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Characterization of an *in vitro* engineered ligament model

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ABSTRACT

In vivo tendon and ligament research can be limited by the difficultly of obtaining tissue samples that can be biochemically analyzed. In this study, we characterize the most widely used *in vitro* engineered ligament model. Despite previous works suggesting multiple passages change gene expression in 2D primary tenocytes, we found no relationship between passage number and expression of classical tendon fibroblast markers across different biological donors. When engineered into 3D ligaments, there was an increase in maximal tensile load between 7 and 14 days in culture, that corresponded with an increase in collagen content. By contrast, percent collagen increased logarithmically from Day 7 to Day 14, and this was similar to the increase in the modulus of the tissue. Importantly, there was no relationship between passage number and mechanical function or collagen content in the two independent donors tested. These results suggest that the model develops quickly and is reliable across differing passage numbers. This provides the field with the ability to 1) consistently determine functional changes of interventions out to passage number 10; and 2) to time interventions to the appropriate developmental stage: developing/regenerating (Day 7) or mature (Day 14) tissue.

Introduction

Ligaments and tendons are fibrous connective tissues made by specialized fibroblasts that, during development, express the basic helix-loop-helix transcription factor scleraxis (SCX) [1] and reside within a dense extracellular matrix (ECM). These SCX^+ fibroblasts synthesize and maintain the ECM, which largely consists of aligned type I collagen.

Even though ligaments and tendons are generally grouped together due to similarities in molecular composition, structure, and function [2], there is an important functional difference between the two. Ligaments connect two stiff tissues (bone to bone), whereas tendons connect a stiff tissue to a dynamic compliant tissue (bone to muscle). This functional difference, results in mechanical variation along the length of a tendon that is not present in ligament [3]. Therefore, when materials of the same stiffness are at either end, it is appropriate to describe the tissue as a ligament. Acknowledging this fundamental difference between the tissues, all structures containing aligned collagen as the primary component of the tissue that have been engineered to date should be referred to as ligaments, even though they can likely model the development and adaptation of either tissue.

In vivo, tendons and ligaments are composed of an extensive extracellular matrix and low cell number which can make biochemical analyses such as qPCR or western blotting difficult [4]. Further, protein turnover within adult human tendons may be very low resulting in difficulties quantifying changes in total protein following interventions [5]. Engineered ligaments were developed to overcome these issues and produce an *in vitro* model that could provide cellular, developmental, and biochemical insights into the response of tendon/ligament cells to stress without compromising the ability to determine the mechanical characteristics of the tissue.

There are several ways in which *in vitro* engineered ligament models may not truly represent the native tissue. In a two-dimensional (2D) cell culture setting, increasing passage number may cause de-differentiation of the primary cells [4,5], resulting in cells that do not reflect the native tissue. In the three-dimensional (3D) setting, the tissues have more cells and less extracellular matrix than adult tendons *in vivo* [6], they express more developmental isoforms of collagen [7], have uniform small collagen fibrils [8], and as a result are much weaker than adult tissues [9]. In many ways, engineered ligaments are therefore more comparable to developing ligaments than their adult counterparts [10]. Even with these limitations, engineered ligaments have similar responses to nutrients [9], growth factors [11], hormones [12], cytokines [13], and

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exercise [14,15] to those reported for native tendons or ligaments. These data suggest that engineered ligaments may be a good model for understanding the physiology of these complex connective tissues if they were better characterized.

Despite demonstrated effectiveness of engineered ligaments, further characterization of the effect of cell passage number and developmental stage of the organoid is still needed. In this study, we set out to further characterize this model by investigating the effect of increasing passage number on gene expression in a 2D environment as well as mechanical function of 3D engineered ligaments. Furthermore, we characterized the development of human engineered ligament matrix and mechanical properties over time. Ultimately, our objective was to provide insight into the methodology so that engineered ligaments could better model native tissues *in vivo*.

Methods

The human anterior cruciate ligament (ACL) remnants used for cell isolation were obtained following informed consent and all procedures and experiments were approved by the University of California Davis Institutional Review Board (IRB# 779755- A tissue collection study for patients undergoing anterior cruciate ligament (ACL) reconstruction).

Human ACL fibroblast isolation

Fibroblast isolation was performed as previously described [9]. Briefly, the remnants of human ACLs were placed into a sterile 50 ml tube and washed 5 times with 25 ml of Dulbecco's phosphate buffered saline (DPBS). The tissue was then transferred to a sterile 50 ml tube and stored in 25 ml 0.5x antibiotic/antimycotic solution at 4 °C for 1 h. The remnant was then transferred to a sterile 50 ml tube containing 25 ml of a 0.1 % Collagenase Type II Solution (Dulbecco's Modified Eagle Medium (DMEM), 20 % fetal bovine serum (FBS), 1 % penicillin, 0.1 % collagenase type II) and incubated at 37 °C overnight. The following day the tube was centrifuged for 5 min at 2000 x g, the supernatant aspirated, and washed with 10 ml of growth media (GM; DMEM containing 10 % FBS). Washing with GM twice before the cells were plated on 15 cm cell culture treated plates. The freshly isolated cells were considered passage zero (P0).

