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Adipogenic differentiation of adipose-derived stem cells in 3-dimensional spheroid cultures (microtissue): Implications for the reconstructive surgeon☆

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Summary *Background:* Adipose-derived stem cells (ADSC) can be readily extracted from adipose tissue, expanded in vitro, and have the capacity to differentiate into multiple cell lineages. This makes this cell type of great interest to the field of regenerative medicine. This study focuses on the isolation and characterisation of ADSC and their differentiation into adipocytes in a 3D microtissue model.
Methods: Human ADSC were isolated from abdominal adipose tissue and characterised using multiparameter flow cytometry. ADSC were then expanded in culture and used to produce 3D scaffold-free micro-tissue. Adipogenic differentiation potential of micro-tissue constructs were subsequently characterised using Oil Red O staining.
Results: Flow cytometric analysis showed ADSC were uniformly positive for CD34, CD73, CD90, and CD105, and negative for CD19, CD14, and CD45. The cells were functionally induced into adipocytes in the presence of appropriate conditioned media.

☆ Conceived the research topic ISW. Designed the experiments ZX. Performed the experiments NN, CW, WF, ZX, CT. Analyzed the data: NN, AS, CT, ISW, ZX. Contributed consumables ZX. Wrote the paper. NN, ISW, CT, ZX. Read and approved the final manuscript for submission NN,CW, WF, AS, CT, ISW, ZX.

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Conclusion: We have demonstrated that adipose-derived stem cells have the ability to form of microtissue and survive in vitro. We postulate that in the future this will result in an ADSC population which is injectable and can extend the delivery options of current stem cell-based therapies.

Introduction

Adipose tissue is used as an autologous filler for soft-tissue defects, following trauma, birth defects, and oncologic resection.[1,2] Autologous fat transfer has the advantage of using the patient's own tissue with minimal donor-site morbidity and eliminating the issues of foreign body reaction or rejection.

In recent years, fat transfer has become increasingly interesting to the plastic surgeon, used for indications such as facial volume replacement,[3] cosmetic breast augmentation,[4] correction of congenital breast abnormalities,[5] chronic wounds[6] and cutaneous radiation syndromes.[7] Fat transfer has also been shown in an animal model to accelerate revascularisation and decrease fibrosis following thermal injury.[8] The ASAPS/ASPS position statement in 2012[9] gives a succinct overview of the issues surrounding contemporary fat transfer and stem cell therapies, and acknowledges the need for increased efforts into both basic science investigations and translation into evidence based clinical therapies.

When replacing volume, fat graft survival is one of the major problems, with long term follow up studies revealing 20—70% volume loss.[10—15] The lack of revascularisation of grafts commonly underpins the problem, and this is particularly a problem in breast reconstruction where larger volumes of fat are required. The result is that multiple procedures are required, with increased direct and hidden financial and emotional costs.

Adipose-derived stem cells (ADSC) are an area of interest for plastic and reconstructive surgeons at present as they can differentiate to specialized cells,[16—18] enhance angiogenesis and minimise inflammation.[19] ADSC have similar characteristics to bone marrow-derived mesenchymal stem cells (BMSC),[20—24] contain 100—1000X more MSC per cm^3[26] and are easier to isolate. ADSC are identified by plastic adhesion, immunophenotypic profile, cell morphology, and multilineage differentiation.[16,25] Due to these factors, ADSC have been the focus of much investigational work in recent years,[27,28] differentiated down all mesenchymal cell lines[28] and are easily expanded in *vitro*.[29] Current lines of investigation include techniques to improve graft survival and methods of delivery. Scaffold-free cell delivery is an area of interest for regenerative medicine research including three main techniques: single cell, cell sheet engineering and micro-tissue.[30] Micro-tissue is a 3D scaffold free cell aggregate using spheroid cultures, and has been referred to as multi-cellular spheroids, micromasses or microspheres in the literature.[31] Engineered micro-tissue constructs have been developed for targeted nervous system reconstruction with living axons, dermal substitution,[35] and vessel[36] and cartilage[37] reconstruction. The basic advantages of multi-cellular spheres over 2D monolayer cultures are the cell-to-cell and cell-to-matrix interactions.[31] Kelm and Fussennegger suggest that 'microtissue maintains tissue specific functionality and supports seamless integration of implants into host tissues'.[38] Compared to monolayer cultures, there seems to be an accelerated differentiation of cells in microtissues.[31]

The induction of adipogenic differentiation in spheroid cultures is new, therefore in this study we aimed to induce adipogenic differentiation in 3D culture systems in vitro as a proof of concept. The differentiation capacity between umbilical cord and adipose-derived stem cells was also investigated in a similar fashion to that of Hildner's study of micromass cultures.[42]

Materials and methods

Adipose tissue samples ($n = 5$) were obtained from the supra-Scarpa's fascia abdominal tissue of patients undergoing Deep Inferior Epigastric Perforator (DIEP) flap reconstruction (age range 31—58 years, mean = 46 years). Umbilical cord mesenchymal stem cells (UC-MSC) were isolated from umbilical cord of full term placentas ($n = 4$). Approval for this study was from the South Wales Research Ethics Committee; all participants gave written informed consent.

