



Higher Pericyte Content and Secretory Activity of Microfragmented Human Adipose Tissue Compared to Enzymatically Derived Stromal Vascular Fraction

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Key Words. Adipose stem cell • Mesenchymal stem cell • Cytokine • Pericyte • Secretome

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Received March 7, 2018; accepted for publication July 19, 2018; first published September 26, 2018.

<http://dx.doi.org/10.1002/sctm.18-0051>

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ABSTRACT

Autologous adipose tissue is used for tissue repletion and/or regeneration as an intact lipoaspirate or as enzymatically derived stromal vascular fraction (SVF), which may be first cultured into mesenchymal stem cells (MSCs). Alternatively, transplant of autologous adipose tissue mechanically fragmented into submillimeter clusters has recently showed remarkable efficacy in diverse therapeutic indications. To document the biologic basis of the regenerative potential of microfragmented adipose tissue, we first analyzed the distribution of perivascular presumptive MSCs in adipose tissue processed with the Lipogems technology, observing a significant enrichment in pericytes, at the expense of adventitial cells, as compared to isogenic enzymatically processed lipoaspirates. The importance of MSCs as trophic and immunomodulatory cells, due to the secretion of specific factors, has been described. Therefore, we investigated protein secretion by cultured adipose tissue clusters or enzymatically derived SVF using secretome arrays. In culture, microfragmented adipose tissue releases many more growth factors and cytokines involved in tissue repair and regeneration, noticeably via angiogenesis, compared to isogenic SVF. Therefore, we suggest that the efficient tissue repair/regeneration observed after transplantation of microfragmented adipose tissue is due to the secretory ability of the intact perivascular niche. *STEM CELLS TRANSLATIONAL MEDICINE* 2018;7:876–886

SIGNIFICANCE STATEMENT

The fat collected during a liposuction contains tissue-repairing cells used diversely to erase wrinkles, reconstruct breasts, or treat arthritic joints. These cells, dissociated after enzymatic digestion of the fat, secrete diverse regenerative molecules playing important roles in tissue repair. Results of this study show that keeping the micro-architecture of the fat intact, by physical fragmentation into sub-millimetric units in the absence of enzyme treatment, guarantees optimal maintenance of regenerative cells and dramatically improves factor secretion thereby. These results likely explain the observed therapeutic superiority of micro-fragmented adipose tissue and suggest it should be preferred to routinely used, enzymatically produced single cell suspensions.

INTRODUCTION

Pioneered at the end of the nineteenth century for face and breast reconstruction, adipose tissue (AT) grafting later met with scepticism and mostly fell out of favor until new successful attempts reinvigorated this surgical approach in the 1980s [1,2]. First used intact or minimally processed as a filler for tissue contouring and augmentation, AT became a genuine cell therapy product following the demonstration that it contains multilineage progenitor cells that behave as mesenchymal stem cells (MSCs) [3]. Routine processing of lipoaspirates (LPA) for therapeutic purposes consists of enzymatic dissociation into

single-cell suspensions, subsequent elimination of adipocytes by centrifugation, and collection of the remaining stromal vascular fraction (SVF), which can be used immediately or following in vitro cell expansion. The latter protocol involving long-term SVF culture allows isolation from AT of bona fide MSCs, also named adipose-derived stem cells, which are morphologically, antigenically, and developmentally similar to their bone marrow-derived counterparts (reviewed in [4]), and can equally well control graft-versus-host reaction via their immunosuppressive properties [5]. MSCs are isolated through enzymatic digestion and are defined by

specific criteria: plastic adhesion once cultured; expression of markers as CD73, CD90, CD105, and ability to differentiate *in vitro* into osteocytes, adipocytes and chondrocytes under appropriate culture stimuli [6]. Bone marrow and AT are no exceptions because MSCs can be derived from multiple other organs after enzymatic dissociation and culture selection, such as dental pulp [7], lung [8], skeletal muscle [9], and endometrium [10], to cite but a few.

Vascularized tissues in general have the capacity to give rise to MSCs in culture, and fittingly peri-endothelial cells, namely pericytes that enclose capillaries and microvessels [11–17], and adventitial stromal cells that surround larger arteries and veins [18–22], were identified as *in vivo* progenitors of cultured MSCs. This view is supported by the fact that both pericytes and adventitial cells express MSC markers *in vivo* and are endowed with mesodermal differentiation abilities upon culture [13,23–27]. Moreover, similarity in gene expression profile between MSCs and pericytes has recently been reported [28]. Analysis of transcriptomes in single perivascular cells purified from human AT confirmed this progenitor status, as well as revealing a hierarchical organization whereby adventitial stromal cells are developmentally more primitive than pericytes [29].

It is, however, important to note that although resident pericytes can replenish different cell lineages *in vivo*, such as adipocytes [30], dental pulp [31,32], satellite cells and myofibers [33], follicular dendritic cells [34], myofibroblasts [35–38], and yield MSCs in culture, their behavior as bona fide MSCs (i.e., immunosuppressive and secretory cells endowed with osteo-, chondro-, adipo-, and myogenic developmental potentials) *in situ* has not been documented. In addition, distinguishing properties can be observed in isolated MSCs depending on the tissue of origin and the isolation technique used [26,27], thus suggesting the presence of a heterogeneous population of progenitor cells in most adult organs [39–42]. Therefore, according to current knowledge, although purified pericytes and adventitial cells have been proven to yield MSCs in culture, whether they are endowed with the same exact potential *in situ* is unknown.

In summary, AT is used therapeutically for the treatment of different conditions, either as an intact tissue or enzymatically derived SVF, used either immediately or cultured into MSCs. Alternatively, undissociated AT transplants have been supplemented with MSC injections to gain higher therapeutic efficiency (reviewed in [43]).

