Directing iPSC differentiation into iTenocytes using combined scleraxis overexpression and cyclic loading

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Abstract

Regenerative therapies for tendon are falling behind other tissues due to the lack of an appropriate and potent cell therapeutic candidate. This study aimed to induce tenogenesis using stable Scleraxis (Scx) overexpression in combination with uniaxial mechanical stretch of iPSC-derived mesenchymal stromal-like cells (iMSCs). Scx is the single direct molecular regulator of tendon differentiation known to date. Bone marrow–derived (BM-)MSCs were used as reference. Scx overexpression alone resulted in significantly higher upregulation of tenogenic markers in iMSCs compared to BM-MSCs. Mechanoregulation is known to be a central element guiding tendon development and healing. Mechanical stimulation combined with Scx overexpression resulted in morphometric and cytoskeleton-related changes, upregulation of early and late tendon markers, and increased extracellular matrix deposition and alignment, and tenomodulin perinuclear localization in iMSCs. Our findings suggest that these cells can be differentiated into tenocytes and might be a better candidate for tendon cell therapy applications than BM-MSCs.

KEYWORDS

stem cells, tendon, tissue engineering
1 | INTRODUCTION

Tendon and ligament injuries are the main reason for all musculo-skeletal consultations worldwide and represent a significant concern in sports medicine, as well as the general population. Tendon injuries are associated with a high morbidity, prolonged disabilities, and painful rehabilitation periods, while secondary tendon ruptures often ensue. Current treatment modalities are inadequate since following treatment, functional recovery of repaired tendons is usually incomplete. The mechanical and structural properties of repaired tissue are permanently altered and fail to reach the level of functionality achieved before injury. Development of a cell therapy approach, which could be applied to an injured tendon or ligament site, could dramatically alter patient outcomes.

Adult stem/progenitor cells that have been identified in the tendon niche and characterized by their marker profile are considered promising cell sources for tendon tissue engineering. However, they are very scarce in vivo and cannot be expanded in vitro for therapeutic applications due to phenotypic drift in culture. Mesenchymal stromal cells (MSCs) derived from bone marrow (BM-MSCs) or adipose tissue (ASCs) have been explored as a potential tendon repair and tissue engineering strategy due to their abundance, multipotency, and regenerative potential in vivo. Several animal models have shown improved functional outcomes and enhanced tendon healing via utilizing MSCs. However, their major disadvantages are their limited self-renewal capacity, phenotypic heterogeneity, and potential for ectopic bone or cartilage formation. These attributes may limit their clinical application due to the need for in vitro expansion in adequate numbers and thorough characterization to meet regulatory standards before clinical use. Additionally, their tenogenic potential has been shown to be restricted. Developing an off-the-shelf cell source that can be efficiently differentiated to tenogenic progenitor cells is a prerequisite for tendon cell-based therapy success.

The discovery of induced pluripotent stem cells (iPSCs) through nuclear reprogramming of somatic cells revolutionized the field of regenerative medicine, because of their high self-renewal capacity and unparalleled developmental plasticity. iPSCs have been successfully differentiated to MSC-like cells (iMSCs) by our group and others. One of the main advantages of using iMSCs is that they potentially represent an unlimited source for tenocytes. Rodent tendon repair models utilizing iPSCs have shown improved functional outcomes. Thus, iMSCs derived from iPSCs may offer an unlimited off-the-shelf allogeneic source for tendon cell therapy applications.

Development of tenocytes occurs in at least two stages: First, tenocyte progenitors (tenoblasts) express scleraxis (Scx), which is the single direct molecular regulator of tendon differentiation known to date. Second, tenocyte maturation results in tissue formation. However, ectopic overexpression of Scx in BM-MSCs was not found sufficient to drive tenogenesis. Tendon and other tissues grow and remodel in response to changes in their environment. At the cell level, spatial distribution of dynamic mechanical cues can affect developmental, maintenance, and healing responses. Mechanoregulation has been shown to be a central element guiding embryonic tendon development and healing. Fully differentiated cells, such as tenocytes and fibroblasts, can actively sense both the external loading and the stiffness of their environment. Mechanical cues can also affect the differentiation of multipotent cells such as BM-MSCs. Uniaxial cyclic stretching has been used to drive tenogenic differentiation in vitro, as it is considered more relevant physiologically. Therefore, mechanical stimulation may be essential for the differentiation of multipotent cells toward tenogenic progenitors, as well as for the maturation of the tenocyte phenotype.

