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A Comparative Analysis of the Biomechanics and Biochemistry of Cell-Derived and Cell-Remodeled Matrices: Implications for Wound Healing and Regenerative Medicine

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A Comparative Analysis of the Biomechanics and Biochemistry of Cell-Derived and Cell-Remodeled Matrices: Implications for Wound Healing and Regenerative Medicine

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ABSTRACT

The purpose of this research was to study the synthesis and remodeling of extracellular matrix (ECM) by fibroblasts with special emphasis on the culture environment (media composition and initial ECM composition) and the resulting mechanical integrity of the ECM. This was investigated by culturing fibroblasts for 3 weeks in a variety of culture conditions consisting of collagen gels, fibrin gels, or media permissive to the self-production of ECM (Cell-Derived Matrix), and quantifying the mechanics of the resulting ECM. The mechanical characteristics were related to the biochemistry of the resulting ECM, notably in terms of collagen accumulation and collagen fibril diameters. The ultimate tensile strength (UTS) of the collagen gels and fibrin gels at the end of the 3-week period was 168.5 ± 43.1 kPa and 133.2 ± 10.6 kPa, respectively. The ultimate tensile strength of the cell-derived matrices was 223.2 ± 9 kPa, and up to 697.1 ± 36.1 kPa when cultured in a chemically-defined medium that was developed for the rapid growth of matrix in a more defined environment. Normalizing the strength to collagen density resulted in a UTS / Collagen Density in these groups of 6.4 ± 1.9 kPa/mg/cm^3, 25.9 ± 2.4 kPa/mg/cm^3, 14.5 ± 1.1 kPa/mg/cm^3, and 40.0 ± 1.9 kPa/mg/cm^3, respectively. Cells were synthetically more active when they produced their own matrix than when they were placed within gels. The resulting matrix was also significantly stronger when it was self-produced than when the cells rearranged the matrix within gels that corresponded to a significantly larger fraction of non-acid and pepsin extractable collagen. These studies indicate that cell-derived matrices have potential both as *in vitro* wound healing models and as soft connective tissue substitutes.

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Thesis Objectives

The purpose of this Master's Thesis was to study the synthesis and remodeling of extracellular matrix (ECM) by fibroblasts with special emphasis on the culture environment (media composition and initial ECM composition) and the resulting mechanical integrity of the ECM. This was investigated by culturing fibroblasts for 3 weeks in a variety of culture conditions consisting of collagen gels, fibrin gels, or media permissive to the self-production of ECM, and quantifying the mechanics of the resulting ECM. The mechanical characteristics were related to the biochemistry of the resulting ECM, notably in terms of collagen accumulation and collagen fibril diameters. The hypothesis was: Cell-derived matrices (CDMs) are stronger than fibroblast populated collagen gels and fibroblast populated fibrin gels due to a greater collagen density and fibril diameter of the self-produced collagen in the CDM. To accurately determine these differences, all groups were grown in parallel with the same number and type of cells, fed with the same media at the same frequency, started and grown for the same period of time, grown next to each other on the same 6-well plates, and overall maintained in the same environment.

Due to the slow growth and development of self-produced ECM, a method of quickly growing strong and thick cell-derived matrices *in vitro* was developed. This involved seeding low passage cells at "superconfluent" densities, or densities where the cells were stacked next to and top of each other, on TransWells™ and feeding the constructs with a chemically-defined medium that was developed. The components and their individual concentrations in this chemically-defined medium were chosen according

to whether they significantly increased the thickness and/or strength of cell-derived matrices. The relatively few components in this chemically-defined medium aided in gaining insight into the effects of these components on the growth and strengthening of cell-derived matrices. This provided insight into how different nutritional components influence the mechanisms of growth and strengthening of cell-derived matrices.

Introduction

Living tissue equivalents (LTE), notably cell-seeded collagen and fibrin gels, have been used extensively as *in vitro* wound-healing models as well as models for tissue remodeling (for review see (Grinnell, 2003)). More recently, tissue-equivalents have also started to gain considerable attention as replacements for lost or damaged connective tissue (e.g., Apligraf™ from Organogenesis, Inc.). Living tissue-equivalents have several advantages over synthetic alternatives including being a natural cell substrate, allowing cellularity to be achieved directly, and being conducive to cell spreading and extracellular matrix (ECM) formation (Neidert *et al.*, 2002).

Over the last two decades, models of fibroplasia that are completely cell-derived have also been developed. This has allowed the study of tissue formation solely from cells *in vitro*. Several research groups have demonstrated that, given a permissive environment, fibroblasts can be induced to produce a thin, 3-dimensional sheet of extracellular matrix material *in vitro* (Grinnell *et al.*, 1989; Clark *et al.*, 1997; Ishikawa *et al.*, 1997; L'Heureux *et al.*, 1998; Ohgoda *et al.*, 1998). These cell-derived matrices (CDM) are produced by slowly proliferating multilayered fibroblasts that become surrounded by the dense extracellular matrix that is composed in large part by supermolecularly organized collagen (Grinnell *et al.*, 1989; Ishikawa *et al.*, 1997). The fibroblasts in this self-produced environment assume a synthetic phenotype (Kessler *et al.*, 2001) characterized by low cell proliferation, high collagen accumulation, fibrillar fibronectin organization, and the formation of actin stress fibers and focal adhesions.

Although the biological and biochemical characteristics of these matrices have been studied to some extent, little is known about their mechanical characteristics and how it relates to the biochemical composition of these materials. From studies by Clark *et al.* (1997) and L'Heureux *et al.* (1998), their structure and composition appears to more closely approximate native tissue than "reconstituted" ECM (e.g., collagen gels). Indeed, Auger and colleagues (1998) have demonstrated significantly higher burst strength of completely biological human blood vessel equivalents (L'Heureux *et al.*, 1998) produced by laminating several of these "self-assembled matrices" into a tubular geometry than has been obtained with collagen or fibrin tubes. Preliminary studies of linear "tendon equivalents" produced using this technology also indicate that these cell-derived matrices have strengths approaching native tissues (Calve *et al.*, 2003). Tissue equivalents of high strength, such as CDMs, are in high demand since current shortages of load-bearing tissue has created a need for man-made tissues that can withstand *in vivo* mechanical forces (Gildner and Hocking, 2003). As with native connective tissues, the mechanics of CDM produced *in vitro* depends not only on the features of individual cells and matrix molecules, but also on the complex organizational and reciprocal interactions of collections of cells and their surrounding matrix (Grinnell, 2003). The biochemical composition and arrangement of the ECM by fibroblasts influence the mechanical characteristics of the ECM. Thus, CDM provides a powerful tool for studying the functional (mechanical) outcomes of these cell-matrix interactions in a controlled environment has similarities to *in vivo* fibroplasia and the early stages of wound-healing. However, a major limitation of CDMs is that they are very thin and take a long time to

grow, generally in the order of months (Auger *et al.*, 2000), whereas collagen gels and fibrin gels can be developed in only a few days (Grinnell, 1994).

The aim of the current study was to compare the biomechanical and biochemical properties of CDMs, collagen gels and fibrin gels to gain insight into the synthesis and remodeling of ECM by fibroblasts. Additionally, to facilitate more rapid studies, we sought to develop a chemically-defined medium permissive to quick self-production of strong and thick ECM *in vitro* that may also be of value for developing functional soft connective tissue substitutes.

Materials and Methods

All components from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Cells

Human neonatal foreskin fibroblasts (HFFs) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HFF cultures were maintained in T-300 tissue culture flasks (BD Biosciences, Bedford, MA) with high glucose Dubelco's modified Eagle' medium (DMEM, Mediatech, Herndon, VA) supplemented with 10% bovine calf serum (BCS, Hyclone, Logan, UT), and 1% penicillin/streptomycin/ ampothericin B (Invitrogen, Carlsbad, CA). HFFs were incubated at 37°C in humidified, 10% CO_2 conditions. Cells were harvested at 90% confluency with a 10min application of 0.25% trypsin/0.05% EDTA solution (Mediatech). Two million, passage 5 cells were used for each sample in all experiments.

Standard Serum-Supplemented Medium

The collagen gels, fibrin gels and cell-derived matrices (CDMs) were fed with a serum-supplemented medium consisting of DMEM with 10% fetal bovine serum (FBS, ATCC), 150µg/ml (519µM) L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako Pure Chemicals, Japan), and 1% penicillin/streptomycin/ampothericin B (Invitrogen). Long-lasting L-ascorbic acid phosphate magnesium salt *n*-hydrate (Ishikawa *et al.*, 1997) was added to stimulate collagen synthesis (Grinnell *et al.*, 1989; Hata and Senoo, 1989; Huang *et al.*, 1993; Kurata *et al.*, 1993; Ishikawa *et al.*, 1997; Girton *et al.*, 2000; Hoerstrup *et al.*, 2000; Laplante *et al.*, 2001; Pouliot *et al.*, 2002). The L-ascorbic acid concentration was 80% higher than the 50µg/ml (289µM) of L-ascorbate used by Grinnell *et al.* (1989).

Collagen Gel, Fibrin Gel and Cell-Derived Matrix Preparation

Fibroblast-populated collagen gels (CGs) were prepared according to the methods of Elsdale and Bard (1972) by mixing 0.2ml of collagen stock solution (5mg/ml of 5mM HCl-extracted rat tail tendon collagen in 5mM acetic acid), 0.05ml 5X DMEM, 0.65ml DMEM (Mediatech) with cells, 0.1ml fetal bovine serum (FBS, ATCC), 150µg L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako Pure Chemicals) and 1% penicillin/streptomycin/ampothericin B (Invitrogen) at room temperature. One milliliter of the resulting solution was added into each 24mm diameter well. The initial collagen concentration was 1.0mg/ml, and the initial cell concentration was 2,000,000 cells/ml in 10% FBS.

Fibroblast-populated fibrin gels (FGs) were prepared based on the methods of Tuan *et al.* (1996) and Neidert *et al.* (2002). Briefly, HFFs in DMEM with 10% fetal bovine serum, 150µg/ml L-ascorbic acid phosphate magnesium salt *n*-hydrate, and 1% penicillin/streptomycin/ampothericin B were added to a fibrinogen (Sigma F4753 type IV) solution. One ml samples were mixed with 4 units of bovine thrombin (Sigma T7513) at room temperature. One ml of the resulting solution was added into each 24mm diameter well. The initial fibrinogen concentration was 1.0mg/ml, and the initial cell concentration was 2,000,000 cells/ml in 10% FBS.

Standard cell-derived matrices (CDMs) were prepared by mixing the 2 million, passage 5 HFFs with DMEM, 10% FBS, 150µg/ml L-ascorbic acid phosphate magnesium salt *n*-hydrate, and 1% penicillin/streptomycin/ampothericin B (serum-supplemented stock medium) at room temperature into a final volume of 1ml per sample. The initial cell concentration was 2,000,000 cells/ml in 10% FBS.

The 1ml samples of collagen gels, fibrin gels, and CDMs were pipetted onto 24mm diameter, porous inserts (0.4µm TransWells™, Corning Life Sciences, Acton, MA) suspended above standard 6-well plates, and allowed to sit undisturbed at room temperature. The semi-permeable culture surfaces allowed a greater volume of nutrient media to be used at each feeding without compromising the oxygen diffusion so that the media could be changed less frequently than once per day required for these high density cultures when cultured on standard plastic plates. Furthermore, the membranes provided a shorter diffusion distance for gases and nutrients for cells on the basal side of the matrices allowing cell-derived matrices to grow more than 50% thicker than in regular 6-well plates (data not shown). After a 1 hour period, 3 ml of standard serum-supplemented

medium was carefully added below and 1 ml above each sample and the samples were incubated at 37°C in humidified, 10% CO_2 conditions. Samples were fed every other day (3 ml below and 2ml above) with the standard serum-supplemented medium for 3 weeks. To reduce experimental variability, all groups were grown in parallel with the same number and type of cells, fed with the same standard serum-supplemented media at the same time, started and grown for the same period of time, grown next to each other on the same 6-well plates, and maintained in the same environment.

Chemically-Defined Media

To efficiently study cell-derived models of wound healing and tissue development *in vitro*, a method of growing CDMs quickly and economically with sufficient thickness and strength was needed. To study the effects of growth factors and media components on CDM growth without the confounding effects of serum (undefined and complex composition), a chemically-defined media was developed to maximize CDM growth. Since CDMs might be one day implanted into humans, it was also desirable to eliminate any non-human components. Chemically-defined media have been formulated for fibroblasts (Bettger *et al.*, 1981; Shipley and Ham, 1983), epidermal cells (Parenteau, 1994; Vaccariello *et al.*, 1999) and dermal equivalents (Bettger *et al.*, 1981; Shipley and Ham, 1983; Parenteau, 1994; Vaccariello *et al.*, 1999), but none of these were designed to promote a high synthetic rate in fibroblasts.

*CDM: Cell-derived matrix fed with the chemically-defined medium developed to induce fibroblasts to synthesize extensive ECM (see Appendix 4). Cells were plated at high density on porous inserts and fed with a chemically-defined medium consisting of a

base of 3:1 ratio of DMEM (high glucose (4.5g/L); with L-glutamine and sodium puryvate, Mediatech) and Ham's F12 (Invitrogen) with the addition of 5µg/ml insulin, 5ng/ml selenious acid, 10^{-4}M ethanolamine, 150µg/ml L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako Pure Chemical), 2.5ng/ml epidermal growth factor (EGF, BD Biosciences, in 5µg/ml human serum albumin, EMD Biosciences, San Diego, CA), 5ng/ml basic fibroblast growth factor (bFGF, BD Biosciences), 1.0 x 10^{-7}M dexamethasone**,** 2 x 10^{-10}M L-3,3',5-Triiodothyronine, 4 x 10^{-3}M of Glutamax™ (Invitrogen), 1µg/ml Glutathione (reduced), and 1% Penicillin/Streptomycin/ Amphotericin B (Invitrogen). (See Appendix 1 for protocol on how to make cell-derived matrix.)

**CDM: Cell-derived matrix fed with the chemically-defined medium with growth factors added to the stock medium at the start of the 3-week experiment (not fresh at every feeding). A slight modification to the chemically-defined medium, entailing the addition of the growth factors (2.5ng/ml EGF and 5.0ng/ml bFGF) fresh into the entire stock medium only at the start of the experiment rather than mixing fresh growth factors separately with the stock media every time the samples are fed, was used to study the effects of decreasing growth factor concentration over time on the growth and development of cell-derived matrix. Growth factors are known to decrease over time in wounds (Clark, 1996; Diegelmann and Evans, 2004). An attempt at roughly simulating this involved adding growth factors at the start of the experiment and taking advantage of the natural loss of growth factor activity in media over time (personal communication with BD Biosciences). Alternatively, adding one-tenth the amount of fresh growth factors

at each feeding was also attempted; however, the CDM did not grow sufficiently thick to perform mechanical testing (data not shown).

To minimize variability and maximize validity of comparison, the chemically-defined medium fed CDMs (*CDMs) and the modified chemically-defined medium fed CDMs (**CDMs) were grown in an identical fashion alongside the standard serum-supplemented medium fed CDMs (CDMs), collagen gels (CGs), and fibrin gels (FGs). Collagen gels fed with the developed chemically-defined medium (*CGs) and modified chemically-defined medium (**CGs) could not be studied because the samples contracted into balls within 24 hours after being fed with the chemically-defined medium (data not shown).

Mechanical Testing

After 3 weeks in culture, the samples were exposed to ddH_2O for 1h to lyse the cells and thus eliminate the contractile forces produced by the fibroblasts, and then equilibrated in phosphate buffered saline (PBS, Mediatech) for biomechanical testing. The thickness, failure tension, failure strain, and ultimate tensile strength (UTS) of the samples were determined by a novel tissue inflation device (Billiar, 2000). Briefly, this device measures the displacement and pressure at which a sample bursts when inflated with PBS at a constant rate of 1 ml/min. The sample is circularly clamped at and inflated through a 1cm diameter opening, thus causing the sample to form a spherical cap before failing. The increasing pressure applied to the sample is measured by an on-board pressure transducer (model PX102-025GV, Omega Engineering, Stamford, CT). The displacement of the center of the cap is measured with a laser displacement system (LDS-

080, Keyence, Woodcliff Lake, NJ). The LSD-080 also measures the thickness of each sample after being slightly compressed by a small reflective disk (1.3g, 1.3cm diameter) for 1 minute. The maximum membrane tension, T, is calculated using the Law of Laplace for a spherical membrane:

$$T = \tfrac{1}{2} PR, \qquad \text{(eq. 1)}$$

where P is the pressure when the tissue bursts and R is the corresponding radius at the point of rupture, calculated assuming a spherical cap geometry by:

$$R = (w^2 + a^2) / 2w, \qquad \text{(eq. 2)}$$

where a is the radius of the clamp (5mm) and w is the displacement at the center of the sample at failure measured by the laser.

The ultimate tensile strength (UTS) is calculated by:

$$UTS = T / t, \qquad \text{(eq. 3)}$$

where t is the initial thickness of the specimen before inflation. The actual thickness at the time of bursting is less than this value. Thus the calculated UTS (engineering stress) is less than the true stress at failure, since the thickness of the specimen decreases as it is being inflated.

The ultimate tensile strength per collagen density (UTS / Collagen Density) is calculated by:

$$\begin{aligned} UTS / Collagen\ Density &= UTS / (Total\ Collagen / \pi t(D/2)^2) \\ &= T / (Total\ Collagen / \pi(D/2)^2), \end{aligned} \qquad \text{(eq. 4)}$$

where D is the diameter of the constructs (2.4cm). This calculated index represents the strength of the constructs normalized per unit of collagen and is independent of the thickness of the samples.

The failure strain is determined from the equibiaxial strain at the pole estimated from the displacement data using the approximate relationship from (Winston *et al.*, 1989):

$$\textit{Failure Strain} = 2/3\ (w / a)^2 - 2/15\ (w / a)^4 + 2/35\ (w / a)^6. \quad \text{(eq. 5)}$$

Biochemical Analysis

Following biomechanical testing, the samples were weighed (wet weight), lyophilized overnight, and then weighed again (dry weight). Each lyophilized sample was solubilized in 1ml pepsin (1 mg/ml in 0.5M acetic acid) and incubated overnight at 20°C with rotation. This extraction step was repeated to achieve complete extraction of the pepsin and acid soluble fraction of collagen. The samples were then centrifuged at 14000 rpm for 1 hour at 15°C, and the supernatant was combined with samples from the first extraction and used for determining non-cross-linked collagen content using the Sircol™ Assay (Biocolor, Belfast, N. Ireland). The Sircol™ Assay quantified the content of intact collagen monomers in the solution, and does not detect degraded collagen (these amounts were 5-10% of the actual collagen content (data not shown) determined by a hydroxyproline assay (see methods below)). Total non-collagenous protein content of each extract was determined with the Tp-Blue™ Total Protein Assay (Biocolor) using Coomassie brilliant blue G (Bradford, 1976). Total protein content was obtained by adding this value to the total amount of collagen obtained for each sample. The remaining pellets were digested with Proteinase K (Invitrogen), 50µg in 500µl solution of 10mM EDTA and 0.1 M sodium phosphate (pH 6.5)(Fisher) overnight at 60°C. A 100µl aliquot of the digest was used for determining sulfated glycosaminoglycan and proteoglycan content (which does not include hyaluronic acid) with the Blycan™ Assay (Biocolor). A

10µl aliquot of the digest was then used to determine DNA content, and thus cell number (assuming 8pg of DNA per cell), with Hoechst 33258 dye (Amersham Biosciences, Piscataway, NJ) on a DyNA Quant 200 fluorometer (Amersham Biosciences). 100-200 µl aliquots of the Proteinase K digests were used to determine the non-acid and pepsin extractable collagen content (insoluble collagen fraction) with a hydroxyproline assay. The hydroxyproline assay was based on the methods of Edwards and O'Brien (1980) and consists of hydrolyzing each sample in 6.0 M HCl for 16 hours at 110^{o}C, followed by drying of the samples under vacuum, reconstituting to 2.0ml with assay buffer (consisting of 5g/l citric acid, 1.2ml/l glacial acetic acid (EMD Chemicals, Gibbstown, NJ), 12g/l sodium acetate (VWR, Bridgeport, NJ), and 3.4g/l sodium hydroxide (VWR)), mixing with 1.0ml of Chloramine-T reagent (made from 62mM chloramine-T solution (VWR) in 20.7% ddH_2O, 26% *n*-propanol (VWR) and 53.3% of assay buffer) for 20 minutes at room temperature, adding 1.0ml of freshly prepared dimethylaminobenzaldehyde reagent (made from 15g *p*-dimethylaminobenzaldehyde in 60ml of *n*-propanol (VWR) and 26ml of 60% perchloric acid (VWR)) and incubating each sample at 60^{o}C for 15 minutes, cooling each sample in tap water for 5 minutes, and measuring the absorbance of each sample at 550 nm within 45 minutes. Absorbance readings were correlated with collagen amount using a standard curve and a conversion factor of 10µg collagen to 1µg 4-hydroxyproline. The standard curve was created and the conversion factor determined with known amounts of Trans-4-hydroxy-L-proline and rat tail type I collagen.

(See Appendix 3 for complete protocol.)

Histology and Transmission Electron Microscopy

One sample from each group was prepared for histological evaluation by fixing in 10% zinc formalin (VWR) for 1 hour, followed by washing and storing in 4°C ethanol. The samples were embedded onto paraffin blocks, sectioned into 10µm thick sections, and stained with hematoxylin and eosin (H&E). The stained sections were imaged and photographed at 200x with a Nikon Eclipse E600 microscope and Spot digital camera (Diagnostic Instruments, Sterling Heights, MI).

One sample from each group (except **CDM) was fixed for transmission electron microscopy (tEM) according to the methods of Gibson and Lang (1979). Briefly, the samples were fixed in 2% glutaraldehyde for 1 hour, rinsed 3 times in sodium cacodylate buffer, fixed in osmium tetroxide for 10 minutes, and dehydrated in 10 minute ethanol (VWR) steps followed by two washes in propylene oxide (all from Electron Microscopy Sciences, Hatfield, PA). The samples were then embedded in epon-araldite resin (Electron Microscopy Sciences) according to the methods of Karnovsky (1965). Briefly, the samples were embedded in a 1:1 ratio of propylene oxide to Epon Araldite for 2 hours, followed by embedding in 100% Epon Araldite for 3 hours, and then transferring each sample to embedding molds with fresh 100% Epon Araldite and cured in a 60°C oven overnight. 60-90nm gold- and silver-colored sections were stained for 5 minutes with urinyl acetate saturated in 50% ethanol, followed by staining for 20 minutes in lead citrate according to the methods of Venable and Coggesmall (1965). The stained sections from each of the samples were then imaged with a ZEISS electron microscope for observation of cell, collagen, and ECM morphology. The average collagen fibril diameter and density were calculated from the average measured diameters and number of

collagen fibrils per square micrometer from 5 randomly chosen fields taken at high magnification (57,000X or 88,000X). The collagen diameters were measured at the thinnest point of cross-sectioned collagen fibrils with a 5x magnified ruler. If a randomly chosen field fell on a grid, then another field continued to be randomly chosen until it fell solely on the sample.

Statistics

Error bars indicate one standard deviation in all figures. Statistical differences between groups were determined using ANOVA with Tukey HSD post hoc analysis (SPSS, Inc., Chicago, IL). Differences were considered significant with $p<0.05$ (see Appendix 5 for complete analysis). Linear regression including all the samples was performed with Tension and Failure Strain as the dependent variables and Total Protein, Total Collagen, Non-Acid and Pepsin Extractable Collagen Fraction, Total Proteoglycans and Glycosaminoglycans, and Cell Number as the independent variables, and the adjusted R^2-values were obtained for determining the degree of association between the independent and dependent variables (SPSS, Inc.).

Results

All samples were sufficiently thick and strong at the end of the 3-week culture period to be handleable and clamped in the mechanical testing device. All samples burst in the center. The collagen and fibrin gels started out at a thickness of 2.2 mm (1ml on a 24mm diameter well) and contracted to a thickness 83 ± 5 μm and 218 ± 5 μm, respectively, as the cells reorganized the initially sparse protein gels, whereas the CDMs started out as slightly more than a single cell layer thick and grew to 125 ± 6 μm during the 3-week period. The CDMs were substantially stronger (UTS = 223 ± 9 kPa) than the collagen gels (169 ± 43 kPa) and significantly stronger than the fibrin gels (133 ± 11 kPa, $p<0.01$). The collagen gels were significantly less extensible than the fibrin gels.

