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Original Article

The Adrenal Pheochromocytoma Cell Line PC12 Efficiently Promotes the Regeneration Capability of Adipose Tissue-Derived Mesenchymal Stem Cells in Myogenesis: A Particular Approach to Improving Skeletal Muscle Cell Regeneration

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Abstract

Background: Researchers are looking for a way to improve the myogenic differentiation of stem cells. Adipose-derived stem cells (ADSCs), known for their multipotency and regenerative capabilities, have been extensively studied for their therapeutic potential. Meanwhile, PC12 cells, derived from rat pheochromocytoma, have been found pivotal in neuroscience research, particularly as a neuronal model system. The current study investigated the effect of the PC12 adrenal pheochromocytoma cell line on the myogenic differentiation of ADSCs.

Methods: This experimental study was conducted during 2019-2022 (Ahvaz, Iran). Differentiation of ADSCs was induced by using 3 µg/mL 5-azacytidine for 24 hours. Then, the culture media was changed with Dulbecco's Modified Eagle-High Glucose (DMEM-HG) containing 5% horse serum (HS) and kept for 7 days. Different percentages of differentiated ADSCs and PC12 (100:0, 70:30, 50:50, 30:70) were cocultured for 7 days in DMEM-HG containing 5% HS. PC12 was labeled with cell tracker C7000. The real-time polymerase chain reaction and Western blotting techniques were utilized to assess gene and protein expression. All experiments were repeated three times. Data were analyzed using GraphPad Prism 8.0.2 software with a one-way analysis of variance. $P<0.05$ was considered statistically significant.

Results: PC12 visualization confirmed the accuracy of the co-culture process. The differentiated cells showed an aligned, multinucleated shape. The differentiated ADSCs revealed significantly elevated levels of *Myh1*, *Myh2*, and *Chrn-α1* gene expression compared with undifferentiated ADSCs ($P<0.0001$). The ADSCs cocultured with PC12 cells showed significantly higher *Myh1*, *Myh2*, and *Chrn-α1* gene expression than differentiated ADSCs ($P<0.001$). ADSCs cocultured with 50% PC12 revealed significantly higher MYH and nAchR protein expression than the differentiated group ($P<0.01$ and $P<0.001$).

Conclusion: Coculturing PC12 cells and ADSCs improves the efficiency of myogenic differentiation. However, the effectiveness of myogenic differentiation depends on the proportions of administered PC12 cells.

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Keywords ● Mesenchymal stem cells ● PC12 Cells ● Musculoskeletal development ● Coculture techniques

What's Known

- Adipose-derived stem cells (ADSCs) can differentiate into myoblasts. The development of stem cells into muscle cells depends on neurotransmitter release.

What's New

- The impact of PC12 on ADSCs' ability to differentiate into muscle has not been studied yet. PC12 cells contribute to the release of neurogenic factors and may promote the myogenesis of ADSCs.

Introduction

The central nervous system governs skeletal muscle contractile activity by sending action potentials from motor neurons to muscle fibers.[1] The neuromuscular junction (NMJ), also known as the motor endplate, is a highly specialized chemical synapse where transmission occurs. When the NMJ does not function properly, it causes muscle weakness or paralysis. The NMJ is affected by disorders such as myasthenia gravis, Lambert-Eaton myasthenic syndrome, botulism, Charcot-Marie tooth, amyotrophic lateral sclerosis, spinal muscular atrophy, and muscular dystrophies. Even though these diseases are uncommon, severe forms of all of these diseases are potentially fatal.[1, 2] There are currently no established methods to reverse muscle loss. As a result, muscle regeneration remains a significant therapeutic requirement. The high regeneration potential of skeletal muscle makes it a suitable target for regenerative therapy. However, muscle tissue's ability is insufficient for muscle regeneration in extensive muscle damage. This condition causes progressive loss of skeletal muscle fibers. Some research has proposed cell therapy for considerable muscle regeneration. Myogenic cell-based therapy is based on using stem cells with a high capacity for myogenic regeneration.[3, 4] Mesenchymal stem cells (MSCs) can be passaged several times without losing their ability to differentiate into particular tissues. The paracrine effects of MSCs are intimately related to their inductive activities. MSCs are considered to have paracrine effects through secreting VEGF, HGF, and FGF2. The secreted paracrine factors help in angiogenesis, cell proliferation, and cell viability. Adipose-derived mesenchymal stem cells (ADSCs) are one of the promising candidates in regenerative medicine. ADSC can be acquired readily using non-invasive techniques, and they have great proliferation and cryopreservation capacity. ADSCs can successfully be differentiated into adipogenic, osteogenic, and chondrogenic lineage cells.[5] Liu and others claimed that the differentiation process of ADSCs toward myoblasts can be achieved using various induction media including dexamethasone, hydrocortisone, 5-azacytidine (5-Aza), and horse serum (HS).[4] One restriction in this approach is the small percentage of ADSCs that differentiate into reliable functional muscle cells. According to certain research, coculturing ADSC with other cells can increase their ability to differentiate into myoblasts.[3, 6] A study has shown that ADSC coculture enhances the cells' capacity to develop into myogenic cells capable of producing transverse myotubes.[6] Further investigation revealed that the coculture of ADSCs with differentiated human Duchenne skeletal muscle cells led to the production of myotubes and dystrophin. The creation of contractile muscle tissue is the primary goal of skeletal muscle tissue engineering.[3]

