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BRIEF REPORT

A novel composition for the culture of human adipose stem cells which includes complement C3

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Abstract Adipose tissue is an easily accessible and abundant source of stem cells. Adipose stem cells (ASCs) are currently being researched as treatment options for repair and regeneration of damaged tissues. The standard culture conditions used for expansion of ASCs contain fetal bovine serum (FBS) which is undefined, could transmit known and unknown adventitious agents, and may cause adverse immune reactions. We have described a novel culture condition which excludes the use of FBS and characterised the resulting culture. Human ASCs were cultured in the novel culture medium, which included complement protein C3. These cultures, called C-ASCs, were compared with ASCs cultured in medium supplemented with FBS. Analysis of ASCs for surface marker profile, proliferation characteristics and differentiation potential indicated that the C-ASCs were similar to ASCs cultured in medium containing FBS. Using a specific inhibitor, we show that C3 is required for the survival of C-ASCs. This novel composition lends itself to being developed into a defined condition for the routine culture of ASCs for basic and clinical applications.

Keywords Stem cells · Tissue specific stem cells · Defined medium · Complement

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Introduction

Mesenchymal stem cells (MSCs) are multipotent stromal progenitor cells which were first cultured from bone marrow (Friedenstein et al. 1966) and have since been identified in other tissues including adipose tissue, umbilical cord blood, muscle, placenta and synovium (Erices et al. 2000; Zuk et al. 2001; Fukuchi et al. 2004; Sakaguchi et al. 2005). MSCs are being explored as treatment options for several disease conditions including graft versus host disease (GvHD) (Yanez et al. 2006), Crohn's disease (Gonzalez et al. 2009), autoimmune encephalomyelitis (Constantin et al. 2009), osteogenesis imperfecta (Horwitz et al. 2002), breast cancer (Qiao et al. 2008), metabolic diseases (Koc et al. 2002), myocardial infarction (Chen et al. 2004) and hematopoietic recovery after

hematopoietic stem cell (HSC) transplantation (Koc et al. 2000).

Adipose tissue is an attractive source of somatic stem cells as it is abundantly available and easily accessible (Zuk et al. 2001). Adipose stem cells (ASCs) are known to be similar to bone marrow derived MSCs (BM-MSCs) in surface marker phenotype (Wagner et al. 2005; McIntosh et al. 2006), and can be directed towards bone, cartilage, adipocyte, myocyte or neuronal lineages (Zuk et al. 2001; Planat-Benard et al. 2004; Ning et al. 2006). Adipose tissue serves as a reliable source of multipotent adult stem cells with substantial therapeutic potential. ASCs have found clinical applications in the field of regenerative medicine (Garcia-Olmo et al. 2009) and transplantation (Fang et al. 2007), as well as gene therapy and cellular immunotherapy (Kucerova et al. 2007; Zuk 2010).

MSCs and ASCs are typically cultured in media supplemented with 10% FBS (Suga et al. 2007). Disadvantages of FBS use include its undefined nature, inherent variations in active components, and the risk of contamination with known and heretofore unknown adventitious agents. Media additives and supplements that have been proposed instead of FBS include human platelet lysate (HPL), autologous and allogeneic human sera (Schallmoser et al. 2008; Bieback et al. 2009; Perez-Ilzarbe et al. 2009), commercially available serum replacements, and 'defined' media with proprietary compositions (Wu et al. 2006; Agata et al. 2009; Lindroos et al. 2009; Lund et al. 2009; Baer et al. 2010). As MSCs grown in 5% HPL have low immune suppressive activity, they could be better suited for regenerative cell therapies than MSCs grown in serum supplemented medium (Bernardo et al. 2007). A serum free medium supplemented with serum substitute (ULTROSER) allowed better ex vivo expansion of MSC compared to α-MEM medium supplemented with FBS, in terms of greater colony-forming unit-fibroblast (CFU-f) progenitors and expansion rate (Meuleman et al. 2006). Others have employed defined medium to test the effect of individual growth factors on adipose precursor cells (Vassaux et al. 1994; Suga et al. 2007).

In this report we describe a novel culture condition in which human ASCs are isolated de novo from adipose tissue, propagated, and maintained in culture without using FBS. Human ASCs were cultured in the novel culture medium which included complement protein C3, and these cultures were called C-ASCs. We have characterized these cultures by describing surface markers with flow cytometry, and have determined the population doubling time (PDT) and CFU-f profiles. Functionally, we have shown that C-ASCs can undergo in vitro differentiation into cartilage, bone and adipose lineages. Thus, the C-ASC culture condition is an important step towards a fully defined composition which yields adipose stem cell cultures.

Materials and methods

Materials

MCDB 201 medium, minimum essential medium Eagle (αMEM), serotonin hydrochloride, dexamethasone, linoleic acid albumin, 2-mercaptoethanol, Indomethacin, 3-isobutyl-1-methylxanthine, L-proline, L-ascorbic acid-2-phosphate sesquimagnesium salt hydrate, human complement serum, complement C3 deficient serum (human), Trypsin inhibitor type I from soybean, Histopaque 1077, Brilliant Blue R, Oil Red-O, TRI reagent and β-glycerol phosphate disodium salt pentahydrate were procured from Sigma–Aldrich; DMEM/F12 powder, N2 supplement, L-Glutamine, Sodium pyruvate, DMEM (with glutamax) medium, penicillin–streptomycin (Pen-Strep), 0.05% Trypsin–EDTA, SuperScript II Reverse Transcriptase, Oligo-dT primer, 10 mM dNTP mix, 0.1 M DTT, RNaseOUT recombinant ribonuclease inhibitor and recombinant human basic fibroblast growth factor (bFGF) were from Invitrogen; recombinant human epidermal growth factor (EGF) and recombinant human transforming growth factor (TGFβ III) were purchased from R&D Systems, ITS Premix (ITS: insulin, transferrin, selenium) and tissue culture dishes from BD Biosciences, Low-Tox-R complement from Cedarlane Labs; Sodium bicarbonate, Formaldehyde solution, DPX mountant, Hematoxylin, Eosin and Safranin O from Qualigens; 0.1% Gelatin and Hydrocortisone from Stem Cell Technologies; phosphate buffered saline (PBS) and fetal bovine serum (FBS) from Hyclone, Trypan blue from Fluka; GeneRuler 100 bp DNA ladder from Fermentas; Taq DNA Polymerase from Bangalore Genei; RT-PCR primers from Integrated DNA Technologies; Calcium Quantitative Kit from Pointe Scientific Inc; swine serum from Dako, Impact DAB and diluents from Vector Laboratories, Inc., Streptavidin peroxidise from Biogenic,

Compstatin from Tocris, CryoSure DMSO from Wak–Chemie Medical, and Collagenase type I from Worthington.

