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Induced differentiation of adipose-derived stem cells enhance secretion of neurotrophic factors.

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Keywords: differentiated ADSCs; Schwann cells; neurotrophic factors; P2X7; nerve damage.

Abstract. Adipose-derived stem cells (ADSCs) could be ideal seed cells for repairing nerve injury as they have the potential for multidirectional differentiation. However, it is still unclear whether the undifferentiated or the differentiated ADSCs have priorities in promoting axonal regeneration and myelin formation. In this study, the primary ADSCs from rats were cultured and differentiated. The morphology, differentiation potential, and secretion of neurotrophic factors of ADSCs were compared before and after induction. Undifferentiated ADSCs (uADSCs) were aggregated into bundles containing reticular, star, and polygonal structures. They contained a large number of lipid droplets and were positive for Oil red O staining. After differentiation, differentiation ADSCs (dADSCs) become long and spindle-shaped with decreasing protrusions around the cells, spiraling growth, and were negative for Oil red O staining. When comparing the groups the flow cytometer analysis showed: similar CD29 and CD45 surface markers in both groups; and CD44 and CD90 markers were very low in the undifferentiated groups. The levels of neurotrophin 3 (NT-3) and neuregulin 1 (NRG-1), and their receptors tropomyosin receptor kinase C (TrkC) and receptor protein-tyrosine kinase erbB-4 (ErbB-4) in dADSCs were higher than those in uADSCs. While the expressions of myelin protein zero (P0), myelin-associated glycoprotein (MAG), and purine receptor P2X7 (P2X7) were not significantly different before and after differentiation. It may be speculated that the dADSCs have enhanced abilities in nerve repairment which is associated with increased expression of neurotrophic factors.

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La diferenciación inducida de las células madre derivadas del tejido adiposo aumenta la secreción de factores neurotróficos.

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Palabras clave: ADSC diferenciadas, células de Schwann, factores neurotróficos, P2X7, daño nervioso.

Resumen. Las células madre derivadas del tejido adiposo (ADSCs) podrían ser una semilla ideal de células para la reparación de lesiones nerviosas, ya que tienen el potencial de diferenciación multidireccional. Sin embargo, aún no está claro si las ADSCs indiferenciadas o diferenciadas tienen prioridades en la promoción de la regeneración axonal y la formación de mielina. En este estudio, ADSCs primarias de las ratas fueron cultivadas y diferenciadas. Se compararon la morfología, el potencial de diferenciación y la secreción de los factores neurotróficos de las ADSCs antes y después de la inducción. Las ADSCs indiferenciadas (uADSCs) se encontraban agregadas en haces que contenían estructuras reticulares, estrelladas y poligonales. Contenían un gran número de gotitas de lípidos y fueron positivas para la tinción de Aceite Rojo O. Después de la diferenciación, las ADSCs (dADSCs) se vuelven largas y en forma de huso con un número decreciente de protuberancias alrededor de las células, crecimiento en espiral, y fueron negativas para la tinción de Aceite Rojo O. Cuando se compararon los dos grupos, análisis del citómetro de flojo muestra que los dos grupos de marcadores superficiales CD29 y CD45 eran similares; y los marcadores CD44 y CD90 eran muy bajos en el grupo indiferenciado. Los niveles de neurotrofina 3 (NT-3) y neuregulina 1 (NRG-1) y sus receptores, el receptor de tropomiosina quinasa C (TrkC) y el receptor de proteína tirosina quinasa erbB-4 (ErbB-4) en dADSC fueron más altos que los de uADSC. Mientras que las expresiones de proteína cero de mielina (P0), glicoproteína asociada a mielina (MAG) y receptor de purina P2X7 (P2X7) no fueron significativamente diferentes antes y después de la diferenciación. Se puede especular que las dADSC tienen capacidades mejoradas en la reparación nerviosa que se asocia con una mayor expresión de factores neurotróficos.

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INTRODUCTION

Regeneration and functional recovery after peripheral nerve damage are foci of research in neuroscience and a problem in clinical surgery [1]. Schwann cells (SCs) are the primary myelin-forming cells in the peripheral nervous system and play a prominent role in neuron survival and function. Schwann cells promote nerve regeneration by secreting neurotrophic factors and adhesion molecules [2]. However, the clinical use of Schwann cells is limited by the difficulty in obtaining adequate quantities. To overcome this, the ability of various types of stem cells to differentiate into Schwann cells is under investigation. Stem cells as seed cells combined with vector scaffolds can be used to

construct tissue-engineered nerves with biological activity and functionality; indeed, this research focuses on peripheral nerve repair. Adipose-derived stem cells (ADSCs) can be induced to differentiate into nerve cells [3], astrocytes, osteoblasts, and myofibroblasts in the appropriate type of medium [4-9]. Adipose tissue has the largest storage capacity in the body and is easily harvested and cultured. ADSCs are genetically stable, have low tumorigenicity, low immunogenicity, and show rapid expansion in vitro [10]. Therefore, ADSCs can be used to repair peripheral nerve damage.

