Unravelling the retention of proliferation and differentiation potency in extensive culture of human subcutaneous fat-derived mesenchymal stem cells in different media

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Abstract

Objectives: This study has intended to investigate longevity of subcutaneous fat-derived mesenchymal stem cells (SF-MSCs) under extensive culturing. It has also focused on optimization of culture media for them over prolonged periods *in vitro*.

Materials and methods: We evaluated SF-MSCs with reference to phenotypic characterization, proliferative ability, karyotype stability and differentiation potency with early (P3) and late passage (P20) conditions, using four different media, DMEM-LG, ALPHA-MEM, DMEM-F12 and DMEM-KO.

Results: This study unravels retention of SF-MSC characteristics in facets of phenotypic expression profile (CD 90, CD 105, CD 73, CD 34, CD 29, CD 54, CD 49d, CD 117, HLA-DR, CD 166, CD 31, CD 44), proliferative characteristics, karyotyping and differentiation potency prolonged culturing to P25 in all media. Population doubling time (PDT) in Alpha MEM, DMEM LG, DMEM F 12, DMEM KO were identified to be (1.81, 1.84, 1.9, 2.08 days) at early passage and (2.93, 2.94, 3.12, 3.06 days) at late passage. As a corollary, Alpha MEM and DMEM LG serve as appropriate basal media for SF-MSC when proliferative potency is considered.

Conclusions: In research, it is imperative that SF-MSC uphold their expansion potency in the aforesaid attributes in all media over extensive culturing, thereby transforming their colossal *in vitro* potency, with the aim of curing a wide horizon of diseases.

Introduction

Subcutaneous depots of adipose tissue are advantageous for obtaining stem cells – considering aspects of accessibility, abundance and replenishing ability when compared to their primeval source, the bone marrow. Subcutaneous fat serves as a reliable source of stem cells which have the reputation of being colossally potent (1,3,4); large quantities of stem cells can be harvested from fat in a single operation, making them one of the best sources of stem cells. Additionally, SF-MSCs win over their counterparts in relation to their proliferative capacity and plasticity (5–8). Owing to these advantages (9–11), it has been reported that SF-MSCs can be used directly in a variety of therapeutic doses for treating a wide range of diseases (12–16). Based on this hypothesis, recently there has been a great deal of interest in use of autologous stem cells from subcutaneous adipose tissue for therapeutic applications (17–19). However, utilizing SF-MSCs for clinical approaches demands considerable proof of their ability to retain their vital characteristics in extensive culture conditions, thereby providing evidence for their efficacy for use over the long-term. In accordance with this, a number of reports have focused on exploring functional stability of both rat and human adipose tissue-derived mesenchymal stem cells over prolonged culture conditions (8,20). Despite favourability of reports, there are concerns related to potential malignant transformation and loss of multi-differentiation potency of human SF-MSCs, over extensive, repetitive proliferation (21–23).

There are uncertainties in validation of prolonged functional stability of SF-MSC over extensive culture conditions, due to existing contradictory reports. Further research on establishing thorough proof of retention of their characteristics with no adverse effects over time before use in clinical transplantation, is mandatory. Hence, our present study has aimed at exploring longevity of subcutaneous fat-derived mesenchymal stem cells

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with facets of morphology, phenotype, proliferative ability, multilineage differentiation capacity, karyotype stability and phenotypic characterization under extensive culturing, thereby providing concrete evidence for SF-MSC as a potential stem cell source for regenerative medicine.

Materials and methods

Sampling

Obese patients undergoing abdominoplasty at the Lifeline Multispeciality Hospital, Chennai, India, were recruited for this study. Protocols were reviewed and approved by the Ethical Committee of the Lifeline Multispeciality Hospitals and written informed consent from the patients undergoing surgery was obtained. Subcutaneous fat of 25–50 g was obtained from 3 subjects ($n = 3$) whose age group ranged between 35 and 55 years, with BMI $28.7 \pm 4.8 \text{ kg/m}^2$ after completion of surgery. Tissues were examined and fully processed within 4 h of collection.

