Lineage depletion of stromal vascular fractions isolated from human adipose tissue: a novel approach towards cell enrichment technology

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Abstract The therapeutic rationale for tissue repair and regeneration using stem cells is at its infancy and needs advancement in understanding the role of individual component's innate capability. As stem cells of adipose tissue reside in a more heterogeneous population of stromal vascular fractions, cell separation or sorting becomes an eminent step towards revealing their unique properties. This study elucidates the comparative efficacy of lineage depleted adipose derived stromal vascular fraction (SVF) and their innate ability using magnetic activated cell sorter (MACS). To this end, isolated SVF from human adipose tissue was lineage depleted according to the manufacturer's instructions using specific antibody cocktail through MACS. The enriched lineage negative (lin[−]) and lineage positive (lin⁺) cell fractions were cultured, phenotypically characterized for the panel of cell surface markers using flowcytometry and subjected to osteoblastic and adipogenic differentiation. The expression profile obtained for lin[−] cells was CD34[−]/CD45[−]/HLADR[−]/CD49d[−]/CD140b[−]/CD31[−]/CD90⁺/CD105⁺/CD73⁺/CD54⁺/CD166⁺/CD117[−] when compared to Lin⁺ cells expressing CD34⁺/CD45⁺/HLADR[−]/CD49d[−]/CD140b⁺/CD31[−]/CD90⁺/CD105⁺/CD73⁺/CD54⁺/CD166⁺/CD117⁺ (CD

—cluster of differentiation). These results, thus, advances our understanding on the inherent property of the individual cell population. Furthermore, both the fractions exhibited mesodermal lineage differentiation capacity. To conclude, this research pursuit rationalized the regenerative therapeutic applicability of both lin[−] and lin⁺ cultures of human adipose tissue for disorders of mesodermal, haematological and vascular origin.

Keywords Stromal vascular fraction · Human adipose tissue · Magnetic activated cell sorting · Flowcytometry · Lineage depletion

Introduction

Stem cell therapy is still at its infancy and attributed with several hurdles in regenerative applicability due to the lack of an ideal source of stem cell that accounts for the functional improvement of the diseased. Recent advancements on the isolation and applicability of stem cells from adipose tissue outweigh the existing uncertainties, albeit failures do prevail. The justification for this failure in terms of stem cell survival, proliferation and regeneration remains unclear. Current techniques employed for stem cell isolation from adipose tissue involves enzymatic digestion method, resulting in a heterogeneous crude mixture consisting of preadipocytes, loose connective tissue matrix, endothelial cells, vascular smooth muscle cells,

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Fig. 1 Cell yield of lineage depleted cultures. The cell yield of lin[−] and lin⁺ cells at P1 and P3 was defined as number of cells per microlitre (μl)

pericyte, leucocytes, mast cells, mesenchymal stem cells (MSC) and immune cells such as resident hematopoietic progenitor cells and macrophages (Zuk et al. 2001; Gimble et al. 2007; Prunet-Marcassus et al. 2006). Thus, the failure might be attributed to the fact that stem cells of adipose tissue reside in a more

heterogeneous population of stromal vascular fraction. Hence, comprehensive understanding of the individual component's innate capability is of utmost important for demarcation and development of cell based therapies. The challenges of cell enrichment methods such as cell separation and sorting are decisive in their biomedical applications such as Diagnostics and regenerative medical Therapeutics (Gossett et al. 2010).

A typical cell enrichment method includes, fluorescence activated cell sorter (FACS), an active sorting method which utilizes complementary fluorochrome conjugated antibodies to label cells of interests. The sorted cells obtained from FACS can be utilized for diagnostics and experimental purposes but not for therapeutics due to its confounded safety and efficacy. Contemporary interests to resolve cell separation challenges can be exploited for regenerative medicine applications (Mimeault et al. 2007; Mays et al. 2007). This brings in a more persuasive technique which is entitled to be used in therapeutics (Allan et al. 2005;

Fig. 2 Morphology of lineage depleted cultures. Morphological appearance of lineage depleted cell cultures derived from SVF of adipose tissue: lin[−] cells at P1 (a) and P3 (b); lin⁺ cells at P1 (c) and P3 (d); scale bar = 20 μm

