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Xenogen-free isolation and culture of human adipose mesenchymal stem cells

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ABSTRACT

Background: Adipose-derived Stem Cells (ASCs) present great potential for reconstructive procedures. Currently, isolation by enzyme digestion and culturing using xenogenic substances remain the gold standard, impairing clinical use.

Methods: Abdominal lipo-aspirate and blood samples were obtained from healthy patients. A novel mechanical isolation method for ASCs was compared to (the standard) collagenase digestion. ASCs are examined by flow-cytometry and multilineage differentiation assays. Cell cultures were performed without xenogenic or toxic substances, using autologous plasma extracted from peripheral blood. After eGFP-transfection, an *in vivo* differentiation assay was performed.

Results: Mechanical isolation is more successful in isolating $CD34^+/CD31^-/CD45^-/CD13^+/CD73^+/CD146^-$ ASCs from lipo-aspirate than isolation *via* collagenase digestion ($p < 0.05$). ASCs display multilineage differentiation potential *in vitro*. Autologous plasma is a valid additive for ASCs culturing. eGFP-ASCs, retrieved after 3 months *in vivo,* differentiated in adipocytes and endothelial cells.

Conclusion: A practical method for human ASC isolation and culturing from abdominal lipo-aspirate, without the addition of xenogenic substances, is described. The mechanical protocol is more successful than the current gold standard protocol of enzyme digestion. These results are important in the translation of laboratory-based cell cultures to clinical reconstructive and aesthetic applications.

1. Introduction

The ultimate goal in plastic reconstructive surgery is to replace like with like. Implant material is prone to complications, and autologous reconstruction is restricted by tissue scarcity or donor site morbidity. Adipose-derived stem cells (ASCs) are first described by Zuk et al. (2002). Since there are about 100 times more ASCs in adipose tissue than mesenchymal stem cells (MSCs) in bone marrow, and the cells have a greater potential for proliferation (Lee et al., 2004), there is an enormous interest in these cells for autologous cell therapy (Banyard et al., 2015; Trepsat, 2009; Puissant et al., 2005) as well as tissue-engineering experiments (Correia et al., 2012; Guilak et al., 2004; Hong et al., 2005). They can be easily obtained through liposuction of subcutaneous adipose tissue, a minimal invasive procedure. Classically, the lipo-aspirate is then enzymatically digested to isolate the stromal vascular fraction (SVF) from the buoyant adipocytes. There are many different enzymatic products available, such as trypsin, clostripain or dispase, the gold standard being collagenase (from *Clostridium histolyticum*).

Despite the efficiency of this isolation method, significant drawbacks (i-v) are incurred with the use of these xenogenic products for SVF and/or ASCs isolation in human clinical trials. Risks inherent to the manufacturing process of enzyme products should be considered: (i) inconsistency in protease activity and (ii) batch-to-batch variations. Regarding patient safety, potential risks with enzymatically isolated SVF/ASCs samples administered to humans include (iii) residual

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collagenase activity (Raposio et al., 2014) and (iv) transferring xeno-antigens into the patient. Further, (v) manipulation of lipo-aspirate *via* enzymatic digestion may alter or disrupt cell surface receptors, which may reduce the regenerative potential of the SVF (Guo et al., 2015; Olenczak et al., 2017).

Although recently a human trial with clinical grade collagenase (NB 6 GMP grade, SERVA Electrophoresis GmbH, Heidelberg, Germany) has been performed (Kølle et al., 2013), mechanical isolation methods have been developed to overcome these risks. However, these methods including sonication (Amirkhani et al., 2016; Packer et al., 2018), explant culture (Priya et al., 2014) and centrifugation protocols (Shah et al., 2013; Condé-Green et al., 2010; Markarian et al., 2014) have failed to deliver a similar yield in ASCs as enzyme digestion.

Standard media for culture and proliferation of the ASCs are α-modified Eagle's medium (αMEM), Dulbecco's MEM (DMEM) and McCoy medium. Culture conditions however, as highlighted by Caplan et al. (Lennon et al., 1995), need an addition to the basic medium to ensure adhesion and proliferation of ASC's. Classically, ASCs are cultured with fetal bovine serum (FBS), in concentrations varying from 10 to 20%.

To ensure the highest possible safety for patients, all steps of autologous ASC isolation and culture should be precisely defined and reproducible, thereby eliminating xenogenic substances. Therefore, we aim to create a useful, high-yield isolation and culture method, composed without xenogenic additives. Further, we assess the regenerative potential of the xenogen-free isolated ASCs *in vivo* by analyzing eGFP-transfected ASCs, 90 days after transplantation into immunodeficient mice.

