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Research Article

"Synapse-like" Connections between Adipocytes and Stem Cells: Morphological and Molecular Features of Human Adipose Tissue

Cristina Bertolotto[1], Juan Carlos Rosillo[2,3], Bibiana Botti[4], and Anabel S. Fernández[3*]

[1]Founder CBV-XStem, Inc. 2916 Oakhurst Ave. Los Angeles, USA

[2]Dpto. de Histología y Embriología, Facultad de Medicina,UdelaR Montevideo, Uruguay

[3]Instituto de Investigaciones Biológicas Clemente Estable- Facultad de Ciencias, UdelaR, Montevideo, Uruguay

[4]Clínica de Cirugía plástica Dr. R. Wolff-Montevideo-Uruguay

[*]**Corresponding authors**: Dr. Anabel S. Fernández, Instituto de Investigaciones Biológicas Clemente Estable- Facultad de Ciencias, UdelaR, Montevideo, Uruguay; E-mail: afernandez@iibce.edu.uy, anabelsonia@gmail.com

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Abstract

Adult mesenchymal stem cells are a heterogeneous population of stem cell that is not completely defined. The importance of increasing knowledge of this cell population will allow us to understand how to use them in multiple treatments for various diseases. Adipose tissue is a rich source from which to easily obtain a great amount of stem cells. We explore and study adipose stem cells (AdSCs) from human abdominal tissue by immunohistochemical analysis, transmission (TEM) and scanning (SEM) electron microscopy. The focus of this study is on the cell population that surrounds each adipocyte. In these populations of cells are included the adipose tissue stem cells (AdSCs) with different potentiality. We observe that the putative AdSCs have an intimate relationship involving close contact with adipocytes which we define here as "synapse-like." We show "synapse-like" connections between adipocytes and small cells to be mediated by connexin43 (Cx43). Our data suggest AdSCs constitute a heterogeneous population both in size and expression of different stem cell markers. We found that some of the AdSC attached to adipocytes are positive for Sox2, Pax6 and Nestin by immunostaining methods. TEM and SEM analysis demonstrate that small cells and adipocytes are surrounded by a compact mesh of collagen fibers that maintain physical adhesion between these cells. TEM also shows structural characteristics of putative stem cells and a presence of vesicles in synapse-like contact. SEM images moreover exhibited a large variation and quantity of cell sizes coexisting with adipocytes. Direct cell-to-cell communication could serve at least two purposes: a) as a survival strategy to maintain cells as stem cells, and b) as a source of signaling for differentiation into a new cell type.

Keywords: human, adipose tissue, stem cells, Cx43

Introduction

Mesenchymal stem cells (MSCs) were studied in multiple types of tissue and one was defined as Multilineage-differentiating stress-enduring (Muse) cells [1]. These cells correspond to a population of MSCs initially isolated from bone marrow, and a few years later were described in adipose tissue [2-6]. Muse cells are able to generate all cell types of endodermal, mesodermal, and ectodermal lineages; they are self-renewable and express

pluripotency markers. Muse cells can be considered as a small percentage of MSCs and are nontumorigenic [6-12]. It is known that most MSCs do not have the pluripotent-like characteristics of Muse cells. The precise composition of MSCs remains unclear and their diverse effects are not fully understood. The main problem in MSCs basic research has been that within most of these cells, there is a difficulty identifying pluripotency characteristics. Previous reports show that Muse cells can migrate specifically to damaged tissue, survive and differentiate spontaneously leading to tissue restoration and repair [13,14]. MSCs are generally stress tolerant, and while normally dormant are activated by damaged tissue stimuli [7,15]. They enter the cell cycle following activation and differentiate into cells like those in the damaged tissue thereby contributing to tissue regeneration and repair. These cells therefore fulfill the criteria for pluripotent stem cells. Muse cells are distributed sporadically in connective tissue of nearly every organ and do not seem to associate with a structural niche [7]. Importantly, cells other than Muse cells that comprise MSCs do not express pluripotency genes, nor do they cross oligolineage boundaries such as between mesoderm, ectoderm and endoderm [6,7,16,17]. Adipose tissue Muse cells are well known to be resistant to multiple stress conditions, in culture they express embryonic markers such as Nanog, Oct¾, Nestin, Sox2, Pax6 and human pluripotent stem cell marker, stage-specific embryonic antigen-3 (ssea-3) [6,8,10]. Little is known about the morphology of these pluripotent stem cell types and their physiological role in adipose tissue. Meanwhile, we describe morphological and molecular characteristics of AdSC. We show for the first time a “synapse-like” contact between human AdSCs and adipocytes. A detailed analysis of the morphological and molecular aspects of adipose tissue can lead to a new understanding of what is "synapse-like" and can elucidate the function of adipocytes for the survival of stem cells. The experimental approach used here may provide additional data obtained from prior studies done with cell cultures only. This information could help us better understand how pluripotent or multipotent stem cells survive under stress conditions. The “synapse-like” structure strongly suggests that AdSCs receive signalling via gap junctions for two purposes: a) to maintain pluri or multipotenciality and b) to allow for differentiation into other cell types.

