TISSUE ENGINEERING: Part A
Volume 16, Number 5, 2010

DOI: 10.1089/ten.tea.2009.0529

Isolation and Characterization of Multipotent Rat Tendon-Derived Stem Cells

Yun-Feng Rui, M.Phil.,[1,2] Pauline Po Yee Lui, Ph.D.,[1,2,3] Gang Li, Ph.D.,[1,2,3]
Sai Chuen Fu, M.Phil.,[1,2] Yuk Wa Lee, M.Phil.,[1,2] and Kai Ming Chan, M.D.[1,2,3]

Stem cells have recently been isolated from humans and mice but not from rat tendon tissue. This study reports the isolation and characterization of stem cells from rat tendon. Nucleated cells isolated from rat flexor tendon tissues after collagenase digestion were plated at a low cell density to allow the selective proliferation of tendon-derived stem cells. About 1–2% of the cells isolated under this optimized culturing condition showed clonogenicity, high proliferative potential at low seeding density, and osteogenic, chondrogenic, and adipogenic multidifferentiation potential. These cells were $CD44^+$, $CD90^+$, $CD34^-$, and $CD31^-$. Although they shared some common properties with mesenchymal stem cells, they also exhibited their unique characteristics by expressing tenogenic and chondrogenic markers. There was expression of tenogenic markers, including α-smooth muscle actin, tenascin C, and tenomodulin, but not collagen type I at passage 0 (P0) and P3. Expression of a chondrogenic marker, aggrecan, was observed at P0 and P3, whereas expression of collagen type II was observed in few cells only at P3. The successful isolation of tendon-derived stem cells under the optimized growth and differentiation conditions was useful for future stem-cell-based tissue regenerative studies as well as studies on their roles in tendon physiology, healing, and disorders using the rat model.

Introduction

Mesenchymal stem cells (MSCs) are multipotent adult stem cells capable of differentiating into osteoblasts, chondrocytes, adipocytes, fibroblasts, tenocytes, neuronal tissues, and myoblasts. They are easy to collect and are considered as a promising cell source for various tissue repair and regeneration.[1–3] In addition to bone marrow,[2] MSCs can be isolated from other adult mesenchymal tissues such as synovium,[4] periosteum,[5] skeletal muscle,[6] adipose tissue,[7] and cartilage.[8] Recently, several lines of evidence suggested that multipotent stem cells were also present in tendon tissues.[9–11] Specific cell lines that were established from single-cell clones derived from murine tendon cells could differentiate into a variety of mesenchymal phenotypes under appropriate stimuli.[9] de Mos *et al.* also showed that explanted human tendon cells exhibited multidifferentiation potential.[10] Bi *et al*. identified a unique cell population, termed tendon stem/progenitor cells (TSPCs), in human and mouse tendons with universal stem cell characteristics, including clonogenicity, multipotency, and self-renewal capacity.[11] All these findings have demonstrated the existence of stem cells in tendon tissues of humans and mice. Nevertheless, no studies to date have reported and characterized tendon-derived stem cells (TDSCs) in rat tendon tissues.

Although MSCs from different species and tissue sources have similar characteristics in part, some data suggested that variations existed among species and tissue sources.[12–14] MSCs from human bone marrow were relatively easy to harvest and to expand in culture,[15] whereas rat MSCs have proven to be more difficult,[1,16–18] although this was not without controversy.[19] There were differences in yield, expansion, and multipotent differentiation potential for MSCs isolated from bone marrow, synovium, periosteum, adipose tissue, and muscle.[14] Better understanding of the characteristics of stem cells from different sources will help us identify a better cell source for tissue engineering including tendon tissue. Several studies have also suggested that resident stem cells may have roles in tendon physiology, healing, and pathological processes such as tendinopathy. Chondrocyte phenotype/markers were expressed in clinical samples of tendinopathy and calcifying tendinopathy.[20–23] Erroneous differentiation of healing tendon cells during injury was suggested.[24] Aberrant differentiation of resident stem cells was also reported in arterial calcification, skin calcification, and skeletal calcification.[25–27] Bi *et al.* have also demonstrated that TPSCs from

[1]Department of Orthopaedics and Traumatology and [2]The Hong Kong Jockey Club Sports Medicine and Health Sciences Centre, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong SAR, China.
[3]Stem Cells and Regeneration Program, School of Biomedical Science, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong SAR, China.

biglycan and fibromodulin knockout mice with ectopic tendon calcification were more sensitive to bone morphogenetic protein-2 signaling.[11] The ability to isolate stem cells from tendon tissue therefore will facilitate studies on the role of TDSCs in tendon physiology, healing, and nonhealing.

Small animals such as rabbits, rats, and mice are popular animal models for biomedical research due to their small size, lower cost, and similarity in genetic composition to humans. Rats are considered the appropriate model for many researches, particularly for surgical operation and diseases that cannot be easily done in mice due to their small size.[28] For instance, rats are currently the smallest animal model for anterior cruciate ligament (ACL) reconstruction. The ability to isolate stem cells from rats and to study their promotional effect in a rat surgical model therefore will open up new treatment options. Many pathological animal models are also established using rats such as animal models of tendinopathy and acute tendon injury.[29,30] The ability to isolate stem cells from rats therefore will facilitate studies on the pathogenesis of tendon diseases.

In this study, we aimed to isolate and characterize TDSCs from the flexor tendon of adult rat. Specifically, we investigated their proliferation potential, clonogenicity, and also the change in cell morphology at different passages. Expression of stem cell markers or markers of different cell lineages was examined by fluorescence-activated cell sorting (FACS) analysis and immunocytochemical staining. The osteogenic, adipogenic, and chondrogenic differentiation potential of cells were investigated using histology, immunohistochemical staining, and gene expression analysis.

Materials and Methods

Study design

Stem cells were isolated from the rat flexor tendon and the optimal plating density as indicated by the highest number of distinguishable colonies per tendon-derived nucleated cell was used for all subsequent experiments. Characterization of isolated cells included their (1) self-renewal and proliferative potential, (2) expression of stem cell and lineage-specific markers, and (3) multidifferentiation potential.

Isolation and culture of rat TDSCs

Three 8-week-old Sprague-Dawley rats, weighing 250–300 g, were used. All experiments were approved by the Animal Research Ethics Committee, the Chinese University of Hong Kong. After euthanasia, the whole piece of intact flexor tendon was excised from both limbs of each rat. Care was taken that only the midsubstance tissue but not the tissue in the bone–tendon junction was collected. Peritendinous connective tissue was carefully removed, and the samples were stored in sterile phosphate-buffered saline (PBS). The tissues were minced, digested for 2.5 h at 37°C with type I collagenase (3 mg/mL; Sigma-Aldrich, St. Louis, MO), and passed through a 70 μm cell strainer (Becton Dickinson, Franklin Lakes, NJ) to yield single-cell suspension. The released cells were washed in PBS by centrifugation at 300 *g* for 5 min and resuspended in Dulbecco's modified Eagle's medium (Gibco BRL; Life Technologies, Invitrogen, Carlsbad, CA), 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA). The isolated cells were plated at different cell densities and cultured at 37°C, 5% CO_2 to form colonies. At day 2 after initial plating, the cells were washed twice with PBS to remove the non-adherent cells. At day 7, they were trypsinized and mixed together as passage 0 (P0). Cells from passages 1–3 (P1–P3) were used for the other experiments. Culture medium was changed every 3 days during experiments.

