Original Article

Optimizing Tenogenic Differentiation of Equine Adipose-Derived Mesenchymal Stem Cells (eq-ASC) Using TGFB3 Along with BMP Antagonists

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Abstract

Objective: Tendon repair strategies usually are accompanied by pathological mineralization and scar tissue formation that increases the risk of re-injuries. This study aimed to establish an efficient tendon regeneration method simultaneously with a reduced risk of ectopic bone formation.

Materials and Methods: In this experimental study, tenogenic differentiation was induced through transforming growth factor- β3 (TGFB3) treatment in combination with the inhibiting concentrations of bone morphogenetic proteins (BMP) antagonists, gremlin-2 (GREM2), and a Wnt inhibitor, namely sclerostin (SOST). The procedure's efficacy was evaluated using real-time polymerase chain reaction (qPCR) for expression analysis of tenogenic markers and osteo-chondrogenic marker genes. The expression level of two tenogenic markers, *SCX* and *MKX*, was also evaluated by immunocytochemistry. Sirius Red staining was performed to examine the amounts of collagen fibers. Moreover, to investigate the impact of the substrate on tenogenic differentiation, the nanofibrous scaffolds that highly resemble tendon extracellular matrix was employed.

Results: Aggregated features formed in spontaneous normal culture conditions followed by up-regulation of tenogenic and osteogenic marker genes, including *SCX, MKX, COL1A1, RUNX2,* and *CTNNB1. TGFB3* treatment exaggerated morphological changes and markedly amplified tenogenic differentiation in a shorter period of time. Along with TGFB3 treatment, inhibition of BMPs by GREM2 and SOST delayed migratory events to some extent and dramatically reduced osteo-chondrogenic markers synergistically. Nanofibrous scaffolds increased tenogenic markers while declining the expression of osteo-chondrogenic genes.

Conclusion: These findings revealed an appropriate *in vitro* potential of spontaneous tenogenic differentiation of eq-ASCs that can be improved by simultaneous activation of TGFB and inhibition of osteoinductive signaling pathways.

Keywords: Bone Morphogrnic Protein, Horse, Mesenchymal Stem Cells, Tenogenic Differentiation, Transforming Growth Factor Beta

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Introduction

Tendon injuries are considered the most frequent orthopedic challenges of all musculoskeletal injuries in horses. Among them, superficial digital flexor tendon (SDFT) injuries commonly occur in racehorses, which is associated with considerable time and cost (1). Tendon is a hypo-vascular and hypo-cellular tissue naturally leading to a low metabolic activity rate which, in turn, slows down spontaneous tendon repair. Autonomous tendon regeneration is accompanied by abnormalities, not only in cellular phenotypes but also in extracellular matrix (ECM) composition. These abnormalities may result in scar formation and ectopic calcification, leading to elastic mismatch and mechanical complications followed by tendon rupture (2).

Conventional medical interventions are mainly intended to control pain or destroy damaged tissue that results in loss of functionality. In recent years, cell therapy strategies using stem or progenitor cells have received a great deal of attention to improve the efficiency of tendon regeneration, particularly in horses (3). However, the relatively high risk of ectopic bone formation, and potential migration of transplanted mesenchymal stem cells (MSCs) from the site of transplantation, suggests that *in vitro* tenogenic induction before transplantation can improve the healing process and avoid the risks mentioned above (4). Adipose-derived mesenchymal stem cells (ASCs) have attracted a great deal of attention

for cell-based regeneration strategies because of their abundance and easy accessibility and their ability to secrete both tendon ECM components and cytokines (5). Indeed, different growth factors [such as TGFB, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor-I (IGF1)] have been applied extensively to overcome challenges of routine cell therapy strategies (6). Platelet-rich plasma (PRP) has become a popular approach in cell-based therapies, both in humans and animals, because of its exceptional level of growth factors (7).

Several lines of evidence support the pivotal regulatory role of transforming growth factor B (TGFB) superfamily either during the development of musculoskeletal differentiation or tissue repair via TGFB and/or bone morphogenetic proteins (BMP) signaling pathways. Three well-known ligand isoforms of the TGFB family (TGFB1, TGFB2, and TGFB3) affect cellular activities via canonical (SMADs 2/3-dependent) or non-canonical pathways (8). The precise regulation of these alternative pathways is highly dependent on cell type, culture conditions, and possible cross-talk with other signaling pathways, which in turn control the overall performance of stem cells (9).

TGFB is a pivotal factor for tendon generation in such a way that mutation in either the ligands (TGFB2 and TGFB3) or the receptor (TBR2) prevents tenogenesis during the early stages of embryogenesis (10). During natural tendon healing, TGFB1 normally reaches the peak of expression earlier, while TGFB3 is expressed through the late stages (11). Given the importance of the TGFB family in tendon development and repair, most of the applied regenerative approaches trigger the TGFB signaling pathway as the critical regulator of musculoskeletal lineage (12). All three known isoforms of TGFB revealed a positive effect on viability and cologne synthesis during tendon healing (13). Furthermore, it is evident that TGFB1 causes scar tissue formation, whereas TGFB3 reduces its creation (14).

Although TGFB has many important roles in tendon development and differentiation, it also regulates chondrogenic and osteogenic differentiation in which the balance between TGFB and BMP signaling is important (15). In general, BMP signaling restricts SCX expression as the key trigger signal for tenogenic differentiation. Phosphorylation of BMP receptors activates intracellular downstream SMADs (SMADs 1/5/8) that form a stable complex associated with SMAD4, which then translocate into the nucleus and regulate several target genes such as runt-related transcription factor 2 (*RUNX2*). BMP signaling plays a crucial role in bone formation, and the knockdown of its receptor (*BMPR2*) inhibits osteogenesis in MSCs (16). The positive impact of some BMP family members (BMP11, 12, and 13) on tendon differentiation is also reported (17).