Materials and Methods

Tissue

Discarded human adipose tissue from abdominal liposuction was obtained from eight adult patients aged between 40 and 56 years old, who underwent local anesthesia for esthetic surgery at the Dr. R. Wolff Clinic. The patients were consulted and an informed consent, which clarified what type of research would be done, was signed by each of them. All the samples were processed for immunohistochemical analysis, and three were processed for both transmission and scanning electron microscope analysis. Each sample was dissected then separated into fragments in order to be processed using different techniques.

Transmission electron microscopy (TEM) study

Adipose tissue of three samples was washed 3 times with saline solution (NaCl 0.9%) and fixed with 2.5% glutaraldehyde (Sigma-Aldrich) plus 2% paraformaldehyde (PFA) (Sigma-Aldrich) in 0.1 M phosphate buffer pH (PB) 7.2 overnight at 4°C. Then, tissue was transferred to PB and rinsed 3 times. Subsequently, tissue was post-fixed in 2% osmium tetroxide for 1.5 h and rinsed with PB three times before dehydration. Dehydration was done with an ascending ethanol series (50, 75, 95 and 100%), and then with anhydrous acetone before embedding in araldite resin (Durcupan, Sigma). Semithin sections (0.5-1 micron) were obtained using a Leica ultramicrotome (PowerTomeXL) and stained with 1% Boraxic Methylene Blue (BMB) before being observed in a Nikon Eclipse E 200 microscope. Images were taken with a Nikon COOLPIX 8400 digital camera. Ultrathin (60 nm) sections were cut with a glass knife, mounted on Formvar-coated slot grids (2x1 mm) and contrasted with 2% uranyl acetate and lead citrate in accordance with Reynolds´s method [18]. Individual cell types were characterized by examination using both a

Jeol100CX-II and a JeolJEM1010 TEM equipped with 4000 AM DVC and HAMAMATSU C-4742-95 digital cameras, respectively.

Scanning electron microscopy (SEM) analysis

Adipose tissue was rinsed with saline solution then fixed overnight by immersion in 2.5% glutaraldehyde at 4°C. After that, tissue was washed 3 times in 0.1 M PB pH 7.2 and postfixed in 2% osmium tetroxide for 1.5 h. The tissue was then rinsed 3 times with PB over 1 h and dehydrated in increasing ethanol concentrations and finally with acetone. Tissue was then subjected to critical point drying with CO2 in a Denton SPC-1 Critical Point Vacuum Drying Apparatus. Gold plating was then done in a Denton Vacuum Desk-II for 120 seconds. Samples were analysed in a Jeol-5900 LV SEM that acquires and displays the video signal with the X-Stream Imaging System.

Immunohistochemistry

Ovaries samples were obtained during surgery and processed for immunohistological analysis and were obtained from a previous study [29]. In brief, tissues were processed, and paraffin embedded as previously described. Slides containing 5 μm sections hematoxylin-eosin (H & E) stained were used for histomorphology analysis. Additionally, series of sections in parallel were processed for immunohistochemistry assays as previously described [29].

