MULTI-DIFFERENTIATION POTENTIAL IS NECESSARY FOR OPTIMAL TENOGENESIS OF TENDON STEM CELLS

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ABSTRACT

Background

Tendon injury is a significant clinical problem, and regenerative treatments are limited by our understanding of endogenous tendon stem cells. Recent evidence suggests that tendon stem cells are diverse in phenotypic character, and may in fact exist on a spectrum of differentiation capacities. However, the functional significance of each differentiation phenotype is poorly understood. Toward this end, we performed a comprehensive assessment of differentiation capacity toward four connective tissue lineages (adipose, bone, cartilage and tendon) with clonal tendon stem cell lines to: 1) evaluate the differences, if any, in tenogenic potential, and 2) identify the relationships in differentiation phenotype and proliferation capacity.

Methods

Tendon stem cells were derived from whole equine flexor tendons for this study (N=3). Clonal tendon stem lines were generated by low-density cell plating, and subjected to standard assays of tri-lineage differentiation and population doublings. For tenogenesis, a previously engineered three-dimensional hydrogel construct was incorporated. Differentiation was quantified by the relative gene expression of lineage-specific markers, and confirmed with lineage-specific cell staining. Tenogenesis was further analyzed by hydrogel contraction and histomorphometry. Statistical significance was determined using analysis of variance and post-hoc Tukey's tests.

Results

Three distinct tendon stem cell phenotypes were identified, namely differentiation toward: 1) adipose, bone, cartilage and tendon, 2) bone, cartilage and tendon, or 3) adipose, cartilage and tendon. Further, a positive correlation was found in the ability to differentiate toward all four lineages and the generation of a robust, composite tendon-like construct upon tenogenesis,

manifested by the strongest expressions of scleraxis and mohawk, and parallel alignment of tenocyte-like cells with elongated cell morphologies. Significantly increased numbers of cumulative cell population doublings were seen in the absence of adipogenic potential in clonal tendon stem cell lines.

Conclusions

Our study strengthens reports on the heterogeneous character of tendon stem cells and identifies key differences in their differentiation and proliferative potentials. Isolation of potent tendon stem cell populations from tendon stromal fractions may yield improved therapeutic benefits in clinical models of repair and promote a native, regenerative phenotype in engineered tendons. Future studies may be targeted to understanding the functional contributions of each tendon stem cell phenotype *in vivo*, and identifying additional cell phenotypes.

KEYWORDS

Mesenchymal stem cell; tendon; tendon stem/progenitor cells; collagen gel; differentiation; tissue engineering

INTRODUCTION

Tendon injuries are debilitating, and significantly impact quality of life. Over 32 million cases of musculoskeletal injuries involving the tendon are reported every year in the United States, and the incidence of tendon injuries is increasing[1]. Acute and chronic injuries of the Achilles, patellar and rotator cuff tendons are most prevalent in the adult population. Acute injuries are common in athletes, whereas chronic injuries usually arise from tendon overuse or aging[2, 3]. Tendons heal poorly[4], and surgical intervention is often necessary to augment healing.

However, the incidence of re-injury following surgical repair can be as high as 20-60%[5]. In recent times, regenerative therapies, specifically mesenchymal stem cells (MSCs) have shown promise in the restoration of native tendon structural and functional properties. Among these, bone marrow MSCs are generally preferred; they are easily accessible, well characterized and have been efficacious in *in vivo* models[6]. However, bone marrow derived MSCs are also predisposed to form bone, may not differentiate into tenocytes and may require pre-transplantation conditioning in bioreactors or with growth factors to facilitate their roles in tendon repair. This suggests that an alternate MSC source such as tendon may be better suited for regenerative tendon healing[7].

Endogenous tendon stem cells (TSCs) arise from the tendon progenitor niche that is predominantly a collagen I-rich extracellular matrix[8]. TSCs are the principal mediators of key processes involved in tendon repair, such as control of the inflammatory response and the synthesis and remodeling of collagen[9, 10]. Like bone marrow MSCs, TSCs express stem cell markers, have high proliferative capacity and can differentiate to non-tendon lineages[11] *in vitro*. Recent studies suggest that TSCs from anatomically different regions of tendon such as the peritenon and the tendon core exhibit differences in their morphologies and tendon healing potentials[10, 12]. For example, TSCs from the core express higher message levels of scleraxis and tenomodulin than those from the peritenon, whereas the reverse was shown for the pericyte marker CD133[7]. This suggests that more than one phenotype of TSCs may reside in tendon tissue. However, a comprehensive analysis of TSC lines generated from individual TSC clones is required to understand the molecular basis of these differences, and their contribution to tendon healing *in vivo.*

The presence of phenotypic variation in TSCs challenges the current paradigm of one phenotype that encompasses all MSCs, and hence, the criteria proposed by Dominici *et al*. may not define TSCs[13, 14]. The source of this phenotypic variation is not well understood. Asymmetrical cell division[15], and culture conditions such as pH, temperature, media additives and cell culture surface type may contribute to heterogeneity in culture. The study of clonal TSC lines has improved our understanding of TSC heterogeneity. Bi *et al*. were the first to report tri-, bi- and unipotential TSCs, or tendon stem/progenitor cells, based on their results from tri-lineage differentiation of human clonal TSC lines[8]. However, benchtop assays of tri-lineage differentiation may be poor indicators of tenogenic potential, and it is unknown whether individual TSCs are differently committed to the tendon fate from each other.

Our goal in this study was to determine the proliferative and differentiation potentials of clonal TSC lines toward four lineages: adipose, bone, cartilage and tendon, to identify TSC phenotypes based on trilineage differentiation and relate them to proliferative and tenogenic capacity. In addition to standard population doublings and trilineage differentiation assays, we incorporated a previously designed tenogenesis assay[16] comprising type I collagen hydrogel and tenogenic growth factors, to assess the TSC's ability to form their tissue of origin. Differentiation was quantified by the gene expression analysis of lineage-specific markers, and tenogenesis was further quantified by gel contraction and histomorphometry. We hypothesized that TSCs would exhibit heterogeneous differentiation in culture. We further hypothesized that TSCs that differentiate to four connective tissue lineages would also generate a composite tendon phenotype upon tenogenesis.

MATERIALS AND METHODS

Experimental design

TSCs from three juvenile horses (N=3) were included in this study. Each parent TSC line was analyzed for the gene expression of the stem cell markers CD90, CD105, GNL3 and Oct-4 on day 0 and at confluence. Thirty clonal TSC lines were generated by plating cells at low density on culture vessels and expanding in culture for two passages (Fig 1)[17]. Clonal lines were analyzed for gene expression of the transcription factor Oct-4 in monolayer cultures on day 21. Cell population doubling assays, trilineage differentiation assays and a tenogenesis assay were performed on clonal TSC lines. Positive differentiation was determined based on upregulation of gene expression (gene amplification) of Runx2 for osteogenesis, fatty acid binding protein-4 (FABP4) for adipogenesis, Sox9 and aggrecan for chondrogenesis, and collagen I, III, scleraxis, mohawk, decorin, biglycan, tenascin C, Axin2 and fibroblast-specific protein-1 (FSP1) for tenogenesis after cells were grown under differentiation conditions.

Clonal TSC lines in this study were categorized based on their differentiation potentials, which were determined from the results of each lineage-specific differentiation assay. Hence, each clonal TSC line was designated with a letter for each positive differentiation outcome; specifically, A = Adipogenesis positive, O = Osteogenesis positive, C = Chondrogenesis positive and T = Tenogenesis positive. Each cell line was grouped with other cell lines exhibiting the same differentiation potential. Between-group comparisons were performed for statistical analysis of gene expression, gel contraction, cell alignment and population doublings.

