Role of integrin in influencing differentiation of PC12 cell grown on PLLA-aligned nanofiber: a mRNA–microRNA–protein integrative study

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Abstract

The aim of this study is to unveil the role of integrin in influencing the differentiation of PC12 cell grown on PLLA aligned nanofibers by integrative study of cDNA microarray, proteomics technology and microRNA sequencing technology. First, PLLA-aligned nanofibers were prepared by electrospinning, and the chemical composition and surface morphology of the nanofibers were examined. Then, morphology and neurite length of PC12 cells and GRGDS-treated PC12 cells on PLLA-aligned nanofibers (AF and GA group) were measured by high-content analysis system. Subsequently, protein expression profile was analyzed by iTRAQ proteomics technology and 250 differentially expressed proteins were screened in the GA group. Integrative analysis of mRNA-microRNA-protein data showed that 'MAPK signaling pathway' was the key affected pathway after the function of integrin were interfered by GRGDS. Other seven pathways were also influenced, and the differentiation of PC12 cell grown on PLLA aligned nanofibers was finally inhibited.

Keywords: integrin; aligned nanofibers; cell differentiation; mRNA–microRNA–protein integrative analysis

Introduction

One of the goals of neural tissue engineering is to find an effective way to guide neural cell differentiation and maturation, in which the most common way is using varieties of favorable biocompatible materials to produce cell growth scaffold [1, 2]. Nanofibers with many advantages, such as large surface area, small size and quantum size effect, have wide application in many areas. Meanwhile, since the nanofibers can simulate the extracellular matrix (ECM) and are conducive to cell growth, differentiation, they provide good scaffold materials for tissue engineering [3, 4]. Studies have shown that morphology of aligned nanofibers, which is similar with ECM can impact the behavior of nerve cells differentiation [5]. Lim et al. [6] found that under the synergy of retinoic acid, the differentiation characteristics of neural stem cells which were grown on the aligned nanofibers were more apparent than those grown on random nanofibers and amorphous surface. Il Cho et al. [7] found that after grown on the surface of aligned nanometer fiber, mRNA marker of neural differentiation in bone marrow mesenchymal stem cells (rMSCs) of rats increased significantly, while on the surface of random nanofibers, the expression of these markers was not obvious. These studies show that aligned nanofibers can effectively induce neural differentiation [8]. The same phenomenon was also observed in our previous studies that poly l-lactic acid (PLLA)-aligned nanofibers could more available induce PC12 cell differentiation than random nanofibers [9]. Furthermore, the underlying molecular mechanism of how PLLA-aligned nanofibers induced PC12 cell differentiation was investigated by using cDNA microarray, microRNA (miRNA) sequencing technology and integrated analyses of above two biomics data by us [10].

Our research found that integrin played a very important role in PLLA aligned nanofibers induced PC12 cell differentiation [9]. Integrin is a kind of transmembrane glycoprotein that is widely distributed on the cell surface. As membrane molecules which transmits mediated signal, integrin regulates cell differentiation, migration, immune adhesion, and multiple biological functions via triggering downstream signaling pathways. Researches had shown that surface
mortality of nanofibers can affect the type and conformation of adsorbed serum proteins, adsorbed proteins will combine with integrin on the surface of cells by Arg–Gly–Asp (RGD) sequence, leading to a corresponding change in the intracellular pathway and affect associated cells function [11, 12]. To evaluate whether integrin plays a role in mediating the effects of aligned nanofibers on behavior of nerve cells, Mukhatyar et al. [13] used cyclo-Gly–Arg–Gly–Asp-Ser (GRGDS) to interfere the interaction between integrin and fibronectin and investigated the influence on cells migration and neurite growth on the cellular level. The results showed that Schwann cells migration and neurite growth was significantly reduced on polyacrylonitrile methylacrylate fibers. Thus the effectiveness of GRGDS interference to integrin function was verified. The reason is that integrin can identify proteins which containing RGD sequence. GRGDS also has RGD sequence which can first competitively combine with integrin, then the interaction between integrin and adsorbed proteins (such as fibronectin and vitronectin) which contains RGD sequence was blocked [14, 15].

