Biomaterials 53 (2015) 716–730

Contents lists available at ScienceDirect

Biomaterials

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

# Well-aligned chitosan-based ultrafine fibers committed teno-lineage differentiation of human induced pluripotent stem cells for Achilles tendon regeneration

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A R T I C L E I N F O

*Article history:*
Received 10 November 2014
Received in revised form
10 February 2015
Accepted 13 February 2015
Available online

*Keywords:*
Well-aligned chitosan ultrafine fibers
Human induced pluripotent stem cells
Stepwise substrate change
Tendon differentiation
Tissue regeneration

A B S T R A C T

Physical property of substrates such as stiffness and topography have been reported to induce mesenchymal stem cells differentiation into bone, muscle and neuron lineages. Human-induced pluripotent stem cells (hiPSCs) are a highly promising cell source for regenerative medicine. However, physical properties have not yet been reported to successfully induce pluripotent stem cells into specific lineages. This study aimed to develop a robust, stepwise topographic strategy to induce hiPSCs differentiate into teno-lineage. A novel spinning approach termed stable jet electrospinning (SJES), is utilized to fabricate continuous well-aligned ultrafine fibers (891 $\pm$ 71 nm), which mimic the native tendon's microstructure and mechanical properties. hiPSCs are first differentiated into MSCs on smooth plastic surface as confirmed by the differentiations into three mesenchymal lineages and expression of characteristic MSC surface markers through an EMT (Epithelial–Mesenchymal Transition) process. Subsequently, the hiPSC derived MSCs are seeded onto well-aligned fibers to differentiate into tenocyte-like cells through activating mechanic-signal pathway. The *in situ* tendon repair study further confirms that aligned fiber scaffold with hiPSC-MSCs had significant effect on improving the structural and mechanical properties of tendon injury repair. These findings indicate that the stepwise physical substrate change strategy can be adopted to induce hiPSCs differentiation for tendon tissue regeneration.

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## 1. Introduction

Tendons and ligaments play crucial roles in stress transfer and joint stability. Tendon injuries occur frequently during sports and other rigorous physical activities, which often lead to instability and abnormal joint movement [1]. Due to their limited vascularity, innervation and cellular content, natural healing of tendon tissue is extremely inefficient [2,3]. Current therapeutic options for

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http://dx.doi.org/10.1016/j.biomaterials.2015.02.051
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repairing tendon injuries include autografts, allografts, xenografts, suture techniques, and tendon prostheses [4]. However, the aforementioned therapeutic modalities have several inherent limitations such as donor site morbidity, poor graft integration and high rates of recurrent tearing [5,6]. More recently, tendon tissue engineering has emerged as a promising alternative therapeutic strategy for tendon repair.

Seed cells and scaffolds are essential elements of tendon tissue engineering. The seed cells widely used in tendon tissue engineering are mesenchymal stem cells and tenocytes. Utilizing tenocytes may not be practical for clinical therapy due to the need of harvesting autologous tendon tissues. Mesenchymal stem cells, which possess multilineage potential represent an important renewable cell source for tissue engineering [7,8]. MSCs initially isolated from adult bone marrow (BM-MSCs) were the main source of MSCs for clinical application. However, their limited capacity to proliferate, the significant variability in cell quality derived from different donors and a rapid loss of their differentiation potential upon *ex vivo* culture [9,10], severely limit the clinical application of these cells. Hence, establishing an adequate cell source that retains the ability to differentiate and controlling the specific cellular differentiation into tendon-forming cells remain a formidable challenge.

iPSCs, that can be generated from any adult cell in the body has spawned an excitement in the field of regenerative medicine. iPSCs not only can provide an inexhaustible source of autologous cells but also resemble embryonic stem cells (ESCs) in the pluripotent state [11] and bypass immunological rejection. These superiorities make it a valuable cell source for cell therapies. However, limited studies had been designated to investigate the potential utility of hiPSCs in tendon tissue engineering [12,13] and there is no protocol for inducing hiPSCs differentiate into teno-lineage.

On the other hand, it has been reported that both the matrix-elasticity and the substrate patterning influence the specification of stem cell lineage [14,15]. These studies highlight the importance of physical substrate in the regulation of cell fate. It is known that tenocytes reside within a niche that comprises primarily of parallel collagen fibers, and this niche plays an important role in regulating their function and differentiation [16]. This forms the rationale of engineering tendon tissues with well-aligned fibers and comparable fiber fineness as that of natural collagen fibers (preferably in the nanoscale) [17–19]. Such kinds of biomimetic scaffolds would provide an extracellular matrix resembling microenvironment to enable cells functioning naturally, which ultimately leads to improved efficacy in tissue repair and regeneration. Here, by using a novel spinning technique termed SJES [20] we are able to readily fabricate continuous well-aligned ultrafine fibers of chitosan-based composite system, which is suitable for regulating hiPSC-MSCs orientation and differentiation.

Based on our previous studies [17,21] and known biological similarities between hESCs and hiPSCs, we hypothesize that hiPSCs can be induced to undergo stepwise differentiation on different physical substrates. To test this hypothesis, we induced hiPSC-MSCs from hiPSCs on a gelatin coated smooth plastic plate, which was confirmed by expression of specific MSC markers, and differentiation assays for adipogenesis, osteogenesis, and chondrogenesis. Additionally, the putative hiPSC-MSCs exhibited a high capacity for proliferation without losing their self-renewal capacity and MSC characteristics. Teno-lineage differentiation was then carried out on the well-aligned chitosan-based ultrafine fiber scaffold. Throughout the process of teno-lineage differentiation, we studied molecular changes in the cells, and assessed the impact of the multi-step physical substrate change on regenerative upon transplantation in a rat Achilles tendon repair model.

