The promising regenerative potential of pulsed electromagnetic fields toward tendon differentiation

a hamstring tendon explant culture study

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Aims

Hamstring tendons are commonly used autografts for anterior cruciate ligament (ACL) reconstruction. They represent an optimal source for in vitro testing of new approaches that may improve tendon regeneration and, likely, ligamentization after ACL reconstruction. We assessed ex vivo, in a 3D explant culture, the anabolic effect of pulsed electromagnetic fields (PEMF) on hamstring tendons in an experimental setting as close as possible to that of common clinical applications, suggesting a potential new therapeutic strategy to improving tendon remodelling.

Methods

We exposed tendon explants from nine donors to PEMF for 21 days, eight hours/day, with an intensity similar to that used in clinical practice, and evaluated specific gene expression by immunofluorescence and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses.

Results

Increased expression of tendon-related markers (scleraxis, collagen types I and VI) and important players of tenogenic differentiation (c-Fos and mammalian target of rapamycin) was evidenced by immunofluorescence analysis upon PEMF exposure and confirmed by qRT-PCR analysis.

Conclusion

Our results demonstrate that PEMF enhances tendon differentiation, which suggests that PEMF exposure could have clinical relevance as a new non-invasive adjuvant treatment for improving the early phases of hamstring autograft remodelling after ACL reconstruction, as well as possibly for hastening the repair of injured tendons.

Article focus

- Investigate the potential of pulsed electromagnetic field (PEMF) exposure to improve tendon remodelling after anterior cruciate ligament reconstruction in an ex vivo model.

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Key messages

- PEMF exposure acted as a positive stimulus to promote the expression of markers related to tendon matrix production and tenogenesis.
- PEMF represents a non-invasive regenerative strategy to enhance tenogenic commitment and tendon anabolism.

Strengths and limitations

- The ex vivo model allowed us to assess the tenogenic effect of PEMF in an experimental setting as close as possible to the clinical application.
- Further studies are required to assess the effect of PEMF on degenerative tendons and under mechanical stimulation.

Introduction

Tendons are essential components of the musculoskeletal system with a predominantly mechanical function: transmitting forces from muscle to bone in order to translate muscular contractions into joint movement. A mature tendon is a highly organized connective tissue characterized by a specific spatial organization of collagen type I fibres, arranged into tendon fascicles, which is responsible for the mechanical properties required for tendon function. This hierarchical collagen structure is interlaced with a network of non-fibrillar molecules and interspersed with tenoblasts and tenocytes. Collagen type VI is a well-known non-structural component of the tendon extracellular space with mechanoceptor activity. Tenocytes are specialized fibroblasts typically arranged in longitudinal rows between collagen fibrils and are the main resident cells of the tendon. They can sense loads from the extracellular matrix (ECM) and actively respond to changes in force, in turn, modulating the ECM composition and organization through the expression of tenogenic genes.[1]

Improving tendon anabolism may be advantageous for the early ligamentization phases after anterior cruciate ligament (ACL) reconstruction by soft-tissue grafts. Hamstring tendons are among the most commonly used autografts, and the success of this procedure largely depends on the graft maturation. This process follows the well-known three stages of the ligamentization process named early healing, proliferation, and maturation. This complex and intertwined sequence of biological events relies mainly on the migrating cells that repopulate the graft and the tendon-derived cells embedded in the original graft structure.[2] Tenocytes have the essential role of producing collagen and ECM proteins that support the in situ graft tension, and help confer the required biomechanical properties. The retention of the tenogenic commitment is, therefore, a prerequisite for tenocytes to optimize this postoperative intra-articular graft remodelling. Different solutions have been proposed to support graft maturation. Orthobiologics have gained popularity in the last 20 years, and there has been an increasing interest in their use to improve tendon repair.[3] Coating the surface of the graft with platelet-rich fibrin,[4] or pre-treating the graft with intratendinous injections of growth factor- and platelet-rich plasma,[5] have shown positive effects, as well as intra-articular injections with autologous adipose tissue-derived stromal vascular fraction.[6] Lu et al[7] demonstrated that the conditioned medium from the direct co-culture of ACL remnant cells and bone marrow stromal cells significantly enhanced the expression of collagen synthesis and the expression of tenogenic genes in hamstring tenocytes. However, the role of mesenchymal stem cells (MSCs) as an adjuvant to tendon metabolism has been questioned recently in different settings, as in preclinical models of rotator cuff repair, in which the addition of MSCs has led to unsatisfying results.[8] Promoting the expression of tenogenic markers may represent an efficient strategy to enhance tendon regeneration; thus, transduction represents another possible solution. In a rat rotator cuff repair model, the scleraxis gene has been transduced in bone marrow-derived mesenchymal stem cells (BMDMSCs), showing satisfactory results,[9] although this approach has many ethical concerns for a future possible clinical application due to the stem cell manipulation procedure. On the other hand, biophysical and environmental interventions, such as postoperative exposure to hyperbaric oxygen,[10] low-intensity pulsed ultrasound,[11] pulsed electromagnetic fields (PEMF),[12–15] or extracorporeal shock wave therapy (ESWT),[16] may exert a positive effect on the cell metabolism with few ethical limitations for a possible clinical perspective.

PEMF are one of the most common non-invasive therapeutic biophysical stimuli. In the past, PEMF have been applied clinically to promote bone healing for at least three decades, and they are clinically approved by the Food and Drug Administration (FDA) as an adjuvant treatment for bone disorders in both animals and humans. These include lumbar and cervical spine fusion, pseudoarthrosis, osteoporosis, and the treatment of delayed union and nonunion fractures.[17] PEMF has shown promising effects on tendinopathy since 1984, when Binder et al[18] described the beneficial use of PEMF in rotator cuff tendinitis. Following these observations, several clinical studies suggested using PEMF to reduce pain in lateral epicondylitis,[19] Achilles tendinopathy,[20] or after rotator cuff repair.[21] Correspondingly, multiple preclinical studies reported similar findings in small animal models. Lee et al[22] demonstrated the potential role of PEMF in the healing process of Achilles tendonitis in rats, noting reductions in inflammation and a return to normal histology in the tendon. In a similar rat model, Strauch et al[23] analyzed PEMF effects after Achilles transection and repair, observing an increase in tensile strength of up to 69% at the repair site compared to non-stimulated control animals. These clinical and preclinical results support the early anabolic effects of PEMF stimulation on overall repair, ultimately influencing the mechanical properties of tendon tissue. Recently, Marmotti et al[24] evaluated in vitro the effect of low-frequency PEMF on the tenogenic differentiation of mesenchymal stem cells (MSCs) isolated from human umbilical cords (UC-MSCs). UC-MSCs cultured with fibroblast growth factor 2 (FGF-2) and stimulated with PEMF showed increased production of collagen type I and scleraxis, suggesting that PEMF exposure provides a positive preconditioning stimulus that may enhance UC-MSC tenogenic potential. The positive effects of PEMF exposure are also evident in tenocytes derived from tendon tissue: in another study, the same authors showed that PEMF exposure for eight hours per day resulted in increased production of collagen types I and VI, as well as scleraxis.[25] Overall, these findings indicate that prolonged PEMF exposure can significantly improve the tenogenic potential of both MSCs and tendon-derived cells. The effects of PEMF may involve interactions with A2A adenosine receptors and other

intracellular pathways, such as the mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) pathway. Activation of these pathways, and potentially others, leads to the upregulation of scleraxis, vascular endothelial growth factor A (VEGF-A), and collagen type I gene expression. Additionally, PEMF exposure correlates with increased release of interleukins IL-6 and IL-10, transforming growth factor β (TGF-β), and the production of elastin and fibronectin by tendon cells.