Antibodies PE (Phycoerythrin) conjugated mouse anti-human CD29, PE conjugated mouse anti-human CD44, PE conjugated mouse anti-human CD73, PE-Cy5 conjugated mouse anti-human CD90, FITC (fluorescein isothiocyanate) conjugated mouse anti-human CD45, PE conjugated mouse anti-human CD34, PerCP (Peridinin chlorphyll protein) conjugated mouse anti-human CD14 and the negative control mouse IgG antibodies for the three fluorochromes PE, FITC and PerCP were all obtained from BD Biosciences. FITC conjugated mouse anti-human CD105 was acquired from AbD Serotec; Rabbit polyclonal antibody to collagen type 2 from AbCam, and polyclonal swine anti-rabbit antibody from Dako.

Isolation and culture of human ASCs

Human subcutaneous adipose tissue was obtained as waste material from patients undergoing elective surgical procedures after obtaining informed consent, as per the regulatory guidelines of the Institutional Review Board of Christian Medical College. A little in excess of the required quantity of the tissue was minced and transferred to a 50 mL tube under sterile conditions. The adipose tissue was washed several times with sterile PBS until it was relatively clear of blood, and digested with an equal volume of 0.1% collagenase type I, with agitation, at 37 °C for 1 h. After centrifugation at 1,200 rpm for 5 min at room temperature, the supernatant was aspirated along with the floating layer of oil and adipocytes. The stromal vascular fraction (SVF), which is the cell pellet, was resuspended in the growth medium and plated in flasks such that the seeding density was approximately 0.16 mL of tissue/cm^2 (Mitchell et al. 2006).

C-ASC cultures were seeded in tissue culture dishes/flasks coated with 0.1% gelatin. Media containing 60% DMEM/F12/N2 and 40% MCDB 201 was used, as a similar composition was previously shown to support the culture of MSCs (Jiang et al. 2002). This was supplemented with 10 μM L-ascorbic acid-2-phosphate, 1 μL/mL ITS premix, 10 μL/mL linoleic acid, 1% Low-Tox-R Complement, 20 ng/mL EGF, 15 ng/mL bFGF, 1 nM dexamethasone, 30 nM serotonin hydrochloride, and Pen-Strep at the concentration of 100 units/mL of penicillin/100 μg/mL streptomycin. After 24 h non-adherent cells and tissue were aspirated and the medium was changed. Thereafter half the medium was changed every other day, while the cultures were fed daily with EGF and bFGF to a final concentration of 20 and 10 ng/mL respectively. Cultures were passaged at 70% confluency by trypsinization, neutralized with an equal volume of 1 mg/mL of type 1 trypsin inhibitor, and reseeded at a density of 4,600 cells per cm^2. ASCs were cultured either in DMEM supplemented with 10% FBS, 2 mM L-glutamine and Pen-Strep; or αMEM supplemented with 10% FBS. 0.36 mg/mL of hydrocortisone and 1 μL/mL of 99% 2-mercaptoethanol, 2 mM L-glutamine and Pen-Strep.

Phenotypic analysis using flow cytometry

ASCs were resuspended in sterile PBS at a minimum concentration of 50,000 cells/tube. Appropriately labelled fluorescent antibodies of the MSC markers were added either singly or in combination based on the fluorochromes. Following incubation of cells with the antibody in the dark for 15–20 min, the cells were washed with sterile PBS to remove unbound antibody and gently centrifuged at 1,500 rpm for 5 min. The supernatant was discarded and the cell pellet was suspended in about 350 μL of PBS. The labelled cells were acquired immediately and analyzed using a BD FACS Calibur instrument, and data analysis performed with Cell Quest Pro software.

Population doubling time assay

The PDT assay was initiated by seeding 10,000 cells/well into 12 well tissue culture plates. Cells were trypsinized and counted every 24 h from triplicate wells, over 8 days. A zero time point count was obtained about 3 h after seeding the cells. PDT values are expressed as the number of hours per doubling (n) and calculated using the formula $n = 1/[X/(T2 - T1)]$, where $X = \text{Log}(Nf/N0)/\text{Log}2$. N0 and Nf are the initial and final number of cells in the exponential phase of the growth curve respectively. T1 and T2 are the corresponding time points in hours (Andley et al. 1994; McAteer and Davis 1994).

Colony forming unit-fibroblast (CFU-f) assay

5,000, 2,500, 500, 250, 50 and 25 cells from C-ASC cultures or ASC cultures were plated in 60 mm tissue

culture dishes, in ASC medium. After 8 days the colonies were fixed with 10% buffered formalin for 1 h and stained with 0.025% Brilliant Blue R (prepared in 40% methanol and 7% acetic acid) for 10 min. This was followed by destaining (with 40% methanol and 7% acetic acid) for 10 min. Counting was done by means of macroscopic observation. Colonies containing a minimum of about 50 cells were counted as a CFU-f colony. Dishes plated with 5,000 and 2,500 cells resulted in fused colonies which could not be counted.

In vitro differentiation assays

Adipogenic and osteogenic differentiations were performed in 6 well tissue culture plates (Pittenger et al. 1999). Adipogenic differentiation was induced in 90% confluent cultures with 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 10 μg/mL bovine insulin and 200 μM indomethacin in DMEM which was supplemented with 10% FBS, Pen-Strep, and 3.5 mg/mL of D-glucose. The maintenance medium comprised of DMEM supplemented with 10% FBS, 10 μg/mL bovine insulin, Pen-Strep and 3.5 mg/mL of D-glucose. Cultures were fixed at the end of 3 rounds of alternate induction (3 days) and maintenance (1 day) cycles. Lipid droplets formed inside the adipocytes were stained with Oil Red-O after fixing the cells with 10% buffered formalin (pH 7.1–7.3) for 1 h at room temperature.