The results from the mechanical and biochemical analyses of CDMs, collagen gels and fibrin gels are summarized in Table 1. The net total protein synthesized by the CDMs was significantly more (1.25 ± 0.06 mg) than the net increase in total protein in the collagen gels (0.08 ± 0.03 mg, $p<0.02$) and fibrin gels (0.58 ± 0.04 mg, $p<0.05$). Collagen was a major constituent of the total amount of protein. Observation by transmission electron microscopy (tEM) revealed that the diameter of the collagen fibrils in the CDMs, collagen gels and fibrin gels were 46 ± 5 nm, 52 ± 10 nm and 47 ± 5 nm, respectively, while the collagen density within 3μm around the cell surface was significantly greater for the CDMs (79 ± 2 fibrils/μm^2) and collagen gels (81 ± 4 fibrils/μm^2) than for the fibrin gels (28 ± 4 fibrils/μm^2, $p<0.01$). Although most of the collagen in the collagen gel consisted of large, approximately 56 nm diameter fibrils, there was a small presence of approximately 46 nm diameter fibrils; presumably newly

synthesized collagen (Fig. 4), indicating that some of the original collagen in the gel had been degraded since the total amount of collagen in the collagen gels remained unchanged over the 3-week period. The CDMs had a significantly ($p<0.01$) greater fraction of non-acid and pepsin extractable collagen (insoluble collagen fraction) than the collagen and fibrin gels. The UTS / Collagen Density was significantly greater (14.5 ± 1.1 kPa/mg/cm^3) for the CDMs than the collagen gels (6.4 ± 1.9 kPa/mg/cm^3, $p<0.01$). The high value of UTS / Collagen Density for the fibrin gels (25.9 ± 2.4 kPa/mg/cm^3) appears to be due to ECM proteins other than collagen, such as fibrin, in the gel.

Table 1. Results from mechanical and biochemical analysis of cell-derived matrices (CDMs), collagen gels and fibrin gels containing human foreskin fibroblasts. Numbers indicate mean +/- SD. Numbers in parentheses indicate approximate initial amount. Collagen fibril diameters and densities were measured in proximity to cell surfaces.

	Collagen gel	**Fibrin gel**	**CDM**
Ultimate Tensile Strength	168.5 ± 43.1 kPa	133.2 ± 10.6 kPa	223.2 ± 9 kPa
Thickness	83 ± 5 μm (2,220 μm)	218 ± 5 μm (2,220 μm)	125 ± 6 μm (~30 μm)
Failure Strain	0.13 ± 0.02	0.21 ± 0.03	0.18 ± 0.03
Total Protein	1.08 ± 0.03 mg (1.0 mg)	1.58 ± 0.04 mg (1.0 mg)	1.25 ± 0.06 mg
Total Collagen	0.99 ± 0.02 mg (1.0 mg)	0.51 ± 0.03 mg	0.85 ± 0.03 mg
Insoluble Collagen Fraction	1.5 ± 0.1 %	1.3 ± 0.1 %	2.3 ± 0.2 %
Collagen Density	26.5 ± 1.5 mg/cm^3	5.2 ± 0.2 mg/cm^3	15.1 ± 0.9 mg/cm^3
UTS / Collagen Density	6.4 ± 1.9 kPa/mg/cm^3	25.9 ± 2.4 kPa/mg/cm^3	14.5 ± 1.1 kPa/mg/cm^3
Collagen Fibril Diameter	52 ± 10 nm	47 ± 5 nm	46 ± 5 nm
Collagen Fibril Density	81 ± 4 fibrils/μm^2	28 ± 4 fibrils/μm^2	79 ± 2 fibrils/μm^2
Proteoglycans & Glycosaminoglycans	38.4 ± 0.7 μg	45.4 ± 0.9 μg	31.3 ± 0.5 μg
Wet Weight / Dry Weight	16.4 ± 0.6	20.1 ± 0.4	13.0 ± 0.5
Cell Number	2.8 ± 0.1 million (2.0 million)	4.0 ± 0.1 million (2.0 million)	2.8 ± 0.1 million (2.0 million)

The *CDMs and **CDMs were also grown in parallel next to the CDMs, collagen gels and fibrin gels in the same 6-well plates, but they were fed with a chemically-defined medium instead of a serum-supplemented medium. To compare the effects of the chemically-defined medium on matrix growth, the *CDMs and **CDMs were compared to CDMs (that were fed with the standard serum-supplemented medium). The chemically-defined medium, on the other hand, caused the contractile forces within the collagen gels to become so great that they detached from the wells and contracted into themselves, preventing a comparison of the effects of the chemically-defined medium and the serum-supplemented medium on the development of gels.

The collagen in the cell-derived matrices organized into several alternating layers that were at right-angles to each other, resembling native soft connective tissue (see Appendix 6). Cells produced a more than 3 times thicker matrix in the chemically-defined medium (*CDMs at 395 ± 6 μm) than in the standard serum-supplemented medium (CDMs at 125 ± 6 μm). One-time addition of growth factors at the start of the 3-week period resulted in a significantly thinner matrix (**CDMs at 225 ± 7 μm) than when the growth factors were added fresh at every feeding. The thicknesses correlated with total protein content (adjusted R^2=0.900), which in turn correlated with total cell number (adjusted R^2=0.874). However, the **CDMs had significantly more total collagen than *CDMs, although the *CDMs had almost twice as much total protein as the **CDMs. The **CDMs were almost twice as strong as the *CDMs (**CDMs at 697 ± 36 kPa significantly stronger ($p<0.01$) than *CDMs at 315 ± 7 kPa), which in turn were significantly stronger than the CDMs (UTS = 223 ± 9 kPa, $p<0.01$) (see Table 3). Cell-derived matrices grown for longer periods of time further increased in thickness and

strength (data not shown). For comparison, the thickness of the samples obtained from histological sections were 110μm, 465μm, and 240μm, for CDM, *CDM, and **CDM, respectively (see figure 1). The failure strains for the *CDMs and **CDMs were almost twice as great as for the CDMs.

The results from the mechanical and biochemical analysis of CDMs, *CDMs and **CDMs are summarized in Table 2. The CDMs and **CDMs had about a 2x greater fraction of their total protein as collagen than *CDMs. Observation by transmission electron microscopy (tEM) revealed that CDMs and *CDMs had approximately the same diameter collagen fibrils at 46 ± 5 nm and 48 ± 5 nm, and the same collagen density at 79 ± 2 fibrils/μm^2 and 80 ± 2 fibrils/μm^2, respectively, measured within 3μm around the cell surface. The *CDMs and **CDMs had more than a five times greater fraction of non-acid and pepsin extractable collagen (insoluble collagen fraction) than the CDMs. The UTS / Collagen Density reflected the high insoluble collagen fraction values and was significantly greater for the *CDMs (40.3 ± 0.4 kPa/mg/cm^3) and **CDMs (40.0 ± 1.9 kPa/mg/cm^3) than for the CDMs (14.5 ± 1.1 kPa/mg/cm^3, $p<0.01$).

Table 2. Results from mechanical and biochemical analysis of CDMs, *CDMs and **CDMs. Numbers indicate mean +/- SD. Numbers in parentheses indicate approximate initial amount. Collagen fibril diameters and densities were measured in proximity to cell surfaces. Standard serum-supplemented medium fed CDM data from Table 1 reported for comparison.

	CDM	***CDM**	****CDM**
Ultimate Tensile Strength	223.2 ± 9 kPa	314.5 ± 7.2 kPa	697.1 ± 36.1 kPa
Thickness	125 ± 6 μm (~30 μm)	395 ± 6 μm (~30 μm)	225 ± 7 μm (~30 μm)
Failure Strain	0.18 ± 0.03	0.33 ± 0.03	0.31 ± 0.06
Total Protein	1.25 ± 0.06 mg	4.40 ± 0.08 mg	2.65 ± 0.07 mg
Total Collagen	0.85 ± 0.03 mg	1.40 ± 0.04 mg	1.78 ± 0.06 mg
Insoluble Collagen Fraction	2.3 ± 0.2 %	12.8 ± 0.5 %	13.1 ± 0.3 %
Collagen Density	15.1 ± 0.9 mg/cm^3	7.8 ± 0.2 mg/cm^3	17.4 ± 0.1 mg/cm^3
UTS / Collagen Density	14.5 ± 1.1 kPa/mg/cm^3	40.3 ± 0.4 kPa/mg/cm^3	40.0 ± 1.9 kPa/mg/cm^3
Collagen Fibril Diameter	46 ± 5 nm	48 ± 5 nm	-
Collagen Fibril Density	79 ± 2 fibrils/μm^2	80 ± 2 fibrils/μm^2	-
Proteoglycans & Glycosaminoglycans	31.3 ± 0.5 μg	64.3 ± 0.6 μg	38.4 ± 0.4 μg
Wet Weight / Dry Weight	13.0 ± 0.5	19.5 ± 0.2	20.3 ± 0.6
Cell Number	2.8 ± 0.1 million (2.0 million)	6.1 ± 0.1 million (2.0 million)	3.6 ± 0.1 million (2.0 million)

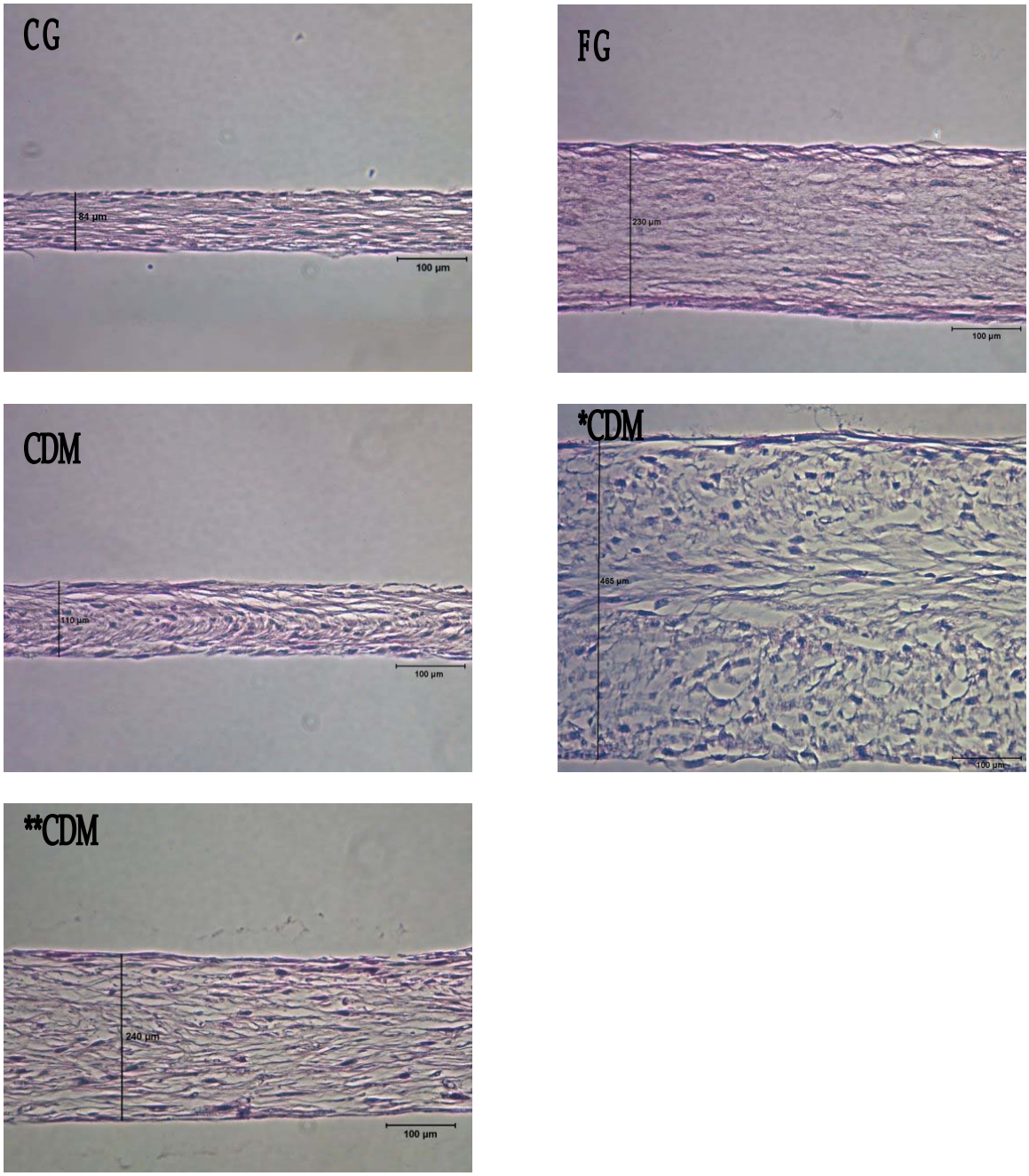

Figure 1. Hematoxylin and Eosin (H&E) stained sections. Thickness measured by digital image analysis: Cell-derived matrices (CDMs) were 110µm thick, collagen gels (CGs) were 84µm thick, fibrin gels (FGs) were 230µm thick, *CDMs were 465µm thick, and **CDMs were 240µm thick. All micrographs taken at 200x. Scale bars = 100µm.

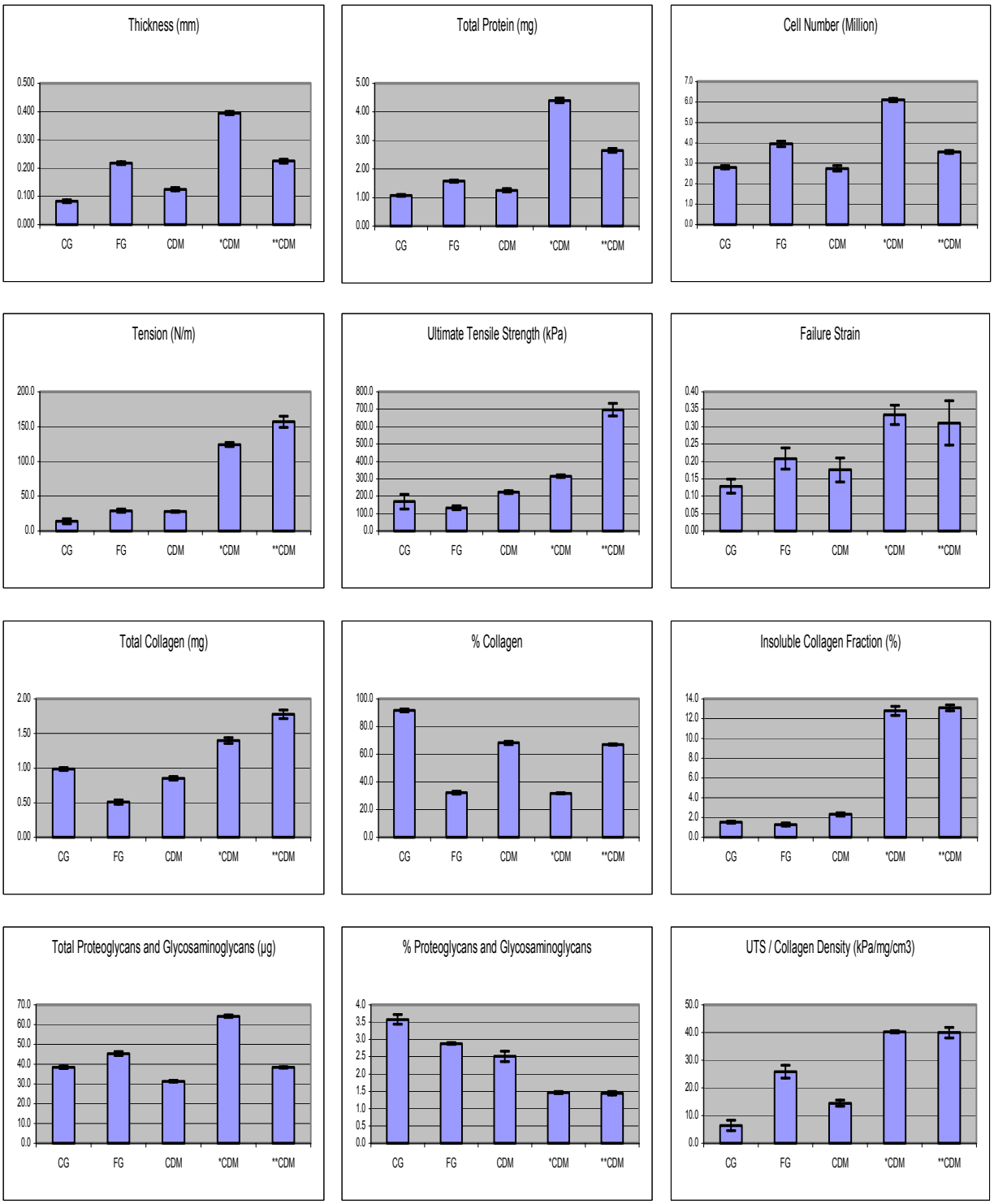

Figure 2. Results from biomechanical and biochemical analysis. All types of the cell-derived matrices were stronger than collagen gels (CG) and fibrin gels (FG). *CDMs and **CDMs were significantly thicker and contained far more total protein than their serum-supplemented companions (CDMs, collagen gels and fibrin gels), but had only half the

percentage of proteoglycans and glycosaminoglycans. *CDMs and **CDMs also contained a significantly higher fraction of non-acid and pepsin extractable collagen (insoluble collagen fraction), and were stronger per microgram of collagen (UTS/Collagen Density) than collagen gels or fibrin gels. CDMs were more than twice as strong as collagen gels per microgram of collagen, indicating the possibility that the collagen in the CDMs was more organized for strength than in collagen gels. In addition to collagen, a substantial proportion of the strength of *CDMs and fibrin gels was also due to other ECM proteins. *CDMs and **CDMs were significantly more extensible than CDMs, collagen gels and fibrin gels.

Table 3. Statistical comparison between each group of specimens. *CDMs were significantly different than CDMs and collagen gels in all parameters tested. *CDMs and **CDMs had the least number of significantly different parameters than any other pair of groups. Statistical differences between groups were determined using ANOVA with Tukey HSD post hoc analysis, with differences with $p<0.05$ being considered significant. The same 12 parameter shown in Figure 2 were used. The parameters between groups that were NOT significantly different from each other are indicated with a letter.
Key: a = thickness, b = total protein, c = cell number, d = tension, e = ultimate tensile strength (UTS), f = failure strain, g = total collagen, h = % collagen, i = fraction of non-acid and pepsin extractable collagen, j = total proteoglycans and glycosaminoglycans, k = % proteoglycans and glycosaminoglycans, l = UTS / Collagen Density.

	Collagen Gel	Fibrin Gel	CDM	*CDM	**CDM
Collagen Gel	-	e,i	c,e,f		j
Fibrin Gel	e,i	-	d,f	h	a
CDM	c,e,f	d,f	-		h
*CDM		h		-	f,i,k,l
**CDM	j	a	h	f,i,k.l	-

The results from linear regressions of all the samples with Tension as the dependent variable and Total Protein Amount, Total Collagen Amount, Non-Acid and Pepsin Extractable Collagen Fraction, Total Proteoglycan and Glycosaminoglycan Amount, and Cell Number as the independent variables are shown in Table 4, along with the adjusted R^2-values. The adjusted R^2-value indicates the fraction of the total variance in "Tension" explained by the regression equation. The adjusted R^2-values are always less than the coefficient of determination R^2. These results indicate that there is a high

degree of association between the "Non-Acid and Pepsin Extractable Collagen Fraction" and "Tension" for collagen gels, fibrin gels, and cell-derived matrices (adjusted R^2 = 0.977). With the addition of the "Total Collagen Amount" independent variable, the adjusted R^2 value increases to 0.992. Further addition of the "Total Protein Amount" independent variable increases the adjusted R^2-value by an insignificant amount to 0.993. A scatterplot of Tension vs. Total Collagen Amount and Non-Acid and Pepsin Extractable Collagen Fraction is shown in Figure 3. Since the area of all the samples were essentially identical, and if each variable was divided by the thickness of the samples, the adjusted R^2-values in Table 4 would be the same for UTS (UTS = Tension / thickness of sample) as the dependent variable and Protein Density, Collagen Density, Non-Acid and Pepsin Extractable Collagen Density, Proteoglycan and Glycosaminoglycan Density, and Cell Density as the independent variables (see table 5).

Table 4. Adjusted R^2-values indicating the fraction of the total variance in Tension of the collagen gel, fibrin gel, and cell-derived matrix samples explained by the Total Protein Amount, Total Collagen Amount, Non-Acid and Pepsin Extractable Collagen Fraction, Total Proteoglycan and Glycosaminoglycan Amount, and Cell Number of the samples. Columns with an asterix (*) indicate that the adjusted R^2-value given in that column have those independent variables added to them.

Independent Variables:	**Tension (dependent variable)**				
Total Protein Amount (mg)	0.681	0.857		0.977	0.993
Total Collagen Amount (mg)	0.713	*	0.992		*
Non-Acid and Pepsin Extractable Collagen Fraction (mg)	0.977		*	*	*
Total Proteoglycan and Glycosaminoglycan Amount (μg)	0.214				
Cell Number (Million)	0.355				

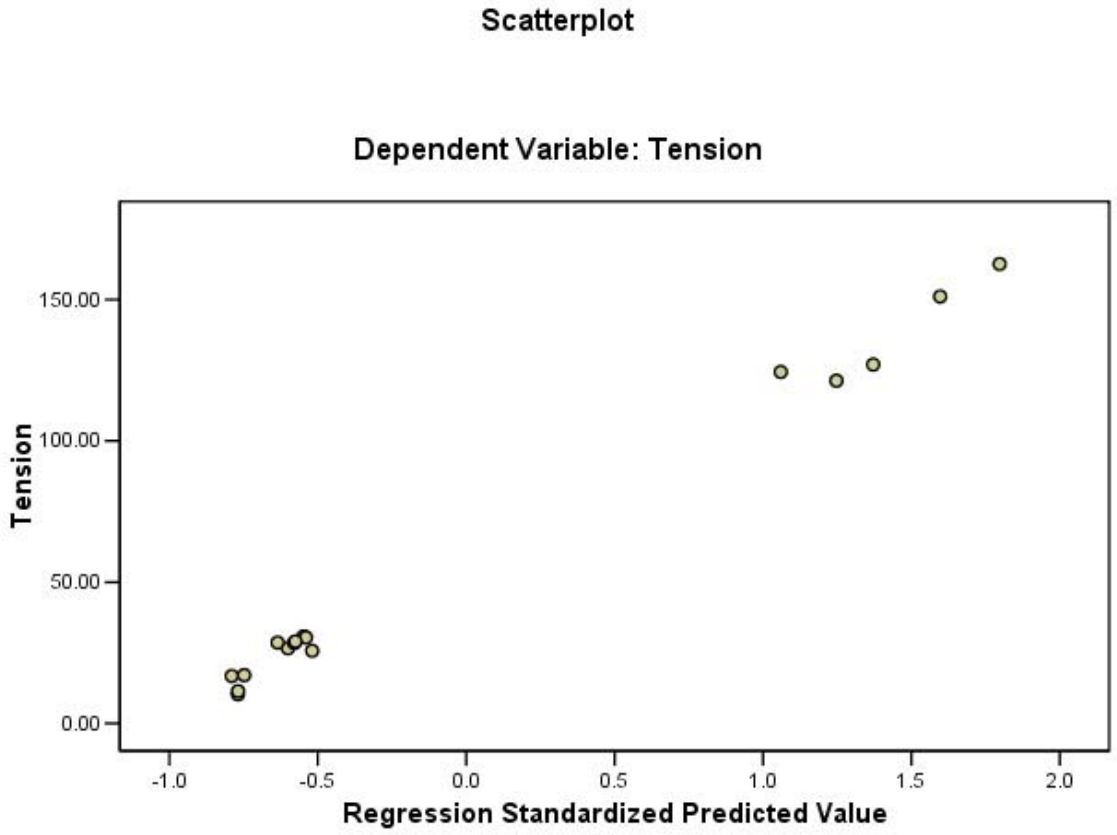

Figure 3. Scatter Plot of Tension vs. Total Collagen Amount and Non-Acid and Pepsin Extractable Collagen Fraction. Adjusted R^2-value = 0.992. Output from SPSS 12.0 for Windows (SPSS, Inc., Chicago, IL).

Table 5. Adjusted R^2-values indicating the fraction of the total variance in Tension of the collagen gel, fibrin gel, and cell-derived matrix samples explained by the Protein Density, Collagen Density, Non-Acid and Pepsin Extractable Collagen Density, Proteoglycan and Glycosaminoglycan Density, and Cell Density of the samples. Columns with an asterix (*) indicate that the adjusted R^2-value given in that column have those independent variables added to them.