In the present study, we took a step further by investigating the tenogenic behaviour of hamstring tendon explants exposed to PEMF with an intensity similar to that used in clinical practice. Assessing the positive effect of PEMF on the explants may have clinical relevance, suggesting PEMF exposure as a non-invasive regenerative strategy to enhance tenogenic commitment and tendon anabolism. This may have a positive effect on the early phases of hamstring graft remodelling after ACL reconstruction, ultimately improving the ligamentization process and, likely, in other settings such as tendon repair and tendinopathy.

Methods

Tendon explants culture

The discarded free ends of gracilis and semitendinosus tendons were collected from nine healthy donors (aged 15 to 34 years) who underwent ACL reconstruction with autologous hamstrings. The consent for the use of discarded material was orally obtained during each patient's preoperative clinical examination before signing the written consent for the surgical procedure. The tendon explants were cut into four to eight pieces about 2 cm long, depending on the explant size, and cultured in Dulbecco's Modified Eagle's Medium containing 10 mM Glutamax and 4.5 mg/ml glucose (DMEM Glutamax, GIBCO Invitrogen Life Technologies, Thermo Fisher Scientific, USA), supplemented with 10% FBS, 1 mM sodium pyruvate, 25 mM HEPES pH 7.4, 100 µg/ml gentamicin, and 5 ng/ml basic fibroblast growth factor (b-FGF). The explants were maintained in a humidified atmosphere with 5% CO_2 at 37°C. The culture medium was changed every three days. For each sample, two equal groups were defined: a control group, not exposed to PEMF, and an experimental group, exposed to PEMF. PEMF stimulation was carried out as previously described by de Girolamo et al.[26] Specifically, tendon explants were cultured with differentiation medium plus or minus exposure to PEMF, eight hours per day for 21 days. PEMF were generated by two electrical coils, connected with the PEMF generator system IGEA (intensity of magnetic field = 1.5 mT, frequency = 75 Hz).

Histological assessment

At day 0 and day 21 tendon explants were formalin-fixed (FF) overnight, paraffin-embedded (PE), cut into 5 µm sections, and haematoxylin and eosin (H&E)-stained for standard histology observations. Samples were examined using light microscopy with 10× magnification (Olympus IX70 microscope; Olympus, Japan).

Immunohistochemistry

The FFPE sections of tendon explants (day 21) were deparaffinized and rehydrated in three changes of xylene (5 minutes each), hydrated in two changes of 100% ethanol (5 minutes each), two changes of 95% ethanol (5 minutes each), and rinsed in distilled water. Antigen retrieval was obtained by boiling the samples in hot citrate buffer (pH 6.0, 10×, Antigen Retriever; Merck Millipore, Italy). 5% Bovine Serum Albumin (BSA) was used as a blocking solution to prevent non-specific binding. Immunohistochemistry stainings were performed using the Caspase-3 Cleaved (Asp175) antibody #9661 (Cell Signaling Technology, USA) with the avidin-biotin-peroxidase techniques (Anti-Mouse HRP-DAB Cell & Tissue Staining Kit, R&D Systems, USA). Slides were counterstained with haematoxylin. Samples were examined using light microscopy with 20× magnification (Olympus IX70 microscope; Olympus).

Sample preparation and immunofluorescence analysis

The FFPE sections of tendon explants (day 21) were prepared for immunofluorescence analysis as follows: sections were deparaffinized in three changes of xylene (5 minutes each), hydrated in two changes of 100% ethanol (5 minutes each), 95% ethanol (5 minutes), and rinsed in distilled water. Antigen retrieval was obtained by boiling the samples in hot citrate buffer (pH 6.0, 10×, Antigen Retriever, Merck Millipore). BSA was used as a blocking solution to prevent non-specific binding. Expression analysis for the specific listed genes was performed using the following antibodies: scleraxis sc-518082 (Santa Cruz Biotechnology, USA); collagen type I MAB3391, collagen type VI AB7821, c-Fos ABE457 and the mammalian target of rapamycin (mTOR) 05-1564 (Merck Millipore); proliferating cell nuclear antigen (PCNA) sc-25280 and β-catenin SC-7199 (Santa Cruz Biotechnology). Primary monoclonal antibodies were diluted at 1:200 in phosphate-buffered saline (PBS)-1% BSA and incubated overnight at 4°C. No primary antibody was added for negative control sections. The fluorescent-labelled secondary anti-Rabbit DyLight 488 antibody 202-03-15-16 (KPL, Kirkegaard & Perry Laboratories, USA) and anti-mouse DyLight 488 antibody ab96879 (Abcam, UK) diluted 1:80, were incubated for one hour at room temperature. Nuclei were counter-stained with DAPI 1 µg/µl diluted at 1:1,000 in dH_2O. An Apotome fluorescence microscope (Zeiss, Germany) and the Leica THUNDER imager were used to visualize stained fluorescent sections. Digital images were collected using × 10, × 20, or × 40 objectives within one to seven days after labelling.

Evaluation of fluorescence intensity

Four cellular fields were randomly chosen for each section in each slide, for a total of four to 12 fields for each sample, depending on the size of the explant. The acquired images were analyzed using FIJI software.[27] For PCNA analysis, we evaluated the number of PCNA-positive nuclei over the total number of cells. For collagen type I and collagen type VI, being both intracellular and extracellular, the difference in total fluorescence intensity was evaluated between the different study groups. The software-generated numerical semiquantitative evaluation corresponds to the mean of fluorescence intensity (MFI) of each image examined. For scleraxis, β-catenin, c-Fos, and mTOR, the images were analyzed by selecting one cell at a time in an image and measuring the area, integrated density, and mean gray value. Using the calculation for corrected total cell fluorescence (CTCF) = integrated density – (area of selected cell × mean fluorescence of background readings), as described by McCloy et al,[28] the

fluorescence intensity of each cell was calculated using Excel (Microsoft, USA) and then used for statistical analyses. For each image, four background areas were used to normalize against autofluorescence.

