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The effect of mechanical stimulation on the maturation of TDSCs-poly(L-lactide-co-e-caprolactone)/collagen scaffold constructs for tendon tissue engineering

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ABSTRACT

Mechanical stimulation plays an important role in the development and remodeling of tendons. Tendon-derived stem cells (TDSCs) are an attractive cell source for tendon injury and tendon tissue engineering. However, these cells have not yet been fully explored for tendon tissue engineering application, and there is also lack of understanding to the effect of mechanical stimulation on the maturation of TDSCs-scaffold construct for tendon tissue engineering. In this study, we assessed the efficacy of TDSCs in a poly(L-lactide-co-ε-caprolactone)/collagen (P(LLA-CL)/Col) scaffold under mechanical stimulation for tendon tissue engineering both *in vitro* and *in vivo*, and evaluated the utility of the transplanted TDSCs-scaffold construct to promote rabbit patellar tendon defect regeneration. TDSCs displayed good proliferation and positive expressed tendon-related extracellular matrix (ECM) genes and proteins under mechanical stimulation *in vitro*. After implanting into the nude mice, the fluorescence imaging indicated that TDSCs had long-term survival, and the macroscopic evaluation, histology and immunohistochemistry examinations showed high-quality neo-tendon formation under mechanical stimulation *in vivo*. Furthermore, the histology, immunohistochemistry, collagen content assay and biomechanical testing data indicated that dynamically cultured TDSCs-scaffold construct could significantly contributed to tendon regeneration in a rabbit patellar tendon window defect model. TDSCs have significant potential to be used as seeded cells in the development of tissue-engineered tendons, which can be successfully fabricated through seeding of TDSCs in a P(LLA-CL)/Col scaffold followed by mechanical stimulation.

1. Introduction

Tendons have a poor self-regenerative capacity due to their low cellularity and vascularity. Tendon regeneration after injury remains a formidable challenge [1]. Developments in tissue engineering may provide a promising alternative therapy for tendon injuries [2].

A long-standing focuses in tissue engineering is to recover the damaged tissues with full regeneration of their biological functions. Three elements are necessary to develop functional tissue-engineered tendons with good biological activity and mechanical properties: (1) seeded cells which can retain good proliferation and differentiation toward tenogenic lineage; (2) a biological scaffold which can provide a three dimensional (3D) space enough for cell growth; and (3) proper mechanical or chemical stimulation [3]. In recent years, studies on tendon tissue engineering have entered into a phase of clinical research [4,5]. However, difficulty in finding appropriate seeded cells hinders the further development of tendon tissue engineering [6]. Tendon-derived stem cells (TDSCs) are a new type of cells and have been isolated successfully from human adults [7], rats [1], mice [8] and rabbits [9]. TDSCs have general characteristics of stem cells, such as colony formation, self-duplication, multi-directional differentiation potential, etc [1]. TDSCs are superior to mesenchymal stem cells (MSCs) from the same individual source in the aspect of multi-directional differentiation and self-renewal capacities, and TDSCs can express more tenogenic differentiation-related mRNA (such as, tenomodulin, scleraxis, type I collagen (Col I), decorin and biglycan) than MSCs

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[10]. The fibrin glue construct with TDSCs can repair the injured tendons earlier and preferable than that without TDSCs [11], thus TDSCs can be used as seeded cells for tendon regeneration. At present, there are few studies to focus on the utilization of TDSCs as seeded cells in tendon tissue engineering or to explore the vast potential of TDSCs for tendon regeneration.

Mechanical microenvironment can affect cell proliferation, migration, differentiation and apoptosis, as well as tissue development [12]. Tendons are subjected to a continuous action of mechanical load (mainly tensile load) generated by muscle contraction in the body [13]. The growth, development and regeneration of tendons are closely related with mechanical stimulation [14]. Low-intensity mechanical stimulation promotes the proliferation and tenogenic differentiation of TDSCs isolated from rabbit patellar tendons, while high-intensity mechanical stimulation induces the adipogenic, osteogenic, and chondrogenic differentiation of TDSCs [15]. It has been found that the collagen synthesis capacity of TDSCs isolated from mice after 1-week treadmill exercise is increased significantly [16]. Therefore, it is feasible to induce the tenogenic differentiation of TDSCs with mechanical stimulation. Different roles of mechanical stimuli in the developing of tissue-engineered tendons have been widely studied [5,17,18]. Mechanical stimulation significantly increased cell proliferation over the 14-day culture period by at least eightfold in the tissue-engineered tendons [19]. In addition, mechanical stimulation contributed to the formation of collagen fibers and the production of extracellular matrix (ECM) along the loading direction [20]. And mechanical stimulation can improve or optimize the mechanical properties of tissue-engineered tendons, including rigidity, Young's modulus, and maximum tensile stress [21–23]. Therefore, mechanical stimulation undoubtedly plays an important role in successfully developing tissue-engineered tendons.

In the preliminary studies, we investigated the utilization of 3D aligned poly(L-lactide-co-ε-caprolactone)/collagen (P(LLA-CL)/Col) scaffolds in tendon tissue engineering. The results have shown that P(LLA-CL)/Col scaffolds provide a positive environment for cell adhesion, alignment and infiltration, and also provide desirable mechanical properties for tissue-engineered tendons [24]. Aligned nanofibers provide an instructive microenvironment for the tenogenic differentiation of TDSCs than random nanofibers [1]. Meanwhile, mechanical stimulation leads to significant changes in the fibroblastic differentiation potential of MSCs [25]. However, the tenogenic differentiation potential of TDSCs cultured on the aligned scaffold under mechanical stimulation was not studied extensively. Also, the effects of the use of dynamically cultured TDSCs-scaffold construct for repairing the injured tendons have not been studied.

This study aimed to investigate the maturation of tissue-engineered tendons formed by TDSCs and 3D aligned P(LLA-CL)/Col under mechanical stimulation *in vitro* and *in vivo*, and the effects of dynamically cultured tissue-engineered tendons in promoting the regeneration of injured rabbit patellar tendons.

2. Materials and methods

2.1. Scaffold fabrication

P(LLA-CL)/Col scaffolds were fabricated using a dynamic water flow system by electrospinning based on our preliminary studies [24]. Briefly, the dynamic water flow system was composed by a basin with an 8 mm hole on its bottom to produce a vertex flow and a pump to recycle water drained into the tank below the basin. P(LLA-CL) (LA:CL = 50:50, Mw = 300,000, Nara Medical University, Japan) and Type I collagen (Col I) (Ming-Rang Co. Ltd. Chengdu, China) were dissolved into 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Da-Rui Co. Ltd. Shanghai, China) to give a blended solution (8 w/v %) of 90:10, then the blended solution was sprayed at 1.0 mL/h under a high voltage of 15 kV, 15 cm above the vertex flow. After HFIP was evaporated, electrospun nanofibers were formed and stacked on the water surface, then they were twisted into yarns under the action of vertex flow, and collected by a rotating mandrel (60 r/min) to form P(LLA-CL)/Col scaffolds. The obtained scaffolds (about 150 μm thick) were cryopreserved at −80 °C for 2 h, then freeze-dried overnight and preserved in a vacuum container. Macroscopic evaluation and scanning electron microscopy (SEM, Fig. 1B) showed the morphology coincident to our preliminary study results [24].

2.2. Cell isolation and culture

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Third Military Medical University. Primary TDSCs were harvested from the bilateral patellar tendons of Oryctolagus cuniculus, isolated according to the previously described procedures [9,26], and then resuspended in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) (HyClone) medium containing 20% fetal bovine serum (FBS; Invitrogen) and 1% penicillin and streptomycin (HyClone). The resultant suspension was diluted to obtain a 1 cell/μl single-cell suspension which was dropped into a 96-well plate (Corning) and cultured for 8–10 days at 37 °C and 5% CO_2 till TDSCs clones were observed. Individual cell clones were isolated by the topical application of trypsin (Gbico) under the microscope (Olympus BX51). The isolated TDSCs clones were collected and transferred with a micro-pipette into a T25 culture flask (Corning) for further culture. The culture medium was replaced in every three days, and the cells were digested with 0.25% trypsin and passaged after reaching 90% confluence.

2.3. In vitro study

2.3.1. Static and dynamic culture of TDSCs-engineered tendons

The scaffold samples (length × width: 4.5 × 2.5 cm) were placed in tissue-culture polystyrene plates (TCPSs, Costar) and sterilized with 70% ethanol for 30 min, then rinsed with sterile phosphate-buffered saline (PBS), and subsequently immersed in DMEM/F12 medium overnight. TDSCs (passage 3, Fig. 1A) were seeded on the scaffolds (1×10^5 cells/scaffold). The cell-seeded scaffolds were cultured in an incubator (37 °C, 5% CO^2) for 4 h to promote cell adhesion. Then, the culture medium was added into the wells.

After the cell-scaffold constructs (Fig. 1C) were subjected to static culture for 24 h, they were curled into concentric 3D constructs along their 4.5 cm long axis, and then fixed on the two opposing tissue fixing columns of the culture chamber by the nylon threads (Fig. 1D, green arrows (in web version)) under sterile conditions. There were totally 3 cell-scaffold constructs/culture chamber. About 80 ml culture medium was added into each culture chamber. The mechanical stretch was performed with a mechanical traction stimulation system developed in our preliminary studies [27]. For the dynamic mechanical stimulation group, the cell-scaffold constructs were placed into a chamber, and then stretched at 4% elongation in length and 0.5 Hz [15], 2 h per day for a total of 14 days. The cell-scaffold constructs in the static group was cultured statically within another chamber under the same conditions.

