

RESEARCH ARTICLE

Open Access



Effects of the pulsed electromagnetic field PST[®] on human tendon stem cells: a controlled laboratory study

Pietro Randelli^{1,2*†}, Alessandra Menon^{1†}, Vincenza Ragone¹, Pasquale Creo¹, Umberto Alfieri Montrasio³, Carlotta Perucca Orfei³, Giuseppe Banfi^{3,4}, Paolo Cabitza¹, Guido Tettamanti¹ and Luigi Anastasia^{1,2*}

Abstract

Background: Current clinical procedures for rotator cuff tears need to be improved, as a high rate of failure is still observed. Therefore, new approaches have been attempted to stimulate self-regeneration, including biophysical stimulation modalities, such as low-frequency pulsed electromagnetic fields, which are alternative and non-invasive methods that seem to produce satisfying therapeutic effects. While little is known about their mechanism of action, it has been speculated that they may act on resident stem cells. Thus, the purpose of this study was to evaluate the effects of a pulsed electromagnetic field (PST[®]) on human tendon stem cells (hTSCs) in order to elucidate the possible mechanism of the observed therapeutic effects.

Methods: hTSCs from the rotator cuff were isolated from tendon biopsies and cultured in vitro. Then, cells were exposed to a 1-h PST[®] treatment and compared to control untreated cells in terms of cell morphology, proliferation, viability, migration, and stem cell marker expression.

Results: Exposure of hTSCs to PST[®] did not cause any significant changes in proliferation, viability, migration, and morphology. Instead, while stem cell marker expression significantly decreased in control cells during cell culturing, PST[®]-treated cells did not have a significant reduction of the same markers.

Conclusions: While PST[®] did not have significant effects on hTSCs proliferation, the treatment had beneficial effects on stem cell marker expression, as treated cells maintained a higher expression of these markers during culturing. These results support the notion that PST[®] treatment may increase the patient stem cell regenerative potential.

Keywords: Tendon stem cells, Pulsed electromagnetic fields, Pulsed signal therapy, Rotator cuff

Background

Rotator cuff tendinopathy is a degenerative process causing pain and disability [1, 2]. Generally, a non-operative management is regarded as the first-line treatment prior to a surgical intervention [3]. Nonetheless, given the rather inconsistent results of either approaches, new strategies have been explored, especially in the rising field of tissue engineering [4–10]. The discovery that also the human rotator cuff tendons possess a reservoir of progenitor cells, the human tendon stem cells (hTSCs), has clearly opened

up new perspectives for developing novel therapies [11–15]. In fact, hTSCs could be used in cell therapy approaches and, maybe most importantly, they could potentially be activated in situ, in order to regenerate the affected tendon without the need of harvesting them from biopsies, growing them in vitro, and re-injecting them. Actually, growing evidences seem to support the notion that when adult stem cells are injected to regenerate a tissue, like the heart or a tendon, only a negligible fraction of cells can survive and differentiate into the desired cell phenotype [16]. In fact, the majority of the injected stem cells simply secrete factors (most of them still unknown) that stimulate resident progenitor cells, which are really the ones that repair the affected tissue. Therefore, in

* Correspondence: pietro.randelli@unimi.it; luigi.anastasia@unimi.it

†Equal contributors

¹IRCCS Policlinico San Donato, San Donato Milanese, Milan, Italy
Full list of author information is available at the end of the article

the last few years, researchers have focused their attention on identifying these secreted factors, to use them instead of injecting cells [16–19]. On the other hand, other approaches have been attempted to stimulate self-regeneration, including biophysical stimulation modalities, such as low-frequency pulsed electromagnetic fields (PEMFs), which are alternative and non-invasive methods that seem to produce satisfying therapeutic effects also on a wide range of orthopedics diseases, including osteoarthritis [20, 21], non-united fractures [22, 23], failed arthrodesis [24], and soft tissue injuries [25]. However, little is known about the real mechanism by which these effects are obtained. A plausible explanation is that these electromagnetic fields can stimulate the self-healing processes of the affected tissues by activating their resident stem cells. Among others, a specific low-frequency pulsed electromagnetic field (commercially known as PST[®], by Global Munich Germany) is a modification of other low frequency PEMFs, and it has been developed to ideally correspond to the body's own stimulatory energy parameters. The PST[®] device consists in a cylindrical solenoid in which the patient can insert the affected area (including the shoulder) during treatment. It employs direct current with unidirectional low frequencies in the range of 10–30 Hz. The waveform is quasi-rectangular, with measured field strengths predominantly in the 0.5–1.5 mT range. Various frequency-amplitude combinations are automatically switched over and transmitted under continuous control during the treatment period, which is generally set to 1 h.

Based on these premises, the aim of this study was to evaluate the effects of PST[®] on primary cultures of hTSCs and to test whether the application of this pulsed electromagnetic field could alter their phenotype, for instance by increasing their stemness and/or their regenerative capacity, with the ultimate goal of clarifying the mechanism of the observed therapeutic effects.

Methods

Isolation, characterization, and culture of human tendon stem cells

Human tendon stem cells (hTSCs) were isolated from supraspinatus tendon specimens collected during arthroscopic rotator cuff repair, according to a previous procedure (Fig. 1a) [11]. The study protocol has been approved by the Hospital Ethical Committee (authorization number 2642; Sept 19, 2011; ASL Milano 2, Melegnano, Milan) and patients received and signed an informed consent. Briefly, samples from supraspinatus tendons (4–8 mm wide) were collected from six patients, kept in HypoThermosol (BioLife Solutions) at 4 °C, and processed separately within

24 h, according to the procedure described below. Samples were washed with phosphate-buffered saline (PBS) (Euroclone), cut into small pieces, and digested for 90 min with collagenase type I (3 mg/mL; Worthington) and dispase (4 mg/mL; Gibco, Life Technologies) in PBS at 37 °C. After centrifugation, cell pellets were resuspended in the following culture medium: α -Minimal Essential Medium (α -MEM) (Sigma-Aldrich) supplemented with 2 mM glutamine (Euroclone), 1 % antibiotic-antimycotic mixture (Euroclone) and 20 % (v/v) fetal bovine serum (FBS) (HyClone, Thermo-Fisher Scientific). Cells were then filtered with a cell strainer (70 μ m; BD Falcon) and plated in 150-cm² dishes. Adherent cells were cultured at 37 °C with a humidified atmosphere of 5 % CO₂. The medium was changed every 2–3 days. The isolated hTSCs at passage three were characterized by flow cytometry for the expression of key stem cell markers and to test the level of contamination with other cell types (Additional file 1: Figure S1). Flow cytometry analysis was performed on 1×10^5 cells/sample. Briefly, aspecific binding sites were blocked with a blocking solution (50 % 1x PBS, 50 % FBS) for 30 min at room temperature, and washed twice with PBS. Cells were stained with fluorochrome-conjugated mouse anti-human antibodies at the optimal concentration (1:20 dilution) in PBS for 10 min at 4 °C, and washed twice with PBS at 4 °C. Cell characterization was performed using the following antibodies: α CD9 FITC, α CD73 FITC, α HLA-DR FITC, α CD13 PE, α CD29 PE, α CD44 PE, α CD45 PE, α CD90 PE, α CD105 PE, α CD106 PE, α CD34 PerCP-eFluor710, α CD166 PerCP-eFluor710, and α SSEA-4 PE (all from eBioscience); α Lineage Cocktail FITC, α CD18 PE, α CD146 PE, and α Stro-1 Alexa Fluor 647 (all from BioLegend); and α CD117 PE (Miltenyi Biotec). The respective isotype antibodies were used as controls. Samples were acquired with a Navios flow cytometer (Beckman Coulter), and data were processed with Kaluza 1.2 software (Beckman Coulter). Cells at passage three were used for all the experiments.