Isolation of human adipose-derived stem cells (ADSC)

ADSC were isolated according to the method described by Zuk et al.[16] with modifications. Following removal of fibrous tissue and visible blood vessels, samples were cut into small pieces (3 mm^3) and digested in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) containing 300 U/ml crude collagenase II (Invitrogen, Life Technologies Ltd, Paisley, UK) for 30 min in an incubator (37 °C, 5% CO_2). The dispersed material was filtered through 70 μm Cell Strainers (BD Biosciences, Oxford, UK). After centrifugation (290× G, 5 min), the ADSC-rich cell preparation formed a pellet at the bottom of the tube. The supernatant was removed and the pellet re-suspended. The number of viable cells was determined by cell counting on a haemocytometer and Trypan blue exclusion. At passage zero 1×10^6 cells per test tube (12 in total) were reserved for flow cytometry analysis and the remaining cells expanded in culture.

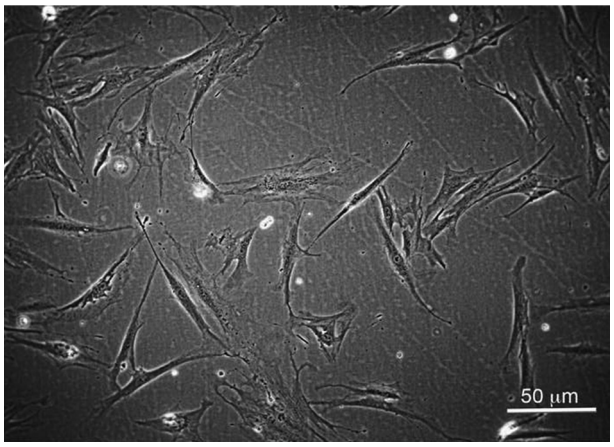

Figure 1 Light microscope image of sub-confluent ADSC at passage 2. 10× magnification showing spindle-shaped plastic-adherent ADSC.

Table 2 Flow cytometry voltage configurations. FSC: forward scatter, SSC: side scatter, FITC: fluorescein isothiocyanate, PE: phycoerythrin, APC: allophycocyanin, PerCP: peridinin-chlorophyll proteins, BDV500: BD Horizon™ V500.

Flow Cytometry Configuration	
Parameters	Voltage
FSC	0
SSC	269
FITC	385
PE	440
APC	610
Alexa Fluor 780	632
BDV500	341
eFluor 450	390
PerCP-Cy 5.5	571
PE-Cy7	786

Isolation of human umbilical cord mesenchymal stem cells (UC-MSC's)

UC-MSC were isolated according to the method described by S. Azandeh et al. with modifications. Exposed veins and arteries were removed from the Whartons Jelly (WJ). The extracted WJ was washed twice with carrying solution and cut into 3—5 mm^3 pieces. For explant growth 6—9 pieces were seeded into 25 cm^2 T-flasks, containing DMEM/F-12 with 10% FBS and 1% antibiotic and incubated (37 °C, 5% CO_2).

Culture and expansion

Cells were cultured for up to 2 passages (Figure 1). At each subsequent passage, cells were seeded to sub-confluence in 75 cm^2 culture flasks for 7—10 days at a cell density of 3×10^4 per cm^2. When the cells reached approximately 80% confluence, subculture was performed through trypsinization. The cell suspension was centrifuged (290× *g*, 5 min), the pellet was resuspended and cells were counted as before. After each passage, 5×10^5 cells per test tube were reserved for flow cytometry (12 in total for ADSC only) and the remaining cells seeded in new 75 cm^2 flasks at 3×10^5 cells per flask and incubated (37 °C, 5% CO_2).

