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Extracorporeal shock waves trigger tenogenic differentiation of human adipose-derived stem cells

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(Article begins on next page)

1 **Extracorporeal shock waves trigger tenogenic differentiation of human adipose-derived stem**
2 **cells**

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21 **Running Head:** Shockwaves and tendon differentiation

22 **Abstract**

23 **Purposes:** Incomplete tendon healing impairs the outcome of tendon ruptures and tendinopathies.
24 Human Adipose-derived Stem Cells (hASCs) are promising for tissue engineering applications.
25 Extracorporeal Shock Waves (ESW) are a leading choice for the treatment of several
26 tendinopathies. In this study we investigated the effects of ESW treatment and tenogenic medium
27 on the differentiation of hASCs into tenoblast-like cells. **Materials and Methods:** hASCs were
28 treated with ESW generated by a piezoelectric device and tenogenic medium. Quantitative real-time
29 PCR was used to check mRNA expression levels of tenogenic transcription factors, extracellular
30 matrix proteins, and integrins. Western blot and immunofluorescence were used to detect collagen 1
31 and fibronectin. Collagen fibers were evaluated by Masson staining. Calcium deposition was
32 assessed by Alizarin Red staining. **Results:** The combined treatment improved the expression of the
33 tendon transcription factors scleraxis and eyes absent 2, and of the extracellular matrix proteins
34 fibronectin, collagen I, and tenomodulin. Cells acquired elongated and spindle shaped fibroblastic
35 morphology; Masson staining revealed the appearance of collagen fibers. Finally, the combined
36 treatment induced the expression of alpha 2, alpha 6 and beta 1 integrin subunits, suggesting a
37 possible role in mediating ESW effects. **Conclusions:** ESW in combination with tenogenic medium
38 improved the differentiation of hASCs towards tenoblast-like cells, providing the basis for ESW
39 and hASCs to be used in tendon tissue engineering.

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44 **Keywords:** tendons; adipose-derived stem cells; extracorporeal shock waves; tenoblast
45 differentiation; tissue engineering

46

47 **Introduction**

48 Tendon injuries and diseases are very common. Unfortunately, due to low cellularity and
49 vascularization, tendon healing is often incomplete (1), and, to date, surgical treatments remain
50 unsatisfactory (2). To overcome these limitations, cell therapy-based tissue engineering has now
51 emerged as a potential alternative approach in the treatment of tendon diseases. Adult mesenchymal
52 stem cells (MSCs) are multipotent lineages capable of differentiating into specialized tissues such
53 as bone, cartilage, tendon, and ligaments; and for this reason, they have been proposed for tissue
54 engineering to enhance tendon healing (3). MSCs can be extracted from bone marrow; but other
55 sources have also been identified. Among these, adipose tissue represents an abundant source of
56 MSCs with potential regenerative properties (4). Human Adipose-derived Stem Cells (hASCs) may
57 therefore be considered as a powerful tool for treating tendinopathies (5), and very recently it has
58 been demonstrated that a tenomodulin positive subpopulation of ASC is a promising source of
59 tendon progenitor cells (6). Moreover, tenocytes respond to mechanical loading by modulating the
60 extracellular environment through the formation and degradation of matrix proteins via a process
61 termed mechanotransduction (7). Extracorporeal Shock Waves (ESW) are transient short-term
62 acoustic pulses with high peak pressure and a very short rise time to peak pressure of the order of
63 magnitude of nanoseconds and short pulse duration. ESW technology has been used in clinical
64 practice since the 1980s, when it was first employed to break up kidney stones (8). More recently,
65 shockwave therapy has emerged as a leading choice for the treatment of several orthopedic diseases
66 (9) and different disorders such as proximal plantar fasciopathy, lateral elbow tendinopathy, calcific
67 tendinopathy of the shoulder and patellar tendinopathy are tendon diseases where ESW treatment is
68 being used successfully (10).

69 Based on these observations, the aim of the present study was to investigate the effects of ESW
70 treatment on the differentiation of hASCs into tenoblast-like cells.

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Materials and methods

Isolation, characterization and tenogenic differentiation of hASCs

hASCs were isolated from the waste subcutaneous-adipose tissue of 5 healthy female donors [range 30–50 years, body mass index (BMI) <30 without any pathological obesity], undergoing elective liposuction, after written consent and Institutional Review Board authorization. Primary cultures were established following the procedure of Zuc et al 2001 (4). Briefly, after digestion of raw lipoaspirates (50–100ml) with 0.075% type I collagenase (Sigma–Aldrich, Saint Louis, MO, USA) for 30 min, hASCs were separated by centrifugation (2100 x g for 10 min), filtered and plated in basal medium, consisting of Dulbecco’s modified Eagle’s Medium (DMEM/F12) (Lonza; Switzerland) plus 10% fetal bovine serum (FBS) and with the addition of 50 U/mL penicillin and 50 µg/mL streptomycin (all from GIBCO, Invitrogen Corp., Grand Island, NY, USA).

hASCs at passage 4 were investigated by flow cytometry analysis for the expression of surface antigens. In details, cells were detached by 0.5% trypsin/0.2% EDTA (Sigma–Aldrich, Saint Louis, MO, USA); thereafter, the cells were washed with cold PBS (Phosphate Buffer Saline) (Sigma–Aldrich, Saint Louis, MO, USA) and then resuspended in cold PBS plus 0.1% BSA (Bovine Serum Albumin) (Sigma–Aldrich, Saint Louis, MO, USA). The cells were incubated for 30 minutes in ice, using the following phycoerythrin (PE) or fluorescein isothiocyanate (FITC)-conjugated antibodies: mouse anti-human CD13, CD14 and CD34 (BD Biosciences, Franklin Lakes, NJ, USA); mouse anti-human CD45, CD90, CD105 and CD44 (Immunotech, Marseille, France). At the end of the incubation, two washings with cold PBS plus 0.1% of BSA were performed. Ten thousand events were acquired for each surface marker on flow cytometer (EPICS XL, Coulter Corp., Hialeah, FL, USA).

To induce tendon cell differentiation, hASCs were cultured in tenogenic medium consisting of basal medium supplemented with 50ng/ml human Insulin-like Growth Factor-I (IGF-1) and 10ng/ml human Transforming Growth Factor beta 1 (TGF-β1) (11). hASCs cultured in basal medium were used as controls.

98 ***Primary cultures of human tenocytes: isolation and expansion***

99 Discarded fragments of semitendinosus tendons were collected from 2 healthy young donors (mean
100 age = 23 ± 7 y) who underwent arthroscopic anterior cruciate ligament reconstruction with
101 autologous hamstrings at our hospital. All patients gave written consent to the procedure and all
102 procedures were carried out with institutional review board approval. Tenocytes were isolated
103 following the procedure by Kraus et al. (12) with minor modifications. Tendon tissues were minced
104 and enzymatically digested with 0.25% trypsin in DMEM/F12 overnight at 4°C. Subsequently, they
105 were treated with 0.3% type I collagenase in DMEM/F12 with continuous agitation for 1h at 37°C.
106 The isolated nucleated cells were then cultured in basal medium consisting of DMEM/F12 plus
107 10% FBS, with 50 U/mL penicillin and 50 µg/mL streptomycin. Cell cultures were maintained at
108 37°C in a humidified atmosphere with 5% CO₂; the culture medium was changed every 3 days.
109 When cells reached 80–90% confluence, they were detached by incubation with 0.5% trypsin/0.2%
110 EDTA (Sigma–Aldrich, Saint Louis, MO, USA) and then expanded at a density of
111 5×10^3 cells/cm². RNA and proteins were extracted from tenocytes at passages 2 to 4, to be used as
112 positive controls in gene expression, western blots and immunofluorescence experiments.

