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Evaluation of the Effect of NT-3 and Biodegradable Poly-L-lactic Acid Nanofiber Scaffolds on Differentiation of Rat Hair Follicle Stem Cells into Neural Cells In Vitro

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Abstract Recent improvement in neuroscience has led to new strategies in neural repair. Hair follicle stem cells are high promising source of accessible, active, and pluripotent adult stem cells. They have high affinity to differentiate to neurons. Aside from using cell–scaffold combinations for implantation, scaffolds can provide a suitable microenvironment for cell proliferation, migration, and differentiation. NT-3 is the most interesting neurotrophic factors being an important regulator of neural survival and differentiation. Since treatment duration in neural repair is very important, this study aims to evaluate the effect of NT-3 and poly-L-lactic acid (PLLA) on differentiation time of bulge stem cells of rat hair follicle to neural-like cells. HFSCs of rat whisker was isolated and cultured on PLLA and differentiated with 10 ng/mL NT-3. Biological features of cultured cells were evaluated with immunocytochemistry and flowcytometry methods by using CD34, nestin, and βIII-tubulin markers. For cell viability and morphological assessment, MTT assay and SEM were performed. Our results showed that bulge stem cells of hair follicle can express CD34 and Nestin before differentiation. By using NT-3 during differentiation process, the cells showed positive reaction to βIII-tubulin antibody. MTT results demonstrated that PLLA significantly increased cell viability. Finally, HFSCs adhesion was confirmed by SEM results. The results indicate that 10 ng/mL NT-3 and PLLA have significant effect on differentiation time of rat HFSCs to neural cells even in 10 days.

Keywords Bulge · Hair follicle stem cell · CD34 · Nestin · βIII-tubulin · NT-3 · Scaffold PLLA

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Introduction

Tissue engineering is a new field in the contemporary human health care administration and in regenerative medicine. The aim of regenerative medicine is to repair or replace damaged

or injured tissues in the human body (Yang et al. 2005; Lim and Mao 2009).

The most attention has been on the development of cell transplantation strategies; for example, stem cells can be transplanted to the site of injury with a supportive scaffold to fill the site of damage and assist in tissue repair. Aside from using cell–scaffold combinations for implantation, another potential application of biomaterials in regenerative medicine is the development of ex vivo techniques for efficient expansion and differentiation of stem cells (Lim and Mao 2009). A lot of investigations have been done in a variety of biomaterials, especially polymers, for their suitability in tissue engineering application. Among them, poly-L-lactic acid (PLLA) is a promising scaffold material because of its biocompatibility and biodegradability (Yang et al. 2005).

Neurotrophins are a family of polypeptide growth factors that influence proliferation, differentiation, survival, and death of neuronal cells during development and are essential for the health of the nervous system (Chao et al. 2006). They include the following: nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 (NT-3), and neurotrophin-4. Among these neurotrophins, NT-3 is the most interesting being an important regulator of neural survival, development, function, and neuronal differentiation. NT-3 has two functions: dendritic growth and axonal guidance which is an important role for the neurotrophins in the CNS (McAllister 2001).

Recently, adult stem cells have been a focus for both research and clinical applicationsbecause they do not possess some of the problems associated with embryonic and fetal stem cells, such as immunological incompatibility (Amoh et al. 2005a, b). Hair follicle stem cells (HFSCs) are high promising source of accessible, active, and pluripotent adult stem cells. They are very suitable for regenerative medicine because: (1) they are easily available, (2) they are highly pluripotent, (3) they can be easily cultured, (4) they have high affinity to differentiate to neurons, and (5) they do not associate with ethical issues like embryonic and fetal stem cells (Hoffman 2006). For the first time, the bulge area of hair follicle was introduced in 1903 by "Stöhr" a German morphologist who called it "der Wulst" to describe the eminent structure near the location of the arrector pili muscle attachment in human hair follicles. Its structure appears at the beginning of the first anagen stage and is accompanied by the initiation of CD34 expression in the bulge area (Jaks et al. 2010). In addition, cell populations which have been proven to resist in bulge area are nestin positive (an intermediate filament that is a neural stem cell marker) (Nobakht et al. 2010; Amoh et al. 2009; Amoh et al. 2008). In a recent study, these cells were shown to lose their nestin positivity and have a high affinity to differentiate to neurons and oligodendrocytes (Nobakht et al. 2010). Considering the importance of time in tissue regeneration and to decrease differentiation time of HFSCs, this study investigates the effect of NT-3 and biodegradable nano-scaffold PLLA on HFSCs differentiation time to neural-like cells.

Materials and Methods

Hair Follicle Isolation

Male Wistar rats (n=40) weighing 180–200 g were purchased from Pasture Institute Animal Facility. All animal experiments were carried out according to the guidelines of the Iranian Council for Use and Care of Animals and were approved by the Animal Research Ethical Committee of Tehran University of Medical Sciences. The rats were permitted free access to food and water at all times and were maintained under light–dark cycles (Yang et al. 1993). The rats were killed with ether, and the whisker follicles were dissected as described by Amoh et al. (2005a, b) with a slight modification. Briefly after disinfection with betadine and 70 % ethanol, the upper lip was completely shaved. To isolate the whole intact follicle of vibrissa, the upper lip containing the vibrissa pad was cut and its inner surface was exposed. The samples were incubated in collagenaseI/ ispaseII solution (Sigma-Aldrich) in incubator. Then, most of the connective tissue and dermis around the follicles was removed, and the whisker follicles were plucked gently by the neck with a fine forceps. The follicles were transferred into another sterile 35-mm dish. To dissect the bulge region, two transversal cuts at the site of the enlargement spots of ORS were made with a fine needle, and the collagen capsule was incised longitudinally. About 20–30 isolated bulges immersed in amphotericin B. Then, the follicles were cut into smaller pieces and finally plated into 25 cm^2 tissue culture flasks (TCFs) precoated with collagen type I (Sigma-Aldrich). Prior to cultivation, the flasks were preincubated with medium which was then removed suspending the bulges in a 3:1 supplemented mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) containing 10 % fetal bovine serum, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), 10 ng/mL epidermal growth factor (Sigma-Aldrich), 10^{-9} M cholera toxin (Sigma-Aldrich), 0.5 mg/mL hydrocortisone, and 0.1 U/mL insulin.