Immunophenotypic characterization of ADSC

ADSC from passage 0 to 2 were used for immunophenotypic characterization using flow cytometry. ADSC were stained with antibodies for different CD (cluster of differentiation) antigens and HLA-DR (Table 1 and Table 2). None of the antibodies were further diluted. 6×10^6 cells at passage 1 onwards and 1.2×10^7 cells at passage 0 were suspended in 0.2 ml PBS and incubated with the antibodies for 30 min on ice and protected from light. At each analysis a separate tube was used for every antibody to provide compensation controls, one sample tube contained all antibodies, 2 sample tubes contained unstained cells as controls, and one tube contained the isotype controls. Subsequently, Easy-Lyse solution was used to lyse the red blood cells — 3 mls of 1:20 Erythrocyte-Lysing Reagent EasyLyse (BD Biosciences, Oxford, UK) in distilled water was added to each tube, left in the dark at room temperature for 15 min, and then centrifuged at 515× *g* for 7 min at 5 °C. The supernatant was then removed, 3 mls of FACS buffer (PBS/0.2% BSA/0.05% sodium azide) added, and each tube was centrifuged at 515× *g*, 5 °C for 7 min again. The supernatant was again removed and 200 μl of 1:3 diluted FACS fix solution (BD Biosciences, Oxford, UK) in distilled water was added. The samples were acquired by flow cytometry (FACSAria I; BD Biosciences, Oxford, UK) within 24 h. Kaluza software (version 1.2; Beckman Coulter, USA) was used to analyse the data.

Micro-tissue production

UC-MSC and ADSC were seeded into 75 cm^2 tissue culture flasks in DMEM-F12 containing glutamate (Life Technologies

Table 1 Flow cytometry antibodies. FITC: fluorescein isothiocyanate, PerCP: peridinin-chlorophyll proteins, APC: allophycocyanin, PE: phycoerythrin.

	CD45	CD34	CD19	HLA-DR	CD14	CD73	CD90	CD105
Source	BD Biosciences, New Jersey, USA		eBioscience, San Diego, USA		Miltenyi Biotec, Germany	eBioscience, San Diego, USA		
Fluorescent dye	FITC	PerCP	APC-eFluor 780	PerCP-eFluor 710	VioGreen	eFluor 450	APC	PE
Excitation wave length (nm)	490	482	633	488	405	409	650	496

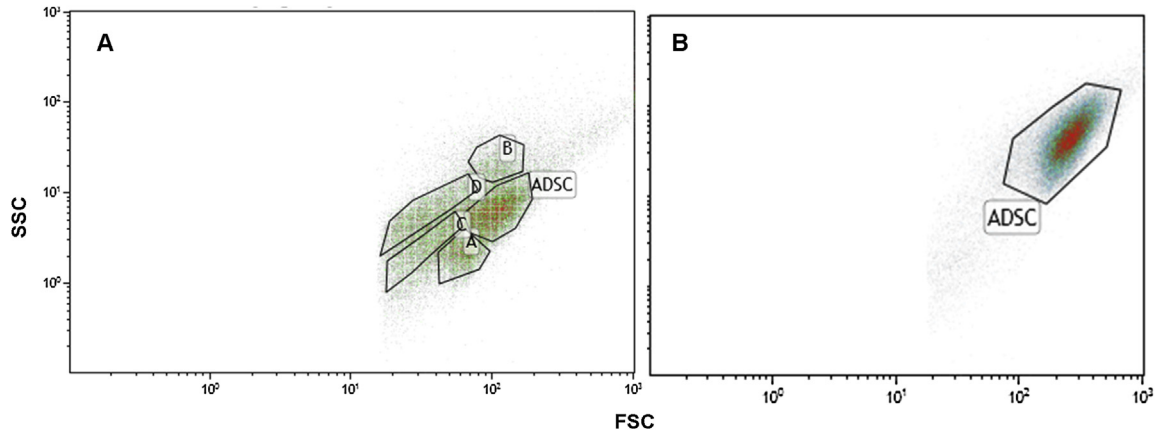

Figure 2 Flow cytometry analysis at P0 and P1. A) At P0 five separate cell populations were identified. B) At P1 only 1 population remained identifiable.

Ltd, Paisley, UK), 10% FCS, 1% antibiotic and incubated (37 °C, 5% CO_2). Cultures were fed every 3—5 days until cells reached 70—80% confluence. Cells were then trypsinised and resuspended into 60 well Terasaki plates (Griener BioOne, Stonehouse, Gloucestershire, UK) at the seeding density of 2 × 10^3 cells (in 20 μl) per well and incubated upside-down overnight to form microtissue in hanging drops (Figure 4).