Osteogenic induction began when cultures were at 70–80% confluency. Growth medium was replaced with DMEM supplemented with 100 nM dexamethasone, 50 μM L-ascorbic acid-2-phosphate, 10 mM β-glycerol phosphate, 10% FBS, and Pen-Strep. After three rounds of induction for 3 days each, the cells were lysed with 500 μL/10 cm^2 of 0.5 N HCl, followed by mixing at 500 rpm for 4 h at 4 °C to solubilize the calcium, and centrifugation at 1,000*g* for 5 min (Gastens et al. 2007). 20 μL of the supernatant was used for calcium estimation per manufacturer's instructions (Calcium Quantitative Kit, Pointe Scientific Inc). Control media used for both adipogenic and osteogenic differentiations comprised 10% FBS and Pen-Strep in DMEM medium.

Chondrogenic differentiation was performed as described previously (Mackay et al. 1998) On the 22nd day, the pellet culture was fixed using 10% buffered formalin for 1 h, embedded in paraffin blocks and 3 μm sections were cut using a Leica semi-automated microtome. The sections were stained with Safranin O, which stains anionic proteoglycans present in the extracellular matrix of cartilage and counterstained with fast green and Weigert's Iron hematoxylin. Collagen type 2 was detected in the pellets by immunohistochemical staining. Color development was based on a Streptavidin conjugated HRP DAB reaction. These sections were counterstained with Harris hematoxylin. Hematoxylin-Eosin staining was also performed on these sections so that nuclei are stained blue and cytoplasm pink. Photographs were taken using Leica DFC290 camera attached to Leica DM1000 microscope.

Representative results of adipogenic and osteogenic differentiation are shown; experiments were performed on three independent C-ASC cultures in passages 1, 2, or 3. Each experiment was performed in duplicate. Chondrocyte differentiation was performed in two C-ASC cultures in passages 2 or 3. In all cases, control cultures were C-ASC cultures which were not induced to differentiate. Differentiation assays were also performed with ASC cultures grown in FBS medium (data not shown).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA from differentiated and control cells was extracted using TRI reagent according to manufacturer's instructions. cDNA was prepared with Superscript II RT enzyme. Primer sequences used and their respective annealing temperatures are described in Table 1. PCR conditions were as follows: initial denaturation for 2 min at 94 °C, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing for 30 seconds and extension at 72 °C for 30 seconds except for PER, AGG and COL where 30 cycles were performed. Final extension was carried out for 10 min at 72 °C. PCR products were analyzed on ethidium bromide-stained 2% agarose gels. Results were documented using GE ImageQuant400. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) RNA was used for the normalization of RNAs.

Cell viability assay

C-ASCs were seeded in a 96-well plate at a density of 3,000 cells/well in the presence of the respective

Table 1 Details of PCR primers

Gene (abbreviation)	Gene symbol accession #	Forward primer sequence	Reverse primer sequence	Annealing temp (°C)
Lipoprotein lipase (LIP)	LPL	5′-CTTCTGTTCTA	5′-TGCTGTGTAGA	55
	NM_000237.2	GGGAGAAAGTG-3′	TGAGTCTGATT-3′	
Adipocyte protein 2 (aP2)	FABP4	5′-TTGTAGGTACC	5′-AAGGTTATGGT	49
	NM_001442.2	TGGAAACTTGTC-3′	GCTCTTGACTT-3′	
Peroxisome proliferator-activated receptor (PPAR)	PPARA	5′-TCGGTTTCA	5′-AACGAATGGT	48
	NM_138711.3	GAAATGCCTTG-3′	GATTTGTCTGT-3′	
Glycerol-3-phosphate dehydrogenase 2 (G3P)	GPD2	5′-TGACTTTGCA	5′-GAGCTGATAA	49
	NM_000408.3	AAACACATCTGA-3′	ATGGGGAGCA-3′	
Osteocalcin (OSC)	BGLAP	5′-ATGAGAGCCC	5′-CAAGGGGAAG	50
	NM_199173.3	TCACACTCCT-3′	AGGAAAGAAG-3′	
Osteopontin (OSP)	SPP1	5′-GAAGGACAGTT	5′-AACATAGACAT	48
	NM_000582.2	ATGAAACGAGT-3′	AACCCTGAAGC-3′	
Parathyroid hormone 1 receptor (PTHR)	PTH1R	5′-AACTACTACTG	5′-CTCCAAGATTT	48
	NM_000316.2	GATTCTGGTGG-3′	CTTGATCTCAG-3′	
Collagen type2 (COL2)	COL2A1	5′-CCCTGGAGAA	5′-GAAGTCCCTG	52
	NM_001844.4	AGAGGTGCTC-3′	GAACCCAGAT-3′	
Perlecan (PER)	HSPG2	5′-GGACATAGAG	5′-CACATCCAGC	50
	NM_005529.5	ACCGTCACAGC-3′	TCCACAAAAA-3′	
Aggrecan (AGG)	ACAN	5′-TACCAGGGC	5′-GCTGGACAA	52
	NM_013227.2	CACTGTTACC-3′	ACCCCTCTGT-3′	
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	GAPDH	5′-CATGACCACAGT	5′-TGAGGTCCACCA	57
	NM_002046.3	CCATGCCATCACT-3′	CCCTGTTGCTGTA-3′	
Complement Receptor 1 (CR1)	CR1	5′-GTGTGCGTC	5′-TCTGCATTTTC	58.5
	NM_000651.4	ATGTGAAACC-3′	CAAGCTCTTC-3′	
Complement Receptor 2 (CR2)	CR2	5′-ACCCCTAACG	5′-TCCATCCATAT	58.5
	NM_001877.3	GGAACCATAC-3′	CTGCTGGTG-3′	
Complement component 3a receptor 1 (C3aR1)	C3AR1	5′-GTGGTGGCTT	5′-GTTGGAAGAC	58.5
	NM_004054.2	TTGTGATGTG-3′	AGTGGGGACT-3′	
Low density lipoprotein-related protein 1 (LRP1)	LRP1	5′-GACTGGCTGA	5′-ATGTCACAGC	59.4
	NM_002332.2	CAGGCAACTT-3′	GTTCCACCTT-3′	
Complement component receptor 3 Alpha (CR3A)	ITGAM	5′-TTATGACCTGG	5′-CGTTCCCACC	58.5
	NM_000632.3	CTCTGGACTC-3′	AGAGAGAAGT-3′	
Complement component 5a receptor 1 (C5aR1)	C5AR1	5′-GTGGGAGTG	5′-ATGAGGGAG	62
	NM_001736.3	CTGGGCAAT-3′	GGCAGGATG-3′	