The bulges were allowed to attach to the collagen for 5–7 days. Cells were incubated at 37 °C and 5 % CO_2. All surgical procedures and cultivation were done in a sterile environment and circumstances.

Preparation of PLLA Scaffolds

Aligned PLLA fibers were fabricated by electrospinning technique under optimum conditions (Yang et al. 2005). Polymer solution was prepared by dissolving the PLLA (Mw=152,000, Sigma-Aldrich, USA) into chloroform and

dimethylformamide at a volume ratio of 1.5:8.5. To fabricate PLLA scaffold, 5 mL of the polymer solution was fed into a plastic syringe controlled by a syringe pump at a feeding rate of 0.5 mL/h under a voltage of 21 kV. The syringe was connected to a 21-G needle by a tube. The distance between the syringe needle tip and the collector was adjusted to15 cm. To obtain aligned fibers, a rotating disk was used. The linear rate of the rotating disk at the edge was set to 1,000 rpm. PLLA fibers were spun onto a grounded aluminum foil target and then transferred to 24-well cell culture plates after sterilization by 70 % ethanol under a UV lamp (30 W, 25 nm).

Structural Morphology of PLLA Scaffolds

The nanostructural morphology of PLLA scaffolds was studied by scanning electron microscopy (SEM; Hitachi (Japan), S-4160, FESEM) at an accelerating voltage of 15 kV. Prior to observation, the samples were coated with gold using a sputter coater (Polaran SC7620 sputter coater). The average diameter of the electrospun nanofibers was measured using the SEM photographs by measuring at least 100 fibers using image analysis software.

The porosity of scaffold was calculated by the following formula:

$$P = \left(1 - \frac{\rho}{\rho_0}\right) \times 100$$

Where P is porosity, ρ is the density of electrospun scaffold and ρ_0 is the density of bulk polymer (Wang et al. 2005).

Scaffold Cell Seeding

Prior to seeding the HFSCs, the scaffold samples were prewetted with sterile phosphate-buffered saline (PBS; 0.2 M) and 70 % ethanol for the minimum period of 10 min to penetrate the PBS and cell culture medium into the pores. The samples were then sterilized as described above. The scaffolds were incubated in culture medium DMEM/F12 at 37 °C and 5 % CO_2 for at least 24 h. To perform cell seeding, cells were detached from the cell culture flask and viable cells were counted by trypan blue assay. The detached cells were seeded on prepared scaffolds at a density of 5×10^4 cells.

Cell Culture and Differentiation

HFSCs were cultured as previously described (Nobakht et al. 2010; Nobakht et al. 2011). In differentiation assay, HFSCs were cultured in three indicated time points into four groups: (1) in 25 cm^2 tissue culture flasks (TCFs) with defined medium (as control group); (2) in TCFs with defined medium and daily addition of NT-3 (10 ng/mL) for 3 days; (3) on PLLA scaffold with defined medium; and (4) on PLLA scaffold with defined medium and daily addition of NT-3 (10 ng/mL) for 3 days. HFSCs were cultured for 7, 8, and 9 days. After first passage on mentioned days, cells were treated in the four groups. Finally, differentiated cells were assessed on the 10th, 11th, and 12th days after cultivation, respectively.

MTT Assay

To assess the viability of HFSc seeded on PLLA nanofiber scaffolds, 3-(4,5-dimethylthiazol-yl)2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay was performed. At indicated time points, the culture medium of the cells from each of the four groups in 24-well plates was replaced with defined medium; 100 μL MTT was added to each well (5 mg/mL final concentration). After 4 h of incubation in the dark at 37 °C, MTT solution was aspirated and the purple formazan reaction products, produced by active mitochondria were dissolved by addition of 1 mL dimethyl sulfoxide (DMSO). After shaking the plates, the optical density of the formazan solution was determined at 570 nm using a spectrophotometer.

Immunocytochemical Staining

At indicated time points culture medium aspirated. Cells were fixed in 4 % paraformaldehyde for 20 min. The fixed cells were then washed with PBS (0.1 M). Cells were then permeabilized using 0.1 % Triton X-100 (Fluka, USA) and blocked in 0.3 % Normal Goat Serum (Sigma-Aldrich, USA) for 1 h at room temperature. Subsequently, the cells were incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: mouse monoclonal βIII-tubulin (1:200; Sigma-Aldrich, USA), mouse anti-CD34 (1:75), mouse anti-nestin monoclonal antibody (1:200, Millipore, USA), and monoclonal anti-K15 (1:15). Cells were washed the following day with PBS for 15 min to remove unbound primary antibodies. Finally, they were incubated for at least 1 h in the dark and at room temperature with the following secondary antibody: sheep anti-mouse fluorescein isothiocyanate (FITC) conjugate IgG (1:1,400; Sigma-Aldrich, USA). For nuclear counterstaining, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:1,000; Invitrogen) was used. Labeled cells were identified using fluorescent microscopy (Olympus Ax70). Triplicate samples were used for each group. Five fields were randomly chosen from each of the four groups, and those cells which were βIII-tubulin positive were counted.

