EXPERIMENTAL STUDY

In vitro changes in human tenocyte cultures obtained from proximal biceps tendon: multiple passages result in changes in routine cell markers

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Abstract

Purpose Results of in vitro cell models are commonly used to promote new therapies (e.g., platelet-rich plasma), and clinicians have to be aware of the specific limitations of such models. To gain a sufficient and effective cell load, many current in vitro models use cells multiplied through various passages. This is especially important in tendon-like cell (TLC) models, since native tendon tissue is not available unlimited and contains limited amount of tenocytes. The purpose was to determine the occurrence of phenotypic changes following extended monolayer culture of TLCs, according to cell-passage number.

Methods Tendon samples were obtained from 15 healthy patients undergoing biceps tenodesis. Tendons were digested and cultured (monolayer) for six passages. Tendon-specific markers (collagens I and III, decorin, tenascin-C, and tenomodulin) and their histology were analyzed using gene expression and protein content assays. Native cells, the cells cultured and cells passaged one to six times were analyzed at each passage.

Results Gene expression of types I and III collagen of cultured TLCs significantly decreased after two passages. Gene expression of decorin, tenascin-C, and tenomodulin exhibited a trend of decreased gene expression with increased passage. Protein levels of types I and III collagen and decorin decreased after four passages.

Conclusions The significant findings let conclude that tenocyte-like cells obtained from human LHB can be maintained in monolayer culture at low passages, before the signs of phenotypic drift are present. But researchers must be aware of rapid phenotypic drift at higher passage numbers. Therefore, only cells within the first 3 passages should be used as a precaution for in vitro monolayer cell models, and one has to be aware of the phenotypic changes if TLCs passaged multiple times are used. The clinical relevance of this data is that understanding of in vitro TLC models, and their limitations may finally help the clinician to judge the potential of experimental data of new biologic treatment options.

Keywords Tenocytes · Cell passage · Cell culture · Phenotypic drift

Introduction

In vitro models of human tenocytes or more precisely named tenocyte-like cells (TLC) are frequently performed to evaluate specific effects of treatment methods on cells, since they offer a valuable platform for the manipulation of tenocytes using drugs or various biomaterials [4–6, 10–13, 20, 23–25]. Results of such models are commonly used to promote new therapies (e.g. platelet-rich plasma), and clinicians have to be aware of the specific limitations of such models. Understanding TLC models and their limitations may finally help the clinician to judge the potential of experimental data of new biologic treatment options [8, 11, 15, 23].

The isolation of TLC from human tissue (specifically supraspinatus (SSP) and long head of the biceps (LHB) tendons) obtained in routine shoulder surgery has been shown to produce sufficient results for characterization of cells passaged three times [13]. However, TLCs are difficult to characterize in vitro, which is mainly due to the

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variation in existing tendon markers for gene expression and protein analysis [13]. Components of the extracellular matrix that are specifically produced by tendon cells in the human body offer potential phenotypic markers for tenocytes. Therefore, combinations of different molecular markers are currently used to evaluate tendon cells [2, 5]. The combined use of gene expression markers for collagens I and III, decorin, tenomodulin, scleraxis, and tenascin-C seems to be adequate to define TLCs.

However, besides the difficulties in cell characterization all in vitro models have in common that a certain overall number of tenocytes is needed to yield valuable results. Therefore, passaged TLC cultures with multiplied cell numbers are commonly used for in vitro experiments. It is known that multiple passaging of cells leads to phenotypic drift and this poses a problem in tenocyte research because freshly cultured tenocytes are not manifold available in a sufficient amount. Studies have shown the potential of tenocyte phenotypic drift after prolonged maintenance in monolayer cell culture [1, 3, 10, 16, 19, 24]. Tenocytes maintained in monolayer culture stop producing tendon-specific proteins at the levels present in freshly cultured tenocytes [1, 10, 24]. Therefore, a timeline (passage number) is needed to show how these isolated cells behave and what effects have to be expected regarding the tendon markers compared to non-cultured cells. Such changes must be considered when in vitro cell models are used. Although already reported for cells isolated from Achilles tendons from patients with primary tendon ruptures, these timelines should be established for each specific tissue type and location [24].