BMP antagonizing appears to increase *SCX* expression, accompanied by reduced calcification (18). Among various BMP antagonists, gremlin-2 (GREM2) presents the notable potential to bind and inhibit BMP-2 and BMP-4 efficiently and block the activation of Smad1/5/8. Furthermore, sclerostin (SOST) is also a BMP antagonist with a higher binding affinity to BMP 5/6/7 than BMP2/4 (19). However, SOST is not accepted as a classical BMP inhibitor but more propounded as an antagonistic ligand for Wnt coreceptor LDL-receptor-related protein5 and 6 (LRP5 and LRP6). Indeed, SOST can effectively promote catenin beta1 destruction and down-regulation of the canonical Wnt target genes (20). Wnt signaling pathway provides effective crosstalk with the BMPs and is involved in bone formation and homeostasis. It has also been reported that canonical Wnt mediators are overexpressed along with tendinopathy injuries (21).

Besides chemical stimulation, scaffolds also provide a promising strategy for regulating various cellular behaviors, such as survival, proliferation, and differentiation (22). There are two common members of the poly (hydroxyalkanoates) (PHAs): Poly (3-hydroxybutyrate) (PHB) and its copolymer with 3-hydroxyvalerate (PHBV). They are known as biological polyesters produced by microorganisms and used as the intracellular energy source. So far, PHB/PHBV nanofibrous membranes have been widely investigated to regenerate connective tissues (23). In fact, PHB /PHBV nanofibrous scaffolds attract significant attention to tendon tissue engineering due to their adaptable mechanical properties, long-term biodegradation, and relatively similar structure to tendon ECM (24). It has also been reported that incorporating collagen into nanofibrous scaffolds effectively improves the biological responses involved in tenogenesis (22).

Our recent data demonstrated that eq-ASCs had propounded inherent potential to differentiate towards musculoskeletal lineage amplified in response to TGFB3 treatment (25). Regarding the critical role of TGFB during the tendon development and repair, we investigated how inhibition of the BMP signaling pathway along with TGFB activation can modulate the exclusive tenogenic differentiation of eq-ASCs with the elimination of undesirable osteo-chondrogenesis. This goal was followed through the stepwise treatment of eq-ASCs by TGFB3 and a combination of GREM2 and SOST. Furthermore, we investigated the impact of PHB /PHBV/ COL1 nanofibrous scaffold on improving the tenogenic differentiation status of eq-ASCs.

Materials and Methods

Eq-ASCs isolation, Culture, and Characterization

Subcutaneous adipose tissue samples were obtained from a 2-years-old female horse, and ASCs were isolated, characterized, and cultured under normal conditions (5% CO_2 and 37°C), as previously described. Briefly, harvested fat tissue was washed with PBS solution and enzymatically digested using 0.075% collagenase type I. Cell suspension were filtered through the 40 μm nylon

mesh and cultured under Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% P/S. Eq-ASCs were characterized in passage 3, considering the minimal criteria of MSCs (25). For further analysis, ADSCs in passages 3–5 were applied. The procedures used in this study to obtain primary adipose stem cells from the horse were approved by the Ethics Committee of the Royan Institute (IR.ACECR.ROYAN.REC.1395.9). All methods were performed in accordance with relevant guidelines and regulations. Unless otherwise noted, all chemical reagents were obtained from Gibco (Paisley, UK).

Appropriate dose selection for bioactive factors

The effective dosages of recombinant human TGFB3 (R&D System, Minneapolis, Minnesota, USA) were assessed based on its ability to induce *SXC* expression following one and three days treatment with 2.5-10 ng/ml of TGFB3 (26). Also, to determine the proper concentration of BMP antagonists for eq-ASCs, dose-response studies were conducted concerning related reports on the different types of MSCs (27, 28). Following one day of treatment with TGFB3 (2.5 ng/ml), the inhibitory potential of recombinant mouse GREM2 (R&D System, Minneapolis, Minnesota, USA) was evaluated at concentrations of 100 and 200 ng/ml. Also, recombinant human SOST (R&D System, Minneapolis, Minnesota, USA) was treated at the range of 250 to 2250 ng/ml for further two days following TGFB treatment.

Tenogenic induction in eq-ASCs

In order to induce tenogenic differentiation, cells were plated at a density of 2×10^4 cells/cm^2 in standard culture conditions. On a sub-confluent state (70-80% confluency), eq-ASCs were treated with the various combination of factors described in Table S1 (See Supplementary Online Information at www.celljournal.org). The morphological feature of cells was precisely monitored during 10 days of induction using an inverted microscope (Olympus, Tokyo, Japan), and cells were harvested for further analysis after 3, 7, and 10 days.

RNA isolation and gene expression analysis

RNA extraction of the cells was carried out using TRI reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and complementary DNA (cDNA) was synthesized by cDNA Synthesis kit (Fermentas, Waltham, Massachusetts, USA) according to the manufacturer's description. Subsequently, the qRT-PCR assay was performed on the ABI Step One Real-Time PCR System (Applied Biosystem, Foster City, California, USA) with SYBR green master mix (TaKaRa Bio Inc, Kusatsu, Shiga Prefecture, Japan). Data analysis was conducted using the $2^{-\Delta\Delta Ct}$ method. *GAPDH* was used as the internal control gene, and the gene expression level was normalized to untreated cells (day 0). The information for specific primers used to evaluate the target genes is provided in Table S2 (See Supplementary Online Information at www.celljournal.org) .

Immunocytochemical assay

Cells were fixed in 4% paraformaldehyde on days 3, 7, and 10,, permeabilized with 0.2% triton x-100, and were blocked with 10% goat serum prior to incubation. So, they were washed and incubated with the primary antibodies against SCX (Abcam, Cambridge, UK), MKX (Aviva Systems Biology, San Diego, California, USA) as well as control immunoglobulin G (Abcam, Cambridge, UK). Specific stained antigens were then labeled with FITC-conjugated secondary antibody (Sigma-Aldrich, St. Louis, Missouri, USA). In addition, nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, Missouri, USA). Further imaging was performed under fluorescence microscopy (BX51, Olympus, Tokyo, Japan). For quantitative SCX and MKX, corrected total cell fluorescence (CTCF) was determined using Image J and calculated through subtracting the background using the following formula:

CTCF=Integrated density-(area of interest×mean background fluorescence).