Additionally, Scx is a crucial factor for tenogenic differentiation. Therefore, combinatory mechanical and biological stimulation may be essential for tenogenesis. We hypothesized that iMSCs can be efficiently differentiated into tenocytes by combination of stable overexpression of Scx and mechanical stimulation in vitro. In this study we investigated (a) the ability of Scx stable overexpression to induce tenogenic differentiation in iMSCs compared to BM-MSCs, and (b) the effect of mechanical stimulation to guide tenogenic differentiation in vitro with and without Scx overexpression. To accomplish this, Scx was overexpressed using lentiviral vectors. iMSCs, and iMSC\textsuperscript{SCX+} cells were grown on mechanovariant substrates. Cyclic uniaxial stretching was applied on iMSCs and iMSC\textsuperscript{SCX+} using the CellScale MCFX bioreactor system. The effect of Scx overexpression with and without mechanical stimulation was assessed with gene expression analyses and immunocytochemistry for tendon markers, collagen deposition, and morphometric analysis of cytoskeletal orientation following uniaxial stretching.

2 | METHODS

Detailed methods are provided in the Supplemental Materials.

2.1 | Primary cell isolation and expansion

Human bone marrow was acquired from Lonza (Allendale, NJ) and BM-MSCs were isolated as previously reported. Media were switched to standard MSC culture media (low glucose DMEM, 1% AA, 10% FBS, 2 mM L-glutamine) and were changed every 3 days. Cells (≤P4) were split upon confluence in a 1:3 ratio.

2.2 | iMSC derivation and expansion

Normal human iPSC lines were obtained from the Cedars-Sinai iPSC core facility, expanded on Matrigel™-coated plates (Corning) with chemically defined mTeSR™Plus media (StemCell Technologies). iMSCs were differentiated from human iPSCs and expanded in vitro using our previously published method. iMSCs (≤P5) were maintained following the same protocol and media as described for BM-MSCs.
2.3 Genetic engineering of BM-MSCs and iMSCs to overexpress Scx

BM-MSCs and iMSCs were engineered via lentiviral transduction to overexpress Scx-GFP under the constitutively active CMV promoter coupled to its enhancer using a lentiviral vector (BM-MSC\(^{\text{scx}}\)-GFP+, iMSC\(^{\text{scx}}\)-GFP+). Transfections were conducted using the BioT method with a 1.5:1 ratio of BioT (µl) to DNA (µg). Transduction titers were determined using flow cytometry for GFP and verified using RT-qPCR for Scx.

2.4 Flow cytometry

For assessment of lentiviral transduction efficiency, transduced cells were lifted and washed with FACS buffer containing 2% bovine serum albumin (BSA, A4503, Sigma) and 0.1% sodium azide (S2002, Sigma) in 1x PBS. Titers were acquired on a BD LSR Fortessa analyzer (BD Biosciences) and were analyzed using Flowjo software (Flowjo LLC).

2.5 Cell culture on various stiffness surfaces

BM-MSCs and iMSCs were seeded at 2.5 x 10^4/cm^2 in plates of varying stiffness (Cytosoft\(^{\text{®}}\) Advanced Biomatrix) which are pre-coated with polydimethylsiloxane (PDMS), and before seeding, coated with Collagen-1 (Puracol\(^{\text{®}}\), Advanced Biomatrix), following the manufacturer’s recommendations. Cells were grown for 12 days in tenogenic media (low glucose DMEM supplemented with 2 mM glutamine, 10% FBS, 1% AAS, and 50 µg/ml ascorbic acid). After 12 days, cells were collected and stored at -80°C until processing for gene expression analysis.

2.6 Mechanical loading

Cells were seeded at a density of 2 x 10^4/cm^2 on fibronectin-coated silicone plates (CellScale Biomaterials Testing) and underwent an optimized cyclic stretching protocol of 4% sinusoidal strain, 0.5 Hz, 2 h/day using the CellScale MCFX bioreactor (CellScale Biomaterials Testing). Cells seeded on the same fibronectin-coated silicone plates were grown in parallel and served as static controls. iMSCs and iMSC\(^{\text{scx}}\) were stretched for up to 7 days, and media were changed daily. Cells were harvested on Days 0, 3, and 7 (n = 8/timepoint).