Immunohistochemistry

Adipose tissue was rinsed in saline and fixed in 4% PFA for immunohistochemical analysis. Tissue was spread onto microscope slides and air dried. Adipose tissue was strongly attached to glass slides. Prior to incubation with the primary antibodies the slides were rinsed with PB. Antibodies were placed in drops on the glass slides, and incubations were done overnight at 4°C in a humid chamber. Polyclonal rabbit anti-Sox2 1:500 dilution (abcam); polyclonal rabbit anti-Pax6 1:250 dilution (abcam); polyclonal chicken anti-Nestin (abcam) at 1:500 dilution; and polyclonal rabbit anti-Cx43 (abcam) at 1:1000 dilution was used. Primary and secondary antibodies were diluted in 0.3% triton X 100 in PB (PBT).

After rinsing with PB (3 times for 10 min), recognition of each label was done with secondary antibodies conjugated to fluorescent Alexa 633 (RED) (Invitrogen, CA, USA), both diluted 1:1000 in PBT for 1.5 h in darkness at room temperature. The slides were counterstained with DAPI.All slides were mounted with glycerol mounting medium. Images were obtained using a confocal FV300 Olympus microscope with Fluoview5.0 software. Sequential imaging and multi-plane view analysis were done using 20 X (0.50 N. A), 40 X (0.75 N. A) and 60 X (1.42 N. A) lenses.

Results

“Synapse-like” communication between AdSCs and adipocytes

Examination of a drop of fresh adipose tissue just diluted with physiological saline by light microscopy showed several small cells attached to adipocytes (Figure 1A). Upon placing a glass coverslip on the same drop of adipose tissue with saline, cells surrounding the larger adipocyte began to separate away from the adipocyte. The smaller cells moved toward the edges of the preparation and also formed clusters (Figure 1B). Adipose tissue samples that were prepared for TEM analysis, had small round cells of 2 to 4 microns in diameter that remain strongly attached to the adipocyte surface (Figure 1C & 1D). We examined the expression of gap junction protein Cx43 in fixed adipose tissue to better understand the strong link between adipocytes and the small cells that surround them. Gap junction channels are formed by paired connexons or hemichannels, each made up of six Cx43 subunits.

Immunostaining with anti-Cx43, in white abdominal adipose tissue, revealed the significant presence of Cx43 around all adipocyte membranes. Stacking of confocal images in successive planes of focus allowed us to

reconstruct portions of the surface of some adipocytes (Figure 1E). This stack of 20 microns showed that Cx43 expression covers the entire adipocyte surface. Cx43 signal was detected in the form of points around the adipocyte membrane. The surface contact between adjacent adipocytes was 10 to 20 microns (Figure 1E). Stacked confocal images of multiple planes also allowed for detection of Cx43 expression between small stem cells in intimate contact with larger adipocytes. Plane-to-plane analysis of stacked images, taken with a large numerical aperture objective, showed that the entire membrane of small stem cells was completely covered with Cx43 (Figure 1F).

