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Phenotypic Drift in Human Tenocyte Culture

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ABSTRACT

Tendon ruptures are increasingly common, repair can be difficult, and healing is poorly understood. Tissue engineering approaches often require expansion of cell numbers to populate a construct, and maintenance of cell phenotype is essential for tissue regeneration. Here, we characterize the phenotype of human Achilles tenocytes and assess how this is affected by passaging. Tenocytes, isolated from tendon samples from 6 patients receiving surgery for rupture of the Achilles tendon, were passaged 8 times. Proliferation rates and cell morphology were recorded at passages 1, 4, and 8. Total collagen, the ratio of collagen types I and III, and decorin were used as indicators of matrix formation, and expression of the integrin β_1 subunit as a marker of cell–matrix interactions. With increasing passage number, cells became more rounded, were more widely spaced at confluence, and confluent cell density declined from 18,700/cm^2 to 16,100/cm^2 ($p = 0.009$). No change to total cell layer collagen was observed but the ratio of type III to type I collagen increased from 0.60 at passage 1 to 0.89 at passage 8 ($p < 0.001$). Decorin expression significantly decreased with passage number, from 22.9 ± 3.1 ng/ng of DNA at passage 1, to 9.1 ± 1.8 ng/ng of DNA at passage 8 ($p < 0.001$). Integrin expression did not change. We conclude that the phenotype of tenocytes in culture rapidly drifts with progressive passage.

INTRODUCTION

RUPTURE OF TENDONS, especially of the Achilles tendon, is increasingly common.[1–3] Repair of these injuries is difficult with many patients never returning to the levels of activity they undertook prior to injury. Tissue engineering has the potential to improve the quality of ligaments and tendons during the healing process by manipulating the cellular and biochemical processes and to improve tissue remodeling.[4,5] To try to understand this process, *in vitro* culture systems, in which mechanical and biochemical stimuli can be regulated and monitored, offer the potential to study in detail the mechanisms by which tenocytes orchestrate the properties of tendon in relation to its load-bearing function and respond to trauma.[6–8] Although primary or early passage cells will logically offer the best approximation to the *in situ* cell, it may be desirable to define a range of passages for which there is minimal or no phenotypic drift. This may be particularly relevant for tenocytes which, because of the small amount of tissue that can be retrieved during surgery and the inherently low cellularity of tendon,[9] are not easily obtained in large numbers. Thus, more prolonged maintenance in culture may allow greater flexibility in the number of experiments that may be performed from a single tissue source. However, studies on avian and rabbit tenocytes indicate that phenotypic drift may manifest at early passage.[10,11]

Fibrillar and nonfibrillar components of the extracellular matrix and mediators of matrix–cell interaction offer potential as both markers for phenotypic drift and tenocyte stress response.[7,10–12] Approximately 95% of collagen in normal tendon is type I, with types III and V present in small amounts.[9] However, ruptured tendon contains a significantly greater proportion of type III collagen, and this is believed to affect deleteriously the mechanical properties of the tissue.[7,13–15] In addition, noncollagenous proteins and

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proteoglycans (PGs) interact with the fibrillar collagen network.[9,16–18] The predominant tendon PG is the small leucine-rich decorin, which belongs to a family of structurally related extracellular matrix PGs. Decorin binds to collagen with high affinity and appears to retard the formation of collagen fibrils, and results in the formation of thinner collagen fibers.[19,20] Changes in collagen and decorin are likely to affect the mechanical functioning of connective tissues by altering the nature of the fibers and stress transfer from matrix to fiber.[21]

A further marker of phenotype is the integrin family,[22,23] cell membrane glycoproteins that mediate cell–extracellular matrix adhesion and also participate in cell–cell adhesion and signal transduction.[24] Integrins consist of 2 subunits, α and β, and to date 17 α subunits and 9 β subunits have been recognized, forming 23 distinct heterodimers. Extracellular ligands in connective tissues include fibronectin, laminin, and various collagens. The β_1 subunit, in combination with different α subunits, is common to the integrin receptor for all of these ligands. Upregulation of integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ has been reported during healing and repair of intrasynovial flexor tendon.[25]

Here, as a model for studying wound and reparative responses, applicable to tissue engineering approaches to ruptured tendon, we describe the culture and characterization of phenotypic drift within human tenocytes derived from patients undergoing surgery for rupture of the Achilles tendon.

