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Electrospun scaffolds for multiple tissues regeneration *in vivo* through topography dependent induction of lineage specific differentiation

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ABSTRACT

Physical topographic cues from various substrata have been shown to exert profound effects on the growth and differentiation of stem cells due to their niche-mimicking features. However, the biological function of different topographic materials utilized as bio-scaffolds *in vivo* have not been rigorously characterized. This study investigated the divergent differentiation pathways of mesenchymal stem cells (MSCs) and neo-tissue formation trigged by aligned and randomly-oriented fibrous scaffolds, both *in vitro* and *in vivo*. The aligned group was observed to form more mature tendon-like tissue in the Achilles tendon injury model, as evidenced by histological scoring and collagen I immunohistochemical staining data. In contrast, the randomly-oriented group exhibited much chondrogenesis and subsequent bone tissue formation through ossification. Additionally, X-ray imaging and osteocalcin immunohistochemical staining also demonstrated that osteogenesis *in vivo* is driven by randomly oriented topography. Furthermore, MSCs on the aligned substrate exhibited tenocyte-like morphology and enhanced tenogenic differentiation compared to cells grown on randomly-oriented scaffold. qRT-PCR analysis of osteogenic marker genes and alkaline phosphatase (ALP) staining demonstrated that MSCs cultured on randomly-oriented fiber scaffolds displayed enhanced osteogenic differentiation compared with cells cultured on aligned fiber scaffolds. Finally, it was demonstrated that cytoskeletal tension release abrogated the divergent differentiation pathways on different substrate topography. Collectively, these findings illustrate the relationship between topographic cues of the scaffold and their inductive role in tissue regeneration; thus providing an insight into future development of smart functionalized bio-scaffold design and its application in tissue engineering.

1. Introduction

Stem cell survival, self-renewal and differentiation are governed by local biochemical and mechanical factors within their microenvironmental niche [1]. The key niche components include soluble factors, other cells, and extracellular matrix molecules. While the role of biochemical signals is well-documented, the importance of biophysical cues has received more recognition and attention only in the last decade. Current advances in microfabrication technologies have enabled the generation of substrates with nano/micro-scale topographies to study the effects of biophysical signals on cellular function. A number of studies have demonstrated that the physical properties of substrata have profound effects on the cellular functions of pluripotent and multipotent stem cells, including cell

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adhesion, morphology, proliferation, migration and differentiation [2–6]. Based on the concept of contact guidance, we designed a biomimetic aligned nanofiber scaffold modelled on the parallel collagenous fibers of tendon extracelluar matrix; and subsequently demonstrated that alignment within the scaffold regulate tendon stem cell orientation and induce specific teno-lineage differentiation [7]. Meanwhile, both random orientation and nanoscale disorder have been demonstrated to induce ossification of human multipotent stem cells *in vitro*, even in the absence of osteogenic media [6]. Although there are promising results in various studies that have attempted to control stem cell fate *in vitro* through modification of substrate physical properties, there is a dire need to move from culture substrate to implantable scaffolds with direct applications in tissue engineering [8].

Conventional scaffolds are designed and fabricated according to the basic requirements of biocompatibility, structural support as well as cell delivery, and have already been widely utilized in various tissue engineering applications [9]. Modern bio-scaffolds not only just serve as a carrier for seed cells, but also provide an appropriate microenvironment for stem cells and mediates biological functions. Further microstructural refinement of current scaffold biotechnology will enhance the progress of tissue engineering in the future [10]. However, the three-dimensional microenvironment *in vivo* represents a much more complicated milieu that encompasses a much more diverse multitude of signaling cues compared to an *in vitro* culture system. Under physiological conditions, stem cells naturally encounter a variety of different signaling cues that can potentially influence cell fate. It is essential and necessary to use the results of *in vitro* studies to aid the rigorous characterization of the functionality of tissue engineered scaffolds *in vivo*. This prompted our investigation on the inductive effects of scaffold topographic cues on stem cell differentiation pathways and lineage fate.

This study aims to characterize the biophysical effects of scaffold topography on tissue regeneration *in vivo* within a 3D microenvironment, utilizing aligned and randomly-oriented fibrous scaffolds. We tested the hypothesis that topographic cues from the aligned fibrous scaffold can enhance tendon-like tissue formation, and that there would be a higher degree of osteogenesis and tissue ossification with the randomly-oriented fiber scaffold. Additionally, the role of cytoskeletal organization in topography driven differentiation of mesenchymal stem cells was also investigated *in vitro*. We believe that the data presented here would be beneficial to the design and application of future biomaterials.

2. Materials and methods

2.1. Fabrication of PLLA scaffolds

Both aligned (1068 ± 190 nm) and randomly-oriented PLLA scaffolds (739 ± 129 nm) were fabricated using the electrospinning technique as previous reported. The polymer solution was prepared by dissolving PLLA (Ji'nan Daigang Biomaterial Co., Ltd) in a mixture of chloroform/ethanol (3:1) at a concentration of 4% (aligned) or 3% (random). The solution was then fed into a 12-ml plastic syringe, which was controlled by a syringe pump at a rate of 2 ml/h. A high voltage (12 kV) was applied to the needle tip, which was placed 10 cm above the collector. A flat aluminum plate was used to collect the random fibers. The collector for aligned fibers was a disk rotating at 4000 rpm. The resulting scaffolds were then transferred to cover slips and sterilized with ethanol and UV overnight before they were utilized for cell culture. Nanofibers were collected for 2–3 h, resulting in a fiber mat ranging in thickness from 0.14 to 0.17 mm. The aligned and randomly-oriented scaffolds utilized in this study were of similar thickness and distribution.