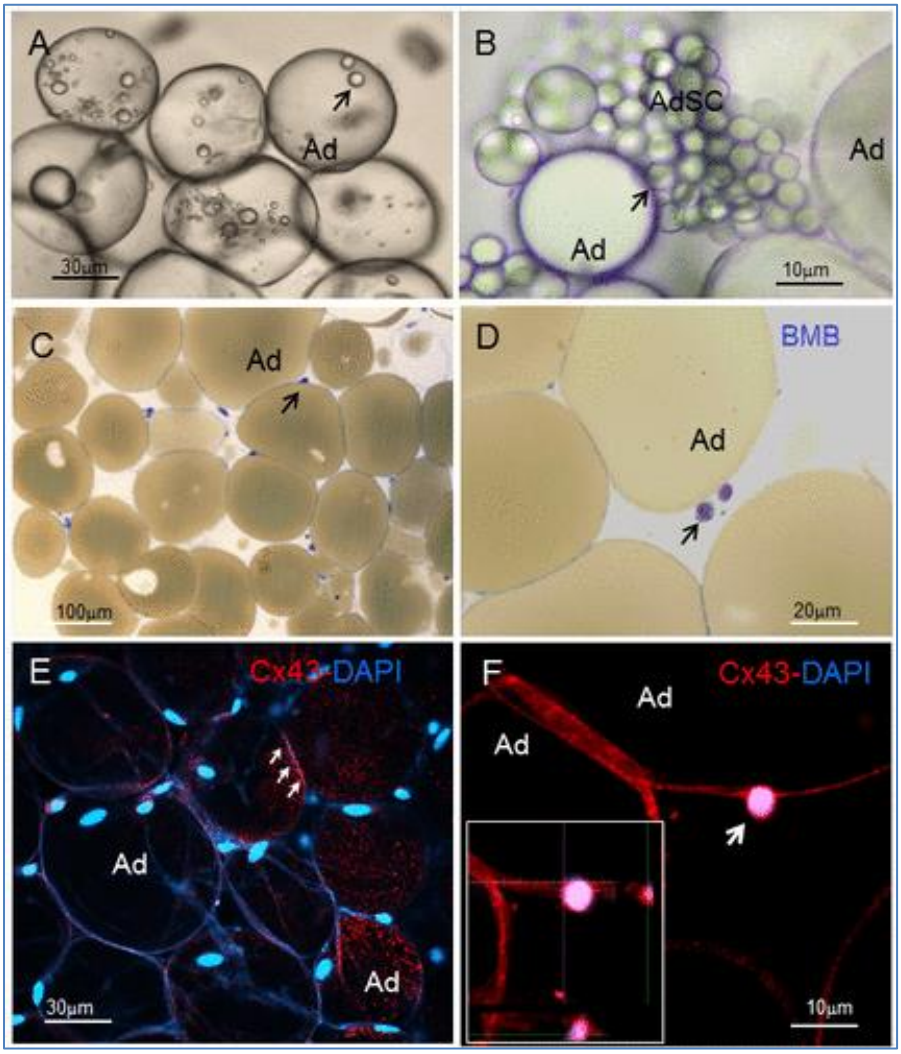

Figure 1: AdCS cells are attached to adipocytes (Ad). **(A)** Fresh adipose tissue one hour after liposuction with a drop of saline. Muse Cells are around adipocyte cells. **(B)** The same fresh preparation shows how they are separated by size when a glass coverslip is placed over the drop with cells. **(C)** Panaromic view and **(D)** higher magnification of semithin-section of fragment of adipose tissue stained with BMB. Shown are small round cells closely attached to adipocyte membrane (black arrows). **(E)** Immunohistochemistry showing Cx43+ expression on adipocyte surfaces. Confocal image obtained after stacking 20 microns of planes shows the adipocyte surface covered with points, i.e, Cx43+ in red. Nuclei are counterstained with DAPI. **(F)** Higher magnification of one cell in contact with an adipocyte. A 7 micron stack allows detecting "gap junction" contact between an adipocyte and small round cells (white arrow). The insert in F shows confocal orthogonal planes that illustrate distribution of Cx43 around the whole cell

Immunohistochemistry of stem cells markers

Immunohistochemical characterization and gene expression studies on the pluripotencial capacity of Muse cells, has been done mainly in cell culture. In our present study, it was important to detect putative stem cell populations in adipose tissue. This approach gives us information on stem cell characteristics in contact with adipocytes. We explored the presence of Sox2, Pax6 and Nestin in adipose tissue. Sox2 and Pax6 are transcription

factors widely expressed in embryonic vertebrate central nervous system progenitor cells, and Nestin is an intermediate filament protein established as a neural stem/progenitor cell marker. Immunohistochemical analysis of adipose tissue showed a population of cells that are in contact with adipocytes, and which express some stem cell markers previously mentioned above (Figure 2). Interestingly, when we counterstained with DAPI, not all cells attached to the adipocyte were positive for these stem cell markers. The cell population positive for Sox2, Pax6 and Nestin were round cells 4 to 6 microns in diameter.