A recent innovation has been the use of mechanically fragmented AT, thus avoiding any enzymatic processing, for the treatment of diverse medical conditions. One of the most commonly used procedures to mechanically dissociate AT is Lipogems technology. Lipogems is a device used to process manual LPA into microfragmented adipose tissue (MAT) clusters through a mild mechanical size reduction using a sequence of sieves and steel marbles. It is a full immersion closed system that can be used directly in the operating room, reducing contamination risk that results from tissue exposure and/or extended processing methods. The generated AT clusters are a few hundred micrometers in diameter and free from blood and free lipids. Autologous transplantation of such MAT has been used with success in multiple indications, spanning cosmetics, orthopaedics, proctology, and gynaecology [44–54].

To further investigate the regenerative potential of MAT compared to that of enzymatically derived SVF, we characterized the perivascular cell distribution and *in vitro* protein secretion of Lipogems processed human AT versus isogenic collagenase digested LPA. We show how mechanical fragmentation of LPA modifies the resulting perivascular cell content of the tissue, and additionally that enzymatic dissociation negatively influences growth factor and cytokine secretion *in vitro*.

MATERIALS AND METHODS

Human Tissues

AT was collected with prior written informed consent from healthy female patients (26–71 years old) undergoing cosmetic liposuction or abdominoplasty. Ethical approval for the use of human tissues in research was obtained from the South East Scotland Research Ethics Committee (reference: 10/S1103/45).

Subcutaneous Abdominal Fat Collection

Subcutaneous AT from abdominoplasty samples was injected with 50 to 100 ml of 0.9% NaCl solution, warmed at 37°C, using a disposable tissue infiltration cannula (17Gx185 mm-VG 17/18). LPA were obtained either manually using a 10-cc luer lock syringe connected to a disposable liposuction cannula (LGI 13Gx185 mm - AR 13/18) or using a standard vacuum pump-assisted liposuction technique. All instruments used in the manual lipoaspiration procedure were provided in the Lipogems Surgical Kit (Lipogems, Milan, Italy).

Microfragmentation of Adipose Tissue

A total of 60 ml of manual lipoaspirate was processed each time with the Lipogems 60 device following manufacturer's instructions. Briefly, the system was connected to a 0.9% NaCl solution supply until the cylinder was completely filled and no air was present in the system. First, 30 ml of the manual lipoaspirate were pushed into the cylinder through the blue size reduction filter inlet. The cylinder was shaken for 1 minute to emulsify oil. During the whole process, blood components and emulsified oil residues were removed by the flow of saline. When the solution inside the cylinder appeared clear, floating MAT was expelled from the cylinder through the gray size reduction filter outlet into 10 ml syringes connected to the device. This procedure was repeated, until 60 ml of lipoaspirate were fully processed, yielding from 20 to 30 ml of MAT.

Cell Isolation

Fresh AT specimens (LPA and MAT) were dissociated enzymatically to obtain SVF. Briefly, samples were digested with type-II collagenase (1 mg/ml collagenase in DMEM, both from Gibco, Thermofisher Scientific, Waltham, MA) for 45 minutes at 37°C in a shaking water bath. Samples were then washed with 2% FCS/PBS (Sigma Aldrich, St Louis, MO) and filtered sequentially through 100- and 70- μ m cell strainers (BD Falcon, Corning, NY). After centrifugation, pellets were resuspended in erythrocyte lysis buffer (155 mM NH₄Cl, 170 mM Tris, pH 7.65, all from Sigma-Aldrich) for 15 minutes at room temperature. Cells were washed again with 2% FCS/PBS and filtered through 40- μ m cell strainers (BD Falcon) to obtain single cell suspensions. Viable

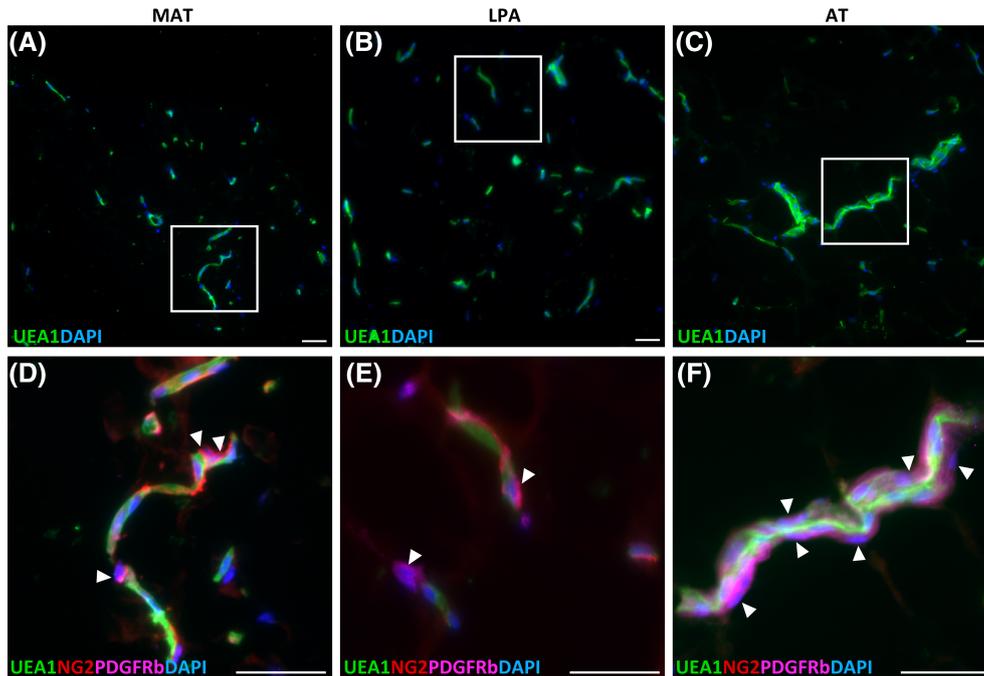


Figure 1. Vasculature in unprocessed and microfragmented adipose tissue. (A, B, C): Endothelial cells are stained with UEA-1. From left to right: microfragmented adipose tissue (MAT), lipoaspirate (LPA), adipose tissue (AT). Larger vessels were observed only in LPA and AT. (D, E, F): Boxed areas in A, B, C are showed enlarged in D, E, F respectively. Arrowheads indicate pericytes, which have been stained using antibodies against PDGFR β and NG2. Scale bar: 50 μ m.

cells were counted following trypan blue staining (BioRad, Hercules, CA) on a haemocytometer.