Being aware of the recent literature, the goal of this study was to evaluate cell passage-dependent changes in the specific marker profile of TLCs obtained from LHB tissue samples. The hypothesis was that tenocyte-like cells cultured in monolayer obtained from LHB tendon tissue will present a phenotypic drift and cells passaged multiple times express a different profile of routine markers than cells passaged one or two times.

Materials and methods

Tissue sample collection

The total number of included samples was 15 tendons obtained from discarded LHB tendons during subpectoral LHB tenodesis performed in the context of rotator cuff repair or non-repairable SLAP lesions (mean age: 51.3 ± 9 years; 9 men and 6 women) at the University of Connecticut Health Center and Farmington Surgical Center (IRB # 07-224). Basic exclusion criteria were hepatitis, HIV, and any specimen lacking complete clinical,

histological, and molecular analysis. Tendon specimens were immediately brought to the laboratory and processed. The senior author analyzed each tendon thoroughly to make sure that the sample appeared macroscopically healthy. Specimens that were torn, discolored, highly vascularized, or not representative of normal tendon were discarded and not used in this study.

Tenocyte cell culture

TLC culture was obtained in accordance with previously published methods [13]. The proximal first 1 cm of the biceps tendon was discarded in an attempt to obtain cells from the distal tendon, which is supposed to be less mechanically affected tissue [7]. If tendons appeared macroscopically damaged or demonstrated any higher grade of degenerations, they were excluded. The tendon sheath was cut away to avoid isolation of cells from connective tissue other than the LHB tendon (Fig. 1). Tendons were cut into 5×5 mm pieces. The tissue was digested in 2 mg/ml collagenase (Sigma-Aldrich, St. Louis MO) for approximately 3.5 h at 37°C until digestion was completed. The digested solution was filtered through a 70-µm nylon mesh cell strainer (Fisher Scientific, Waltham MA) to remove undigested tissue and centrifuged for 10 min at 1,500 rpm. The supernatant was removed, and the pelleted tenocytes were washed, centrifuged, and resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Invitrogen, Carlsbad CA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). Cells were plated at a density of 5,000 cells/cm^2 and fed twice per week with supplemented DMEM media (Passage 1). Cells were grown to confluence in a humidified environment containing 5% carbon dioxide at 37°C before passage. Cells were passed six times using 1 mL of 0.01%, trypsin-0.04% EDTA for 15 min at 37°C. A sample culture plate of each passage was isolated for gene expression and protein analysis as described below. Assays were performed on cell primary to seeding and after each cell passage from passage 1 to 6. Time between passages was approximately 1 week. Cell morphology was analyzed at the same time points using a Nikon Eclipse inverted microscope (Nikon Corporation, Tokyo, Japan).

