measured in triplicate at 570 and 690 nm using a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA, US). The number of adhered cells per day was calculated by comparing the net absorbance (A_{570}-A_{690}) with net absorbance for a standard curve derived from known cell numbers. The standard curves were created by measuring net absorbance from triplicate wells seeded at 0.1, 0.5, 1.0, 2.0, and 4.0x10^5 cells/well. Since different cell lines might metabolize MTT differently, standard curves for both cell types were created to verify any difference in absorbance for the same cell number.
4.2.3 Adhesion assays

Cell adhesion to collagen and fibronectin coated surfaces was analyzed for both cell types. To evaluate time dependent adhesion to collagen, cells were plated in triplicate in 12-well tissue culture plates coated with type I collagen (BD Biosciences, Franklin Lakes, NJ) at an initial seeding density of $1.5 \times 10^5$ cells/well ($3.9 \times 10^4$ cells/cm$^2$). After 0, 5, 10, 30, 60, 90, and 120 min, the culture media was aspirated to remove any unadherent cells, and 1 ml of media was added to the specific wells. The number of the adherent cells at each timepoint was measured using the MTT assay and normalized to the number of cells adhered at the final timepoint.

To evaluate the effect of cell concentration on the adhesion to collagen, cells were plated in triplicate wells at 0.1, 0.5, 1.0, 2.0, and $4.0 \times 10^5$ cells/well ($0.3, 1.3, 2.6, 5.3,$ and $10.5 \times 10^4$ cells/cm$^2$). After 1.5 h, the MTT assay was used to determine the number of adhered cells as previously described.

The above studies (time and concentration dependence) were also performed using fibronectin coated plates (BD Biosciences) in order to determine if the role of decorin in cell adhesion was substrate-dependent. Although identical seeding densities were used, the experimental times were reduced to 60 min for the time dependence study and 30 min for the varying cell concentration study since both cell types adhered to fibronectin more rapidly than to type I collagen. Fibronectin substrate studies were also performed in triplicate wells.

Another study tested the ability of exogenously added decorin to restore the Dcn$^{-/-}$ cells to wild-type behavior. The cells were plated in triplicate in a 12-well tissue culture plate coated with type I collagen at an initial seeding density of $2.0 \times 10^5$ cells/well. Immediately
after seeding the cells, exogenous decorin (0, 1, 5, 10, or 20 μg/ml) was added to each well. After 1 h, the MTT assay was used to determine the number of adhered cells. The number of adhered cells for each concentration of decorin was normalized to the data from the control wells, to which no exogenous decorin was added.

4.2.4 Contributions of α2 and β1 integrin subunits to cell adhesion

To determine the influence of α2β1 integrins on MEF adhesion to different matrix substrates, antibodies against the α2 and β1 subunits (rabbit integrin α2 and β1 from the Integrin β1 Antibody Kit, Chemicon International, Temecula, CA) were used in a competitive binding study. The optimal antibody concentration required to cause an effect on cellular adhesion for both cell types on each substrate was determined to be 1:500. To perform this study, 1 ml of media containing 1.5x10^5 Dcn^-/- or wild-type cells (3.9x10^4 cells/cm^2) and the antibodies against either α2 or β1 integrin subunits was added to triplicate wells of 12-well plates coated with either type I collagen or fibronectin. Controls were performed without antibodies. After 1.5 h (collagen) or 30 min (fibronectin), the number of adhered cells was measured using the MTT assay as previously described. Each study was performed in triplicate wells and repeated three times. The numbers of adhered cells were normalized to the number of adhered cells in the control wells.

4.2.5 Migration study

Cell migration on collagen-coated or uncoated tissue culture plates was determined for both cell types. The migration study was designed based on the migration studies by DeLong et al., Ura et al. and Vernon et al. [180-182] One cloning cylinder (10 mm diameter
x 10 mm high, Fisher Scientific, Pittsburgh, PA) was placed at the center of each well of 6-well tissue culture plates, either uncoated or coated with type I collagen (BD Biosciences). Cells were seeded inside the cloning ring at 45,000 cells/well (in a volume of 50 μl to avoid leakage from the cloning rings) and incubated at 37°C. After four hours, the cloning ring was removed and the wells were carefully washed twice with PBS to remove unadhered cells. Three ml of fresh media containing mitomycin C (0.5 μg/ml, Sigma) was then added to each well. Each well was viewed with an inverted microscope to verify that the cells were adherent and confined within the circular region previously created by the cloning cylinder. After incubating the cells for 24 hours, the number of cells that migrated out of the original circular region (based on comparison with an equivalent size circle drawn on a transparency) was counted. The study was performed in triplicate wells for both cell types on collagen-coated and uncoated surfaces.

4.2.6 Statistical analysis

Replicate analyses were averaged to obtain means and standard deviations. Statistical evaluations were performed using Sigmastat software (SPSS, Chicago, IL) for 1, 2 and 3 factor ANOVA. When a significant difference was observed, post-hoc testing was performed as suggested by the software for pair-wise comparisons. A value of p<0.05 was considered significant.
4.3 Results

4.3.1 Greater proliferation for Dcn\textsuperscript{+} cells

Since decorin inhibits cell growth for a variety of cell types [5, 6, 47], the cell growth on regular and collagen-coated tissue culture plates were determined for both cell types. The 2-D growth studies performed on both surfaces confirmed the general observation that the Dcn\textsuperscript{+} fibroblasts had higher growth rate than the wild-type cells (p<0.001, Figure 4-1 (a) & (b)). Because there was no significant difference in MTT metabolism between the Dcn\textsuperscript{+} and wild-type cells (p=0.572), there was no need to prepare separate standard curves for the two cell lines.

![Graph](image)

Figure 4-1: In 2-D culture, Dcn\textsuperscript{+} cells grew faster than wild-type cells. The cells were grown on uncoated or collagen coated surfaces. The Dcn\textsuperscript{+} cells demonstrated significantly higher proliferation than the wild-type cells (p<0.001) in both 2-D culture studies. Data represents average values (n=3) at each time point and error bar represents standard deviation.
4.3.2 *Greater adhesion for Dcn<sup>−/−</sup> cells*

Since cell division is reportedly dominated by cell adhesion [173, 174], the adhesion studies investigated which cell line demonstrated a faster adhesion rate and greater overall adherence to collagen and fibronectin. For the varying time experiment, there were differences in adhesion between the cell types after 10 minutes for both collagen and fibronectin substrates, with the maximum differences in adhesion at the 30 and 60 minute timepoints (Figure 4-2).

![Graphs showing adhesion on collagen and fibronectin](image)

**Figure 4-2**: Dcn<sup>−/−</sup> cells demonstrated greater cellular adhesion on (a) collagen-coated, and (b) fibronectin-coated 12-well plates. Cell adhesion at various time point is normalized to 150x10<sup>3</sup> cells (i.e. total cells). Data represents average values (n=9) at each time point and error bar represents standard deviation. There was a statistically significant difference between the cell-types (p<0.001) for both collagen and fibronectin substrate.
Regardless of the substrate, the Dcn<sup>−/−</sup> cells had a greater overall adhesion than wild-type cells (p<0.001 for cell type and for both substrates). Both types of MEFs demonstrated greater adhesion to collagen than to fibronectin, although their initial rates of adhesion were faster on fibronectin. The varying concentration experiment determined if cellular adhesion was dependent on cell density. For the varying concentration experiment, adhesion was observed to be dependent on the number of seeded cells on both substrates (Figure 4-3, p<0.001 for both substrates). However, at the higher cell concentrations tested (2 and 4x10<sup>5</sup> cells/well), the Dcn<sup>−/−</sup> cells showed greater adhesion to collagen-coated plates than did the wild-type cells (p<0.005). For fibronectin-coated plates, no significant difference was observed between the cell types at any tested cell concentration (p=0.265).
Figure 4-3: Cellular adhesion to (a) collagen-coated, and (b) fibronectin-coated 12-well plates was dependent on cell concentration for both cell-types. Data represents average values (n=3) for each cell density and error bar represents standard deviation. A significant increase in cellular adhesion was observed for cell concentration for both cell-types (p<0.001) for both collagen and fibronectin substrates. However, a significant difference in adhesion between the Dcn−/− and wild-type cells were also observed only for the collagen-coated plates (p<0.001).

Exogenous decorin study verified the direct effect of decorin on cellular adhesion on collagen and fibronectin substrates. For the exogenous decorin study, Dcn−/− MEF adhesion to collagen or fibronectin substrates was measured after 60 min. In this study, cell adhesion was shown to be proportional to the concentration of exogenous decorin (Figure 4-4a).
Figure 4-4: Reduced cellular adhesion by Dcn⁻/⁻ cells after addition of exogenous decorin on (a) collagen-coated, and (b) fibronectin-coated 12-well plates, when normalized to 2x10⁵ cells (no added decorin). Data represents average values (n=3) for each concentration of decorin and error bar represents standard deviation. There was a significant effect of the concentration of exogenously added decorin on cellular adhesion on fibronectin substrates (p<0.001), but not for collagen.

The Dcn⁻/⁻ cells demonstrated decreased adherence to collagen and fibronectin with increasing concentrations of exogenous decorin. Even though the overall trend was not significant for collagen (p=0.132) due to large standard deviations, a significant difference in cell adhesion was observed between the control wells (no decorin) and the wells treated with 20 µg/ml of exogenous decorin (p=0.021). There was a similar concentration-dependent effect of exogenous decorin on the adhesion of Dcn⁻/⁻ cells to fibronectin substrates (Figure 4-4b, p<0.001 for effect of concentration, p<0.05 between control and 20 µg/ml of exogenous decorin). However, at the highest concentration tested (20 µg/ml), the exogenous
decorin had a greater inhibitory effect on Dcn−/− cell adhesion to collagen than on adhesion to fibronectin (p<0.05 for substrate effect).

4.3.2 Blocking α2β1 integrin limited adhesion to collagen, but not to fibronectin

Since β1 integrins are reported to contribute to fibroblast adhesion to collagen type I and fibronectin [183, 184], cell adhesion after blocking with antibodies against the α2 and β1 integrin subunits was assessed using the MTT assay. For collagen-coated plates, blocking the α2 and β1 subunits caused a significant reduction in adhesion of both cell types when compared to control wells untreated with antibodies (Figure 4-5a, p<0.05). Although there was no significant difference in the reduction of adhesion between the cell types for either antibody, blocking the integrin subunits had a more pronounced effect on the Dcn−/− cells (p<0.005 vs. controls) than on the wild-type cells (p<0.05 vs. control) with their respective controls.