Some researchers have suggested stem cells and neural cell coculturing to improve the myogenic differentiation procedure. Schwann cells have been established to stimulate Mb/ADSC's myogenic development.[7] Arifuzzaman and others have shown that the presence of neuronal progenitor cells, such as embryonic motor neurons or slices of newborn rat neurons, results in the establishment of neuromuscular connections, myotube maturation, and neurotization.[8] The adrenal pheochromocytoma cell line (PC12) is isolated from rat adrenal medulla pheochromocytoma.[9] The PC12 cells exhibit a well-known model for the study of neuronal signaling pathways and neuronal function. It has been confirmed that PC12 improves C2C12 myogenic differentiation.[9] As a result, it appears that the presence of motor neurons improves myotube maturation by enhancing cytoskeletal structure.[8] PC12 cells contribute to skeletal muscle regeneration by secreting specific proteins that affect myoblast behavior, nerve function, and angiogenesis.[1] The study of PC12 cell functioning provides insight into the relationships between the nervous system and the muscles. One of these interactions is the NMJ.[9] The NMJ is a synaptic chemical connection that exists between the terminal end of a motor nerve and a muscle. Because of its ease of access and simplicity, the NMJ has attracted a lot of interest as a synaptogenesis model.[1] The NMJ in the skeletal muscle membrane is distinguished by the presence of a high concentration of acetylcholine receptors. Nicotinic acetylcholine receptors (nAChRs) are a kind of ligand-gated pentameric ion channels that are essential for acetylcholine neurotransmission at the NMJ.[2, 10] The agrin signaling pathway is required to develop and maintain NMJs.[11] After being released, it binds again to LRP4 on the postsynaptic muscular membrane and activates MuSK factors. This signaling pathway regulates post-synaptic differentiation at the NMJ. The agrin-LRP4-MuSK signal is transmitted downstream, causing AChR clustering by the scaffold protein rapsyn.[2] nAChRs were shown to serve two crucial functions in the formation of skeletal muscle fibers and their innervation. The first one, chronologically, involves encouraging myoblast fusion into myotubes. The second

occurs during synaptogenesis when nAChRs have been postulated to identify membrane locations for nerve-muscle interactions.[11] According to prior research, freshly isolated satellite cells do not express nAChRs. However, proliferating myoblasts have high nAChR levels. Following the creation of nerve-muscle contact, Ca^{2+} influx contributes to the orchestration of the signaling required for the correct development of the NMJ.[10] Several investigations have suggested that nAChR has a role in myoblast fusion.[10, 12] Human myoblast fusion is likewise affected by nAChR activity.[12] The mechanism of nAChR-mediated myoblast fusion is unknown, but it could be connected to the calcium influx induced by this receptor.[10]

In general, it can be concluded that the presence of nerve impulses through neurotransmitter release promotes the development of stem cells into muscle cells. PC12 cells are not thought of as motor neurons, but they contribute to the release of neurogenic factors and may promote myogenesis. The impact of PC12 on ADSCs' ability to differentiate into muscle has not been studied yet. In this regard, the impact of PC12 cells on the myogenic differentiation capability of ADSCs was the aim of this study.

Materials and Methods

Animals

The experimental study was conducted during 2019-2022 at the Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. All the animals were kept according to the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences (National Institutes of Health Publication No. 86-23).[13] This study was approved by the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.ABHC.REC.1399.007) for Experimental Animals. Adult Sprague–Dawley rats (male, eight weeks old) were obtained from Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Isolation and Culture of ADSCs

Adult Sprague–Dawley rats were deeply anesthetized using 100 mg/mL ketamine (Ketavet®, Pfizer, Germany)/20 mg/mL xylazine (Rompun® 2%, Bayer, Germany). The adipose tissue was extracted from the testicular fat pads under sterile conditions. The ADSCs were isolated using the enzymatic digestion method.[14] Adipose tissue samples were washed three times in cold phosphate-buffered saline (PBS) (Invitrogen, USA), containing 3% penicillin/streptomycin (Pen/Strep) (Gibco, USA). Then, the tissues were mechanically cut into small pieces. The digestion protocol included a 30-min incubation at 37 °C in 0.2% collagenase type I (Gibco, USA). The suspensions were immediately centrifuged to separate the floating adipocytes from the stromal vascular fraction. The supernatant was removed and the stromal vascular fraction cells were resuspended in DMEM-HG medium (Sigma-Aldrich, USA) with 15% fetal bovine serum (FBS) (Gibco, Heat-inactivated, USA) and 1% pen/strep (Sigma, USA) and kept at 37 °C and 5% CO_2. Four days later, the culture medium was refreshed to eliminate non-adherent cells. The cells in passage three were used for further experiments.

Multilineage Differentiation Assays

Adipogenic Differentiation: Differentiation assays were performed to determine the multipotentiality of the ADSCs. After culturing cells in an adipogenic medium containing 1 µM dexamethasone (Sigma-Aldrich, USA), 500 µM isobutyl methyl xanthine (Sigma-Aldrich, USA), 60 µM indomethacin (Sigma-Aldrich, USA), and 5 µg/mL insulin (Sigma-Aldrich, USA), they were kept at 37 °C and 5% CO_2 for three weeks. Adipogenic differentiation of cells was determined using oil red O staining (Sigma-Aldrich, USA). The differentiated cells were visualized using an inverted microscope.[15]

Osteogenic Differentiation: The cells were cultured in an osteogenic differentiation medium containing 50 µM ascorbate-2 phosphate (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma Aldrich), and 0.1 µM dexamethasone and kept 37 °C and 5% CO_2 for three weeks. The mineralized colonies resulting from the osteogenic differentiation of cells were visualized with alizarin red S staining using an inverted microscope.[15]

PC12 Cell Line Culture: PC12 cells (C153) were obtained from the cell bank of the Pasteur Institute (Tehran, Iran). The cells were grown in 25 cm^2 tissue culture flasks in RPMI-1640 medium (Sigma-Aldrich, USA) containing 10% FBS, 1% pen/strep, 1% L-glutamine (Gibco, USA), and 1% non-essential amino acids (Sigma-Aldrich, USA). Cultures were maintained according to standard protocols at 37 °C in a 95% humidified incubator with 5% CO_2. The culture media was renewed every 48 hours.[9]

Cell Culture Plate Coating: The coating method was used to attach suspended PC12 cells before the coculture process. A fresh collagen solution was prepared by solving the

collagen Powder type 1 (Pasteur Institute, Iran) in ice-cold 0.1 M acetic acid (Merck, Germany) to a final concentration of 2 mg/mL. After that, the solution was diluted 10 times in ice-cold 50 mM HEPES (Merck, Germany) (pH=8.5) to yield a final concentration of 0.2 mg/mL. Then, 3 mL of collagen solution was added to each T25 flask and incubated overnight at 37 °C with shaking in the dark. After 24 hours of incubation, the collagen solution was removed. The flasks were dried at room temperature in sterile conditions. Subsequently, the flasks received UV illumination for 90 min. The flasks were then rinsed with 1x PBS for 5 min.[16]

Myogenic Differentiation: The ADSCs were cultured with DMED-HG containing 10% FBS and 3 µg/mL 5-azacytidine (Sigma, USA) for 24 hours. Then, the culture media was changed with DMEM-HG containing 5% horse serum (HS) (Gibco, USA) and kept for 14 days. Additionally, 4′,6-diamidino-2-phenylindole (DAPI) staining was performed to confirm that cultured ADSCs exhibit an aligned, multinucleated morphology expected during myogenic differentiation. Differentiated ADSCs were immediately examined under a confocal microscope, and the images were acquired for further image processing using ImageJ software, version 1.49 (National Institutes of Health, USA).[17]