ADSCs can be induced to differentiate into Schwann-like cells in vitro, which involves a changing from a flat to an elongated spindle shape and expressing s-100, GFAP, and P75. In coculture with spinal dorsal root ganglion (DRG) neurons, induced ADSCs promoted the axonal growth of DRG neurons and myelin sheath formation [11,12], indicating that the induced ADSCs had the phenotype and functionality of Schwann cells.

Repair by ADSCs of injured nerves has been confirmed in vivo. In mice with sciatic nerve injury, intravenous injection of ADSCs significantly increased the growth of sciatic nerve axons and ameliorated the inflammatory response [13]. ADSCs were transferred into artificial nerve conduits made of collagen [7], silica gel [8,9], PCL [14], and fibroin/collagen [15] and transplanted into sciatic nerve defects of rats. The regenerated axon of the sciatic nerve in the transplantation group was longer, and the walking gait, muscle weight, and nerve conduction velocity were significantly improved compared to that in the control group [7,15-17].

The mechanism by which ADSCs repair peripheral nerve injury is unclear. After differentiation, the mRNA and protein levels of the purine receptor P2X7 increased significantly in ADSCs, stimulating Ca2+ inflow and inhibiting the P2X7 receptor to prevent ATP-induced cell death [18].

The therapeutic effect and the underlying mechanisms of uADSCs and dADSCs on nerve injury are unclear. It is crucial to determine the phenotypic changes of ADSCs before and after differentiation is induced. Repair of peripheral injured nerves involves the secretion of neurotrophic factors, axon growth, and myelin sheath formation. Schwann cells secrete multiple growth factors that promote axonal regeneration, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) [19]. Stem cells repair damaged tissue by releasing several trophic factors in situ, which alters the local microenvironment [3]. To compare the efficacy of uADSCs and dADSCs in treating peripheral nerve injury, we investigated the effect of induction of differentiation on the differentiation potential, morphology, proliferation, and levels of Schwann cell-related proteins of ADSCs.

MATERIALS AND METHODS

Extraction, isolation, and culture of ADSCs

Male Sprague-Dawley (SD) specific-pathogen-free rats, of approximately 300 g of weight were euthanized by cervical dislocation and soaked in 75% ethanol for 10 min. Adipose tissue under the skin of the abdomen was dissected and placed in a Petri dish containing phosphate-buffered saline (PBS, pH=7.2) (Solarbio, Beijing, China). The cells were washed with PBS three times to remove blood and vessels. The adipose tissue was cut into one mm^3 pieces, digested with 0.075% type I collagenase (Invitrogen, Carlsbad, California, USA), and incubated at 37°C for 90min. After centrifugation, the upper undigested adipose tissue and the supernatant were discarded, and the pellet was washed in Dulbecco's modified Eagle's medium (DMEM)/F12 (containing 1% penicillin/streptomycin and 10% fetal bovine serum). The cells were centrifuged, filtered through a 200-mesh sieve, and transferred to a $75cm^2$ culture flask. After 24h, half of the medium was replaced, and the cells were passaged at a ratio of 1:2. Then

they were cultured to the third generation to achieve the purity of the isolated cells, and the cell morphology (CKX41, Olympus, Tokyo, Japan) was examined. When the cells were grown at their best, they were digested and centrifuged with 0.25% trypsin, and collected in DMSO: FBS: DMEM/F12=1:2:7, and mixed with cryopreserved solution, which was added into the cryopreservation tube after resuspension, and placed at 4°C for 1 hour, -20°C for 4 hours, and -80°C overnight. Finally, they were transferred to the liquid nitrogen tank for cryopreservation for later use. The experiment was conducted in three batches of cells.

Cell viability assay

Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. ADSCs were cultured in a 96-well plate, and 20μL (0.6mM) of MTT solution was added to each well. The cells were incubated at 37°C for 4h, 150μL of dimethyl sulfoxide was added to each well, and the plates were shaken for 10 min to ensure that crystals were dissolved. The optical density was measured at 570nm. The experiment was conducted in three batches of cells.

