Research Article

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Regenerative Medicine

Multidifferentiation potential of human mesenchymal stem cells from adipose tissue and hamstring tendons for musculoskeletal cell-based therapy

Deborah Stanco[1], Marco Viganò[1], Carlotta Perucca Orfei[1], Alessia Di Giancamillo[2], Giuseppe M Peretti[2,3], Luciano Lanfranchi[2] & Laura de Girolamo*[,1]

[1]Orthopaedics Biotechnology Lab, IRCCS Galeazzi Orthopaedic Institute, Via R. Galeazzi 4, 20161 Milan, Italy
[2]IRCCS Galeazzi Orthopaedic Institute, Via R Galeazzi 4, 20161 Milan, Italy
[3]Biomedical Science for Health, University of Milan, Milan, Italy
*Author for correspondence:
Tel.: +39 02 6621 4059
Fax: +39 02 6621 4048
laura.degirolamo@grupposandonato.it

Aim: Adipose-derived stem cells (ASCs) have been deeply characterized for their usefulness in musculoskeletal tissue regeneration; recently, other mesenchymal stem cell (MSC) sources have also been proposed. This study compares for the first time human tendon stem/progenitor cells isolated from hamstring tendons with human ASCs. **Materials & Methods:** Human TSPCs and ASCs were isolated from hamstring tendon portions and adipose tissue of healthy donors undergoing ACL reconstruction or liposuction, respectively (n = 7). Clonogenic ability, immunophenotype and multidifferentiation potential were assessed and compared. **Results:** Both populations showed similar proliferation and clonogenic ability and expressed embryonic stem cell genes and MSC surface markers. Tendon stem/progenitor cells showed lower adipogenic and osteogenic ability, but after the chondrogenic differentiation, they produced more abundant glycosaminoglycans and expressed higher levels of aggrecan with regards to ASCs. The tenogenic induction with BMP-12 upregulated *SCX* and *DCN* gene expression in both populations. **Conclusion:** Our results demonstrate that waste hamstring tendon fragments could represent a convenient MSC source for musculoskeletal regenerative medicine.

Keywords: adipose-derived stem cells • bone morphogenetic protein-12 • multidifferentiation potential • musculoskeletal pathologies • regenerative medicine • tendon • tendon stem progenitor cells • tenogenic differentiation

Although bone marrow is still the most common source of mesenchymal stem cells (MSCs), in recent years, driven by a constant quest for the 'most convenient' source, researchers have demonstrated that these cells can be identified in several alternative sites. In particular, MSCs have been found in tissues that are discarded after surgical interventions, including adipose tissue, periodontal ligaments and deciduous teeth. Many studies have been performed to compare the features of MSCs from different origins, with the final aim of identifying the best possible MSC tissue source for a given clinical situation. The choice of the most appropriate cell type is crucial since, although MSCs deriving from different tissues share common properties, it is known that they are influenced by the surrounding microenvironment and tissue-specific characteristics that, ultimately, may influence the final treatment outcome. Moreover, age, site, gender and pathological conditions have been shown to affect the number, proliferation potential and differentiation capacity of MSCs [1–4]. Recently, it has been shown that discarded tendon fragments derived from knee ligament reconstruction [5] or tendon cuff repair [6] also contain MSC populations, named tendon stem/progenitor cells (TSPCs) [7]; this observation has enabled remarkable advancements in understanding the physiopathology of this tissue, as well as the possibility of using this cell source as a potential tool for tendon regenerative treatments.

In this study, the *in vitro* immunophenotype, embryonic stem cell marker expression, clonogenic and proliferation ability and

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multilineage differentiation potential, including the tenogenic potential, of TSPCs and adipose-derived stem cells (ASCs) were compared for the first time. Indeed, ASCs have already been well characterized and demonstrated to be potentially useful in musculoskeletal cell-based therapy, but to date, very few *in vitro* studies have investigated their *in vitro* tenogenic potential [8–10]. Thus, the main aim of this study was to provide further knowledge regarding the potential use of TSPCs in musculoskeletal tissue regeneration strategies, and our hypothesis was that it would be more advantageous to use this cell source for tendon regenerative purposes in comparison with ASCs.

Materials & methods

TSPC & ASC isolation & culture

All of the procedures were carried out at Galeazzi Orthopedic Institute (Italy) with Institutional Review Board approval (M-SPER-014.ver7 for the use of surgical waste). All of the donors gave their written consent for the use of their surgical waste material for research purposes. TSPCs were isolated from discarded fragments of semitendinosus and gracilis tendons collected from seven donors (mean age: 33 ± 14 years) who underwent anterior cruciate ligament reconstruction; ASCs were isolated from raw lipoaspirates of seven donors (mean age: 47 ± 14 years) who underwent esthetic liposuction (Table 1). The samples of tendon tissue were minced into small pieces (0.5–0.8 cm), placed in 100-mm Petri dishes and covered with control medium (CTRL medium; Table 2). During the first 10 days in culture, tendon cells migrated from tissue and started to proliferate [11,12]; at approximately 3 weeks later, they reached 80–90% confluence.

ASCs were isolated as previously described [2]. Briefly, adipose tissue was washed with phosphate-buffered saline and digested with 0.075% type I collagenase (Worthington Biochemical Corp., NJ, USA) at 37°C for 30 min. The stromal vascular fraction was centrifuged (1200 *g*, 10 min) and then filtered through a 100-µm nylon cell strainer (Becton, Dickinson and Co., NJ, USA); the collected stromal vascular fraction cells were plated in CTRL medium at 10^4 cells/cm^2 of density and reached 80–90% confluence in approximately 7 days. TSPCs and ASCs were maintained in culture, changing the medium every 3 days.

Cell doubling time

For TSPCs and ASCs, cell doubling time (DT) was calculated from passages 2 to 4. The cells were plated at a density of 3×10^3 cells/cm^2 in CTRL medium. Fresh medium was supplied every 3 days, and at 80–90% confluence, the cells were split using trypsin/EDTA (0.5% trypsin/0.2% EDTA; Sigma-Aldrich (MO, USA). DT was calculated according to the following formula: $DT = CT/\ln(N_f\text{-}N_i)/\ln 2$, where CT is the cell culture time (hours), N_f is the final number of cells and N_i is the initial number of cells [13].

Fibroblast colony forming unit assay

The clonogenic ability of ASCs and TSPCs from passages 2 to 4 was evaluated by colony forming unit assay as previously described [2]. Briefly, cells were plated in six-well plates at low density by limiting dilution (starting dilution: 100 cells/cm^2; ending dilution: 13 cells/cm^2) and cultured in CTRL medium supplemented with 20% fetal bovine serum for 14 days. Then, the cells were fixed with 4% paraformaldehyde solution and counted after 2.3% Crystal Violet staining (Sigma-Aldrich) for 10 min at room temperature. The frequency of CFU-F (colony forming unit-fibroblasts) was established by scoring the individual colonies composed of at least 30 cells and expressed as a percentage relative to the number of the initial seeded cells.

Cell metabolic activity

For both TSPCs and ASCs, 1.5×10^4 cells at passage 4 were seeded into 96-well plates and the cell metabolic activity assay was performed at 1, 3, 7 and 14 days. Briefly, a final concentration of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) was added to the culture medium and incubated for 4 h at 37°C. After medium removal, 100% DMSO was added to each

Table 1. Characteristics of patients and cellular yields at passage 1.

	TSPCs	ASCs
Number of patients	7 (two female, five male)	7 (six female, one male)
Age (years)	33 ± 14	47 ± 14
Tissue from each donor (g)	1.7 ± 0.9**	21.5 ± 13.3
Number of cells at passage 1/gram of tissue	2.2 ± 1.8 × 10^{5*}	0.7 ± 0.5 × 10^5

*p < 0.05; **p < 0.01 vs ASCs.
ASC: Adipose-derived stem cell; TSPC: Tendon stem/progenitor cell.

Table 2. Composition of differentiation media.

Medium	Main components	Serum	Supplements
CTRL	HG-DMEM, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.29 mg/ml L-glutamine	10% FBS	–
Adipogenic			
– Induction	CTRL	10% FBS	1μM dexamethasone, 10 μg/ml insulin, 500 μM IBMX, 200 μM indomethacin
– Maintenance	CTRL	10% FBS	10 μg/ml insulin
Osteogenic	CTRL	10% FBS	10 nM dexamethasone, 10 mM glycerol-2-phosphate, 150 μM L-ascorbic acid-2-phosphate, 10 nM cholecalciferol
Chondrogenic	CTRL	1% FBS	1 mM sodium pyruvate, 1% ITS+1, 0.1 μM dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate, 10 ng/ml TGF-β1
Tenogenic	CTRL	1% FBS	50 ng/ml BMP-12

CTRL: Control; FBS: Fetal bovine serum; HG-DMEM: High-glucose Dulbecco's Modified Eagle's Medium; IBMX: 3-isobutyl-1-methyl-xanthine; ITS+1: 1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin, 0.5 μg/ml sodium selenite, 50 mg/ml bovine serum albumin and 470 μg/ml linoleic acid.

well in order to solubilize the formazan precipitate. The absorbance of this solution was read at 570 nm (Victor™ X3 microplate; Perkin Elmer, MA, USA).