2.7 Cell morphology and morphometric analysis

To perform immunocytochemistry (ICC), at all time-points, cells were fixed with 10% buffered formalin, permeabilized in PBS with 0.3% Triton X-100 (PBS-T) and stained with Phalloidin-iFluor555 reagent (Abcam) to identify the cytoskeleton F-actin, and Tnmd (HPA055634, Sigma) or Collagen-1 (1310001, Bio-Rad). Cells were then washed and incubated for 2 h at RT with the secondary antibody donkey anti-rabbit Alexa Fluor\(^{\text{®}}\) 647-conjugated AffiniPure (Jackson Immunoresearch). Nuclei were counterstained with DAPI (Thermofisher). Samples were imaged with an Inverted Revolve fluorescent microscope (Revolve, Echo) with a 200x magnification objective using a 3 x 3 grid to capture nine nonoverlapping images for analysis. ImageJ was used to quantify Tenomodulin (Tnmd) staining intensity in unprocessed single channels to extract relative pixel intensity per field of view. No primary antibody-treated samples (only secondary antibody) were used to normalize this analysis. Last, to quantify the organization of the cytoskeleton in each cell, the ImageJ directionality tool was used to assess actin filament angle distributions.

Single-cell morphometric analysis was conducted using a custom image analysis pipeline developed in MATLAB (Matlab Natic, MA), as previously described. For this analysis, n = 7 wells/group with five nonoverlapping high-powered fields of view per well were used. Nuclear orientation and ImageJ directionality data were displayed as relative frequency distribution rose plots using MATLAB, with a bin width of 5° and 10°, respectively. A nuclear orientation or actin direction of 90° indicates that the nucleus or actin fiber angled perpendicular to the direction of stretch.

2.8 Gene expression analysis

Differentiation to iPSC-derived Tenocytes (iTenocytes) was defined based on expression of tendon-specific markers (Supporting Information: Table S1) using RT-qPCR with TaqMan\(^{\text{®}}\) gene expression. Total RNA was isolated using the RNeasy plus mini kit (Qiagen) and then was reverse-transcribed with the high-capacity cDNA reverse transcription kit (Applied Biosystems). The threshold cycle (Ct) value of 18S rRNA was used as an internal control using the TaqMan\(^{\text{®}}\) gene expression FAM/MGB probe system (4333760F, Thermofisher). The Livak method was used to calculate ΔΔCt values and fold change was calculated as 2^-ΔΔCt, as previously described and published.

2.9 Assessment of collagen deposition

Newly synthesized collagen was quantified using the Sirius red total collagen assay detection kit (Chondrex). Optical density at 535 nm was determined for samples and assay standards. Experimental samples (n = 8) and assay standards were conducted in technical triplicates.

2.10 Statistical analysis

All data are presented as mean ± standard deviation from the mean. Normally distributed data were analyzed with unpaired t-test (for two groups), or nonrepeated measures analysis of variance followed by Tukey-Kramer HSD post hoc analysis when more than two groups were compared. Nonparametric data were analyzed using the
Mann–Whitney and Kruskal–Wallis tests. To compare gene expression levels over time, a mixed repeated measures model was used, followed by Tukey’s post hoc tests for between group comparisons. Last, to analyze the orientation of nuclei and actin filaments between the static and stretched conditions, medians were compared using the Mann–Whitney test, while the two frequency distributions were compared using the Kolmogorov–Smirnov test. Statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Confirmation of stable lentiviral-driven Scx overexpression in BM-MSCScx+ and iMSCScx+

We generated lentiviral vectors expressing Scx tagged with GFP at the C-terminus and under the constitutively active CMV promoter, as previously described. Absolute GFP fluorescence, was similar for the highest titers (Supporting Information: Figure S1A). A dose–response effect of lentiviral load in transduction efficiency was observed when flow results were presented as percentage of GFP+ cells (Supporting Information: Figure S1B). We expanded BM-MSCs and iMSCs transduced with Scx-GFP lentivirus vector (BM-MSCScx+ and iMSCScx+) and assessed the expression of Scx at 4 weeks of regular culture without sorting (Figure 1C,D). Scx expression was significantly upregulated in BM-MSCScx+ and iMSCScx+ showed stable overexpression of Scx at Week 4 (Supporting Information: Figure S1D).

3.2 | Tenogenic marker gene expression of BM-MSCs, BM-MSCScx+, iMSCs and iMSCScx+ after 12 days of static culture