2.3.2. Cell viability and morphology

The viability and morphology of cells were evaluated with Live/Dead stain (Invitrogen) according to the manufacturer's instructions [28]. The stained samples were photographed at an excitation wavelength of 488/594 nm under laser confocal microscope (LSM 510, Zeiss, Germany). The number of viable cells and the total number of cells were counted from the images by imageJ software (ImageJ 1.46 r; NIH), and then their ratio was calculated ($n = 3$).

2.3.3. Cell proliferation

The content of total DNA was measured by PicoGreen dsDNA assay (Invitrogen) [25]. The harvested samples were homogenized by Triton-X (Sigma) and ultrasound. The fluorescence was detected at an excitation/emission wavelength of 485/535 nm using a microplate reader (Model 550; Bio-Rad, USA). The standard curve was plotted with DNA concentration-dependent fluorescence intensity, and was used to calculate the number of cells in the samples.

2.3.4. Histological analysis

The cell-scaffold constructs were harvested and then immediately fixed in 4% neutral formalin, gradient-dehydrated with alcohol and embedded into paraffin. The longitudinal sections (5 μm) were stained with hematoxylin and eosin to evaluate cell growth and infiltration.

2.3.5. Real-time polymerase chain reaction (PCR) analysis

In order to clarify the effects of mechanical stimulation on the differentiation of TDSCs, the levels of Col I, type III collagen (Col III), decorin, tenascin C, biglycan (tenogenic differentiation-related genes), Runx2 (osteogenic gene), and type II Collagen (Col II), aggrecan (chondrogenic genes), were detected in dynamic group and static group after 7 and 14 days of culture, respectively. TDSCs collected immediately prior to scaffold seeding served as controls. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the instructions. 500 ng RNA was put into a 20 μL reaction system for reverse transcription with PrimeScript™ RT reagent kit with gDNA eraser (RR047A, Takara) according to the manufacturer's instructions. Real-time PCR was performed using 2× SYBR® Green PCR Master Mix (Applied Biosystems) on a Real-Time PCR System (Applied Biosystems 7500). All primer sequences (Sangon Biotech Co., Ltd., China) were designed using primer 5.0 software and summarized in Table 1. Each sample was tested for three times and 3 PCR cycles

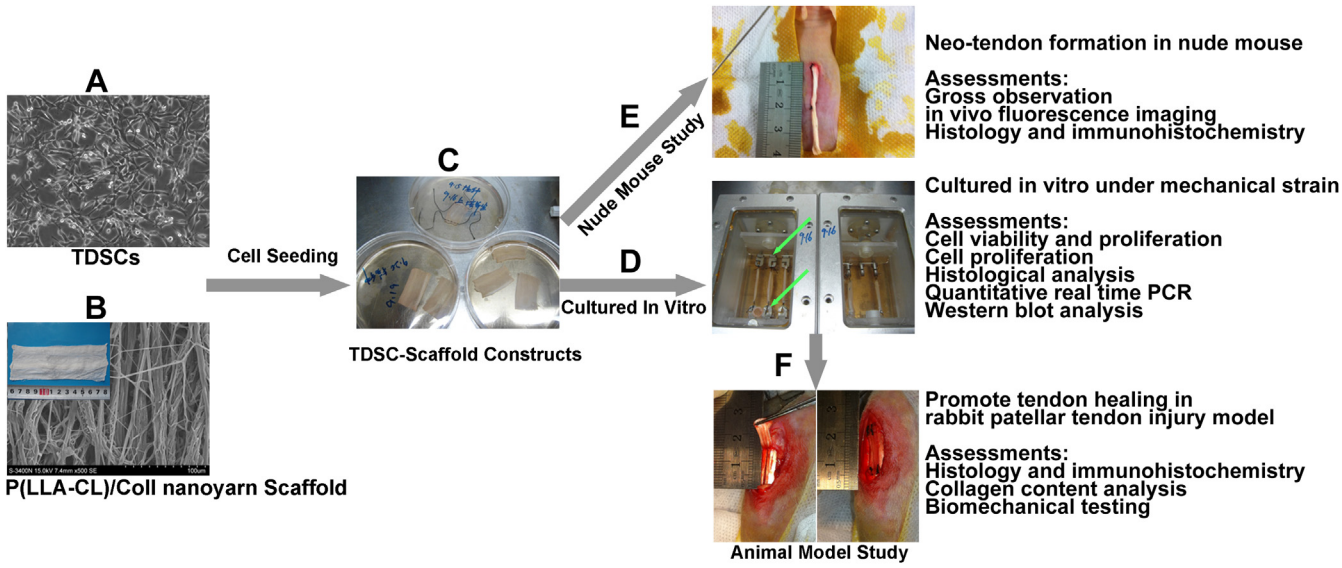

Fig. 1. TDSCs (A) seeded on P(LLA-CL)/Col scaffolds (B) formed TDSCs-scaffold constructs (C). The TDSCs-scaffold constructs were then curled and cultured under mechanical strain to form tissue-engineered tendons *in vitro* (D), and sutured on the back of nude mice (E) to form neo-tendons *in vivo*. The TDSCs-scaffold constructs or scaffolds were sutured to patellar bone and tibia tuberosity to promote tendon healing in a rabbit patellar tendon window injury model (F).

were tested for each gene. GAPDH served as internal reference, and the expression level of target gene was calculated as $2^{-\triangle\triangle Ct}$.

2.3.6. Western blotting analysis

The cell-scaffold constructs were put into liquid nitrogen for grinding immediately after harvest, then total protein was extracted with 100 μl RIPA lysis buffer (P0013B, Beyotime) containing PMSF (ST506, Beyotime), and the protein concentration was measured with BCA protein test kit (P0010S, Beyotime). 20 μg protein from each sample was added into 12% (for 26 kDa Tenascin-c) or 10% (for 136 kDa Col I, and 160 kDa Col III) SDS-PAGE for gel electrophoresis. The isolated protein was transferred onto PVDF membrane (Tiangen Biotech) at 80 V and in 120 min, then PVDF membrane was blocked for 2 h with 5% BSA and thereafter incubated overnight at 4 °C using Col I (1:1000, ab90395, abcam), Col III (1:250, AF5810, Acris Antibodies Gmbh), tenascin C (1:500, LS-C39575, Lifespan biosciences) and GAPDH (1:5000, KC-5G4, Kangchen Biotech) monoclonal antibodies, followed by incubation with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2000, CW0102, cwbiotech). The membranes were washed with TBST three times for 15 min, and finally the protein on PVDF membrane was colored with Super Signal West Femto Maximum Sensitivity Substrate (34095, Thermo Scientific). And the protein level was quantified and normalized to GAPDH bands by densitometry in ImageJ software ($n = 3$).

2.4. In vivo neo-tendon formation by TDSCs-engineered tendons in nude mice ectopic implantation

2.4.1. Cell labeling and ectopic implantation of tissue-engineered tendons

Before *in vivo* ectopic transplantation, TDSCs were pre-stained with CM-Dil (C7000, invitrogen) [29]. Briefly, the cells were incubated with 2 μg/ml CM-Dil at 37 °C, for 5 min, and then for an additional 15 min at 4 °C, after labeling, cells were washed with PBS and resuspended in fresh medium, and then inoculated on the scaffolds with the previous method.

A nude mouse model was utilized as previously reported [26] to detect the potential of ectopic tendon formation with the tissue-engineered construct under *in vivo* mechanical stimulation (Fig. 1E). Totally fifteen nude mice were anesthetized with 0.05% pentobarbital sodium, and then a 3 cm incision was made on their median back. To provide *in vivo* mechanical stimulation with the natural movement of the mouse back, two ends of 2.5 cm tissue-engineered constructs (total length: 4.5 cm) were sutured with Ethicon 6-0 suture onto the back ligaments of nude mice under subcutaneously; the remaining 2 cm tissue-engineered constructs were implanted subcutaneously without suturing, which served as controls. At week 2, 4 and 8 after operation, the transplanted tissues were harvested for *in vivo* fluorescence imaging examination, HE staining, masson staining and immunohistochemistry.

2.4.2. Macroscopic evaluation and in vivo fluorescence imaging

The fluorescence images of neo-tendon tissues at each time point were acquired by IVIS 200 imaging system (Caliper Life Sciences) [30]. After anesthesia with 0.05% pentobarbital sodium, the nude mice were immediately placed into the sample chamber of IVIS 200 imaging system. CM-Dil-expressing TDSCs at the operative site were visualized using the CM-Dil filter for excitation (445–490 nm) and emission (515–575 nm) at an exposure time of 10 s. Data were presented on a color scale overlaid on a grayscale photograph of mice and quantified as total radiant efficiency ([photons/s/sr]/[μW/cm^2]) using Living Image analysis software (Caliper Life Sciences).