Flow Cytometry Analysis

The SVF was stained with the following antibodies: CD31-V450 (1:400) or CD144-PerCP Cy5.5 (1:100), CD34-PE (1:100), CD45-V450 (1:400) or CD45-APC Cy7 (1:100), and CD146-BV711 (1:100) (all from BD Biosciences, San Jose, CA). Cells were stained for 30 minutes at 4°C in the dark, followed by washing with 2% FCS/PBS. Analysis was performed on a BD LSR Fortessa 5-laser flow cytometer (BD Biosciences) using Diva software (v.6.0, BD Biosciences). Single stained beads were used as compensation controls. Data were analyzed using FlowJo (v.10.0, FlowJo, Ashland, OR). Forward scatter area (FSC-A) versus side scatter area (SSC-A) gate was used to identify cells, followed by FSC-A versus forward scatter height (FSC-H) to select single cells. Viable cells were gated as negative for 4',6-diamidino-2-phenylindole (DAPI, Life Technologies, Carlsbad, CA) staining. Hematopoietic and endothelial cells were excluded by gating on CD31 and CD45 negative cells. Perivascular cells were identified as pericytes (CD146⁺ CD34⁻) or adventitial cells (CD146⁻ CD34⁺).

Fluorescent Immunohistochemistry

AT specimens (unprocessed AT, LPA and MAT) were fixed in 4% buffered paraformaldehyde (PFA) at 4°C overnight. Samples were immersed for 24 hours in 15% sucrose in PBS (w/w), then embedded in 15% sucrose and 7% gelatin in PBS. After 4 hours at 37°C, samples were transferred to 4°C. After 24 hours, samples were frozen on dry ice. Embedded samples were stored at -80°C and cryosectioned at 8–10 μ m thickness.

Sections were fixed in 4% PFA prior to staining. Nonspecific antibody binding was blocked with 10% goat serum in PBS (Sigma-Aldrich) for 1 hour at room temperature. The following uncoupled primary antibodies were used: mouse anti-human-NG2 (1:100; ref. 554275, BD Biosciences), rabbit anti-human-PDGFR β (1:100; ref. 32570, Abcam, Cambridge, UK). All primary antibodies were diluted in antibody diluent (Life Technologies, Carlsbad, CA) and incubated at 4°C overnight. After washing with PBS, sections were incubated for 1 hour at room temperature with species-specific secondary antibodies diluted 1:300. The following fluorochrome-conjugated secondary antibodies were used: anti-mouse-Alexa 555 IgG, anti-rabbit-Alexa 647 IgG, and streptavidin conjugated 488 (all from Life Technologies). Directly biotinylated *Ulex europaeus* lectin (UEA-1) was used as an endothelial cell marker for long-term cultured cells (1:200; Vector-B1065, Vector Laboratories, Burlingame, CA). Nuclei were stained with DAPI (Life Technologies) for 10 minutes at room temperature. Slides were mounted using Fluoramount G (SouthernBiotech, Birmingham, AL) and images were acquired using a fluorescence microscope (Zeiss Observer, Zeiss, Oberkochen, Germany; Olympus BX61, Olympus, Tokyo, Japan). Images were processed using Fiji software [55] or ZEN Blue lite version (Zeiss).

Tissue Culture and Medium Collection

SVF cells derived from MAT or LPA were plated at a density of 6,000 cells/cm² and cultured in basal medium, consisting of DMEM Glutamax (Gibco) supplemented with 100 μ g/ml streptomycin (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich) and 20% heat-inactivated foetal calf serum (Sigma-Aldrich). 200 mg (corresponding to 200 μ l of MAL) were plated in each

well of a six-well plate and cultured in basal medium. After 8 days in culture under standard conditions (37°C, 5% CO₂) culture media from SVF and MAT were collected and stored at -20°C.

Secretome Arrays

Secretomes were analyzed using the Proteome Profiler Human XL Cytokine Array kit (ARY022b) and Human Angiogenesis Array kit (ARY007), following manufacturer's instructions (R&D Systems, Minneapolis, MN). Conditioned media collected from cultured SVF and MAT were centrifuged at 500g for 5 minutes at room temperature to remove debris, filtered through a 70-µm cell strainer to get rid of adipocytes/small residues of MAT, and incubated with both arrays. The signal was detected using the LiCOR Odyssey Fc apparatus (LICOR, Lincoln, NE), exposing array membranes for 10 minutes. Positive signals on the membranes were quantified using Image Studio Lite Software (LICOR). The average signal (pixel density) of the duplicate spots corresponding to each protein was normalized on the average signal of paired spots on the negative control. Normalized signals of each protein were then used for comparative analysis.

Statistics

Statistical analysis was performed by using the Student's *t* test using Microsoft Excel or GraphPad Prism5 software. Results are presented as means ± SEM. A *p* value of less than .05 was considered statistically significant.

RESULTS

The Perivascular Niche Is Preserved in Microfragmented Fat

Detection of the endothelial cell marker *Ulex europaeus* agglutinin 1 (UEA-1) receptor on sections of MAT, LPA, and AT illustrated the vascular network present in AT, with microvessels located between adipocytes. Larger vessels were observed principally in the unprocessed AT and LPA, while MAT was mainly characterized by the presence of small, capillary-like vessels (Fig. 1A–1C).

Staining for pericyte markers revealed that after AT mechanical fragmentation, pericytes expressing NG2 or PDGFRβ are normally distributed, still ensheathing endothelial cells in microvessels (Fig. 1D). The same was observed in AT and LPA specimens, suggesting that microfragmentation is not affecting the perivascular cell compartment in microvessels (Fig. 1E, 1F).