Western blotting

T/G and T/G/S treated cells were lysed after 3, 7, and 10 days using TRI reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer protocol. Total protein concentration was measured by Bradford assay. Afterward, equal amounts of each sample (30 μg) were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and blotted onto PVDF membrane (Bio Rad). Blots were blocked with 10% skim milk (Millipore) and incubated with anti-SCX (Abcam, ab58655), anti-MKX (Aviva Systems Biology, ASB-ARP32574-P050), and anti-β-Actin (Santa Cruz, sc-47778) antibodies for 2 hours at room temperature. Then, membranes were incubated for 1 hour at room temperature with an appropriate secondary antibody: horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Dako) or HRP-conjugated mouse anti-rabbit IgG (Santa Cruz). Finally, the protein bands were visualized by an Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare).

Sirius red staining

For Sirius Red staining, induced eq-ASCs at three-time points (3, 7, and 10 days) were rained with PBS and fixed with 4 % paraformaldehyde. Next, deposited collagens were stained with 0.1% Sirius Red F 3B (Direct Red 80, Sigma-Aldrich, St. Louis, Missouri, USA) in a saturated aqueous solution of picric acid for 1 hour at room temperature. In order to quantify, nodules were solubilized with 1:1 (v/v) 0.1% NaOH and absolute methanol for 30 minutes at room temperature, and related absorbance was measured at 540 nm by a spectrophotometric plate reader (Stat Fax 3200, Awareness Technology, Inc., Palm City, Florida, USA).

Phalloidin staining

The morphology of the eq-ASCs on the scaffolds was evaluated with phalloidin staining of the actin cytoskeleton. Briefly, cells were fixed in formaldehyde 4%, permeabilized using triton 0.2%, and sequentially stained with the phalloidin-TRITC label (Tetramethylrhodamine B isothiocyanate; Sigma) and 4′, 6-diamidino-2-phenylindole (DAPI, Sigma). Finally, fluorescence images were obtained using fluorescence microscopy (Olympus, BX51, Japan).

MTS assay

The viability and proliferation rate of the cells on the nanofiber scaffold was evaluated using MTS assay after one, three, and five days. To assess the metabolic activity at each time point, the medium was replaced with the fresh medium containing 10% MTS stock solution (Promega, WI, USA) and incubated at 37˚C for 3.30 hours. Finally, net absorbance was measured at 450 nm with a Microplate Reader (Fluostar Optima, BMG Lab Technologies, Germany).

Cell seeding on the scaffold

A previously characterized aligned PHB /PHBV/Col electrospun nanofiber scaffold (a kind gift from Dr. Elaheh Masaeli, Royan institute for biotechnology, Isfahan, Iran) (29) was used to mimic the natural microenvironment of tendon tissue. The scaffolds were sterilized by immersion in 70% ethanol for 2 hours, washed twice with PBS, and incubated overnight in the basic cell culture medium. To evaluate the potential of scaffolds for amplifying tenogenic response in eq-ASCs, the cells were seeded at a density of 2.5×10^4 cells/cm^2 and induced with the optimum concentrations of T/G/S factors. The eq-ASCs cultured on the tissue culture plate (TCP) and treated under similar conditions were considered as control. Tenogenesis was assessed after 10 days via analysis of the expression of specific tenogenic markers (*SCX*, *MKX*, *COL1A1*) and osteo-chondrogenic genes (*SOX9*, *BMPR2*, *RUNX2*, *CTNNB*).

Statistical analysis

At least three independent experiments were performed for all assays. Statistical analysis was carried out using SPSS 16.0 software (Inc., Chicago, USA) by two-way analysis of variance (ANOVA) followed by 'Tukey's post hoc test. P<0.05 were considered statistically significant for all the experiments.

Results

Finding the proper concentration of inducing factors

Regarding the specific function of TGFB3 in triggering musculoskeletal specification, we assessed the most effective concentration of TGFB3 on eq-ASCs based on the expression of *SCX*. 2.5 ng/ml was defined as the optimal dose for TGFB3, resulting in maximal expression of *SCX* on dayone (Fig.1A). In addition, we demonstrated that pre-treatment with 2.5 ng/ml of TGFB3 followed by 200 ng/ml of GREM2 preserves the increased *SCX* expression level on day 3 (Fig.1B). In contrast, in the absence of this pre-treatment with TGFB3, GREM2 significantly reduced the *SCX* expression (P<0.05, Fig.S1A, See Supplementary Online Information at www.celljournal.org). Moreover, a high *SCX* level was achieved by 1250 ng/ml SOST treatment accompanied by significant inhibition of *CTNNB1,* as a sign of osteogenesis (Fig.1C, D). Therefore, treatment with 2.5 ng/m of TGFB3, 200 ng/m of GREM2, and 1250 ng/ml of SOST were chosen as the optimal condition to achieve the highest *SCX* expression and used for the remaining experiments.