Independent Variables:	**UTS (dependent variable)**				
Protein Density (mg)	0.681	0.857		0.977	0.993
Collagen Density (mg)	0.713	*	0.992		*
Non-Acid and Pepsin Extractable Collagen Density (mg)	0.977		*	*	*
Proteoglycan and Glycosaminoglycan Density (μg)	0.214				
Cell Density (Million)	0.355				

The results from linear regressions of all the samples with Failure Strain as the dependent variable and Total Protein Amount, Total Collagen Amount, Non-Acid and Pepsin Extractable Collagen Fraction, Total Proteoglycan and Glycosaminoglycan Amount, and Cell Number as the independent variables are shown in Table 6, along with the adjusted R^2-values. The adjusted R^2-value indicates the fraction of the total variance in the "Failure Strain" explained by the regression equation. These results indicate that there is some degree of association between the "Failure Strain" and "Total Protein" (adjusted $R^2 = 0.742$) or "Non-Acid and Pepsin Extractable Collagen Fraction" (adjusted $R^2 = 0.709$) for collagen gels, fibrin gels, and cell-derived matrices. Together, these two independent variables results in an adjusted $R^2 = 0.787$. With the addition of the "Total Collagen Amount" independent variable to the "Non-Acid and Pepsin Extractable Collagen Fraction", an adjusted $R^2 = 0.884$ is obtained. Conversely, the independent variables "Non-Acid and Pepsin Extractable Collagen Fraction", "Total Proteogycan and Glycosaminoglycan Amount" and "Cell Number" together results in an adjusted R^2-value of 0.864. A scatterplot of Failure Strain vs. Total Collagen Amount and Non-Acid and Pepsin Extractable Collagen Fraction is shown in Figure 4.

Table 6. Adjusted R^2-values indicating the fraction of the total variance in Failure Strain of the collagen gel, fibrin gel, and cell-derived matrix samples explained by the Total Protein Amount, Total Collagen Amount, Non-Acid and Pepsin Extractable Collagen Fraction, Total Proteoglycan and Glycosaminoglycan Amount, and Cell Number of the samples. Columns with an asterix (*) indicate that the adjusted R^2-value given in that column have those independent variables added to them.

Independent Variables:	**Failure Strain (dependent variable)**							
Total Protein Amount (mg)	0.742	0.787	0.761					0.797
Total Collagen Amount (mg)	0.332				0.884			
Non-Acid and Pepsin Extractable Collagen Fraction (mg)	0.709	*		0.823	*	0.775	0.864	
Total Proteoglycan and Glycosaminoglycan Amount (μg)	0.396		*			*	*	*
Cell Number (Million)	0.583			*			*	*

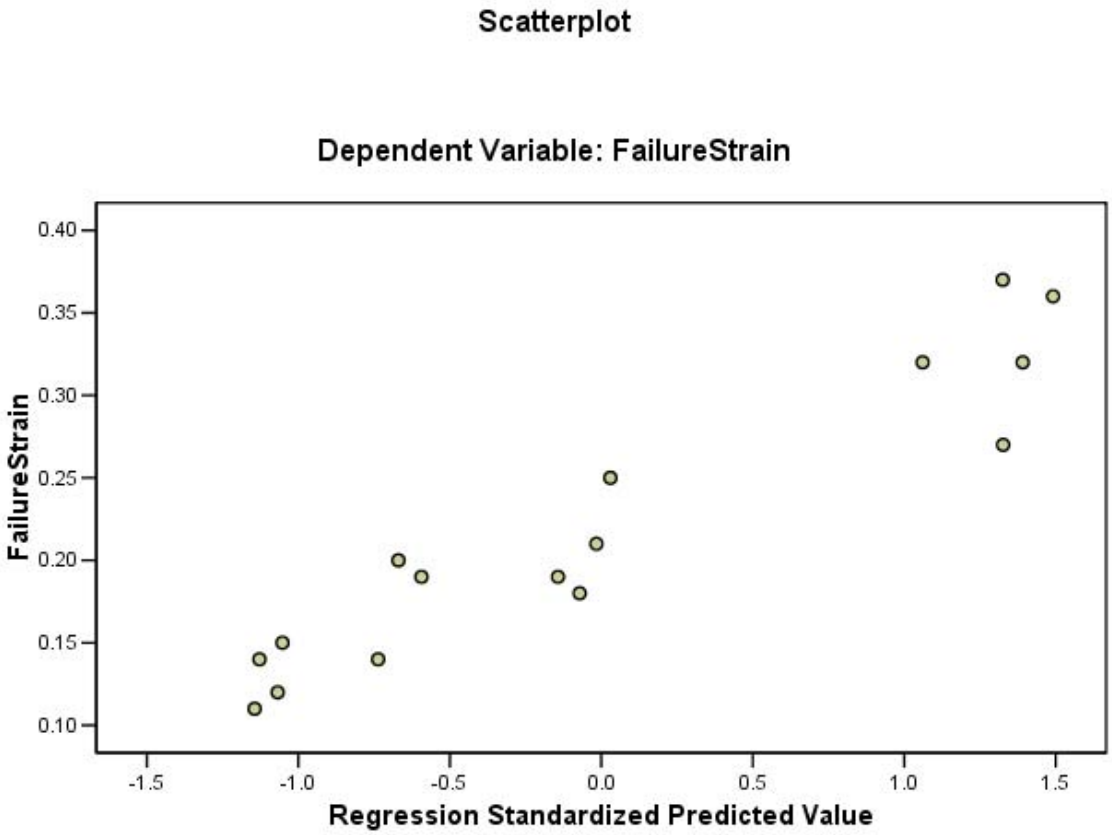

Figure 4. Scatter Plot of Failure Strain vs. Total Collagen Amount and Non-Acid and Pepsin Extractable Collagen Fraction. Adjusted R^2-value = 0.884. Output from SPSS 12.0 for Windows (SPSS, Inc., Chicago, IL).

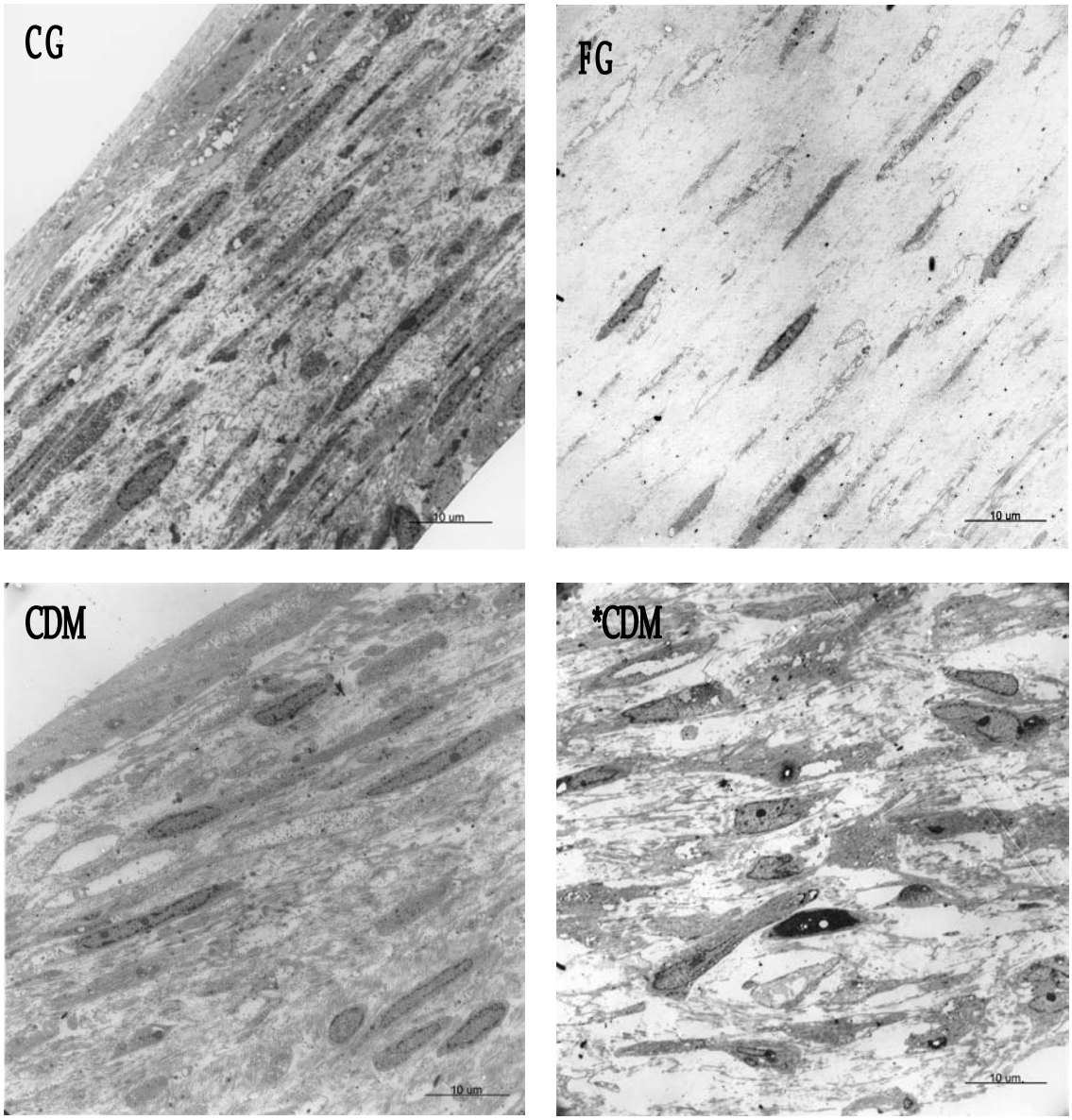

Figure 5. Sample morphology observed with tEM. The fibroblasts within the *CDM appeared to be slightly larger than in the other samples. Fibrin gels had a cell density that was significantly higher than in the other samples. All micrographs taken at 3,000x. Scale bar = 10μm.

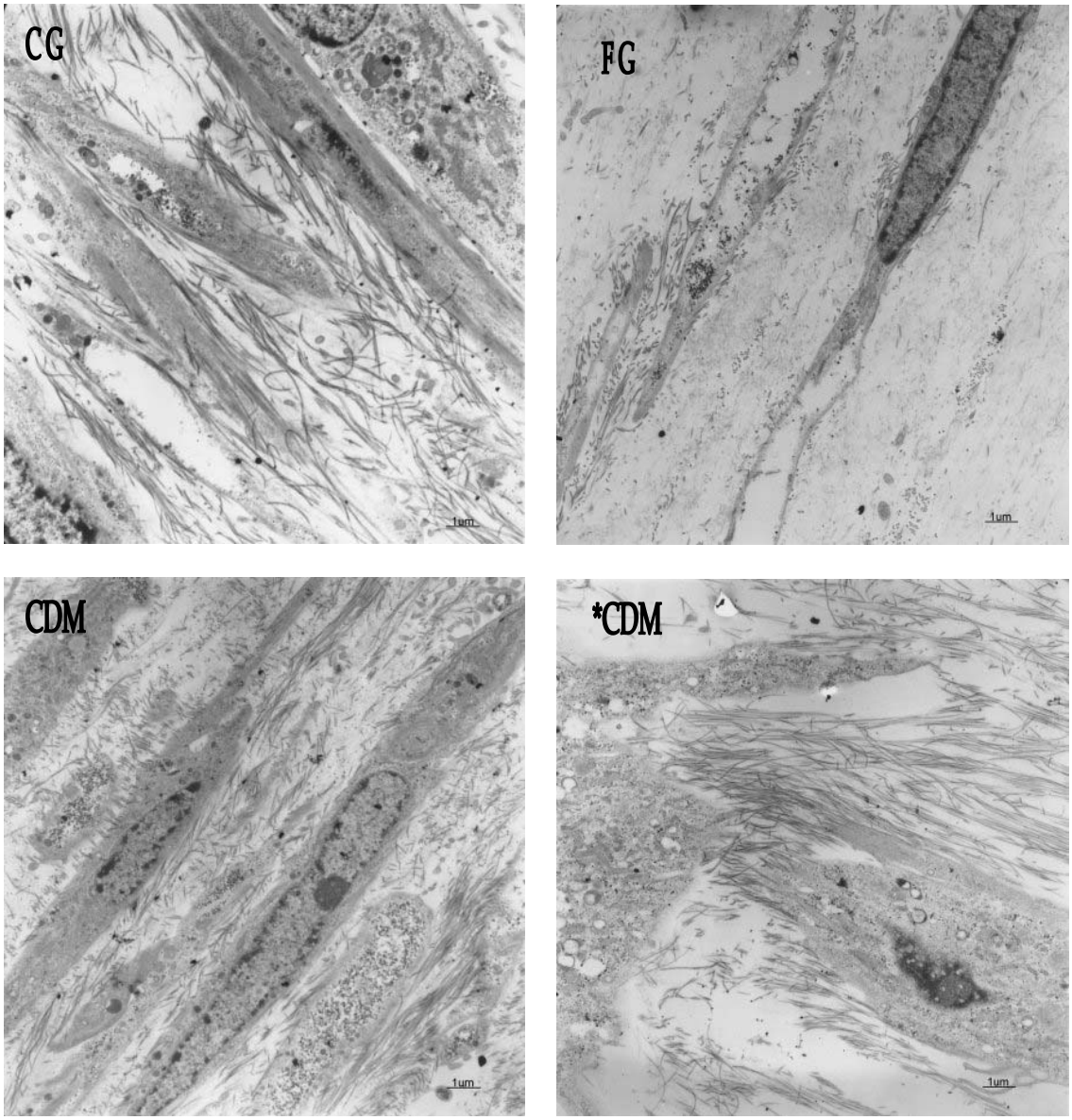

Figure 6. Distribution of collagen fibrils. CDMs, collagen gels, and *CDMs had a more than 2.5 times greater density of collagen fibrils around the cell surface at around 80 fibrils/μm^2 than fibrin gels at 28 fibrils/μm^2. The collagen fibrils of the cell-derived matrices appeared more parallel and to consist of more parallel alternating layers than any of the other groups. The collagen fibrils of the collagen gels appeared somewhat parallel but consisted of only a few alternating layers, while the collagen fibrils of the fibrin gels appeared only parallel in small regions of the ECM. The collagen gels had a large majority of collagen fibrils about 56 nm in diameter due to the collagen gel, and a small number of collagen fibrils about 46 nm in diameter that had presumably been secreted by the fibroblasts. All micrographs taken at 12,000x. Scale bar = 1μm.

Discussion

The data from the present study suggest that tissue-equivalents could be *grown* stronger and thicker when they are self-produced – in other words, when the cells are allowed to grow and develop their own mechanical environment rather than being supplied with a mechanical environment as in gels. To gather more insight into the differences between fibroblast-populated collagen gels, fibrin gels, and cell-derived matrices, it was desirable to reduce experimental variability by growing all groups next to each other on the same 6-well plates for 3-weeks, seeded with the same number of cells from the same batch, and fed with the same standard serum-supplemented medium at the same frequency. While the collagen and fibrin gels contracted more than an order of magnitude (2.2 mm to a thickness 83 ± 5 μm and 218 ± 5 μm, respectively) over the 3-week culture period as the cells reorganized the initially sparse protein gels, the CDMs quadrupled in thickness (~30μm to a thickness of 125 ± 6 μm) and were significantly stronger (UTS = 223 ± 9 kPa) than the collagen gels (169 ± 43 kPa, $p<0.05$ LSD) and the fibrin gels (133 ± 11 kPa, $p<0.01$). The net increase in protein accumulated by the CDMs was significantly more (1.25 ± 0.06 mg) than the net increase in protein accumulated by the collagen gels (0.08 ± 0.03 mg, $p<0.02$) and fibrin gels (0.58 ± 0.04 mg, $p<0.05$).

The collagen in collagen gels is known to signal embedded fibroblasts to drastically slow down their proliferation and synthesis of ECM proteins (Clark, 1996). However, the synthesized collagen in the CDMs and fibrin gels (Neidert *et al.*, 2002) did not appear to have this effect. The amount of collagen in the CDMs (0.85 ± 0.03 mg) was essentially the same as in the collagen gels (0.99 ± 0.02 mg) at the end of the 3-week

culture period. CDMs also contained far more non-collagenous protein (0.40 ± 0.03 mg) than the collagen gels (0.09 ± 0.01 mg, $p<0.01$). The lack of any collagen in the fibrin gels at the start of the culture did not present any inhibitory signal towards collagen synthesis as in the case of the collagen gels, but fibrin is known to downregulate fibroblast procollagen mRNA (Clark, 1996) which possibly explains the observation that the fibroblasts within the fibrin gel ended up synthesizing only half as much collagen (0.51 ± 0.03 mg) as the CDMs during the 3 week period. Interestingly, the net total non-collagenous protein synthesized in the fibrin gels was also very low (0.07 ± 0.01 mg).

The UTS / Collagen Density was also significantly greater (14.5 ± 1.1 kPa/mg/cm^3) for the CDMs than the collagen gels (6.4 ± 1.9 kPa/mg/cm^3, $p<0.01$). Each microgram of collagen in the CDMs was thus more than twice as strong as each microgram of collagen in the collagen gels, possibly due to higher collagen cross-link density and/or more organized arrangement of collagen in the CDMs. The UTS / Collagen Density values correlated with the non-acid and pepsin extractable collagen fraction for the CDMs and collagen gels ($R^2 = 0.925$), but not for the fibrin gels ($R^2 = 0.109$). The high value of UTS / Collagen Density for the fibrin gels (25.9 ± 2.4 kPa/mg/cm^3) appears to be due to ECM proteins other than collagen, such as fibrin, in the gel, that contributed substantially to the strength.

Collagen gels have been studied extensively for the last several decades as *in vitro* wound-healing models, and much effort has gone into developing stronger collagen gels for use as tissue equivalents (Grinnell, 2003). Recently, Feng *et al.* (2003) measured the UTS of collagen gels at 250kPa at 10 weeks when allowed to contract freely. Earlier development and characterization of tissue equivalents made from collagen gels

(ligament-equivalent fibroblast-populated collagen matrices) by Huang *et al.* (1993) resulted in an ultimate tensile strength (UTS) of 140 kPa in 12 weeks when mechanically stimulated uniaxially. The cell number tripled in 2 weeks from an initial cell density of 1 million cells/ml and remained at this cell number for the rest of the period, compared to an almost tripling in cell number in 3 weeks for the collagen gels in this study. The total weight of the constructs decreased from 12mg at week 1 to 10mg after week 3, while the sulfated glycosaminoglycan content increased steadily over the 12 week period. In comparison, the total weight of the collagen gels in this study remained fairly constant during the 3-week period, and the total sulfated proteoglycan and glycosaminoglycan content was 38.4 ± 0.7 µg at the end of the 3-week culture period.

Recent developments in engineering strong fibrin gels by Neidert *et al.* (2002) with an initial fibrinogen concentration of 3.3 mg/ml and the addition of factors such as TGF-β1 and insulin in addition to 10% fetal bovine serum has produced tissue equivalents with a tensile strength (UTS) of 240 kPa over a 51 day growth period. These fibrin gels contracted 1500 ± 115µm to 200 ± 72µm within 4 days, and contained about 100µg/cm^2 of collagen at day 21 and about 230µg/cm^2 of collagen at day 51 which represented 33 ± 5.4 % of the total dry weight. The fibrin gels in this study had similar results and contained 113 ± 10 µg/cm^2 collagen at day 21 which represented 32.2 ± 1.2 % of the total protein in the constructs. Neidert *et al.* (2002) also found that the UTS of fibrin gels are correlated to the total collagen amount in the constructs (R^2 = 0.7686 at day 21), while this study found that the UTS correlated to the collagen density (R^2 = 0.733 at day 21) not just in fibrin gels but also in collagen gels and cell-derived matrices.

However, the UTS was found to correlate even more closely to the non-acid and pepsin extractable collagen fraction ($R^2 = 0.993$) in all of these specimens.

The cell-derived matrices cultured with the standard serum-supplemented medium had similar, but higher, values for thickness and amount of collagen (125 ± 6 μm; 0.189 ± 0.007 mg/cm^2 collagen in 3 weeks) as the self-produced matrices produced by Grinnell *et al.* (1989)) that grew multiple cell layers thick with 0.118 ± 2 mg/cm^2 collagen in 5 weeks. These differences might be due to the much higher starting cell density of $4.4x10^5$ cells/cm^2 wells (compared to $5x10^4$ cells/cm^2 wells by Grinnell *et al.* (1989), feeding of the samples every other day, the use of porous inserts, and 80% greater concentration of L-ascorbate, in the more stable form of L-ascorbic acid phosphate magnesium salt *n*-hydrate (1 week stability vs. 24h for L-ascorbate). Ishikawa *et al.* (1997) have grown cell-derived matrices that synthesized 0.327 ± 0.66 mg collagen in 4 weeks. L'Heureux *et al.* (1998) have made matrices of 120μm in thickness in 4 weeks. Clark *et al.* (1997), on the other hand, have produced matrices that were 3-5 cell layers thick in 2 weeks, but 8-16 cell layers thick in 2 weeks when TGF-β1 was added into the serum-supplemented medium.

As with many native connective tissues, much of the strength of these cultured tissues can be attributed to the collagen density (Hukins, 1982; Roeder *et al.*, 2002). However, while the CDMs had 68.2 ± 1.1 % of their total protein as collagen, they were significantly weaker (UTS = 223.2 ± 9.0 kPa, $p<0.01$) than the cell-derived matrices cultured with the chemically-defined medium, or *CDMs (UTS = 314.5 ± 7.2 kPa) that had only 31.8 ± 0.5 % of their total protein as collagen and the **CDMs (UTS = 697.1 ± 36.1 kPa) that had 67.0 ± 0.6 % of their total protein as collagen. The collagen gels, in

turn, with 91.8 ± 1.0 % of their total ECM proteins as collagen were even weaker than this (UTS = 168.5 ± 43.1, $p<0.05$). The UTS / Collagen Density values for the *CDMs and **CDMs (40.3 ± 0.4 kPa/mg/cm^3 and 40.0 ± 1.9 kPa/mg/cm^3, respectively) were thus significantly higher than for the CDMs (14.5 ± 1.1 kPa/mg/cm^3). This difference could be due to the high tensile strength of collagen being largely attributable to the presence of intra-molecular covalent cross-links between the collagen fibrils (Parry, 1988). The non-acid and pepsin extractable collagen fraction was closely correlated to the UTS / Collagen Density values for all these groups (see Table 3). The non-acid and pepsin extractable collagen fraction might thus represent collagen cross-links in these samples or some other property leading to significant stability of the collagen fibrils and strengthening of the matrix. Strength also increases with increasing collagen fibril diameter and density (which typically range from 20 – 120 nm in diameter in native tissue) (Hukins, 1982; Huang *et al.*, 1993; Christiansen *et al.*, 2000; Roeder *et al.*, 2002), but the collagen fibril diameters and densities for all these samples were essentially the same, so this does not appear to be a significantly contributing factor to the differences in strength.

Although the collagen gels had by far the greatest percentage of collagen, the largest collagen fibril diameters and were allowed to be contracted by the fibroblasts in order to increase their strength (Huang *et al.*, 1993; Seliktar *et al.*, 2000; Roeder *et al.*, 2002; Feng *et al.*, 2003), they were substantially weaker than the CDMs grown under identical conditions. The ratio of UTS to Collagen Density of the collagen gels was less than half the value of the CDMs. This might be due to the collagen being more organized for strength in the CDMs, 50% greater fraction of non-acid and pepsin extractable

collagen, and larger amounts of other supporting ECM proteins, including the possibility of fibronectin. (Gillery *et al.*, 1986; Klueh *et al.*, 2001; Gildner and Hocking, 2003) have shown that strength increases with the addition of fibronectin which increases actin organization and regulates the composition of the ECM. For example, Gildner and Hocking (2003) have shown that fibronectin induces a 5-fold increase in the ultimate tensile strength of collagen gels.