Cell isolation and clonal cell line preparation

Whole digital flexor tendons (N=3) were isolated from horses aged one month (horse 211), 3 months (horse 961) and 12 months (horse 755) with IACUC approval and available on site. After euthanasia (performed for reasons other than this study), whole tendons were excised, minced

and digested with collagenase overnight at 37°C. The next day, tendon digests were strained with a cell strainer to generate single-cell suspensions, which were centrifuged at 800g for 10 minutes to obtain cell pellets. Cells were plated into tissue culture dishes at a high density of 6,666 cells/cm^2 to derive parent TSC lines and at a low density of 111 cells/cm^2 to obtain single-cell derived clonal colonies. All cell lines were cultured in growth media comprising high glucose DMEM (Thermo Scientific), 10% Cellect^{TM} Silver fetal bovine serum (MP Biomedicals, Santa Ana, CA) and 1% penicillin-streptomycin solution (Thermo Scientific). Confluent colonies were isolated within 10mm cloning cylinders (0.8 x 0.8 cm, Corning, Inc., Corning, NY) [17] and detached using trypsin-EDTA[18]. Tendon samples from each horse were processed independently to generate three independent parent tendon stem cell lines and thirty clonal tendon stem cell lines (10 per horse). For the experiment, clonal colonies were expanded in culture for two successive passages before plating for differentiation assays and population doublings. A complete cell culture workflow is illustrated in Fig 1.

Cell proliferation

Ten thousand cells per cell line were plated in individual wells of 24-welled tissue culture dishes (Nunc™, Thermo Scientific), trypsinized at 70-80% confluence, and counted with an automated cell counter (Beckman Coulter, Brea, CA). Population doubling numbers (DN) and doubling time (DT) for each passage, and cumulative doubling numbers (CDN) over 3 successive passages were calculated using the following formulae:

DN = $\log_2$(cell number at confluence/cell number at seed)

DT (days) = culture duration*log(2)/log(cell number at confluence/cell number at seed)

CDN = sum of DNs from each passage

Tenogenesis assay

Gels for tenogenesis were generated using a previously published method[16]. One million cells were suspended in 5 milliliters of tenogenic growth media comprising high glucose DMEM (Thermo Scientific), 10% Cellect^{TM} Silver fetal bovine serum (MP Biomedicals), 37.5 μg/ml L-ascorbic acid (Sigma-Aldrich, St. Louis, MI), 1% penicillin G (Sigma-Aldrich) and 0.8 mg/ml rat tail collagen I solution (Corning Life Sciences, Tewksbury, MA). Cell/gel suspensions were plated immediately following preparation in individual wells of 4-welled, rectangular dishes (Nunc™, 12.8 x 8.6 cm, Thermo Scientific) affixed with two sterile cloning cylinders (0.8 x 0.8 cm, Corning Inc.) set 3 centimeters apart from each other along the longitudinal midline of the well, and held in place by sterile silicone on day 0. Gels were maintained at 37°C, with 5% CO_2 and 90% humidity. On day 1, growth media was replaced with media containing 50ng/ml BMP-12 (recombinant human, Sigma Aldrich) and 10ng/ml IGF-1 (recombinant human, BioVision, San Francisco, CA). Media was changed on alternate days over a 10-day period.

Gel histology and analysis of cell alignment

Longitudinal sections of each gel were fixed in 4% paraformaldehyde overnight at 4°C, washed in phosphate buffered saline the next day and submitted for histology to a commercial service (Laudier Histology, New York, NY). Two, 6 micron thick longitudinal slices per sample section were stained with Masson's trichrome stain, and images were acquired with a microscope (Olympus Corp, Center Valley, PA) and digital camera (Motic North America, Richmond, BC). Cell alignment was quantified using ImageJ software analytical tools[19]. Fifty cellular angles per histological section and two sections/sample were measured relative to the longitudinal gel axis. Parallel alignment to the longitudinal axis was assigned 0º, and angles of each cell relative to 0º (0º-90º) were averaged for each sample to draw comparisons between samples.

Gel contraction

Digital images of each gel were taken on days 1, 3, 5, 7 and 10 to determine the percentage of contracted area at each time point relative to the gel area at day 0. Images were analyzed using ImageJ software analytical tools.

Tri-lineage differentiation

Tri-lineage differentiation potential was assessed using standard benchtop assays of adipogenesis, osteogenesis and chondrogenesis. For adipogenesis and osteogenesis, cells were plated at high (21,000 cells/cm^2) and low densities (4,000 cells/cm^2) respectively in tissue culture dishes (Nunc™, Thermo Scientific), and cultured in growth media comprising high-glucose DMEM and 10% fetal bovine serum (Thermo Scientific). At 70-80% culture confluence, media was replaced with differentiation media and maintained for 21 days. For chondrogenesis, 200,000 cells per cell line were centrifuged at 800g for 10 minutes to obtain a pellet. Pellet cultures were maintained in growth media comprising high glucose DMEM and 1% insulin-transferrin-selenium mix (Gibco™, Thermo Scientific) for 2 days prior to differentiation for 21 days. Differentiation media comprised the following: for adipogenesis, StemPro™ adipogenesis differentiation medium (Thermo Scientific), for osteogenesis, growth media supplemented with 10mM beta-glycerophosphate, 50µg/ml ascorbate 2-phosphate and 100nM dexamethasone (all Sigma-Aldrich), and for chondrogenesis, growth media supplemented with 37.5µg/ml ascorbate 2-phosphate, 100nM dexamethasone (both Sigma Aldrich) and 10ng/ml TGF-β3 (recombinant human, R&D Systems, Minneapolis, MA). On day 21, cultures were either fixed with 10% formalin (Sigma Aldrich) or frozen for gene expression analysis. Positive results from gene expression analysis were confirmed with Oil Red O (for adipogenesis) and Alizarin Red S (for osteogenesis) staining (both Sigma Aldrich), and images were acquired using an inverted microscope (Olympus Corp, Center Valley, PA).

Gene expression

RNA isolation was performed using the TRIzol™ method (Thermo Scientific). RNA pellets were subjected to RNeasy® spin columns for removal of genomic DNA contamination (QIAGEN Inc., Germantown, MD) and purified RNA was quantified using a NanoDrop™ 2000c spectrophotometer. cDNA was synthesized using a commercial kit (High-Capacity RNA-to-cDNA kit, Thermo Scientific). Real-time qPCR (7500 Real-Time PCR System, Thermo Scientific) was performed using custom TaqMan®-MGB probes and primers (Thermo Scientific) included in Table 1. TaqMan® gene expression assays for equine-specific Runx2 (Assay ID: Ec03469741_m1), Sox9 (Assay ID: Ec03469763_s1), Axin2 (Assay ID: APT2CHG) and FSP-1 (Assay ID: APU643E) were obtained from Thermo Scientific. The comparative threshold cycle method ($2^{-\Delta\Delta Ct}$) was employed for the relative quantification of gene expression[20]. Data was normalized to GAPDH. Tenogenic marker expression is reported as fold change with respect to an equine juvenile tendon reference control. Tri-lineage marker expression is reported as fold change relative to monolayer controls cultured in growth media on day 21. Stem cell marker expression is reported as fold-change relative to an adult equine muscle negative control for parent TSC lines, and relative to day 0 parent TSC lines (monolayer culture) for clonal TSC lines.