2.4.3. Histology and immunohistochemistry

HE staining of neo-tendon tissues was performed with the previous method. In addition, the formation of collagens was detected according to the instructions of Masson Kit (HT15, sigma). In order to analyze the expression levels of tendon-related proteins in neo-tendons, immunohistochemical staining was performed as described in previous literature [28]. Briefly, the sections were deparaffined, hydrated, inactivated with endogenous peroxidase, and repaired with antigens, and thereafter blocked with 5% normal goat serum and then incubated overnight at 4 °C using Col I (1:1000), Col III (1:250), tenascin C (1:500) and GAPDH (1:5000) monoclonal antibodies, followed by incubation with goat anti-mouse HRP-conjugated secondary antibody (1:2000) for 1 h respectively. Sections were lightly counterstained with Harris hematoxylin, dehydrated, and mounted with p-xylene-bis-pyridinium bromide (DPX) mounting medium (Sigma). Primary antibody was replaced with blocking solution in the negative controls. The sections were examined under light microscope (Olympus BX51).

2.5. In situ rabbit patellar tendon repair model

2.5.1. Patellar tendon injury and repair animal model

Fifty-four adult female Oryctolagus cuniculus were used in the present study. Tendon injury was made with the method reported in previous literature [23]. Left full-length, full-thickness, 3 mm wide central defects were created between distal patella and tibial tuberosity. The implants were divided into three groups: (1) scaffold control group; (2) statically cultured tissue-engineered tendon group; and (3) dynamically cultured tissue-engineered tendon group. The implants were placed on tendon injuries and then sutured with Ethicon 6-0 suture onto patella and tibial tuberosity (Fig. 1F). Thereafter, the animals were fed in the cage freely. At week 4 and 12 after operation, regenerative tendon samples were collected from each group for HE staining, masson staining, polarized microscopy and immunohistochemistry, collagen content assay and mechanical test.

2.5.2. Histology and immunohistochemistry

After rabbits were enthanized with excessive pentobarbital sodium. The repaired tendons in each group were randomly selected for histological sections and then HE staining, masson staining and immunohistochemistry were performed according to the previous method. Collagen fiber alignment was examined under polarized light according to previous study [13].

2.5.3. Determination of the collagen content of repaired tendons

The collagen content in regenerative tendons was determined with collagen quantification kit (Nanjing Jiancheng, China) according to the manufacturer's instructions.

Table 1
Real time PCR primers used in this study. Primers for type I collagen, type III collagen, decorin, tenascin C, biglycan, Runx2, type II Collagen, aggrecan and GAPDH were designed from Oryctolagus cuniculus gene sequences obtained from the NCBI GenBank and RefSeq databases using Primer 5.0 software.

Gene	GenBank ID	Sequence	
Type I collagen	NM_001195668	FORWARD	CCAGAGTGGAGCAGTGGTTAC
		REVERSE	GCAGGTTTCGCCAGTAGAGA
Type III collagen	XM_002712333	FORWARD	TCAGGGTGTCAAGGGTGAA
		REVERSE	ATCCAGGGTTTCCGTCTCTT
Decorin	NM_001082330	FORWARD	CACCTTCGAGTTGTCCAGTG
		REVERSE	AGGTCCAGTAGCGTCGTGTC
Tenascin C	XM_002720513	FORWARD	GGAACTCAAATGTCCCAACAG
		REVERSE	CCAGTGTAGCCCTCATCACA
Biglycan	NM_001195691	FORWARD	TTCTGGACCTGGCTTCATTC
		REVERSE	CGGTGTGTGTGTGAGAGTAAGAG
Runx2	AY598934	FORWARD	GCAGTTCCCAAGCATTTCAT
		REVERSE	CTGGCGGGGTGTAAGTAAAG
Type II collagen	NM_001195671	FORWARD	CGCTCAAGTCCCTCAACAAC
		REVERSE	TAGTCACCGCTCTTCCACTC
Aggrecan	XM_002723376	FORWARD	GTCCACCATTCGGCATAACT
		REVERSE	CCAGGTCAGGGATTCTGTGT
GAPDH	NM_001082253	FORWARD	CTGGCAAAGTGGATGTTGTC
		REVERSE	CCGTGGGTGGAATCATACTG

2.5.4. Mechanical testing

In order to evaluate the mechanical properties of regenerative tendons, the ultimate stress and Young's modulus were measured using an Instron tension/compression system with Fast-Track software (H5K-S, Hounsfield, UK) [26]. The regenerative tendons in the center of patellar tendon repair window were fixed on the test bench with clamps and then subjected to traction with a preload of 0.1 N at 20 mm/min till breakage. The ultimate stress and Young's modulus were calculated with the real-time recorded stress–strain curve. The percentages of testing values in the regenerative tendons to the same portion of the contralateral healthy patellar tendons were also calculated.

2.6. Data analysis

All quantitative data were presented as mean ± SD. Single factor analysis of variance (ANOVA) was used to assess the statistical significance of results between groups. Differences of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Cell viability and morphology

The TDSCs were cultured on the 3D aligned P(LLA-CL)/Col scaffolds in a loading bioreactor. In terms of cell morphology, an elongated fibroblast-like morphology was maintained on aligned scaffolds with no change in the static group and dynamic group by day 14 (Fig. 2A–F). It was observed that cells remained viable in both groups by day 14 (Fig. 2A–F). There were no significant differences in the proportion of the number of viable cells in the total number of cells between static group and dynamic group at each time point (Fig. 2G).

3.2. Cell proliferation

There was consistent proliferation in both static groups and dynamic groups throughout the 14-day culture period (Fig. 2H).

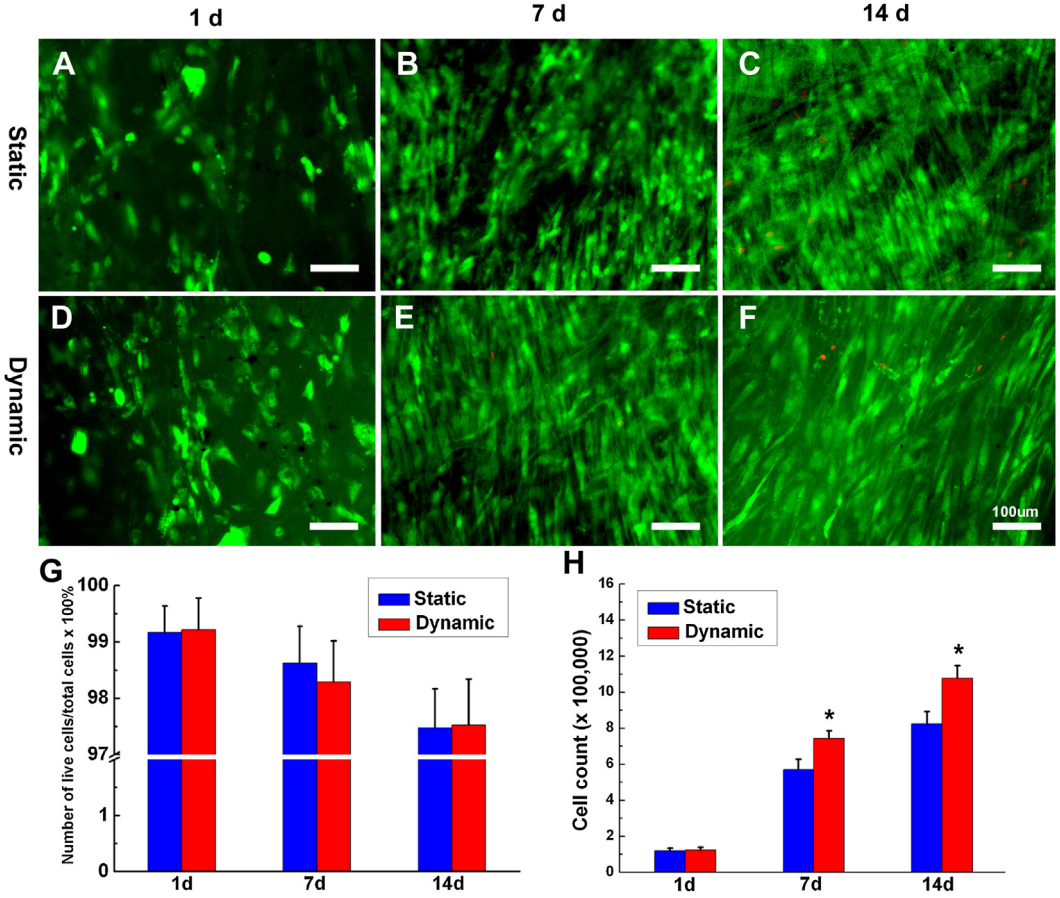

Fig. 2. LSCM images showing the cell morphology and viability of TDSCs cultured on P(LLA-CL)/Col scaffolds in static (A–C) and dynamic (D–F) groups on day 1, 7 and 14 as measured by live/dead assay. Magnification: 200×, scale = 100 μm. G. The ratio of the number of viable cells/the total number of cells was counted. No significant differences were detected between the two groups. H. Proliferation of TDSCs cultured on P(LLA-CL)/Col scaffolds in static and dynamic groups measured by PicoGreen dsDNA assay on day 1, 7 and 14. Data were expressed as mean ± SD ($n = 3$). The samples indicated with (*) had a significant difference between the two groups ($p < 0.05$).

After being cultured for 1 day, there was no significant difference in the number of cells between the groups. However, the number of TDSCs in the dynamic group was significantly increased compared with the static group by day 7 and 14 ($p < 0.05$).