First, we examined the effect of Scx overexpression on BM-MSCs and iMSCs in vitro. The four cell groups BM-MSCs, BM-MSCScx+, iMSCs, and iMSCScx+ were generated and cultured in vitro on standard TCP culture plates for a duration of 12 days (Figure 1A). Findings were summarized in a heat map showing changes compared to baseline (Figure 1B) and as bar graphs displaying differences between all cell types (Figure 1C). Tenogenic marker genes (Scx, Mkkx, Thbs4, and Tnmd) and extracellular matrix (ECM) proteins previously associated with tenogenesis (Bgn, Col3a1, and Dcn) were significantly upregulated in iMSCScx+ compared to their own baseline (Day 0) whereas in BM-MSCScx+ only Scx, Bgn, Thbs4, and Dcn were significantly upregulated compared to their own baseline (Figure 1B). Mkkx and Tnmd showed significant downregulation in BM-MSCScx+ and in BM-MSCs. Scx was demonstrated to be significantly overexpressed in BM-MSCScx+ compared to BM-MSCs and iMSCs after 12 days (Figure 1C). When compared to other cell types, in the iMSCScx+ group, expression of Mkkx, Bgn, Thbs4, and Tnmd was significantly higher. Thbs4 was significantly higher in BM-MSCScx+ versus BM-MSCs. However, Tnmd was significantly higher in BM-MSCs compared to BM-MSCScx+. Dcn was significantly upregulated in iMSCScx+ but was lower compared to both BM-MSCs and BM-MSCScx+ (Figure 1C).

3.3 | Static culture of BM-MSCs, BM-MSCScx+, iMSCs, and iMSCScx+ under differential substrate stiffness conditions

First, we examined the effect of substrate stiffness on the BM-MSC and BM-MSCScx+ tenogenic differentiation potential. Tenogenic marker expression was increased in the TCP group. Col1a1, Mkkx, Thbs4, Bgn, and Dcn were significantly higher in BM-MSCScx+ cultured on TCP compared to the two softer substrates (Supporting Information: Figure 2). Last, the effect of differential substrate stiffness on iMSC and iMSCScx+ differentiation was assessed (Figure 2A). Tnmd and Bgn were significantly higher in iMSCs and iMSCScx+ cultured on TCP compared to the lower stiffness substrates in iMSCs (Figure 2B). Tenogenic markers Scx, Mkkx, Tnmd, and Thbs4 were significantly upregulated in iMSCScx+ cultured on TCP compared to iMSCs and iMSCScx+ cultured on all other substrates. Last, Col1a1 was close to baseline expression levels in iMSCScx+ cultured on TCP (data not shown). Zero net secretion of collagen was found in BM-MSCs, BM-MSCScx+, iMSCs and iMSCScx+ compared to their baseline levels on the three different substrates after 12 days (data not shown).

3.4 | Dynamic culture of iMSCs and iMSCScx+ using cyclic uniaxial stretching

Tenogenic marker expression of iMSCs and iMSCScx+ cultured on silicon plates in static conditions or cyclic stretching was analyzed at 3 and 7 days. Findings were summarized in a heat map showing changes compared to baseline (Figure 3A and Supporting Information: Figure S3). At 3 days, Scx, Mkkx, and Bgn were upregulated in the iMSC stretched group compared to baseline and Scx and Mkkx in the iMSC static group. At Day 7, Scx, Col3a1, Pdgfra, Tppp3, Tnmd, and Tnc were significantly upregulated in the iMSC static group. Cyclic stretching of iMSCScx+ for 3 days resulted in an upregulation of Scx, Mkkx, Bgn, and Thbs4. At Day 7, Thbs4 was still upregulated, Sox9 was also significantly upregulated, while Mkkx dropped to baseline levels (Figure 3A). Interestingly, in iMSCScx+ baseline expression of Col1a1 and Col3a1 was significantly downregulated compared to iMSC baseline expression (Supporting Information: Figure S5). Newly synthesized secreted collagen was significantly higher after 7 days of cyclic stretching in iMSCScx+ compared to iMSCs that were also stretched for the same time, as well as both cell types that were cultured for 7 days in the silicone plates (static condition, Figure 3C).

Cellular morphology and orientation of iMSCs and iMSCScx+ were examined after 7 days of uniaxial loading or static culture via
FIGURE 1 (See caption on next page)
phalloidin staining of the actin fibers of the cytoskeleton. Actin filaments aligned perpendicular to the axis of the load in the stretched plates, whereas a stochastic cytoskeleton alignment was observed in the static culture (Figure 4A). Additionally, an unbiased morphometric analysis was conducted on both culture conditions in iMSCS and iMSCSCX+. Nucleic orientation assessment showed that uniaxial cyclic loading for 7 days significantly affected the frequency distribution of nuclear orientation in iMSCS and iMSCSCX+, which was quantified as an angle from the axis of loading (Figure 4B). In iMSCS after 7 days of cyclic stretching, 54% of stretched nuclei were 65–90°.