Self-renewal and proliferative potential

Colony assay. To determine the optimal initial plating density for the isolation and culture of stem cells from tendon tissue, nucleated cells derived from flexor tendon of three rats were plated in triplicate at 50, 500, 5000, and 50,000 cells/cm^2 in 21 cm^2 dishes each and cultured for 7 days. The cells were stained with 0.5% crystal violet (Sigma, St. Louis, MO) for counting the number of cell colonies. Colonies less than 2 mm in diameter and faintly stained were ignored. The number of colonies per nucleated cells was calculated. The optimal initial cell density was chosen based on the following criteria: (1) the colony size was not affected by colony-to-colony contact inhibition and (2) the greatest number of colonies per tendon-derived nucleated cells was obtained.

Cell proliferation assay. Tendon-derived cells at P0 at the determined optimal seeding density were plated at 100, 500, and 1000 cells/cm^2 in triplicate for each indicated time point and incubated at 37°C and 5% CO_2. At days 2, 4, 6, 8, 10, and 12, the number of cells in each well were counted with a hemocytometer. The number of fold changes in cell number was calculated by dividing the cell number at each indicated time point by the initial plating cell number. The experiment was repeated twice in two rats.

Expression of stem cell and lineage-specific markers

Flow cytometry assay. Tendon-derived cells (5×10^5) at P3 were incubated with 1 μg of phycoerythrin-conjugated or fluorescein-isothiocyanate-conjugated mouse anti-rat monoclonal antibodies (R&D Systems, Minneapolis, MN) for 1 h at 4°C. After washing with PBS at 400 *g* for 5 min, the stained cells were re-suspended in 500 μL of ice-cold PBS (with 10% fetal bovine serum and 1% sodium azide) and subjected to FACS analysis (Becton Dickinson, Franklin Lakes, NJ). About 10^4 events were counted for each sample. The percentage of cells with positive signal was calculated using the FACSCAN program (Becton Dickinson, San Jose, CA). Anti-CD34 (sc-7324; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD31 (ab33858; Abcam, Cambridge, United Kingdom), anti-CD44 (ab23396; Abcam), and anti-CD90 (ab33694; Abcam) were the antibodies used in this study. They were replaced with phycoerythrin-conjugated or fluorescein-isothiocyanate-conjugated isotype-matched IgG1 in negative controls (IC002P or IC002F; R&D Systems). Triplicates of cells from three rats were examined in this assay.

Immunocytochemical staining. Immunocytochemical staining was performed according to the procedures described earlier.[31] Five thousand tendon-derived cells at P0 and P3 were seeded on poly-L-lysine–precoated 22×22 mm^2 glass coverslips at 37°C, 5% CO_2 overnight. The cells were then fixed in 4% paraformaldehyde, quenched with 10% H_2O_2 in methanol for 20 min, blocked with 5% normal goat or rabbit serum, and incubated with primary antibody for 30 min at

room temperature. The primary antibodies and the titers used were as follows: mouse monoclonal anti-human alpha-smooth muscle actin (α-SMA) (1:100, CBL 171; Chemicon International, Temecula, CA), goat polyclonal anti-rat tenascin C (1:100, sc-9872; Santa Cruz Biotechnology), goat polyclonal anti-rat aggrecan (1:100, sc-8433; Santa Cruz Biotechnology), goat polyclonal anti-rat tenomodulin (TNMD) (1:100, sc-49325; Santa Cruz Biotechnology), mouse polyclonal anti-rat collagen type I (Col I) (1:100, c-2456; Sigma), and mouse monoclonal anti-rat collagen type II (Col II) (1:100, MS-235-P; Neomarkers, Lab Vision, Fremont, CA). Primary antibody was replaced with blocking solution in the negative controls. After washing with PBS, the cells were incubated with biotinylated anti-mouse/rabbit IgG antibody (1:100; Dako, Glostrup, Denmark) or biotinylated anti-goat IgG antibody (1:100; Chemicon International) for 20 min at room temperature. The classical antibody binding complex streptavidin–biotinylated horseradish peroxidase method was used to amplify the specific binding signal (Dako; AB reagent diluted at 1:500 in PBS for 30 min). Finally, 3,3′ diaminobenzidine tetrahydrochloride (Dako) was used to develop the color in the presence of H_2O_2. The cells were rinsed in distilled water, counterstained in Harris hematoxylin, dehydrated through graded alcohol, and mounted with *p*-xylene-*bis*-pyridinium bromide (DPX) (Sigma-Aldrich). For good reproducibility and comparability, all incubation times and conditions were strictly controlled. The cells were examined under light microscopy (Leica DMRXA2; Leica Microsystems, Wetzlar, Germany). Triplicates of cells from two rats were examined for each marker.

Multidifferentiation potential

We investigated the osteogenic, adipogenic, and chondrogenic differentiation potential of tendon-derived cells at P3 according to de Mos *et al*.[10] and Sakaguchi *et al*.[32] with some modifications.

Osteogenic differentiation assays. Tendon-derived cells were plated at 4×10^3 cells/cm^2 in a six-well plate and cultured in complete culture medium until the cells reached confluence. They were then incubated in complete medium or osteogenic medium, which was complete culture medium supplemented with 1 nM dexamethasone, 50 mM ascorbic acid, and 20 mM *β*-glycerolphosphate (all from Sigma-Aldrich) for 28 days for the assessment of mRNA expression of Runt-related transcription factor 2 (*RUNX2*), osteopontin (*OPN*), and osteocalcin (*OCN*) by real-time reverse transcription–polymerase chain reaction (PCR) (see below) as well as calcium nodule formation by alizarin red staining. For alizarin red staining, the cells were fixed in 70% ethanol for 10 min and stained with 0.5% alizarin red (pH 4.1; Sigma-Aldrich) for 30 min.

Adipogenic differentiation assays. Tendon-derived cells were plated at the same density as that indicated for the osteogenic assays. The medium was replaced with complete medium or adipogenic medium, which was complete culture medium supplemented with 500 nM dexamethasone, 0.5 mM isobutylmethylxanthine, 50 μM indomethacin, and 10 μg/mL insulin (all from Sigma-Aldrich). The cells were cultured for an additional of 21 days for the assessment of mRNA expression of *C/EBPα* and *PPARγ2* by real-time PCR (see below) as well as the presence of oil droplets by oil red-O staining. The presence of oil droplets was confirmed by staining the cells with 0.3% fresh oil red-O solution (Sigma-Aldrich) for 2 h after fixation with 70% ethanol for 10 min.

Chondrogenic differentiation assays. For chondrogenic differentiation, a pellet culture system was used. About 8×10^5 cells were pelleted into a micromass by centrifugation at 450 *g* for 10 min in a 15-mL conical polypropylene tube and cultured in complete medium or chondrogenic medium at 37°C, 5% CO_2, which contained low-glucose Dulbecco's modified Eagle's medium (Gibco, Invitrogen Carlsbad,

Table 1. Primer Sequences and Condition for Real-Time Reverse Transcription–Polymerase Chain Reaction