Induction of differentiation of ADSCs

We hypothesized that adipose-derived stem cells are more conducive to axonal regeneration and myelination after differentiation. In order to verify this, this study mainly investigated the differences between undifferentiated and differentiated ADSCs in cell morphology and in promoting neurotrophic factor secretion and myelin-related protein expression. ADSCs were digested with 0.25% trypsin/ EDTA (Invitrogen, USA) at the third passage, centrifuged, resuspended in DMEM/F12, and transferred to a six-well plate (2×10^5/mL). After 24h, 2mL of 1mM β-mercaptoethanol (Sigma-Aldrich, USA) was added, followed by fresh medium containing 35ng/mL all-trans-retinoic acid (Sigma-Aldrich, USA). The cells were washed in PBS after cultivation for 72h, and 2mL of ADSC differentiation medium (DMEM/F12 containing 5 ng/mL platelet-derived growth factor, 10 ng/mL basic fibroblast growth factor, 14μM forskolin, and 200ng/mL heregulin) was added. The experiment was conducted in three batches of cells, the cells were maintained for 1 week under the same conditions and fresh medium was added at 48–72h intervals. The morphological characteristics of uADSCs and dADSCs were observed under a microscope.

Oil red O staining

uADSCs and dADSCs were cultured in six-well plates (2×10^5/mL), the medium was discarded, and the cells were washed in PBS three times and fixed in 10% formaldehyde for 40min. Next, the cells were washed three times in PBS, stained with Oil Red O (Worldbio, China), and incubated at room temperature for 40min. The cells were rinsed with 75% alcohol to remove excess dye. The cells were sealed with glycerin gelatin, and cell morphology was observed under a microscope. Three batches of cell morphological maps were collected and analyzed.

Flow cytometry

uADSCs and dADSCs were digested with 0.25% trypsin/EDTA, centrifuged, and the supernatant was discarded. The cells were washed three times in 2% bovine serum albumin (BSA; abcbio, China). The pellet was resuspended in 5% BSA and subjected to cell counting. The cell density was adjusted to 1×10^7/mL, and the cells were incubated with 10μL of antibodies against CD29, CD44, CD90, and CD45 (Bio-Rad) for 30min on ice in the dark. The cells were washed in 5% BSA, centrifuged (1500 rpm) for 5min, and resuspended in PBS for flow cytometry. Three batches of cells were collected and analyzed.

Western blotting

Cells were rinsed in 0.01M PBS and lysed in radioimmunoprecipitation assay lysis buffer. The lysates were centrifuged at

4°C at 12,000 rpm for 5min. The supernatant was collected and stored at -20°C. The protein concentration was quantified using a BCA Protein Assay Kit (Beyotime Biotechnology, China). The proteins were resolved by sodium dodecyl sulfate-polyvinylidene fluoride gel electrophoresis and transferred onto polyvinylidene fluoride membranes. Primary antibodies against NRG-1 (1:1000, Affinity), NT-3 (1:500, Servicebio), P0 (1:500, Affinity), MAG(1:1000,Bioss), TrkC (1:500, GeneTex), ErbB-4 (1:500, GeneTex), and P2X7 (1:1000, ab109054, Abcam), were added and the membranes were incubated at 4°C for 24h. Next, the secondary antibody was added, followed by incubation for 2h.β-actin was used as the loading control for normalization. The grey values of the target protein were analyzed by Image J. The results were calculated by the ratio of accumulated gray values of target protein to the bands of β-actin, which represented the relative expression level of target protein. Bands of each target protein where appeared three times, were sorted out and analyzed.

Statistical analysis

Prism 5.0 was used for statistical analysis (GraphPad Systems, Inc., La Jolla, CA, USA), performed by t test. The data are means ± standard deviation. $P<0.05$ was considered indicative of statistical significance.

RESULTS

Morphological characteristics of ADSCs

ADSCs were isolated from subcutaneous abdominal fat of male SD rats and cultured in DMEM/F12. After 48h, the cells began to grow rapidly (Fig. 1A), adhered to the wall, and formed a short fusiform, star-shaped structure and irregular polygonal structure. After 5–7 days, the ADSCs were spindle-shaped and growing vigorously (Fig. 1B).