RNA isolation and qRT-PCR for mRNA detection

Total RNA was isolated from the formalin-fixed, FFPE tendon explants using the RNeasy FFPE Kit (Qiagen, Netherlands). For mRNA detection, 200 ng total RNA was retrotranscribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR analyses were carried out using gene-specific primers and SYBR Green PCR Master Mix, using a QuantStudio 6 Pro Real-Time PCR System (Thermo Fisher Scientific). Quantitative normalization was performed on 18 S expression. The relative expression levels between samples were calculated using the comparative delta CT (threshold cycle number) method (2-ΔΔCT) with a control sample as a reference point. QuantiTect Primer Assays for 18 S, collagen type I, scleraxis, and PCNA: Hs_RRN18S_1_SG, Hs_COL1A1_1_SG, Hs_SCX_2_SG, Hs_PCNA_1_SG. Gene-specific primers for collagen type VI, c-Fos, mTOR, and β-catenin are reported in Table I.

Table I. Sequences of gene-specific primers for collagen type VI, c-Fos, mammalian target of rapamycin (mTOR), and β-catenin.

Gene	Primer sequence
Collagen type VI	F 5′ AGCAAGTGTGCTGCTCCTTC 3′
	R 5′ CTTCCAGGATCTCCGGCTTC 3′
c-Fos	F 5′ GGAGAATCCGAAGGGAAAGGA 3′
	R 5′ AGTTGGTCTGTCTCCGCTTG 3′
mTOR	F 5′ GACGAGAGATCATCCGCCAG 3′
	R 5′ ACAAGGGACCGCACCATAAG 3′
β-catenin	F 5′ CGTGCACATCAGGATACCCA 3′
	R 5′ GGCTCCGGTACAACCTTCAA 3′

Statistical analysis

All data are provided as means and standard error of the mean (SEM). Statistical analysis was carried out with the statistical software package GraphPad Prism 5.0 (GraphPad Software, USA). The Shapiro-Wilk test was performed to confirm normal data distribution. Grubbs' test was performed using the GraphPad Outlier Calculator to detect possible outliers. A two-tailed independent-samples *t*-test was used for comparison. Statistical significance was set at $p < 0.05$.

Results

Histological assessment of tendon explants

For histological evaluation, tendon explant sections were prepared at day 0 and day 21 and stained with H&E. As shown in Figure 1, tissue integrity was maintained after 21 days in culture compared to controls at day 0, and no difference in tissue morphology was observed between tendons either exposed or not exposed to PEMF. Apoptosis was evaluated by immunohistochemistry analysis for cleaved caspase-3 (CC3). No significant difference in the percentage of CC3-positive cells in PEMF-exposed samples was observed, compared to the untreated control (Figure 2).

Immunofluorescence analysis of tendon-related markers

Gene expression for scleraxis, collagen types I and VI, c-Fos, m-Tor, PCNA, and β-catenin was evaluated in tendon samples at day 21, with or without PEMF stimulation, via immunofluorescence analysis. All samples showed some level of staining for each gene, compared to negative controls lacking the primary antibody, suggesting that the tendon culture was still healthy and retained the original phenotype. However, a statistically significant increase in fluorescence intensity was revealed in PEMF-stimulated samples for scleraxis (Figure 3), collagen type I (Figure 4), collagen type VI (Figure 5), c-Fos (Figure 6), and mTOR (Figure 7), compared to samples which were not exposed to PEMF. The observed results suggest that the stimulation of tendons with PEMF leads to increased expression of tendon differentiation and regeneration genes.

However, we did not find any statistically significant modulation for PCNA staining following PEMF exposure compared to controls (Figure 8), which suggests that cell proliferation was not influenced. As for β-catenin, able to suppress tenogenic genes, no significant difference in fluorescence intensity between the two groups was observed; eventually, a general lower staining was observed (Figure 9).

The mean of CTCF values for each patient for scleraxis, c-Fos, mTOR, and β-catenin, along with the MFI values for collagen type I and collagen type VI and the ratios of PCNA-positive cells versus the total number of cells, are reported in Supplementary Table i, which also summarizes the mean of all patients and the corresponding SEM for each marker.

mRNA expression analysis of tendon-related markers

In addition to immunofluorescence stainings, gene expression was analyzed by qRT-PCR analysis, and similar results were found. In fact, as shown in Figure 10, a statistically significant increase for scleraxis, collagen type I, collagen type VI, and mTOR mRNA levels was observed under PEMF stimulation, compared to untreated controls, at day 21. Moreover, a tendency toward increased c-Fos expression was observed following PEMF stimulation, although this was not statistically significant. As for PCNA and β-catenin, no expression difference was revealed between the two study groups.

Discussion

This study demonstrates the positive anabolic effect of prolonged exposure to PEMF on hamstring tendon explant cultures. The ex vivo model of tendon explants was suitable for assessing the tenogenic effect of PEMF in an experimental setting as close as possible to that of an in vivo clinical application. Indeed, patient-derived tendon explants were cultured without any further manipulation, retaining their 3D tendon structure, and exposed to PEMF with a similar intensity and time of exposure to those used in clinical practice. PEMF exposure acted as a positive biophysical stimulus, promoting the expression of tenogenic markers related to tendon matrix production and tenogenesis without substantially altering tissue morphology or tendon cell proliferation. This may have clinical relevance, suggesting PEMF as a potential adjuvant therapy for improving the early phases of tendon remodelling

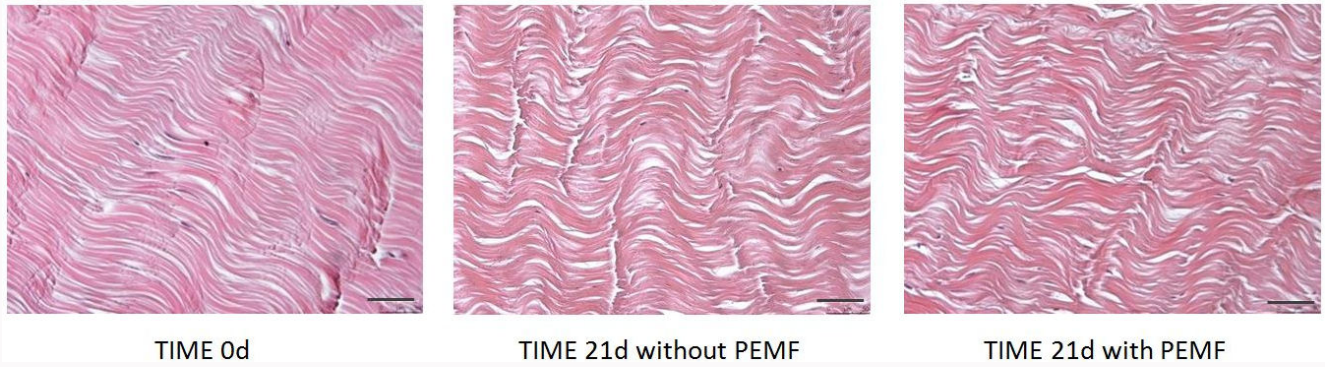

Fig. 1
Haematoxylin and eosin-stained tendon sections a) at the beginning of the experiment (day 0), b) after 21 days of culture without pulsed electromagnetic field (PEMF) stimulation, and c) with eight hour/day PEMF stimulation. Images were acquired using a 10× magnification. Representative images are shown. Scale bar: 50 μm; d = days.