Direct Coculture: On day 7 of differentiation, differentiated ADSCs and PC12 cell lines were correctly combined at varied densities and cultivated in collagen-coated (0.2 mg/mL) T25 flasks for an additional 7 days. To detect the presence of both types of cells until the end of the differentiation day, PC12 cell lines were stained with Cell Tracker CM-DiI tracking dye that binds to plasma membranes (Cell Tracker, C7000, Thermo Fisher Scientific, USA) as manufacturer recommendation.[17]

Experimental Groups: The experimental groups were divided into five groups labeled G1-G5. The control group (G1) was comprised of undifferentiated ADSCs, and the G2 group received 5-azacytidine and 5% HS for myogenic differentiation. Group G3 was made up of 70% ADSCs and 30% PC12. Group G4 contained 50% ADSCs and 50% PC12, while Group G5 included 30% ADSCs and 70% PC12.

Staining of Differentiated Cells: The differentiated cells were fixed using paraformaldehyde (Sigma-Aldrich, USA). The cells were washed thrice with PBS. A sufficient amount of 300 nM DAPI stain solution (Sigma, USA) was added, and the cells were incubated at room temperature in the dark for 5 min. The stain was removed, and the cells were washed thrice with PBS and visualized under an inverted fluorescence microscope.[17]

Real-Time Reverse Transcriptase-Polymerase Chain Reaction

RNA Extraction: Ribonucleic Acid (RNA) extraction was performed using the RNA extraction kit (Pars Tos, Iran) according to the manufacturer's protocol. The quality and concentration of isolated RNA were evaluated using a NanoDrop instrument. (Thermo, Canada). OD260/OD280 ratios between 1.8 and 2 indicated acceptable quality of the RNA.[17]

cDNA Synthesis: For complementary deoxyribonucleic acid (cDNA) synthesis, a concentration of 1 µg of RNA was used according to the instructions of the Easy cDNA Synthesis Kit (Pars Tous Company, Iran). The cDNA synthesis reaction was performed in a volume of 20 µL in a thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Germany).[17]

Real-Time PCR: The primers were designed using the Primer 3 online software. The sequence accuracy of the primers was confirmed using the bioinformatic online software. The characteristics of the primers are given in table 1. The real-time PCR reaction was performed in a volume of 12.5 µL. The chemical composition included 6.25 µL of Syber Green qPCR Master Mix 2× (Pars Tos Company, Iran), 0.25 µL of each of the primers, 2.75 µL of water, and 3 µL (300 ng) of cDNA. The reaction program consisted of initial denaturation: temperature of 94 °C for 5 min and 45 cycles as follows: 94 °C for 15 sec, 60 °C for 15 sec, and 72 °C for 30 sec. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as a calibrator. Two negative control samples were considered, one without cDNA and the other without RNA. The expression levels of various genes were compared based on the $2^{-\Delta\Delta Ct}$ method.[17]

Western Blot Analysis: Following treatment on the 14th day of differentiation, the cells were harvested and washed with cold PBS and then lysed using the lysis buffer. Following centrifugation, the supernatant was collected, and the protein concentration was determined using the Bradford protein assay kit (Thermo Scientific, USA) according to the manufacturer's instructions. Equal amounts of proteins were denatured by boiling at 100 °C for 5 min in 2× Laemmli sample buffer (Bio-Rad Laboratories, USA). The protein samples were separated on 8% or 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked by 5% non-fat dry milk (BD Biosciences, USA) for one hour at room temperature.

Table 1: Primer sequences of the examined genes

Gene	Primer sequencing (5´-3´)
Myh1	F: 5´-TGCATCCCTAAAGGCAGACT-3´ R: 5´-GCTTGTTCTGAGCCTCGATT-3´
Myh2	F: 5´-GGCTGGCTGGACAAGAACA-3´ R: 5´-CCACCACTACTTGCCTCTGC-3´
Chrn-α1	F: 5′-GAATCCAGATGACTATGGAG-3′, R: 5′-GACAATGATCTCACAGTAGC-3′;
GAPDHF	F: 5´-TGCTGGTGCTGAGTATGTCGTG-3´ R: 5´-CGGAGATGATGACCCTTTTGG-3´

The membranes were then incubated with various primary antibodies, including mouse monoclonal anti-MYH (B-5) (1:100) (Santa Cruz Biotechnology Inc., USA), rabbit polyclonal to nicotinic acetylcholine receptor gamma (1:500) (Abcam, USA) at 4 °C overnight. After being thoroughly washed with Tris-buffered saline with 0.1% Tween® 20 detergent (TBST) buffer (Cell Signaling Technology, USA), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, USA) for 1 hour at room temperature. Antigen-antibody complexes were detected by the Enhanced chemiluminescence (ECL) detection system (GE Healthcare, USA). Quantitative analysis of markers was performed using the ImageJ software. Protein expression levels were normalized to that of the GAPDH.[18]

Statistical Analysis

Statistical analysis was performed by GraphPad Prism 8.0.2 software (San Diego, CA, USA). The results were expressed as the means±SEM of three separate experiments. Data were analyzed by one-way ANOVA and Student *t* test followed by Tukey's test for each pair-wise comparison. Values of $P<0.05$ were considered to indicate statistically significant differences.

Results

Characterization of the Isolated ADSCs

Morphology of the Cultured ADSCs: Primary isolated ADSCs were a heterogeneous population of small round cells with a strong tendency to adhere to the culture dish surface. The initial round ADSC form gradually developed into a spindle-like shape. The cells were extremely proliferative (figure 1A). In passage three, ADSCs displayed a flat polygonal fibroblast-like appearance. During expansion, the cells became long spindle-shaped and colonized the whole culturing surface (figure 1B). The morphology of isolated ADSCs resembled mesenchymal cells' typical form.

Cell Differentiation Potential Assessment: After the third passage, cells were grown in the adipogenic or osteogenic media to verify the multipotent capacity of ADSCs. The outcomes supported ADSC's differentiation into osteocytes and adipocytes. Alizarin red staining showed the development of osteocytes and calcium deposits (figure 1C). Oil Red O staining revealed the lipid vacuoles, which helped to identify the establishment of adipocytes (figure 1D). The results confirmed the differentiation ability of isolated ADSCs.