Blocking adhesion to fibronectin-coated plates produced very different results from the collagen-coated plates. Even though β1 integrins are reported to inhibit cell adhesion to fibronectin, blocking the β1 or α2 integrin subunits did not cause any reduction in adhesion for either cell type compared with the controls (Figure 4-5b). Compared to the Dcn−/− cells, however, the wild-type cells demonstrated a slight reduction in adhesion when the α2 or β1 integrin subunits were blocked (p<0.05 for α2 and p<0.001 for β1 integrin between the cell types).
4.3.3 Greater cell migration for Dcn^{-} cells

Cell migration on uncoated and collagen-coated plates was determined by counting the number of cells migrating outwards from the edge of a circular area (previously confined by a cloning cylinder) in wells of a 6-well tissue culture plate (Figure 4-6). Even though Dcn^{-} cells demonstrated a trend of greater migration compared to wild-type cells on both uncoated and collagen-coated plates (Figure 4-7), there was a significant difference in cell migration between the cell types only on regular tissue culture plate (p<0.005), but not for...
collagen-coated plates (p=0.1) due to large standard deviations. Furthermore, there was a significant difference in the number of cells migrating on the different tissue culture substrates (p<0.05). In the collagen-coated plates, cell migration did not advance much further than the outline of the cloning cylinder, whereas in the uncoated plate, cells migrated a greater distance away from the edge of the cloning cylinder.

Figure 4-6: Dcn<sup>−/−</sup> cell migration on uncoated and collagen-coated plates. Panel (a) shows adhered cells confined within the area defined by the cloning cylinder, (b) and (c) shows migrating cells on uncoated and collagen-coated plates. A greater number of cells migrated on uncoated plates than on collagen-coated plates (p<0.05).

Figure 4-7: Greater cell migration by Dcn<sup>−/−</sup> cells as compared to wild-types on uncoated and collagen-coated 6-well plates. Data represents average values (n=3) for each cell type for both substrate and error bar represents standard deviation. There was a significant difference in cell migration for cell-type (p<0.005) and substrate (p<0.05).
4.4 Discussion

The main findings of this study were that Dcn' cells demonstrated significantly greater cell adhesion, migration, and proliferation than wild-type cells. In addition, both of these types of MEFs demonstrated affinity for adhesion to collagen and fibronectin substrates. More specifically, adhesion of these cells to collagen appears to be modulated by a decorin-α2β1 integrin complex. The surface-dependent behavior of these two cell types shown in this study also provides insight into the cell-induced organization of the matrix within 3-D collagen gels [178]. Collagen gel contraction assays are useful in comparing the behavior of different cell types, but can also be used to study specific interactions between cells and collagen because the cells seeded within collagen gels will migrate and align themselves in the direction of tension via α2β1 and other integrins [53-55]. In fact, cells expressing α2β1 integrins preferentially bind to collagen type I [185]. Our previous study, in which we demonstrated that Dcn' cells expressed more α2β1 integrins and caused greater contraction of 3-D collagen type I gels than did wild-type cells [178], provided the motivation to examine these cells' adhesion to type I collagen. Overall, the trends in cell adhesion, proliferation, and migration observed in this 2-D study support and clarify the findings of our previous 3-D investigation.

Decorin can inhibit cell growth for a variety of cell types [5, 6, 47] by binding to and sequestering TGF-β[4] as well as by binding to the epidermal growth factor receptor [6, 41-43]. Correspondingly, we previously showed that Dcn' cell-seeded collagen gels had greater cell densities than control wild-type cell-seeded gels after 4 weeks of culture [178]. To support these observations, this study demonstrated that Dcn' cells grow faster on both uncoated and collagen-coated tissue culture plates. While the finding from this 2-D study
might have been influenced by growth factor effects [4, 178], reports that decorin influences cell adhesion to a variety of matrix proteins [168-170], suggest that differential adhesion by the two cell types might have also contributed to this difference in cell growth rate [174, 186].

Because the influence of decorin on cell adhesion and migration *in vivo* is relevant to numerous cell types [8, 170, 177], we investigated the adhesion of the Dcn<sup>−/−</sup> and wild-type cells to collagen and fibronectin, two of the most abundant matrix components. Although MEF adhesion to collagen was of primary interest, fibroblasts also demonstrate high affinity to fibronectin [187] and reportedly bind via β<sub>1</sub> integrins [183, 184]. Therefore, fibronectin was also investigated to determine if the different cell types showed substrate-dependent adhesion. Here, the Dcn<sup>−/−</sup> cells exhibited greater adhesion to both collagen and fibronectin than wild-type cells, which is consistent with previous reports where external addition of decorin inhibited fibronectin or collagen-dependent human skin fibroblast adhesion [177, 188]. Although both types of cells adhered more rapidly to fibronectin than to collagen in the first few minutes, their cumulative adhesion to collagen was approximately twice that to fibronectin, suggesting that these cells may have a higher affinity for collagen binding than fibronectin binding, perhaps utilizing different types of integrins.

The addition of exogenous decorin to wells containing the Dcn<sup>−/−</sup> cells reduced their adhesion to levels closer to those demonstrated by the wild-type cells. These results confirm that decorin contributes to the inhibition of adhesion to these substrates by wild-type cells, as was previously reported for human skin fibroblast and macrophages [174, 188]. For the highest dose of exogenous decorin (20 µg/ml), adhesion to collagen was reduced approximately 40% and adhesion to fibronectin was reduced approximately 20%. This
differential effect of exogenous decorin may imply that decorin plays a less significant role in fibroblast adhesion to fibronectin. Several previous investigations have suggested that integrin components are involved in decorin-mediated cell adhesion [183, 184]. If fibroblast adhesion to collagen vs. fibronectin indeed involves different integrins, as speculated above, these results could imply that decorin has variable affinity for different integrins.

Because integrins play critical roles in cell migration, cellular adhesion to various matrix molecules, and cell growth and differentiation [174], we investigated the effect of blocking selected integrin classes on adhesion by these MEFs. Several previous investigations have suggested that integrin components are involved in decorin-mediated cell adhesion [183, 184], but there is some controversy regarding the exact mechanism of this decorin-integrin interaction. Merle et al. demonstrated the inhibitory effect of decorin on cell migration (which is in turn dependent upon adhesion) was alleviated when the dermatan sulfate GAG chains were enzymatically cleaved [8]. The dermatan sulfate GAG chains by themselves, however, were unable to block migration, suggesting that both the GAG chains and core protein are necessary to for the inhibition. Another study contradicted the observations by Merle et al. and suggested that the decorin-mediated adhesion occurs by non-β1 integrin receptors [174]. It remains unclear whether adhesion is prevented by the decorin molecule (either the GAG chain or core protein) binding to the substrate or to the cell surface adhesion receptor (such as an integrin) or a combination of multiple bonds by the different parts of the molecule [8].

Here, we found several interesting differences between Dcn-/- and wild-type MEFs concerning their α2β1 integrin-dependent adhesion to type I collagen and fibronectin. Blocking the α2 and β1 integrin subunits reduced adhesion to collagen by the wild-type and
Dcn⁻ cells by 30% and 50%, respectively, demonstrating that adhesion is strongly mediated by this integrin family. The greater inhibitory effect on the Dcn⁻ cells is consistent with our previous report that these cells express more α₂β₁ integrins than do wild-type cells [178], and implies that in the absence of decorin, numerous α₂β₁ integrins are available for binding to collagen. The finding that blocking the same integrins in wild-type cultures also reduced adhesion implies that not all α₂β₁ integrins in the wild-type cell are bound to decorin. The adhesion studies involving fibronectin produced quite different results. Even though the small effect of α₂ integrin subunits on adhesion was expected, since adhesion to fibronectin has been reported to occur predominantly via other β₁ integrins such as α₅ integrins [189]. To our surprise, blocking the β₁ integrins also had no effect on the Dcn⁻ cells’s adhesion to fibronectin, which may suggest that other integrins are involved in that binding. Based on our results from the exogenous decorin study, we believe that decorin may bind to those non α₂-, non β₁-integrins as well.

With respect to cell migration, greater numbers of Dcn⁻ cells migrated across both type I collagen-coated and uncoated tissue culture plates than did the wild-type cells, even though a significant difference was observed for uncoated plates only (p<0.005). This finding supports the work of Merle et al., who showed that the addition of decorin to migrating osteosarcoma cells slowed their migration on type I collagen and fibronectin [8]. Overall, these 2-D studies suggest that the faster migration by the Dcn⁻ cells, in combination with their increased adhesion to matrix molecules, would enable them to initiate remodeling of 3-D collagen matrices earlier than would be possible by the wild-type cells.

There were a number of limitations to this study. With respect to the study motivation, we acknowledge that the observed patterns in cell adhesion, proliferation, and
migration found in this 2-D study may differ from these same processes within 3-D matrices and \textit{in vivo} tissues. The cell migration investigated in this study represents random migration by the cells and does not reflect migration dictated by any specific chemoattractant. This study also did not investigate the role of the different structural components of the decorin molecule (i.e., core protein or GAG chain) on these outcomes. Further investigation regarding the matrix binding structural components of decorin will continue to shed light on the unique characteristics of these Dcn\textsuperscript{−/−} cells and their study in 2-D and 3-D cultures. Investigation of other β₁ integrin subunits, such as α₅ and α₁₁ [190, 191] that are reportedly involved in cell-matrix adhesion, would continue to reveal the multifaceted role of decorin in cell-matrix adhesion.

4.5 Conclusion

Although the role of decorin in cell adhesion to fibronectin has been widely investigated, only a few studies have examined its influence in cell adhesion to collagen. Furthermore, no previous study has conclusively demonstrated substrate-specific, decorin-mediated cell behavior. Here, we report that decorin deficient murine embryonic fibroblasts show greater proliferation, adhesion, and migration than wild-type cells. In addition, we observed unique substrate-specific effects that suggest interactions between decorin and different classes of integrins, and demonstrate that the adhesion of these cells to fibronectin is largely mediated by non-β₁ integrins. These unique decorin-dependent interactions suggest that decorin actively modulates the cell-mediated matrix organization of engineered and native tissues.
CHAPTER 5

Optimization of 3-D Collagen Gels

Before the static tension collagen gels were prepared for Aim 2 (Chapters 6-7), several small studies were performed to optimize the collagen gels. Since this study investigated the decorin-mediated collagen fibril organization, the logical choice for the scaffold was type I collagen. For the optimization study, the molds for preparing the collagen gels were designed and then the collagen concentration and cell density were optimized for the cell types used in this research work.

5.1 Mold and counter mold design

For the static culture of the gels under uniaxial tension, a countermold was designed to create silicone rubber molds of precise geometries. The molds contained wells in which the collagen gels were grown. I worked with Rice undergraduate student Martin Bost on the mold and anchor design (section 5.2 below). Since the highest modulus has been reported for gels grown in wells with an aspect ratio of 8 [192], our first mold consisted of 4 rectangles, each 40 mm long, 5 mm wide, and 5 mm deep, on an aluminum block (Figure 5-1). The volume of each well was thus 1 ml. Stainless steel pins were inserted vertically about 3 mm away from the longitudinal edge at the end of the wells. However, after our first set of gels was grown with this mold, we observed that the gels had difficulty attaching to the anchors on the pins due to the limited space available around the holders.
To correct this problem, we redesigned our mold with the ends being extended by a circle of radius 6 mm. This redesign changed the volume of the wells to 2.13 ml, without altering other dimensions. To fit 4 wells in the same mold, the dog-bone shaped molds were staggered (Figure 5-2).
The countermold was made using computer aided design software (Pro-Engineer, Needham, MA) and stereolithography (Laser Reproductions, Columbus, OH), and silicone rubber molds were made from the countermold.

