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Original Article

Promoting and accelerating muscle regeneration through cell therapy in a mouse model

Marwa I. Salman, PhD [a], Eman G. Khalil, PhD [b], Aous K. Almzaien, MSc [c], Ali A. Hadi, PhD [d,e], Aysar A. Ahmed, BSc [c], Hiba K. Shaker, BSc [c] and Ahmed M. Al-Shammari, PhD [c,*]

[a] *Biotechnology Department, College of Science, Baghdad University, Baghdad, Iraq*
[b] *Biomedical Engineering Department, Engineering College, Al-Nahrain University, Baghdad, Iraq*
[c] *Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University, Baghdad, Iraq*
[d] *Department of Physical Education and Sports Sciences, Dijlah University College, Baghdad, Iraq*
[e] *Individual Sports Department, College of Physical Education and Sports Sciences, University of Baghdad, Iraq*

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الملخص

أهداف البحث: تعد إصابات واضطرابات العضلات الهيكلية تحديات سريرية عالمية ذات آليات مباشرة وغير مباشرة وتأثيرات متبقية ملحوظة، مثل الألم الشديد الذي يستمر لفترة طويلة والإعاقة الجسدية. الخلايا الجذعية هي أداة مبتكرة للعلاج بالخلايا ضد الاضطرابات العضلية الهيكلية والتي يمكن أن تعزز على وجه التحديد تجديد العضلات والهيكل العظمي. تهدف الدراسة الحالية إلى دراسة استخدام الخلايا الجذعية الوسيطة وخلاياها العضلية المتمايزة لإعادة بناء العضلات الهيكلية التالفة أو المريضة كعلاج قائم على الخلايا لتعزيز تجديد العضلات.

طريقة البحث: تم عزل الخلايا الجذعية الوسيطة للنخاع العظمي من النخاع العظمي للفئران البالغة وتم تربيتها في رقائق زراعة الأنسجة. كانت الخلايا الجذعية الوسيطة للنخاع العظمي إيجابية لـ سي د 90 و سي دي 105، وسالبة لـ سي دي 45 و سي دي 34. تم تحفيز هذه الخلايا في المختبر إلى سلالة خلايا عضلية هيكلية من خلال وسط تمايز محدد في 7 أيام. تم توصيف تمايز العضلات الهيكلية عن طريق صبغة الهيماتوكسيلين والأيوسين وفحصها عن طريق المسح المجهري الإلكتروني لدراسة التشكل. تم إجراء الصبغ المناعي لـ العامل العضلي 6، وسلسلة الميوسين الثقيلة، وعوامل محددة لتطور العضلات الهيكلية، لتأكيد تمايز العضلات الهيكلية. في دراسة على الجسم الحي، تم إنشاء نموذج إصابة العضلات الذي يستخدم لتقييم العلاج بالخلايا باستخدام الخلايا الجذعية الساذجة والخلايا العضلية المتمايزة.

النتائج: لقد أثبتنا أن الفئران المستزرعة أن الخلايا الجذعية الوسيطة للنخاع العظمي كانت إيجابية بالنسبة لـ سي د 90 و سي دي 105، وسلبية بالنسبة لـ سي دي 45 و سي دي 34. تم تطوير هذه الخلايا إلى عضلات هيكلية تظهر إمكانات تمايز قوية للعضلات الهيكلية تم تأكيدها بشكل فعال بواسطة الكيمياء المناعية لعلامات العامل العضلي 6 وسلسلة الميوسين الثقيلة وديزمين. أظهرت الخلايا العضلية المتمايزة تعزيزا أفضل للإصلاح من الخلايا الجذعية غير المتمايزة عند زرعها في نموذج فأر لعلاج ضمور العضلات الهيكلية.

الاستنتاجات: يمكن تضمين الخلايا العضلية المشتقة من الخلايا الجذعية الوسيطة للنخاع العظمي في علاج ضمور العضلات كاستراتيجية بيولوجية لإدارة أمراض وإصابات العضلات الهيكلية من أجل تطوير العلاجات السريرية القائمة على الخلايا.

الكلمات المفتاحية: الخلايا الجذعية الوسيطة؛ العامل العضلي 6؛ العلاج الخلوي؛ العضلات الهيكلية؛ المسح المجهري الإلكتروني

Abstract

Objectives: Skeletal muscle injuries and disorders are universal clinical challenges with direct and indirect mechanisms and notable residual effects, such as prolonged, intense pain and physical disability. Stem cells, an innovative tool for cell therapy for musculoskeletal disorders, specifically promote skeletal muscle regeneration. This study was aimed at investigating the use of mesenchymal stem cells (MSCs) and their differentiated myocytes as a cell-based therapy to promote regeneration in damaged or diseased skeletal muscle.

* Corresponding address: Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University, Baghdad, Iraq.
E-mail: alshammari.am@uomustansiriyah.edu.iq (A.M. Al-Shammari)

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Methods: Bone marrow mesenchymal stem cells (BM-MSCs) were isolated from the bone marrow of adult mice and grown in tissue culture flasks. The BM-MSCs were positive for CD90 and CD105, and negative for CD45 and CD34. These cells were induced with specific differentiation medium *in vitro* to differentiate into a skeletal muscle cell lineage over 7 days. Skeletal muscle differentiation was characterized according to morphology through hematoxylin and eosin staining, and scanning electron microscopy. Immunostaining for Myf-6, myosin heavy chain (MHC), and desmin—specific factors for skeletal muscle development—was performed to confirm skeletal muscle differentiation. An *in vivo* study in a muscle injury model was used to evaluate cell therapy based on naïve stem cells and differentiated myocytes.

Results: Cultured mouse BM-MSCS were positive for CD90 and CD105, and negative for CD45 and CD34. These cells developed into skeletal muscle with strong skeletal muscle differentiation potential, as confirmed by immunohistochemistry for the markers Myf6, MHC, and desmin. The differentiated myocytes showed better repair enhancement than undifferentiated stem cells after transplantations into a mouse model of skeletal muscle atrophy.

Conclusions: Myocytes derived from BM-MSCs may be incorporated into muscular atrophy treatment as a biological strategy for managing skeletal muscle diseases and injuries, thus advancing cell-based clinical treatments.