Fluorescence-activated cell sorting analysis

TSPCs and ASCs at passage 4 were detached and washed twice in cold fluorescence-activated cell sorting buffer (phosphate-buffered saline without Ca/Mg^{2+}, 2% fetal bovine serum and 0.1% NaN_3). A total of 2.5×10^5 cells were incubated with the antihuman primary monoclonal antibodies: fluorescein isothiocyanate-conjugated CD90, CD13, CD31, CD44, CD45 and CD166 (Ancell Corp., MN, USA) ; biotinylated-conjugated CD29, CD34, CD54, CD71 and CD105 (Ancell Corp.); and phycoerythrin-conjugated CD73 (Miltenyi Biotec, Germany). After incubation, streptavidin–phycoerythrin and fluorescein isothiocyanate-conjugated goat antimouse antibodies (Ancell Corp.) were used as secondary antibodies for cells stained with biotinilated antibodies. Background fluorescence was established by negative controls and data on 10,000 cell fluorescence events were acquired by flow cytometry using a FACSCalibur™ flow cytometer (BD Biosciences, NJ, USA) and analyzed by CellQuest™ software (BD Biosciences).

Adipogenic differentiation

Both cell populations at passage 4 were seeded at 10^4 cells/cm^2 and then induced to differentiate towards an adipogenic lineage for 21 days using a repeated pulsed protocol, consisting of 3 days in adipogenic induction medium followed by 3 days in adipogenic maintenance medium (ADIPO medium; Table 2). To quantify the lipid vacuoles, cells were rinsed and fixed in 10% neutral buffered formalin for 1 h, stained with Oil Red O (Sigma-Aldrich) for 15 min, then unstained with 100% isopropanol. Absorbance was read at 490 nm.

Osteogenic differentiation

Cells at passage 4 seeded at 10^4 cells/cm^2 were differentiated into an osteogenic lineage by culturing in osteogenic medium (OSTEO medium; Table 2). Cells were assessed for alkaline phosphatase (ALP) activity and extracellular calcified matrix deposition [2]. Briefly, after 14 days of differentiation, cells were lysed with 0.1% Triton™ X-100 (Sigma-Aldrich) and the enzymatic ALP activity was determined by incubating cellular lysates at 37°C with 1 mM p-nitrophenylphosphate (Sigma-Aldrich) in alkaline buffer (100 mM diethanolamine and 0.5 mM $MgCl_2$, pH 10.5). The absorbance was read at 405 nm (Victor X3 microplate reader) and the ALP activity was normalized against the determined total protein content (Pierce™ BCA protein assay kit; Pierce Biotechnology, IL, USA).

The extracellular calcified matrix deposition was evaluated after 21 days of differentiation. Briefly, the cells were stained with 40 mM Alizarin Red S (pH 4.1; Fluka-Sigma Aldrich, MO, USA) for 15 min. The dye was extracted with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.0) and the absorbance was read at 550 nm.

Chondrogenic differentiation

At passage 4, 5.0×10^5 TSPCs and ASCs were centrifuged (250 *g*, 5 min) in order to obtain cell pellets. Pel-

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lets were cultured in chondrogenic medium (CHONDRO medium; Table 2) for 21 days. For histological analysis, pellets were fixed for 24 h in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 µm. Sections were stained with hematoxylin–eosin (Sigma-Aldrich) and safranin O in order to evaluate the extracellular matrix and glycosaminoglycan (GAG) deposition [14]. GAGs were also quantified by digesting the pellets (16 h, 60°C) in 500 µl of PBE buffer (Phosphate Buffer/EDTA; 100 mM Na_2HPO_4, 10 mM NaEDTA, pH 6.8) containing 1.75 mg/ml of L-cystein (Sigma-Aldrich) and 14.2 U/ml of papain (Worthington). Samples were incubated with 16 mg/l dimethylmethylene blue (Sigma-Aldrich) and absorbance was read at 500 nm (Victor X3 microplate reader). The same samples were used for DNA quantification using a CyQUANT® Kit (Life Technologies, NY, USA). The amount of GAG produced for each sample was normalized against the DNA content and expressed as micrograms of GAG per microgram of DNA.

Tenogenic differentiation

Both TSPC and ASC populations were seeded at passage 4 and at a cell density of 10^4 cells/cm^2. After 7 and 14 days of tenogenic differentiation in an inductive medium (Table 2), the expression of tendon-related genes was assessed by real time-PCR.

Gene expression

The total RNA was purified from the cell lysates using the RNeasy® Mini Kit (Qiagen, Germany) and reverse transcripted to cDNA (5 min at 25°C, 30 min at 42°C and 5 min at 85°C) using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA). A total of 10 ng of cDNA were incubated with a PCR mix (50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min) containing TaqMan® Universal PCR Master Mix and Assays-on-Demand Gene expression probes (Life Technologies) for the following genes: *LEP* (Hs00174877_m1), *RUNX2* (Hs00231692_m1), *COL1A1* (Hs01076777_m1), *COL3A1* (Hs00943809_m1), *SOX9* (Hs00165814_m1), *ACAN* (Hs00153936_m1), *SCX* (Hs03054634_g1), *DCN* (Hs00370385_m1), *POU5F1* (Hs04260367_gh) and *KLF4* (Hs00358836_m1). Reactions were performed with Applied Biosystems® StepOnePlus™ (Life Technologies). The fold change in expression was normalized against the expression of the housekeeping *GAPDH* gene (Hs99999905_m1).

Statistical analysis

Data are expressed as means ± standard deviations. The normal distribution of values was assessed by the Kolmogorov–Smirnov normality test. Statistical analyses were performed using the Student's t-test for data with a normal distribution and the Wilcoxon test for data with a non-normal distribution (GraphPad Prism® v5.00; GraphPad Software, CA, USA). The level of significance was set at $p < 0.05$ (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The correlation between age and each parameter was assessed by Pearson's correlation test.

Results

Undifferentiated TSPCs & ASCs show similar stem cell features

TSPCs and ASCs were harvested from seven different donors each (Table 1). The mean donor ages of the two cell populations were different, but the large variability within each group makes this difference not significant (NS). A regression analysis was performed to assess the possible correlation between age and all of the parameters evaluated and revealed no significant correlations (data not shown).

Although the weight of the starting tendon fragments was significantly lower with respect to lipoaspirates ($p < 0.001$), the numbers of cells at passage 1 normalized per gram of tissue was $2.2 \pm 1.8 \times 10^5$ and $0.7 \pm 0.5 \times 10^5$ for TSPCs and ASCs, respectively ($p < 0.05$). During passages in culture, TSPCs showed a typical fibroblastic-like morphology and proliferated with a rate that was similar to that of ASCs (Figure 1A & B). Indeed, at passage 2, the average DT was 87.0 ± 7.6 h for TSPCs and 70.8 ± 23.6 h for ASCs (NS); then, the TSPC and ASC proliferation rates progressively decreased until reaching average DTs of 176.7 ± 108.1 h and 190.3 ± 65.8 h at passage 4, respectively (NS). The clonogenic ability was also similar between the two cell populations in all of the passages analyzed; in particular, the highest number of colonies was observed at passage 4 for TSPCs (4.4 ± 1.9%) and at passage 3 for ASCs (3.5 ± 1.9%; Figure 1C). In order to evaluate the *in vitro* cell viability of TSPCs and ASCs, which is considered to be an important parameter concerning primary cell survivorship after isolation and during culture, we observed their metabolic activity using the MTT assay. No significant differences were observed in terms of cell metabolic activity at passage 4 between TSPCs and ASCs (Figure 1D).

The expression of stemness-specific transcription factors *KLF4* and *POU5F1* of TSPCs was also comparable to that observed for ASCs (NS; Figure 1E). The cell size and granularity of both populations were also similar (Figure 2). Moreover, TSPCs were found to possess the characteristic mesenchymal immunophenotypic profile, without any significant difference with regards to ASCs. Indeed, they were both highly positive for CD13, CD73, CD90, CD29, CD44, CD105,

CD166 and CD54, and both cell populations did not express hematopoietic markers such as CD31, CD34, CD45 and CD71 (Figure 2).

TSPCs have a lower adipogenic potential than ASCs

TSPCs cultured for 14 days in adipogenic medium only showed a slight intracellular lipid vacuole increase compared with cells maintained in noninductive medium (+40.3%, NS; Figure 3A & B). The lipid vacuole content observed in TSPCs was significantly lower than that observed in ASCs ($p < 0.05$) and, when cultured in adipogenic medium, they showed a significant increase in comparison with control ASCs (+136.3%; $p < 0.05$). However, both differentiated cell populations expressed significantly higher levels of *LEP* in comparison with their respective control cells ($p < 0.05$) and without any differences between them (Figure 3C).