Gene	*Primer nucleotide sequence*	*Product size (bp)*	*Annealing temperature (°C)*	*Accession no.*
β-Actin	5′-ATC GTG GGC CGC CCT AGG CA-3′ (forward) 5′-TGG CCT TAG GGT TCA GAG GGG-3′ (reverse)	243	52	NM_031144
RUNX2	5′-CCGATGGGACCGTGGTT-3′ (forward) 5′-CAGCAGAGGCATTTCGTAGCT-3′ (reverse)	74	60	XM_346016
OPN	5′-TCCAAGGAGTATAAGCAGCGGGCCA-3′ (forward) 5′-CTCTTAGGGTCTAGGACTAGCTTCT-3′ (reverse)	200	55	AB001382.1
OCN	5′-GGTGCAAAGCCCAGCGACTCT-3′ (forward) 5′-GGAAGCCAATGTGGTCCGCTA-3′ (reverse)	199	60	M23637
C/EBPα	5′-AAGGCCAAGAAGTCGGTGGA-3′ (forward) 5′-CAGTTCGCGGCTCAGCTGTT-3′ (reverse)	189	55	NM_012524.2
PPARγ2	5′-CGGCGATCTTGACAGGAAAG-3′ (forward) 5′-GCTTCCACGGATCGAAACTG-3′ (reverse)	174	59	AB019561
COL2A1	5′-ATGACAATCTGGCTCCCAACACTGC-3′ (forward) 5′-GACCGGCCCTATGTCCACACCGAAT-3′ (reverse)	364	55	BT007205
Aggrecan	5′-CTTGGGCAGAAGAAAGATCG-3′ (forward) 5′-GTGCTTGTAGGTGTTGGGGT-3′ (reverse)	158	58	J03485
SOX9	5′-AGAGCGTTGCTCGGAACTGT-3′ (forward) 5′-TCCTGGACCGAAACTGGTAAA-3′ (reverse)	66	60	AB073720

OCN, osteocalcin; OPN, osteopontin; Runx2, Runt-related transcription factor 2.

CA), supplemented with 10 ng/mL transforming growth factor-β3 (R&D Systems), 500 ng/mL bone morphogenetic protein-2 (R&D Systems), 10^{-7} M dexamethasone, 50 μg/mL ascorbate-2-phosphate, 40 μg/mL proline, 100 μg/mL pyruvate (all from Sigma-Aldrich), and 1:100 diluted ITS+Premix (6.25 mg/mL insulin, 6.25 mg/mL transferrin, 6.25 mg/mL selenous acid, 1.25 mg/mL bovine serum albumin, and 5.35 mg/mL linoleic acid) (Becton Dickinson, Franklin Lakes, NJ). At day 21, the pellet was either fixed for histology and immunohistochemical staining of Col II or mRNA expression of *COL2A1*, *aggrecan*, and *SOX9* as described below.

Histological assay

The cell pellet was fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections were cut at a thickness of 5 μm and were stained with safranin-O/fast green after deparaffination[33] and viewed using a LEICA Q500MC microscope (Leica Cambridge, Cambridge, United Kingdom). Three cell pellets from three rats were examined for each condition.

Immunohistochemical staining

Immunohistochemical staining was performed as previously described.[24,29,30,34,35] Briefly, paraffin-embedded sections were deparaffinized in xylene and dehydrated through graded series of alcohol. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 20 min at room temperature. Antigen retrieval was then performed with 2 mg/mL protease (Calbiochem, Bie and Berntsen, Rødovre, Denmark) at 37°C for 30 min for Col II detection.

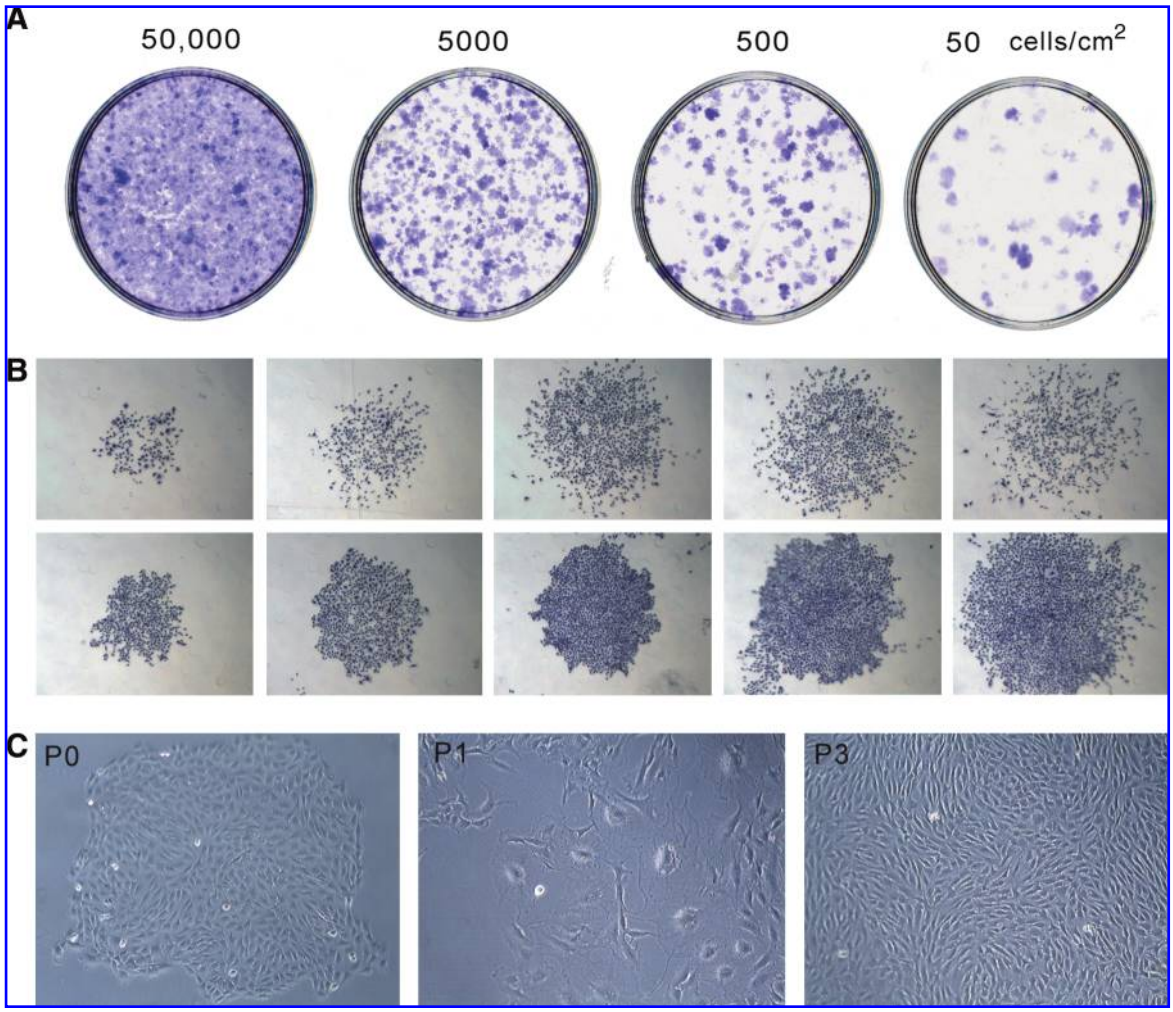

FIG. 1. Photomicrographs showing the clonogenicity and cell morphology of tendon-derived cells at different passages. (**A**) Formation of cell colonies of tendon-derived cells after culturing for 7 days at different initial seeding densities. Distinctive colonies meeting the criteria described in Materials and Methods were observed at the initial seeding density of 50 and 500 cells/cm^2. Magnification: ×1. (**B**) Ten photographs showing the different sizes and densities of colonies at passage (P0). Magnification: ×50. (**C**) The cells exhibited different morphologies at different passages. At P0, large polygonal and star-shaped cells were observed, and flat cells and slender fibroblast-like cells were observed at P1. At P3, homogeneous populations of fibroblast-like cells were observed. Magnification: ×100; stain: crystal violet. Color images available online at www.liebertonline.com/ten.