The present data also suggest that serum-free media can be developed that induce fibroblasts to produce a stronger and thicker ECM. When cell-derived matrices were fed with the chemically-defined medium, they grew significantly thicker and stronger than their serum-supplemented counterparts. When collagen gels were fed with the chemically-defined medium, the contractile forces within the collagen gels became so great that they detached from the wells and contracted into themselves within 24h of feeding them with the chemically-defined medium. The reason for the contraction of the collagen gels when fed with the chemically-defined medium is the subject of further studies. Apart from the necessary components of insulin, selenious acid and a lipid precursor (ethanolamine, in this case) in serum-free media (Freshney, 2000) (these components are present in serum), the chemically-defined medium was similar to the serum-supplemented medium (less the serum) except for the addition of basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), dexamethasone and L-3,3',5-Triiodothyronine. The addition of these relatively few components (and the lack of the numerous other components present in serum), resulted in a doubling (**CDMs) or tripling (*CDMs) in thickness and a 40% (*CDMs) to 210% (**CDMs) increase in the

ultimate tensile strength over identical standard serum-supplemented samples. It also resulted in a doubling of the failure strain, a quadrupling of the proliferation rate, and a quintupling of the fraction of non-acid and pepsin extractable collagen. These large differences could also be as a result of certain components in fetal bovine serum (FBS) having an inhibitory role on the proliferative and synthetic rate of fibroblasts.

Epidermal growth factor (EGF) was added since it has been shown to stimulate the growth and production of non-collagenous proteins and other ECM components such as proteoglycans and glycosaminoglycans (Kurata and Hata, 1991) that contribute to the mass and thickness of cell-derived matrices. Basic fibroblast growth factor (bFGF), on the other hand, plays an important role in normal tissue repair and wound healing, and is a potent mitogenic and chemotactic factor for fibroblasts, regulating proliferation and extracellular matrix (ECM) production by these cells (Milewski *et al.*, 1998; Aktas and Kayton, 2000), and was found to increase the strength and thickness of cell-derived matrices in pilot tests. The major reason for the addition of the growth factors into the chemically-defined medium was to stimulate the production of numerous non-collagenous ECM proteins in addition to collagen (Kurata and Hata, 1991) in order to produce tissue that might more closely resemble native soft connective tissue. L-ascorbate was added since it has been found to significantly increase ECM synthesis by upregulating the expression of proalpha(1)(I) and proalpha(2)(I) collagen (Grinnell *et al.*, 1989; Hata and Senoo, 1989; Huang *et al.*, 1993; Kurata *et al.*, 1993; Girton *et al.*, 2000; Hoerstrup *et al.*, 2000; Laplante *et al.*, 2001; Pouliot *et al.*, 2002). Since L-ascorbate breaks down within 24h in culture, a more stable form (L-ascorbic acid phosphate magnesium salt *n*-hydrate) with a more than one week stability in culture was used

(Ishikawa *et al.*, 1997). Ishikawa *et al.* (1997) found the optimum concentration of this form of L-ascorbate to be 1mM for fibroblast cultures; however, half of this amount, 150μg/ml (519mM), was found to results in a more desirable combination of strength and thickness for the cell-derived matrices in pilot tests. Dexamethasone was added since it has been shown to significantly increase fibroblast cell proliferation in serum-free media (Bettger *et al.*, 1981; Shipley and Ham, 1983) by upregulating the PDGF-alpha receptor mRNA and protein (Warshamana *et al.*, 1998), and to promote actin stress fiber formation and increased fibronectin matrix accumulation that aid in the more effective organization of the ECM (Zoppi *et al.*, 1998; Brenner *et al.*, 2000). Also, the increase in collagenase and stromelysin mRNA by EGF that degrade collagen has been shown to be inhibited with the addition of dexamethasone (Delany and Brinckerhoff, 1992). Insulin, selenious acid, and a lipid precursor (ethanolamine, in this case) were added since they are essential standard components of any serum-free media (Freshney, 2000). Insulin also increases the residence time of procollagen mRNA and upregulates latent TGF-β1 synthesis (Ghahary *et al.*, 1999). Interestingly, transferrin, another essential standard component of many serum-free media (Freshney, 2000), was not needed and had no effect on the growth or strengthening of cell-derived matrices in pilot studies. Further studies would be needed to determine the reason for this. L-3,3',5-Triiodothyronine was added to increase the rate of cell metabolism. Due to the high synthetic rate of the fibroblasts, extra L-glutamine, which is an energy carbon source for nucleic acid synthesis, was added in the form of the more stable Glutamax™ that does not easily degrade to ammonia, a compound that adversely affects cell cultures. An antioxidant (glutathione (reduced)) was added to prevent oxidative stress in the cells. Most of these

components were added only after they had been determined to either significantly increase the strength or thickness of cell-derived matrices in pilot studies. The near-optimum concentrations of these components for producing the best combination of increased strength and thickness were determined separately in preliminary studies (data not shown), although a full combinatorial media optimization study was beyond the scope of this study.

The much higher strength of the **CDMs compared to the *CDMs appears to have resulted from the lowered concentration of growth factors (bFGF and especially EGF) in the feeding media over time. This effect was not duplicated by adding a low concentration of fresh growth factors at every feeding instead (data not shown). It is possible that the matrices developed mostly in thickness for the first part of the growth period followed by development in strength for the remainder of the growth period, since, although EGF stimulates the growth and production of non-collagenous proteins, it inhibits the transcription of type 1 collagen genes and high bFGF concentrations favor enhanced cell proliferation over enhanced ECM strengthening (Kurata and Hata, 1991). Thus a decrease in growth factor concentration over time could increase the strength of the ECM by the resulting increase in collagen production. This leads to a hypothesis that cell-derived matrices that grow thicker have a greater fraction of proteins and other ECM components other than collagen, while cell-derived matrices that grow stronger but thinner in the same period of time have a greater fraction of collagen. In this study, although the fibroblasts in the *CDMs synthesized significantly more ECM than the fibroblasts in the **CDMs, the **CDMs had significantly more total amount of collagen than the *CDMs. Further studies would need to be performed to determine if similar

results might be obtained if a relatively high concentration of growth factors promoting growth is used during the first part of the culture period, followed by a much lower concentration of these growth factors and possibly even the addition of growth factors that promote strengthening such as TGF-β1. Many growth factors, including bFGF, can accumulate and retain their activity for relatively long periods of time within ECM (Aktas and Kayton, 2000; Bottaro *et al.*, 2002), and thus some of the growth factors might have accumulated in the ECM during the first part of the culture period and used later as the matrix developed. The lowered growth factor concentration over time may also approximate what occurs naturally *in vivo* during the wound healing response (Xing *et al.*, 2003; Diegelmann and Evans, 2004), where the high concentration of growth factors in a fresh clot decreases over time as it is invaded and reconstructed by fibroblasts.

The cell-derived matrices produced in this study have potential in being used as soft connective tissue substitutes since they are produced solely from cells fed with a chemically-defined medium that does not contain any animal components, and they could be made strong and thick enough for certain tissue replacement applications within a matter of weeks. Tissue substitutes produced solely from fibroblasts have been developed by other groups, such as the completely biological human blood vessels developed by Auger *et al.* (L'Heureux *et al.*, 1998; Auger *et al.*, 2000), but these matrices have taken several months to grow, are fed with serum, and usually involve stacking several matrices together, thus changing some of the self-produced aspects of the matrix. The higher thermal and enzymatic stability and mechanical integrity of cell-derived matrices over

both collagen and fibrin gels may allow them to retain their structural integrity longer in *in vivo* conditions. Their high strength and stability could allow them to one day be used as alternatives to soft connective tissue and load-bearing tissue. Cell-derived matrices might also serve as *in vitro* biological models for the effects of nutritional components and pharmaceutical products on the growth and development of soft connective tissue, as well as for numerous *in vivo* conditions and processes such as wound healing, connective tissue formation, and development and interaction of different cells and tissues in a soft connective tissue environment.

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Appendix 1: Cell-Derived Matrix Protocol

Thawing Cells (for 1M cells):

1. Add 16ml of ***Growth Media*** into a T75 flask and place into 10% CO_2 incubator for 30min.
2. Add 3ml of ***Growth Media*** to a 15ml polypropylene *conical tube*, and place in water bath
3. Thaw cryovial w/ cells in 37ºC waterbath + agitate in hand
4. Slowly add some ***Growth Media*** from *conical tube* to just thawed cells in cyovial
5. Mix, pipette out, and place cells in *conical tube* + repeat (rinse cryovial w/ more media)
6. Place the cells into T75 flask in the incubator
7. Replace media with 15ml of fresh ***Growth Media*** after 24h (see **Feeding Cells** below)

Feeding & Passaging Cells:

1. Feed cells 2x / week with same amount of ***Growth Media*** (replace) until 90% confluent.
2. When 90% confluent (takes ~1 week), aspirate media + add 1/3 of media amount (5ml into a T75 flask; 8ml into a T150 flask) of 0.05% Trypsin-EDTA
3. Place in incubator until cells have detached (5-15min) + crack lid to vent
4. Denature Trypsin with same amount of ***Growth Media*** and put entire solution into a 50ml polypropylene *tube*
5. Repeat for all flasks + go back thru and rinse all flasks with 10ml of ***Growth Media***
6. Do a ***Cell Count*** on each *tube*
7. Centrifuge *tube*s at 1,000rpm for 6min
8. Aspirate supernatant

9. Based on ***Cell count***, dilute cells w/ ***Growth Media*** to a concentration of ~ 1M / 25ml (1M / 25ml in a T150 flask) and seed into flasks

Freezing Cells:

Identical to Steps 2-8 of **Feeding & Passaging Cells** Protocol, then:

9. Based on ***Cell count***, dilute cells w/ ***Cryo Media*** to a concentration of 1M / ml

10. Put 1ml of solution / cryovial

11. Place cryovials in Styrofoam and put into -80ºC freezer for a few days, and then store in liquid nitrogen

Cell-Derived Matrix (CDM):

Identical to Steps 2-8 of **Feeding & Passaging Cells** Protocol, then:

9. Based on ***Cell count***, dilute cells w/ ***CDM Media*** to a concentration of: a) *for wells:* 2M / 5ml; b) *for TransWells:* 2M / 2ml.

10. a) *For wells*: put 5ml of ***CDM Media*** **w/ cells** into the wells; b) *For TransWells:* Put 3ml of ***CDM Media*** under wells, and 2ml of ***CDM Media*** **w/ cells** onto the TransWells.

11. Feed cells every 2 days with same amount of ***CDM Media***: a) *for wells:* 5ml; b) *for TransWells:* 2ml above, 3ml below.

Growth Media: *DMEM + 10% BCS + 1% Pen/Strep/AmphotericinB (37ºC)*

Cryo Media: *DMEM + 10% BCS + 10% DMSO*

CDM Media – 500ml:

- *3:1 DMEM : Ham's F12 + 1% Pen/Strep/AmphotericinB –* ***360ml : 120ml + 5ml***
- *4 x 10^{-3}M Glutamax (10ml/500ml) -* ***10ml***
- *8.61 x 10^{-7}M Insulin (0.25ml/500ml) -* ***250µl***

From -20ºC Freezer:

- *1.0 x 10^{-4}M Ethanolamine (500X = 3µl/ml).*
- *5.2 x 10^{-4}M L-ascorbic acid phosphate magnesium salt n-hydrate (500X = 75mg/ml).*
- *3.3 x 10^{-6}M Glutathione (reduced) (500X = 500µg/ml)*
- *1.0 x 10^{-7}M Dexamethasone (500X = 19.7µg/ml).*
- *3.9 x 10^{-8}M Selenious Acid (500X = 2.5µg/ml).*
- *2.0 x 10^{-10}M L-3,3',5-Triiodothyronine(dissolve in EtOH first) (500X = 67.5ng/ml).*
- *2.8 x 10^{-10}M bFGF (500X = 2.5µg/ml in 1X PBS) -* ***10µg in 4ml 1X PBS.***
- *4.2 x 10^{-10}M EGF (500X = 1.25µg/ml in 1X PBS with 2.5g/L Serum Albumin) -* ***100µg + 0.2g Human Serum Albumin in 80ml 1X PBS.***

<u>Cell Count:</u>

- *Pipette 100µl of cell suspension + 100µl of 0.04% trypan blue into an Eppendorf tube*
- *Pipette out 10µl of mixture and put into hematocytometer*
- **Cell count**: # of cells / ml = 20,000 x Average # of cells / Quadrant

Appendix 2: BIOMECHANICAL ANALYSIS PROTOCOL

1. Remove media from wells and fill wells with ddH_2O. After 5 minutes, remove ddH_2O and add new ddH_2O for 30 minutes. (The ddH_2O lyses the cells due to the high osmotic pressure built up in the cells due to the low osmolarity of the ddH_2O relative to the inside of the cells. This eliminates the contractile forces otherwise produced by the fibroblasts, and allows the samples to be mechanically tested without contracting into themselves. The lysing of the cells releases lysosomes into the ddH_2O that might negatively affect the matrix. The ddH_2O is therefore changed after 5 minutes to decrease the concentration of the lysosomes.)
2. After 30 minutes, remove ddH_2O from wells and replace with room temperature 1X phosphate buffered saline (PBS). (This equilibrates the samples for biomechanical testing.)
3. Measure and record the thickness of each sample with the laser displacement system of the mechanical membrane inflation device after being slightly compressed by the small reflective disk (1.3g, 1.3cm diameter) for 1 minute. (This is to reduce the error due to the hydration state of the samples. The software program can be made to display the thickness by setting the 0 value for the small reflective disk on the flat stage *before* placing any samples between the disk and the flat stage. Make sure the laser is centered on the reflective disk.)
4. After all thicknesses have been measured, remove the flat stage and adjust the height of the laser so that the faceplate gives a reading of 0 height. Center the laser over the 1cm diameter opening in the faceplate. Place one sample at a time

over the 1cm diameter opening for mechanical testing. Make sure that the sample lies flat and over the O-ring channel. Place O-ring over the sample without moving it, and then clamp it down. (During clamping, the entire circumference of the sample is forced into the O-ring channel on the faceplate, which will prevent "waffling" of the sample in the test region. An even clamping force is created around the circumference of the sample by the clamping plate, which is also guided and kept from rotating relative to the sample by two pins.)

5. Start data acquisition on the computer, and inflate the samples with PBS at a constant rate of 1 ml/min (thinner samples need a higher rate, while a lower rate can be used for thicker samples for greater accuracy). When the sample bursts, stop the data acquisition and infusion pump. Record the size and location of the burst in the sample before replacing it with the next sample. (The tissue inflation device measures the displacement and pressure at which a sample bursts. The increasing pressure applied to the sample is measured by an on-board pressure transducer (model PX102-025GV, Omega Engineering, Stamford, CT). The displacement of the center of the cap is measured with a laser displacement system (LDS-080, Keyence, Woodcliff Lake, NJ).)
6. Measure the Wet Weight of each sample, and then lyophilize overnight and measure the Dry Weight. Place in –80°C freezer for later biochemical analysis. (The –80°C temperature will help to prevent any collagen degradation.)

The sample is circularly clamped at and inflated through a 1cm diameter opening, thus causing the sample to form a spherical cap before failing.

- The maximum membrane tension, T, is calculated using the Law of Laplace for a spherical membrane:

  $$T = ½\ PR,$$

  where P is the pressure (conversion factor: (133.3 N/m^2)/mmHg) when the tissue bursts and R is the corresponding radius at the point of rupture, calculated assuming a spherical cap geometry by:

  $$R = (w^2 + a^2) / 2w,$$

  where a is the radius of the clamp (5mm) and w is the displacement at the center of the sample at failure measured by the laser.

- The ultimate tensile strength (UTS) is calculated by:

  $$UTS = T / t,$$

  where t is the thickness of type-matched specimens before inflation. Since the thickness measurements are obtained after the samples have been isotonically compressed by a 1.3g weight for 1min, the error due to the hydration state of the samples is reduced. The actual thickness at the time of bursting is less than this value since the samples are inflated.

- The ultimate tensile strength per collagen density (UTS / Collagen Density) is calculated by:

  $$UTS / Collagen\ Density = UTS / (Total\ Collagen / \pi t(D/2)^2)$$
  $$= T / (Total\ Collagen / \pi(D/2)^2),$$

where D is the diameter of the constructs (2.4cm for TransWells). This value gives an accurate measure of strength of the constructs per unit of collagen since it is independent of the thickness of the samples.

- The failure strain is determined from the equibiaxial strain at the pole estimated from the displacement data using the approximate relationship:

$$\textit{Failure Strain} \approx 2/3\ (w / a)^2 - 2/15\ (w / a)^4 + 2/35\ (w / a)^6.$$

Appendix 3: BIOCHEMICAL ANALYSIS PROTOCOL

1. Obtain wet weight of samples, lyophilize overnight, and then measure dry weight. Place lyophilized samples in –86ºC freezer (to prevent collagen degradation) for later biochemical analysis, or go to next step.

2. Solubilize each lyophilized sample in 1ml pepsin (Sigma # P-6887)(1 mg/ml in 0.5M acetic acid (EMD Chemicals # AX0073P-5) and incubate overnight at 20°C with rotation. Centrifuge samples at 14000 rpm for 1 hour at 15°C, and transfer supernatant into separate 2ml Eppendorf tubes. (The pellet contains the remains of cells and ECM. The supernatant contains the soluble collagen. Collagen is soluble, so it will not go into the pellet. A high salt concentration (4M NaCl for samples with neutral pH, and 2M NaCl for samples in acetic acid) would be needed to precipitate the collagen out of solution.) Repeat this extraction step to achieve complete extraction of the pepsin and acid soluble fraction of collagen.

3. Combine the supernatants from the first and second extractions, and use 100µl of this to determine non-cross-linked collagen content with the Sircol™ Assay (Biocolor, Belfast, N. Ireland). To confirm results, a 200 µl aliquot of the soluble collagen extract can be used to determine collagen content by hydroxyproline assay (NOTE: this value will be higher since it will include collagen that was degraded during growth).

4. Use a 50µl aliquot of the extract to determine total non-collagenous protein content with the Tp-Blue™ Total Protein Assay (Biocolor). Generate standard

curve by adding 5ml of reagent dye per tube, mixing with 0-500µl of the included bovine serum albumin, and reading at 595nm. Add the 50µl aliquot of the extract to 5ml of reagent dye, read at 595nm, and use standard curve to determine total protein content less collagen. Add this amount to the total collagen amount that will be obtained to get total protein amount.

5. Digest the remaining pellets with Proteinase K (Invitrogen # 25530-049), 50µg in 500µl solution of 10mM EDTA and 0.1M sodium phosphate (pH 6.5)(0.1M sodium phosphate is made by mixing 30ml 1M Na_2HPO_4 (Fisher # BP332-1), 70ml 1M NaH_2PO_4 (Fisher # BP329-1) and 900ml H_2O) overnight at 60°C. (Proteinase K is a serine protease with broad specifity towards aliphatic, aromatic and other hydrophobic amino acids. It digests all proteins into small peptides and makes them soluble. After digestion of the proteins, the DNA, proteoglycans and glycosaminoglycans are released into the solution.)

6. Use a 100µl aliquot of the ***supernatant*** to determine sulfated glycosaminoglycan and proteoglycan content (not including hyaluronic acid) with the Blycan™ Assay (Biocolor, Belfast, N. Ireland).

7. Use a 10µl aliquot of the ***supernatant*** to determine DNA content. The number of cells per sample can be calculated from the measured amount of DNA using Hoechst 33258 dye (Amersham Biosciences # 80-6226-87) and a DyNA Quant 200 fluorometer (Amersham, designed specifically for the quantification of low DNA concentrations using Hoechst 33258 dye, with a fixed excitation bandpass source (365 nm) and an emission bandpass filter (460 nm)) based on the known DNA content per cell of 8pg. Add 10µl sample to the cuvette with 2ml TNE

Buffer (10mM Tris, 200mM NaCI, 1mM EDTA, pH 7.4), mix, place cuvette in flurometer and read the concentration. Calibrate DyNA Quant 200 using 10ng Calf thymus DNA (add 10ng of standard DNA to the cuvette with 2ml TNE Buffer, mix, place cuvette in flurometer and press CALIB).

8. Use a 200µl aliquot of the ***supernatant*** to estimate cross-linked collagen content with a hydroxyproline assay:

    a) Hydrolyze each 200µl sample in 200µl of 12M HCl (final HCl concentration: 6.0M) in 1.5ml screw cap tubes for 16 hours at 110^{o}C, followed by drying of the samples under vacuum.

    b) Reconstitute samples (1-5 µg of hydroxyproline) to 2.0ml with **Assay Buffer**.

    Assay Buffer consists of: 5g/l citric acid (Sigma # C1909), 1.2ml/l glacial acetic acid (EMD Chemicals # AX0073P-5), 12g/l sodium acetate (VWR # MK776804), and 3.4g/l sodium hydroxide (VWR # MK770810). (Stock Buffer contains 50g of citric acid (H_2O) (Sigma # C1909), 12ml of glacial acetic acid (EMD # AX0073P-5), 120g of sodium acetate (VWR # MK776804), $3H_2O$ and 34g of NaOH (VWR # MK770810) in 1.0 liter of solution. Stock Buffer solution is diluted tenfold with H_2O to make **Assay Buffer**.)

    c) Add 1.0ml of **Chloramine-T Reagent** and stand for 20 minutes at room temperature.

    Chloramine-T Reagent is made from: 1.41g of chloramine-T (VWR # VW3506-0) dissolved in 20.7ml of H_2O and mixed with 26ml of *n*-

propanol (VWR # MK303508) and 53.3ml of Stock Buffer. This reagent is stable at 4°C for 2 weeks.

d) Add 1.0ml of freshly prepared **Dimethylaminobenzaldehyde Reagent** and mix throroughly.

Dimethylaminobenzaldehyde Reagent: 15g of *p*-dimethylaminobenzaldehyde (Sigma # D-2004) is suspended in 60ml of *n*-propanol (VWR # MK303508), and 26ml of 60% perchloric acid (VWR # EM-PX0396D-4) is added *slowly*. (Use a fume hood with protective goggles!)

e) Incubate tubes at 60°C for 15 minutes, followed by cooling in tap water for 5 minutes.

f) Measure the absorbance of each sample at 550nm within 45 minutes. Correlate absorbance readings with collagen amount using a standard curve and a conversion factor of 10µg collagen to 1µg 4-hydroxyproline. Create standard curve with known amounts of Trans-4-hydroxy-L-proline (Sigma # 56250) and hydrolyzed rat tail Collagen, Type 1 (Biocolor, Belfast, N. Ireland).

Appendix 4: Steps in Development of Chemically Defined Medium

A serum-free base medium was first developed based on a number of serum-free media from literature (Bettger *et al.*, 1981; Shipley and Ham, 1983; Parenteau, 1994; Vaccariello *et al.*, 1999) (see chart on next page). The effects on the tension, thickness, ultimate tensile strength (UTS) and failure strain of a number of components after 3 weeks in culture was determined in series. Apart from the last two sets of experiments, all samples were grown on the bottom of regular 6 well plates, unless otherwise indicated. Growth factors were added into the base medium at the beginning of each 3-week study. When a particular component was found to significantly increase the strength and thickness of the CDMs at a particular concentration, it continued to be added into the media at that concentration throughout the study. The chemically defined media formulation that was developed from all these studies is shown below (Ahlfors, 2003).