Coculture Identification

Morphological Changes in ADSCs During the Coculture Process: On the 14th day of differentiation, the cells grew longer. ADSCs converged and appeared as multinucleated parallel cells (figures 1E, 1F). The modifications to ADSC morphology suggest that they have differentiated into functional myoblasts.

Morphological Changes in PC12 Cells During the Coculture Process: PC12 cells appeared rounded and suspended, with a strong tendency to proliferate and form colonies (figure 2A). After being adhered to the bottom of the flask, PC12 cells appeared as single, amorphous polyhedral cells (figure 2B). On the 14th day of differentiation, the neurites that enabled PC12 cells to communicate with differentiated ADSCs or their counterpart cells were noticed (figure 2C). This finding shows that ADSCs stimulated neurite outgrowth in PC12 cells. As a result, the coculture procedure may produce an NMJ between PC12 cells and ADSCs, enhancing PC12 cells' myogenic effects on ADSCs.

Cell Tracking: In culture plates, PC12 cells were marked with C7000 fluorescent dye and mixed with ADSCs. Red fluorescent dyes were used to visualize the PC12 cells. The microscopic image revealed the correctness of the coculture of ADSCs and PC12 (figures 2D, 2E).

Changes in the Expression of Genes Associated with Myogenesis after Myogenesis Differentiation: According to the results of real-time PCR findings, differentiated ADSCs exhibited significantly higher amounts of nAChR subunit α1 (*Chrn-α1)*, myosin heavy chain 1 (*Myh1)*, and myosin heavy chain 2 (*Myh2)* mRNA than undifferentiated ADSCs ($P<0.0001$) (figure 3).

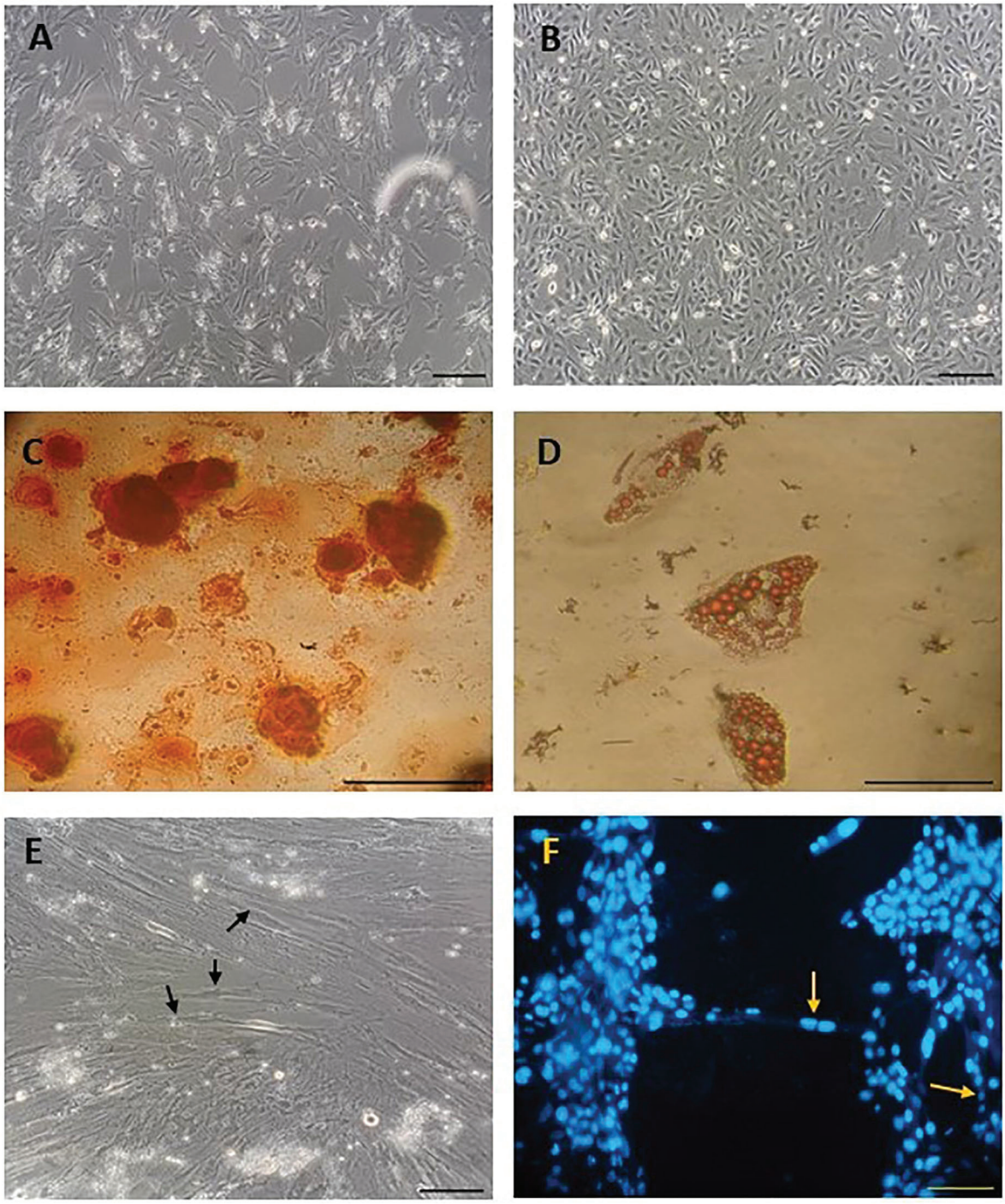

Figure 1: The morphological characteristics of isolated ADSCs are illustrated. A: The primary ADSCs with round shape. B: ADSCs show spindle-like morphology at passage 3. C: Osteogenic and adipogenic differentiation. Osteogenic differentiation was confirmed by Alizarin Red staining. D: Adipogenic differentiation. Adipogenic differentiation was confirmed by Oil Red O staining. E: Myogenic Differentiation. The differentiated ADSCs On the 14th day of differentiation show aligned morphology (black arrows). F: DAPI staining. The differentiated ADSCS On the 14th day of differentiation show a multinucleated appearance (yellow arrows). The yellow and black arrow represents an aligned, multinucleated morphology of ADSCs that was expected during myogenic differentiation. The morphology of the cells was evaluated using the phase contrast microscope with a 200 µm Scale.

