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Original Paper

Human Adipose-Derived Pericytes: Biological Characterization and Reprogramming into Induced Pluripotent Stem Cells

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Key Words
PCs • multipotent stem cells • adipose tissue stem cells • induced pluripotent stem cells (iPSCs)

Abstract
Background/Aims: Pericytes (PCs) are multipotent vascular precursors that play a critical physiological role in the development and maintenance of blood vessel integrity. In this study, we aim to characterize PCs isolated from human abdominal adipose tissue and develop an integration-free induced pluripotent stem cells (iPSCs) using episomal vectors. ***Methods:*** The ultrastructure of adipose tissue-derived PCs was determined using scanning and transmission electron microscopy. The expression of mesenchymal stem cells (MSCs) and pericyte markers were examined using flow cytometry and PCR analysis. PCs were induced to adipogenic, osteogenic and myogenic lineages, and their angiogenic potential was determined using tube formation assay. We further established pericyte reprogramming protocol using episomal vectors. ***Results:*** Our data showed that human adipose tissue-derived PCs uniformly expressed MSCs, CD105 and CD73, and PCs markers, desmin, and alpha smooth muscle actin (α-SMA), while lacked the expression of HLA-DR and the hematopoietic markers CD34, CD11b and CD45. Ultrastructure analysis showed typical internal structure for the PCs with a characteristic prominent eccentric nuclei and cytoplasmic invaginations forming a caveolar system. Functional analysis showed efficient differentiation into adipocytes, osteocytes, and myocyte-like cells. Adipose tissue-derived PCs showed angiogenic potential using tube-forming assay. To determine further application of these cells for personalized therapy, we reprogrammed PCs into induced pluripotent stem cells (iPSCs) using episomal vectors. Reprogrammed cells gradually lost their fusiform shape, acquired the epithelial cell morphology and formed colonies. Furthermore, reprogrammed cells successfully expressed the pluripotency markers

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OCT4, Nanog, SSEA-4, and β-catenin, an early reprogramming marker. ***Conclusion:*** The accessibility and abundance of human fat supports the application of adipose derived PCs as a novel and promising source of cell therapy and regenerative medicine.

Introduction

Mesenchymal stem cells (MSCs) are adult stem cells with self-renewal capacity and the potential to undergo multipotent differentiation into adipocytes, osteocytes and chondrocytes [1]. MSCs were first isolated in the sixties by Friedenstein et al. from rodent bone marrow as adherent fibroblastoid cells [2, 3]. The term "mesenchymal stem cells" was later coined by Caplan [4]. MSCs were later isolated from different tissues, such as adipose tissue [5], umbilical cord [6], umbilical cord blood [7], placenta [8], amniotic fluid [9], muscle [10], dental pulp [11], and tendons [12]. While MSCs from marrow and umbilical cord origin showed promise for clinical purposes [13-15], adipose tissue-derived MSCs are showing superior regenerative capacities in the pre-clinical setting [16]. General criteria for MSCs reported by the Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) include the expression of CD105, CD73 and CD90 markers, and a lack of expression of the haematopoietic stem cells markers CD45, CD34, CD14, CD11b, CD79α, CD19, and HLA-DR. MSCs differentiate into chondrocytes, osteocytes and adipocytes *in vitro* and adhere to plastic [17]. MSCs have also been isolated from the vascular tissue in vascularized organs [18]. Perivascular cells, including pericytes (PCs) and adventitial cells (ACs), present with similar characteristics to MSCs, such as multipotent differentiation potential (adipogenic, chondrogenic, osteogenic and myogenic), surface expression of the CD73, CD90 and CD105 antigens, and a lack of expression of endothelial cell and hematopoietic surface markers (CD34, CD45, and CD31) [19, 20]. According to Crisan et al., PCs derive from a perivascular origin and are the *in vivo* counterparts of MSCs [18]. PCs are also called mural cells or Rouget cells by their discoverer Charles Rouget, who described them as non-pigmented adventitial cells in 1837 [21, 22]. In 1923, Zimmermann, a German anatomist, coined the term "pericytes" [22, 23]. Most PCs originate from the mesoderm, but others are derived from the neural crest, such as the PCs in the brain and retina [24]. PCs also develop directly from endothelial cells (ECs) within the bone marrow [25, 26]. PCs were named after their function in wrapping the endothelial cell in microvessels and blood capillaries (peri: around, cyte: cell [18]). An analysis of pericyte morphology shows fibroblast-like cells with a distinct nucleus, small cytoplasmic content, and several long processes surrounding the endothelial wall [27, 28]. In addition to small capillaries, venules and arterioles, PCs have been isolated from the subendothelial region of large vessels [29, 30]. PCs to regulate the differentiation and proliferation of the endothelial cells [31, 32], and have important roles in maintaining the integrity and stability of blood vessels and in establishing and maintaining both angiogenesis and vasculogenesis [33]. In the present study, we isolated and characterized PCs from human adipose tissue and induced them to differentiate into cells of multiple lineages. We further reprogramed PCs into induced iPSCs using episomal vectors, without the need for viral integration. Reprogrammed PCs might thus prove to be a valuable tool in regenerative medicine as they are abundant and are easily obtained and isolated from various tissues.

Materials and Methods

Cell isolation and culture

Adipose tissue was collected as a byproduct from patients who underwent abdominal surgery at Al-Sheikh-Zayed Specialized Hospital after obtaining the informed consent and the approval of the Ethical Committee of the Hospital. All methods were performed in accordance with the relevant guidelines and regulations. The experimental design used to characterize the human adipose tissue-derived PCs and to generate an integration-free iPSCs using episomal vectors was illustrated in (Fig. 1). PCs were isolated