Component	Reason	Bettger et al., 1981	Shipley and Ham, 1983	Ommell et al., 1998	Huang et al., 1993	Parenteau, 1994	Vaccariello et al., 1999	Pouliot et al., 2002	Murphy and Ronfard, 2002	Calve et al., 2003	Advanced DMEM	Base Media	Ahlfors, 2003
Base Media	***basic nutrients + water***	MCDB 110	MCDB 402	MEM	DMEM	3:1 DMEM:F12	3:1 DMEM:F12	3:1 DME:F12	3:1 DMEM:F12	F12 -> DMEM	DMEM	3:1 DMEM:F12	3:1 DMEM:F12
Fetal Bovine Serum	*Wide range of necessary nutrients*			10%	10% Nu-Serum		10%	10%		20% -> 7%			
Glucose	*Energy source*	1.8 mg/ml (10mM)	1 mg/ml (5.55mM)?	1 mg/ml (5.55mM)	4.5 mg/ml (25mM) Fructose	4.5 mg/ml (25mM)	4.5 mg/ml (25mM)	4.5 mg/ml (25mM)	4.5 mg/ml (25mM)	1.8mg/ml (10mM) -> 4.5mg/ml (25mM)	4.5 mg/ml (25mM)	4.5 mg/ml (25mM)	4.5 mg/ml (25mM)
L-Glutamine	*energy & carbon source for nucleic acid synthesis*	2.5 mM	2-4 mM?	2 mM	4 mM	7.25 mM	7.25 mM	3.25 mM	3.25 mM + 4 mM Glutamax	1 mM -> 4 mM		3.25 mM + 4 mM Glutamax	3.25 mM + 4 mM Glutamax
Hepes	*pH stability*	30 mM	50 mM?	20 mM									
Liposome B	*comprehensive lipid source*	10.26 µg/ml	5 µg/ml										
Albumin	*enzyme stabilizer & carrier protein*					20 µg/ml (0.30µM)?					400 µg/ml (6.1µM)		
Epidermal Growth Factor	*cell proliferation; upregulates ECM protein synthesis (except collagen)*	30 ng/ml (5.0nM)	5 ng/ml (0.83nM)			10 ng/ml (1.67nM)		10 ng/ml (1.67nM)	5 ng/ml (0.83nM)			10 ng/ml (1.67nM)	2.5 ng/ml (0.42nM)
Basic Fibroblast Growth Factor	*mitogenic protein & enhances cell proliferation; upregulates DNA synthesis*		25 ng/ml										5 ng/ml (0.28nM)
Platelet Derived Growth Factor	*principal mitogen for cells synthesizing connective tissue*		25 ng/ml										
Progesterone	*enhanced transcription of certain genes*						(2nM)						
Insulin	*glucose & amino acid uptake*	0.95 µg/ml (16.4µM)	500 ng/ml (8.6µM)			5 µg/ml (86.1µM)	5 µg/ml (86.1µM)	5 µg/ml (86.1µM)	5 µg/ml (86.1µM)		10 µg/ml (172µM)	5 µg/ml (86.1µM)	5 µg/ml (86.1µM)
Dexamethasone	*cell proliferation; increases fibronectin synthesis; upregulates PDGF receptor mRNA*	196.3 ng/ml (500nM)	1963 ng/ml (5µM)										39.3 ng/ml (100nM)
Hydrocortisone	*proliferation of certain cell lines, including HeLa cells and keratinocytes*		362.5 ng/ml (1µM)			400 ng/ml (1.1µM)	?	400 ng/ml (1.1µM)	400 ng/ml (1.1µM)			400 ng/ml (1.1µM)	
L-3,3',5-Triiodothyronine	*increases rate of cell metabolism*		no effect			13.5 pg/ml (20pM)	13.5 pg/ml (20pM)	1346 pg/ml (2nM)	13.5 pg/ml (20pM)			13.5 pg/ml (20pM)	135 pg/ml (200pM)
Transferrin	*iron transport regulation*		no effect			5 µg/ml (62.5nM)	5 µg/ml (62.5nM)	5 µg/ml (62.5nM)	5 µg/ml (62.5nM)		7.5 µg/ml	5 µg/ml (62.5nM)	
Ethanolamine	*widely used lipid; inositol pathway precursor*		no effect			6.1 µg/ml (100µM)	6.1 µg/ml (100µM)		6.1 µg/ml (100µM)		1.9 µg/ml (31.1µM)	6.1 µg/ml (100µM)	6.1 µg/ml (100µM)
O-Phosphorylethanolamine	*lipid; inositol pathway precursor*		no effect			14.1 µg/ml (100µM)	14.1 µg/ml (100µM)		14.1 µg/ml (100µM)			14.1 µg/ml (100µM)	
Selenious Acid	*increases glutathione peroxidase activity (lack of inhibits DNA and RNA synthesis)*					6.8 ng/ml (53nM)	6.8 ng/ml (53nM)		6.8 ng/ml (53nM)		5 ng/ml (39nM)	5 ng/ml (39nM)	5 ng/ml (39nM)
Ascorbic Acid	*increases transcription of collagen genes*			50 µg/ml (289µM)	50 µg/ml (289µM)			50 µg/ml (289µM)	50 µg/ml (173µM)	100 µg/ml (577µM)	2.5 µg/ml (14.5µM)	50 µg/ml (173µM)	150 µg/ml (519µM)
L-Proline	*collagen building block*								214 µg/ml (1.86mM)				
Glycine	*collagen building block*								114 µg/ml (1.52mM)				
L-Cysteine	*creates disulfide bridges in & between ECM proteins*												
Copper Sulfate	*essential metal, but causes oxidative stress*				21 µM						1.25 ng/ml		
Sodium Sulfate	*increases electrochemical gradient across cell membranes*				820 µM								
Dithiothreitol	*stabilizes enzymes and other proteins containing sulfhydryl groups*	6.5 µM											
Phosphoenolpyruvate	*precursor for ADP, pyruvate, and oxaloacetate*	10 µM											
Prostaglandin E1	*activates adenylate cyclase activity*	25 nM											
Prostaglandin F2 α	*stimulates cAMP formation*	2 µM											
Glutathione (reduced)	*antioxidant; protects cells from DNA damage and oxidative stress*	199.7 ng/ml (650nM)									1 µg/ml (3.25µM)	1 µg/ml (3.25µM)	1 µg/ml (3.25µM)
Adenine	*ATP/ADP component*					180 µM	180 µM	24.3 µg/ml					
Strontium Chloride	*extracellular calcium-sensing receptor agonist*					1 mM							
Ammonium Metavanadate	*regulates mitogenic effects of insulin*										300 µg/ml		
Manganous Chloride	*modulates oxidative events in cells*										50 pg/ml		
Cholera Toxin	*stimulates adenylate cyclase pathway*							100 pM					
Fibroblast Source						Dermis	Dermis	Dermis	Human neonatal foreskin	Bovine Achilles Tendon			Human neonatal foreskin
Designed For		Fibroblast proliferation	Fibroblast proliferation	3-D ECM growth	Ligament equivalent FPCL	Fibroblasts + Keratinocytes	Fibroblasts + Keratinocytes	Fibroblasts + Keratinocytes	Fibroblasts + Keratinocytes	Tendon equivalent	Serum-free Growth		Fast 3-D ECM growth + Strength
Growth Time		-	-	4-6 weeks	12 weeks	-	-		2 - 5 weeks	14 days -> 25 days	-		3 weeks
Thickness									60 - 120+ µm				230 µm (400 µm)
UTS					140 kPa				365 N/m	800 kPa -> 2.2 MPa			700+ kPa (300 kPa)
Fibril Diameter				70 nm						60 nm			48 nm
Mechanical Stimulation				NO	UNIAXIAL				NO	CYCLIC STRAIN; UNIAXIAL			NO

The first component that was tested was **L-ascorbic acid phosphate magnesium salt *n*-hydrate** (Wako Pure Chemicals, Japan). L-ascorbate at 50 μg/ml (289μM) has been found to significantly increase fibroblast growth and ECM synthesis by upregulating the expression of proalpha(1)(I) and proalpha(2)(I) collagen (Grinnell *et al.*, 1989; Hata and Senoo, 1989; Huang *et al.*, 1993; Kurata *et al.*, 1993; Girton *et al.*, 2000; Hoerstrup *et al.*, 2000; Laplante *et al.*, 2001; Pouliot *et al.*, 2002). However, since L-ascorbate breaks down within 24h in culture, a more stable form of L-ascorbate, L-ascorbic acid 2-phosphate has been adopted in several studies (Hata and Senoo, 1989; Geesin *et al.*, 1993; Kurata *et al.*, 1993; Senoo and Hata, 1994; Ohgoda *et al.*, 1998; Hoerstrup *et al.*, 1999). A more stable form is L-ascorbic acid phosphate magnesium salt *n*-hydrate, which has a more than one week stability in culture and has been found to be most effective for the production of ECM at a 1mM concentration for fibroblast cultures (Ishikawa *et al.*, 1997).

The three concentrations of L-ascorbic acid phosphate magnesium salt *n*-hydrate tested were: 50 μg/ml (173μM), 150 μg/ml (519μM), and 300 μg/ml (1,038μM). After 4 weeks in culture, the wet weights (and dry weights) of these groups were:

	Sample 1 (mg)	Sample 2 (mg)	Sample 3 (mg)	Sample 4 (mg)	Average (mg)
50 μg/ml	31.7 (1.3)	30.7 (1.2)	32.1 (1.3)	31.3 (1.3)	31.5 (1.3)
150 μg/ml	32.6 (1.6)	31.8 (1.5)	32.8 (1.7)	32.4 (1.6)	32.4 (1.6)
300 μg/ml	30.3 (1.5)	29.8 (1.6)	30.7 (1.4)	30.4 (1.5)	30.3 (1.5)

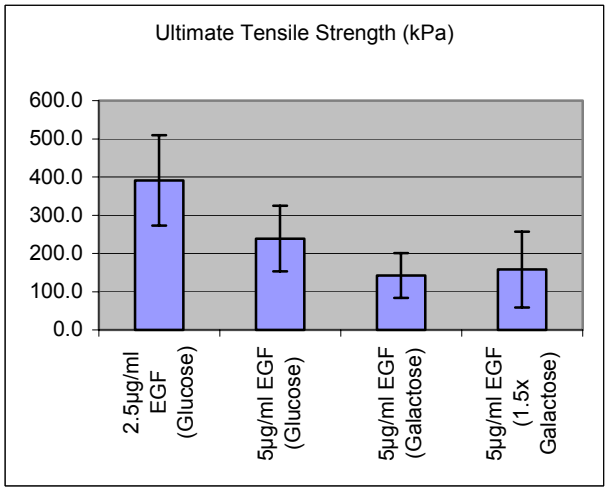
According to the above results, 150 μg/ml (519μM) of L-ascorbic acid phosphate magnesium salt *n*-hydrate appeared to cause the cell-derived matrix to produce the most extensive ECM. The relatively high wet weight to dry weight ratio of for the 50 μg/ml (173μM) concentration indicated that these samples were more hydrated, while the other

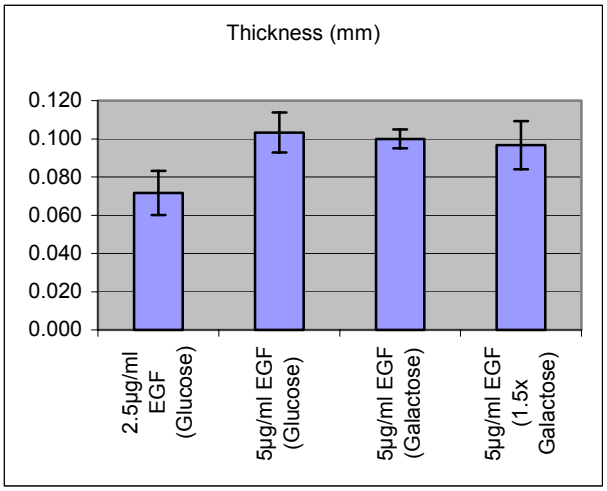
two groups had more dense ECM presumably due to a greater fraction of the ECM being composed of collagen. Mechanical testing on these samples was not performed because the samples started curling up at around 2 weeks (experiment was repeated again, with the same results of curling) in culture due to polyethylene terephthalate treated cell culture wells (BD Biosciences) that were later determined not to be a good attachment surface for CDMs. From here onwards, 150 μg/ml (519μM) of L-ascorbic acid phosphate magnesium salt *n*-hydrate was used in the medium.

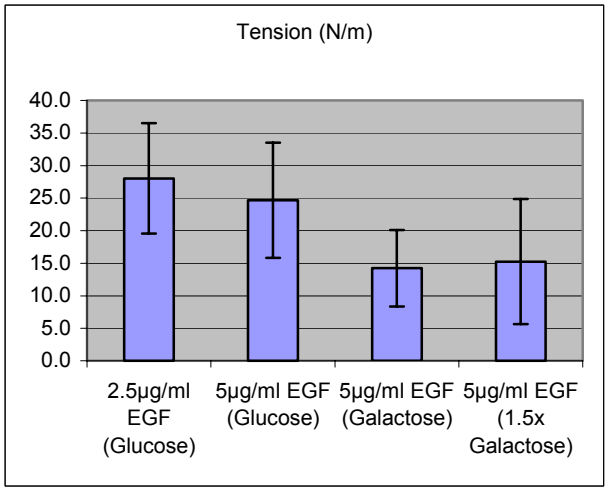
The next two components that were tested were Galactose and Epidermal Growth Factor (EGF). For cultures grown over long periods of time, fructose or galactose has been used instead of glucose since fructose and galactose reduce the production of acidic metabolites since they do not break down to lactic acid and thus permit higher density cell cultures (Leibovitz, 1963; Huang *et al.*, 1993). High glucose (4.5 mg/ml) DMEM was compared to low glucose (1 mg/ml) DMEM supplemented with 3.5 mg/ml galactose (galactose has same molarity as glucose) or 5.75 mg/ml galactose (due to high activity, cultures were expected to require more sugars). EGF, on the other hand, stimulates the growth and production of non-collagenous proteins, but also inhibits the transcription of type 1 collagen genes and thus a lower EGF concentration should increase the strength of the ECM (Kurata and Hata, 1991). A previous study had already indicated that 0.5 μg/ml of EGF resulted in the strongest cell-derived matrices, but they were so thin that they were difficult to handle and test. Ten times this amount, or 5 μg/ml of EGF, resulted in a weaker but much thicker cell-derived matrix, and had the best combination of strength and thickness of the all the groups. Double of this amount, or 10 μg/ml of EGF, resulted in slightly thicker but much weaker samples. The following experiment tested the effects of five times the 0.5 μg/ml EGF amount, or 2.5 μg/ml of EGF, versus the 5 μg/ml of EGF that had the best combination of strength and thickness in the previous experiment. Samples with 0.5 μg/ml EGF and 1.0 μg/ml EGF were also grown in parallel with the above samples, but they were sill too thin after 3 weeks to be mechanically testable.

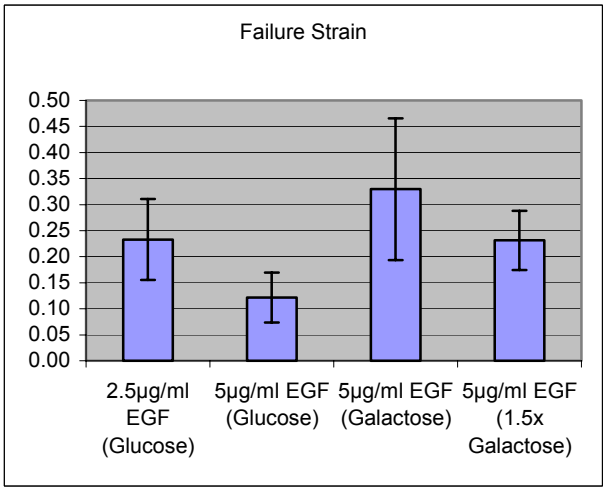
The results below indicate that galactose did not improve the thickness and significantly reduced the UTS of cell-derived matrices, and thus was not kept in the media formulations. The reduced UTS appears to be due to the high glucose in the other

samples inducing collagen cross-linking through the process of glycation. The 2.5 µg/ml EGF concentration was thinner than the 5 µg/ml concentration, but much stronger. Since both the tension and failure strain were also higher for the 2.5 µg/ml EGF samples, it was determined that this concentration caused the best combination of properties, and was used in the medium from this point onwards.









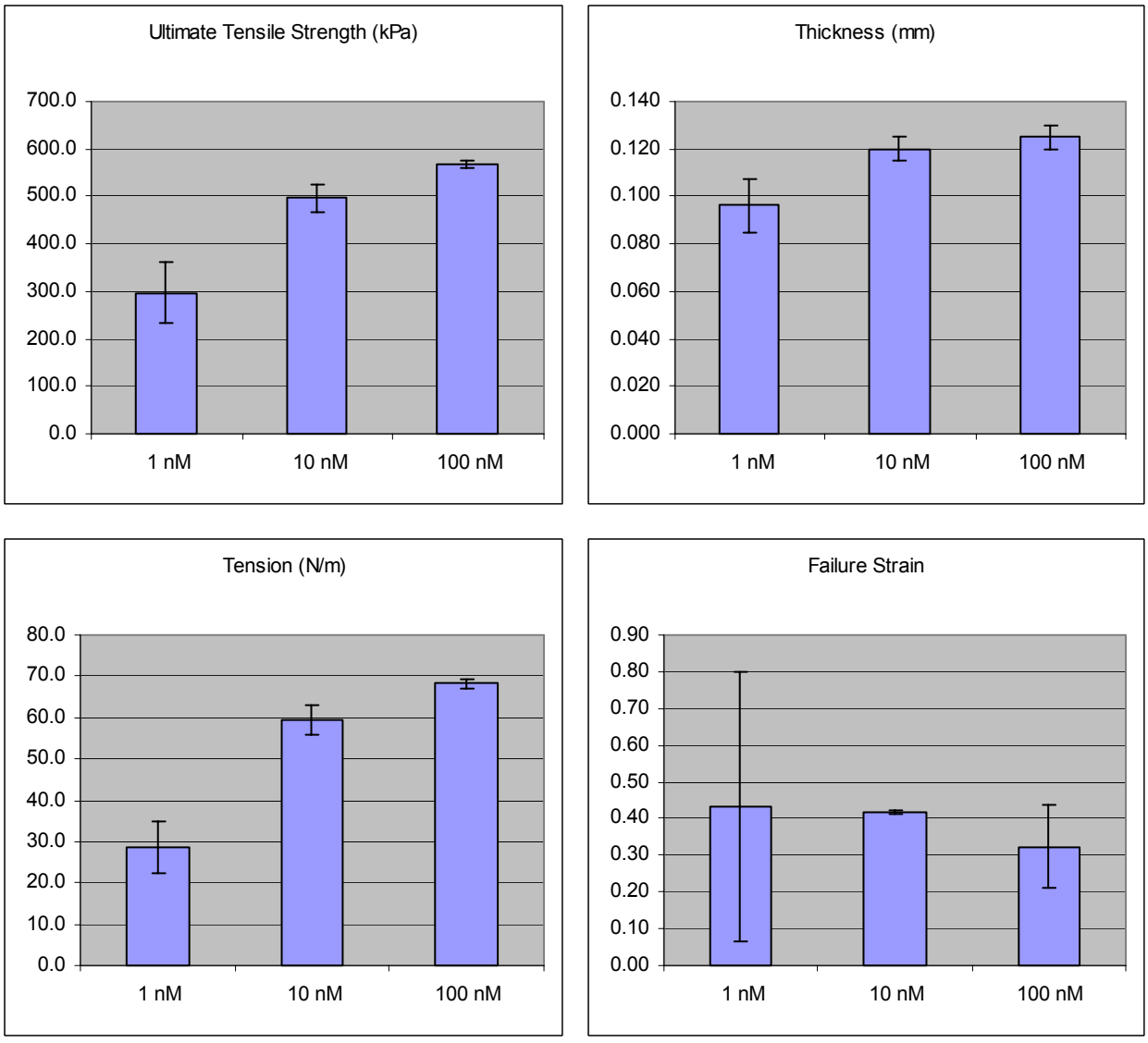
Sample	Thickness (mm)	Displacement (mm)	Tension (N/m)	Ultimate Tensile Strength (kPa)	Failure Strain
1a	0.085	2.40	37.5	523.7	0.15
1b	0.065	3.49	21.3	296.9	0.30
1c	0.065	3.19	25.3	352.4	0.25
4a	0.115	2.62	22.7	219.8	0.17
4b	0.100	1.76	31.9	308.7	0.08
4c	0.095	2.07	19.4	187.7	0.11
5a	0.095	4.46	15.1	150.7	0.47
5b	0.105	3.56	8.0	79.9	0.31
5c	0.100	2.84	19.7	196.6	0.20
6a	0.095	2.80	12.5	128.9	0.20
6b	0.085	2.81	7.3	75.9	0.20
6c	0.110	3.47	25.9	268.4	0.30

	Thickness (mm)	Tension (N/m)	Ultimate Tensile Strength (kPa)	Failure Strain
2.5µg/ml EGF (Glucose)	0.072	28.0	391.0	0.23
5µg/ml EGF (Glucose)	0.103	24.7	238.7	0.12
5µg/ml EGF (Galactose)	0.100	14.2	142.4	0.33
5µg/ml EGF (1.5x Galactose)	0.097	15.2	157.7	0.23
Standard	0.012	8.5	118.2	0.08
Deviations	0.010	8.8	85.6	0.05
	0.005	5.9	58.8	0.14
	0.013	9.6	99.5	0.06

The fourth component that was tested was Dexamethasone. Dexamethasone at 1nM –100nM concentrations has been shown to significantly increase fibroblast cell proliferation in serum-free media (Bettger *et al.*, 1981; Shipley and Ham, 1983) by upregulating the PDGF-alpha receptor mRNA and protein (Warshamana *et al.*, 1998), which has been shown to significantly extend the dendritic network of fibroblasts in 3D matrices (Grinnell, 2003). Dexamethasone has also been shown to be involved in increasing ECM growth and strength in FPCLs (Siddiqui *et al.*, 1992; Ramshaw *et al.*, 1999). This appears to be due to the ability of dexamethasone to promote actin stress fiber formation and increased fibronectin matrix accumulation that aid in the more effective organization of the ECM (Zoppi *et al.*, 1998; Brenner *et al.*, 2000). Also, the increase in collagenase and stromelysin mRNA by EGF that degrade collagen is inhibited with the addition of dexamethasone (Delany and Brinckerhoff, 1992). However, dexamethasone also starts to downregulate the expression of proalpha(1)(I) collagen at around 100nM and higher concentrations, suggesting that the strength of the ECM produced by fibroblasts will start decreasing at higher than 100nM dexamethasone concentrations (Zoppi *et al.*, 1998). The dexamethasone concentration should thus be kept at or below 100nM to retain the ECM growth and strengthening effects of dexamethasone. Also, since dexamethasone is a glucocorticoid as is hydrocortisone, hydrocortisone was removed from the medium in order to keep it simple.

The 3 concentrations of Dexamethasone that were tested were 1nM, 10nM, and 100nM. The 100nM concentration significantly increased the tension, thickness, and UTS of cell-derived matrices, and was thus added into the medium from this point onwards.

Interestingly, the 1nM concentration of Dexamethasone increased the thickness but not the tension of the samples, thus causing a decrease in the UTS of cell derived matrices.

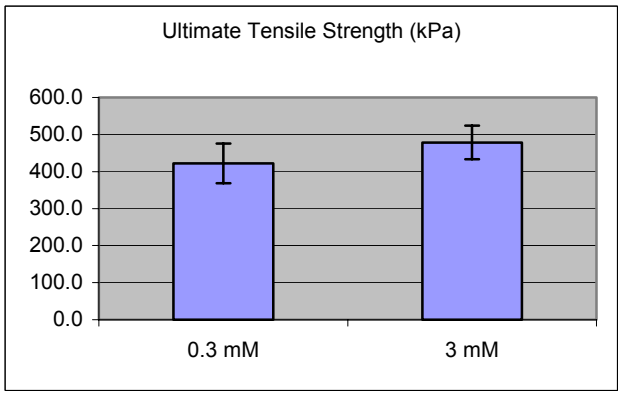


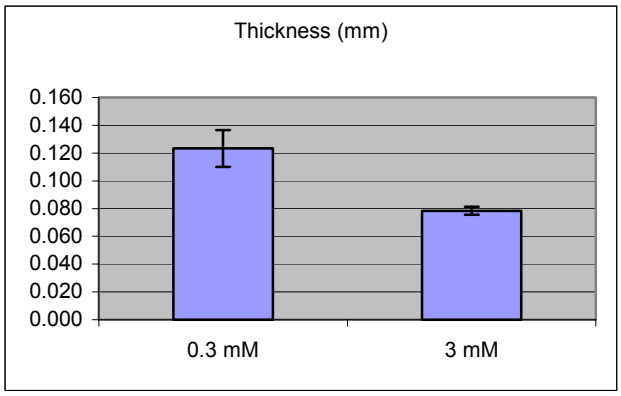
Sample	Thickness (mm)	Displacement (mm)	Tension (N/m)	Ultimate Tensile Strength (kPa)	Failure Strain	Dry Weight (mg)
J1	0.100	3.516	34.5	358.9	0.30	4.5
J2	0.110	2.739	33.4	347.1	0.19	4.7
J3	0.085	3.18	22.0	229.0	0.25	5.5
J4	0.090	6.372	24.5	254.6	0.98	5.2
K2	0.120	4.176	55.9	466.1	0.42	5
K3	0.115	4.176	59.7	497.5	0.42	5.6
K4	0.125	4.146	63.2	526.5	0.41	5.8
L1	0.120	2.769	69.3	577.6	0.19	4.8
L3	0.130	3.897	67.7	564.2	0.37	5
L4	0.125	4.104	67.6	563.3	0.41	5.2

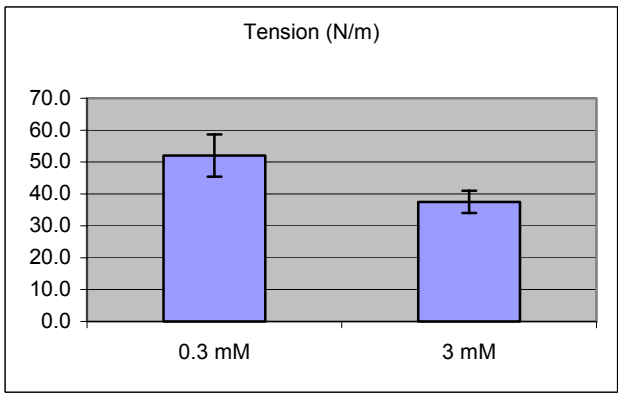
Dexamethasone	Thickness (mm)	Tension (N/m)	Ultimate Tensile Strength (kPa)	Failure Strain	Dry Weight (mg)
1 nM	0.096	28.6	297.4	0.43	5.0
10 nM	0.120	59.6	496.7	0.42	5.5
100 nM	0.125	68.2	568.4	0.32	5.0
Standard	0.011	6.3	65.2	0.37	0.5
Deviations	0.005	3.6	30.2	0.00	0.4
	0.005	1.0	8.0	0.11	0.2

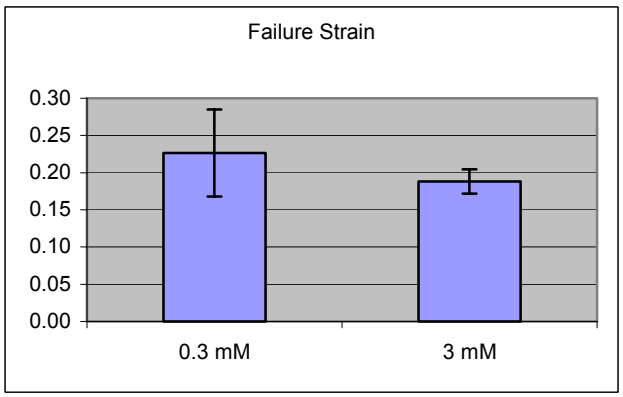
The next set of components that were tested were the amino acids L-Proline and Glycine. The addition of L-Proline and Glycine would be expected to increase the synthesis of collagen in CDMs since L-Proline and Glycine are the major components of collagen (in a 100:68 ratio of Gly:Pro) that is the major structural protein that provides strength to extracellular matrices (Ramshaw *et al.*, 1996). In fact, Organogenesis, Inc. has been adding Glycine and L-proline (in an approximate 1:1 ratio) to their media that they use to produce Apligraf™ and other products containing fibroblasts (Murphy and Ronfard, 2002).