Growth of ADSCs

The ADSC growth curve was S-shaped. The ADSCs entered the logarithmic growth phase after 72h, and growth peaked at day 5 and decreased thereafter (Fig. 1C). In addition, ADSCs at passage 3 had the highest growth rate and those at passage 13 the low-

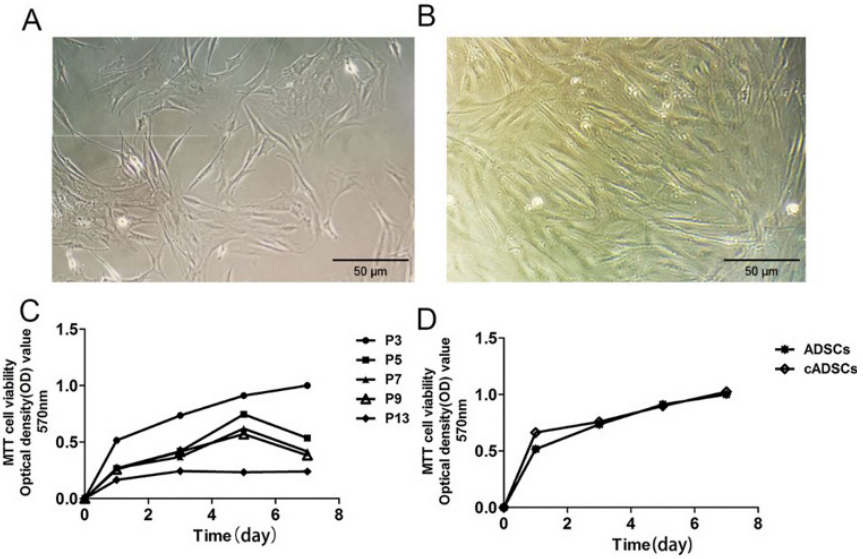

Fig. 1. Morphological characteristics and proliferation changes of ADSCs. A. After 48h, ADSCs were short and fusiform, with a star-shaped structure and irregular polygonal structure (×10). B. After 5–7 days, the primary ADSC cells exhibited a typical fibroblast-like morphology (×10). C. Growth curves of ADSCs at passages 3, 5, 7, 9 and 13. D. Growth curves before and after cryopreservation (cADSCs, ADSCs after cryopreservation).

est; therefore, the growth rate decreased with increasing passage number. At the initial stage of culture, the growth rate of cryogenically preserved ADSCs was similar to that of freshly prepared ADSCs (Fig. 1D).

Morphological characteristics of uADSCs induced to differentiate into dADSCs

The uADSCs aggregated into bundles containing reticular, star structures, and polygonal structures (Fig. 2A). After differentiation the cells were long and spindle-shaped, the number of protrusions around the cells decreased, spiraling growth, and showing a Schwann-like morphology (Fig. 2B).

Oil red O staining

The uADSCs contained a small number of lipid droplets (Fig. 3A). The dADSCs are Schwann cell-like cells and did not exhibit lipid droplets (Fig. 3B).

Expression of cell surface factors

Flow cytometry was performed to examine CD29, CD44, CD90 (stem-cell markers), and CD45 expression levels. In ADSCs, 96.0% expressed CD29, 60.1% expressed CD44, and 74.2% expressed CD90, indicating ADSCs have mesenchymal stem cell-related surface markers and have the potential of multi-differentiation of stem cells (Fig.4). As a marker of hematopoietic cells, the positive rate of CD45 was less than 50% (only 32.5%), suggesting they were uADSCs but could not differentiate into hematopoietic cells. After induction and differentiation into SCs, in dADSCs, 99.1% expressed CD29, 91.4% expressed CD44, 96.8% expressed CD90, and only 31.2% expressed CD45 (Fig.4). Compared with uADSCs, the expression levels of each marker in dADSCs were significantly increased, suggesting that the differentiation potential of ADSCs induce to differentiate into SCs was enhanced (Fig. 5).

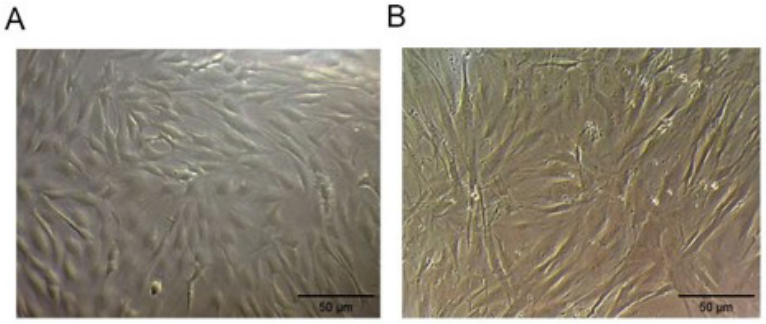

Fig. 2. Morphological changes of ADSCs after induced differentiation. A. Morphology of uADSCs. B. Morphology of dADSCs. After induction, the cells were long and spindle-shaped, the number of protrusions around the cells decreased, and spiraling growth (×10).