Passaging cells

When the cells reached approximately 70 % confluence, they were expanded by trypsin-triggered cell dissociation and passaging onto new plates. The growth media was replaced every 3 days until the cells were ready to passage. At each point, cells were passaged 1:5 with one plate at each passage used for gene expression analysis and four for expansion/engineering ligaments. For engineered ligaments, cells were passaged until sufficient plates 20–25 were produced.

RNA Extraction/qPCR

Human ACL cells were grown in a 2D environment and collected from plates from passage zero until passage seven. Cells were collected in Trizol and vortexed to release RNA. After centrifugation for five minutes at 10,000xg, chloroform extraction of the aqueous solution phase, precipitation with isopropanol, and washing with ethanol, RNA was resuspended and quantified. One microgram of RNA was reverse transcribed (MultiScribe RT, 10x RT buffer, 10x Primers, dNTPs, RNase inhibitor; Applied Biosystems, Foster City, CA) and then diluted to 3 ng/µl prior to qPCR. Samples were amplified in triplicate with 3 µl cDNA, 5 µl SYBR green (BioRad, Hercules, CA; PCRbio.com, Wayne, Pennsylvania), and 2 µl of 10 uM primer (Invitrogen, ThermoFisher, Waltham, MA). qPCR reactions were performed on a CFX384 Touch Real-Time PCR Detection System (BidRad, Hercules, CA). GAPDH was used as housekeeping control. Absolute CT for GAPDH was not different between groups. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Construct plate preparation

Minutien pins (0.2 mm) were placed into 1.5 mm tear drop anchor molds. The solid phase (β-tricalcium phosphate powder, Plasma Biotal Limited, Derbyshire, UK) was mixed in equal parts (1 g:1ml) with ice cold liquid phase (3.5 M *ortho*-phosphoric acid with 100 mM citric acid) and then pipetted into molds. Molds were centrifuged for 1 min at 2250 x g at 4 °C, then left overnight to set. The next day, the anchors were removed from the molds and pinned 12 mm apart into Sylgard PDMS (Dow, Midland, MI) coated 35 mm plates. Plates were sterilized in 70 % ethanol for 1 h.

Engineering ligaments

Constructs were engineered as previously described [9]. For each construct, $2.5x10^5$ cells were suspended in 714 µl growth medium containing 5.8 Units of thrombin, 20 µg aprotinin, and 200 µg 6-aminohexanoic acid. This mixture was added to a 35 mm plate with brushite anchors and shaken to make sure the solution covered the plate. To the thrombin/cell mixture, 286 µl of fibrinogen (20 mg/ml) was added and the plate was incubated at 37 °C with 5 % CO_2 for 15 min to allow fibrin-gel formation. After 15 min, 2 ml of feed media (GM supplemented with 200 µM ascorbic acid, 50 µM proline, and 5 ng/ml of transforming growth factor-β1(TGF-β1)) was added to each plate. Feed media is replaced every other day until testing (Fig. 2).

Mechanical testing

On day of testing, constructs were removed from the incubator, and the length measured using digital calipers as the distance between the inside points of the teardrop anchors. The media was aspirated, the plates rinsed with Dulbecco's modified phosphate buffered saline (DPBS) and the width and thickness of the tissue were determined by spectral-domain optical coherence tomography using a OQ Labscope (Lumedica, Durham, NC). After measuring width and depth, 2 ml of DPBS were added back to the plate to keep ligament hydrated until testing. Ligaments still attached to anchors were loaded into 3D printed grips in the test space of a Model 68SC-1 single column tensile tester (Instron, Norwood, MA) containing a 10 N load cell. The grips were printed to secure each brushite anchor with an appropriate opening for the tissue to fit through. At later timepoints when anchor degradation became a limitation, constructs that were mechanically tested were those whose anchors were still able to be loaded into grips without failing. The samples were submerged in 37 °C saline within the temperature controlled BioBath, and mechanically tested using 10 cycles of preconditioning to 0.10 N at a rate of 0.25 mm/s prior to loading to failure at a constant rate of 0.25 mm/s. From the test, the maximum tensile load (MTL), failure stress, modulus, and cross-sectional area (CSA) were determined (Fig. 2). MTL is the maximal load measured at failure in Newtons. Failure stress was calculated by normalizing the MTL by CSA (width x depth) and the Young's modulus was calculated as the maximal slope of the stress–strain (displacement divided by the initial length) curve. The position of graft failure was noted, and this data is presented as the % of grafts tested that failed at either the anchor or midsubstance. After testing, ligaments were removed from anchors and dried on a glass plate at 120 °C for 30 min. Dry mass was measured, and samples were left at room temperature until processed for hydroxyproline.

