Induction of Tendon-Specific Markers in Adipose-Derived Stem Cells in Serum-Free Culture conditions

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Abstract

Differentiation of stem cells as a cell-based therapy for repairing, replacing or restoring damaged tissues such as bone, cartilage, and tendon is becoming increasingly attractive within the field of musculoskeletal tissue engineering. Towards this end, there are numerous published and well-defined protocols to differentiate stem cells towards cartilage and bone tissues, but the protocols towards tendon tissue are still emerging and thus less developed. Recent studies focused on the induction of tendon-specific markers in cultured stem cells using different Growth Factors (GFs) including Bone Morphogenetic Proteins (BMPs) and Transforming Growth Factor (TGF) isoforms. However, the inclusion of serum in relatively high concentration across these studies is less favorable, since the components within serum may interfere with the induction of the markers. Alternatively, *in vitro* studies with low concentration or absence of serum would be ideal. In this study, we assessed the induction effect of BMP-12 and TGF-β1 on tendon-specific markers in Adipose-Derived Stem cells (ADSCs), in serum-free conditions. Specifically, we investigated the temporal and dosing effects of both GFs on several markers. Our results demonstrate that BMP-12 induces late expression of the transcription factors Scleraxis (SCX) and Mohawk (MKX), whereas TGF-β1 induced their earlier expression. Moreover, BMP-12 induced Decorin (DCN) but was inhibited by TGF-β1. Other markers such as Collagen Iα1 (COL1A1) likewise showed this pattern. Importantly, the protein analysis generally supported the gene expression data. Interestingly, differences were observed in the cellular localisation of SCX between BMP-12 and TGF-β1 stimulations. Furthermore, the addition of Ascorbic Acid (AA) with either BMP-12 or TGF-β1 resulted in increased deposition of Collagen I. Our results enhance the existing protocols for the differentiation of ADSCs towards the tenogenic lineage in serum-free conditions and contribute to the understanding and the development of tenogenic induction protocols.

Statement of significance

Herein we describe the tenogenic effect of BMP-12 and TGF-β1 in cultured ADSCs in serum-free conditions. This culture system provides an insight into serum-free culture conditions in stem cell

differentiation protocols. A positive response of the ADSCs to the tenogenic induction was observed. In particular, the different Growth Factors used in this study displayed notable differences both on the gene and on the protein expression of the tendon-specific markers. The results underline the positive outcome of the serum removal in tenogenic differentiation protocols, contributing to the development of future cell-based therapies for tendon regeneration and repair.

INTRODUCTION

Tendon injuries are a common clinical problem affecting millions of patients worldwide. In less severe cases, conservative treatments target clinical symptoms such as pain or inflammation. However, in severe cases such as tendon rupture surgical interventions are needed, and this may result in prolonged rehabilitation processes. In either case, the affected tissue often fails to fully recover or regain the native tendon functions, leading to pain and inferior tissue quality (1). Moreover, the prevalence of these injuries is likely to set to increase, particularly in the sports community or in the elderly population (2). Therefore, alternative treatments focusing on tissue regeneration, in particular, cell-based therapies, are becoming attractive revenues for next-generation therapeutics.

Towards this end, the application of autologous adult mesenchymal stem cells for tissue regeneration is becoming increasingly popular within the musculoskeletal field (3). This is because of the l ability of stem cells to differentiate to other phenotypes of the same embryologic origin *in vivo*, or under the influence of physical and chemical cues *in vitro* (4, 5). Over the past decade, numerous reports have been published on the application of stem cells in musculoskeletal research, with a particular focus on methods for describing stem cells differentiation and their *in vitro* characterisation, with specific attention to bone and cartilage tissues. However, the methods for the differentiation of stem cells towards tendon tissue are less understood and still emerging.

Recently, several studies have focused on the induction of stem cells toward tenogenic lineage, demonstrating promising results on this front. For example, reports have shown the upregulation of common tendon-specific markers such as Scleraxis (SCX) in *in vitro* cultured Bone marrow-derived Mesenchymal Stem Cells (BMSCs) with a range of Growth Factors (GFs), including Bone Morphogenetic Proteins (BMPs) or Transforming Growth Factor (TGF) isoforms, thus indicating the tenogenic potential of BMSCs (6, 7). However, for the BMSCs to become a clinically viable source of cells, a high volume of them is required. This could pose some concerns, since obtaining BMSCs

requires an invasive surgical procedure (8) and very often the amount of cells collected in each sample is relatively low in numbers, although these can be further expanded in *in vitro* conditions (9).

To address these concerns, researchers have shifted the focus onto the use of Adipose-Derived Stem cells (ADSCs) as an alternative to BMSCs (10). ADSCs can be obtained via lipoaspirates (11) in high volumes (12-15). Preliminary studies have shown the induction of tenogenic-specific markers in cultured ADSCs stimulated with BMPs and TGF isoforms, demonstrating their differentiation potential towards a tenogenic lineage. For example, tenogenic markers including SCX and Mohawk (MKX) were shown to be upregulated in ADSCs induced with BMP-12 and TGF-β1, and the deposition of Collagen type I and several proteoglycans has been reported (6, 14-17). Notably, the use of serum in the published studies is a common feature, raising concerns around the interference of the mixture of proteins and GFs in unknown quantities within serum. Moreover, the use of serum represents a limitation for the clinical applicability of the cell-based therapies to humans. Therefore, and despite the promising results reported so far, the emergence of a well-defined method for differentiation of ADSCs towards tendon tissue is still lacking (6).

Herein, in serum-free conditions, we have investigated the differentiation potential of ADSCs stimulated with BMP-12 and TGF-β1 tenogenic GFs. In particular, we expanded the study characterising the effect of dosing and time-intervals on a variety of tendon-specific markers. Parameters such as the induction of tendon markers and the deposition of a tenogenic Extracellular Matrix (ECM) by the cultured ADSCs were investigated and subsequently characterised by RT-qPCR, Immunostaining (ICC) and Western Blotting techniques, expanding our understanding on the protocols for the differentiation of ADSCs in serum-free conditions towards a tenogenic lineage.

MATERIALS AND METHODS

Cell culture

Human ADSCs from one donor (Catalogue number PT-5006, Lot number 0000439846) were supplied by the manufacturer (Lonza), under ethical conditions after the donor consent. Cells were used in passage 3 for the experiments and grown as monolayers in ADSCs basal Media supplemented with 10% FBS, 1% of L-Glutamine and 0.1% of Gentamicin-Amphotericin as specified by the manufacturer (Catalogue number PT-4505, Lonza). During expansion, ADSCs were grown in Nunc EasYFlasks of 175 and 75 cm^2 culture area (Thermo Fisher Scientific) until 80% of cell confluence was reached, refreshing 70% of the media every four days, in order to supply fresh nutrients to the cells but preserving secreted Growth Factors necessary for cell communication. For the cell seeding, after washing the cells in PBS, cells were detached with Trypsin/EDTA Solution (Lonza) for about 3 minutes at 37 °C and subsequently neutralised with Trypsin Neutralisation Solution (Lonza). Cells were centrifuged prior to counting in order to remove residual Trypsin. For the experiments, three independent cell culture replicas were used.

Study design and ADSCs stimulation

Cells were left to attach for 24h in basal media containing FBS (10%). Monolayers were then washed with PBS and starved overnight (0% FBS). BMP-12 or TGF-β1 were added (10 or 50 ng/ml) in the presence or absence of Ascorbic Acid (50 μg/ml) for determined time intervals. Unstimulated ADSCs grown in starved media were used as a control in all the experiments. All supplements were refreshed every 3 days. All the experiments were performed at the same hour in order to avoid potential circadian variation in the cell response. Schematic representation of the study design is shown in Supplementary Figure 1. Cultures were monitored and photographs were taken using a phase contrast microscope (EVOS XL Core, Thermo Fisher Scientific) with a 10x magnification and processed in ImageJ software.

MTS assay

Cells were cultured in 96-well cell culture plates (Nunc™ surface, Thermo Fisher Scientific) in specific densities (4000 cells/0.3cm^2) and treated with 10 or 50 ng/ml of BMP-12 or TGF-β1 in serum-free media for one, five, seven and fourteen days. 10% of MTS (Promega) was added and incubated at 37°C in the dark for 3 h. Absorbance was measured at 490 nm with a microplate reader (CLARIOSTAR, BMG Labtech). Data are presented as Optical Density (OD) values.

Live and dead Viability Assay

Cells were cultured in in 24-well cell culture plates (Nunc™ surface, Thermo Fisher Scientific) in specific densities (28000 cells/1.9cm^2) and treated with 10 or 50 ng/ml of BMP-12 or TGF-β1 in serum-free media for one, five, seven and fourteen days. Monolayers were washed with PBS and freshly prepared Calcein AM-Ethidium homodimer-1 (Thermo Fisher) solution was added to the monolayers following manufacturer's guidelines. Imaging was performed using a Zeiss Axiovert 200M microscope using Axiovision software. Pictures were taken with a 20x magnification Plan-Neofluar (0.5 NA) objective and a Zeiss AxioCam HRm CCD camera. Fluorescence was excited either at 494-517 nm (Calcein, green) or 528-617 nm (Ethidium homodimer-1, red). Merged images were created in Image J software. Live and dead cells were counted in every picture, using minimum three random locations per sample.

