Acta Biomaterialia 10 (2014) 1360–1369

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Platelet-derived growth-factor-releasing aligned collagen–nanoparticle fibers promote the proliferation and tenogenic differentiation of adipose-derived stem cells

XingGuo Cheng [a,*], Christopher Tsao [a], Victor L. Sylvia [b], Douglas Cornet [b], Daniel P. Nicolella [c], Todd L. Bredbenner [c], Robert J. Christy [d]

[a] Microencapsulation and Nanomaterials Department, Southwest Research Institute, 6220 Culebra Rd, San Antonio, TX 78238, USA
[b] Department of Orthopedics, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA
[c] Materials Engineering Department, Southwest Research Institute, 6220 Culebra Rd, San Antonio, TX 78238, USA
[d] Extremity Trauma and Regenerative Medicine Program, US Army Institute of Surgical Research, 3698 Chambers Pass, Fort Sam Houston, TX 78234, USA

ARTICLE INFO

Article history:
Received 23 July 2013
Received in revised form 17 October 2013
Accepted 20 November 2013
Available online 27 November 2013

Keywords:
Collagen
Nanoparticle
Growth Factors
Stem cell
Tendon

ABSTRACT

In order to enhance the healing potential of an injured tendon, we have prepared a novel biomimetic aligned collagen–nanoparticle (NP) composite fiber using an electrochemical process. The aligned collagen–NP composite fiber is designed to affect the cellular activity of adipose-derived stem cells (ADSCs) through two different ways: (i) topographic cues from the alignment of collagen fibril and (ii) controlled release of platelet-derived growth factors (PDGFs) from the NPs. PDGF released from collagen–NP fibers significantly enhanced the proliferation of ADSCs when tested for up to 7 days. Moreover, compared to random collagen fibers with PDGFs, aligned collagen–NP fibers significantly promoted the desirable tenogenic differentiation of ADSCs, as evidenced by an increased level of tendon markers such as tenomodulin and scleraxis. On the other hand, no undesirable osteogenic differentiation, as measured by the unchanged level of alkaline phosphatase and osteocalcin, was observed. Together, these results indicate that the aligned collagen–NP composite fiber induced the tenogenic differentiation of ADSCs through both a topographic cue (aligned collagen fibril) and a chemical cue (PDGF released from NPs). Thus, our novel aligned collagen–NP composite fiber has a significant potential to be used for tendon tissue engineering and regeneration.

1. Introduction

Tendon/ligament injuries are among the most common orthopedic injuries to soldiers due to repeated exercise, heavy-duty work and battlefield injuries [1,2]. Tendon/ligament injuries are also common among civilians due to aging, sports and other rigorous activities, with ~800,000 patients needing surgical treatment in the USA each year [3–5]. Injured tendons/ligaments are difficult to heal due to their low cellularity and lack of blood supply. With current treatment options being limited, developing a new tendon/ligament repair and regeneration technology to treat such injuries will have substantial military and public benefit. The demand for a clinically efficient synthetic tendon healing material is substantial.

Many novel biomaterial scaffolds, such as collagen [3,6–9], silk [10–14] and poly(lactic-co-glycolic acid) (PLGA) [15–18] fibers, have been investigated for tendon/ligament tissue engineering. Alignment of nanofibrils in these synthetic scaffolds not only improves the biomechanical properties but also improves cell behavior. For example, our previous work and other work has shown that pure aligned collagen fibers prepared by an electrochemical process have improved mechanical properties and result in improved mesenchymal stem cell (MSC) behavior compared with random collagen fibers [19,20].

However, since tendons/ligaments inherently have low cellularity and vascularity, the performance of biomaterial scaffolds needs to be further enhanced with either growth factors and/or cells. In the current study, we chose a platelet-derived growth factor (PDGF) as a growth factor for loading into aligned collagen fiber since it acts as a chemotactic agent for both connective tissue cells and stem cells. PDGF is a highly promising candidate for the promotion of tendon healing [21–23]. However, the dosage and release of PDGF must be carefully controlled so that it will not be cleared from the biomaterial implant too quickly. We also chose well-characterized, readily available adipose-derived stem cells (ADSCs) as a practical and viable source of stem cells for tendon tissue engineering. Since tenocytes or tendon-derived stem cells are

* Corresponding author. Tel.: +1 210 522 6354; fax: +1 210 522 4565/4632.
E-mail address: xcheng@swri.org (X. Cheng).

1742-7061/$ - see front matter © 2013 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.actbio.2013.11.017

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 MAR 2014	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Platelet-derived growth-factor-releasing aligned collagen-nanoparticle fibers promote the proliferation and tenogenic differentiation of adipose-derived stem cells	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Cheng X., Tsao C., Sylvia V. L., Cornet D., Nicolella D. P., Bredbenner T. L., Christy R. J.,	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
US Army Institute of Surgical Research, JBSA Fort Sam Houston, Texas	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**10**	

limited to tissue regeneration of tendons, the use of ADSCs would be a viable and practical approach for tendon/ligament repair and regeneration. The key is to control the desirable differentiation of ADSCs into the preferred tenocytes, rather than an osteoblast lineage. We hypothesized that the biomaterial's topography (e.g. alignment of collagen fibrils) and the incorporation of a growth factor, such as PDGF, could act together to promote the proliferation and preferred differentiation of ADSCs.

In the current study, we synthesized PDGF-containing nanoparticles (NPs) and loaded them directly into aligned collagen fibers by using an electrochemical process. Scanning electron microscopy (SEM), compensated polarized optical microscopy and fluorescence microscopy were used to confirm the loading of NPs inside aligned collagen fibers and an enzyme-linked immunosorbent assay (ELISA) was used to examine the release profile of PDGF. We examined the ADSCs proliferation, tenogenic and osteogenic differentiation on aligned collagen–NP fibers containing PDGF and no PDGF. We also examined the topographic effect of aligned collagen–NP fibers on cell differentiation and biomechanical strength, comparing them to random collagen fibers which contain PDGF and no PDGF. Specifically, we demonstrated that PDGF released from aligned collagen–NP fiber significantly enhanced the cell proliferation, and both the topographic cues from aligned collagen fibers and the sustained PDGF release contributed to the preferred tenogenic differentiation of ADSCs and scaffold stiffness while preventing the undesirable osteogenic differentiation. This study indicated the importance of topographical control and incorporation of a growth factor in biomaterials for stem cell (e.g. ADSCs) proliferation and differentiation. This study also demonstrated the promising use of aligned PDGF-containing collagen–NP fibers with ADSCs for tendon regeneration.

2. Materials and methods

2.1. Nanoparticle synthesis, characterization and PDGF release

Poly(lactic-co-glycolic acid)-monomethoxy-poly(ethylene glycol) (PLGA-m-PEG, with 5% PEG at 5000 Da, Boehringer Ingelheim, Germany) NPs were fabricated using a standard water–oil-water double emulsion technique as described previously [24]. Briefly, 200 mg of PLGA-m-PEG was dissolved in 4 ml of CH_2Cl_2 (Sigma–Aldrich), and then 200 μl of PDGF (50 μg ml^{-1} reconstituted PDGF-BB, Mw = 12.3 KDa, R&D Systems, MN) solution was emulsified in the above oil phase. This water-in-oil emulsion was further emulsified in 40 ml of 1% sodium cholate. NPs formed after the solvent was evaporated for 3 h. Washed NPs were collected by centrifugation at 20,000 rpm for 30 min and lyophilized into powder for determination of drug loading. Using the same method, rhodamine 6G fluorescent dye (Mw = 479, Sigma) loaded NPs and empty PLGA-m-PEG NPs (control) were also fabricated by substituting the PDGF water phase with dye solution or pure water. The dye-loaded NPs were used to visualize the NP incorporation inside the collagen fiber by optical imaging and fluorescence imaging. NPs were characterized for particle size using Brookhaven ZetaPlus analyzer (Brookhaven Instruments, Holtsville, NY). A drop of washed NPs was also deposited on Au coated substrates and imaged by an environmental scanning electron microscope (ESEM, Zeiss, EV050).