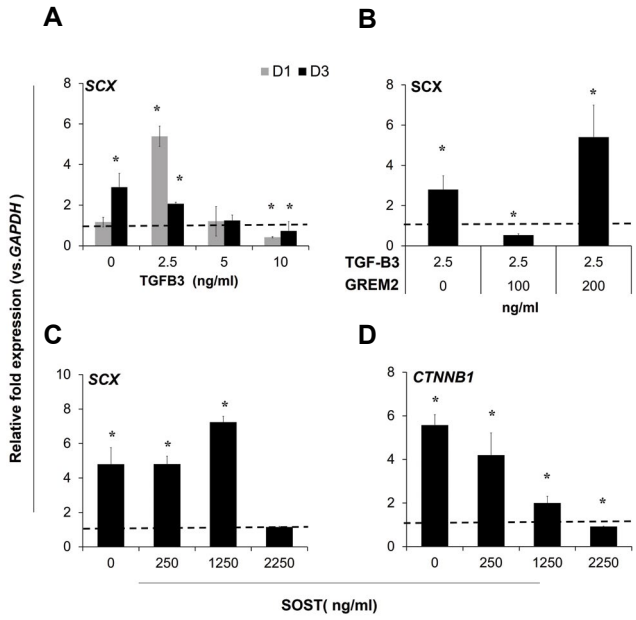

Fig.1: Concentration-dependent effect of bioactive factors used to induce tenogenic differentiation. **A.** qRT-PCR analysis of *SCX* following treatment of eq-ASCs with different concentrations of TGFB3 (0-10 ng/ml) for one and three days and **B.** GREM2 (100 and 200 ng/ml) for a further two days, **C.** *SCX* revealed the highest expression level in 2.5 ng/ml TGFB3 and 200 ng/ml of GREM2. In response to serial five-fold dilution of SOST (250-2250 ng/ml), *SCX* peaked at 1250 ng/ml, and **D.** The expression level of *CTNNB1* gradually decreased. All data were normalized to *GAPDH* and presented as mean ± SD. *; Present significant changes vs. day 0 untreated cells (dashed line), P<0.05 and qRTPCR; Real-time polymerase chain reaction.

Effect of different factors on tenogenic differentiation of eq-ASCs

The untreated cells started to migrate on day 7 and formed compact aggregations after 10 days of being cultured under normal conditions (Fig.2A). However, after TGFB3 treatment (T) the aggregates were observed on day 3, and followed by cell death. In contrast, following pre-treatment for one day with TGFB3 and then with GREM2 treatment (T/G), cells spread out of aggregates and formed a monolayer. We also found that following pre-treatment with TGFB3

for one day and then with GREM2 and SOST (T/G/S), an intermediate morphological feature between T and T/G was observed after 10 days.

Assessment of expression of genes related to tenogenesis showed a significant up-regulation of *SCX* on day 3 in T/G group (P<0.05), which gradually declined over time (Fig.2B). *MKX* expression on day 3 was significantly lower in T compared to the control group (0.2 vs. 0.86, P<0.05), while *MKX* expression significantly increased in T/G and T/G/S compared to the control group (2.23 vs. 0.86, P<001; 3.8 vs. 0.86, P<0.0001 respectively, Fig.2C). *MKX* expression was also significantly reduced on days 7 and 10 in T/G/S group compared to the control group (0.35 vs. 1.6 P<0.0001, and 0.21 vs. 0.99 P<0.004). *COL1A1* expression was significantly increased on days 3, 7, and 10 in the T/G/S group in comparison with the control group (1.3 vs. 0.2, P<0.0001; 1.35 vs. 0.37, P<0.000, 0.79 vs. 1.27, P<0.002). While, COL1A1 expression in T/G group was significantly higher than the control group on day 3 (0.84 vs. 0.2, P<0.0001), the expression of this gene gradually declined by days 7 and 10. Despite this reduction, *COL1A1* expression was still significantly higher than the control group on day 7 (0.73 vs. 0.37, P<0.05). Although, it was significantly decreased and reached to a values lower than the control group by day 10 (0.02 vs. 1.27, P<0.000, Fig.2D). Nevertheless, *TNMD* expression could not be detected in any of the groups.

Regarding chondrogenic markers, *SOX9* expression level remained just below the baseline in standard culture conditions at all-time points. Although its expression fluctuated in response to TGFB3, except for a slight increase in T/G group on day 3, the value of *SOX9* did not markedly change in T/G and T/G/S (Fig.2E). The expression of *CTNNB* showed a gradual increase in the control and T/G group, while it dropped sharply in the T group and relatively in T/G/S group (Fig.2F). Figure 2G reveals a low level of *BMPR2* expression with a periodic mode in the control group, while it leveled out in the T group and dramatically decreased in T/G. Surprisingly, we found a primary increase in *BMPR2* in response to T/G/S induction, which returned to its basal level before induction. *RUNX2* exhibited a similar expression pattern to *BMPR2* in standard culture conditions, which was amplified by TGFB3 treatment and leveled out in response to GREM2 (T/G group). In addition, it was strongly inhibited by T/G/S treatment over time from day 3 to day 10 (Fig.2H).

Interestingly, treatment of cells with GREM2 without pre-treating with TGFB3, not only reduced the expression level of *SCX,* as a tenogenic marker (Fig. S1A, See Supplementary Online Information at www.celljournal.org), but also significantly increased osteo-chondrogenic markers as the time went by (Fig.S1B, See Supplementary Online Information at www.celljournal.org). Additionally, cellular aggregation in this group (Fig.S1C, See Supplementary Online Information at www.celljournal.org) was more than that in the control group, while it was delayed compared to the other groups like T, T/G, and T/G/S (Fig.2A).