Real-Time quantitative PCR (RT-qPCR)

Cells were cultured in 6-well cell culture plates (Nunc™ surface, Thermo Fisher Scientific) in specific densities (150000 cells/9.6 cm^2) and treated with 10 or 50 ng/ml of BMP-12 or TGF-β1 in serum-free media for one, five, seven and fourteen days. Monolayers were lysed with Tri Reagent solution (Thermo Fisher). Total RNA was separated into the aqueous phase with 1-bromo-3-chloropropane (Sigma), precipitated with Isopropanol and washed with Ethanol at 4°C. DNA contaminations were removed using DNA-*free*™ DNA Removal Kit (Thermo Fisher) following manufacturer's guidelines. RNA purity was assessed by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). First strand complementary DNA (cDNA) was synthetised using High-Capacity cDNA Reverse Transcription

Kit (Thermo Fisher) in a PTC-100 Thermal Cycler (Biorad) (10 minutes at 25°C, 120 minutes at 37°C and 5 minutes at 85°C). 10 ng of cDNA were used in RT-qPCR reactions in a 20 µL volume, with a SYBR Green PCR Master Mix (Sigma) in a Rotor-Gene Q 2plex Platform (Qiagen) (initial denaturation 94°C for 2 minutes, 40 cycles of denaturation at 94°C and annealing and extension at 60°C). Data were analysed using the threshold cycle (Ct) – relative quantification method ($2^{-\Delta Ct}$), between the target gene(s) and the housekeeping gene GAPDH and expressed as a relative expression. Primers used for RT-qPCR experiments were obtained from published literature, their specificity was confirmed using NCBI Primer Blast and purchased from Thermo Fisher Scientific (Supplementary Table 1).

Immunocytochemical staining (ICC)

Cells were cultured on glass coverslips in 24-well cell culture plates (Nunc™ surface, Thermo Fisher Scientific) in specific densities (28000 cells/1.9cm^2) and treated with 10 or 50 ng/ml of BMP-12 or TGF-β1 with or without Ascorbic Acid in serum-free media for one and seven days. Monolayers were fixed in 4% paraformaldehyde for 10 minutes and washed with PBS. Cell membranes were permeabilised or not for 3 minutes with 0.5% NP-40 (Thermo Fisher) and blocked with 3% BSA in PBS for one hour at room temperature. Coverslips were then incubated overnight at 4°C in a humidified chamber with anti-SCXA (1:100, Abcam, ab58655, Rabbit polyclonal) or anti-C1A1 (1:100, Abcam, ab34710, Rabbit polyclonal). Monolayers were then incubated with 3% BSA in PBS for 10 minutes and subsequently incubated with Goat anti-Rabbit IgG (H+L) (1:400, Invitrogen, A-11034) for 1 h in the dark. Coverslips were then washed with PBS and mounted with mounting media containing DAPI (Vector Laboratories, H-1500). Imaging was performed using a Zeiss Axioplan 2ie microscope using Axiovision software. Pictures were taken with a 20x magnification PlanApochromat (0.6 NA) objective and a Zeiss AxioCam HRm CCD camera. Fluorescence was excited either at 300-400 nm (DAPI) or 500-560 nm (AlexaFluor 488). Merged images of DAPI (blue) and Collagen (green) or Scleraxis (red) were created and fluorescence was quantified in Image J software. Data is expressed as mean of the fluorescent intensity, using minimum three random locations per sample.

Western blotting

Cells were cultured in 60x15 mm Dishes (Nunc™ surface, Thermo Fisher Scientific) in specific densities (300000 cells/21.5 cm^2) and treated with 10 ng/ml of BMP-12 or TGF-β1 in serum-free media for one and seven days. Media was removed from monolayers, washed three times with chilled PBS and lysed for 5 minutes at 4°C with RIPA lysis buffer (Thermo Fisher), freshly supplemented with Phenylmethanesulfonyl fluoride (Sigma Aldrich) and Protease inhibitor Cocktail (Sigma Aldrich). Cell lysates were collected and centrifuged for 30 minutes at 4°C. Supernatants were quantified for protein concentration using Pierce BCA Protein Assay (Thermo Fisher) following manufacturer's instructions. For Collagen 1A1 and Scleraxis detection, 5µg of cell lysates were separated in 8% (120V, 70 minutes) or 12% (200V, 50 minutes) SDS-PAGE gels respectively, under reducing conditions. Gels were wet transferred onto a 0.45 µM pore size PVDF membrane (GE Healthcare) for 18 h at 15V for Collagen 1A1 or for 2 h at 90V for Scleraxis at 4°C. Membranes were blocked with milk protein (5%) in TBS-T buffer for 1 h and then incubated with anti-C1A1 (1:1000, Abcam, ab34710, Rabbit Polyclonal), anti-SCXA (1:250, Abcam, ab185940, Rabbit Polyclonal) or anti-GAPDH (1:1000, R&D Systems, AF5718, Goat Polyclonal) for overnight at 4°C. For the secondary antibodies incubation, membranes were incubated with anti-goat for GAPDH (1:1000, R&D systems) or anti-rabbit for Collagen 1A1 (Collagen type I) and Scleraxis (both dilution 1:1000) (Abcam), conjugated with horseradish peroxidase. Membranes were analysed using ECL Plus Detection System (Amersham Biosciences) using Image Quant LAS 4000 platform (GE Healthcare).

Statistical analysis

Two-way ANOVA statistical analysis was used with two main variables, dose of Growth Factors and time of stimulation. In RT-qPCR data, Tukey's multiple comparison tests was applied for displaying significant differences of the doses in between the time points and Dunnet's multiple comparison tests was applied for displaying significant differences of the doses compared to un stimulated cells. In MTS, OD values are expressed as mean $\pm$ SD (n=3 independent cell culture replicates). In Live and Dead assay, counted live and dead cells per picture is expressed as mean $\pm$ SD (n=3 independent cell culture

replicates). In RT-qPCR, fold change compared to control data are expressed as mean ± SD (n=3 independent cell culture replicates). In ICC, for permeabilised ADSCs, data are expressed as mean of fluorescence intensity ± SD (n=3 independent cell culture replicates) and normalised to cell number. For non permeabilised cells, fluorescence intensity without cell number normalisation is reported as mean of fluorescence intensity ± SD (n=3 independent cell culture replicates).).

RESULTS

Effect of serum-free conditions and tenogenic media in the viability of ADSCs

Due to the serum-free conditions, morphology and viability of the cultured ADSCs were monitored. In serum-free conditions, ADSCs presented a star-shaped morphology with a less elongated cytoplasm, compared to standard culture conditions containing serum (Fig. 1A). Tenogenic media containing BMP-12 did not reverse the observed morphology (Supplementary Figure S2) but the addition of TGF-β1 generated a more elongated cytoplasm (Supplementary Figure S3). Interestingly, no significant differences were found in metabolic activity (Fig. 1B), although it was found to be slightly higher and constant with TGF-β1. To further confirm the viability of the cultures, we then stained and quantified the live and dead cells (Fig. 1C, 1D and 1E). BMP-12 stimulation did not change the ADSCs morphology during the time course experiment (Fig. 1C), and both the number of live and dead cells remained stable with no significant variations (Fig. 1E). In contrast, we detected enlarged cells in the presence of TGF-β1, which became visible 5 days after the induction (Fig. 1D). However, no significant increase in the number of live cells was detected (Fig. 1E).