2. Materials and methods

2.1. Isolation of ASCs and culture in autologous medium

All procedures were carried out in accordance with the UZ Gent guidelines and regulations, and all experimental protocols were approved by the UZ Gent ethical committee. After obtaining written informed consent from all included patients using an UZ Gent ethical review board-approved protocol, adipose tissue was acquired from healthy female patients ($n = 7$) undergoing liposuction of the abdomen between 35 and 50 years of age (mean age 42.3; mean BMI: 28.7). During the procedure, 50 mL of peripheral blood was drawn from each patient, and 100 IU of heparin were added. After infiltration with Klein's solution, a 3-mm blunt cannula (Mentor, Santa Barbara, Cal, USA) was used in combination with device aspiration at −1.5 atm. The lipo-aspirate was centrifuged in 10 mL Luer-Lok™ syringes (Becton Dickinson, Franklin Lake, NJ, USA) at 3000 rpm for 1 min. After centrifugation, the oily and fluid phases were discarded, and the fat graft was transferred to produce 8 syringes filled with exactly 10 mL centrifuged fat. Next, the syringes were randomly assigned: 40 mL of fat graft was then processed according to a classic enzyme digestion protocol with collagenase (Collagenase-isolated Adipose-derived Stem Cells; cASCs), and 40 mL according to our new mechanical protocol (Mechanically isolated Adipose-derived Stem Cells; mASCs).

For enzyme digestion isolation a sterile filtered aliquot of collagenase type I 0.3% (Sigma-Aldrich, St. Louis, MO, USA) in Phosphate Buffered Saline (PBS), and 1% penicillin/ streptomycin (P/S) (Thermo Fisher, Waltham MA, USA) was added. The mixture was incubated for 45 min at 37 °C on a shaker at 15 rpm. FBS was added to a final concentration of 10% to stop enzyme activity, followed by centrifugation at 1000 rpm for 5 min. The overlying fluid and adipose phases were aspirated and discarded. The SVF pellet was resuspended in culture medium.

For mechanical isolation, a 10 mL syringe filled with 5 mL fat graft was connected Luer-to-Luer with another 10 mL syringe filled with 5 mL PBS and mixed. The mix was forcefully pushed back-and-forth 30 times and emulsified. The resulting 80 mL of PBS-diluted, emulsified fat graft was then centrifuged again at 3000 rpm for 10 min.

The blood samples were processed to extract plasma. The samples were centrifuged at 4000 rpm for 10 min, after which the supernatant plasma, without the buffy coat, was obtained. Culture medium was created with 90% DMEM-hepes (Sigma-Aldrich, St Louis, MO, USA), 10% human plasma, 1% P/S and 100 IU of heparin. Both the cASCs and mASCs were cultured at 37 °C and 5% CO2 in an incubator. After 48 h, the medium was replaced to sort the cells for adherence. After 3 more days, the medium was replaced again.

2.2. Cell count

After 8 days of culture, cells were released using TrypLE Select (Thermo- Fisher, Waltham MA, USA). The P1 viable cells of both the mASC and the cASC group were counted using propidium iodide (Sigma-Aldrich, St Louis, MO, USA) and resuspended in PBS for flowcytometric analysis or replated for multiple lineage differentiation assays.

2.3. Flowcytometric analysis

Flowcytometric analysis of the collected cell cultures was performed ($n = 7$). To identify different (sub)populations, samples of the isolated cell populations were labeled for specific phenotypic markers.

Following subpopulations were identified: (i) $CD34^+$ progenitor cells (ii) pre-adipocytes, defined as $CD34^+/CD45^-/CD31^-/CD146^-$, (iii) transitional cells, defined as $CD34^+/CD45^-/CD31^-/CD146^+$, (iv) EPCs, defined as $CD34^+/CD45^-/CD31^+/CD146^+$ and (v) true ASCs, defined as $CD34^+/CD31^-/CD45^-/CD13^+/CD73^+/CD146^-$.

Samples of collected cASCs and mASCs were labeled with CD34-PE, CD45-VioGreen, CD31-APC, CD13-APC-Vio700, CD73-PE-Vio770, and CD146-VioBright515, purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Samples were acquired on a four-laser flowcytometry system (BD LSR II, Becton-Dickinson, Franklin Lake, NJ, USA). Fc-receptor reagent was added to avoid unspecific labelling of cells *via* Fc-receptors.

2.4. Differentiation assays

A part of the cASCs and mASCs were seeded on thermanox cover slips (Nunc, Roskilde, Denmark) for trilineage differentiation. Adipogenic differentation was performed using basic culture medium with addition of dexamethasone, insuline, indomethacine and 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St Louis, MO, USA). Differentiation was confirmed by Oil Red O staining. Osteogenic differentiation was performed using basic culture medium with addition of dexamethasone, ascorbic acid and β-glycerophosphate (Sigma-Aldrich, St Louis, MO, USA). Differentiation was confirmed by Von Kossa staining. Chondrogenic differentiation was performed using Chondromax® medium (Sigma-Aldrich, St Louis, MO, USA) in pellet cultures. Differentiation was confirmed by Alcian blue staining, combined with Hematoxyline-Eosine staining (H-E staining). Digital imaging was performed using an Olympus inverted microscope, and CellˆM software (Olympus Europe, Hamburg, Germany).