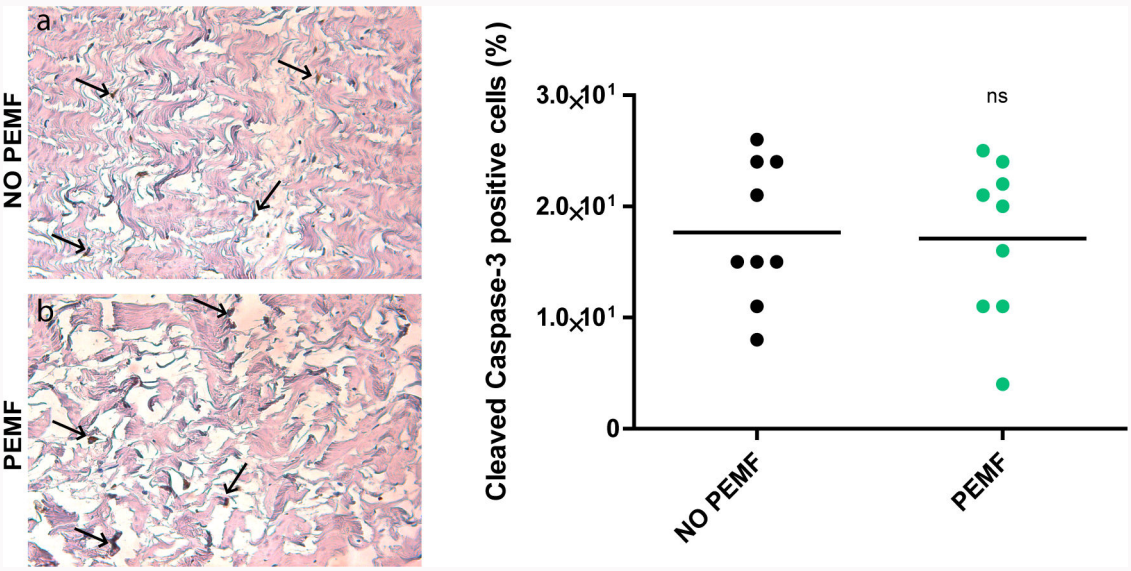

Fig. 2
Untreated and pulsed electromagnetic field (PEMF)-exposed tendon sections were stained with cleaved caspase-3 (CC3) antibody by immunohistochemistry (IHC), and nuclei were counterstained with haematoxylin. Left: representative images for a) no PEMF and b) PEMF groups. b) shows a similar CC3 signal compared to a). Right: graph presenting the percentage of positive versus total cells in the two experimental groups, including the statistical analysis. Black arrows in the images indicate representative localization of CC3 signal. Images were acquired using a 10× magnification. ns, not significant.

and maturation after ACL reconstruction with hamstring tendon autografts.

Graft healing in ACL reconstruction is a complex and long process, leading to the so-called 'ligamentization', during which a tendon undergoes different biomechanical and biochemical stimuli in the knee joint microenvironment.[29] Enhancing the ligamentization process may allow for faster and better healing of the tendon graft after surgery. From this perspective, our study showed that hamstring grafts are not mere scaffolds, but viable tenogenic constructs in which the tenocytes maintain the ability to express tendon-related genes, and that PEMF exposure strongly improves the anabolic behaviour and tenogenic commitment of tendon resident cells. Indeed, we demonstrated the viability of the hamstring tendon resident cells and their commitment toward tenocytic lineage after the surgical manipulation. No detectable cellular impairment was caused despite the application of repetitive stress on the tendons during the harvesting procedure, which is followed by prolonged exposure to the external atmosphere and by pretensioning maneuvers to obtain a graft suitable for the introduction into the knee joint. Not only did tenocytes survive the manipulation, but it was also possible to culture the explants for 21 days without losing the original phenotype of the resident cells. Although our study only offers a short-term analysis, we believe that the observed tenogenic effect of PEMF may positively influence the proceedings of ligamentization, the initial phase of which involves a regenerative anabolic effect and tenogenic commitment of the graft. In this early healing period, graft maturation begins in the joint microenvironment, in which the inflammatory response is associated with the release of cytokines and chemokines that lead to the production of growth factors, migration and proliferation

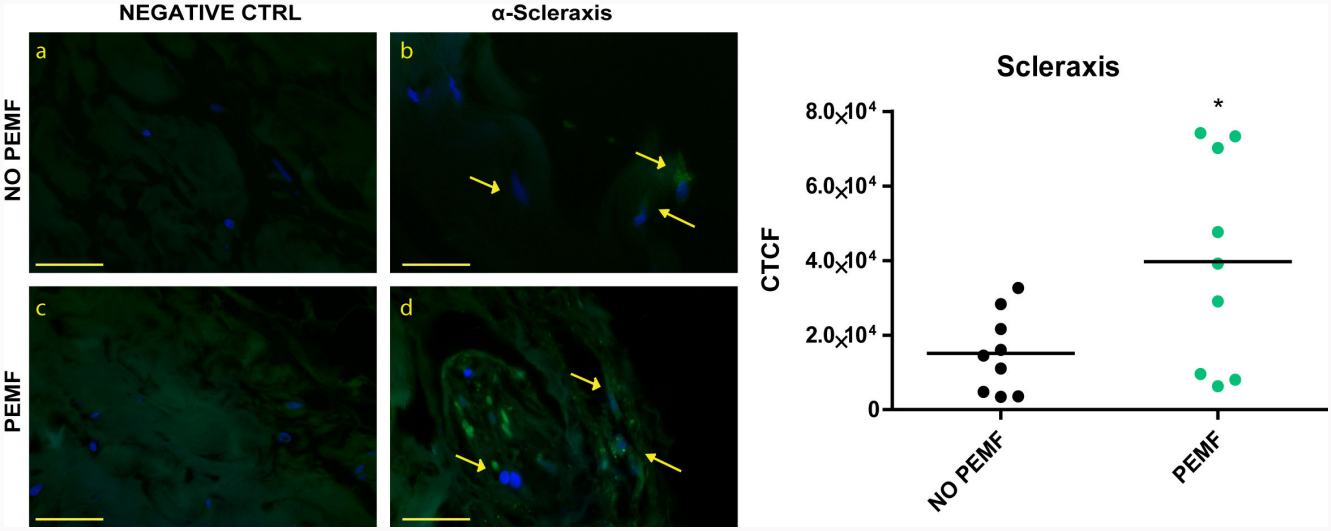

Fig. 3

Scleraxis expression and fluorescence quantification in hamstring tendon explant sections with or without exposure to pulsed electromagnetic fields (PEMF) for 21 days, 8 hr/day. Representative images are shown. Left: representative immunostaining images for b) no PEMF and d) PEMF groups and their negative controls (a and c). Image d shows increased fluorescence intensity compared to image b. Yellow arrows in the images indicate representative localization of the specific markers. Scale bar: 25 μm. Right: graph depicting the results for each marker including the statistical analysis. Results are shown as mean (standard error of the mean) of corrected total cell fluorescence (CTCF). *Statistically significant p-value (< 0.05) in a two-tailed independent-samples *t*-test. Nine samples were evaluated.