**FIGURE 2** Lower substrate stiffnesses do not promote tenogenic differentiation in iMSCS with and without Scleraxis (Scx). (A) Cells (iMSCS and iMSCSCX+) were differentiated in vitro for 12 days on surfaces with different substrate stiffnesses (2 kPa, 32 kPa, and TCP i.e., ~Gpa). (B) Gene expression analyses were performed. Data are mean ± SD and they are normalized to d0 unperturbed iMSC controls (nontransduced); n = 6 replicates/group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**FIGURE 1** iMSCSCX+ show higher tenogenic potential than other cell types. (A) Graphical representation of the generation of four cell types that were used in the static culture differentiation experiments. BM-MSCS and iMSCS were transduced with lentiviral vectors to overexpress Scx stably. Cells (BM-MSCS, BM-MSCSCX+, iMSCS, and iMSCSCX+) were maintained in vitro for 12 days and then gene expression analyses were performed. (B) Tenogenic marker expression levels are displayed as heat maps; n = 6, *p < 0.05, (significant upregulation compared to baseline, i.e., d0 unperturbed BM-MSCS or iMSCS, respectively); $p < 0.05 (significant downregulation compared to baseline). (C) Gene expression levels of tenogenic markers were different between the four cell types after 12 days of culture in vitro. Data are mean ± SD and are normalized to d0 unperturbed BM-MSC or iMSC controls, respectively (nontransduced). Between-group comparisons were performed using one-way analysis of variance with Tukey’s post hoc; n = 6, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
from the axis of load compared to 33.7% nuclei in the static condition in the same range. Additionally, 36% of static culture nuclei angles ranged from 0–35°, unlike the stretched condition with 17.6% nuclei in the same range. (Figure 4B; left panel). In iMSCSCX+ after 7 days, 42.5% of stretched nuclei displayed an angle of 65–90°, as opposed to only 26.5% of the nuclei of the static culture (Figure 4B; right panel). In contrast, 30.2% of the static culture nuclei displayed an angle of 0–20°, while only 15.1% in the stretched plate were found in the same angular range (Figure 4D). Furthermore, the frequency distribution of nuclear angles was skewed toward 90°, as opposed to the static culture, which showed a stochastic alignment of nuclei in all directions. Actin filaments displayed a different orientation distribution in the static culture versus the stretched condition in both iMSCs and iMSCSCX+ after 7 days of cyclic stretching. In stretched iMSCs, 54.1% of actin fibers were found to be aligned in 60–90° angles from the axis of stretch, compared to 33.5% of the static condition. Similarly, in the stretched condition of iMSCSCX+, >50% (50.8%) of fibers were found to be aligned in 60–90°, while in the static condition, there were similar fractions of fibers aligned in each directionality cluster. For both cell types, the frequency distributions

![Figure 3](image-url)
**FIGURE 4** (See caption on next page)
between the two conditions were shown to be significantly different. Cell density was similar for both conditions and cell types (Figure 4D). Stretched iMSCs resulted in significantly lower aspect ratios (Figure 4E). The frequency distribution of the nuclei in iMSCSCX+ showed that in both conditions, 76% of all nuclei had aspect ratios between 0.55 and 0.8 (data not shown) but the distributions or medians were not significantly different (Figure 4E).

Further, phalloidin staining of actin fibers and ICC against Tnmd and DAPI in iMSCs and iMSCSCX+ was performed (Figure 5). Following 7d of uniaxial stretching, quantitative assessment of median Tnmd intensity showed no difference between any of the groups for both cell types (data not shown).

Finally, Collagen-1 staining was performed for iMSCs and iMSCSCX+ following 7d of cyclic loading and compared to the static condition (Figure 6). For both cell types, Collagen-1 fiber deposition seemed to be in parallel to the actin fibers, unlike the static conditions, where the matrix displayed a more random pattern. Collagen-1 quantification showed a significant increase in total staining intensity in iMSCSCX+ compared to static and stretched iMSC groups (Figure 6C).