MAT Is Enriched in Pericytes Compared to Lipoaspirate

AT samples (MAT and LPA) were digested using collagenase and analyzed by flow cytometry. The average yield of nucleated cells in the SVF was $27 \times 10^3 \pm 15 \times 10^3$ cells per milliliter of MAT (*n* = 7) and $69 \times 10^3 \pm 56 \times 10^3$ cells per milliliter of LPA (*n* = 7). Viable cells were selected excluding debris, dead cells, and doublets. Endothelial cells and leukocytes were excluded from the analysis using CD31 and CD45, respectively. Pericytes were identified as CD146⁺CD34⁻, and adventitial cells as CD34⁺CD146⁻ cells [56]. MAT was observed to be enriched in pericytes compared to LPA. On an isogenic specimen analysis, pericytes and adventitial cells in LPA account for 8.39% and 51.5% of the cells, respectively, in agreement with

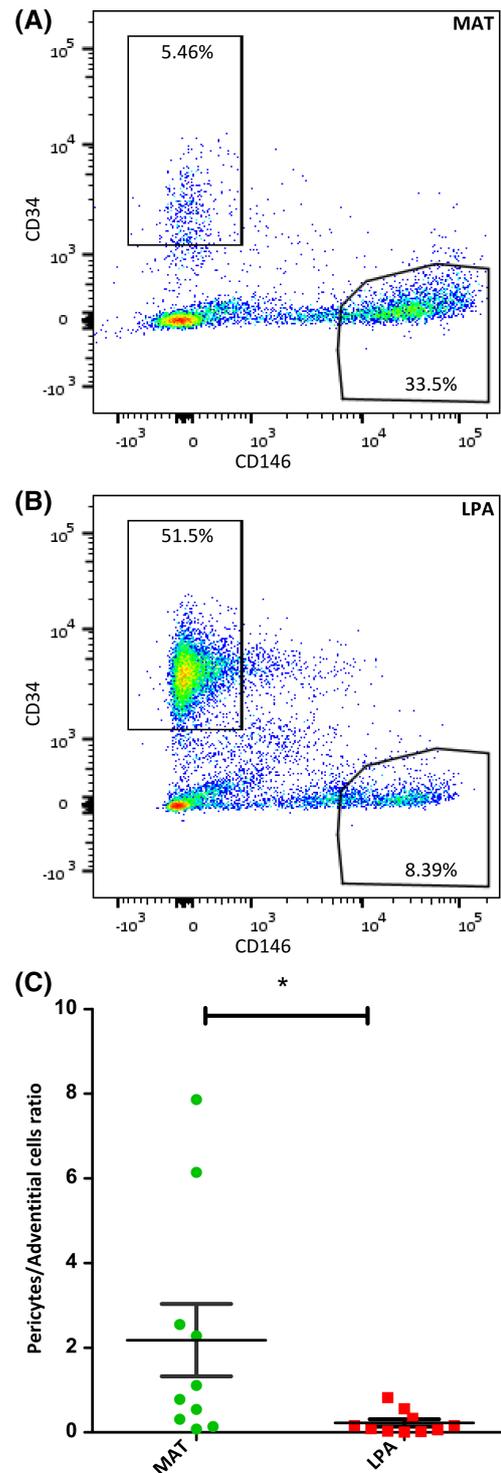


Figure 2. Respective abundances of perivascular adventitial cells and pericytes in MAT and LPA. **(A, B):** Dotplots showing adventitial cells (CD34⁺CD146⁻) and pericytes (CD146⁺CD34⁺) populations in MAT and LPA from the same donor. **(C):** Quantitative distribution of pericytes and adventitial cells in LPA and MAT, *n* = 10, **p* < .05.

previously observed values [13,18,56] (Fig. 2B). In the MAT counterpart, pericytes and adventitial cells amounted to 33.5% and 5.46%, respectively (Fig. 2A). This difference between LPA and MAT, regarding pericyte and adventitial cell numbers, was observed to be significant (*p* < .05, *n* = 10; Fig. 2C).

