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Growth of outgrowth endothelial cells on aligned PLLA nanofibrous scaffolds

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Abstract Tissue engineering holds great promise in providing vascular grafts as substitutes for damaged small-diameter blood vessels. Two of the key factors in vascular tissue engineering are biocompatible scaffolds that mimic the effects of extracellular matrix and the source of seeding cells. Synthetic poly-L-lactic acid (PLLA) nanofibers has been shown to be excellent scaffolds for tissue engineering. Outgrowth endothelial cells (OECs) isolated from human peripheral blood could also be expanded in vitro and stably maintain the differentiated phenotypes and could be used as the seeding cells for engineering autologous vascular crafts. Here we tested the possibility of combining these two together. We found that PLLA nanofibers are not only biocompatible with OECs originally isolated from rabbit peripheral blood, the aligned PLLA fibers actually promoted and guided their sustained proliferation. These results suggest that aligned PLLA could be excellent both as the scaffolds and as a promoter of cell growth during vascular tissue engineering.

1 Introduction

Atherosclerosis and heart disease are still the leading causes of morbidity and mortality worldwide. The lack of suitable autologous grafts has produced a need for artificial grafts, which have been in use for more than half a century. Conduits of synthetic Dacron or expanded polytetrafluoroethylene (ePTFE) have proven excellent artery substitutes in high-flow, large-diameter environments. However, due to frequently occurring occlusion, small-diameter artificial grafts (<5–6 mm in inner diameter) have not been successfully used in clinical settings despite many years of research and numerous approaches [1]. Such occlusion mainly results from thrombus formation during early phase of implantation [2, 3]. Excessive tissue ingrowth (intimal hyperplasia) at the anatomized site also leads to stenosis-induced thrombus formation and occlusion of small-diameter artificial grafts during the chronic phase of implantation [4]. As an alternative, completely biologically tissue-engineered vascular grafts are promising substitutes for damaged blood vessels.

The extracellular matrix (ECM) play important roles in controlling both cell behavior and tissue formation within living systems [5]. The ECM is composed of nanometer-sized networks of proteins and glycosaminoglycans. The ECM serves as scaffolds for organizing cells and provides environmental cues for cell proliferation, differentiation, and interaction [6–9]. It is increasingly appreciated that synthetic nanofibers with dimensions similar to natural ECM and biocompatibility could be excellent scaffolds in small vascular tissue engineering. Synthetic poly-L-lactic acid (PLLA) nanofibers made by electrospinning have been successfully used as scaffolds in other tissue engineering processes [10–12]. These nanofibrous scaffolds are structurally similar to natural extracellular matrix (ECM). Their sizes can be defined, ranging from a few to hundreds of nanometers in diameter. Their pore sizes, porosity, patterns and alignments can also be defined to meet various specific requirements in tissue engineering. Such nanofibers have

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been shown to promote biological responses of seeded cells, including controlled cell adhesion, cell proliferation, cell shapes, and cell functions [8, 13]. Also due to its lack of toxicity, low cost and suitable degradation period, the PLLA nanofibers are model scaffolds both for biomedical applications in patients and for tissue engineering.

Particularly important for vascular tissue engineering is sufficient number of seeding cells. Expansion of a small subset of mononuclear cells (MNCs) isolated from rabbit peripheral blood could result in so-called outgrowth endothelial cells (OECs), which are similar to fully differentiated endothelial cells with respect to cellular morphology and expression of cellular markers such as the ability to uptake ac-LDL and to bind UEA-I [14–16]. These OECs maintain their proliferation capacity and differentiated status during a period of several months, suggesting that they could be an excellent source of seeding cells for engineering autologous vascular grafts [17, 18].

In a context of tissue engineering, here we show that OECs obtained from rabbit peripheral blood adhered to and grew along the directions dictated by the aligned PLLA nanofibers. Moreover, the proliferation capacity of these OECs was further promoted by the presence of these PLLA fibers and correlated with their directionality. Importantly, these cells grown on PLLA scaffolds also maintained their differentiated endothelial phenotypes and angiogenic potential during the entire culturing period. These results thus have laid a solid foundation for future vascular tissue engineering involving both PLLA and OECs.