TSPC osteogenic potential is lower than that of ASCs

After 14 days of culture in osteogenic medium, both TSPCs and ASCs showed significant increases in terms of ALP activity of 173 and 177%, respectively ($p < 0.05$) in comparison with control cells (Figure 4A).

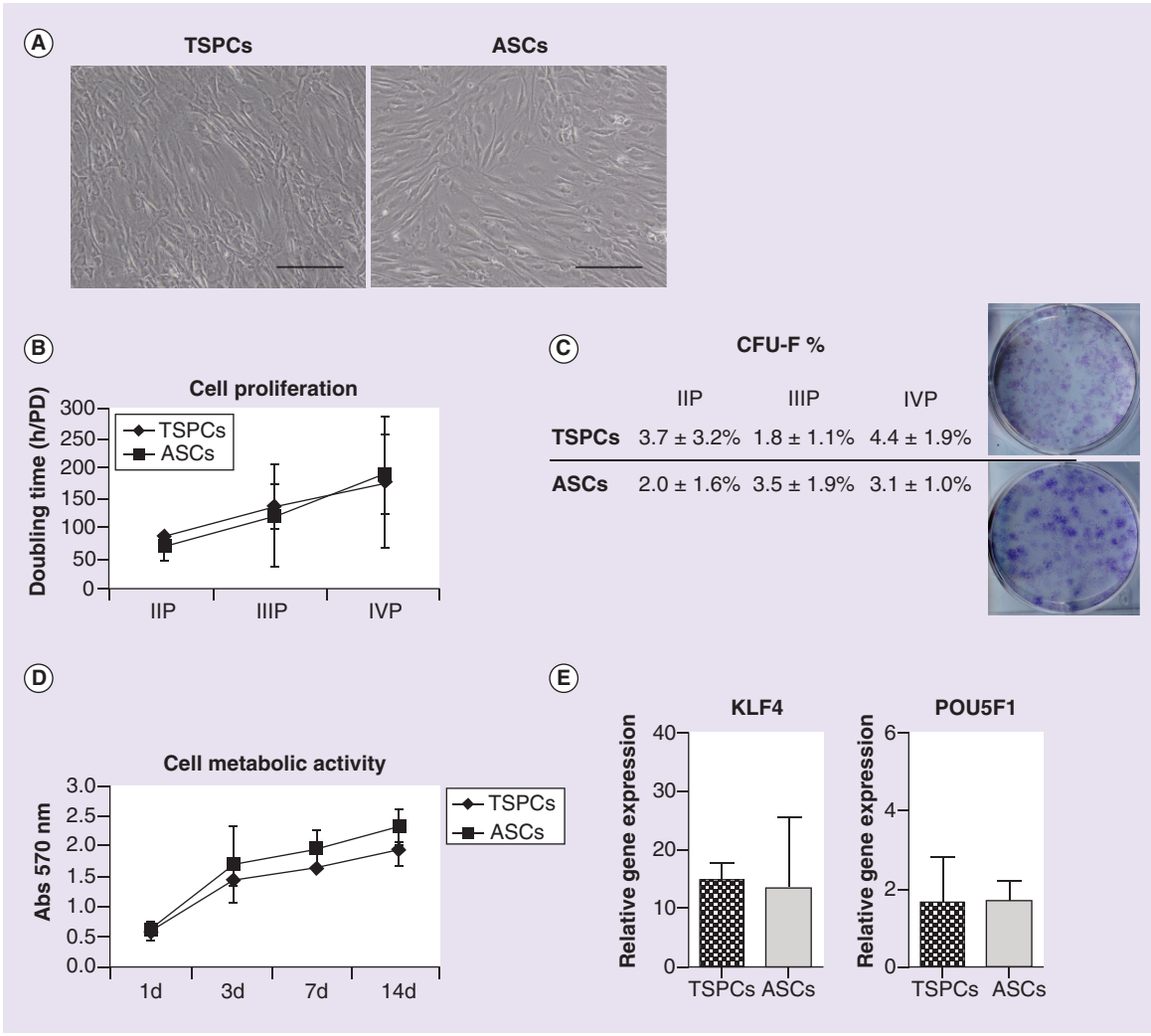

Figure 1. Features of tendon stem/progenitor cells and adipose-derived stem cells. (A) Cells in culture at passage 4 (optical microscopy: 10×; scale bar: 200 μm). **(B)** Proliferation ability during passages in culture expressed as mean of doubling times. **(C)** Percentage of clonogenic cells from passages 2 to 4 (scored colonies were normalized against number of seeded cells) and representative images of stained colonies for both cell population at passage 4. **(D)** Cell viability of tendon stem/progenitor cells and adipose-derived stem cells at passage 4 at several time points (1, 3, 7 and 14 days of culture). **(E)** Evaluation of *KLF4* and *POU5F1* gene expression determined by quantitative real-time PCR in both cell populations. Data were normalized against the expression of the housekeeping *GAPDH* gene.

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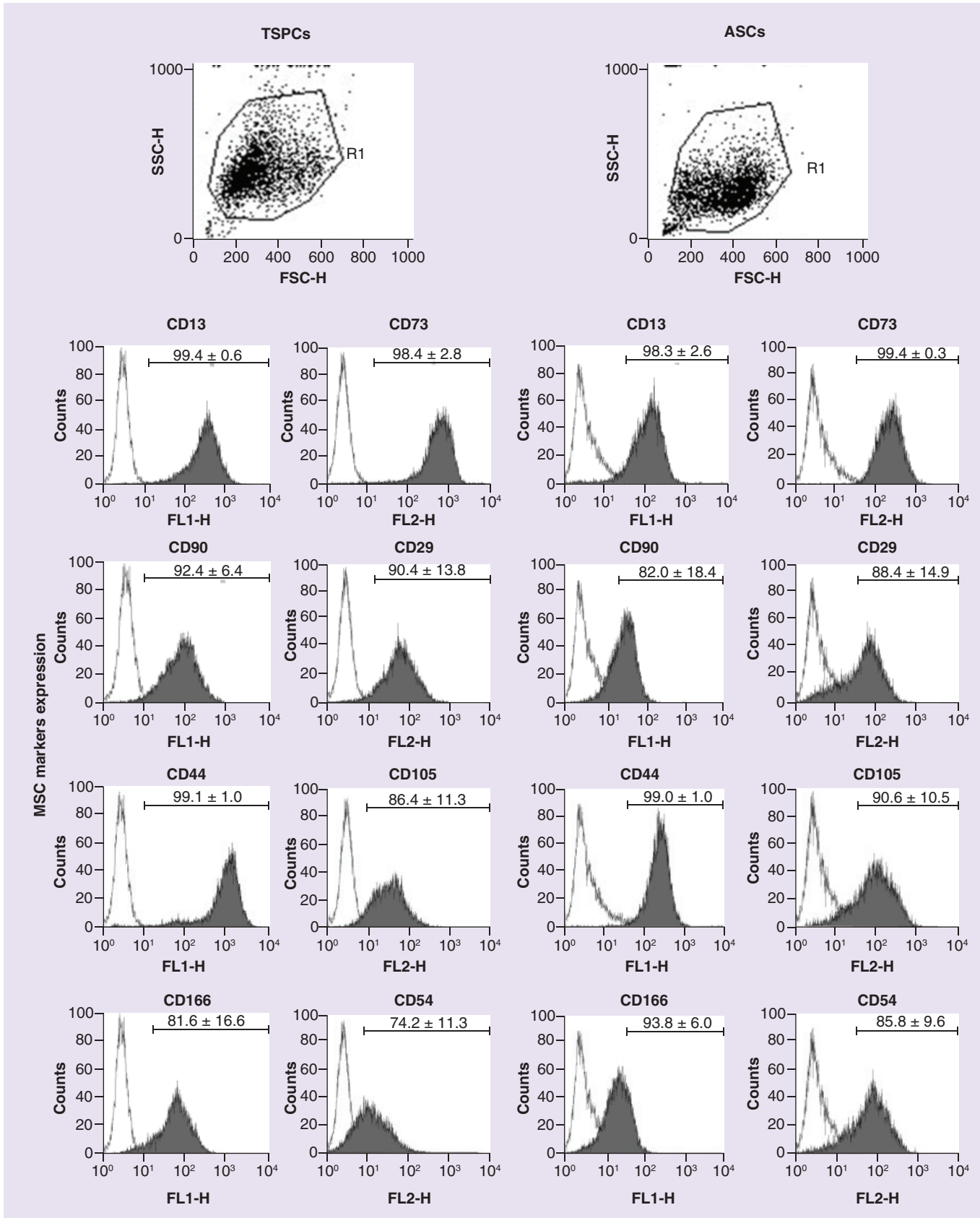
TSPCs
ASCs
SSC-H
FSC-H
R1
MSC markers expression
Counts
FL1-H
FL2-H
CD13
99.4 ± 0.6
CD73
98.4 ± 2.8
CD13
98.3 ± 2.6
CD73
99.4 ± 0.3
CD90
92.4 ± 6.4
CD29
90.4 ± 13.8
CD90
82.0 ± 18.4
CD29
88.4 ± 14.9
CD44
99.1 ± 1.0
CD105
86.4 ± 11.3
CD44
99.0 ± 1.0
CD105
90.6 ± 10.5
CD166
81.6 ± 16.6
CD54
74.2 ± 11.3
CD166
93.8 ± 6.0
CD54
85.8 ± 9.6