Table 1. Custom designed equine primer and probe sequences

	FORWARD	REVERSE	PROBE
GAPDH	CAAGTTCCATGGCACAGTCAAG	GGCCTTTCCGTTGATGACAA	CCGAGCACGGGAAG
Scleraxis	CGCCCAGCCCAAACAG	TTGCTCAACTTTCTCTGGTTGCT	TCTGCACCTTCTGCC
Collagen I	GCCAAGAAGAAGGCCAAGAA	TGAGGCCGTCCTGTATGC	ACATCCCAGCAGTCACCT

Collagen III	CTGCTTCATCCCACTCTTAT	ATCCGCATAGGACTGACCA	AACAGGAAGTTGCTGAAGG
Decorin	AAGTTGATGCAGCTAGCCTG	GGCCAGAGAGCCATTGTCA	ATTTGGCTAAATTGGGACTG
Biglycan	TGGACCTGCAGAACAATGAGAT	AGAGATGCTGGAGGCCTTTG	TCTGAGCTCCGAAAGG
FABP4	AAAATCCCAGAAACCTCACAAAAT	TCACTGGCGACAAGTTTCCA	TGTGATGCATTTGTAGGCA
Aggrecan	GACCACTTTACTCTTGGCGTTTG	GTCAGGGTCTGAAACGTCTACTGA	ACTCTGAGGGTCATCAC
GNL3	TTCGGGAAGCTGAGCTAAGG	CTGTCAAGCTTCTGCTGCTGTT	AACAGCGGCTTGAAG
CD90	GGCAGACCAGAGCCTTCGT	ATGGGTGTGGCGGTGGTAT	TGGACTGCCGCCATG
CD105	TCCACATCCTCTTCCTGGAGTT	GGACCTTTGGATAGTCAGCTTCA	CCAAGGGATGTGTCAGAG
Oct-4	CAGCTCGGGCTCGAGAAG	TTCTGGCGACGGTTGCA	ACGTGGTACGAGTGTGG
Mohawk	CCCACCAAGACGGAGAAGATACT	CACCTGCACTAGCGTCATCTG	TTGGCGCTCGGCTC
Tenascin C	GTTGGACTCCTGTACCCATTCC	GGCCCGAGGTCGTGTCT	TCCCAAGCGATGCTG

Statistical analysis

Non-normal data was log transformed prior to analysis. Clonal TSC line differentiation potential was classified and grouped as described in the experimental design. Group means were compared to each other using a one-way ANOVA and post hoc Tukey's tests for gene expression, population doublings (number and time) and cell alignment. Significant differences in contraction were assessed using a one-way MANOVA with a repeated measures design and post hoc Tukey's tests. A *p*-value of less than 0.05 was considered significant. Computation was performed in JMP Pro 15 (SAS Institute, Cary, NC) and MS Excel 11 (Microsoft, Redmond, WA).

RESULTS

Clonal TSC lines proliferate in two and three-dimensional culture

All parent TSC lines used in this study expressed the stem cell markers CD90, CD105, GNL3 and Oct-4 in monolayer culture at day 0 and at confluence (Fig 2). Of the thirty clonal colonies

isolated and expanded in culture, twenty-six yielded a million cells or more at confluence, and were seeded in 3D hydrogels for analysis of tenogenic potential. Of these, fifteen clonal TSC lines were additionally seeded for tri-differentiation assays and cultured in growth media to assess population doublings. Thirteen out of fifteen clonal TSC lines expressed Oct-4 in monolayer culture on day 21 (Additional Fig 1). TSCs successfully adhered to tissue culture-treated plastic, formed 3D pellets for chondrogenesis, and expanded to confluence in successive monolayer and 3D cultures. Data from fifteen clonal TSC lines was analyzed for comparisons of differentiation and proliferative potentials, since enough cells could be procured from a single passage of each of these cell lines to enable four differentiation assays and one assay of population doublings.

Three distinct TSC phenotypes can be found in tendon

No unipotent or bipotent clonal TSC lines were obtained in the experiment, as each cell line differentiated toward three or four tissue types. All of the fifteen clonal TSC lines exhibited both chondrogenic and tenogenic potential, evident by the positive upregulation of all markers of the respective lineages upon differentiation. Eight out of fifteen lines differentiated toward adipose, bone, cartilage, and tendon lineages (quadrapotent AOCT phenotype). Five of fifteen were positive for osteogenesis, chondrogenesis and tenogenesis (OCT phenotype) but not adipogenesis, and did not express FABP4 ($Ct \geq 35$, no amplification) (Fig 3A). The difference in FABP4 expression between AOCT and OCT reached significance ($p = 0.0002$) and between ACT and OCT approached significance ($p = 0.0599$). Two of fifteen were positive for adipogenesis, chondrogenesis and tenogenesis (ACT phenotype) but did not undergo osteogenesis, and did not express Runx2 ($Ct \geq 35$) (Fig 3C). Runx2 expression was significantly

decreased in the ACT group and compared to the AOCT (p = 0.0010) and OCT (p = 0.0179) groups. Oil Red O staining confirmed the presence of intracellular oily droplets in adipogenic cultures of the AOCT and ACT groups (Fig 3B). Likewise, osteogenic differentiation was confirmed with the formation and staining of calcium nodules and a calcified matrix with Alizarin Red S in cultures of the AOCT and OCT groups (Fig 3D).

Tissue marker gene expression levels and proliferative capacity of TSCs correlates with the ACT, OCT and AOCT phenotypes

Gene expression of chondrogenic markers significantly differed among the three phenotypic groups (Fig 4). Expression of chondrogenic Sox9 was significantly increased in the AOCT group compared to ACT and OCT (p = 0.0167 and 0.0017) on day 21 of chondrogenesis, whereas message levels of aggrecan were the highest in the OCT group, and significantly greater then AOCT (p = 0.029). There were no significant differences in Runx2 expression between the AOCT and OCT groups (Fig 3C) or in FABP4 expression between the AOCT and ACT groups (Fig 3A).

The OCT group exhibited significantly greater numbers of cumulative population doublings over three passages compared to the AOCT and ACT groups (p = 0.0105 and 0.0392) (Fig 5). Population doubling numbers decreased in passages 4 and 5 relative to passage 3 in all three groups, and this difference reached significance with the AOCT group.

Quadra-differentiation potent TSCs differentiate to express the optimal composite tendon phenotype

Significant differences were noted in tendon gene expression between the AOCT and OCT groups (Fig 6). Specifically, scleraxis and mohawk were expressed ≥3-fold higher in the AOCT group compared to the OCT group (p = 0.0454 for scleraxis and 0.0431 for mohawk). Expression of collagen type III was significantly elevated in the OCT group compared to AOCT (p = 0.0315). In contrast, collagen type I expression remained unaffected by TSC differentiation phenotype. No between group differences in tenascin C, Axin2 or FSP1 expressions were observed.

All groups contracted collagen matrix in three dimensions albeit to different degrees over 10 days (Fig 7). Significant differences were noted in contracted gel area at two time points between groups (Fig 7A). Specifically, the ACT group was significantly less contracted than the AOCT and OCT groups on day 5 (p = 0.0004 and 0.0008) and day 7 (p = 0.0031 and 0.0026).

All groups exhibited a uniform distribution of TSCs in three dimensions on day 10 (Fig 8). A greater proportion of TSCs (>90%) in all groups were highly aligned to the axis of tension (Fig 8A), and exhibited elongated cell morphologies. No significant differences in cell alignment were observed between the three groups (Fig 8B).

DISCUSSION

The goal of this study was to determine whether clonal TSC lines obtained from individual TSCs are diverse in their phenotypic character, and the functional contributions of each phenotype to *in vitro* differentiation and proliferation. Three distinct TSC phenotypes were identified, and the most significant differences correlated to the presence or lack of adipogenic potential. TSCs of the AOCT phenotype strongly differentiated to a composite tendon-like construct, most remarkably by a significant increase in their expression of scleraxis and mohawk. In contrast,

TSCs of the OCT phenotype exhibited significantly increased proliferative capacity compared to the AOCT and ACT groups.

This is the first study to investigate the tenogenic potentials of individual TSCs or multipotent clonal TSC lines from any species. In the absence of tendon-specific markers, we evaluated a range of markers that collectively identify the tendon phenotype[11, 21]. Significant increases in message levels of the bHLH transcription factor scleraxis[22] in the AOCT group can be attributed to tendon neogenesis[23]. Further, scleraxis and mohawk (a member of the TALE superclass of homeobox genes[24]) are co-expressed in mature, differentiated tendons[25, 26], which may explain their overlapping expression patterns observed in this study. Collagen type I is the predominant tendon collagen[27], and the ratio of collagen type III protein to type I protein is a determinant of the pathological state of tendons[28]. The higher collagen type III to type I ratio message level observed in the OCT group (compared to AOCT), may be suggestive of disorganized collagenous matrices undergoing active remodeling[28, 29], as opposed to relatively mature tendons of the AOCT group. The small leucine-rich proteoglycans decorin and biglycan are functionally similar and likely compensate for each other *in vivo*[30]. However, in this study, we did not achieve significant differences in their expression patterns.