Hydroxyproline assay

To determine collagen content, a hydroxyproline assay was performed [16]. Briefly, 200 µl of 6 N HCl were added to previously dried samples in 1.7 ml snap-cap tubes. Samples were hydrolyzed on a heat

block at 120 °C for two hours (using micro-tube cap locks to prevent cap from opening during boiling). After two hours, the samples were transferred to a heat block in a laminar flow hood for another 90 min, this time with the tube lids opened to allow evaporation. After drying the samples, 200 µl of hydroxyproline buffer were added to each sample. Samples were further diluted 1:20 in hydroxyproline buffer and 150 µl of 14.1 mg/ml Chloramine T solution was added to each sample before vortexing and incubating at room temperature for 20 min. Aldehyde-perchloric acid containing 60 % 1-propanol, 5.8 % perchloric acid and 1 M 4-(dimethylamino)benzaldehyde (150 µl) was added to each sample before vortexing and incubating at 60 °C for 15 min. Once removed from heat and allowed to cool at room temperature, 200 µl of each standard and sample were loaded in duplicate on a 96 well plate and absorbance was read at 550 nm on an Epoch Microplate Spectrophotometer (BioTek Instruments Limited, Winooski, VT). Hydroxyproline content of samples was calculated using a standard curve and then converted to total collagen by assuming hydroxyproline makes up 13.7 % of the dry mass of collagen. The collagen content was then divided by the dry mass of each sample to determine the percent collagen.

Statistical analysis

For all assays, a technical replicate was a single engineered ligament or 2D tissue culture well within a group at a given time. Biological

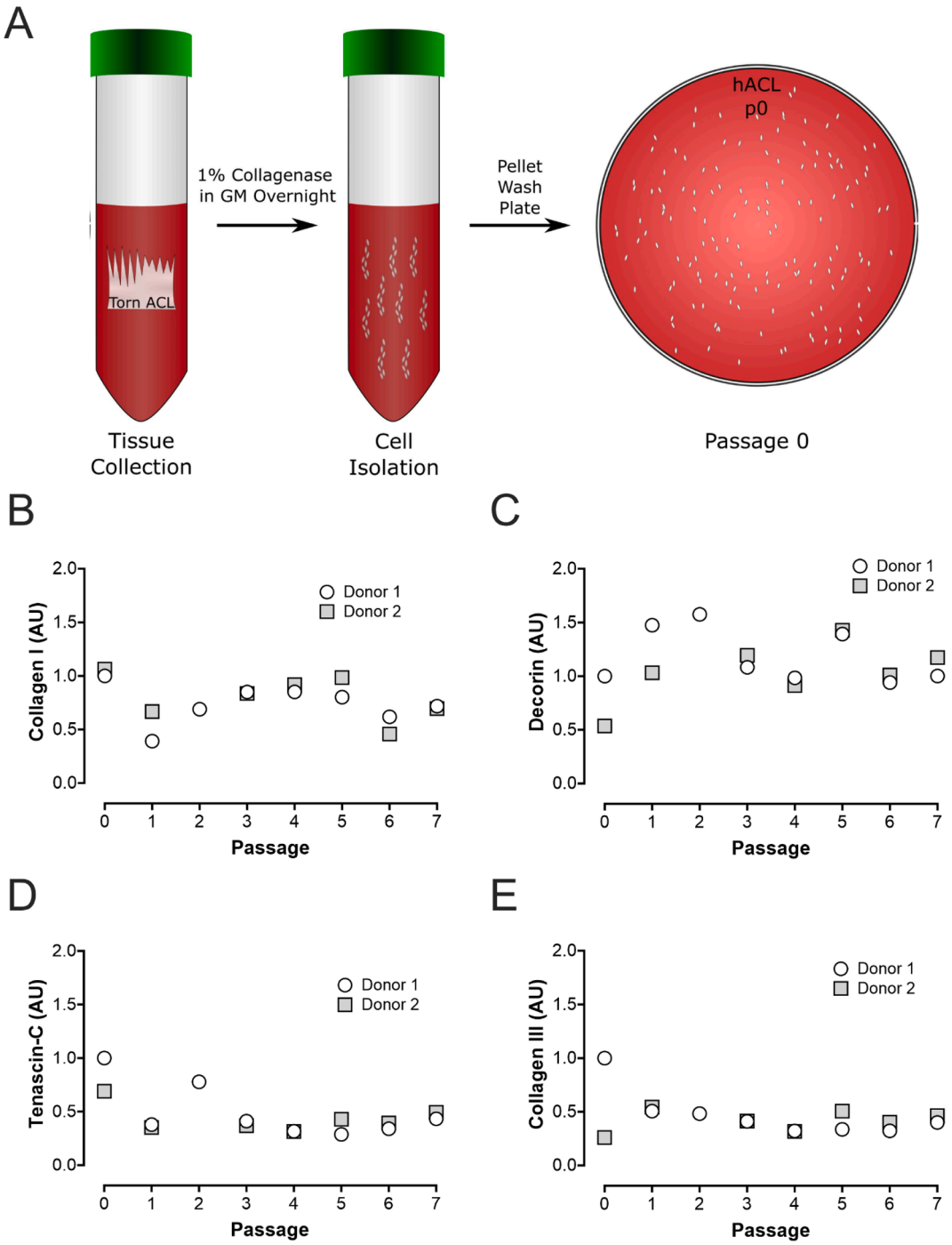

Fig. 1. A) Schematic of the Isolation of Human ACL Cells and Gene Expression o as a Function of Passage Number. Relative gene expression did not change as a function of passage number for **B)** Decorin, **C)** Tenascin-C, **D)** Collagen I, **E)** Collagen III. n = 2 biological replicates.

replicates reflect that the experiments were repeated using a different cell donor or a separate vial of cells from the same donor. Each experiment presented here therefore represents a single biological replicate that was analyzed independently using a one-way ANOVA with time as the primary factor of interest using GraphPad Prism v9. Where statistical differences were detected, a Tukey's honestly significant difference test was used for post-hoc analysis since all groups demonstrated equal variance. Linear regressions were performed separately and both linear and non-linear fit were calculated using Prism. Statistical analyses and the type I error was maintained at $\alpha < 0.05$ for all comparisons.