To determine the amount of PDGF drug loading, 10 mg of washed, lyophilized NPs were digested in 1 ml of α-minimal essential medium, then 205 μl of 1 N NaOH was added to the above suspension. The particles were dissolved by stirring for 60 min. Next, 205 μl of 1 N HCl was added to adjust the pH to the acidic condition, as acidic pH stabilizes PDGF for quantification. After centrifugation at 10,000 rpm for 15 min, the PDGF concentration in the supernatant was measured by human PDGF-BB ELISA assay (R&D Systems, MN).

PDGF drug release from washed, lyophilized PLGA NPs was evaluated by following a release procedure developed for PLGA-PDGF microparticles developed by Wei et al. [25]. Sodium dodecyl sulfate (SDS) was added to prevent PDGF electrostatic attachment to PLGA NPs, and bovine serum albumin (BSA) was added to reduce the PDGF binding to the plastic tube. The drug release solution was prepared by mixing 10 ml of phosphate-buffered saline (PBS) with 0.158 ml of 10% SDS and 1 ml of 1% BSA (final concentrations of the release medium, 50 mM SDS, 0.1% BSA). NP suspension (1 mg ml^{-1}) was released at 37 °C in an orbital shaker (1 Hz). At designated time points, the tube was centrifuged and the PDGF in the supernatant was assayed. Fresh release medium was added and this process continued for 42 days. Afterwards, the remaining NP residue was also digested and assayed for remaining PDGF. The cumulative release profile was obtained based on calculating the amount of PDGF released over time.

2.2. Aligned collagen–NP fiber formation and microscopic analysis

To retain the NP nanosize, prevent the lost of activity of PDGF from centrifugation and lyophilization and increase the overall PDGF loading inside collagen, unwashed NP suspension containing encapsulated PDGF and non-encapsulated free PDGF were equally mixed with acidic collagen extracted from fetal bovine skin (8.2 mg ml^{-1}, Type I, Collagen Solutions LLC, San Jose, CA) and dialyzed at 5 °C for 48 h (molecular weight cut-off, or MWCO of dialysis tubing, 3500 Da, Spectrum Laboratories, CA). The dialyzed collagen/NP mixture was used for synthesis of collagen–NP fibers. The collagen–NP mixture (final concentration: collagen 4.2 mg ml^{-1} and NP 2.5 mg ml^{-1}, total volume 300 μl) was loaded inside the electrochemical cell to form an aligned NP–collagen fiber. The procedure to form an aligned collagen–NP composite fiber was described in our previous publication [19,24,26,27]. The DC voltage between two electrodes was 3 V and the distance between electrodes was 1.5 mm. The fiber formed and collected nearer the cathode side after 1 h.

The alignment of collagen inside collagen–NP fibers was confirmed by using compensated polarized microscopy using a previously described procedure [19]. Briefly, fiber was placed on a rotating stage of a compensated polarized optical microscope (Olympus BX51, Melville, NY, USA) with the application of first order wavelength gypsum plate. The sample was rotated to the direction either parallel or perpendicular to the slow axis of the gypsum plate and the interference color of aligned collagen–NP fiber was observed. For a positive birefringent material such as collagen, molecules which are aligned along the slow axis of this plate appear blue while those molecules which are aligned perpendicular to the slow axis appear yellow [19].

To observe the morphology of aligned collagen fibril, fibers were dehydrated via graded series of ethanols (70%, 80%, 95%, 100% and 100% ETOH) for a minimum of 20 min per step. Samples were then viewed in an ESEM (Zeiss EV050).

2.3. PDGF drug loading and release from aligned collagen–NP fibers

Because collagenase (Type I, Worthington, Inc.) digestion affected PDGF quantification by ELISA, the loading of PDGF inside aligned collagen–NP fibers was determined using two different methods:

(1) In the indirect method, the left-over liquid from each electrochemical cell used for fiber formation was directly assayed. Based on this, the loading of PDGF on each aligned collagen–NP fiber was calculated.

(2) In the direct method, the aligned collagen–NP fibers were put in HCl solution (adjusted to pH 4) for 24 h to dissolve the collagen fibers. The solution was centrifuged and the supernatant was assayed for PDGF by ELISA.

For PDGF release from aligned collagen–NP fiber, non-cross-linked fibers were immersed in 1 ml of 1× PBS at 37 °C for various time points, the solution was centrifuged and the supernatant was assayed for PDGF release by ELISA.

2.4. ADSC culture and proliferation on aligned collagen–NP fibers (24 and 48 h)

ADSCs derived from male Lewis rats between 8 and 9 weeks old were provided frozen using an approved protocol. The characterization of these ADSCs as a stem cell source (differentiation into adipogenic- and osteogenic-like precursors) has been previously completed in the same lab [28,29]. Cells were thawed and placed in T-25 culture flasks with MesenPRO RS® medium (2% serum) and growth supplement, L-glutamine 200 mM and mycotics (all Invitrogen). Cells were incubated (37 °C, humidified air, 5% CO_2) and passed twice into T-75 flasks with medium. For investigation of cell proliferation and differentiation, serum-free medium was used.

Aligned collagen–NP fibers were placed inside 6-well cell culture plates ($N = 3$ per well, total three wells and nine fibers per group). In the control fiber group, NPs have no PDGF loaded. In the experiment fiber group, NPs were loaded with PDGF. ADSCs were seeded in each well at a density of 20,000 cells per well. Plates were incubated at 37 °C at 5% CO_2 in serum-free media for either 24 or 48 h. At designated time points, ADSCs were removed from the plate using 750 μl of 0.25% trypsin–EDTA for 5 min, followed by the addition of 2 ml of medium. Samples were centrifuged at 4500 rpm for 5 min, the supernatant was removed and the pellet was re-suspended in 500 μl of MesenPro® medium. After light vortexing, 10 μl of cell suspension was added to 10 μl of Trypan Blue and the mixture placed into a cell counting slide. A Countess Automated Cell Counter (Invitrogen) displayed proliferating cell numbers.

2.5. Effect of aligned collagen–NP fibers on cell morphology and proliferation (7 days' culture)

A similar cell culture procedure as above was followed on aligned collagen–NP fibers placed in 6-well culture plates ($N = 3$ per well, total three wells and nine fibers per group). Each plate was seeded with ~89,500 Passage 2 cells and returned to the incubator. After 7 days of incubation, substrates (aligned collagen–NP fibers with or without PDGF loading) were removed and stained with four drops of NucBlue™ (Life Technologies) nucleus stain. Substrates were placed on a Nikon Eclipse TE2000-S fluorescent microscope and imaged with a 20× objective with NIS-Elements BR (v3.00) software. A UV dicrotic mirror was used to observe cell nuclei. Four observations were conducted per fiber (three fibers per well, total nine fibers per group). The number of live cells in each field of view (FOV) was recorded. The FOV for cell counting was measured with slide grid. Exactly three grids spanned the FOV at 200× total magnification. Grid spacing was 0.33 mm, yielding a FOV of 0.785 mm^2. After live cell counts were complete, cells were then fixed with glutaraldehyde, stained with DAPI (D3511, Lot 983827, Invitrogen) and Phalloidin Alexa Fluor 488 (A12379–3000U, Lot 808465, Invitrogen) and viewed with a FITC filter. Images were taken on an Olympus IX81 inverted fluorescence microscope with a halogen light source and an Olympus DP72 camera.

2.6. Effect of collagen alignment and PDGF from either aligned collagen–NP fibers or random collagen fibers on tenogenic differentiation and osteogenic differentiation of ADSCs

Random collagen fibers were created by heat gelation at 37 °C using dialyzed collagen solution supplemented with 10× PBS and inserting it into a mold. After the fibers were dry, a small amount of soluble PDGF with same overall nominal loading as aligned collagen–NP fiber (50 ng per fiber) was loaded inside for the PDGF group. Four groups of collagen fibers were examined by seeding cells on various fibers using 6-well plates (three fibers per well, total three wells and nine fibers per group and per time point): random fiber; random fiber with PDGF; aligned collagen–NP fiber; and aligned collagen–NP fiber with PDGF.