Cell differentiation

hTSCs were induced to differentiate toward adipocytes, osteoblasts, and chondroblasts in vitro to test for their multi-lineage potential, according to the following procedures (Fig. 1b).

Adipogenic differentiation

hTSCs were plated at a concentration of 3×10^4 cells/cm² in normal growth medium, and then switched to DMEM-low glucose (Sigma-Aldrich), 10 % FBS (HyClone, Thermo-Fisher Scientific), 4 mM L-glutamine (Euroclone), 1 % antibiotic-antimycotic mixture (Euroclone), with the addition of the mesenchymal stem cell adipogenesis

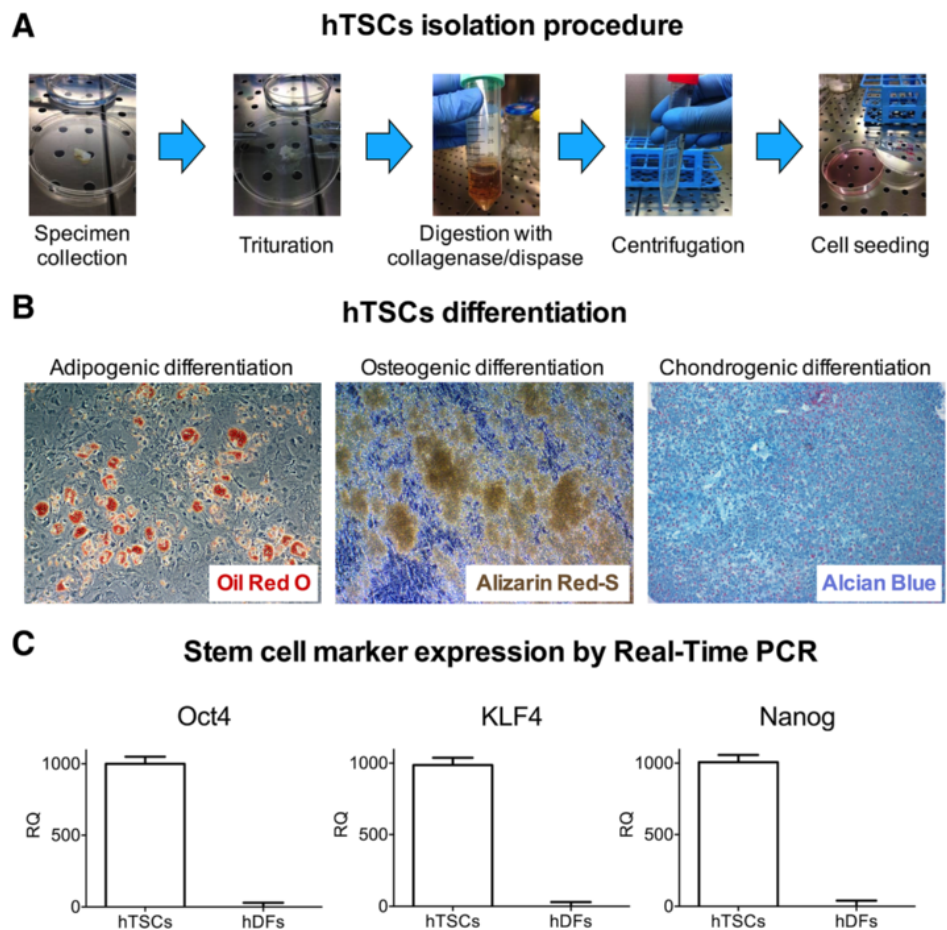


Fig. 1 Isolation and characterization of human tendon stem cells (hTSCs). **a** Schematic representation of the protocol used to isolate hTSCs. **b** In vitro differentiation of hTSCs toward the adipogenic, the osteogenic, and the chondrogenic phenotypes. Lipid intracellular droplets (red) in the adipocytes were stained with Oil Red O solution. Alizarin Red-S staining revealed the presence of calcium deposits 2016 (yellowish-brown). Alcian Blue staining detected/assessed the proteoglycan content. Typical results are shown. Original magnification x10. **c** Gene expression of stem cell marker (Oct4, KLF4, and Nanog) by Real-Time PCR in hTSCs and human dermal fibroblasts (hDFs). Data are expressed as means ± SD of three different experiments

kit (Millipore) for 21 days, according to the manufacturer’s instructions. At day 21, Oil Red O solution (Millipore) was used to stain lipid droplets of derived adipocytes, according to the manufacturer’s procedures. All photomicrographs were acquired with an Axiovert 40 microscope (Zeiss) equipped with a Moticom 2300 camera (Motic). The adipogenic medium was changed every 2–3 days.

Osteogenic differentiation

hTSCs were plated at a concentration of 3×10^4 cells/cm² in normal growth medium, and then switched to the osteogenesis induction medium, which was constituted of DMEM-low glucose (Sigma-Aldrich), 10 % FBS (HyClone, Thermo-Fisher Scientific), 4 mM L-glutamine (Euroclone), 1 % antibiotic-antimycotic mixture (Euroclone), supplemented with 0.1 μM dexamethasone, 50 μg/ml L-ascorbic acid-2-phosphate,

and 10 mM β-glycerophosphate (all reagents from Sigma-Aldrich) for 17 days. At day 17, Alizarin Red solution (Millipore) was used to detect calcium deposition in derived osteoblasts, according to the manufacturer’s instruction. All photomicrographs were acquired with an Axiovert 40 microscope (Zeiss) equipped with a Moticom 2300 camera (Motic). The osteogenic medium was changed every 2–3 days.

Chondrogenic differentiation

hTSCs were maintained in a 3D culture by growing them in cell pellets (1×10^6 cells/pellet) in AdvanceSTEM chondrogenic differentiation medium (HyClone, Thermo Scientific), according to the manufacturer’s instructions. After 28 days of differentiation, matrix deposition by derived chondroblasts was detected with Alcian Blue staining (Sigma-Aldrich), according to the manufacturer’s instruction. All photomicrographs were

acquired with an Axiovert 40 microscope (Zeiss) equipped with a Moticam 2300 camera (Motic). The chondrogenic medium was changed every 2–3 days.