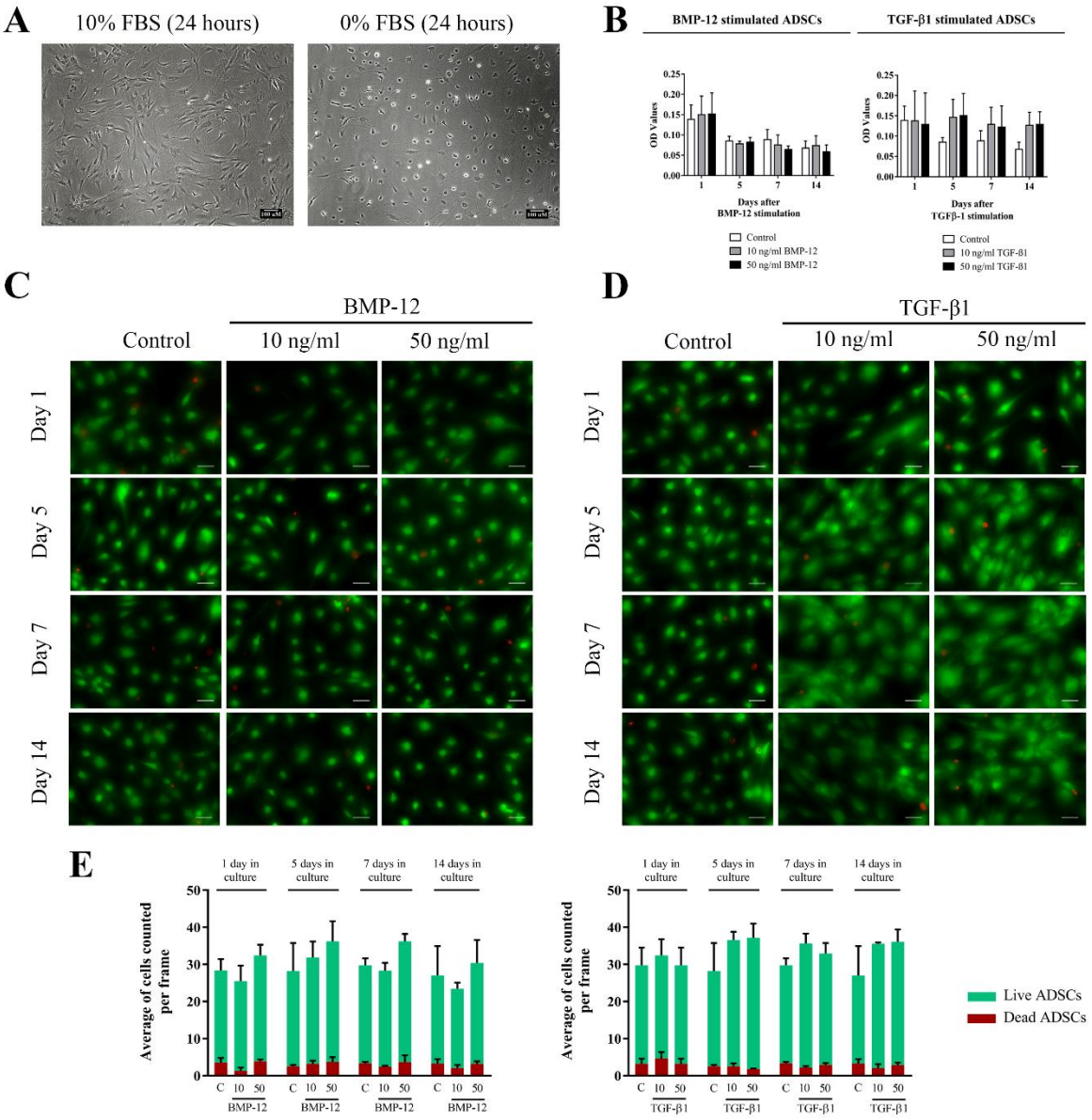

FIG.1. Viability of ADSCs in serum-free media and stimulated with BMP-12 or TGF-β1. Cell morphology and general status of cultured ADSCs were monitored by bright field. (**A**) Image of ADSCs taken 24 hours after culture under standard conditions (10% FBS) or 24 hours after serum starvation (0% FBS). Scale bar is 100 µM (10x magnification). Metabolic activity of cultured ADSCs was analysed by MTS assay (**B**) for over one, five, seven and fourteen days after stimulation with BMP-12 or TGF-β1. Unstimulated cells were used as a control. Data is presented as Optical Density (OD) values ± SD (n=3 independent cell culture replicates). Live and dead ADSCs were stained with Live/Dead Staining cytotoxicity kit (**C** and **D**) for over one, five, seven and fourteen days after stimulation with BMP-12 (**C**) or TGF-β1 (**D**). Unstimulated ADSCs were used as a control. Live ADSCs were stained with Calcein AM (Green). Dead ADSCs were stained with Ethidium Homodimer-1 (Red). Scale bar is 50 µM (20x magnification). Live and dead cells were counted (**E**) in each condition. Data is presented as average of counted cells ± SD (n=3 independent cell culture replicates) in three random locations, therefore using a total of 9 images per sample. C: Control; 10: 10 ng/ml BMP-12 or TGF-β1; 50: 50 ng/ml BMP-12 or TGF-β1.

BMP-12 tenogenic media moderately induces the expression of tenogenic markers in ADSCs

To detect changes in the gene expression, RT-qPCR was used in ADSCs cultured in serum-free conditions with BMP-12 refreshed every three days (Fig. 2). The analysis revealed a late and mild effect of BMP-12 on the expression of almost every tendon marker. *Collagen 1α1* (COL1A1), *Collagen 1α2* (COL1A2), *Collagen 3α1* (COL3A1), *Tenascin C* (TNC), *Decorin* (DCN), *Mohawk* (MKX), *Thrombospondin-4* (THB-4) and *Cartilage Oligomeric Matrix Protein* (COMP) mRNA levels were upregulated at the end of the stimulation period (14 days), being barely detectable beforehand. Among those, DCN, MKX, THB-4 and especially COMP were significantly induced. Interestingly, the latter displayed the highest mRNA levels by BMP-12 addition, in particular with the highest dose administrated (50 ng/ml). On the other hand, variable *Scleraxis* (SCX) expression was observed over the time course, being considerably upregulated 7 and 14 days after induction.

TGF-β1 tenogenic media highly induces the expression of tenogenic markers in ADSCs

Contrary to the observed induction by BMP-12 (Fig. 2), TGF-β1 showed an earlier upregulation in all the tenogenic markers except for DCN (Fig. 3). Five days after the induction, tendon markers such as COL1A1 and MKX were significantly upregulated and observed to decrease thereafter. However, the expression was maintained for over 14 days of induction in the case of COL1A1 and barely detected in the case of MKX. The mRNA levels of other markers such as THB-4, TNC and COL3A1 were upregulated and maintained since the beginning of the induction, increasing thereafter. Moreover, TGF-β1 showed significant upregulation and maintenance of the markers SCX and COMP throughout all the time course experiment. In the case of SCX, high mRNA levels were detected from the first time interval. In the case of COMP, a large increase in expression was observed. Interestingly, none of the tested conditions induced the expression of DCN, which was continuously downregulated.

Media containing BMP-12 and TGF-β1 mildly induce the expression of cartilaginous markers in ADSCs, while having little or no effect on the expression of the osteogenic marker

The cartilage marker *Aggrecan* (ACAN) showed to be significantly upregulated by BMP-12 at a later time of induction (Fig. 4A), whereas TGF-β1 had almost no effect in the upregulation of this marker, only weakly expressed 14 days after the induction (Fig. 4B). In addition, *Sex-determining region Y-box 9* (SOX9) was selected as another marker indicating a cartilaginous differentiation. In contrast to what was detected with ACAN, we observed that SOX9 was significantly upregulated by TGF-β1 since the first time point of induction. However, SOX9 expression significantly decreased during the time course experiment, showing mRNA levels closer to basal at the end of the stimulation time (Fig. 4B). On the other hand, BMP-12 did not induce SOX9 expression (Fig. 4A). The expression of the osteogenic marker *Osteocalcin* (BGLAP) was also analysed. Neither of the tested tenogenic Media, containing BMP-12 or TGF-β1 (Figures 4A and 4B, respectively), increased the expression of BGLAP compared to the control.