Residual enzymatic activity was removed by washing with PBS. After blocking with 5% goat serum for 20 min at room temperature, the sections were incubated with mouse monoclonal antibody against rat Col II (Neomarkers-Biogen, Lab Vision; Fremont, CA; 1:100 dilution with 5% goat serum in PBS containing 1% bovine serum albumin) overnight at 4°C. The spatial localization of Col II was observed by incubating with goat anti-mouse IgG horseradish-peroxidase-conjugated secondary antibody (Chemicon International) for an hour at room temperature, followed by 3,3′-diaminobenzidine tetrahydrochloride (Dako) in the presence of H_2O_2. Afterward, the sections were rinsed, counterstained with hematoxylin, dehydrated with graded series of ethanol and xylene, and mounted with DPX. Primary antibody was replaced with blocking solution in the negative control. Articular cartilage of femora condyle was used as positive control. The sections were examined under light microscopy (Leica DMRXA2; Leica Microsystems).

Quantitative real-time reverse transcription-PCR

Real-time PCR was performed as previously described.[24,34] The tendon-derived cells were harvested and homogenized for RNA extraction with RNeasy mini kit (cat. no. 74104; Qiagen GmbH, Hilden, Germany). RNA was reverse transcribed to cDNA by the First Strand cDNA kit (Promega, Madison, WI), following the manufacturer's protocol. The concentration of cDNA was determined by measuring the optical density at 260 nm/280 nm. The condition for PCR was optimized in a conventional PCR machine (GeneAmp 9700; Applied Biosystems, Foster City, CA) for the primers specific for *β-actin*, *RUNX2*, *OPN*, *OCN*, *C/EBPα*, *PPARγ2*, *COL2A1*, *aggrecan*, and *SOX9* as shown in Table 1 at various annealing temperatures. Optimized results were transferred to the real-time PCR protocol. Cycling conditions were as follows: denaturation at 95°C for 10 min, 45 cycles at 95°C for 10 s, optimal annealing temperature (Table 1) for 15 s, 72°C for 20 s, and finally at 60–95°C with a heating rate of 0.1°C/s. Calibrator was used as positive control to validate the primers, and replacement of cDNA template with water was used as negative control. The real-time PCR machine, the reaction kits, and the software used in the experiments were purchased from Roche (LightCycler, Roche Diagnostics, Penzberg, Germany). Results were analyzed using Relative Quantification Software (Roche Diagnostics). Expression of the target gene was normalized to that of *β-actin* gene. Relative gene expression was calculated according to the $2^{-\Delta\Delta CT}$ formula. The experiment was run in duplicate using cells from three rats to confirm the consistency of the findings.

Data analysis

The cell number and colony number were reported as mean plusmn; standard deviation. The immunocytochemical, histological, and immunohistochemical data were qualitatively described. Representative images were shown. The mRNA data were presented in box-plots. To compare the mRNA level of induction groups with control groups, the ratio of target gene expression in induction group and control groups was calculated. A ratio of 1 indicated no difference of osteogenic, adipogenic, or chondrogenic differentiation potential of tendon-derived cells in induction medium compared to that in expansion medium and was tested using Mann–Whitney *U*-test. All the data analysis was done using SPSS (Chicago, IL; version 16.0). $p < 0.05$ was regarded as statistically significant.

Results

Clonogenicity and cell morphology of tendon-derived cells at different passages

The tendon-derived cells formed colonies after *in vitro* culture for 7 days as indicated by crystal violet staining (Fig. 1A). These colonies were heterogeneous in size and cell density, potentially reflecting differences in the rate of cell proliferation (Fig. 1B). At P0, large polygonal and star-shaped cells were observed (Fig. 1C). At P1, flat cells and slender fibroblast-like cells were observed (Fig. 1C). At P3, a homogeneous population of fibroblast-like cells was observed (Fig. 1C). To determine the optimal initial plating density that gives the maximum number of cell colonies, we plated the cells isolated from tendon tissue at different densities. Colonies formed at 5000 or 50,000 cells/cm^2 were small and indistinct, possibly due to contact inhibition (Fig. 1A). Plating of cells at 50 cells/cm^2 was found to yield the highest number of single cell colonies per tendon-derived nucleated cell (12.33 ± 1.53 colonies/10^3 nucleated cells at 50 cells/cm^2 group, and 8.5 ± 0.89 colonies/10^3 nucleated cells at 500 cells/cm^2 group, $p = 0.020$) and was used as the optimal initial seeding density for all subsequent experiments.

Proliferative potential of tendon-derived cell colonies

Tendon-derived cell colonies were trypsinized and plated at different cell densities, and the cell number was counted

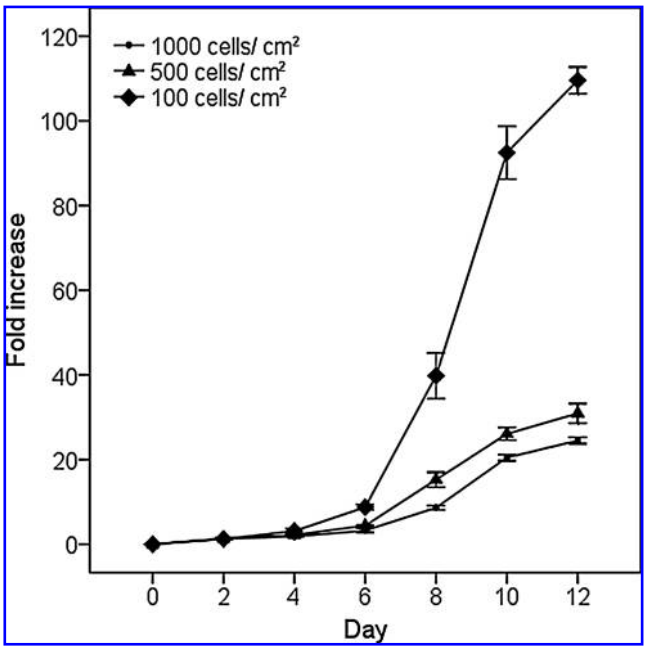

FIG. 2. Graph showing the proliferative potential of tendon-derived cells at P1 at different seeding densities. Cells seeded at the lowest density (100 cells/cm^2) showed the highest fold increase in cell number than those seeded at higher densities. The results shown were mean ± standard deviation of three Petri dishes for each time point.