113 ***ESW treatment***

114 The shockwave generator utilized for hASC treatments is a piezoelectric device (Piezoston 100;
115 Richard Wolf, Knittlingen, Germany), especially designed for clinical use in orthopaedics and
116 traumatology. The experimental set-up has been reported previously (13). Briefly, 1 ml aliquots of
117 hASC cell suspension adjusted to 1×10^6 cell/ml were placed in 20mm polypropylene tubes (Nunc,
118 Wiesbaden, Germany), which were then completely filled with culture medium. Subsequently, cells
119 were gently pelleted by centrifugation at 250 x g in order to minimize motion during shockwave
120 treatment. Each cell-containing tube was placed in vertical alignment with the focal area and was
121 adjusted so that the central point of the focal area corresponded to the centre of the tube bottom.
122 The shockwave unit was kept in contact with the cell containing tube by means of a water-filled
123 cushion. Common ultrasound gel was used as a contact medium between cushion and tube. hASC

124 cells were treated as follows: 1) control cells maintained in DMEM/F12 plus FBS 10% (Basal); 2)
125 ESW-treated cells (Energy Flux Density, EFD=0.32mJ/mm²; peak positive pressure 90 MPa)
126 receiving a number of 1000 shots (frequency = 4 shocks/s) and maintained in DMEM/F12 plus FBS
127 10% (ESW); 3) cells maintained in tenogenic medium (TEN0); 4) ESW-treated cells
128 (EFD=0.32mJ/mm²; 1000 shots) maintained in tenogenic medium (TEN0 ESW).

129 ***Cell viability***

130 After treatments as above, cells were seeded at 3×10³ cells/well in 96-well plates (Corning, New
131 York, NY, USA). At 1, 3, 5, 7 and 9 days, cell viability was assessed using the Cell Proliferation
132 Reagent WST-1 (Roche Applied Science, Penzberg, Germany), following the manufacturer's
133 instructions. This is a colorimetric assay for the quantification of cell viability and proliferation,
134 based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases of viable
135 cells. Briefly, 10 µl of WST-1 were added to each well. After 1-h incubation, absorbance at 450 nm
136 was measured using a plate reader (Model 680 Microplate Reader; Bio-Rad, Laboratories S.r.l.,
137 Milan, Italy). Four replicate wells were used to determine each data point.

138 ***Cell Morphology***

139 After 72 hours of treatment, cell morphology was observed under Diavert inverted light microscope
140 (Leitz, Wetzlar, Germany); photos were taken at x200 magnifications by Leica DC100 digital
141 camera system (Microsystems, Wetzlar, Germany) using Leica Qwin as software for image
142 processing.

143 ***Gene expression***

144 At defined times after treatment, total RNA was extracted from hASCs and primary tenocytes using
145 TRIzol Reagent (Invitrogen Ltd, Paisley, UK). DNase I was added to remove remaining genomic
146 DNA. 1 µg sample of total RNA was reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad
147 Laboratories S.r.l., Milan, Italy) following manufacturer's protocol. Primers (Table S1) were
148 designed using Beacon Designer 5.0 software according to parameters outlined in the Bio-Rad
149 iCycler Manual. The specificity of primers was confirmed by BLAST analysis. Real-time

150 polymerase chain reaction was performed using a BioRad iQ iCycler Detection System (Bio-Rad
151 Laboratories S.r.l., Milan, Italy) with SYBR green fluorophore. Reactions were performed in a total
152 volume of 25 µl containing 12.5 µl IQ SYBR Green Supermix (Bio-Rad Laboratories S.r.l., Milan,
153 Italy), 1 µl of each primer at 10 µM concentration, and 5 µl of the previously reverse-transcribed
154 cDNA template. The protocol used is as follows: denaturation (95°C for 5 min), amplification
155 repeated 40 times (95°C for 15 s, 60°C for 30 s). A melting curve analysis was performed following
156 every run to ensure a single amplified product for every reaction. All reactions were carried out at
157 least in triplicate for each sample. Results were normalized using the geometric mean for three
158 different housekeeping genes (β -actin and ribosomal protein L13A and RPLPO) and expressed as
159 relative expression fold vs untreated controls (Basal).

160 ***Immunofluorescence microscopy***

161 After different treatments, cells (2×10^3) were seeded in 96-well plates (Corning, New York, NY,
162 USA). After 1 and 4 weeks, cells were fixed in acetone/methanol (1:1) at 4°C for 20 minutes,
163 permeabilized with PBS containing 0.5% Triton X-100, 0.05% NaN_3 and incubated with the
164 following antibodies: polyclonal sheep anti-type I collagen (COL1, 2 µg/ml, R&D Systems,
165 Minneapolis, MN, USA) and polyclonal rabbit anti-fibronectin 1 (FN1, 1:500, Sigma–Aldrich,
166 Saint Louis, MO, USA), at 4°C overnight. Detection with secondary antibodies was as follows: for
167 COL1, anti-sheep conjugated with Alexa Fluor 594 (1:500, Invitrogen, San Diego, CA, USA) and
168 for FN1, anti-rabbit conjugated with cy3 (1:1000, GE Healthcare Europe, Milan, Italy). Nuclear
169 staining was obtained by Hoechst 33258 [500 ng/ml in dimethylsulphoxide (DMSO)] in PBS. Cells
170 were washed twice with distilled water. Cells were observed by inverted microscope Leica DMI
171 4000 B (Leica Microsystems, Wetzlar, Germany) and photos of single channels and overlays at
172 x200 magnifications, were taken by Leica DCF340 FX digital camera system (Leica Microsystems,
173 Wetzlar, Germany). Fluorescence was quantified using ImageJ (version 1.48, NIH, Bethesda,
174 Maryland) imaging software.

175

176 ***Immunoblotting***

177 At different times after treatments, cell culture media were recovered and both hASC cells and
178 tenocytes were scraped from the flask in the presence of 1 mL lysis buffer (50 mM Tris-HCl pH
179 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% sodium deoxycholate, 1% Nonidet P-40,
180 0.1% SDS, 10 mg/mL PMSF, 30 U/mL aprotinin, and 100 mM sodium orthovanadate). Cell lysates
181 were incubated in ice for 30–60 minutes. At completion, tubes were centrifuged at 4 °C for 20
182 minutes at 15,000 x g. Media and cell lysates were separated on SDS-PAGE, transferred to PVDF
183 and probed with the following antibodies: polyclonal anti-COL1 (1:800, R&D Systems,
184 Minneapolis, MN), for culture media; polyclonal anti-FN1 (1:10000, Sigma-Aldrich, Saint Louis,
185 MO, USA), for cell lysates. The membrane for FN1 was then stripped and reprobed with a mouse
186 monoclonal anti-Tubulin antibody (1:10,000, Sigma-Aldrich, Saint Louis, MO, USA) to check
187 protein loading. Red ponceau was used to confirm equal loading for type I collagen membrane.
188 Proteins were detected with Pierce Super Signal chemiluminescent substrate. Bands were
189 photographed and analyzed using Kodak 1D Image Analysis software.