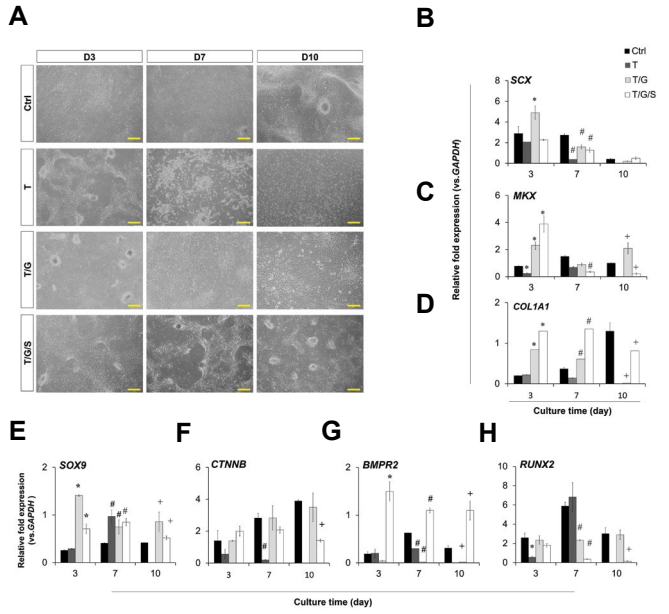

Fig.2: Morphological and gene expression analysis in response to the various combination of inducing factors. **A.** Phase-contrast photographs on three-time points (day 3, 7, and 10) presented notable morphological changes in eq-ASCs in all test groups (T, T/G, and T/G/S) as well as in control (scale bar: 400 µm). **B-D.** Commonly, migratory features consist of aggregate formation and appearance of empty spaces, which were different between groups and at different times. qPCR analysis for the evaluation of tenogenic-related genes (*SCX*, *MKX,* and *COL1A1*) in eq-ASCs cultured under different conditions revealed a remarkable expression level, particularly in T/G/S group. **E-H.** Analysis of the expression of chondrogenic (*SOX9*) and osteogenic-related genes (*CTNNB*, *RUNX2,* and *BMPR2*) showed a dramatic reduction in response to T/G/S treatment, except for *BMPR2.* Data were normalized to *GAPDH* and presented as mean ± SD. *, #, +; Present significant (P<0.05) changes vs. T/G group at the day 3, 7, and 10, respectively, T; Treatment with TGFB3 (2.5 ng/ml) for one day, T/G; Pretreatment with TGFB3 (2.5 ng/ml) followed by GREM2 (200 ng), T/G/S; Pretreatment with TGFB3 (2.5 ng/ml) followed by GREM2 (200 ng/ml) and SOST (1250 ng/ml), and qPCR; Real-time polymerase chain reaction.

Expression of tendon-associated proteins in T/G vs. T/G/S culture conditions

In addition, the protein profile of two specific markers related to tenogenesis, SCX, and MKX, were investigated following T/G and T/G/S treatment which had the most significant effect on the tenogenic differentiation status of eq-ASCs. Figure 3A, B shows the strong nucleus signal (green stain) of SCX on day 3, both in T/G and T/G/S groups, which gradually declined until day 10; this reduction appeared to delay in the T/G/S. On the other hand, the expression of MKX was facilitated in T/G/S compared to the T/G group (Fig.4A, B), which was verified by quantification of SCX and MKX intensity (Figs.C3, C4). The results are consistent with the expression pattern of SCX and MKX presented by western blot analysis (Figs.3D, 4D). The targeted protein bands were visualized at 22 KDa for SCX and 39 KDa in the case of MKX and β-actin.

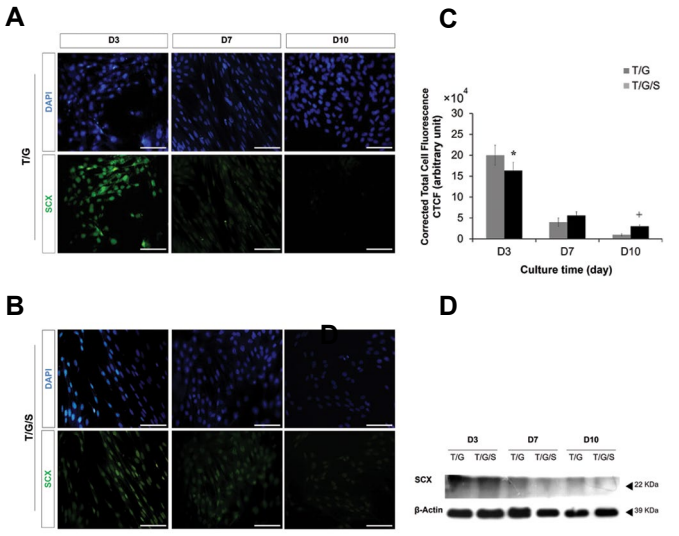

Fig.3: Immunocytochemistry assay to assess expression pattern of SCX. **A, B.** Immunofluorescence images showed a considerable level of SCX (green) positive cells, especially on day 3, in both T/G and T/G/S treated groups, which was gradually reduced by day 10. **C.** Nuclei were counterstained with DAPI (scale bar: 50 µm). Quantification assay results are presented as corrected total cell fluorescence (CTCF) for SCX in both T/G and T/G/S treated groups and presented as mean ± SD, *, +; Present significant (P<0.05) changes vs. T/G group at the day3 and 10, respectively. **D.** Western blotting analysis confirmed the expression pattern of SCX in T/G and T/G/S during the treatment period. T/G; Pretreatment with TGFB3 (2.5 ng/ml) for one day followed by GREM2 (200 ng/ml), T/G/S; Pretreatment with TGFB3 (2.5 ng/ml) followed by GREM2 (200 ng/ml) and SOST (1250 ng/ml).

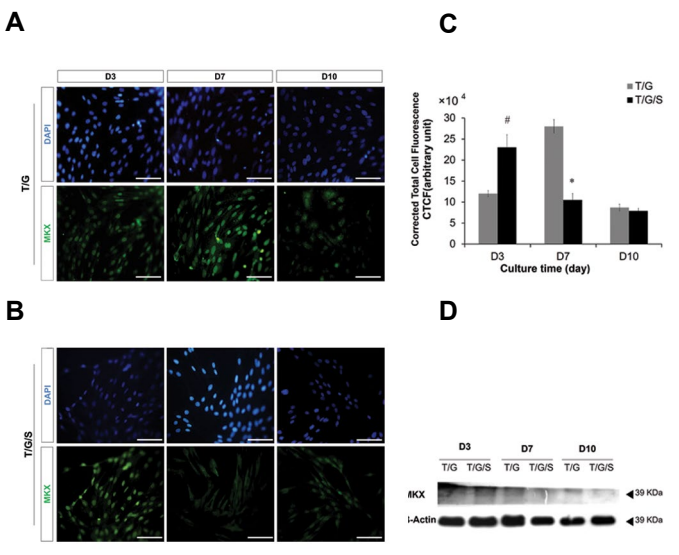

Fig.4: Immunocytochemistry assay to assess expression pattern of MKX. **A.** Immunofluorescence images showed that MKX (green) reached the maximum level on day 7 in T/G group (scale bar: 100 µm), **B.** but was extensively expressed on day 3 in T/G/S treated eq-ASCs. Nuclei were counter-stained with DAPI (scale bar: 100 µm). **C.** Quantification assay results are presented as corrected total cell fluorescence (CTCF) for MKX in both T/G and T/G/S treated groups(c) and presented as mean ± SD. *, #, +; Present significant (P<0.05) changes vs. T/G group at days 3, 7 and 10 respectively. **D.** The western blotting analysis confirmed the expression pattern of MKX in T/G and T/G/S during the treatment period. T/G; pretreatment with TGFB3 (2.5 ng/ml) for one day followed by GREM2 (200 ng/ml), T/G/S; pretreatment with TGFB3 (2.5 ng/ml) followed by GREM2 (200 ng/ml) and SOST (1250 ng/ml).