Sequences, annealing temperatures and gene accession numbers used in the PCR reactions shown in Figs. 2 and 3 are shown

complement reagents, growth factors, and inhibitor, as described in the legends of Figs. 3 and 4. Viability of cultures was measured on day 6 and was estimated by a 3-(4,5-dimethylthiozol-2-yl)-2,5,-diphenyl tetrazolium bromide (MTT) assay which measures metabolic activity. Cultures were incubated for 4 h with 0.5 mg/mL MTT, and cells were lysed in 10% SDS made in 0.01 N hydrochloric acid for 3 h at 37 °C. Absorbance was read at 550 nm using an ELISA plate reader (Thermo Multiskan EX), with a reference filter of 620 nm.

Statistical analysis

Statistical analysis for all relevant data was performed using the Mann–Whitney U test with the

program SPSS16.0. Data are represented as arithmetic mean ± standard deviation (SD). p values of significant differences are indicated in the respective figures or figure legends.

Results

Establishment and characterization of C-ASC cultures

Five independent C-ASC cultures were established. These cultures propagated well and were amenable to cryopreservation and subsequent thawing (Fig. 1a, b). We determined the expression of the surface antigens CD73, CD29, CD90, CD44, CD105, CD34, CD14 and CD45 on C-ASCs using flow cytometry. As is characteristic of bone marrow derived MSCs (BM-MSCs), C-ASCs expressed high levels of CD73, CD29, CD90, CD44 and CD105 and negligible levels of CD34, CD14 and CD45 (Fig. 1c, Table 2). Shaded histograms are isotype controls and open histograms show surface markers. A similar profile was obtained for ASC cultures (data not shown). The population doubling time of C-ASCs was determined during the initial 96 h of exponential expansion, and was found to be about 28 h (Fig. 1d). PDT of ASCs isolated and cultured from the same donor tissue in FBS supplemented medium was estimated to be about 26 h (Fig 1e). We also determined the profile of the colony forming unit-fibroblast (CFU-f) fraction of ASC and C-ASC cultures in triplicate (Fig. 1f). 37 ± 6 (mean ± SD) colonies were formed when 500 cells of C-ASC culture were plated, while 112 ± 4 colonies were formed with ASC culture. There is a linear correlation between the number of cells plated and the number of CFU-f generated from both C-ASCs and ASCs, consistent with each colony arising from a single CFU-f. Our data suggest that there are differences in adhesion and/or survival of clones from the CFU-f between C-ASC and ASC culture conditions. This is not surprising as cell adhesion, growth and survival promoting factors are abundant in FBS, which might result in the selection of different populations of cells in the ASC and C-ASC cultures. CFU-f assays were not interpretable when seeded with primary P0 cultures, as adipose tissue did not yield a homogeneous cell suspension during initial plating.

Differentiation potential of C-ASC cultures

We assessed the ability of C-ASCs to differentiate in vitro into adipocytes, osteoblasts and chondrocytes by using methods established for BM-MSCs (Mackay et al. 1998; Pittenger et al. 1999). Induction of adipocyte differentiation was confirmed by using Oil Red O to stain lipid droplets formed inside cells (Fig. 2a, b). A micromass pellet culture system was used to induce C-ASCs to differentiate into chondrocytes; this resulted in the deposition of an extra cellular matrix (ECM) rich in acidic proteoglycans and Type II collagen (a marker of hyaline cartilage). Chondrocyte differentiation was identified by sectioning differentiated pellet cultures of C-ASCs, and detection of collagen Type II with immunohistochemistry (Fig. 2c–e), and acidic proteoglycans with Safranin O staining (Fig. 2f). A serial section was stained with hematoxylin/eosin (Fig. 2g) to show the distribution of cells within the ECM. Osteoblast differentiation was estimated by quantitating calcium deposition (Table 3).

In addition, lineage specific markers of adipocyte (Fig. 2h), chondrocyte (Fig. 2i, lanes 1–6), and osteoblast (Fig. 2i, lanes 7–12) differentiation were identified by performing RT-PCR on RNA which was extracted from control and differentiated cultures at the end of the respective treatments. The following markers were investigated: adipocyte markers-lipoprotein lipase (LIP), adipocyte protein 2 (aP2), glycerol 3 phosphate dehydrogenase 2 (G3P) and peroxisome proliferator activated receptor (PPAR); chondrocyte markers-aggrecan (AGG), collagen (COL2) and perlecan (PER); osteoblast markers-osteocalcin (OSC), osteopontin (OSP) and parathyroid hormone 1 receptor (PTHR). GAPDH was used for normalization of RNA in all samples (bottom panels of (H) and (I)).