190 ***Masson Trichrome staining***

191 After different treatments, cells (25×10^4) were seeded on sterilized coverslips (22 x 22 mm) placed
192 in 6 cm Petri dishes and kept in a humidified incubator with 5% CO₂ at 37°C.

193 After 4 weeks, the cells grown on coverslips were fixed in 4% neutral-buffered formalin for 10 min
194 and dehydrated through a series of alcohols up to absolute alcohol. The slides were first stained in
195 modified Harris's haematoxylin for 5 min; then, rinsed in lithium carbonate solution until the
196 sections changed colour to blue. Subsequently, the slides were washed and stained in scarlet-acid
197 fuchsine solution for 7-10 min. The slides were rinsed in a 1% acetic acid solution and immersed in
198 phosphomolybdic acid for 4-5 min. The sections were transferred to Light Green and stained for 7-
199 10 min. After that, they were placed in 1% acetic acid solution. The slides were then dehydrated
200 through 95% ethyl alcohol, absolute ethyl alcohol and cleared in xylene before being mounted.
201 Slides were observed by microscope Leica DM2000 (Leica Microsystems, Wetzlar, Germany) and

202 photos were taken by Leica ICC50 HD digital camera system (Leica Microsystems, Wetzlar,
203 Germany) at x200 final magnification. ImageJ (version 1.48, NIH, Bethesda, Maryland) imaging
204 software was used to analyze Masson trichrome digital images and quantify the amount of collagen.

205 *Alizarin Red staining*

206 Calcium deposition was evaluated at 4 weeks by staining with 40 mM Alizarin Red-S (ARS, pH
207 4.1). After fixation in ethanol and washing with Tris-buffered saline, cells were stained with ARS
208 for 15 min, washed with Tris-buffered saline and observed under light microscopy. Cells were
209 observed under inverted microscope Leica DMI 3000 B (Leica Microsystems, Wetzlar, Germany)
210 and photographed by Leica DCF310 FX digital camera system (Leica Microsystems, Wetzlar,
211 Germany) at x400 final magnification.

212 *Statistical analysis*

213 Data are expressed throughout the text as means \pm SD. The means were calculated from n=5 for
214 hASCs, and n=2 for primary tenocytes, respectively. 3 technical replicates were used for each
215 donor. Comparison between groups was performed with analysis of variance (two-way ANOVA)
216 and the threshold of significance was calculated with the Bonferroni test. Statistical significance
217 was set at $p < 0.05$.

218

219 **Results**

220 *hASC characterization*

221 More than 95% of hASCs expressed CD13, CD44 and CD105, whereas a very low expression of
222 CD14 (1%) and no expression of CD34 and CD45 was detected (Table S2). Moreover, a positivity
223 of at least 95% for the same markers was also maintained in subsequent steps (data not shown). As
224 we reported elsewhere (14,15), hASCs were able to differentiate into osteogenic and adipogenic
225 lineages under specific culture conditions, confirming their differentiating potential.

226 *ESW effect on cell viability and morphology*

227 The energy level and number of shots used for ESW treatment (EFD =0.32 mJ/mm²; peak positive
228 pressure 90 MPa, 1000 shots) allowed a viability > 80% soon after treatment (Table S2) and did not
229 modify cell growth up to 9 days (Figure 1, panel A).

230 Light microscopy was used to evaluate the differences in cell morphology in response to treatments
231 (Figure 1, panel B). After 72 hours, cells in basal conditions exhibited a polygonal phenotype; on
232 the contrary, combined treatment with tenogenic medium and ESW determined the appearance of
233 an elongated and spindle shaped fibroblastic morphology.

234 *Effects on tenogenic gene expression*

235 As shown in Figure 2 (panels A and B), after 48 hours' treatment, tenogenic medium alone
236 significantly increased the expression of the two transcription factor *scleraxis* (*SCX*) (panel A,
237 TENO vs Basal: 3.5 times, p<0.001) and *eyes absent 2* (*EYA2*) (panel B, TENO vs Basal: 2.5 times,
238 p<0.001); but, the greatest effect was obtained by using the combined treatment with tenogenic
239 medium and ESW (for *SCX*, panel A: TENO ESW vs Basal: 5.8 times, p<0.001; TENO ESW vs
240 TENO: 1.8 times, p<0.001; for *EYA2*, panel B: TENO ESW vs Basal: 3.0 times, p<0.001; TENO
241 ESW vs TENO: 1.2 times, p<0.001).

242 As both *SCX* and *EYA2* regulate the expression of the tendon extracellular matrix proteins (16,17),
243 we evaluated the expression of *fibronectin* (*FNI*), *type I collagen* (*COL1*) and *tenomodulin* (*TNMD*)
244 at 7 days after treatment. Tenogenic medium significantly increased the expression of *FNI* (Figure

245 2, panel C, TENO vs Basal: 2.7 times, $p < 0.001$), *COL1* (Figure 2, panel D, TENO vs Basal: 1.22
246 times, $p < 0.05$), and *TNMD* (Figure 2, panel E, TENO vs Basal: 2.9 times, $p < 0.01$); again the
247 combined treatment determined the best effect (for *FN1*, panel C: TENO ESW vs Basal: 4.25 times,
248 $p < 0.001$; TENO ESW vs TENO: 1.6 times, $p < 0.001$; for *COL1*, panel D: TENO ESW vs Basal:
249 1.36 times, $p < 0.01$; TENO ESW vs TENO: 1.1 times, $p < 0.05$; for *TNMD*, panel E: TENO ESW vs
250 TENO: 4.5 times, $p < 0.001$). Moreover, as far as tenomodulin expression is concerned, ESW
251 treatment alone significantly increased its expression (panel E: ESW vs Basal: 2.5 times, $p < 0.05$).
252 Notably, after the combined treatment, levels of expression of both transcription factors as well as
253 of the extracellular matrix proteins were comparable and even greater than those observed in
254 primary culture of tenocytes, used as positive controls.

255 ***Effects on extracellular matrix protein deposition***

256 The effect on the extracellular matrix proteins FN1 and COL1 was further confirmed by the
257 evaluation of protein deposition.

258 ***Effects on FN1***

259 After 7-day-treatments, tenogenic medium significantly increased FN1 production, as demonstrated
260 by both immunofluorescence (Figure 3, panel A, TENO vs Basal, $p < 0.01$) and western blotting
261 (Figure 3, panels B and C, TENO vs Basal, $p < 0.05$); but was the combination of tenogenic medium
262 and ESW that gave the best result in terms of FN1 induction (immunofluorescence, Figure 3, panel
263 A, TENO ESW vs Basal, $p < 0.001$; TENO ESW vs TENO, $p < 0.05$; western blotting, Figure 3,
264 panels B and C, TENO ESW vs Basal, $p < 0.001$; TENO ESW vs TENO, $p < 0.01$).