PST® device and cell treatments

The PST® treatment was performed by placing the cell culture plate at the center of PST® device in order to have the magnetic field vector perpendicular to the plate surface (Fig. 2). hTSCs were plated at a concentration of 2.6×10^3 cells/cm² in normal growth medium. Twenty-four hours after seeding, cells were either treated with PST® for 1 h (PST), or were kept outside the incubator for the same amount of time (control) (Fig. 2c). PST and control cells were then returned to the incubator and grown for other 10, 24, or 48 h.

Cell morphology and proliferation experiments

To assess whether PST® stimulation could affect hTSCs phenotype, cell morphology was examined

with a phase-contrast microscope (Axiovert 40 CFL, Zeiss, equipped with a Moticam 2300 camera, Motic) after 0, 10, 24, and 48 h of PST® exposure. For cell viability analyses, hTSCs were subjected to PST® stimulation, as described before. PST and control cells were analyzed at each time point after harvesting with Trypsin-EDTA solution (Sigma-Aldrich) by counting with a Countess Cell Counter (Invitrogen, Life Technologies), according to the manufacturer’s procedure. Cell viability was determined by trypan blue dye exclusion assay. The number of viable cells in each sample was expressed as a percentage of the total untreated cells number at day 0. All assays were carried out in triplicates for each sample.

Cell viability by MTT assay

hTSCs were plated in 12-well plates (1×10^4 cells/well) and were subjected to PST® stimulation, as previously described. At each time point (0, 10, 24, and 48 h), two hours before

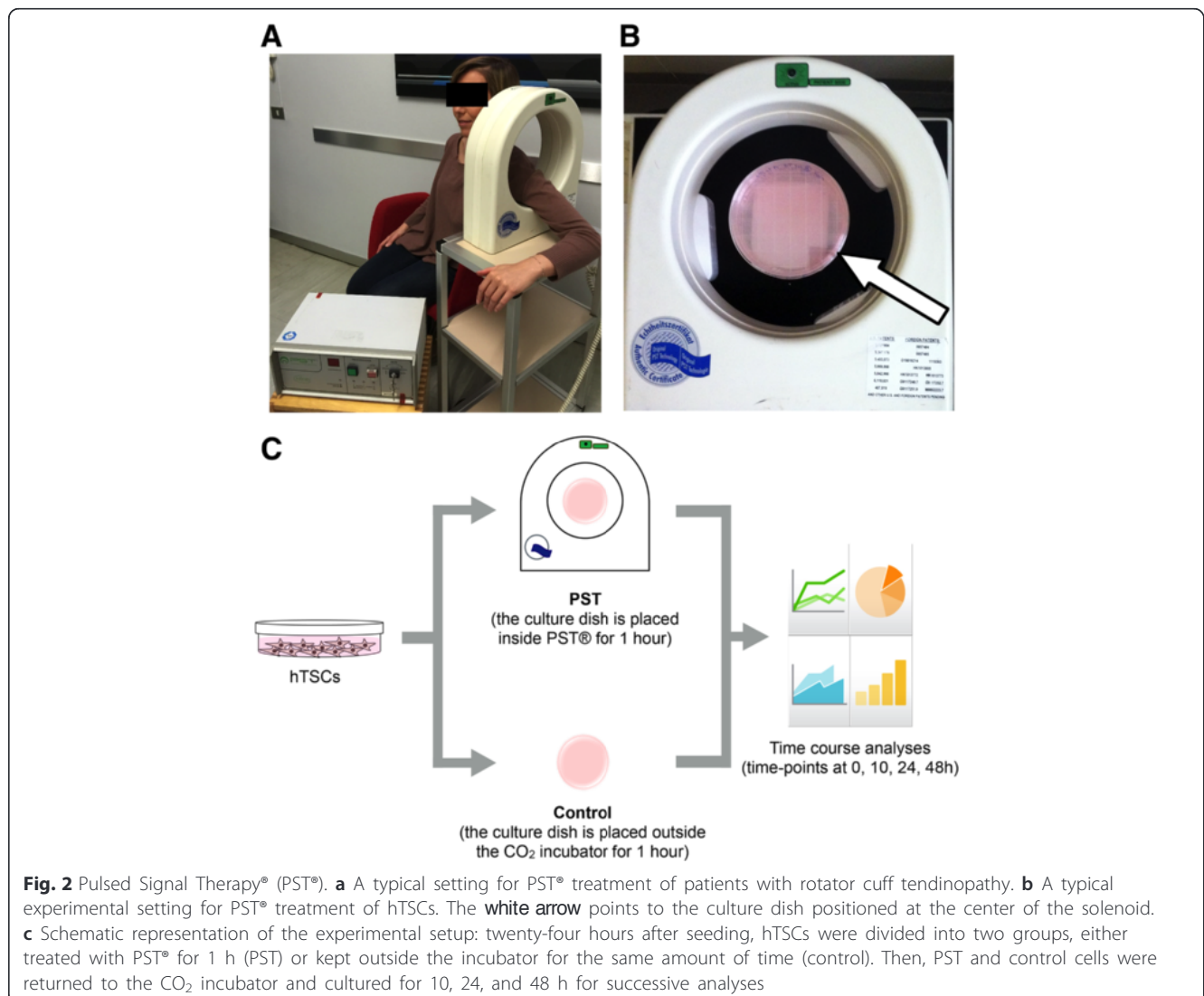


Fig. 2 Pulsed Signal Therapy® (PST®). **a** A typical setting for PST® treatment of patients with rotator cuff tendinopathy. **b** A typical experimental setting for PST® treatment of hTSCs. The **white arrow** points to the culture dish positioned at the center of the solenoid. **c** Schematic representation of the experimental setup: twenty-four hours after seeding, hTSCs were divided into two groups, either treated with PST® for 1 h (PST) or kept outside the incubator for the same amount of time (control). Then, PST and control cells were returned to the CO₂ incubator and cultured for 10, 24, and 48 h for successive analyses

collection, the reconstituted 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (5 mg/ml in PBS; Sigma) was added to the medium (10 % of the final volume). Following a 2-hour incubation at 37 °C, PST and control cells were lysed by adding an amount of MTT Solubilization Solution equal to the original culture medium volume, gently pipetting to completely dissolve the MTT formazan crystals. The MTT reduction was spectrophotometrically measured at a wavelength of 570 nm.

Cell migration by wound-healing assay

Wound-healing assay was performed as previously described [26]. hTSCs were grown to confluence in 6-well plates and were subjected to PST[®] stimulation or were kept outside the incubator for the same amount of time (controls). A sterile P200 pipet tip was used to create a scratch across the cell monolayer. Then, cultures were washed once with 1 ml of growth medium to remove the damaged and detached cells. After replacing the medium, hTSCs were allowed to grow for 48 h. At different time points, cell cultures were examined with a phase-contrast microscope (Axiovert 40 CFL, Zeiss, equipped with a Moticam 2300 camera, Motic) and images of the same scratch fields were acquired at time 0 and after 5, 20, 24, and 30 h from the scratch. The gap area between the cells was calculated in each acquired image using software ImageJ. The migration rate was based on the measure of the recovered wound area (experimental data expressed in percentage). All assays were carried out in triplicates for each sample.