3.3. Histological analysis

TDSCs growth and infiltration on the porous structures of scaffolds were assessed by histological observations. Longitudinal HE-stained sections of the cell-cultured scaffolds revealed that TDSCs could infiltrate into nearly half of the scaffolds in both groups by day 4 (Fig. 3A, D). Furthermore, the cells infiltrated into nearly the entire scaffolds in both groups by day 7 (Fig. 3B, E) and 14 (Fig. 3C, F). Nevertheless, compared with the static group, the tissue matrix of the constructs in the dynamic group is denser, and the cell distribution throughout the P(LLA-CL)/Col scaffold was found to be more uniform (Fig. 3E and F).

3.4. PCR analysis

We compared the tenogenic differentiation-related mRNA (Col I, Col III, decorin, tenascin C and biglycan) and non-tenogenic differentiation-related (Runx2, Col II and aggrecan) mRNA expression levels of TDSCs between the two groups on day 7 and 14 (Fig. 4). The results showed that the expression levels of all tenogenic differentiation-related mRNA in the dynamic group were increased compared with the static group, though only Col I and Decorin were significantly upregulated on day 7 (1.91-fold, $p < 0.05$; 2.75-fold, $p < 0.05$). Furthermore, the expression levels of Col I, Col III, decorin, tenascin C and biglycan were all significantly higher on day 14 (1.43-fold, $p < 0.05$; 1.49-fold, $p < 0.05$; 1.33-fold, $p < 0.05$; 2.30-fold, $p < 0.05$; and 1.67-fold, $p < 0.001$, respectively) in the dynamic group compared with the static group. By contrast, the dynamic group had downregulated expression of Runx2, Col II and aggrecan on day 7 (0.63-fold, $p < 0.05$; 0.51-fold, $p < 0.05$; 0.71-fold, $p < 0.05$) and 14 (0.32-fold, $p < 0.05$; 0.35-fold, $p < 0.05$; 0.54-fold, $p < 0.05$) compared with the static group.

3.5. Expression of tendon-related proteins

The expression levels of specific tendon-related proteins were assayed with Western blotting (Fig. 5). After 7 days of culture, a greater amount of Col III expressed was deposited in the dynamic group compared with the static group. Furthermore, compared with the static group, mechanical stimulation elicited a significant increase in the protein expression levels of Col I, Col III, and tenascin C in the dynamic group by day 14.

3.6. Neo-tendon formation after tissue-engineered tendon implantation in nude mice

3.6.1. In vivo fluorescence imaging and gross observation

As shown in Fig. 6, the *in vivo* fluorescence imaging results showed that the transplanted CM-Dil positive cells were present in the window wound at week 2 and 4 (Fig. 6A–B), and were decreased at week 8 (Fig. 6C). The implanted tendon tissues became slightly larger at the end of week 4 (Fig. 6E) and 8 (Fig. 6F) than week 2 (Fig. 6D) following implantation. Compared with the dynamic group, neo-tendon tissues in the static group got shorter gradually with the increase of time (Fig. 6E–F, arrows).

3.6.2. Histology and masson staining

HE staining revealed that TDSCs could penetrate into the internal space of the scaffolds well in both groups. Compared with the static group, the ECM of the dynamic group exhibited parallel alignment to the direction of mechanical stress at week 2 (Fig. 7D); though the scaffolds degraded gradually, the ECM of the dynamic group still exhibited aligned morphology at week 4 and 8 (Fig. 7E–F, arrows). In order to evaluate the maturation level of collagens, masson staining revealed that more collagen fibers were formed in the dynamic group compared with the static group at week 2 (Fig. 7J), which was more obvious at week 4 and 8 (Fig. 7K–L), indicating more collagen deposition.

3.6.3. Immunohistochemistry staining

Immunohistochemistry staining for Col I, Col III, tenascin C was performed in the neo-tendon tissues at week 2, 4 and 8 following implantation. At week 2 and 4, the expression of Col I, Col III, tenascin C in the neo-tendon showed no difference between dynamic group and static group (Fig. 8B1-4, C1-4, D1-4). Except Col III (Fig. 8C5-6), the expressions of Col I (Fig. 8B5-6) and tenascin C (Fig. 8D5-6) were stronger in the dynamic group than in the static group at week 8. No positive signal was found in each negative control groups (Fig. 8A1-6).

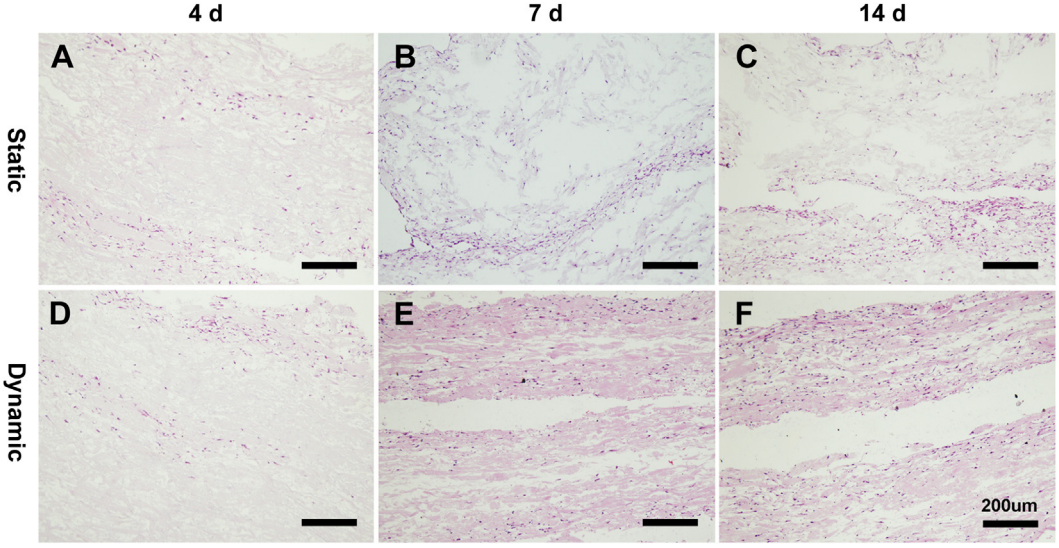

Fig. 3. Histology assay of statically and dynamically cultured TDSCs-P(LLA-CL)/Col constructs after 1(A, B), 7(C, D), 14(E, F) days. Magnification: 100×, scale = 200 μm.

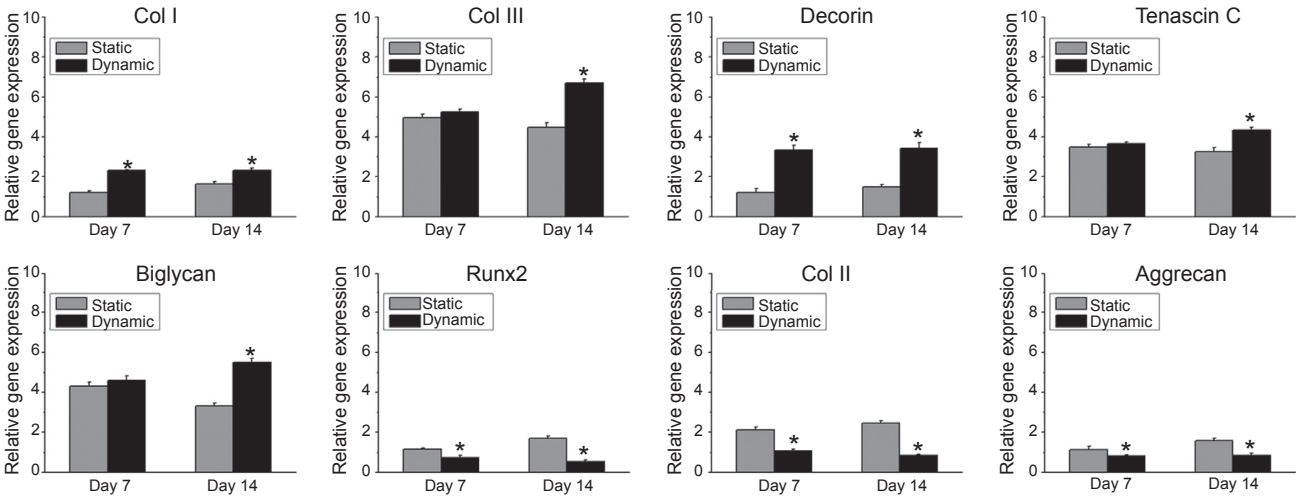

Fig. 4. Cell differentiation: the expression of tenogenic differentiation-related (Col I, Col III, decorin, tenascin C and biglycan) and non-tenogenic differentiation-related (Runx2, Col II and aggrecan) mRNA makers of TDSCs cultured in static group and dynamic group was detected on day 7 and 14. Gene expression levels were normalized to the reference genes GAPDH relative to day 0. Data were expressed as mean ± SD. The samples indicated with (*) had a significant difference between the two groups ($p < 0.05$, $n = 3$).