Flowcytometry

To determine the percentage of cell expressing special markers at indicated times, cells were detached from the culture flasks and scaffolds as described above and incubated in the same

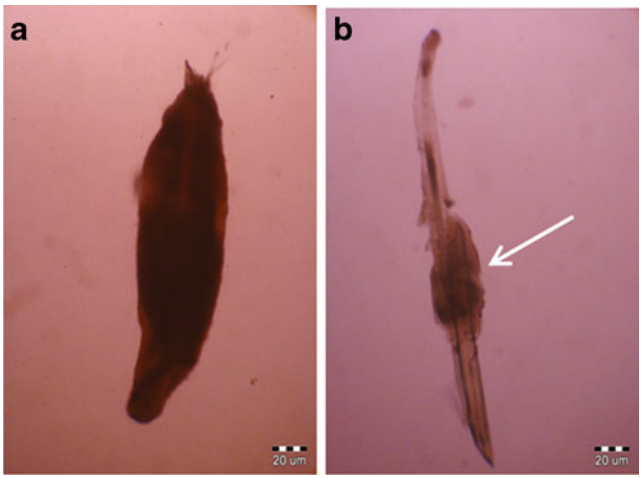

Fig. 1 **a** A dissected hair follicle of whisker pad of rat. **b** Bulge region (*arrow*) shown after longitudinal incision of collagen capsule. *Scale bars*=20 μm

primary antibodies for 1 h at room temperature. After washing with PBS (0.1 M), they were incubated for 1 hour in the dark and at room temperature with secondary antibody FITC (1:1,400). Labeled cell were analyzed using flowcytometry technique.

Cell Morphology on PLLA Scaffold

The SEM was employed to study the morphology of cells grown on the scaffolds. The scaffolds were taken out after 1 day of cell seeding and treated with fixation procedure. Samples were fixed in 4 % paraformaldehyde for 30 min. After washing with PBS (0.1 M), samples were dehydrated with a graded concentration of ethanol subscript as 50, 70, 80, 95, and finally 100 % ethanol for 40 min. Subsequently, the samples were treated with hexamethyldisilazane (HMDS; Fluka), a specimen drying agent. A combination of ethanol and HMDS was used for 45 min and kept in a fume hood for air drying. Finally, the samples were mounted onto a stub and coated with gold using sputter coating for the observation of cell morphology.

Statistical Analysis

All the experiments were performed in triplicate, and the data are expressed as mean ± SD. Statistical analysis was performed by independent-samples *t* test and one-way ANOVA followed by Tukey's post hoc test to evaluate the statistical significance between different groups. A value of $p<0.05$ was considered statistically significant.

Results

Hair Follicle Isolation and Cell Culture

In this study, a modified method was successfully used to isolate and culture the bulge cells from dissected rat hair follicles. One isolated follicle is shown in Fig. 1a, b.

Within 3–4 days, stem cells started to outgrowth from the isolated bulges (Fig. 2a). Given the rapid proliferation after 8–10 days, the bulge cells compacted around the bulge fragment and formed dome-like cell layers (Fig. 2b). Finally, cells at the edges of the domes started to migrate (Fig. 2c).

The bulge area stem cells of rat vibrissa follicles were isolated and suspended in DMEM/F12. Bulge cells were nestin and CD34 positive and K15 negative. These data suggested that these cells were primitive stem cells (Fig. 3a–c).

Structural Morphology of PLLA Scaffolds

To investigate the effects on the differentiation of HFSCs, aligned PLLA nanofibers were fabricated using electrospinning technique. Figure 4 shows SEM image of aligned PLLA nanofibers. The fiber diameter was determined. Most of the fibers had a diameter ranged from 300 to 500 nm and is shown in Fig. 5.

Furthermore, the scaffolds were revealed to be highly porous with an average porosity of 74 %.

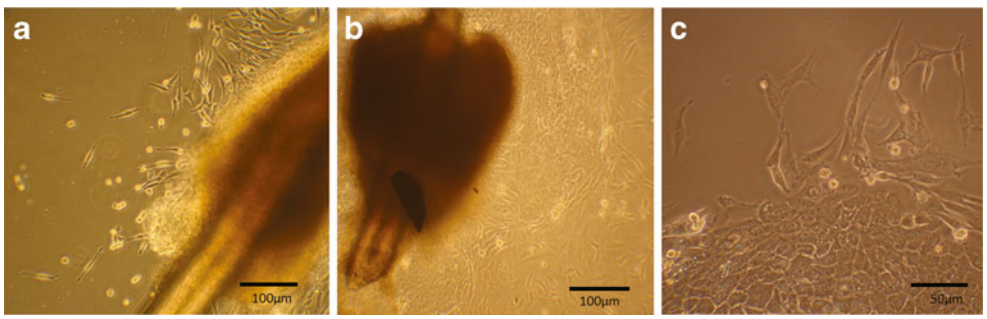

Fig. 2 Rat hair follicle bulge-derived neural crest stem cells in culture; **a** 3 or 4 days postexplantation of the bulge region, stem cells surrounded it. **b** Within 8 to 10 days, stem cells accumulated around the bulge region and makes a dome-shaped population of cells which gradually start to migrate. **c** Cells which are migrating. *Scale bars*=100 (**a**, **b**) and 50 μm (**c**)