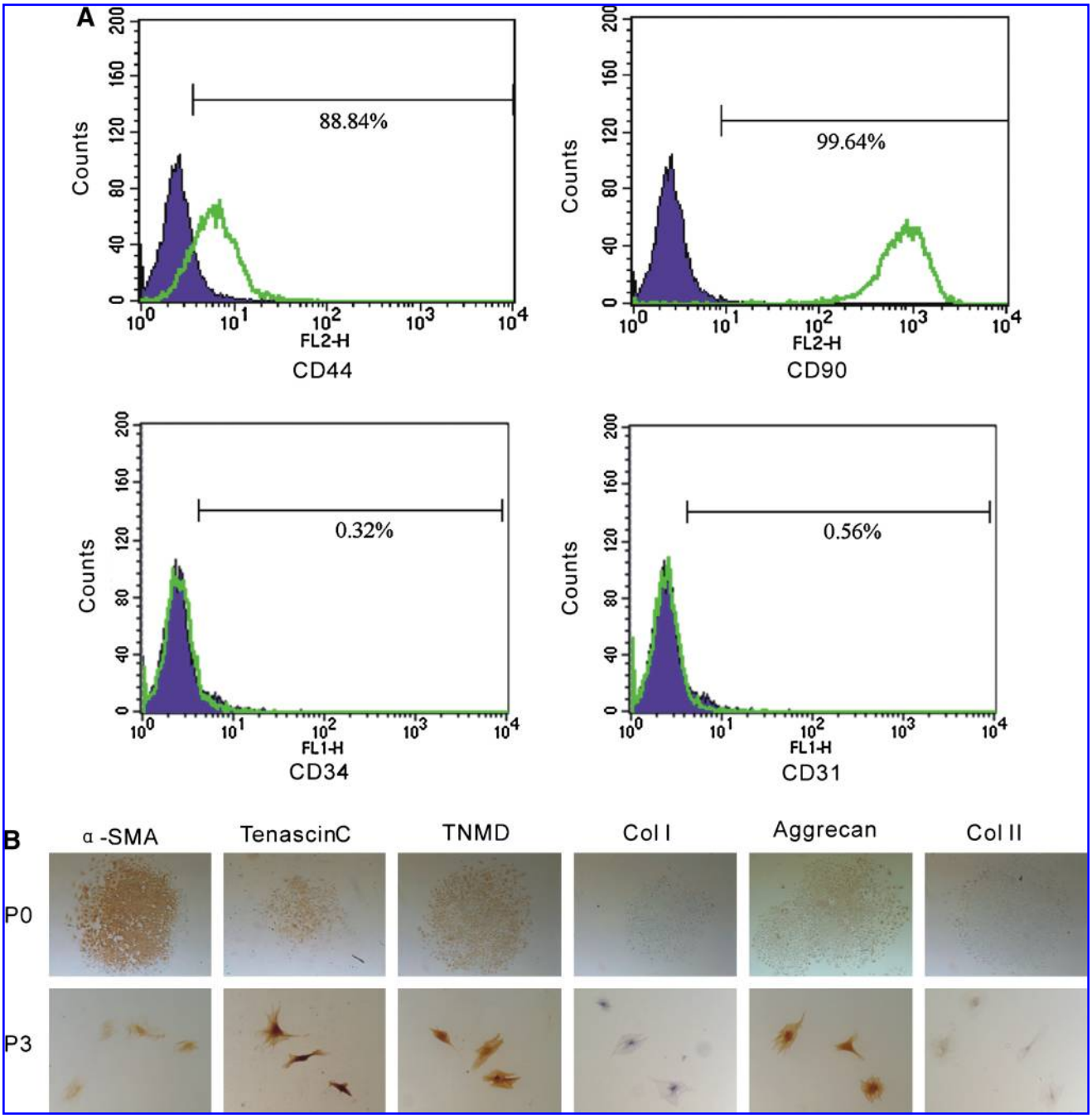

FIG. 3. (**A**) Graphs showing expression of mesenchymal stem cell markers (CD44 and CD90), hematopoietic stem cell marker (CD34), and endothelial cell marker (CD31) on cells derived from tendon-derived cell colonies. High percentage of cells expressed CD44 and CD90, whereas only few cells expressed CD34 and CD31. (**B**) Photomicrographs showing expression of immunocytochemical staining of tenogenic (α-SMA, tenascin C, TNMD, and Col I) and chondrogenic (aggrecan and Col II) markers at P0 and P3. All tendon-derived cells expressed α-SMA, tenascin C, TNMD, and aggrecan at P0 and P3. There was no immunopositive signal of Col I in these cells at both P0 and P3. There was no immunopositive signal of Col II at P0, but there was some faint positive staining in some cells at P3. Magnification: ×50 (P0) and ×100 (P3). α-SMA, alpha-smooth muscle actin; TNMD, tenomodulin; Col I, collagen type I; Col II, collagen type II.

every 2 days for 12 days (Fig. 2). Our results showed that the cell number increased with time up to day 12 and cells seeded at lower density (100 cells/cm^2) proliferated much faster than those seeded at higher densities. The number of cells could increase by more than 100-fold after culturing for 12 days at the lowest seeding density tested (Fig. 2).

Flow cytometric analysis of MSC markers

To confirm that the tendon-derived cells were stem cells, we examined expression of MSC surface markers on the cells using flow cytometric analysis. Our results showed that over 88% and 99% of tendon-derived cells were positive for the

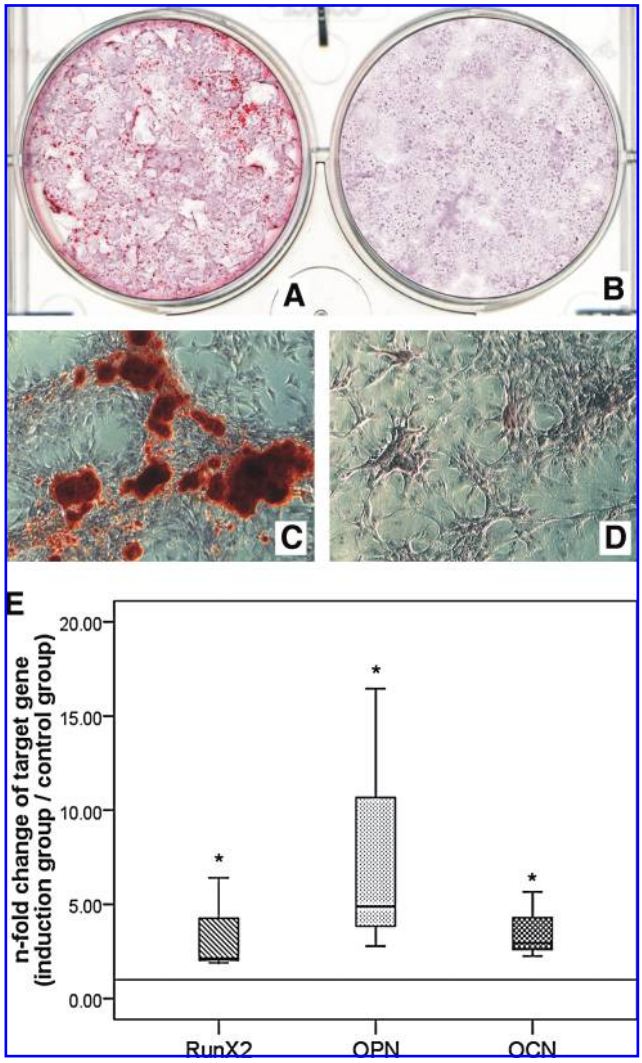

FIG. 4. Osteogenic differentiation potential of cells from the tendon-derived cell colonies *in vitro*. (**A–E**) Alizarin red S staining of cells after culturing for 28 days in osteogenic (**A, C**) or control (**B, D**) media. Calcium deposition was seen in osteogenic medium (**A, C**), but not in control medium (**B, D**). (**E**) Graph showing the ratio of fold change of target gene in osteogenic medium and control medium. There was significantly higher expression of Runx2, OPN, and OCN at day 28 in osteogenic medium compared with that in control medium (**E**). $*p < 0.05$ indicates statistical significance in osteogenic group compared with that in control group. Magnification: ×1 (**A, B**) and ×100 (**C, D**). *OPN*, osteopontin; *OCN*, osteocalcin; *RUNX2*, Runt-related transcription factor 2. Color images available online at www.liebertonline.com/ten.