Keywords: Cellular therapy; MSCs; Myf-6; SEM; Skeletal muscle

Introduction

Several diseases affecting muscles, including Duchenne muscular dystrophy, cancer, traumatic injury, and congenital disabilities, cause irreversible muscular atrophy, inflammation, and fibrosis, and ultimately lead to death.[1,2] These diseases may be treated to restore the full regenerative potential of the muscle. Bone marrow mesenchymal stem cells (BM-MSCs) have the potential to repair muscle through tissue engineering, and therefore are promising candidates for reconstructing damaged or destroyed skeletal muscle.[3] *In vitro* studies examining the development of skeletal muscle cells have revealed the essential steps of myogenesis, including cell differentiation mechanisms, morphogenesis, and cell—matrix interactions.[4,5] This process is regulated by signals that are secreted by the affected fibers and activate dormant satellite cells. This population of myogenic stem cells is largely responsible for skeletal muscle regeneration and repair.[6] Adult stem cells are emerging as promising sources for tissue repair and regeneration in musculoskeletal tissue regenerative medicine.[7,8] BM-MSCs, because of their superior proliferation *in vitro* and minimal immunogenicity *in vivo*, are a potential cell source for skeletal muscle tissue engineering.[9] The healing process of injured skeletal muscle is characterized by the presence of several bioactive molecules, such as proinflammatory cytokines and fibroblast growth factor (FGF), which promote fibroblast proliferation and regeneration of healthy skeletal muscle tissue,[10] and facilitate mitogenesis in mesenchymal stem cells.[11] Epidermal growth factor promotes fibroblast migration and proliferation while also controlling angiogenesis and maintaining the equilibrium of the extracellular matrix.[12] These signaling molecules are mitogenic stimuli that activate precursor cells, thereby accelerating the regeneration of damaged muscle tissue.[11] Mesenchymal stem cells (MSCs) play important roles in skeletal muscle regeneration, because they easily differentiate into myocytes and fuse with local muscle cells while secreting bioactive molecules that participate in skeletal muscle regeneration.[13] Our study was aimed at rebuilding damaged or diseased skeletal muscles by using myocytes derived from MSCs as a cell-based therapy to promote muscle regeneration and accelerate functional recovery.

Materials and Methods

Animals

The Iraqi center for cancer and medical genetic research (ICCMGR) at Mustansiriyah University, Baghdad, Iraq provided the Swiss Albino male mice used in this study. These mice were 8 weeks old and weighed 20—25 g. The mice were housed in groups of four per cage in a facility maintaining a constant temperature, and were given unrestricted access to food and water. Animal testing was conducted in accordance with the guidelines established by the ICCMGR for the care and use of experimental animals. The ICCMGR Scientific Committee approved all procedures involving experimental animals.

Isolation and characterization of BM-MSCs

BM-MSCs were freshly isolated from the whole bone marrow of 8-week-old mice, then resuspended in primary culture medium comprising minimum essential medium (USBiological, United States) with 15% FBS (Cellgro, USA), 100 IU/ml penicillin, and 100 g/ml streptomycin.[14] After 24 h, adherent MSCs were selected and maintained in growth medium, which was changed three times per week. Cultures were observed daily until they reached 70—80% confluence (Figure 1A), at which point the cells were detached with trypsin—EDTA (USBiological, USA) and used in immunocytochemical analyses of MSC markers (CD90, CD105, and negative MSCs CD45 and CD34).[14]

In vitro *differentiation of MSCs into the skeletal muscle cell lineage*

MSCs were induced to differentiate into skeletal muscle *in vitro*. The MSCs were grown in tissue culture flasks in a chemically defined medium (skeletal muscle differentiation medium, Promocell, Germany) for 7 days. This medium consisted of fetal bovine serum (50 µl/ml), recombinant

epidermal growth factor (10 ng/ml), recombinant basic FGF (1 ng/ml), insulin (recombinant human; 10 μg/ml), and dexamethasone (10 μg/ml). The cells formed confluent monolayers and were imaged daily under an inverted microscope with a Micros camera (Micros, Austria).

Characterization of skeletal muscle differentiation

Morphological study with hematoxylin and eosin staining

Every day for 7 days, cultured differentiated and undifferentiated cells were fixed in 4% buffered formalin at 4C° for 30 min, then dehydrated in ethanol and stained with H&E (Sigma, USA) to visualize the morphology of the differentiated cells.

Immunostaining

After plating of BMMSCs at a density of 30.000 cells/well on coverslips in six-well plates overnight, the cells were differentiated into skeletal muscle cells for 7 days in the presence of skeletal muscle differentiation medium. For immunochemical staining, the cells were washed twice with cold PBS and fixed in 4% paraformaldehyde/PBS at 4 °C for 10 min. The staining was performed with a anti-mouse ABC staining system (Santa Cruz Biotechnology; Santa Cruz, CA) according to the manufacturer's procedure. The cells grown on plates were blocked with 1.5% blocking serum dissolved in PBS, then incubated overnight at 4 °C with anti-mouse MYF-6, anti-mouse myosin heavy chain (MHC), and anti-mouse desmin. The slides were washed and incubated with biotinylated secondary antibody at room temperature for 30 min. The preparation of the secondary antibody with avidin and biotinylated horseradish peroxidase was required for 30 min to determine its identity. Subsequently, incubation with peroxidase substrate was performed until the desired color intensity developed. After counterstaining with hematoxylin for 10 s, the slides were viewed under a light microscope and photographed.

Scanning electron microscopy examination

Differentiated and undifferentiated cells were cultured on coverslips. We prepared samples for the SEM examination every 24 h for all 7 days of the differentiation period. The cultured cells were washed with PBS, soaked with 2% glutaraldehyde, and stored at 4 °C overnight. The samples were then postfixed for 10 min with a final concentration of 2% osmium tetroxide and placed on ice during fixation. Dehydration of the samples was performed through with a graded alcohol series (25%, 50%, 75%, 95%, and 100% v:v).[15] The samples were examined with Phenom G2 Pro SEM (Thermo Fisher, the Netherlands).

Animal model

Muscle injury model and in vivo *cell transplantation therapy*

In vivo transplantation of newly formed BM-MSC-derived skeletal muscle cells was performed to investigate their function in 6-week-old female mice with a defect (visible lysis) induced by ischemic injury with a metal probe (1 cm diameter) cryofrozen in liquid nitrogen. This metal probe was applied directly to the femoris of the biceps (anterior, BFA; posterior, BFP) after an incision was made in the skin of the femur. This muscular damage led to atrophy of the affected area. The animals were divided into three groups of five mice each. The first group was injected with 1×10^6 BM-MSCs, the second group was injected with 1×10^6 differentiated cells, and the third group was left untreated. After 4 weeks, we sacrificed the animals and prepared histological sections. Muscle samples were preserved with 4% paraformaldehyde-PBS at 4 °C 2 days before H&E staining. The tissues were embedded in paraffin and sectioned to a thickness of 5 μm. Standard procedures for H&E staining were used.[16]

Results

Isolation and characterization of BM-MSCs

Isolated cells were grown *in vitro* and started to form a monolayer within 3 days of culture. Cultured cells were positive for the MSC markers CD90 and CD105, and were negative for CD45 and CD34.

Skeletal muscle induction

The myogenic capacity of BM-MSCs was confirmed through morphology and IHC *in vitro* after induction of myogenic differentiation. The functionality of newly formed myocytes was demonstrated *in vivo* by transplantation of differentiated BM-MSCs into animal models.

Morphological analysis

Cytological analysis of newly generated myocytes from BM-MSCs after 7 days of differentiation (*in vitro*) showed clear evidence of morphological transformation and formation of skeletal muscle myotubes.