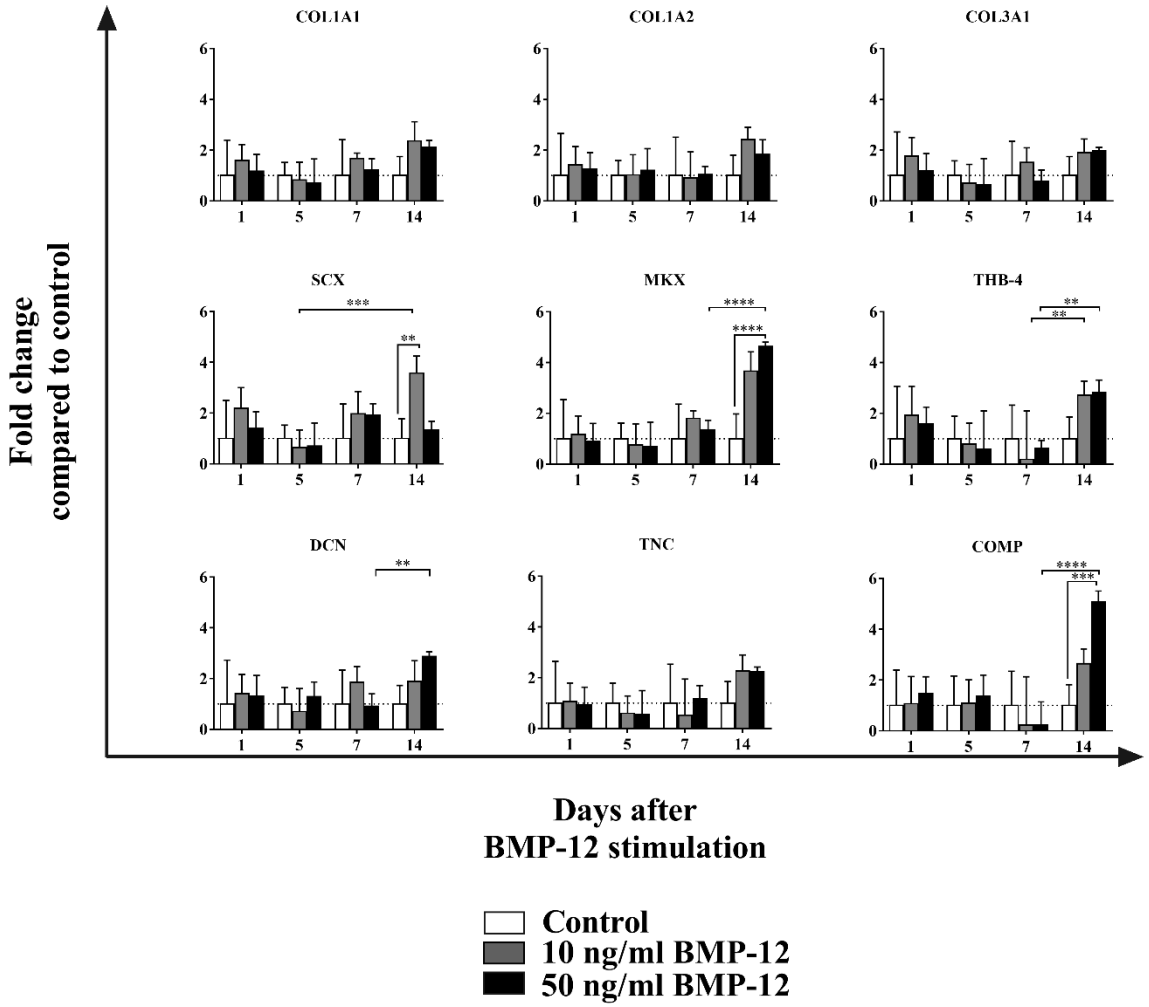

FIG.2. Characterisation of tenogenic induction in ADSCs stimulated with BMP-12. ADSCs were cultured in serum-free media for one, five, seven and fourteen days and stimulated with 10 or 50 ng/ml of BMP-12. Unstimulated ADSCs were used as a control. mRNA levels were analyzed by q-RT PCR. The dashed line represents baseline equal to 1, calculated with DCt values from unstimulated ADSCs at each time point. Data is expressed as mean of fold change ± SD (n=3 independent cell culture replicates), compared to control (unstimulated ADSCs). Statistical analysis 2-way ANOVA with Tukey's multiple comparison test was applied for displaying significant differences of the doses in between the time points and Dunnet's multiple comparison test was applied for displaying significant differences of the doses compared to unstimulated cells. *p 0.03, **p 0.002, ***p 0.0002, ****p < 0.0001. COL1A1 (*Collagen 1α1*); COL1A2 (*Collagen 1α2*): COL3A1 (*Collagen 3α1*); SCX (*Scleraxis*); MKX (*Mohawk*); THB-4 (*Thrombospondin –4*); DCN (*Decorin*); TNC (*Tenascin C*); COMP (Cartilage Oligomeric Matrix Protein): GAPDH (*Glyceraldehyde- 3-phosphate dehydrogenase*).

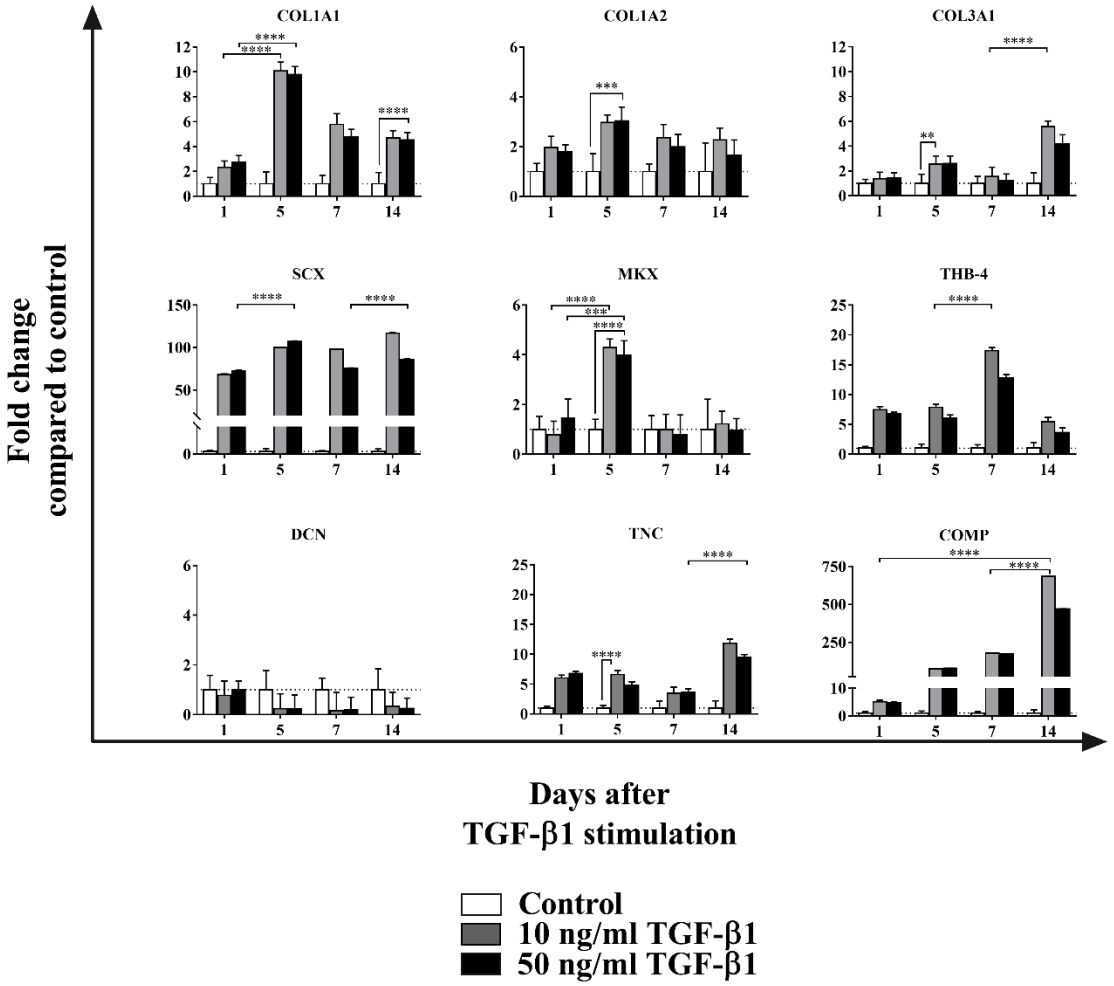

FIG. 3. Characterisation of tenogenic induction in ADSCs stimulated with TGF-β1. ADSCs were cultured in serum-free media for one, five, seven and fourteen days and stimulated with 10 or 50 ng/ml of TGF-β1. Unstimulated ADSCs were used as a control. mRNA levels were analyzed by q-RT PCR. The dashed line represents baseline equal to 1, calculated with DCt values from unstimulated ADSCs at each time point. Data is expressed as mean of fold change ± SD (n=3 independent cell culture replicates), compared to control (unstimulated ADSCs). Statistical analysis 2-way ANOVA with Tukey's multiple comparison test was applied for displaying significant differences of the doses in between the time points and Dunnet's multiple comparison test was applied for displaying significant differences of the doses compared to unstimulated cells. *p 0.03, **p 0.002, ***p 0.0002, ****p < 0.0001. COL1A1 (*Collagen 1α1*); COL1A2 (*Collagen 1α2*): COL3A1 (*Collagen 3α1*); SCX (*Scleraxis*); MKX *(Mohawk*); THB-4 (*Thrombospondin – 4*); DCN (*Decorin*); TNC (Tenascin C); COMP (*Cartilage Oligomeric Matrix Protein*): GAPDH (*Glyceraldehyde- 3-phosphate dehydrogenase*).