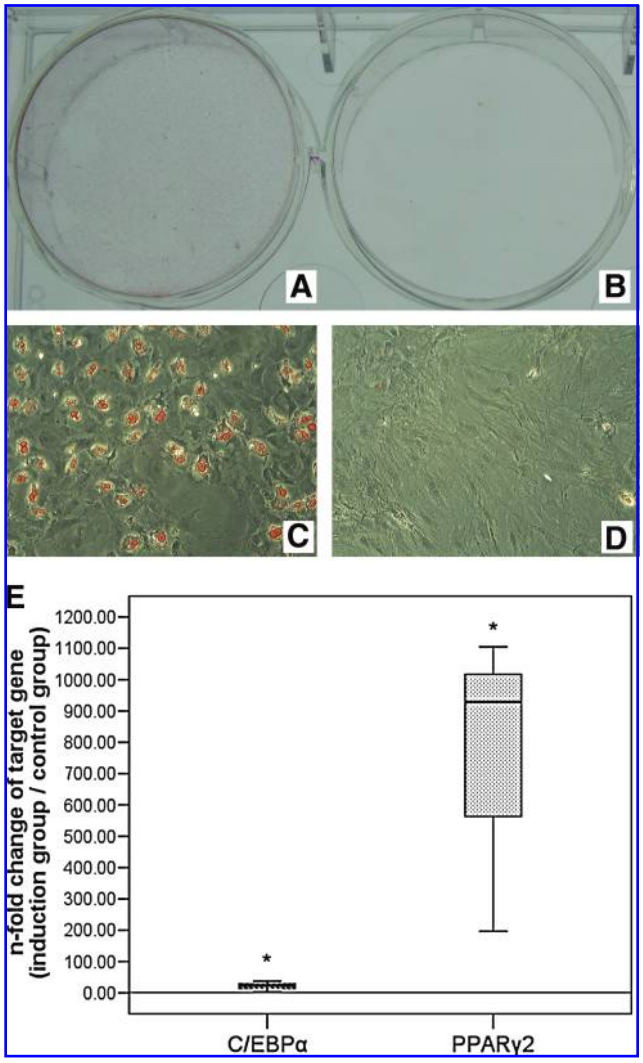

FIG. 5. Adipogenic differentiation potential of cells from the tendon-derived cell colonies *in vitro*. (**A–E**) Oil red-O staining of cells after culturing for 21 days in adipogenic (**A, C**) or in control medium (**B, D**). Oil red-O–positive lipid vacuoles were seen in adipogenic medium (**A, C**), but not in control medium (**B, D**). (**E**) Graph showing the ratio of fold change of target gene in adipogenic medium and control medium. There was significantly higher expression of *C/EBPα* and *PPARγ2* at day 21 in adipogenic medium compared with that in control medium (**E**). $*p < 0.05$ indicates statistical significance. Magnification: ×1 (**A, B**) and ×100 (**C, D**). Color images available online at www.liebertonline.com/ten.

MSC marker CD44 and fibroblastic marker CD90, respectively (Fig. 3A). They were negative for the hematopoietic stem cell marker CD34 and for the endothelial cell marker CD31, thus verifying the absence of contaminating hematopoietic cells and endothelial cells (Fig. 3A).

Immunocytochemical staining of tenogenic and chondrogenic markers

All tendon-derived cells expressed α-SMA, tenascin C, TNMD, and aggrecan but not Col I at P0 and P3 (Fig. 3B). The tendon-derived cells had no expression of Col II at P0, but there was faint positive staining in some cells at P3 (Fig. 3B).

Multidifferentiation potential of tendon-derived cell colonies

Osteogenic differentiation assay. The osteogenic differentiation potential of the tendon-derived cell colonies was determined *in vitro*. Alizarin-red-positive calcium nodules were observed after osteogenic induction of the cells for 28 days (Fig. 4A, C), which were negative in the control group

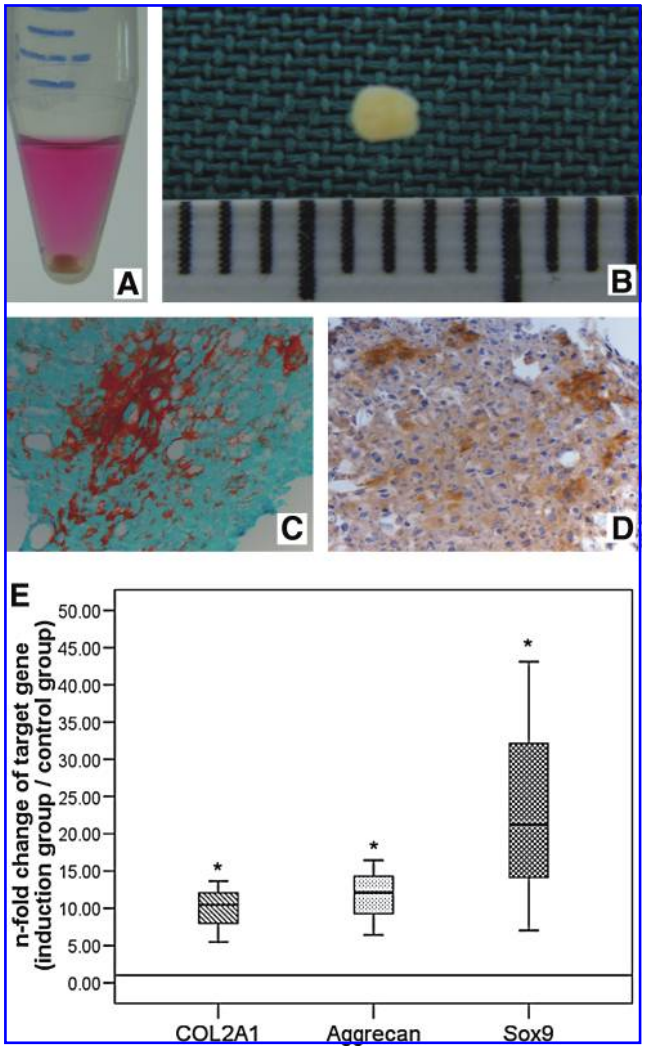

FIG. 6. Chondrogenic differentiation potential of cells from the tendon-derived cell colonies *in vitro*. (**A–E**) Chondrogenic differentiation of cells after cultured in chondrogenic medium for 21 days (**A**). Cell pellet was formed *in vitro* (**B**). There was expression of proteoglycan and Col II as indicated by safranin-O/fast green staining (**C**) and immunohistochemical staining (**D**), respectively. (**E**) Graph showing the ratio of fold change of target gene in chondrogenic medium and control medium. There was also significantly higher expression of *COL2A1*, *aggrecan*, and *SOX9* at day 21 in chondrogenic medium compared with that in control medium (**E**). $^*p < 0.05$ indicates statistical significance. Magnification: ×1 (**A, B**) and ×100 (**C, D**). Color images available online at www.liebertonline.com/ten.

(Fig. 4B, D). There was also significant upregulation of mRNA expression of *RUNX2*, *OPN*, and *OCN* at day 28 after osteogenic induction (Fig. 4E) (all $p = 0.037$).

Adipogenic differentiation assay. Lipid droplets were formed after incubating the cells in complete medium with adipogenic supplements for 21 days (Fig. 5A, C). This was not observed in the control group (Fig. 5B, D). mRNA expression of *C/EBPα* and *PPARγ2* were significantly upregulated at day 21 after adipogenic induction (Fig. 5E) (all $p = 0.037$).

Chondrogenic induction assay. A micromass was formed after culturing the tendon-derived cells in chondrogenic medium for 21 days (Fig. 6A, B). The micromass expressed proteoglycan (Fig. 6C) and Col II (Fig. 6D), as indicated by safranin-O/fast green and immunohistochemical staining, respectively. There was also increased expression of *COL2A1*, *aggrecan*, and *SOX9* mRNA after chondrogenic induction for 21 days (Fig. 6E) (all $p = 0.037$).