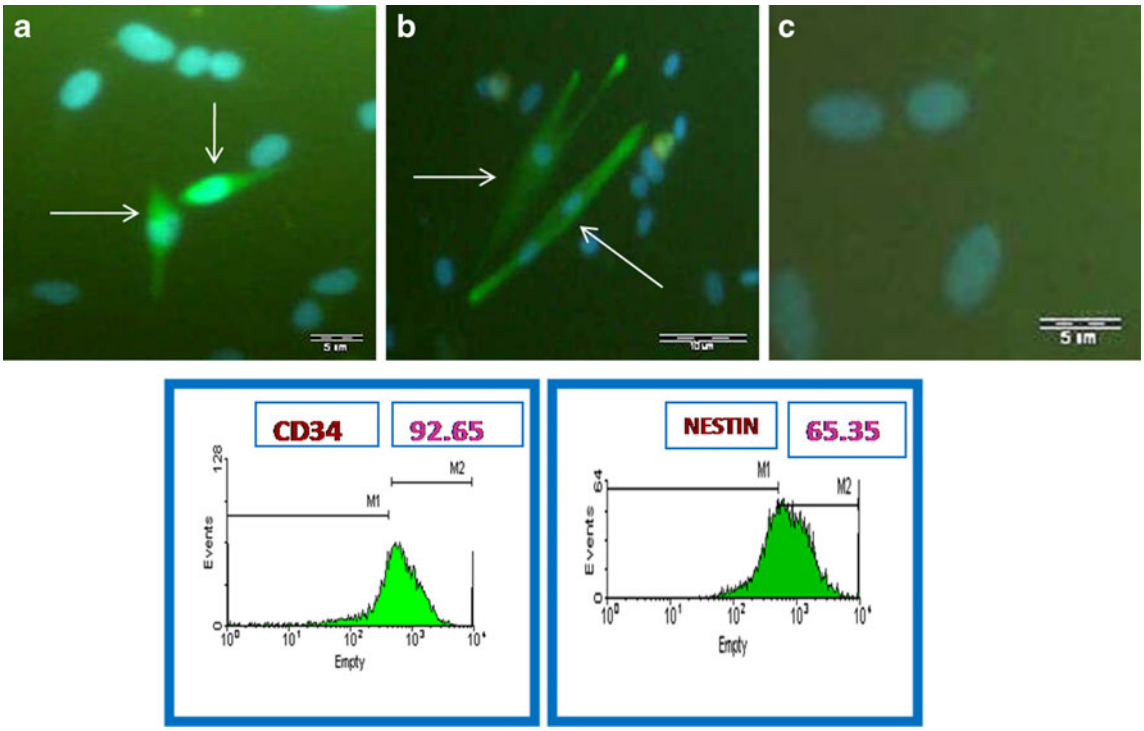

Fig. 3 Probing bulge-derived cells with specific antibodies before differentiation is necessary to determine if they are primarily stem cells and originated from neural crest. **a** Staining with CD34 antibody (*arrows*) and DAPI nuclear stain represents stem cells. **b** Staining with Nestin antibody (*arrows*) and DAPI nuclear stain represent neural stem cells that originated from neural crest. **c** Staining with K15 antibody and DAPI nuclear stain. No positive reaction was seen with this antibody. This demonstrated that bulge-derived neural stem cells are K15 negative. Flowcytometery results show percentage of CD34 and Nestin-positive cells. *Scale bars*=5 (**a**, **c**) and 10 μm (**b**)

MTT Assay

MTT chromometry assay was used to determine cell viability. The results showed that after 3 days of co-culture, the PLLA scaffold and daily addition of NT-3 on PLLA scaffold groups had higher cell viability than the control and daily addition of NT-3 in TCFs groups (one-way ANOVA, test of homogeneity of variances, and Tukey's test; $p<0.05$) (Fig. 6).

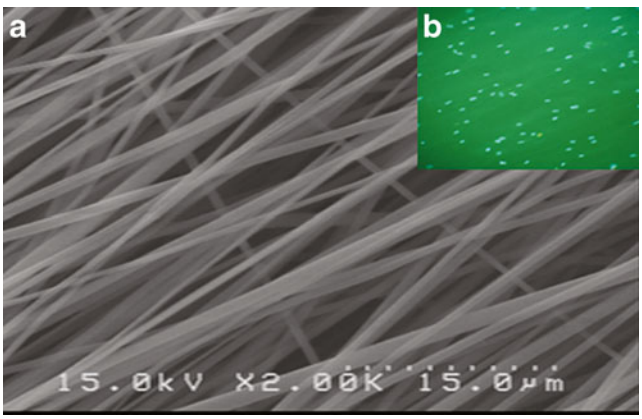

Fig. 4 **a** SEM micrograph of PLLA nonofiber fabricated by electospinning technique. **b** Micrograph of nuclear staining with DAPI at the top right shows direction of cell seeded on PLLA which is parallel to the direction of fibers. *Scale bar*=15 μm

Quantitative Analysis of the Cells Differentiating from HFSCs

The effect of nanofiber scaffold on HFSCs differentiation was investigated in the presence of 10 ng/mL NT-3, and HFSCs have been shown to differentiate into neurons. To quantify the number of differentiated cell of the four co-culture groups,