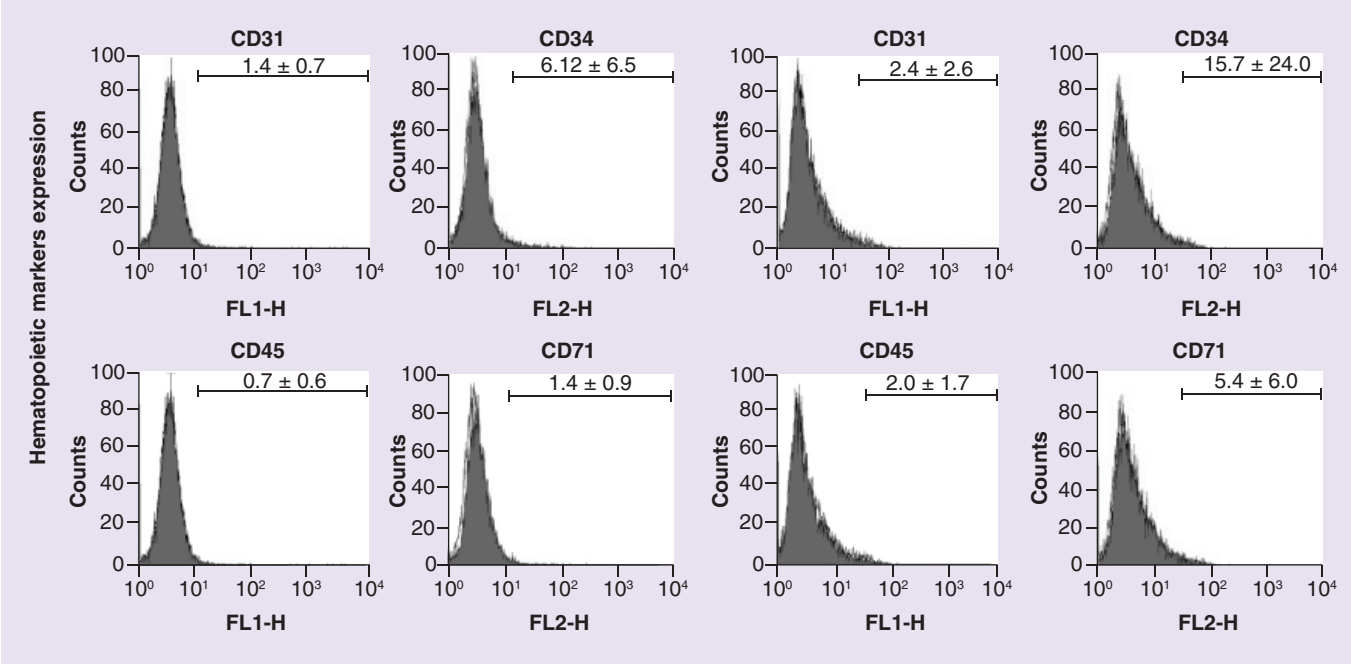

Figure 2. Mesenchymal stem cell surface marker patterns of expression (see facing page and above). Tendon stem/progenitor cell and adipose-derived stem cell distribution based on forward scatter and side scatter and the representative expression of the typical mesenchymal stem cell surface markers and hematopoietic markers for both populations at passage 4 (markers are represented as gray histograms and isotype control antibodies are represented as white histograms).

Significant upregulation of *RUNX2* expression was observed in both osteogenic-differentiated TSPCs (+35%; $p < 0.05$) and ASCs (+113%; $p < 0.05$) in comparison with the respective control cells. Comparing the two cell types, significantly higher mRNA levels of *RUNX2* were observed in differentiated ASCs compared with TSPCs (+136% for ASCs vs TSPCs; $p < 0.01$; Figure 4B). After 21 days of culture in osteogenic medium, the calcified matrix deposition, as revealed by Alizarin Red S staining and extraction, was significantly higher in comparison with control cells for both TSPCs and ASCs (+46 and +410%, respectively; both $p < 0.05$; Figure 4C & D). However, the amount of calcified matrix produced by differentiated TSPCs was very similar to that of undifferentiated ASCs, which, after the osteogenic induction, produced significantly higher amounts of calcified matrix compared with TSPCs (+ 411%; $p < 0.001$). All of these data suggest a lower capacity of TSPCs to differentiate towards the osteogenic lineage.

TSPC chondrogenic potential is higher than that of ASCs

Both cell populations were cultured in pellets in chondrogenic medium for 21 days. As revealed by the histological evaluation, the pellets cultured in noninductive medium were smaller and less organized in comparison to those cultured in chondrogenic medium for both types of cells (Figure 5A). Moreover, only the chondrogenic-differentiated pellets of TSPCs showed a marked extracellular matrix production, which was intensively stained by Safranin O dye. Chondrogenic-induced TSPCs also showed a significant increase in terms of GAG deposition compared with undifferentiated cells (+83%; $p < 0.01$); however, undifferentiated ASCs showed significantly higher levels of GAG in comparison with undifferentiated TSPCs (+96%; $p < 0.05$). Both *SOX9* and *ACAN* were strongly upregulated in chondrogenic-induced pellets (+251 and +44% for *SOX9* and +1279 and +145% for *ACAN* in terms of TSPCs and ASCs, respectively; Figure 5C & D) in comparison with control pellets. Moreover, both undifferentiated and differentiated TSPCs showed higher *ACAN* expression compared with ASCs (+1208 and +7256% increases for undifferentiated and differentiated TSPCs vs the respective ASCs). However, all of these differences, due to the wide interdonor variability, were NS.

BMP-12 increases *SCX* & *DEC* expression in both TSPCs & ASCs

The BMP-12 dosage used in this study (50 ng/ml) was chosen according to previously experiments carried on ASCs (data not shown).

The gene expression of specific tendon markers was evaluated after 7 and 14 days in undifferentiated and

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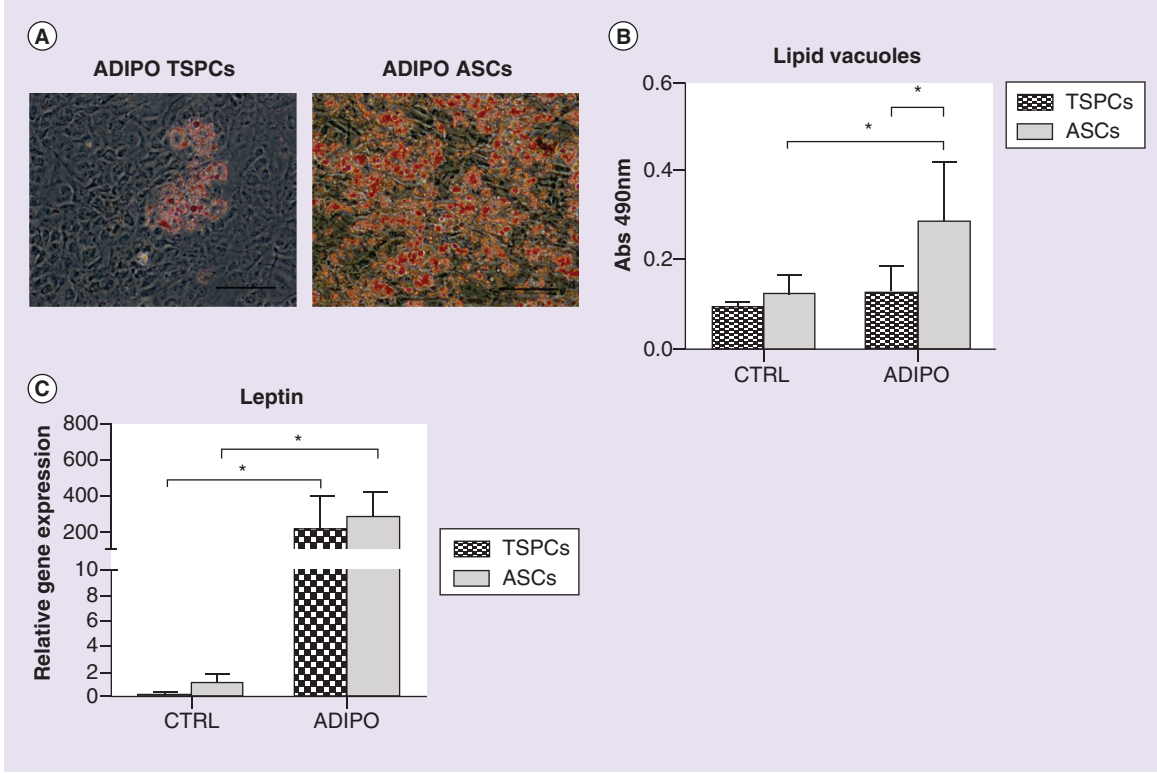