2.5. eGFP-transduction of ASCs

To enable tracking of the ASCs during the *in vivo* experiment, eGFP-transduction of the ASCs was performed as described before. 293 T cells were cultured in DMEM (41965039, ThermoFisher) with 10% FCS and 2 mM L-Glutamine (BE17-605F, Lonza) and transfected with lentiviral envelope plasmid pMD2.G, packaging plasmid psPAX2 and lentiviral eGFP expression plasmid pLenti6-eGFP. The medium was removed and replaced with fresh medium 8 h post transfection. The virus was harvested 48 h post transfection and filtered through a 0.45 μm PES filter (Merck- Millipore, Burlington, Massachusetts, USA). P1 mASCs were

cultured in DMEM with 10% FCS and 2 mM L-Glutamine until a density of approximately 60% was reached. The medium was removed and replaced by pLenti6-eGFP virus containing medium for 24 h. After 10 days the eGFP positive cells were sorted with the BD FACSAria III cell sorter. This resulted in 45.3% eGFP-positive ASCs before sorting, and 95% after sorting.

2.6. Animal model

On the day of the *in vivo* experimental procedure, abdominal subcutaneous adipose tissue was again obtained *via* an aesthetic liposuction procedure in a healthy female (age 30 years), as described above. 50 mL of fat graft was digested with collagenase as described above. Now, the overlying adipose phase was carefully isolated from the digested fat graft mixture. 10 Eight-week-old T-cell deficient nude mice (BALB/c-nude; Envigo, Huntingdon, UK) were used in this study. All of the surgical instruments were sterilized, and surgical procedures were performed under laminar flow. Immediately before the procedure, 5 mL peptide hydrogel was gently mixed with 5×10^5 eGFP-positive ASCs in 250 µL PBS and 5 mL mature adipocytes (*eGFP-ASC/adipocyte construct*). Animal anaesthesia was achieved with isoflurane vaporizer 3.5% for induction, and 1% for maintenance, with non-rebreather mask. After anaesthesia, the surgical site on the mouse skin was sterilized with chlorhexidin digluconate 0.5% in aqua. A skin puncture was made with a 19-gauge needle, and 1 mL of *eGFP-ASC/adipocyte construct* was placed subcutaneously on the dorsal side. The puncture wounds were covered with poly-urethane bandage (Tegaderm®, 3M, Minneapolis, US).

2.7. Histology

After 90 days, the mice were sacrificed, and the *eGFP-ASC/adipocyte constructs* were carefully dissected out. 5 µm-paraffin sections of the grafts were made through the centre area. The areas composed of signet-ring cells indicating reticular fat tissue were evaluated on 5 sections through the largest diameter region of the spherical constructs. Additionally, sections from the *eGFP-ASC/adipocyte constructs* were examined with GFP staining: tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed by heating the sections in 10 mM sodium citrate buffer (pH 6.0) (Vector laboratories, Burlingame, US) in an electric pressure cooker. Blocking of endogenous peroxidase occurred in 3% H_2O_2 in methanol. Sections were then treated with 5% goat serum in PBS + 1% BSA, followed by an overnight incubation with primary GFP-antibody (Clone D5.1, Cell Signalling Technology, US) at 4 °C. Detection was done with a biotin-conjugated secondary antibody followed by Avidin-Biotin complex (ABC) and developed with diaminobenzidine (DAB) (Vector Laboratories, Burlingame, US). The sections were stained with H-E staining and photographed by light microscopy (Olympus BX50, Hamburg, Germany). The scores were determined by two independent reviewers using ImageJ software (National Institutes of Health, Maryland, USA).

For control, we also performed immunofluorescent staining with the following primary antibodies: rabbit anti-human perilipin (dilution 1:1000; Invitrogen, PA5-55046) and chicken anti-eGFP (dilution 1:50; Abcam, ab13970), incubated overnight at 4 °C. The following secondary antibodies were used: For perilipin, Alexa-fluor 555-conjugated donkey anti-rabbit (dilution 1:500, Thermo Fisher, A31572) incubated for 30 min at room temperature. For GFP, detection was done with a biotinylated secondary antibody (dilution 1:200, Abcam, ab6876), for 30 min at room temperature, followed by incubation with third antibody Streptavidin/HRP (dilution 1:200, Dako, P0297) for 30 min at room temperature.

The sections were viewed and photographed with fluorescence microscopy, and CellˆM software (Olympus Europe, Hamburg, Germany).

2.8. Statistical analysis

Statistical analysis was performed on isolated cASCs and mASCs of 7 patients ($n = 7$). The following outcomes were recorded *via* cell counts and quantitative flowcytometric analysis for both isolation methods: (i) Total (absolute) number of isolated cells, (ii) Percentage of $CD34^+$ cells, (iii) Percentage of 4 subsets of cells (pre-adipocytes, transitional cells, pericytes and true ASCs). Statistical analysis determined whether there was a significant difference in the cell-yield between the two procedures, for the outcomes listed above. A Wilcoxon signed rank test was carried out using SPSS Statistics 24. This is the non-parametric version of the paired student's *t*-test, testing the null hypothesis that within each patient, there is (on average) no difference in outcome between the two treatments. A result was considered significant with $p < 0.05$.

3. Results

3.1. ASC yield

Cell cultures in medium supplemented with autologous plasma were without adverse events. After 48 h both cASCs and mASCs became spindle-shaped and started rapid expansion. Although there was a large inter-patient variability in cell yield, a higher cell count was found in all samples of the mASC group, significant at a 5% level ($p = .018$). After 8 days of cell culture, median cell count was 3.0×10^5 cells (min.4.0×10^4-max.6.0×10^5) in the cASC group and 1.2×10^6 (min.2.0×10^5-max.4.0×10^6) in the mASC group (Table 1) (Fig. 1).