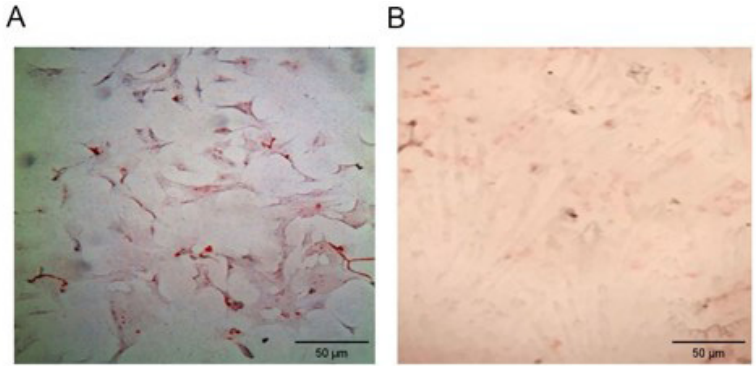

Fig. 3. Morphological changes after Oil red O staining. A.Oil red O staining of uADSCs. B.Oil red O staining of dADSCs (×10).

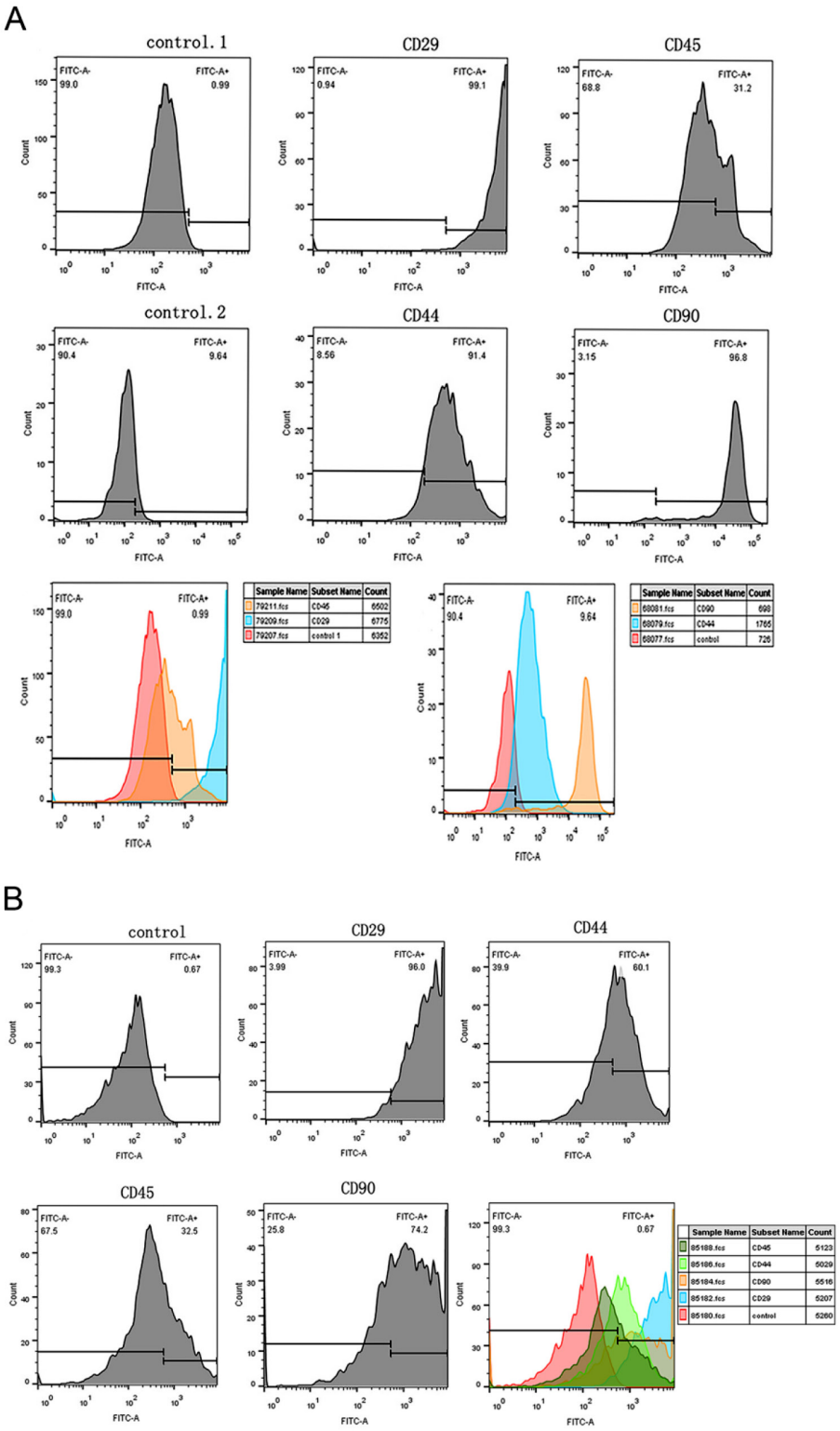

Fig. 4. CD29, CD44, CD45, and CD90 expression in uADSCs and dADSCs by flow cytometry. A. Expression of CD29, CD44, CD90, and CD45 in uADSCs. B. Expression of CD29, CD44, CD90, and CD45 in