5.2 Anchor selection

To improve anchorage of the gels to the vertical pins, we tried a variety of holder materials such as nylon and polyester mesh, perforated plastic canvas, and cellular rigid polyurethane foam holders of different porosity. The small grade nylon and polyester meshes acted as poor anchors, and the gels tended to slip from the anchor and cling onto the metal holder and thus tear in less than a week. The perforated plastic canvas showed similarly poor results. Holders cut from the high porosity cellular rigid polyurethane foam (from Sawbones Worldwide, Vashon, WA) and looser weave polyester mesh could hold the gels for four weeks. Of these latter two materials, we selected the polyurethane foam holders since they were easier to handle. The dimensions for the Sawbone foam holder was chosen to be 3 mm x 3 mm x 3 mm [192].

5.3 Optimization of cell density and collagen concentration

The collagen constructs were grown with two variables: cell numbers (0.2, 0.5, 0.75, 1, and 2 million cells per ml) and collagen concentration (1, 2, and 3 mg collagen per ml). The constructs were grown in multiples (2 to 4) for each condition for 4 weeks. Images of the constructs were taken every 2 days for 2 weeks for contraction measurements. After 4 weeks, the collagen gels were sacrificed and used for mechanical tests.
After the cell numbers and collagen concentrations were optimized for the collagen gels, the optimum TGF-β concentration for Ch. 6 was determined to be 1 ng/ml from 3 dilutions (0.1, 1, 5 ng/ml) of TGF-β for 1 million cells/ml, since the highest concentration of TGF-β was observed to cause reduced contraction of the collagen gels from 2 days onwards.

5.3.1 Collagen gel preparation

The collagen gels were prepared using an established protocol in our lab. For the collagen gel scaffolds, we used acid-soluble type I collagen (from rat tail, BD Biosciences, Franklin Lakes, NJ) that was diluted with acetic acid so that the final collagen gel would have the desired concentration. The desired amount of cells suspended in medium was added to the collagen solution. The pH of this collagen-cell mixture was brought to biological pH and was then suspended in our uniaxial silicone rubber molds (Figure 5-3). The collagen-cell mixture solidified in 1-2 hours. The gels were cultured in the incubator (37°C, 5% CO2 and 95% humidity), and the medium was changed every alternate day. The collagen gels with varying cell numbers (0.2, 0.5, 0.75, 1, and 2 million cells per ml) were grown with a fixed collagen concentration of 2 mg/ml, while the gels with varying collagen concentrations (1, 2, and 3 mg collagen per ml) were grown with a fixed cell density of 1 million cells per ml.

Figure 5-3: Uniaxial collagen gels in static tension.
5.3.2 Contraction measurement

The contraction was measured using ImagePro software. The constructs contracted consistently along the length. Three random measurements across the length of constructs were taken for diameter measurement, averaged, and then compared with the initial average diameters (well diameter) to calculate the percentage contraction for each sample. The average value for percentage contraction was then calculated for all of the samples (n ranges from 2 to 4) for each day and plotted to determine the contraction trend.

By the end of 2 weeks, the collagen constructs containing at least 0.75 million cells/ml contracted to approximately 80% of the original diameter. More contraction was observed for collagen gels containing higher cell concentrations (p<0.001, Figure 5-4). In the cell concentration study, there was no significant difference in contraction between the decorin deficient (Dcn\(^{-/-}\)) and wild-type constructs. With respect to collagen concentration, the 1 mg/ml construct contracted the most (ANOVA p<0.001), but became very thin and fragile by the end of 4 weeks and could not be mechanically tested. The 3 mg/ml constructs contracted very slowly at the initial stage and also had contracted the least amount after 2 weeks. The 2 mg/ml construct showed rapid contraction and were suitable for mechanical testing. In the collagen concentration analysis, there was significantly more contraction for the Dcn\(^{-/-}\) cells (p<0.001). Overall, collagen concentration seemed to have a larger effect on contraction than the cell concentration. The resulting range of cross-sectional areas for the constructs is shown in Table 5-1.
Effect of cell concentration on diameter contraction

Figure 5-4: Comparison of contraction between wild type (WT) and decorin deficient (DCN) collagen gels; error bars indicate standard deviation.
Table 5-1: Range of mean cross-sectional areas for collagen gels after 2 weeks

<table>
<thead>
<tr>
<th>Cell Concentration</th>
<th>Mean cross-sectional area in (mm²) for Wild-Type</th>
<th>Mean cross-sectional area in (mm²) for Den&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75 million cells/ml</td>
<td>0.84</td>
<td>0.87</td>
</tr>
<tr>
<td>1 million cells/ml</td>
<td>0.9</td>
<td>0.77</td>
</tr>
<tr>
<td>2 million cells/ml</td>
<td>0.41</td>
<td>0.49</td>
</tr>
<tr>
<td>Collagen Concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg/ml collagen</td>
<td>0.22</td>
<td>0.39</td>
</tr>
<tr>
<td>2 mg/ml collagen</td>
<td>0.9</td>
<td>0.77</td>
</tr>
<tr>
<td>3 mg/ml collagen</td>
<td>2.1</td>
<td>1.18</td>
</tr>
</tbody>
</table>

5.3.3 Material parameters

The ELF3200 tensile mechanical tester (EnduraTec, Minnetonka, MN) was used to perform load-elongation test on the constructs after they were grown for 4 weeks. The constructs were separated from the holders, clamped between the test grips (Figure 5-5), and kept hydrated using phosphate buffered saline (PBS) solution. Samples were subjected to displacement cycles of 0.5 mm with displacement increasing in 0.5 mm increments until failure occurred. The load and displacement test data were exported to Microsoft Excel for further analysis.

![Figure 5-5: Construct clamped in grips](image)

From the data, we observed greater load on repeated tests with the same construct (Figure 5-6), meaning that the constructs showed strain hardening. In addition, we found that the constructs showed increased extensibility with successive cycles (in which samples were stretched 0.5 mm further each time), because the load-displacement curve tended to shift to
the right with repeated tests (Figure 5-7) suggesting that each cycle induced permanent stretching of the collagen gels. We also observed that collagen gels containing higher cell densities were more extensible, with one exception: the 1 million cells/ml constructs were more extensible than the 2 million cells/ml constructs. With regard to collagen concentration, we observed that the constructs became more extensible with greater collagen concentration. The 3 mg/ml constructs, in fact, were most extensible for both cell types, possibly due to the higher amount of collagen limiting the ability of the cells to contract the gel. In addition, the 1 mg/ml constructs could not be subjected to tensile tests due to their extreme fragility. These optimization tests also showed that the wild type constructs were found to sustain fewer grams of loading during the incremental displacement testing than did the decorin deficient constructs (Figure 5-7).

![Load-Displacement curve showing repeated test with same construct](image)

**Figure 5-6: Load-Displacement curve for 0.75 million cells/ml with 2 mg/ml collagen for decorin deficient constructs showing strain hardening with repeated test**

With regard to cell concentration, we observed greater displacements with greater cell concentration, with one exception: the 1 million cells/ml constructs could sustain greater displacement (i.e. were more extensible) than the 2 million cells/ml constructs. With regard to collagen concentration, we observed that the constructs were more extensible with greater
collagen concentration. The 1 mg/ml constructs were excluded from the tensile tests due to their extreme fragility. The 3 mg/ml constructs, on the other hand, were more extensible for both cell types, possibly due to higher amount of collagen in the constructs, since tissues with more collagen are in general more extensible. Overall, the wild type constructs were more extensible or could sustain more displacement increments during the mechanical testing than the decorin deficient constructs for both cell numbers and collagen concentrations.
Figure 5-7: Load-Displacement curve for 1 million cells/ml with 2 mg/ml collagen for wild type and decorin deficient cell constructs

5.3.4 Conclusions from optimization study

Based on our observations from contraction measurement and mechanical testing, we selected 1 and 2 million cells/ml as the desired cell densities and 2 mg/ml as the desired collagen concentration for the static tension collagen gels. Also, because the collagen gels showed strain hardening and increased deformation with repeated tests, we decided to do one
single failure test on the collagen gels in future studies instead of conducting repeated load-elongation tests on the same gel.
CHAPTER 6

Decorin-TGF-β Interaction Regulates Matrix Organization and Mechanical Characteristics of 3-D Collagen Matrices

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 gestational aged decorin null (Dcn<sup>−/−</sup>) 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<sup>−/−</sup> 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<sup>−/−</sup> 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-beta (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<sup>−/−</sup> 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.

Part of this chapter is published in the Journal of Biological Chemistry 2007; 2:35887-98
6.1 Introduction

Decorin, a member of the small leucine-rich proteoglycan (SLRP) family [193], regulates a myriad of functions in the extracellular matrix (ECM) including collagen fibrillogenesis [2], collagen degradation [3], cell growth [5-7] and extracellular signaling [41, 194]. Decorin consists of a core protein of approximately 40 kDa attached to a single chondroitin/dermatan sulfate glycosaminoglycan (GAG) chain [79, 195]. 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 [196]. Decorin also binds to other collagens including types II, III, VI, and XIV, thereby affecting a variety of extracellular matrix components [167].

Decorin also participates in many important intracellular and extracellular signaling processes, including ligation of the epidermal growth factor receptor [6, 41-43], which upregulates 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 beta (TGF-β1, -β2, -β3) [167, 197, 198], even when bound to collagen. As with collagen, this binding takes place via the protein core and not the GAG chains [6, 199]. Conversely, the degradation of decorin by matrix metalloproteases, such as during tissue repair processes, releases the bound TGF-β [200, 201]. 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 [5, 6, 47], 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 [47]. 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 [202]. Similarly, exogenously added decorin sequesters active TGF-β and results in lower expression of TGF-β receptors [44].

Many significant advances in determining the functional roles of decorin have been made possible through the decorin knockout (Dcn<sup>−/−</sup>) mouse model [15]. The Dcn<sup>−/−</sup> 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 [14]. One of the most distinctive phenotypic characteristics of the Dcn<sup>−/−</sup> 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 due to abnormal lateral fusion [15, 48]. When treated with exogenous decorin, dermal Dcn<sup>−/−</sup> fibroblasts grown in 3-D culture recovered their wild-type phenotype and generated collagen fibrils with more uniform diameters [51].

Although investigations of the Dcn<sup>−/−</sup> 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, 3-D collagen gels seeded with Dcn<sup>−/−</sup> 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 3-D environment to cells than do 2-D surfaces [53-55]. It has been shown that fibroblasts seeded in collagen gels
migrate within the gel using $\alpha_2\beta_1$ and other integrins [53-55]. Upon binding of these integrins to ECM components, a cascade of signals is initiated to trigger motor proteins to power cell movement [53]. 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 2-D 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 [56]. 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. Since 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. Furthermore, since anchored (restrained) collagen gels can respond differently to growth factors than do unanchored ("floating") gels [54, 61], some of these assays were also conducted on unanchored collagen gels.