Moreover, Sirius Red staining was applied to investigate the collagen content as the major component of the tendon matrix. All groups were positive for this staining (Fig.5A). However, the quantitative analysis of the stained imagesrevealed that the maximum collagen content was observed in the T/G/S group, which was significantly higher than the control group on days 3 and 7 (P<0.05). In contrast, collagen content significantly decreased in the T/G group compared to the control group by day 10 (Fig.5B). Furthermore, the considerable amount of collagen accompanied by results of gene expression profile analysis in the control group confirmed the spontaneous tenogenic differentiation potential of eq-ASCs. Expression of tenogenic markers in the control group showed up-regulation of tendon progenitor marker (*SCX*) at mRNA level on day 3 versus day 1 (1.17 vs. 2.8, P<0.02) and late differentiation marker (*MKX)* on day 3 versus day 7 (0.77 vs. 1.6, P<0.002) followed by increasing of *COL1A1* expression on day 10. However, their tenogenic potential is limited due to the up-regulation of osteo- chondrogenic genes (Fig.2F-H).

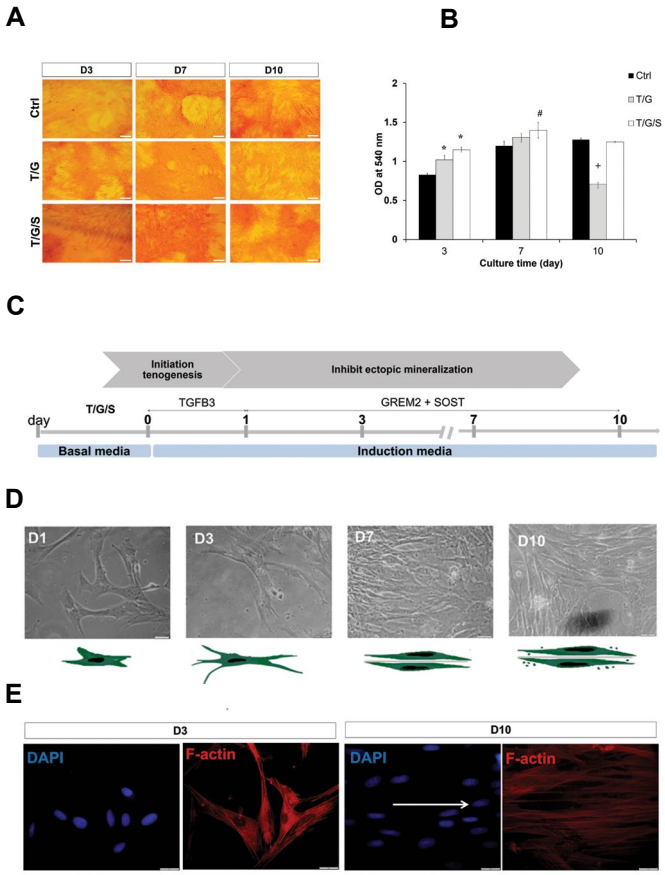

Fig.5: Eq-ASCs during stepwise tenogenic differentiation. **A.** Accumulation of collagen fibers in the extracellular matrix was shown in red, which was gradually increased over induction time, specifically in T/G/S group (scale bar: 200 µm). **B.** Quantification analysis of Sirius Red stained samples at 540 nm by spectrophotometric plate reader significantly showed the highest collagen secretion in T/G/S. Data are presented as mean ± SD. *; Present significant changes vs. control sample of the same day, P<0.05. Ctrl; control, T/G; Pretreatment with TGFB3 (2.5 ng/ml) followed by GREM2 (200 ng), T/G/S; Pretreatment with TGFB3 (2.5 ng/ml) followed by GREM2 (200 ng/ml) and SOST (1250 ng/ml). **C.** Flow chart illustrates the experimental design for stepwise induction of Eq-ASCs into tenocytes through biological factors stimulation. **D.** Morphological changes of eq-ASCs during differentiation to tenocytes under an inverted microscope with their schematic presentation. **E.** Fluorescence images of TRITC-phalloidin staining showed morphological changes of eq-ASCs at the early (day 3) and late (day 10) time point of induction. The arrow highlights the alignment of the cell (scale bar: 20 µm).

Expression of tendon-specific markers is associated with morphological changes of eq-ASCs

In addition to tenogenic marker expression, the morphologic features for tenocytes were also assessed in the best group (T/G/S) according to mRNA and protein expression profiles. Phase-contrast photographs at different time points (day 1, 3, 7, and 10) indicated that we achieved tenocytes based on cell shape appearance within 7 days of induction by treatment with 2.5 ng/m of TGFB3 for one day, followed by 200 ng/m of GREM2 and 1250 ng/ml of SOST, as stepwise tenogenic differentiation approach (Fig.5C, D). Furthermore, phalloidin staining exhibited an ovoid nucleus with large cytoplasmic and protrusive structures at the early stage of induction (day 3). Elongated nuclei and thin cytoplasmic are observed at the late time point (day 10). The cells displayed a few prolonged stress fibers on day 3 which gradually became longer along with the aligned cell morphology (Fig.5E), which confirmed morphological changes under inverted microscopy (Fig.5D).

Cell - cytotoxicity assay for scaffold using eq-ASCs

The cell viability, cell growth, and cell morphology were used as parameters to determine the cytotoxic effect of the scaffolds. Visualization of the F-actin cytoskeleton to assess the morphology was done by phalloidin staining after 3 days of culture. The fluorescence images demonstrated that eq-ASCs had well attached and spread on the scaffold; additionally, an aligned cell orientation was observed (Fig.6A,B). Interestingly, MTS assay after 1, 3, 5 days of cell culture on nanofiber displayed no significant difference ($P<0.05$) compared with tissue culture plates (TCP) (Fig.6C).