3.7. Tissue-engineered tendons promoted tendon healing in the rabbit patellar tendon injury model

3.7.1. Histology and collagen content assay

Histology results showed that there was no heterotopic bone and fibrocartilage formation in three groups at week 4 and 12 after operation (Fig. 9A–F). In three groups, the number of cells in regenerative tendon tissues was significantly lower at week 12 (Fig. 9B, D, F) than at week 4 (Fig. 9A, C, E) postoperatively, while matrix deposition was greater; compared with other groups, more longitudinally-arranged spindle-shaped healing tendon cells were observed embedded between parallel collagen fibers in the dynamic group at week 12 (Fig. 9F arrows). Masson staining (Fig. 9A1-F1) showed that the collagen content was significantly increased as time went on in three groups, and collagens were stained evidently strongest in the dynamic group at all time points (Fig. 9E1-F1). The

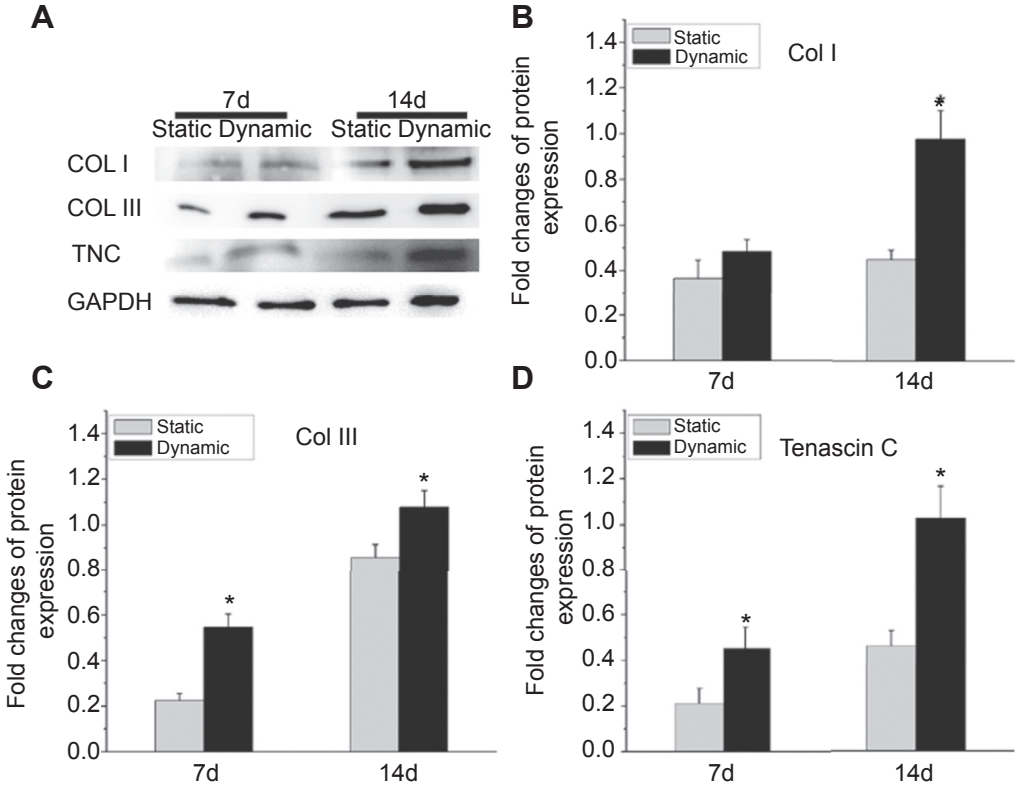

Fig. 5. Western blotting analysis of tendon/ligament-related ECM proteins, Col I, Col III, and tenascin C, produced by TDSCs cultured on the scaffolds (statically and dynamically) for 7 and 14 days (A). The results (B–D) were normalized to the data obtained from static culture for 7 days and evaluated on a relative basis for comparison between different samples. Data were expressed as mean ± SD. The samples indicated with (*) had a significant difference between the two groups ($p < 0.05$, $n = 3$).

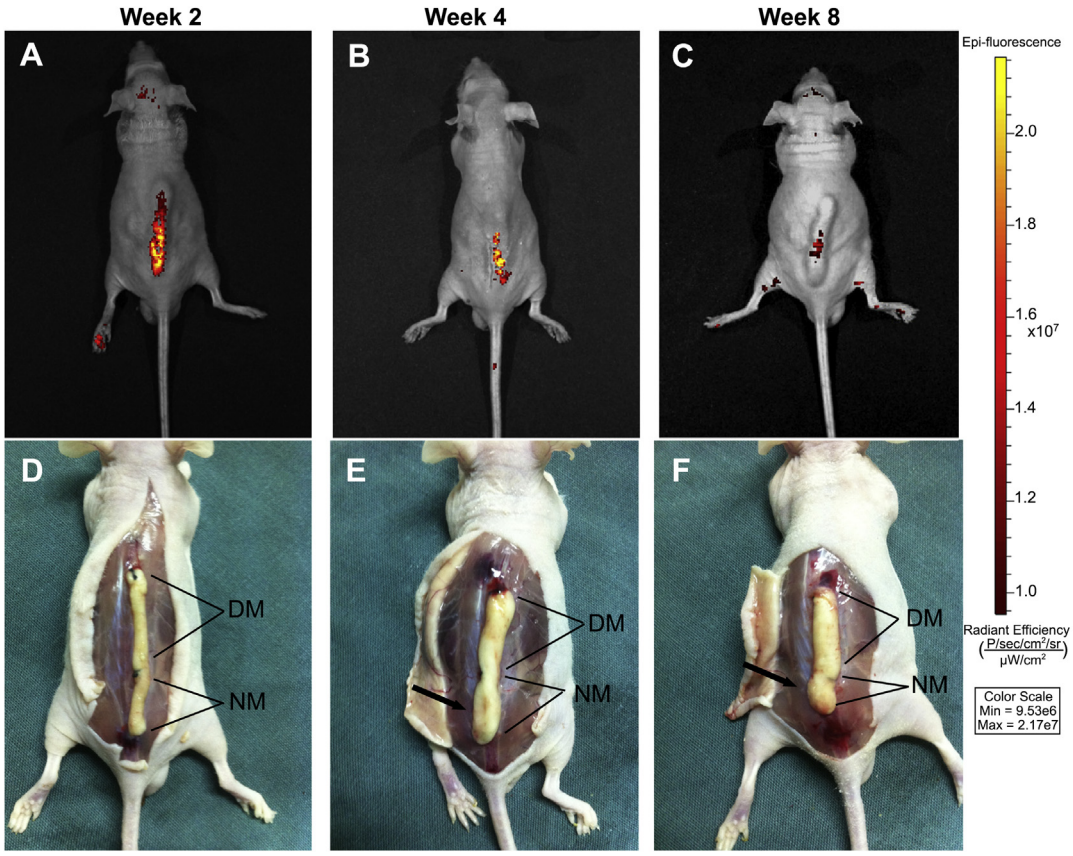

Fig. 6. Photographs showed gross observations and *in vivo* fluorescence imaging of the neo-tendon formation by TDSCs-P(LLA-CL)/Col constructs in nude mice. (A–C) *in vivo* fluorescence imaging at week 2, 4 and 8 after implantation; (D–F) gross observations at week 2, 4 and 8 after implantation, DM: dynamic group, NM: static group; black arrows: static group.

collagen birefringence was low at week 4 and increased with time in all groups (Fig. 9A2-F2). Highest collagen fibers with typical birefringence of tendon were observed at week 4 (Fig. 9E2) and week 12 (Fig. 9F2) in the dynamic group compared with other two groups. The collagen content (Fig. 9H) was lowest in control group and highest in dynamic group at all time points. At week 12, the collagen content in dynamic group reached 77.76 ± 6.82% of that in contralateral healthy patellar tendons (174.31 ± 13.89 μg/mg), significantly greater than those in static group (64.46 ± 5.04%, $p < 0.05$) and control group (47.24 ± 4.35%, $p < 0.05$).

3.7.2. Expression of tendon specific ECM markers in the regenerative tendon tissues

Immunohistochemistry staining for Col I, Col III, and tenascin C was performed in the regenerative tendon tissues at week 4 and 12. Col I, Col III, and tenascin C were positive expressed in the native tendons (Fig. 10B–D). Except control group (Fig. 10B1, B4), the expression of Col I was positive at week 4 in both dynamic group and static group (Fig. 10B2-3), and became stronger at week 12 (Fig. 10B5-6). The expression of Col I in the dynamic group was stronger (Fig. 10B3, B6) than that in the static group (Fig. 10B2, B5) and the control group (Fig. 10B1, B4) at each time point, indicating more mature neo-tendons regenerated. At week 4, the expression of Col III and tenascin C was weak in all groups (Fig. 10C1-3, D1-3), and these two kinds of protein expression were still very weak in the static group and the control group (Fig. 10C4-5, D4-5), and increased significantly in the dynamic group at week 12 (Fig. 10C6, D6). No positive signal was detected in all the negative controls for immunostaining (Fig. 10A, A1-6).

3.7.3. Mechanical testing

The repaired tendons from three groups were carefully fixed for mechanical testing with mechanical testing machine (Fig. 11). The results showed that the ultimate stress and Young's modulus were increased with time in all groups. Compared with control group, these two values were significantly higher at week 4 and 12 both in dynamic group and static group; and compared with static group, these two values were significantly higher only at week 12 in dynamic group. At week 12, the ultimate stress measured for the dynamic group was 59.58 ± 7.81% of that in normal rabbit patella tendons (57.28 ± 3.56 Mpa), compared with the static group (43.18 ± 6.58%, $p < 0.05$) and the control group (32.43 ± 5.27%, $p < 0.05$). Additionally, the implantation in the dynamic group restored Young's modulus to 51.99 ± 7.16% of that in normal rabbit patella tendons (426.69 ± 17.31 Mpa), compared with the static group (34.76 ± 4.75%, $p < 0.05$) and the control group (23.30 ± 3.83%, $p < 0.05$).