dADSCs. Blank control, cells treated with PBS but not anti-CD antibody.

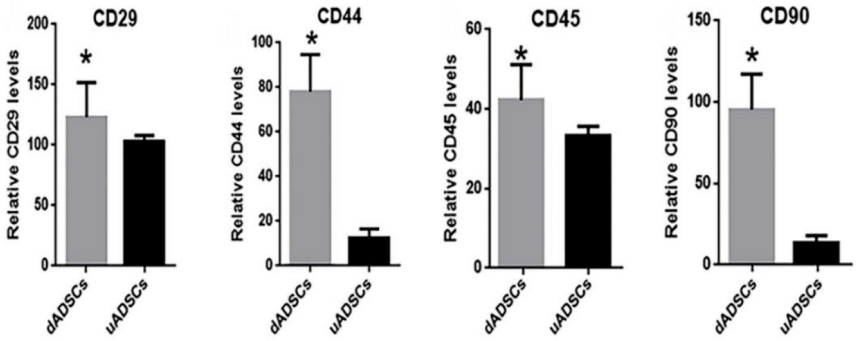

Fig. 5. Expression levels of CD29, CD44, CD45, and CD90 in uADSCs were lower than those in dADSCs (*p <0.05, n = 3).

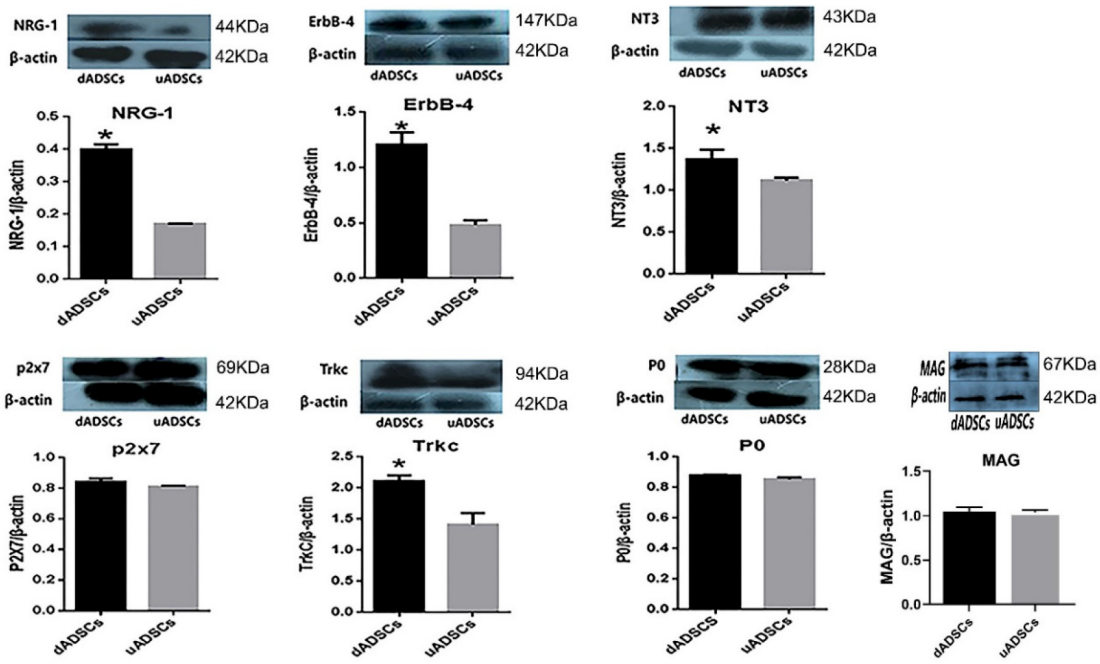

Fig. 6. Changes in NRG-1, NT3, TrkC, ErbB-4, P0, MAG and P2X7 protein levels. β-actin was used as the loading control (*p<0.05, n = 3).