3.2. Flowcytometric analysis

Flowcytometric analysis *via* population-specific phenotypic markers showed a larger number of $CD34^+$ cells in the mASC group, significant at a 5% level ($p = 0.028$) (Fig. 1). Median percentage of $CD34^+$ cells found in cASC is 4.7% (min.0.7%–max.19.8%) as minimum and maximum percentage resp.), median percentage of $CD34^+$ cells found in mASC is 21.3% (min.2.8%-max.62.1%) (Table 2).

Different subpopulations (pre-adipocytes, transitional cells, EPCs and true ASCs) were investigated to detect the presence of specific progenitor cells. The yield of primary culture pre-adipocytes and true ASCs was larger in the mASC group than in the cASC group, significant at a 5% level (p = 0.028 for both subpopulations) (Table 2) (Fig. 1).

There was no significant difference in yield of primary culture transitional cells and EPC cells (*Data not shown*).

3.3. Differentiation potential

Trilineage differentiation potential of the ASCs was evaluated with a standard *in vitro* differentiation assay. Both the cASCs and the mASCs displayed similar differentiation potential to adipose, osteogenic and chondrogenic lineage (Fig. 2). Adipogenic differentiation became obvious after 1 week, with cells transforming into a round shape, and presence of cytoplasmatic lipid droplets as confirmed by Oil Red O staining. Osteogenic differentiation occurred more slowly over 3 weeks' time, with presence of calceiform material, stained by Von Kossa's stain. Chondrogenic differentiation also occurred over 3 weeks' time, with

Table 1
Descriptive statistics of the Total cell count for collagenase-isolated and the mechanically isolated samples (n = 7). Cell count was significantly higher in the mASC group.

Sample	Median	Minimum	Maximum
Collagenase total cell count ($\times 10^3$ cells)	300	40	600
Mechanical total cell count ($\times 10^3$ cells)	1200	200	4000

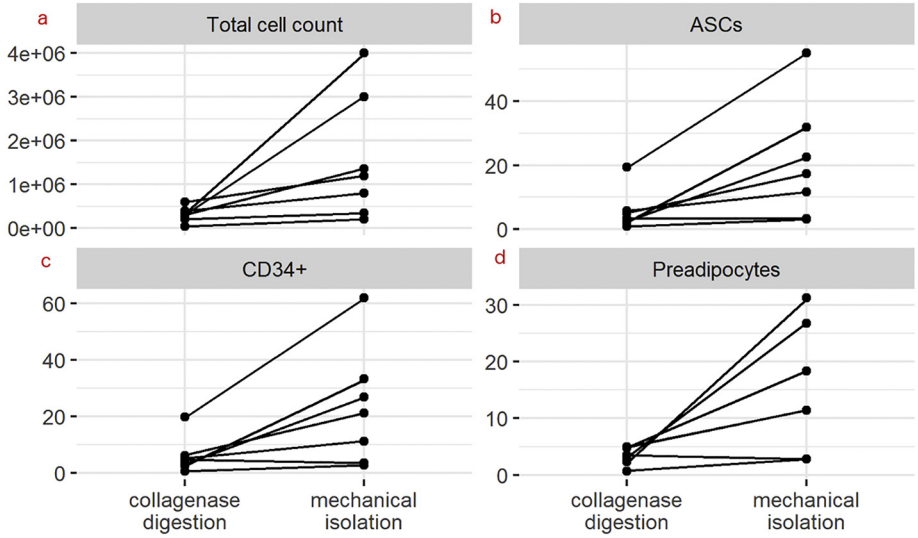

Fig. 1. Graphs a-d. a. Total cell count of the collagenase (left) *versus* the mechanical cultures (right) of all patients ($n = 7$). After 8 days of cell culture, median cell count was 3.0×10^5 cells in the cASC group and 1.2×10^6 in the mASC group. A higher cell count was found in all samples of the mASC group, significant at a 5% level.
b.–d. Percentage of b. ASCs ($CD34^+/CD31^-/CD45^-/CD13^+/CD73^+/CD146^-$), c. $CD34^+$ stromal cells and d. Preadipocytes, present in the collagenase (left) *versus* the mechanical cultures (right) of all patients (n = 7). For all 3 subpopulations, a significant higher cell-yield was found in the mASCs group ($p < .05$).

Table 2
Descriptive statistics of different subpopulations ($CD34^+$, pre-adipocytes, true ASCs) present in collagenase-isolated and mechanically isolated samples (n = 7). For all 3 subpopulations, a significantly higher cell-yield was found in the mASCs group.

Sample	Median	Minimum	Maximum
$cCD34^+$ (%)	4.7	0.7	19.8
$mCD34^+$ (%)	21.3	2.8	62.1
cPreadipocytes (%)	3.5	0.7	5.0
mPreadipocytes (%)	11.4	2.8	31.3
cASCs (%)	3.4	0.9	19.4
mASCs (%)	17.4	3.2	55.0

Alcian blue staining the sulfated proteoglycans.