6.2 Experimental Procedures

6.2.1 Cell Culture

Embryonic fibroblasts were isolated from euthanized Dcn$^{-/-}$ or wild-type Balbc mouse embryos (12.5 to 13.5 gestational days old) using an established protocol (http://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 DNAase (2 mg) and collagenase III (1 mg per 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 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.

6.2.2 Gel Preparation

The collagen gel matrices were prepared using acid-soluble type I collagen (from rat tail tendon, BD Biosciences, Franklin Lakes, NJ) using an established protocol [203]. The collagen matrices contained a final collagen concentration of 2 mg/ml and final cell concentrations of either 1 or 2x10⁶ cells/ml. The collagen-cell mixture was poured into uniaxially oriented wells (40 mm x 5 mm x 5 mm) within custom designed silicone rubber molds (Figure 6-1a,b). 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, Minneapolis, MN) 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, since the gels seeded with wild-type cells at the $1 \times 10^6$ cells/ml concentration had very delayed contraction, contraction in those gels was initiated by supplementing with recombinant human TGF-β1 (only on day 2).

Figure 6-1: Cell-seeded collagen gels in 6-well plate and in silicone rubber mold. The anchored collagen gels were grown in uniaxial wells in silicone rubber molds under static tension. Part (a) shows the solidified collagen gels in the silicone rubber mold 1 day after preparation and part (b) shows the contracted gel in PBS after 4 weeks. Part (c) shows the unanchored collagen gels grown in 6-well plates after 2 weeks.
To study the effects of the external factors in a less complex system, unanchored collagen gels containing $1 \times 10^6$ cells/ml were also prepared for both cell types. Two ml of the collagen-cell mixture were poured into each well of 6-well tissue culture plates, detached from the bottom of the plate after polymerization occurred, and grown for 2 weeks in the incubator under identical conditions as the anchored (static tension) gels. The gels were divided into 3 groups and were grown in triplicates. The first group was treated with 1 ng/ml of recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) throughout the culture period. For the second group, TGF-β1 was added only on day 0 to determine if continuous supplementation of TGF-β1 was required to influence remodeling of the gels. The third group (controls) was cultured with regular media (Figure 6-1c). It should be noted that the static controls for the anchored gels underwent internal dynamic strain as the cells contracted and organized the matrix and were not truly as "static" as the unanchored gels.

Finally, to verify the influence of TGF-β on contraction of the collagen gels seeded with the Dcn<sup>−/−</sup> cells, a group of unanchored and anchored gels containing Dcn<sup>−/−</sup> cells were cultured in media supplemented with the TGF-β receptor kinase inhibitor SB431542 (Sigma, St. Louis, MO) [204]. SB431542 thus prevents TGF-β from activating Smad-2 and its associated gene regulation. The unanchored gels were treated with a range of dilutions (0.1, 0.3, 1 and 3 μM) of SB431542, prepared from 3 mM stock solution of SB431542 dissolved in dimethyl sulfoxide (DMSO) and the optimal concentration required to inhibit TGF-β for the unanchored gels were determined to be 3 μM. The concentration of SB431542 was optimized for the anchored gels to be 10 μM and was prepared from 20 mM stock solutions of SB431542 dissolved in DMSO. The unanchored gels were treated with a lower concentration of SB431542 because they were detached and freely floating within the
medium soon after gelation. For the anchored gels, media could only be added to the top surface of the gels (Figure 6-1) and could not reach other surfaces until contraction had initiated; therefore, higher concentration of SB431542 was required to affect contraction of the anchored gels. The control gels for this group were cultured with media containing DMSO (10 and 5 μl for 10 ml of media respectively).

6.2.3 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, Uppsala, Sweden) as previously described [92, 203]. 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 PGs were eluted with four column volumes of 7 M urea buffer containing 3 M NaCl, ethanol precipitated, treated with chondroitinase ABC containing 0.1% BSA, 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, NIH, [205]) and goat anti-rabbit horseradish peroxidase-linked secondary antibodies (1:20000 dilution, Amersham Biosciences UK Limited, Buckinghamshire, UK). The bands for decorin were detected using chemiluminescent exposure to radiographic film.

6.2.4 Gel Contraction Measurement
To monitor the anchored collagen gel contraction, the gels were digitally photographed every 2 days for the first two weeks, since most of the contraction occurred during this period. Because significant contraction was observed during the first 72 h, pictures of the gels were also taken every hour for several hours each day during this period. The diameter of each anchored contracted gel was measured in triplicate using Image-Pro Express (Media Cybernetics, San Diego, CA) and then compared with original diameter (5 mm) to calculate percentage contraction. The rates of contraction were calculated from the slopes of the 2-week contraction curves. For the unanchored gels, the areas of the contracted gels were measured and compared with the area per well (9.6 cm²) of the 6 well plates to determine percentage contraction.

6.2.5 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 alpha actin (SMαA) [59] and α5β1 integrins [60] between the wild-type and Dcn⁻/⁻ cells. The cells were trypsinized, and 2x10⁶ 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% Na Azide in PBS, Fisher Chemical, Fairlawn, NJ) was added to each sample and the cells were centrifuged (5 min, 1500 xg). The pelleted cells were permeabilized in 100 μl of 0.1% Triton X100 buffer (Perkin Elmer, Boston, MA) 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 beta 1 antibody kit, Chemicon International, Temecula, CA), while the controls were kept in buffer at 0°C. Each sample was treated with appropriate secondary antibody (SMαA: 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 FACSscan device running BD Cell Quest Pro software (Becton Dickinson) to collect median forward scatter (FSC), side scatter (SSC) and fluorescence light (FL) data.

6.2.6 Mechanical Testing

Mechanical testing was performed on the anchored gels using an ELF 3200 uniaxial tensile tester with a 250 gram load cell (EnduraTec, Minnetonka, MN); the resolution of the load cell was within 0.02%. After 4 weeks, the anchored 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 minute). 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.

6.2.7 Electron Microscopy

Mature anchored 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 et al. [206]. 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 Image J software (NIH) to calculate the percentage area fraction of the collagen fibrils and the average collagen fibril diameter.

6.2.8 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 (anchored) or 2 weeks (unanchored) 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 weight and dry weight of the samples to the wet weight.

6.2.9 GAG Content

Fluorophore-assisted carbohydrate electrophoresis (FACE) was used to determine the amount of GAGs secreted by the cells and retained within the mature anchored collagen gels for both cell types (n=2 for each cell type and cell concentration) [92]. Briefly, the harvested gels were digested in proteinase-K solution (10 mg/ml in ddH2O, Invitrogen, Carlsbad, CA) and vacuum concentrated. The samples were then treated with chondroitinases ABC and ACII (3 mU each, Associates of Cape Cod, Falmouth, 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 analyzed to quantify the various sulfated and unsulfated disaccharides.

6.2.10 Growth Study

A DNA assay was used to quantify the cell density in the mature collagen gels [207]. Briefly, 3 gels were harvested each week for up to 4 weeks (anchored) or at 2 weeks (unanchored), lyophilized, and 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 to facilitate release of DNA. After the samples were fluorotagged with Hoechst 33258 dye (Sigma), fluorescence emission was measured at 458 nm and compared with double stranded DNA standards (Sigma). This study was then repeated two more times (n=6-10).
6.2.11 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 anchored collagen gel matrices were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5 μm sections [208]. 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 minutes and eosin Y (Richard-Allen Scientific) for 1 minute. 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.

6.2.12 Immunohistochemistry (IHC)

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 mU/ml of chondroitinase ABC (in 0.05 M Tris, 0.06 M Na 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) [205] 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, Burlingame, CA).
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 one hour, and treated in the same procedure as described above.

6.2.13 Statistical Analysis

Replicate analyses were averaged to obtain mean values for each sample. Data were presented as mean and standard deviations. Statistical evaluations (1, 2 and 3 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 $\alpha=0.05$.

6.3 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 [209], western blotting was performed to verify that the wild-type cells were actually synthesizing decorin.

![Western blot](image)

*Figure 6-2: Western blot verified that only wild-type cells synthesized decorin in primary cell culture.*
Conditioned medium from cultures of the wild-type cells showed a positive band for decorin that was absent in the medium from the Dcn⁻ cells (Figure 6-2).

Contraction analysis as a measure of matrix organization for the collagen gels

To determine differences in the cell-mediated remodeling of the anchored 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, Figure 6-3a) and throughout 2 weeks (p<0.001, Figure 6-4).

Figure 6-3: Dcn⁻ cells contracted the anchored collagen matrices more than wild-type cells during the first 72 hours. Greater contraction was observed for the Dcn⁻ cell-seeded collagen gels during the first 72 h (n=4–8, p<0.001)). The values represent mean ± SD. Part (a) shows the initial time points from part (b) demonstrating that the contraction of collagen gels populated with Dcn⁻ cells was initiated much earlier than for those populated with the wild-type control cells.
The Dcn<sup>-/-</sup> cells initiated contraction of the anchored collagen gels hours before contraction was observed in the collagen gels containing the wild-type cells (Figure 6-3b). From the 2 week contraction measurements (Figure 6-4), it was observed that even the gels seeded with Dcn<sup>-/-</sup> cells at a concentration of 1x10<sup>6</sup> cells/ml showed higher amounts of contraction than gels seeded with either wild-type cell concentrations at 2 weeks.

![Graph](image)

*Figure 6-4. Dcn<sup>-/-</sup> cells contracted the anchored collagen matrices more than wild-type cells throughout 2 weeks. Parts (a) and (c) show contraction for 1 and 2 x10<sup>6</sup> cell concentrations respectively. Greater contraction was observed for the Dcn<sup>-/-</sup> 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 1x10<sup>6</sup> cells/ml density only (p=0.016). Part (b) and (d) shows the rate of contraction over 2 weeks corresponding to the gels from part (a) and (c) respectively. The values represent mean ± SD.*
This trend persisted through 4 weeks (Table 6-1, p<0.01 for both cell type and cell concentration). Similarly, the rate of contraction plots demonstrated that most of the contraction occurred between 0 to 4 days before reaching a plateau. Although both types of collagen gels showed similar contraction kinetics, those containing the wild-type cells lagged or showed a slower rate of contraction as compared to the Dcn、“ cells.

As shown for the anchored collagen gels, the unanchored gels populated with Dcn、“ cells showed greater contraction than wild-type cells after 2 weeks (Table 6-2, p<0.001). The unanchored collagen gels for both cell types showed the greatest contraction when the gels were treated with TGF-β1 (Table 6-2, n=3, p<0.001 for both cell type and TGF-β1), whereas lower contraction was observed for the untreated and initial TGF-β1 treated group. No significant difference in contraction was observed between the control and initial TGF-β1 treated group.