4. Discussion

Tendon tissue engineering aims to induce the self-regeneration of tendon tissues *in vivo* or to produce functional alternative tissues *in vitro* which can then be implanted into the body [31]. This study focused on the successful production of functional tissue-engineered tendons by promoting the tenogenic differentiation of TDSCs inoculated on 3D aligned P(LLA-CL)/Col scaffolds under mechanical stimulation. Our study revealed that TDSCs up-regulated the expression of tenogenic differentiation-related mRNA while down-regulated the expression of osteogenic and

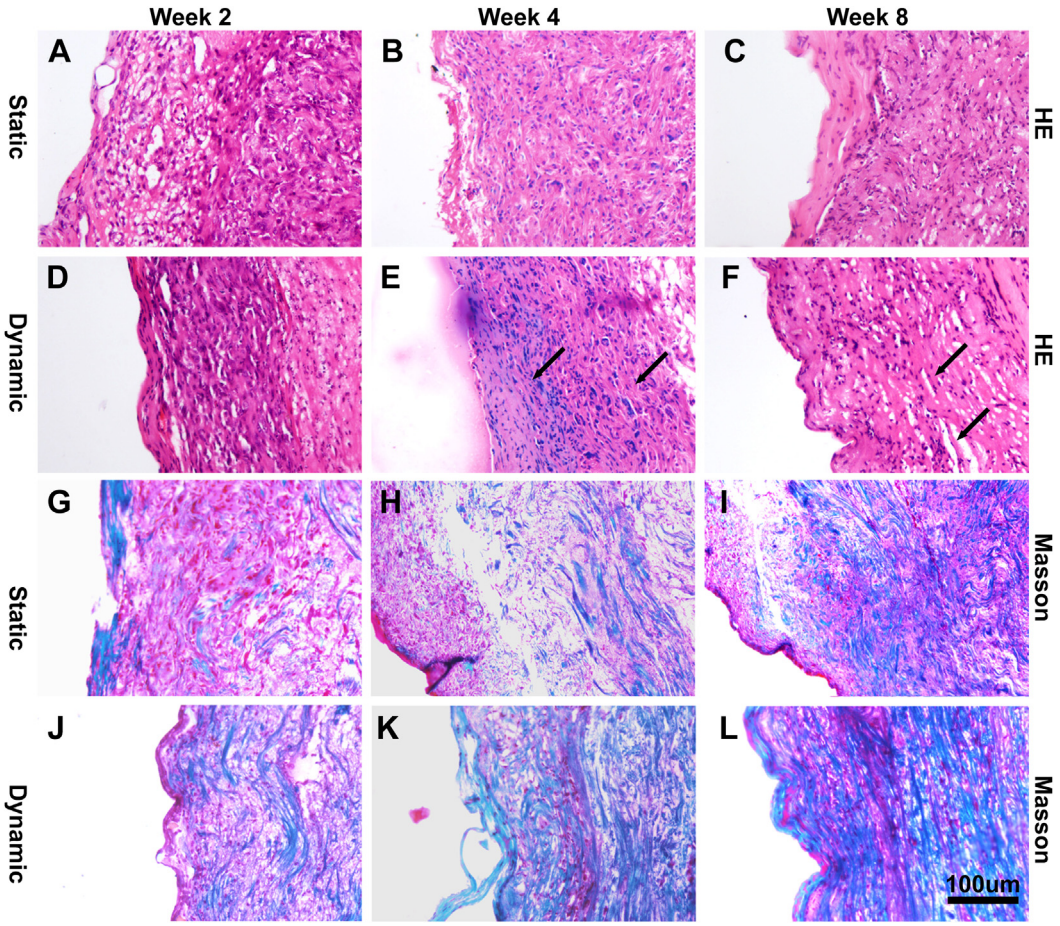

Fig. 7. Photomicrographs showed HE staining (A–F) and masson staining (G–L) of neo-tendon tissue formation by TDSCs-P(LLA-CL)/Col constructs at week 2 (A, D; G, J), week 4 (B, E; H, K), and week 8 (C, F; I, L) in static group and dynamic group in the nude mice. Magnification: 200×, scale = 100 μm ($n = 3$).

chondrogenic differentiation-related mRNA under mechanical stimuli; meanwhile, dynamically cultured TDSCs-P(LLA-CL)/Col constructs enabled more positive expression of tendon-related proteins *in vitro*. Furthermore, after transplanting the cell-scaffold constructs into nude mice, CM-Dil-labeled TDSCs survived for a long time and high-quality neo-tendon tissues were formed under physiological mechanical stimulation *in vivo*, including collagen synthesis and tendon-specific protein expression. Last but not least, compared with statically cultured constructs and scaffold control, dynamically cultured constructs significant promoted the maturation of regenerative tendon tissues and enhanced the mechanical properties of injured rabbit patellar tendons, showing its potential to promote tendon repair.

Both mechanical stimulation direction and fiber orientation of the scaffold substrate can affect cell morphology and induce uniaxial cellular alignment [25]. Our study indicated that TDSCs exhibited a fusiform shape in both dynamic group and static group (Fig. 2), because of giving the direction of mechanical loading along with the fiber orientation of P(LLA-CL)/Col scaffolds. Besides, TDSCs in both two groups maintained good cell viability after 14 days of culture (Fig. 2G); however, the cell proliferation in dynamic group was significantly higher than that in static group after 7 and 14 days of culture (Fig. 2H). This indicates that mechanical stimulation can well promote the proliferation of TDSCs. In other studies, mechanical stimulation could promote the proliferation of tenocytes [32], ligament fibroblasts [33], MSCs [1], and TDSCs [15]. Mechanical stimulation might have a direct or an indirect effect on the cells, affecting surface receptors, activating ion channels and improving nutrient transport, which led to an increase in proliferation rate [4,19]. HE staining showed that P(LLA-CL)/Col scaffolds provided a good microenvironment applicable for infiltrative growth of TDSCs (Fig. 3). TDSCs completely infiltrated into the inside of scaffolds at day 7 after inoculation in both groups, and tissue-engineered tendons in dynamic group (Fig. 3E–F) demonstrated a firmer and more ordered tissue structure than those in static group, resembling the tissue structure of native tendon tissues. Mechanical stimulation resulted in more mature tissue-engineered tendons characterized by a firmer tissue texture, highly compacted and organized tissue structure [13].

Col I is the most abundant tendon ECM protein which is crucial for repair and regeneration of the fibrous tissues. Col III is also typically over-expressed in the early stage of tendon repair [34]. Beside, decorin, tenascin C and biglycan are the important tendon proteoglycans for regulating collagen fibrils structure and function [35]. In our study, the expression of tenogenic differentiation-related ECM genes (Col I, Col III, decorin, tenascin C and biglycan) was significantly up-regulated in dynamic group by day 14 (Fig. 4). While the expression of osteogenic (Runx2) and chondrogenic (Col II and Aggrecan) differentiation-related ECM genes was significantly down-regulated in dynamic group, which indicates that the tenogenic differentiation of TDSCs is promoted under mechanical stimuli, but the osteogenic and chondrogenic differentiation of TDSCs are inhibited. More importantly, our findings also demonstrated that TDSCs in dynamic group enabled more positive