Results

After obtaining human ACL remnants from two different biological donors, cells were isolated and passaged multiple times (Fig. 1A). At each passage, starting at 0, one plate of cells was collected to quantify gene expression of classical markers of tendon differentiation. We found that there was no change in expression of decorin, tenascin-C, collagen I, or collagen III as a function of passage number in either donor (Fig. 1).

To determine the optimal time to mechanically test engineered ligaments, we characterized the cellular and mechanical development of

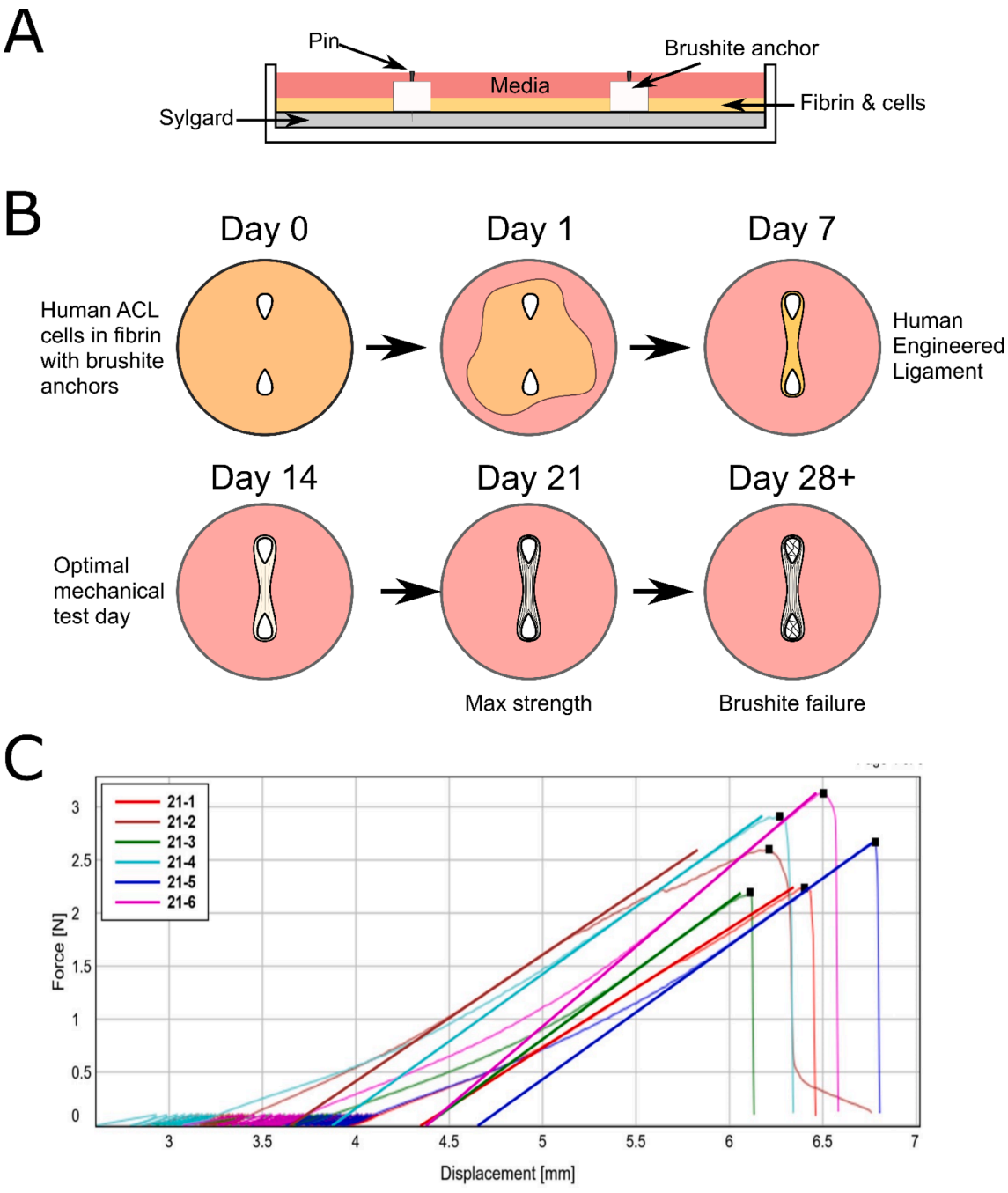

Fig. 2. Schematic of the Formation of Engineered Ligaments. **A)** Ligaments are formed in a 35 mm plate by combining a fibrin/cell mixture and plating this mixture on PDMS substrate containing two brushite anchors. Once the fibrin gel forms, the grafts are fed 2 ml growth media. **B)** The fibrin gel is contracted by the cells around the brushite anchors within the first 4–5 days. Between Day 7 and Day 14 the cells digest the fibrin matrix and replace it with an endogenously produced native collagen matrix (represented by a shift from yellow (fibrin) to white (collagen)). Finally, the resorption of the brushite within the anchor is depicted at Day 28. **(C)** The load – elongation curves for the 21-day-old engineered human ligaments reported in Fig. 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