Molecular analysis

RNA was isolated from cultured cells using TRIzol® reagent (Invitrogen, Carlsbad CA) according to the manufacturer's instructions. Frozen tendon samples were processed similarly using a homogenizer. RNA quantity and purity (A260/280) were measured using uQuant software. RNA was reverse transcribed into cDNA using 1 ug of mRNA and a High Capacity Reverse Transcription kit
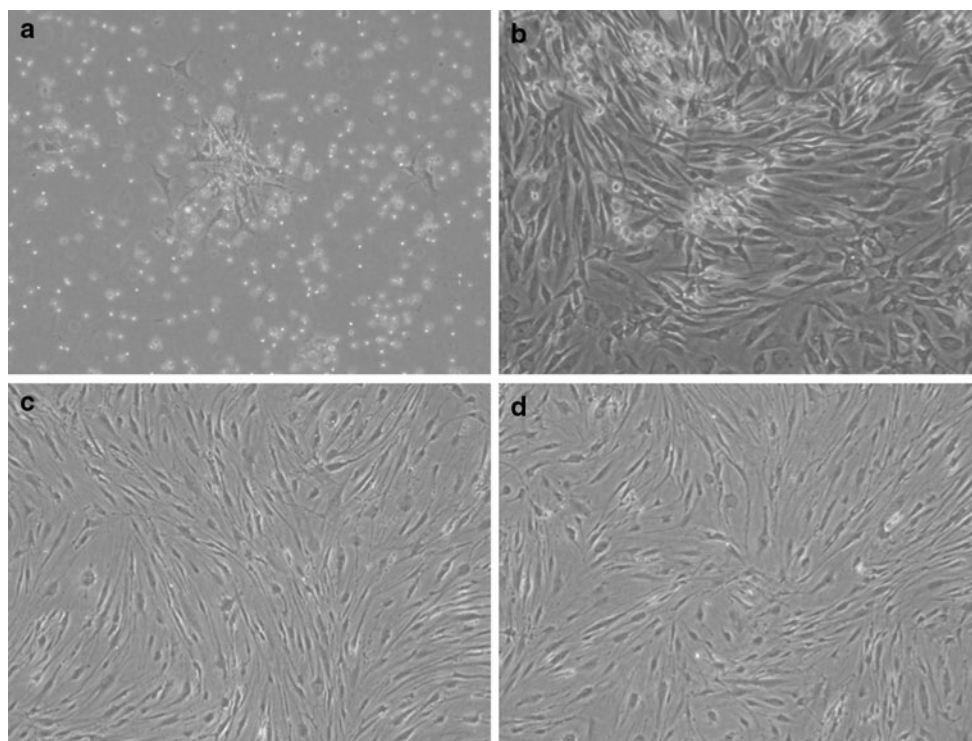
Fig. 1 Tenocyte-like cells' (TLC) morphology for passage 0 (**a**), 2 (**b**), 4 (**c**), and 6 (**d**) displayed in 10× magnification. Passage 0 cells appear fibroblastic with larger nuclei and thick cell bodies and processes. Freshly cultured biceps TLC appear fibroblastic in shape.

The morphologic appearance of TLCs changed with increasing cell passage and appeared less specific for TLC at higher passage numbers. Passage 6 cells have small, rounded cell bodies with thin cellular processes

(Invitrogen, Carlsbad CA). Real-time polymerase chain reaction was performed using 10–100 ng of cDNA as template and the StepOne Real-Time PCR System (Applied Biosystems, Foster City CA). Resultant cycle threshold (Ct) values were normalized to the endogenous control, GAPDH and analyzed using the standard curve method. TaqMan Gene Expression Assays were obtained for the following sequences: Type I collagen, type III collagen, Tenascin-C, decorin, and scleraxis relative to GAPDH as the endogenous control (Table 1).

Western blot analysis

Protein was extracted from cultured TLC by washing adherent cells twice with PBS and lysing them with radio-immunoprecipitation assay (RIPA) buffer containing 50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.004% sodium azide, and protease inhibitor cocktail (Sigma-Aldrich) by scraping with a rubber policeman. The homogenates were centrifuged at 10,000 g for 10 min at 4°C. The protein concentration in the supernatant was determined by bicinchoninic acid assay according to the manufacturer's instructions (Pierce, Rockford, IL). Equal

amounts of protein sample (50 μg/well) were subjected to SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel) electrophoresis using 4–15% gradient gels (Bio-Rad Laboratories, Hercules, CA). Precision Plus Kaleidoscope Protein Standards (Bio-Rad Laboratories) were used as molecular weight markers on each gel. Proteins were electrophoretically transferred to Polyvinylidene Difluoride (PVDF) membranes (Millipore, Billerica MA) and incubated for 60 min at room temperature in blocking buffer containing TTBS (20 mM Tris, 150 mM NaCl (pH 7.5), 0.05% Tween-20 (Sigma), and 5% non-fat dry milk (Bio-Rad Laboratories). After rinsing, the membranes were incubated overnight at 4°C in primary antibodies against the analyte of interest in dilutions ranging from 1:200 to 1:1,000 dependent upon antibody specifications. Following a rinse, membranes were washed extensively with TTBS and incubated in horseradish peroxidase–conjugated secondary antibodies (Santa-Cruz Biotechnology, Santa Cruz Ca) for 1 h at room temperature at dilutions between 1:10,000 and 1:20,000. Antibody binding was detected using an enhanced chemiluminescence kit (Pierce Supersignal, Rockford Il.). Membranes were exposed to Biomax MR film (Eastman Kodak, Rochester, NY), and the autoradiographs were generated and scanned to measure protein expression as represented by band intensity in units of pixel intensity (Adobe Systems Incorporated, San