265 ***Effects on COL1***

266 The same behavior was also observed for COL1 production. In fact, after 7 days, already tenogenic
267 medium alone significantly increased COL1 (immunofluorescence, Figure 4, panel A, TENO vs
268 Basal, $p < 0.001$; western blotting, Figure 4, panels B and C, TENO vs Basal, $p < 0.001$); again, was
269 the combination of tenogenic medium and ESW that gave the best results in terms of COL1
270 production (immunofluorescence, Figure 4, panel A, TENO ESW vs Basal, $p < 0.001$; TENO ESW

271 vs TENO, $p < 0.05$; western blotting, Figure 4, panels B and C, TENO ESW vs Basal, $p < 0.001$;
272 TENO ESW vs TENO, $p < 0.01$).

273 In addition, the increased COL1 production was maintained up to five weeks, as demonstrated by
274 immunofluorescence (Figure 5, panels A and B: TENO vs Basal $p < 0.01$; TENO ESW vs Basal
275 $p < 0.001$; TENO ESW vs TENO $p < 0.001$). Interestingly, at this time point the combined treatment
276 (Figure 5, panel A) determined the formation of parallel collagen fibers, which play an important
277 role in regulating tenoblast function and differentiation (18). Finally, the appearance of collagen
278 fibers (arrows) was confirmed by Masson trichrome staining, as shown in Figure 5, panels C and D
279 (TENO vs Basal $p < 0.01$; TENO ESW vs Basal $p < 0.001$; TENO ESW vs TENO $p < 0.001$).

280 *Effects on integrin expression*

281 To get insights into the mechanisms involved in the effect of ESW in tenogenic differentiation, the
282 expression of integrins involved in mechanotransduction was assessed. We evaluated the expression
283 of *integrins* $\alpha 1, 2, 5, 6, 10, 11$ and V and of *integrins* $\beta 1, 3, 5,$ and 8 ; the most expressed in tendons
284 and ligaments (19). Among these, after 72 hours, the expression of $\alpha 2$ (*ITG* $\alpha 2$), $\alpha 6$ (*ITG* $\alpha 6$) and $\beta 1$
285 (*ITG* $\beta 1$) integrins, was affected by our experimental treatments, as shown in Figure 6. The
286 expression of *ITG* $\alpha 2$ (Figure 6, panel A) was significantly increased by ESW alone (ESW vs Basal:
287 4.7 times, $p < 0.001$), by tenogenic medium (TENO vs Basal: 2.1 times, $p < 0.05$) and by the
288 combined treatment (TENO ESW vs Basal: 8 times, $p < 0.001$) respectively. The latter was the most
289 effective (TENO ESW vs ESW: 1.7 times, $p < 0.001$; TENO ESW vs TENO: 3.8 times, $p < 0.001$).

290 The expression of *ITG* $\alpha 6$ (Figure 6, panel B) was significantly increased by ESW alone (ESW vs
291 Basal: 4.7 times, $p < 0.001$), by tenogenic medium (TENO vs Basal: 1.9 times, $p < 0.01$) and by the
292 combined treatment (TENO ESW vs Basal: 5.9 times, $p < 0.001$) respectively. The latter was the
293 most effective (TENO ESW vs ESW: 1.3 times, $p < 0.01$; TENO ESW vs TENO: 3.1 times, $p < 0.01$).

294 The expression of *ITG* $\beta 1$ (Figure 6, panel C) was significantly increased by ESW alone (ESW vs
295 Basal: 1.7 times, $p < 0.01$) and by the combined treatment (TENO ESW vs Basal: 2.3 times,

296 p<0.001). The latter was again the most effective (TENO ESW vs ESW: 1.4 times, p<0.05; TENO
297 ESW vs TENO: 2.1 times, p<0.001).

298 ***Effects on osteogenic differentiation***

299 As we reported (14) that ESW treatment, especially when combined with specific osteogenic
300 medium, elicited the differentiation of hASCs towards osteoblast-like cells, we assessed whether the
301 combined treatment could lead to the osteogenic differentiation of hASCs. As reported in Figure 7,
302 the combined treatment with tenogenic medium and ESW had no effect on *bone morphogenetic*
303 *protein-2, BMP-2* (panel A), *runt-related transcription factor 2, Runx2* (panel B) and *alkaline*
304 *phosphatase, ALP* (panel C) expression and determined no production of calcium deposits (panel
305 D). Moreover, present data confirm our previous results (14) about the positive effect of ESW
306 treatment alone on the expression of *BMP-2, Runx2* and *ALP* and on calcium deposits.

307

308 **Discussion**

309 The present study demonstrates that ESW potentiate the effects of tenogenic medium on the
310 differentiation of hASCs towards tenoblast-like cells, as evidenced by the up-regulation of specific
311 gene markers, spindle-shaped cell morphology and extracellular matrix fiber deposition. These
312 findings support the potency of combining stem cells, mechanical forces and biochemical factors to
313 induce and sustain tendon differentiation and regeneration.

314 Since their first description by Zuk et al. (4), it is well known that ASCs are at least as efficient as
315 bone marrow-derived stem cells when used in tissue engineering; moreover, the low donor site
316 morbidity and high rate of growth during culturing, make them an ideal cell type to be used in
317 regenerative medicine. ASCs have been successfully used in *in vivo* models of tendon repair (20)
318 and some Authors reported that ASCs work better than bone marrow stem cells in *in vivo* tendon
319 injury models (21). Moreover, it has been described that ASC source can be biochemically induced
320 towards tenogenic commitment, validating its potential for tendon regeneration strategies (22).
321 Accordingly, in our *in vitro* model, tenogenic medium increased the expression of specific tendon
322 genes and determined collagen and fibronectin deposition.

323 Here we demonstrate that mechanical stimulation with ESW enhances the biochemical effects of a
324 containing-growth factor specific medium to drive hASCs towards a tenoblast-like phenotype. Our
325 results are in line with previous reports about the role of mechanical forces in tendon healing and
326 stem cell differentiation towards tenoblasts. Pulsed electromagnetic fields influence the
327 proliferation, tendon-specific marker expression and release of anti-inflammatory cytokines and
328 angiogenic factors of healthy human tendon cells (23). Cyclic tension promotes fibroblastic
329 differentiation of stem cells seeded on fibrous collagen-based scaffolds (24) and stem cell
330 differentiation toward tenocytes (25, 26). Moreover, the combination of the tendon transcription
331 factor scleraxis and mechanical stress synergistically drives differentiation of human embryonic
332 stem cells to induce teno-lineage commitment (27).

333 As far as the biological effects of ESW therapy on tendon healing are concerned, several
334 experimental studies on animal models have been performed (28-34), suggesting a role of ESW in
335 determining the activation of a complex network of molecules acting on collagen synthesis and
336 angiogenesis. On the contrary, to date only two studies from the same group described the effects of
337 ESW *in vitro* on human healthy and ruptured tendon-derived tenocytes (35, 36) and another study
338 demonstrated that soft-focused ESW treatment was able to induce positive modulation of cell
339 viability, proliferation and tendon-specific marker expression, as well as release of anti-
340 inflammatory cytokines (37). However, to date no other study investigated the possibility of ESW
341 to facilitate the differentiation of human mesenchymal stem cells towards tenocytes.