Table 6-1: Contraction and % water content for anchored collagen gels grown with or without TGF-β1 at 4 weeks

<table>
<thead>
<tr>
<th></th>
<th>1x10^6 cells/ml</th>
<th>2x10^6 cells/ml</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No TGF-β1</td>
<td>TGF-β1</td>
</tr>
<tr>
<td>Contraction (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>75.1±3.2</td>
<td>84.2±1.6</td>
</tr>
<tr>
<td>Dcn-/-</td>
<td>87.6±1.2</td>
<td>85.7±3.0</td>
</tr>
<tr>
<td>Water Content (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>93.7±1.8</td>
<td>92.6±0.2</td>
</tr>
<tr>
<td>Dcn-/-</td>
<td>91.5±2.1</td>
<td>89.6±0.9</td>
</tr>
</tbody>
</table>

*p<0.001 for cell type, cell density, and TGF-β1 effects
Table 6-2: Contraction, DNA content, and % water content for unanchored gels grown with or without TGF-β1 at 2 weeks

<table>
<thead>
<tr>
<th></th>
<th>No TGF-β1</th>
<th>Initial TGF-β1</th>
<th>TGF-β1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraction (% at 2 weeks)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>86.4±0.2</td>
<td>87.5±0.6</td>
<td>94.2±0.5</td>
</tr>
<tr>
<td>Dcn/−</td>
<td>91.2±0.1</td>
<td>92.0±0.7</td>
<td>98.8±0.1</td>
</tr>
<tr>
<td>Water Content (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>95.8±0.2</td>
<td>94.6±0.1</td>
<td>92.9±0.4</td>
</tr>
<tr>
<td>Dcn/−</td>
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<td>92.7±0.7</td>
<td>86.2±1.4</td>
</tr>
<tr>
<td>DNA (ng)/ dry wt. (mg)†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>3160±290</td>
<td>3720±230</td>
<td>3980±115</td>
</tr>
<tr>
<td>Dcn/−</td>
<td>5365±1250</td>
<td>5790±860</td>
<td>6150±1400</td>
</tr>
</tbody>
</table>

*p<0.001 for both cell type and TGF-β1 effects
†p=0.01 for combined effect of cell type and TGF-β

Data values represent mean ± SD

Role of TGF-β1 in contraction of collagen gels

At 2 weeks, TGF-β1 improved the amount and onset of contraction for the 1x10^6 cells/ml anchored collagen gels containing wild-type cells, but did not significantly alter contraction for 2x10^6 cells/ml gels (p<0.001 for cell number and TGF-β1 interaction, Figure 6-4); 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 6-1, p<0.001 for cell type, cell density, and TGF-β1 effects) when compared to 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, anchored gels seeded with wild-type cells (1x10^6 cells/ml) were treated with 5 and 10 ng/ml of TGF-β1. Both TGF-β1 concentrations resulted in early contraction comparable to the gels seeded with Dcn/− cells (Figure 6-5a). On the other hand,
when the Dcn\textsuperscript{-/-} cell-seeded gels were treated with a TGF-\(\beta\) receptor kinase inhibitor, a reduction in the initial rate of contraction as well as the overall amount of contraction was observed (\(p=0.001\), Figure 6-5b, 6-5c, and 6-5d). Similarly, the unanchored Dcn\textsuperscript{-/-} collagen gels treated with SB431542 demonstrated reduced contraction as compared to untreated control gels in a dose-dependent manner. Gels treated with 3 \(\mu\)M of SB431542 demonstrated the least amount of contraction (Figure 6-6).

Figure 6-5: Applying or blocking TGF-\(\beta\)1 regulated contraction of anchored collagen gels for the different cell types. For part (a), early contraction comparable to collagen gels containing Dcn\textsuperscript{-/-} was achieved when the gels containing wild-type cells (1x10\(^6\) cells/ml) were treated with 5 and 10 ng/ml TGF-\(\beta\)1 (\(n=4\) per group). For part (b), (c) and (d), collagen gels containing Dcn\textsuperscript{-/-} cells seeded at 1x10\(^6\) cells/ml were treated with 10 \(\mu\)M of the TGF-\(\beta\) receptor kinase inhibitor SB431542. SB431542 reduced both early and 2-week contraction for the Dcn\textsuperscript{-/-} cell-seeded gels (\(n=4\), \(p=0.001\)). Part (b) shows the initial time points from part (c). Part (d) shows the rate of contraction over 2 weeks for the gels from part (c). The values represent mean \(\pm\) SD.
Figure 6-6: Blocking TGF-β regulated contraction of the unanchored collagen gels containing Dcn⁻/⁻ cells. Part (a) demonstrates the difference in contraction between control and 3 μM SB431542 treated unanchored gels (both group of gels prepared from the same cell-collagen mixture). Part (b) shows the 2-week contraction for the above unanchored control and SB431542 treated gels (n=5, p=0.001). The values represent mean ± SD.

Greater expression of α2β1 integrin and SMαA by Dcn⁻/⁻ cells

Compared to wild-type cells, the Dcn⁻/⁻ cells showed higher median fluorescence intensities for both SMαA and α2β1 integrin (2.3 vs. 8.5 for SMαA and 2.98 vs. 7.3 for α2β1 integrin, for wild-type and Dcn⁻/⁻ respectively). The same trend was observed for median FSC (7.4 vs. 10.2 for SMαA and 8.3 vs. 12 for α2β1 integrin, for wild-type and Dcn⁻/⁻ respectively) and SSC (62.3 vs. 78 for SMαA and 66.7 vs. 97.4 for α2β1 integrin, for wild-type and Dcn⁻/⁻ respectively) values. The higher FSC and SSC 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 anchored collagen gels, the gels were stretched up to 6 mm, and the resulting load and displacement data were converted to stress-strain curves. Figure 6-7a shows representative stress-strain curves (each showing raw data from
one tensile testing measurement) used to obtain the material parameters of the collagen gels from both cell types.

![Graphs showing tensile testing results for Dcn-/ and WT cells](image)

**Figure 6-7:** After 4 weeks, anchored collagen gels populated with Dcn^v^ cells had higher ultimate tensile strength and elastic modulus than gels populated with wild-type cells. Part (a) shows two representative load-displacement curves, one from each type of cell-seeded collagen gels. The dots in each plot are the data from one sample exported from the materials testing system; the grey dots were used to calculate the slope. Note the difference in y-axis scale between the cell types. Part (b) represents stress at failure or if the gels did not fail, the stress at maximum strain. Part (c) represents modulus calculated by determining the post-transition slopes of the stress-strain curves. The gels containing the Dcn^v^ cells had higher ultimate tensile strength and elastic modulus than the gels containing wild-type cells for both cell concentrations (p<0.001). The addition of TGF-β1 improved material parameters for the wild-type group only (p<0.005 for combined effects of cell type and TGF-β1). Values represent mean ± SD (n=8–12).

Collagen gels containing Dcn^v^ cells demonstrated greater stiffness, maximum load, elastic modulus, and strength compared to the gels containing the wild-type cells. This difference was most significant for strength and elastic modulus (Figure 6-7b and 6-7c, ~9.5 fold higher for both cell concentrations, p<0.001) due to the higher contraction of the matrix by Dcn^v^.
cells, since elastic modulus and strength are normalized to 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 anchored gels seeded with wild-type cells, an effect that was greater for the lower cell concentration (p<0.001, Figure 6-7b and 6-7c). 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 anchored collagen gel matrices and to 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 did 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 (Figure 6-8).
Figure 6-8: TEM demonstrated greater collagen fibril abundance in the pericellular matrix of Dcn<sup>−/−</sup> cells. The Dcn<sup>−/−</sup> cells were more elongated with large amounts of collagen fibrils in the pericellular matrix as compared to wild-type cells. An initial cell density of 2×10<sup>6</sup> cells/ml was used in these gels. The image magnification for part (a), (b), and (c) is 5000X, 10,000X, and 30,000X respectively. Arrows in part (c) indicate collagen fibrils in the pericellular matrix.

The collagen gels containing Dcn<sup>−/−</sup> 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, Figure 6-9). 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<sup>−/−</sup> cells (lower cell density only) when compared to untreated gels (p<0.001). However, within the TGF-β1 treated group, the gels containing Dcn<sup>−/−</sup> cells had greater collagen area fractions than wild-type controls (p<0.001, Figure 6-9b). The fibrils from collagen gels containing Dcn<sup>−/−</sup> cells showed a trend of slightly higher average diameter (Figure 6-9c); however, no significant difference was observed between collagen fibril diameters among cell types or cell concentrations, with or without TGF-β1.
Figure 6-9: Anchored collagen gels populated with Dcn$^{-/}$ cells had greater collagen content and organization than collagen gels containing wild-type cells. Part (a) shows representative longitudinal section images taken using transmission electron microscopy (TEM); image magnification 30,000X. Part (b) shows area fraction for collagen fibrils calculated from the TEM 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. Part (c) shows collagen fibril diameter measured from the TEM images for all of the groups. The graph legend is same as in part (b). The values represent mean ± SD.

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 [16] since greater hydration usually results in greater spacing between fibrillar matrix components. 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 anchored collagen gels with Dcn" cells had lower percent water content compared to the gels with wild-type cells at 4 weeks for both untreated (p=0.001) and TGF-β1 treated conditions (Table 6-1, p<0.001 for cell type and p=0.05 for TGF-β1). Similarly, for the unanchored collagen gels, TGF-β1 treatment resulted in significantly lower water content for both cell types, whereas the initial one-time TGF-β application did not alter water content (Table 6-2).

*GAG synthesis in collagen gels depended on initial cell density for both cell types*

To determine if there was a relation between hydration and GAG content, FACE 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 anchored collagen gels seeded with either type of cell was dependent upon the initial seeding density and greater for the higher initial density (2x10^6 cells/ml), as shown in Table 6-3 (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.
Table 6-3: GAG content within collagen gels at 4 weeks, as measured by FACE analysis

<table>
<thead>
<tr>
<th></th>
<th>1x10^6 cells/ml</th>
<th>2x10^6 cells/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>Dcn/-</td>
</tr>
<tr>
<td>Total GAG (pmol/ng DNA)*</td>
<td>0.85±0.16</td>
<td>0.66±0.03</td>
</tr>
<tr>
<td>Hyaluronan (pmol/ng DNA)*</td>
<td>0.14±0.00</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>Sulfated GAGs (pmol/ng DNA)^p</td>
<td>0.72±0.16</td>
<td>0.56±0.05</td>
</tr>
</tbody>
</table>

\*p<0.05 and \^p=0.058 for initial cell density; \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.
Data values represent mean ± SD

Greater cell density for collagen gels populated with decorin-deficient cells as compared to those with wild-type cells

A DNA assay was used to determine the final cell concentration within the collagen gels. At 4 weeks, the anchored gels containing the Dcn^- cells had a greater cell density than the gels containing the wild-type cells (Figure 6-10). An interesting observation was an initial decrease in cell concentration in collagen gels, which recovered by 4 weeks in all cases. At 4 weeks, there were more cells in the Dcn^- gels, with the difference being more pronounced for the lower initial cell concentration (higher by 2.8 fold as compared to 1.2 fold for the higher initial cell concentration, p<0.05 for cell type). TGF-β1 influenced the final cell density for both cell types and cell concentrations at 4 weeks (Figure 6-10, p<0.001 for cell type, cell concentration, and TGF-β1 effects). However, even though all gels seeded with 1x10^6 cells/ml showed about 2 to 3 fold increase in final cell density after TGF-β1 treatment, gels seeded with 2x10^6 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 Dcn^- cell-seeded gels. As with the anchored gels, the unanchored collagen gels also showed improved
final cell densities after treatment with TGF-\(\beta\)1 (Table 6-2, \(p=0.01\) for combined effect of cell type and TGF-\(\beta\)1).