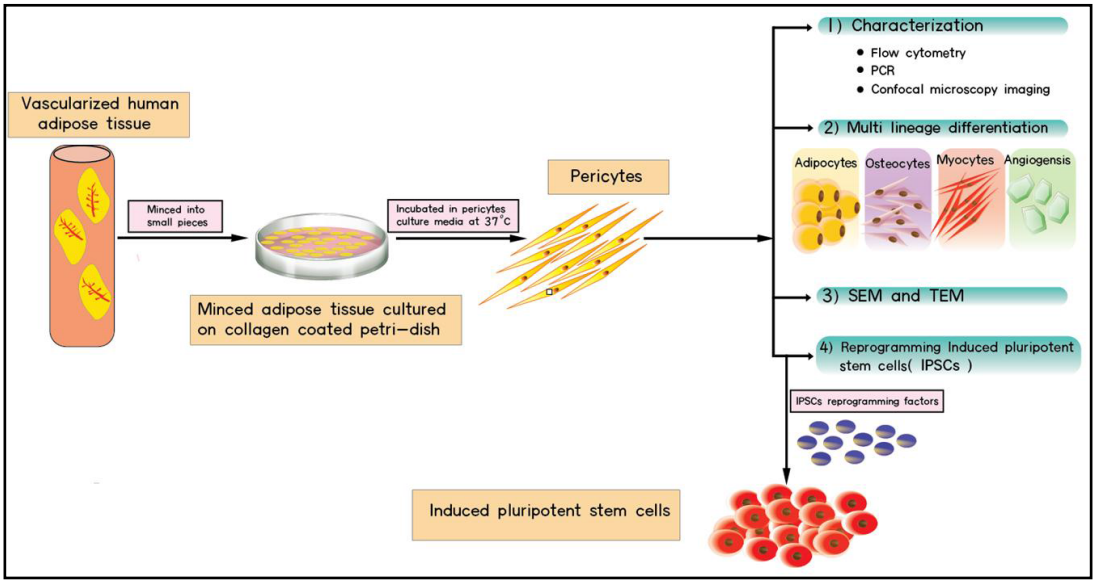

Fig. 1. Schematic diagram of the experimental design used in the current study.

from human adipose tissue using previously described methods [34], with minor modifications. Adipose tissues were dissected into small pieces and cultured on 0.1% collagen-coated 10 cm^2 Petri dishes (Serva, Germany). After at least 3 hours of adhesion, pericyte culture media were added (Dulbecco's Modified Eagle's Medium (DMEM)-mega-cell supplemented with 10% FBS (Sigma Aldrich, USA), 2 mM glutamine, 100 U/ml penicillin-streptomycin amphotericin, 1% non-essential amino acids (Lonza, Switzerland), 0.1 mM β-mercaptoethanol (Serva, Germany) and 5 ng/ml basic fibroblast growth factor (Gibco, USA). After 2 weeks in culture, both floating and adherent cells were transferred to non-coated Petri dishes, which was considered passage one. Cells were trypsinized when they reached 70-80% confluence. Human adipose-derived PCs (up to passage 10) were imaged using an inverted microscope (Leica, DMI8, Germany).

Flow cytometry characterization

PCs were characterized using fluorescence-activated cell sorting (FACSCalibur, Becton Dickinson, USA). For surface markers staining, cells were blocked with 4% bovine serum albumin and stained with the following antibodies: FITC-conjugated CD34, PE-CD11b, APC-HLADR, FITC-CD73, Per-CP-CD105 and APC-CD45 (BD Biolegend, USA). For intracellular markers staining, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 4% BSA and then stained with anti-alpha smooth muscle actin (Abcam, UK), anti-desmin (Abcam, UK), anti-Oct4 (R&D Systems, USA), anti-Sox2 (R&D systems, USA) and anti-Nanog (Bioss Antibodies, USA) antibodies, followed by specific secondary antibodies to examine the expression of intracellular markers using a FACSCalibur flow cytometer (Becton Dickinson, USA) using standard procedures and were analyzed by CellQuest Pro software (Becton Dickinson, USA).

Conventional PCR analysis

Total RNA was extracted from PCs using the PureLink® RNA Mini Kit (Thermo Fisher Scientific, USA). The total RNA was reverse-transcribed into cDNAs using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The amplification of alpha smooth muscle actin (α-SMA) and β-actin was carried out using the following primers: α-SMA sense, 5'-CCGACCGAATGCAGAAGGA-3' and anti-sense, 3'-ACAGAGTATTTGCGCTCCGAA-5'; β-actin sense, 5'-AGAGCTACGAGCTGCCTGAC-3' and anti-sense, 5' AGCACTGTG TTGGCGTACAG-3' (Invitrogen, USA), PCR product was loaded on gel and bands were visualized using a Chemi Doc imaging system.

Angiogenic tube formation assay

PCs diluted in supplemented medium 200 were cultured on Geltrex® LDEV-Free Reduced Growth Factor Basement Membrane Matrix -coated plates (Gibco, USA) at a density of 20,000 cells/cm^2 (angiogenic starter kit, Gibco, USA). Cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2. Twelve hours after seeding, the cells were stained with 2 µg/ml of Calcein AM, then incubated at 37°C in a 5% CO_2 atmosphere for 30 minutes, and imaged at 5X magnification using phase contrast and florescence inverted microscopes (Leica, Germany).

In vitro adipogenic, osteogenic and myogenic differentiation

PCs were cultured in adipogenic differentiation medium consisting of DMEM (Lonza) supplemented with 10% FBS (Sigma), 2 mM L-glutamine, 100 U/ml penicillin-streptomycin amphotericin (Lonza, Switzerland), 0.5 mM isobutyl-methyl-xanthine, 1 µM dexamethasone (Serva, Germany), 10 µM insulin and 200 µM indomethacin (Acros Organics, USA) to induce differentiation into cells of the adipogenic lineage. Lipid droplets were visualized using Oil red O staining (Acros Organics, USA). PCs were cultured in osteogenic differentiation medium consisting of DMEM (Lonza) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin amphotericin (Lonza), 0.1 µM dexamethasone (Serva, Germany), 50 µM L-ascorbic acid and 10 mM β-glycerophosphate (Sigma Aldrich, USA) to induce differentiation into cells of the osteogenic lineage. The extracellular calcified mineral matrix was visualized using Alizarin red staining (Alpha Chemika, India).

PCs were cultured in myogenic differentiation medium (DMEM)-mega-cell, supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin-streptomycin amphotericin, 1% non-essential amino acids (Lonza, Switzerland), 0.1 mM β-mercaptoethanol (Serva, Germany), 5 ng/ml basic fibroblast growth factor (Gibco, USA) and 10 µM 5-Aza-2'-deoxycytidine (5-Aza) (Abcam, UK). After 24 hrs, the myogenic differentiation medium was removed; cells were washed twice with PBS to remove any remaining toxic azacytidine and were cultured for two weeks in normal pericyte culture medium. Cells were imaged at different times using an inverted microscope (Leica, Germany) until the end of the second week.