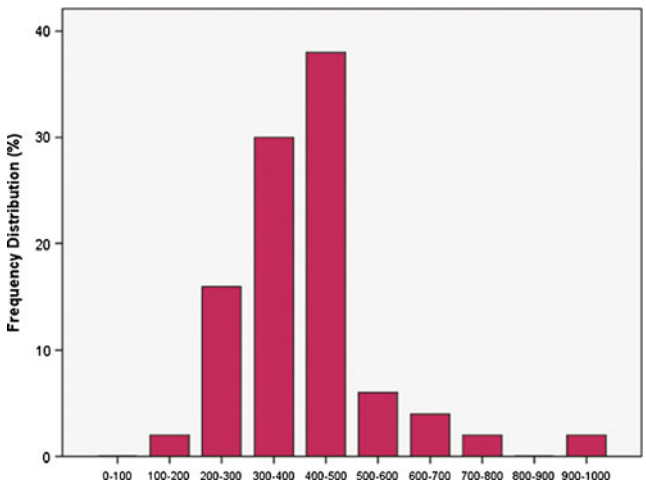

Fig. 5 The average diameter of the fibers was measured for 100 randomly selected fibers per scaffold. As the diagram shows, most of the fibers had a diameter ranged from 300 to 500 nm

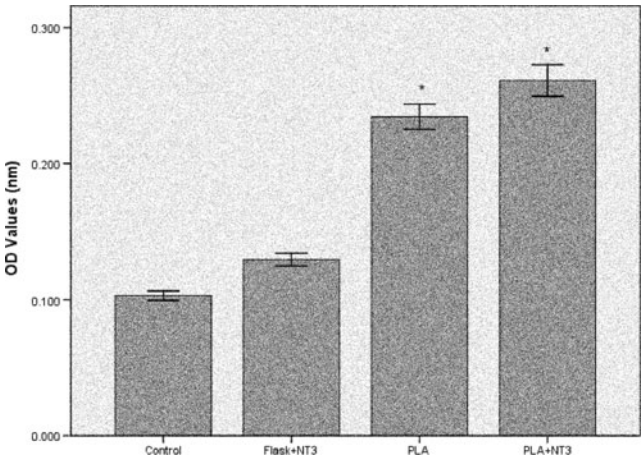

Fig. 6 Three days after co-culture of PLLA and NT-3, cell viability in the four co-culture groups were assayed. *Asterisk* indicates that cell viability of PLLA and PLLA+NT-3 groups was significantly higher than those of the other groups (one-way ANOVA, test of homogeneity of variances, and Tukey's test; $p<0.05$). *Error bars* represent means ± SD ($n=3$)

immunocytochemical staining was used to analyze phenotypes of these differentiated cells at the indicated time points in each group. βIII-tubulin was used as a marker of neuron-like cell expression (Nobakht et al. 2010; Mignone et al. 2007; Mignone et al. 2004; Sieber-Blum and Grim 2004). The differentiation rate of HFSCs cultured on electrospun fiber scaffold was significantly different from that on the TCFs. At the 11th and 12th cultivation days, HFSCs in PLLA groups and PLLA and daily addition NT-3 groups differentiated into neuron-like cells, as shown in Fig. 7d, g, h, k, l. Given the daily addition of NT-3 in TCFs groups, HFSCs differentiated into neurons 10, 11, and 12 days after cultivation (Fig. 7b, f, j). However, the reaction of differentiated cells to βIII-tubulin was so weak on the 10th and 11th days in comparison with differentiated cells on the 12th day, which was confirmed with flowcytometery results. HFSCs in the control groups only started to differentiate into neurons on the 12th day (Fig. 7i) and not on the 10th and 11th days (Fig. 7a–e)

For further quantitative analysis of the differentiated cells, five fields were randomly selected from each of the four co-culture groups, in which βIII-tubulin-positive cells were counted. Figure 8 shows the average number of differentiated cell after 10, 11, and 12 days of co-culture.

Flowcytometery results were nearly similar to interstitial cells of Cajal (ICC) results (Figs. 9 and 10).