Cell apoptosis analysis

Apoptosis was measured by flow cytometry on PST and control cells before a 1-h PST[®] treatment and then 10, 24 and 48 h post treatment, using Annexin V-FITC Apoptosis detection kit (Enzo Life Sciences), according to the Manufacturer's protocol. Briefly, adherent cells were trypsinized, washed in PBS by gentle shaking, and resuspended with 200 µl of a specific Binding Buffer (10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM CaCl₂) containing 5 µl of annexin V-FITC. After incubation for 10 min in the dark at room temperature, cells were washed in PBS, resuspended in 190 µl of Binding Buffer, and then stained with 10 µl Propidium Iodide (20 µg/ml). Samples were acquired with a Navios flow cytometer (Beckman Coulter), and analyzed using Kaluza 1.2 software (Beckman Coulter).

Gene expression analysis

Stem cell, tendon-related marker, and vascular endothelial growth factor expression was tested by Real-Time

PCR. Total RNA was extracted from PST and control cells at 0, 10, 24 and 48 h using TRIzol[®] Reagent (Ambion, Life Technologies) and 1 µg of extracted RNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (BioRad), according to the Manufacturer's instructions. Real-Time PCR was performed in a 96-well plate with 10 ng of cDNA as template, 0.2 µM primers, and 1× Power SYBR[®] Green PCR Master Mix (Applied Biosystems, Life Technologies) in a 20 µl final volume per well, using a StepOnePlus[™] Real-Time PCR System (Applied Biosystems). The mRNA levels of octamer-binding transcription factor 4 (Oct4), kruppel-like factor 4 (KLF4), Nanog homeobox (Nanog), Tenascin C, collagen type I alpha-1 (COL1A1), and vascular endothelial growth factor (VEGF) were assessed. Somatostatin-14 peptide (S14) was used as the housekeeping gene in quantitative analysis. Primer sequences: S14, forward 5'-GTGTGACTGGTGGGATGAAGG-3' and reverse 5'-TTGATGTGTAGGGCGGTGATAC-3'; Oct4, forward 5'-AGGA GAAGCTGGAGCAAAA-3' and reverse 5'-GGTCGAA TACCTTCCCAA-3'; KLF4, forward 5'-GACTTCCCC CAGTGCTTC-3' and reverse 5'-CGTTGAACTCCT CGGTCTC-3'; Nanog, forward 5'-GGTCCCAGTCAA GAAACAGA-3' and reverse 5'-GAGGTTTCAGGATGT TGGAGA-3'; Tenascin C, forward 5'-CGGGGCTATA GAACACCAGT-3' and reverse 5'-AACATTTAAGTT TCCAATTTTCAGGTT-3'; COL1A1, forward 5'-GGGAT TCCCTGGACCTAAAG-3' and reverse 5'-GGAACAC CTCGCTCTCCA-3'; VEGF, forward 5'-CAACATCAC CATGCAGATTATGC-3' and reverse 5'-TCGGCTTG TCACATTTTTCTTGT-3'.

Amplification protocol: an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 5 s each at 95 °C and 30 s at 57 °C. Relative quantification of target genes was performed in triplicates, analyzed using the 2^{-ΔΔCt} method and normalized to the corresponding S14 values.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v 6.0 software (GraphPad Software Inc.). Data were typical results from three replicate experiments for each of the four patients-derived cell lines, and were expressed as the mean ± standard deviation (SD). Paired comparisons were performed by two-tailed t test. When data was not normally distributed, the Wilcoxon matched-paired test was performed. The significance level was set at p value lower than 0.05.

Results

To mimic the standard PST[®]-treatment procedure on patients' rotator cuff (Fig. 2a), hTSCs cells were cultured in 150 mm² dishes, placed for 1 h inside the

PST[®] solenoid (Fig. 2b), and then returned to the CO₂ incubator (Fig. 2c), as described in the Methods. Control hTSCs were kept outside the incubator for 1 h during the PST[®]-treatment time.

Isolation, characterization, and culture of hTSCs

Isolation of hTSCs was performed according to the protocol described in the Methods section and summarized in Fig. 1a.

hTSCs were cultured to passage three, and then subjected to immunophenotyping by flow cytometry, revealing positivity for mesenchymal antigens CD73, CD90, CD105, CD166, CD106, and CD146 and negativity for hematopoietic antigens, CD19, CD34, CD45, and HLA-DR, as expected (Additional file 1: Figure S1A). As shown in Additional file 1: Figure S1B, hDFs were phenotypically characterized by flow cytometry as controls for comparison with hTSCs, revealing that the immunophenotype of hDFs does not significantly differ from that of hTSCs.

Both cell populations resulted positive for mesenchymal lineage markers like CD73, CD90 and CD105, and negative for hematopoietic lineage markers like CD19, CD34, CD45 and HLA-DR (Additional file 1: Figure S1).

To confirm their stemness, isolated hTSCs were induced to differentiate in vitro toward adipocytes, osteoblasts, chondrocytes by treatment with the proper differentiation media. Results confirmed that hTSCs could be efficiently induced to differentiate towards these cell phenotypes (Fig. 1b).

Conversely, analysis of stem cell markers Oct4, KLF4 and Nanog, measured by quantitative Real-Time PCR, confirmed the expression of the genes in hTSCs, while hDFs showed almost undetectable levels, as expected for terminally differentiated cells (Fig. 1c).

Effects of PST[®] treatment on hTSCs morphology, proliferation, and viability

Cell morphology was analyzed by phase contrast microscopy before a 1-h PST[®] treatment and then 10, 24, and 48 h post treatment. Results showed no noticeable differences between PST and untreated control cells at all time points (Fig. 3a). In addition, proliferation analyses revealed an exponential cell growth in both groups, with no significant differences at all time points (Fig. 3b, $p > 0.05$). These results were confirmed by MTT cell metabolic activity assay, where no significant differences between PST and control cells could be observed at all time points (24, 48, and 72 h) (Fig. 3c).

Effects of PST[®] treatment on hTSCs migration

In order to evaluate the effects of PST[®] treatment on the repairing capacity of hTSCs, an in vitro wound-healing assay was performed. The wound was completely closed in all conditions within 45–48 h, and PST and control cells

showed a similar rate of wound closure (a representative image of PST and control hTSCs moving into the wound space is shown in Fig. 3d for both groups at 0 and 24 h after scratching). Quantitative analyses indicated no significant differences in cell migration velocity between PST and control cells at all-time points ($p > 0.05$, Fig. 3d, e).

Effects of PST[®] treatment on hTSCs apoptosis

Apoptosis was measured by flow cytometry before a 1-h PST[®] treatment and then 10, 24 and 48 h post treatment using Annexin V-FITC, and compared to control untreated cells at the same time points (Fig. 4). Results revealed no significant apoptosis in all tested samples (always below 1 %), with no statistically significant differences between PST- and control- groups.