The mean amount of mRNA expression showed 21921±635.7, 46.31±2.09 and 21921±635.7 increases for *Myh1, Myh2* and *Chrn-α1* compared with undifferentiated group.

Changes in the Expression of Genes Associated with Myogenesis Following Coculture with PC12 Cells

Changes in Myh1 Gene Expression: The findings demonstrate that coculturing ADSC cells with PC12 cells substantially improved the expression of the *Myh1* gene.

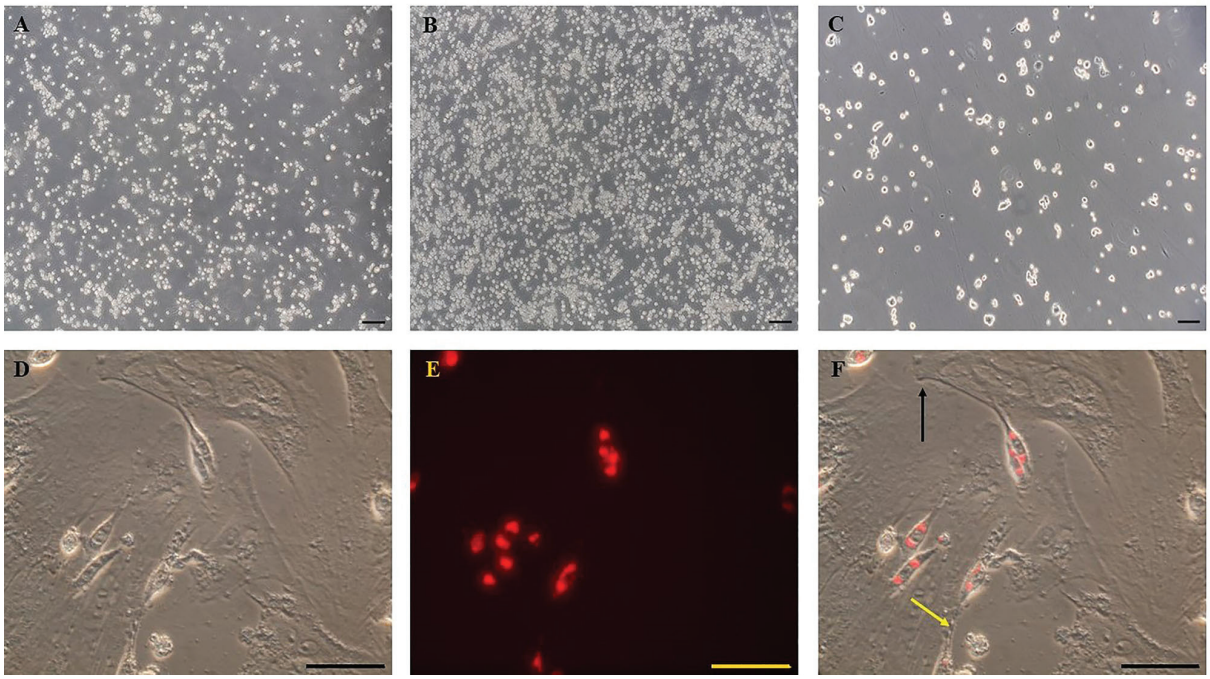

Figure 2: The morphology of the PC12 cell line was examined when cocultured with differentiated ADSCs. A: PC12 cell line with low density. B: PC12 cell line with high density. C: Transformation of suspended cells into adherent cells. PC12 cell line was adhered to a plastic surface using 0.2 mg/mL collagen. D-F: visualization of adhered PC12 cells. The adhered PC12 cells were visualized using a C7000 cell tracker. The PC12 cells are shown with red colour. The junction between ADSCs and PC12 cells is shown using a black arrow. The connection between two PC12 cells is shown using the yellow arrow. The morphology of the cells was evaluated using the phase contrast microscope with a 200 µm Scale.

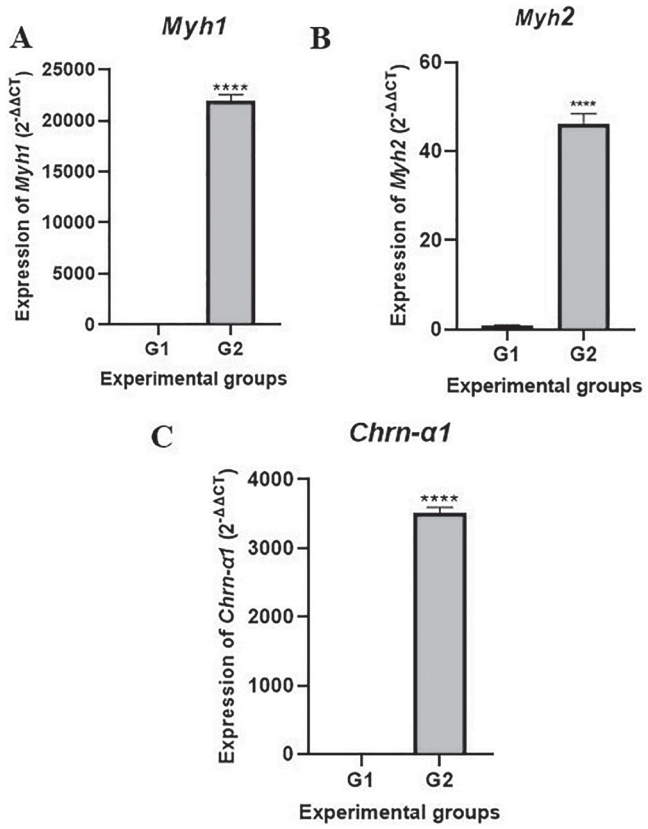

Figure 3: The effects of the myogenic differentiation process on *Chrn-α1*, *Myh1*, and *Myh2* mRNA expression are illustrated with Real-time RT-PCR. A, B, C: The expression of *Myh1*, *Myh2* and *Chrn-α1* mRNAs in differentiated ADSCs (G2) showed significant elevation compared to undifferentiated ADSCs (G1) ($P<0.0001$). Data were normalized to *GAPDH*. Data are presented as mean±SEM of three separate experiments. ****$P<0.0001$

A maximal *Myh1* gene expression was seen in Group G5, which was composed of 70% PC12 cells and 30% ADSCs (figure 4A). The mean value of *Myh1* mRNA expression showed 1.96±0.33, 2.84±0.19, 4.22±0.25 increase in G3, G4, and G5 groups compared with G2, respectively.