However, very little is known about the effects of integrin on the expressions of mRNA, microRNA, proteins, and their functions in the interaction between biomaterials (including aligned nanofibers) and neuronal cells. In our previous work, pentapeptide GRGDS interference was first performed to hamper the function of integrin of PC12 cells, then the effect on morphologies and neurites outgrowth of cells grown on PLLA-aligned nanofibers were studied on the cellular level. Furthermore, cDNA microarray and miRNA SOLID sequencing technology were utilized to compare mRNA and miRNA expression profiles of PC12 cell with and without GRGDS treatment, so as to explore the role of integrin in PLLA aligned nanofiber-PC12 cell interaction on the mRNA and miRNA level [16]. The results showed that after GRGDS interference, pathways including ‘MAPK signal pathway’ and ‘Regulation of actin cytoskeleton pathway’ in PC12 cells grown on PLLA aligned nanofiber were affected, resulted in morphologies change and neurites outgrowth inhibition.

As we know, proteins are the executors of physiological function, and direct embodiments of the life phenomena. Therefore, the aim of this study is first to use proteomics technology to compare protein expression profile in PC12 cells and GRGDS-treated PC12 cells grown on PLLA-aligned nanofibers, and then integrate protein data and mRNA, miRNA data from our previous study to finally unveil the mechanism of integrin in influencing PC12 cell differentiation on PLLA-aligned nanofibers systematically.

**Materials and methods**

**Preparation and characterization of PLLA-aligned nanofiber**

As described in our previously study [9], PLLA (M<sub>n</sub> = 400–600 kDa, Daigang, China) was dissolved in 1,2-dichloroethane/N,N-dimethylformamide (vol:vol = 3:1) to prepare electrospinning solution (1.8 wt%). Then the solution was electrospun into aligned nanofibers at a rate of 4 ml/h and a voltage of 10 kV, and placed in oven at 37 °C for 2 h. The morphology was observed by S-3000N scanning electron microscope (SEM) (Hitachi, Japan). The average diameter of the nanofibers was measured with Image J software. Chemical composition of PLLA powders and aligned nanofibers were determined by Nicolet 5700 Fourier Transform Infrared Spectrometer (FTIR) (Thermo, USA).

**PC12 cell culture and pentapeptide GRGDS treatment**

Undifferentiated PC12 cells (purchased from the Type Culture Collection of Chinese Academy of Sciences) were cultured with the reported method [17]. For differentiation study, undifferentiated PC12 cells were cultured by differentiation medium for 24 h. Differentiation medium components were 96% high-glucose DMEM (Gibco, USA), 2% horse serum (Gibco, USA), 1% fetal bovine serum (Sijijing, China), 1% penicillin–streptomycin solution (Sigma, China) and 50 ng/ml nerve growth factor (R&D, USA). Differentiating PC12 cells were seeded on PLLA aligned nanofibers at a density of 5000 cells/cm<sup>2</sup> and cultured for 24 h. This group was designated as the AF group. According to the reported method [13], differentiating PC12 cells were incubated with differentiation medium in an addition with 0.5 mg/ml pentapeptide GRGDS (Sangon, China) in 37 °C for 30 min, and then cultured on PLLA-aligned nanofibers with the same cell density for another 24 h. This experimental group was designated as the GA group.

**Cell morphology observation and neurite length count**

At 24 h, after PC12 cells seeded on PLLA substrates, samples were taken out and rinsed with PBS twice (pH 7.2), then fixed in 4% glutaraldehyde for 15 min, dyed with 5 µg/ml FITC-phallolidine (Sigma, USA) for 30 min and DAPI for 4 min. Samples were observed and neurite length was counted by Cellomics ArrayScan VTI High Content Scanning (HCS) (Thermo Fisher, USA). Every sample was randomly observed at three different fields.

**Proteomics analysis**

PC12 cells of AF and GA group were collected and proteins were extracted by SDT lystate. Subsequent iTRAQ protein detection experiments were performed by Shanghai Applied Protein Technology Company (Shanghai, China). The prepared samples were labeled with 8-plex isotope labeling relative quantitative kit (iTRAQ<sup>TM</sup>) and analyzed by the protocol of capillary high-performance liquid chromatography/Q-Exactive mass spectrometry (Thermo Finnigan, USA). Then, the iTRAQ data were searched with software Mascot 2.2 and Proteome Discoverer 1.3 (Thermo, USA) for protein identification and quantification. The experiment was carried out in three independent runs. Filter parameters were Peptide FDR < 0.01. Compared with the AF group, proteins in the GA group with fold changes of >1.2 were considered to be differentially expressed.