human engineered ligaments as a function of days in culture. All samples reported at each timepoint were made on the same day with the same batch of fibrin, thrombin, and media. The experiment was repeated on three separate occasions and the results were qualitatively identical. At Day 7, three of the six ligaments in the reported cohort failed during preconditioning and one failed before precondition, indicating that at this early time point the ligaments are extremely weak. Mechanical testing revealed a rapid increase in the maximal tensile load at failure (MTL; $r^2 = 0.917$ for a linear fit and 0.932 for an exponential fit) with over a 10-fold increase in mean MTL from Day 7 to Day 14 and a further 2.5-fold increase in mean from Day 14 to Day 21 (Fig. 3A). After day 21, the anchors became mechanically unstable resulting in a drop in MTL due to poor sample gripping. There was a similar rapid increase in failure stress ($r^2 = 0.951$ for a linear fit and 0.939 for an exponential fit) with over a 15-fold increase from Day 7 to Day 14 and a further 2-fold increase from Day 14 to Day 21 (Fig. 3B). We did not include Day 28 or Day 35 in statistical analysis of MTL or failure stress due to the degradation of the anchors and the resulting lowering of the absolute strength of the tissue. The increase in modulus ($r^2 = 0.904$ for a linear fit and 0.85 for an exponential fit), a mechanical property that is measured at ~ 25 % maximal load, followed a slightly different pattern increasing 6-fold between Day 7 to Day 14 and a further 1.5-fold increase from Day 14 to Day 21 (Fig. 3C). Since the stability of the anchor is less important for modulus, we were able to observe a plateau in modulus between days 21 and 35. With maturation, the location of graft failure changed from at/near the anchor at days 7 and 14 to the midsubstance of the tissue at days 21 and 28 (Fig. 3E).

In parallel with the increase in mechanical function with time, there was an increase in collagen content and concentration. Collagen content increased nearly 10-fold from Day 7 to Day 21 ($R^2 = 0.958$ for a linear fit and 0.947 for an exponential fit) and this increase appeared to be associated with the increase in MTL ($R^2 = 0.958$). Unlike MTL, collagen content continued to increase from Day 21 to Day 28 but did not change between Day 28 and Day 35 (Fig. 3F). The percent of the dry mass of the tissue that was collagen (percent collagen) showed a rapid increase from Day 7 to Day 14 and then plateaued between Day 14 and 35 (Fig. 3G). Dry mass of the tissue tended to increase linearly with time ($R^2 = 0.759$) increasing 2-fold from Day 7 to Day 35 (Fig. 3H).

From the developmental data, we felt that day 14 was the best day to test engineered ligaments since mechanics and collagen were high but had yet to reach maximum tensile strength. We felt this was important since it would allow us to observe interventions that improved and/or impaired function and collagen accumulation. To determine the effect of passage number on human engineered ligament structure/function, ligaments were engineered for six consecutive passages for the two different donors and tested 14 days after plating. There was no significant change in MTL, failure stress, and modulus as a function of passage number in either the female (Fig. 4A–C) or male (Fig. 4G–I) donor. Similarly, there was no change in total collagen, percent collagen, or tissue dry mass as a function of passage number in either the female (Fig. 4D–F) or male (Fig. 4J–L) donor.

Discussion

Despite previous studies demonstrating a shift in gene expression of primary cells *in vitro* [4,5], we found that through multiple passages (0–7) human ACL cells continue to express common tendon cell markers. When engineering ligaments, the tissues develop rapidly from a mechanically weak, collagen poor, tissue at day 7 to strong, collagen dense tissue at day 21. From day 21 to 35, the tissues continue to add collagen; however, the brushite anchors used in the current work begin to dissolve, limiting the strength of the full tissue. When ligaments are tested at day 14 for six contiguous passages there is no loss in either collagen or mechanics, indicating that human ACL cells can be passaged at least 11 times before the function of the ligaments are altered.

Previous studies in the field demonstrated multiple passages of cells isolated from tendons and ligaments and then cultured in a 2D environment showed changes in gene expression [6,7]. When we repeated this work, we did not find that human ACL cells de-differentiated in culture. Not only did the cells not de-differentiate at the level of gene expression, but we also did not detect any noticeable change in proliferation rate; however, this was not actively quantified. There are a few possible explanations for the differences between our findings and those of others. First, our cells were isolated from the remnants of human ACLs following rupture. These tissues are typically collected 2–3 months after injury. The result is that the cells that we are isolating may be different than those taken from an intact ACL or hamstring tendon. We have previously isolated cells from cadaveric human ACLs and hamstring