Confocal immunofluorescence imaging

PCs were cultured on coverslips for 24 hours, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 4% BSA. Cells were then stained with anti-desmin and anti-alpha smooth muscle actin antibodies (Abcam), labeled with the appropriate secondary antibodies, and stained with Hoechst 33342. Cells were mounted using ProLong gold antifade mounting medium (Molecular Probes, USA) and imaged using a 60x objective with a Nikon A1R inverted laser scanning confocal microscope (Nikon Microsystems, France).

Transmission electron microscopy (TEM) imaging

PCs were rapidly fixed with 0.1 M cacodylate-buffered 2% glutaraldehyde for 2 hours at 4°C. Cells were then washed with equal volumes of 0.4% sucrose and 0.2% cacodylate for 2 hours before fixation with equal volumes of 0.2% cacodylate and 2% osmic acid for 1 hour. Afterwards, PCs were washed twice with distilled water. Dehydration was performed by incubating cells with an ascending series of ethyl alcohol for 5 min each; then, the cells were cleared with propylene oxide. Finally, PCs were embedded in epoxy resin. The polymerized resin blocks were cut into semithin sections (1 µm thick) with an ultramicrotome (American Optical Co., USA). These sections were then routinely stained with methylene blue and azure mix for examination under a light microscope. Ultrathin sections (60 nm thick) were obtained using a diamond knife and the same ultramicrotome, and then mounted on perforated copper grids (Electron Microscopy Science, Hatfield, USA). Finally, the ultrathin sections were stained with uranyl acetate and lead citrate. Examinations and imaging were performed using TEM (TEM, Philips EM 208S, Tokyo, Japan) at an 80 kV acceleration voltage.

Scanning electron microscopy (SEM) imaging

PCs were processed using the same procedure as described for TEM until dehydration with an ascending series of ethyl alcohol (30%, 50%, 70% and 90%) for 5 min each. Cells were then rinsed with absolute ethyl alcohol 3 times for 3 min each and were examined on the Formvar coating grids by an Environmental SEM (Inspect S50; FEI, Holland).

Induced reprogramming of PCs using an episomal plasmid vector

PCs were transfected as previously described [35]. Briefly, bacteria expressing plasmids carrying reprogramming factors, Addgene: 27077 (pCXLE-hOct3/4-shp53-F), 27078 (pCXLE-hSK), and 27080 (pCXLE-hUL), were thawed and incubated at 37°C in Luria broth media (LB media) overnight; the plasmids were then purified using the PureYield™ Plasmid Miniprep System (Promega). In total, $6x10^6$cells were trypsinized, resuspended in 1 ml of optimum medium, and centrifuged at 400xg to remove any traces of the culture medium, which may affect cell viability during electroporation. Finally, cells were resuspended in 200 µl of optimum medium and elctroporated with 2 µg of purified plasmids in 0.4 cm Gene Pulser cuvettes (Bio-Rad) using a Gene Pulser electroporator (Bio-Rad). All transfection parameters were adjusted to a voltage of 400 V and a duration of 17 msec. After transfection, cells were incubated at room temperature for 2-3 minutes to allow them to recover from electroporation and then cultured in 20% FBS-supplemented pericyte culture medium on gelatin-coated six-well plates. Twenty-four hours after transfection, the culture medium of the transfected cells was changed and refreshed every other day. On day 5, some of the observed colonies were picked up under the stereomicroscope (Zeiss, stemi508, Germany) and cultured for 24 hours on a coverslip for immunofluorescence staining. Attached colonies were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 4% BSA and stained with anti-Oct4 (R&D Systems, USA), anti-Nanog (Bioss Antibodies, USA) and anti-β-catenin (Cell Signalling Technology, USA) antibodies, labeled with specific secondary antibodies, and imaged using a Leica inverted florescence microscope (Leica, DMI8, Germany). On day 13, transfected cells were stained with anti-SSEA-4 and data were acquired with a FACSCalibur flow cytometer (Becton Dickinson, USA). After two weeks, transfected cells were cultured in mouse embryonic fibroblast-conditioned medium (DMEM F-12, Lonza) supplemented with 20% knock-out serum (Gibco, USA), 2 mM glutamine, 100 U/ml penicillin-streptomycin amphotericin, 1% non-essential amino acids (Lonza), 0.1 mM β-mercaptoethanol and 5 ng/ml basic fibroblast growth factor.

Results

Generation of fibroblast-like cells from human adipose tissues

After 5 days of culture of human adipose tissue on collagen-coated Petri dishes, fibroblast-like cells began to be released. The floating cells were discarded, and the adherent cells were transferred to uncoated Petri dishes and passaged when they reached 70-80% confluence, for up to 10 passages. Adherent cells cultured in pericyte-specific medium showed a spindle-shaped fibroblast-like morphology with a prominent eccentric nucleus and cytoplasmic projections (Fig. 2A).

Cells isolated from human adipose tissue express pericyte markers

Fibroblast-like cells isolated form human adipose tissue expressed common pericyte markers, as determined by intracellular staining and flow cytometry. Cells were positive for desmin and alpha smooth muscle actin as showed by confocal microscopy imaging and flow cytometry (Fig. 2B-D). Also, cells expressed CD73 and CD105 specific markers of MSCs, but not the hematopoietic markers CD34, HLADR, CD11b and CD45 (Fig. 2C). Furthermore, cells expressed the intracellular pluripotency markers Oct4, Sox2 and Nanog (Fig. 2D).

Differentiation of PCs into cells of adipogenic, myogenic and osteogenic lineages

The adipogenic and osteogenic differentiation potential of PCs was qualitatively determined on day 17 after induction. Differentiated PCs showed positive staining for Oil red O (adipocyte-specific staining) and Alizarin red (osteocyte-positive staining) (Fig. 3A and C). Myogenic potential was determined by treating PCs with azacytidine for 24 hrs. The cells started to elongate on day 5 of culture in normal pericyte culture medium, and developed into myocyte-like cells at day 14 (Fig. 3B).