Discussion

In this study, we have successfully isolated a unique cell population with stem cell characteristics from adult rat flexor tendon tissue. This is the first work that isolated and characterized rat TDSCs *in vitro*. These tendon-derived cells had universal MSC characteristics, including clonogenicity, high proliferative potential at low seeding density, MSC marker expression, and multidifferentiation potential including osteogenesis, adipogenesis, and chondrogenesis. We termed these tendon-derived cells as TDSCs.

Our results showed that the TDSCs exhibited higher proliferative capacity at lower seeding density and the colonies formed were heterogeneous in size and density. This was consistent with previous studies that lower seeding density favored the enrichment and proliferation of stem cells[36,37] and stem cells from the same source also exhibited differences in proliferation and differentiation potential.[11] As confluency was reached only after 10 days when seeding at higher densities (i.e., 500 and 1000 cells/cm^2) and we replaced with fresh medium every 3 days during experiment, it was unlikely that nutrient was a limit factor for cell growth. The TDSCs isolated expressed stem cell markers CD44 and CD90. Expression of high CD44 and CD90 was also reported in TSPCs isolated from mouse and human tendons.[11] Expression of CD44 and CD90 was also similar to previous reports describing the expression of surface markers in rat MSCs.[19,37,38] There was minimal contamination with hemopoietic and endothelial cells in our culturing system as there was less expression of CD34 and CD31 in the cultured cells. Based on optimal seeding density in this study, about 1–2% of nucleated cells isolated were stem cells, comparable to the percentage of stem cells in Bi *et al.*'s study[11] and other solid mesenchymal tissues in rats.[14] There were mixture of cells with different cell morphologies at P0 and P1; however, a homogeneous population of fibroblast-like cells was observed at P3. Our findings suggested that cells from P3 are suitable for *in vitro* stem cell differentiation and tissue engineering studies.

In addition to the stem cell markers, we also studied expression of lineage-specific markers in TDSCs. Immunocytochemistry staining confirmed the unique phenotype of the isolated TDSCs. Specifically, there was expression of α-SMA, tenascin C, aggrecan, and TNMD at P0 and P3. There was no immunopositive signal of Col I in these cells at both P0 and P3. The tendon-derived cell colonies had no expression of Col II at P0, but there was some faint positive staining in some cells at P3. Positive expression of tenascin C and α-SMA as well as negative expression of Col II in our rat TDSCs was consistent with results on mouse TSPCs in Bi *et al.*'s study.[11] However, our findings regarding expression of α-SMA were different from that reported by de Mos *et al.*,[10] who showed no expression of α-SMA in human tendon-derived progenitor cells. Our findings on negative expression

of Col I in TDSCs were also different from that reported by Bi *et al*.,[11] who showed high level of Col I in mouse TSPCs. Differences in species and age of animals as well as cell isolation method might account for the differences. As the TDSCs expressed tendon-related markers, including tenascin C and TNMD,[38] a unique population of stem cells that was different from BMSCs was present in tendon tissue.[39–41] Expression of aggrecan at P0 and P3 and Col II at P3 suggested that the cells also showed some chondrogenic characteristics. Indeed, the transdifferentiation of tendon fibroblasts to chondrocytes including the up-regulation of cartilage-associated genes and down-regulation of tendon-associated genes in rat supraspinatus tendon[42] and in horse superficial digital flexor tendon[43] after overuse injury has been reported, supporting that tendon fibroblasts have the machineries for transdifferentiation under appropriate biological and mechanical stimuli.

In conclusion, we have isolated a population of stem cells from tendon tissue in rat, which exhibited universal stem cell characteristics, including clonogenicity, proliferative capacity, multidifferentiation potential, and MSC marker expression. In addition, these TDSCs also expressed tendon-related and some chondrogenic-related markers that make them a unique cell population. The ability to isolate stem cells from rat tendon tissues would open up new opportunities of studying TDSCs for tendon tissue engineering and improve our understanding of the role of resident tendon stem cells in tendon physiology, healing, and pathological processes such as tendinopathy in rat models.

Acknowledgment

This work was supported by equipment/resources donated by the Hong Kong Jockey Club Charities Trust, the Restructuring and Collaboration Fund from University Grant Council, and the CUHK Direct Grant (ref. no. 2007.1.065).

Disclosure Statement

No competing financial interests exist.

References

1. Friedenstein, A.J. Precursor cells of mechanocytes. Int Rev Cytol **47,** 327, 1976.
2. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. Science **284,** 143, 1999.
3. Prockop, D.J. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science **276,** 71, 1997.
4. De Bari, C., Dell'Accio, F., Tylzanowski, P., and Luyten, F.P. Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum **44,** 1928, 2001.
5. De Bari, C., Dell'Accio, F., and Luyten, F.P. Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. Arthritis Rheum **44,** 85, 2001.
6. Cao, B., Zheng, B., Jankowski, R.J., Kimura, S., Ikezawa, M., Deasy, B., Cummins, J., Epperly, M., Qu-Petersen, Z., and Huard, J. Muscle stem cells differentiate into haematopoietic lineages but retain myogenic potential. Nat Cell Biol **5,** 640, 2003.
7. Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P., and Hedrick, M.H. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell **13,** 4279, 2002.
8. Barbero, A., Ploegert, S., Heberer, M., and Martin, I. Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis Rheum **48,** 1315, 2003.
9. Salingcarnboriboon, R., Yoshitake, H., Tsuji, K., Obinata, M., Amagasa, T., Nifuji, A., and Noda, M. Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. Exp Cell Res **287,** 289, 2003.
10. de Mos, M., Koevoet, W.J., Jahr, H., Verstegen, M.M., Heijboer, M.P., Kops, N., van Leeuwen, J.P., Weinans, H., Verhaar, J.A., and van Osch, G.J. Intrinsic differentiation potential of adolescent human tendon tissue: an *in-vitro* cell differentiation study. BMC Musculoskelet Disord **8,** 16, 2007.
11. Bi, Y., Ehirchiou, D., Kilts, T.M., Inkson, C.A., Embree, M.C., Sonoyama, W., Li, L., Leet, A.I., Seo, B.M., Zhang, L., Shi, S., and Young, M.F. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nat Med **13,** 1219, 2007.
12. Martinez-Lorenzo, M.J., Royo-Canas, M., Alegre-Aguaron, E., Desportes, P., Castiella, T., Garcia-Alvarez, F., and Larrad, L. Phenotype and chondrogenic differentiation of mesenchymal cells from adipose tissue of different species. J Orthop Res **27,** 1499, 2009.
13. Peister, A., Mellad, J.A., Larson, B.L., Hall, B.M., Gibson, L.F., and Prockop, D.J. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood **103,** 1662, 2004.
14. Yoshimura, H., Muneta, T., Nimura, A., Yokoyama, A., Koga, H., and Sekiya, I. Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. Cell Tissue Res **327,** 449, 2007.
15. Sekiya, I., Larson, B.L., Smith, J.R., Pochampally, R., Cui, J.G., and Prockop, D.J. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells **20,** 530, 2002.
16. Simmons, D.J., Seitz, P., Kidder, L., Klein, G.L., Waeltz, M., Gundberg, C.M., Tabuchi, C., Yang, C., and Zhang, R.W. Partial characterization of rat marrow stromal cells. Calcif Tissue Int **48,** 326, 1991.
17. Aubin, J.E. Osteoprogenitor cell frequency in rat bone marrow stromal populations: role for heterotypic cell-cell interactions in osteoblast differentiation. J Cell Biochem **72,** 396, 1999.
18. Dobson, K.R., Reading, L., Haberey, M., Marine, X., and Scutt, A. Centrifugal isolation of bone marrow from bone: an improved method for the recovery and quantitation of bone marrow osteoprogenitor cells from rat tibiae and femurae. Calcif Tissue Int **65,** 411, 1999.
19. Javazon, E.H., Colter, D.C., Schwarz, E.J., and Prockop, D.J. Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells. Stem Cells **19,** 219, 2001.
20. Corps, A.N., Robinson, A.H.N., Movin, T., Costa, M.L., Hazleman, B.L., and Riley, G.P. Increased expression of aggrecan and biglycan mRNA in Achilles tendinopathy. Rheumatology **45,** 291, 2006.
21. Chard, M.D., Cawston, T.E., Riley, G.P., Gresham, G.A., and Hazleman, B.L. Rotator cuff degeneration and lateral epicondylitis—a comparative histological study. Ann Rheum Dis **53,** 30, 1994.