Figure 6-10: Anchored collagen gels containing Dcn\(-/-\) cells had a greater final cell density than gels containing wild-type cells at 4 weeks. At 4 weeks, there were greater cell densities in the gels containing the Dcn\(-/-\) cells, with the difference being more pronounced for the 1x10^6 cells/ml samples (\(p<0.05\) for effect of cell type for non-TGF-\(\beta\)1 treated gels). The addition of TGF-\(\beta\)1 increased final cell density in the gels containing either initial concentration of wild-type cells. However, a more significant increase in cell density was observed for the gels containing the Dcn\(-/-\) cells. The values represent mean ± SD (n=6–10, \(p<0.001\) for combined effect of cell type and TGF-\(\beta\)1).
To verify these differences in cell density at 4 weeks, the numbers of cells within an imaged region of interest were also measured for H&E stained anchored collagen gels. There was a significantly higher cell density in Dcn\textsuperscript{-/-} cell-seeded gels for both initial seeding densities when compared to wild-type cell-seeded gels (Figure 6-11a and 6-11b, p<0.001). The cell nuclei also appeared more elongated in the Dcn\textsuperscript{-/-} cell-seeded gels.
Figure 6-11: Anchored collagen gels containing Dcn\(^{-/-}\) cells had a greater final cell density than gels containing wild-type cells. Part (a) shows cell density in mature anchored collagen gels determined by counting the number of cells within a defined region of interest in images of H&E stained histological sections. Part (b) and (c) shows example images of collagen gel tissue sections for both cell types stained for H&E and proliferating cell nuclear antigen (PCNA), respectively. Scale bar is 100 \(\mu\)m.
To determine whether the cells measured using the H&E and DNA were proliferating, cells expressing PCNA were examined using IHC (Figure 6-1c). PCNA was found to stain ~80% of the nuclei within the histological sections of the collagen 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 SLRP is knocked out, the synthesis of other SLRPs tends to be upregulated to compensate for this deficiency [166, 179]. To determine if such compensation was occurring in these 3-D collagen gel matrices, IHC was used to demonstrate the abundance of biglycan within the collagen gels. Overall, the collagen gels seeded with the Dcn<sup>−/−</sup> cells showed stronger staining for biglycan than did the gels seeded with the wild-type cells (Figure 6-12).
Figure 6-12: The collagen gels seeded with Dcn" cells demonstrated stronger immunohistochemical staining for biglycan than those seeded with wild-type cells. Part (e) shows the negative control (Dcn" cell-seeded gel at 2x10^6 cells/ml density), which was not treated with primary antibody, but only with secondary antibody. Image magnification is 10X.

6.4 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, 47, 48], these data support some recent trends reported for Dcn" tendons [14] and immature tendons in particular [179]. 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<sup>−/−</sup> 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, 210]. Delayed contraction of collagen matrices has also been reported for acellular collagen-GAG scaffolds in which the GAG component was replaced by decorin [211], and for collagen gels seeded with either cells transfected to overexpress decorin or control cells in the presence of exogenously added decorin [212]. 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 [213]. 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. While 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. Since decorin inhibits cell growth [5, 6, 214], the absence of decorin highly stimulated cell growth for the Dcn<sup>−/−</sup> cells at 4 weeks in 3D engineered tissues, similar to observations from Dcn<sup>−/−</sup> periodontal tissues [214]. However, in the 3D growth study, we observed an initial decrease in cell concentration until 2 weeks for both cell types. It is worth mentioning that suppressed cell proliferation in collagen gels has also been observed by
many research groups [215-218]. Nonetheless, in our gels, the cell concentrations tended to recover or stabilize from the 3rd week and approached or surpassed the 1-week cell density by 4 weeks. The recovery in cell growth depended on the growth rate of the cell type used as well as the initial cell concentration used in the gels. 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. Likewise, the high collagen density and low cross-sectional area of these gels containing Dcn<sup>−/−</sup> cells then contributed to their superior mechanical strength and elastic modulus as compared to 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 [20], and has been shown to be upregulated during the growth of murine Dcn<sup>−/−</sup> tendons [179]. The excess biglycan in these collagen gel matrices containing Dcn<sup>−/−</sup> cells may have compensated for the absent decorin in regulating collagen fibrillogenesis.

**Comparison of 3-D collagen gels with adult and juvenile native tissues**

These Dcn<sup>−/−</sup> cell-seeded gels behaved differently than originally expected based on reported characteristics of knockout mouse tissues. Dcn<sup>−/−</sup> tissues from postnatal knockout mice have been characterized as having larger collagen fibril diameters, irregular fibril outlines, and loose fibril packing, leading to lower mechanical strength [15]; 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. measured a higher elastic modulus for Dcn<sup>−/−</sup> tissues for both patellar
and flexor digitorum longus (FDL) tendons [14]. In another study, Zhang et al. investigated the mechanical characteristics of Dcn−/− FDL tendons at different postnatal days (P) [179]. They observed that mature Dcn−/− tendons (P150) had significantly lower strength and stiffness, but at the end of the fibril growth phase (P60), there was no significant difference between Dcn−/− and wild-type tendons. In fact, their data at P60 showed a trend of higher stiffness, maximum stress, and elastic modulus for the Dcn−/− tendons. Since 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 P60 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 3-D collagen gels

To determine whether the significant differences in collagen gel contraction between the wild-type and Dcn−/− 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 Dcn−/− 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, Dcn−/− 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 [219, 220], increased collagen synthesis [221, 222] and suppressed collagenase activity [223, 224] by fibroblasts cultured from various tissues. TGF-β causes fibroblasts to differentiate into myofibroblasts, resulting in their greater expression of SMαA [59, 225] and α2β1 integrins [226], which as shown here would cause greater contraction of the collagen matrix. Similarly, many of the effects of TGF-β, such as cell migration [227, 228], hyaluronan synthesis [228, 229], PG synthesis [230], 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-β has been attributed to either improved binding of TGF-β by the fibroblasts [231] or increased expression of TGF-β receptors [232].

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 Dcn−/− 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 since TGF-β1 treatment has been reported to increase the mechanical strength and modulus of tissues regenerated from patellar tendon [233] or collagen gels prepared with smooth muscle cells [234]. Because these wild-type cells are capable of synthesizing decorin, which sequesters TGF-β [167, 197], 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 Dcn<sup>−/−</sup> 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 Dcn<sup>−/−</sup> 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 Dcn<sup>−/−</sup> 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 Dcn<sup>−/−</sup> cells with TGF-β1.
Since 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 dependency 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 ECM components in addition to increasing the contraction of the collagen gels. However, since 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. [235]. Furthermore, since collagen gels demonstrate viscoelastic behavior, as reported by Wagenseil et al. [236], 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.

6.5 Conclusions

This study demonstrated that in the absence of decorin, free TGF-β greatly influenced the contraction, final cell density, and mechanical strength of 3-D cell-seeded collagen gel matrices, resulting in unexpected behavior of the collagen gels seeded with Dcn<sup>-/-</sup> cells. This study shed 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.
Exogenous Decorin Treatment Induces More Wild Type-like Mechanical Behavior in Collagen Gels with Decorin Deficient Cells

Decorin deficient (Dcn-/-) embryonic fibroblast cells demonstrated increased proliferation, contraction, collagen content, and mechanical strength and modulus when compared to wild type (WT) control cells in 3-D collagen gels grown under static tension (Chapter 6). We further studied the influence of exogenous decorin with the hypothesis that the application of exogenous decorin would revert the above-mentioned properties of the decorin deficient collagen gels back to normal. The contribution of tension was also studied by growing the collagen gels supplemented with exogenous decorin under static tension or no tension.

7.1 Methods

7.1.1 Unanchored collagen gels

The collagen-cell mixture was prepared using 1x10^6 cells/ml and 2 mg/ml of acid soluble type I rat-tail collagen as previously described. Two ml of the collagen-cell mixture were then poured onto each well of a 6-well tissue culture plate (n=3 to 6). After the collagen gels solidified, the gels were carefully detached from the bottom of the wells and allowed to float in the culture medium. The gels were divided into 3 groups: (a) gels containing WT cells, (b) gels containing Dcn^-/- cells, and (c) gels containing Dcn^-/- cells treated with exogenous bovine decorin (10 μg/ml of culture media, Sigma) throughout the entire culture
period. The gels were cultured as floating gels in the tissue culture incubator for 2 weeks. For these unanchored collagen gels, gel area contraction and DNA content were measured as described in chapter 5.

7.1.2 Anchored collagen gels

For the anchored collagen gels, the collagen-cell mixture were poured into the silicone rubber mold, as described in Chapter 6. The collagen gels were prepared with two different cell densities of the Dcn-/- cells - 1 and 2 x 10^6 cells/ml. The collagen gels were treated with exogenous bovine decorin at 10 μg/ml throughout the culture duration and these gels were compared with collagen gels cultured with regular media for both cell-types (Chapter 6). The gel diameter contraction was measured every 2 days for the first 2 weeks. The collagen gels were harvested at 4 weeks and mechanical testing (n=12 per group) and DNA assays (n=3 per group) were performed to determine material parameters and cell density of the collagen gels. Four additional collagen gels (1 of each cell density and each cell types were also cultured with exogenous decorin and then processed for TEM. The TEM images thus obtained were analyzed to determine the area fraction of collagen fibrils and the mean collagen fibril diameter as described in Chapter 6. A small subset of collagen gels were also cultured under dynamic conditions with and without exogenous decorin and were processed for TEM to obtain area fractions for collagen fibrils. A detailed description of the dynamic gel preparation is provided in the next chapter (Chapter 8).
7.2 Results and discussion

Since the Dcn⁻/⁻ cell-seeded collagen gels demonstrated greater gel contraction, tensile strength, elastic modulus, cell density, and collagen fibril density, treatment of these gels with exogenous decorin were expected to result in opposite trends and to make the properties more similar to those shown by WT cell-seeded gels. Both the unanchored and the anchored gels demonstrated some expected behavior after treatment with exogenous decorin, but most of the parameters did not demonstrate significant differences when compared to Dcn⁻/⁻ cell-seeded collagen gels cultured with regular media. There was no difference in gel area contraction between Dcn⁻/⁻ cell-seeded unanchored gels with or without exogenous decorin (Table 7-1). For the cell density in the Dcn⁻/⁻ cell-seeded unanchored gels, there was a trend of lower cell density with exogenous decorin, but the difference was not significant (p=0.3, Figure 7-1).