Table 1 Table of primer sequences for the tendon-specific genes analyzed using RT-PCR

Gene	Primer sequences (5′–3′) Sense–antisense	PubMed ID
Decorin	CTAGCCCCACAGACCCTTCC CAATGGAGTCCTGGCTGTCC	18694758
Tenascin	GTGCCAGGAGACCGTACCAC CTTTGGCTGGGTTGCTTGAC	18305139
Type I collagen	GGCCACGACAAAGCAGAAAC CCCGGTGACACATCAAGACA	18353721
Type II collagen	TCACACTCCTCGCCCTATTGG GGGCAAGGGGAAGAGGAAAGA	17254772
GAPDH	GGGCTGCTTTTAACTCTGGT GCAGGTTTTTCTAGACGG	18708368

The National Institute of Health PubMed ID numbers is also included for each gene

Jose CA). Analyses were obtained for the following proteins: type I collagen, type III collagen, tenascin-C, and decorin. GAPDH was used as the endogenous control.

Statistical analysis

Statistical tests were performed using the Statistical Package for the Social Sciences (SPSS). One-way analysis of variance (ANOVA) was used to compare group means followed by Bonferroni post hoc tests for experiments with a statistically significant difference in means. A P value of $P < 0.05$ was used to determine statistical significance.

Results

Tenocyte culture (Fig. 1)

Cells isolated from human biceps tendon samples took approximately 2 weeks to reach confluence for further passaging. Cellular morphology was analyzed for all tendon samples at time point zero and at each passage. TLCs appeared fibroblastic with multiple thick, elongated processes and a large cell body. Cell morphology of the TLCs changed after extended passages. Cells appeared to have more rounded cell morphologies with progressive passage. Cells in the sixth passages no longer had the fibroblastic cell characteristics observed in the first two passages. In addition, the cellular processes were longer and thicker in early passage cells as compared to cells in their sixth passage.

Molecular analysis (Fig. 2)

Collagens I and III: Gene expression levels remained the same between the first two passages for type I collagen

(n.s.) and type III collagen (n.s.). However, gene expression levels dropped significantly between the second and third passages for both type I collagen ($P = 0.004$) and type III collagen ($P = 0.008$). Although significance was not reported, type I and III collagen gene expression levels continued to decrease slowly between passages three and six. Decorin gene expression levels remained relatively constant throughout the six passages. A trend of decreasing decorin transcript levels was observed with increased passages; however, no significance was noted. Tenascin-C was the only molecular marker that was not significantly decreased in cultured tenocytes when compared to the native biceps tendon tissue. Tenascin-C also followed a similar trend with decreased transcript levels with increased passage; however, significance was only obtained between passage one and passage five ($P = 0.03$). Tenomodulin gene expression also decreased with increased number of passages; however, the significance was not noted. Scleraxis gene expression decreased with increased number of passages without significant differences.

Western blot analysis (Fig. 3)

Western blot analysis showed that type I collagen and type III collagen significantly decreased after passage four. Decorin protein content was similar to the collagen content with a significant decrease observed after four passages. Tenascin-C protein content, on the other side, remained about the same throughout all six passages. GAPDH was used as the endogenous control, and Western blot analysis showed that all passages contained equal amounts of protein.