2 Materials and methods

2.1 Cell isolation and culture

Peripheral blood from a rabbit was diluted with 1× phosphate-buffered saline (PBS) at a ratio of 1:1 and overlayed on Lymphoprep (Tianjin Hanyang Biologicals Technology Co., Ltd., China). Cells were isolated by density gradient centrifugation at 2,000 rpm for 30 min. The resulting rabbit peripheral blood mononuclear cell (PBMC) were collected and washed three times in 1× PBS. After the final wash, cells were resuspended in endothelial basal medium (EBM-2, Cambrex Corp.,USA) supplemented with EBM-2-SingleQuots (Clonetics) that contain bovine brain extracts (BBE, 12 μg/ml), human endothelial growth factor (hEGF, 10 ng/ml), hydrocortisone (1 μg/ml), GA-1000 (1 μg/ml), human vascular endothelial growth factor(VEGF, 50 ng/ml), human insulin-like growth factor-1 (IGF-1, 50 ng/ml) and fetal bovine serum (15%). 2×10^6 of PBMCs were immediately plated on fibronectin-coated (5 μg/cm^2) 25 cm^2 culture plates and incubated for 3 days. Non-adherent cells were discarded by gentle washing with 1× PBS. Fresh medium was added to the attached cells, which were continually cultured with complete EBM-2 medium for 3 weeks, with a change of medium every 3 days. Typically, cells were passaged when reaching 80–90% confluence. To confirm OEC generation during culturing, morphological changes of adherent cells were visualized with Olympus phase-contrast microscopy (Olympus Optical Co. Ltd, Tokyo, Japan).

2.2 OEC characterization

After 16 days of culture in vitro, the attached cells were cultured with complete EBM-2 medium to obtain OECs. Binding of FITC-UEA-I(Fluorescein isothiocyanate-ulex europaeus agglutinin I, Sigma Corp.,USA) and uptake of DiI-ac-LDL (DiI labeled acetylated low-density lipoprotein,Molecular Probes, USA), characteristics specific to endothelial cells, were determined to verify OECs [19]. Cells were either incubated with Ac-LDL (10 μg/ml) that was labeled with the fluorochrome DiI (Harbor Bioproducts, Norwood, USA) at 37°C for 4 h and washed three times in 1× PBS and fixed with 2% paraformaldehyde for 20 min or incubated with FITC-conjugated UEA-I (10 μg/ml) at 4°C for 1 h. Incorporation of DiI-labelled Ac-LDL and binding of FITC-UEA-I was determined by fluorescent microscopy.

2.3 Fabrication of PLLA nanofibrous scaffolds

The electrospinning solution was prepared by dissolving 0.2 g PLLA (DaiGang CO. Ltd, Jinan, China) in 10 ml of methylenechloride/n, n-dimethylformamide (volume ratio: 3/1). Subsequently, the solution was fed into a 5 ml syringe with a 6-gauge needle and continually driven by an advancing pump (SiLuGao CO. Beijing. China) at a speed of 3 ml/h. A DC voltage (High DC power supply) of 1 kV/cm was applied between the syringe needle and an insulated high-speed roller collector covered with parallel steel sticks at a fixed distance of 10 cm. The random, aligned and super-aligned PLLA nanofibers were prepared by rotating at 0 rpm, 1,000 rpm and 2,500 rpm, respectively. Finally, the electorspun PLLA nanofibers was peeled off the collector and fixed onto 34 mm coverslips and stored in a vacuum at room temperature before use. For culturing cells, the PLLA scaffolds were cut into pieces of 3 mm × 3 mm × 1 mm and fitted into 96-well plates or into pieces of 24 mm × 24 mm × 1 mm and fitted into 6-well plates.

2.4 Cell culture in the presence of PLLA scaffolds

PLLA nanofibers were washed with ethanol and de-ionized water for seven times and dried in vacuum at room temperature before each use. The PLLA nanofibers were treated with a PDC-002 plasma system (Harrick Plasma,