342 As far as gene expression is concerned, a panel of genes typically expressed by tendons was
343 increased by the combined treatment. We observed an enhanced expression of the transcription
344 factor scleraxis, which is the only direct molecular regulator of tenocyte differentiation identified
345 and expressed in tendon progenitors (38). Moreover, the combined treatment with tenogenic
346 medium and ESW induced the expression of the tendon extracellular matrix proteins fibronectin
347 and type I collagen. Type I collagen is the most abundant protein in healthy tendon tissue (39).
348 Notably, the combined treatment antagonized the ESW-induced BMP-2 expression, which is known
349 to stimulate osteogenesis in both hASCs (18) and in tendon-derived stem cells (40). As a
350 consequence, no increase of Runx2 and ALP and no production of calcium deposits were observed.
351 Nevertheless, being the present an *in vitro* study, only future work in animal models will
352 definitively exclude any potential effect of the combined treatment on ectopic calcification of
353 tendons.

354 Integrins mediate cell–matrix interactions and are the transducers of the mechanochemical
355 information between extracellular and intracellular compartments (41). Present work suggests an
356 involvement of integrins as possible mediators of ESW effects at cellular and molecular levels. In
357 fact, ESW increased the expression of $\alpha 2$, $\beta 1$ and $\alpha 6$ subunits. $\alpha 2/\beta 1$ integrin is a receptor for
358 laminin, collagen, and fibronectin (19). It is responsible for cell adhesion to collagens, modulation

359 of collagen and collagenase gene expression, force generation and organization of newly
360 synthesized extracellular matrix (42). The $\alpha 6$ subunit, expressed in vascular structures in the healing
361 ligaments (43) and in the healing tendons (44), is involved in vasculogenesis.

362 In conclusion, the novelty of the present work is the ability of ESW to boost the process of hASC
363 differentiation towards tenoblast-like cells induced by tenogenic medium, supporting a role for
364 ESW as a new tool to be used in combination with ASC in tendon tissue engineering.

365

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369

370 **Declaration of interests**

371 The authors report no conflicts of interest. The authors alone are responsible for the content and
372 writing of the article.

373

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377

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504

505 **Figure Legends**

506 **Figure 1. Effects on cell viability (panel A).** Basal, ESW, TENO and TENO ESW-treated h ASC
507 cells were cultured up to 9 days. At 1, 3, 5, 7 and 9 days, cell viability was determined by the
508 WST-1 method, and expressed as ratio vs day 0. **Effects on cell morphology (panel B).**
509 Morphology of Basal, ESW, TENO and TENO ESW-treated hASC cells. Magnification x200.

510 **Figure 2. Effects on tendon-specific gene expression.** mRNA expression of SCX (panel A),
511 EYA2 (panel B), FN1 (panel C), COL1 (panel D) and TNMD (panel E) was evaluated by RT-PCR.
512 Results are normalized for three different housekeeping genes (β -actin, RPLPO and L13A) and
513 expressed as relative expression fold vs Basal. Significance vs Basal: *, $p < 0.05$; ***, $p < 0.001$.
514 Significance TENO ESW vs TENO: °, $p < 0.05$; °°, $p < 0.001$.

515 **Figure 3. Effects on fibronectin.** Immunofluorescence for FN1 (panel A) in Basal, ESW, TENO
516 and TENO ESW-treated hASC cells after 7 days. Magnification x200. FN1 fluorescence
517 quantification. Significance vs Basal: ***, $p < 0.001$. Significance TENO ESW vs TENO: °, $p < 0.05$.
518 Immunoblotting for fibronectin (panel B) in Basal, ESW, TENO and TENO ESW-treated hASC
519 cells. Anti-Tubulin antibody was used to confirm equal loading. Blot is representative of five
520 independent experiments. Semiquantitative analysis of immunoblotting results (panel C).
521 Significance vs Basal: *, $p < 0.05$; ***, $p < 0.001$. Significance TENO ESW vs TENO: °°, $p < 0.01$.
522 Tenocytes were used as positive controls.

523 **Figure 4. Effects on type I collagen after 7 days.** Immunofluorescence for COL1 (panel A) in
524 Basal, ESW, TENO and TENO ESW-treated h ASC cells. Magnification x200. COL1 fluorescence
525 quantification. Significance vs Basal: ***, $p < 0.001$. Significance TENO ESW vs TENO: °, $p < 0.05$.
526 Immunoblotting for COL1 (panel B) in cultured media from Basal, ESW, TENO and TENO ESW-
527 treated hASC cells. Red Ponceau was used to confirm equal loading. Blot is representative of five
528 independent experiments. Semiquantitative analysis of immunoblotting results (panel C).
529 Significance vs Basal: ***, $p < 0.001$. Significance TENO ESW vs TENO: °°, $p < 0.01$. Tenocytes
530 were used as positive controls.

531 **Figure 5. Effects on type I collagen after 5 weeks.** Immunofluorescence for COL1 (panel A) in
532 Basal, ESW, TENO and TENO ESW-treated hASC cells. Magnification x200. COL1 fluorescence
533 quantification (panel B). Significance vs Basal: **, p<0.01 ***, p<0.001. Significance vs TENO:
534 °°, p<0.001. Masson trichrome staining (panel C) of Basal, ESW, TENO and TENO ESW-treated
535 hASC cells. Collagen fibers are indicated by arrows. Magnification x200. Masson trichrome
536 quantification (panel D). Significance vs Basal: **, p<0.01 ***, p<0.001. Significance TENO ESW
537 vs TENO: °°, p<0.001.

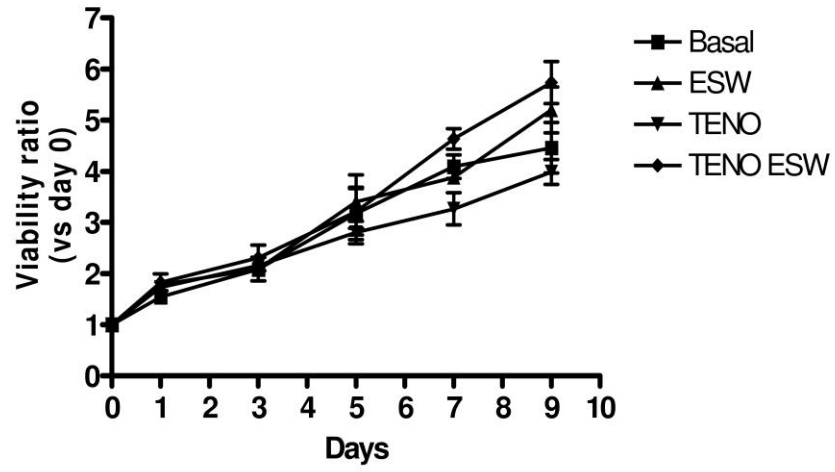
538 **Figure 6. Effects on integrin gene expression.** mRNA expression of ITGα2 (panel A), ITGα6
539 (panel B), ITGβ1 (panel C) was evaluated by RT-PCR. Results are normalized for three different
540 housekeeping genes (β-actin, RPLPO and L13A) and expressed as relative expression fold vs Basal.
541 Significance vs Basal: *, p<0.05; **, p<0.01; ***, p<0.001. Significance vs ESW: #, p<0.05; ##,
542 p<0.01; ###, p<0.001. Significance TENO ESW vs TENO: °, p<0.01; °°, p<0.001.