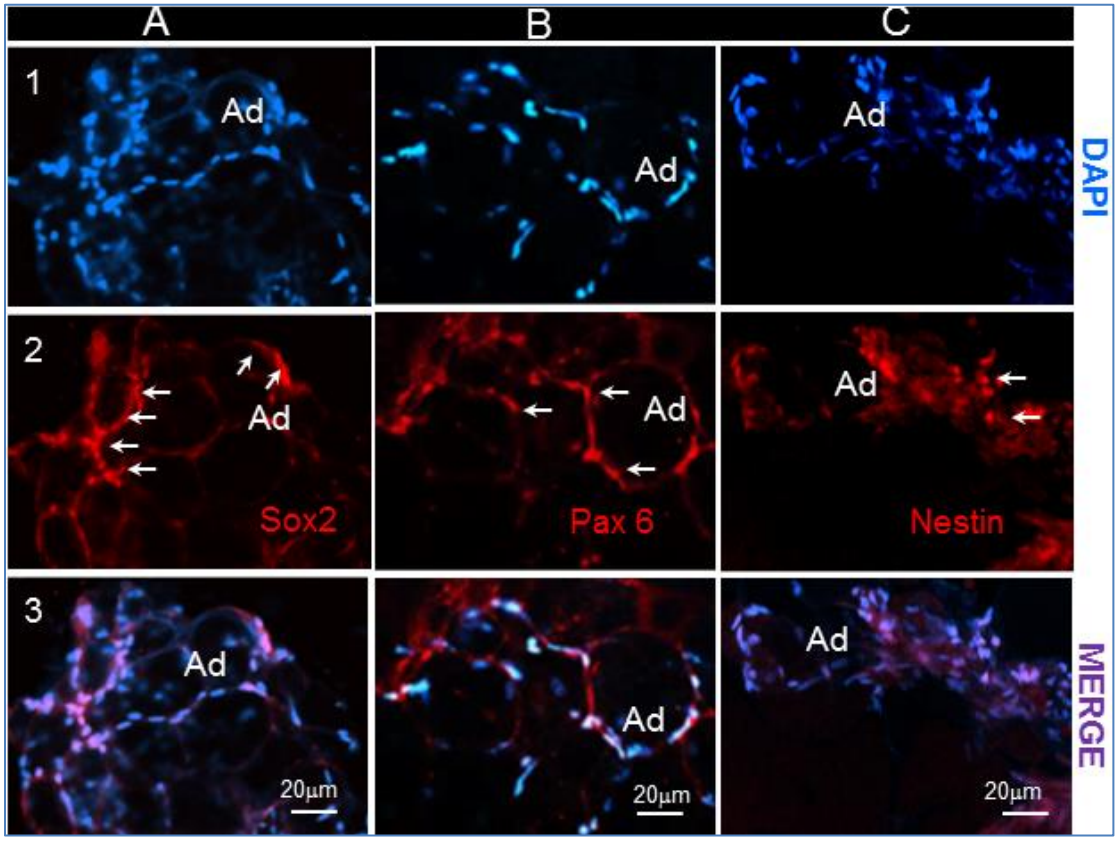

Figure 2: Immunohistochemistry of stem cell markers. Line 2 of columns A, B and C shows the expression of different markers of stem cells that were analyzed in fixed adipose tissue. Sox2, Pax 6 and Nestin are expressed in some cells, which are in contact with Ad. The white arrows indicate cells positive for different markers for mother cells in contact with the Ad. In line 1 is shown only cells stained with DAPI. Merged images are shown in line 3

TEM study of adipocyte stem cells

Thin sections for TEM analysis showed that small cells were attached to adipocytes or were located in spaces between them (Figure 3A). These cells were mostly about 4 microns in diameter, however, smaller cells were also present. These cells had little cytoplasm around the nucleus, which was rounded or oval in shape and occupied a central position in the cell. Nuclei exhibited heterochromatin in the periphery and euchromatin in the centre. This arrangement was variable, and heterochromatin was predominating. It was also common to observe cells compressed between two adipocytes (Figure 3D). When cells were seen in spaces between adipocytes but free and not attached to adipocytes, the cytoplasm had pseudopodia, perhaps facilitating cell migration (Figure 3E & 3F). Also, nuclei occasionally had indentations (Figure 3D-3F). Around each adipocyte there was a substantial amount of collagen that appeared to act as a mesh. In addition to connections between small cells and adipocytes via gap junctions, at high magnification we observed clusters of small vesicles, close to adipocyte membranes and located close to membranes of small cells (Figure 3A). This finding reinforces the idea of synaptic linkages between these cells.