Figure 3. Adipogenic differentiation. (A) Micrographs of tendon stem/progenitor cells and adipose-derived stem cells differentiated towards the adipogenic lineage after Oil Red O staining (scale bars: 200 μm). **(B)** Quantification of lipid vacuoles in undifferentiated (CTRL) and adipogenic-differentiated (ADIPO) cells. **(C)** Gene expression of *LEP* normalized against *GAPDH*. *p < 0.05.

differentiated cells in both cell populations. In undifferentiated cells, *SCX* was more expressed in TSPCs than in ASCs (+461% [p < 0.05] and +344% at 7 and 14 days, respectively; Figure 6A, left column). The tenogenic medium was able to induce a further increase in the expression of this transcription factor in both populations; indeed, differentiated TSPCs and ASCs showed higher levels of *SCX* in comparison with undifferentiated cells both at 7 days (+86 and +89%, respectively) and at 14 days of culture (+50 and +24%, respectively), although these differences were not statistically significant (Figure 6A, right column). Gene expression of *DCN*, *COL1A1* and *COL1A3,* components of the tendon extracellular matrix, were evaluated . After 7 days of culture in undifferentiated medium, TSPCs showed higher levels of *DCN* in comparison with ASCs (+93%; NS); however, at 14 days of culture, this difference was not present anymore, as ASC *DCN* expression increased with time in culture (Figure 6B, left column). Tenogenic medium induced a significant upregulation of this marker in both populations already at 7 days of differentiation compared with undifferentiated cells (+50%, p < 0.05 and +164%, p < 0.01, respectively). However, after 14 days of differentiation, the *DCN* expression in ASCs significantly decreased compared with the previous time point, whereas TSPCs maintained a similar expression level (Figure 6B, right column). Unexpectedly, undifferentiated ASCs showed much higher levels of *COL1A1* and *COL3A1* expression compared wtih TSPCs (+1296%, p < 0.01 and +1279%, p < 0.01, respectively; Figure 6C & D, left column). Moreover, both cell populations did not show any further increase after tenogenic induction compared with undifferentiated cells (Figure 6C & D, right column).

Discussion

To the best of our knowledge, this is the first study comparing stem cell properties and the multilineage differentiation potential of human TSPCs and ASCs. The main finding of our study is that TSPCs and ASCs show similar cell proliferation, viability and clonogenic ability, as well as stem cell marker expression. On the other hand, although both MSC populations showed multidifferentiation potential, some significant differences were observed that were partially related to their tissue origin, supporting the idea that a given MSC tissue source could be more appropriate for a given clinical situation.

Little is known about tendon biology, and there is also scarce knowledge of the cell populations that are

resident in tendon tissue. It has recently been demonstrated that tendons contain stem/progenitor cells (i.e., TSPCs) that can provide a new tool for studying tendon physiology, pathology and possible innovative tendon therapies based on their properties [7]. Indeed, TSPCs are supposed to play a primary role in maintaining tissue homeostasis and in promoting repair after injury, and they could be addressed as a new potential therapeutic target. In particular, TSPCs could be the main effector of *in vitro* biophysical stimuli, such as pulsed electromagnetic fields and extracorporeal shock waves that have been proposed as promising alternatives for the treatment of tendinopathies [15–17]. Another perspective might involve the use of TSPCs as a cell source for cell-based therapy approaches to improve the regeneration of tendons or of other tissues of mesodermal origin, such as bone and cartilage. Currently, adipose tissue is considered to be one of the best and most convenient sources of MSC isolation due to its wide availability as surgical waste material [18], and for this reason, in recent years, several cell/tissue banks have focused their activity on adipose tissue preservation and of its related MSC population. Similarly, as already demonstrated and confirmed by this study, it is possible to efficiently isolate TSPCs from surgical waste tendon fragments, including hamstring tendons that are used in ligament reconstruction. Considering that each year approximately 200,000 anterior ligament reconstruction procedures are performed worldwide [19], it could be noteworthy to ameliorate the knowledge of the potential of these cells to possibly exploit them for future allogeneic applications [20]. In this study, we have compared the *in vitro* behavior and the stemness features and multidifferentiation potential of human TSPCs with those of ASCs. The number of cells is crucial for some regenerative medicine applications; in our study, the cellular yield of TSPCs was higher compared with ASCs. Since in the first passages in culture the tendon cell population is composed not only of TSPCs, but also of terminally differentiated tenocytes, the yield could have been influenced by this factor. However, tenocytes lose their phenotype *in vitro* with time and passages in culture and, although specific markers have yet to be found, they differ from TSPCs in terms of morphology, proliferation potential and expression of stem cells mark-

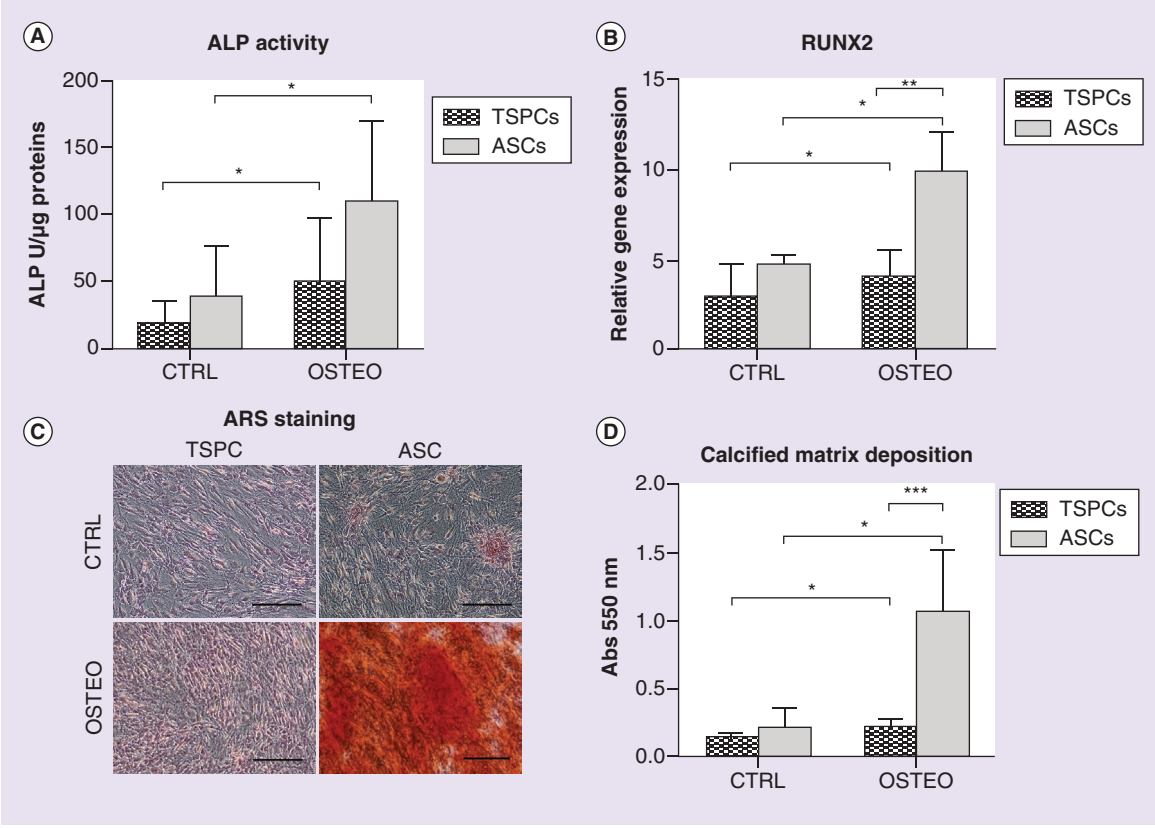

Figure 4. Osteogenic differentiation. (A) Alkaline phosphatase activity determined at 14 days of culture in undifferentiated (CTRL) and osteogenic-differentiated (OSTEO) cells. **(B)** Gene expression of *RUNX2* normalized against *GAPDH* at 14 days of culture. **(C)** Micrographs of CTRL and OSTEO cells stained by Alizarin Red S (scale bars: 200 µm). **(D)** Quantification of calcified matrix deposition by Alizarin Red S staining and extraction. *p < 0.05; **p < 0.01; ***p < 0.001.