3.4. Assessment of the fat graft in vivo

Grafts were harvested after 90 days. Overall graft retention was poor. The average volume of the *eGFP-ASC/adipocyte construct* was 0.015 ± 0.009 mL, with on average 30% viable fat tissue and large oil cyst formation. On H-E staining, large cyst formation was observed, as well as reticular adipose tissue composed of signet-ring like cells, connective tissue and blood vessels with red blood cells in the lumen (Fig. 3). No foreign material was visualized. Interestingly, all retrieved adipocytes in the *eGFP-ASC/adipocyte constructs* stained positive for DAB, suggesting that no grafted adipocytes or differentiated adipocytes from the recipient were present. By evaluating sequential 5 µm coupes by H-E and DAB-staining, vascular structures were found to be delineated by both DAB-positive and DAB-negative stained cells. The cyst walls were monocellular and equally composed of DAB-positive and DAB-negative stained cells. These findings were confirmed by immunofluorescent staining for perilipin, a specific staining for viable adipocytes, and for eGFP (Fig. 4).

4. Discussion

We present a novel and practical method for human ASC isolation and culture from abdominal adipose tissue. The isolation protocol delivers large numbers of ASCs in 8 days, without the addition of xenogenic substances, thus further closing the gap between laboratory-based tissue engineering assays and their clinical application. Furthermore, we compare this novel isolation method to the gold standard, collagenase digestion, and find it to be more efficient in ASC yield for autologous plasma-supplemented culture.

However, large variations in harvested stromal cell counts are seen between the included patients, as described before by other groups (Bellei et al., 2017).

To avoid biases, only patients undergoing abdominal liposuction are included in the study. Not included in the study are two patients (female, ages 45 and 48) with lipoedema of the legs for which liposuction was performed, and two healthy patients who had liposuction of the flanks and the upper thighs. In these four patients, despite an identical experimental protocol, mechanical isolation does not appear to be superior to collagenase digestion. Therefore, further research is needed to explain the large variability in stromal cell harvest between patients, and the success of our method in other indications or anatomical regions.

In the pioneering works of Friedenstein et al. (1966), Caplan (1991) and Owen (1988), isolation, culture and osteogenic differentiation of bone marrow-derived progenitor cells are described, and a new research field for mesenchymal stem cells (MSCs) is unveiled. Twenty years later, the presence of MSCs is described in nearly all adult tissues, including adipose tissue: adipose-derived stem cells (ASCs) (Baer and Geiger, 2012).

The International Society for Cellular Therapy (ICTS) suggests that the term 'ASCs' is used only for the subset of progenitor cells that meets three minimal criteria: (i) plastic adherence, (ii) expression of $CD34^+/CD45^-/CD31^-/CD13^+/CD73^+/CD90^+$, (iii) trilineage differentiation potential. These criteria are stated in a conjoined effort with the International Federation for Adipose Therapeutics (IFATS) in 2013 (Dominici et al., 2006).

Currently, there is evidence that ASC preparations are heterogeneous cell cultures comprising subsets of stem cells and more differentiated progenitor cells, the first presenting very attractive candidates for clinical applications to repair or regenerate damaged tissues, and tissue engineering purposes.

With regard to cellular composition and therapeutic potential, it is important to emphasize the difference between the stromal vascular fraction (SVF) and the ASCs (sub)population(s) after culturing. Some studies report results attributed to 'ASCs' while in fact the SVF is used and not isolated, cultured stem cells. Although the rationale behind the use of SVF preparations in those studies relies on the presence of stem cells within this fraction, it is known that the SVF contains a diminutive percentage of stem cells among various other cell populations (Dykstra et al., 2017). Therefore, it is valuable to define what cellular product(s) are examined when reporting on new research. This may lead to more adapted and varied tools for cell-based therapies, whereby autologous regenerative cells or cell populations are selected according to the procedural indication (Guo et al., 2015). In this study, both mASCs and