Isolation of SVF

Solid fat tissue was washed three times, vigorously, in tubes containing $1 \times$ phosphate-buffered saline (PBS) (HiMedia, Mumbai, India) containing 1% antibiotics, for 5–10 s and allowed to settle. They were then minced gently into very fine pieces 2–3 mm in diameter and minced tissue was digested using type I collagenase (HiMedia) which was incubated for 1 h at 37 °C. Collagenase activity was finalised by addition of 10% FBS (Invitrogen, NY, USA). Digested tissues were centrifuged at 600 *g* for 10 min to remove oil and mature adipocytes and pellets were further washed in PBS at 600 *g* for 10 min at 20 °C. Suspended pellets were passed through a 100- μm cell strainer (BD Falcon, NC, USA) and further filtered using a 40- μm cell strainer (BD Falcon) to remove debris and undigested tissue; RBCs contaminating SVF were lysed using 0.7% ammonium chloride solution (Sigma, MO, USA). Cells were resuspended in PBS and centrifuged at 300 *g* for 10 min. Supernatants were discarded and pellets were resuspended in DMEM containing 1% antibiotics, reaching required volume, for further use.

Culturing SF-derived MSCs

Cells were seeded in culture flasks with seeding density of $1.2 \times 10^4 \text{ cells/cm}^2$ all then being incubated in a 5% CO_2 incubator at 95% humidity and 60 psi pressure; media were replaced twice a week. In particular, cells expressing

haematopoietic and endothelial markers were found to be lost by passage 3. Primary cell populations were subcultured once they attained 80–90% confluence (cell concentration being determined using haemocytometer), and seeded into culture flasks at split ratio of 1:2.

Immunophenotype analysis

SF-MSCs were examined for surface antigen profile using flowcytometry, and BD FACS Aria apparatus, see Table 1 for list of antibodies plus fluorochrome. 1×10^5 cells in suspension with appropriate antibodies were incubated for 20 min. Cells were washed in BD FACS wash buffer and after centrifugation, pellets were resuspended in 500 μl sheath fluid/BD FACS flow cytometer, and vortexed. All samples were recorded with minimum of 10 000 events, and characterized. Appropriate isotype controls were used for analysis (BD Biosciences, CA, USA).

Proliferation potency of SF-MSC

Growth curves were plotted to evaluate population growth characteristics of the isolated cells, in four different media (DMEM-LG (Invitrogen), α -MEM (Invitrogen), DMEM-F12 (Invitrogen) and in DMEM-KO (Invitrogen), each supplemented with 10% FBS (Invitrogen) and 1% antibiotic–antimycotic solution), and characterization was performed at P3 and P20 on 12-well plates in duplicates, with seeding density of 3×10^5 cells per plate at day 0. Cells were harvested and counted every day, until day 10. Results were plotted on a log-linear scale and population doubling time (PDT) was calculated using the formula:

$$\text{PDT} = \frac{\text{days at exponential phase}}{(\log N_2 - \log N_1)/\log 2}$$

Table 1. Details of antibodies along with its fluorochrome and catalogue number

Antibody	Conjugate	CAT number
CD90	PERCP	CatNo: 15-0909-73, e-Biosciences
CD105	APC	CatNo: 17-1057-73, e-Biosciences
CD73	PE	Cat No: 550257, BD Biosciences
CD29	PE	Cat No: 555443, BD Biosciences
CD44	FITC	CatNo: 555478, BD Biosciences
CD166	PE	Cat No: 559263, BD Biosciences
CD34	PE	Cat No: 348057, BD Biosciences
CD31	FITC	Cat No: 555445, BD Biosciences
HLA-DR	PER-CP	Cat No: 347364, BD Biosciences
CD49d	PE	CatNo: 12-0499-73, e-Biosciences
CD54	PER-CP	Cat No: 555512, BD Biosciences
CD117	APC	CatNo: 17-1179-73, e-Biosciences

Where N1 was number of cells at the beginning of the exponential growing phase and N2 was number of cells at the end of the exponential growing phase.

Osteogenic and adipogenic differentiation

Multilineage differentiation ability of the SF-MSCs was established in all media at early and late passages. 80–90% confluent cells at P3 and P20 were cultured in osteogenic induction medium (0.1 μ M dexamethasone, 10 mM, β -glycerophosphate 2 mM Ascorbic). Mineralization of aggregated cells was evident by observing dense refractile Ca^{2+} deposits confirmed by von Kossa staining as well as using the alizarin red method. Similarly, the SF-MSCs were cultured in adipogenic induction media (1 μ M dexamethasone, 0.5 mM isobutyl methyl xanthine, 10 μ g insulin, 200 μ M indomethacin) and accumulation of lipid droplets were stained using oil red O.

Karyotyping

Karyotype stability of SF-MSCs in Alpha MEM, DMEM LG, DMEM-F12 and DMEM-KO at late passage was analysed using G banding of metaphases. A minimum of 20–30 banded metaphases were captured, using image analysis, through a CCD camera. Metaphases were karyotyped using software cytovision.