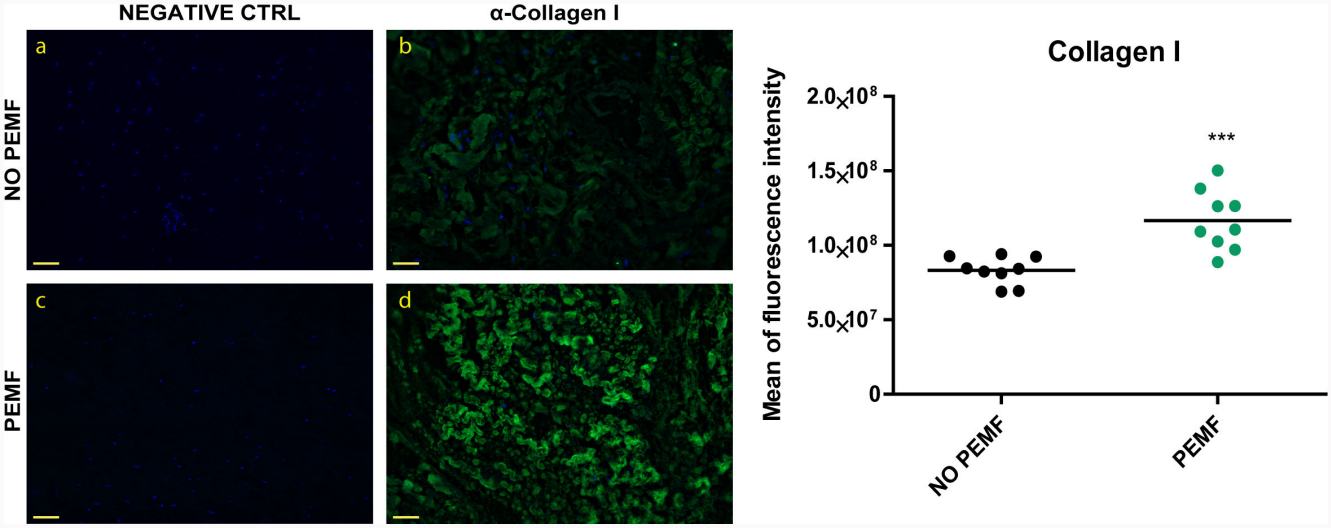

Fig. 4

Collagen type I expression and fluorescence quantification in hamstring tendon explant sections with or without exposure to pulsed electromagnetic fields (PEMF) for 21 days, 8 hr/day. Representative images are shown. Left: representative immunostaining images for b) no PEMF and d) PEMF groups and their negative controls (a and c). Image d shows increased fluorescence intensity compared to image b. Scale bar: 25 μm. Right: graph depicting the results for each marker including the statistical analysis. Results are shown as mean (standard error of the mean) of fluorescence intensity (MFI). ***Statistically significant p-value (< 0.001) in a two-tailed independent-samples *t*-test. Nine samples were evaluated.

of cells, revascularization, and synthesis of ECM components. In this context, the increased collagen and tenogenic gene expression obtained from PEMF exposure may be associated with more efficient ligamentization of the graft in the long term. This concept is also suggested in different in vitro settings: Lu et al[7] showed that remnants of ACL mixed with bone marrow MSCs could improve the tenogenic commitment and expression of tenogenic genes in hamstring tendon graft during ACL reconstruction, and ultimately help the ligamentization process. Cardona-Ramirez et al[30] suggested a positive influence of tenogenic commitment as a key element for graft maturation and positive outcome of ACL reconstruction, analyzing the different expressions of scleraxis and collagen in different graft sources under mechanical stimulation. Similarly, Park et al[31] also suggested a positive role of collagen types I and III at an early stage. Future in vivo studies at a medium-term follow-up will allow confirmation of the improvement of ligamentization by early exposure to PEMF after ACL reconstruction.

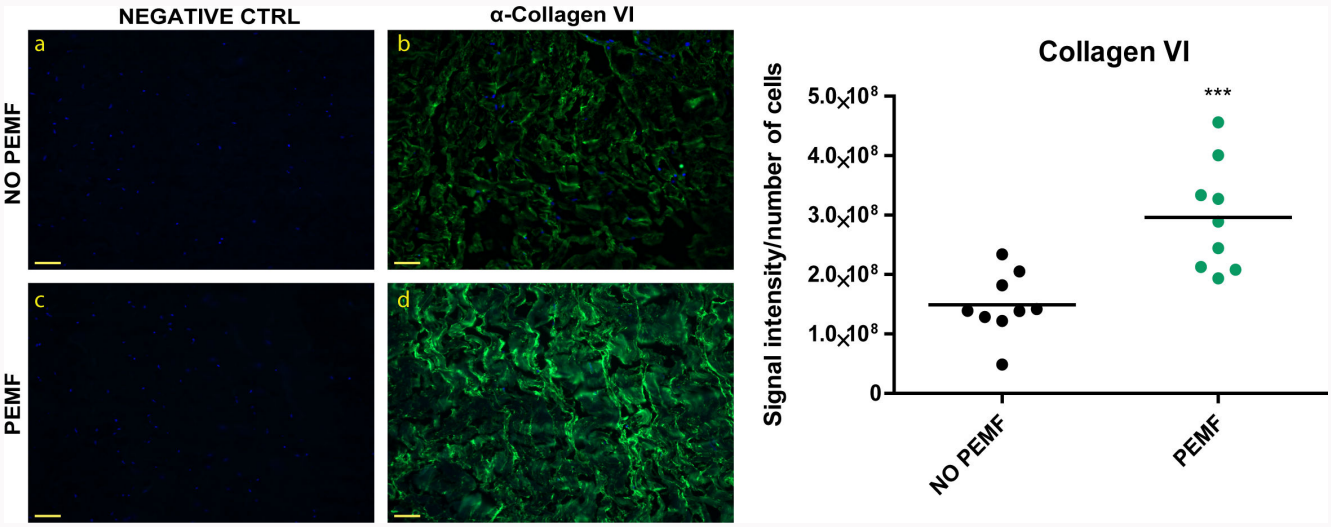

Fig. 5

Collagen type VI expression and fluorescence quantification in hamstring tendon explant sections with or without exposure to pulsed electromagnetic fields (PEMF) for 21 days, 8 hr/day. Representative images are shown. Left: representative immunostaining images for b) no PEMF and d) PEMF groups and their negative controls (a and c). Image d shows increased fluorescence intensity compared to image b. Scale bar: 25 μm. Right: graph depicting the results for each marker including the statistical analysis. Results are shown as mean (standard error of the mean) of fluorescence intensity. ***Statistically significant p-value (< 0.001) in a two-tailed independent-samples *t*-test. Nine samples were evaluated.