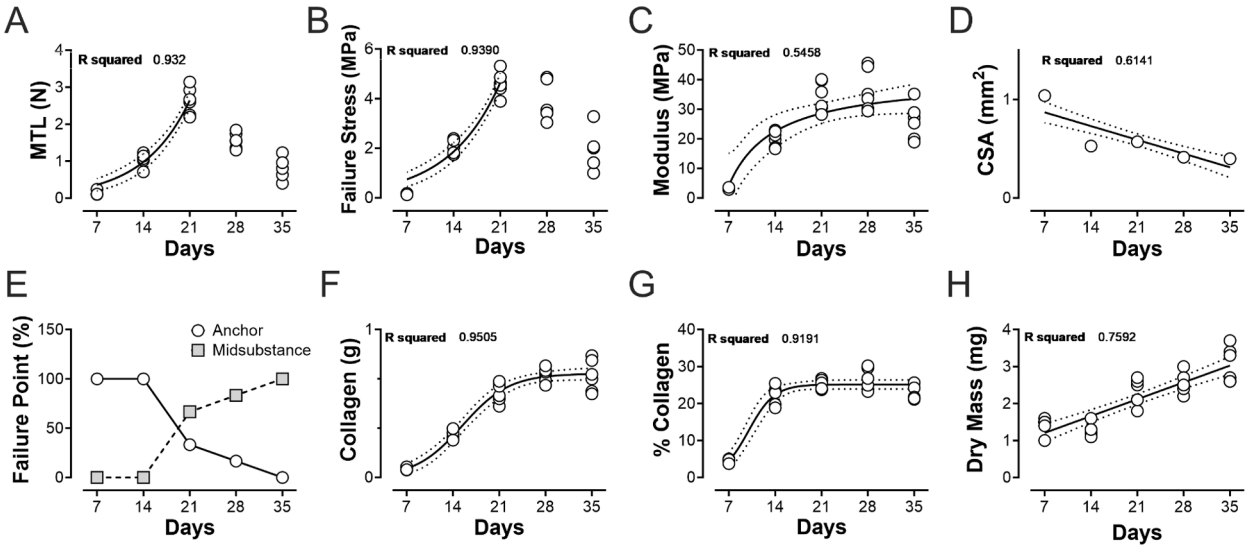

Fig. 3. Construct Development Over Time. Mechanical **(A)** maximal tensile load (MTL) and material **(B)** failure stress and **(C)** modulus show a rapid increase from Day 7 to Day 21 before regressing or plateauing. **(D)** Cross sectional area (CSA) decreases linearly as a function of time in culture. **(E)** Failure location, the location within the tissue where failure occurred changes with time. Collagen expressed either as **(E)** total content or **(F)** percent of dry mass rapidly increases before plateauing at day 21 and 14, respectively. **(G)** Dry mass increases linearly as a function of time in culture ($R^2 > 0.759$). Dotted lines represent 95 % confidence interval. Dots are the technical replicates (n = 6) at each time point. Qualitatively identical data were seen in three biological replicates of the experiment using at least two different donors.