Complement C3 is a required component of the C-ASC condition

We then identified components of the medium that supported the isolation and proliferation of the C-ASC cultures. Viability of C-ASC cultures in the absence of bFGF, EGF or complement was estimated as absorbance values obtained by the MTT metabolic activity assay. C-ASCs were cultured in the absence of either Low-Tox-R complement, growth factors, or

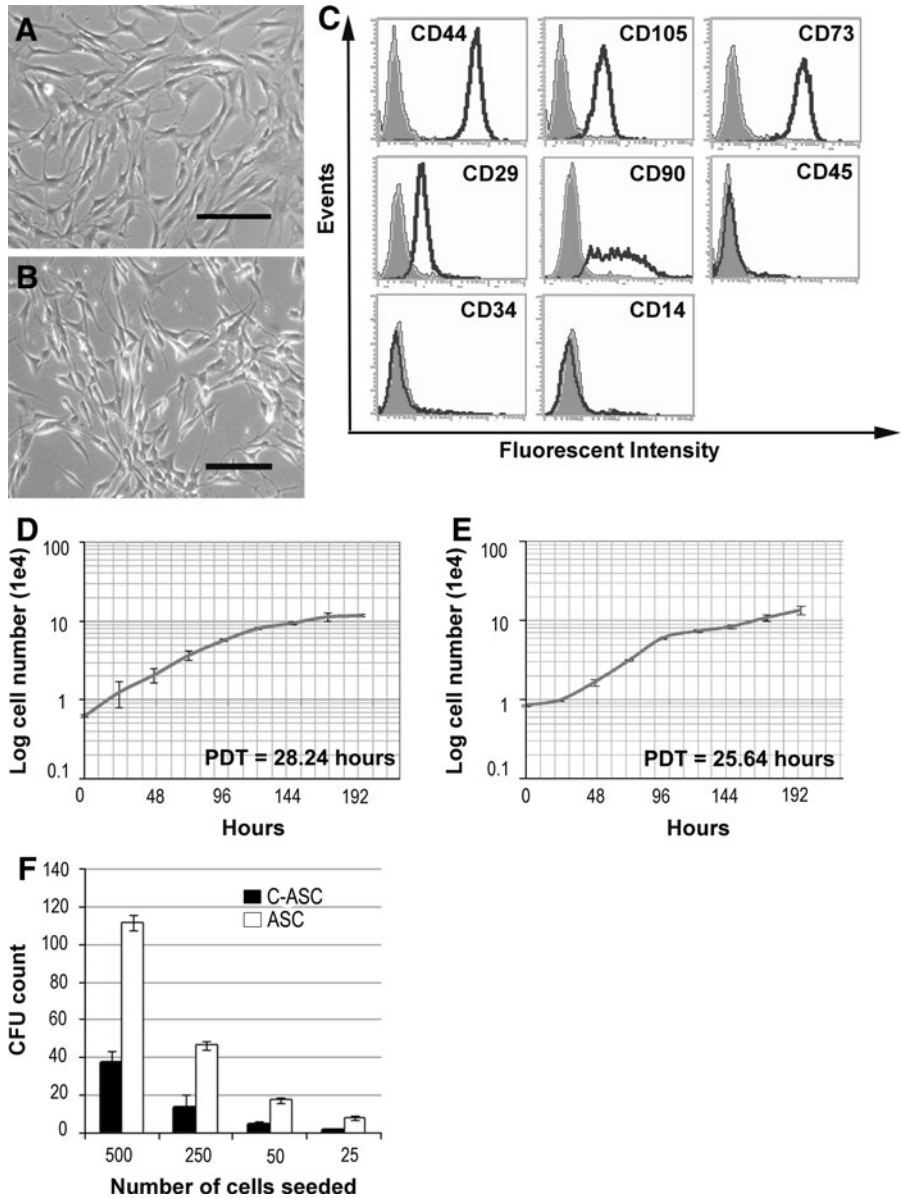


Fig. 1 Characterization of C-ASC cultures. Phase-contrast images showing morphology of **a** ASCs and **b** C-ASCs, scale bar represents 10 μm. **c** Phenotypic characterization of C-ASCs by flow cytometry: *Shaded histograms* are isotype controls, *open histograms* show surface markers (representative experiment, number of independent experiments (n) = 5, also see Table 2). **d** Growth curve of C-ASCs with the calculated PDT (representative experiment; each experiment done in triplicate, mean ± SD, n = 2). **e** Growth curve of ASC with calculated PDT (experiment done in triplicate, mean ± SD). **f** Bar graph showing the number of CFU-f obtained with C-ASCs and ASCs (representative experiment done in triplicate, mean ± SD, total n = 4). PDT and CFU-f assays were performed in cultures at passages P2 or P3. The probability (p value) of similar CFU-f counts occurring between the ASC and C-ASC cultures was $p = 0.05$ when 500 cells were plated; $p = 0.05$ for 50 cells; $p = 0.043$ for 250 cells; and $p = 0.046$ for 25 cells

both. Survival of the cultures was assayed. Absence of the growth factors (bFGF + EGF) or complement reduced survival of the culture to 50–60%. The absence of both led to about 20% survival of C-ASC cultures after 5 days as quantitated by the MTT metabolic-activity assay (Fig. 3a). EGF, bFGF and complement were each essential for successful C-ASC culture.

Table 2 Phenotypic characterization of 5 independent C-ASC cultures: Cultures 1 through 5 were initiated independently from human adipose tissue obtained from 5 individual donors

	% Positive cells				
	1	2	3	4	5
CD34	5.22	9.65	15.82	9.17	4.22
CD14	5.15	5.62	0.7	2.08	2.8
CD45	3.77	2.24	1.59	1.34	2.53
CD73	93.36	99.69	99.58	99.92	99.87
CD29	91.2	80.81	55.99	85.4	61.94
CD90	98.9	74.04	99.43	99.35	85.6
CD44	86.08	99.7	99.3	99.8	99.71
CD105	91.44	70.94	90.13	91.1	97.17

The table shows the percentage of cells in the gated population which were positive for the indicated cell surface markers. The cultures were characterised at passage 3 (P3), P4, P1, P2 and P1 for cultures 1 through 5, respectively. Histograms shown in Fig. 1c correspond to culture 5