USA) under the following conditions: input power, 40 W; pressure, 20 Pa; treatment time, 40S; NH_3 flow rate, 20 ml/min. Subsequently, the PLLA nanofibers was disinfected with ethylene oxide and soaked in 3 mg/ml of collagen I solution (Sigma, USA) for 24 h to allow attachment of collagen I. First passage OECs were seeded onto sterilized PLLA nanofiber scaffolds (3 mm × 3 mm) in 96-well tissue culture plates at a density of 2×10^4 cells/well. Medium was changed every other day. To determine the growth curves, cells were taken from 4 randomly chosen wells for growth each condition every other day from day 3 until day 17. Briefly, cells on the PLLA scaffolds were treated with 25% trypsin-EDTA mixture, neutralized with EBM-2, and washed off the PLLA scaffolds, which were subsequently removed from wells. Cells were cultured for another 24-h period in the absence of the PLLA scaffolds. After discarding the medium, cells were incubated in 20 μl of MTT (Thiazolyl blue, 5 mg/ml, Sigma, USA) at 37°C for 4 h and subsequently resuspended in 150 μl of DMSO Dimethyl sulfoxide, Sigma, USA) with shaking. The absorbance A representing cell density in each well was measured with a ELISA Immune Detector (TECAN Corp.,Switzerland) at a wavelength of 490 nm.

Cell proliferation was also investigated directly on the PLLA scaffolds. Third passage adherent cells were seeded at a density of 1×10^5 cells/well. After incubation for 4 h, 12 h, 24 h, 3 days, and 7 days, respectively, PLLA nanofiber scaffolds were randomly taken from 3 wells for each condition. Non-adherent cells were washed away with 1× PBS for three times. Cells attached to the PLLA scaffolds were then treated with 25% trypsin-EDTA mixture and counted. The rate of cell proliferation was calculated by dividing the number of cells after incubation with the number of cells originally seeded.

2.5 Microscopy

Colony-forming cells with cobblestone-like morphology were designated as OECs according to their endothelial morphology and their ability to uptake of ac-LDL and to bind UEA-I (Dual positive). The third passage cells adherent to PLLA scaffolds were labeled with 2 μg/ml of Cell Tracker chloromethyl-1,1′-dioactadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (CM-DiI; Molecular Probes, Eugene, OR) at 37°C for 5 min and subsequently at 4°C for 15 min. The labeled cells were treated with 25% trypsin-EDTA mixture. EBM-2 was added to neutralize the medium. The labeled Cells were collected by density gradient centrifugation at 2 × 1,000 rpm for 10 min, washed 2 times with 1× PBS, and resuspended in EBM-2. A total of 3×10^5 cells/well were seeded on the PLLA scaffolds. After incubation for 4 h, 24 h, and 72 h, respectively, non-adherent cells were washed away with 1× PBS for three times. Cells adhered to PLLA scaffolds were observed with contrast phase microscopy and fluorescent microscopy. OECs incubated in the presence of PLLA scaffolds for 72 h were washed three times in 1× PBS and fixed with 2.5% glutaraldehyde for 2 h and 30–100% gradient ethanol dehydration. Samples were critical point dried, sputtered with gold, and observed with SEM.

2.6 Statistical evaluation

Data were presented as means and standard error of means. Statistical analysis was performed using SPSS 11.5 One-Way ANOVA test with a P value 0.05.

3 Results

3.1 Isolation and characterization of OECs

As a potential cell source for vascular tissue engineering, we first isolated mononuclear cells from rabbit peripheral blood and continually cultured them in endothelial cell medium for 3 weeks until the appearance of flattened and cobblestone-like or spindle-shaped endothelium-like cells. These cells tend to form clusters or colonies (Fig. 1a). They can take up acetylated low-density lipoprotein (Ac-LDL) from the medium (Fig. 1b) and bind to UEA-I (Fig. 1c, d), indicating that they are indeed endothelial cells. These results are similar to previous findings [9, 11]. These endothelium-like cells can be expanded for about 5–7 passages each month with a passage proportion of 1:3 (data not shown), indicating relatively robust growth potential. Importantly, these cells stably maintained the cobblestone-like morphology during the passages, making them potentially an excellent cell source for vascular tissue engineering.

3.2 Fabrication and characterization of PLLA nanofibers

Anisotropic electrospinning is an excellent technique for fabricating oriented fibers with a high length/diameter ratio and well-defined configuration [20]. We used this method to fabricate the PLLA nanofibers as three different formats-randomly oriented (Fig. 2a), modestly aligned (Fig. 2b), or super-aligned (Fig. 2c). The diameters of these fibers ranged from 300 nm to 400 nm. Their porosities were more than 90% (data not shown).