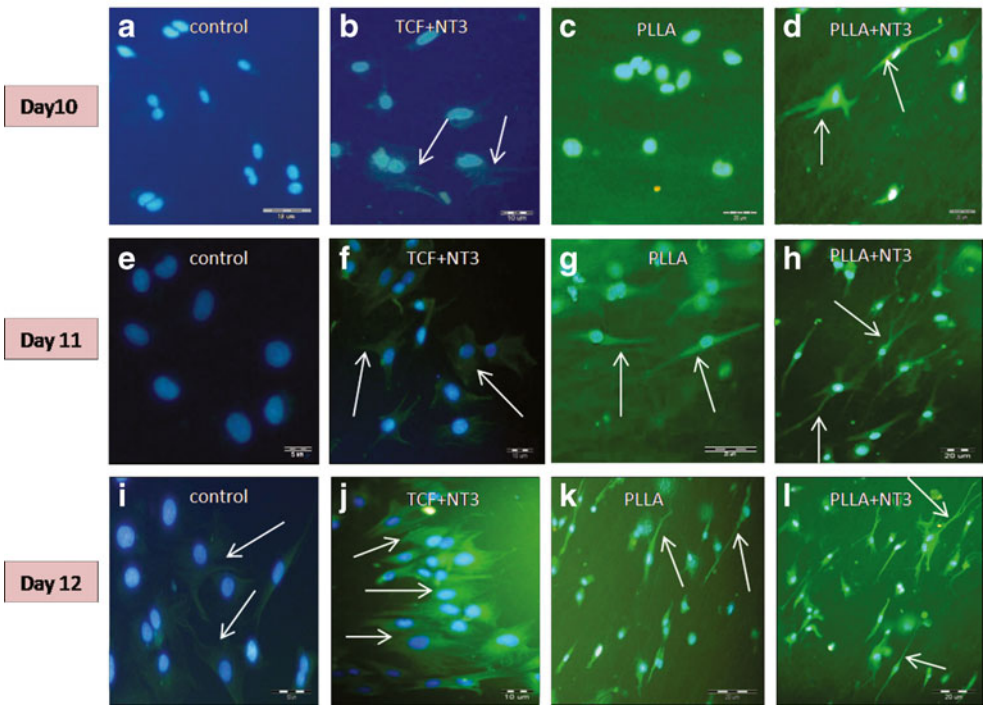

Fig. 7 To investigate the effect of PLLA nonofiber and NT-3 on HFSCs differentiation, HFSCs were cultured in four groups: (1) in TCFs with defined medium (as control group); (2) in TCFs with defined medium and daily addition of NT-3 (10 ng/mL) for 3 days (TCF+NT3); (3) on PLLA scaffold with defined medium (PLLA); and (4) on PLLA scaffold with defined medium and daily addition of NT-3 (10 ng/mL) for 3 days(PLLA+NT3). After first passage on the 7th, 8th, and 9th days, cells were treated in above four groups. Finally, differentiated cells were assessed by immunocytochemical staining using βIII-tubulin antibody on the 10th, 11th, and 12th days after cultivation, respectively. **a**–**d** On the 10th day, differentiated cells (*arrows*) were observed in TCF+NT3 and PLLA+NT3 groups, **b**, **d** whereas no differentiated cell was detected in the control and PLLA groups. **e**–**h** On the 11th day, no differentiated cell was detected in the control group. **i**–**l** on the 12th day, differentiated cells were detected in all groups. *Scale bars*=10 μm (**a**, **b**, **f**, **j**), 20 (**c**, **d**, **g**, **h**, **k**, **l**), 5 (**e**), 50 μm (**i**)

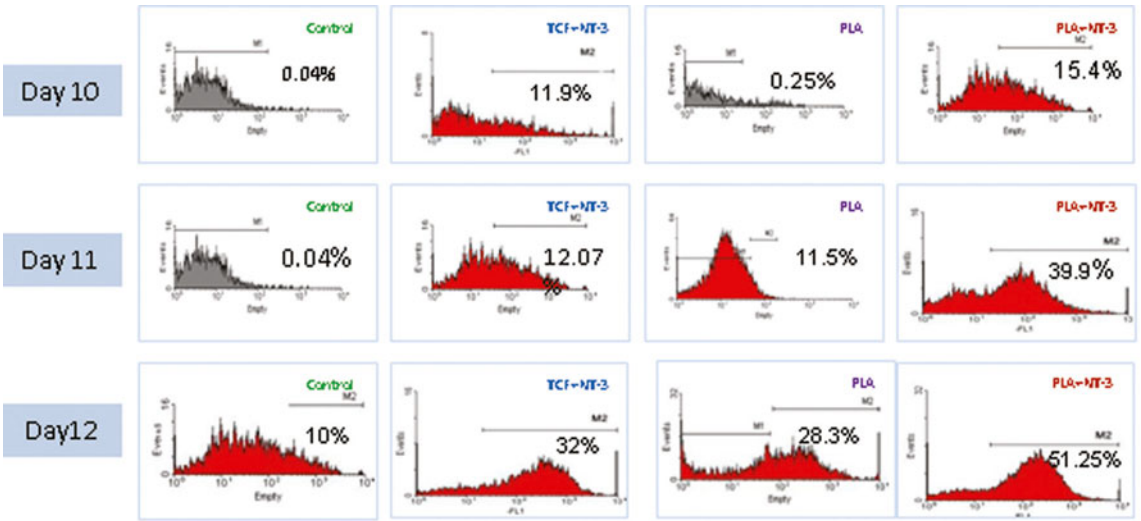

Fig. 8 The average number of differentiated cell after 10, 11, and 12 days of co-culture. *Asterisk* indicates that the number of the neurons differentiating from the HFSCs in groups with daily addition of NT-3 for 3 days was more than the other groups not treated with NT-3 (one-way ANOVA, test of homogeneity of variances, and Tukey's test; $p<0.05$). Moreover, PLLA+NT-3 groups on the 11th and 12th days have more differentiated cells than TCFs+NT-3 groups. *Error bars* represent means ± SD ($n=5$)

Morphological Features of HFSCs on PLLA Scaffolds

Figure 11 represents the ICC micrographs of HFSCs cultured for 3 days on aligned PLLA scaffold. Our results indicated that the direction of HFSCs elongation and neurite outgrowth was exactly parallel to the direction of PLLA fibers. Whereas the neurites in TCFs were randomly orientated and the cells extended multiple processes unlike the HFSCs on aligned fibers, Fig. 12 shows a HFSC cultured for 1 day on the scaffold. However, the overall results demonstrated that HFSCs were well attached on the scaffold and the cells showed elongated and spindle-like shape.

Discussion

Investigation has demonstrated that stem cells can be found in most organs of the body, including the heart, musculoskeletal, adipose tissue, liver, nervous system, hematopoietic system, and epidermal system (Mimeault and Batra 2008). Cell replacement using stem cells is thought to be a potent strategy to promote tissue repair after nervous system injuries. The optimal source of stem cells for regenerative medicine is a major question. The hair follicle is dynamic, cycling between growth (anagen), regression (catagen), and resting (telogen) phases throughout life (Hoffman 2000; Taylor et al. 2000). In this study, HFSCs were isolated and characterized and shown to express the stem cell marker CD34 but not the keratinocytes marker (keratin-15) (Amoh et al. 2005a, b; Amoh et al. 2008; Amoh et al. 2009). In addition, resident stem cells in the bulge region of mice and human hair follicle are nestin expressing cells (Mignone et al. 2007; Li et al. 2003). According to the results of the present and previous studies, proliferated cells derived from the bulge region of the rat hair follicle can also be nestin positive (Nobakht et al. 2010; Esmaeilzade et al. 2012). These stem cells are multipotent and since nestin can be expressed, in both neural and HFSCs, they can differentiate to various neural cells (Esmaeilzade et al. 2012; Amoh et al. 2005a, b; Mignone et al. 2004).