2.2. Morphology of PLLA scaffolds

The scaffold samples were sputter-coated with gold, and then their structure was observed under scanning electron microscopy (SEM) (Hitachi S3000N) at an accelerating voltage of 15 kV. After the micrographs were obtained, image analysis software (Image-Pro Plus) was used to measure the average diameter of the nanofibers (n = 3). For each sample, an average of 50 fibers were counted.

2.3. SEM imaging

C3H10T1/2 cells (mouse multipotent mesenchymal stem cell line) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were seeded onto PLLA scaffolds at 2×10^4 cells/cm^2 and cultured in Dulbecco's modified Eagle's medium (DMEM, low glucose; Gibco, Grand Island, NY, http://www.invitrogen.com) with 10% (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, http://www.invitrogen.com-Gibco) and 1% (v/v) penicillin-streptomycin (Gibco). The medium was changed once every 3 days. Three days after seeding, the cell morphology and distribution were visualized using SEM. Specimens were fixed in 0.25% glutaraldehyde solution, and then rinsed 3 times in PBS, 30 min each time. The specimens were immersed in OsO_4 for 40 min and then rinsed 3 times in PBS, 30 min each time, followed by dehydration in increasing concentrations of acetone (30–100% v/v). After drying, the specimens were mounted on aluminum stubs and coated with gold, then viewed under a Hitachi S-3000N SEM at an accelerating voltage of 15 kV. For quantification of cell morphology on different scaffolds, a minimum of fifty cells for each SEM image were selected randomly as ROI (Regions of Interest). The area and radius ratio were then quantified using the Image-Pro Plus software.

2.4. Alkaline phosphatase (ALP) staining

C3H10T1/2 cells (10^4/cm^2) were seeded onto scaffolds and cultured in osteogenic induction medium, in the presence of 10 mM β-glycerol phosphate (Sigma), 0.1 mM dexamethasone (Sigma), and 50 mg/ml ascorbic acid (Sigma) supplemented in DMEM-high glucose medium containing 10% (v/v) FBS and 1% (v/v) penicillin–streptomycin. After 7 days, ALP activity was assayed using a BCIP/NBT alkaline phosphatase color development kit (Beyotime Institute of Biotechnology). DAPI (Beyotime Institute of Biotechnology) was used to stain nuclei and observed under a light microscope (Olympus IX71).

2.5. Quantitative PCR

Total cellular RNA was isolated by lysis in TRIzol (Invitrogen). The expression levels of tendon-specific genes and osteogenic markers in cells cultured on aligned and randomly-aligned fibrous scaffolds were assessed by quantitative PCR. PCR was performed using a Brilliant SYBR Green qPCR Master Mix (TakaRa) on a Light Cycler apparatus (ABI 7900HT). The PCR cycling consisted of 40 cycles of amplification of the template DNA with primer annealing at 60 °C. The relative expression levels of each target gene was then calculated using the 2-ΔΔCt method. The amplification efficiencies of primer pairs were validated to enable quantitative comparison of gene expression. All primers (Generay) were designed using primer 5.0 software and are summarized in the Supplementary Table 1.

2.6. Animal model

The Zhejiang University Institutional Animal Care and Use Committee approved the study protocol. *In situ rat Achilles tendon repair model*: Twenty hind limbs of skeletally mature female rats weighing 200–220 g were utilized for this experiment. Under general anesthesia, a gap wound was created and the Achilles tendon was removed to create a defect of 6 mm in length. Aligned and random fibrous scaffolds (8 mm × 8 mm, thickness = 100 um) were folded about 2 mm from the bottom of the membrane upwards. This was followed with fan-folding the next 2 mm to the back. Fan-folding of the scaffold was continued until it was completely folded. This was followed by binding the center of the strip using a suture and subsequent placement into the gap wound. Suturing to the remaining Achilles tendon was then carried out using a non-resorbable suture material (Nylon6). The wound was then irrigated and the skin was closed. The animals were allowed free cage activity after surgery. At 2, 4, and 8 weeks post-implantation, samples from each group were harvested for the evaluation of histology, transmission electron microscopy imaging, mechanical testing, as well as collagen content determination (Supplementary Table 2).

2.7. Immunofluorescence

Briefly, cells were fixed in 4% (w/v) paraformaldehyde for 10 min at room temperature, permeabilized, and blocked for 30 min with 1% (w/v) bovine serum albumin, and then permeabilized with 0.1% (w/v) Triton X-100. Fixed cells were washed and incubated with a primary antibody against SCX (Abcam Inc.), Vinculin (Millipore), or control IgG (BD) at 4 °C overnight. Cells were then incubated with Alexa fluor 488-conjugated secondary antibody (Invitrogen) for 2 h and the nuclei were stained with DAPI. TRITC-phalloidin (Millipore) staining was used to visualize the cytoskeleton. The imaging was then performed with confocal microscopy (Zeiss LSM-510).

2.8. Histological evaluation and staining

Harvested specimens were immediately fixed in 10% (v/v) neutral buffered formalin, dehydrated through an alcohol gradient, cleaned, and then embedded within paraffin blocks. Histological sections (7 um) were prepared using a microtome and subsequently stained with hematoxylin and eosin. In addition, Masson trichrome staining was performed according to standard procedures to examine the