Changes in Myh2 Gene Expression: Real-time PCR results showed that introducing the PC12 cell line to ADSCs significantly enhanced *Myh2* expression. When compared to the differentiated ADSCs group, the group that was cocultured with 70% of the PC12 cell line displayed the highest amount of *Myh2* expression ($P<0.0001$). Additionally, the groups treated with 30% or 50% of the PC12 cell line showed a significant increase in *Myh2* expression compared with the differentiated ADSCs group ($P<0.0001$) (figure 4B). The mean value of *Myh2* mRNA expression showed 2.835±0.15, 3.04±0.12 and 3.89±0.37 increase in G3, G4, and G5 groups compared with G2, respectively.

Changes in Chrn-α1 Expression: The expression of the *Chrn-α1* gene was much higher in the coculture groups than in the differentiated ADSCs group. The application of more PC12 cells certainly resulted in higher gene expression of *Chrn-α1* when compared to group G2. The greatest *Chrn-α1* expression was found in Group G5 (figure 4C). The mean value of *Chrn-α1* mRNA expression showed 1.57±0.11, 3.11±0.34, 3.23±0.11 increases in G3, G4, and G5 groups compared with G2, respectively. Overall, the considerable increase in *Myh1*, *Myh2*, and *Chrn-α1* gene expression demonstrated that PC12 cells induce myogenesis in ADSCs.

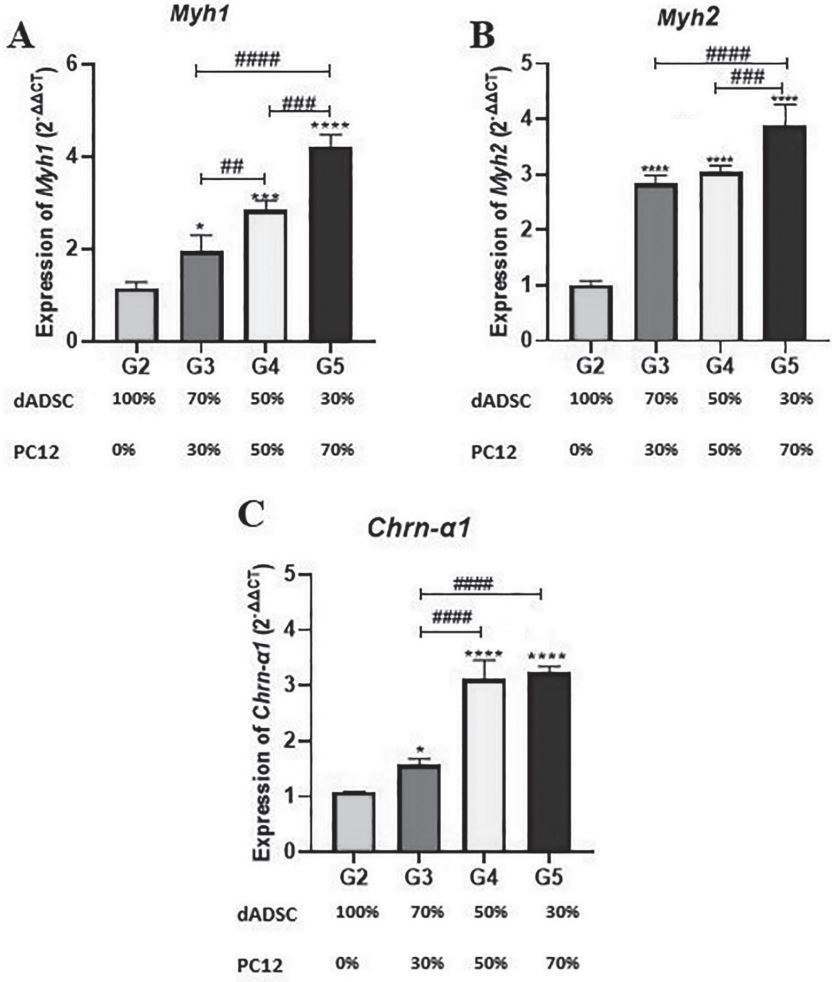

Figure 4: Real-time PCR of *Myh1*, *Myh2*, and *Chrn-α1* genes is illustrated. The coculture process induced a significant increase in *Myh1*, *Myh2* and *Chrn-α1* mRNA expression compared to differentiated ADSCs (A, B, C). P value for *Myh1*; G2 vs. G3: P=0.0134, G2 vs. G4: P=0.0001, G2 vs. G5: P<0.0001, G3 vs. G4: P=0.0080, G3 vs. G5: P<0.0001, G4 vs. G5: P=0.0005, P value for *Myh2; G2 vs. G3:* P<0.0001, G2 vs. G4: P<0.0001, G2 vs. G5: P<0.0001, G3 vs. G4: P=0.5230, G3 vs. G5: P<0.0001, G4 vs. G5: P=0.0005 and P value for *Chrn-α1*; G2 vs. G3: P=0.0498, G2 vs. G4 P<0.0001 G2 vs. G5 P<0.0001 G3 vs. G4 P<0.0001 G3 vs. G5 P<0.0001 G4 vs. G5: P=0.8585. Data are presented as mean±SEM of 3 separate experiments. * and # symbols indicate significant differences between the experimental groups G2 group, and coculture groups, respectively. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; #P<0.05; ##P<0.01; ###P<0.001 and ####P<0.0001. G2; differentiated ADSCs, G3; 30% PC12 cells and 70% of differentiated ADSCs, G4; 50% of the PC12 cells and 50% of differentiated ADSCs, G5; 70% of the PC12 cells and 30% of differentiated ADSCs. Data are presented as mean±SEM of three separate experiments.