MATERIALS AND METHODS

Materials

Fetal calf serum (FCS) was purchased from Globepharm (Surrey, UK) and Dulbecco's modified Eagle's medium (DMEM) from Gibco-BRL (Paisley, UK). Tissue culture flasks were supplied by Greiner Labortecknik (Gloucestershire, UK). Phycoerythrin (PE)-conjugated anti-integrin β_1 mouse monoclonal IgG_1 and the corresponding isotype control IgG_1–PE conjugate were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-decorin mouse IgG_1 monoclonal antibody was supplied by R&D Systems, Oxford, UK. Horseradish peroxidase (HRP) conjugated anti-mouse IgG_1, protein molecular weight markers and Precision Strep–Tactin–HRP conjugate were purchased from Bio-Rad (Nottingham, UK). A chemiluminescence detection kit (Pierce) was obtained from Perbio Science UK (Cheshire, UK). Decorin core protein and collagen types I and III were supplied by R&D Systems and Sigma Chemical (Dorset, UK), respectively. All other reagents were purchased from Sigma Chemical unless stated otherwise.

Tissue samples and primary cell culture

One sample of tendon was obtained from each of 6 patients receiving emergency surgery for first rupture of the Achilles tendon. Local Ethics Committee approval was obtained for the study, and all patients gave their written informed consent. Histology identified features of degeneration as described by Maffulli *et al.*[14,26,27] Samples of tendon were cut into approximately $2\times2\times2\,mm^3$ pieces, placed in a flask, and subjected to overnight collagenase (10% w/v) digestion in DMEM containing 10% (v/v) FCS on an orbital shaker at 37°C. Medium was replaced, without collagenase, and tenocytes allowed to grow until about 95% confluent in a humidified atmosphere of 5% carbon dioxide (CO_2), 37°C before passage. Cells (passage 1) were then cultured in 75-cm^2 flasks in DMEM containing 10% (v/v) FCS, 1% (v/v) penicillin–streptomycin (Gibco-BRL), and 0.15 mM ascorbic acid at 5% CO_2, 95% relative humidity at 37°C until approximately 95% confluent before passaging. Time to passage was typically about 4 weeks, with medium changed every 3 days. This cycle was repeated 8 times to obtain cells up to passage 8.

Cell morphology and proliferation

To determine proliferation rates, cells were seeded in 6-well plates at a density of 3000/cm^2 and were counted from 2 wells each day for 14 days. Cells were harvested by trypsin digestion and counted using a hemocytometer (Improved Neubauer). In addition, alamar blue reduction, which is a measure of the cellular reducing environment and thereby indicative of innate metabolic activity, was determined by measuring absorbance using a Dynatech MR 5000 plate reader.[28] At confluence, photomicrographs of 3 or 4 representative fields were recorded using a Zeiss Axiovert S100 (Carl Zeiss UK, Hertfordshire, UK) inverted fluorescence microscope equipped with a "Cool Snap" camera (Image Associates, Bicester, UK). Cultures were observed under both bright-field and phase-contrast illumination.

Protein extraction

After the culture medium was removed, the cell layer was frozen at −80°C and subsequently extracted by adding 1 mL of 4 M guanidine hydrochloride (GuHCl)) containing 1% (v/v) general protease inhibitor cocktail (Sigma) and placing on an orbital shaker at 4°C for 24 h. An assessment of cell number, to determine electrophoretic gel loading, was obtained by measuring total DNA content.[29] Cell layer protein was precipitated with absolute ethanol.

Collagen analysis

Hydroxyproline (Hyp) content was determined as a measure of total collagen using the conversion factor of 14 g Hyp/100 g of collagen.[30] The ratio of collagen types I and III was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with delayed reduction.[31] Post-electrophoresis, proteins were silver or Coomassie blue stained, and protein band composition was recorded and analyzed using Bio-Rad Fluor-S™

MultiImager. Standard mixtures of collagen types I and III in the range 0.2 to 2 μg for total combined collagen were used to determine linearity of response and validate the efficacy of ratio determination.