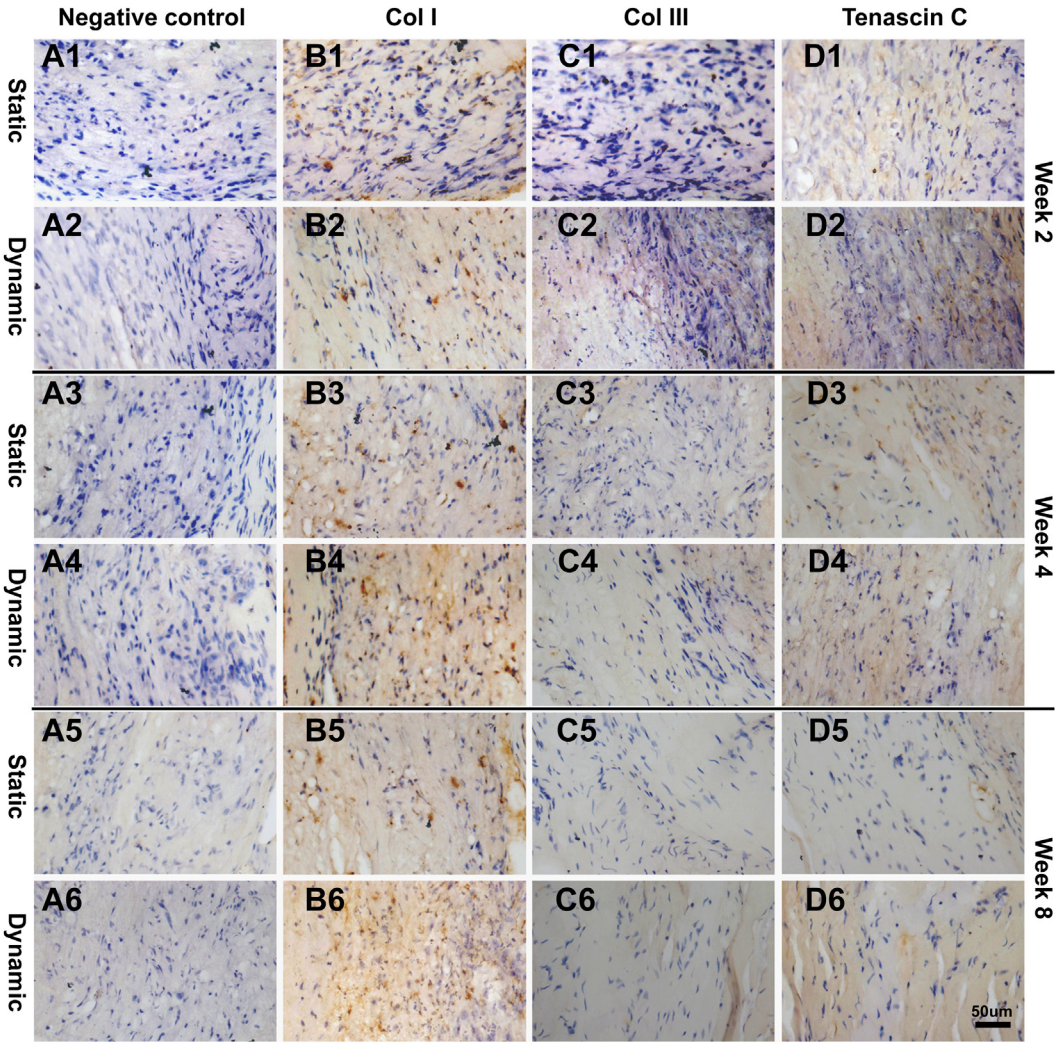

Fig. 8. Photomicrographs showed the immunohistochemistry staining for Col I (B1-6), Col III (C1-6) and tenascin C (D1-6) of neo-tendon tissue formation by TDSCs-P(LLA-CL)/Col constructs at week 2 (B1-2, C1-2, D1-2), week 4 (B3-4, C3-4, D3-4) and week 8 (B5-6, C5-6, D5-6) in static group and dynamic group in the nude mice. A1-6 were negative controls for immunostaining. Magnification: 400×, scale = 50 μm ($n = 3$).

expression of tendon-related ECM (Col I, Col III, tenascin C) proteins examined by Western blotting by day 14 (Fig. 5), suggesting that dynamic mechanical stimuli provide a good microenvironment to promote tenogenic differentiation, which is beneficial to construct tissue-engineered tendons *in vitro*. Protein expression of Col I, Col III, and tenascin C were higher when MSCs were cultured on aligned scaffolds under dynamical conditions after 7 and 14 days [36].

In the present study, we also evaluated the effects of native mechanical stimulation on the maturation of neo-tendons by the heterotopic transplantation of TDSCs-P(LLA-CL)/Col constructs in nude mice. *In vivo* imaging results showed CM-Dil-labeled TDSCs could survive in nude mice for a long time (Fig. 6A–C). Compared with static group, tissue-engineered tendons in dynamic group still maintained a long cylindrical over time (Fig. 6E–F), and more matrixes arranged parallel to the direction of mechanical stress were formed (Fig. 7E–F). Additionally, masson staining showed that there were more collagen matrixes deposited in dynamic group (Fig. 7J–L), indicating more compact neo-tendon formation. The longitudinal arrangement of collagen fibers plays an essential role in maintaining the physiological structure of normal tendons and bearing the mechanical load [37]. The main proteins (Col I, Col III and tenascin C) in tendon ECM were significantly more expressed in dynamic group than in static group (Fig. 8). It reveals that mechanical stimulation plays an important role in cell-scaffold constructs forming neo-tendon tissues after heterotopic transplantation. A similar phenomenon was observed in another study [38].

The early stage of tendon tissue engineering is likely to be the most critical in establishing tendon development to resemble native tendons [2]. To simulate the internal environment of native tendons, TDSCs-P(LLA-CL)/Col constructs were cultured under dynamical and statical conditions before implantation, and then were implanted into a rabbit patellar tendon injury repair model. Our results showed no evidences of heterotopic bone and fibrocartilage formation in three groups at week 12 after operation (Fig. 9A–F), suggesting that tendon repair with TDSCs-p(LLA-CL)/Col constructs is safe. Tendon healing exhibited a typical wound-healing course [35,39]: a short inflammatory phase (from 24 h to 5 days) was followed by a proliferative phase (from 5 days to a few weeks), then was followed by a remodeling phase (from 6 weeks to 9 months), during the remodeling phase, cellularity decreases and collagen and other ECM components deposit at the wound site to increase its strength. HE staining, masson staining, polarized

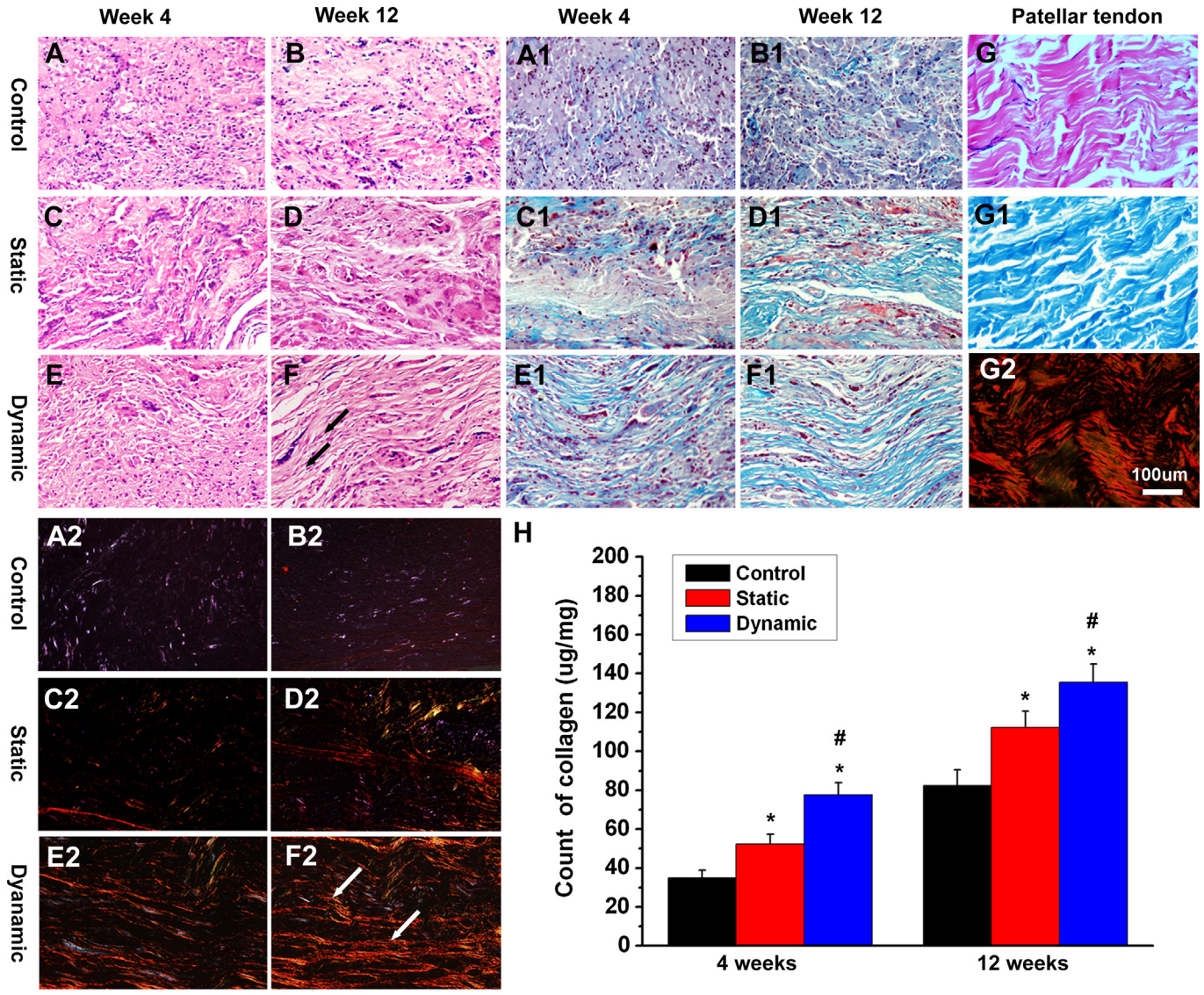

Fig. 9. Photomicrographs showed HE staining of regenerative tendon tissue formation by implants at week 4 and week 12 in control group (A–B), static group (C–D) and dynamic group (E–F) in rabbits; masson staining of regenerative tendon tissue formation by implants at week 4 and week 12 in control group (A1-B1), static group (C1-D1) and dynamic group (E1-F1) in rabbits; the polarized microscopic images of regenerative tendon tissue formation by implants at week 4 and week 12 in control group (A2-B2), static group (C2-D2) and dynamic group (E2-F2) in rabbits. G, G1 and G2 were the images of HE staining, masson staining and polarized microscopy for normal rabbit patellar tendon tissues. Magnification: 200×, scale bar = 100 μm. H. The deposited collagens in the three groups at week 4 and 12 after implantation were quantified by collagen content assay. Data were expressed as mean ± SD. $^{*}p < 0.05$, vs. the scaffold group; $\#p < 0.05$, vs. the static group ($n = 3$).