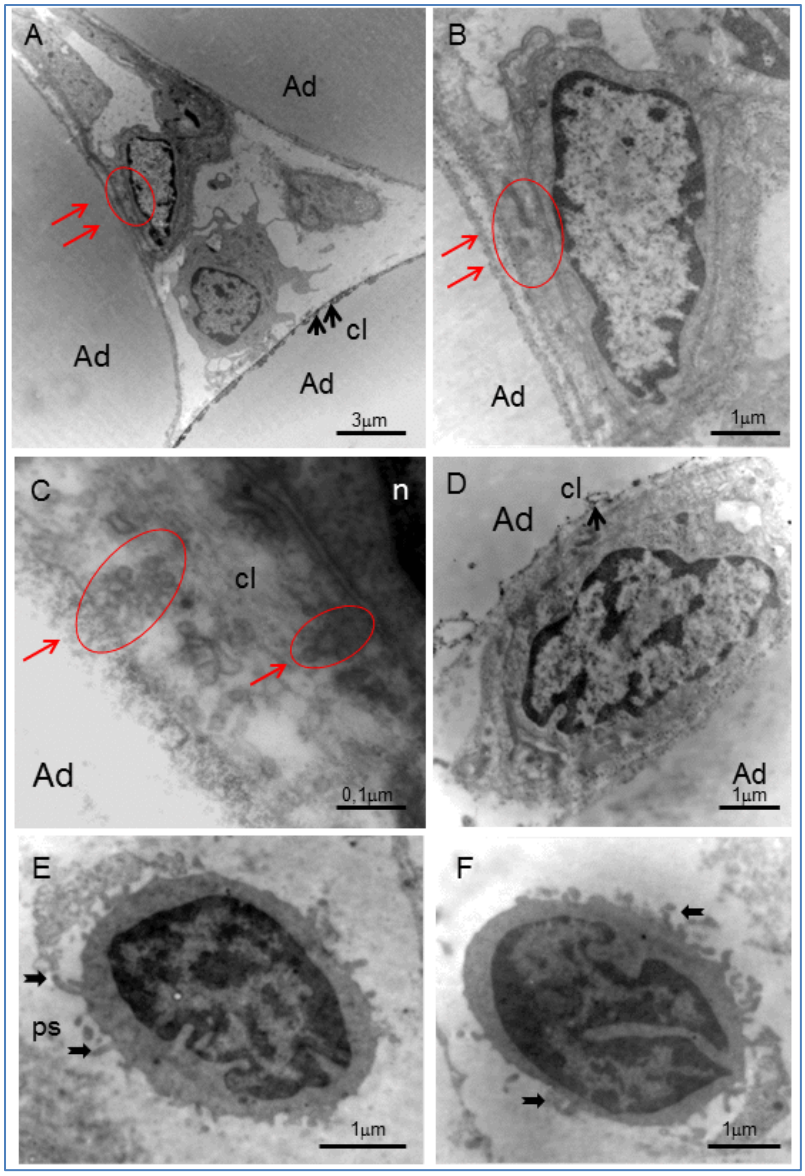

Figure 3: AdSCs cell analysis by TEM. **(A)** Panoramic image, where 3 adipocytes converge, and small cells linked to them are observed (red circles and red arrows) the black arrows indicate collagen (cl) bundles around Ads. **(B)** Higher magnification of the region where one of the cells maintains contact with one of the adipocytes (red circles). **(C)** Higher magnification of contact zone between Ad and AdSCs cell. The red circle indicates clustered vesicles adjacent to Ad membrane and Muse cell membrane. **(D)** A cell between two Ad in intimate contact. The adipocytes comprise the AdCSs. The black arrow points to collagen (cl) fibers. **(E&F)** Muse cells found close to adipose tissue

SEM analysis of adipose tissue

A fragment of whole adipose tissue without any manipulation was studied using SEM techniques. SEM analysis gave us different information than did the TEM analysis. SEM study showed three-dimensional images in adipose tissue and how small cells surrounded adipocytes. We were able to observe how adipocyte and all the other cells were organized, wrapped and protected by connective tissue. Small windows where the connective tissue was broken, allowed us to better observe the relationship between the adipocytes and the small cells. The small cell population seems quite heterogeneous in cell size, ranging between 2 to 10 microns in diameter (Figure 4A). We found that some small cells were in close contact with adipocytes, and others were immersed or trapped in a network of collagen fibres. We further observed the amount of collagen fibres surrounding each adipocyte and between the

small cells (Figure 4B). When analysing different samples, we observed a predominance of cells smaller than 5 microns. With this methodology, cells were always observed to be round and, in many cases, seemed to be in contact with each other (Figure 4C). Small cells that were in contact with only one adipocyte had a perfectly round shape (Figures 4C-4F). However, when the cells were between 2 adipocytes, they were compressed and oval in shape as shown in (Figure 1E). This same phenomenon was observed many times by TEM.