3.3 Aligned PLLA scaffolds promote growth of OECs

We then tested the effects of the different PLLA scaffolds on the proliferation rates of OECs. The OECs were grown in the absence or presence of random, modestly aligned, or

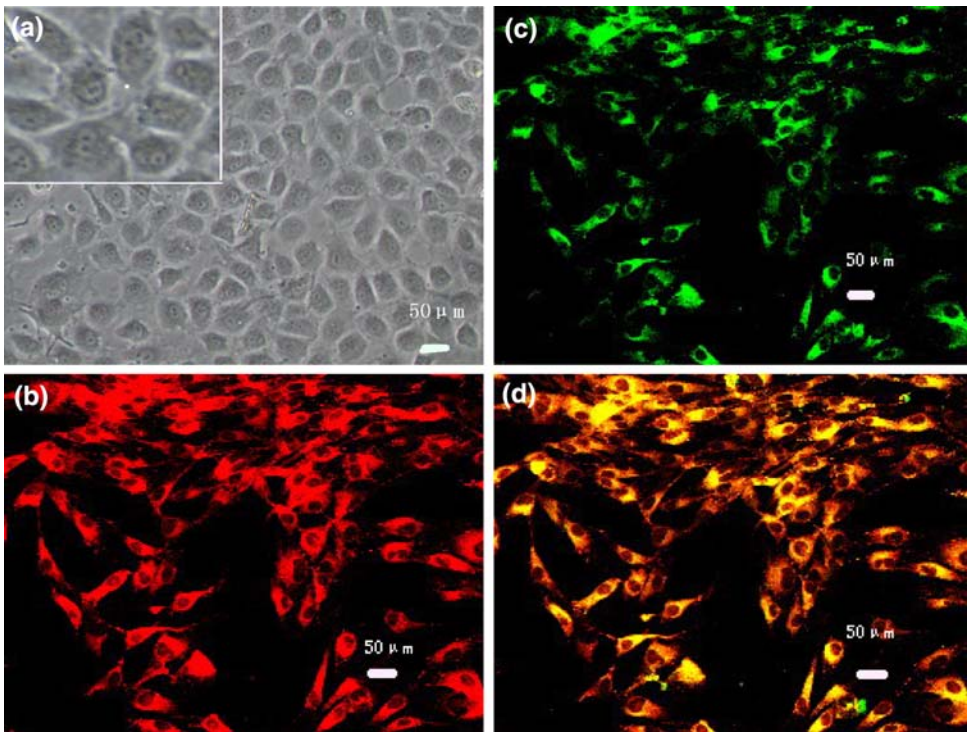

Fig. 1 Characteristics of the outgrowth endothelial cells (OECs) derived from the rabbit peripheral blood. **a** MNCs from rabbit peripheral blood were cultured for 2–3 weeks and photographed (LM, 100×). **b** Up-take of acetylated low-density lipoprotein (Ac-LDL) as red fluorescence from the medium by the OECs. **c** Binding of UEA-1 as green fluorescence by the OECs. **d** Merging of the fluorescence images displayed in **b** and **c** (100×)