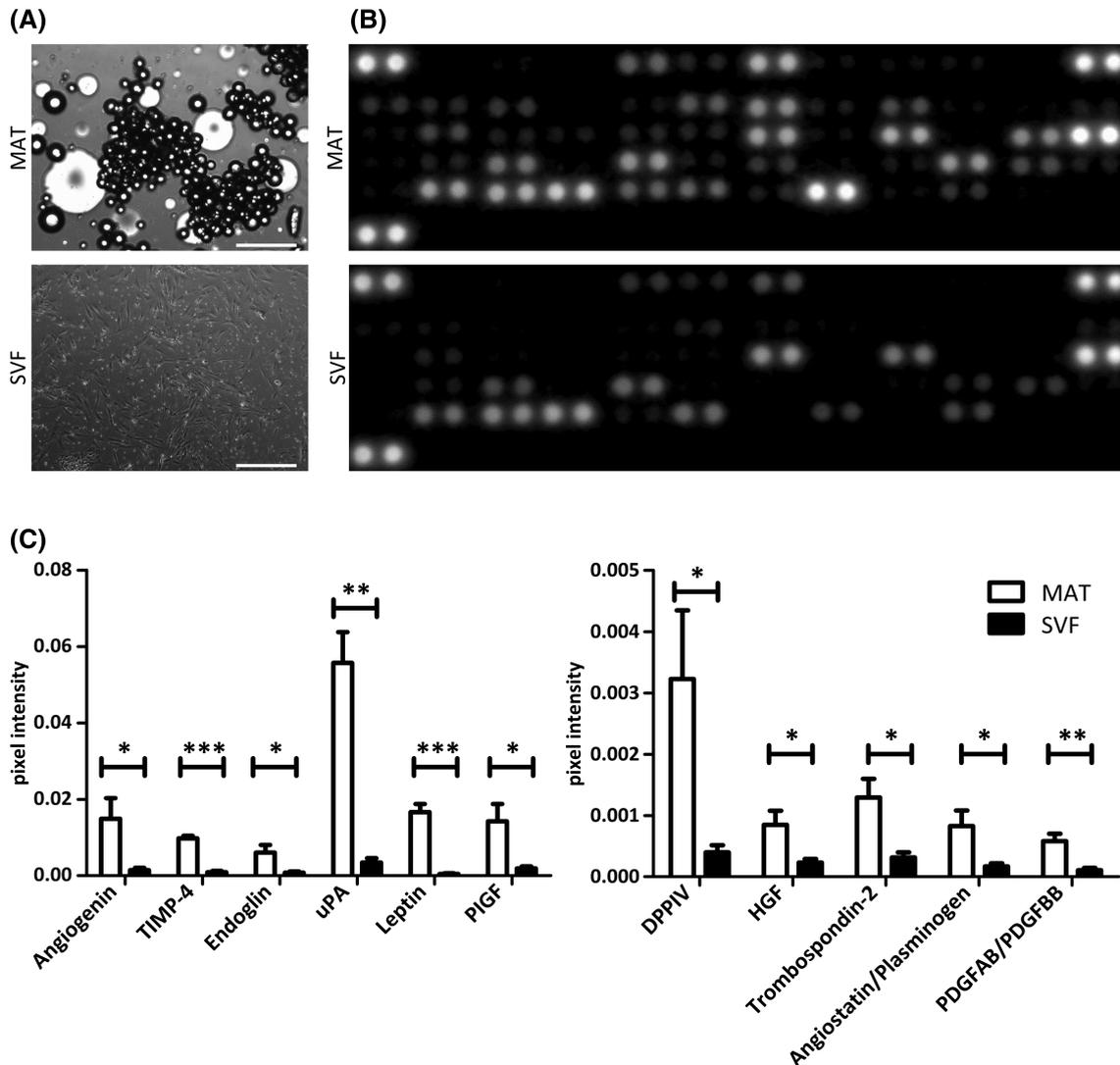


Figure 3. Angiogenic protein secretion by cultured MAT and isogenic SVF. **(A):** MAT and SVF cultured in basal medium. Scale bar: 500 μm . **(B):** Angiogenesis proteomic array showing secreted proteins from MAT and SVF after 8 days in culture. Capture antibodies are spotted in duplicate, each dot doublet represents a detected protein. **(C):** Secretion level of different angiogenic proteins measured as the average of the pixel intensity of the doublets and normalized to the negative control. Statistical analysis was performed on pooled secretion values detected in four separate donors. * $p < .05$; ** $p < .01$; *** $p < .001$.

Microfragmented Fat Secretes in Culture a Higher Number and Higher Amounts of Proteins than Isogenic SVF

MSCs are known to secrete growth factors and cytokines, either free or via microvesicle cargoes, involved in tissue repair and regeneration [57,58]. Assuming that native perivascular cells, the *in vivo* progenitors of MSCs, present in AT are responsible for its regenerative potential, we aimed to compare the secretome of MAT to that of enzymatically derived SVF. Isogenic MAT and SVF isolated from four different donors were cultured for 8 days in basal medium (Fig. 3A). Conditioned media were then analyzed, using proteome profiler commercial assays, for the presence of a range of cytokines and growth factors. Four independent experiments revealed that MAT secretes a greater number of cytokines and angiogenic growth factors than SVF (Figs. 3B and 4A). Moreover, comparative analysis on data derived from independent

experiments conducted on four different biological samples revealed that most cytokines and angiogenic factors secreted by both MAT and SVF were more abundant in the supernatant of the former (Figs. 5 and 6). These results suggest that collagenase digestion reduces the secretory activity of AT stromal vascular cells, both qualitatively and quantitatively. To directly test this hypothesis, MAT was digested with collagenase, and the derived SVF was placed in culture. Both intact and enzymatically dissociated MAT were cultured in parallel for 8 days in basal medium and the resulting conditioned media were analyzed as described above. Enzymatic treatment of microfragmented fat dramatically reduced secretory activity, which became comparable to that observed from conventional SVF (Figs. 3B, 4A, 7A and 7B).

On detailed analysis of secreted angiogenic growth factors, we found higher secretion by MAT of angiogenin, angiostatin/plasminogen, DPPIV, endoglin, hepatocyte growth factor (HGF),