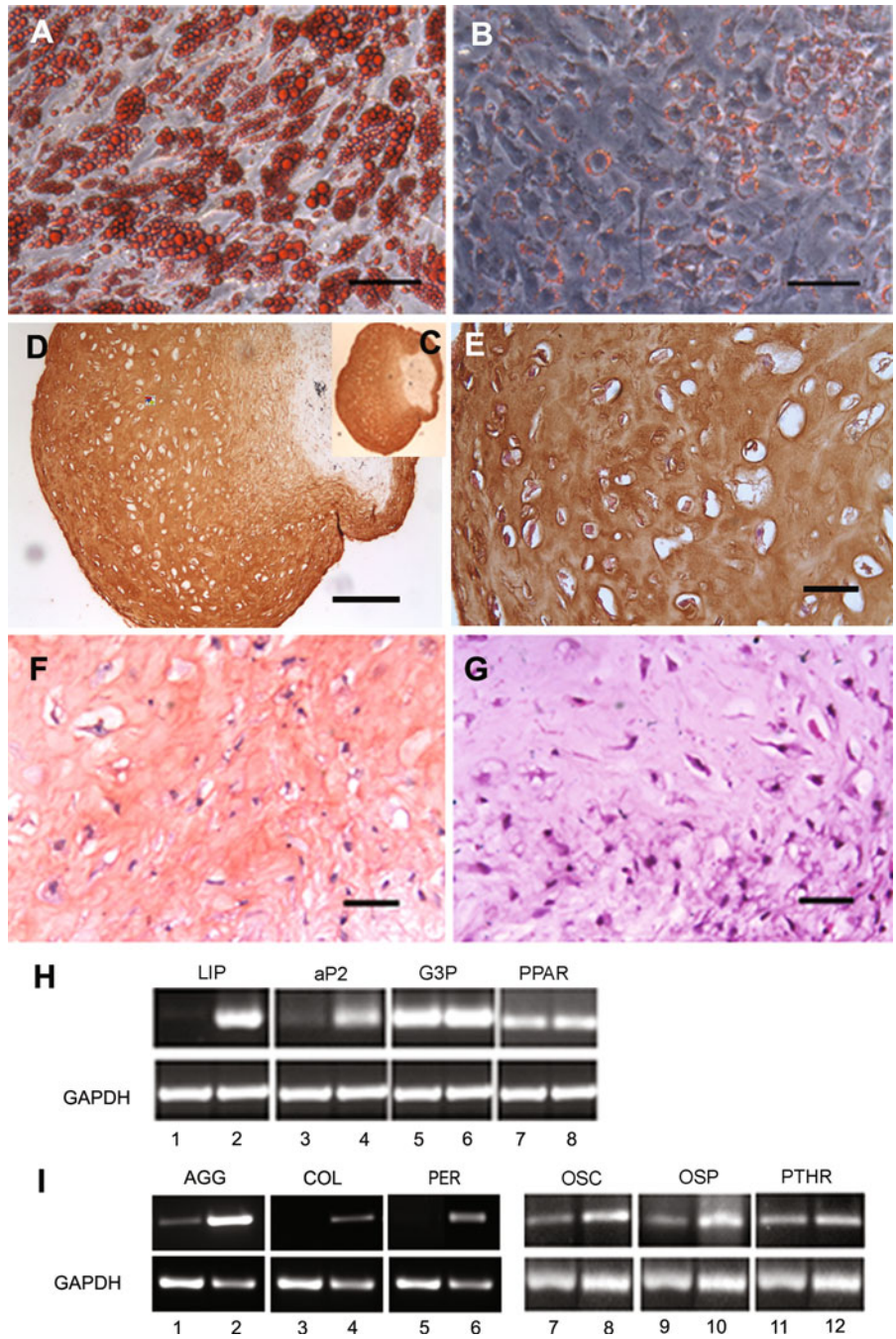
Fig. 2 Differentiation of C-ASCs: Adipocyte differentiation was estimated by staining intracellular oil droplets with Oil Red-O. Phase-contrast images showing stained C-ASC cultures in **a** induced and **b** control conditions. Chondrocyte differentiation was identified by sectioning and staining pellet cultures for Collagen type II shown in panels **c**; whole pellet (**d**), and (**e**). Panel **f** shows a section stained with Safranin O which detects acidic proteoglycans, and panel **g** shows a section stained with Hematoxylin/Eosin. *Scale bar* in **a**, **b** and **d** represents 10 μm; **e**–**g** represents 2 μm. Osteoblast differentiation was quantified by estimating calcium depositions within the cells (see Table 3). Lineage-specific markers of differentiation were identified by performing RT-PCR on RNA which was extracted from control and differentiated cultures. **h** RT-PCR analysis for adipocyte markers—LIP, aP2, G3P, and PPAR in undifferentiated cultures (*top panels*, *lanes 1, 3, 5, 7*) and differentiated cultures (*top panels*, *lanes 2, 4, 6, 8*). **i** RT-PCR analysis for chondrocyte markers AGG, COL2, and PER in undifferentiated cultures (*top panels*, *lanes 1, 3, 5*) and differentiated cultures (*top panels*, *lanes 2, 4, 6*). RT-PCR analysis for osteoblast markers OSC, OSP, and PTHR in undifferentiated cultures (*top panels*, *lanes 7, 9, 11*) and differentiated cultures (*top panels*, *lanes 8, 10, 12*). Corresponding GAPDH bands are shown in the bottom panels of **h** and **i**

In order to identify molecules within the complement fraction that might participate in C-ASC survival and proliferation, we screened ASC and C-ASC cultures for the expression of complement-related receptors (Fig. 3b). C3 and its derivatives bind the receptor molecules complement receptor 1 (CR1), complement receptor 2 (CR2), complement C3a receptor 1 (C3aR1) and complement receptor C3a (CR3a), C1q binds low density lipoprotein related protein 1 (LRP1), and C5 binds C5a receptor 1 (C5aR1). RT-PCR assays indicated that CR1, C3aR1, LRP1 C5aR1 were present on the C-ASCs (lanes 3, 9, 15, and 18), while CR2 and CR3a were undetected (lanes 6 and 12). RNA extracted from peripheral blood mononuclear cells (PBMNCs) was used as a positive control for all primer sets, and yielded PCR products of the anticipated size for each primer set.