Live-cell fluorescent staining

The relative locations of DNA in all cells, intra-plasmatic esterase in live cells, and nucleic acids in dead cells within micro-tissues were visualized via staining with the fluorescent dyes Hoechst 33342, Calcein AM, and Propidium Iodide (PI), respectively (Life Technologies Ltd, Paisley, UK). The micro-tissue samples were incubated with serum-free DMEM-F12 containing 5 μl/ml of 5 mg/ml Hoechst 33342, 0.5 μl/ml of 4 mM Calcein AM, and 1 μl/ml of 1 mg/ml PI fluorescent dyes for 15 min. Stained cells were imaged by confocal microscopy with an excitation/detection of 352/461 nm, 495/515 nm, and 535/617 nm, respectively.

Adipose differentiation, Oil Red O and DiI staining

Adipose tissue differentiation media containing Dexamethasone (1 μM), 3-isobutyl-1-methylxanthine (0.5 mM), Indomethacin (60 μM), Insulin (10 μg/ml) and Hydrocortisone (0.5 μM) was used for the experiments. For adipose differentiation, the microtissues formed from hanging drops were moved to a U-bottom 96 well plate (Griener BioOne, Stonehouse, UK). Adipose differentiation was assessed at 7, 10, and 14 days using Oil red O staining and light microscopy. Due to the possibility of microtissue dissociation in 2D or U-bottom plate cultures, a rotator was used to sustain 3D culture. The contents of twenty wells containing micro-tissues were transferred from the Terasaki plates in a centrifuge tube and incubated in a Ruskinn

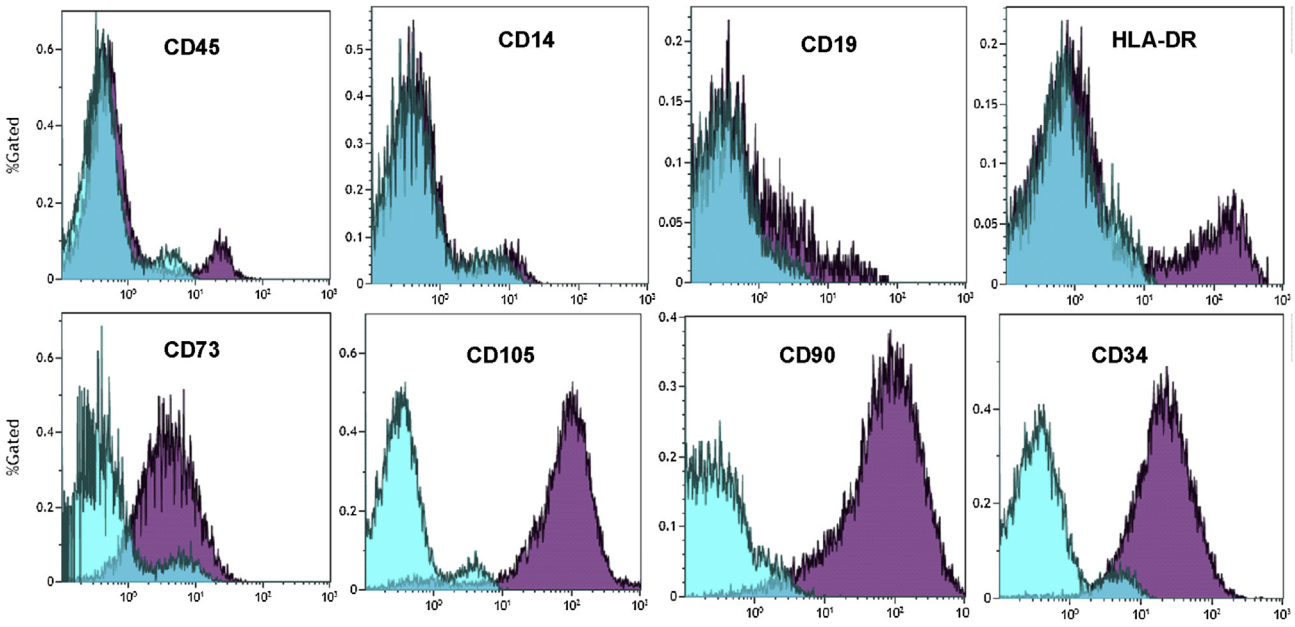

Figure 3 CD antigen expression at P1. ADSC expressed CD73, CD90, CD105, CD34, and lacked CD14, CD19, CD45 and HLA-DR expression. A small population of contaminating macrophages was observed which was CD45 and HLA-DR positive.