**Western blot analysis**

Based on the proteomics experiment, four proteins (namely, Mapk12, Rhob, Egfr and Smad1) were selected for western blot analysis. β-Tubulin was used as an internal standard. Total protein was extracted from PC12 cells of AF group and GA group. Fifty microgram protein extracts were fractionated on 10% SDS-PAGE gels and electrophoretically transferred to positively charged nylon membrane. The membranes were blocked with 5% bovine serum albumin (BSA, Sigma) at room temperature for 1 h and incubated with primary antibody overnight at 4°C. Membranes were washed, probed with 1:5000 diluted secondary antibody, then the bands were scanned and analyzed using Image J software.

**mRNA and microRNA expression profiles analysis and miRNA target prediction**

mRNA and miRNA data were obtained from our previous work [16]. Briefly, cDNA microarray experiment was conducted as follows: after cultured on PLLA aligned nanofibers for 24 h, PC12 cells
of the AF group and the GA group were harvested and total RNA was extracted. cDNA microarray was employed and 3030 differentially expressed mRNAs in the GA group were filtered. SOLiD sequencing experiment was conducted as follows: small RNAs were extracted and conversed to construct cDNA library. Sequencing experiments were performed and 96 differentially expressed known miRNAs were screened out.

The target genes of differentially expressed known miRNAs were predicted and the repression rate from dysregulated miRNAs for each target gene was calculated. Finally, 1140 target genes (807 up-regulated and 333 down-regulated) predicted to be significantly dysregulated in the GA group were obtained [16].

Biological pathway analysis of three biomics data

Biological pathway enrichment analysis of differentially expressed proteins, mRNAs and predicted significantly dysregulated miRNA target genes was performed using DAVID (http://david.abcc.ncifcrf.gov/) [18].

Identification of matched miRNA targets

Differentially expressed mRNAs and differentially expressed proteins were matched with predicted significantly dysregulated miRNA target genes [16]. The mRNA and protein that had the same names and the same expression trend as the predicted significantly dysregulated miRNA target genes were identified as the matched miRNA target mRNA and target protein.

Screening of biological pathway containing matched miRNA targets

By searching pathways that differentially expressed mRNAs and proteins affected, the pathways that contained matched miRNA targets were identification.

Statistical analysis

Data were presented as mean ± SD. Unless otherwise stated, statistical analysis was carried out using one-way ANOVA in Origin 6.1 software. A value of $P < 0.05$ was considered as statistically significant. All the experiments were repeated at least three times.

Results and discussion

Characterization of PLLA substrates

SEM observation showed that PLLA-aligned nanofibers were uniform and unbeaded with a good orientation. The average diameters of PLLA aligned nanofibers were 269.30 ± 4.27 nm (Fig. 1). IR spectra analysis of PLLA powders and the as-prepared aligned nanofibers showed that the two materials shared three characteristic absorption peak of PLLA (PLLA-aligned nanofibers at 1758.31 cm⁻¹, 1183.03 cm⁻¹ and 1089.34 cm⁻¹; PLLA powders at 1758.07 cm⁻¹, 1186.99 cm⁻¹ and 1092.29 cm⁻¹) [19] (Fig. 2): These results indicated that PLLA chemical composition did not change after being electrospun into nanofibers.

Morphology observation of PC12

Fig. 3 shows the fluorescent images of PC12 cells on the AF and the GA group. It was easy to find that the neuritis of PC12 cells in the PLLA-aligned nanofibers group extended out paralleled with the orientation of the aligned nanofibers, while the neurites of PC12 cells in the GA group were hard to find under the interference of pentapeptide GRGDS. Fig. 4 is the bar chart of average neurite length of every cell in the AF and the GA group which was counted by HCS. The average neurite length of each cell in the GA group was much shorter (6.34 μm) than the AF group (21.29 μm). The results in Figs. 3 and 4 indicated that after the interference of integrin by pentapeptide GRGDS, the differentiation of PC12 cell has been inhibited.

Proteomics analysis

The protein expression profiles were analyzed by iTRAQ proteomics technology. Results showed that compared with the AF group, 250 differentially expressed proteins (57 up-regulated and 193 down-regulated) were found in the GA group (see Supplementary Table 1).

Western blot analysis

To verify the proteomics data, four proteins (Mapk12, RhoB, Egfr and Smad1) which play a role in nerve cell differentiation were selected for western blot analysis. The results showed that the expression trends of the four proteins were similar with that in proteomics data, which indicated the good reliability and repeatability of the proteomics technology (Fig. 5).