Effects of PST[®] treatment on hTSCs stem cell/tendon-related markers and VEGF

The relative mRNA levels of stem cell markers Oct4, KLF4 and Nanog were measured by quantitative Real-Time PCR analyses before a 1-h PST[®] treatment and then 10, 24 and 48 h post treatment, and compared to the initial expression values before treatment (Fig. 5a–c). A significant reduction in stem cell marker expression at 48 h was observed in the control group, as compared to the PST cells. Oct4, KLF4 and Nanog expressions were reduced of 44 ± 7 % ($p = 0.009$), 33 ± 7 % ($p = 0.01$) and 49 ± 36 % ($p = 0.04$), respectively (Fig. 5a–c). Otherwise, stem cell marker expression of PST group was similar to that of untreated cells ($p > 0.05$). Oct4 expression at 10 h in the PST group was also significantly reduced compared to the untreated cells of 41 ± 13 % ($p = 0.03$) (Fig. 5a).

Tenascin C and COL1A1 expression levels were comparable to untreated cells (day 0) at each time point in both groups ($p > 0.05$) (Fig. 5d, e). COL1A1 expression at 48 h in the PST group was also significantly reduced compared to the untreated cells of 33 ± 19 % ($p = 0.04$) (Fig. 5e).

Analysis of the VEGF, a key marker involved in vasculogenesis and angiogenesis, by Real-Time PCR revealed no significant differences between the PST and control group in VEGF mRNA expression as compared to untreated cells (day 0) at each time point in both groups ($p > 0.05$) (Fig. 5f).

Discussion

The possibility of activating tendon healing through electromagnetic stimulation has become increasingly popular. However, little is known about the origin of the beneficial effects that have been observed, although the possibility of stem cells involvement in the process has been often speculated. Thus the main goal of this work was to evaluate the effects of a

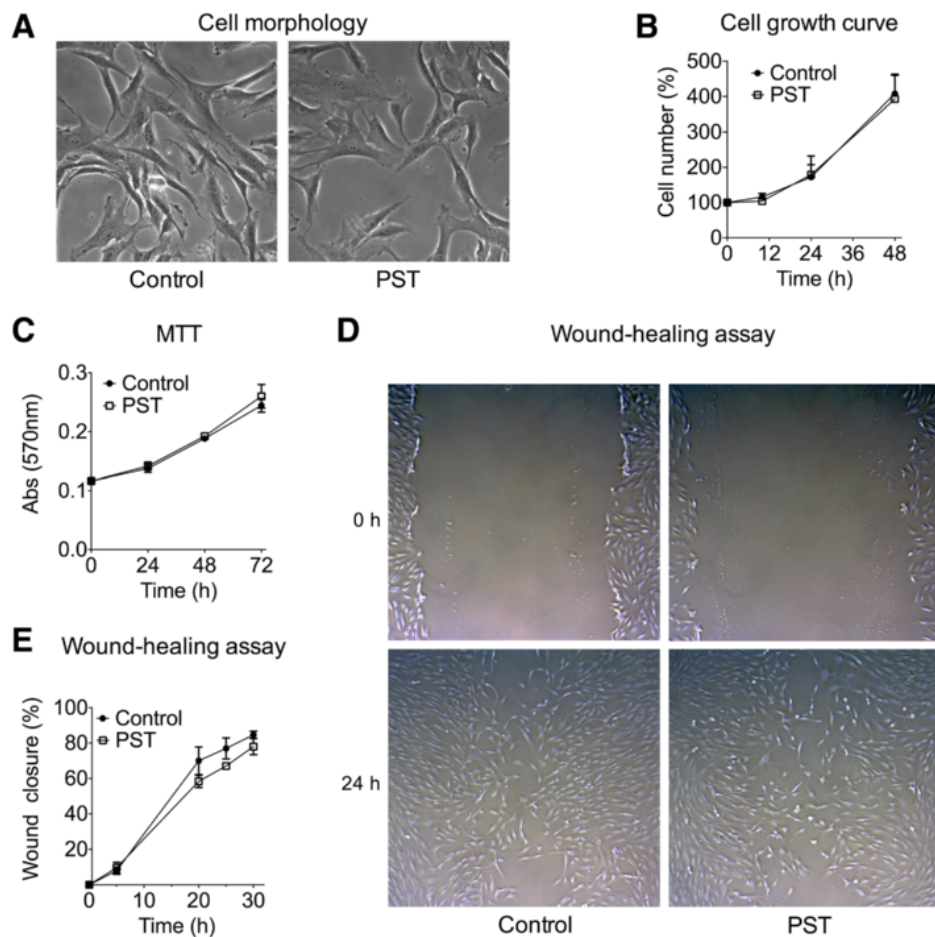


Fig. 3 Effects of PST® treatment on hTSCs morphology, proliferation, viability, and migration. **a** Phase-contrast microphotographs (original magnification x10, at 48 h after treatment), **b** cell growth curves of hTSCs before a 1-h PST® treatment and at 10, 24, and 48 h post treatment. **c** MTT assay of hTSCs before a 1-h PST® treatment and at 24, 48, and 72 h post treatment. Control cells were cultured outside the incubator for 1 h during PST® treatment. **d, e** Effect of PST® treatment on hTSCs migration. **d** Representative time-lapse migration images of PST and control cells. Images were acquired at 0 and 24 h in in vitro wound-healing assay. Original magnification x5. **e** The migration rate was measured by quantifying the total area of the wounded region lacking cells. The average percentages of recovered area obtained from three different experiments at 5, 10, 20, 24, and 30 h post treatment, as compared to control cells. All experiments were performed in triplicates. **Error bars** show the mean ± SD of three different experiments. Only **p-values** <0.05 are indicated, as compared to control cells

pulsed electromagnetic field, PST®, on human tendon stem cells isolated from patients undergoing surgeries. A critical issue that had to be taken into consideration in designing this in vitro study was to maximize the cell exposure to the field. In fact, several authors have shown that the electrical field depends on the cross-sectional area of the culture media seen by the magnetic field [27–29]. Therefore, in this study, the PST® treatment was performed by placing the cell culture plate at the center of the PST® device, parallel to the ground, in order to have the magnetic field vector perpendicular to the plate surface.