Table 1 Categorization of cell surface marker expression profile

Markers	SVF	Lin+		Lin–	
		P1	P3	P1	P3
CD34	H	H	M	L	S
CD45	L	L	L	S	S
HLA-DR	H	S	S	S	S
CD49d	S	S	L	S	L
CD140b	M	L	M	S	L
CD31	S	L	S	S	S
CD90	H	R	R	H	R
CD105	H	R	R	H	R
CD54	H	R	R	M	H
CD166	S	M	M	M	M
CD117	L	M	H	S	S
CD73	R	R	R	R	R

Expression of cell surface markers were 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#

Hager et al. 2005). Magnetic activated cell sorting (MACS) is a commonly used immuno-magnetic separation technique which employs antibody-conjugated magnetic beads to bind specific proteins on cells of interest (Miltenyi et al. 1990). MACS allow elimination of highly differentiated cells, leaving behind a heterogeneous cell fraction, enriched for stem and progenitor cells. MACS can also be coupled with flowcytometry for further sorting if necessary. Thereby, reducing the

number of cells undergoing sorting and intensifying the efficiency of cells acquired per sorting. The cell sorting speed of FACS ranges from 10,000–50,000 cells per second whereas MACS can sort cells up to speed of 10 million cells per second. In a clinical setting safety criterion is of utmost importance and biodegradable magnetic beads used in MACS overcome this bottleneck. The separation based on magnetic activated cell sorting (MACS) technology, thus, wins over its counterpart, FACS on grounds of speed, safety and cost (Miltenyi et al. 1990).

Thus, to obviate the uncertainties and to advance therapeutic strategies for pervasive diseases, the present work focuses on an alternative, novel technique for the isolation of adipose derived stem cells that is implicated towards understanding the innate capability of the individual components. This involves lineage depletion of the isolated stromal vascular fraction (SVF) of human adipose tissue using a specific antibody cocktail through Magnetic activated cell sorter. These depleted lin– and lin+ fractions were subjected to culturing, subsequent phenotypic characterization and differentiation.

Materials and methods

Sampling

The adipose tissue was collected from patients undergoing abdominoplasty (n = 3) with age group 34–40 years with a mean body mass index of $29 \pm 2.08 \text{ kg/m}^2$. 25 g of sample was collected after an informed consent

Table 2 Comparative expression profile analysis of SVF and lineage depleted cell cultures

Markers	SVF	Lin+		Lin–	
		P1	P3	P1	P3
CD34	57.52 $\pm$ 0.34	61.1 $\pm$ 0.1	57.8 $\pm$ 0.6	20.8 $\pm$ 0.2	10 $\pm$ 0.3
CD45	37.68 $\pm$ 1.0	22.7 $\pm$ 0.2	11 $\pm$ 1.0	5.7 $\pm$ 0.21	1.1 $\pm$ 0.3
HLA-DR	60.11 $\pm$ 0.23	6.7 $\pm$ 0.4	4.4 $\pm$ 0.16	2.4 $\pm$ 0.9	0.7 $\pm$ 1.0
CD49d	7.4 $\pm$ 0.8	5.7 $\pm$ 0.4	10.63 $\pm$ 0.3	5.4 $\pm$ 0.11	15.56 $\pm$ 0.14
CD140b	49.4 $\pm$ 0.2	38.5 $\pm$ 0.5	65.4 $\pm$ 0.7	3.9 $\pm$ 0.2	11 $\pm$ 0.12
CD31	10.4 $\pm$ 0.65	15.2 $\pm$ 0.12	2.56 $\pm$ 0.5	7.6 $\pm$ 0.6	0.13 $\pm$ 0.1
CD90	56.67 $\pm$ 0.5	98.8 $\pm$ 0.4	99.1 $\pm$ 0.2	83.1 $\pm$ 0.3	90.96 $\pm$ 0.1
CD105	63.44 $\pm$ 0.2	99.53 $\pm$ 0.11	99.6 $\pm$ 0.24	88.1 $\pm$ 0.9	92.03 $\pm$ 0.2
CD54	44.96 $\pm$ 0.13	90.9 $\pm$ 0.23	94.86 $\pm$ 0.12	66 $\pm$ 0.8	77.86 $\pm$ 0.7
CD166	5.94 $\pm$ 0.7	54.4 $\pm$ 0.5	69.46 $\pm$ 0.5	45.2 $\pm$ 0.67	56.53 $\pm$ 0.9
CD117	24.96 $\pm$ 0.6	67.7 $\pm$ 0.1	81.03 $\pm$ 0.2	1.5 $\pm$ 0.12	4.53 $\pm$ 0.11
CD73	32 $\pm$ 0.8	99.2 $\pm$ 0.4	99.8 $\pm$ 0.5	95.8 $\pm$ 0.7	96.56 $\pm$ 1.0

Surface antigen expression of SVF and cultured lin– and lin+ cells represented in the form of mean $\pm$ SEM

was signed by each patient in addition to the ethical committee approval of Lifeline Multispecialty Hospital, Chennai, India. The samples were transported in normal saline (0.9 % NaCl) and processed within 2 h of collection.