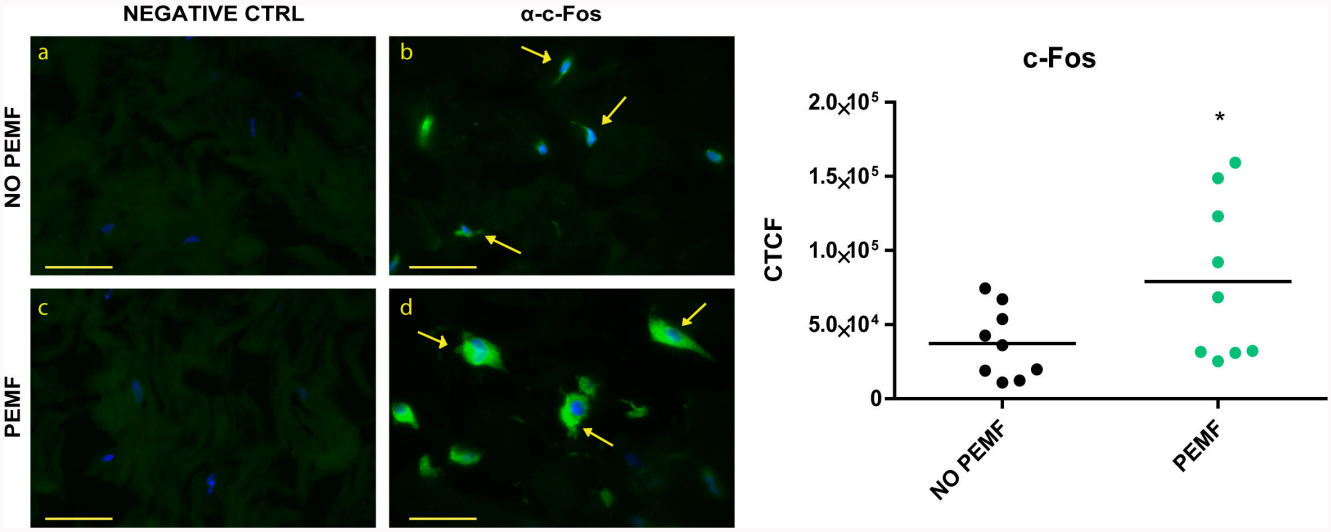

Fig. 6

c-Fos expression and fluorescence quantification in hamstring tendon explant sections with or without exposure to pulsed electromagnetic fields (PEMF) for 21 days, 8 hr/day. Representative images are shown. Left: representative immunostaining images for b) no PEMF and d) PEMF groups and their negative controls (a and c). Image d shows increased fluorescence intensity compared to image b. Yellow arrows in the images indicate representative localization of the specific markers. Scale bar: 25 μm. Right: graph depicting the results for each marker including the statistical analysis. Results are shown as mean (standard error of the mean) of corrected total cell fluorescence (CTCF). *Statistically significant p-value (< 0.05) in a two-tailed independent-samples *t*-test. Nine samples were evaluated.

In the present study, we examined tendon explants exposed to PEMF for eight hours per day for 21 days. We found increased expression of tenogenic markers such as scleraxis, collagen type I, and collagen type VI, suggesting increased tenogenic behaviour. In particular, collagen type VI, in addition to being a matrix structural molecule, is also a mechanoreceptor that plays a critical role in tendon matrix organization and cell behaviour.[32–34] The PEMF-dependent increase in collagen type VI expression may represent a possible mechanism for the anabolic effects of PEMF on tendon cells, through a direct action on tenocytes' pericellular matrix. One of the cellular receptors involved in PEMF signal transduction is the adenosine A2a receptor (A2aR), as previously shown by Colombini et al.[35] In line with these findings, our immunostaining results are evidence that PEMF exposure increases c-Fos expression, which was shown to be stimulated by A2aR in several studies.[36–38] Moreover, our data are consistent with the results of De Mattei et al,[39] who described increased c-Fos levels in osteoblast-like cells exposed to PEMF, and with the study of Chen et al,[40] who showed that Fos promotes

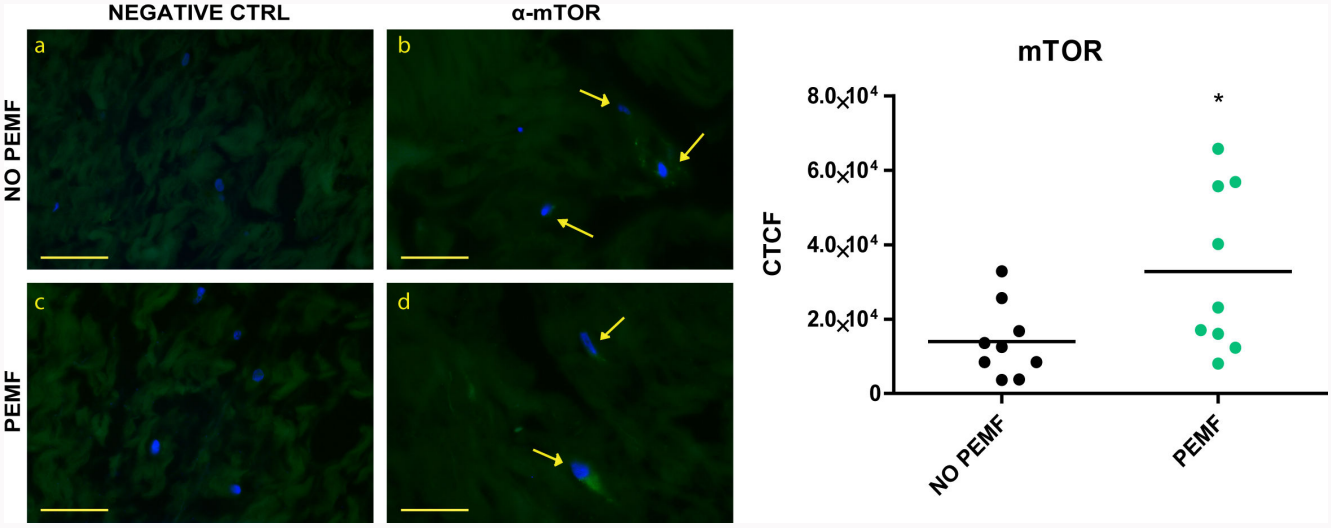

Fig. 7

Mammalian target of rapamycin (mTOR) expression and fluorescence quantification in hamstring tendon explant sections with or without exposure to pulsed electromagnetic fields (PEMF) for 21 days, 8 hr/day. Representative images are shown. Left: representative immunostaining images for b) no PEMF and d) PEMF groups and their negative controls (a and c). Image d shows increased fluorescence intensity compared to image b. Yellow arrows in the images indicate representative localization of the specific markers. Scale bar: 25 μm. Right: graph depicting the results for each marker including the statistical analysis. Results are shown as mean (standard error of the mean) of corrected total cell fluorescence (CTCF). *Statistically significant p-value (< 0.05) in a two-tailed independent-samples *t*-test. Nine samples were evaluated.