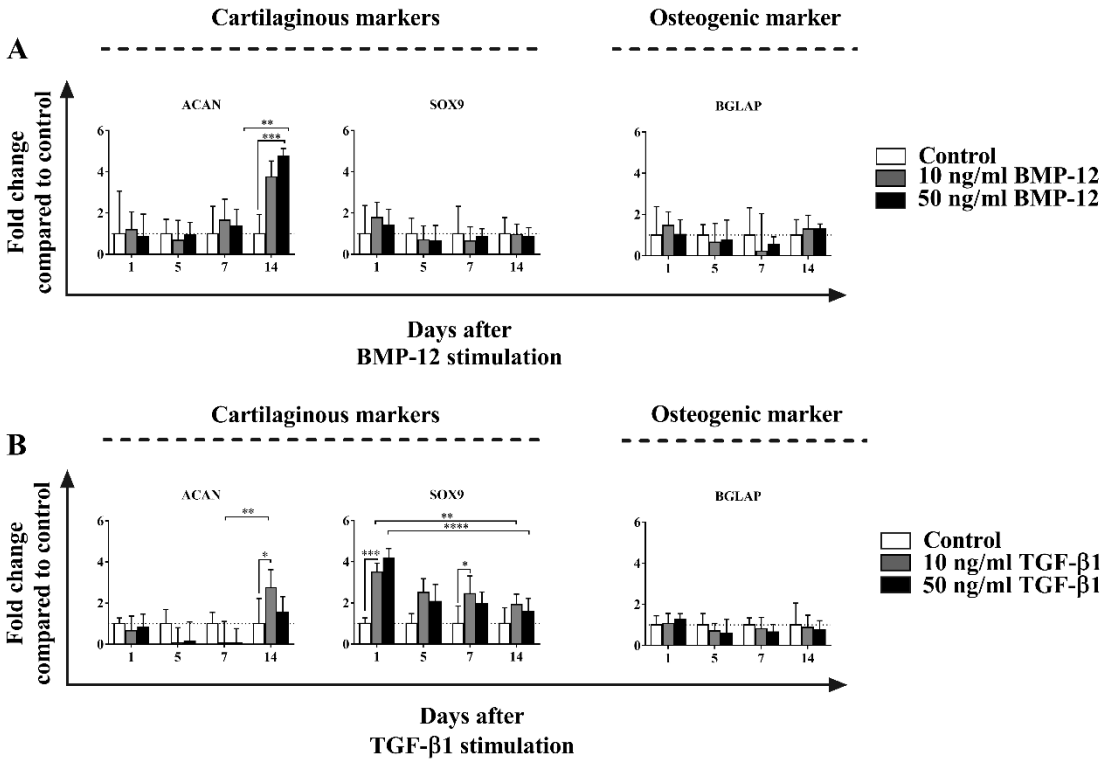

FIG.4. Characterisation of cartilaginous and osteogenic induction in ADSCs stimulated with BMP-12 or TGF-β1. ADSCs were cultured in serum-free media for one, five, seven and fourteen days and stimulated with (**A**) 10 or 50 ng/ml of BMP-12 or (**B**) 10 or 50 ng/ml of TGF-β1. Unstimulated ADSCs were used as a control in A and B. mRNA levels were analyzed by q-RT PCR. The dashed line represents the baseline equal to 1, calculated with DCt values from unstimulated cells at each time point. Data in A and B are expressed as mean of fold change ± SD (n=3 independent cell culture replicates), compared to control (unstimulated ADSCs) Statistical analysis 2-way ANOVA with Tukey's multiple comparison test was applied for displaying significant differences of the doses in between the time points and Dunnet's multiple comparison test was applied for displaying significant differences of the doses compared to unstimulated cells. *p 0.03, **p 0.002, ***p 0.0002, ****p < 0.0001. ACAN (*Aggrecan*); SOX9 (*Sex-determining region Y-box 9*); BGLAP (*Osteocalcin*); GAPDH (*Glyceraldehyde- 3-phosphate dehydrogenase*).

Effect of tenogenic media and Ascorbic Acid on Collagen type I protein expression in permeabilised ADSCs

One and seven days after the tenogenic induction with BMP-12 or TGF-β1, localisation of *Collagen type I* (COL1) was assessed and quantified by Immunocytochemical techniques (ICC) (Fig. 5A and 5B) in permeabilised ADSCs and protein levels were confirmed by Western Blotting (Fig. 5C). Moreover, Ascorbic Acid was added to the tenogenic media and the effect on COL1 fibril formation was observed. Overall, COL1 was detected in the presence of all the basal and tenogenic media (Fig. 5A and 5B) and was visible in the form of a cytoplasmic distribution. However, the addition of Ascorbic Acid allowed for the formation of Collagen fibrils, secreted into the extracellular space between cells. We observed that the few fibrils generated by the combination of BMP-12 and Ascorbic acid (Fig. 5A) were mostly detectable seven days after the tenogenic induction. In contrast, the mixture of TGF-β1 and Ascorbic acid (Fig. 5B) strongly induced the accumulation of the fibrils from the first day of induction, maintained over the time course. These results were confirmed by quantification of the fluorescent intensity (Fig. 5B). Furthermore, analysis by Western blotting (Fig. 5C) showed COL1 protein levels were detected in basal and tenogenic media, but strongly upregulated by TGF-β1, especially at early time points. Seven days after the stimulation, we detected a decrease in the intensity of the signal both in basal and BMP-12 containing media. At the same time, TGF-β1 stimulation increased COL1 protein levels compared to BMP-12, displaying the most intense signal from all the time point (Fig. 5C). Full-length version of the gels is available in supplementary section (Fig. S4).

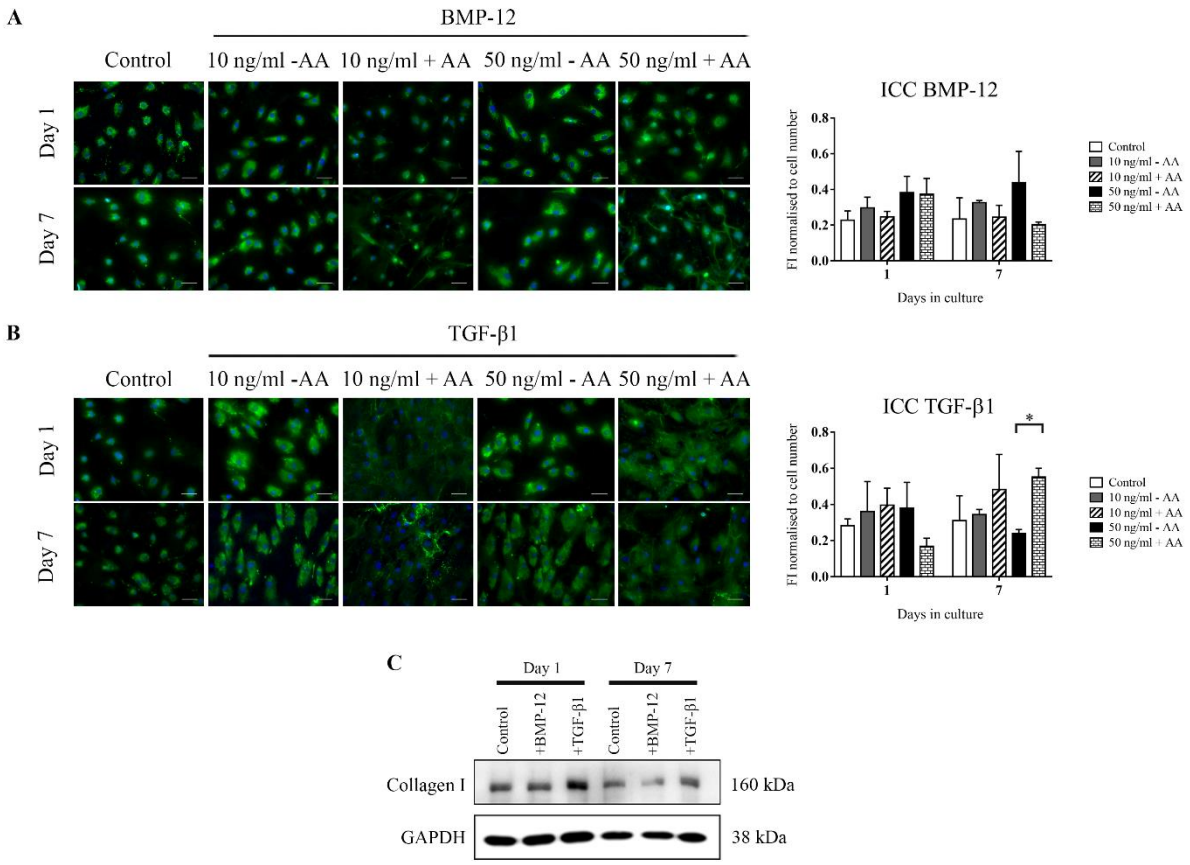

FIG.5. Collagen type I protein immunolocalisation in permeabilised ADSCs stimulated with BMP-12 or TGF-β1. Collagen 1 protein was detected by immunocytochemical techniques in permeabilised ADSCs stimulated with (**A**) 10 or 50 ng/ml of BMP-12 or (**B**) TGF-β1 with or without Ascorbic Acid (50µg/ml) in serum-free media for one and seven days. Collagen 1 was coloured in green and chromatin was stained with DAPI (blue). Colouring and merged images of both colour channels were created with ImageJ software. Scale bar represents 50 µm (20x magnification). Quantification of fluorescence intensity is shown in bar charts. Data is presented as Fluorescence Intensity (FI) ± SD (n=3 independent cell culture replicates) normalised to cell number, quantified in three different images per condition. FI was quantified with ImageJ software. Statistical analysis 2-way ANOVA with Tukey's multiple comparison test was applied. *p 0.03. (**C**) ADSCs were cultured in serum-free media for one and seven days, stimulated with 10 ng/ml of BMP-12 or TGF-β1 and Collagen I protein translation was confirmed by Western Blotting against loading control GAPDH. Representative image is shown from three separate electrophoresis. Images have been cropped for clarity purposes. Full length gels are available in Supplementary Figure S4. -AA: Without Ascorbic Acid; +AA: Stimulated with 50 µg/ml of Ascorbic Acid; ICC: Immunocytochemistry; FI: Fluorescence intensity; GAPDH (*Glyceraldehyde- 3-phosphate dehydrogenase*).

Effect of tenogenic media and Ascorbic Acid on Collagen type I deposition in non permeabilised ADSCs

Following the results seen in Fig.5, we then next assessed the Collagen I localisation in non permeabilised ADSCs. We observed both basal and BMP-12 containing media were not able to induce large quantities of deposited Collagen, and mostly without a defined arrangement (Fig. 6A). Few fibrils were detected in the presence of Ascorbic Acid, although only in a significant manner at early time points, as confirmed by the quantification analysis. The mixture of TGF-β1 and Ascorbic Acid also generated Collagen fibril deposition without a defined orientation (Fig. 6B), however in larger quantities, significantly increased at day 7, compared to the previous time point.

Evaluation of Scleraxis expression as an effect of the administered tenogenic media in ADSCs

The presence of SCX in cultured ADSCs was assessed next by ICC and protein levels confirmed by Western Blotting (Fig. 7). Overall, we detected differences in the distribution of SCX depending on the tenogenic media used. BMP-12 containing media displayed a positive SCX signal in early induction time points, with levels closer to basal. SCX displayed a subcellular localisation, with strong staining in the cell nuclei. However, the longer induction time resulted in a complete loss of the SCX signal, barely above the background, as confirmed by the quantification of the fluorescence intensity (Fig. 7A). On the other hand, SCX was detected both in the cell nuclei and in the cytoplasm of ADSCs induced with TGF-β1, regardless of the dose or time point (Fig. 7B). In addition, the intensity of the signal was higher than both basal and BMP-12 containing media (Fig. 7A), remaining constant in late induction times (Fig. 7B). Consistent with the ICC results, protein levels analysed by Western Blotting showed a decrease in SCX protein seven days after the induction, only being maintained and upregulated in tenogenic media containing TGF-β1 (Fig. 7C). Full-length version of the gels is available in supplementary figure S5.