Decorin analysis

Ethanol-precipitated protein was reconstituted in 0.1 M acetate–Tris buffer (pH 6.8) and digested with 2 U/mL of chondroitinase ABC at 37°C for 4 h.[32] Aliquots corresponding to 0.3 ng of DNA were electrophoresed under reducing conditions (SDS-PAGE, 4% stacking gel, 12% resolving gel). Standards of 2 ng, 5 ng, 10 ng, and 20 ng per lane decorin core protein were treated identically and loaded on the same gel. Following electrophoresis, protein was transferred to polyvinylidene fluoride (PVDF) membrane by semidry blotting. The membrane was blocked using 5% (w/v) nonfat milk powder in 50 mM Tris-buffered saline (130 mM NaCl), 0.1% (v/v), and Tween-20 (TBST) for 30 min. The membrane was incubated with anti-decorin antibody (1.5 μg/mL in TBS) for 2 h at room temperature. The membrane was washed 3 times in TBST and incubated with anti-mouse IgG_1–HRP conjugate secondary antibody (1:5000 H&L) and Precision Strep-Tactin-HRP conjugate solution (1:5000; Bio-Rad) for 1 h at room temperature. After they were washed twice with TBST, the protein bands were visualized using a Chemiluminescent Detection Kit according to the manufacturer's instructions (Pierce, Perbio Science) and analyzed using a Bio-Rad Fluor-S™ Multi-Imager. For each replicate, band intensity was compared with decorin standards on the same gel.

Integrin analysis

The β_1 component of the integrin receptor was analyzed using flow cytometry at day 8, maximum growth rate, and day 12, confluent. Tenocytes were incubated with anti-integrin β_1 mouse monoclonal IgG_1–PE conjugate (20 μL, in 100 μL PBS) or isotype IgG_1–PE conjugate for 1 h at room temperature. Unlabeled cells were also examined. Flow cytometry was performed with a FACS Calibur flow cytometer (Becton Dickinson, Oxford, UK) using a 488-nm argon ion laser with emission monitored at 585 nm (FL-2 detector). Signals were processed using a logarithmic amplifier and fluorescence distributions plotted on a 4-decade logarithmic scale (1024 channels). Ten thousand events were counted and the median linear fluorescence values were calculated using Cell Quest Software (Becton Dickinson). Isotype-labeled cells were used to determine changes in and correct for nonspecific binding.

Statistics

Data are presented as means ± standard deviation. Variability between passages was assessed by analysis of variance (ANOVA) or Student's *t*-test, to compare primary or first passage with passage 8, using SigmaStat version 3 software (SPSS). A p value < 0.05 was chosen to represent a significant difference.

RESULTS

Culture morphology and proliferation

Alterations to culture growth were observed in primary cells through to passage 8. The final cell density at confluence fell from 18,700/cm^2 to 16,100/cm^2 ($p = 0.009$, *t*-test; Fig. 1A). A decline in alamar blue reduction by confluent cells also occurred between primary culture and passage 8 ($p < 0.05$, *t*-test; Fig. 1B). However, the doubling time of 3.9 days was not significantly affected within this period of passage. The decrease in confluent culture cell density was reflected in the cell morphology. Primary and

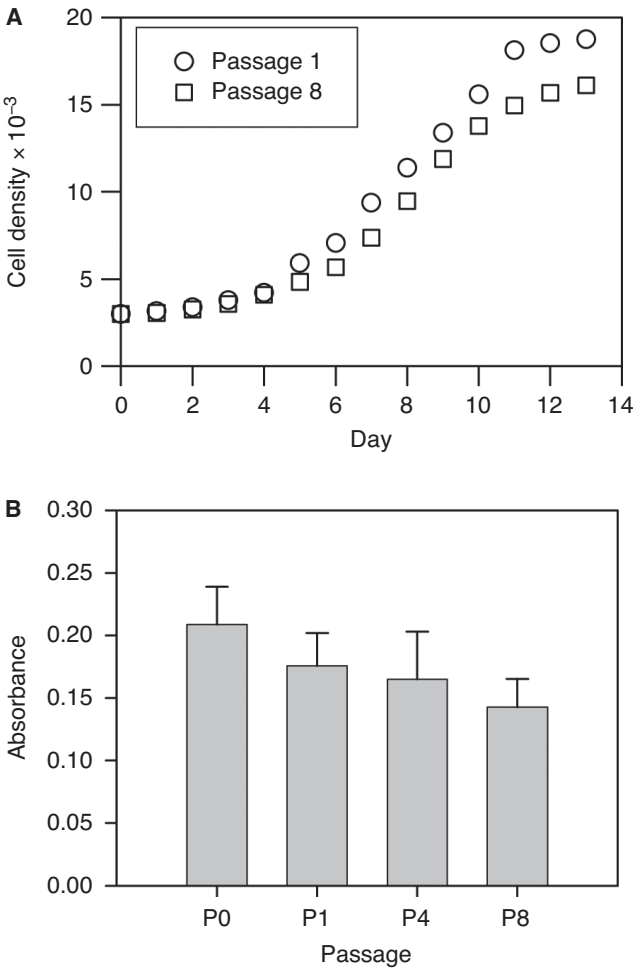

FIG. 1. Tenocyte proliferation. Growth curves (**A**) and alamar blue reduction at confluence (**B**), from a starting density of 3000 cells/cm^2, demonstrate the decline in confluent cell density with increasing passage number (P0 represents the initial outgrowth). Data are means ± SD ($n = 6$).