The effects of 3 different concentrations of L-proline (and the corresponding concentration of Glycine to maintain a 100:68 ratio of Gly:Pro) were tested over a 3 week period: 0.3mM, 1mM, and 3mM. Unfortunately, all the 1mM samples (6 samples in all) curled up into little balls after approximately 1 week in culture. Although the 3mM samples had a slightly higher UTS, they had a significantly smaller tension, thickness, and failure strain than the 0.3mM samples. However, the 0.3mM samples had a much reduced UTS from the previous set of experiments (568.4 ± 8.0 kPa versus 422.1 ± 53.4 kPa), indicating that the addition of L-proline and Glycine caused the UTS of cell-derived matrices to decrease significantly. This observation indicated that the 1mM concentration most likely as well caused a decrease in the UTS from the previous set of experiments, even though the contraction of all the samples into balls indicated the possibility of a high presence of collagen. Thus, L-proline and Glycine were not added into the media.









Sample	Thickness (mm)	Displacement (mm)	Tension (N/m)	Ultimate Tensile Strength (kPa)	Failure Strain	Dry Weight (mg)
G1	0.135	2.77	50.9	412.6	0.19	4.4
G2	0.140	3.58	56.8	460.6	0.31	5
G3	0.130	3.38	62.6	507.5	0.28	4.8
G4	0.115	2.75	50.3	407.6	0.19	4.4
G5	0.110	2.55	47.1	382.0	0.17	4.2
G6	0.110	2.90	44.7	362.2	0.21	4.7

H1 - H6 All 1 mM samples contracted in the first week

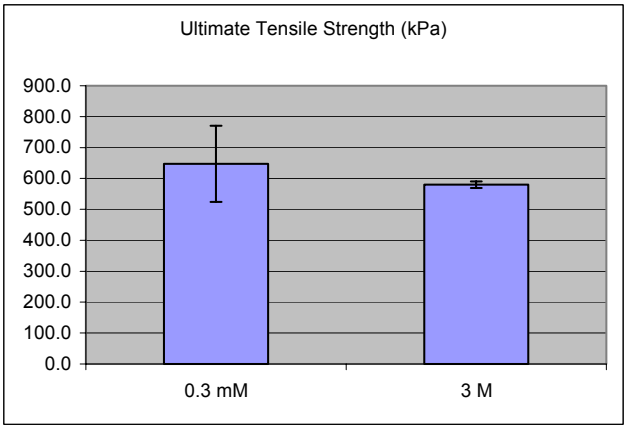
I1	0.080	2.84	39.3	501.8	0.20	3.8
I3	0.075	2.74	33.4	426.5	0.19	4
I4	0.080	2.60	39.8	507.5	0.17	4

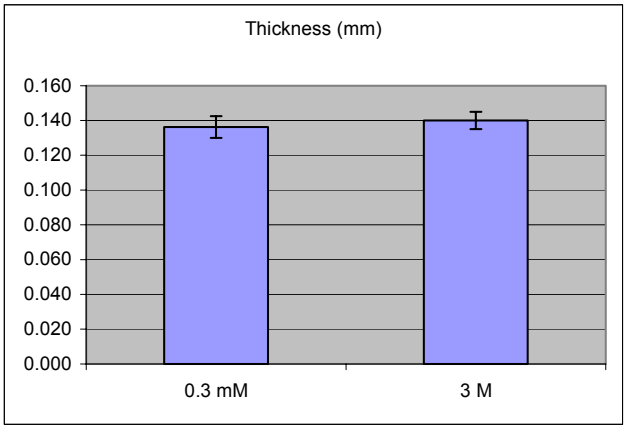
100:60 Glycine:L-Proline

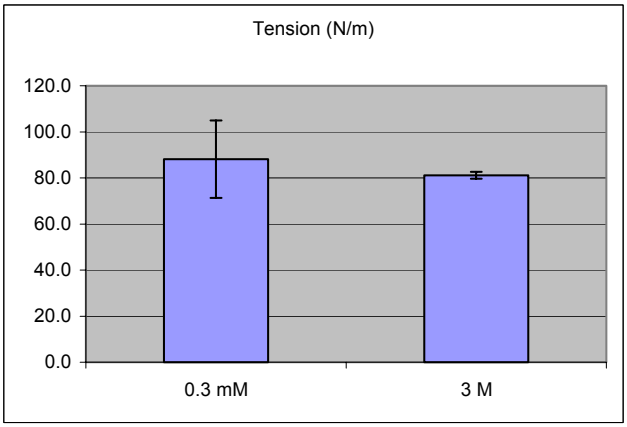
L-Proline	Thickness (mm)	Tension (N/m)	Ultimate Tensile Strength (kPa)	Failure Strain	Dry Weight (mg)
0.3 mM	0.123	52.1	422.1	0.23	4.6
3 mM	0.078	37.5	478.6	0.19	3.9
Standard	0.013	6.6	53.4	0.06	0.3
Deviations	0.003	3.5	45.2	0.02	0.1

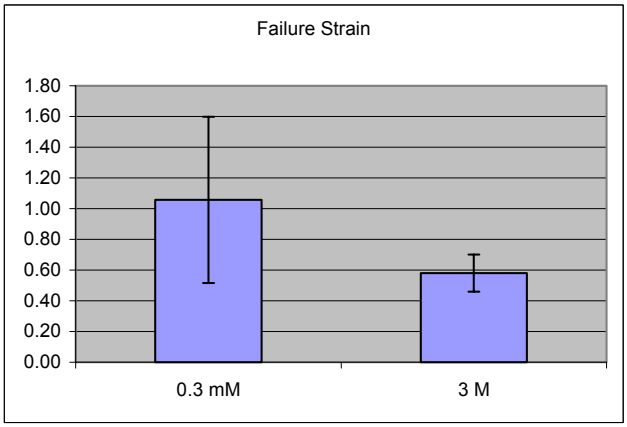
The seventh component that was tested was the amino acid L-cysteine. L-cysteine would be expected to increase the mechanical strength of CDMs since it is known to increase ECM cross-linking through disulfide bridges and is involved in modulating the synthetic phenotype of fibroblasts (Sannes *et al.*, 1997; Basu *et al.*, 1999; Zhu *et al.*, 1999; Carlson *et al.*, 2003). It is also found in high concentrations in the protein SPARC that increases the tensile strength of collagen fibers by directing formation and maintaining the stability of these fibers (Bradshaw *et al.*, 2003).

The effects of the 3 different concentrations of L-cysteine that were tested were: 0.1mM, 0.3mM, and 3mM. These 3 groups were grown in parallel on 12-well plates (1M cells / well) for 3 weeks. Unfortunately, all the 1.0mM samples got infected with fungus. However, the results for the 0.3mM and 3mM samples indicate that the tension, thickness, and UTS might be fairly constant throughout the range of concentrations. Although these values were higher than in previous experiments, these increases appeared to be due to the higher density of cells used on the 12-well plate (1M/3.8cm^2 vs. 2M/9.6cm^2), and thus the addition of L-cysteine did not appear to have any significant effect on these values on cell-derived matrices, and thus was not added to the medium being developed. However, the failure strain approximately doubles with the addition of 0.3mM of L-cysteine. Thus, the addition of L-cysteine could come in handy when the failure strain of cell-derived matrices would need to be significantly increased.







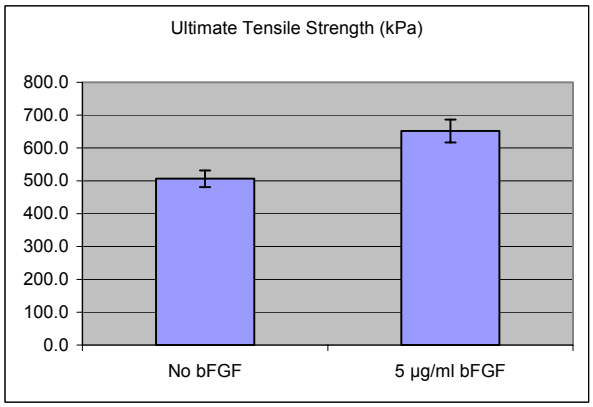


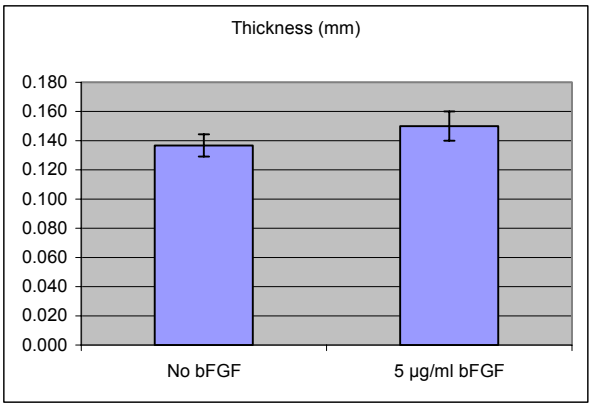
Sample	Thickness (mm)	Displacement (mm)	Tension (N/m)	Ultimate Tensile Strength (kPa)	Failure Strain	Dry Weight (mg)
M1	0.145	6.50	99.6	730.8	1.02	4.3
M2	0.130	8.06	104.0	763.1	1.83	5.6
M3	0.135	5.41	67.6	496.5	0.69	4.2
M4	0.135	5.39	81.4	597.2	0.68	5.4
N1 - N4	All 1 mM samples got infected during last week of culture					
O1	0.140	5.00	-	-	0.59	5.3
O2	0.145	5.44	82.2	587.0	0.70	4.6
O3	0.135	4.37	80.1	572.0	0.46	4.8

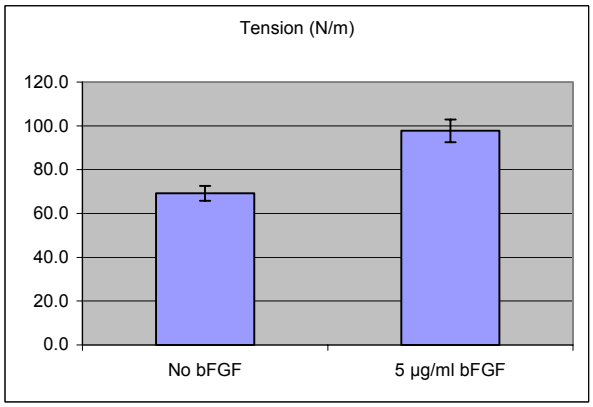
L-Cysteine	Thickness (mm)	Tension (N/m)	Ultimate Tensile Strength (kPa)	Failure Strain	Dry Weight (mg)
0.3 mM	0.136	88.1	646.9	1.06	4.9
3 M	0.140	81.1	579.5	0.58	4.9
Standard	0.006	16.8	123.3	0.54	0.7
Deviations	0.005	1.5	10.7	0.12	0.4

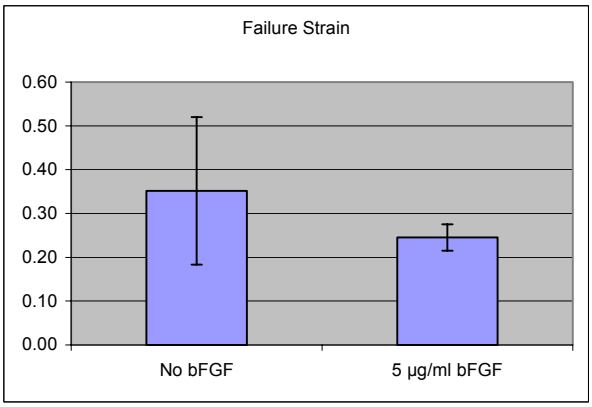
The eighth component that was tested was basic fibroblast growth factor. Basic fibroblast growth factor (bFGF) at 5-25ng/ml concentrations has been show to stimulate collagen production by fibroblasts in monolayers, and thus increasing the strength of the ECM produced by these fibroblasts (Shipley and Ham, 1983; Gospodarowicz *et al.*, 1987; Butt *et al.*, 1995). bFGF is thought to play an important role in normal tissue repair and wound healing, and is a potent mitogenic and chemotactic factor for fibroblasts, regulating proliferation and extracellular matrix (ECM) production by these cells (Milewski *et al.*, 1998; Aktas and Kayton, 2000). It has also been shown to mediate fibroblast migration (Kondo *et al.*, 1993). It is also a potent inducer of tenascin expression, which is involved in cell motility, cell proliferation, and tissue modeling (Tucker *et al.*, 1993). Also, since the mechanisms underlying the effects of epidermal growth factor (EGF) overlap with those of bFGF, the addition of bFGF allows a lower concentration of EGF to be used without diminishing any of the EGF's effects on ECM development (Abe *et al.*, 1990).

The effect of 5 μg/ml of bFGF on the growth of cell-derived matrices was investigated over a 3-week time period. This is a standard amount used in media containing bFGF. Higher concentrations generally have more beneficial effects, but bFGF is very expensive. The addition of 5 μg/ml of bFGF significantly increased the tension and UTS of cell-derived matrices, but not the thickness. Basic fibroblast growth factor was thus included in the medium from this point onwards.









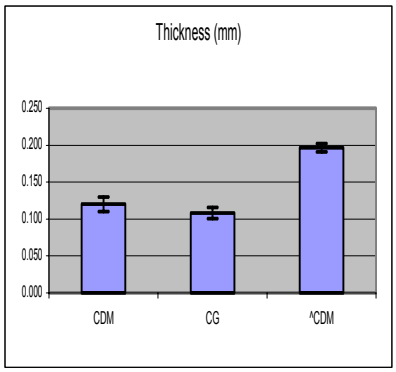
Sample	Thickness (mm)	Displacement (mm)	Tension (N/m)	Ultimate Tensile Strength (kPa)	Failure Strain
A	0.145	3.2	73.0	534.2	0.25
B	0.135	3.2	68.3	499.4	0.25
C	0.130	4.8	66.3	485.4	0.55
D	0.150	2.9	103.6	690.5	0.21
E	0.140	3.2	96.0	639.9	0.25
F	0.160	3.3	93.7	624.4	0.27

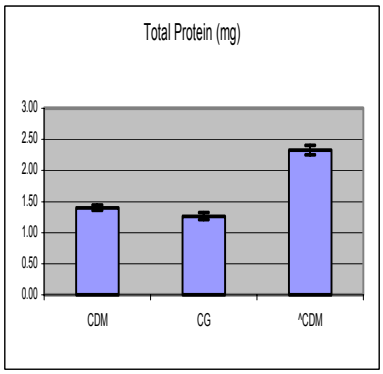
	Thickness (mm)	Tension (N/m)	Ultimate Tensile Strength (kPa)	Failure Strain
No bFGF	0.137	69.2	506.4	0.35
µg/ml bFG	0.150	97.7	651.6	0.25
Standard	0.008	3.4	25.2	0.17
Deviations	0.010	5.2	34.6	0.03

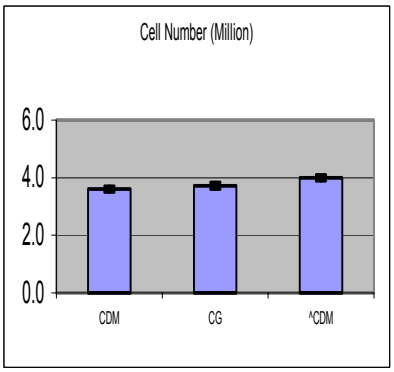
The table below summarizes all the modification that were made to the medium and in what order. L-Cysteine (*) was grown in 12-well plates at a 20% higher density which resulted in higher values. The resulting medium was later simplified further by getting rid of the transferrin and O-Phosphorylethanolamine, since there was no solid literature to support the need of these components for the growth of cell-derived matrices. The concentration of L-3,3',5-Triiodothyronine was also increased by ten times to support the necessary increased rate of cell metabolism of the fibroblasts within cell-derived matrices.

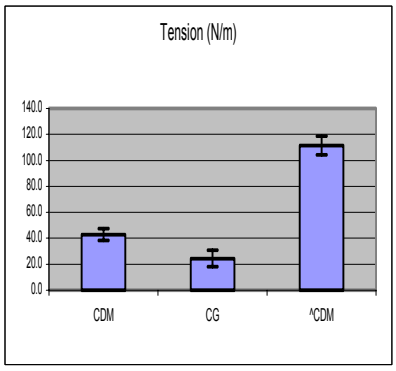
Component	Base Media	L-Ascorbate	Galactose	EGF	Dexamethasone	100:68 Glycine:L-Proline	L-Cysteine*	bFGF	Ahlfors, 2003
Base Media	3:1 DMEM:F12	3:1 DMEM:F12	3:1 DMEM:F12	3:1 DMEM:F12	3:1 DMEM:F12	3:1 DMEM:F12	3:1 DMEM:F12	3:1 DMEM:F12	3:1 DMEM:F12
Fetal Bovine Serum									
Glucose	4.5 mg/ml (25mM)	4.5 mg/ml (25mM)	**1.0mg/ml + 3.5mg/ml Galactose**	4.5 mg/ml (25mM)	4.5 mg/ml (25mM)	4.5 mg/ml (25mM)	4.5 mg/ml (25mM)	4.5 mg/ml (25mM)	4.5 mg/ml (25mM)
L-Glutamine	3.25mM + 4mM Glutamax	3.25mM + 4mM Glutamax	3.25mM + 4mM Glutamax	3.25mM + 4mM Glutamax	3.25mM + 4mM Glutamax	3.25mM + 4mM Glutamax	3.25mM + 4mM Glutamax	3.25mM + 4mM Glutamax	3.25mM + 4mM Glutamax
Hepes									
Liposome B									
Albumin									
Epidermal Growth Factor	10 ng/ml (1.67nM)	10 ng/ml (1.67nM)	5 ng/ml (0.83nM)	**2.5 ng/ml (0.42nM)**	2.5 ng/ml (0.42nM)	2.5 ng/ml (0.42nM)	2.5 ng/ml (0.42nM)	2.5 ng/ml (0.42nM)	2.5 ng/ml (0.42nM)
Basic Fibroblast Growth Factor								**5 ng/ml (0.28nM)**	5 ng/ml (0.28nM)
Platelet Derived Growth Factor									
Progesterone									
Insulin	5 µg/ml (86.1µM)	5 µg/ml (86.1µM)	5 µg/ml (86.1µM)	5 µg/ml (86.1µM)	5 µg/ml (86.1µM)	5 µg/ml (86.1µM)	5 µg/ml (86.1µM)	5 µg/ml (86.1µM)	5 µg/ml (86.1µM)
Dexamethasone					**39.3 ng/ml (100nM)**	39.3 ng/ml (100nM)	39.3 ng/ml (100nM)	39.3 ng/ml (100nM)	39.3 ng/ml (100nM)
Hydrocortisone	400 ng/ml (1.1µM)	400 ng/ml (1.1µM)	400 ng/ml (1.1µM)	400 ng/ml (1.1µM)	-				
L-3,3',5-Triiodothyronine	13.5 pg/ml (20pM)	13.5 pg/ml (20pM)	13.5 pg/ml (20pM)	13.5 pg/ml (20pM)	13.5 pg/ml (20pM)	13.5 pg/ml (20pM)	13.5 pg/ml (20pM)	13.5 pg/ml (20pM)	**135 pg/ml (200pM)**
Transferrin	5 µg/ml (62.5nM)	5 µg/ml (62.5nM)	5 µg/ml (62.5nM)	5 µg/ml (62.5nM)	5 µg/ml (62.5nM)	5 µg/ml (62.5nM)	5 µg/ml (62.5nM)	5 µg/ml (62.5nM)	-
Ethanolamine	6.1 µg/ml (100µM)	6.1 µg/ml (100µM)	6.1 µg/ml (100µM)	6.1 µg/ml (100µM)	6.1 µg/ml (100µM)	6.1 µg/ml (100µM)	6.1 µg/ml (100µM)	6.1 µg/ml (100µM)	6.1 µg/ml (100µM)
O-Phosphorylethanolamine	14.1 µg/ml (100µM)	14.1 µg/ml (100µM)	14.1 µg/ml (100µM)	14.1 µg/ml (100µM)	14.1 µg/ml (100µM)	14.1 µg/ml (100µM)	14.1 µg/ml (100µM)	14.1 µg/ml (100µM)	-
Selenious Acid	5 ng/ml (39nM)	5 ng/ml (39nM)	5 ng/ml (39nM)	5 ng/ml (39nM)	5 ng/ml (39nM)	5 ng/ml (39nM)	5 ng/ml (39nM)	5 ng/ml (39nM)	5 ng/ml (39nM)
Ascorbic Acid	50 µg/ml (173µM)	**150 µg/ml (519µM)**	150 µg/ml (519µM)	150 µg/ml (519µM)	150 µg/ml (519µM)	150 µg/ml (519µM)	150 µg/ml (519µM)	150 µg/ml (519µM)	150 µg/ml (519µM)
L-Proline						**25.9 µg/ml (225µM)**			
Glycine						**8.7 µg/ml (116µM)**			
L-Cysteine							**30.5 µg/ml (250µM)**		
Copper Sulfate									
Sodium Sulfate									
Dithiothreitol									
Phosphoenolpyruvate									
Prostaglandin E1									
Prostaglandin F2 α									
Glutathione (reduced)	1 µg/ml (3.25µM)	1 µg/ml (3.25µM)	1 µg/ml (3.25µM)	1 µg/ml (3.25µM)	1 µg/ml (3.25µM)	1 µg/ml (3.25µM)	1 µg/ml (3.25µM)	1 µg/ml (3.25µM)	1 µg/ml (3.25µM)
Adenine									
Strontium Chloride									
Ammonium Metavanadate									
Manganous Chloride									
Cholera Toxin									
TENSION	(23.1% increase	24.7 ± 8.8 N/m	14.2 ± 5.9 N/m	28.0 ± 8.5 N/m	68.2 ± 1.0 N/m	52.1 ± 6.6 N/m	88.1 ± 16.8 N/m	97.7 ± 5.2 N/m	156.9 ± 8.1 N/m
THICKNESS	in Dry Weight) →	103 ± 10 µm	100 ± 5 µm	72 ± 12 µm	125 ± 5 µm	123 ± 13 µm	136 ± 6 µm	150 ± 10 µm	225 ± 7 µm
UTS		238.7 ± 85.6 kPa	142.4 ± 58.8 kPa	391.0 ± 118.2 kPa	568.4 ± 8.0 kPa	422.1 ± 53.4 kPa	646.9 ± 123.3 kPa	651.6 ± 34.6 kPa	697.1 ± 36.1 kPa
FAILURE STRAIN		0.12 ± 0.05	0.33 ± 0.14	0.23 ± 0.06	0.32 ± 0.11	0.23 ± 0.06	1.06 ± 0.54	0.25 ± 0.03	0.31 ± 0.06

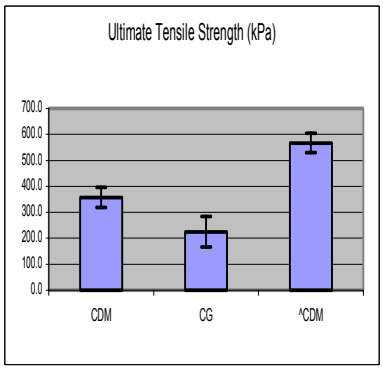
The results for cell-derived matrices seeded with 2.5million HFFs (for faster growth) on TransWells™ and fed with the resulting medium for 3 weeks are shown below (as ^CDM). These are compared to CDMs and collagen gels that were grown in parallel to the ^CDMs and fed with the serum-supplemented medium. The results below show higher thickness but lower UTS values than for the previous experiment with bFGF (where the media was identical), indicating that TransWells promote the growth of thick CDMs but do not appear to increase the UTS. Starting cell number did not appear to have a significant effect (as long as cells are seeded at "superconfluent" densities) on the growth and strengthening of the chemically-defined medium fed samples. However, the higher starting cell number appear to cause a significant increase in the UTS of the serum-supplemented samples (both CDMs and collagen gels) – compare to CDMs and collagen gels in the reported (next) experiments where starting cell number was 2M.