C3 is a central component of the complement functions, and the effect of a C3-specific small molecule inhibitor on the survival of C-ASC cultures was determined. Compstatin is a cyclic peptide that inhibits the convertase-mediated cleavage of C3 into C3a and C3b with an IC50 of 28 μM. C-ASCs were cultured for 5 days in medium supplemented with 1% Low-Tox-R complement (LT in Fig. 4), 1% human complement serum (HCS in Fig. 4), or 1% C3 deficient human complement serum (C3defHS in Fig. 4). EGF and bFGF were present in all conditions. At 29 mg/mL, the protein concentration of LT was about half that of HCS and C3defHS. Compstatin used at a concentration of 128.9 μM for 5 days caused a 45% reduction in survival of C-ASC cultures (Fig. 4a). As expected, human complement serum (HCS) could substitute for Low-Tox-R complement in our FBS-free culture conditions. 128.9 μM Compstatin caused a 30% reduction in metabolic activity of C-ASCs supplemented with HCS (Fig. 4a), as did human complement serum deficient in C3. This is consistent with the notion that C3 is required for a successful culture. A dose response with compstatin on survival of C-ASC cultures showed a linear correlation of metabolic activity of the culture with concentration of the inhibitor, suggesting that the effect is specific (Fig. 4b). However, the requirement that compstatin be used at levels above the IC50 for inhibition of C3 activation (32.2 μM) suggests that a novel C3-mediated event is responsible for the inhibitor's effect. Our experiments indicate that C3 supports C-ASC survival and proliferation in vitro.

Discussion

We describe a novel culture condition in which human ASCs are isolated and maintained in culture without FBS. Culture medium containing 60% DMEM/F12/N2 and 40% MCDB 201 was used since MSCs have previously been successfully grown in



similar medium (Jiang et al. 2002). Small-molecule supplements were selected for their potentials to enhance survival or promote proliferation. Ascorbate serves as an anti-oxidant, while selenium is a cofactor for enzymes that ameliorate oxidative stress. Dexamethasone, a steroid, promotes cell growth, as do linoleic acid and serotonin.

Certain proteins are also commonly used in the culture of adherent cells in the absence of serum. Insulin promotes survival via activation of the insulin and IGF2 receptors. Transferrin is needed for iron uptake. bFGF and EGF were found to be required for optimal survival and proliferation of the C-ASC cultures (Suga et al. 2007; Hebert et al. 2009;

Table 3 Osteoblast differentiation of C-ASCs

Sample	Calcium value (mg)
1	2.99 ± 0.05
2	2.76 ± 0.273
3	1.351 ± 0.031

Quantification of calcium formed during osteoblastic differentiation of three independent C-ASC cultures, each performed in duplicate. The value shows the quantity of calcium formed when C-ASCs were cultured and differentiated in one well of a 6-well culture dish. Negligible calcium was deposited in dishes by control cultures

Iwashima et al. 2009). Lastly, the role of complement is the subject of this study.

C-ASCs are grown on gelatin-coated plastic as a substrate. Treatment of tissue-culture plastic is usual for adherent cultures maintained in serum-free conditions, to compensate for the absence of adhesion molecules contributed by FBS. The results of the CFU-f assay (Fig. 1f) suggest that the clonogenic fraction is smaller in the C-ASC cultures than in the ASC cultures. This could also be the result of the FBS-supplemented ASC medium being able to select a larger population of cells in the initial passages when compared to the C-ASC medium, which is presumably less abundant in factors which promote cell survival, cell proliferation and adhesion. The C-ASC cultures have a similar surface antigen profile to ASCs cultured in FBS (Mitchell et al. 2006, and data not shown), have a PDT similar to ASCs, and can differentiate in vitro into fat, bone and cartilage lineages, a property that is characteristic of ASC cultures.

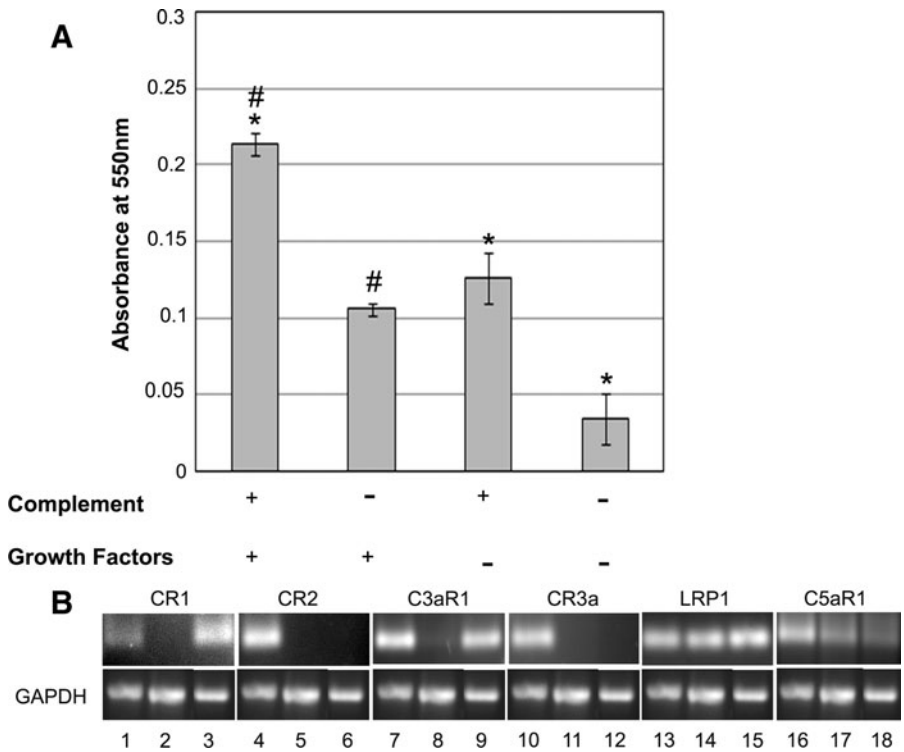


Fig. 3 Requirement of growth factors and complement for survival of C-ASC cultures. **a** Both complement and the growth factors (EGF and bFGF) are required for maintenance of C-ASC cultures. *Bar graph* shows the viability of C-ASC cultures as absorbance values obtained by the MTT metabolic activity assay (mean ± SD). C-ASCs were cultured for 5 days in the absence of either Low-Tox-R complement (n = 4), growth factors (n = 3) or both (n = 2) and the survival of the cultures assayed. Representative results are shown, experiments were performed in triplicate or quadruplicate. C-ASCs grown in the presence of complement and growth factors served as the positive control, $^{*}p = 0.034$; $^{\#}p = 0.05$ as compared to the positive control. **b** Cohort of complement receptors present on C-ASCs. Receptors of C3, C1q and C5 and their derivatives were identified by performing RT-PCR on RNA extracted from PBMNCs (positive control; *lanes 1, 4, 7, 10, 13, 16*), ASC cultures maintained in FBS (*lanes 2, 5, 8, 11, 14, 17*) and C-ASC cultures (*lanes 3, 6, 9, 12, 15, 18*). *Bottom panels* show the corresponding GAPDH bands