Table 7-1: Contraction and DNA content for unanchored and anchored gels grown with or without exogenous decorin

<table>
<thead>
<tr>
<th></th>
<th>Initial cell density</th>
<th>Wild-type</th>
<th>Dcn⁻/⁻</th>
<th>Dcn⁻/⁻, exogenous decorin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area contraction – unanchored gels (%; at 2 weeks)</td>
<td>1x10⁶ cells/ml</td>
<td>86.4±0.2</td>
<td>91.2±0.1</td>
<td>91.9±0.6</td>
</tr>
<tr>
<td>Diameter contraction – anchored gels (%; at 4 weeks)</td>
<td>1x10⁶ cells/ml</td>
<td>75.1±3.2</td>
<td>87.6±1.2</td>
<td>87.6±2.2</td>
</tr>
<tr>
<td></td>
<td>2x10⁶ cells/ml</td>
<td>77.4±6.4</td>
<td>92.2±0.7</td>
<td>84.8±4.9</td>
</tr>
<tr>
<td>DNA (µg)/ dry wt. (mg)</td>
<td>1x10⁶ cells/ml</td>
<td>1.5±0.8</td>
<td>4.1±3.3</td>
<td>5.9±3.4</td>
</tr>
<tr>
<td></td>
<td>2x10⁶ cells/ml</td>
<td>2.0±0.9</td>
<td>2.5±1.7</td>
<td>6.0±4.1</td>
</tr>
</tbody>
</table>
The anchored collagen gels behaved similar to the unanchored collagen gels. The Dcn" cell-seeded gels treated with exogenous decorin showed no significant difference in contraction and cell density as compared to those cultured with regular media. However, these exogenous decorin treated Dcn" cell-seeded gels showed expected behavior for the material parameters in that they demonstrated significantly lower strength and elastic modulus (p<0.001 for both strength and elastic modulus) than those cultured with regular media. In other words, the material behavior of these Dcn" cell-seeded gels became similar to those containing WT cells after treatment with exogenous decorin (Figure 7-2).
Figure 7-2: The Dcn<sup>-/-</sup> cell-seeded gels treated with exogenous decorin showed significantly lower strength and elastic modulus than those cultured with regular media (n=12).

The TEM images obtained for anchored collagen gels under static tension were analyzed only for fibril diameter and not for collagen content as they were processed differently (gels cultured with exogenous decorin were stained for GAGs, whereas the gels cultured with regular media were not). Therefore, the collagen content was determined for the gels cultured under dynamic condition as they were processed indentically. The addition of exogenous decorin resulted in a lower collagen content only for the wild-type cell-seeded
gels for both static and dynamic condition, as measured from the TEM images (p＜0.05 for wild-type cells, Figure 7-3 (a)), but did not have a significant effect for those containing Dcn⁻/⁻ cells. Likewise, for the collagen fibril diameter, no significant difference was observed between collagen gels cultured with and without exogenous decorin. However, within the collagen gels cultured with exogenous decorin, there was a significant increase in fibril diameter for the Dcn⁻/⁻ cell-seeded gels as compared to those with wild-types at 1x10⁶ cells/ml initial density (p＜0.001, Figure 7-3 (b)), but no significant difference in fibril diameter was observed between the cell types at 2x10⁶ cells/ml initial density. Interestingly, exogenous decorin treatment resulted in smaller average fibril diameter for both wild-type and Dcn⁻/⁻ cells, even though this trend was not significant.
Figure 7-3: Exogenous decorin treatment induced (a) significantly lower collagen fibril density only for wild-type cells and (b) no significant change in fibril diameter for both cell types.
The unexpected results from this experiment could have been due to a number of reasons. The exogenous addition of decorin might not be sufficient to restore endogenous production of decorin or a greater concentration of decorin might be needed to bring the collagen gels back to normal (wild-type like) state, especially for the different initial cell densities. In fact, the inconsistency in trends observed between 1 and 2x10^6 cells/ml initial densities in several studies suggest that a larger concentration of external decorin might be needed for the higher cell density. It is also possible that the commercially available bovine decorin from Sigma did not contain the entire decorin molecule (~88% pure [237]) and thus could restore only partial behavior of the collagen gels. Thus, the mechanical behavior was restored, but the other properties of the collagen gels were not. To test the above explanations in future studies, mouse decorin could be purified from the wild-type cells or tissues and then could be used in place of the bovine decorin from Sigma. This experiment could examine whether the source of decorin influenced the parameters measured in this study, or whether there is a difference in how exogenously and endogenously secreted decorin modulate matrix components.
CHAPTER 8

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

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 deficient (Dcn<sup>−/−</sup>) and wild-type murine embryonic fibroblasts were seeded within collagen gels and mechanically conditioned using a Flexcell<sup>®</sup> Tissue Train<sup>®</sup> 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<sup>−/−</sup> cells. Although cyclic strain caused an elevation in proteoglycan expression regardless of cell type, the type of proteoglycan differed between groups: the Dcn<sup>−/−</sup> 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.

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8.1 Introduction

Decorin is a small-leucine rich proteoglycan (SLRP) that "decorates" collagen fibrils and mediates collagen fibrillogenesis in native and engineered tissues [16]. Decorin consists of a core protein of approximately 40 kDa and a single glycosaminoglycan (GAG) chain of repeating chondroitin/dermatan sulfate disaccharides [79, 195]. Decorin binds to a variety of collagens, including types I, II, III, VI, and XIV [3, 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 [48].

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 [238]. 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 [239-241]. Similarly, collagenous matrices that have been grown under mechanical stimulation demonstrate improvements in cell alignment and collagen fibrillar orientation and packing [242]. 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 [71, 238, 243]. 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 [76, 238]. 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 [238], alteration in specific PG expression and their contribution towards engineered tissue organization has not been extensively investigated.

Because decorin sequesters transforming growth factor beta (TGF-β), we recently described how 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 [244]. 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 [71, 238, 243, 245], 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.
8.2 Materials and methods

8.2.1 Cell isolation and collagen gel preparation

Primary cell culture was isolated from euthanized 12.5 to 14.5 gestational day old wild-type or Den<sup>−</sup> mouse embryos, using established protocol for feeder cells [244]. 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% CO<sub>2</sub>, 95% humidity). Cells from passages P4-P6 were used for preparing the collagen gels [244].

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 1x10<sup>6</sup> cells/ml, as previously described [244]. The collagen gels were formed in the incubator using the Flexcell® Tissue Train® culture system, which has been previously described by Garvin et al. [242]. 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 trough.
(Figure 8-1a), 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 (Figure 8-1a). 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, Figure 8-1b). 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, Figure 8-1b) 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 8-1c shows mature collagen gels in phosphate buffered saline (PBS) at day 8.
Figure 8-1: 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.

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 mm$^3$ sections and processed to stain for collagen fibrils and proteoglycans [206, 244]. 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$_2$ [246]. The samples were then immersed in 0.5% Na$_2$WO$_4$ in acetate
buffer for 1 hour and then overnight in 0.5% Na$_2$WO$_4$ 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 10,000X 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.
8.2.3 Mechanical testing

After 8 days, the culture plates containing intact collagen gels designated for mechanical testing were placed on the posts containing troughs and were digitally photographed. The collagen gel diameter was then measured using Image-Pro Express software (Media Cybernetics, San Diego, CA) [244] using the dimensions of the trough (3 mm) to calibrate the image. While the collagen gels were initially rectangular shaped, the gel contracted uniformly throughout the length of the gel as the cells organized within the collagen matrix. Therefore, collagen gels were assumed to be cylindrical in shape with circular cross-section, 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) [244] 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 (Figure 8-1c). 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 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 (Figure 8-2). 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.

![Load-displacement plot](image-a)

![Stress-strain plot](image-b)

Figure 8-2: 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.

8.2.4 Cell density

Cell density within the collagen gels cultured under static tension or cyclic strain was measured using a DNA assay [244]. 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 μl 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.

8.2.5 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<sup>−/−</sup> cells.

The media samples were processed for western blotting as previously described [92, 203, 244]. 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 [205]) 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 μl, which resulted in a large biglycan band from the media samples collected from the dynamically cultured Dcn<sup>−/−</sup> 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-amino hexanoic acid, 0.08% benzamidine HCl, 10 mM N-ethyl maleimide, 1 mM phenylmethylsulfonyl fluoride (PMSF); 1 ml per 25 mg tissue dry weight) [92, 203]. 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 Cybernatics) 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<sup>−/−</sup> samples in each gel and the exposure time to radiographic film were kept to be around 2 min to avoid experimental bias.
8.2.6 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 $\alpha=0.05$.

8.3 Results

8.3.1 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 (Figure 8-3a and 8-3b). 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 8-3: 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.

Figure 8-4a 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 [246]. 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 (Figure 8-4b). 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 further 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 (Figure 8-4c, 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 (Figure 8-4d). 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).
Figure 8-4: 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.

8.3.2 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 (Figure 8-5a and 8-5b). 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 (Figure 8-5c and 8-5d). When considering only the gels grown under the
dynamic strain condition, the collagen gels containing Dcn<sup>−/−</sup> 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.

(a) Load  (b) Stiffness

Figure 8-5: Normalized material parameters for statically and dynamically cultured
collagen gels containing either Dcn<sup>−/−</sup> or wild-type cells at 8 days: (a) Load normalized to
static tension wild-type (8.67±1.74); (b) Stiffness normalized to static tension wild-type
(3.18±0.79); (c) Strength normalized to static tension wild-type (104.34±37.26); and (d)
Elastic Modulus normalized to static tension wild-type (429.50±189.46). * 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 ± SD (n=13 to 18 gels for each group).
8.3.3 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 (Figure 8-6, p<0.004 for the combined effect of cell type and mechanical condition). 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.

![DNA content in collagen gels](image)

Figure 8-6: 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).

8.3.4 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<sup>−/−</sup> cells. Figure 8-7a 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 (Figure 8-7b and 8-7c, 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<sup>−/−</sup> cells undergoing cyclic strain (p<0.001).
8.4 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 [29, 73, 74, 242]. 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.

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 [247] or native tissues [248] 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 [29] via their core protein [3] as well as their GAG chains [29]. 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. 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 [249]. 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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 [5]. Even though decorin and biglycan are often found in different regions of the extracellular matrix and respond differently to growth factors [5], biglycan competes with decorin for the same binding site on type I collagen fibrils [5]. Thus, compensatory behavior of biglycan has been reported in Dcn<sup>−/−</sup> cell-seeded collagen gels and native tissues [166, 178, 179].

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<sup>−/−</sup> cell-seeded gels. These results suggest that the biglycan and decorin, the predominant SLRPs in the Dcn<sup>−/−</sup> 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 [29].
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 [15]. 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 [14] and postnatal mice [179] as well as decorin deficient engineered gels [244], compared to wild-type controls. In addition, greater than normal decorin expression has been reported in prolapsed mitral valve [10] in which the tissue is weakened [250], and our lab has histochemically demonstrated that excess decorin is present valves that were exposed to altered shear stress [251]. 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 [244], free cell-secreted TGF-β is also expected to have influenced some of the differences observed between the Dcn<sup>−/−</sup> and wild-type cell-seeded gels in this study. In fact, TGF-β had been reported to upregulate the expression of chondroitin/dermatan sulfate PGs [252] and elongate their GAG chains [249] in static 2-D cultures. Unlike our previous study where TGF-β had a strong effect, in this study the endogenously produced free TGF-β did not cause as many noticeable differences between the two cell types within the statically-cultured collagen gels. Thus, we believe that the differences reported in this study were mostly influenced by the combined interaction between decorin, TGF-β, and mechanical stimuli and not only due to free TGF-β.