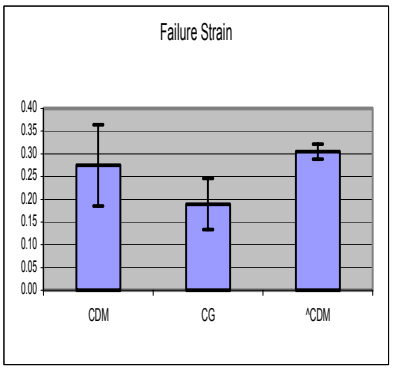

Thickness (mm)
0.250
0.200
0.150
0.100
0.050
0.000
CDM
CG
^CDM

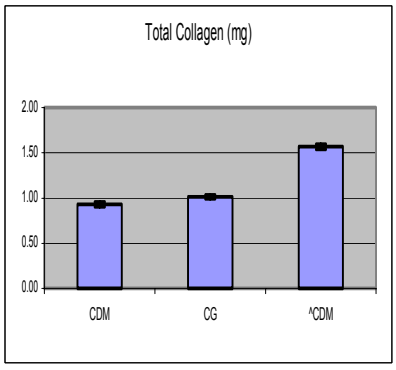

Total Protein (mg)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
CDM
CG
^CDM

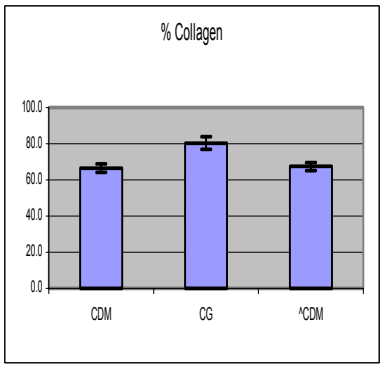

Cell Number (Million)
6.0
4.0
2.0
0.0
CDM
CG
^CDM

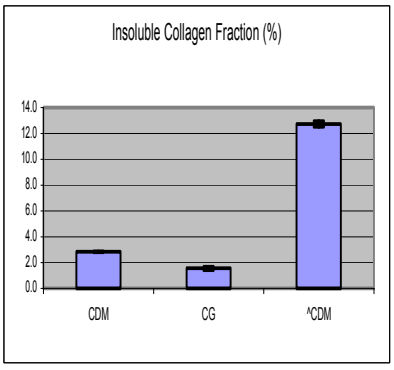

Tension (N/m)
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
CDM
CG
^CDM

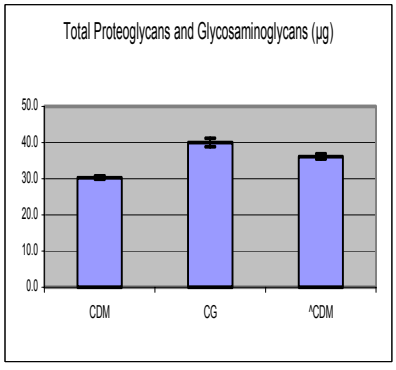

Ultimate Tensile Strength (kPa)
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
CDM
CG
^CDM

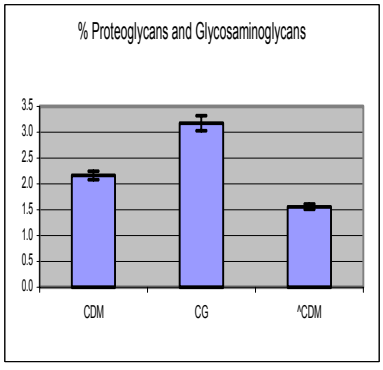

Failure Strain
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
CDM
CG
^CDM

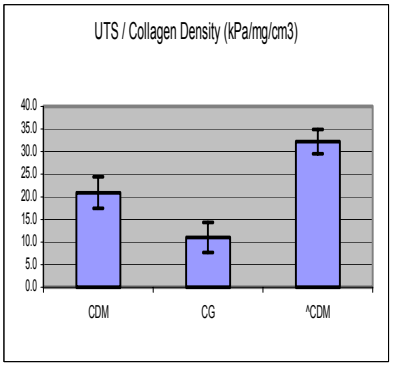

Total Collagen (mg)
2.00
1.50
1.00
0.50
0.00
CDM
CG
^CDM


% Collagen
100.0
80.0
60.0
40.0
20.0
0.0
CDM
CG
^CDM


Insoluble Collagen Fraction (%)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
CDM
CG
^CDM


Total Proteoglycans and Glycosaminoglycans (µg)
50.0
40.0
30.0
20.0
10.0
0.0
CDM
CG
^CDM


% Proteoglycans and Glycosaminoglycans
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CDM
CG
^CDM


UTS / Collagen Density (kPa/mg/cm3)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
CDM
CG
^CDM

Sample	Thickness (mm)	Displacement (mm)	Tension (N/m)	Ultimate Tensile Strength (kPa)	Failure Strain	Wet Weight (mg)	Dry Weight (mg)
[D1]	0.110	3.91	38.5	320.8	0.37	24.7	1.8
[D2]	0.120	2.78	42.3	352.5	0.20	23.6	1.9
[D3]	0.130	3.22	47.7	397.5	0.26	24.5	1.9
[C1]	0.100	3.29	11.9	109.8	0.27	29.7	1.8
[C2]	0.110	2.55	27.8	256.6	0.17	28.4	1.7
[C3]	0.120	2.83	29.3	270.5	0.20	28.8	1.6
[C4]	0.100	2.33	26.7	246.5	0.14	29.6	1.6
[C5]	0.110	3.08	27.4	252.9	0.24	31.6	1.7
[C6]	0.110	2.20	23.2	214.2	0.12	30.4	1.7
[A1]	0.190	3.63	104.8	532.9	0.32	87.0	3.0
[A2]	0.200	3.50	110.5	561.9	0.30	92.4	3.1
[A3]	0.200	3.43	119.2	606.1	0.29	88.1	3.2

		Thickness (mm)	Tension (N/m)	Ultimate Tensile Strength (kPa)	Failure Strain	Wet Weight (mg)	Dry Weight (mg)
10% FBS CDM	CDM	0.120	42.8	356.9	0.27	24.3	1.9
10% FBS FPCL	CG	0.108	24.4	225.1	0.19	29.8	1.7
Ahlfors CDM	^CDM	0.197	111.5	566.9	0.30	89.2	3.1
Standard		0.010	4.6	38.5	0.09	0.6	0.06
Deviations		0.008	6.4	59.5	0.06	1.1	0.08
		0.006	7.3	36.9	0.02	2.9	0.10

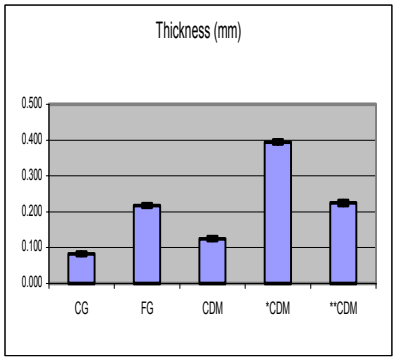
Wet Weight / Dry Weight	Total Protein (mg)	Total Collagen (mg)	% Collagen	Collagen Density (mg/cm3)	UTS / Collagen Density (kPa/mg/cm3)
13.7	1.4	0.92	68.1	18.5	17.4
12.4	1.4	0.91	63.9	16.8	21.0
12.9	1.4	0.96	67.4	16.3	24.4
16.5	1.4	1.02	75.6	22.5	4.9
16.7	1.3	1.04	81.2	20.8	12.3
18.0	1.2	0.98	81.7	18.1	15.0
18.5	1.2	1.03	85.4	22.7	10.9
18.6	1.3	1.03	80.8	20.7	12.2
17.9	1.3	0.99	77.6	19.9	10.8
29.0	2.3	1.54	68.4	17.9	29.7
29.8	2.3	1.60	68.8	17.7	31.8
27.5	2.4	1.56	65.0	17.2	35.2

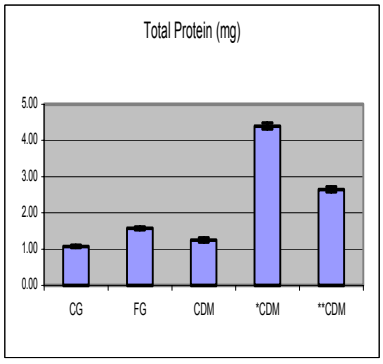
Wet Weight / Dry Weight	Total Protein (mg)	Total Collagen (mg)	% Collagen	Collagen Density (mg/cm3)	UTS / Collagen Density (kPa/mg/cm3)
13.0	1.40	0.93	66.5	17.2	20.9
17.7	1.26	1.01	80.4	20.8	11.0
28.8	2.33	1.57	67.4	17.6	32.2
0.7	0.04	0.03	2.3	1.1	3.5
0.9	0.06	0.02	3.4	1.7	3.4
1.2	0.08	0.03	2.1	0.3	2.7

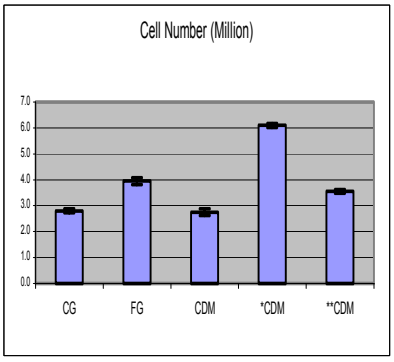
Insoluble Collagen Fraction (mg)	Insoluble Collagen Fraction (%)	Total Proteoglycans and Glycosaminoglycans (µg)	% Proteoglycans and Glycosaminoglycans	Cell Number (Million)
0.026	2.8	30.4	2.3	3.5
0.026	2.9	29.8	2.1	3.6
0.027	2.8	30.7	2.2	3.7
0.017	1.7	39.2	2.9	3.7
0.016	1.5	40.5	3.2	3.8
0.014	1.4	39.3	3.3	3.6
0.018	1.8	38.8	3.2	3.7
0.015	1.5	40.1	3.1	3.9
0.015	1.5	42.0	3.3	3.6
0.199	12.9	35.5	1.6	4.1
0.200	12.5	37.0	1.6	4.0
0.199	12.8	36.0	1.5	3.9

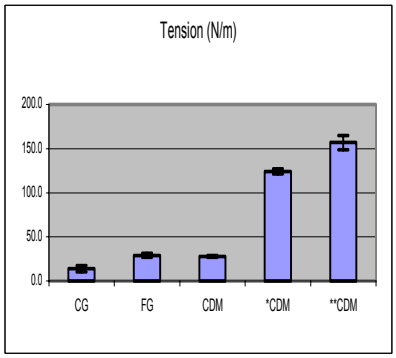
Insoluble Collagen Fraction (mg)	Insoluble Collagen Fraction (%)	Total Proteoglycans and Glycosaminoglycans (µg)	% Proteoglycans and Glycosaminoglycans	Cell Number (Million)
0.026	2.8	30.3	2.2	3.6
0.016	1.6	40.0	3.2	3.7
0.199	12.7	36.2	1.6	4.0
0.001	0.0	0.5	0.1	0.1
0.001	0.1	1.2	0.1	0.1
0.001	0.2	0.8	0.0	0.1

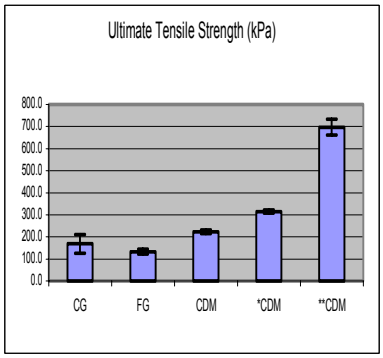
The resulting medium was simplified further by getting rid of the transferrin and O-Phosphorylethanolamine, since there was no solid literature to support the need of these components for the growth of cell-derived matrices. The concentration of L-3,3',5-Triiodothyronine was also increased by ten times to support the necessary increased rate of cell metabolism of the fibroblasts within cell-derived matrices. The results for cell-derived matrices (*CDMs, **CDM, and BTCs) seeded with 2.0 million HFFs (0.5 million less than above) on TransWells™ and fed with this medium for 3 weeks are shown below. This chemically defined medium increased the thickness and UTS. The decreased cell number resulted in a significant decrease in the tension, thickness, total protein, and UTS of CDMs and collagen gels.

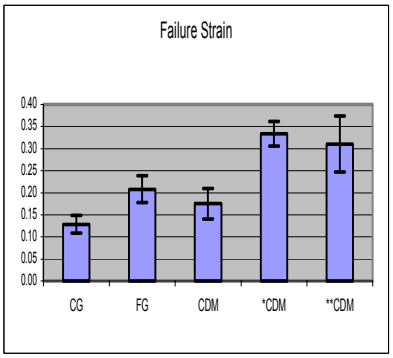

Thickness (mm)
0.500
0.400
0.300
0.200
0.100
0.000
CG
FG
CDM
*CDM
**CDM

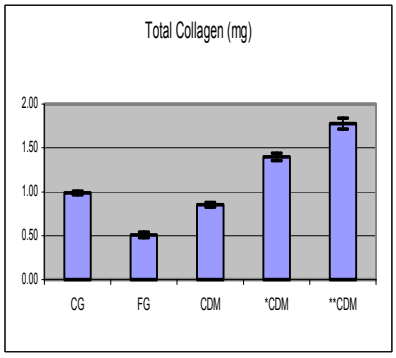

Total Protein (mg)
5.00
4.00
3.00
2.00
1.00
0.00
CG
FG
CDM
*CDM
**CDM

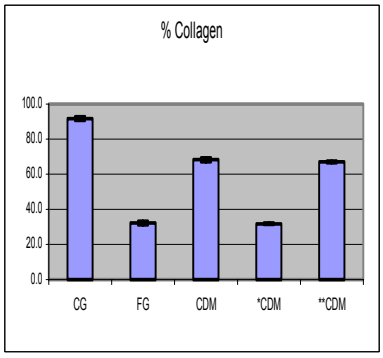

Cell Number (Million)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
CG
FG
CDM
*CDM
**CDM

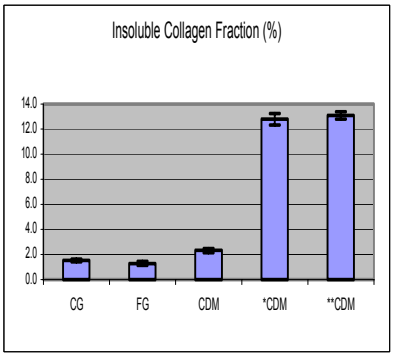

Tension (N/m)
200.0
150.0
100.0
50.0
0.0
CG
FG
CDM
*CDM
**CDM

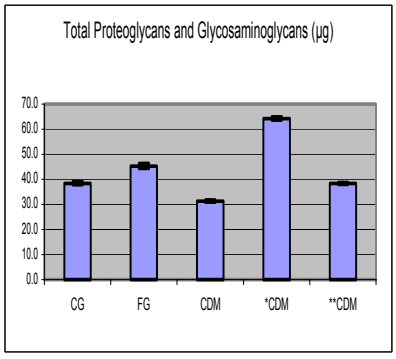

Ultimate Tensile Strength (kPa)
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
CG
FG
CDM
*CDM
**CDM

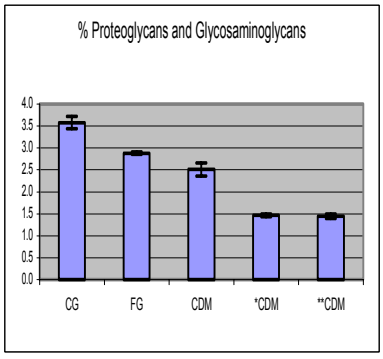

Failure Strain
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
CG
FG
CDM
*CDM
**CDM

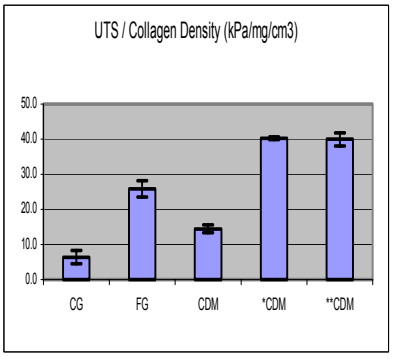

Total Collagen (mg)
2.00
1.50
1.00
0.50
0.00
CG
FG
CDM
*CDM
**CDM


% Collagen
100.0
80.0
60.0
40.0
20.0
0.0
CG
FG
CDM
*CDM
**CDM


Insoluble Collagen Fraction (%)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
CG
FG
CDM
*CDM
**CDM


Total Proteoglycans and Glycosaminoglycans (µg)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
CG
FG
CDM
*CDM
**CDM


% Proteoglycans and Glycosaminoglycans
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CG
FG
CDM
*CDM
**CDM


UTS / Collagen Density (kPa/mg/cm3)
50.0
40.0
30.0
20.0
10.0
0.0
CG
FG
CDM
*CDM
**CDM

Sample	Thickness (mm)	Displacement (mm)	Spherical Cap Area (mm2)	Estimated Burst Thickness (mm)	Tension (N/m)
[CDM1]	0.130				
[CDM2]	0.120	2.81	103.3	0.091	26.6
[CDM2]	0.130	2.30	95.2	0.107	28.6
[CDM4]	0.120	2.75	102.3	0.092	28.5
[CG1]	0.080	2.30	95.2	0.066	10.3
[CG2]	0.080	2.45	97.4	0.065	17.1
[CG3]	0.090	2.05	91.7	0.077	16.8
[CG4]	0.080	2.12	92.7	0.068	11.4
[FG1]	0.210	3.17	110.1	0.150	25.7
[FG2]	0.220	2.69	101.3	0.171	30.8
[FG3]	0.220	2.89	104.8	0.165	30.4
[FG4]	0.220	2.74	102.1	0.169	29.0
[*CDM1]	0.400	3.88	125.8	0.250	121.3
[*CDM2]	0.390	3.59	119.0	0.257	124.4
[*CDM3]	0.390				
[*CDM4]	0.400	3.61	119.5	0.263	127.0
[**CDM1]	0.220	3.27	112.1	0.154	151.1
[**CDM2]	0.230	3.82	124.4	0.145	162.6

		Thickness (mm)	Displacement (mm)	Spherical Cap Area (mm2)	Estimated Burst Thickness (mm)	Tension (N/m)
10% FBS FPCL	CG	0.083	2.23	94.2	0.069	13.9
10% FBS FPFL	FG	0.218	2.87	104.6	0.164	29.0
10% FBS CDM	CDM	0.125	2.62	100.3	0.097	27.9
Ahlfors CDM	*CDM	0.395	3.69	121.4	0.257	124.2
Ahlfors+ CDM	**CDM	0.225	3.55	118.3	0.150	156.9
Standard	CG	0.005	0.18	2.6	0.006	3.6
Deviations	FG	0.005	0.22	4.0	0.010	2.3
	CDM	0.006	0.28	4.5	0.009	1.1
	*CDM	0.006	0.16	3.8	0.007	2.9
	**CDM	0.007	0.39	8.7	0.006	8.1

Ultimate Tensile Strength (kPa)	Estimated True UTS (kPa)	Failure Strain	Wet Weight (mg)	Dry Weight (mg)	Wet Weight / Dry Weight	Total Protein (mg)
			23.8	1.8	13.2	1.2
212.8	274.6	0.20	24.1	1.9	12.7	1.3
228.8	295.2	0.14	23.9	1.9	12.6	1.3
228.0	294.2	0.19	24.4	1.8	13.6	1.2
124.8	149.6	0.14	23.1	1.4	16.5	1.1
207.3	248.4	0.15	24.5	1.5	16.3	1.1
203.6	244.0	0.11	23.6	1.5	15.7	1.1
138.2	165.6	0.12	23.9	1.4	17.1	1.1
118.2	157.1	0.25	44.3	2.2	20.1	1.5
141.6	188.2	0.18	46.1	2.3	20.0	1.6
139.8	185.8	0.21	45.3	2.2	20.6	1.5
133.3	177.2	0.19	44.9	2.3	19.5	1.6
307.1	472.6	0.37	122.3	6.3	19.4	4.4
314.9	484.7	0.32	122.6	6.2	19.8	4.3
			122.8	6.3	19.5	4.4
321.5	494.8	0.32	124.4	6.4	19.4	4.5
671.6	1009.6	0.27	76.8	3.7	20.8	2.6
722.7	1086.5	0.36	75.5	3.8	19.9	2.7

Ultimate Tensile Strength (kPa)	Estimated True UTS (kPa)	Failure Strain	Wet Weight (mg)	Dry Weight (mg)	Wet Weight / Dry Weight	Total Protein (mg)
168.5	201.9	0.13	23.8	1.45	16.4	1.08
133.2	177.1	0.21	45.2	2.25	20.1	1.58
223.2	288.0	0.18	24.1	1.85	13.0	1.25
314.5	484.1	0.33	123.0	6.30	19.5	4.40
697.1	1048.0	0.31	76.2	3.75	20.3	2.65
43.1	51.6	0.02	0.6	0.06	0.6	0.03
10.6	14.2	0.03	0.8	0.06	0.4	0.04
9.0	11.6	0.03	0.3	0.06	0.5	0.06
7.2	11.1	0.03	0.9	0.08	0.2	0.08
36.1	54.3	0.06	0.9	0.07	0.6	0.07

Total Collagen (mg)	Total 4-Hydroxyproline (mg)	% Collagen	Collagen Density (mg/cm3)	UTS / Collagen Density (kPa/mg/cm3)	Insoluble Collagen Fraction (mg)
0.83	0.088	68.8	14.0		0.018
0.87	0.090	67.2	16.1	13.2	0.021
0.88	0.091	67.4	14.9	15.4	0.019
0.84	0.087	69.6	15.4	14.8	0.021
1.01	0.108	91.8	27.9	4.5	0.016
0.98	0.104	92.9	26.9	7.7	0.016
1.00	0.106	90.5	24.4	8.3	0.014
0.97	0.108	91.9	26.7	5.2	0.014
0.48	0.054	31.1	5.0	23.4	0.007
0.52	0.057	32.5	5.3	26.9	0.007
0.49	0.054	31.6	4.9	28.6	0.006
0.54	0.059	33.7	5.5	24.4	0.006
1.38	0.143	31.4	7.6	40.3	0.180
1.37	0.145	31.8	7.7	40.7	0.166
1.39	0.144	31.6	7.9		0.177
1.46	0.150	32.4	8.1	39.8	0.193
1.73	0.183	66.6	17.4	38.6	0.223
1.82	0.188	67.4	17.5	41.3	0.242