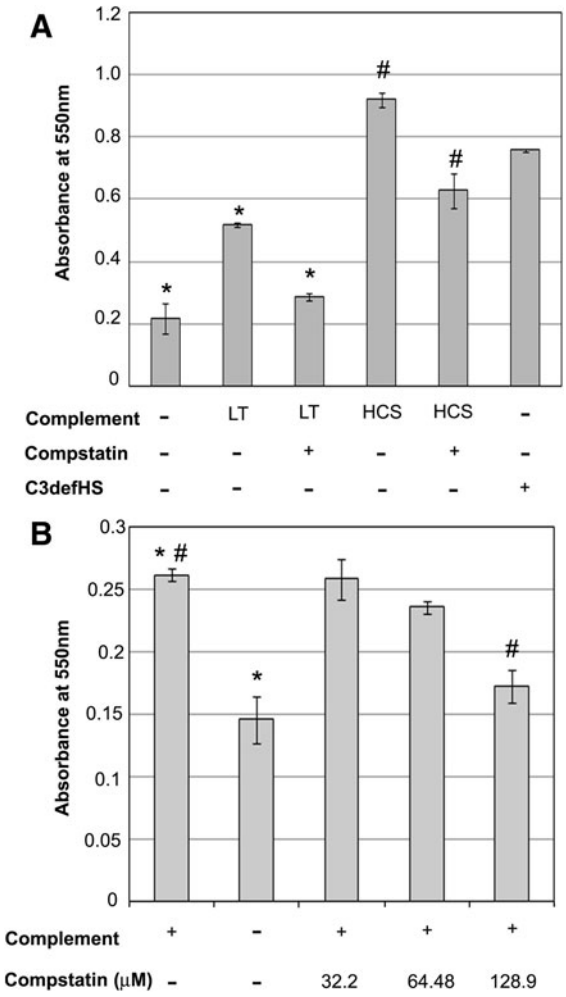


Fig. 4 Complement protein C3 promotes survival of C-ASC cultures. **a** *Bar graph* showing the viability of C-ASC cultures as absorbance values obtained in an MTT metabolic activity assay. C-ASCs were cultured for 5 days in medium supplemented with 1% Low-Tox-R complement (LT, representative experiment, n = 4), 1% human complement serum (HCS, n = 1) or 1% C3 deficient human complement serum (C3defHS, representative experiment, n = 4). The absence of complement served as the negative control. Experiments were performed in triplicate or quadruplicate. The data are shown as mean ± SD; * $p = 0.05$ within the LT group, and # $p = 0.05$ within the HCS group. Effect of C3defHS on survival of C-ASCs was variable depending on the culture used. **b** Compstatin causes dose-dependent reduction in cell viability of C-ASC cultures. *Bar graph* showing the viability of C-ASC cultures as absorbance values obtained in an MTT assay. C-ASCs were cultured for 5 days in the presence of increasing concentrations of compstatin. EGF and bFGF were present in all conditions. Absence of both compstatin (μM) and complement served as the negative control. Experiment was performed in triplicate, n = 1. The data are shown as mean ± SD; *, # $p = 0.05$ as compared to the positive control (presence of complement only)

This is the first report which establishes complement C3 as an essential medium component for the culture of adipose-derived stem cells. Our composition is defined with the exception of complement, which we use at the modest concentration of 1%. The C-ASC cultures express the mRNAs of the C3 receptors CR1 (which binds to the ligand C3b/4b) and C3aR1 (binds C3a). We did not detect the expression of CR2 (binds C3dg) or CR3a (binds C3b). C-ASCs also express the C1q receptor LRP1, and the C5a receptor C5aR1. Phenotypic and differentiation criteria establish the striking similarities of C-ASCs to ASCs and to MSCs which have been cultured in FBS-containing media (Mackay et al. 1998; Pittenger et al. 1999; McIntosh et al. 2006).

The notion that C3 supports stem cell survival and proliferation in culture has immediate practical implications in basic and translational stem cell biology. Complement C3 and its cleavage products are known to have multiple biological effects. They induce dose dependent proliferation in Raji cells, a B-cell line, by specific interaction with the CR2 receptor (Hatzfeld et al. 1988). In bone marrow cells, C3 promotes osteoclast development by potentiating Vitamin D3 and macrophage-colony stimulating factor (M-CSF) dependent proliferation, and the induction of osteoclast differentiation (Sato et al. 1993). The C3a/C3aR axis plays an important role in retaining hematopoietic stem/progenitor cells in bone marrow by increasing their responsiveness to SDF-1 (Ratajczak et al. 2004). C3a also modulates SDF-1α induced differentiation and migration of neural progenitor cells (Shinjyo et al. 2009), and is involved in neurogenesis (Rahpeymai et al. 2006). Farther afield, C3a and C5a are essential for liver regeneration, through their interaction with cytokines IL-6 and TNF via the canonical signalling pathways JAK1/STAT3, PI3 K/Akt/mTOR, and NF-κB (Strey et al. 2003; Markiewski et al. 2009).

The media typically used to isolate and culture ASCs contain 10% FBS (Suga et al. 2007). FBS is poorly defined, complex, and heterogeneous; lot to lot differences are known to affect MSC phenotype (Caterson et al. 2002). In addition, FBS may harbor unknown risks when used as a culture additive for cells destined for human therapeutics. Efforts to replace FBS as a culture supplement have led to the use of replacements including human serum and human platelet lysate, both of which are poorly defined, and

other 'defined' medium compositions, some of which are proprietary (Meuleman et al. 2006; Schallmoser et al. 2008; Agata et al. 2009; Perez-Ilzarbe et al. 2009). The culture condition we have described here is a novel composition which yields highly proliferative stem cell cultures, and can be readily developed into a defined composition.

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Conflict of interest None.

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