In recent years, most of research have focused on the factors that affect on the proliferation and differentiation of neural stem cells. These factors including those diffusible from the medium may stimulate or inhibit proliferation and

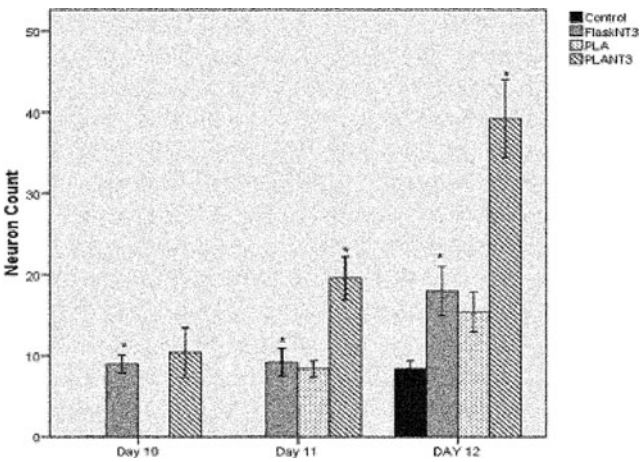

Fig. 9 Three days after co-culture in the four groups at indicated time points, differentiated cells were assessed by flowcytometery technique using βIII-tubulin antibody. Flowcytometery results show percentage of differentiated cells

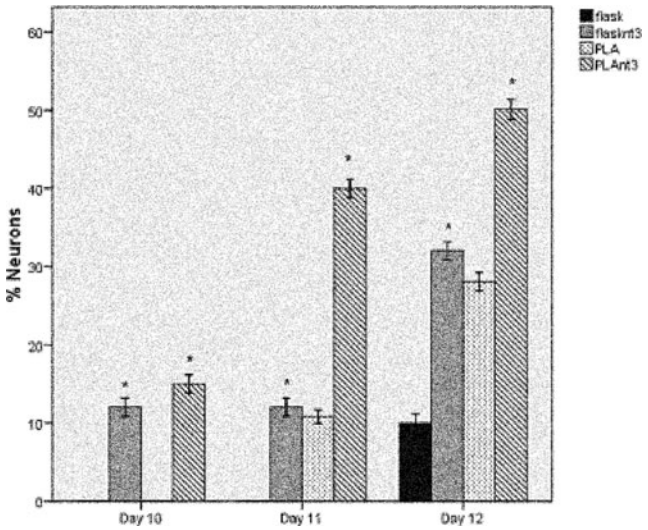

Fig. 10 The average percentage of differentiated cell after 10, 11, and 12 days of co-culture. *Asterisk* indicates that the percentage of the neurons differentiating from the HFSCs in groups with daily addition of NT-3 for 3 days was more than other groups which not treated with NT-3 (one-way ANOVA, test of homogeneity of variances, and Tukey's test; $p<0.05$). Moreover, PLLA+NT-3 groups on the 11th and 12th days have more differentiated cells than TCFs+NT-3 groups. *Error bars* represent means ± SD ($n=5$)

differentiation of neural stem cells (Cattane and Mckay 1990; Tzeng 2002). Neural growth factors, especially NT-3, are the best choices for clinical therapy because of its multipotency to stimulate the survival, proliferation, and differentiation of neural stem cells (Tzeng 2002; Lachyankar et al. 1997). Considering the use of growth factors, recent studies focus on advantages of various kinds of scaffolds to mimic natural environment in the human body. As HFSCs have the multipotential characteristic and may respond to varying environments differently, this study established four different co-culture groups to be compared with specify the most efficient one for more rapid differentiation of HFSCs into neurons, which would then be used in vivo.

NT-3 has been reported to increase the preferential differentiation of neural stem cells into neurons (Lim et al. 2007). In a previous study, research showed that 100 ng/mL NT-3 was the most suitable concentration to promote axonal growth in vitro (Taylor et al. 2004). Moreover, 20 and 50 ng/mL NT-3 have been demonstrated to have prominent effects on differentiating neural stem cells (Lim et al. 2007; Li et al. 2009). Furthermore, 20 ng/mL NT-3 not only promote axonal growth but also enhance the differentiation percentage from neural stem cells into neurons (Tzeng 2002). Therefore, the present experiment used 10 ng/mL NT-3 and observed the same result as Li et al. (2009) between control and daily addition of NT-3 group.

However, in this study, the differentiation percentage from HFSCs into neurons in the daily addition of NT-3 group was lower than in the NT-3 on PLLA group. Thus, the fibrous scaffold has been shown to have a considerable effect on stem cells differentiation as anticipated. Extracellular matrix is composed of growth factors and different proteins, such as collagen, laminin, other fibrils, and proteoglycans in a nanometer scale, which are responsible for cellular functions like cell adhesion, proliferation, migration, differentiation, and cell shape. Electrospun nanofiber matrix has morphological similarities to the natural ECM. Their interconnected highly porous structures increase cell migration and nutrients and metabolic wastes transport (Kumbar et al. 2008). Moreover, previous literature suggests that hydrophilic surface of nanofibrous scaffold improves cellular adhesion (Geunhyung and Wandoo 2007).