Light microscope examination

Under an inverted light microscope, we observed morphological changes during the differentiation of BM-MSCs to skeletal muscle cells *in vitro*. On the first day of differentiation, no clear morphological changes were observed, and cells still resembled the original stem cells; most cells were round, and a few cells had started to become elongated (Figure 1A). On the second day of differentiation, cell proliferation was observed, with slight changes in shape as the elongated cells become more evident (Figure 1B). On the third day, most cells showed morphological changes, including the presence of spindle shaped cells, cell proliferation, and myoblast formation before myotube formation (Figure 1C). On the fourth day of differentiation, we observed more drastic changes toward a myoblast morphology characterized by increased elongation and enlarged spindles (Figure 1D). On the fifth day, the myoblasts showed maturation and proliferation, and started to form myocytes (Figure 1E). On day 6 of differentiation, the myoblasts transformed into myocytes and started to align and fuse to form myotubes (Figure 1F). On the seventh day, the newly generated mature skeletal muscle was

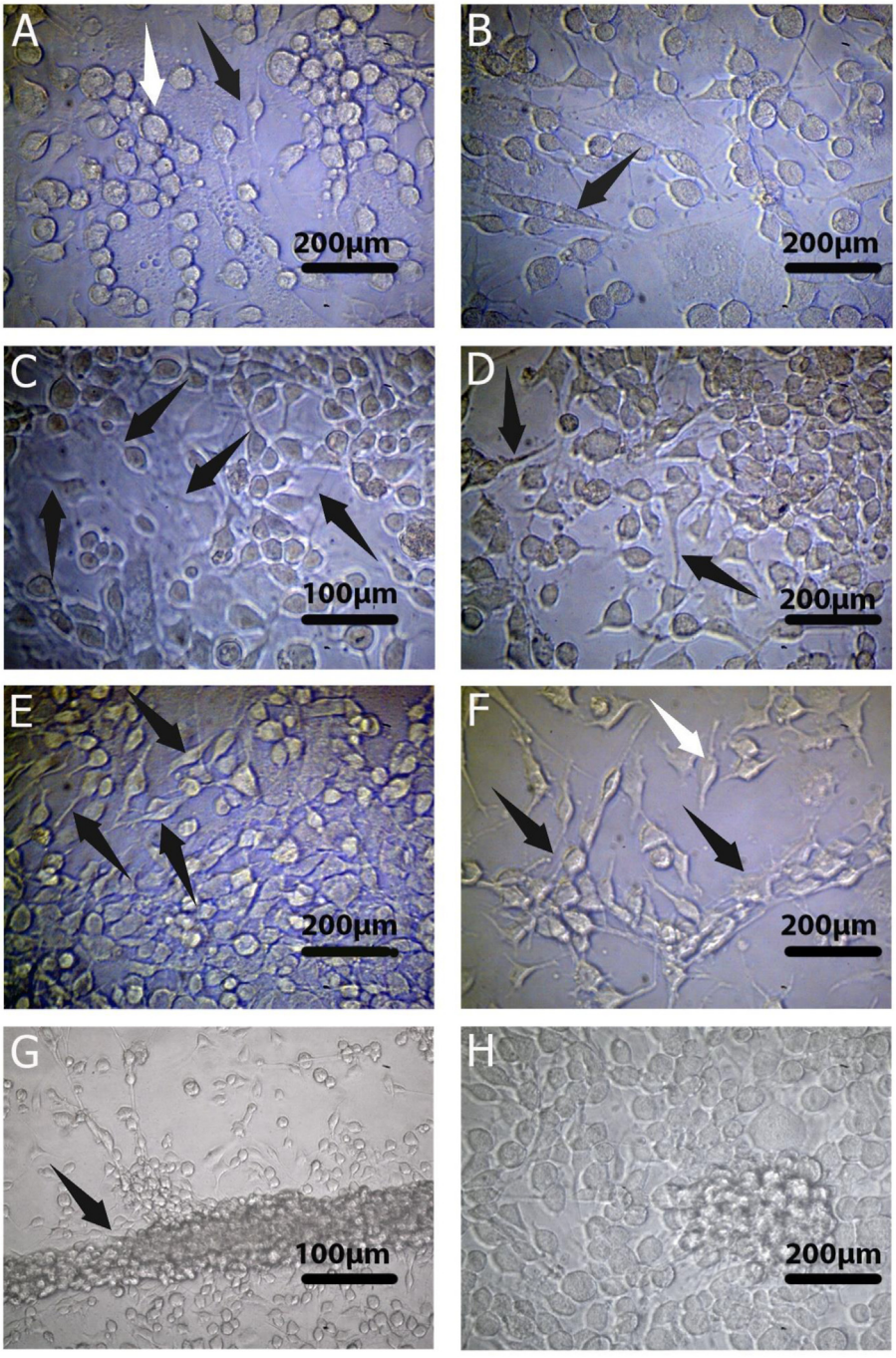


Figure 1: Morphological changes during the differentiation of BM-MSCs into skeletal muscle (*in vitro*), viewed under an inverted light microscope at ×40 magnification. (A) On the first day of differentiation, no clear morphological changes were observed, and the cells still resembled the original stem cells: most cells were round (white arrow), and several cells had started to elongate (black arrow). (B) On the second day of differentiation, proliferation of BM-MSCs, and slight changes in cell shapes were observed, as the elongated cells become more evident (black arrow). (C) On the third day, most cells showed morphological changes including the presence of spindle shapes, greater cell proliferation, and myoblast formation before myotube formation (black arrows). (D) On the fourth day of differentiation, further morphological changes in myoblasts were characterized by increased elongation and enlarged spindles (black arrows). (E) On the fifth day, myoblast maturation and proliferation, and myocyte formation (black arrows) were observed. (F) On day 6 of differentiation, myoblasts had transformed into myocytes (white arrow) and started to align (black arrows) and subsequently fuse to form myotubes. (G) On day 7, full generation of myocytes to form myotubes was observed, with the formation of myofibers with multiple nuclei through cell migration to form multinuclear tubes (black arrow). (H) In control undifferentiated stem cells, no myotube-like cells were observed in BM-MSCs grown in medium without growth factors.