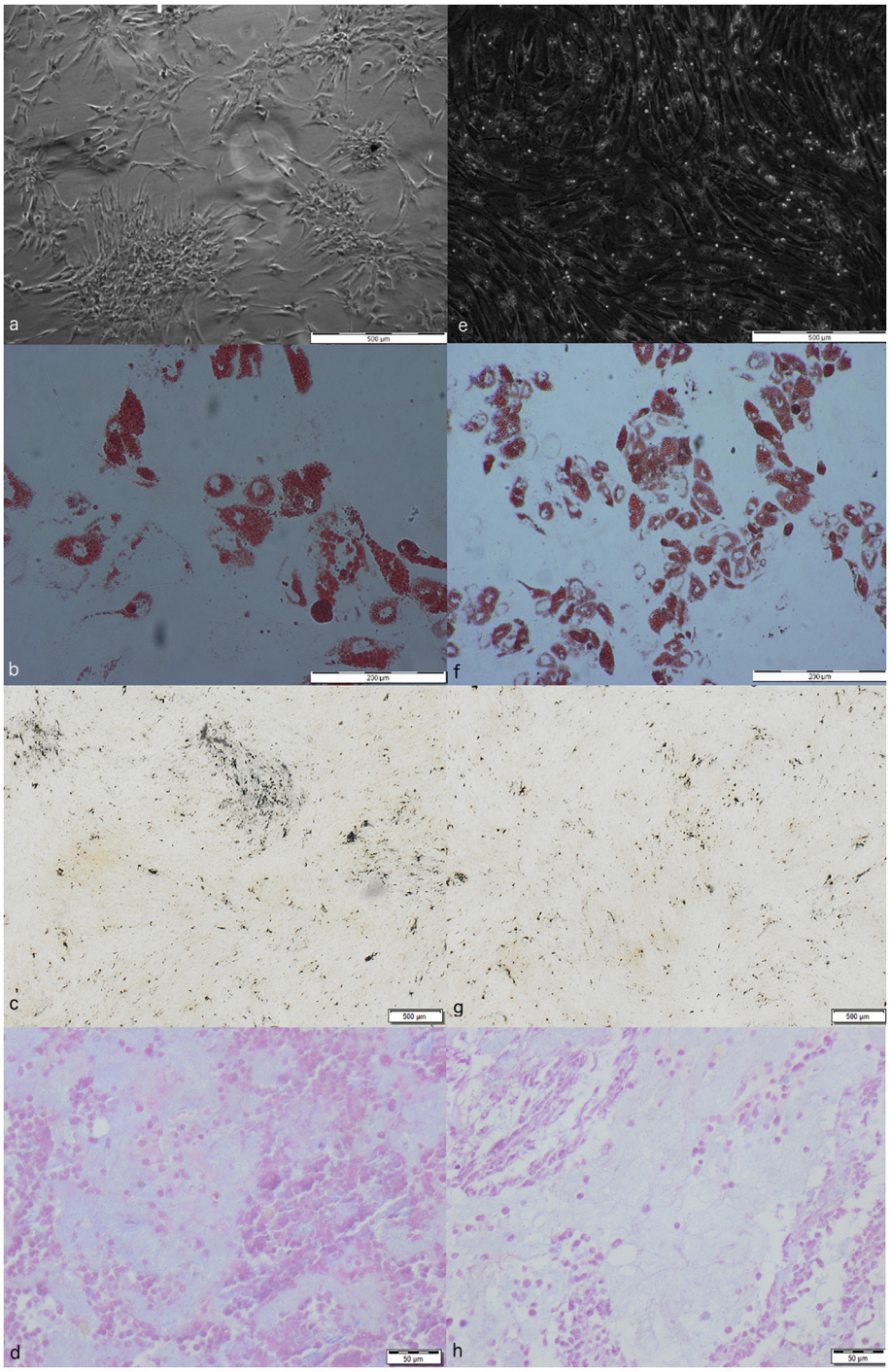

Fig. 2. Trilineage differentiation assays. Inverted microscopy, magnification 10×. Left column: cASCs cultures, Right column: mASCs cultures.
a. cASCs culture day 8, light microscopy b. cASCs adipogenic differentiation day 10, Oil Red O staining: detection of cytoplasmatic lipid-filled vacuoles (Red) c. cASCs osteogenic differentiation day 24, Von Kossa staining: detection of extracellular mineralization (Black) d. cASCs chondrogenic differentiation day 21, Alcian blue & Hematoxyline-Eosine staining (H-E staining) (Fuchsia): detection of extracellular GAGs (Blue) e. mASCs culture day 8, light microscopy f. mASCs adipogenic differentiation day 10, Oil Red O staining g. mASCs osteogenic differentiation day 24, Von Kossa staining h. mASCs chondrogenic differentiation day 21, Alcian blue & H-E staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cASCs are plastic adherent and are able to differentiate into adipose, osteogenic and chondrogenic lineages. Besides a larger cell yield in the mASC group, significantly more true ASCs ($CD34^+/CD31^-/CD45^-/CD13^+/CD73^+/CD146^-$) are present in the cultures.

To assess the regenerative potential of the mASCs *in vivo*, we co-transplanted mASCs with mature adipocytes into immunodeficient mice. To recover the mASCs after 90 days *in vivo*, they are transfected with eGFP, enabling us to distinguish host tissue from donor mASCs after the mice are sacrificed.

Previously published research from our group has demonstrated that co-culture of ASCs and mature adipocytes incite proliferation and adipogenic differentiation *in vitro* of ASC (Doornaert et al., 2012), hence the idea of co-transplantation. However, isolation of mature adipocytes is again performed *via* collagenase digestion of lipo-aspirate and collection of the buoyant fraction of the adipocytes. Mature adipocytes are fragile cells, difficult to manipulate and to our knowledge, enzymatic isolation is the only feasible method for isolation of mature adipocytes.

Ongoing research of our group focuses on a stabilizing carrier gel, supporting adipogenic (and angiogenic) differentiation, for co-injection with the ASCs (Van Nieuwenhove et al., 2017; De Moor et al., 2018). This would render the use of mature adipocytes obsolete.

After 90 days, eGFP-ASCs are retrieved as having differentiated into mature adipocytes and endothelial cells. These findings further support the effectiveness of our method to isolate and culture true ASCs. Furthermore, our *in vivo* study supports the *graft replacement theory,* as documented by Eto, Yoshimura et al. (Eto et al., 2012; Doi et al., 2015).