At each time point (3, 7 and 14 days) of culture, aligned collagen–NP fibers as well as random collagen fibers were removed from the culture plate. ADSCs were removed from the substrate following laboratory protocol. The cell–trypsin solution was analyzed for markers indicative of fibroblastic, tenocytic and osteoblastic differentiation, described briefly below.

Scleraxis, Tenomodulin (TNMN; tenocyte markers) and Tenascin C were quantified using real time-PCR (rt-PCR). ADSCs cultured in standard plastic plates for the same duration were used as baseline control. Briefly, cells cultured on either aligned collagen–NP fibers or random collagen fibers were treated with Trizol (Invitrogen/GIBCOBRL 15596-018) and the total RNA for each group was extracted. 20 ng of RNA from each group was put into wells in triplicate in an Applied Biosystems (ABI) 96-well plate. After adding 1.25 μl of primer-probe mixture (specific Assay-on-Demand™ product corresponding to TNMN, Tenascin C and Scleraxis), 0.625 μl of Multi-Scribe reverse transcriptase (Applied Biosystems, Foster City, CA), 12.5 μl of Master Mix containing AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and DEPC-treated water were sequentially added to each well to bring the total final volume to 25 μl. Each specific gene was detected and quantified using ABI PRISM™ 7000. Standard curves for each gene was constructed using known quantities of RNA and all genes were compared to 18S rRNA (ABI cat # 4319413E) as a normalizer.

2.6.1. Alkaline phosphatase (ALP) and osteocalcin (OCN)

ADSCs were washed in lysis buffer contained in an ALP kit (SynsoLyte™, Anaspec, San Jose, CA), then placed in lysis buffer + Triton-X 100 and agitated at 4 °C for 10 min. The suspension was centrifuged at 2500 rpm and the supernatant was saved for marker assays. ALP, a mineralization marker, was quantified. Osteocalcin (OC; osteoblast marker) was assayed using ELISA specific for rat osteocalcin.

2.7. Biomechanical analysis of aligned collagen–NP fibers and random collagen fibers after 21 days' cell culture

After the 21-day cell culture, aligned collagen–NP composite fibers and random collagen fibers were dehydrated and tested to determine their tensile mechanical properties. Both ends of dried bundles were fixed between thin PVC tabs using cyanoacrylate, and the fibers were rehydrated in PBS. Prior to testing, rehydrated specimen thickness was measured using a calibrated digital microscope (VHX-100, Keyence Corp., Elmwood Park, NJ). Substantial variation in width was observed along the length of each specimen. In order to accurately determine initial specimen width, photographic images of the specimens were collected by digital microscope (prior to testing) in order to allow initial specimen width to be determined at the approximate point of specimen failure following tensile testing. Specimens were mounted in a moving magnet linear motor testing frame (ElectroForce 3330, Bose

Corporation, Eden Prairie, MN) and loaded monotonically under displacement control (10 mm min^{-1}) to tensile failure. Crosshead displacement was measured using the testing frame LVDT and load was measured using a 5 lb (22.24N) load cell (Transducer Techniques, Temecula, CA). A total of six random collagen fibers and seven aligned collagen–NP fibers were successfully tested. After testing, specimen width was determined from the calibrated photographs obtained prior to the testing at the approximate point of failure. Initial specimen length was determined based on detection of a non-zero tensile load. Specimen strain was determined by dividing the displacement data by the initial specimen length, and stress was determined by dividing the load data by the cross-sectional area calculated as the product of specimen thickness and width at the point of failure. The elastic region was identified within the stress–strain data by using a moving window approach (minimum window size of 0.45 s = 45 data points) to detect any reduction in the slope of the stress–strain data. The elastic modulus for each specimen was determined as the slope of the stress–strain data within the elastic region. Peak tensile stress and strain at the point of peak tensile stress were also determined.

2.8. Statistical analysis

The results for cell proliferation in each well ($N = 3$ per group) and cell viability on various aligned collagen–NP fibers ($N = 9$ per group) were analyzed using the Mann–Whitney U test. For comparisons of three or more numeric data sets (gene expression data), variances within the data was compared with a Bartlett's test. If the variances were not significant, a one-way ANOVA was performed accompanied by Tukey's post hoc test for group discrimination. If the variances were found to be significantly different, a Kruskal–Wallis test was performed accompanied by Dunn's post hoc test for group discrimination. Statistical significance was set at $p < 0.05$.

Non-parametric Wilcoxon rank-sum tests were used to evaluate the statistical significance of differences between elastic modulus, peak stress and strain at peak stress for the collagen and reinforced collagen fibers.

3. Results

3.1. PDGF drug loading and drug release from NPs

SEM image (Fig. 1A) showed that PLGA-m-PEG NPs tend to aggregate together but each particle is ~150 nm or less. The freshly prepared NP suspension, based on laser light scattering, showed a narrow particle size distribution with an effective diameter of 149.6 nm (Fig. 1B). The typical PDGF loading was determined to be 6.6 ng PDGF mg^{-1} of washed, dried PLGA-m-PEG NPs. At 37 °C, the hydrolysis of PLGA NPs resulted in PDGF release. There is a burst release within the first 24 h, followed by a sustained release up to 42 days (Fig. 1C), with 30% remaining at that time.

3.2. Aligned collagen–NP fiber formation

Collagen–NP fiber formation is shown in Fig. 2A–C. To visualize the NP incorporation inside the aligned collagen fiber, rhodamine dye-loaded NPs were prepared at the same conditions as PDGF-loaded NPs. As shown in Fig. 2A, the collagen–NP mixture showed uniform red color due to the loading of dye in NPs. After applying the DC voltage for 1 h, the electrochemical process resulted in the formation of a collagen–NP fiber, which appeared solid red close to the cathode side (Fig. 2B and C). Based on the color change, it was determined that the majority of NPs were incorporated inside the collagen fiber by the electrochemical process.

The incorporation of NPs inside aligned collagen fibers were further verified by fluorescence microscopy. Two different aligned collagen fibers were placed next to each other. One collagen fiber was loaded with dye-loaded NPs (red arrow); another one was loaded with empty NPs (control, white arrow). Both fibers are clearly seen using optical microscopy (Fig. 3A). However, only the fiber containing dye-loaded NPs was visible using fluorescence microscopy (red arrow, Fig. 3B). This clearly confirmed the presence of NPs inside aligned collagen fibers.

The blue and yellow interference color indicated that collagen was aligned along the fiber direction (Fig. 3C and D). Due to NPs

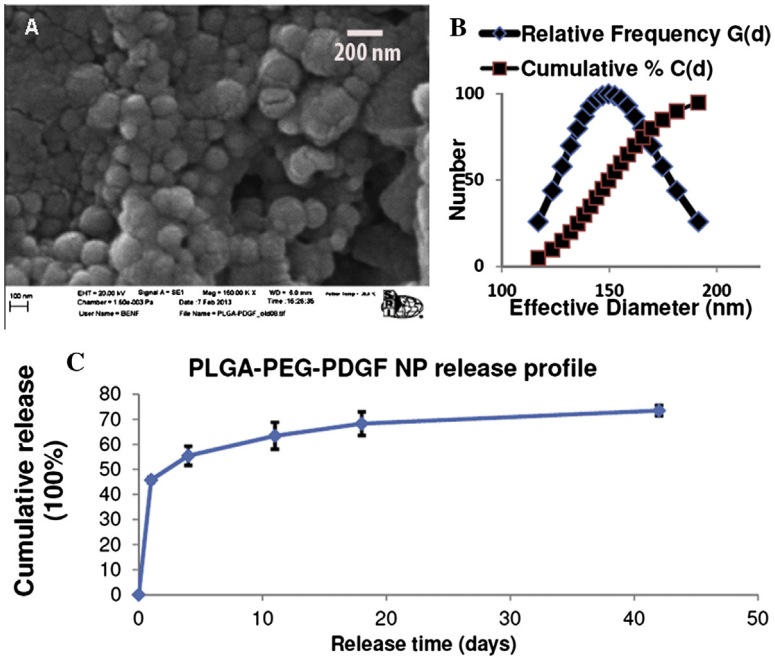

Fig. 1. (A) SEM image of dried PDGF-loaded PLGA-m-PEG NPs. (B) Particle size analysis of freshly prepared and washed NPs. (C) Release of PDGF from NPs in PBS at 37 °C.