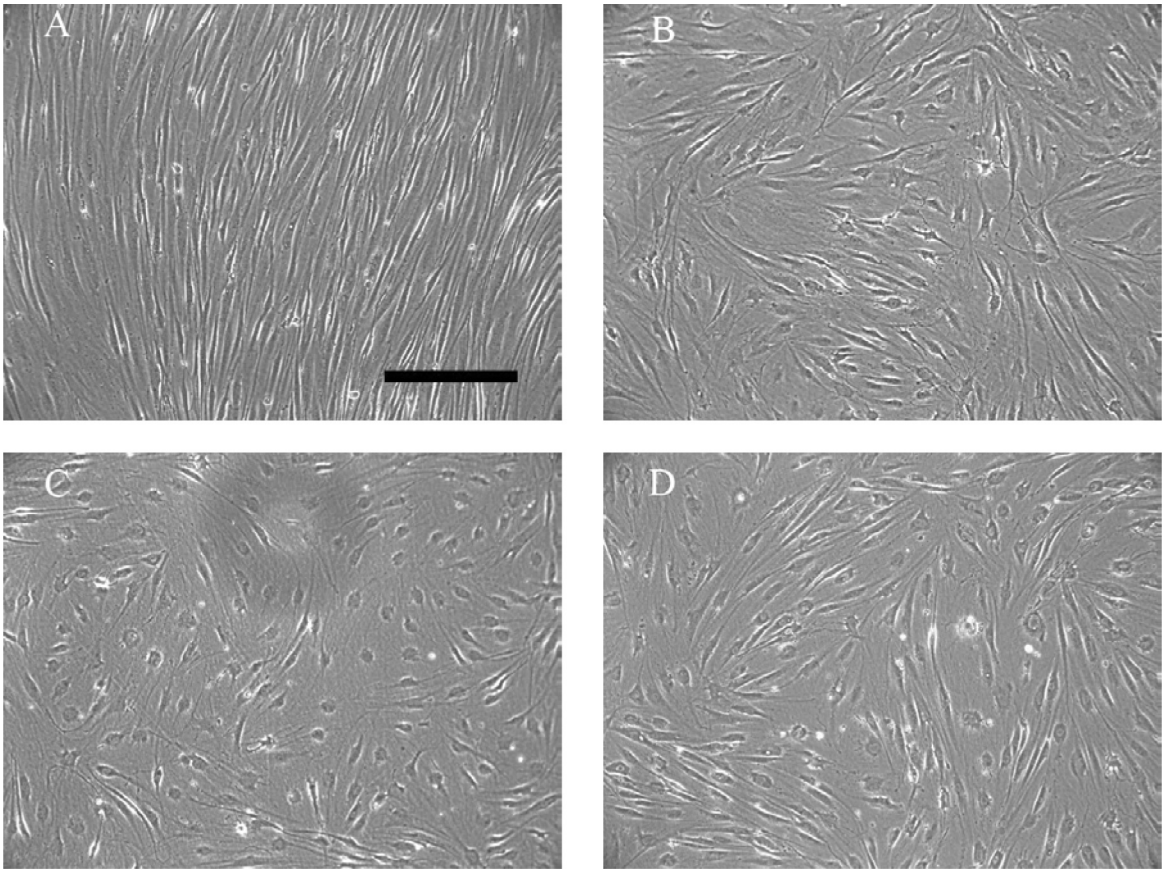

FIG. 2. Culture morphology. Primary (P0) (**A**), passage 1 (**B**), passage 4 (**C**), and passage 8 (**D**) tenocytes observed at confluence, using phase-contrast microscopy. Close cell apposition appears to be lost early, from P0 to P1, with "rounding" becoming prominent at passage 4. Images are representative of 3 or 4 fields observed for each of 6 cultures derived from independent biopsy samples. Scale bar = 300 μm.

passage 1 cells were spindle-shaped, but by passage 8 many cells were rounded and were not found in such close apposition (Fig. 2).