A recent study revealed the presence of a highly proliferative subpopulation of Axin2 and scleraxis co-expressing TSCs at the site of healing in injured tendons, suggesting this subpopulation of cells may be the first responders to tendon injury *in vivo*[31]. Our results suggest that TSCs of the highly proliferative, Axin2-expressing OCT phenotype may represent this subpopulation. In contrast, scleraxis expression was significantly greater in the AOCT compared to the OCT group. This result is in support of the hypothesis that Wnt signaling

suppresses tenogenic differentiation in TSCs, specifically by downregulating the expression of scleraxis[32].

In response to uniaxial strain along a longitudinal axis, TSCs can contract a disorganized collagenous matrix to an anisotropic, tendon-like structure with parallel-aligned cells and collagen fibers[33-36]. Our tenogenesis assay successfully and consistently generated tendon-like constructs with aligned cells of elongated morphologies on day 10 of culture. The reduced contraction of the ACT group in this study suggests that downregulation of Runx2 expression in TSCs may impede tendon repair *in vivo*. One study suggests that overexpression of Runx2 may augment tendon-to-bone healing, by inducing site-specific rather than heterotopic bone formation[37].

Expansion of single-cell derived colonies is notoriously difficult *in vitro*, and limited this study to fifteen clonal TSC lines. Feeder layers, growth factors or alternative methods to supplement cell growth were not used in this study to avoid potential interference with study outcomes. However, sufficient cell numbers were acquired from fifteen TSCs to enable gene expression analysis as a superior outcome of differentiation as opposed to conventional stain absorbance and microscopy. In contrast to a previous study on trilineage differentiation of TSCs [8], we did not identify clones with solely adipogenic, osteogenic or chondrogenic potential. This may be attributed to species-related differences, or the requirement of highly proliferative clones to simultaneously assess trilineage, tenogenic and proliferative capacities.

Surprisingly, the group with the highest differentiation capacity, AOCT, did not have the highest proliferation capacity in our study. The AOCT group exhibited significantly lower numbers of cumulative cell doublings compared to the OCT group. This observation is in contrast to the proposed model of hierarchical loss of potential in bone marrow MSCs, wherein

multi-differentiation and proliferative potential are positively correlated[38, 39]. In this model, adipogenic potential is reduced with successive population doublings[40]. In tendon, we propose that lack of adipogenic potential may signify 1) increase in proliferative potential, and 2) decrease of tendon-related gene expression upon tenogenesis as observed in TSCs of the OCT phenotype. Further, it is also possible that in contrast to proposed models of lineage commitment, distinct TSC phenotypes identified in this study represent independent tendon cell populations rather than ones derived from a common ancestor. Future investigation is warranted to further understand the relationships in TSC characteristics and the molecular mechanisms underlying loss or gain of potential *in vitro*.

CONCLUSIONS

Of the three TSC phenotypes identified in this study, TSCs of the AOCT phenotype had optimal tenogenic capacity, evident by the strongest expression of scleraxis and mohawk, progressive contraction over 10 days and elongated cell morphologies in histologic sections of tendon constructs. In contrast, TSCs of the OCT phenotype may exhibit reduced osteogenesis, and thus offer select advantages in tendon healing such as a reduced proclivity to ectopic bone formation. Future studies may be targeted to the identification of additional TSC phenotypes, and the contributions of each phenotype to functional tendon repair *in vivo*.

DECLARATIONS

Ethics approval and consent to participate

Animal subjects used in this study were euthanized for reasons other than stated in this study, and with Virginia Tech's Institutional Animal Care and Use Committee approval.

Consent for publication

Not applicable

Availability of data and materials

All data generated and analyzed during this study are included with this submitted article (and its supplementary information files).

Competing interests

The authors declare that they do not have any competing interests.

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Author contributions

IR and JGB designed the study. IR performed the experiments, analyzed the data and wrote the manuscript. Both authors edited the manuscript and approved the final submitted version.

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LIST OF ABBREVIATIONS

1) TSC: tendon stem cell

2) MSC: mesenchymal stem cell

3) FABP4: fatty acid binding protein-4

4) FSP-1: fibroblast specific protein-1

5) DN: doubling number

6) DT: doubling time

7) CDN: cumulative doubling number

8) 3D: three-dimensional

9) Oct-4: Octamer-4

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ADDITIONAL FILE INFORMATION

File name: Additional Fig. 1

File format: .pdf (Additional Fig. 1.pdf)

Title: Gene expression of the transcription factor Oct-4 in clonal TSC lines.

FIGURE LEGENDS

Fig 1. Cell culture workflow

Fig 2. Gene expression of stem cell markers in parent TSC lines: 211, 755 and 961 are individual subject identifiers. CD90 and CD105 are cell surface proteins, GNL3 is a nuclear protein and Oct-4 is a transcription factor.

Fig 3. Adipogenesis assay (A, B) and osteogenesis assay (C, D) results. Gene expression of FABP4 relative to undifferentiated monolayer cells on day 21 (A), visualization of oily droplets in representative AOCT and ACT cultures by Oil Red O staining (B), gene expression of Runx2 relative to undifferentiated monolayer cells on day 21 (C) and visualization of calcium nodules and calcified matrices in representative AOCT and OCT cultures by Alizarin Red S staining (D). Lines and asterisk indicate groups with significant differences in them. Images are at a 10X magnification. A = Adipogenesis positive, O = Osteogenesis positive, C = Chondrogenesis positive and T = Tenogenesis positive.

Fig 4. Chondrogenesis assay results. Gene expression of Sox9 and aggrecan relative to uninduced monolayer cells on day 21. Lines and asterisk indicate groups with significant differences in them.

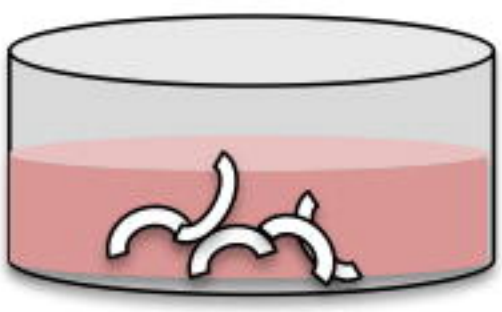
Fig 5. Population doubling assay results**.** Cumulative clonal tendon stem cell lines' doubling numbers (top), number of doublings (bottom left) and doubling time (bottom right) over 3 successive passages. Lines and asterisks indicate groups with significant differences.

Fig 6. Tenogenesis assay results. Gene expression of tendon-related genes relative to equine juvenile tendon on day 10. Lines and asterisks indicate groups with significant differences.

Fig 7. Percentage contracted gel area relative to day 0 gel area at different time points over 10 days of culture (A), and digital images of constructs at harvest (B). The ACT group had

534 contracted the least at culture endpoint. Asterisks indicate groups with significant between-group
535 differences in contraction at time point. Scale bar on images represents 0.5 centimeters.
536 **Fig 8.** Masson's trichrome staining of 6 micron thick histological sections of representative
537 constructs (A), and average cellular angle of deviation from the longitudinal axis (B). For cell
538 alignment, an angle of 0° demarcates perfect parallel alignment. Scale bar on images represents
539 250 microns.
540
541
542

Excise whole digital flexor tendons



Digest tendon pieces with collagenase, overnight at 37°C

Plate tendon cells at high density to get a mixed cell population (parent TSC line)