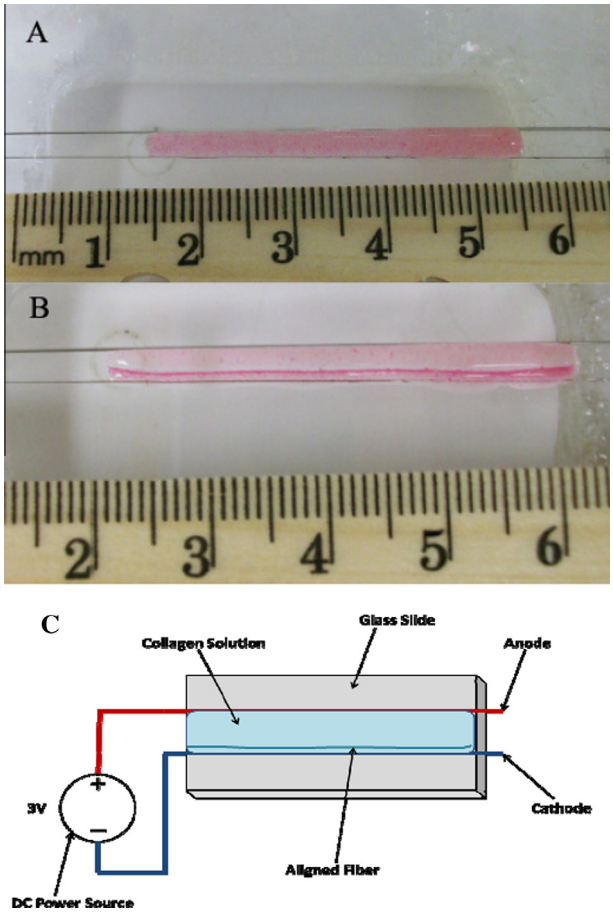

Fig. 2. Fabrication of aligned collagen–NP fibers using electrochemical process. (A) Collagen and dye-loaded NP mixture before the electrochemical process and (B) aligned collagen–NP fiber formed close to the cathode after 1 h. (C) Schematic of electrochemical alignment apparatus to form aligned collagen–NP fiber.

incorporation, the collagen fiber lost some transparency, resulting in reduction in intensity of interference color. Based on the non-uniform interference color, we can deduce that NPs slightly affect the alignment and thickness of collagen fiber. The alignment of collagen fibrils was evident based on high resolution SEM imaging (Fig. 4A and B).

3.3. PDGF loading and release from aligned collagen–NP fibers

Theoretically, there should be 50 ng PDGF loaded per fiber. However, the electrochemical process did not result in 100% NP and PDGF loading inside collagen. Using an indirect method that measures the leftover PDGF in the electrochemical chamber, we confirmed that PDGF loading per fiber was 37.5 ng per fiber (65% PDGF loading efficiency). For each aligned collagen–NP fiber, 5 ng of PDGF was loaded inside the NPs, while 32.6 ng of free PDGF was outside the NPs.

PDGF release from aligned collagen–NP fibers was dependent on two major factors: hydrolysis of PLGA NPs and diffusion of free PDGF from the collagen fiber matrix. Within the first 24 h of incubation, nearly 20.0% of the total PDGF contained within the fiber was released. After 3 days of incubation, ~50.0% of the total PDGF is released from the fiber. After the 7-day incubation period, the total cumulative release of PDGF from the aligned collagen–NP fiber was 53.26% (Fig. 4C).

3.4. ADSCs cell proliferation on aligned collagen–NP fibers (24 and 48 h)

At each time point (24 and 48 h), there was a significant difference ($p < 0.05$, Fig. 5A) in the number of proliferating cells in the well which contained aligned collagen–NP fibers loaded with PDGF than in the well with fibers that contained only empty NPs (control).

3.5. ADSCs cell proliferation and morphology on aligned collagen–NP fibers (7 days)

Direct cell imaging and counting from FOV on aligned collagen fibers was done on a 7-day culture group. Again, there is a significant increase in cell proliferation (at least twofold, $p < 0.05$) on aligned collagen–NP fibers that contained PDGF than aligned collagen–NP fibers control (Fig. 5B, $N = 9$ per group). The blue colors in Fig. 5C and D showed the nucleus of the cells, while the green colors corresponded to the cytoskeletons (mainly the actin filament) of the cells. ADSCs on aligned collagen–NP (control) fibers (Fig. 5C) and on aligned collagen–NP fibers which contained PDGF (Fig. 5D) displayed elongated cell morphology in both nuclei and cytoskeleton.

3.6. ADSCs differentiation (random collagen fiber vs. aligned collagen–NP fibers)

Tenomodulin, a marker for tenogenic differentiation of MSCs, was significantly higher on aligned collagen–NP fiber compared to that on random collagen fiber at each time point. Moreover, tenomodulin was also significantly higher on aligned collagen–NP fibers that contained PDGF than on control collagen–NP fibers (Fig. 6A). Scleraxis gene expression of ADSCs was the highest at day 3 or day 7 on aligned collagen–NP fiber that contained PDGF within the four groups studied (Fig. 6B). However, this effect disappeared on day 14. For each type of fiber (random or aligned) and at each time point, the Scleraxis gene expression was slightly higher on fibers containing PDGF than on control fibers (non-significant). Tenacin C gene expression increased in all groups as times progressed (Fig. 6C). There is no significant difference between all four groups at each time point for Tenacin C.

On the other hand, the ALP activity and OCN levels were measured at three different time points: 3, 7 and 16 days to examine the undesirable osteogenic differentiation of ADSCs. Regardless of collagen alignment, PDGF loading and time of incubation, ADSCs did not show significant increase or decrease in ALP and OCN levels (Fig. 7).

3.7. Biomechanical analysis of aligned collagen–NP fibers

Biomechanical testing indicates that after 21 days' cell seeding, PDGF-releasing aligned collagen–NP fibers have significantly higher modulus ($p < 0.05$, Fig. 8A) and higher peak stress ($p < 0.05$, significant, Fig. 8B) compared to random collagen fibers which contain PDGF.