Effect of scaffold on the expression of tenogenic markers in eq-ASCs treated with T/G/S

To evaluate the influence of the substrate on the tenogenic differentiation status, we analyzed the expression levels of the genes mentioned above in eq-ASCs treated with T/G/S in the presence and absence of scaffold. According to our findings, tenogenesis was induced on PHB/PHBV/COL1 membranes under sequential treatment with T/G/S factors. In comparison with T/G/S group, we showed a relatively lower level of expression of tenogenic markers at the earliest time point (day 3) in the scaffold group, while it provided higher and more stable expression on days 7 and 10 (Fig.6D-F). It is also interesting to note that the expression of osteo-related genes (BMPR2, RUNX2, and CTNNB) had significantly reduced in the scaffold group. The only exception was the expression of the chondrogenic marker *SOX9* which had fluctuated (Fig.6G-J).

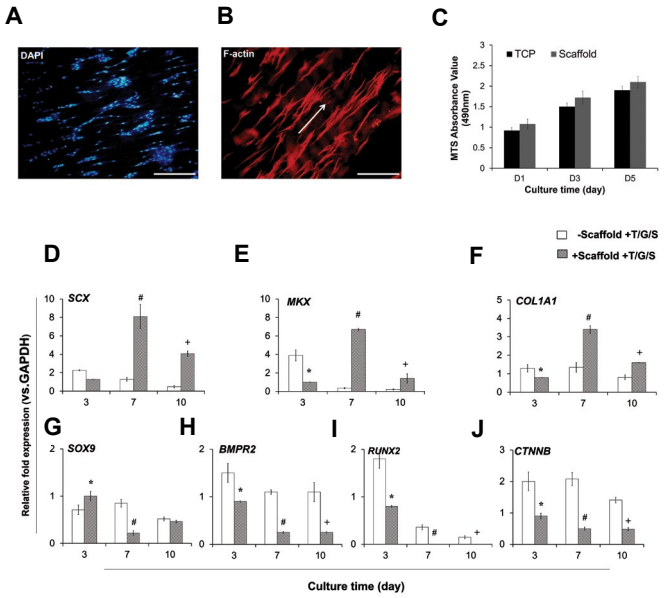

Fig.6: Cell culture and tenogenic differentiation of eq-ASCs on nanofibrous PHB/PHBV/COL1 scaffold. **A, B.** The phalloidin staining of the actin cytoskeleton (red) after 3 days of culture showed the attachment and growth of the eq-ASCs on the scaffolds. Nuclei were counterstained with DAPI (blue) (scale bar: 200 µm). **C.** MTS assay indicated that there was no significant difference in viability of eq-ASCs in the presence and absence of nanofiber scaffold on day 1, 3, and 5 of the culture. The arrow highlights the alignment of cells. Data were normalized to the ratio absorbance of scaffold and medium without cells and presented as mean ± SD. *P<0.05. **D-F.** QPCR analysis of tenogenic markers expression and **G-J.** The osteochondrogenic markers in eq-ASCs treated with T/G/S for 10 days. eq-ASCs were seeded on the scaffold and treated with T/G/S showed increased expression of *SCX*, *MKX*, *COL1A1, SCX*, *MKX,* and *COL1A1,* and decreased expression of *SOX9, CTNNB*, BMPR2, and *RUNX2* compared to T/G/S group without scaffold. Data are presented as mean ± SD. *, #, +; Present significant (P<0.05) changes *vs.* control at days 3, 7, and 10, respectively. T/G/S; Pretreatment with TGFB3 (2.5 ng/ml) followed by GREM2 (200 ng/ml) and SOST (1250 ng/ml).

Discussion

Despite recent advances in cell therapy for tendon injuries, conventional strategies for regenerating functional tendons are often inefficient. Therefore, *in vitro* modification for improving the efficacy of cell therapy appears to be highly reasonable before clinical application. We recently showed that eq-ASCs have inherent differentiation capacity to musculoskeletal-lineage (26). In the present study, we focused on investigating an efficient procedure for specific tenogenic induction of eq-ASCs with particular attention to reducing the chance of osteogenesis.

During embryogenesis, transcription factors, such as scleraxis (SCX) and sex-determining region Y-box 9 (SOX9), play a crucial role in the formation of tendon progenitors, whereas Mohawk (MKX) and early growth response protein 1/2 (EGR1/2) are required for terminal tendon differentiation. These transcription factors are involved in tenocyte specification and coding ECM components such as collagen type I (*COL1A1*) and tenomodulin (*TNMD*) as a late marker of tendon development (30). Therefore, we initially estimate the effective concentration of TGFB3 that induced higher expression of *SCX*. Since the highest expression level of

SCX was achieved on day 1, for further experiments, we treated the cells with TGFB3 only for one day.

Literature supports the critical impact of TGFB3 ligands on the up-regulation of tendon-associated markers, including *MKX* and *COL1,* via up-regulation of SCX (31). The relative expression of these markers was decreased upon the continuation of treatment with TGFB3 within the following days. This is not unexpected as the expression of SCX was reduced by further treatment with TGFB3 (after day one). Indeed, similar to our results, it has been shown that a high concentration of TGFB over time negatively affects tenogenesis (32). Therefore, we decided to treat the eq-ASCs only with TGFB3 for one day based on these results.

These results confirmed that TGFB3 efficiently triggered tendon specification of eq-ASCs while achieving a stable and final tenogenic differentiation status requires complementary signals. It is important to note that ectopic mineralization, as a major hurdle to tendon regeneration, takes place in the absence of these secondary signals. Besides, BMP treatment has an inhibitory effect on *SCX* expression, which was almost reversed by noggin (18). Hence, we focused on inhibiting the BMP signaling pathway by using two well-known BMP antagonists, GREM2 and SOST, following one-day treatment or induction with TGFB3.