Strain tendon digests and centrifuge at 800g for 10 minutes to get cell pellets

Isolate confluent colony with trypsin and cloning cylinder

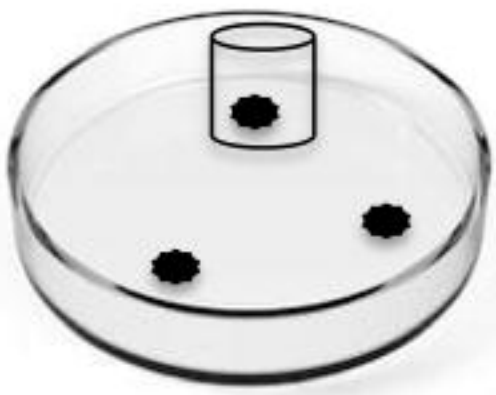
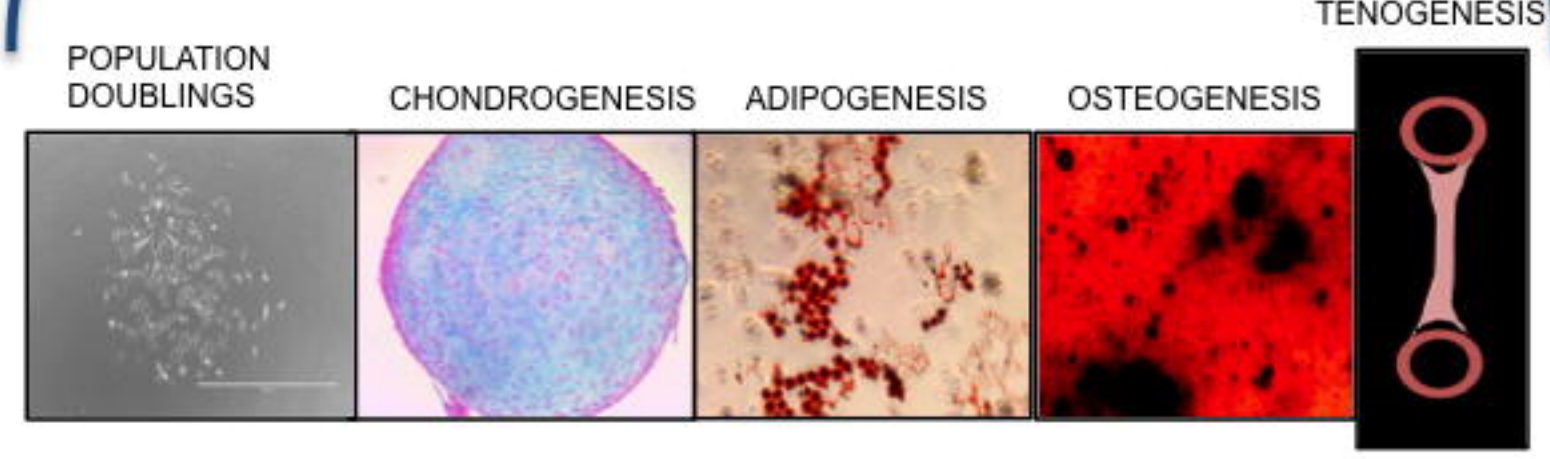


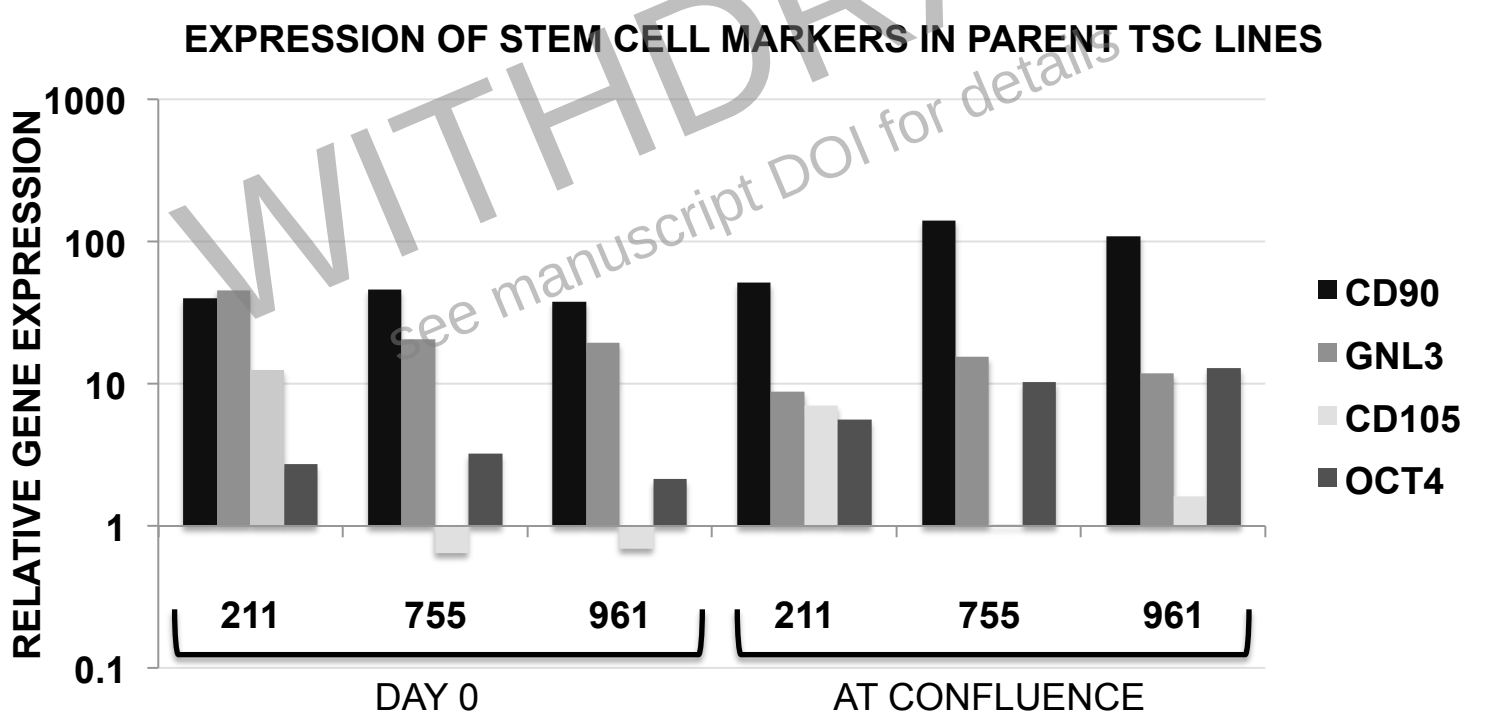
Plate tendon cells at low density to get single-cell derived colonies