4. Discussion

This study has demonstrated that NPs can be loaded inside aligned collagen fibers using an electrochemical process. The loading of NPs (and PDGF) inside aligned collagen fibers provides a novel approach for controlled release of a drug (i.e. PDGF) from an aligned scaffold. Our method to load a growth factor directly or using nanoparticles inside aligned collagen material is highly

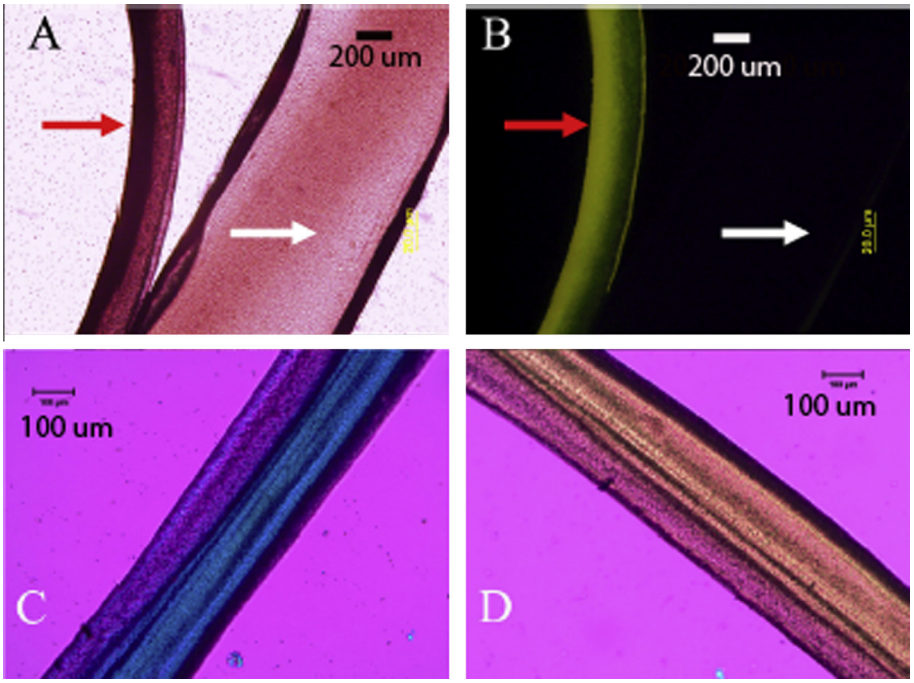

Fig. 3. Confirmation of NP incorporation and alignment of collagen within collagen–NP fibers using optical microscopy. (A) Optical image of two collagen–NP fibers, one loaded with rhodamine dye (red arrow), another loaded with empty NPs (white arrow). (B) Fluorescence imaging confirmed that dye-loaded NPs were distributed inside collagen. (C, D) Compensated polarized optical microscopy images of an aligned collagen fiber that was placed at two different directions perpendicular to each other. The interference colors (blue and yellow) indicate the alignment of collagen.

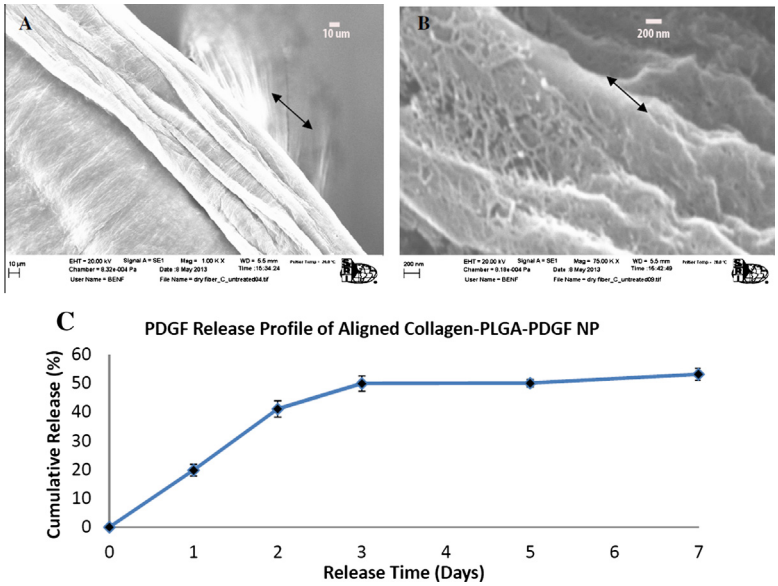

Fig. 4. SEM image of aligned collagen–NP fibers and PDGF drug release profile. (A) Low magnification image without any digestion of collagen. (B) NPs and aligned collagen fibrils are observed (double arrow indicates fiber direction). (C) PDGF release profile of aligned collagen–NP fiber.

innovative. It has a distinct difference from the method used by Lynch et al., where they directly loaded PDGF inside a regular collagen gel matrix by simple immersion [30]. Their immersion method resulted in the release of almost all PDGF in the first few hours, while our collagen–NP fiber showed a sustained PDGF release profile, with only 40% total loaded PDGF released within 48 h. Our method is also distinctively different from two other studies by Jin et al. [23] and Wei et al. [25], where they describe a method to directly load PLGA microspheres into fibrous scaffolds for controlled delivery of PDGF.

Thomopoulos et al. [21] has investigated the sustained delivery of PDGF for tendon repair using a non-load-bearing fibrin/heparin gel matrix delivery system. It was found that sustained delivery of PDGF resulted in increased cell proliferation and matrix remodeling in vivo. However, improvements in tensile properties were not achieved in vivo, possibly due to suboptimal release kinetics or other factors [31]. Our delivery method for PDGF is distinctly different from his. First, we deliver it from an aligned collagen fiber matrix. The topography (aligned fibril) and strength of our fiber differ from their delivery system. The modulus and strength of

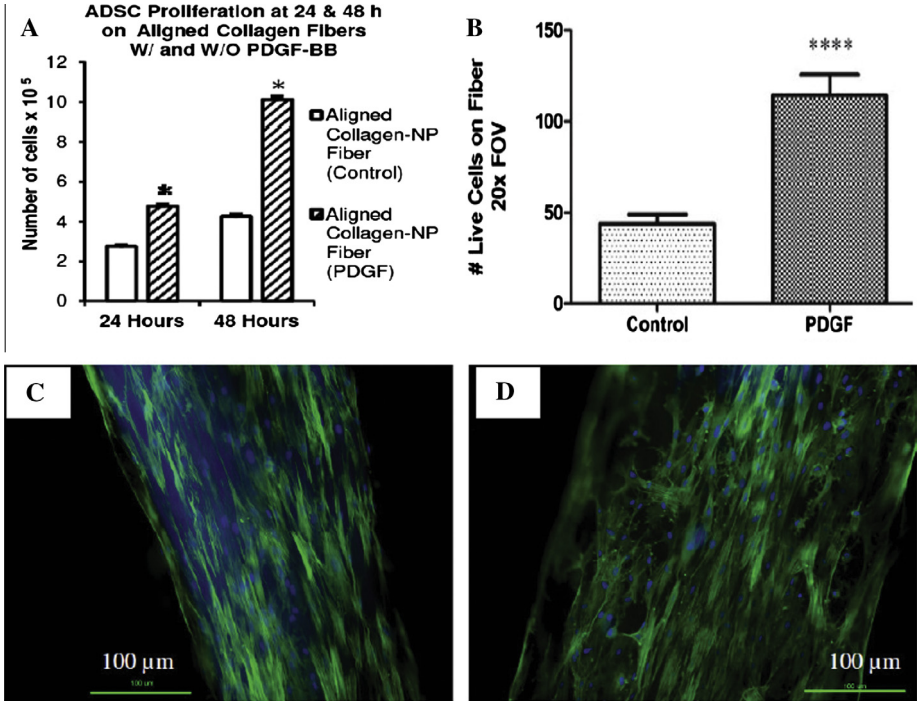

Fig. 5. Cell proliferation and morphology on aligned collagen–NP fibers (control and PDGF). (A) Aligned collagen–NP fiber that contains PDGF enhanced the proliferation of ADSCs by at least two fold compared to the control. (B) Cell number directly counted on aligned collagen–NP fiber (control vs. PDGF) after 7 days, culture. (C) Morphology of ADSCs and proliferating on aligned collagen–NP fiber (control). (D) ADSCs on aligned collagen–NP fiber which release PDGF.