Initially, we showed that short-term treatment with GREM2 for one day and assessment of SCX on day 3 did not improve SCX expression compared to the control group. Therefore, we decided to evaluate the effect of TGFB3 for one day, followed by GREM2 from days 2-10. Our result revealed that the expression of SCX, MKX, and COL1A1 was increased on day 3, followed by a reduction during additional days. The only signal in osteo-chondrogenic differentiation was reduced through treatment with TGFB3 followed by GREM2 was RUNX2. Therefore, these results suggest that other secondary signals are required to improve tenogenic differentiation.

Several lines of evidence verified SOST as an impressive BMP antagonist, which also negatively regulates the Wnt signaling pathway and inhibits osteogenesis (32). Therefore, it was not surprising that the severe inhibition of BMPR2 induced by GREM2 was partially reversed in the presence of SOST, which seemed to recover the BMP pathway to some extent. This is consistent with the observed drastic down-regulation of *RUNX2* by the combinational treatment with G/S over time (day 10), along with the reduced *CTNNB* level. Although SOX9 is known as a cartilage-associated marker, previous studies have suggested that a basal level of *SOX9* expression is required in a distinct subtype of tendon progenitor cells (33). We also found a primary overexpression of *SOX9* in T/G and T/G/S groups, which declined over time.

Regarding tenogenic related genes, T/G treated cells showed the highest expression level of SCX, while MKX showed its highest expression in T/G/S group, both on day 3. Considering the primary high COL1A1 expression level in untreared cells, T/G/S showed aoitential to keep this level constant which underscored the importance of SOST in tenogenic induction. These results are consistent with the data obtained by Sirius Red staining showing almost the same manner for the secretion of collagen fibers as the major extracellular content in the tendon.

SCX is a basic helix-loop-helix transcription factor that plays a critical role in the early stage of development in tenocytes and other dense connective tissues, like periodontal ligaments and heart valve (34). Regarding the force transmitting function of these cells, SCX is required for modulating cytoskeletal tension via triggering the expression of proteins linked between actin filaments and integrin molecules (35). Nichols et al. (36) reported that equine tenocytes transfected with *SCX* siRNA faced a remarkable decrease in cytoskeletal stiffness and inability to migrate on soft surfaces. Moreover, several tendon-specific genes, such as *MKX*, *COL1,* and *TNMD,* are positively regulated in tenocytes by SCX (37).

Unlike SCX, different studies have noticed some severe issues with using TNMD as a reliable marker for tenogenic differentiation in equines, especially at mRNA level. For instance, an almost similar expression level of *TNMD* was detected by Taylor et al. (38) in the 'horse's bone and tendon. Also, Barsby and Guest (26) provided some valuable evidence about the high degradation of TNMD mRNA in TGFB -treated equine ESCs. Similarly, we also did not find TNMD expression in the control and tested groups.

Gene expression analysis was further confirmed at the protein level, which revealed a more stable expression of SCX and facilitated the expression of MKX as the result of T/G/S treatment compared to T/G treatment. Since the morphological feature of cells is still noted as an important sign of their state, the spindle-like shape and aligned arrangement are typically considered for tenocytes, in contrast with the more rounded shape of tenoblasts with a large and oval nucleus. At the end of the induction time, we observed spindle-like cells with elongated nuclei and thin cytoplasmic protrusions.

Overall, based on the results of gene expression analysis, protein expression data, and morphological analysis, we propose a stepwise protocol to induce tenogenic differentiation of eq-ASCs within 7 days which is far shorter than what has been previously reported (14 days) by transient induction with TGFB3 followed by co-inhibition of BMP and Wnt signaling pathways. In addition, one of the main strengths of this procedure was a severe reduction in levels of ectopic osteo and chondrogenesis markers. It has been highlighted in several studies in which the expression of tendon-associated markers were shown to be down-regulated in mature tenocytes (39). However, reaching stable mature tenocytes with proper function in the long term and preventing cellular dedifferentiation requires further modification of the protocol. Therefore, we subsequently examined the effects of nanofibrous PHB /PHBV/COL1 scaffolds on the efficiency and long-

term stability of reported sequential tenogenic induction on eq-ASCs.

Both enhanced and more stable expression of tenogenic markers and the potent inhibition of osteo-chondrogenesis on PHB /PHBV/COL1 membrane proposed the scaffold as a suitable substrate for cell transplantation and tendon regeneration. It seems that incorporating collagen type 1, as the major content of natural tendon ECM, in addition to the fibrous and porous topology of PHB/PHBV, provides a positive impact on tenogenesis (24).

It is interesting to note that the most successful cell therapy approaches were reported in the cases of intra-lesional injection of a cell suspension due to granular tissue and the enclosed nature of core lesions. The use of MSC-derived tenocyte progenitors revealed a notable positive impact on increasing the efficacy of cell therapy for tendon repair (40). Although, for other forms of tendon-related disorders (like eccentric lesions), further studies are required to optimize the condition ,including the use of different types of scaffolds or self-organizing tendon structures (3D tendon-like tissue constructs).

For the first time, we investigated the tenogenic differentiation of equine adipose stem cells under the stepwise inductive approach which provides a safe, valuable alternative for un-induced MSCs for future intra-lesional injection. This result was verified mainly by using the scaffold to maintain a tenogenic status. However, future studies need to be conducted regarding the damaged condition to choose a scaffold which can be practicaly delivered into equine tendon injury.

Conclusion

The present study demonstrated a fast and effective procedure for reducing the risk of ectopic osteogenesis in tendon regeneration strategies. We recently provided evidence that confirmed the remarkable potential of eq-ASCs for spontaneous differentiation towards musculoskeletal lineage due to the high expression of endogenous TGFB1. Here, we established a stepwise inductive approach to induce tenogenesis of eq-ASCs using TGFB3, GREM2, and SOST to overcome this limitation to some degree and achieve tenocytes in a short time. The current study provides new findings for future cell-based therapies to avoid the risk of ectopic bone formation after injection of MSCs in tendon injuries.

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Authors' Contributions

A.Sh., Carried out the experiments and analyzed data. F.E., Conceived the project, interpreted the data, and drafted the manuscript. A.P., M.H.N.-E.; Supervised the research, interpreted the data, and critically revised the manuscript. All authors read and approved the final manuscript.

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