Collagen, decorin, and integrin expression

Total collagen in the cell layer appeared to decrease slightly after the first passage, and thereafter remained constant (Fig. 3A), though this failed to reach significance ($p = 0.46$, ANOVA). There was, however, a significant increase in the ratio of type III to type I collagen, from 0.60 at passage 1 to 0.89 at passage 8 ($p < 0.001$, ANOVA, Fig. 3B). The amount of decorin, normalized by DNA content, fell significantly from 22.9 ± 3.1 ng/ng of DNA at passage 1, to 9.1 ± 1.8 ng/ng of DNA at passage 8 ($p < 0.001$, ANOVA, Fig. 3C). Expression levels of the β_1 component of the integrin receptor were unchanged when compared in confluent and nonconfluent cultures (Fig. 3D). There was a slight trend toward reduced expression with passage number, after correcting for day of culture, but this did not reach significance (Fig. 3D, $p = 0.095$, ANOVA).

DISCUSSION

Phenotypic drift can rapidly occur in cultured rabbit and avian tenocytes[10,11] but has not been widely investigated for human tenocytes.[10,11,22] The present study has identified small but significant changes in the phenotype of human Achilles tenocytes over 8 passages in culture. Changes are reflected in growth characteristics and alterations to the composition of the extracellular matrix.

Notably, there is an increase in the type III:I collagen ratio. As there is no change in total collagen, this is likely an increase in type III at the expense of type I collagen production. Collagen production also changes during avian and rabbit tenocyte culture. Chick embryo tendons contain predominantly type I collagen and, although type I collagen production remained constant, tenocytes produced type III collagen in about 10% of cells within 3 days of culture, increasing to around 80% of cells at passage 3.[11] Culture of juvenile rabbit tenocytes results in a decrease in type I collagen transcript levels after the first passage.[10] In our study, a decline in decorin accompanies the increased