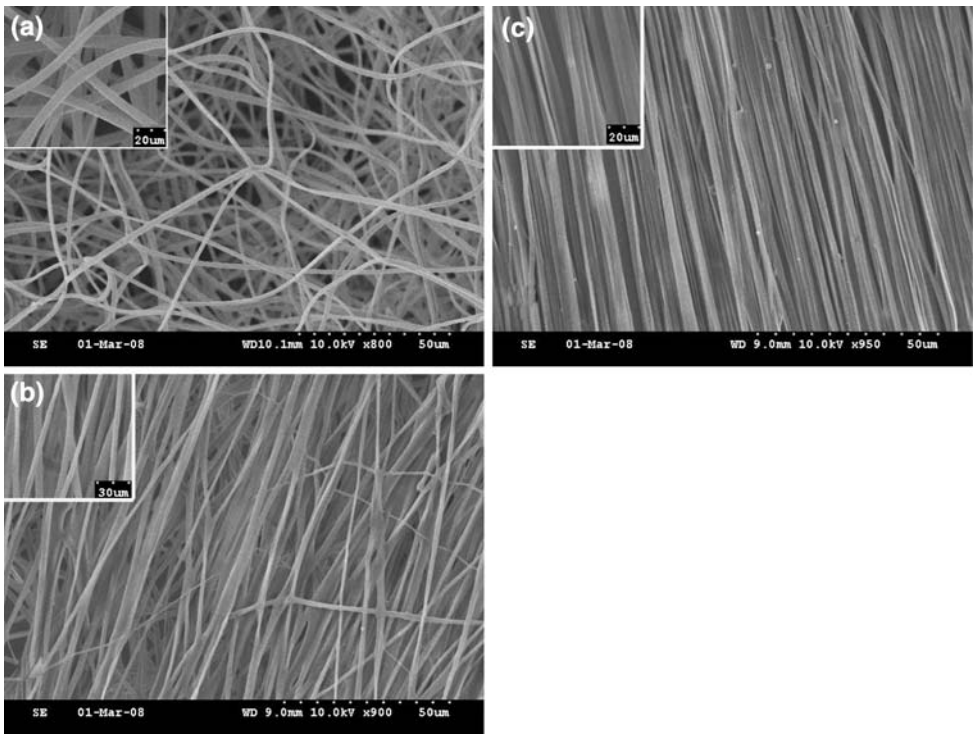

Fig. 2 Structures of different electrospun PLLA scaffolds observed with scanning electron microscopy. **a** Scaffolds of randomly oriented PLLA nanofibers. **b** Scaffolds of modestly aligned PLLA nanofibers with most nanofibers aligned along the longitudinal axis. **c** Scaffolds of super-aligned PLLA nanofibers with almost all nanofibers aligned along the longitudinal axis

super-aligned PLLA nanofibrous scaffolds. The growth curve of each culture was determined by measuring cell density as absorbance A. Although random PLLA scaffolds did not have detectable effect on the growth OECs, both modestly aligned and super-aligned PLLA nanofibrous scaffolds significantly enhanced their growth ($P < 0.05$) (Fig. 3). These results indicate that not only the PLLA scaffolds are biologically compatible with the OECs, the aligned nanofibers actually promote growth of OECs. This conclusion was further corroborated by the observations with OECs grown on and attached to PLLA scaffolds. Here we found that within 4 h, more cells adhered to the random

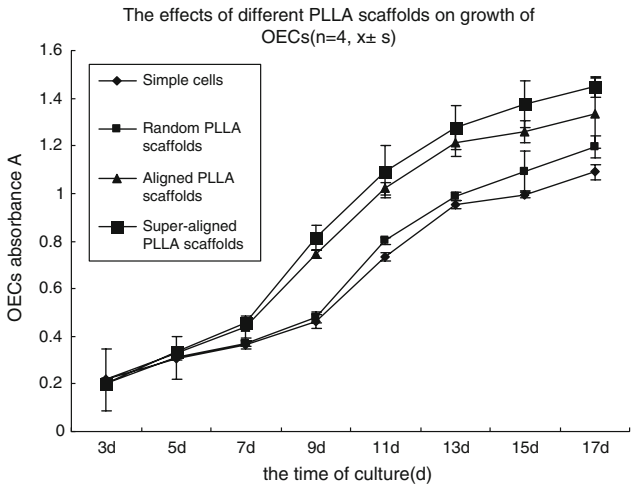

Fig. 3 The effects of different PLLA scaffolds on growth of OECs. For each experimental condition, samples were taken every other day from day 3 to day 17 after the initial inoculation. The relative cell density in each sample was measured using the MTT colorimetric method and presented as absorbance A value. Each data point depicted was the average result of four independent samples

PLLA nanofibers (Table 1). However from the 12th hour until day 7, more cells were attached to and grew in the presence of both modestly aligned and super-aligned PLLA nanofibers, with the effects of the later being more prominent (Table 1).

3.4 PLLA scaffolds provide directionality for cell orientation

We next investigated whether the PLLA scaffolds might direct the orientation and dictate the morphology of OECs. When grown in composite cultures, we found that OECs maintained the spindle-shaped morphology in the presence of all types of PLLA scaffolds (Fig. 4). Importantly, cell orientations correlated with the structures of the scaffolds—they were randomly oriented in the presence of random scaffolds and aligned reasonably well along the aligned and super-aligned scaffolds (Fig. 4), indicating that the scaffolds provided directional cues to the OECs. Among all three different scaffolds, super-aligned nanofibers provides the best directionality (Fig. 4). This was further supported by the observation of fluorescence-labeled OECs (Fig. 5).