8.5 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.
CHAPTER 9

Conclusions and Future Directions

Even though decorin knockout mouse tissues have been characterized throughout the past decade, very few follow-up studies have been performed to provide more insight into the tissue behavior. This novel research work combined in vitro tissue engineering methodologies with decorin deficient (Dcn^{-/-}) cells from these knockout mice to reproduce native tissue-like properties that could be investigated systemically. The objective of this research work was to understand the contributions of the proteoglycan decorin to matrix organization using Dcn^{-/-} cells seeded within 3-D collagen matrices. The major findings from this work consist of several unique and novel observations, primarily that the properties of the Dcn^{-/-} cell-seeded 3-D collagen gels were very different from native Dcn^{-/-} tissues from adult mice and were heavily influenced by free transforming growth factor beta (TGF-β). The collagen gels prepared with either cell types also showed strain-dependent behavior, in that cyclic strain induced greater collagen fibril density, PG density, maximum load, and stiffness regardless of the cell type used. The Dcn^{-/-} cell-seeded collagen gels also demonstrated unique strain-dependent parameters; cyclic strain induced increased cell density, collagen fibril diameter, GAG chain length, and biglycan expression in these gels only. The Dcn^{-/-} cells also demonstrated significantly greater proliferation, cellular adhesion and migration on collagen substrates compared to wild-type cells, which supports the explanation of altered cell-induced organization in the Dcn^{-/-} cell-seeded 3-D collagen gels. The cell types also showed substrate dependent behavior, meaning that adhesion of these
cells to collagen appeared to be modulated by a decorin-α2β1 integrin complex. In contrast, cellular adhesion by wild-type and Dcn<sup>−/−</sup> cells to fibronectin appeared to be mediated via different integrin subunits.

In Chapter 4, the cell proliferation, adhesion and migration properties in 2-D culture were reported for the Dcn<sup>−/−</sup> and wild-type cells. The cell behavior on collagen and fibronectin substrates from this 2-D study was expected to mimic matrix organization by both cell types in 3-D gels. In this research work, the Dcn<sup>−/−</sup> cells demonstrated greater proliferation and cell migration on collagen-coated tissue culture plates than did wild-type cells. The Dcn<sup>−/−</sup> cells also showed greater adhesion on both collagen and fibronectin substrates. Since fibroblasts show high affinity to fibronectin [187] and bind to fibronectin as well as collagen type I via β<sub>1</sub> integrins [183, 184], the fibronectin substrate was used as an alternate matrix to determine collagen-specific behavior of the Dcn<sup>−/−</sup> cells. The results from this study thereby suggested that in 3-D culture, the Dcn<sup>−/−</sup> cells would proliferate, adhere to the collagen and migrate more rapidly than the control wild-type cells and would thus facilitate superior matrix organization of the resulting 3-D collagen gels. This study also investigated the contributions of α<sub>2</sub> and β<sub>1</sub> integrin subunits on cell adhesion. Blocking the α<sub>2</sub> and β<sub>1</sub> integrin subunits reduced adhesion to collagen for both cell types demonstrating that adhesion is strongly mediated by this integrin family. The finding that blocking the same integrins in wild-type cultures also reduced adhesion has two possible explanations. The first explanation is that decorin binds to the α<sub>2</sub> or β<sub>1</sub> subunit in a manner that does not block the antigenic site. A second explanation could be that not all α<sub>2</sub>β<sub>1</sub> integrins in the wild-type cell are bound to decorin. The adhesion studies involving fibronectin produced quite different results. Blocking the α<sub>2</sub> and β<sub>1</sub> integrin subunits had no effect on the Dcn<sup>−/−</sup> cells's adhesion
to fibronectin, which suggests that other integrins are involved in the binding of these cells to fibronectin. The wild-type fibroblasts demonstrated only a minimal reduction adhesion to fibronectin when the α2 and β1 integrin subunits were blocked. Possible explanations for this result could be that α2β1 integrins are only marginally responsible for wild-type cell adhesion to fibronectin, or that almost all of the α2β1 integrins are bound by decorin and therefore unavailable for binding. Given that these cells’ adhesion to collagen was significantly reduced by these same blocking antibodies, the former explanation is the more plausible. Investigation of other β1 integrin subunits, such as α3 and α5 [189] that are reportedly involved in cell-matrix adhesion, would continue to reveal the multifaceted role of decorin in cell-matrix adhesion.

In Chapter 6, the contributions of Dcn−/− cells to collagen gel contraction, cell proliferation, material parameters, collagen synthesis and collagen organization were elucidated for 3-D collagen gels cultured under static tension. The Dcn−/− cell-seeded gels showed very distinct behavior from those containing wild-type cells and demonstrated increased gel contraction, matrix organization, ultimate tensile strength and elastic modulus. The behavior of these Dcn−/− cell-seeded gels supported trends reported for native embryonic tissues [179], but were opposite to the trends reported for native adult Dcn−/− tissues [47]. The results from the 2-D studies supported these improved matrix organization by the Dcn−/− cells as well. Further investigation of these unexpected results demonstrated that free cell-secreted TGF-β as well as TGF-β from the serum in cell culture media contributed significantly to the properties of the Dcn−/− cell-seeded gels. Addition of TGF-β increased gel contraction for the wild-type gels, while blocking TGF-β reduced contraction for those containing Dcn−/− cells. Moreover, addition of TGF-β to the wild-type cell-seeded gels increased collagen content,
cell proliferation, tensile strength and elastic modulus, that is, made them similar to Dcn^+ cell-seeded gels.

In Chapter 7, partial recovery of the mechanical strength and elastic modulus was reported when the 3-D Dcn^+ cell-seeded gels under static tension were cultured with media containing exogenous decorin. Surprisingly, several other parameters measured in this study did not show any significant difference from the gels cultured with regular media. Since the source of the exogenous decorin was bovine and not murine, separating decorin from the wild-type cells could verify whether the source of the exogenous decorin hindered recovery of the behavior of the Dcn^+ cell-seeded gels. Otherwise, it is possible that the properties measured in this study were dependent on endogenous secretion of decorin and could not be reversed by exogenous addition of decorin.

In Chapter 8, the contributions of mechanical stimulation to collagen gel contraction, cell proliferation, material parameters, proteoglycan density, collagen synthesis and organization for both cell-types were explained. This study is the first to investigate proteoglycan length and content in engineered tissues in response to cyclic strain and demonstrated an increase in both of these parameters in response to cyclic strain. Mechanical stimulation also increased several parameters including collagen fibril density, and material strength for both cell-types. In contrast, cell density, collagen fibril diameter, and biglycan content increased only in the Dcn^+ cell-seeded gels under dynamic culture. These results indicate that decorin-mediated tissue organization is dependent upon the mechanical condition imparted on tissues. Further investigation regarding the activated signaling pathways for both cell-types and mechanical conditions might provide insight on the strain-dependent response of decorin.
This novel research work was the first to combine tissue engineering techniques with knockout cells to investigate the contributions of a matrix protein, in this case decorin, to the synthesis and organization of various matrix components. This research work not only provided insight on the roles of decorin, but has important implications for tissue engineering as well. Since decorin participates in collagen fibrillogenesis and thus influence material parameters, a common belief in tissue engineering is that adding decorin to engineered tissue would improve their strength. As demonstrated in this work, decorin has a variety of biological and biomechanical roles in the extracellular matrix and thus decorin’s influence on tissue strength would depend on the combined effect of all these interactions. Similarly, when external factors are added to modify certain properties of the engineered tissues, the factor might contribute to the characteristics of the engineered tissue via several different interactions and thus, all possible interactions of external factors need to be considered for designing engineered tissues. As observed in this work, the unbound cell-secreted TGF-β modified many properties of the Dcn−/− cell-seeded collagen gels.

While this research work presented several exciting and unexpected observations, it also opens the door for several new experiments to support the results observed in this study. For the 2-D studies, investigation regarding whether the core protein or the glycosaminoglycan chain of decorin is responsible for the cell-to-matrix adhesion would provide information about the mechanism facilitated by this molecule. Similarly, comparison of mRNA levels for various collagen types and proteoglycans in both 2-D cultures and 3-D gels would reveal any compensatory mechanism employed by the Dcn−/− cells. This investigation would also show whether the cells inherently synthesize different amounts of the above matrix proteins or whether the 3-D environment stimulates differential protein
expression by the cell-types. Since decorin interacts with various matrix metalloproteinases (MMPs), investigation of MMP secretion by the Dcn<sup>−/−</sup> cells would give insight on collagen turnover by these cells [201]. Decorin has also been reported to bind to several components of elastin such as tropoelastin and fibrillin microfibrils [253]. Therefore, the Dcn<sup>−/−</sup> cell-seeded gels might provide an excellent model to investigate the role of decorin on elastin organization as well.

Even though this research work mainly investigated the role of decorin on matrix organization for tissues under uniaxial tension, decorin is also heavily expressed in tissues under compression, such as cartilage. Therefore, investigation of decorin in tissues under compression would be expected to reveal unique strain dependent behavior of this molecule.

In the 3-D gels under static tension, we determined a significant influence of free TGF-β in matrix organization for the Dcn<sup>−/−</sup> cell-seeded gels. However, the amount of TGF-β secreted by the Dcn<sup>−/−</sup> cells was not determined. The number of TGF-β receptors expressed by the cell types could be measured by immunohistochemistry and flow cytometry, where the amount of TGF-β secreted by the cells could be measured by biochemical assays. These future studies would allow us to quantify the contribution of TGF-β in the Dcn<sup>−/−</sup> cell-seeded gels.

Since decorin expression in native tissues has been reported to vary with age [209, 254], preparation of the collagen gels with adult dermal Dcn<sup>−/−</sup> fibroblasts might explain some of the results that were anomalous to the trends reported for the adult mouse tissues. The collagen gels in this study contained embryonic fibroblasts and appeared to mimic developing tissues. Similarly, if the collagen gels with adult cells mimic native adult tissues, it would suggest an age-dependent behavior of decorin.
Since we observed a significantly greater collagen content in the Dcn⁺⁺ cell-seeded gels than those containing wild-types, it would be interesting to create engineered tissues where the collagen expression could be selectively upregulated in the wild-types by inducible gene modifications. This could then verify the contributions of collagen content on the parameters measured in this research work, in particular on the material parameters of the collagen gels.

Overall, decorin is a fascinating molecule with diverse functions in the extracellular matrix. Further research on this proteoglycan will continue to reveal more facets of its role in tissue organization and should lead to a better understanding of the causes of various connective tissue disorders as well as acquired diseases.
References:


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