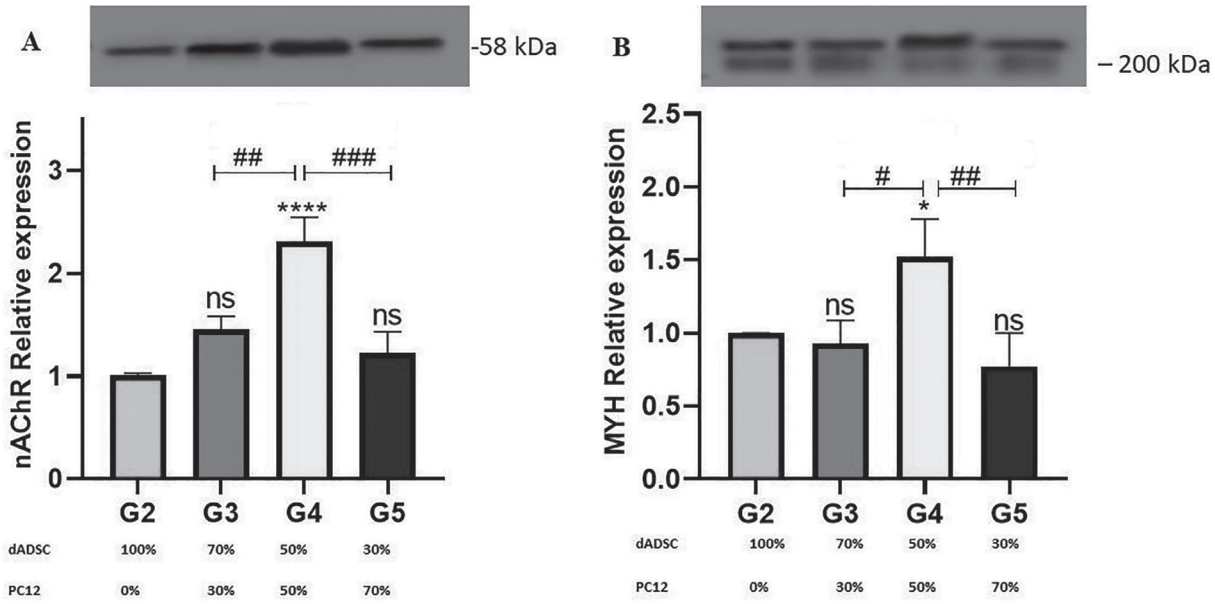

Figure 5: Western blot of MYH and nAChR proteins is illustrated. The expression of MYH, and nAChR proteins in differentiated ADSCs cocultured with PC12 cell line. A, B: The expression of MYH and nAChR proteins in group G4 showed significant elevation compared to the control group (G2). However, the expression of MYH and nAChR proteins showed no significant difference with group G2. Protein loading was confirmed by total protein visualization. For MYH; G2 vs. G3: P=0.9630, G2 vs. G4: P=0.0395, G2 vs. G5: P=0.4944, G3 vs. G4: P=0.0206, G3 vs. G5: P=0.7541, G4 vs. G5: P=0.0056 and for nAChR; G2 vs. G3: P=0.0532, G2 vs. G4: P<0.0001, G2 vs. G5: P=0.4603, G3 vs. G4: P=0.0013, G3 vs. G5: P=0.4203, G4 vs. G5: P=0.0003. Data was reported as Mean±SEM for three separate experiments. G2; differentiated ADSCs, G3; 30% of the PC12 cells cocultured with 70% of differentiated ADSCs, G4; 50% of the PC12 cell line cocultured with 50% of differentiated ADSCs, G5; 70% of the PC12 cell line cocultured with 30% of differentiated ADSCs. *P<0.05; ****P<0.0001

Changes in Myogenesis-Related Protein Expression Following Myogenesis Differentiation

Changes in nAChR Protein Expression: Data from nAChR protein expression revealed that the group received 50% of the PC12 cell line had considerably elevated levels of nAChR protein expression compared to the group of differentiated ADSCs (P<0.0001). However, the expression of the nAChR protein in the groups cocultured with 30% or 70% of the PC12 cell line was not significant compared with the group of differentiated ADSCs (P=0.86) (figure 5A). Overall, the significant increase in nAChR protein expression shows that PC12 cells stimulate myogenesis in ADSCs. Furthermore, the differentiated cells seem to resemble the functional features of adult myoblasts. The mean value of nAChR protein expression showed 1.45±0.13, 2.30±0.24, and 1.22±0.20 increase in G3, G4, and G5 groups compared with G2, respectively.

Changes in MYH Protein Expression: The greatest MYH expression was found in Group G4 compared to control differentiated cells (P=0.0395). However, the groups that received 30% or 70% of PC12 cells showed no significantly different levels of MYH protein compared with control differentiated cells (figure 5B). These results indicate that ADSC-derived myogenic progenitors were induced to differentiate into myotubes by culturing with PC12 in the DMEM-HG containing 5% HS medium. The mean value of MYH protein expression was 0.93±0.16, 1.52±0.26, and 0.77±0.23-fold of the G2 group in the G3, G4, and G5 groups, respectively.

Discussion

The differentiation procedure successfully promoted ADSCs to differentiate into myoblast-like cells. The differentiated cells expressed significantly higher amounts of myogenesis-related genes than ADSCs. According to our findings, PC12 treatment effectively causes myogenic differentiation of ADSCs. ADSCs cocultured with varying percentages of PC12 cells revealed considerably increased expression of myogenesis-related genes in a dose-dependent manner. The group that was treated with 50% PC12 exhibited significantly higher levels of nAchR and MYH protein expression.

Stem cell therapy is among the most promising treatments for muscular dystrophy.[3] The ability of stem cells to differentiate into several cell lineages and their propensity for long-term self-renewal are among their distinguishing characteristics.[5] MSCs' inductive behaviors are closely related to their paracrine effects. MSCs are thought to exert paracrine effects through VEGF, HGF, and

FGF2 secretion. The secreted paracrine factors contribute to angiogenesis, cell proliferation, and cell viability. The release of exosomes and extracellular vesicles by stem cells has also been documented in the literature. Exosomes contain a variety of bioactive chemicals, including microRNAs (miRNAs), messenger RNA (mRNA) molecules, peptides, proteins, and cytokines, which may help to induce differentiation. ADSCs lack immunologically surface antigens. ADSCs' favorable immunological behavior allows for transplantation without significant immunosuppression.[3, 5, 19]

Some studies focus on myogenesis differentiation by employing a differentiation-induced media. Others, on the other hand, use ADSCs cocultured with myoblasts to induce myogenesis differentiation.[4] However, the efficiency of the established protocols is extremely low. According to Liu and others' study, the differentiation-inducing medium, which contains horse serum, dexamethasone, and hydrocortisone, causes myogenic differentiation.[4] Horner and others discovered that Schwann cells cocultured with hiPSC-derived motoneurons and C2C12 myoblasts and given horse serum enhanced myotube growth.[20] According to multiple research, the coculture of ADSCs with C2C12 myoblasts alongside treatment with 5-Aza resulted in effective myogenesis differentiation.[9, 21] In the Zhou and others' study, a combination of 10 μM 5-azacytidine and 2% horse serum successfully generated muscle progenitors from chick bone marrow stem cells and enhanced muscle regeneration *in vivo*.[22] In line with previous research, our findings showed that ADSCs treated with a combination of horse serum and 5-Aza successfully differentiated into myoblast-like cells with much higher *Myh1, Myh2*, and *Chrn-α1* levels than the control group.