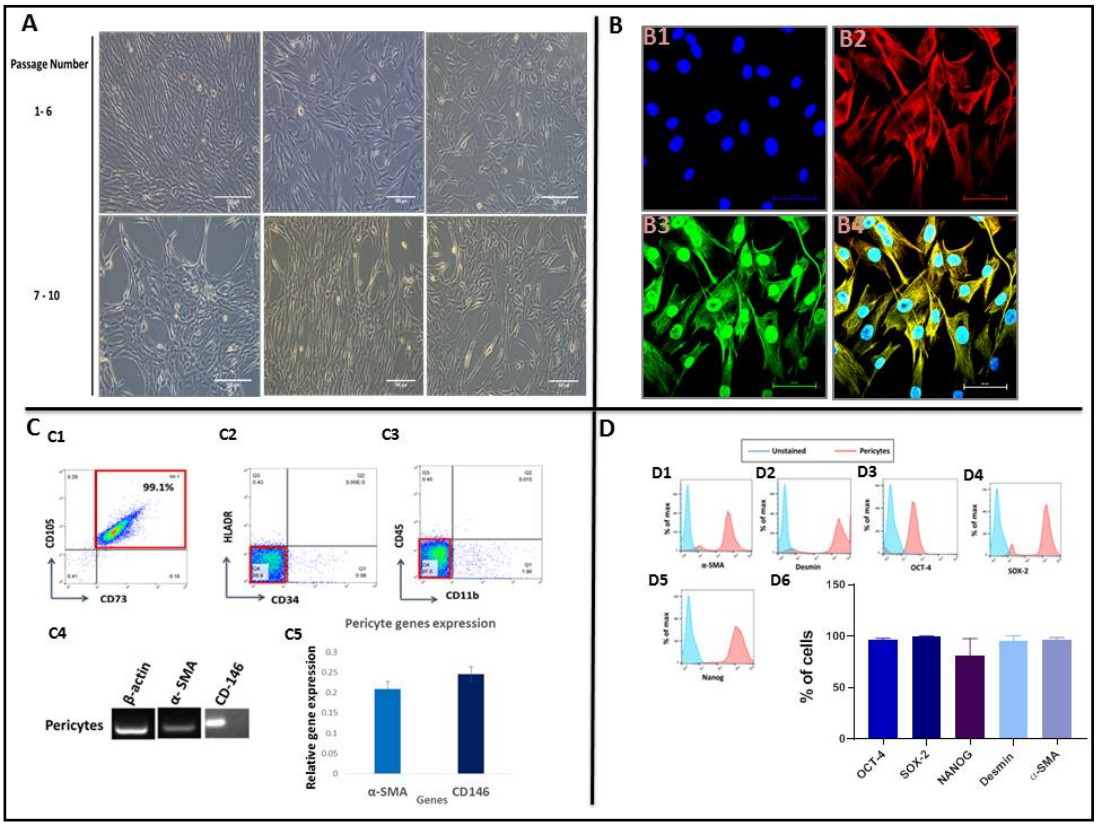

Fig. 2. Morphological, phenotypic and genotypic analyses of pericytes. A. Images of human adipose-derived pericytes at different passages captured using an inverted microscope: (A-C) p1-p6, and (D-F) p7-p10. B. Confocal microscope images showing the expression of alpha smooth muscle actin and desmin. B1. nuclear staining using Hoechst, B2. alpha smooth muscle actin expression, B3. desmin expression, and B4. merged image from B1-B3. C. Flow cytometry analysis of human adipose tissue-derived pericytes. (C1-C3) showing expression of the mesenchymal stem cells markers CD105 and CD73, but not HLA-DR, or the hematopoietic stem cells markers CD34, CD45 and CD11b. C4,5. Conventional PCR analysis showing the expression of alpha smooth muscle actin and CD146. D. Flow cytometry analysis of human adipose tissue-derived pericytes. (D1-D6) showing expression of the pericyte markers desmin and alpha smooth muscle actin and the pluripotency markers Oct4, Sox2 and Nanog.

Angiogenic potential of PCs, as determined by the tube formation assay

Based on the fundamental function of PCs in the formation of new vessels, we analyzed the angiogenic differentiation potential of PCs using an angiogenesis differentiation kit. Our data showed robust tube formation compared to control cells (Fig. 3D).

TEM and SEM imaging of PCs

An examination of the ultrathin sections by TEM showed elongated cell bodies with longitudinal cytoplasmic processes. The cell surface showed a characteristic caveolar system composed of plasma membrane invaginations near the cell membrane and clearly visible cytoplasmic organelles. These organelles included smooth and rough endoplasmic reticulum, the Golgi apparatus, and mitochondria, which were located around the nucleus of each pericyte. Many intermediate filaments had assembled in the cytoplasm, while dense granules were appeared. Large vesicles were observed within the cytoplasm and in close proximity to the cytoplasmic processes. The cells showed prominent nuclei with prominent chromatin filaments and distinct nucleoli (Fig. 4A-F). SEM imaging showed a pericyte topography with many cytoplasmic processes, for which the diameter ranged from 19.90 µm to 25.70 µm (Fig. 4G-I).