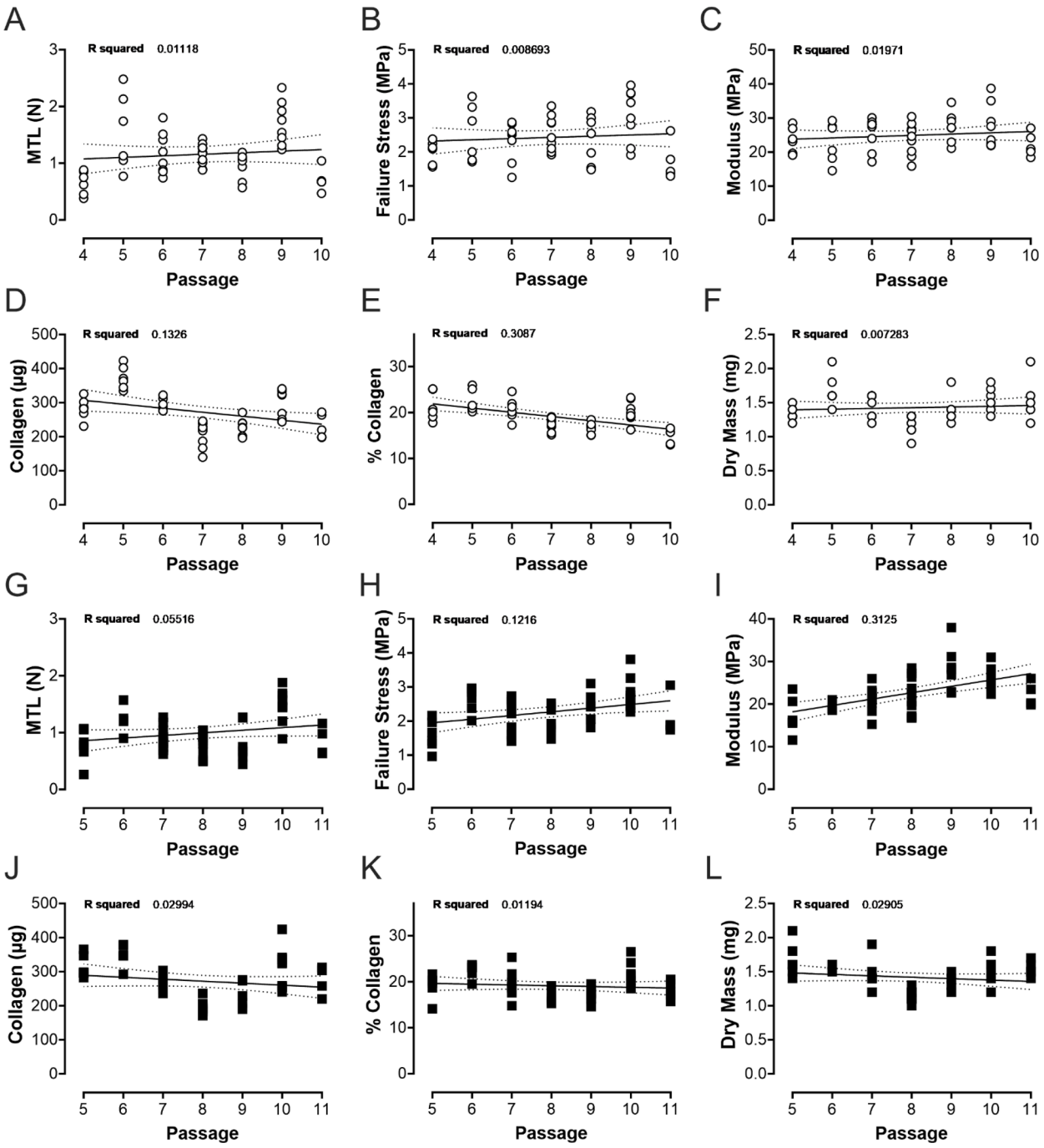

Fig. 4. Construct Mechanics and Collagen as a Function of Passage Number. **A-F)** Are data from a 23-year-old female donor, whereas **G-L)** were collected on constructs made from a 31-year-old male donor. **A-C, G-I)** Mechanical and material properties do not change as a function of passage number. **D-F, J-L)** Total collagen content, percent collagen, and dry mass also do not change as a function of passage number. Dots represent the technical replicates (n = 5–10) at each passage. Solid lines are lines of best fit (R^2 is reported for the line used). Dotted lines represent 95 % confidence interval.

tendons collected during ACL reconstruction and these cells produce qualitatively normal engineered ligaments. Further, Herchenhan and others from the Kjaer group have similarly produced engineered human ligaments from human hamstring tendons [7,17,18]. Therefore, we do not feel that the cell type is the only issue underlying the difference in cell identity as a function of passage number. Other differences between the current work and previous experiments include the fact that previous studies used 100 mg/ml streptomycin in the culture medium and grew their cells to a higher confluence before passaging. In their manuscript, Mazzocca and colleagues show plates prior to passaging (Fig. 1 in [4,5]) and the cells are completely confluent. A key component of our culturing process is not to let the cells get over 70 % confluent. It is possible that passaging at a relatively low confluence may help maintain cellular function.

It is important to understand the development of human engineered ligaments as a function time to better use them to model native tissues. Therefore, we measured collagen content and mechanical properties from Day 7 to Day 35. Interestingly, the shifts in mechanics and collagen that we observed between days 7 and 21 mirrored what was recently shown in the development of rat patellar tendon from Day 7 to Day 28 [19]. *In vivo,* development of the patellar tendon showed a rapid increase in collagen content and concentration that was coupled with

improved mechanical properties. For example, between day 7 and 28 the collagen content and MTL of the rat patellar tendon both increased 9-fold [19]. In the current work, the engineered ligament collagen content similarly increased 9-fold from day 7 to 21. Although the engineered ligaments in this model demonstrated similar mechanical development to what was previously described in rat, these engineered ligaments are still significantly smaller and weaker than an adult human ACL. The average cross-sectional area and mechanical properties of an adult male cadaver ACL are: CSA = 72.9 mm; MTL = 1818 N; failure stress = 26 MPa; Modulus = 128 MPa [20]. Compared to the average Day 21 values of engineered ligaments, these measurements are 128-fold, 692-fold, 5.5-fold, and 4-fold higher, respectively. Interestingly, the material properties indicate that the tissues are within 5-fold of the native ACL. Despite the high material properties in the engineered ligaments, *in vivo* collagen accounts for approximately 80 % of the dry mass of a human ACL, whereas the engineered ligaments plateaued at approximately 25 %. Potential explanations for this are that the *in vitro* ligaments maintain a higher cell mass than the ~ 10 % value seen *in vivo*; residual fibrin within the matrix may accounts for some of the dry mass; and the density and diameter of the collagen fibrils are likely significantly lower than native tissue. Further research will be needed to investigate matrix organization and composition with maturation of the engineered ligaments. Currently, the engineered ligaments are smaller and weaker with a less developed matrix and greater cellularity. Despite these differences, the data presented demonstrates similarities in development to an *in vivo* tissue even in the absence of the active (movement) and passive (bone growth) forces seen *in vivo*. Additionally, the greater cellularity of engineered ligaments may make interventions and biochemical analysis easier, adding to the usefulness of such a reliable *in vitro* ligament model.

The material properties reported here are some of the highest for engineered tendons/ligaments. For example, Puetzer and colleagues have engineer elegant tendon/ligaments with an integrated compressed interface [21]. Those tissues are larger than the ligaments reported here. Using advanced imaging, they demonstrate that the collagen fibrils within the tissue are well aligned, and like this model max out at ~ 35 nm [6,8]. However, the material properties of their tissues are an order of magnitude lower (modulus = 1 MPa and failure stress = 0.4 MPa at 6 weeks) than those reported here. There are a few possible explanations for these differences. Most importantly, Puetzer uses a collagen gel to form ligaments. Using a collagen gel can affect the development of an engineered ligament since a high-density collagen gel is significantly stiffer than the fibrin used in the current model, and this may decrease the tensional strain on the cells and limit endogenous collagen production. A second issue that arises when using a collagen gel is that the large collagen molecules formed during gelation are likely difficult for cells to modify. By contrast, tendon and ligament cells within a fibrin gel create an endogenous matrix using fibropositors to align the matrix along the line of force [6]. A last possible issue is that within a collagen gel the cells lack developmental cues. Since fibrin is the matrix present when cells begin to form/regenerate a tendon in vivo, the stiffness, structure, and biochemistry likely provide proliferative cues. As this developmental matrix is replaced by a stiffer, cell-derived, collagen matrix, new cues for maturation likely help the cells quickly differentiate and drive the production and stabilization of collagen necessary to increase material properties.