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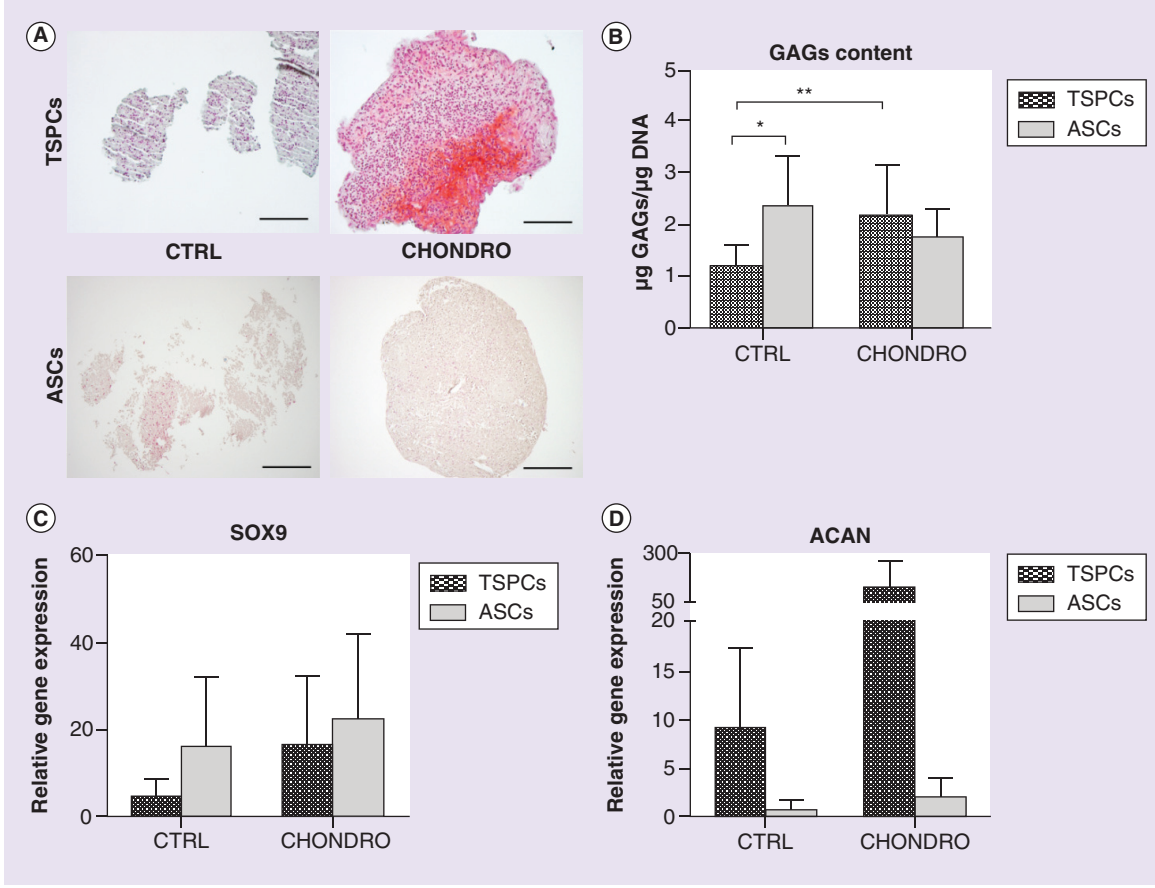

Figure 5. Chondrogenic differentiation. (A) Histological evaluation using hematoxylin–eosin and Safranin O staining in undifferentiated (CTRL) and chondrogenic-differentiated (CHONDRO) pellets of both tendon stem/progenitor cells and adipose-derived stem cells (scale bars, 200 µm). **(B)** DNA and GAG content in tendon stem/progenitor cell and adipose-derived stem cell pellets. **(C & D)** Gene expression of *SOX9* and *ACAN* normalized against *GAPDH*.
*p < 0.05; **p < 0.01.

ers such as OCT4 (*POU5F1*) [20–24]. For all of these reasons, the experiments were performed starting from cells at passage 4 (after ~5 weeks of culture), when it was observed that the cell population possessed features that were peculiar to progenitor cells, including the expression of transcription factors that are essential for self-renewal maintenance and pluripotency in embryonic stem cells, such as *POU5F1* and *KLF4* [25] and, in agreement with the minimal criteria for defining multipotent MSCs [26], the expression of specific MSC markers in standard culture conditions, without any difference with ASCs. Clonogenic assays for fibroblast-like colonies revealed a very similar frequency of cells able to form colonies in TSPCs to that observed in ASCs. All TSPC and ASC populations did not show differences in cell metabolic activity, suggesting similar cell viability when they are cultured *in vitro*. Moreover, we also observed that TSPCs and ASCs possessed a comparable DT of approximately 3 days at passage 2. These results are consistent with previous results indicating that human and mouse TSPCs proliferated faster than bone marrow stromal cells (BMSCs) [7,27], which have been demonstrated to possess a lower proliferation ability than ASCs [28,29], thus explaining the comparable behavior observed between ASCs and TSPCs.

The differences in terms of multidifferentiation potential of MSCs from different sources have been deeply investigated: previous studies reported a higher multidifferentiation potential of mouse and rat TSPCs compared with BMSCs [7,27]; others found that human ASCs were able to more efficiently differentiate into the adipogenic lineage, but not into the chondrogenic lineage in comparison with BMSCs [28–30]. In our study, TSPCs and ASCs also presented some differences in terms of multidifferentiation potential. In particular, although adipogenic- and osteogenic-differentiated TSPCs showed significant increases in the related tissue-specific marker expression compared with undiffer-

entiated cells, they possessed lower adipogenic and osteogenic potential compared with ASCs The more pronounced adipogenic potential of ASCs could be attributed to their precommitment, whereas the more marked osteogenic potential is also indicated by the very high type I collagen expression that we observed in undifferentiated ASCs, as has already been reported in previous studies [31,32]. Quite unex-

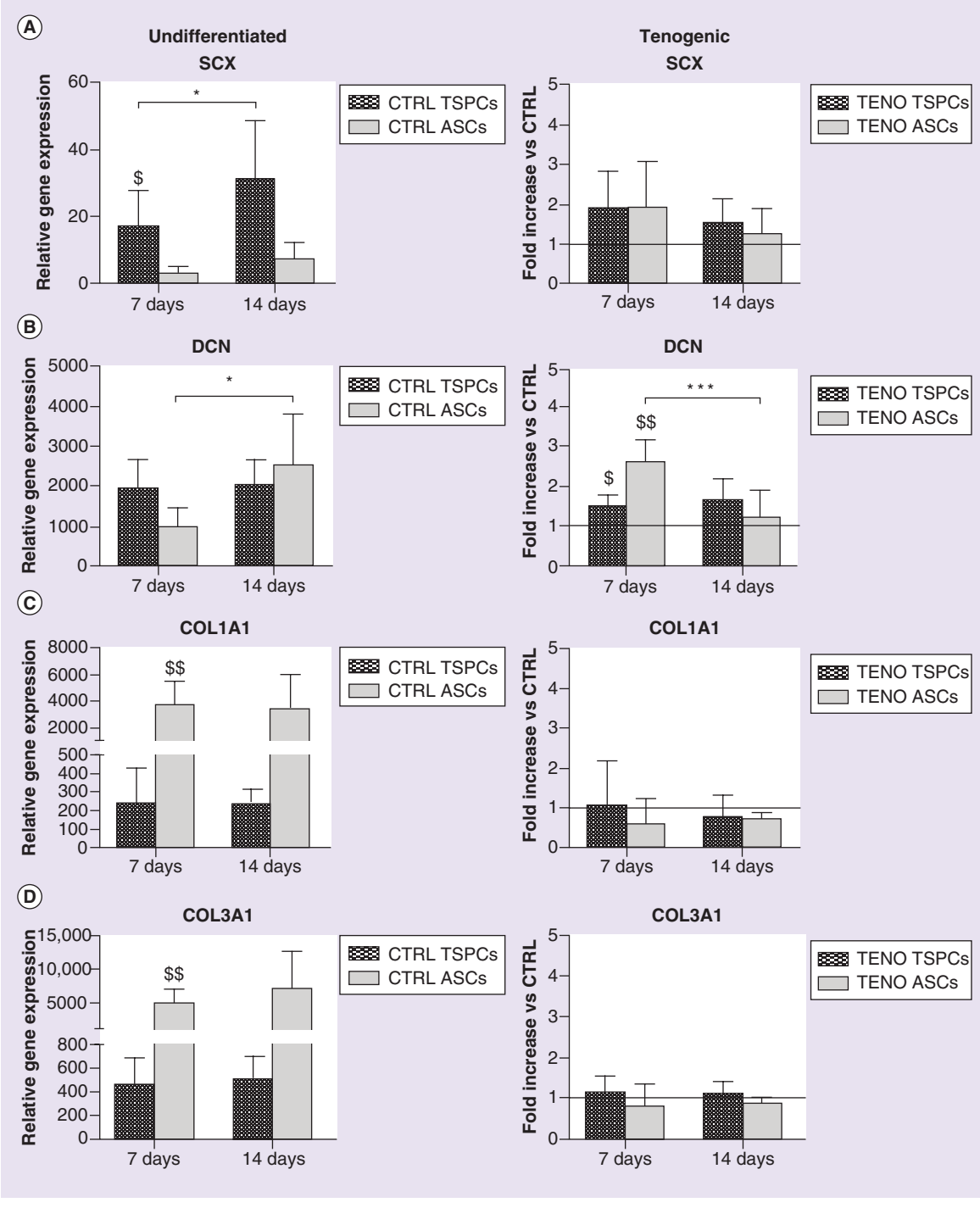

Figure 6. Tenogenic differentiation. (A–E) Gene expression of *SCX*, *DCN*, *COL1A1* and *COL1A3* normalized against *GAPDH* in undifferentiated (CTRL, left panel) and tenogenic-differentiated (TENO, right panel) tendon stem/progenitor cells and adipose-derived stem cells at 7 and 14 days of culture. Data for the TENO cells are expressed as average fold increase ± standard deviation compared with undifferentiated cells (fixed line set at 1). *p < 0.05, **p < 0.01 for adipose-derived stem cells versus tendon stem/progenitor cells.