Biological pathway analysis of three biomics data

DAVID analytical results showed that 250 differentially expressed proteins in the GA group were involved in 43 KEGG pathways (see Supplementary Table 2). About 3030 differentially expressed
mRNAs and 1140 predicted significantly dysregulated miRNA target genes in the GA group were involved in 174 (see Supplementary Table 3) and 137 (see Supplementary Table 4), respectively. Thirty-eight pathways were common in above three groups (see Supplementary Table 5). Among them, six pathways were directly related to cell differentiation (Table 1), including ‘MAPK signaling pathway’, ‘Neurotrophin signaling pathway’, ‘Adherens junction’, ‘T cell receptor signaling pathway’, ‘Insulin signaling pathway’ and ‘ErbB signaling pathway’. Fourteen pathways were related to above differentiation-related pathways, with eight pathways related to cell adhesion and cytoskeleton (such as ‘Focal adhesion’ and ‘Regulation of actin cytoskeleton’) and other six pathways (‘Apoptosis’ and ‘Fc epsilon RI signaling pathway’) involved in cell metabolism, apoptosis, and so (Fig. 6). Therefore, after the activation of integrin was blocked by GRGDS, cell behaviors such as adhesion, cytoskeleton, metabolism and apoptosis of PC12 cells in the GA group were widely affected, and cell differentiation might be further inhibited.

**Identified matched miRNA targets**

As miRNAs play their biological functions via their target genes, identification of miRNAs’ target genes accurately is the first and most important task to functionally characterize miRNAs [16]. However, it is difficult to obtain miRNA target genes accurately using software prediction only, so cDNA microarray and proteomics technologies become two effective methods to verify the prediction results of miRNAs [20].

Here, to obtain reliable miRNAs target genes and analyze the roles of miRNAs, an integrative analysis of mRNA, protein expression data and miRNA expression data were performed according to negatively correlated relationships [16]. By matching 3030 differentially expressed mRNAs and 250 differentially expressed proteins with 1140 predicted significantly dysregulated miRNA target genes respectively, 44 matched miRNA target mRNAs, 8 matched miRNA target proteins and one matched miRNA target mRNA/protein pair (Casp7) were matched in the GA group finally, with a total of 53 different matched miRNA targets (see Supplementary Table 6).

**Screened biological pathway containing matched miRNA targets**

By searching pathways that differentially expressed mRNAs and proteins affected (see Supplement Tables 2 and 3), 53 matched miRNA targets were found involved in 37 pathways (see Supplementary Table 7). Among them, eight pathways might be relate to PC12 cell differentiation, including ‘MAPK signaling pathway’, ‘Neurotrophin signaling pathway’, ‘mTOR signaling pathway’, ‘Tight junction’, ‘Regulation of actin cytoskeleton’, ‘Insulin signaling pathway’, ‘Calcium signaling pathway’ and ‘Apoptosis’. The miRNA-mRNA and miRNA-protein target pairs contained in these eight pathways are listed in Table 2 and the relationships are summarized in Fig. 7. It could be deduced from Fig. 7 that ‘MAPK signaling pathway’ was the key pathway in integrin mediated PLLA-aligned nanofiber–PC12 cell interaction. The main molecular mechanisms of PC12 cells differentiation induced by PLLA-aligned nanofibers after the function of integrin was interfered with pentapeptide GRGDS were discussed here in detail.

The ‘MAPK signaling pathways’ transduce various external signals, leading to a variety of cellular responses, such as cell growth, differentiation [21, 22] and apoptosis. Ribosomal protein S6 kinase A6 (Rps6ka6) belongs to the ribosomal S6 kinase family and was down-regulated by four miRNAs in the GF group. Rps6ka6 mRNA was found down-regulated in blocked C2C12 myoblast differentiation [23]. Related RAS viral (r-ras) oncogene homolog 2 (Rras2) was up-regulated by two miRNAs in the GF group. It was found to be involved in regulating actin cytoskeleton and cell migration [24]. Thus, the down-regulation of Rps6ka6 protein and up-regulation of Rras2 mRNA indicate that GRGDS disruption might affect the
morphology and differentiation of PC12 cells on PLLA-aligned nanofiber.