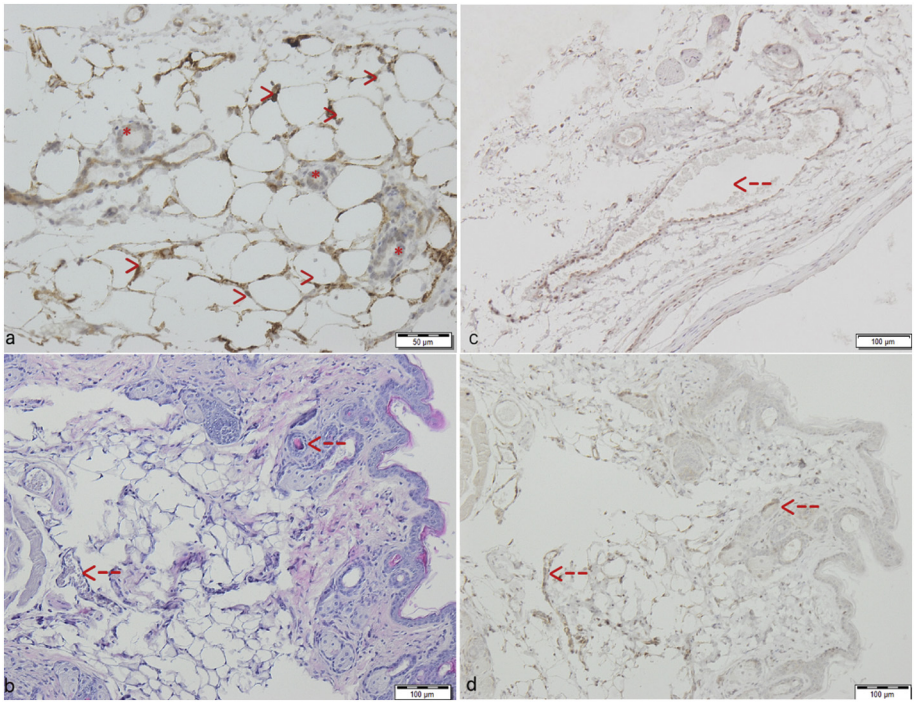

Fig. 3. Histology of eGFP-ASC/adipocyte constructs after 3 months *in vivo*. Light microscopy, Cell^M software.
a. Magnification 20×, immunohistochemical staining (IHC staining) for eGFP, visualization with DAB of *eGFP-ASC/adipocyte construct*: detail of reticular adipose tissue. Arrow-points: eGFP-positive adipocytes. Asterixes: Interlying vascular structures, eGFP-negative.
c. Magnification 40×, IHC staining for eGFP, visualization with DAB of *eGFP-ASC/adipocyte construct*. Arrow: vascular structure with inlying red blood cells, lined by eGFP-positive cells.
b. and d. Magnification 40×, respectively Hematoxyline-Eosine staining and IHC staining with DAB of eGFP of subsequent coupes. Arrows: vascular structures composed of both eGFP-positive and negative cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Hong et al. co-transplant donor ASCs from DsRed-expressing mice with donor fat from GFP-expressing mice into recipient mice to trace the eventual fate of donor cells in the graft. They find that newly differentiated fat from donor DsRed-ASCs and surviving donor GFP-fat integrate with recipient tissue. They also describe donor ASCs to participate in new vessel formation (Hong et al., 2018). These results corroborate those of Fu et al., who also find convincing evidence that the donor SVF cells participate in adipogenesis and angiogenesis (Fu et al., 2013). It must be noted that these findings indicate that the true, *in vivo* potential of human ASCs lies far beyond their defining, *in vitro* criteria as described by the ICTS and IFATS.

We have adopted the idea of inter-syringe shuffling from Tonnard et al., who apply this processing step to create Nanofat (Tonnard et al., 2013). Their protocol focuses on clinical applications, creating a fat graft with fat particles sufficiently small for a controllable injection through fine cannulas.

In search for a mechanical isolation protocol for ASCs, Chaput et al. describe a lesser yield of nucleated cells with inter-syringe processing compared to enzyme digestion (Chaput et al., 2016). Inter-syringe shuffling is further examined by Banyard et al., who also find a three-fold upregulation of $CD34^+$ cells, a universal marker of stem cell activity, isolated through inter-syringe shuffling (Banyard et al., 2016).

Our protocol differs from the protocols of Chaput et al. and Tonnard et al. in a number of ways. In this study (i) the lipo-aspirate is centrifuged first to obtain 10 mL syringes with identical density lipo-aspirate to allow unbiased comparison between the mASC and cASC groups, (ii) no filters are used in the process and, (iii) PBS is added to the lipo-aspirate prior to inter-syringe shuffling. By adding PBS, the efficiency of centrifugation to separate particles of different mass, size and shape, is vastly improved. The volume fraction of the solids, the viscosity and the density difference between the particles and the liquids are altered, allowing for a more efficient centrifugal release of the stromal cell pellet.

In the works of Tonnard and Banyard, the shuffled fat grafts are further subjected to collagenase digestion to analyze, measure and report their resulting ASC yield after processing *via* inter-syringe shuffling, rather than to provide the ASC yield from the inter-syringe shuffling *per se*.

Raposio et al. find a lesser cell yield with mechanical dissociation with vibrant shaking, compared to collagenase digestion, but no anatomical liposuction regions are mentioned, nor the viability assay that is used (Raposio et al., 2017). Shah and all find a lesser cell yield by washing and shaking the adipose tissue, compared to collagenase digestion (Shah et al., 2013).