The first finding of this study was that the PST® treatment (0.5–1.5 mT, 10–30 Hz, for 1 h, as in the standard treatment administered to patients) had no cytotoxic effects on hTSCs in terms of cell viability, proliferation,

and migration. These results are in agreement with other studies made with different pulsed electromagnetic fields on various cell types, including human tenocytes [30–33]. However, these studies reported contrasting results on cell proliferation, which was sometimes significantly increased upon stimulation [31–35]. Nonetheless, the time of treatment, often exceeding twelve consecutive hours, could be considered rather incompatible with a realistic therapeutic application. Also, changes in cell morphology, size and orientation were occasionally reported upon pulsed magnetic field stimulation [28]. Still, in our experimental settings, hTSCs morphology was not altered by PST® exposure. Then, we tested the effects of PST® treatment on stem cell and tendon-related marker expression. Along this line, it has been observed that a single prolonged

Apoptosis/necrosis analyses with Annexin V-FITC and Propidium iodide staining

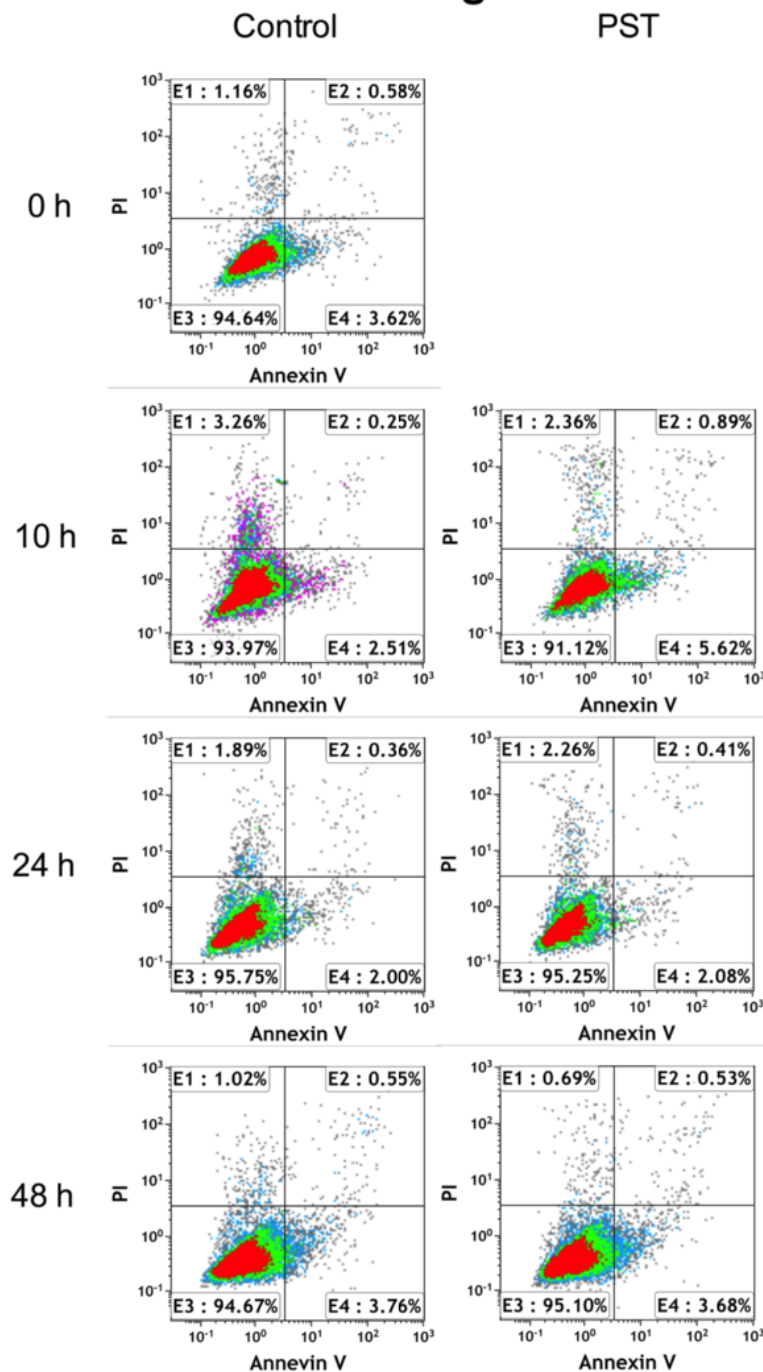


Fig. 4 Effects of PST® treatment on apoptosis. Flow cytometric analysis of hTSCs survival rate before a 1-h PST® treatment and then 10, 24 and 48 h post treatment (right panel), as compared to control cells (left panel), through double staining with Annexin V-FITC and PI. Early apoptotic cells (Annexin V-positive/PI-negative) are localized in the lower right region, late apoptotic and necrotic cells (Annexin V-positive/PI-positive) in the upper regions, and vital cells (double negative) in the lower left region

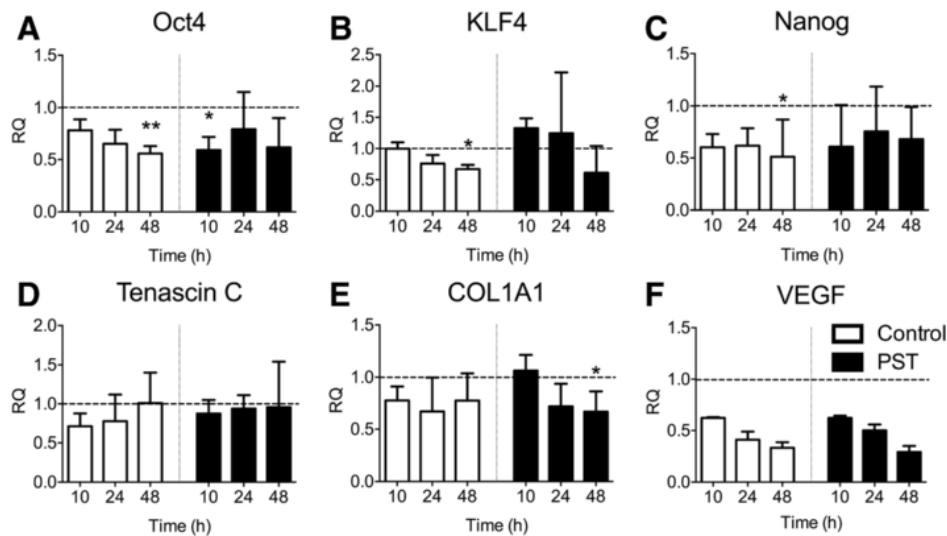


Fig. 5 Effects of PST[®] treatment on stem cell marker (Oct4, KLF4, and Nanog) (a–c), tendon marker (Tenascin C and COL1A1) (d, e), and VEGF (f) expression by Real-Time PCR before a 1-h PST[®] treatment and at 10, 24, and 48 h post treatment, as compared to untreated controls. Values are expressed as fold-changes relative to untreated cells at time zero (dotted line set at 1). Data are expressed as means ± SD of three different experiments. p-values were calculated using T student test or Wilcoxon test according to data distribution. Only p-values <0.05 are indicated: *, p < 0.05; **, p < 0.01

exposure to PEMFs (8 to 12 h) can positively influence the expression of tendon-specific markers (COL1A1 and SCX) in a dose-dependent manner [31, 32]. However, a 1-h PST[®] treatment on hTSCs had no significant effects on the expression of Tenascin C and COL1A1. Similar results were observed by Du et al. on human umbilical cord MSCs, where a 2-h treatment per day for 15 days did not cause any significant changes in the expression of several tendon and osteogenic markers, including COL1A1 [36]. On the other hand, PST[®] treatment seems to have a positive effect on stem cell marker expression. In fact, while the expression of stem cells markers (OCT4, Nanog, and Klf4) significantly decreased in control hTSCs, as expected during a 48 h in vitro cell culture, PST[®] treatment seems to maintain hTSCs in a more undifferentiated state, as no significant decrease in the same stem cell markers could be observed for 48 h after the 1-h treatment. These findings are in agreement with previous literature reports on other stem cells, which showed that the gene expression profiles regulating osteogenic and neuronal differentiation were altered after subjecting cells to an electromagnetic field treatment [37, 38]. Regarding the variability of stem cell marker expression in the PST group, this could be due to the fact that stem cells isolated from different patients were used in the study. In fact, high inter-individual differences in stem cell response could be due to a variety of parameters, including the patient's age, co-morbidities, or the different degree of tendon injury. This could be also the cause of the discrepancies observed in the literature, where

electromagnetic fields of different types were used on a variety of cells (differentiated or stem cells), which could indeed respond very differently to the treatment. Moreover, PST[®], in contrast to other PEMF, uses a unique rectangular pulsed as the stimuli, which varies in amplitude and frequency [39]. Therefore, it is difficult to compare the results of the current study with previous reports, as many parameters, including the time of treatment, are different.