Figure 5 Confirmation of tenogenic differentiation on 2D bioreactor using immunocytochemistry. Cytoskeleton visualization using phalloidin staining of actin filaments (red), nucleic staining with DAPI (blue), and immunocytochemical staining for Tnmd (cyan) was performed in the static culture ("static") and cyclic stretch ("stretched") conditions in iMSCs (A) and iMSCSCX+ (B). Cells were seeded into silicone deformable plates with the same density. In the stretched condition, they were stretched uniaxially for 7 days in a 2D bioreactor, while for the static condition they were placed in the incubator for the same timeframe. Channels are shown individually and merged for the same field of view. A merged higher magnification image is also shown on the right for each cell type and condition. Dotted line displays the long axis of the silicone plate which was the axis of the longitudinal stretch that was applied with this bioreactor system. Two different magnifications are shown for the two conditions. Scale bars represent 130 µm (yellow) and 60 µm (white).
In the present study, we investigated the effect of combined stable Scx overexpression and biomechanical stimulation to induce tenogenic differentiation in iMSCs. We demonstrated the following: (a) lentiviral vector-mediated stable overexpression induced tenogenic differentiation in vitro (Figure 1); (b) when altering substrate stiffness, cell culture on softer substrates did not promote tenogenic differentiation of iMSCs or BM-MSC controls compared to standard tissue culture polystyrene with and without Scx overexpression (Figure 2); (c) uniaxial cyclic stretching was able to induce upregulation of tenogenic markers in both iMSCs and iMSCSCX+ at Day 7, with significantly higher levels of expression in the iMSCSCX+ group (Figure 3); (d) uniaxial stretching resulted in changes in nuclear orientation and cytoskeleton alignment in iMSCs and iMSCSCX+ cells (Figure 4); and (e) Scx overexpression in iMSCs combined with stretching resulted in Collagen-1 fiber alignment and Tnmd deposition, indicating complementing effects of biological and mechanical cues to induce tenogenic differentiation in iMSCSCX+ compared to iMSCs (Figures 5 and 6). To our knowledge, this is the first study to demonstrate tenogenic differentiation processes in iMSCSCX+ under 2D cyclic tension.

Scx stable overexpression resulted in a significant upregulation of earlier and later tendon markers in vitro, especially in iMSCSCX+. These findings are consistent with prior literature reporting that Scx is an important transcription factor that regulates syndetome specification and tendon formation.22,30 Specifically, we demonstrated that BM-MSCSCX+ cells significantly upregulated Bgn, Thbs4, and Dcn after 12 days of culture compared to their own baseline. Compared to naïve BM-MSCs, Thbs4 was increased and Tnmd decreased in BM-MSCSCX+ cells (Figure 1C). In contrast to our study, Alberton et al. demonstrated Scx transduction of the SCP-1 hTERT immortalized BM-MSC cell line to induce Tnmd expression in a static in vitro culture system.13 These differences may be a result of the cell source, since they used an immortalized cell line while our study tested primary low passage BM-MSCs.13

In this study, iMSCSCX+ showed a significant upregulation in a larger number of tenogenic markers, including Tnmd as well as Mkx, Tnc, Bgn, and Thbs4 compared to baseline and the other cell types that were assessed. The stronger response of the iMSCs to Scx overexpression might be due to differences in the cell origin: while BM-MSCs are obtained from different donors of different ages displaying phenotypic variability that are known to potentially affect their in vitro performance, iMSCs are considered a more homogeneous cell type, since they are generated and expanded from a single origin.31 Moreover, iMSCs are derived from embryonic-like iPSCs and were reported to have a rejuvenation gene signature.32 In addition, iMSCs were shown to have superior survival and engraftment after transplantation,33 higher proliferative capacity, lower senescence rate,34 and higher immunomodulation ability34,35 compared to BM-MSCs.
The effect of substrate stiffness on the differentiation of multiple adult stem cells has been studied extensively.\textsuperscript{36-38} Mechanovariant substrates and selected ligands have been used in the past as a strategy to achieve directed differentiation of BM-MSCs toward tendon.\textsuperscript{39,40} In the present study, use of mechanovariant substrates (stiffnesses of E=2 kPa, 32 kPa and ~GPa) did not promote expression of tenogenic differentiation markers compared to control on standard TCP in BM-MSC and iMSC with and without Scx overexpression after 12 days of static culture (Figure 2B). These results are in contrast with a previous study using BM-MSCs cultured at E=40kPa for 2 weeks, showing an upregulation of Scx, Tnmd, and Tnc.\textsuperscript{39} Differences between the results may be due to differences in donor or method of cell expansion before the experiments.\textsuperscript{41} For example, Li et al. found that priming of primary rat BM-MSCs on a stiff surface resulted in mechanical memory that lasted for more than 2 weeks through a micro-RNA-21-controlled mechanism. \textsuperscript{42} They showed that miR-29 expression was established after mechanical priming for three passages and this effect could be maintained for at least two passages after switching to substrate with a higher or lower stiffness.\textsuperscript{42} The cells that were used for our experiments, BM-MSCs and iMSCs, were initially expanded on TCP plates (up to P4). Therefore, it is likely our initial expansion cycles on TCP could have primed the cells toward a stiffer phenotype that masked the effect of the softer substrates over the course of 12 days of our in vitro static culture.