Statistical analysis

All quantitative data are represented as mean $\pm$ SEM. Statistical analysis was carried out using software statistical package for social science, SPSS 15.0 (SPSS Inc., Chicago, IL, USA) and significance was assessed by one-way analysis of variance along with the Dun-

can multiple range test. In all comparisons, *P*-values <0.01 and <0.05 were considered to be statistically significant.

Results

Cell culture

Subcutaneous fat-derived mesenchymal stem cells were cultured with seeding density of 1.2×10^4 cells/cm² and were expanded through extensive proliferation up to P25, in four different media – Alpha MEM (Invitrogen), DMEM LG (Invitrogen), DMEM F 12 (Invitrogen) and DMEM KO (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% antibiotic–antimycotic solution. They were isolated based on their ability to adhere to tissue culture plastic and were identified as elongated and of spindle-shaped fibroblastic morphology, in both early and later passages. This morphology was maintained in all four different media with 90% viability until P25 (Fig. 1).

Immunophenotypic expression profile analysis

The SF-MSCs (donor $n = 3$) were assessed for expression of cell surface markers at early (P1, P3, P5) and late passages (P9, P12, P15, P20, P25) using flow cytometry (Figs 2 and 3). A group of 12 proteins considered to be stem cell markers was selected for characterization of the cells when cultured in DMEM-LG, ALPHA-MEM, DMEM-F12 and DMEM-KO. Expression profiles of all markers at specific time points were categorized in accordance with our preceding studies (25); values are tabulated with expression range (Table 2) and mean $\pm$ SEM values detailed as

Figure 1. Morphology of SF-MSC at early and late passages. Morphology of cultured human subcutaneous fat-derived MSCs at early (P3) and late (P20) passages of all media is as follows: DMEM-LG at early passage (a) and late passage (e); α -MEM at early passage (b) and late passage (f); DMEM-F12 at early passage (c) and late passage (g); DMEM-KO at early passage (d) and late passage (h).

Figure 2. Immunophenotypic analysis for expression profiles of cell surface markers of SF-MSC at early passage. Immunophenotypic expression profile of cell surface markers of SF-MSC analysed using FACS at early passage (P3).

Figure 3. Immunophenotypic analysis for expression profiles of cell surface markers of SF-MSC at late passage. Immunophenotypic expression profile of cell surface markers of SF-MSC analysed using FACS at late passage (P20).

supplementary data (Table S1). Remarkable expression profiles of greater than 90% for CD 90, CD 105, CD 73, CD 29, CD 44, CD 166, and sparse expression of less than 10% expression for CD 34, CD 31 and

HLA-DR, were observed throughout the cultures. Expressions of these proteins were consistent in all media, throughout expansion at early and late passages, however, inconsistency was identified in expression

Table 2. Expression profile comparison of cell surface markers in all media

Markers	DMEM-LG								ALPHA-MEM							
	PI	P3	P5	P9	P12	P15	P20	P25	PI	P3	P5	P9	P12	P15	P20	P25
CD 90	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
CD105	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
CD73	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
CD29	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
CD44	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
CD166	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
CD34	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
CD31	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
HLA-DR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
CD49d	M	M	H	H	H	H	H	H	L	M	H	H	M	H	H	H
CD54	H	H	M	H	H	R	R	R	H	H	M	M	H	H	R	R
CD117	M	M	H	H	H	H	H	M	M	M	H	H	H	H	H	M
MARKERS	DMEM-F12								DMEM-KO							
	PI	P3	P5	P9	P12	P15	P20	P25	PI	P3	P5	P9	P12	P15	P20	P25
CD 90	R	R	R	R	R	H	R	R	R	R	R	R	R	R	R	R
CD105	R	R	R	R	R	H	R	R	R	R	R	R	R	R	R	R
CD73	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
CD29	R	R	R	R	R	H	R	R	R	R	R	R	R	R	R	R
CD44	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
CD166	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
CD34	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
CD31	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
HLA-DR	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
CD49d	H	M	M	M	H	H	R	R	H	M	M	M	M	M	H	R
CD54	H	M	M	M	H	H	R	R	H	M	M	M	M	M	H	R
CD117	M	H	H	H	H	H	H	R	M	M	M	H	H	H	H	H

Expression of cell surface markers in all media was categorized as follows: >90% (Remarkable expression – R); 75–89% (High expression – H); 40–74% (moderate expression – M); 11–39% (Low expression – L); 1–10% (Sparse expression – S). Cells characterized at each passage numbers were specified as P#

profile of CD 54, CD 49d and CD 117, at all passages of all four media. Expression of CD 54 was found to be high at early passage with gradual shift in expression attaining more than 90% beyond P20. Varying moderate-to-higher expression of CD 49d ranging from 40% to 85% was recorded, however, in DMEM-KO there was lower expression of it compared with other media. CD 117 had a rise in expression through early passages and sustained its higher level (more than 80%) at later passages, with some variations at P25.