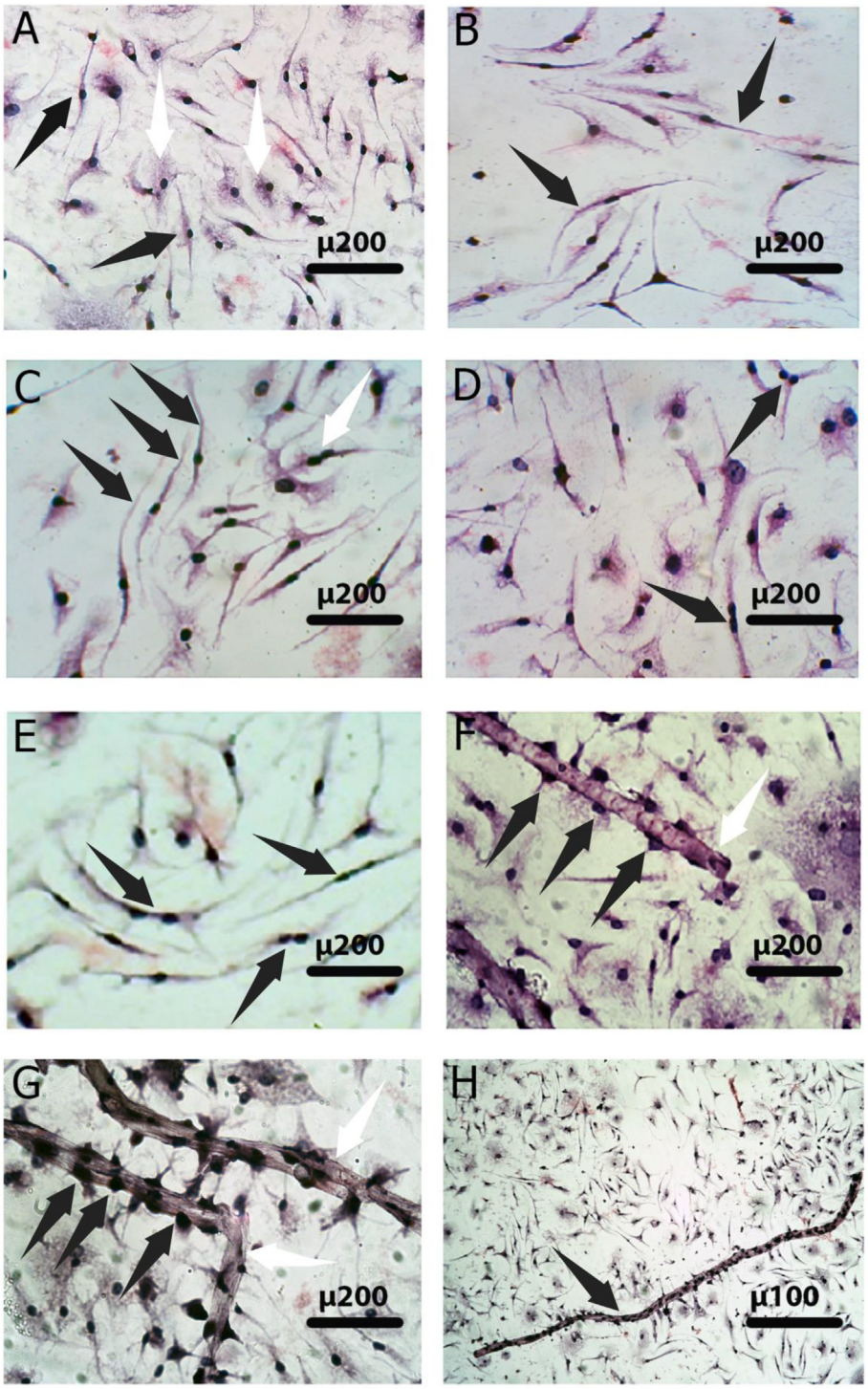


Figure 2: Myogenesis differentiation, revealed by H&E staining. Myogenic differentiation was observed from before the formation of myotubes through fusion into muscle fibers. H&E staining revealed more details than light microscopy alone. (A) On the first day of differentiation, several morphological changes in stem cells were observed; some cells had started to elongate to form myoblasts (black arrow), whereas other cells remained round and still resembled the original stem cells (white arrow). (B) On the second day of differentiation, the morphological changes involved elongation of more cells (black arrow), whereas few cells remained round (white arrow). (C) On the third day, spindle shaped cells were observed, indicating myocyte alignment before fusion. Three cells in parallel (black arrows) with the presence of fused cells with two nuclei (white arrow) were clearly observed. (D) On the fourth day of differentiation, more cells had two nuclei (black arrows). (E) On the fifth day of differentiation, most myocytes had fused into multinucleated cells that would later form myotubes. (F) On day 6 of differentiation, the fused myocytes formed myotubes (white arrow) with multiple nuclei (black arrows). (G) On day 7, generation of myotubes was observed, with the formation of myofibers with multiple nuclei (black arrows). (H) A lower magnification image on the seventh day shows the entire myotube length.

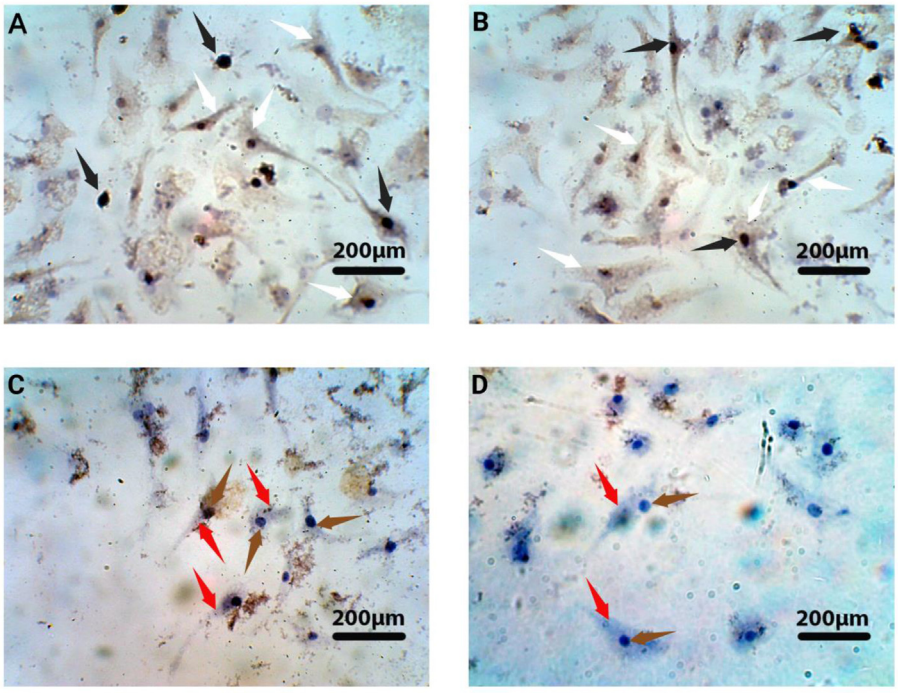


Figure 3: Immunocytochemical staining for the specific expression of MYF-6, a marker of skeletal muscle differentiation, after 7 days of differentiation. A and B) Nucleo-cytoplasmic positive (brown) anti-MYF-6 staining in differentiated myocytes. Positive nuclear staining (black arrows) and simultaneous presence of positive cytoplasmic staining (white arrows) are shown. C and D) Negative control, showing blue nuclei with no brown staining (brown arrows), cytoplasm showing no brown staining, and blue staining from the counterstain only (red arrows).

composed of myofibers containing myonuclei located primarily at the center and periphery in multinucleated myotube-like cells (Figure 1G). In contrast, no myotube-like cells were observed in the control group of BM-MSCs without growth factors (Figure 1H).

Furthermore, we used H&E staining to observe the myogenic differentiation of skeletal muscle cells before the formation of myotubes in greater detail (Figure 2). On the first day of differentiation, several morphological changes in stem cells were observed. Some cells started to elongate and form myoblasts, whereas others resembled the original stem cells, showing primarily round cells with several cells starting to become elongated (Figure 2A). On the second day of differentiation, morphological changes were observed, in which more cells had become elongated, although several cells remained round (Figure 2B). On the third day, the cells showed spindle-like shapes, thus indicating that myocytes had aligned in preparation for fusion. Three cells were observed in parallel with the presence of fused cells with two nuclei (Figure 2C). On the fourth day of differentiation, more cells with two nuclei were observed (Figure 2D). The fifth day of differentiation was characterized by fusion of most myocytes into multinucleated cells that would later form myotubes (Figure 2E). On day 6 of differentiation, the fused myocytes formed myotubes with multiple nuclei (Figure 2F). Day 7 was characterized by the generation of myotubes, with the formation of myofibers with multiple nuclei (Figure 2G). An illustration of the complete length of myotubes is shown in Figure 2H.