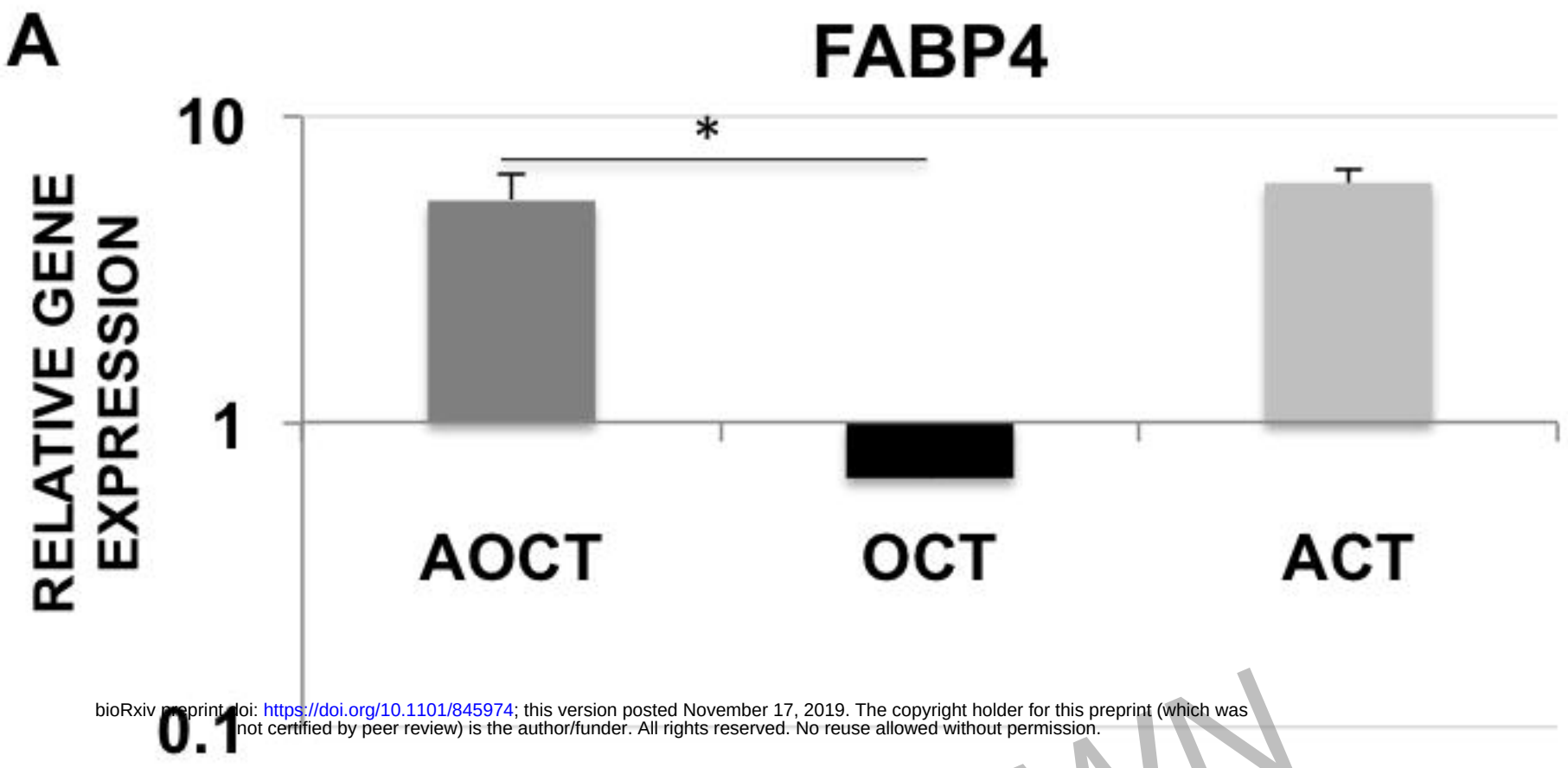
Expand colony for two passages

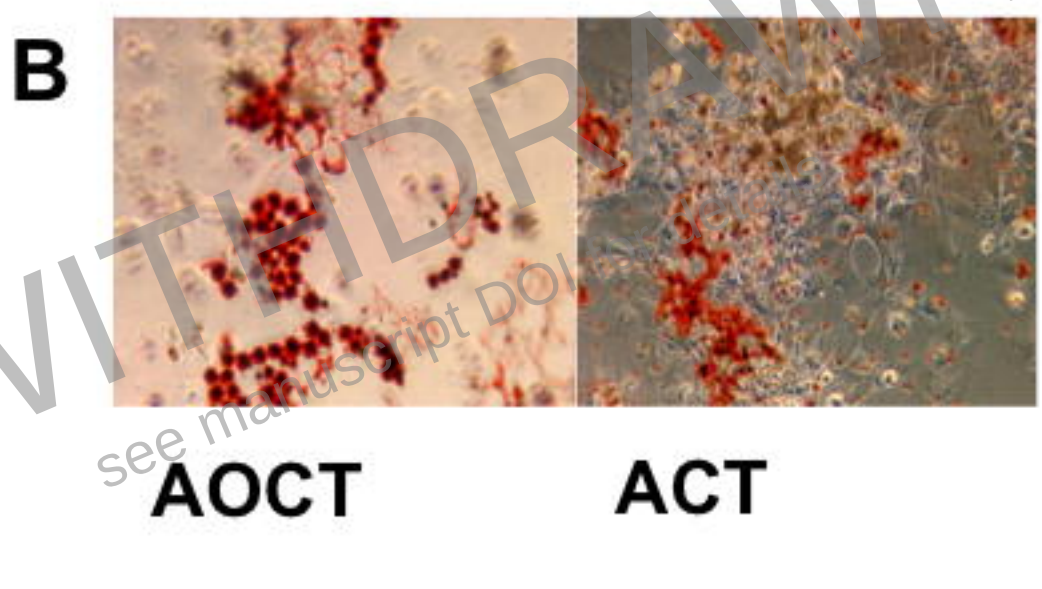
Trypsinize confluent culture and centrifuge at 800g for 10 minutes to get cell pellets

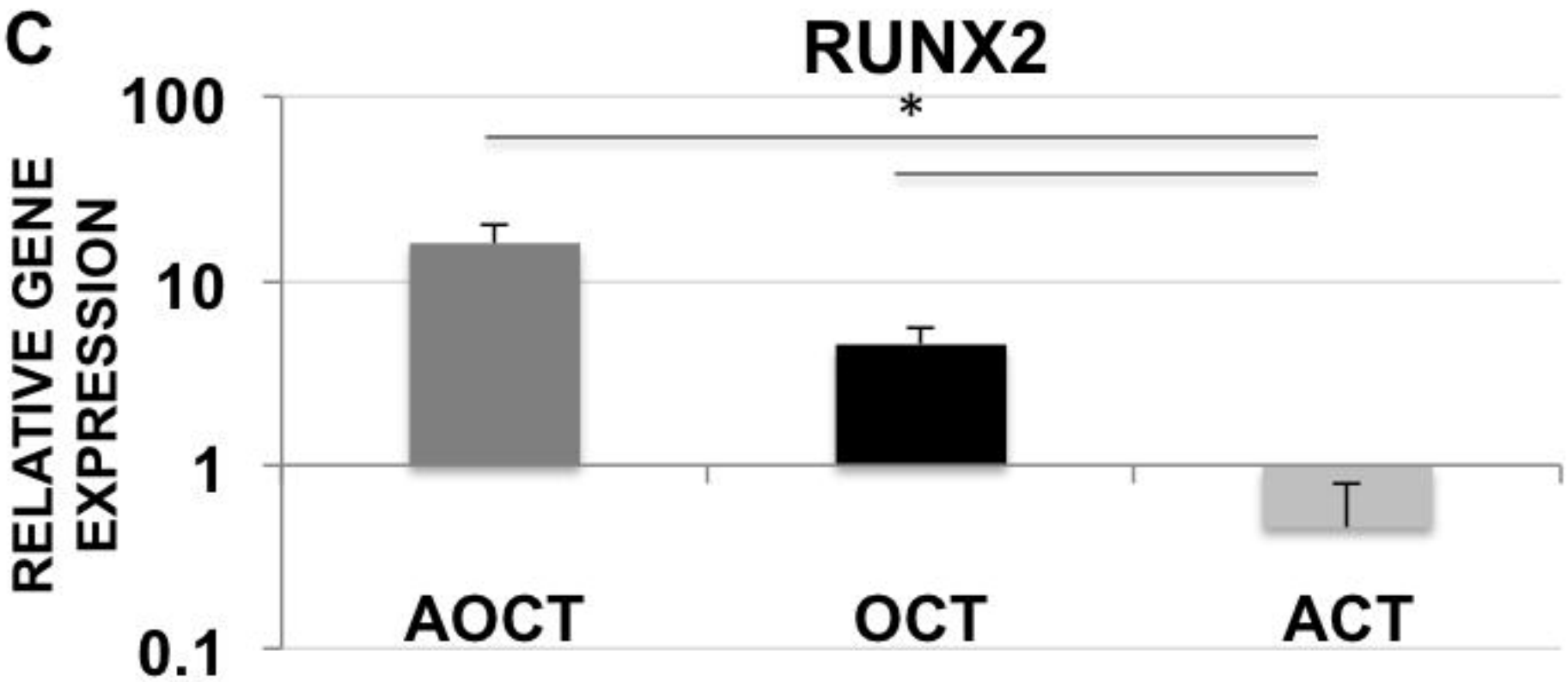
Plate cells for assays (P3)