Differentiation potency

SF-MSCs were examined for their differentiation ability into osteogenic and adipogenic lineages at early (P3) and late passage (P20) in the four different media. Specific induction factors were used for differentiation into respective lineages and differentiation was confirmed using well defined staining techniques. Undifferentiated MSC without addition of induction medium, served as negative controls.

Osteoblast differentiation

SF-MSC differentiation at early and late passages was detected by deposition of mineralized calcium deposits, over a period of 15 days, in all media. On day 21, appearance of osteogenic morphology was confirmed by positive staining obtained by both von Kossa and alizarin red techniques (Fig. 4). However, comparatively lower differentiation capacity was detected in cells cultured in DMEM F12.

Adipocyte differentiation

Adipogenic differentiation was induced in cells cultured in all the 4 media, at early and later passages, using adipogenic induction factors. Adipogenic phenotype was demonstrated within 10–12 days by accumulation of lipid vacuoles – lipid droplets appearing from day 15 onwards. Adipogenic differentiation was confirmed on day 18 by positive oil red O staining in all media at both early and late passages (Fig. 5). Similar to

Figure 4. Osteogenic differentiation capacity of SF-MSC. Von Kossa staining of osteogenic differentiated SF-MSCs in early passage: control (a), Differentiated cells in; DMEM LG (b); Alpha MEM (c); DMEM F 12 (d); DMEM KO (e); von Kossa staining of osteogenic differentiated SF-MSCs in late passage: control (f), Differentiated cells in; DMEM LG (g); Alpha MEM (h); DMEM F 12 (i); DMEM KO (j); alizarin red staining of osteogenic differentiated SF-MSCs in early passage: control (k), Differentiated cells in; DMEM LG (l); Alpha MEM (m); DMEM F 12 (n); DMEM KO (o); alizarin red staining of osteogenic differentiated SF-MSCs in late passage: control (p), Differentiated cells in; DMEM LG (q); Alpha MEM (r); DMEM F 12 (s); DMEM KO (t).

osteogenic differentiation, less adipogenic differentiation capacity was detected in DMEM F12.

Growth characteristics

Analysis of growth characteristics of MSC at early (P3) and late (P20) passages was carried out in a 12-well plate, in duplicates, with seeding density of 3×10^5 cells/plate, in all four media, at day 0 and expansion of cells was calculated up to day 10, on a daily basis. Growth curves

symbolized cells' exponential rise from day 2 to day 7, and existence of lengthy stationary phases, with minuscule lag phases (Fig. 6). Population doubling times (Fig. 7) were found to be lowest (1.81 days) in ALPHA MEM, (1.84 days) in DMEM LG, (1.9 days) in DMEM F 12 and highest (2.08 days) in DMEM-KO at early passages. However, decrease in proliferation rate was seen by late passages with PDT highest (3.12 days), in DMEM-F12 (3.06 days), in DMEM KO (2.94 days), in DMEM LG and lowest (2.93 days) in Alpha MEM.

Figure 5. Adipogenic differentiation capacity of SF-MSC. Oil red O staining of adipogenic differentiated SF-MSCs in early passage: control (a), Differentiated cells in; DMEM LG (b); Alpha MEM (c); DMEM F12 (d); DMEM KO (e); oil red O staining of adipogenic differentiated SF-MSCs in late passage: control (f), Differentiated cells in; DMEM LG (g); Alpha MEM (h); DMEM F12 (i); DMEM KO (j).

Karyotyping

Numerical and structural chromosome stability, in view of long-term culture of SF-MSC at late passage in Alpha MEM, DMEM LG, DMEM KO and DMEM-F12 was carried out using GTG banding. There were no

Figure 6. Growth curve of SF-MSC. Growth curve analysis (in days) of SF-MSC at early and late passages with respect to different media: (a) early passage (P3); (b) late passage (P20).

Figure 7. Population doubling time analysis of SF-MSC. PDT (days) analysis of SF-MSC in different media at early and late passages

structural nor numerical anomalies identified throughout P20 in all the media (Fig. 8).