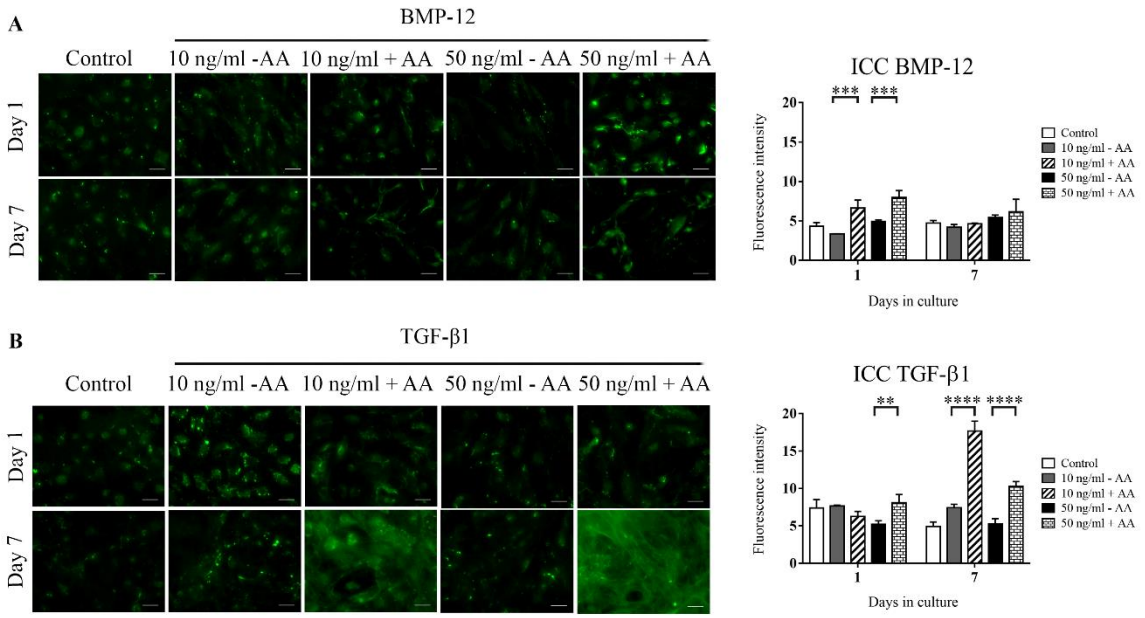

FIG.6. Collagen type I protein immunolocalisation in non-permeabilised ADSCs stimulated with BMP-12 or TGF-β1. ADSCs were cultured in serum-free media for one and seven days and stimulated with (**A**) 10 or 50 ng/ml of BMP-12 or (**B**) TGF-β1 with or without Ascorbic Acid (50µg/ml) in serum-free media for one and seven days. Collagen 1 protein was detected by immunocytochemical techniques and coloured in green. Colouring was created with ImageJ software. Scale bar represents 50 µm (20x magnification). Quantification of fluorescence intensity is shown in bar charts. Data is presented as Fluorescence Intensity (FI) ± SD (n=3 independent cell culture replicates), quantified in three different images per condition. FI was quantified with ImageJ software. Statistical analysis 2-way ANOVA with Tukey's multiple comparison test was applied. **p 0.002; ****p < 0.0001. -AA: without Ascorbic Acid; +AA: Stimulated with 50 µg/ml of Ascorbic Acid; ICC: immunocytochemistry; FI: Fluorescence intensity.

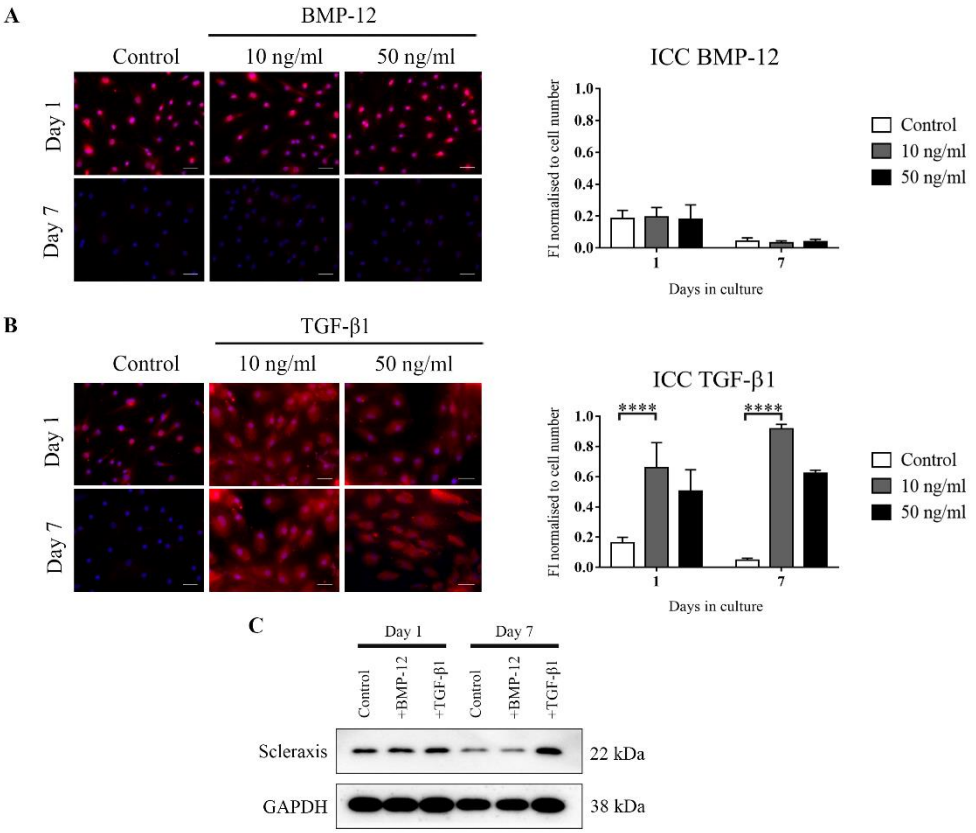

FIG.7. Scleraxis protein expression in ADSCs stimulated with BMP-12 or TGF-β1. Scleraxis protein was detected by immunocytochemical techniques in ADSCs stimulated with (**A**) 10 or 50 ng/ml of BMP-12 or (**B**) TGF-β1 in serum-free media for one and seven days. Scleraxis was coloured in red and chromatin was stained with DAPI (blue). Colouring and merged images of both colour channels were created with ImageJ software. Scale bar represents 50 µm (20x magnification). Quantification of fluorescence intensity normalised to cell number is shown in bar charts. Data is presented as Fluorescence Intensity (FI) ± SD (n=3 independent cell culture replicates) normalised to cell number, quantified in three different images per condition. FI was quantified with ImageJ software. Stati stical analysis 2-way ANOVA with Tukey's multiple comparison test was applied. ****p < 0.0001. (**C**) ADSCs were cultured in serum-free media for one and seven days, stimulated with 10 ng/ml of BMP-12 or TGF-β1 and Scleraxis protein translation was confirmed by Western Blotting against loading control GAPDH. Representative image is shown from three separate electrophoresis. Images have been cropped for clarity purposes. Full length gels are available in Supplementary Figure S5. ICC: Immunocytochemistry; FI: Fluorescence intensity; GAPDH (*Glyceraldehyde- 3-phosphate dehydrogenase*).

DISCUSSION

In the present study, we report a comprehensive analysis of tenogenic differentiation induced by BMP-12 and TGF-β1 in cultured ADSCs, varying doses as well as different time intervals. Most importantly, the study was conducted in serum-free conditions in order to eradicate the endogenous effect of the serum.

The culture conditions did not cause ADSCs cytotoxicity, although a morphology change was observed. Instead of the well-described fibroblast morphology (10), ADSCs displayed a smooth-edged configuration. This shift was not reversible upon addition of BMP-12 but effectively reversed by TGF-β1, in agreement with previous studies performed with low serum concentrations (18).

Overall, the results revealed a delayed effect of BMP-12 in the induction of the tenogenic markers. In contrast, the addition of TGF-β1 demonstrated a predominant role not only in the early activation of the markers but in the maintenance of their expression across the time intervals, both at gene and protein level. The tenogenic media containing TGF-β1 rapidly activated SCX and MKX, two transcription factors that have key mediator roles in tendon development (6, 19). Although MKX was found to be significantly upregulated five days after the induction, SCX expression was strongly upregulated throughout all the time intervals of TGF-β1 induction. On the contrary, it scarcely achieved significance in tenogenic media containing BMP-12. Moreover, the contained nuclear distribution of SCX upon addition of BMP-12 was observed both in the nuclei and in the cytoplasm of the ADSCs after addition of TGF-β1, indicating a possible effect of TGF-β1 in the accumulation of SCX. However, the morphology of the cultured ADSCs in tenogenic media containing TGF-β1 was notably enlarged compared to the rest of the conditions, therefore the accumulation of SCX in the cytoplasm could be related to the changes in cell morphology and should be addressed further. Nonetheless, these results suggest that TGF-β1 is more effective at activating and preserving SCX expression, in agreement with other studies (19-21).