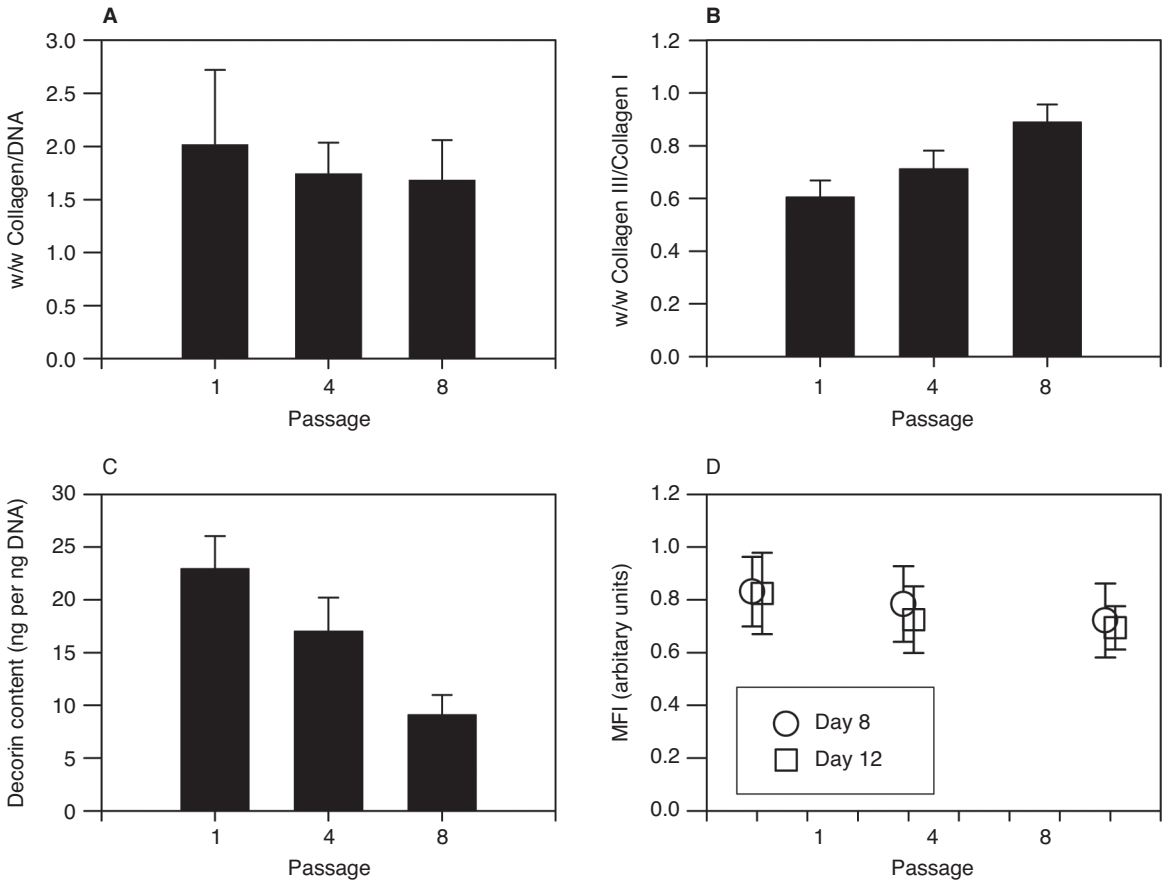

FIG. 3. Matrix and cell-matrix proteins. Total cell layer collagen was unchanged ($p = 0.46$) (**A**) but the collagen III:I ratio was increased ($p < 0.001$) (**B**) in confluent monolayers. Decorin protein also reduced ($p < 0.001$) (**C**). The integrin subunit β_1 (**D**) expressed as the median of the fluorescence intensity distribution (MFI) did not change significantly ($p = 0.095$) either during maximal proliferation (day 8) or at confluence (day 12) (shown slightly offset for clarity). Ten thousand events were analyzed per sample. All data are means ± SD ($n = 6$).

collagen III:I ratio. Conversely, β_1 integrin expression was not significantly affected by passage and, in contrast to observations on human tenocytes in high-density culture, no change to the expression of the β_1 component of the integrin receptor was observed during any single passage.[22]

Thus, the human Achilles tendon tenocyte phenotype drifts rapidly in culture. That the tenocytes were obtained from ruptured tendon is perhaps reflected in the relatively strong presence of collagen III within the primary and first passage culture. The shift in collagen ratio is also reminiscent of that found in cell wounding experiments *in vitro*[7] and *in vivo* such changes may well be important for the reduced mechanical strength underlying tendon rupture.[7,13] The alteration to decorin expression may also be indicative of a wound or degeneration response phenotype.[12,33,34] We therefore hypothesize that the movement to the culture environment might further exacerbate a wound or stress response phenotype.

In vivo, changes in collagen and decorin are likely to affect the mechanical functioning of connective tissues by altering the nature of the fibers and the ways in which stresses are transferred from matrix to fiber. Decorin binds to collagen with high affinity and affects collagen fibril formation. A reduction in decorin might act in 2 ways. If it mediates the interaction between collagen and the surrounding proteoglycan gel, it would lead to a reduction in the effectiveness of stress transfer from the weak matrix to the strong fibers.[21,35] In addition, it could also lead to an increase in the fiber diameter.[19,20] If there is no corresponding increase in fiber length, this will lead to a reduction in the ratio of fiber length/diameter, which, again, will have the effect of reducing the strength of the composite.[21]

There is clearly a requirement to study the mechanism of tenocyte responses to mechanical loading and cell wounding *in vitro*, with the aim of developing tissue engineering approaches to improving healing and optimizing surgical repair of ruptured tendons.[7,22] However, it seems vital to establish the stability of any such model given the apparent susceptibility of tenocytes from many species to undergo rapid phenotypic drift. High-density three-dimensional culture may stabilize the tenocyte phenotype, and this approach seems highly promising.[22] However, to achieve

high-density culture, it is possible that cells may already be passaged in monolayer beyond their normophenotypic state. This may be particularly relevant for cells removed from tendon tissue that has undergone trauma. The imposition of mechanical cyclical strain as a means of maintaining or influencing differentiation also warrants future investigation. For example, epithelial and chondrocytic cell differentiation, and hence phenotype, is influenced by the physical environment, possibly with changes to the rigidity of the extracellular matrix activating signaling pathways through the intermediacy of the cytoskeleton.[36–38] In addition, analysis of the potential for differential rates of drift from tenocytes from a different tendon origin or with donor age should be evaluated.[39] Here, although markers such as scleraxis might further define the extent and significance of phenotypic drift within our culture system,[22] we would contend that culture and passage in monolayer should be kept to an absolute minimum prior to experimentation, the establishment of culture models or seeding onto scaffolds. Based on this study, significant changes in tenocyte phenotype are manifest by passage 4, and our future experiments, currently investigating the role of oxidative stress in relation to mechanical loading and cell wounding within degenerate tendon,[40] will use only first- and second-passage cells to maintain a phenotype as close as possible to that pertaining *in vivo*.

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