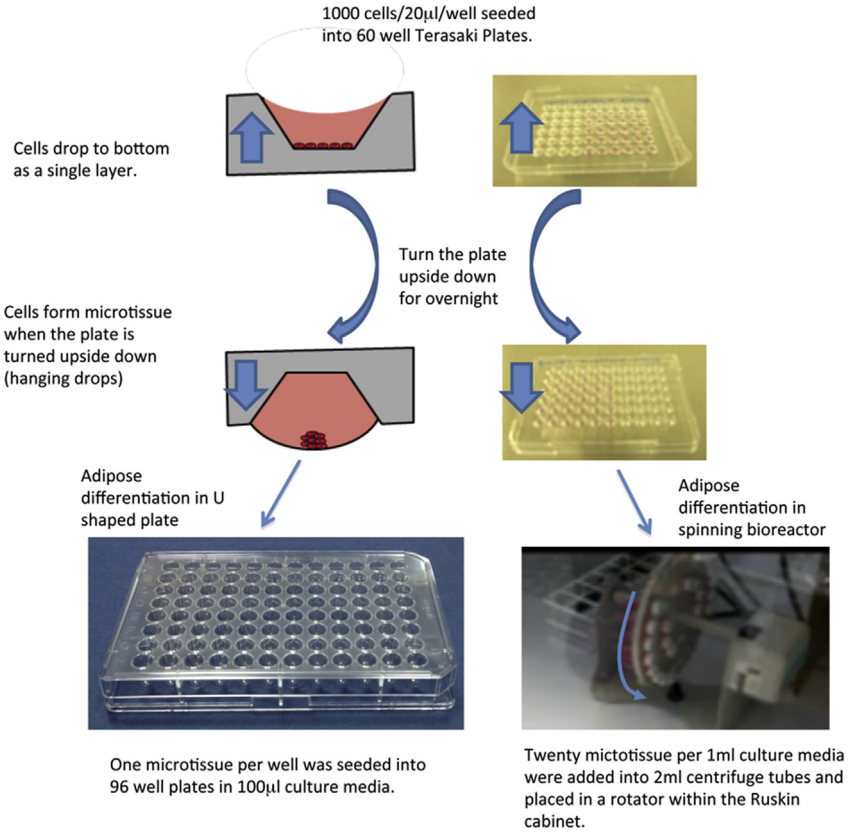

Figure 4 Diagram of microtissue preparation.

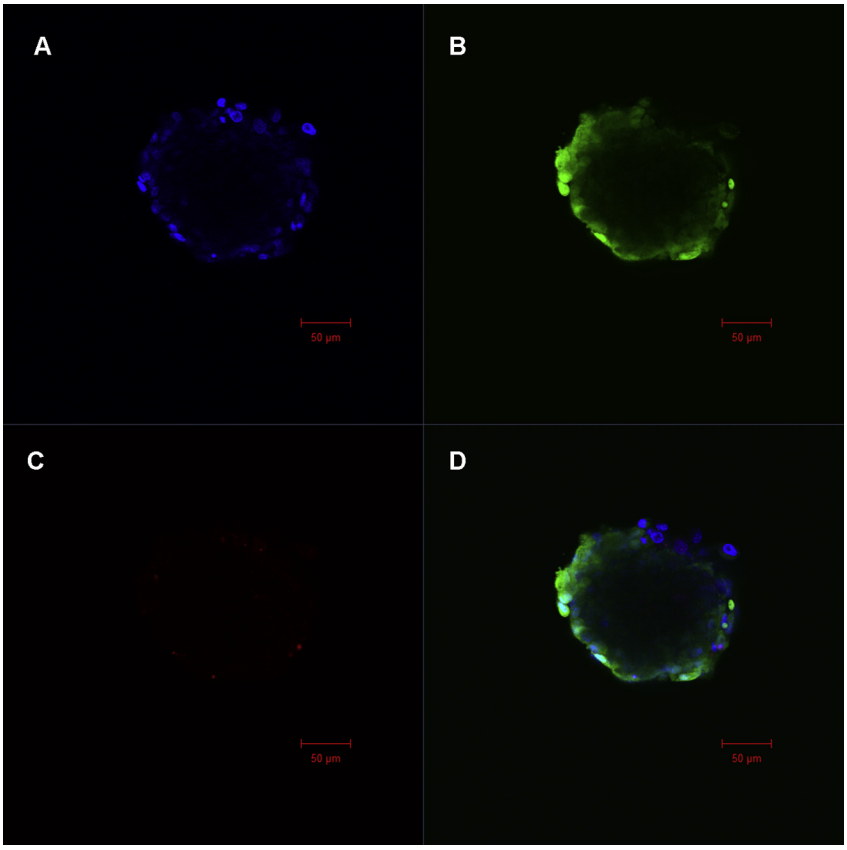

Figure 5 Micro-tissue formation from ADSC 1 day after incubation. Confocal microscopy of A) Hoechst 33342 (blue) for cell nuclei, B) Calcein AM (green) for living cells, and C) PI stained (red) for dead cells. D) Merged image of the three stains.