The results further revealed a potential inhibitory effect of TGF-β1 in DCN expression (22), an ECM component with a role in Collagen fibril formation (23, 24). In contrast, BMP-12 significantly upregulated DCN two weeks after the first induction. These results are in agreement with previous studies (6).

Ascorbic Acid (AA) is a well-known co-factor for the correct assembly of the Collagen triple helix (25). Therefore, the investigation of the role of AA as a supplement in stem cells differentiation media is highly desirable. Initially, we carried out a preliminary study assessing the effect of AA in RT q-PCR, ICC and Western Blotting experiments. However, the addition of AA did not display significant differences at mRNA or protein level, compared to cultures without AA (data not shown). Although the positive effect of AA at mRNA and protein level has been described previously (26), we then considered that the effect of AA in the Collagen deposition is apparent in the ICC experiments, and thus the inclusion of AA is exclusively in ICC in the present study. Collagen fibres in the extracellular space are apparent by immunolocalisation upon addition of AA. Future work including AA in all experimental designs, especially Western Blotting, should be considered and investigated. Overall, upon stimulation with TGF-β1 and BMP-12, a cytoplasmic distribution of Collagen was observed, suggesting a procollagen synthesis prior to the extracellular secretion (27) independent of the GF.. However, the addition of AA clearly showed the extra and intracellular accumulation of fibrils, especially in combination with TGF-β1, confirming the positive role of this co-factor in Collagen biosynthesis and deposition (26, 28), and the beneficial combination of Ascorbic Acid and TGF-β1 in Collagen synthesis, as described previously (26). Further contributing to Collagen fibrillogenesis, COMP is a glycoprotein that is believed to provide integrity to the tendon ECM (7). Interestingly, all tenogenic media displayed an exponential increase of COMP expression. These observations could suggest a potential and gradual effect of these GFs on the accumulation of COMP to the ECM.

The obtained results underline the positive response of the ADSCs to the GF addition in the absence of serum. The selected tendon specific markers were overall upregulated and both BMP-12 and TGF-

β1 revealed to have mild induction in cartilage and no effect on bone markers. Nonetheless, the chosen bone marker (*Osteocalcin*) is considered as a marker of a late osteogenic development, and further analysis with other earlier osteogenic markers such as *Runt-Related transcription factor 2* (RUNX2)_should be carried further in these conditions, although other examples can be found in the literature (14, 15).

Moreover, the experimental design of this study agrees with previously reported studies, since the continuous administration of GFs is a commonly used approach (14, 15, 29). However, recent research explores the stepwise addition of GFs (6, 17), priming the cultured cells with one or several GF before the stimulation. The differences in the effect of the mentioned approaches on the cultured cells address the necessity of consolidation of tenogenic protocols. In addition, the use of no serum or low serum concentrations in stem cell characterisation studies should be explored further. Moreover, other experimental setups during crucial steps for stem cells differentiation protocols such as the initial stem cell expansion could be addressed further. In the present study, the ADSCs were expanded in 10% FBS, and recent literature describes the use of low serum maintenance medium as a beneficial alternative (30) for the later applicability of the stem cells.

Therefore, the significance of this study resides in the utilisation of multiple doses and time intervals of two important tenogenic GFs in serum-free conditions. The overall positive response of the ADSCs, the absence of cell cytotoxicity, and the differences found in the tenogenic profile reinforces the potential exclusion of serum in tenogenic differentiation protocols, although deeper studies should be conducted. In addition, the positive assets of ADSCs venture them as an important stem cell population for regenerative medicine and in particular for tendon regeneration.

We have demonstrated a strong upregulation and maintenance of the tenogenic commitment upon addition of TGF-β1, not only inducing early key tendon markers such as SCX but also promoting the deposition of a tendon-like ECM, especially in combination with Ascorbic Acid. Despite extensive studies probing the effect of BMP-12 in different cell types (15, 29, 31) we found it ineffective to

actively induce tendon commitment in ADSCs. Even so, we address the importance of longer time intervals in order to further test the effect of BMP-12.

Ultimately, this study emphasises the relevance of cell culture microenvironments for the development of more accurate and representative tenogenic differentiation tools for pluripotent stem cells, in order to develop efficient future cell-based therapies for tendon repair.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interest exist.

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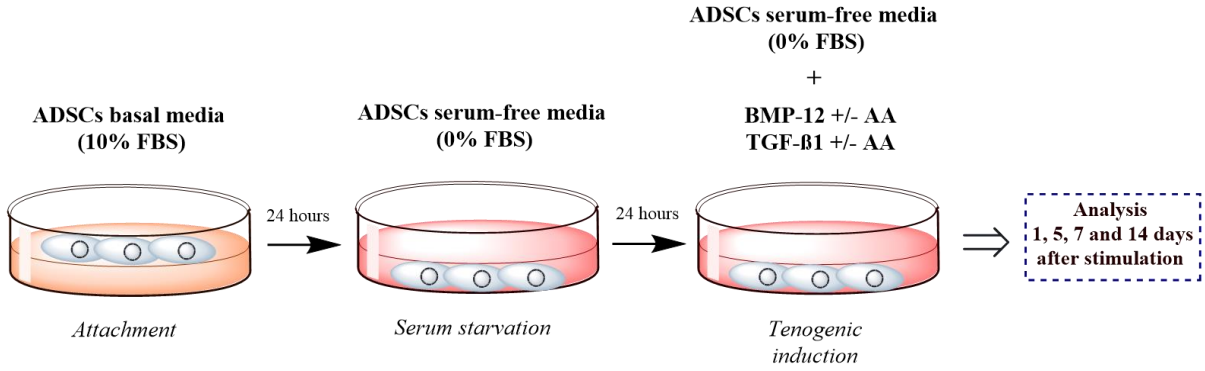
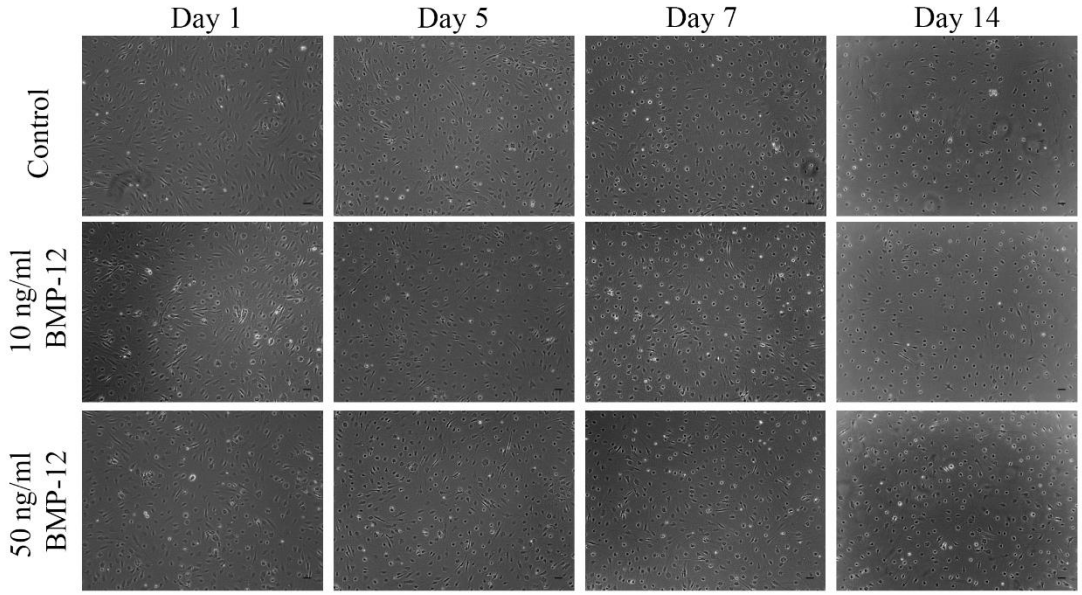
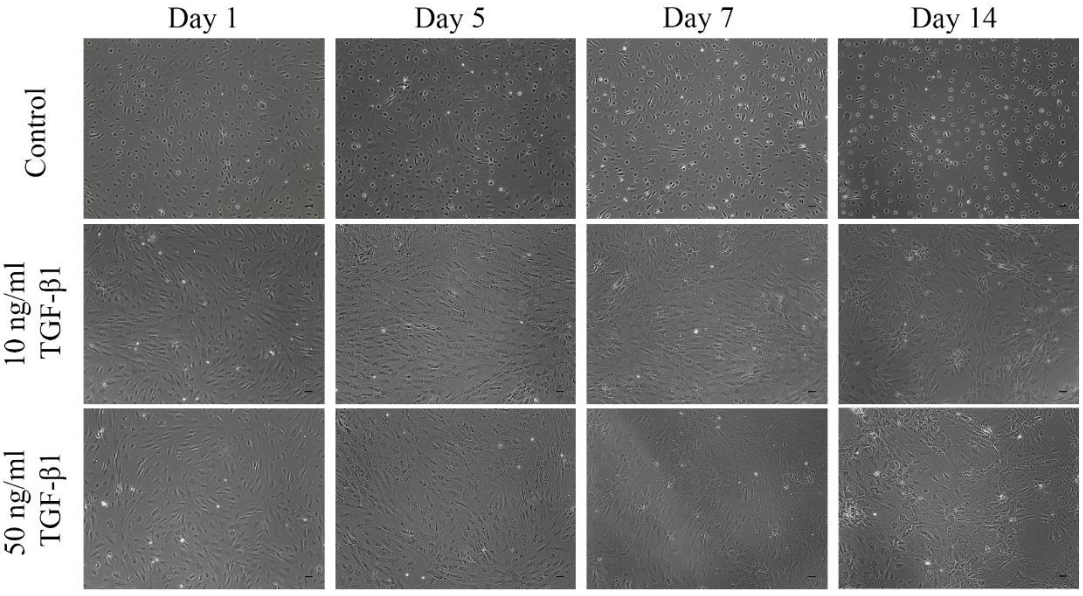
SUPPLEMENTARY INFORMATION