microscopy and collagen content assay results showed at week 12 after operation, more mature and remodeled collagen matrix were formed in dynamic group than in other two groups (Fig. 9), and the collagen content in dynamic group reached almost half of that in contralateral healthy patellar tendons. Furthermore, the expression of tendon-related proteins in regenerative tendons also demonstrated that more Col I, Col III and tenascin C produced in dynamic group than those in other two groups (Fig. 10), which proves that more mature regenerative tendons formed. Proper mechanical stimulation can help to increase collagen synthesis and upregulate proteoglycans expression [40,41]. Last but not least, the ultimate stress and Young's modulus of the regenerative tendons were increased with time in all groups (Fig. 11), indicating injured tendons were being repaired. Optimal rigidity is important for repaired tendons, ensuring that the tissues are not lax and forces are efficiently transmitted across the tissues to the bones [42]. At week 12, the implanted unseeded scaffolds retained only 32% of the ultimate stress and 23% of the Young's modulus of those in normal rabbit patella tendons, lower than statically and dynamically cultured constructs, indicating new ECM deposited by TDSCs contributes to healing of a central patellar tendon defect injury. These two values were the highest in dynamic group at week 12, almost 60% of the ultimate stress and 52% of the Young's modulus of those in normal rabbit patella tendons, indicating previous mechanical stimulation for tissue-engineered tendons *in vitro* significantly improves the mechanical properties of repaired tendon tissues. A previous study also indicated that the linear rigidity and Young's modulus of regenerative patellar tendons in stimulated group were higher than those in non-stimulated group, when BMSCs-collagen-sponge construct was used to repair injured rabbit patellar tendons [43]. Our study indicates that not only the maturation but also the mechanical properties of regenerative tendon tissues will be improved obviously by implanting dynamically cultured TDSCs-P(LLA-CL)/Col constructs.

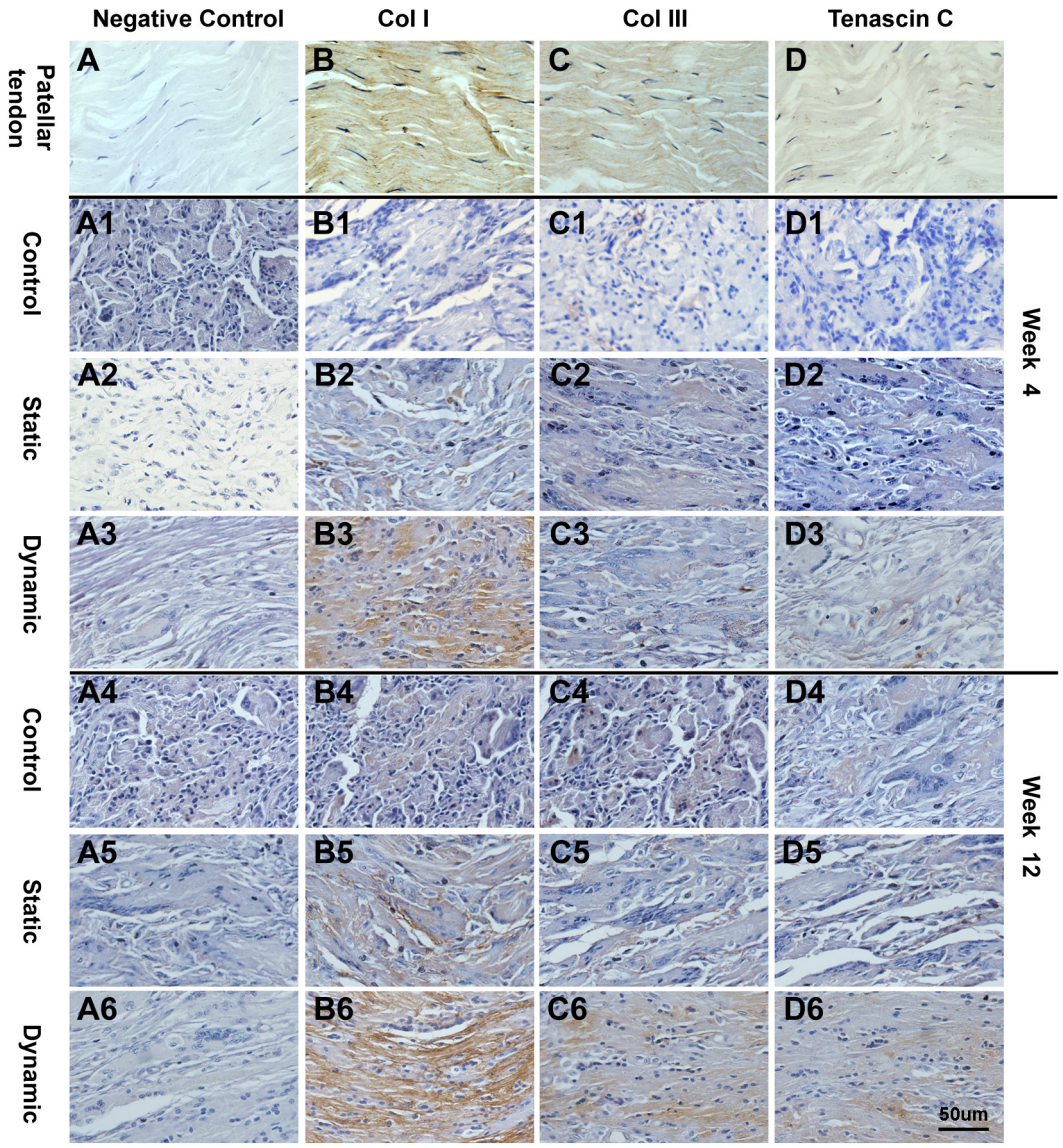

Fig. 10. Photomicrographs showed the immunohistochemistry staining for Col I (B, B1-6), Col III (C, C1-6) and tenscin-C (D, D1-6) in the normal patellar tendons (B–D) and repaired patellar tendon tissues formed in control group (B1-D1; B4-D4), static group (B2-D2; B5-D5) and dynamic group (B3-D3; B6-D6) at week 4 and 12 in rabbits. A, A1-6 were negative controls for immunostaining. Magnification: 400×, scale bar = 50 μm ($n = 3$).

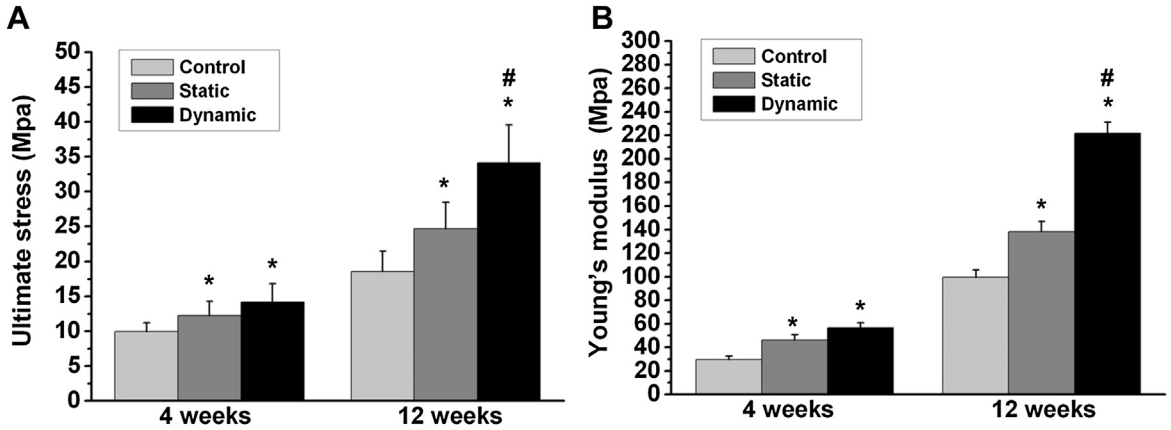

Fig. 11. The ultimate stress (A) and Young's modulus (B) of the repaired tendon tissues from different groups plotted against weeks after operation in rabbits. Data were expressed as mean ± SD. $^{*}p < 0.05$, vs. the scaffold group; $\#p < 0.05$, vs. the static group ($n = 3$).

A limitation of this study is that the signaling pathway of mechanical stimulation influencing the tenogenic differentiation of TDSCs was not clarified, though tissue-engineered tendons were successfully developed with TDSCs-P(LLA-CL)/Col constructs under mechanical stimuli and the repair of injured rabbit patellar tendons was improved. In addition, future studies are required to elucidate the healing mechanism of statically cultured tissue-engineered tendons and dynamically cultured tissue-engineered tendons promoting tendon repair and regeneration.

5. Conclusions

Our study shows that mechanical stimulation can enhance cell proliferation and subsequent tenogeneic differentiation of TDSCs *in vitro*. In addition, native mechanical stimulation can promote neo-tendon tissues formed after TDSCs-P(LLA-CL)/Col constructs implantation in nude mice. More importantly, our study is the first study showing dynamically cultured TDSCs-P(LLA-CL)/Col constructs could significantly promote the repair of injured rabbit patellar tendons with good mechanical properties by enhancing their collagen production and tendon-related proteins expression. Thus this study provides evidences proving the vast potential of TDSCs in tendon regeneration and reveals that dynamic mechanical stimulation could be helpful for the maturation of tissue-engineered tendons.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.12.042.

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