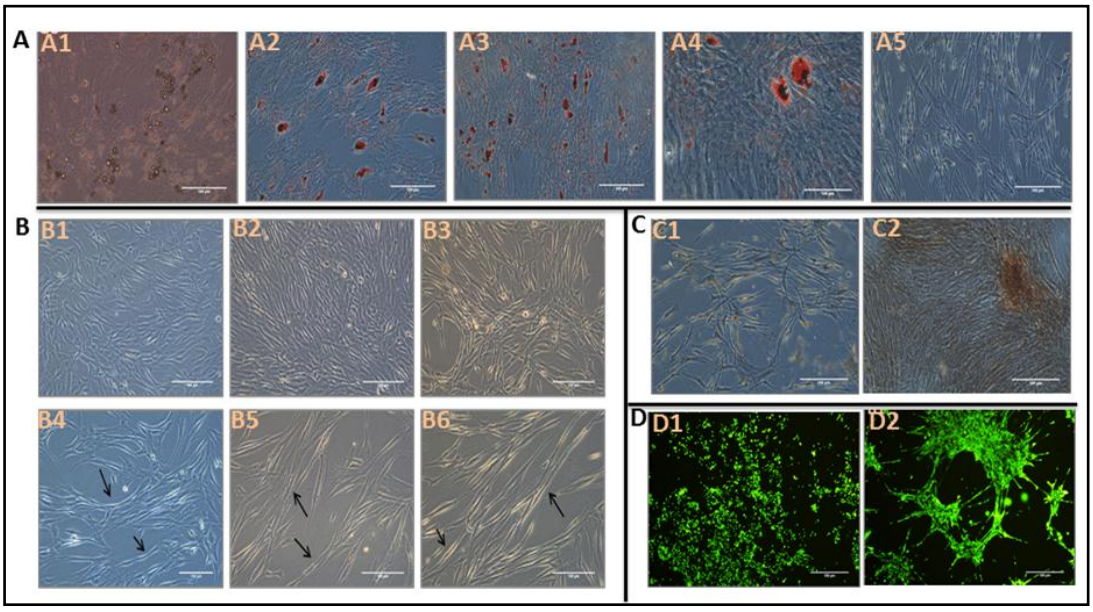

Fig. 3. Inverted microscope images showing multipotent differentiation potential of pericytes. A. Images of the adipogenic differentiation of pericytes on day 17 A1. shows the formation of lipid droplets in response to adipogenic induction before staining, (A2 and 3) showing differentiated pericytes on day 17 after staining with Oil red O, A4. magnified image of A3, A5. control pericytes that were not subjected to adipogenic induction but were stained with Oil red O. B. Images showing the myogenic differentiation of pericytes using 5-azacytidine, (B1-B3) control pericytes that not subjected to myogenic induction; black arrows indicate elongated myoblast-like cells on days 7,13 and 14. C. Images showing the osteogenic differentiation of pericytes on day 17. C1. images of Alizarin red-stained control cells that were not induced to undergo osteogenic differentiation. C2. showing Alizarin red-stained cells after osteogenic induction. D. Angiogenic differentiation (tube formation assay) of pericytes captured using an inverted fluorescence microscope. D1. control cells stained with Calcein AM without angiogenic induction. D2. cells stained with Calcein AM after angiogenic induction.

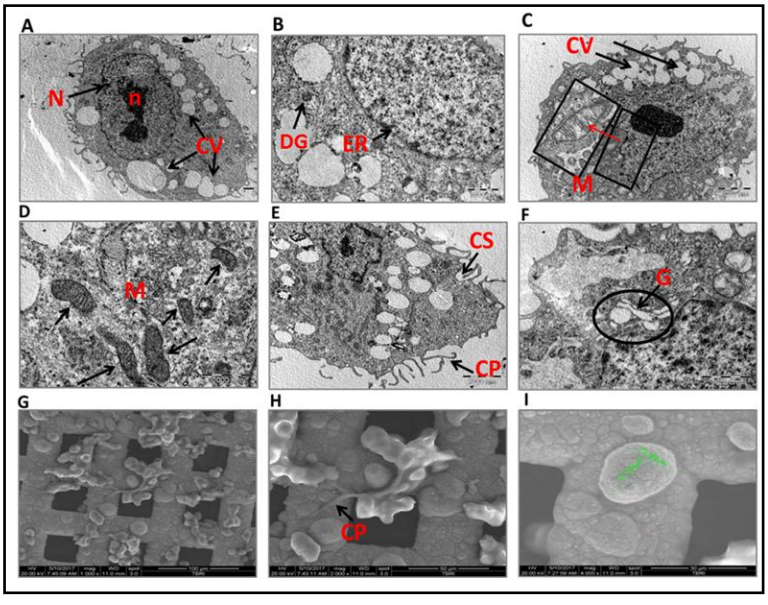

Fig. 4. Electron micrographs of pericytes showing intracellular organelles. A. Nucleus (N), nucleolus (n), cytoplasmic vesicles (CV). B. magnified image showing the endoplasmic reticulum (ER) and dense granules (DG). C and D. Mitochondria (M) are located close to the nucleus; the red arrow shows a magnified mitochondrion with its transverse cristae. E. The cytoplasmic processes (CP) of pericytes form the caveolae system (CS). F. Golgi bodies (G) are located close to the nucleus. G-I. Images showing the pericyte topography. H. Magnification of the image shown in G, (CP), cytoplasmic processes. I. Image showing the pericyte diameter, X4000.

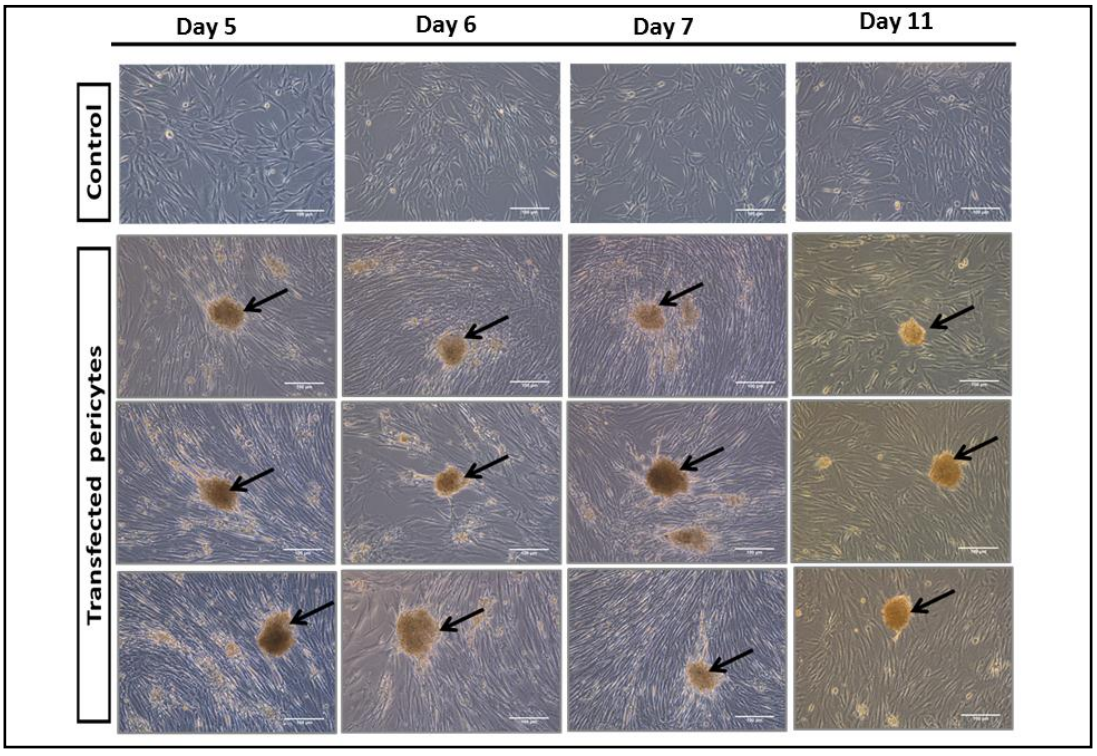

Fig. 5. Microscopic images of reprogrammed pericytes after transfection with episomal vectors using electroporation; black arrows show colonies of transfected cells on days 5-11.