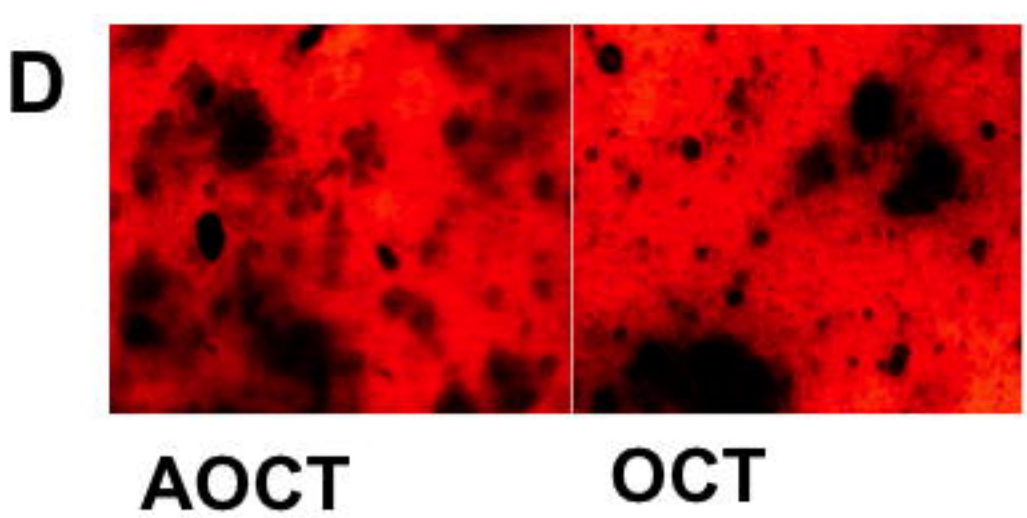


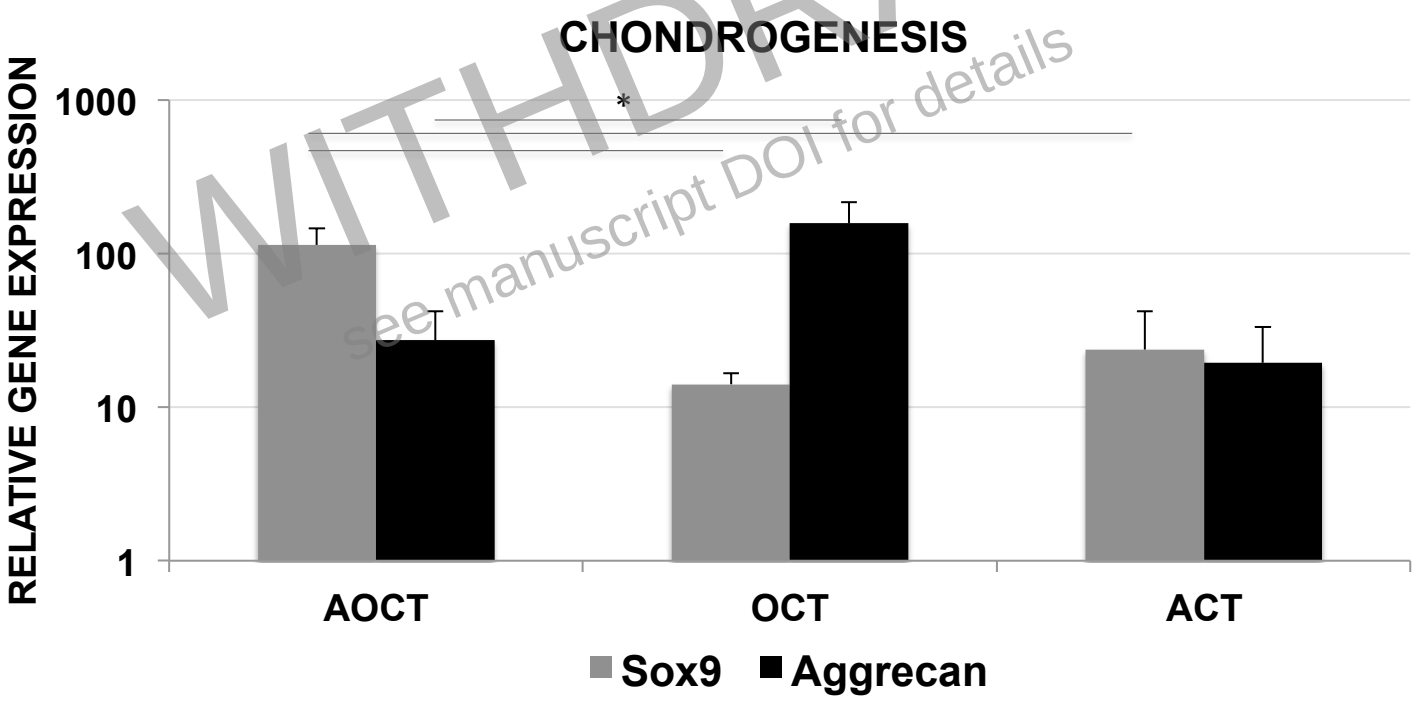


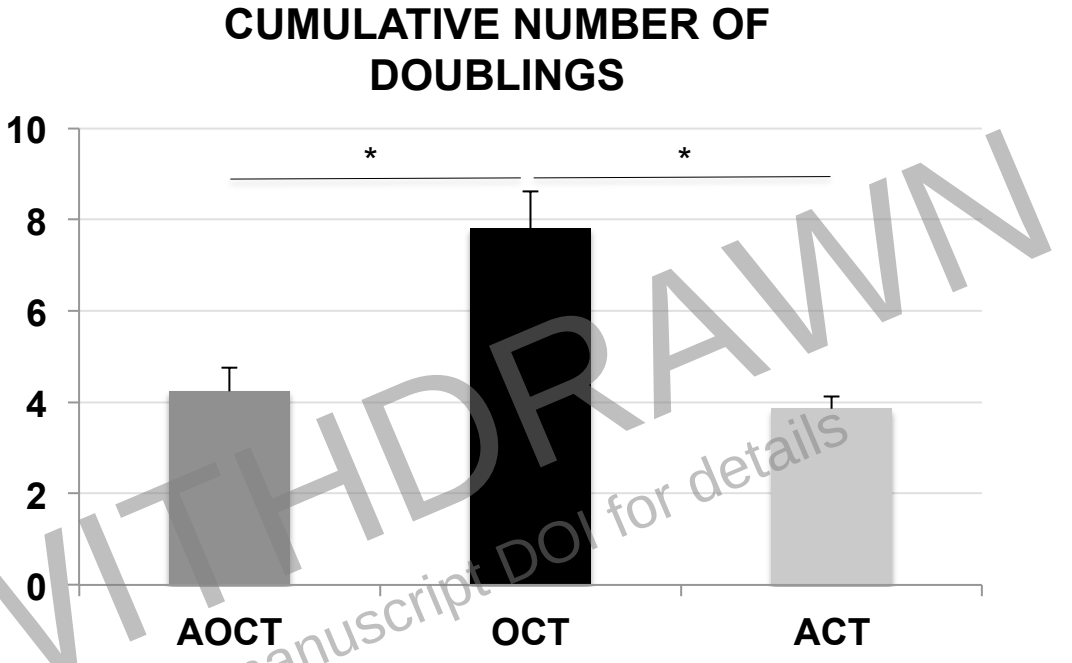


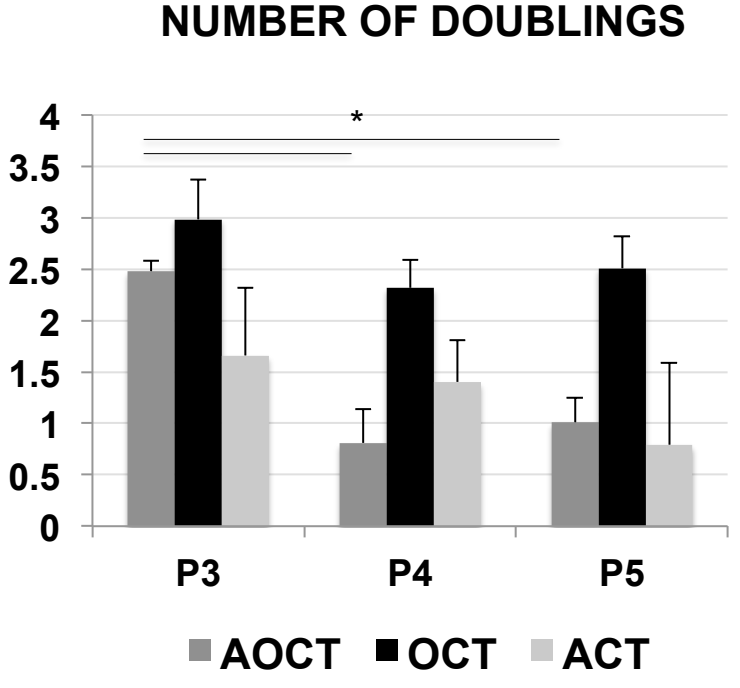


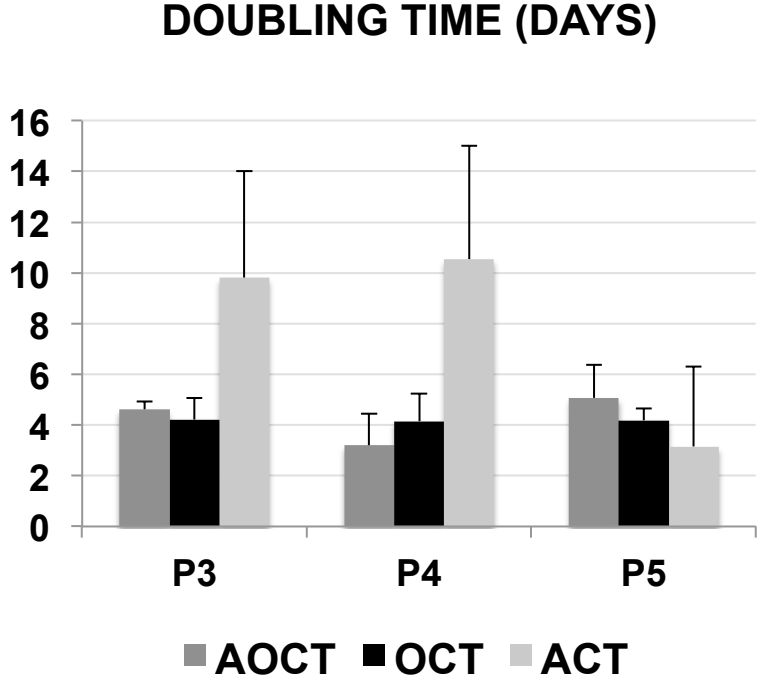