543 **Figure 7. Effects on BMP-2 (panel A), Runx2 (panel B) and ALP (panel C) gene expression.**
544 mRNA expression was evaluated by RT-PCR. Results are normalized for three different
545 housekeeping genes (β-actin, RPLPO and L13A) and expressed as relative expression fold vs Basal.
546 Significance vs Basal: **, p<0.01; ***, p<0.001. Significance TENO ESW vs TENO: °, p<0.05.

547 **Effect on calcium deposition (panel D):** Calcium deposits at 4 weeks (arrows) as revealed by
548 Alizarin Red Staining. Magnification x400. Staining is representative of five independent
549 experiments.

550

A



B

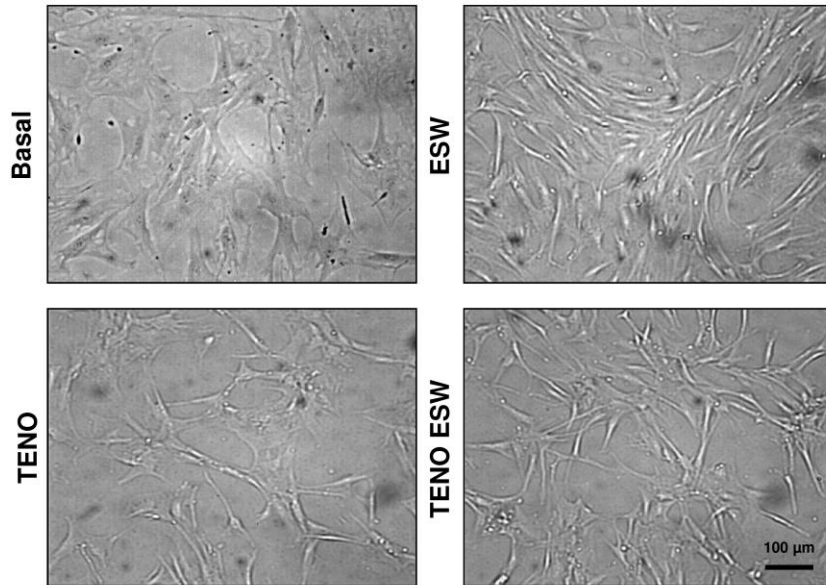


Figure 1

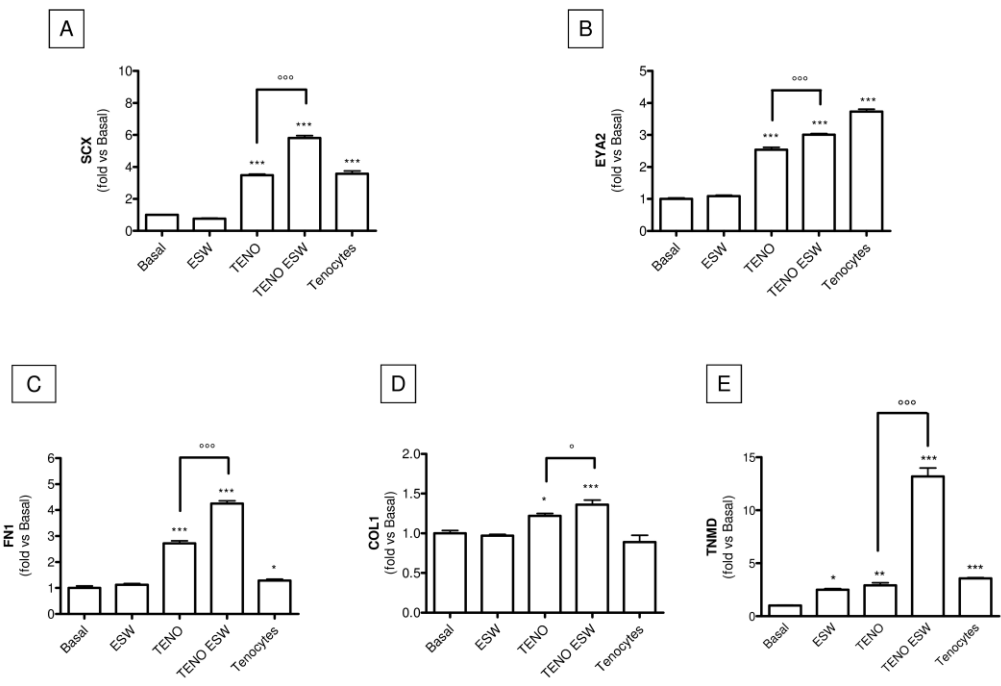


Figure 2

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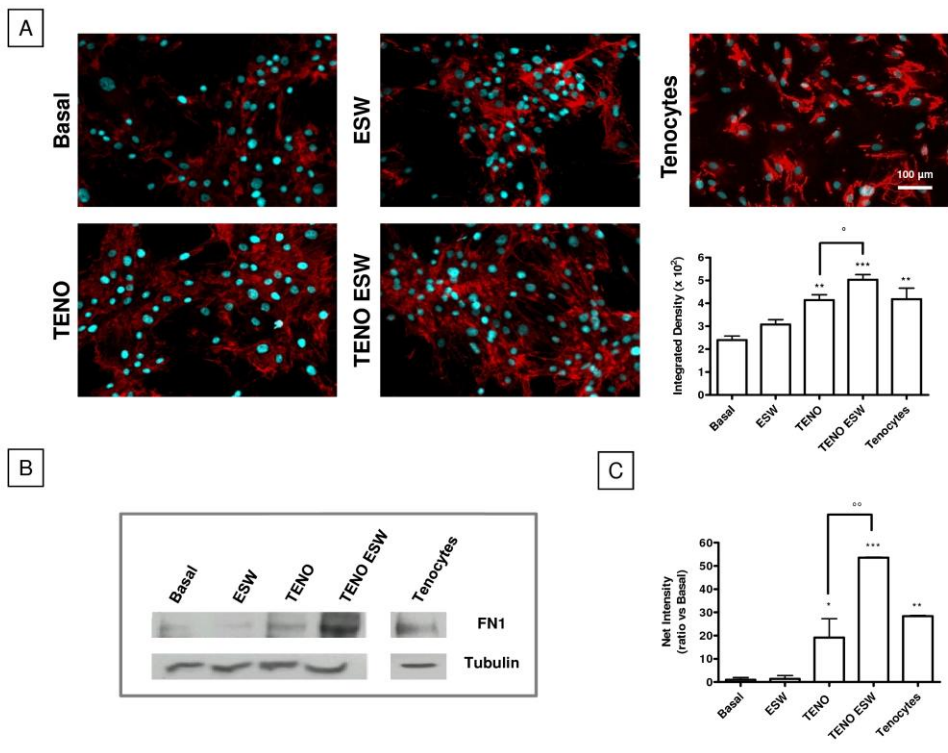