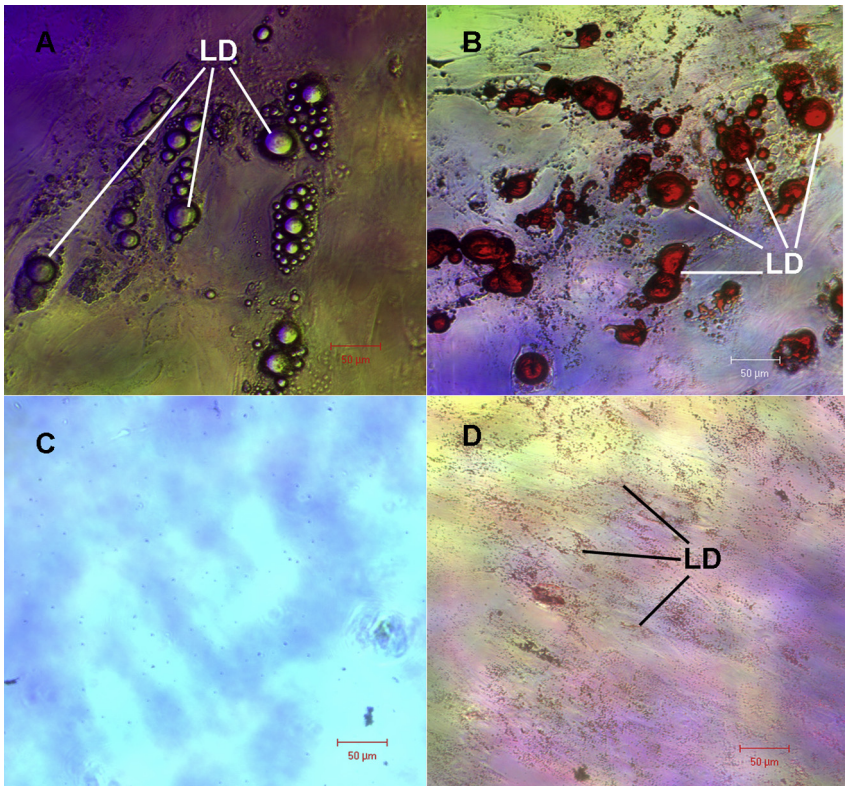

Figure 6 Lipid formation in ADSC compared to UCM-MSC in U-bottom 96 well plate culture system demonstrated using Oil Red O staining and light microscopy. A) ADSC in ADM for 10 days, lipid droplets (LD) are visible. B) Oil red O staining of ADSC in ADM for 10 days showing large red lipid droplets (LD). C) UCM-MSCs in ADM for 14 days, no visible lipid droplets observed. D) Oil red O staining of UCM-MSCs in ADM for 14 days showing only very fine lipid droplets (LD).

SCI-tive Dual system (Ruskinn Technology Ltd, Bridgend, UK). A Mini LabRoller, Labnet (Appleton Woods Ltd, Birmingham, UK) was used to rotate the centrifuge tubes containing microtissue for continuous culture. Adipose differentiation was assessed at day 7 using DiI (Life Technologies Ltd, Paisley, UK) fluorescent staining for lipids and observed by confocal microscopy. All cultures were rinsed in PBS and fixed in 10% buffered formalin for 1 h. A 0.5% stock solution of Oil Red O in 100% isopropanol (50mg/10 ml) was prepared. Cultures were rinsed in 60% isopropanol, immersed in a working solution of Oil Red O for 30 min and subsequently washed several times in distilled water.

For fluorescent staining of microtissues, 5 µl/ml of 5 mg/ml Hoechst 33342, 0.5 µl/ml of 4 mM Calcein AM were used to stain living cells and a lipophilic dye, DiI (Life Technologies Ltd, Paisley, UK) was used to stain lipids for 30 min.

Quantification of Oil Red O staining

Isolated ADSC ($n = 5$) were differentiated into adipocytes or grown in culture in control media. The level of Oil Red O staining was quantified by dissolving the stained fat in 100% isopropanol for 15 min. The plates were centrifuged at 1000 rpm for 10 s and the supernatant taken for spectroscopic analysis using the Polarstar Omega plate reader (BMG Laboratories, UK). Absorbance was read at 500 nm. The student *T*-test (Microsoft Excel software) was used to show statistical significance in the difference of Oil Red O staining between the differentiated ADSC and controls.