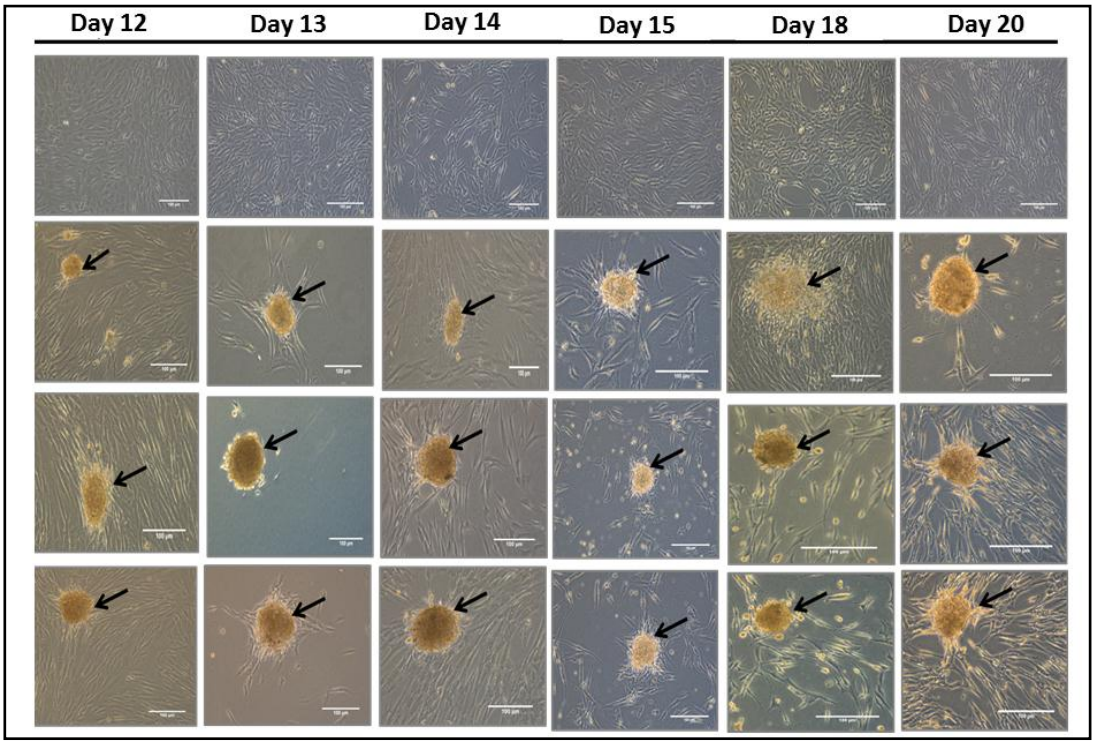

Fig. 6. Microscopic images of reprogrammed pericytes after transfection with episomal vectors using electroporation; black arrows show colonies of transfected cells on days 12-20.