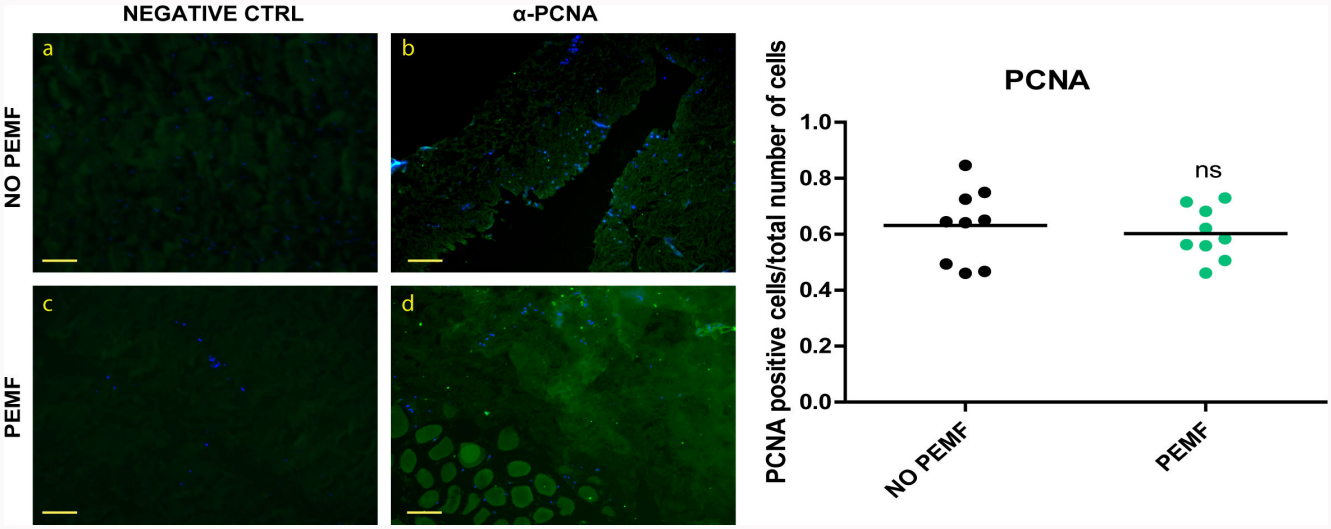

Fig. 8

Proliferating cell nuclear antigen (PCNA) expression and fluorescence quantification in hamstring tendon explant sections with or without exposure to pulsed electromagnetic fields (PEMF) for 21 days, 8 hr/day. Representative images are shown. Left: representative immunostaining images for b) no PEMF and d) PEMF groups and their negative controls (a and c). No significant difference was observed in the number of PCNA-positive cells between the two groups. Scale bar: 25 μm. Right: graph depicting the results for each marker including the statistical analysis. Results are shown as mean (standard error of the mean) of the ratio between the number of PCNA-positive cells and the total number of cells in each field. Nine samples were evaluated. ns, non-significant.

the differentiation of tendon stem/progenitor cells, thereby identifying the Fos gene as an early-stage tenogenic differentiation marker. As far as we are aware, our study demonstrates for the first time a link between PEMF exposure and c-Fos expression in tendon explant cultures. The link between PEMF, mTOR signalling, and tenogenic behaviour was also observed for the first time. This is particularly interesting since mTOR represents the main nutrient sensor able to regulate protein synthesis, metabolism, and cell growth.[41] Different studies suggest a role for mTOR in regulating musculoskeletal stem cell differentiation.[42–44] Cong et al[45] showed that mTOR was upregulated during the tenogenesis of MSCs and its inhibition significantly reduced collagen type I production, impairing tenogenic differentiation. In another study by Liu et al,[46] the CCAAT enhancer-binding protein delta (CEBPD), a known positive regulator of mTOR signalling,[47] was found to be downregulated in lesioned tendons compared to normal tendons. These correlations may represent new hypothetical mechanisms underlying tendon anabolism, which may be investigated in future studies. Finally, our results demonstrate