3.5 Attachment of OECs to PLLA scaffolds

The directionality provided by the PLLA scaffolds prompted us to investigate whether the OECs physically attach to the nanofibers. We found that the OECs could detach from the PLLA nanofibers only when treated with trypsin digestion (data not shown), indicating protein mediated physical interaction between cells and the scaffolds. The formation of contacts was also visible by overlaying the fluorescent images of the OECs and the reflective images of the PLLA nanofibers (Fig. 5). The OECs grown on PLLA nanofibrous scaffolds polarized and formed new focal adhesions at their leading edges in the migratory active state as evidenced by some filament-like structures extending out from the cell bodies of OECs, which likely represent secreted extracellular matrix (Fig. 6). The extracellular matrix attached to the PLLA scaffolds at defined adhesion sites (Fig. 6). The super-aligned nanofibers were advantageous in keeping the fibers oriented along the longitudinal axis, indicating that fiber alignment have considerable effects on the interaction between the OECs and the PLLA nanofibers. As a result, significantly larger areas of the super-aligned scaffolds were covered by the OECs as compared to the random scaffolds (Fig. 6). We also observed intimate intercellular contacts among adjacent cells (Fig. 6), suggesting the formation of tight endothelial cell layers surrounding the fibers of the scaffolding material.

4 Discussion

Synthetic and natural polymers have been used as fabricate scaffolds for regenerating tissues and organs. An ideal scaffold should mimic the structure and biological function of native extracellular matrix (ECM) proteins to provide

Table 1 The effects of PLLA nanofiber structures on the adhesion and growth of OECs

PLLA	4 hours	12 hours	24 hours	3 days	7 days
Random	46.93 ± 7.29	60.27 ± 6.93$^{\#}$	70.57 ± 3.18$^{\#\#}$	123.90 ± 6.01$^{\#\#}$	130.30 ± 6.72$^{\#\#}$
Modestly aligned	34.47 ± 4.61*	82.80 ± 8.75**$^{,\#\#}$	87.20 ± 5.53**$^{,\#\#}$	151.40 ± 2.96*$^{,\#\#}$	171.37 ± 6.36**$^{,\#\#}$
Super-aligned	33.87 ± 3.93**	86.40 ± 5.03**$^{,\#\#}$	94.17 ± 3.04**$^{,\#\#}$	165.30 ± 4.26**$^{,\#\#}$	185.57 ± 9.25**$^{,\#\#}$

Note: Data were presented as means of three independent experiments and standard error of means

* $P < 0.05$, ** $P < 0.01$ indicate the statistic significance in the effects of both aligned PLLA nanofibers on cell adhesion and growth as compared to the random scaffold

$^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ indicate the statistic significance in the effects of incubation time on cell adhesion and growth with each scaffold by comparing the later time points with the 4th hour