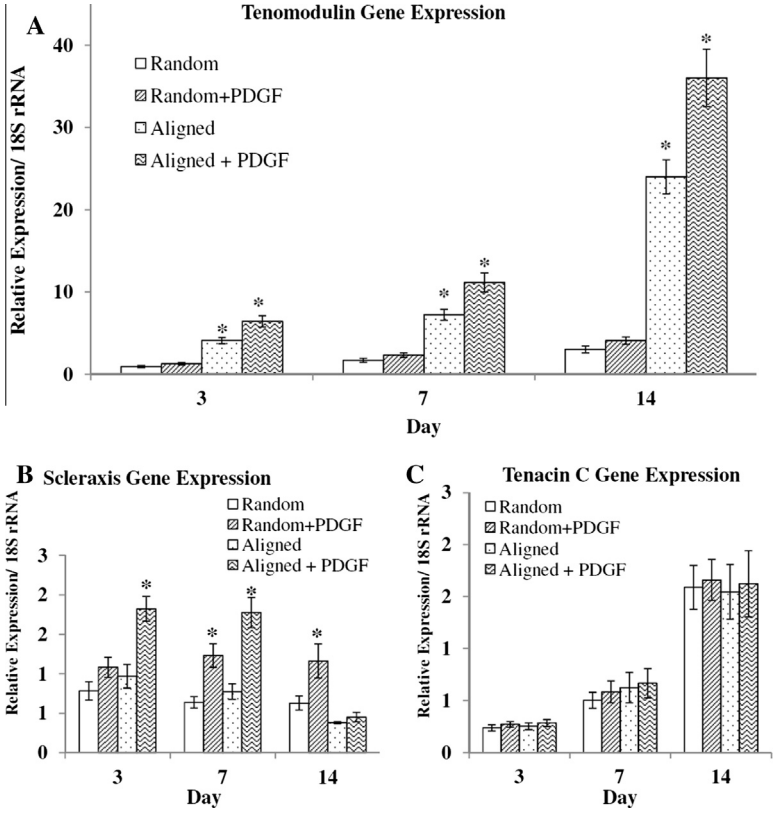

Fig. 6. Assessment of tenogenic differentiation of ADSCs cultured on four different types of fibers using gene expression: (A) Tenomodulin, (B) Scleraxis and (C) Tenascin C.

crosslinked aligned collagen fiber are close to half of those of tendon fibers [19]. Second, part of our PDGF was incorporated inside the NPs. The release of PDGF in vivo will be dependent on at least two factors: collagen degradation and remodeling, and NP hydrolysis. Further optimization of our delivery system might result in sustained release of PDGF over an even longer time period. In fact,

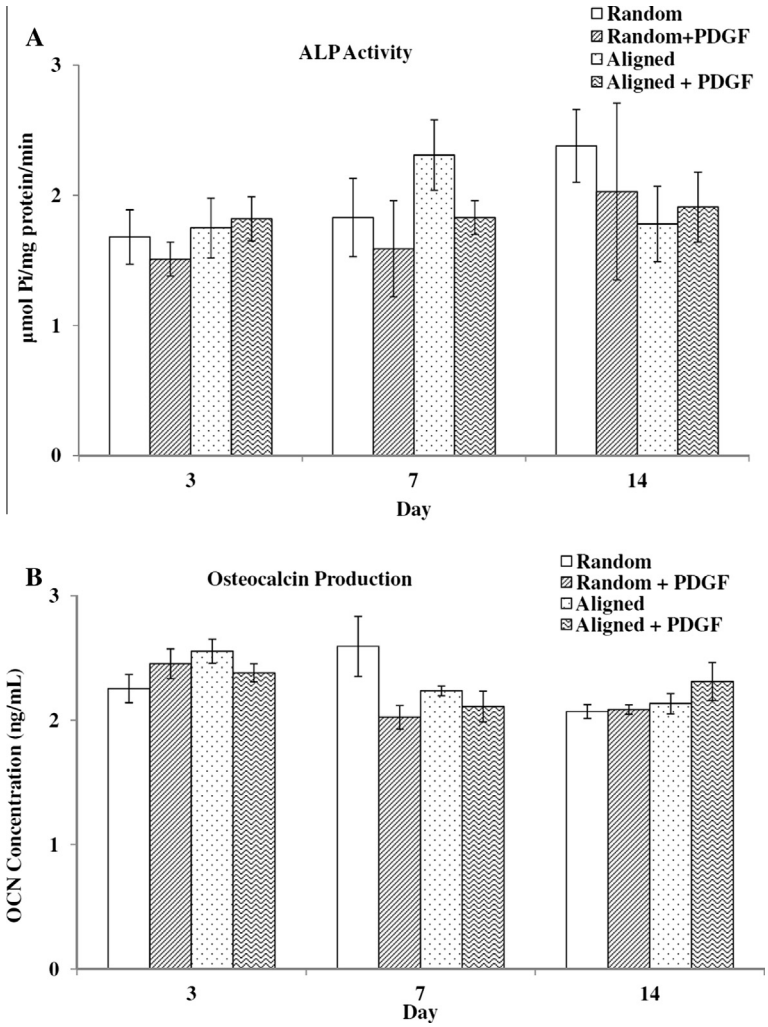

Fig. 7. Assessment of osteogenic differentiation of ADSCs cultured on four different types of fibers: (A) ALP level and (B) OCN level.

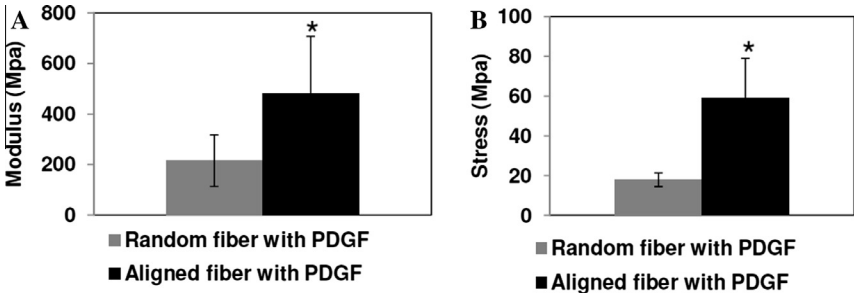

Fig. 8. Assessment of mechanical properties of aligned collagen–NP fibers and random collagen fibers (both contained PDGF) after 21 days' cell culture: (A) modulus and (B) peak stress.

our study is the first one to investigate the cellular effects of both topography and a growth factor from aligned collagen fibers prepared using an electrochemical process.

The PDGF loading inside PLGA-m-PEG NPs was dependent on various parameters: PLGA-m-PEG weight, PDGF solution volume and concentration, sodium cholate volume and concentration, and evaporation condition (e.g. room temperature vs. 5 °C). Increasing the mass of PLGA-m-PEG in the formulation will increase the loading by introducing more polymer to encapsulate PDGF. However, by doing this, it may increase the size of the particles. Literature has reported that much higher PDGF loading inside polymer microparticles can be achieved using high concentrations of PDGF and higher mass of polymers [23]. Sodium cholate, a surfactant, plays a key role in emulsifying and stabilizing the NPs during formation. Thus, a change of sodium cholate volume and concentration will affect the PDGF loading inside NPs. Lastly, it was found that controlling the evaporation temperature during NP formation can drastically affect the PDGF loading of the NPs. The loading of PDGF inside the NPs increases if kept at 5 °C while evaporating dichloromethane out of the solution.

The mechanism of NP incorporation inside aligned collagen fibers is mainly attributed to the movement of collagen due to

isoelectric focusing, as discussed previously in detail [19]. Briefly, the electrolysis of water created a pH gradient (pH value of 4 in the anode side and pH value of 11 in the cathode side). Under the pH gradient, amphiphilic collagen molecules become positively charged in the anode side and move towards an isoelectric line (collagen will have no charge at pH of ~9). Collagen molecules in the cathode side become negatively charged and move toward the isoelectric line as well. Thus, all collagen molecules are congregated and assembled in the isoelectric line, forming a highly aligned collagen fiber. The length of each collagen molecule is ~300 nm in length and 1.5 nm in diameter. The entanglement of a large amount of collagen molecules during movement might result in the size of collagen's structure being much larger than the size of PLGA NPs we used (150 nm). We have observed earlier that even microbubbles trapped inside collagen can be moved along with collagen to be trapped inside the fiber. The isoelectric focusing also causes the microbubble to change from round shape to oval shape. It appeared that NPs (empty NPs, dye-loaded NPs, PDGF-loaded NPs) and/or free PDGF molecules can be assembled along with collagen in a similar manner as microbubbles.