Expression of the myogenic marker Myf-6 in differentiated cells

To confirm skeletal muscle differentiation, we also examined expression of the myogenic marker Myf-6 (also known as MRF-4 or herculin) in differentiated myocytes after 7 days of myogenic differentiation. Clear positive staining for the Myf-6 marker was observed in both the nucleus and cytoplasm (Figure 3A and B), but was absent in the negative control stained with only counterstain (Figure 3C and D). To further confirm the skeletal muscle differentiation, we stained the differentiated cells to detect desmin and myosin heavy chain. We observed positive results after 7 days of myogenic differentiation (Figure 4A and B) but not in the negative control stained with counterstain only (Figure 3C and D).

Scanning electron microscopy and morphometric study of differentiated cultured cells

SEM analysis revealed undifferentiated mesenchymal cells, which were visible as small polygonal cells (Figure 5A). In contrast, differentiated cells showed elongated spindle-like cell shapes, with enlarged cells early during differentiation (Figure 5B). On the second and third days (Figure 5C and D), characteristics of skeletal muscle morphology were observed. SEM revealed clear morphological alterations in cells on days 4 and 5, when differentiated cells migrated and fused with the ends of adjacent cells (Figure 5E and F). Furthermore, differentiated cells became more

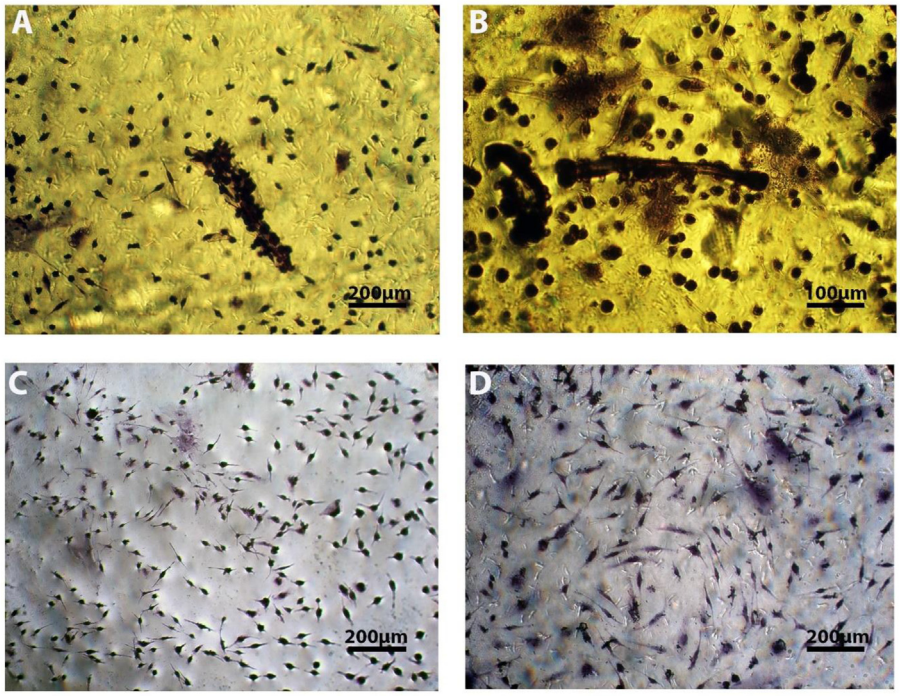


Figure 4: Immunocytochemical staining for desmin and myosin heavy chain, markers of skeletal muscle differentiation, after 7 days of differentiation. A) Nucleo-cytoplasmic positive (brown) anti-desmin staining in differentiated myocytes. B) Nucleo-cytoplasmic positive (brown) anti-myosin heavy chain staining in differentiated myocytes. C and D) Negative control, showing blue nuclei with no brown staining, cytoplasm showing no brown staining, and blue staining from the counterstain only.

elongated and formed myotube shapes at the end of the sixth day (Figure 5G), and many long, thin processes were observed on the seventh day (Figure 5H).

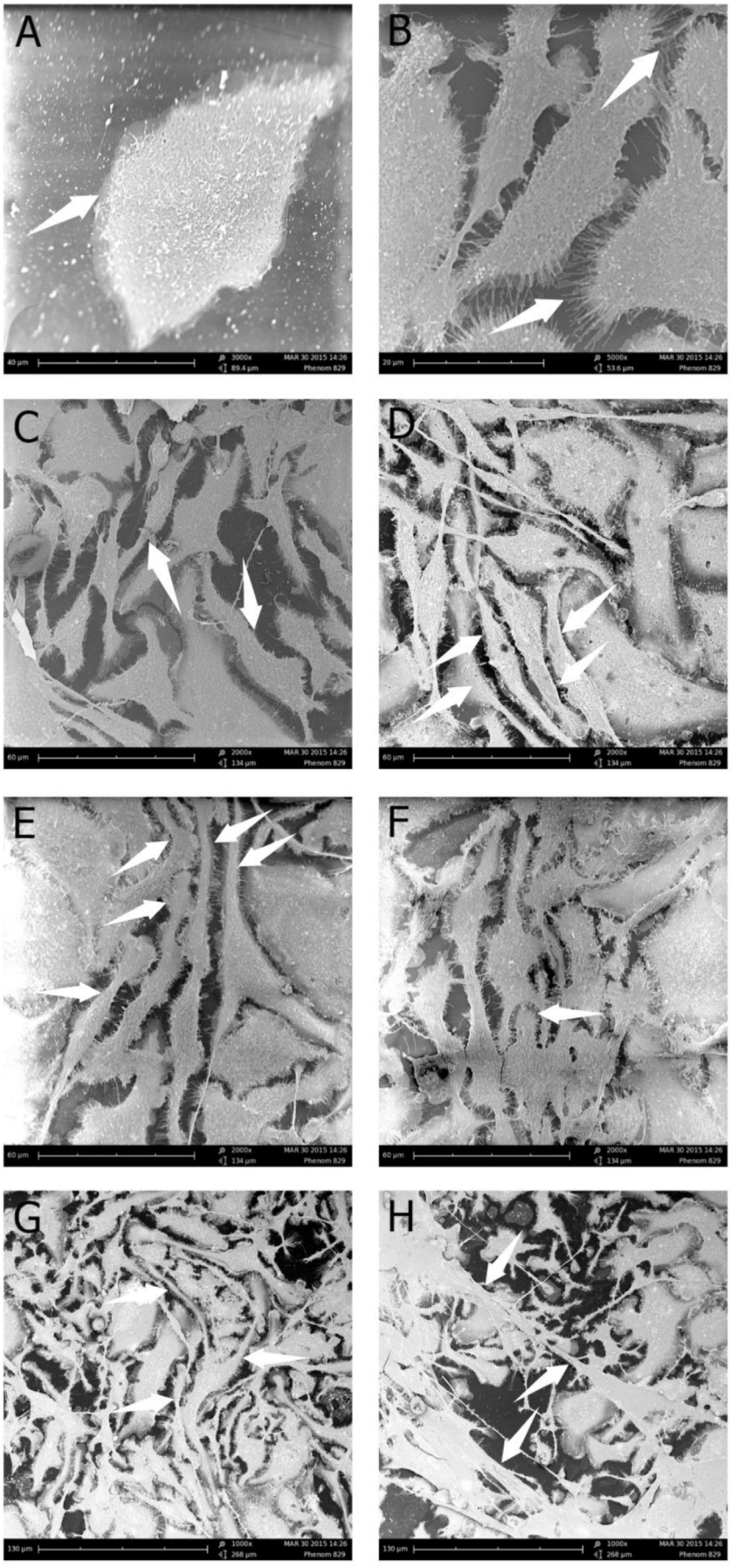
Measurements in Phenom scanning electron microscopy software and ImageJ indicated that the cell length (in µm) had considerably increased. In addition, we measured the maximum and minimum lengths of the cells on the seventh day of differentiation (Figure 6A); after the cells began to amalgamate before developing into myotube cells, an increase in length was observed. We calculated the average percentage change in cell length on each day of growth. The changes in cell length were measured by calculating the average length on day 7. This measure may reflect alterations in the cellular environment, such as extracellular matrix changes. Major changes were observed between days 3 and 6 (Figure 6B).