The high surface area-to-volume ratio and the porous structure of nanofibers might increase the contact area between cells and the fibers. This property facilitates growth factor uptake by cells which lead to faster proliferation, migration, and differentiation (Schofer et al. 2008; Cao et al. 2009), which is confirmed by immunocytochemistry, flwcytometery, and MTT results.

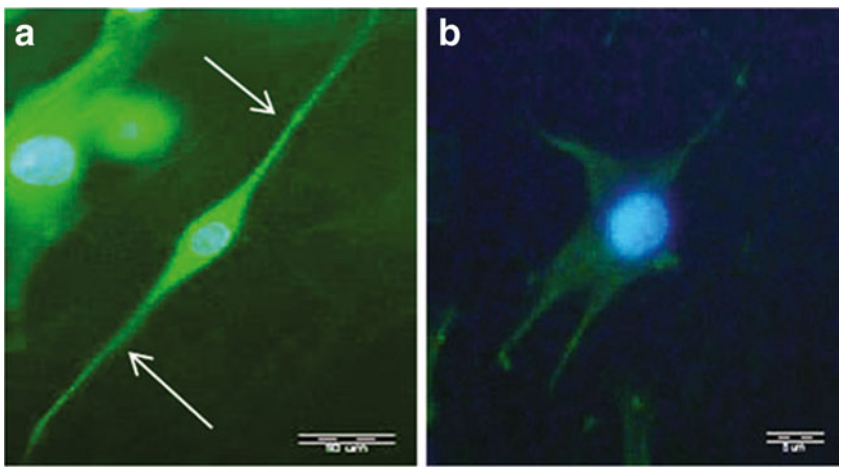

Fig. 11 ICC micrograph of a differentiated HFSC. **a** after differentiation neurite outgrowth is parallel to PLLAA fiber (*arrows*). **b** Randomly orientated and multiple processes in a differentiated cell in TCFs. *Scale bars*=10 μm (**a**), 5 μm (**b**)

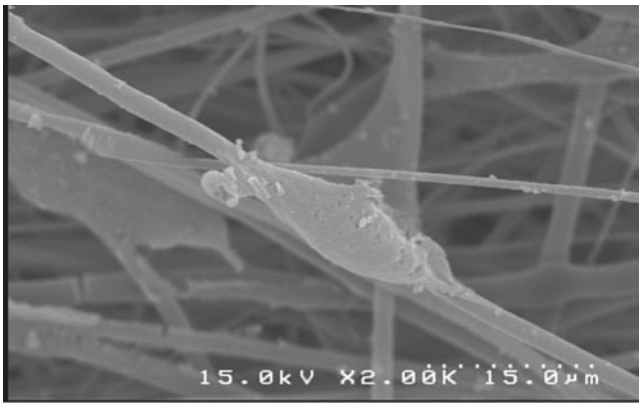

Fig. 12 SEM micrographs of HFSCs seeded on PLLA for 1 day showing the cell–fiber adhesion between the HFSCs and the PLLA fibers. *Scale bar*=15 μm

Researchers demonstrated that ES cultured onto PCL nanofibers were capable of differentiating into neural lineage cells including neurons, oligodendrocytes, and astrocytes (Xie et al. 2009). In a recent study, PCL nanofibers were used also for cultivation of HFSCs. The results showed that PCL is a suitable candidate for tissue engineering (Hejazian et al. 2012). Yang et al. showed that the rate of neural stem cell differentiation on nanofibers was higher than that on microfibers (Yang et al. 2005). Hence, a combination of stem cell and aligned nanofibers could provide a better approach to peripheral nerve repairing because of scaffold ability which provides a more hospitable environment for cell survival (Willerth and Sakiyama-Elbert 2008).

Amoh et al. reported βIII-tubulin expression 2 months after cultivation of ND-GFP cells from the bulge area of vibrissa hair follicles (Amoh et al. 2005a, b). According to previous investigations, isolated bulge cells were nestin negative and could express βIII-tubulin 2 months after first passage without any treatment and 2 weeks after first passage by using silibinin (Nobakht et al. 2010; Nobakht et al. 2011). In this study, the authors demonstrated that by using biodegradable nanoscaffold PLLA and NT-3 in a defined dose, the bulge region stem cells could express βIII-tubulin within 10 days after cultivation. Furthermore, HFSCs showed various differentiation rates in all of the four co-culture groups in the 12th day after cultivation.

In addition, the present study shows that the direction of HFSCs elongation and their neurite outgrowth on PLLA scaffold was parallel to the direction of fiber alignment in comparison to cells in TCFs which was consistent with previous reports (Yang et al. 2005). The advantage of alignment might be valuable in nerve injury repair by restoring the cellular architecture which is lost after injury.

MTT experiments are currently the main method in vitro to test the biocompatibility and cytotoxicity of biomaterials and the different dose of growth factors, being a quantitative assay for cell viability (Thonhoff et al. 2008; Yang et al. 2010). Here, PLLA scaffold were co-cultured with HFSCs to investigate whether PLLA scaffold and defined dose of NT-3 have cytotoxicity to stem cells. The MTT assay result showed that PLLA scaffold and NT-3 had no cytotoxicity but instead cause proliferation and differentiation of HFSCs.

Conclusion

The results indicate that 10 ng/mL NT-3 and PLLA could accelerate the differentiation process of rat HFSCs to neural cells.

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