Results

Isolation and flow cytometry characterisation

Cellular surface markers on passage 0, 1, and 2 cells were analyzed in order to characterize the cell populations. At passage 0, five different cell populations were consistently identifiable (Figure 2A), whilst only a single cell population remained at passages 1 and 2 (Figure 2B). At passage 0 each population showed a different CD expression profile. Only one population remained throughout the passages and this was uniformly $CD34^+/CD73^+/CD90^+/CD105^+/CD19^-/CD14^-/CD45^-$ (Figure 3) and capable of undergoing adipogenesis in vitro based on Oil Red O staining (Figure 6B and D).

Live-cell fluorescent staining of micro-tissues

Confocal microscopy images of Hoechst, Calcein AM, and PI stained micro-tissue formed 1 day after incubation of ADSC showed good cell viability, with micro-tissue diameters of around 100 µm (Figure 5A–D).

Adipose differentiation

ADSC and UCM-MSC micro-tissues were cultured in adipose differentiation medium (ADM), Cells in microtissue were dissociated in U-bottom tissue culture plates during the course of culture. Adipose differentiation was assessed at

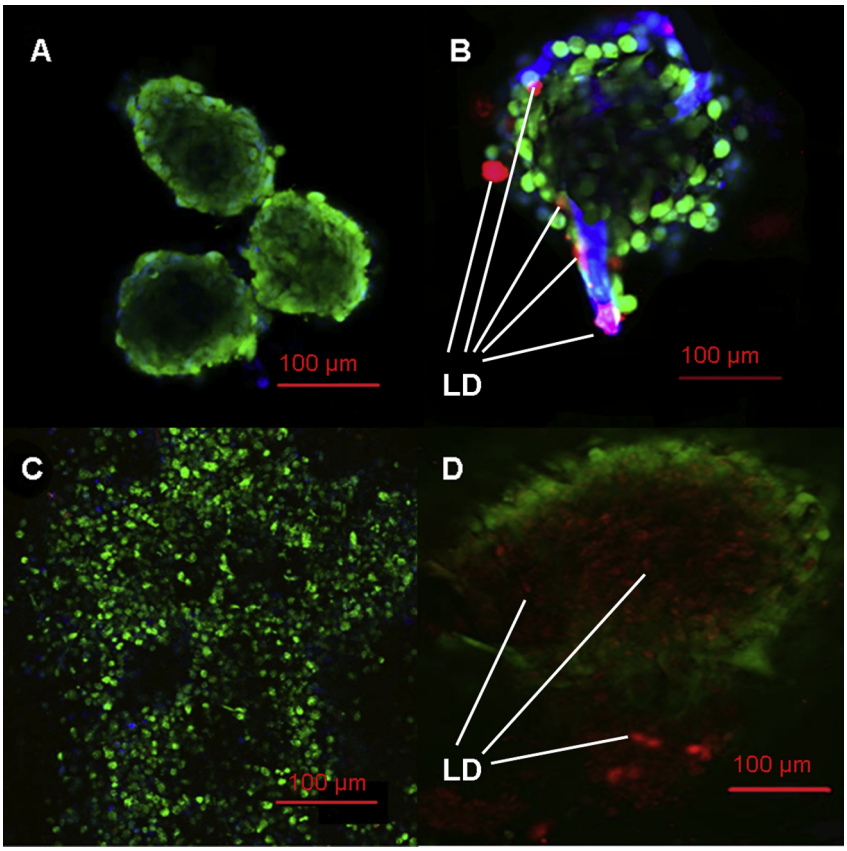

Figure 7 Adipose differentiation in a 3D rotating culture system. A) ADSC micro-tissue in control culture medium for 1 week. B) Lipid staining (red) of ADSCs in ADM for 1 week. Notice the red lipid droplet (LD) formation. C) UCM-MSC in control culture media for 1 week. Micro-tissue grows in size. D) UCM-MSCs in ADM for 1 week. Formation of only very fine lipid droplets (LD) observed.

7, 10, and 14 days using fluorescent Oil red O staining and light microscopy. We have demonstrated that ADSC from micro-tissue can successfully differentiate into adipocytes (Figure 6A and B) and have developed a protocol for adipose tissue differentiation. Our results illustrate that ADSC had a better potential of adipose differentiation (Figure 6A and B) compared to UCM-MSC (Figure 6C and D). In 3D culture using a Ruskinn stem cell station and a rotating bioreactor in vitro, we demonstrated that ADSC differentiated into adipose cells within microtissue using DiI lipophilic fluorescent staining (Figure 7A and B). Using Calcein AM staining for living cells we demonstrated that most cells in microtissue remained viable after 7 days in 3D culture (Figure 7).