Histological analysis for in vivo *cell transplantation*

The ability of damaged muscles to regenerate after injection with new cells *in vivo* was studied. Muscle tissue sections were stained with H&E for histological analysis in mice 4 weeks after skeletal muscle damage and treatment. Damaged muscles were examined and compared with normal tissue (without damage) in the control group (Figure 7A). When compared to damaged muscles that were not treated with any cells, they revealed damaged muscle fibers with inflammatory cell infiltrations at the site of injury (Figure 7B), also as a control (Figure 7C) reveals the groups of mice with damaged tissue treated with undifferentiated stem cells that do not give details of muscle fibers, show good expression of repair or healing but with some abnormal focal dark stained fibers (necrotic fibers), whereas the groups of mice injured and treated with differentiated cells showed comprehensive repair of muscle fibers (Figure 7D).

Expression of the differentiation marker Myf6 in vivo *after transplantation*

The differentiation marker Myf6 is a muscle-specific transcription factor that is normally expressed in skeletal muscle and also is expressed in injured muscle tissue. Expression of Myf6 was induced by the effects of the repair process of transplanted differentiated myoblasts and myocytes (Figure 8). Myf6 was expressed in normal healthy muscle tissue (Figure 8A). Damaged tissue without treatment showed positive abnormal muscle tissue with loss of muscle fiber alignment and fibrosis (black arrows) (Figure 8B). Furthermore, positive Myf6 expression was observed in the MSC treatment group, and an increase in muscle fibers was observed at damage sites (Figure 8C). Sections in the differentiated myocyte treatment group showed more normal structure with positive staining (Figure 8D). In contrast, expression of Myf6 was absent in the negative control (Figure 8E).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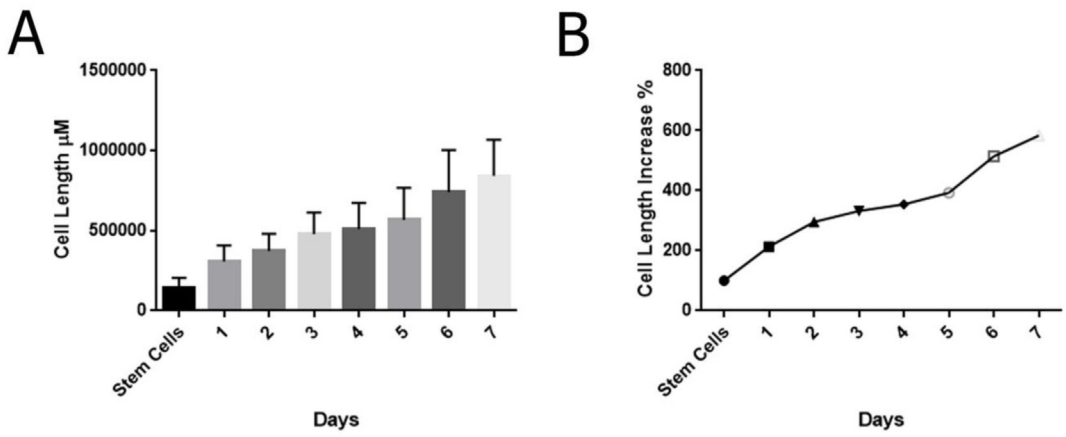

Figure 6: (A) Maximum cell length increases on every day of growth. (B) Average percentage change in cell length on every day of growth.

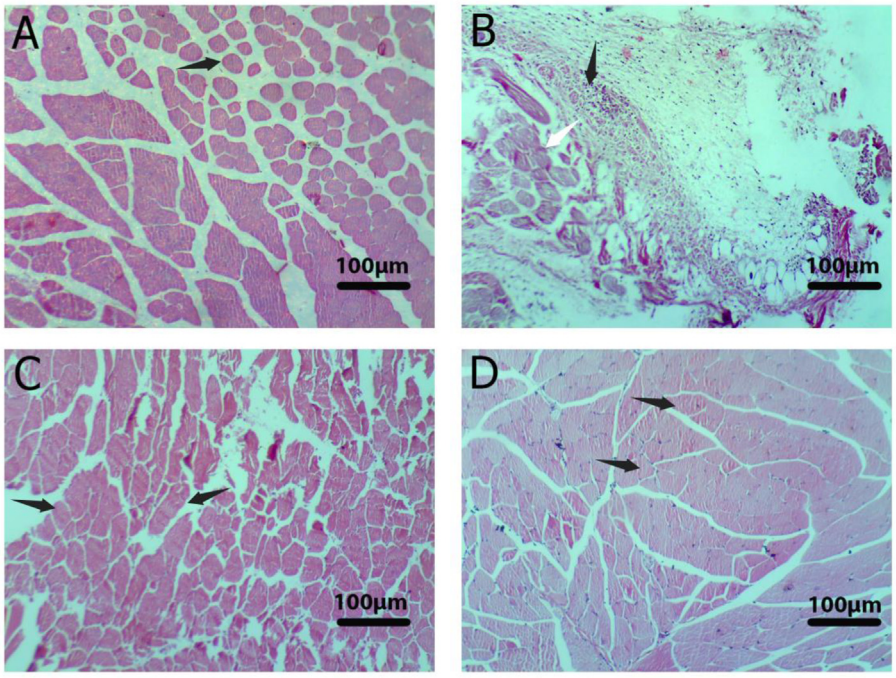

Figure 7: Histological sections for *in vivo* cell transplantation. (A) Normal muscle tissue without damage, as a control (black arrow). (B) Damaged muscle without treatment, showing atrophy of the injured muscle fibers (white arrow), necrotic dead cells, and inflammatory cells at the site of injury (black arrow). (C) Favorable repair in damaged tissue treated with undifferentiated stem cells that do not have any details for muscle fibers (black arrows). (D) Injured muscle treated with differentiated cells, showing the complete repair of muscle fibers (black arrows).