Expression of uADSC- and dADSC-related proteins

Western blotting showed that NRG-1, ErbB-4, NT3, TrkC, P0, MAG and P2X7 were expressed on the surface of uADSCs and dADSCs (Fig. 6). The levels of NRG-1, ErbB4, NT-3, and TrkC in dADSCs were significantly higher than those in uADSCs ($p<0.05$). However, there were no significant differences in the levels of the myelin protein P0, MAG, and the purine receptor P2X7 before and after induction of differentiation.

DISCUSSION

Mesenchymal stem cells (also known as all-powerful mesenchymal stromal cells), as one of the stem cells, have attracted much attention in the field of stem cell therapy and regenerative medicine. ADSCs play an

important role in nerve injury and functional recovery and are considered ideal seed cells for nerve transplantation. However, it is currently unclear whether ADSCs differentiate into dADSCs after being stimulated by the in vivo injury environment or their direct effects on promoting nerve regeneration [20]. In this study, type I collagenase was used to isolate the subcutaneous adipose tissue of rat abdomen to evaluate the differentiation potential, morphology, and protein levels before and after differentiation. When the cells passed to the third generation, the cell growth activity was the best, so the third generation of ADSCs was selected as the research object. Cryopreservation is used for long-term storage of biological materials, such as oocytes, stem cells, vascular tissues, and embryos [21-23]. In this study, revived ADSCs undergoing cryopreservation did not show significant loss of viability or proliferation by MTT assay.

Some scholars compared the roles of dADSCs and uADSCs in nerve transplantation and believed that undifferentiated adipose stem cells (ADSCs) were easy to obtain and had the advantage of a short culture cycle in promoting neurotrophic factors secretion and repairing myelin sheath injury [24,25]. At the same time, studies have pointed out that differentiated adipose stem cells are more conducive to playing the role of stem cells in nerve injury repair [26]. In this study, adipose stem cells were differentiated and cultured to explore the advantages and disadvantages of undifferentiated and differentiated ADSCs in promoting axonal regeneration and myelination. The morphologies of uADSCs and dADSCs were significantly different. uADSCs cells presented star and polygonal structures. After 1-2 w of induction and differentiation by adding specific Schwann-inducing fluid, dADSCs cells presented a spindle shaped Schwann-like cell morphology, and the number of protuberances around the cells decreased. ADSCs expressed mesenchymal stem cell (MSC) markers and have similar properties to MSCs [6].

MSCs derived from some mammals can be transformed into Schwann cells in the presence of inducers or mixtures of growth factors. ADSCs express several stem cell surface molecules such as CD105, CD29, CD44, and CD45 [7]. In the recently published studies, just like our method, only positive markers were detected in the differentiation and identification of primary ADSCs cells, less involving negative markers such as CD34 [27, 28]. High expression of CD29, CD44, and CD90 and low expression of CD45 were found in uADSCs and dADSCs. Jiang *et al.* [29] reported that ADSCs isolated from SD mice show high CD29, CD44, and CD90 expression levels, but low or absent expression of CD45. This agrees with our finding that both uADSCs and dADSCs express MSC-associated surface markers and can undergo differentiation into multiple cell lineages. The uADSCs, but not the dADSCs, were positive for Oil red O staining, indicating that the former had a larger number of lipid droplets than the latter.

Repairing and regenerating damaged nerves involves a complicated pathophysiological process, mainly dependent on regulating various cytokines. When injured, the body can rely on its own nerve regeneration or granulation tissue hyperplasia and scar formation to achieve healing. ADSCs participate in various stages of tissue repair by virtue of their multiple physiological functions [20]. Multidirectional studies have confirmed that ADSCs can secrete various cytokines, which play a vital role in diverse physiological activities of ADSCs.

Schwann cells (SCs) are the most promising seed cells for peripheral nerve tissue engineering [30], which can promote peripheral axon regeneration after peripheral nerve injury (PNI). Recent research has found that salidroside may improve the regeneration effect on the sciatic nerve following a combined application of epimysium conduit and RSC96 Schwann cells in rats [31]. Epothilone B (EpoB) is an FDA-approved antineoplastic agent, which shows the capacity to induce

alpha-tubulin polymerization and improve microtubules' stability. The latest research found the potential therapeutic value of EpoB in enhancing regeneration and functional recovery in cases of PNI [32]. In addition, Schwann cells also play a significant role in promoting the regeneration of PNI. According to the latest research, SCs are integral in the regeneration and restoration of function following PNI. SCs are able to dedifferentiate and proliferate, remove myelin and axonal debris, and are supportive of axonal regeneration [33]. Moreover, 5% gastrodin/PU NGC efficiently promotes nerve regeneration, indicating their potential for use in peripheral nerve regeneration applications [34].