Finally, we tested whether the PST[®] treatment would increase VEGF expression, as the beneficial effects could be due to reduced inflammation and increased neovascularization. However, we could not detect any significant changes in VEGF expression upon PST[®] treatment on hTSCs, which doesn't rule out that the treatment could have effects on other cell types, as endothelial cells, which are more likely to be involved in the process.

Conclusions

In conclusion, we found that a 1-h exposure to pulsed electromagnetic field PST[®] did not cause any significant changes in human tendon stem cell proliferation and morphology. Nonetheless, the treatment seems to have beneficial effects on stem cell marker expression, as treated cells maintain a higher expression of these markers during the in vitro culturing, supporting the preservation of a more undifferentiated status. Clearly, the next step would be to test whether these effects could be observed in vivo in an animal model, which is currently undergoing in our laboratories.

Additional file

Additional file 1: Figure S1. Phenotypic characterization of human dermal fibroblasts (hDFs) and human tendon stem cells (hTSCs) by flow cytometry. (PDF 339 kb)

Abbreviations

COL1A1, collagen type I alpha-1; FBS, fetal bovine serum; hDFs, human dermal fibroblasts; hTSCs, human tendon stem cells; KLF4, kruppel-like factor 4; Oct4, octamer-binding transcription factor 4; PEMFs, pulsed electromagnetic fields; PST, pulsed signal therapy; S14, somatostatin-14 peptide; SCX, scleraxis; SD, standard deviation; VEGF, vascular endothelial growth factor; α -MEM, α -Minimal Essential Medium

Acknowledgments

The authors would like to thank the Department of Biomedical Sciences for Health, University of Milan, Italy for its financial support.

Funding

This work was supported by Grant 15-6-3016000-112 from the Department of Biomedical Sciences for Health, University of Milan, Italy.

Availability of data and materials

All data and materials are included and described within the manuscript.

Authors' contributions

PR, AM, PC, UAM, CPO, GB, GT, and LA designed the experiments; PR, AM, VR, and PC performed the experiments; PR, AM, VR, PC, UAM, CPO, GB, GT, and LA analyzed data; PR, AM and LA wrote the paper. All authors have read and approved the final submitted manuscript.

Competing interests

The authors declare that they have no competing interests and no financial support nor connections with PST[®] manufacturer.

Consent for publication

Written informed consent for the publication of image (Fig. 2a) has been obtained from the patient.

Ethics approval and consent to participate

Research has been performed in accordance with the Declaration of Helsinki. The study protocol has been approved by the Hospital Ethical Committee (authorization number 2642; Sept 19, 2011; ASL Milano 2, Melegnano, Milan), and an informed consent to participate in the study has been obtained from all patients.

Author details

¹IRCCS Policlinico San Donato, San Donato Milanese, Milan, Italy. ²Department of Biomedical Sciences for Health, University of Milan, piazza Malan 2, 20097, San Donato Milanese, Milan, Italy. ³IRCCS Istituto Ortopedico Galeazzi, Milan, Italy. ⁴Università Vita-Salute San Raffaele, Milan, Italy.