Based on observation using fluorescent and optical microscopy using dye-loaded NPs and drug release of collagen–NP fibers, we concluded that the majority of NPs (more than 60%) of PLGA NPs in the collagen–NP mixture was assembled inside the aligned collagen–NP fiber. Thus, this process can efficiently load NPs inside collagen fibers. A SEM image (Fig. 4) and compensated polarized optical image (Fig. 3C and D) also show that NPs might not be evenly distributed across the transverse direction of the fiber, with more NPs on one side compared to the other. We suspect that the charge of NPs (Z-potential) might be a contributing factor but the investigation of the assembly mechanism was not the focus of this work.

The ADSCs cell study further demonstrated the advantage of our growth factor delivery strategy. The effect of released PDGF from aligned collagen–NP fibers on ADSCs can be observed at 24 h, 48 h and up to 14 days (Figs. 5–7). Fig. 5A and B demonstrates that released PDGF from aligned collagen–NP fibers promotes the proliferation of ADSCs. Compared to the method of PDGF release from a collagen gel matrix (released within 24 h) [30], our controlled release method resulted in a longer, sustained effect on cells. This is highly desirable because tendons/ligaments tend to need a longer time to healing due to the lack of vascularity and cellularity.

TNMN and scleraxis are both candidate marker genes for tenocytes (i.e. tenogenic differentiation) [32]. Kishore et al. have previously demonstrated that purely aligned collagen fibers which have been fabricated using the same process, without any growth factors, can promote the tenogenic differentiation [20]. They demonstrated that TNMN was significantly elevated on aligned collagen fibers compared to random collagen fibers. On the other hand, scleraxis was only significantly elevated at 3 days, but not at 7 and 14 days. Our results are consistent with their findings for fibers without PDGFs. TNMN gene expression was much higher on aligned collagen fibers compared to random collagen fibers at all three time points ($p < 0.05$). The loading of PDGF inside aligned collagen–NP fibers also resulted in the highest TNMN expression of ADSCs. Our results demonstrated that both biomaterial alignment and growth factor release have pronounced effect of TNMN. It should be pointed out the effect of PDGF on the TNMN gene was not due to cell proliferation which results in a larger amount of nuclei material, since the same amount of total RNA was used for each group and normalized.

On the other hand, scleraxis expression was highest in the collagen–PDGF fiber group at both 3 and 7 days, but not 14 days. This might be due to the potential negative feedback loop between tenomodulin and scleraxis genes in the late stage (i.e. the increase of one eventually leads to decrease of the other), as reported very recently by Qi et al. [33]. Another marker, Tenascin C, was unchanged among all four groups at all three time points. Tenascin C is an ECM gene that was found abundantly in developing tendons and it binds to fibronectin. Tenascin C is a tendon related gene, but not a specific marker of tenogenic differentiation. Nevertheless, it appeared that both aligned collagen–NP fibers (without and with PDGF) enhanced that tenogenic differentiation of ADSCs. The release of PDGF further significantly enhanced the tenogenic differentiation.

One of the major limitations of using stem cells for tendon/ligament repair is the possible differentiation of stem cells into osteoblasts. This will result in ossified bone formation onto the implant material. It is reported that the MSCs treatment may induce ectopic bone formation in 28% of the tendon/ligament repairs [34]. The biomaterial matrix will play an important role in prevention of osteogenic differentiation. ALP level and OC level were not significantly elevated on our aligned collagen–NP fibers during our investigation period (3, 7 and 14 days, Fig. 7). This indicated that aligned collagen–NP fibers (PDGF and control) did not cause the osteogenic differentiation of ADSCs.

There are several limitations in the current study. First, the loading of PDGF inside PLGA-m-PEG NPs is low (6.6 ng PDGF/mg NP). To increase PDGF loading inside aligned collagen fiber, we loaded both PDGF-containing NPs and free PDGF inside collagen. This resulted in a complex PDGF release mechanism. Further study on fiber prepared using free PDGF or pure PDGF-containing NPs are needed. Second, all collagen fibers used in this study were not crosslinked. Crosslinking will improve the mechanical properties of the fiber, but it might crosslink the PDGF as well. We have observed that crosslinking of aligned collagen–NP fiber using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) decreased the amount of PDGF available. The crosslinking issue might be resolved by braiding nine crosslinked collagen fibers along with one non-crosslinked collagen fiber. Thus, a sufficient amount of PDGF can be delivered from one non-crosslinked collagen fiber, while the mechanical property will remain largely non-compromised using nine crosslinked fibers. A total of ten aligned collagen–NP fibers were braided and bundled by a silk suture to create one 3-D aligned collagen scaffold (Fig. 9) that should be able to fit a 5 mm Achilles tendon gap defect in rat. Third, our aligned collagen fiber needs further investigation before being implanted in vivo. In particular, the braided scaffold in Fig. 9 will be tested for maximum load and strength at wet conditions or tested under dynamic physiological conditions using high replica numbers (e.g. $N = 9$, 1 Hz, wet, 37 °C). A rat Achilles tendon can sustain a maximum force of 32 N. Depending on the outcome of mechanical evaluation, the aligned collagen fiber scaffold can either be used as

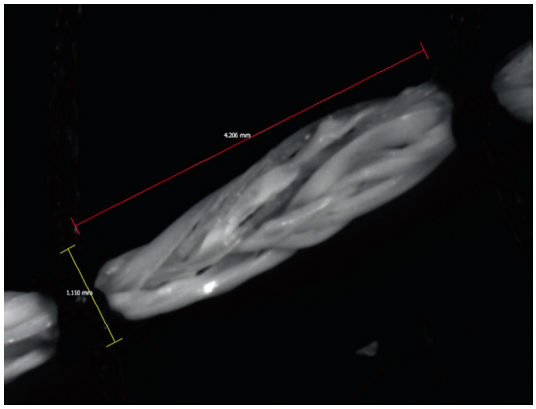

Fig. 9. Image of a braided aligned collagen–NP fiber scaffold 20×.

an augmentation graft (non-load-bearing) or used directly as a load-bearing graft (repairing tendon defect).

Despite the above limitations, our study demonstrated that both topography and PDGF from aligned collagen fibers resulted in favorable tenogenic differentiation. PDGF released from aligned collagen fibers also enhanced the cellular proliferation as well. Since PDGF is also proangiogenic, our study might result in an effective tendon healing strategy using aligned collagen–NP fiber scaffolds, overcoming the slow healing of an injured tendon due to low cellularity and lack of vascularization. Our next objective is to further improve the scaffold and implant aligned collagen–NP fibers (with and without PDGF and/or ADSCs) into a rat model for tendon repair and regeneration. Thus, the role of the biomaterial (aligned collagen fibers), growth factor (PDGF) and cells (ADSCs) can be fully investigated for tissue engineering and regeneration of tendons.

5. Conclusion

In summary, we have shown a novel strategy to directly load PDGF-containing PLGA-m-PEG NPs into aligned collagen fiber biomaterials using an electrochemical process. The loading of NPs and PDGF inside aligned collagen fibers resulted in sustained release of PDGF over a much longer time, compared with conventional methods by simple immersion inside a collagen matrix. Controlled release of PDGF from biocompatible PDGF-containing collagen–NP fibers enhanced the proliferation of ADSCs. Moreover, it also further promoted the tenogenic differentiation of ADSCs from two different cues: topographic cues from the aligned collagen fibrils and released PDGF from the fibers. The undesirable osteogenic differentiation of ADSCs on aligned collagen–NP fiber was not observed. Thus, these novel aligned collagen–NP fiber biomaterial (with or without PDGF and/or ADSCs) are promising for tendon/ligament repair and regeneration.

Acknowledgements

This work is supported by funding from the US Army Med Research ACQ Activity (Contract No. W81XWH-10-1-0986). The authors also acknowledged help from John P. Salcido for editing and grammar checking of this manuscript.

Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1–5, 9 are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2013.11.017.

References

[1] Chattar-Cora D, Perez-Nieves R, McKinlay A, Kunasz M, Delaney R, Lyons R. Operation Iraqi freedom – a report on a series of soldiers treated with free tissue transfer by a plastic surgery service. Ann Plast Surg 2007;58:200–6.
[2] White DW, Wenke JC, Mosely DS, Mountcastle SB, Basamania CJ. Incidence of major tendon ruptures and anterior cruciate ligament tears in US army soldiers. Am J Sports Med 2007;35:1308–14.
[3] Butler DL, Gooch C, Kinneberg KRC, Boivin GP, Galloway MT, Nirmalanandhan VS, et al. The use of mesenchymal stem cells in collagen-based scaffolds for tissue-engineered repair of tendons. Nat Protoc 2010;5:849–63.
[4] Butler DL, Hunter SA, Chokalingam K, Cordray MJ, Shearn J, Juncosa-Melvin N, et al. Using functional tissue engineering and bioreactors to mechanically stimulate tissue-engineered constructs. Tissue Eng Part A 2009;15:741–9.
[5] Butler DL, Juncosa-Melvin N, Boivin GP, Galloway MT, Shearn JT, Gooch C, et al. Functional tissue engineering for tendon repair: a multidisciplinary strategy using mesenchymal stem cells, bioscaffolds, and mechanical stimulation. J Orthop Res 2008;26:1–9.
[6] Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: structure and function. Acta Biomater 2009;5:1–13.
[7] Juncosa-Melvin N, Boivin GP, Galloway MT, Gooch C, West JR, Sklenka AM, et al. Effects of cell-to-collagen ratio in mesenchymal stem cell-seeded implants on tendon repair biomechanics and histology. Tissue Eng 2005;11:448–57.
[8] Juncosa-Melvin N, Boivin GP, Gooch C, Galloway MT, West JR, Dunn MG, et al. The effect of autologous mesenchymal stem cells on the biomechanics and histology of gel–collagen sponge constructs used for rabbit patellar tendon repair. Tissue Eng 2006;12:369–79.
[9] Kishore V, Uquillas JA, Dubikovsky A, Alshehabat MA, Snyder PW, Breur GJ, et al. In vivo response to electrochemically aligned collagen bioscaffolds. J Biomed Mater Res B 2012;100B:400–8.
[10] Chen X, Qi YY, Wang LL, Yin Z, Yin GL, Zou XH, et al. Ligament regeneration using a knitted silk scaffold combined with collagen matrix. Biomaterials 2008;29:3683–92.
[11] Fan HB, Liu HF, Wong EJW, Toh SL, Goh JCH. In vivo study of anterior cruciate ligament regeneration using mesenchymal stem cells and silk scaffold. Biomaterials 2008;29:3324–37.
[12] Fang Q, Chen DL, Yang ZM, Li M. In vitro and in vivo research on using *Antheraea pernyi* silk fibroin as tissue engineering tendon scaffolds. Mater Sci Eng C 2009;29:1527–34.
[13] Hairfield-Stein M, England C, Paek HJ, Gilbraith KB, Dennis R, Boland E, et al. Development of self-assembled, tissue-engineered ligament from bone marrow stromal cells. Tissue Eng 2007;13:703–10.
[14] Liu HF, Fan HB, Wang Y, Toh SL, Goh JCH. The interaction between a combined knitted silk scaffold and microporous silk sponge with human mesenchymal stem cells for ligament tissue engineering. Biomaterials 2008;29:662–74.
[15] Bashur CA, Dahlgren LA, Goldstein AS. Effect of fiber diameter and orientation on fibroblast morphology and proliferation on electrospun poly(D, L-lactic-co-glycolic acid) meshes. Biomaterials 2006;27:5681–8.
[16] Moffat KL, Kwei ASP, Spalazzi JP, Doty SB, Levine WN, Lu HH. Novel nanofiber-based scaffold for rotator cuff repair and augmentation. Tissue Eng Part A 2009;15:115–26.
[17] Sahoo S, Ouyang H, Goh JCH, Tay TE, Toh SL. Characterization of a novel polymeric scaffold for potential application in tendon/ligament tissue engineering. Tissue eng 2006;12:91–9.
[18] Sahoo S, Toh SL, Goh JCH. PLGA nanofiber-coated silk microfibrous scaffold for connective tissue engineering. J Biomed Mater Res B 2010;95B:19–28.
[19] Cheng XG, Gurkan UA, Dehen CJ, Tate MP, Hillhouse HW, Simpson GJ, et al. An electrochemical fabrication process for the assembly of anisotropically oriented collagen bundles. Biomaterials 2008;29:3278–88.
[20] Kishore V, Bullock W, Sun X, Van Dyke WS, Akkus O. Tenogenic differentiation of human MSCs induced by the topography of electrochemically aligned collagen threads. Biomaterials 2012;33:2137–44.
[21] Thomopoulos S, Zaegel M, Das R, Harwood FL, Silva MJ, Amiel D, et al. PDGF-BB released in tendon repair using a novel delivery system promotes cell proliferation and collagen remodeling. J Orthop Res 2007;25:1358–68.
[22] Caliari SR, Harley BAC. The effect of anisotropic collagen-GAG scaffolds and growth factor supplementation on tendon cell recruitment, alignment, and metabolic activity. Biomaterials 2011;32:5330–40.
[23] Jin QM, Wei GB, Lin Z, Sugai JV, Lynch SE, Ma PX, et al. Nanofibrous scaffolds incorporating PDGF-BB microspheres induce chemokine expression and tissue neogenesis in vivo. PLoS ONE 2008:3.
[24] Cheng XG, Tsao C, Saul JM, Sylvia V, Cornet D, Christy R. Comparison of two nanoparticle formulations for localized delivery of platelet-derived growth factor (PDGF) from aligned collagen fibers. Pharm Nanotechnol 2013;1:1–10.
[25] Wei GB, Jin QM, Giannobile WV, Ma PX. Nano-fibrous scaffold for controlled delivery of recombinant human PDGF-BB. J Control Release 2006;112:103–10.
[26] Cheng XG, Poenitzsch V. Aligned polymers including bonded substrates. US patent application No. 12/813,834, 2010.
[27] Cheng XG, Poenitzsch V, Cornell L, Tsao C, Potter T. Electrochemical bioencapsulation of nanomaterials into collagen for biomedical applications. J Encapsulation Adsorp Sci 2013;3:16–23.
[28] Natesan S, Baer DG, Walters TJ, Babu M, Christy RJ. Adipose-derived stem cell delivery into collagen gels using chitosan microspheres. Tissue Eng A 2010;16:1369–84.
[29] Natesan S, Zamora DO, Suggs LJ, Christy RJ. Engineering a bilayered hydrogel to control ASC differentiation. J Vis Exp 2012.
[30] Lynch SE, Wisner-Lynch LA, Kestler HK, Liu YC. Platelet-derived growth factor composition and methods for the treatment of tendon and ligament injuries. US patent application No. 2010/0174368 A1; 2010.
[31] Thomopoulos S, Das R, Silva MJ, Sakiyama-Elbert S, Harwood FL, Zampiakis E, et al. Enhanced flexor tendon healing through controlled delivery of PDGF-BB. J Orthop Res 2009;27:1209–15.
[32] Jelinsky SA, Archambault J, Li L, Seeherman H. Tendon-selective genes identified from rat and human musculoskeletal tissues. J Orthop Res 2010;28:289–97.
[33] Qi J, Dmochowski JM, Banes AN, Tsuzaki M, Bynum D, Patterson M, et al. Differential expression and cellular localization of novel isoforms of the tendon biomarker tenomodulin. J Appl Physiol 2012;113:861–71.
[34] Awad HA, Boivin GP, Dressler MR, Smith FNL, Young RG, Butler DL. Repair of patellar tendon injuries using a cell-collagen composite. J Orthop Res 2003;21:420–31.