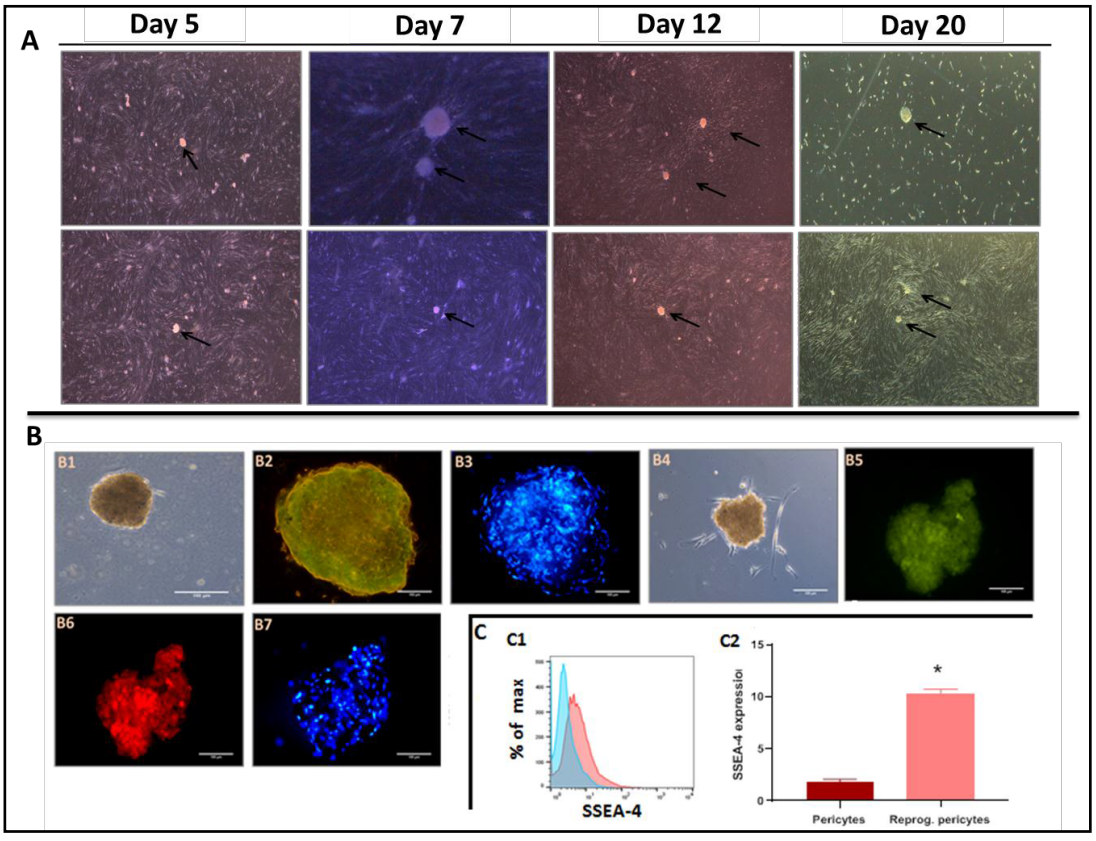

Fig. 7. Characterization of iPSC-like colonies. A. Stereo-microscope images of reprogrammed pericytes showing regular-shaped colonies with sharp boarders (days 5-20). B. Inverted florescence microscope images of pericyte-derived iPSC-like colonies attached to a cover slip after they were picked on day 5. B1. BF (bright field) image of an attached colony. B2. fluorescence image of the same colony showing the expression of b-catenin. B3. nuclear staining with Hoechst 33342. B4. An image of another attached colony. B5 and B6. florescence images of the same colony showing positive expression of the pluripotency markers Oct-4 and Nanog . B7. nuclear staining with Hoechst 33342. C. Flow cytometry analysis of iPSC colonies. C1, C2. Showing the expression of SSEA-4, a highly specific embryonic stem cell marker, before and after reprogramming into iPSCs.

Transfected human adipose-derived PCs showed iPSC-like colonies

Human adipose-derived PCs were reprogrammed into iPSCs as shown by the morphology, formation of colonies, and expression of pluripotency markers. PCs were transfected with plasmid episomal vectors, without the need for viral integration, as described in the above-mentioned protocol. Five days after transfection, microscopic imaging showed that some cells began to lose the fusiform morphology, acquire epithelial cell morphology, and form colonies (Fig. 5). Colony formation became more pronounced and homogenous over time (Fig. 6). On day 5, colonies imaged using stereomicroscopy (Fig. 7A) showed expression of the pluripotency markers Oct4, Nanog and β-catenin (Fig. 7B). Reprogrammed cells also expressed SSEA-4, a pluripotency marker, when analyzed by flow cytometry on day 13 (Fig. 6C). Finally, the reprogramming efficiency was calculated to be 0.007% (number of colonies divided by number of starting cells after electroporation) [36, 37].

Discussion

In this study, we have successfully isolated and characterized PCs from human adipose tissue. These cells displayed similar ultrastructure to PCs derived from other sources, displayed robust angiogenic potential, and could be induced into pluripotent stem cells using bacterial episomal vectors, and without viral integration.

Multipotent perivascular adherent cells (PCs) have been isolated from various tissues using different isolation procedures [18, 38]. Although specific markers of those cells were not identified in most of these reports, PCs from different tissues share the common characteristics we have described in this study. The cultured cells displayed the typical pericyte morphology and expressed surface markers similar to those reported in the literature [39, 40].

Adipose tissue-derived PCs expressed desmin and alpha smooth muscle actin, in addition to mesenchymal stem markers, and lacked hematopoietic and endothelial markers, confirming their pericyte phenotype. One of the most recognized physiological functions of PCs is the formation of new blood vessels to regulate blood flow [41]. They have been shown to enhance the survival and migration of endothelial cells [42-46], and their loss caused vessel destruction [47] and the rupture of capillaries at late gestation [48]. Furthermore, previous studies have reported that PCs ameliorate ischemia after being transplanted into a mouse model of severe hind limb ischemia [49, 50]. They were also found to improve the blood flow in mice during femoral artery ligation [51] indicating their promising therapeutic targets for vascular disorders [52]. Our data showed robust tube formation when adipose tissue-derived PCs were cultured in angiogenesis-inducing medium, further supporting their function in vasculogenesis. As PCs participate in angiogenesis during wound healing [53] and in tumor growth [54], they are also considered promising targets for vascular regeneration, or tumor suppression. It is important however to consider, when designing therapies, that their heterogeneity suggests that different pericyte subpopulations may have different functions, based on the tissue of origin and surface markers [52].

Adipose tissue-derived PCs have been shown to differentiate into adipocytes *in situ* as a result of thermal injury [55]. Thus, although disputed by some reports, PCs may be adipocyte progenitors [56]. The common phenotypic and molecular pathways shared between adipogenesis and vasculogenesis led to the hypothesis that vascular cells may indeed function as adipocyte progenitors [57, 58]. In our study, PCs differentiated into adipocytes under adipogenic differentiation conditions, suggesting multipotency or a propensity for adipogenic lineage. These data are consistent with findings from a previous study [59] reporting a correlation between fat production by vascular cells and atherosclerosis.

Important characteristics of PCs include myogenic differentiation and contribution to skeletal muscle formation [60]. After xenograft transplantation, PCs fused with myoblasts to form myotubes. Similarly, in cases of acute injury or muscle necrotic disease, such as muscular dystrophy, PCs helped regenerate muscles [61]. After muscle injury, a small population of transplanted PCs engrafted beneath the basal lamina of myofibres and express Pax7 [62]. PCs were thus reported to generate satellite cells, the *bona fide* muscle stem cells, leading to the proposal to use them for cell-based therapies to treat muscular dystrophies [63]. Our data support the multilineage differentiation potential of PCs into myocyte-like cells following azacytidine induction [64].

In this study we are analysing the surface and internal structure of human adipose tissue-derived PCs for the first time using scanning and transmission electron microscopy. The structure of PCs was studied in the sixties by Epling [65], who described cells with large nuclei compared to a small cytoplasmic content with dense patches of heterochromatin. Mitochondria were distributed close to the nucleus, and granular endoplasmic reticulum with frequent free ribosomes was also observed. A few years later, in 1968, Bruns and Palade confirmed these data and reported non-prominent Golgi saccules, multivesicular bodies and dense cytoplasmic particles, which were proposed to be glycogen [66]. According to our TEM and SEM data, adipose tissue-derived PCs have a similar morphology and

ultrastructure to muscle, lung and heart-derived PCs from model animals, such as rats, mice, swine and cattle. Adipose-derived PCs exhibited cytoplasmic processes forming caveolae or plasmalemmal vesicles in the perinuclear region and in the roots of processes. Caveolae are plasma membrane invaginations that are abundant in all blood vessels lining endothelial cells and PCs [67, 68]. Caveolae can bud to form cytoplasmic vesicles, which have the ability to act as carriers for the transport of proteins such as albumin to the inside of the cell [68]. These cytoplasmic vesicles have a diameter of 650-750 Å and a membrane similar to the plasmalemma. They are present as single units or are fused together to form short chains of a few vesicles. Each configuration may be completely located within the cytoplasm or continuous with the plasmalemma and open onto the cell surface [66].