Total Collagen (mg)	Total 4-Hydroxyproline (mg)	% Collagen	Collagen Density (mg/cm3)	UTS / Collagen Density (kPa/mg/cm3)	Insoluble Collagen Fraction (mg)
0.99	0.107	91.8	26.5	6.4	0.015
0.51	0.056	32.2	5.2	25.9	0.007
0.85	0.089	68.2	15.1	14.5	0.020
1.40	0.146	31.8	7.8	40.3	0.179
1.78	0.186	67.0	17.4	40.0	0.233
0.02	0.002	1.0	1.5	1.9	0.001
0.03	0.002	1.2	0.2	2.4	0.001
0.03	0.002	1.1	0.9	1.1	0.002
0.04	0.003	0.5	0.2	0.4	0.011
0.06	0.004	0.6	0.1	1.9	0.013

Insoluble Collagen Fraction (%)	Total Proteoglycans and Glycosaminoglycans (µg)	% Proteoglycans and Glycosaminoglycans	Cell Number (Million)
2.2	31.5	2.6	2.7
2.4	30.8	2.4	2.8
2.2	31.1	2.4	2.6
2.5	31.9	2.7	2.9
1.6	38.3	3.5	2.8
1.6	38.0	3.6	2.9
1.4	38.0	3.5	2.7
1.5	39.4	3.8	2.8
1.5	44.7	2.9	3.9
1.3	46.5	2.9	4.0
1.2	44.5	2.9	4.1
1.1	45.7	2.8	3.8
13.0	65.1	1.5	6.1
12.2	63.9	1.5	6.2
12.7	64.3	1.5	6.0
13.2	63.8	1.4	6.1
12.9	38.6	1.5	3.6
13.3	38.1	1.4	3.5

Insoluble Collagen Fraction (%)	Total Proteoglycans and Glycosaminoglycans (µg)	% Proteoglycans and Glycosaminoglycans	Cell Number (Million)
1.5	38.4	3.6	2.8
1.3	45.4	2.9	4.0
2.3	31.3	2.5	2.8
12.8	64.3	1.5	6.1
13.1	38.4	1.4	3.6
0.1	0.7	0.1	0.1
0.2	0.9	0.0	0.1
0.2	0.5	0.2	0.1
0.5	0.6	0.0	0.1
0.3	0.4	0.1	0.1

Appendix 5: One-Way Anova and Tukey HSD Post Hoc Statistical Analysis of Parameters between all Groups

OneWay ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Thickness	Between Groups	.231	4	.058	1879.809	.000
	Within Groups	.000	13	.000		
	Total	.232	17			
Total Protein	Between Groups	29.838	4	7.459	2154.951	.000
	Within Groups	.045	13	.003		
	Total	29.883	17			
Cell Number	Between Groups	29.718	4	7.429	666.088	.000
	Within Groups	.145	13	.011		
	Total	29.863	17			
Tension	Between Groups	46558.721	4	11639.680	921.794	.000
	Within Groups	138.899	11	12.627		
	Total	46697.620	15			
UTS	Between Groups	492785.018	4	123196.254	181.257	.000
	Within Groups	7476.440	11	679.676		
	Total	500261.458	15			
Failure Strain	Between Groups	.098	4	.024	23.008	.000
	Within Groups	.012	11	.001		
	Total	.109	15			
Total Collagen	Between Groups	2.879	4	.720	667.044	.000
	Within Groups	.014	13	.001		
	Total	2.893	17			
Percent Collagen	Between Groups	10535.886	4	2633.972	2919.150	.000
	Within Groups	11.730	13	.902		
	Total	10547.616	17			
Non-Acid and Pepsin Extractable Fraction	Between Groups	502.709	4	125.677	2163.981	.000
	Within Groups	.755	13	.058		
	Total	503.464	17			
Total Proteoglycans and Glycosaminoglycans	Between Groups	2482.114	4	620.528	1396.256	.000
	Within Groups	5.777	13	.444		
	Total	2487.891	17			
Percent Proteoglycans and Glycosaminoglycans	Between Groups	11.810	4	2.953	260.226	.000
	Within Groups	.148	13	.011		
	Total	11.958	17			
UTS per Collagen Density	Between Groups	2816.691	4	704.173	229.485	.000
	Within Groups	33.753	11	3.068		
	Total	2850.444	15			

Tukey HSD Post Hoc

(* The mean difference is significant at the .05 level.)

Dependent Variable	(I) SampleType	(J) SampleType	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Thickness	CDM	Collagen Gel	.04250(*)	.00392	.000	.0301	.0549
		Fibrin Gel	-.09250(*)	.00392	.000	-.1049	-.0801
		*CDM	-.27000(*)	.00392	.000	-.2824	-.2576
		**CDM	-.10000(*)	.00480	.000	-.1151	-.0849
	Collagen Gel	CDM	-.04250(*)	.00392	.000	-.0549	-.0301
		Fibrin Gel	-.13500(*)	.00392	.000	-.1474	-.1226
		*CDM	-.31250(*)	.00392	.000	-.3249	-.3001
		**CDM	-.14250(*)	.00480	.000	-.1576	-.1274
	Fibrin Gel	CDM	.09250(*)	.00392	.000	.0801	.1049
		Collagen Gel	.13500(*)	.00392	.000	.1226	.1474
		*CDM	-.17750(*)	.00392	.000	-.1899	-.1651
		**CDM	-.00750	.00480	.545	-.0226	.0076
	*CDM	CDM	.27000(*)	.00392	.000	.2576	.2824
		Collagen Gel	.31250(*)	.00392	.000	.3001	.3249
		Fibrin Gel	.17750(*)	.00392	.000	.1651	.1899
		**CDM	.17000(*)	.00480	.000	.1549	.1851
	**CDM	CDM	.10000(*)	.00480	.000	.0849	.1151
		Collagen Gel	.14250(*)	.00480	.000	.1274	.1576
		Fibrin Gel	.00750	.00480	.545	-.0076	.0226
		*CDM	-.17000(*)	.00480	.000	-.1851	-.1549
Total Protein	CDM	Collagen Gel	.15000(*)	.04160	.022	.0190	.2810
		Fibrin Gel	-.30000(*)	.04160	.000	-.4310	-.1690
		*CDM	-3.15000(*)	.04160	.000	-3.2810	-3.0190
		**CDM	-1.40000(*)	.05095	.000	-1.5604	-1.2396
	Collagen Gel	CDM	-.15000(*)	.04160	.022	-.2810	-.0190
		Fibrin Gel	-.45000(*)	.04160	.000	-.5810	-.3190
		*CDM	-3.30000(*)	.04160	.000	-3.4310	-3.1690
		**CDM	-1.55000(*)	.05095	.000	-1.7104	-1.3896
	Fibrin Gel	CDM	.30000(*)	.04160	.000	.1690	.4310
		Collagen Gel	.45000(*)	.04160	.000	.3190	.5810
		*CDM	-2.85000(*)	.04160	.000	-2.9810	-2.7190
		**CDM	-1.10000(*)	.05095	.000	-1.2604	-.9396
	*CDM	CDM	3.15000(*)	.04160	.000	3.0190	3.2810
		Collagen Gel	3.30000(*)	.04160	.000	3.1690	3.4310
		Fibrin Gel	2.85000(*)	.04160	.000	2.7190	2.9810
		**CDM	1.75000(*)	.05095	.000	1.5896	1.9104
	**CDM	CDM	1.40000(*)	.05095	.000	1.2396	1.5604
		Collagen Gel	1.55000(*)	.05095	.000	1.3896	1.7104
		Fibrin Gel	1.10000(*)	.05095	.000	.9396	1.2604
		*CDM	-1.75000(*)	.05095	.000	-1.9104	-1.5896
Cell Number	CDM	Collagen Gel	-.05000	.07468	.960	-.2851	.1851
		Fibrin Gel	-1.20000(*)	.07468	.000	-1.4351	-.9649

		*CDM	-3.35000(*)	.07468	.000	-3.5851	-3.1149
		**CDM	-.80000(*)	.09146	.000	-1.0880	-.5120
	Collagen Gel	CDM	.05000	.07468	.960	-.1851	.2851
		Fibrin Gel	-1.15000(*)	.07468	.000	-1.3851	-.9149
		*CDM	-3.30000(*)	.07468	.000	-3.5351	-3.0649
		**CDM	-.75000(*)	.09146	.000	-1.0380	-.4620
	Fibrin Gel	CDM	1.20000(*)	.07468	.000	.9649	1.4351
		Collagen Gel	1.15000(*)	.07468	.000	.9149	1.3851
		*CDM	-2.15000(*)	.07468	.000	-2.3851	-1.9149
		**CDM	.40000(*)	.09146	.006	.1120	.6880
	*CDM	CDM	3.35000(*)	.07468	.000	3.1149	3.5851
		Collagen Gel	3.30000(*)	.07468	.000	3.0649	3.5351
		Fibrin Gel	2.15000(*)	.07468	.000	1.9149	2.3851
		**CDM	2.55000(*)	.09146	.000	2.2620	2.8380
	**CDM	CDM	.80000(*)	.09146	.000	.5120	1.0880
		Collagen Gel	.75000(*)	.09146	.000	.4620	1.0380
		Fibrin Gel	-.40000(*)	.09146	.006	-.6880	-.1120
		*CDM	-2.55000(*)	.09146	.000	-2.8380	-2.2620
Tension	CDM	Collagen Gel	14.00000(*)	2.71401	.002	5.2228	22.7772
		Fibrin Gel	-1.07500	2.71401	.994	-9.8522	7.7022
		*CDM	-96.33333(*)	2.90140	.000	-105.7165	-86.9501
		**CDM	-128.95000(*)	3.24387	.000	-139.4407	-118.4593
	Collagen Gel	CDM	-14.00000(*)	2.71401	.002	-22.7772	-5.2228
		Fibrin Gel	-15.07500(*)	2.51269	.001	-23.2011	-6.9489
		*CDM	-110.33333(*)	2.71401	.000	-119.1105	-101.5562
		**CDM	-142.95000(*)	3.07740	.000	-152.9024	-132.9976
	Fibrin Gel	CDM	1.07500	2.71401	.994	-7.7022	9.8522
		Collagen Gel	15.07500(*)	2.51269	.001	6.9489	23.2011
		*CDM	-95.25833(*)	2.71401	.000	-104.0355	-86.4812
		**CDM	-127.87500(*)	3.07740	.000	-137.8274	-117.9226
	*CDM	CDM	96.33333(*)	2.90140	.000	86.9501	105.7165
		Collagen Gel	110.33333(*)	2.71401	.000	101.5562	119.1105
		Fibrin Gel	95.25833(*)	2.71401	.000	86.4812	104.0355
		**CDM	-32.61667(*)	3.24387	.000	-43.1074	-22.1259
	**CDM	CDM	128.95000(*)	3.24387	.000	118.4593	139.4407
		Collagen Gel	142.95000(*)	3.07740	.000	132.9976	152.9024
		Fibrin Gel	127.87500(*)	3.07740	.000	117.9226	137.8274
		*CDM	32.61667(*)	3.24387	.000	22.1259	43.1074
UTS	CDM	Collagen Gel	54.72500	19.91175	.109	-9.6700	119.1200
		Fibrin Gel	89.97500(*)	19.91175	.006	25.5800	154.3700
		*CDM	-91.30000(*)	21.28656	.009	-160.1412	-22.4588
		**CDM	-473.95000(*)	23.79910	.000	-550.9168	-396.9832
	Collagen Gel	CDM	-54.72500	19.91175	.109	-119.1200	9.6700
		Fibrin Gel	35.25000	18.43470	.366	-24.3682	94.8682
		*CDM	-146.02500(*)	19.91175	.000	-210.4200	-81.6300
		**CDM	-528.67500(*)	22.57780	.000	-601.6921	-455.6579
	Fibrin Gel	CDM	-89.97500(*)	19.91175	.006	-154.3700	-25.5800
		Collagen Gel	-35.25000	18.43470	.366	-94.8682	24.3682
		*CDM	-181.27500(*)	19.91175	.000	-245.6700	-116.8800

		**CDM	-563.92500(*)	22.57780	.000	-636.9421	-490.9079
	*CDM	CDM	91.30000(*)	21.28656	.009	22.4588	160.1412
		Collagen Gel	146.02500(*)	19.91175	.000	81.6300	210.4200
		Fibrin Gel	181.27500(*)	19.91175	.000	116.8800	245.6700
		**CDM	-382.65000(*)	23.79910	.000	-459.6168	-305.6832
	**CDM	CDM	473.95000(*)	23.79910	.000	396.9832	550.9168
		Collagen Gel	528.67500(*)	22.57780	.000	455.6579	601.6921
		Fibrin Gel	563.92500(*)	22.57780	.000	490.9079	636.9421
		*CDM	382.65000(*)	23.79910	.000	305.6832	459.6168
Failure Strain	CDM	Collagen Gel	.04667	.02486	.382	-.0337	.1271
		Fibrin Gel	-.03083	.02486	.730	-.1112	.0496
		*CDM	-.16000(*)	.02658	.001	-.2460	-.0740
		**CDM	-.13833(*)	.02972	.005	-.2344	-.0422
	Collagen Gel	CDM	-.04667	.02486	.382	-.1271	.0337
		Fibrin Gel	-.07750(*)	.02302	.040	-.1519	-.0031
		*CDM	-.20667(*)	.02486	.000	-.2871	-.1263
		**CDM	-.18500(*)	.02819	.000	-.2762	-.0938
	Fibrin Gel	CDM	.03083	.02486	.730	-.0496	.1112
		Collagen Gel	.07750(*)	.02302	.040	.0031	.1519
		*CDM	-.12917(*)	.02486	.002	-.2096	-.0488
		**CDM	-.10750(*)	.02819	.019	-.1987	-.0163
	*CDM	CDM	.16000(*)	.02658	.001	.0740	.2460
		Collagen Gel	.20667(*)	.02486	.000	.1263	.2871
		Fibrin Gel	.12917(*)	.02486	.002	.0488	.2096
		**CDM	.02167	.02972	.945	-.0744	.1178
	**CDM	CDM	.13833(*)	.02972	.005	.0422	.2344
		Collagen Gel	.18500(*)	.02819	.000	.0938	.2762
		Fibrin Gel	.10750(*)	.02819	.019	.0163	.1987
		*CDM	-.02167	.02972	.945	-.1178	.0744
Total Collagen	CDM	Collagen Gel	-.13500(*)	.02323	.000	-.2081	-.0619
		Fibrin Gel	.34750(*)	.02323	.000	.2744	.4206
		*CDM	-.54500(*)	.02323	.000	-.6181	-.4719
		**CDM	-.92000(*)	.02845	.000	-1.0096	-.8304
	Collagen Gel	CDM	.13500(*)	.02323	.000	.0619	.2081
		Fibrin Gel	.48250(*)	.02323	.000	.4094	.5556
		*CDM	-.41000(*)	.02323	.000	-.4831	-.3369
		**CDM	-.78500(*)	.02845	.000	-.8746	-.6954
	Fibrin Gel	CDM	-.34750(*)	.02323	.000	-.4206	-.2744
		Collagen Gel	-.48250(*)	.02323	.000	-.5556	-.4094
		*CDM	-.89250(*)	.02323	.000	-.9656	-.8194
		**CDM	-1.26750(*)	.02845	.000	-1.3571	-1.1779
	*CDM	CDM	.54500(*)	.02323	.000	.4719	.6181
		Collagen Gel	.41000(*)	.02323	.000	.3369	.4831
		Fibrin Gel	.89250(*)	.02323	.000	.8194	.9656
		**CDM	-.37500(*)	.02845	.000	-.4646	-.2854
	**CDM	CDM	.92000(*)	.02845	.000	.8304	1.0096
		Collagen Gel	.78500(*)	.02845	.000	.6954	.8746
		Fibrin Gel	1.26750(*)	.02845	.000	1.1779	1.3571
		*CDM	.37500(*)	.02845	.000	.2854	.4646

Percent Collagen	CDM	Collagen Gel	-23.52500(*)	.67168	.000	-25.6399	-21.4101
		Fibrin Gel	36.02500(*)	.67168	.000	33.9101	38.1399
		*CDM	36.45000(*)	.67168	.000	34.3351	38.5649
		**CDM	1.30000	.82264	.534	-1.2902	3.8902
	Collagen Gel	CDM	23.52500(*)	.67168	.000	21.4101	25.6399
		Fibrin Gel	59.55000(*)	.67168	.000	57.4351	61.6649
		*CDM	59.97500(*)	.67168	.000	57.8601	62.0899
		**CDM	24.82500(*)	.82264	.000	22.2348	27.4152
	Fibrin Gel	CDM	-36.02500(*)	.67168	.000	-38.1399	-33.9101
		Collagen Gel	-59.55000(*)	.67168	.000	-61.6649	-57.4351
		*CDM	.42500	.67168	.967	-1.6899	2.5399
		**CDM	-34.72500(*)	.82264	.000	-37.3152	-32.1348
	*CDM	CDM	-36.45000(*)	.67168	.000	-38.5649	-34.3351
		Collagen Gel	-59.97500(*)	.67168	.000	-62.0899	-57.8601
		Fibrin Gel	-.42500	.67168	.967	-2.5399	1.6899
		**CDM	-35.15000(*)	.82264	.000	-37.7402	-32.5598
	**CDM	CDM	-1.30000	.82264	.534	-3.8902	1.2902
		Collagen Gel	-24.82500(*)	.82264	.000	-27.4152	-22.2348
		Fibrin Gel	34.72500(*)	.82264	.000	32.1348	37.3152
		*CDM	35.15000(*)	.82264	.000	32.5598	37.7402
Non-Acid and Pepsin Extractable Fraction	CDM	Collagen Gel	.80000(*)	.17041	.003	.2634	1.3366
		Fibrin Gel	1.02500(*)	.17041	.000	.4884	1.5616
		*CDM	-10.47500(*)	.17041	.000	-11.0116	-9.9384
		**CDM	-10.77500(*)	.20870	.000	-11.4321	-10.1179
	Collagen Gel	CDM	-.80000(*)	.17041	.003	-1.3366	-.2634
		Fibrin Gel	.22500	.17041	.684	-.3116	.7616
		*CDM	-11.27500(*)	.17041	.000	-11.8116	-10.7384
		**CDM	-11.57500(*)	.20870	.000	-12.2321	-10.9179
	Fibrin Gel	CDM	-1.02500(*)	.17041	.000	-1.5616	-.4884
		Collagen Gel	-.22500	.17041	.684	-.7616	.3116
		*CDM	-11.50000(*)	.17041	.000	-12.0366	-10.9634
		**CDM	-11.80000(*)	.20870	.000	-12.4571	-11.1429
	*CDM	CDM	10.47500(*)	.17041	.000	9.9384	11.0116
		Collagen Gel	11.27500(*)	.17041	.000	10.7384	11.8116
		Fibrin Gel	11.50000(*)	.17041	.000	10.9634	12.0366
		**CDM	-.30000	.20870	.616	-.9571	.3571
	**CDM	CDM	10.77500(*)	.20870	.000	10.1179	11.4321
		Collagen Gel	11.57500(*)	.20870	.000	10.9179	12.2321
		Fibrin Gel	11.80000(*)	.20870	.000	11.1429	12.4571
		*CDM	.30000	.20870	.616	-.3571	.9571
Total Proteoglycans and Glycosaminoglycans	CDM	Collagen Gel	-7.10000(*)	.47139	.000	-8.5843	-5.6157
		Fibrin Gel	-14.02500(*)	.47139	.000	-15.5093	-12.5407
		*CDM	-32.95000(*)	.47139	.000	-34.4343	-31.4657
		**CDM	-7.02500(*)	.57734	.000	-8.8428	-5.2072
	Collagen Gel	CDM	7.10000(*)	.47139	.000	5.6157	8.5843
		Fibrin Gel	-6.92500(*)	.47139	.000	-8.4093	-5.4407
		*CDM	-25.85000(*)	.47139	.000	-27.3343	-24.3657
		**CDM	.07500	.57734	1.000	-1.7428	1.8928

	Fibrin Gel	CDM	14.02500(*)	.47139	.000	12.5407	15.5093
		Collagen Gel	6.92500(*)	.47139	.000	5.4407	8.4093
		*CDM	-18.92500(*)	.47139	.000	-20.4093	-17.4407
		**CDM	7.00000(*)	.57734	.000	5.1822	8.8178
	*CDM	CDM	32.95000(*)	.47139	.000	31.4657	34.4343
		Collagen Gel	25.85000(*)	.47139	.000	24.3657	27.3343
		Fibrin Gel	18.92500(*)	.47139	.000	17.4407	20.4093
		**CDM	25.92500(*)	.57734	.000	24.1072	27.7428
	**CDM	CDM	7.02500(*)	.57734	.000	5.2072	8.8428
		Collagen Gel	-.07500	.57734	1.000	-1.8928	1.7428
		Fibrin Gel	-7.00000(*)	.57734	.000	-8.8178	-5.1822
		*CDM	-25.92500(*)	.57734	.000	-27.7428	-24.1072
Percent Proteoglycans and Glycosaminoglycans	CDM	Collagen Gel	-1.07500(*)	.07532	.000	-1.3122	-.8378
		Fibrin Gel	-.35000(*)	.07532	.003	-.5872	-.1128
		*CDM	1.05000(*)	.07532	.000	.8128	1.2872
		**CDM	1.07500(*)	.09225	.000	.7845	1.3655
	Collagen Gel	CDM	1.07500(*)	.07532	.000	.8378	1.3122
		Fibrin Gel	.72500(*)	.07532	.000	.4878	.9622
		*CDM	2.12500(*)	.07532	.000	1.8878	2.3622
		**CDM	2.15000(*)	.09225	.000	1.8595	2.4405
	Fibrin Gel	CDM	.35000(*)	.07532	.003	.1128	.5872
		Collagen Gel	-.72500(*)	.07532	.000	-.9622	-.4878
		*CDM	1.40000(*)	.07532	.000	1.1628	1.6372
		**CDM	1.42500(*)	.09225	.000	1.1345	1.7155
	*CDM	CDM	-1.05000(*)	.07532	.000	-1.2872	-.8128
		Collagen Gel	-2.12500(*)	.07532	.000	-2.3622	-1.8878
		Fibrin Gel	-1.40000(*)	.07532	.000	-1.6372	-1.1628
		**CDM	.02500	.09225	.999	-.2655	.3155
	**CDM	CDM	-1.07500(*)	.09225	.000	-1.3655	-.7845
		Collagen Gel	-2.15000(*)	.09225	.000	-2.4405	-1.8595
		Fibrin Gel	-1.42500(*)	.09225	.000	-1.7155	-1.1345
		*CDM	-.02500	.09225	.999	-.3155	.2655
UTS per Collagen Density	CDM	Collagen Gel	8.04167(*)	1.33789	.001	3.7149	12.3684
		Fibrin Gel	-11.35833(*)	1.33789	.000	-15.6851	-7.0316
		*CDM	-25.80000(*)	1.43026	.000	-30.4255	-21.1745
		**CDM	-25.48333(*)	1.59908	.000	-30.6548	-20.3119
	Collagen Gel	CDM	-8.04167(*)	1.33789	.001	-12.3684	-3.7149
		Fibrin Gel	-19.40000(*)	1.23865	.000	-23.4058	-15.3942
		*CDM	-33.84167(*)	1.33789	.000	-38.1684	-29.5149
		**CDM	-33.52500(*)	1.51702	.000	-38.4311	-28.6189
	Fibrin Gel	CDM	11.35833(*)	1.33789	.000	7.0316	15.6851
		Collagen Gel	19.40000(*)	1.23865	.000	15.3942	23.4058
		*CDM	-14.44167(*)	1.33789	.000	-18.7684	-10.1149
		**CDM	-14.12500(*)	1.51702	.000	-19.0311	-9.2189
	*CDM	CDM	25.80000(*)	1.43026	.000	21.1745	30.4255
		Collagen Gel	33.84167(*)	1.33789	.000	29.5149	38.1684
		Fibrin Gel	14.44167(*)	1.33789	.000	10.1149	18.7684
		**CDM	.31667	1.59908	1.000	-4.8548	5.4881

**CDM	CDM	25.48333(*)	1.59908	.000	20.3119	30.6548
	Collagen Gel	33.52500(*)	1.51702	.000	28.6189	38.4311
	Fibrin Gel	14.12500(*)	1.51702	.000	9.2189	19.0311
	*CDM	-.31667	1.59908	1.000	-5.4881	4.8548

* The mean difference is significant at the .05 level.

Appendix 6: Arrangement of Collagen in Cell-Derived Matrices

The collagen in the cell-derived matrices organized into several layers of alternating alignment where fibers were parallel to each other within layers, but at right angles to adjacent fibers in layers.