Discussion

The most important finding of this study was that routinely used markers for multiple passaged TLC cultures demonstrated considerable changes according to cell passage. TLCs obtained from human LHB tendons exhibit significantly higher gene expression levels of tendon-specific proteins such as types I and III collagen and tenomodulin within their first two passages. A change in expressions of key structural tendon proteins such as decorin and tenascin-C was also observed which supports the findings of a phenotypic drift, though not significant. The cultured TLCs obtained from human LHB tissue samples showed histological properties of TLC morphology at time point zero and matched accounts described by other authors [13, 24]. Besides the observed changes in cell markers, the morphologic appearance of TLCs changed with increasing cell passage and appeared less specific for TLC at higher passage numbers.
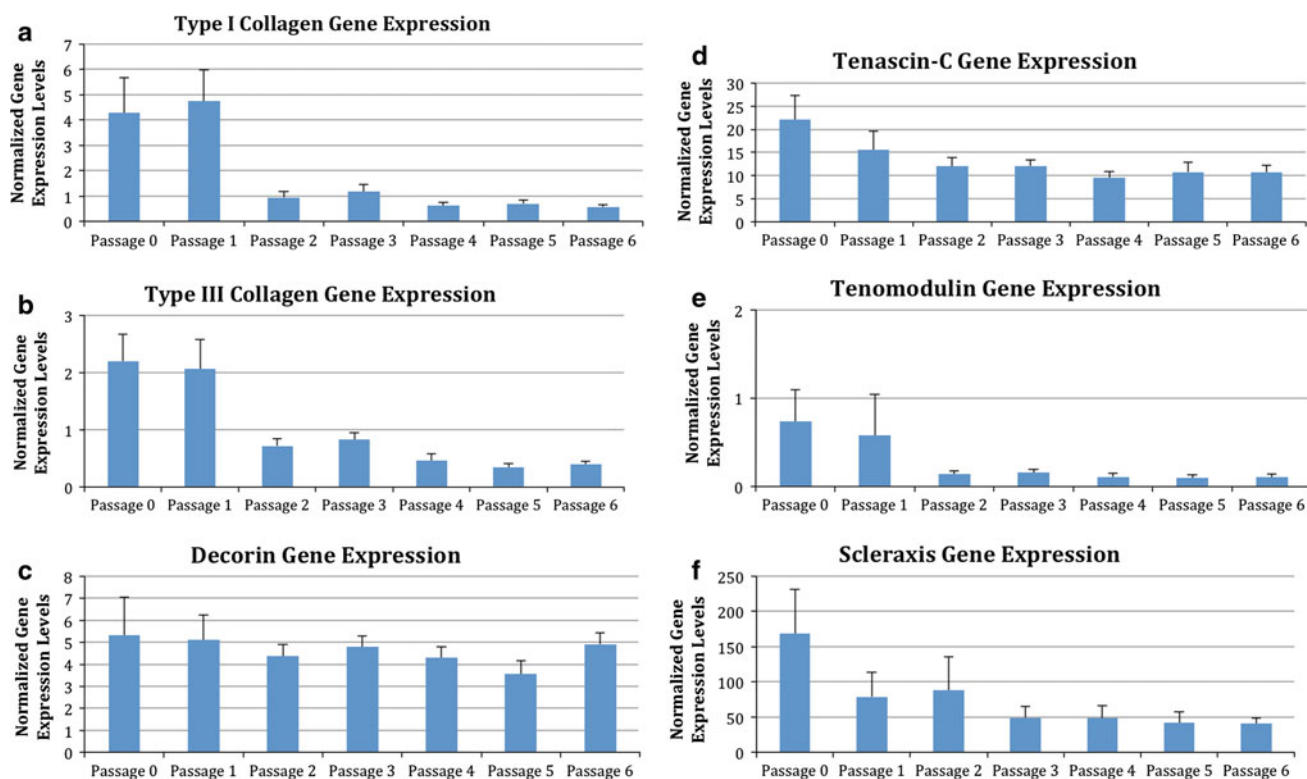
Fig. 2 Gene expression for types I (**a**) and III (**b**) collagen, decorin (**c**), tenascin-C (**d**), tenomodulin (**e**), and scleraxis (**f**) of human biceps tendon cells cultured for six passages. All genes trended toward decreased gene expression as the passage number increased. After two passages, types I and III collagen gene expression levels decreased significantly. Decorin, scleraxis, and tenascin-C levels decreased gradually over 6 passages. All data are shown relative to GAPDH