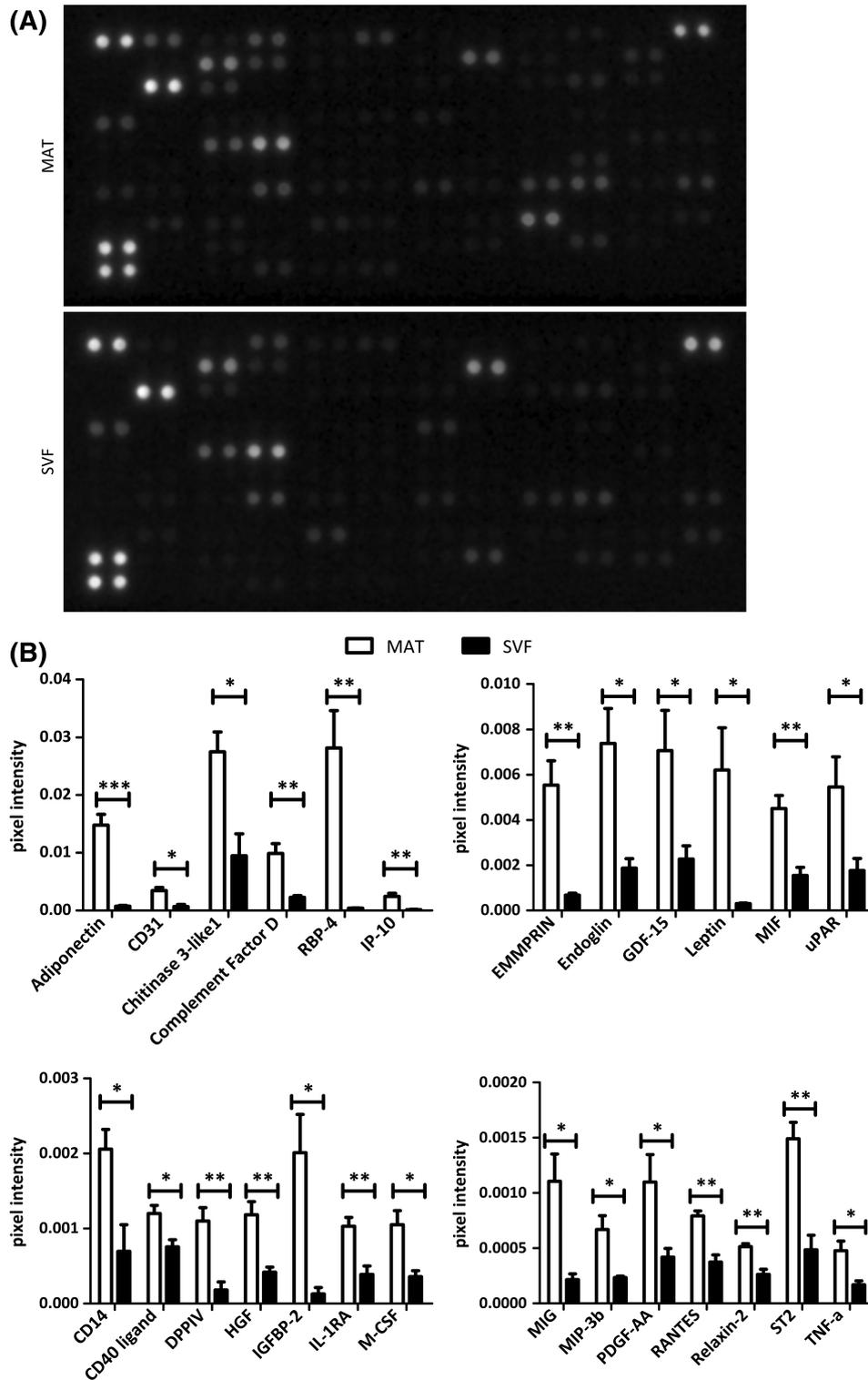


Figure 4. Cytokine secretion by cultured MAT and isogenic SVF. **(A):** Cytokine proteomic array showing secreted proteins from MAT and SVF after 8 days in culture. Capture antibodies are spotted in duplicate, each dot doublet represent a detected protein. **(B):** Secretion level of different cytokines measured as the average of the pixel intensity of the doublets and normalized to the negative control. Statistical analysis was performed on pooled secretion values detected in four separate donors. **p* < .05; ***p* < .01; ****p* < .001.

leptin, PDGFAB/BB, placental growth factor (PIGF), thrombospondin 2, TIMP4, and uPA (Fig. 3C).

Regarding cytokine secretion, adiponectin, CD14, CD31, CD40 ligand (CD154), chitinase 3-like 1, complement factor D,

EMMPRIN (CD147, basigin), GDF-15, IGFBP-2, IL1RA, IP-10, M-CSF, MIF, MIG (CXCL9), MIP-3β (CCL19), PDGFAA, RANTES (CCL5), RBP-4, relaxin-2, ST2, TNF-α, and uPAR (CD87) were significantly more abundant in MAT supernatants compared to

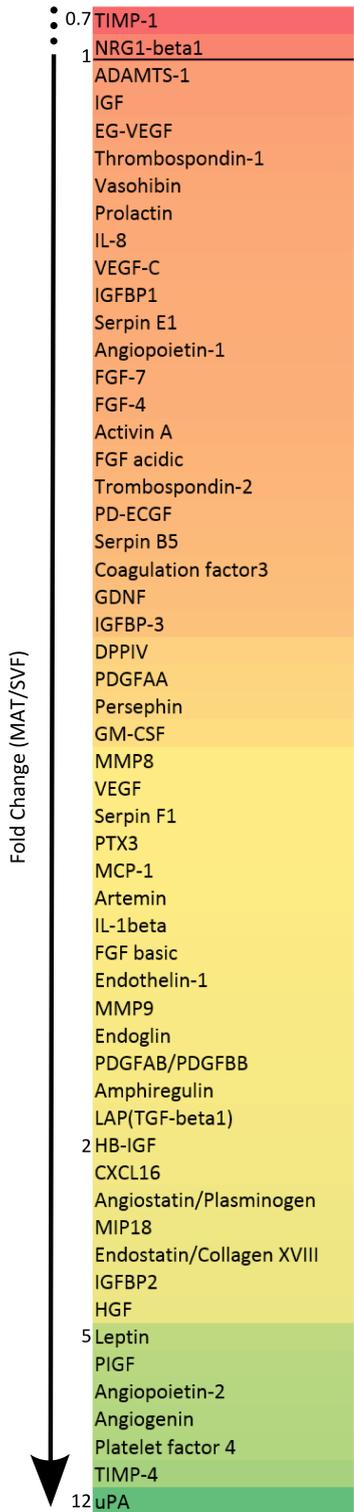


Figure 5. Angiogenic protein secretion levels of MAT compared to SVF. Secretion levels of angiogenic factors detected using the angiogenesis proteomic array, expressed as fold change between MAT and SVF values. The values represent the normalized pixel intensity detected in four separate donors. The numbers at the side of the list indicate fold changes.

SVF ones (Fig. 4B). In the cytokine arrays, the increased secretion of endoglin, HGF, leptin, and DPPIV by MAT compared to SVF observed in the angiogenesis array was replicated (Fig. 4B).