Discussion

The enormous proliferative and multilineage differentiation potential of mesenchymal stem cells has ushered in exciting possibilities for autologous stem cell-based therapies to repair damaged tissues and organs. Great amounts of interest have been shown in recent years concerning subcutaneous fat-derived mesenchymal stem cells for use in clinical transplantation (17–19). In spite

Figure 8. Karyotyping of SF-MSC. Karyotypic analysis of SF-MSC at late passage: (a) DMEM LG; (b) α -MEM; (c) DMEM F12; (d) DMEM KO.

of widespread interest though, there have been controversial reports regarding longevity of SF-MSC *in vitro*, thereby limiting their applicability in curative therapeutics (21–23). Our study described here, hopes to provide a step forward to outweigh some possible controversies regarding SF-MSC. We have isolated and propagated SF-MSCs up to further than passage P25 effectively, and we aimed at optimizing culture media for their extensive expansion. We analysed SF-MSC in view of morphology, expression profile analysis, proliferation and differentiation analysis, and karyotyping, at early and late passages.

Although some studies have already focused on immunophenotypic characterization of adipose-derived stem cells (5,11,14,24,25,26), our work is the first of its kind to compare and analyse these cells expression of certain surface markers, in all four media tested, at early and late passages. We found consistent and remarkable expression of CD 90, CD 105, CD 73, CD 166, CD 29 and CD 44 throughout extensive passages up to P25 in agreement with other literature (1,11,25,26), whereas, certain other studies have reported reduction in expression of CD 73 and CD 29 in prolonged culture conditions of such cells (1,13,27,28). On the other hand, our study has revealed inconsistency in expression of CD 49d and CD 54 with respect to different media at early and late passages. The discrepancy obtained here has

also been reported in other literature (11,13,14,26, 29). Comparatively, expression profile of CD 117 was contradictory to existing literature (1,26), which suggests that CD 117 may be downregulated after extensive passaging. This illustrates that no definitive conclusion could be made concerning expression of CD 54, CD 49d and CD 117. However, it can be stated that CAMs, CD 54 and CD 49d, and progenitor cells, CD 117 were widely expressed throughout the prolonged culture conditions.

Growth characteristics of SF-MSC were obtained and potency revealed in this study was identified to be similar to that found in other publications (3); however, our study is the first to report proliferative potency of SF-MSC in all four media. DMEM-KO, a medium optimized for proliferation of undifferentiated embryonic stem cells, is a proprietary basal medium with reduced osmolality. As DMEM KO has been found to serve as optimal growth medium for long-term culture of bone marrow and Wharton's jelly stem cells (29), it was chosen for our study, for analysing its proliferative capacity for SF-MSC. It is evident from our results that DMEM KO does not support extensive proliferation in relation to its growth characteristics. Furthermore, it can also be concluded that Alpha MEM and DMEM LG are most appropriate for extensive culturing compared with other media based on growth curve and PDT analysis. This

result was in agreement with work carried out by Lund *et al.*, who reported Alpha MEM as an optimal basal medium (2) and was identified that SF-MSC were karyotypically normal at later passages when cultured in DMEM F 12 and DMEM KO.

Differentiation ability of SF-MSC into osteogenic and adipogenic lineages has been reported by many workers (2,6,16,28,30,31). Zhu *et al.* (2) demonstrated ability of SF-MSC to differentiate into osteogenic and adipogenic lineages, at later passages; however, also there are reports stating that SF-MSC lose their differentiation ability at late passages (22). This described here, is the first study to perform differentiation of SF-MSCs into osteogenic and adipogenic lineages at both early and later passages in four different basal media. Differentiation was similar in all media with the exception of slight variations exhibited in cells in DMEM F12, at both early and late passages, in accordance with results of specific staining observed by microscopy. Thus, this study has revealed that subcutaneous fat can be cultured in all the media with reference to differentiation ability.

Conclusion

Today's research on SF-MSC is inconclusive concerning various attributes. For instance, it is clear that prevalence of marker specificity of SF-MSC is yet to be determined. Despite certain initiatives, optimization of culture media and retention of its characteristics in extensive culture for SF-MSC have remained a challenge. The present study, however, has revealed importance of utilization of SF-MSC for curative therapeutics, by eliciting advantages through attributes of expression profiles, proliferation, differentiation and normal karyotype, over extensive culture conditions. Additionally, optimization of basal media appropriate for culturing SF-MSC has been identified, thereby facilitating the researchers to choose their pertinent media. Overall, our results create hope in the light of existing perplexity to bring subcutaneous fat to the frontline as a source of therapeutic stem cells in the field regenerative medicine.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Statistical analysis on phenotypic expression percentage of cell surface markers of omentum fat derived stem cells. *P*-value <0.01 (**) and <0.05 (*) was considered statistically significant.

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