The rapid increase in total and relative collagen (day 7–14) followed by a maturation phase (day 14–35) demonstrated by the plateau in both measures suggests the model can be used to study interventions targeting either a developing/regenerating tissue (days 7–14) or a mature (days 14–21) tissue. During development/regeneration *in vivo,* tissues have increased vascularity [22], cellularity [23], lower collagen content [23], and increased collagen synthesis [24]. This is similar to days 7–14 in the human engineered ligament model. On the other hand, interventions designed to target a more mature tissue, identified by reduced collagen turnover [25] and a more developed matrix, would be better modeled between days 14 and 21.

One interesting finding from the timecourse experiment was that as the engineered ligaments matured, the location of graft failure changed. Early in the process, the ligaments would always fail at the interface between the soft biological tissue and the hard ceramic anchor. The progressive increase in the strength of this interface over time could reflect that as the brushite anchor was beginning to be resorbed the biological matrix of the ligament began to better integrate with the hard calcium phosphate, as we have previously seen by Raman microscopy [9]. As the anchor and the ligament better integrated, the weakness of the tissue shifted to the midsubstance resulting in higher failure rate at the midsubstance, as is often observed *in vivo* where the enthesis is mechanically stronger than the tissue proper [26].

A major limitation to the long-term study (days 28–35) was the degradation of the anchor seen from day 28 onwards. The integrity of the anchor was maintained out to day 21 as evidenced by the high MTLs. However, by day 28 there was noticeable cracking along the anchor surface and the ligaments would pull through the 3D printed grips resulting in lower maximal mechanical properties despite increased total collagen in the construct. The loss of anchor integrity is the result of our use of brushite cement. Originally, when the goal of engineering ligaments was to produce a graft that could be used for ACL reconstruction, we chose a 1:1 (powder:liquid) ratio of βTCP:orthophosphoric acid to make the brushite used as the anchor material for its ability to be rapidly resorbed and replaced by biological tissue *in vivo* [27]. These brushite anchors were never optimized for long-term culture. In fact, FBS is known to accelerate the degradation of 1:1 (P:L) ratio brushite [28]. A simple fix for those looking to keep engineered ligaments for longer than 21 days would be to switch to a 3:1 βTCP:orthophosphoric acid brushite, which loses two thirds less mass in cell culture [27], or calcium deficient hydroxyapatite, which is not passively or actively degraded *in vitro* [29], as the anchor material. This simple change in anchor material would permit much longer studies of *in vitro* tissue development.

We chose day 14 as the best day for determining whether the mechanical properties and collagen content of engineered ligaments changed with increasing passage number. This time point was chosen since it was in the linear range of the increase in both the mechanical properties and collagen content of the organoids. From this point, we would be able to detect both increases and decreases in function as passage number increased. However, when taken together, there were not differences in either mechanics or collagen as a function of passage number. There were fluctuations in mechanics and collagen from passage to passage; however, we attribute this to the variability inherent in the technique as well as small changes in the biological components of the fibrin gel and media. Importantly, the highest average MTL and total collagen were measured at passages 5 and 9 in one biological donor, and passage 6 and 10 in the other. These data demonstrate that any passage out to at least 11 would all be appropriate in future studies utilizing this engineered ligament model.

Overall, we have characterized the development and progression of a popular *in vitro* human engineered ligament model and have shown that it produces reliable and reproduceable data out to at least passage 11. The protocol is straightforward, requiring only basic sterile technique and common cell culture materials. We suggest that using a media without 100 mg/ml streptomycin and passaging cells at lower confluence may prevent de-differentiation of primary human ACL cells and allow researchers to engineer consistent ligaments (mechanical properties and matrix composition) from passage 4 to 11. We propose that this human engineered ligament model can be used to represent a developmental or injured tissue when interventions are performed between days 7 and 14, as well as a more mature tissue when interventions are performed from day 14–21. Lastly, when using a 1:1 βTCP:orthophosphoric acid ratio to make brushite anchors, engineered ligaments should be mechanically tested by Day 21 to ensure anchor integrity. If longer interventions are desired, a different anchor material should be

used.

Summary

Repeated passage of human ACL fibroblasts does not alter cell phenotype or the function of engineered ligaments. The mechanical properties of *in vitro* human engineered ligaments show similar development over time to that of *in vivo* animal tissues, suggesting that this is a robust and reliable *in vitro* model of tendon/ligament.

CRediT authorship contribution statement

Alec M. Avey: Conceptualization, Formal analysis, Methodology, Writing – original draft. **Omar Valdez:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Keith Baar:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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