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pectedly, TSPCs seemed to be more susceptible to chondrogenic medium in comparison with ASCs, above all in terms of GAG production, although they were already high in undifferentiated ASCs. The more dramatic difference observed between TSPCs and ASCs that underwent the chondrogenic differentiation protocol was that of *ACAN* gene expression. Aggrecan is a proteoglycan that is present in cartilage as well as in compressed tendon regions. Thus, the already very high expression of this marker in undifferentiated TSPCs could be partially related to their tissue origin and consequently to their precommitment. In any case, as articular cartilage has only a poor capacity for self-repair [33], it could be important to further investigate this aspect, as hamstrings can be easily harvested during knee surgery, causing minimal additional morbidity to patients who possibly require autologous regenerative treatment for knee-concomitant chondral defects. Collectively, all of these data confirm that MSCs from different sources can respond differently to stimuli, posing the question of whether optimal conditions of differentiation should be properly adapted to the specific MSC type. In order to achieve a more efficient *in vitro* differentiation protocol, it could be useful to further investigate the cell niche factors, as it is widely accepted that several different environmental factors contribute to the overall control of stem cell activity. As little is known about the maturation of tendons and the related tendon cell niche factors, little information is available for developing an efficient tenogenic differentiation medium. In previous *in vitro* studies on embryonic stem cells, ASCs and BMSCs, the tenogenic induction has been attempted using a variety of growth factors, including bone morphogenic proteins (BMP-2 and -5, as well as BMP-12, -13 and -14, also known as GDF-7, -6 and -5, respectively), IGF-1, TGF-β1 and TGF-β3 [8–10,34–37]. The influence of BMP-12 on tenogenic differentiation was evaluated in several types of MSCs [37,38], but only a few studies were conducted on ASCs [8,10] and, to our knowledge, none were conducted on human cells. Here, for the first time, we evaluated the effect of BMP-12 on the tenogenic induction of human ASCs and TSPCs.

Regarding tendon-related marker expression, our results demonstrated that *SCX*, a transcription factor involved in tendon development, and *DEC*, a proteoglycan that stabilize and aligns collagen type I and III fibrils [15,24,39], were more expressed in undifferentiated TSPCs compared with undifferentiated ASCs, in this case probably due to their precommitment. However, BMP-12 was able to induce a strong upregulation of *SCX* expression in both populations, as well as a significant increase of *DEC*. On the other hand, tenogenic induction did not affect either *COL1A1* and *COL3A1* expression in both TSPCs and ASCs, suggesting that BMP-12 alone is not able to induce a complete upregulation of tendon extracellular matrix markers. Our data on ASC tenogenic differentiation confirm what was observed by Shen *et al.*, who demonstrated that BMP-12 is capable of inducing tenogenic differentiation in canine and mouse ASCs [8], but our hypothesis that it could be more advantageous to use TSPCs for tendon regenerative applications in comparison with ASCs was not confirmed, at least with the protocol used here. Other tenogenic factors, such as GDF-5/BMP-14, should been tested on human ASCs and TSPCs in order to assess whether they are able to induce a more evident tenogenic induction, although previous studies reported that GDF-7/BMP-12 induced the tenogenic differentiation of canine ASCs more efficiently in comparison with GDF-5/BMP-14 [8]. Moreover, the identification of more specific targets could enable the development of new factors that are able to induce cells towards tendon differentiation more efficiently.

Since tendon is a mechanoresponsive tissue, it can be speculated that, together with biochemical stimuli, appropriate mechanical loads would be helpful for improving the tenogenic differentiation of progenitor cells, in particular of TSPCs, as the modulation of physical stress response is part of their physiological role in tendon tissue; indeed, external physical stimuli seem to enhance or accelerate the differentiation into tenocyte-like cells [40]. For this reason, further studies are needed in order to clarify the cell response to mechanical stimuli and also how they are able to influence the fate of MSCs. Another criticism in this research field is that the markers usually used to assess tenogenic differentiation are not strictly specific for tendons, as they can be also found in other tissues and cells. For this reason, the evaluation can sometimes be misleading, and thus in order to obtain more reliable results, the identification of more specific tendon markers may provide better insights into *in vitro* tenogenesis.

One of the limitations of this study is the lack of a donor-matched comparison that could have partially reduced the high interdonor variability that often represents a critical point when dealing with primary cells. However, in this case, the use of donor-matched cells would have involved ethical issues, since part of the collected tissue should have been intentionally harvested, whereas in our study, all of the samples used to isolate cells were from surgical waste. Nevertheless, the possibility to obtain data form a donor-matched comparison study could be considered in

future studies in order to better characterize MSCs from different sources. Another limitation of this study is the use of the differentiation protocols that were previously developed and adapted for ASCs, and this could have disadvantaged the TSPC differentiation ability. Moreover, the assessment of only tendon-specific gene expression without the related protein expression and the testing of a single growth factor without any mechanical stimulation to induce the tenogenic differentation represent further limitations of this study. Further comparative studies of MSC immunogenicity could be useful in order to better identify the best cell type for allogeneic use.

Conclusion

Taken together, our results demonstrated that TSPCs do not significantly differ from ASCs in term of clonogenic ability, proliferation and immunophenotypic profile. In comparison with ASCs, TSPCs are less prone to differentiating into adipogenic and osteogenic lineages using our protocols, but are more able to differentiate into chondrogenic- and tenogenic-like cells, potentially opening up the possibility of using TSPCs as a new cell type in some regenerative medicine applications in the musculoskeletal field.

Future perspective

Preclinical studies have shown the possibility of exploiting the properties of ASCs and BMSCs in order to manage tendon disorders. Our findings, together with others concerning TSPCs, allow us to speculate that, in the future, tendons could represent an appealing and sustainable cell source for isolating MSCs for musculoskeletal regenerative purposes. Moreover, thanks to the low immunogenicity of MSCs, it could be possible to preserve TSPCs isolated from surgical waste material for allogeneic use, as is currently done for other MSC types, including umbilical cord- and adipose tissue-derived MSCs.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Adipose tissue is one of the most common source of mesenchymal stem cells for regenerative purposes; however, the search of the 'most convenient' mesenchymal stem cell source is still ongoing.
- In this study, tendon stem.progenitor cells (TSPCs) and adipose-derived stem cells (ASCs) were compared for the first time, with the aim of providing further knowledge regarding the potential use of TSPCs in musculoskeletal tissue regeneration strategies.

Materials & methods

- The *in vitro* immunophenotype, embryonic stem cell marker expression, clonogenic and proliferation ability and multilineage differentiation (adipogenic, chondrogenic, osteogenic and tenogenic) potential of TSPCs and ASCs were compared.

Results

- TSPCs and ASCs show similar *in vitro* proliferation, clonogenic abilities and expression of stem cell markers.
- Upon specific stimulation, TSPCs show lower adipogenic and osteogenic differentiation potential, but higher chondrogenic differentiation potential compared with ASCs.
- BMP-12 is able to upregulate the expression of tendon markers in both TSPCs and ASCs.

Discussion

- Our results demonstrate that TSPCs do not significantly differ from ASCs, potentially opening up the possibility of using TSPCs as a new cell type for musculoskeletal regenerative medicine.
- As ASC and TSPC tenogenic differentiation abilities were not noticeably different, our hypothesis that it could be more advantageous to use TSPCs for tendon regenerative applications in comparison with ASCs was not confirmed, at least with the protocol used here.

10.2217/RME.14.92

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 de Girolamo L, Lopa S, Arrigoni E, Sartori MF, Baruffaldi Preis FW, Brini AT. Human adipose-derived stem cells isolated from young and elderly women: their differentiation potential and scaffold interaction during *in vitro* osteoblastic differentiation. *Cytotherapy* 11(6), 793–803 (2009).

2 de Girolamo L, Stanco D, Salvatori L *et al.* Stemness and osteogenic and adipogenic potential are differently impaired in subcutaneous and visceral adipose derived stem cells (ASCs) isolated from obese donors. *Int. J. Immunopathol. Pharmacol.* 26(1 Suppl.), 11–21 (2013).

3 Schipper BM, Marra KG, Zhang W, Donnenberg AD, Rubin JP. Regional anatomic and age effects on cell function of human adipose-derived stem cells. *Ann. Plast. Surg.* 60(5), 538–544 (2008).