Figure 3

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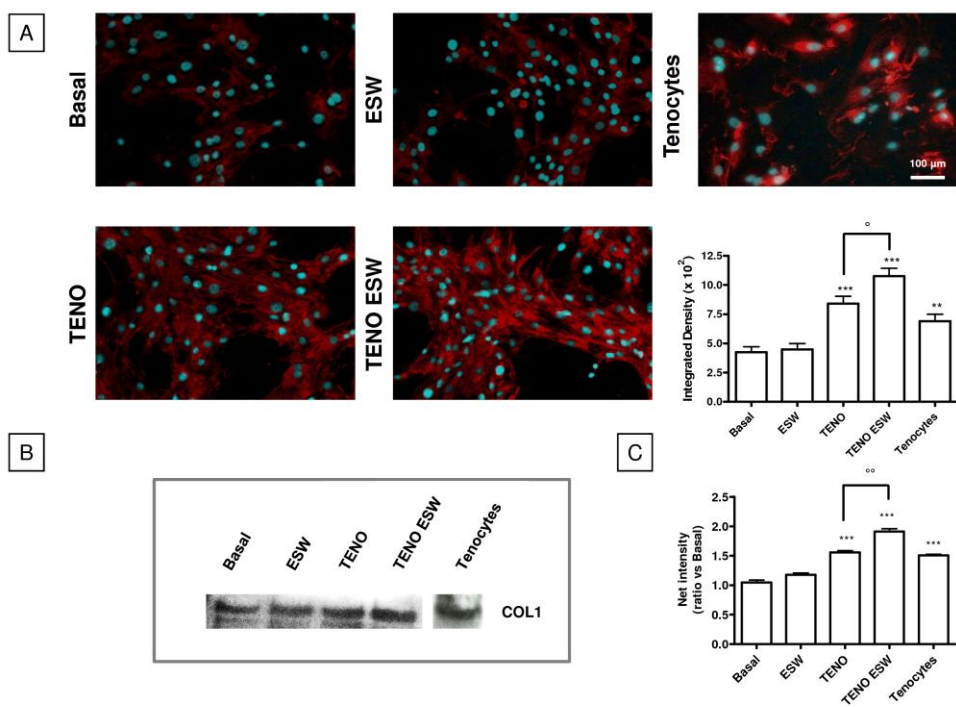


Figure 4

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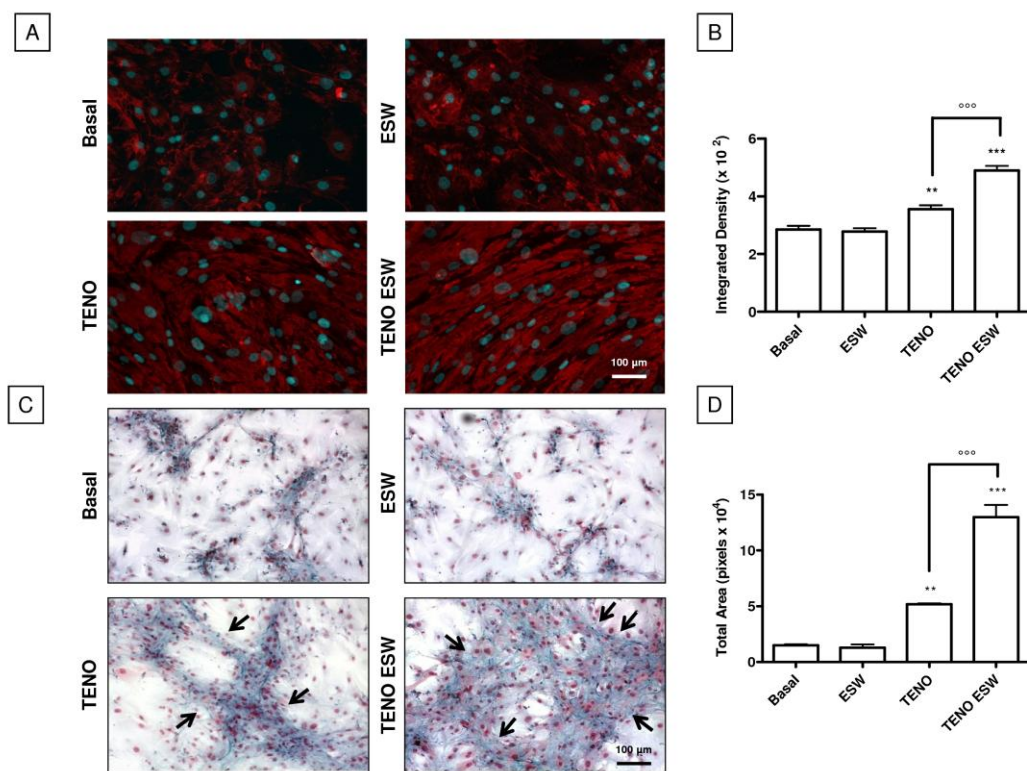
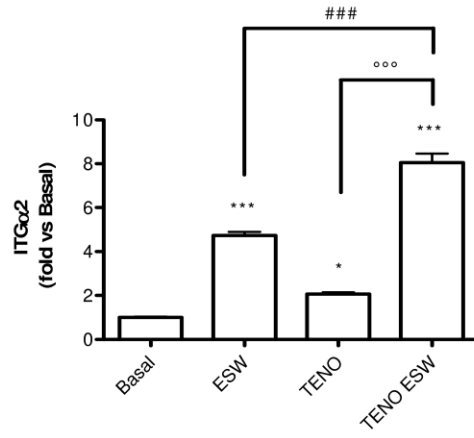