In our study, caveolae of adipose tissue-derived PCs were not motile, consistent with earlier descriptions, and in line with their main functions of regulating microvascular permeability, discharging plasma "cargo" into the intracellular space and regulating pinocytosis. Caveolin-1 and vesicle-associated membrane proteins (VAMPs) were detected in PCs caveolae of human venules. VAMPs comprise a family of v-SNARES (SNAP-Receptors) which main role is to promote vesicle fusion with target membrane-bound compartments [69], such as lysosomes. The detection of VAMPs in pericyte caveolae thus suggests a role in vesicular containers that perform their function while remaining attached to the plasma membrane [70]. Future work aims to determine the role of VAMPs in the structure and function of adipose-derived PCs.

Previous studies have reported that cells which express high level of endogenous transcription factors could be highly useful in cell reprogramming [71]. Adipose-derived PCs were thus tested for the pluripotency marker expression. We determined that adipose-derived PCs express endogenous Oct4, Sox2 and Nanog. This finding was consistent with previous studies on adult adipose-derived stem cells [72, 73]. This expression of endogenous pluripotency markers is thought to be functional in pericyte reprogramming to iPSCs, and could enhance cellular reprogramming [71, 72].

After transfecting adipose tissue-derived PCs with Oct3/4, Sox2, Klf4, and c-Myc; morphological changes, high expression of pluripotency markers and colony formation all indicated successful reprogramming into iPSCs starting from day 5. It has been reported that starting with cells which have more pluripotent epigenomic profile compared to the terminally differentiated cells could ease the iPSCs generation [74]. This proposition was confirmed in our manuscript by the 0.007% reprogramming efficiency of PCs into iPSCs [36, 37]. The use of episomal vector transduction without using viral integration furthers the use of PCs in clinical applications [36]. Recent protocols using a variety of modulators to reprogram somatic cells into iPSCs showed high reprogramming efficiency [75-77]. For example, Kogut et al. showed that a combination of synthetic modified mRNAs encoding reprogramming factors and miRNA-367/302s could synergistically enhance the iPSCs generation from human primary fibroblasts with a reprogramming efficiency of up to 90.7% when started with neonatal fibroblasts [77]. Wang et al. have demonstrated that long-term hematopoietic stem cells have the potential to be reprogrammed into iPSCs with a reprogramming efficiency of ≈50 % using Sendai virus transduction method [78]. Reprogramming pericytes using synthetic molecules, and the safety of these protocols for clinical applications remain to be investigated.

Positive expression of β-catenin on day 5 provides an additional evidence for early-stage reprogramming, as reported by previous studies on the nuclear accumulation of β-catenin during the reprograming of mouse embryonic fibroblasts into iPSCs [79, 80]. Furthermore, it has been reported that β-catenin interacted with the reprograming factors of pluripotency; Oct4, Sox2, Klf4 which could enhance the endogenous expression of the pluripotency markers and activate the pluripotent network at the early stage of reprogramming [81]. Thus, β-catenin is critical for reprogramming but not for the maintenance of iPSCs identity [81, 82]. Reprogrammed cells in this study showed positive expression for Oct-4, Sox-2, nanog and SSEA-4. The expression of these markers, in addition to vimentin, was used by Liu et al. to reprogram peripheral blood using recombinant lentiviruses transfection with Sox2, OCT-4,

Klf4 and c-Myc [83]. Zhang et al. also showed iPSCs generation from PBMCs by Yamanaka factors (KMOS) transfection using Sendai-virus transfection [83]. Because of its availability and low invasiveness, adipose tissue is considered a clinically valuable source of stem cells. Furthermore, the *in-vitro* isolation, characterization and handling of human adipose tissue-derived PCs is simple compared to other adult stem cells. For these reasons we propose the use of adipose tissue derived PCs as source for iPSCs generation. Reprogrammed PCs might be applied to drug screening, and to studies aiming to understand disease mechanisms, toxicology, and regenerative medicine.

Conclusion

Pericytes hold reliable promise in vascular regenerative therapies, due to their physiological contribution to angiogenesis and maintenance of the blood vessel integrity. They are relatively easy to access and isolate from adipose tissue with minimally invasive procedures. Their potential capacity to differentiate into cells of different lineages, in addition to cells of vascular tissue suggests a potentially valuable contribution to treating a diverse array of diseases, and especially in vascular regenerative medicine. Future experimental studies to investigate the pathways by which PCs contribute in the process of angiogenesis during the physiological and pathological conditions are required. Controlling these pathways either by enhancement or inhibition could present an important approach in the treatment of different diseases with based on the vessel pathology. Another area for further research is to define the best extracellular matrix to enhance the angiogenic potential of the PCs, when transplanted in patients which vascular disorders. Lastly, many questions on the different growth factors, cytokines and pathways which control homing of PCs in cell-based therapy remain to be investigated.

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Statement of Ethics

Adipose tissue was collected as a surgical byproduct Al-Sheikh-Zayed Specialized Hospital after obtaining the informed consent and the approval of the Ethics Committee of the Hospital.

Author contributions

Toka A. Ahmed: conceived the idea, designed the experiments, pericytes isolation and characterization, adipogenic and angiogenic differentiation, reprograming of pericytes into iPSCs and wrote the manuscript.

Wafaa G. Shousha: osteogenic differentiation and revised the manuscript.

Sara M. Abdo: myogenic differentiation and revised the manuscript.

Ihab K. Mohamed: electron microscopy imaging and revised the manuscript.

Nagwa El-Badri: conceived the idea, designed and supervised the experiments, wrote and revised the manuscript.

Disclosure Statement

The authors have confirmed that no conflict of interests exits.

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