4 Fraser J, Wulur I, Alfonso Z, Zhu M, Wheeler E. Differences in stem and progenitor cell yield in different subcutaneous adipose tissue depots. *Cytotherapy* 9(5), 459–467 (2007).

5 Ruzzini L, Abbruzzese F, Rainer A *et al.* Characterization of age-related changes of tendon stem cells from adult human tendons. *Knee Surg. Sports Traumatol. Arthrosc.* 22(11), 2856–2866 (2013).

6 Randelli P, Conforti E, Piccoli M *et al.* Isolation and characterization of 2 new human rotator cuff and long head of biceps tendon cells possessing stem cell-like self-renewal and multipotential differentiation capacity. *Am. J. Sports Med.* 41(7), 1653–1664 (2013).

7 Bi Y, Ehirchiou D, Kilts TM *et al.* Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat. Med.* 13(10), 1219–1227 (2007).

•• **Identifies and characterizes mesenchymal stem cells from tendon for the first time, here named tendon stem/progenitor cells.**

8 Shen H, Gelberman RH, Silva MJ, Sakiyama-Elbert SE, Thomopoulos S. BMP12 induces tenogenic differentiation of adipose-derived stromal cells. *PLoS ONE* 8(10), e77613 (2013).

•• **Investigates the effects of BMP12 on canine adipose-derived stem cell differentiation *in vitro*. The results of this study provided the first evidence for adipose-derived stem cell tenogenic differentiation.**

9 Park A, Hogan MV, Kesturu GS, James R, Balian G, Chhabra AB. Adipose-derived mesenchymal stem cells treated with growth differentiation factor-5 express tendon-specific markers. *Tissue Eng. Part A* 16(9), 2941–2951 (2010).

10 Raabe O, Shell K, Fietz D *et al.* Tenogenic differentiation of equine adipose-tissue-derived stem cells under the influence of tensile strain, growth differentiation factors and various oxygen tensions. *Cell Tissue Res.* 352(3), 509–521 (2013).

•• **Provides the first evidence on the ability of adipose-derived stem cells to enhance tendon repair *in vivo*.**

11 Gittel C, Brehm W, Burk J *et al.* Isolation of equine multipotent mesenchymal stromal cells by enzymatic tissue digestion or explant technique: comparison of cellular properties. *BMC Vet. Res.* 9, 221 (2013).

12 de Mos M, van El B, DeGroot J *et al.* Achilles tendinosis: changes in biochemical composition and collagen turnover rate. *Am. J. Sports Med.* 35(9), 1549–1556 (2007).

13 Staszkiewicz J, Frazier TP, Rowan BG *et al.* Cell growth characteristics, differentiation frequency, and immunophenotype of adult ear mesenchymal stem cells. *Stem Cells Dev.* 19(1), 83–92 (2010).

14 Barbero A, Grogan S, Schafer D, Heberer M, Mainil-Varlet P, Martin I. Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. *Osteoarthritis Cartilage* 12(6), 476–484 (2004).

15 de Girolamo L, Stanco D, Galliera E *et al.* Low frequency pulsed electromagnetic field affects proliferation, tissue-specific gene expression, and cytokines release of human tendon cells. *Cell Biochem. Biophys.* 66(3), 697–708 (2013).

16 de Girolamo L, Vigano M, Galliera E *et al. In vitro* functional response of human tendon cells to different dosages of low-frequency pulsed electromagnetic field. *Knee Surg. Sports Traumatol. Arthrosc.* doi:10.1007/s00167-014-3143-x (2014) (Epub ahead of print).

17 de Girolamo L, Stanco D, Galliera E *et al.* Soft-focused extracorporeal shock waves increase the expression of tendon-specific markers and the release of anti-inflammatory cytokines in an adherent culture model of primary human tendon cells. *Ultrasound Med. Biol.* 40(7), 1650–1659 (2014).

18 Gimble JM, Bunnell BA, Guilak F. Human adipose-derived cells: an update on the transition to clinical translation. *Regen. Med.* 7(2), 225–235 (2012).

•• **Comprehensive review focusing on the status of clinical and preclinical studies on adipose-derived cell therapies in regenerative medicine.**

19 Parkkari J, Pasanen K, Mattila VM, Kannus P, Rimpelä A. The risk for a cruciate ligament injury of the knee in adolescents and young adults: a population-based cohort study of 46500 people with a 9 years follow-up. *Br. J. Sport Med.* 42(6), 422–426 (2008).

20 Lui PP, Kong SK, Lau PM, Wong YM, Lee YW, Tan C. Immunogenicity and escape mechanisms of allogeneic tendon-derived stem cells. *Tissue Eng. Part A* 20(21–22), 3010–3020 (2014).

21 Yao L, Bestwick CS, Bestwick LA, Maffulli N, Aspden RM. Phenotypic drift in human tenocyte culture. *Tissue Eng.* 12(7), 1843–1849 (2006).

22 Tan Q, Lui PP, Rui YF. Effect of *in vitro* passaging on the stem cell-related properties of tendon-derived stem cells-implications in tissue engineering. *Stem Cells Dev.* 21(5), 790–800 (2012).

23 Tan SL, Ahmad RE, Ahmad TS *et al.* Effect of growth differentiation factor 5 on the proliferation and tenogenic differentiation potential of human mesenchy stem cells *in vitro*. *Cells Tissues Organs* 196(4), 325–338 (2012).

24 Zhang J, Wang JH. Characterization of differential properties of rabbit tendon stem cells and tenocytes. *BMC Musculoskelet. Disord.* 11, 10 (2010).

25 Nichols J, Zevnik B, Anastassiadis K *et al.* Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 95(3), 379–391 (1998).

26 Dominici M, Le Blanc K, Mueller I *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8(4), 315–317 (2006).

• **Defines for the first time the minimal set of standard criteria for the characterization of mesenchymal stem cells.**

27 Tan Q, Lui PP, Rui YF, Wong YM. Comparison of potentials of stem cells isolated from tendon and bone marrow for musculoskeletal tissue engineering. *Tissue Eng. Part A* 18(7–8), 840–851 (2012).

28 Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 24(5), 1294–1301 (2006).

29 Izadpanah R, Trygg C, Patel B *et al.* Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. *J. Cell Biochem.* 99(5), 1285–1297 (2006).

30 De Ugarte DA, Alfonso Z, Zuk PA *et al.* Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. *Immunol. Lett.* 89(2–3), 267–270 (2003).

31 Lopa S, Colombini A, Stanco D, de Girolamo L, Sansone V, Moretti M. Donor-matched mesenchymal stem cells from knee infrapatellar and subcutaneous adipose tissue of osteoarthritic donors display differential chondrogenic and osteogenic commitment. *Eur. Cell Mater.* 27, 298–311 (2014).

32 Hamid AA, Idrus RB, Saim AB, Sathappan S, Chua KH. Characterization of human adipose-derived stem cells and expression of chondrogenic genes during induction of cartilage differentiation. *Clinics (Sao Paulo)* 67(2), 99–106 (2012).

33 Zhang L, Hu J, Athanasiou KA. The role of tissue engineering in articular cartilage repair and regeneration. *Crit. Rev. Biomed. Eng.* 37, 1–57 (2009).

34 Barsby T, Guest D. Transforming growth factor beta3 promotes tendon differentiation of equine embryo-derived stem cells. *Tissue Eng. Part A* 19(19–20), 2156–2165 (2013).

35 Holladay C, Abbah SA, O'Dowd C, Pandit A, Zeugolis DI. Preferential tendon stem cell response to growth factor supplementation. *J. Tissue Eng. Regen. Med.* doi:10.1002/term.1852 (2014) (Epub ahead of print).

36 Lee JY, Zhou Z, Taub PJ *et al.* BMP-12 treatment of adult mesenchymal stem cells *in vitro* augments tendon-like tissue formation and defect repair *in vivo*. *PLoS ONE* 6(3), e17531 (2011).

37 Gulati BR, Kumar R, Mohanty N, Kumar P, Somasundaram RK, Yadav PS. Bone morphogenetic protein-12 induces tenogenic differentiation of mesenchymal stem cells derived from equine amniotic fluid. *Cells Tissues Organs* 198(5), 377–389 (2013).

38 Mohanty N, Gulati BR, Kumar R *et al.* Immunophenotypic characterization and tenogenic differentiation of mesenchymal stromal cells isolated from equine umbilical cord blood. *In Vitro Cell. Dev. Biol. Anim.* 50(6), 538–548 (2014).

39 Uysal CA, Tobita M, Hyakusoku H, Mizuno H. Adipose-derived stem cells enhance primary tendon repair: biomechanical and immunohistochemical evaluation. *J. Plast. Reconstr. Aesthet. Surg.* 65(12), 1712–1719 (2012).

40 Schiele NR, Marturano JE, Kuo CK. Mechanical factors in embryonic tendon development: potential cues for stem cell tenogenesis. *Curr. Opin. Biotechnol.* 24(5), 834–840 (2013).