The ‘Neurotrophin signaling pathway’ was associated with differentiation and survival of neural cells. Neurotrophin signaling can be regulated by connected signaling cascades such as ‘MAPK signaling pathway’, transmitting signals-like cell survival and growth [25]. The ‘mTOR signaling pathway’ responds to growth factors and controls cytoskeletal organization, metabolism and survival. So the down-regulation of Rps6ka6 protein might weaken signals transmitted to these two pathways to affect cytoskeleton and suppress differentiation of PC12 cells.

The ‘Tight junction’ pathway is related to cell adhesion, and connected to the ‘Regulation of actin cytoskeleton’ pathway which can affect neuronal morphology and axonal outgrowth [26]. So the up-regulation of Rras2 mRNA might disturb adhesion the stability of actin, and then affect cell differentiation.

The ‘Insulin signaling pathway’ regulates energy metabolism, apoptosis, cell proliferation and differentiation. Some signal transduction in this pathway can lead to the activation of MAPK signaling pathway [25]. Protein phosphatase 1, regulatory subunit 3B (Ppp1r3b), was up-regulated by five miRNAs. Ppp1r3b has important roles in hepatic glucose metabolism [27]. Our previous research had stated that in the process of PLLA nanofiber inducing PC12 cell differentiation, the glucose metabolic rate was enhanced while Ppp1r3b mRNA down-regulated [10]. So the up-regulation of Ppp1r3b mRNA implied that the differentiation might decrease in the GF group.

The ‘Calcium signaling pathway’ was found to play a role in apoptosis, cell proliferation, metabolism, etc. Solute carrier family 25 member 4 (Slc25a4) was up-regulated by miR-23a in the GF group. The overexpression of Slc25a4 can induce apoptosis [28] and Slc25a4 is considered as a pro-apoptotic protein [29]. Here, the up-regulation of Slc25a4 mRNA might result in apoptosis of PC12 cells.

The ‘Apoptosis’ pathway is controlled by a variety of connected processes. Apoptosis is a genetically programmed process by the activation of caspases. Here, Caspase 7 (Casp7), the only mRNA target miRNA/protein pair which was co-regulated by two miRNAs (miR-23a/23b), is involved. Casp7 is a hydrolytic enzyme protein and has been confirmed as being associated with apoptosis and induced cell death by activating related pathways [30]. Thus, in the GA group, the interference of GRGDS down-regulated the expression of miR-23a/b, which in turn up-regulated Casp7 and finally induced apoptosis.

Fig. 8 shows the molecular mechanism explanation for how the differentiation of PC12 cells grown on PLLA-aligned nanofibers was inhibited after the function of integrin was interfered by GRGDS. When pentapeptide GRGDS competitively bound with integrin of PC12 cells, the function of integrin to combine with adsorbed proteins was prevented. Then the cell differentiation was inhibited and apoptosis was induced via miR-23a, miR-23b, miR-26b, etc. by regulating Rras2, Rps6ka6, Ppp1r3b, Slc25a4 and Casp7 in the above eight pathways. These results coincide with our previous reports that the morphology and the neurite outgrowth of PC12 cells on PLLA-aligned nanofibers were significantly affected after treated with GRGDS [16].
Conclusion

Through the integrative analysis of mRNA–miRNA-protein expression profiles, the molecular mechanism that how differentiation of PC12 cells was inhibited after the function of integrin was interfered by GRGDS was revealed. The role of integrin in influencing differentiation of PC12 cell grown PLLA aligned nanofiber was confirmed. miRNAs such as miR-23a, miR-23b and miR-26b were induced to regulate target genes (Rras2, Rps6ka6, Casp7, etc.) and thereby affected eight main pathways, including ‘MAPK signaling pathway’, ‘Neurotrophin signaling pathway’, ‘mTOR signaling pathway’, ‘Tight junction’, ‘Regulation of actin cytoskeleton’, ‘Insulin signaling pathway’, ‘Calcium signaling pathway’ and ‘Apoptosis’. Among them, ‘MAPK signaling pathway’ was the key pathway in integrin-mediated PLLA-aligned nanofiber–PC12 cell differentiation-related pathways and connected 14 pathways that differentially expressed proteins, mRNAs, predicted significantly dysregulated miRNA target genes involved simultaneously in the GA group.
interaction. These results were agreed well with our previous results obtained from researches on mRNA and miRNA levels.

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Supplementary data

Supplementary data is available at REGBIO online.

Conflict of interest statement. None declared.

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