Schwann cells (SCs) secrete neurotrophin 3 (NT-3). NT-3 can promote the development and differentiation of neurons, and its binding with tropomyosin receptor kinase C (TrkC) receptor can maintain the survival of neurons [35], inhibit cell apoptosis, and promote the differentiation of SCs into neurons [36,37]. Several studies have shown that in vitro transfection of adenovirus carrying NT-3 (AdvNT-3) gene can promote the differentiation of MSCs into neuron-like cells. The role of NT-3 is mediated by its preferred binding receptor TrkC. In this study, the expression levels of NT-3 and TrkC were significantly increased after the induction of differentiation of ADSCs, consistent with the above. Western blot results showed that dADSCs could promote the expression of NT-3 and its receptor TrkC, thus maintaining the regeneration of injured nerves and reducing nerve apoptosis.

The neuregulin-1 (NRG-1)/receptor/tyrosine protein kinase ErbB (ErbB) system is an endothelium-controlled paracrine system. It has been found that NRG-1 can promote the recovery of nerve function after brachial plexus injury after contralateral C7 nerve root metastasis in rats, and NRG-1 has combined anti-inflammatory and anti-fibrosis effects in different organs, including skin, lung, and heart. There is increasing evidence that the NRG-1/ErbB system is active in various organs throughout the body. NRG-1 not only promotes neuronal activity but also acts on the receptor ErbB-4 in nerve endings. At nerve endings, NRG-1 enters the cell body through axoplasmic countercurrent and promotes the growth of neurons. In this study, NRG-1 and erbB-4 expression levels were significantly increased after induction of ADSC differentiation, consistent with a previous report [38]. Thus, the ability of dADSCs to secrete neurotrophic factors and promote the growth of axons likely explains their ability to repair peripheral nerve injury.

Repair of injured nerves is accompanied by myelin sheath formation, which involves the coordinated synthesis of a group of proteins related to myelin, including the transmembrane glycoprotein P0 and myelin-associated glycoprotein MAG. MAG is a major component of myelin-derived nerve growth inhibitor. MAG shows different functions at different stages of the nervous system development, promoting axon growth during development and inhibiting axon growth during maturation. In this study, the level of the myelin-sheath protein P0 and MAG in uADSCs and dADSCs was not significantly different before and after induction of differentiation. Synthesis by Schwann cells of P0 is dependent on contact with axons [39]. The synthesis of P0 in Schwann cells is regulated by neural developmental growth [40]. Therefore, we can speculate that our results may be related to this cause.

P2X7 is a non-selective cationic channel receptor expressed in neurons and smooth muscle; the ligand of this receptor is ATP [41]. P2X7 acts as a bridge between the nervous and immune systems when nerve damage occurs. After differentiation, the mRNA and protein levels of the purine receptor P2X7 increased significantly in ADSCs, and inhibiting the P2X7 receptor could prevent ATP-induced cell death [18]. However, in this study, the expression of P2X7 in uADSCs and dADSCs was not significantly different. This may suggest that P2X7 plays a fundamental role in maintaining the proliferation and dif-

ferentiation of uADSCs and dADSCs under physiological conditions.

In conclusion, there are no differences in myelin production in the two groups studied, despite the increased neurotrophic factors and their receptors in the dADSCs group being more potent inducers of axonal growth potential than uADSCs. dADSCs and SCs are similar in morphology and function in vitro and in vivo and are readily implanted and proliferate rapidly [42]. Nevertheless, the undifferentiated state of ADSCs enables multiple-lineage differentiation and the establishment of a favorable environment for nerve regeneration. uADSCs are easier to obtain, have shorter incubation periods, and are less costly than dADSCs, suggesting their potential for nerve regeneration. Further studies are needed to assess the potential of uADSCs and dADSCs.

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Conflict of interests

All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

XZ: conceptualization, methodology, software, investigation, formal analysis, writing-original draft; YNL: conceptualization, methodology, formal analysis, writing-original draft; investigation; ZL: resources, writing-original draft; YH: conceptualization, visualization; FL: resources, supervision; SYZ: software, validation; JG: resources, data curation, visualization; LL: conceptualization, funding acquisition, supervision, writing - review & editing.

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