## 2. Materials and methods

### 2.1. Fabrication of fiber scaffolds

A recently emerged stable jet electrospinning (SJES) technique (Fig. 1B and supplementary video) was employed to prepare well-aligned fiber mats and/or scaffolds. Briefly, chitosan (CTS, Mw = 900,000 Da, Zhejiang Golden-Shell Biochemicals) and gelatin (GT, Sigma) were dissolved in trifluoroacetic acid (TFA, Shanghai Darui Finechemical) at a weight ratio of 6:1 to make solution 1 (i.e., CTS/GT, 3 wt%), and poly(L-lactic acid) (PLLA, Mw = 100,000 Da, Polysciences) and poly(ethylene oxide) (PEO, Mw > 5,000,000 Da, Avocado) were dissolved in dichloromethane (Sinopharm Chemical Reagent) at a weight ratio of 3:1 to make solution 2 (i.e., PLLA/PEO, 4 wt%). The two solutions were then mixed to obtain a chitosan-dominant compounded solution system for SJES utilizing a set of variables (Table 1) to produce well-aligned ultrafine composite fibers, which were constituted of CTS, PLLA, GT and PEO at the mass ratios of 62.1, 20.7, 10.3 and 6.9 wt%, respectively. For comparison, the same solution used for SJES was also electrospun under optimized processing parameters into randomly oriented fiber mats by conventional electrospinning (CES) technique (Fig. 1C), which involved a chaotic motion of the jet. A syringe pump (KDS100, KD Scientific) and a high voltage power supply (TXR1020N30-30; Teslaman, Dalian, China) were used during electrospinning to control the solution feeding rate and applied voltage, respectively. Electrospinning proceeded for 2 h to fabricate aligned or random fiber mats with a thickness of 0.11–0.12 mm. The resultant fiber mats were then immersed in 0.5 M sodium hydroxide solution for half an hour, washed 3 times in ethanol, and then transferred to cover slips, prior to being sterilized with ethanol and UV overnight.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.02.051.

### 2.2. Characterization of the fiber scaffolds

Morphology of the electrospun fibers was observed by scanning electron microscopy (SEM, TM-1000, Hitachi) at an accelerating voltage of 15 kV. Prior to imaging, samples mounted on aluminum stubs were subjected to gold coating for better conductivity. Fiber diameters were measured from the SEM images by using ImageJ software.

The degree of fiber alignment was assessed by analyzing SEM images with the 2D fast Fourier transform (FFT) function of the ImageJ program and by applying an oval profile plug-in (designed by Bill O'Connell). In brief, grayscale 8-bit images were cropped to 1590 × 1590 pixels and processed using 2D FFT. The resulting FFT output image was rotated 90° to correct for the inherent rotation of the data induced by 2D FFT analysis. The application of the oval profile plug-in enabled the summing of pixel intensities along the radius for each angle of a circular projection, which was previously placed on the FFT output image by using the ImageJ circular marquee tool. The summed pixel intensities were normalized to a baseline value of 0 and plotted as a function of the corresponding angle in arbitrary units from 0 to 180°.

Tensile properties of the electrospun fibrous mats were determined using a material testing machine (H5K-S, Hounsfield, United Kingdom) equipped with a 50 N load cell. The specimens (dimension: 30 × 10 mm, n = 5) were stretched at a constant cross-head speed of 20 mm/min. Ultimate tensile strength, Young's Modulus and strain at break were computed accordingly from the generated stress–strain curves.

### 2.3. Generation of iPSCs

All cells were cultured in a humidified atmosphere at 37 °C and 5% $CO_2$. We generated human iPSCs from HFF (human foreskin fibroblasts) by recombinant expression of the four classic Yamanaka's factors (Oct3/4 (O), Sox2 (S), c-Myc (M) and Klf4 (K)). 100,000 HFF was seeded on gelatin (0.1%, w/v) coated 3.5 cm dish and cultured overnight, then infected with fresh retrovirus twice in consecutive days. Subsequently, the infected cells were cultured in fresh HFF medium for 24 h, and change to ESC medium, hereafter the medium were changed every two days. Some small colonies appeared around 7 days post the first infection. Some colonies were picked and transferred onto a feeder layer of irradiated mouse embryonic fibroblasts (MEF). Colonies with human ES cell-like morphology (putative iPSC colonies) after 20 days post-transfection were chosen for further study. The ESC medium consisted of DMEM/F12 1:1 (Gibco), supplemented with 20% (v/v) knockout serum replacer (Invitrogen), 1 mM L-glutamine (Invitrogen), 1 × NEAA, 0.1 mM β-mercaptoethanol and 10 ng/ml human recombinant basic fibroblast growth factor (bFGF; Invitrogen).

### 2.4. Derivation of MSCs from hiPSCs

To differentiate the iPSCs to MSCs, we picked undifferentiated iPSC colonies from a 6 well plate and seeded these onto 0.1% gelatin-coated 12 well plates. The original iPSCs growth medium was switched to another medium consisting of

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