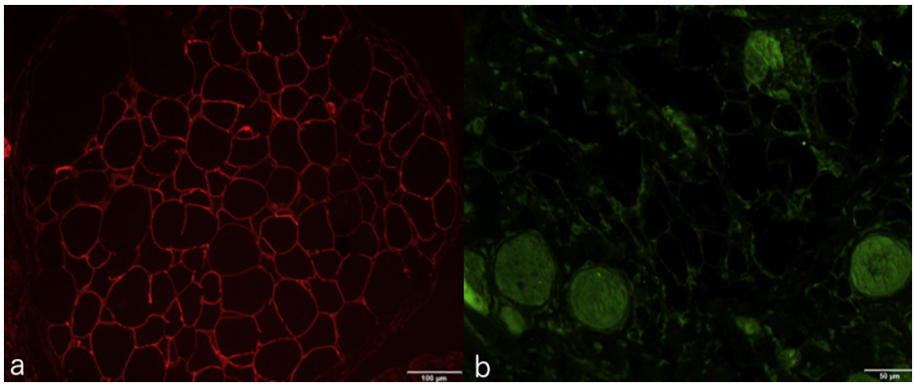

Fig. 4. Immunofluorescent staining of eGFP-ASC/adipocyte constructs after 3 months *in vivo*. Fluorescence microscopy, magnification 40×, Cell^M software.
a. Staining for viable adipocytes at 3 months with Perilipin. Primary antibody, rabbit anti-human perilipin (dilution 1:1000), was incubated overnight at 4 °C. Secondary antibody, Alexa-fluor 555-conjugated donkey anti-rabbit (dilution 1:500) was incubated for 30 min at room temperature.
b. Staining for GFP-positive cells present after 3 months *in vivo* with primary antibody chicken anti-eGFP (dilution 1:50), incubated overnight at 4 °C, biotinylated secondary antibody incubated for 30 min at room temperature, followed by incubation with third antibody Streptavidin/HRP (dilution 1:200) for 30 min at room temperature.

The scientific discussion about the use of collagenase in ASC isolation remains active. Chang et al. describe neutralization of collagenase activity after several washing steps and non-carcinogenity of resulting ASCs (Chang et al., 2013). To reduce xenogens, xenogen-free alternatives for collagenase exist (Carvalho et al., 2013). However, studies also describe the adverse effect of collagenase on human adipocytes, interstitial cell viability, and a very detrimental effect on the fat graft in general (Olenczak et al., 2017; Seaman et al., 2015). Furthermore, 1 g of collagenase costs on average $450, expenses which thus can be saved.

During cell culture, no xenogenic or toxic substances are used, such as FBS, trypsin or red cell lysis buffer. There are many concerns about the practicality of FBS-supplemented medium in cell cultures for human therapeutic approaches, such as infectious complications or host immune reactions (Muller et al., 2006). Others have tested new xenogen-free media (Muller et al., 2006; Rajala et al., 2007; Parker et al., 2007) but find significant differences in surface markers. Autologous plasma however is readily available in large quantities, very cost-efficient, and does not pose these safety issues. During the experiment the platelet-containing buffy coat is not included for standardization. However, in pre-trial experiments, adding of this platelet-rich layer appeared to cause a strong increase in culture growth (unpublished observation), corroborating other studies (Masoudi et al., 2016; Schallmoser and Strunk, 2009).

Because the mechanical extraction and autologous plasma-augmented culture of ASCs is completely xenogen-free, the secretome of the cultured ASCs is available for clinical purposes. After hypoxic culture in particular, ASCs are known to secrete a vast array of growth factors (Braga Osorio et al., 2010).

Any procedure related to Cell-Based Medicinal Products (CBMPs) requires strict control in a cGMP facility (Giancola et al., 2012). These facilities are designed and organized according to Good Manufacturing Practice for Pharmaceutical Manufactures. *Quality control and quality assurance programs* control collection, processing, storage and release of CMBPs developed in these facilities. Among others, all used equipment and materials are specified, controlled, validated, labeled, risk assessed and reviewed. Hence, any effort to reduce the involved materials in the processing is a major cost and time reducing benefit. Furthermore, major risks to CMBPs include microbiological contamination, loss of cell function, immunogenicity, toxicity of media. Again, keeping the procedures simple and close to the clinical practice, vastly reduces these adverse events.

In conclusion, an easy and practical method for human ASC isolation and culture from abdominal liposuction fat, without the addition of xenogenic substances, is described. We compare this novel isolation method to the current gold standard, collagenase digestion, and find it to be more efficient.

Additional information/statements

- This manuscript and every part of it are original, not previously published nor under consideration elsewhere.
- There are no competing/ conflicting interests to declare.
- We agree to make all materials, data and associated protocols in this manuscript promptly available to readers without undue qualifications in material transfer agreements.

Author contributions

Maarten AJ Doornaert: Experimental setup, laboratory experiments, draft writing.
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Julien Colle: Laboratory experiments, cell culture.
Heidi Declercq: Laboratory experiments, review.
Phillip Blondeel: Clinical support, theoretical discussion, review.

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