Received: 8 October 2015 Accepted: 5 August 2016

Published online: 18 August 2016

References

- Meislin RJ, Sperling JW, Stitik TP. Persistent shoulder pain: epidemiology, pathophysiology, and diagnosis. *Am J Orthop (Belle Mead NJ)*. 2005;34(12 Suppl):5–9.
- Factor D, Dale B. Current concepts of rotator cuff tendinopathy. *Int J Sports Phys Ther*. 2014;9(2):274–88.
- Mantone JK, Burkhead Jr WZ, Noonan Jr J. Nonoperative treatment of rotator cuff tears. *Orthop Clin North Am*. 2000;31(2):295–311.
- Randelli P, Randelli F, Ragone V, Menon A, D'Ambrosi R, Cucchi D, et al. Regenerative medicine in rotator cuff injuries. *BioMed Res Int*. 2014;2014:129515. doi:10.1155/2014/129515.
- Beitzel K, Solovyova O, Cote MP, Apostolakis J, Russell RP, McCarthy MB, et al. The future role of mesenchymal stem cells in the management of shoulder disorders. *Arthroscopy*. 2013;29(10):1702–11. doi:10.1016/j.arthro.2013.06.014.
- Beitzel K, McCarthy MB, Cote MP, Russell RP, Apostolakis J, Ramos DM, et al. Properties of biologic scaffolds and their response to mesenchymal stem cells. *Arthroscopy*. 2014;30(3):289–98. doi:10.1016/j.arthro.2013.11.020.
- Chahal J, Van Thiel GS, Mall N, Heard W, Bach BR, Cole BJ, et al. The role of platelet-rich plasma in arthroscopic rotator cuff repair: a systematic review with quantitative synthesis. *Arthroscopy*. 2012;28(11):1718–27. doi:10.1016/j.arthro.2012.03.007.
- Weeks 3rd KD, Dines JS, Rodeo SA, Bedi A. The basic science behind biologic augmentation of tendon-bone healing: a scientific review. *Instr Course Lect*. 2014;63:443–50.
- Mora MV, Iban MA, Heredia JD, Laakso RB, Cuellar R, Arranz MG. Stem cell therapy in the management of shoulder rotator cuff disorders. *World J Stem Cells*. 2015;7(4):691–9. doi:10.4252/wjsc.v7.i4.691.
- Murrell WD, Anz AW, Badsha H, Bennett WF, Boykin RE, Caplan AI. Regenerative treatments to enhance orthopedic surgical outcome. *PM R*. 2015;7(4 Suppl):S41–52. doi:10.1016/j.pmrj.2015.01.015.
- Randelli P, Conforti E, Piccoli M, Ragone V, Creo P, Cirillo F, 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*. 2013;41(7):1653–64. doi:10.1177/0363546512473572.
- Utsunomiya H, Uchida S, Sekiya I, Sakai A, Moridera K, Nakamura T. Isolation and characterization of human mesenchymal stem cells derived from shoulder tissues involved in rotator cuff tears. *The American journal of sports medicine*. 2013;41(3):657–68. doi:10.1177/0363546512473269.
- Tsai CC, Huang TF, Ma HL, Chiang ER, Hung SC. Isolation of mesenchymal stem cells from shoulder rotator cuff: a potential source for muscle and tendon repair. *Cell Transplant*. 2013;22(3):413–22. doi:10.3727/096368912x656090.
- Song N, Armstrong AD, Li F, Ouyang H, Niyibizi C. Multipotent mesenchymal stem cells from human subacromial bursa: potential for cell based tendon tissue engineering. *Tissue Eng A*. 2014;20(1-2):239–49. doi:10.1089/ten.TEA.2013.0197.
- Steinert AF, Kunz M, Prager P, Gobel S, Klein-Hitpass L, Ebert R, et al. Characterization of bursa subacromialis-derived mesenchymal stem cells. *Stem Cell Res Ther*. 2015;6:114. doi:10.1186/s13287-015-0104-3.
- Nesselmann C, Ma N, Bieback K, Wagner W, Ho A, Kontinnen YT, et al. Mesenchymal stem cells and cardiac repair. *J Cell Mol Med*. 2008;12(5b):1795–810. doi:10.1111/j.1582-4934.2008.00457.x.
- Bedi A, Maak T, Walsh C, Rodeo SA, Grande D, Dines DM, et al. Cytokines in rotator cuff degeneration and repair. *J Shoulder Elbow Surg*. 2012;21(2):218–27. doi:10.1016/j.jse.2011.09.020.
- Goncalves AI, Rodrigues MT, Lee SJ, Atala A, Yoo JJ, Reis RL, et al. Understanding the role of growth factors in modulating stem cell tenogenesis. *PLoS One*. 2013;8(12):e83734. doi:10.1371/journal.pone.0083734.
- Molloy T, Wang Y, Murrell G. The roles of growth factors in tendon and ligament healing. *Sports Med*. 2003;33(5):381–94.
- Trock DH, Bollet AJ, Markoll R. The effect of pulsed electromagnetic fields in the treatment of osteoarthritis of the knee and cervical spine. Report of randomized, double blind, placebo controlled trials. *J Rheumatol*. 1994;21(10):1903–11.
- Ryang We S, Koog YH, Jeong KI, Wi H. Effects of pulsed electromagnetic field on knee osteoarthritis: a systematic review. *Rheumatology (Oxford)*. 2013;52(5):815–24. doi:10.1093/rheumatology/kes063.
- Bassett CA. A conversation with C. Andrew L. Bassett, MD. Pulsed electromagnetic fields, a noninvasive therapeutic modality for fracture nonunion. *Orthop Rev*. 1986;15(12):781–95.
- Heckman JD, Ingram AJ, Loyd RD, Luck Jr JV, Mayer PW. Nonunion treatment with pulsed electromagnetic fields. *Clin Orthop Relat Res*. 1981;161:58–66.
- Bassett CA. The development and application of pulsed electromagnetic fields (PEMFs) for ununited fractures and arthrodeses. *The Orthopedic clinics of North America*. 1984;15(1):61–87.
- Glassman LS, McGrath MH, Bassett CA. Effect of external pulsing electromagnetic fields on the healing of soft tissue. *Ann Plast Surg*. 1986;16(4):287–95.
- Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc*. 2007;2(2):329–33. doi:10.1038/nprot.2007.30.
- Razavi S, Salimi M, Shahbazi-Gahruei D, Karbasi S, Kermani S. Extremely low-frequency electromagnetic field influences the survival and proliferation

- effect of human adipose derived stem cells. *Advanced Biomed Res.* 2014;3:25. doi:10.4103/2277-9175.124668.
28. Simko M. Induction of cell activation processes by low frequency electromagnetic fields. *TheScientificWorldJOURNAL.* 2004;4 Suppl 2:4–22. doi:10.1100/tsw.2004.174.
 29. Harland JD, Liburdy RP. Environmental magnetic fields inhibit the antiproliferative action of tamoxifen and melatonin in a human breast cancer cell line. *Bioelectromagnetics.* 1997;18(8):555–62.
 30. Denaro V, Ruzzini L, Barnaba SA, Longo UG, Campi S, Maffulli N, et al. Effect of pulsed electromagnetic fields on human tenocyte cultures from supraspinatus and quadriceps tendons. *Am J Phys Med Rehabil.* 2011;90(2):119–27. doi:10.1097/PHM.0b013e3181fc7bc7.
 31. de Girolamo L, Stanco D, Galliera E, Viganò M, Colombini A, Setti S, et al. Low frequency pulsed electromagnetic field affects proliferation, tissue-specific gene expression, and cytokines release of human tendon cells. *Cell Biochem Biophys.* 2013;66(3):697–708. doi:10.1007/s12013-013-9514-y.
 32. de Girolamo L, Viganò M, Galliera E, Stanco D, Setti S, Marazzi MG, et al. In vitro functional response of human tendon cells to different dosages of low-frequency pulsed electromagnetic field. *Knee Surg Sports Traumatol Arthrosc.* 2014. doi:10.1007/s00167-014-3143-x.
 33. Seeliger C, Falldorf K, Sachtleben J, van Griensven M. Low-frequency pulsed electromagnetic fields significantly improve time of closure and proliferation of human tendon fibroblasts. *Eur J Med Res.* 2014;19:37. doi:10.1186/2047-783x-19-37.
 34. Chang WH, Chen LT, Sun JS, Lin FH. Effect of pulse-burst electromagnetic field stimulation on osteoblast cell activities. *Bioelectromagnetics.* 2004;25(6):457–65. doi:10.1002/bem.20016.
 35. Fu YC, Lin CC, Chang JK, Chen CH, Tai IC, Wang GJ, et al. A novel single pulsed electromagnetic field stimulates osteogenesis of bone marrow mesenchymal stem cells and bone repair. *PLoS One.* 2014;9(3):e91581. doi:10.1371/journal.pone.0091581.
 36. Du L, Fan H, Miao H, Zhao G, Hou Y. Extremely low frequency magnetic fields inhibit adipogenesis of human mesenchymal stem cells. *Bioelectromagnetics.* 2014;35(7):519–30. doi:10.1002/bem.21873.
 37. Zhao D, Wu H, Li F, Li R, Tao C. Electromagnetic field change the expression of osteogenesis genes in murine bone marrow mesenchymal stem cells. *J Huazhong Univ Sci Technolog Med Sci.* 2008;28(2):152–5. doi:10.1007/s11596-008-0209-3.
 38. Ma Q, Deng P, Zhu G, Liu C, Zhang L, Zhou Z, et al. Extremely low-frequency electromagnetic fields affect transcript levels of neuronal differentiation-related genes in embryonic neural stem cells. *PLoS One.* 2014;9(3):e90041. doi:10.1371/journal.pone.0090041.
 39. Fioravanti A, Nerucci F, Collodel G, Markoll R, Marcolongo R. Biochemical and morphological study of human articular chondrocytes cultivated in the presence of pulsed signal therapy. *Ann Rheum Dis.* 2002;61(11):1032–3.

Submit your next manuscript to BioMed Central
and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at
www.biomedcentral.com/submit