In the present study, we stimulated iMSCs with 4% uniaxial strain and 0.5 Hz for 2 h/day in a 2D bioreactor using deformable silicone plates, which is consistent with other studies showing that physiologically relevant loads range from 4% to 6% strain at the cellular level of tendon.\textsuperscript{18} The final seeding density was chosen to avoid monolayer overgrowth on the silicone plate, which has been described to contribute to static tension.\textsuperscript{43} After 7 days of cyclic mechanical stimulation, a significant upregulation of Scx, Tnmd, Tnc, Col3a1, Tnpp3 as well as Pdgfra in stretched iMSCs was detected (Figure 3A). Using the same bioreactor system (CellScale MCFX), but applying higher total strain amplitude and duration (10% at 1 Hz, for 12 h/day), Gaspar et al. were not able to detect changes in gene expression of Scx, Tnmd, and Col1a1 and collagen deposition in BM-MSCs and human tenocytes.\textsuperscript{43} However, they reported significant upregulation of Thbs4 after 3 days. This could reflect the higher strains that were applied in that study, since BM-MSCs aligned parallel to the load which contrasts the big body of research demonstrating perpendicular cell alignment with physiological strains.\textsuperscript{44} In our study, stretched iMSCSCX+ significantly upregulated Scx, Col3a1, Bgn, Thbs4, Sox9, and Pdgfra, but not Tnmd at Day 7 (Figure 3A). Comparable to our study, Chen et al. demonstrated an induction of Col1a1, Col1a2, Col14, and Tnmd and increased ECM deposition in lentiviral-mediated Scx-overexpressing hESC-MSCs that were assembled in multilayered cell sheets and cultured under uniaxial dynamic cyclic load (10% strain, 1 Hz, 2 h/day) for up to 21 days.\textsuperscript{45}

Assessment of new ECM deposition displayed significantly higher total collagen content in the iMSCSCX+ cells stretched for 7 days compared to iMSCs in both static and stretched conditions and to iMSCSCX- in the static culture at the same timepoint (Figure 3C). This indicates a potential synergistic effect of Scx overexpression and uniaxial loading resulting in not only tendon marker expression, but also more importantly secretion of ECM proteins to the media. BM-MSCSCX+ in static culture and cell sheets of Scx+ human ESCs-derived cells under loading have been shown to result in increased collagen deposition.\textsuperscript{13,45} Since we detected significant upregulation of Col3a1 but not Col1a1, we assessed the inflammatory markers II-6, II-1ß, and TNF-α to exclude fibrotic tissue formation. II-6 was significantly upregulated after 12 days of static culture in iMSCSCX+ cultured on the 32 kPa substrate but not in any other groups (Figure 2). Additionally, II-6 was upregulated at Days 3 and 7 of uniaxial stretching in iMSCSCX+ but not in iMSCs (Figure 3B). Inflammatory markers including II-6 have been shown to be upregulated by loading of higher magnitude (8%-12%) in 2D uniaxial stretching systems\textsuperscript{18} and its upregulation has been reported in tendinopathies and ruptured tendons.\textsuperscript{46} However, in this study, II-1ß and TNF-α did not change compared to baseline in iMSCs and iMSCSCX+ cultured on the three substrates, nor when they were stretched in the 2D bioreactor (Figure 3B). II-6 expression did not change following stretching in iMSCs (Figure 3B). In human tendons, II-6 is upregulated following exercise, and it is a potent stimulator of collagen synthesis.\textsuperscript{47} Thus, II-6 upregulation might have contributed to the increased ECM deposition in stretched iMSCSCX+ (Figure 3B).

The distribution of nuclear and cytoskeleton orientation was very distinct between the two conditions, suggesting that the cells re-orientated as a response to the stretch stimulus (Figure 4B, C). While the statically cultured cells displayed stochastic nuclear arrangement in all directions, in the stretched cells the predominant orientation of nuclei was perpendicular to the applied load. Consistent with our findings, previous reports have shown cell orientation perpendicular to the stretching direction when physiological strains (~7%) were applied.\textsuperscript{44,48} Nucleic aspect ratios (width to height) of stretched versus static cells were unaltered in iMSCSCX+ but significantly lower in stretched iMSCs compared to the static condition, pointing to more elongated nuclei (Figure 4E).