Some studies show that neuronal induction affects stem cell differentiation to myoblasts. The process begins with neural cells releasing growth and neurotrophic substances.[9, 11] Neuronal transcription factors bind to stem cell surface-specific receptors to initiate intracellular signaling pathways. These signaling pathways lead to the activation of myogenic transcription factors, including MyoD, Myf5, Myogenin (MYOG), and MRF4.[23] These transcription factors subsequently bind to specific DNA regions in the genome of stem cells, promoting the production of myogenesis-related genes such as muscle-specific structural proteins and enzymes.[23] Myoblasts then go through a series of proliferation and differentiation processes that result in the development of adult muscle fibers. During this phase, myoblasts unite to produce multinucleated myotubes, which eventually mature into functional muscle fibers.[24] According to several research, denervation has a deleterious impact on myogenesis and muscle function, emphasizing the necessity of maintaining adequate nerve-muscle connections for good muscle health.[23]

There is some evidence that various types of neurons have varied impacts on myogenesis induction and muscle regeneration.[9, 18] Some evidence focused on the role of motor neuron-derived factors in skeletal muscle regeneration.[25] Xue and others revealed the importance of neurons in the regulation of myogenesis via VGF and brain-derived neurotrophic factor (BDNF) release.[26] Horner and others found that Schwann cells trigger the Notch pathway to induce myogenesis[20]. Forcina and others identified satellite cells as essential regulators of skeletal muscle regeneration[27]. Some studies postulated the role of Interneurons in myogenesis promotion.[27, 28]

PC12 cells are a cell line created from a pheochromocytoma (adrenal gland tumor). PC12 cells are not regarded as motor neurons. However, PC12 cells have been shown to secrete nerve growth factor (NGF), BDNF, glial cell line-derived neurotrophic factor (GDNF), and neurotrophin-3 (NT-3).[29] Perini and others recognized NGF to be an effective factor in myogenic cell differentiation and proliferation. They found that NGF stimulates L6C5 myogenic cells.[30] According to the Morcuende and others. survey findings, GDNF therapy of rabbit extraocular muscle recovers muscle function.[31] According to current scientific findings, BDNF collaborates in cellular pathways associated with muscle function maintenance and plasticity.[32] Some research shows that neurotrophin-3 stimulates muscle fiber development and neuromuscular connections *in vitro* and *in vivo*.[33,34] Hence, given PC12 cells' ability to secrete neurotrophic compounds, we hypothesized that these cells could stimulate myogenesis in ADSCs. It seems that in our study, PC12 cells promoted the differentiation of ADSCs through the secretion of various factors as well.

Previous research has shown that boosting the expression of acetylcholine receptors on the surface of stem cells or muscle precursors plays an important role in the activation of myogenesis and the repair of muscle processes.[10] In our investigation, the coculture of ADSCs with varied doses of PC12 cells resulted in a dose-dependent manner in *Chrn-α1* gene expression. In addition, the expression of nAchR protein increased considerably in the group of ADSCs

cocultured with 50% PC12 cells. At the same time, the morphology of ADSC cells changed to filamentous and parallel multinucleated forms. Furthermore, microscopic identification revealed the presence of some connections between the developing ADSC cells and PC12 cells. The above findings, coupled with an increase in *Myh* gene and protein expression, could point to the probable formation of an NMJ between ADSC and PC12 cells during coculture.

In the study by Zammit and others, activation of myogenic regulatory factors such as XMyoD, XMyf5, and XMRF4 resulted in the overexpression of the *Chrn-α1* gene.[35] Cai and others investigated myogenic differentiation in primary myoblasts cocultured with mesenchymal stem cells (MSCs) on PCL-collagen I-nano scaffolds under serum-free conditions. They reached the conclusion that MSCs successfully differentiated into myoblasts.[6] The review by Im and others exhibited that the expression of alpha-actinin skeletal muscle 2 and MYOG in differentiated ADSCs was substantially higher than in BMSCs.[36] Ostrovidov and others studied the influence of C2C12 myoblasts on the development of myotubes in ADSCs treated with 5-Aza and FGF-2.[9] Their results showed that the ADSCs successfully differentiated into myotubes and could express MyoD and MYOG.[9] In Cao's study, ADSCs were injected into the gastrocnemius muscle of mdx mice. The mice that received ADSCs showed improved Akt/mTOR pathway activity, as well as high levels of dystrophin and MYOG expression and lower levels of Caspase-3.[37] The researchers concluded that the paracrine effects of ADSCs promote the regeneration and survival of muscle cells *in vivo*.[37]

A review of the literature indicates that ADSCs may be suitable candidates for skeletal muscle regeneration. ADSCs can spontaneously develop into myoblast-like cells. However, the presence of neuronal or muscular-generated growth factors provides the foundation for differentiation towards mature muscle cells. Differentiated myoblast-like cells will be able to secrete MYOG, MyoD, and myosin.[38]

It is necessary to mention that due to funding limitations, we were unable to supply a transwell to the indirect coculture of two different cells. However, the results of the study confirm the hypothesized theory about the direct enhancing effect of ADSCs on myogenesis differentiation.

Conclusion

Our study defined a successful and efficient differentiation strategy for myogenesis induction. The findings suggest that PC12 has an influence on myogenesis stimulation in ADSCs *via* paracrine behaviors or NMJs. This 2D coculture platform offers a low-cost instrumental model for establishing myogenesis procedures. In subsequent steps, PC12 cells and ADSCs can be generated from the same animal model and cocultured to form functional NMJs and alleviate muscular dystrophies.

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Authors' Contribution

Z.ShS; V.B; D.D: Study design, data collection, and evaluation, all experimental work such as cell culture, immunofluorescence staining, RT-qPCR, western blot statistical analysis, and drafting; S.A; M.R: Contributed to all experimental work such as cell culture, immunofluorescence staining, RT-qPCR, western blot, statistical analysis, interpretation of data, and reviewing the manuscript; All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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