One of the basic reasons that in vitro TLC cultures are not as well established as, e.g., osteoblast models is that defining tenocytes in culture has been extremely difficult due to the lack of cellular markers that are specific to the tendon phenotype [13]. Since most markers may be commonly found in other human tissue sources, a combination of markers should be used to identify TLCs [13]. Commonly used markers include collagens I and III, which are extracellular proteins common to tendon, dermis, bone, and ligament, and other non-collagenous proteins such as decorin, tenascin-C, and tenomodulin, which are found in tendon but are also found in other connective tissue such as skin and ligament [9, 14, 21]. Therefore, the combined use of these markers should enable one to compare the phenotypic changes observed in TLC culture according to cell passage. It should be recognized that the cultured cells might not be absolutely purified according to their "tenocyte" phenotype at time of initial culture. However, the evaluated monolayer method of TLCs isolation and culture has to be regarded a commonly used technique and has been shown to have characteristics of TLC. Therefore, this method has to be regarded as the current "gold standard" of isolating TLCs for culture and further in vitro evaluation [4, 13, 24].

Since the physical environment and specific loading conditions may influence each tissue in a different way,

changes in TLC cultures have to be evaluated for each specific tissue [13, 24]. The authors choose to use human LHB tissue as the origin of the cell culture for this study. The rational for using human LHB tendon as an origin for TLC cultures is that it can be easily obtained in shoulder surgery, when LHB tenotomy or tenodesis is performed and a significant amount of tissue is considered surgical waste and discarded. To the authors' knowledge, previous work has shown comparable results of rapid phenotypical drift evaluating animal cell line TLCs obtained from differing origins and only one study examined TLCs obtained from ruptured human achilles tendons [2, 10, 16, 17, 24, 25]. TLC culture may react in a non-physiologic way and show increased phenotypic drift due to low initial tissue quality, if degenerated tissue is used as origin. Therefore, cell cultures should be generated from healthy tendons. The harvested tendon in the current study was evaluated thoroughly for any signs of degeneration or inflammatory changes. Additionally, the intra-articular portion of the tendon has been discarded to use the less biomechanically stressed portion as the source for TLC culture. However, the observations of the current study agree with the results of Yao et al., who showed comparable changes in TLC cultures according to multiple passages in TLCs obtained from ruptured human achilles tendons [24].
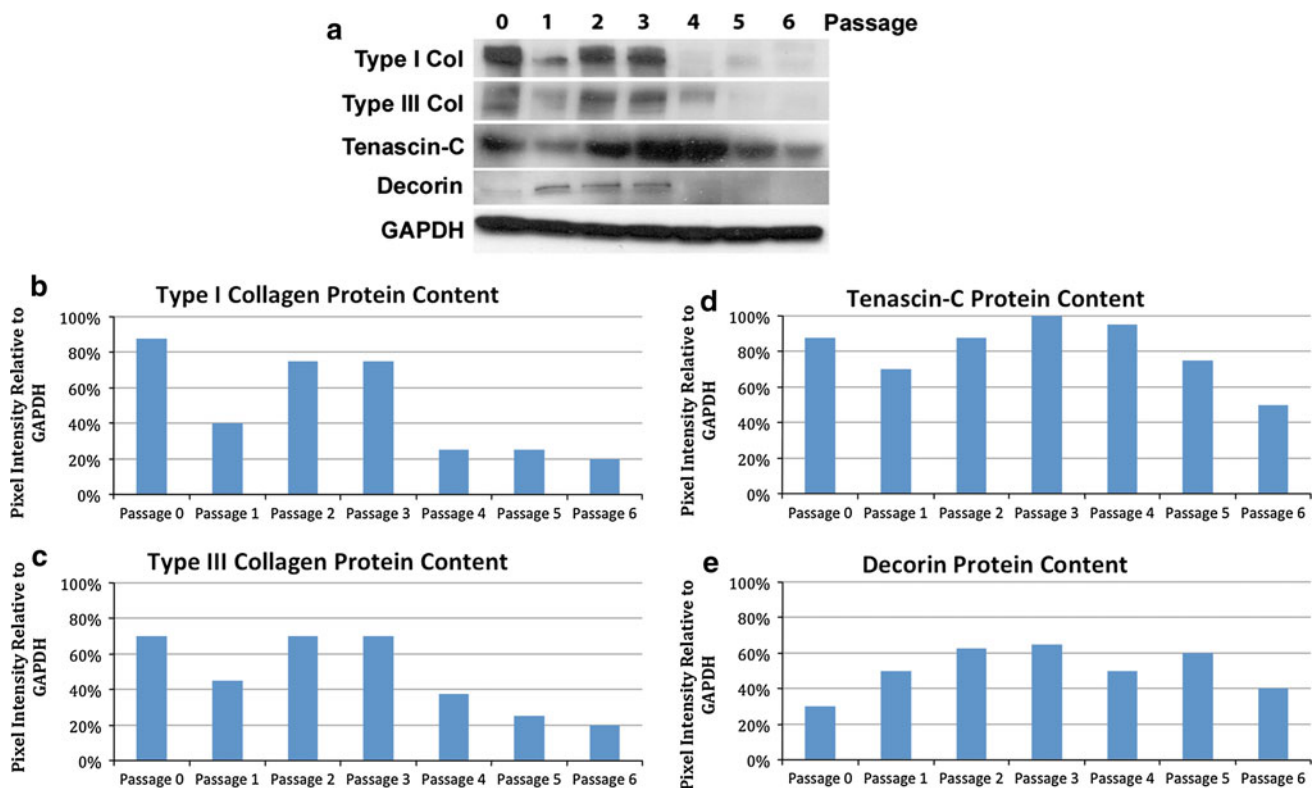
Fig. 3 Western blot (**a**) analysis for types I (**b**) and III (**c**) collagen, tenascin-C (**d**), decorin (**e**) of human biceps TLC cultured for six passages. Protein levels for types I and III collagen, and decorin decreased after 4 passages. Tenascin-C protein content remained similar throughout the six passages. All data are shown normalized to GAPDH