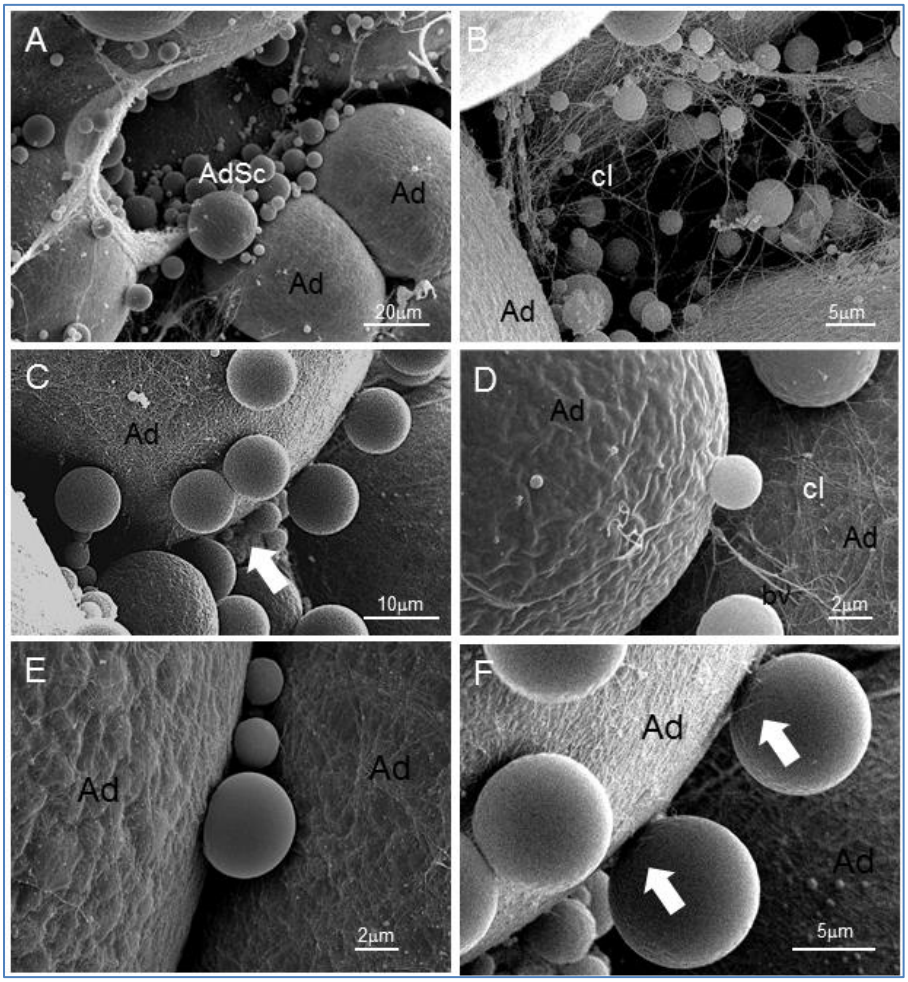

Figure 4: SEM analysis. **(A)** A lager population of small cells (AdSc) is observed around adipocytes (Ad). Intermediate size cells are also observed between adipocytes and small cells, which are presumably pre-adipocytes. Torn conjunctive sheaths are also observed, which allows visualization of enormous number of cells that exists between Ads. **(B)** A collagen fibers (cl) networks support adipose tissue stem cells in the space between adipocytes. **(C)** Small cells of 4 micron in diameter tightly bound to adipose are observed. Some small cells are tightly attached (white arrows). **(D)** An adipocyte surface covered with collagen (cl) fibers and some small blood vessels is observed in greater detail. **(E)** Cells of 2 to 4 microns in diameter are compressed by two adipocytes (Ad). **(F)** Higher magnification of the SEM image allows visualization of collagen fibers that firmly attacj Muse cells to adipocytes (white arrows)