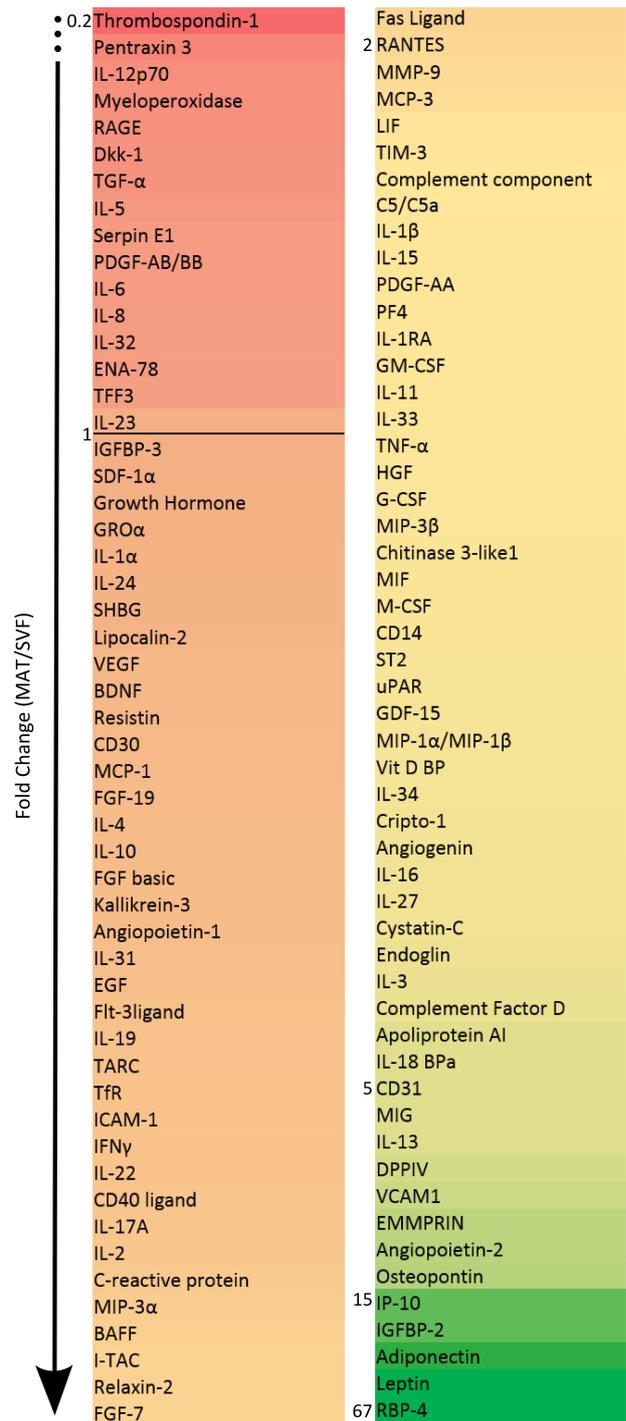


Figure 6. Cytokine secretion levels of MAT compared to SVF. Secretion levels of cytokines detected using the cytokine proteomic array, expressed as fold change between MAT and SVF values. The values represent the normalized pixel intensity detected in four separate donors. The numbers at the side of the list indicate fold changes.

DISCUSSION

AT has long been used in the clinic as a plain, unprocessed tissue graft. Adopting the enzymatically produced adipocyte free SVF was seen as a major improvement, even more so as the AT-

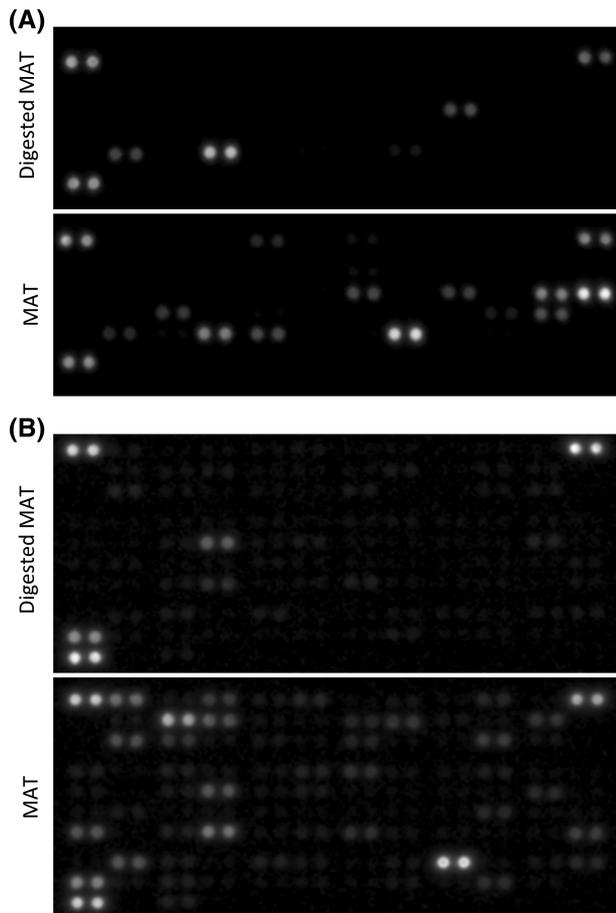


Figure 7. Protein secretion of enzymatically digested MAT and undigested MAT. **(A)**: Angiogenesis proteomic array and **(B)** cytokine proteomic array showing secreted proteins from enzymatically digested MAT and undigested MAT after 8 days in culture in basal medium. Capture antibodies are spotted in duplicate, each dot doublet represent a detected protein.

derived SVF can be grown in culture into therapeutically potent MSCs [59]. However the use of dissociated AT-derived SVF, cultured or not into MSCs, for applications in regenerative medicine remains empirical as the exact mode of action of these cells is still obscure, with progenitor potential, trophic, secretory, and immunomodulatory activities being diversely suggested to mediate tissue regeneration and repair.