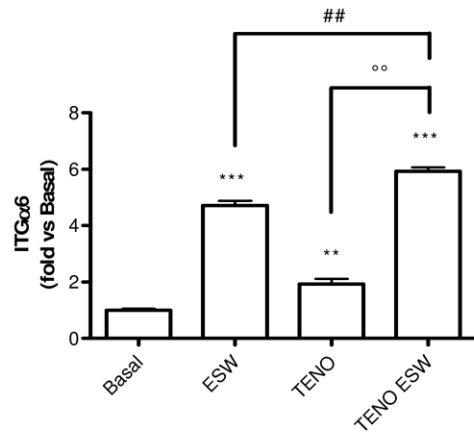
Figure 5

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A



B



C

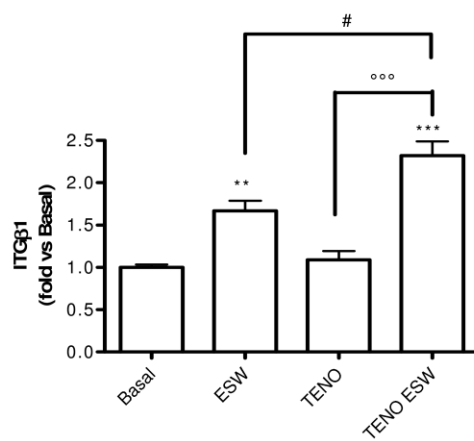


Figure 6

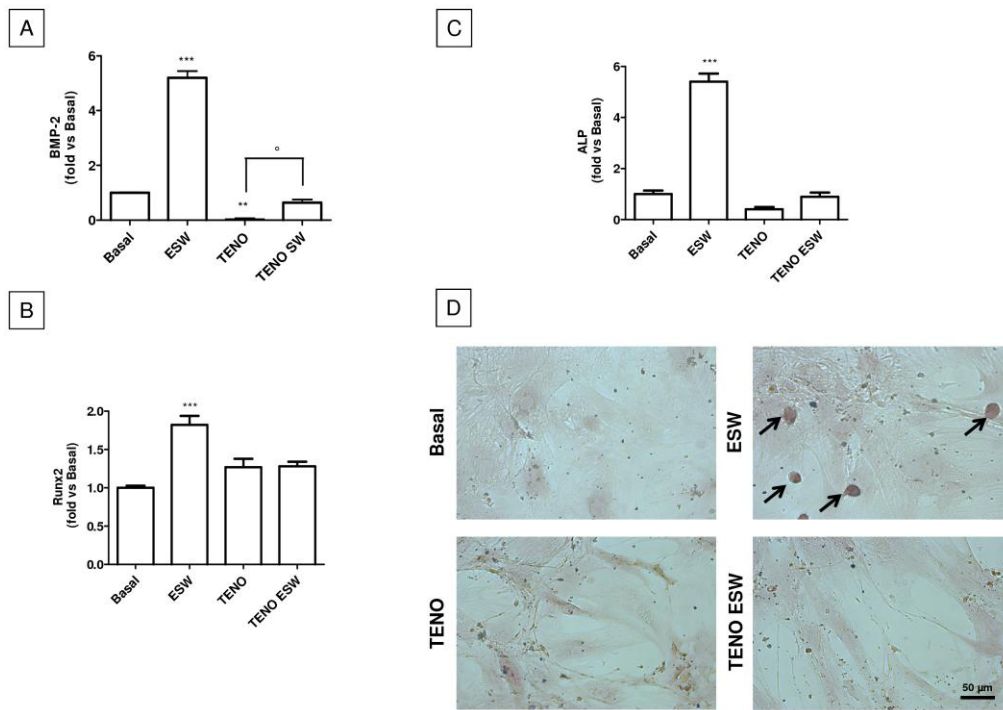


Figure 7

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Table S1 Primers for real-time PCR

Gene	Primer sequence	NCBI Reference
SCX	Sense: 5'-GAA CAC CCA GCC CAA ACA GATC-3' Antisense: 5'-GCG GTC CTT GCT CAA CTT TCT C-3'	NM_001080514.2
EYA2	Sense: 5'-GAT TGA GCG TGT GTT CGT GTG G-3' Antisense: 5'-GTG GTG TCC TTC CCG TAT CTG G-3'	NM_005244.4
FN1	Sense: 5'-CCG TGG CAT TGG GGA GTG G-3' Antisense: 5'-GGG TGG GAG TTG GGC TGA C-3'	NM_212482.2
COL1	Sense: 5'-ATG GAT GAG GAG ACT GGC AAC C-3' Antisense: 5'-GGA AGG GCA GGC GTG ATG G-3'	NM_000089.3
TNMD	Sense: 5'-ACGGATACACTGGCATCTACTTCG-3' Antisense: 5'-GGGACCCAAATCACTGACTGTTC-3'	NM_022144

ITG α 1	Sense: 5'-ACA TGG GAA CAG AGA AGG AGG AG-3' Antisense: 5'-TGA ATT GTG CTG CCG AGA TGA AC-3'	NM_181501.1
ITG α 2	Sense: 5'-TGT AGC AAC ATC CCA GAC ATC CC-3' Antisense: 5'-GCA CTT CGT CGC CCA CCA G-3'	NM_002203.3
ITG α 5	Sense: 5'-CGG GCT CCT TCT TCG GAT TCT C -3' Antisense: 5'-CAC TCC TGG CTG GCT GGT ATT AG -3'	NM_002205.4
ITG α 6	Sense: 5'-TCC TAC CCT GAT GTT GCT GTT GG-3' Antisense: 5'-TCT GGC GGA GGT CAA TTC TGT TAG-3'	NM_001079818.2
ITG α 10	Sense: 5'-GGG AAC CGA AGA GGG CAG TG -3' Antisense: 5'-ACA GGC AGA AGA CAA GGA GAG C -3'	NM_003637.4
ITG α 11	Sense: 5'-GCA GGC AGT GAC AGT AAT GAG C -3' Antisense: 5'-CGT ATT TGA GGT GGA AGC GTA AGG -3'	NM_001004439.1
ITG α V	Sense: 5'-TAG CGT ATC TGC GGG ATG AAT CTG -3' Antisense: 5'-GGC GTG AAC TGG TTA AGA ATG GG -3'	NM_002210.4
ITG β 1	Sense: 5'-TGC CTT GGT GTC TGT GCT GAG-3' Antisense: 5'-CAA CAG TCG TCA ACA TCC TTC TCC-3'	NM_002211.3
ITG β 3	Sense: 5'-CTT CAA TGC CAC CTG CCT CAA C -3' Antisense: 5'-GGA CAG CCT CGC ACC TTG G -3'	NM_000212.2
ITG β 5	Sense: 5'-TGC CTG CTG CTC CAC TCT G -3' Antisense: 5'-AGC CTC CTG GTC ATC TTT CAC G -3'	NM_002213.4
ITG β 8	Sense: 5'-GTG TGT GGA AGG TGT GAG TGC -3' Antisense: 5'-TGA GAC AAA TTG TGA GGG TGA AGG -3'	NM_002214.2
BMP-2	Sense: 5'-CGG GAG AAG GAG GAG GCA AAG -3' Antisense: 5'-GAA GCA GCA ACG CTA GAA GAC AG -3'	NM_001200.3
Runx2	Sense: 5'-CGG AGT GGA CGA GGC AAG AG-3' Antisense: 5'-AGG CGG TCA GAG AAC AAA CTA GG -3'	NM_001024630.3

ALP	Sense: 5'-ATG AGG CGG TGG AGA TGG AC -3' Antisense: 5'-CAT ACA GGA TGG CAG TGA AGG G -3'	NM_000478.5
β -ACT	Sense: 5'-GCG AGA AGA TGA CCC AGA TC- 3' Antisense: 5'- GGA TAG CAC AGC CTG GAT AG-3'	NM_001101.3
L13A	Sense: 5'-GCA AGC GGA TGA ACA CCA ACC-3' Antisense: 5'-TTG AGG GCA GCA GGA ACC AC-3'	NM_012423.3
RPLPO	Sense: 5'-CGA CAA TGG CAG CAT CTA CAA CC-3' Antisense: 5'-CAC CCT CCA GGA AGC GAG AAT G-3'	NM_001002.3

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Table S2. Mesenchymal stem cell marker expression

CD 13	98.2 % \pm 3.5
CD 14	1.3% \pm 0.4
CD 34	NEG
CD 44	99.3 % \pm 0.6
CD 45	NEG
CD 90	96.9 % \pm 0.1
CD 105	96.8 % \pm 2.6

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Table S3 Cell viability

Treatment	Viable cells
Basal	98.3%
ESW	83%
TENO	97.6%
TENO ESW	82.1%