Figure 5: Scanning electron microscopy evaluation of the morphological and morphometric changes in BM-MSCs during the differentiation into skeletal muscle cells. (A) Control stem cells without any differentiation. Small polygonal cells, without elongated shape and clear edges (white arrow), are shown. (B) On the first day, the cells started to elongate, and villi were observed around the edges of the cells (white arrows). (C) On the second day, the cells continued to elongate (white arrows). (D) On the third day, elongated cells started to align with each other (white arrows) to initiate myotube formation. (E) On the fourth day, more aligned cells (white arrows) underwent continuous differentiation and myotube formation. (F) On the fifth day, more elongated differentiated cells had fused with the ends of the nearest neighboring cells (white arrow). (G) On the sixth day, most cells had become longer, presenting a characteristic myotube shape (white arrows). (H) On the seventh day, myotube formation was observed (white arrows).

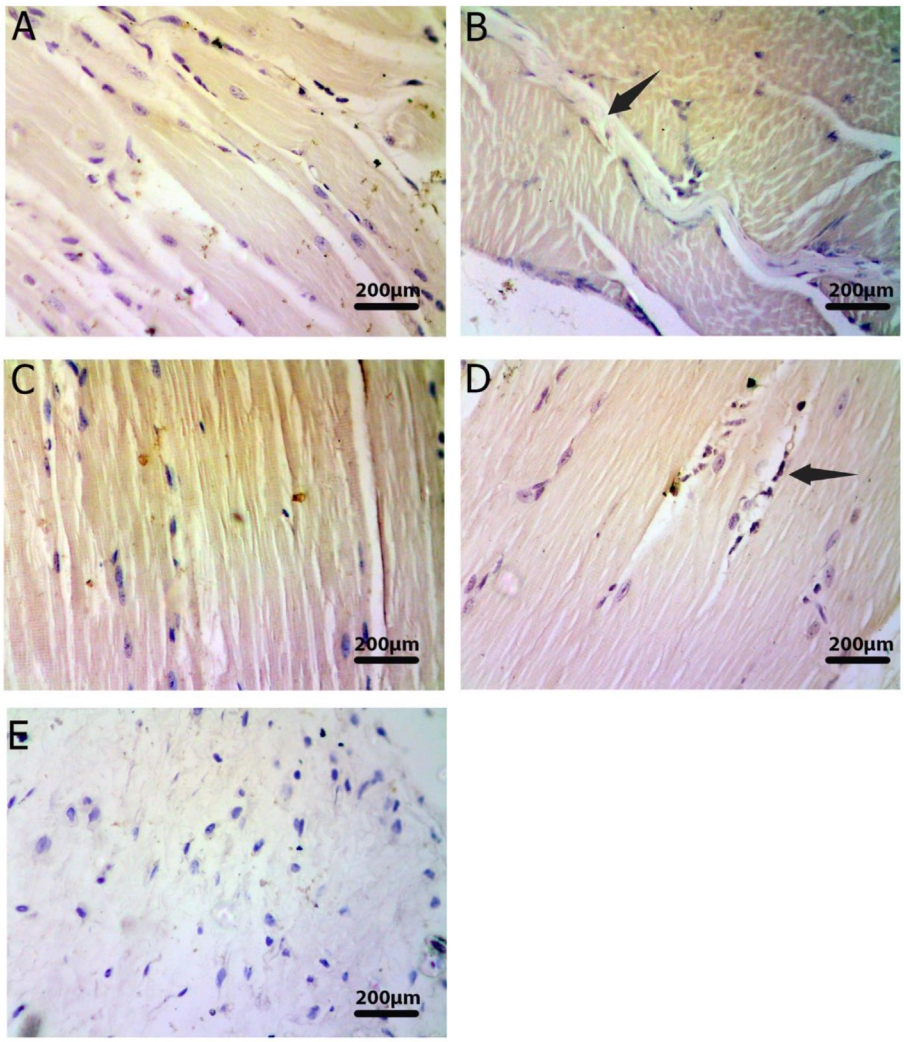

Figure 8: Identification of Myf6 expression after transplantation of newly differentiated muscle cells via engraftment in an *in vivo* mouse model. Expression profile of the Myf6 marker after regeneration following extensive muscular injury. (A) Normal muscular tissue, as a control. (B) Damaged tissue without treatment, showing loss of fiber alignment and fibrosis (black arrows). (C) Section in the mesenchymal stem cell treated group of damaged tissues, showing cells with strong marker positivity. (D) Section in the differentiated myocyte treatment group, showing cells with strong marker positivity and improvements in the damaged muscles. (E) Negative control section without primary antibody, showing no expression of the Myf6 marker, thus demonstrating an absence of nonspecific staining.

Discussion

This study successfully administered a cell-based therapy that rebuilt damaged skeletal muscles by using myocytes derived from MSCs. The transplanted cells were found to promote muscle regeneration. Regenerative medicine is aimed at creating new types of cells, tissues, or organs through stem cell differentiation or adult cell reprogramming.[17] In the case of myogenesis, newly formed cells are incorporated into damaged muscles with myofiber formation.[18]

Many common clinical malfunctions in skeletal muscles occur because of degenerative pathologies, congenital diseases, traumas, cancers, metabolic syndromes, and aging, and subsequently increase the risk of muscle diseases. In adults, skeletal muscle regeneration depends on satellite cell function, which is gradually impaired after continued regeneration during aging.[19] Various cellular therapies are available for muscular dystrophies but have had limited success.[20] Large-scale cultures of stem cells differentiated into myocytes and myoblasts are the most important approach for skeletal muscle regeneration for clinical use.[19]

We created myocytes that formed myotubes *in vitro*, as demonstrated in morphological studies using light microscopy, H&E staining, and scanning electron microscopy. Joshi and colleagues,[21] Carotenuto et al.,[22] and others[23,24] have used H&E staining to study morphological changes in myoblasts differentiating into myotubes in cell culture. Moreover, Clemente et al.[25] have morphologically confirmed the myotube formation in myogenesis through H&E staining. Jarocha and colleagues[26] have studied the fusion potential of cultured myoblasts in various media, and have confirmed the results morphologically through staining with Wright's eosin methylene blue solution.