22. Archer, R.S., Bayley, J.I.L., Archer, C.W., and Ali, S.Y. Cell and matrix changes associated with pathological calcification of the human rotator cuff tendons. J Anat **182,** 1, 1993.
23. Uhthoff, H.K., Sarkar, K., and Maynard, J.A. Calcifying tendinitis: a new concept of its pathogenesis. Clin Orthop Relat Res **118,** 164, 1976.
24. Lui, P.P.Y., Chan, L.S., Cheuk, Y.C., Lee, Y.W., and Chan, K.M. Expression of bone morphogenetic protein-2 in the chondrogenic and ossifying sites of calcific tendinopathy and traumatic tendon injury rat models. J Orthop Surg Res **21,** 27, 2009.
25. Speer, M.Y., Yang, H.Y., Brabb, T., Leaf, E., Look, A., Lin, W.L., Frutkin, A., Dichek, D., and Giachelli, C.M. Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. Circ Res **104,** 733, 2009.
26. Kim, S.Y., Choi, H.Y., Myung, K.B., and Choi, Y.W. The expression of molecular mediators in the idiopathic cutaneous calcification and ossification. J Cutan Pathol **35,** 826, 2008.
27. Lounev, V.Y., Ramachandran, R., Wosczyna, M.N. Yamamoto, M., Maidment, A.D., Shore, E.M., Glaser, D.L., Goldhamer, D.J., and Kaplan, F.S. Identification of progenitor cells that contribute to heterotopic skeletogenesis. J Bone Joint Surg Am **91,** 652, 2009.
28. Warden, S.J. Animal models for the study of tendinopathy. Br J Sports Med **41,** 232, 2007.
29. Lui, P.P.Y., Cheuk, Y.C., Hung, L.K., Fu, S.C., and Chan, K.M. Increased apoptosis at the late stage of tendon healing. Wound Repair Regen **15,** 702, 2007.
30. Lui, P.P., Fu, S.C., Chan, L.S., Hung, L.K., and Chan, K.M. Chondrocyte phenotype and ectopic ossification in collagenase-induced tendon degeneration. J Histochem Cytochem **57,** 91, 2009.
31. Fu, S.C., Cheuk, Y.C., Chan, K.M., Hung, L.K., and Wong, M.W. Is cultured tendon fibroblast a good model to study tendon healing? J Orthop Res **26,** 374, 2008.
32. Sakaguchi, Y., Sekiya, I., Yagishita, K., and Muneta, T. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. Arthritis Rheum **52,** 2521, 2005.
33. Wong, M.W., Qin, L., Tai, J.K., Lee, S.K., Leung, K.S., and Chan, K.M. Engineered allogeneic chondrocyte pellet for reconstruction of fibrocartilage zone at bone-tendon junction—a preliminary histological observation. J Biomed Mater Res B Appl Biomater **70,** 362, 2004.
34. Lui, P.P.Y., Chan, L.S., Lee, Y.W., Fu, S.C., and Chan, K.M. Sustained expression of proteoglycans and collagen type III/type I ratio in a calcified tendinopathy model. Rheumatology. (Oxford) **49,** 231, 2010.
35. Lui, P.P.Y., Chan, L.S., Fu, S.C., and Chan, K.M. Expression of sensory neuropeptides in tendon is associated with failed healing and activity-related tendon pain in collagenase-induced tendon injury. Am J Sports Med, in press.
36. Colter, D.C., Class, R., DiGirolamo, C.M., and Prockop, D.J. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc Natl Acad Sci USA **97,** 3213, 2000.
37. Dezawa, M., Ishikawa, H., Itokazu, Y., Yoshihara, T., Hoshino, M., Takeda, S., Ide, C., and Nabeshima, Y. Bone marrow stromal cells generate muscle cells and repair muscle degeneration. Science **309,** 314, 2005.
38. Shukunami, C., Takimoto, A., Oro, M., and Hiraki, Y. Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. Dev Biol **298,** 234, 2006.
39. Petrigliano, F.A., English, C.S., Barba, D., Esmende, S., Wu, B.M., and McAllister, D.R. The effects of local bFGF release and uniaxial strain on cellular adaptation and gene expression in a 3D environment: implications for ligament tissue engineering. Tissue Eng **13,** 2721, 2007.
40. Omae, H., Zhao, C., Sun, Y.L., An, K.N., and Amadio, P.C. Multilayer tendon slices seeded with bone marrow stromal cells: a novel composite for tendon engineering. J Orthop Res **27,** 937, 2009.
41. Zhang, L., Tran, N., Chen, H.Q., Kahn, C.J., Marchal, S., Groubatch, F., and Wang, X. Time-related changes in expression of collagen types I and III and of tenascin-C in rat bone mesenchymal stem cells under co-culture with ligament fibroblasts or uniaxial stretching. Cell Tissue Res **332,** 101, 2008.
42. Archambault, J.M., Jelinsky, S.A., Lake, S.P., Hill, A.A., Glaser, D.L., and Soslowsky, L.J. Rat supraspinatus tendon expresses cartilage markers with overuse. J Orthop Res **25,** 617, 2007.
43. Clegg, P.D., Strassburg, S., and Smith, R.K. Cell phenotypic variation in normal and damaged tendons. Int J Exp Pathol **88,** 227, 2007.

Address correspondence to:
Pauline Po Yee Lui, Ph.D.
Room 74025, 5/F, Clinical Sciences Building
Prince of Wales Hospital
Shatin
Hong Kong SAR
China

E-mail: pauline@ort.cuhk.edu.hk

Received: July 31, 2009
Accepted: December 8, 2009
Online Publication Date: February 2, 2010

This article has been cited by:

1. Zuping Zhou, Takintope Akinbiyi, Lili Xu, Melissa Ramcharan, Daniel J. Leong, Stephen J. Ros, Alexis C. Colvin, Mitchell B. Schaffler, Robert J. Majeska, Evan L. Flatow, Hui B. Sun. 2010. Tendon-derived stem/progenitor cell aging: defective self-renewal and altered fate. *Aging Cell* **9**:5, 911-915. [CrossRef]
2. Yun Feng Rui, Pauline Po Yee Lui, Ming Ni, Lai Shan Chan, Yuk Wa Lee, Kai Ming Chan. 2010. Mechanical loading increased BMP-2 expression which promoted osteogenic differentiation of tendon-derived stem cells. *Journal of Orthopaedic Research* n/a-n/a. [CrossRef]