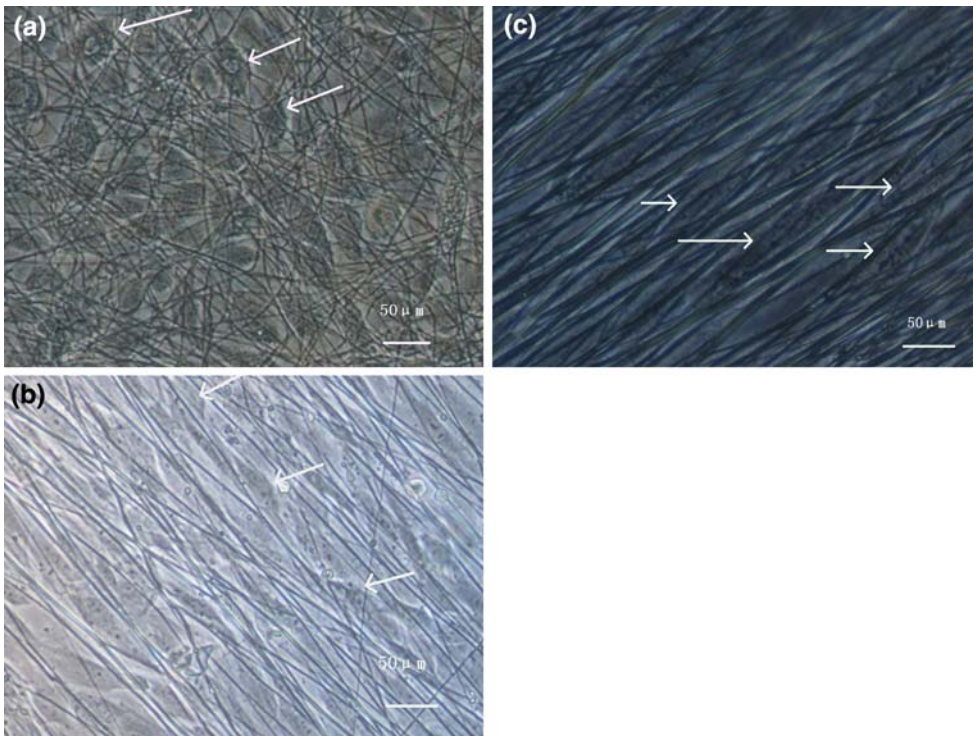

Fig. 4 Growth of OECs along the PLLA scaffolds. OECs grown in composite cultures in the presence of random (**a**), aligned (**b**), and super-aligned (**c**) PLLA scaffolds for 72 h and observed with phase contrast microscopy (100× magnification)

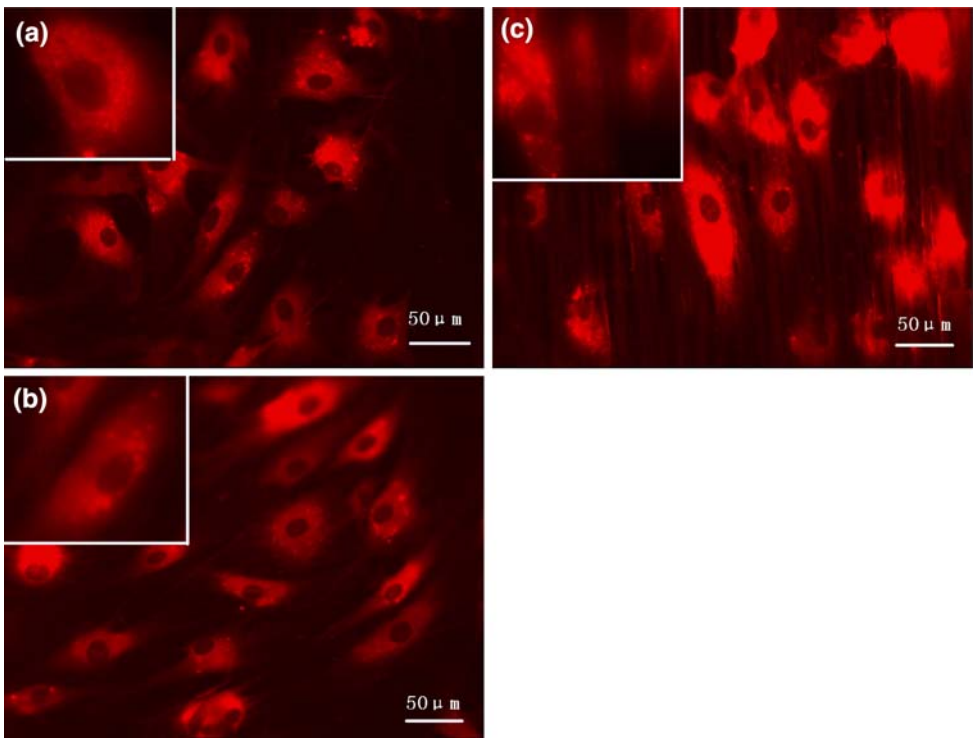

Fig. 5 The effects of different PLLA scaffolds on the morphology of OECs. OECs grown in the grown in the presence of random (**a**), aligned (**b**), and super-aligned (**c**) PLLA scaffolds were labeled with Cell Tracker and observed with fluorescence microspocy after 48 h incubation

mechanical support and to regulate cellular activities. Due to its biocompatibility, controllable length/width ratio, orientation, and porosity, the PLLA nanofiber has been an excellent scaffold in various tissue-engineering applications [21]. For example, Xu et al. [5]. found that human coronary artery smooth muscle cells (SMCs) attached and migrated along the axis of aligned P(LLA-CL) nanofibers and expressed a spindle-like contractile phenotype. They also found that the adhesion and proliferation rate of SMCs on the aligned nanofibrous scaffold was significantly improved