Actin filament staining and ICC for Tnmd in iMSCs and iMSCSCX+ static and stretched cells showed presence of Tnmd in the entire cytoplasm and in close proximity to the nucleus (Figure 5). This is consistent with previous reports that Tnmd isoforms I and II localize to the nuclear envelope, while isoform III is cytoplasmic.\textsuperscript{49} We observed more widespread presence of Tnmd in the cytoplasm of static iMSCSCX+ while in the stretched group there appeared to be a stronger signal in the perinuclear area. Unfortunately, ICC performed on the silicone bioreactor plate wells has limitations since it is not able to discriminate between the different isoforms. Further, the semi-quantitative assessment of staining intensity we ran did not show any difference between static and stretched groups in iMSCs and iMSCSCX+. It remains to be determined whether differential isoform localization could have a functional significance. Intriguingly, Tnmd gene expression was unchanged at Days 3 and 7 with cyclic stretching in iMSCSCX+ cells (Figure 3 and Supporting Information:...
Figure S4). Tnmd is directly regulated by Scx, and previous studies using Scx overexpression in BM-MSCs have reported Tnmd upregulation. However, recent findings using equine ESCs and fetal tenocytes to overexpress Scx have shown Tnmd gene downregulation, suggesting that mRNA and protein levels are differentially regulated and therefore need to be assessed in tandem. Last, Collagen-1 staining in iMSCs and iMSCSCX+ that were loaded for 7 days (Figure 6) showed a more organized collagen fiber network which was in parallel to the actin fibers and perpendicular to the axis of stretch, unlike the static groups, which displayed a more random orientation of fibers. Total Collagen-1 staining intensity in iMSCSCX+ was significantly higher compared to static and stretched iMSC groups but not compared to the static iMSCSCX+ group (Figure 6C). Complemented with our collagen assay data, it can be concluded that iMSCSCX+ that were cyclically loaded for 7 days resulted in a newly formed ECM fiber network whose orientation might have been instructed by mechanical regulation.

This study is not without limitations. It could be postulated that even though our transduction efficiency was close to 100%, there may have been cells that were not transduced with Scx. Thus, the proportion of Scx+ cells may decrease with expansion and some of the transduced cells might have lost Scx expression due to gene silencing. Cell sorting could potentially be used to obtain a purer iMSCSCX+ population. Although, cell sorting was not necessary in this study since we were able to achieve iMSCSCX+ tenogenic differentiation, it may still be needed for future in vivo applications. Further, it has been postulated that the nominal strain applied in a 2D stretching system may be higher compared to the actual strain experienced by the cells. The latter may vary depending on the biomechanical system, cell density and cell type (size, morphology, etc.), which hampers comparison and standardization between different studies. To this end, we performed a series of pilot studies to optimize the stretch protocol and used complementary morphometric analyses to ensure we were stretching the cells within their physiological range. Last, the lack of adequate specific markers to assess temporal tenocyte gene expression changes has resulted in difficulty to characterize differentiation efforts and to discriminate between the effects of biological cues versus biomechanical ones that are crucial for tendon tissues. Therefore, we need additional functional assessments to understand changes following differentiation efforts and the effects of mechanical stimulation on the maturation of the cellular phenotype.

5 | CONCLUSIONS

An appropriate and potent cell therapeutic candidate for tendon regeneration is needed to address today’s challenges in tendon and ligament repair. Our data provide evidence that iMSCs, genetically engineered to express Scx and stimulated by cyclic mechanical stretch to drive the cells into iTenocytes, may be a potential candidate for tendon cell therapy applications. Tenogenic potential of iMSCSCX+ was demonstrated by upregulation of early and late tendon markers, increased collagen deposition and ECM alignment, Tnmd expression, morphometric and cytoskeleton-related changes. Overall, we showed that iMSCs were amenable to Scx overexpression and mechanical stimulation. Further, iMSCs were more responsive to Scx overexpression in vitro static culture, displaying upregulation of more tenogenic markers compared to BM-MSCs. It could be postulated that this might be due to higher plasticity of iMSCs and further studies exploring the potential of the two cell types for tendon cell therapy applications are warranted.

In future research, the functional impact of iTenocytes on tendon and ligament repair should be evaluated in an animal tendon injury model, providing an environment of physiological locomotion.

AUTHOR CONTRIBUTIONS

Angela Papalamprou made a substantial contribution to the conception, design of the work, performed experiments, analyzed data, prepared the original and revised manuscript drafts and edits. Victoria Yu made a substantial contribution to original and revised draft preparation, performed experiments, analyzed data, and prepared rose plots. Angel Chen, Tina Stefanovic, Giselle Kaneda, Khosrowdad Salehi, Chloe Castaneda performed experiments and analyzed data. Arkadiusz Gertych wrote the code for nuclear orientation analysis and contributed to draft review and editing. Juliane D. Glaeser made a substantial contribution to the conception, design of the work and manuscript preparation. Dmitriy Sheyn is the PI and made a substantial contribution to the conception, design of the work, manuscript preparation and edits. All authors have read and approved the final submitted manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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