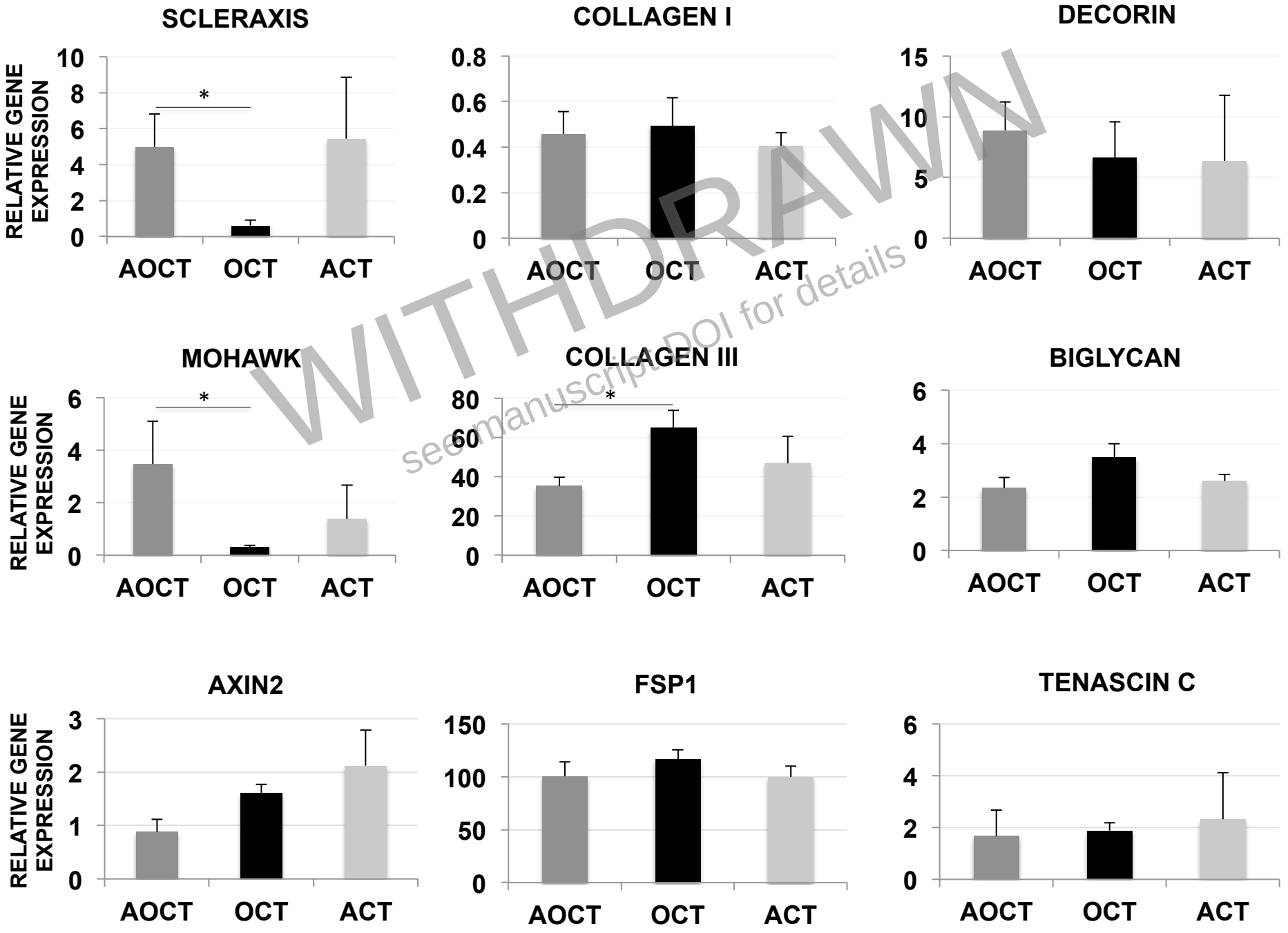












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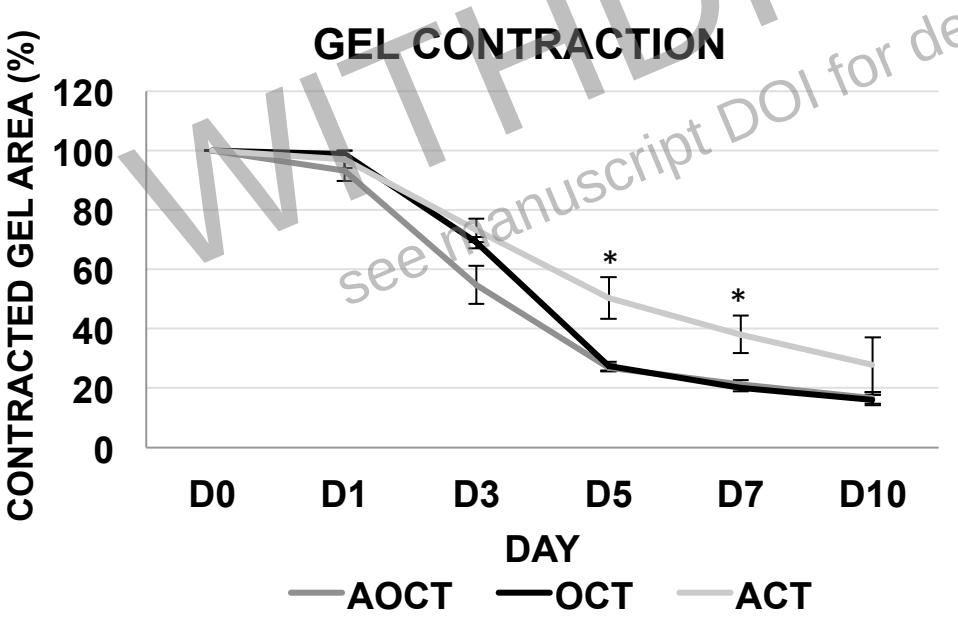

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