Discussion

Previous studies done before identification of Muse cells demonstrated that adipose tissue stem cells were capable of differentiating into nervous system lineages [15]. This finding is relevant when considering the possibility of using this cell type for the repair of neural pathologies. Our analysis aims to clarify morphological and functional aspects of all potential stem cells present in adipose tissue with special emphasis on the cells that can express early markers for neural cell lineages. We have been able to verify that in fresh adipose tissue there is a fairly heterogeneous population of small cells (2-10 microns in diameter). When these cells are placed in physiological saline, they are able

to move away from adipocytes and form clusters as described as Muse cells. Muse cells were detected for the first time by Kuroda et al. [1] in bone marrow and later by Heneidi et al. [6] in adipose tissue. Using immunohistochemical analysis, of stem cell markers we found that small cells which are attached to adipose tissue, express neurogenic lineage factors, such as Nestin, Pax6, and Sox2. Not all cells attached to adipocytes were positive for these three markers unlike Carelli et al. [19] described in which Nestin was expressed in close to 100% of cells when these AdSCs were maintained in culture. We found that a discrete population of cells around adipocytes can express some of these stem cell markers. For example, Pax6 is described as an indispensable factor and is essential for the maintenance and multi-lineage differentiation of neural stem cells, as well as for neuronal incorporation into the adult olfactory bulb [20,21]. Also, Sox2 plays an important role in maintenance of human embryonic stem cell pluripotency [22]. Our results with respect to detection of Nestin, Pax6 and Sox2 in adipose tissue make us think that there are either different cell populations or cells that are in different differentiation states at the same time.

Detection of Cx43 on the adipocyte surface was described by Burke et al. [23] and Zhu et al., [24] where this marker plays a role in transmission of Chagas disease. A connexin-deficient line was made by genetic engineering in which gap junction levels were further decreased in mouse embryonic stem cells, and where cell differentiation was qualitatively or quantitatively compromised [25,26]. Our results showed that Cx43 is present on adipocyte surfaces. We also found that there were gap junctions between adipocytes and also between adipocytes and small cells attached to them. This suggests the importance of communication between adipocytes and potential stem cells. Some investigations demonstrated that fatty acid synthesis is critical for maintenance of stem cell pluripotency via promoting mitochondrial fission [27]. Also, it was demonstrated that preferential distribution of fatty acids was found during neurogenesis in major areas of the adult murine brain. Inhibition or deletion of fatty acid synthesis reduced proliferation of stem/progenitor cells [28]. The aforementioned findings allowed for establishing that lipid metabolism emerges as a bioenergetic control for self-renewal versus differentiation, ensuring lifelong neurogenesis. Lipid metabolism may thus represent a valuable target to promote stem cell function and tissue regeneration. Cx43 was recently reported in induced pluripotent embryonic stem cells from human cord blood [29]. The importance of Cx43 for multi- or pluripotency seems to be a more general phenomenon, as it has also been described for skin-derived stem cells in that their multipotency was dependent on Cx43 [30]. Recent findings have shown that gap junctions, and specifically Cx43, can play a significant role in neural progenitor migration in the developing brain [31]. It was also previously demonstrated that gap junction-mediated intercellular communication is essential in determining proliferation in embryonic stem cells and survival of mouse cortical neural progenitor cells as well as in the dentate gyrus in response to brain injury [32-34]. Our immunohistochemical results with anti-Cx43, and results of other researchers highlights the functional importance that this type of communication implies. Confocal image stacks allowed us to affirm that “Cx43+ gap junctions” are widespread on adipocyte surfaces and in promoting contact between adipocytes and AdSCs. This observation, together with the finding by TEM of the presence of small vesicles near the membranes of the adipocytes, suggests the existence of a "synapse-like" communication link where stem cells may be receiving signals from adipocytes to continue in the pluripotency state [35]. This type of connection could also play a role in cellular latency, or for activation toward a proliferative state when exposed to different stress conditions. These different cellular responses are surely mediated by this "synapse-like" communication. At the same time, adipocytes could be the source of energy for the survival of these valuable cells in the human body. SEM images gave us an idea of the numerous varieties of small cells that reside within adipose tissue, where all cells seem to be intimately connected with adipocytes. We wonder if all these small cells could be cataloged as Muse cells or as a very heterogeneous population of cells with different states of potentiality. To answer these questions, more analysis is

necessary, such as taking into account more morphological aspects, including those different than described in our study.

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