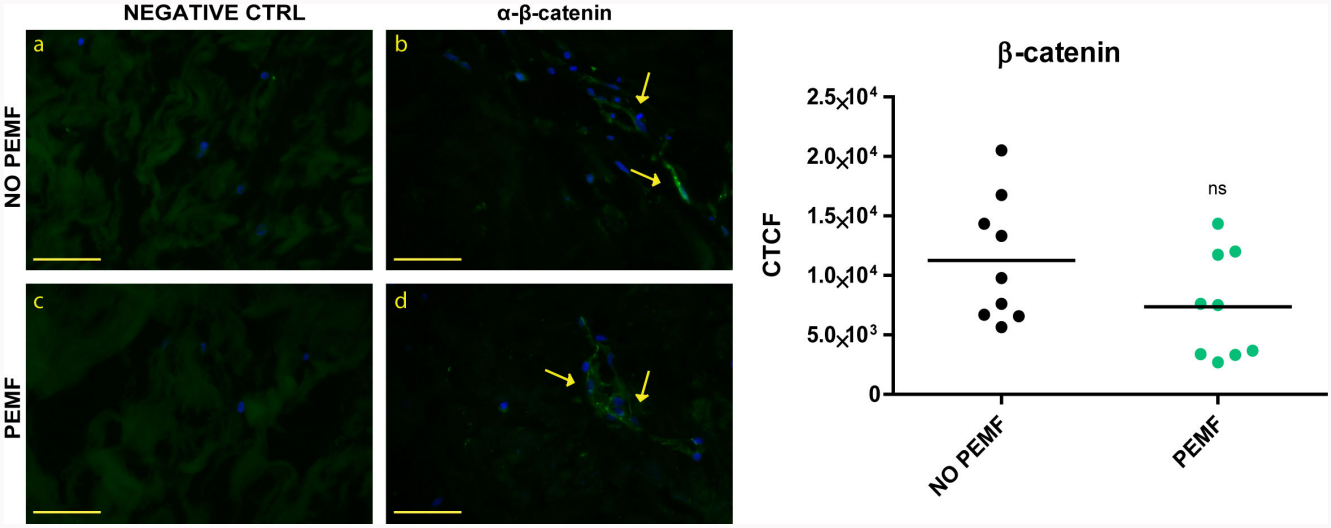

Fig. 9

β-catenin expression and fluorescence quantification in hamstring tendon explant sections with or without exposure to pulsed electromagnetic fields (PEMF) for 21 days, 8 hr/day. Representative images are shown. Left: representative immunostaining images for b) no PEMF and d) PEMF groups and their negative controls (a and c). No significant difference was observed between the two groups. Image d is representative of the tendency toward decreased β-catenin signal. Yellow arrows in the images indicate representative localization of the specific markers. Scale bar: 25 μm. Right: graph depicting the results for each marker including the statistical analysis. Results are shown as mean (standard error of the mean) of corrected total cell fluorescence (CTCF). Nine samples were evaluated. ns, non-significant.

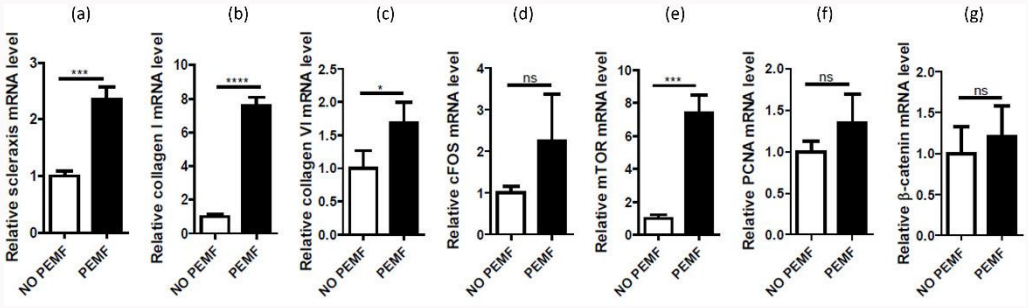

Fig. 10

Relative messenger RNA (mRNA) levels of a) scleraxis, b) collagen type I, c) collagen type VI, d) c-Fos, e) mammalian target of rapamycin (mTOR), f) proliferating cell nuclear antigen (PCNA), and g) β-catenin in tendon explants exposed to pulsed electromagnetic fields (PEMF) for 21 days, 8 hr/day, compared to tendon explants not exposed to PEMF measured by quantitative reverse transcription polymerase chain reaction analysis. Results were calculated as fold changes (mean (SD) of triplicates) relative to controls, normalized on 18S RNA level. *Statistically significant p-value ($p < 0.05$) in a two-tailed independent-samples *t*-test; ***Statistically significant p-value ($p < 0.001$) in a two-tailed independent-samples *t*-test. Three samples were evaluated. ns, non-significant.

that PEMF exposure has no impact on either apoptosis or proliferation. Indeed, we did not observe any increase in CC3-positive cells or the proliferation marker PCNA and β-catenin expression. This is in line with a tenogenic phenotype based on differentiation rather than on proliferation. In keeping with our observations, Kishimoto et al[48] showed that the activation of the Wnt/β-catenin signalling pathway suppresses the expression of the tenogenic gene scleraxis, as well as mohawk homeobox and tenomodulin, in tendon-derived cells isolated from rat Achilles tendons.

This study has some limitations. One possible drawback is linked to the source of tendon explants: we used healthy tendons harvested during ACL reconstructions and, thus, our findings should not be directly translated to clinical settings of tendinosis (i.e. rotator cuff, Achilles tendon, and patellar tendon tendinopathy). In these pathologies, the tendon structure is altered by the degenerative process, and cells might not respond to PEMF as vividly as those from hamstring explants harvested during ACL reconstruction. Further in vitro studies may better clarify the anabolic effect of PEMF on degenerative tendon explants, to support a safe and effective clinical application of PEMF in tendinosis. A second limitation lies in the ex vivo nature of the study design. Even if the setting of the tendon explant cultures is as close as possible to a hypothetical clinical setting, it lacks the physiological peculiar stimuli and growth factors of the complex joint microenvironment. A preclinical animal study may offer a more exhaustive experimental model, allowing for confirmation of our observations about the positive tenogenic effect of PEMF exposure. Finally, the tendons in our study were not subjected to mechanical loading during the culture period, which is a critical factor in tendon adaptation and differentiation. The mechanical stimulation of tendon explants could be simulated, with devices linked to the tendon limbs

and generating an alternate tension-relaxation stress for each explant during the culture period. However, this artifice is non-physiological, and a better mechanical stimulation is realized in a preclinical small animal model, such as a rat model. In our study, we chose to focus on PEMF exposure as the single variable among the study groups. Indeed, both groups were subjected to the same culture conditions, and, most importantly, each sample in the exposed group was associated, as a counterpart, with an unexposed sample derived from the same source of explant in the control group (the same patient). This allowed us to exclude any bias derived from different baseline levels of tenogenic marker expression, and to assume that the alterations observed were due to PEMF exposure. Further preclinical studies may properly clarify the anabolic effect of PEMF exposure under physiological mechanical stimulation, and corroborate our observations.

In conclusion, we demonstrated the positive tenogenic and anabolic effect of PEMF exposure in a 3D healthy tendon explant culture, which is similar to the in vivo clinical setting of the early phases of the hamstring graft maturation process after ACL reconstruction. In this context, we observed a novel PEMF-driven pathway of activation involving c-Fos expression and mTOR signalling. These results have clinical relevance, as PEMF may represent a promising, non-invasive adjuvant treatment for the first postoperative period of ACL reconstruction, to accelerate and enhance the physiological graft maturation and, possibly, the ligamentization process of hamstring tendons. Considering the positive impact of PEMF on tenogenic commitment, we may hypothesize a future clinical translation of our results even for the treatment of other degenerative tendon pathologies, such as Achilles tendinosis and patellar and rotator cuff tendinopathies, in which PEMF may facilitate the tendon regenerative response. Further in vitro and in vivo studies are required to validate this new therapeutic perspective and broaden the applications of PEMF exposure to other tendon pathologies.

Supplementary material

A table showing the mean of the corrected total cell fluorescence values for each patient for scleraxis, c-Fos, mammalian target of rapamycin, and β-catenin; the mean of fluorescence intensity values for collagen type I and collagen type VI; and ratios of proliferating cell nuclear antigen-positive cells versus the total number of cells for nine patients.

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Data sharing

All data generated or analyzed during this study are included in the published article and/or in the supplementary material.

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Ethical review statement

This study was conducted using discarded biological material obtained with patients' informed consent. All samples were irreversibly anonymized, and no identifiable donor information was available to the investigators.

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