Oil Red O staining of differentiated cells was significantly higher ($p = 0.0024$) than that of the undifferentiated control cells (Figure 8).

Discussion

As aforementioned, human ADSC can be readily harvested from adipose tissue using standard techniques[16] and have high proliferation rates for in vitro expansion with multilineage differentiation capacity.[24] There is considerable contemporary interest in the applications of human ADSC, and there are many ongoing clinical trials regarding their use. A variety of methods have been developed to harvest these cells, but the purity of isolated ADSC remains an issue. The Cytori Therapeutics' Celution® system (Cytori Therapeutics Inc., San Diego, CA, USA), for example, isolates a heterogenous population of cells with fractions of ADSC that are 'cell enriched' rather than specifically 'stem cell enriched'.[43] To date there is no ideal method for identification of ADSC from the SVF. Several groups are

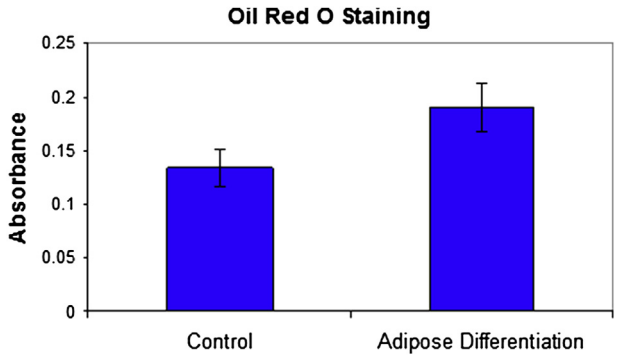

Figure 8 Oil Red O staining quantification of adipogenic differentiated ADSC compared to undifferentiated ADSC controls ($n = 5$) ($p = 0.0024$).

currently investigating identification methods and mechanisms to isolate and purify ADSC.[44–46] We used a simple and effective way to obtain ADSC by using collagenase digestion. Since no *unique* molecular marker for mesenchymal stem cells has been established we used multi-parameter flow cytometry for the identification of ADSC. ADSC showed consistent expression of CD73, CD90, CD105 (known mesenchymal stem cell markers), and CD34 contrasting with the lack of expression of haematopoietic cell markers CD14, CD19, CD45, and HLA-DR. There was a small subset of HLA-DR positive cells present at passage 1 which were identified as microphages and were no longer present at passage 2. CD34 expression in early culture of mesenchymal stem cells is well documented, although loss of expression does occur in later passages.[47–50]

Our results provide preliminary in vitro evidence that mesenchymal stem cells derived from human adipose tissue can be grown as floating micro-tissue spheres through several passages. In the present study, we plated cells from freshly dissociated adipose tissue in preliminary monolayer culture. Our culture process induced the loss of the vast majority of the harvested primary stromal vascular fraction (SVF) cells, while some of the growth factor-responsive cells generated micro-tissues. This was evident from the loss of 3 cell populations after several passages. ADSC have the capacity to proliferate in non-adherent conditions and to form medium sized micro-tissues (100–200 μm in diameter). As we expected, the ADSC showed superior adipogenic differentiation capacity compared to UC-MSCS.

Previous studies support our putative theory that micro-tissue culture of ADSC may enhance their biological activity, and hence be of benefit *in vivo* – the administration of human ADSC increased the rate of diabetic wound healing when ADSC were derived from aggregates, but not when ADSC derived from monolayer culture were delivered.[51] Dromard et al. developed a new culture system, which allows the expansion of ADSC as spheres in a defined medium[40] and these spheres could be passaged several times. They were not only aggregated cells but rather originated from single cells as clonal spheres that can be obtained after seeding at very low density and reform clonal spheres after dissociation. These spheres can revert to monolayer growth when plated in medium containing human plasma and even generate fibroblast-like colonies. Under several differentiation-specific media, sphere-derived ADSC maintained their capacity to differentiate into osteoblasts, endothelial cells and adipocytes. There is a growing interest in designing bioreactor systems that provide safe and controlled high-density cell culture. In this context, our results represent an important step in our ability to isolate and maintain ADSC in culture. Our in vitro study builds on the work of others involving different mesenchymal stem cell lineages and shows that growth of ADSC in micro-tissue is possible. As the induction of adipogenic differentiation in spheroid cultures is relatively new, we believe the ability to induce adipogenic differentiation in 3D culture systems in vitro is of interest, 3D culture systems may enhance biological activity and in timetool to investigate and develop novel injectable micro-tissue preparations from adipose-derived stem cells in the future. If successful, such preparations would have potential as an *in vivo* cell delivery tool in soft tissue regeneration and reconstruction.

Conflict of interest

None.

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