One limitation of this study may be a monolayer cell culture system. It may be hypothesized that growing cells in a 3 D environment or on biomechanically loadable scaffolds would result in more specific tenocyte characterization or would reduce the observed phenotypic drift [18, 22]. Therefore, previous studies attempted to create a better tenocyte model by using three-dimensional culture; however, only collagen I levels increased while other tendon markers remained unchanged. In addition, this study used rat cells and not the more "ideal" human tenocyte culture model [26]. However, it was the aim of the current study to evaluate commonly used procedures such as the monolayer culture and draw conclusions on these applied methods. In this, TLCs have been defined by the use of a specific marker set. No additional markers were used to further diversify the culture and identify subpopulations of osteoblasts or chondrocytes in the resulting cell cultures [13]. Therefore, a certain risk of a mixed population could not be excluded in the cultured cells. Unfortunately, it was beyond the capacities of this study to further specify each possible subpopulation of such cells. However, microscopic evaluation of cell morphology showed cells with TLC-specific properties, and cell isolation has been performed in a commonly used and previously published method [4, 13, 24]. Therefore, this study enabled to evaluate a currently used method of cell culture for its phenotypic drift and to determine the optimal number of cell passages, TLC cultures can undergo before significant changes are to be expected. Understanding these limitations of in vitro tenocyte models may finally help the clinician to judge the potential of experimental data promoting new biologic treatment options.

Conclusion

The results of this study provide reassurance that the in vitro use of TLCs obtained from human LHB tendons can be seen as a valid model only if using freshly isolated human tenocytes at low passage. Multiple passages result in changes in commonly used cell markers due to either phenotypic drift of the TLC by themselves or overpopulation of other cell lines like fibroblasts. If a high cell load is needed for sufficient culture size and a higher passage number cannot be avoided, researchers must be aware of these massive changes in protein expressions observed after multiple cell passages. As a result, freshly isolated TLCs at low passage number need to be used for in vitro

experiments using monolayer cell culture so that cells demonstrate as much physiologic characteristics as possible. Clinicians have to be aware of the specific limitations of such models to judge the results and conclusions gained from in vitro studies. Such human TLC models can then be used to study various types of healing of human tendons, e.g., rotator cuff repairs, and to see how drugs or biologics may affect tendon growth.

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