Myotube induction occurs because of loss of cell anchorage or anchorage to unsuitable and disrupted extracellular matrix proteins, and cells eventually detach from the culture plates.[27] These cells were initially spindle-shaped, but became larger and more rectangular with increasing culture time, whereas the cell morphology did not show any significant changes in the cells cultured in medium without differentiation factors. Alimperti and colleagues have demonstrated that MSCs differentiate into contractile smooth muscle cells when cultured in a growth/myogenic differentiation medium containing basic FGF, owing to the formation of adherents junctions.[28] This process is mediated by cadherin-11, a regulator of myogenesis.[28]

SEM examination revealed that cells increased in length during the differentiation period and showed marked morphological changes characterized by elongation; by the third day, the cells had started to align to each other to begin myotube formation, the most notable myocyte feature. In a SEM study by Barbon and colleagues, stem cells under myogenic stimulation have been found to align in parallel with one another on day 3 of differentiation, form packed bundles at 7 days, and stratify and fuse with one another at 14 days, thereby forming multinucleated structures resembling myotubes.[29]

In our study, the differentiation of MSCs into myogenic progenitors was confirmed by the expression of the regulatory factor Myf-6 in skeletal muscles, as well as desmin, and myosin heavy chain. Myf-6, encoded by the MYF6 gene, is a myogenic regulatory factor also known as MRF4. Myogenic regulatory factor (MRF) expression could be adequate to program satellite cells for injury-induced myogenesis.[30] The expression of the myf6 lineage is critical for myogenesis.[31] Consequently, the positive cells in our study provided a strong confirmation of myogenesis induction. MYF6 is a marker of late muscle differentiation and is responsible for the terminal differentiation of muscle fibers.[32] Our results showed cellular and nuclear localization, in agreement with a report by Thompson and colleagues indicating that the cellular distribution of MYF6 is both cytoplasmic and nuclear.[33] Ferri and colleagues have confirmed MyF6 presence in the cytoplasm by MRF4 (myf6) functions as a positive transcriptional regulator involved in the myotube maturation during terminal differentiation. MRF4 protein is subject to negative regulation via phosphorylation by PKA and PKC both *in vivo* and *in vitro*, thus explaining its preferential cytoplasmic localization. Furthermore, MRFs traffic to the nucleo-cytoplasm and subsequently control muscle differentiation. Thompson and colleagues have proposed that MRFs subcellular localization has a regulatory function in transcriptional control mechanisms.[34] Desmin and MHC were also found to be positive. Romagnoli et al.[35] have described MHC as one of the most important proteins in skeletal muscle, with roles in muscle movement and contraction, and have observed MHC, desmin, and MRF4 positivity after 9 days of myogenic differentiation *in vitro*.

Morphological analysis indicated that stem cells under differentiation continue to proliferate and produce a myoblast population. These muscle precursor cells differentiate into myocytes—mature muscle cells—and lose the MSCs phenotype along with the expression of MHC, desmin, and Myf-6. Myoblasts undergo fusion, thereby forming new multinucleated myofibers and mature skeletal muscle. Dedicated myoblasts are differentiated through the inhibition of tumor suppressor proteins, such as retinoblastoma (Rb) and ARF.[36] The expression of myogenic markers demonstrated the myogenic potential of MSC cells, given that MHC, desmin, and Myf-6 are uniquely expressed in skeletal muscles.[37] Our *in vitro* differentiation study confirmed that MHC, desmin, and Myf-6 were expressed relatively late in the differentiation process. The factor Myf-6 showed two-phase expression, in contrast to the other myogenic regulatory factor genes. Myf-6 and Myf-5 are transiently expressed in the somites throughout the initial steps of myogenesis.[32]

The differentiation cocktail used in our study included several factors that may activate several genes responsible for morphological changes, such as myotube formation. A group of protein kinases, specifically Akt1/2s, with roles in cell growth and protein synthesis, is needed to activate histone acetyltransferases, such as p300 and the CREB binding protein to the chromatin of muscle genes.[38,39] Akt1 activates myotube anabolism under stretching stimuli. In myoblasts *in vitro*, static mechanical stretching causes myotube alignment and fusion, thereby resulting in myotube hypertrophy.[40] Another important component of the differentiation induction cocktail is the glucocorticoid dexamethasone. Dexamethasone improves myogenic differentiation by upregulating specific myogenic transcription factors and the synthesis of dysferlin.[36] Another important differentiation factor, basic FGF, stimulates myoblast proliferation.[41]

The current investigation indicated that myocytes elongated and aligned from day 5, and formation of premyofibers and promyotubes revealed clear details regarding the development of myotubes preliminary with migration of nuclei, after unification of the myocytes with each other to form the final shape of myotubes at days 6 and 7.

MRFs are transcription factors with fundamental roles in controlling skeletal muscle cells' embryonic differentiation and adult myogenesis.[42] These transcription factors promote the expression of several muscle-specific genes and contain a basic helix-loop-helix DNA-binding domain. The MRF family members MyoD and myogenin are two crucial transcription factors in myogenesis,[43] which are upregulated in the initial stages of myogenesis and eventually slow myoblast proliferation and fusion by upregulating cell-cycle inhibitors (p21) and down-regulating cell-cycle activators, such as cyclins and cyclin-dependent kinases.[44] As observed in myogenin-deficient knockout mice, in which muscle generation is halted because of insufficient myoblast fusion, myogenin emerges in later stages of differentiation and functions in the development of multinucleated muscle tissue.[45] These transcription factors are elevated during skeletal muscle formation and can serve as differentiation markers. Muscle fibers comprise cells with a continuous cytoplasm containing several hundred nuclei. Consequently, the regeneration process is believed to involve the fusion of mononucleated precursor cells or the fragmentation of decaying muscle fibers, which may generate new cells.[46] Multinucleated fibers are established through the fusion of single cells, as shown in Figure 2. Satellite cells (also known as muscle stem cells) play crucial roles in muscle repair and renewal. The myofibers are

surrounded by a membrane-enclosed compartment containing these cells, which is formed by the sarcolemma (plasma membrane) and the basal lamina. In their normal state, muscle stem cells are dormant, as discovered by Mauro and colleagues in 1961.[47,48] During *in vitro* differentiation, BM-MSCs have different fates, thus giving rise to Myof-6 expression, and increasing muscle volume and myofiber size. In skeletal muscle regeneration, satellite cells play a central role. In response to injury, satellite cells are activated; proliferate and differentiate into myoblasts; and re-express myogenic regulatory factors such as MyoD, Myfg, myogenin, and MyF6.[49] Our histological findings suggested that myocyte transplantation might result in better tissue recovery than naïve stem cell transplantation; however, functional studies remain necessary to confirm the results and address this limitation of our study. Furthermore, we recommend the use of labeled cells in *in vivo* studies to enhance the tracking of transplanted cells.

Conclusion

This work demonstrates the possibility of generating muscle tissue *in vitro* through cell culture with suitable growth factors and transplantation *in vivo*, as a treatment for muscular atrophy and damage. The differentiated multinucleated skeletal muscle cells expressed MHC, desmin, and Myf-6 protein, and formed myotubes. This work confirmed that muscle tissue can be generated *in vitro*, and used to rebuild damaged skeletal muscles and accelerate tissue recovery, thereby achieving better results than transplantation of undifferentiated stem cells.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

Animal testing was conducted according to the guidelines established by the ICCMGR for the care and use of experimental animals. The ICCMGR Scientific Committee approved all operations involving experimental animals (N.10 on October 24, 2016).

Authors contributions

AMA conceived and designed the study. AMA, AAH, MIS, EGK, AKA, AAA, and HKS conducted research, provided research materials, and collected and organized data. AMA, AAH, and MIS analyzed and interpreted data. AMA, MIS, and EGK wrote the initial and final drafts of the article, and provided logistic support. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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