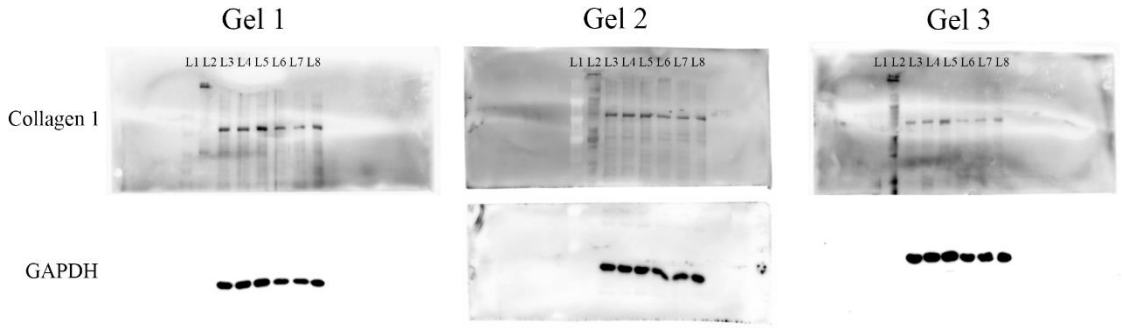
Supplementary Figure S1. Schematic representation of ADSCs preparation and stimulation. ADSCs were seeded in tissue culture plates and left to attach for overnight time, using ADSCs basal media containing 10% FBS. 24 h after, monolayers were washed three times with PBS (1X) and ADSCs serum-free media (0% FBS) was added. After overnight time, ADSCs were stimulated with BMP-12 or TGF- β1 (10 or 50 ng/ml) in the presence or absence of Ascorbic Acid (50 µg/ml). The analysis was performed one, five, seven and fourteen days after the stimulation, depending on the experiment. Cell culture media and supplements were refreshed every three days. All the experiments were performed at the same hour in order to avoid potential circadian variation in the cell response. ADSCs (Adipose Derived Stem Cells), BMP-12 (Bone Morphogenetic Protein-12); TGF-β1 (Transforming Growth Factor- β1), AA (Ascorbic Acid). Schematic representation of the study was performed with ChemDraw Professional Version 15.0.0.106.



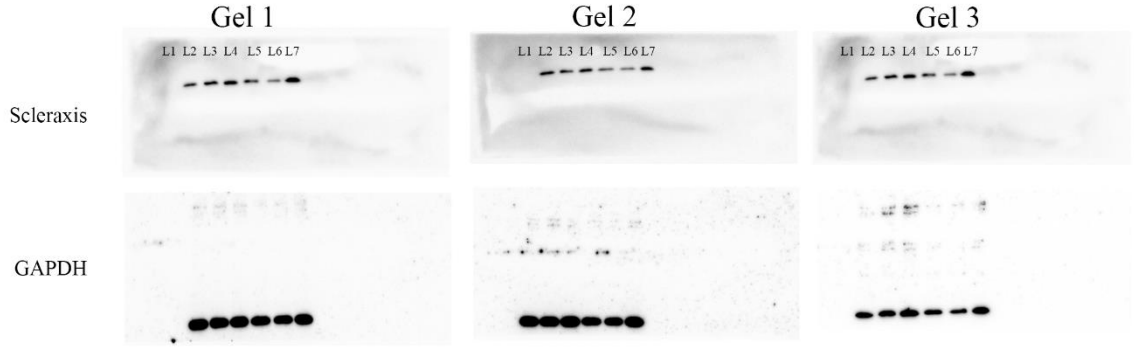
Supplementary Figure S2. Time course experiment of ADSCs stimulated with BMP-12 in serum-free media. Cell morphology and confluence of cultured ADSCs were monitored by bright field. ADSCs were cultured for one, five, seven and fourteen days with 10 or 50 ng/ml of BMP12. Unstimulated ADSCs were used as a control. Scale bar is 50 µM (10x magnification). BMP-12 (Bone Morphogenetic Protein-12).



Supplementary Figure S3. Time course experiment of ADSCs stimulated with TGF-β1 in serum-free media. Cell morphology and confluence of cultured ADSCs were monitored by bright field. ADSCs were cultured for one, five, seven and fourteen days with 10 or 50 ng/ml of TGF-β1. Unstimulated ADSCs were used as a control. Scale bar is 50 µM (10x magnification). TGF-β1 (Transforming Growth Factor-β1).



Supplementary Figure S4. Full length Collagen I and GAPDH gels. ADSCs were cultured in three different replicas and run in three separated electrophoresis. Each gel was wet transferred to a single membrane and subsequently cut by half in order to blot Collagen I and GAPDH and avoid membrane stripping. For figure clarity purposes, Gel 1 was the selected for the representation of the experiment. L1: Molecular Weight marker; L2: Commercial Collagen Standard, L3: unstimulated ADSCs cultured for one day, L4: ADSCs stimulated with 10 ng/ml of BMP-12 for one day, L5: ADSCs stimulated with 10 ng/ml of TGF-β1 for one day, L6: unstimulated ADSCs cultured for seven days, L7: ADSCs stimulated with 10 ng/ml of BMP-12 for seven days, L8: ADSCs stimulated with 10 ng/ml of TGF-β1 for seven days. Images without exposure manipulation were obtained with Image Quant LAS 4000 platform (GE Healthcare).



Supplementary Figure S5. Full length Scleraxis and GAPDH gels. ADSCs were cultured in three different replicas and run in three separated electrophoresis. Each gel was wet transferred to a single membrane and subsequently cut by half in order to blot Scleraxis and GAPDH and avoid membrane stripping. For figure clarity purposes, Gel 2 was the selected for the representation of the experiment. L1: Molecular Weight marker (not detected by exposure), L2: unstimulated ADSCs cultured for one day, L3: ADSCs stimulated with 10 ng/ml of BMP-12 for one day, L4: ADSCs stimulated with 10 ng/ml of TGF-β1 for one day, L5: unstimulated ADSCs cultured for seven days, L6: ADSCs stimulated with 10 ng/ml of BMP-12 for seven days, L7: ADSCs stimulated with 10 ng/ml of TGF-β1 for seven days. Images without exposure manipulation were obtained with Image Quant LAS 4000 platform (GE Healthcare).

Gene	Sequence	Reference
COL1A1	F- GGCAACAGCCGCTTCACCTAC R- GCGGGAGGACTTGGTGGTTTT	(32)
COL1A2	F- CCCAGCCAAGAACTGGTATAGG R- GGCTGCCAGCATTGATAGTTTC	(33)
COL3A1	F- CACGGAAACACTGGTGGACAGATT R- ATGCCAGCTGCACATCAAGGAC	(32)
THB-4	F- GTTGCAGAACCTGGCATTCAG R-CCCTGGACCTGTCTTAGACTTCA	(34)
TNC	F- CCGCAGAGGTGACATGTCAA R-CTCCATTCCAGGGTGATGCT	(35)
SCX	F- CAGCCCAAACAGATCTGCACCTT R- CTGTCTTTCTGTCGCGGTCCTT	(32)
MKX	F- CATCGTCATCAGAACTGAAGGCA R- TCTGTTAGCTGCGCTTTCACCC	(32)
DCN	F- GGTGGGCTGGCAGAGCATAAGT R- TGTCCAGGTGGGCAGAAGTCA	(32)
COMP	F- GTGGTGCTCAACCAGGGAAG R- AAGCCCGCATAGTCGTCATC	(36)
ACAN	F- TGCATTCCACGAAGCTAACCTT R- GACGCCTCGCCTTCTTGAA	(37)
BGLAP	F- ATCAAAGAGGAGGGGAACCTA R- AGGAAGTAGGGTGCCATAACA	(38)
SOX9	F- AGCGAACGCACATCAAGAC R- GCTGTAGTGTGGGAGGTTGAA	(37)
GAPDH	F- TCAACGACCACTTTGTCAAGCTCA R- GCTGGTGGTCCAGGGGTCTTACT	(39)

Supplementary Table 1. **Primer sequences used for qRT-PCR**. COL1A1 (Collagen 1α1); COL1A2 (Collagen 1α2); COL3A1 (Collagen 3α1); THB-4 (Thrombospondin – 4); TNC (Tenascin C), SCX (Scleraxis); MKX (Mohawk); DCN (Decorin); COMP (Cartilage Oligomeric Matrix Protein); ACAN (Aggrecan); BGLAP (Osteocalcin); SOX9 (Sex determining region Y-box 9); GAPDH (Glyceraldehyde- 3-phosphate dehydrogenase).