As a middle path between intact AT and single cell suspensions, mechanical dissociation of AT into microclusters (exemplified by the Lipogems system) has yielded a product of high therapeutic value. In the present study, we used immunohistochemistry to describe MAT clusters and compared those, side by side, with SVF derived from the same donors in terms of perivascular presumptive MSC content and secretory activity in culture. As expected, the microanatomy of MAT is essentially similar to that of intact AT, with capillaries and microvessels organized and distributed between adipocytes, and pericytes wrapped around endothelial cells. However, quantification by flow cytometry of pericytes and adventitial stromal cells revealed that pericytes are encountered at a higher frequency in MAT than in LPA and derived SVF, confirming previous observations [44,60]. Conversely, LPA contains more adventitial stromal cells than MAT preparations do. This

suggests that microvessels are spared by microfragmentation, and preserved in MAT, whereas larger arteries and veins, encircled by the *tunica adventitia*, are partially lost after mechanical dissociation. While both purified pericytes and adventitial stromal cells yield MSCs in vitro [13,18], their respective contributions to long-term conventional MSC cultures are unknown. However, description of gene expression in single cells has revealed that adventitial stromal cells are developmentally more primitive than pericytes [29], although we and other investigators have previously documented the strong regenerative potential of pericytes purified from AT [20,24,61–64]. Therefore, if the therapeutic value of Lipogems is related to pericyte enrichment, those cells might exert their regenerative action more indirectly, via growth factor secretion, than as progenitor cells. This hypothesis was tested by characterizing, qualitatively and quantitatively, growth factors and cytokines produced in culture by MAT preparations and SVFs obtained from the same AT samples. The overall conclusion is that Lipogems-derived MAT produces higher amounts of many tested growth factors and cytokines, as compared with enzymatically dissociated SVF. As a main difference between MAT and SVF, only the former contains adipocytes; therefore, as expected, MAT but not SVF culture supernatants contained factors secreted by adipocytes: adiponectin, which regulates several metabolic cascades [65]; complement factor D [66]; RBP-4, a retinol binding protein [67]; the anti-inflammatory IL-1RA [68]; and leptin, the satiety regulating adipokine [69]. No other molecules detected in MAT supernatants are known to be secreted by adipocytes. Interestingly, several of the factors present in higher amounts in MAT supernatants stimulate angiogenesis, hence may indirectly support tissue regeneration: angiogenin [70], endoglin [71], the VEGF family member PlGF [72], the multifunctional HGF [73], and PDGF [74]. The insulin-like growth factor binding protein IGFBP2, which is much more abundant in MAT supernatants, also positively regulates angiogenesis through modulation of VEGF expression [75], and TNF α , whose secretion is upregulated in MAT cultures, can promote new vessel formation [76]. These data support and extend the results of a previous study in which Lipogems-derived MAT culture supernatants were observed to stimulate endothelial cell (HUVEC) proliferation and tube formation in vitro; accordingly, angiopoietin-1 and -2 were detected in higher amounts in these MAT supernatants, as compared to culture supernatants of AT-derived MSCs [64]. In the present experiments, three inhibitors of angiogenesis were also detected, albeit in very small amounts, in MAT supernatants: IP-10 (CXCL10) [77], angiostatin [78], and thrombospondin 2 [79].

Remarkably, higher amounts of diverse mediators of immuno-inflammation, leukocyte recruitment, and migration were detected in MAT supernatants, as compared to SVF cultures: GDF-15, a regulator of inflammation and biomarker in diverse pathologies [80]; MIF, which controls inflammation and innate immunity [81]; MIG (CXCL9), a T-cell chemoattractant [82]; MIP3 β (CCL19), involved in immune cell migration [83]; RANTES (CCL5), a chemokine chemotactic for multiple leukocytes [84]; and CD40 ligand (CD154), which binds to CD40 on antigen presenting cells [85]. It is known that adipose tissue can support substantial inflammation [86], hence the presence in culture supernatants of these many players of immune-inflammatory responses. However, how these factors

may influence tissue regeneration, following autologous transplantation, is unknown, and interpretation is complicated by the fact that many of these molecules can play multiple distinct roles. For instance, the CCL5 chemokine, which recruits leukocytes at the site of inflammation, can also promote angiogenesis [87]. Chitinase 3-like-1, dramatically overexpressed in MAT supernatants, can stimulate blood vessel formation in tumors, besides playing major roles in inflammation, angiogenesis, and tissue remodeling [88].

Why does MAT secrete more growth factors and cytokines than SVF cultured under the same conditions? Besides adipocytes, secretory cells within fat tissue include hematopoietic cells, responsible for the production of most factors involved in the regulation of immuno-inflammatory reactions, endothelial and perivascular cells, including pericytes, and other cell compartments loosely designated as stromal or “mesenchymal.” The demonstration that mesenchymal stem/stromal cells are of perivascular origin has supported the development of a model whereby pericytes and other perivascular cells can, in adverse pathologic conditions, lose contact with blood vessel walls, migrate away from blood vessels and become reprogrammed into regenerative cells [89], playing this role as tissue progenitors [13,18], niche cells for lineage specific stem cells [24], “medicinal secretory cells” producing trophic factors [57], as well as scarring pro-fibrotic cells [35–38]. The present results suggest that AT resident regenerative cells perform these functions much more efficiently when maintained in the intact perivascular environment, such as that provided by the Lipogems mechanical fragmentation system, than following digestion and culture. Notably, it is known that tissue enzymatic dissociation can cause changes in gene expression [90] and exosome content [91] and this research reveals how severely AT residing native MSCs can be affected when reduced to single cell suspensions. Taken together, these data reveal differences in physically fragmented AT, compared to

SVF, which provide the foundations on which to build an explanation of the former’s therapeutic superiority. Further investigations, not only in culture but also involving experiments in animals, are needed to confirm the effect of enzymatic dissociation on native MSCs, giving more insight into the therapeutic effect of MAT.

ACKNOWLEDGMENTS

We thank Barbara Krutchkoff for helpful discussions. This work was supported by grants from the British Heart Foundation and Lipogems, which supplied adipose tissue processing kits.

AUTHOR CONTRIBUTIONS

B.V.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; I.S.: conception and design, collection and assembly of data; H.L.: collection and assembly of data; L.Y.: provision of study material; N.K.: collection and assembly of data; C.T.: conception and design, provision of study material; B.P.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

L.Y. declared research funding with Small Pump Priming Grant, Royal College of Surgeons Edinburgh. C.T. is the President and founder of Lipogems and declared employment, patent holder and stock ownership. B.P. declared consultancy and researcher funding. All other authors indicated no potential conflicts of interest.

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