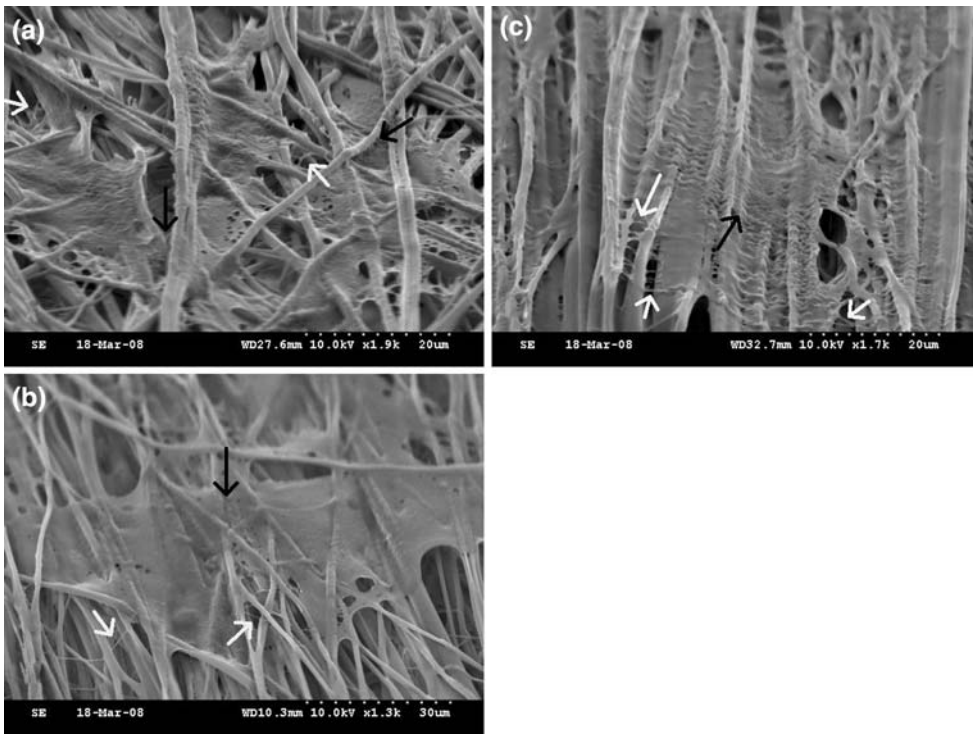

Fig. 6 Scanning electron microscopy image of OECs attached to random (**a**), modestly aligned (**b**), and super-aligned (**c**) PLLA scaffolds. White arrows indicate filament-like structures extended from cells. Black arrows show intercellular contacts among adjacent cells after 72 h incubation

when compared with those on solid polymer films [5]. In the present study, we similarly found that aligned PLLA nanofibers promoted growth of OECs isolated from rabbit peripheral blood. The OECs attached PLLA and grew in directions dictated by the orientations of the fibers (Figs. 4, 5, 6). Moreover, these OECs maintained their shapes and differentiated phenotypes during proliferation on these PLLA scaffolds. These OECs also showed intimate intercellular contacts with neighboring cells (Fig. 6). Given that the OECs could be relatively easily obtained from circulating peripheral blood, these results suggest that growing OECs on aligned PLLA might be an excellent approach towards generating autologous vascular grafts.

Our results are also consistent with previous observations that controlled two-or three-dimension architecture are beneficial to cell differentiation, cell proliferation and their functional longevity [22, 23]. Presumably, migration of the OECs along aligned and super-aligned PLLA scaffolds facilitates spreading of cells and alleviating the crowding problem, which typically leads to apoptotic cell death [24]. Our results also further supported the "contact guidance" theory, which predicts that a cell will most likely migrates in preferred directions when associated with a substratum of particular chemical, structural and/or mechanical properties [25]. Recent studies provided initial evidence that physical or chemical modifications of biomaterials can influence cell reaction [9, 26, 27], providing new opportunities in biomaterial design for tissue engineering or tissue regeneration approaches. This is further supported by our observation that change of the physical directionality of the PLLA nanofibers had significant effects on the proliferation of the OECs and the appearance of the resultant biomaterials (Fig. 4, 5, 6).

5 Conclusions

The study clearly indicates that PLLA nanofibers are not only biocompatible with OECs originally isolated from rabbit peripheral blood, the aligned PLLA fibers actually promoted and guided their sustained proliferation. These results suggest that aligned PLLA could be excellent both as the scaffolds and as a promoter of cell growth during vascular tissue engineering.

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