SVF isolation

The SVF was isolated from subcutaneous fat according to our previously published protocol (Dhanasekaran et al. 2012a). The isolated SVF was analysed for a cell viability testing and lineage depleted using human lineage cell depletion kit (Cat No.: 130-092-211; Miltenyi Biotec, Bergisch Gladbach, Germany) by magnetic activated cell sorting (MACS) technique according to the manufacturer's instructions for the isolation of both lin[−] and lin⁺ fractions. The present study involves the use of LS column for the separation of these fractions. The enriched lin[−] population, representative of the purified stem cells were collected as the cells pass through the column and the retained cells in the column (lin⁺ population), representatives of the other heterogeneous population were collected using a syringe filter. Both these populations were subjected to culturing and further analysed in our study.

Cell culture

The lineage depleted lin[−] and lin⁺ fractions obtained from MACS were seeded with a density of $3 \times 10^4/\text{cm}^2$ in T-25 flasks (Nunc, Roskilde, Denmark) and cultured in α -MEM (Invitrogen, Carlsbad, CA, USA) with 10 % FBS (Invitrogen) and 1 % antibiotic-antimycotic (10,000 units Penicillin, 10 mg Streptomycin

and 25 μg Amphotericin B per ml in 0.9 % normal saline) solution. The cells were maintained for 2–4 days before first medium change. Standard culture conditions of 37 °C, 5 % CO₂ and 95 % humidity were maintained. 60–70 % confluent cells were harvested using Trypsin-EDTA and were maintained till passage 3.

Flowcytometry characterization

Freshly isolated SVF cells and the cultured lineage depleted fractions cultured until P3 were analysed for surface marker expression using BD (Becton–Dickinson, Franklin Lakes, NJ, USA) FACS–DIVA Software as illustrated. About 1×10^6 cells were treated with fluorochrome conjugated antibodies such as CD34-PE (Cat No.: 348057, BD Biosciences, San Jose, CA, USA), CD45-FITC (Cat No.: 347463, BD Biosciences), CD31-FITC (Cat No.: 555445, BD Biosciences), HLA-DR-PERCP (Cat No.: 347364, BD Biosciences), CD90-PERCP (Cat No.: 15-0909-73, e-Biosciences, San Diego, CA, USA), CD105-APC (Cat No.: 17-1057-73, e-Biosciences), CD73-PE (Cat No.: 550257, BD Biosciences), CD117-APC (Cat No.: 17-1179-73, e-Biosciences), CD166-PE (Cat No.: 559263, BD Biosciences), CD54-PERCP (Cat No.: 555512, BD Biosciences), CD49d-PE (Cat No.: 12-0499-73, e-Biosciences), CD140b-PE (Cat No.: 558821, BD Biosciences). The cells were labelled by incubating in dark for 20 min at 37 °C. The incubated cells were washed thrice with wash flow buffer (phosphate buffer supplemented with 2 % (v/v), FBS (Sigma Aldrich, St. Louis, MO, USA) and 0.1 % (w/v) sodium azide, NaN₃ (Sigma Aldrich)) and resuspended in BD FACS flow.

Table 3 Statistical interpretation of cell surface marker expression

Surface markers	SVF versus lin ⁺	SVF versus lin [−]	Lin ⁺ versus lin [−]
CD34	—	**	**
CD45	**	**	*
HLA-DR	**	**	**
CD49d	**	**	**
CD140b	**	**	**
CD31	**	**	*
CD90	**	**	**
CD105	**	**	**
CD54	**	**	**
CD166	**	**	**
CD117	**	**	**
CD73	**	**	**

— no significant difference;
 * significant difference
 ($p < 0.05$); ** significant
 difference ($p < 0.01$)

Mesodermal differentiation

The mesodermal differentiation ability of these lin[−] and lin⁺ fractions was analysed based on their ability to differentiate into osteoblast and adipocyte using the specific induction factor. The differentiation was confirmed by defined staining techniques. The undifferentiated MSC without addition of induction medium served as negative controls. The cells were treated with an osteogenic medium consisting of α -MEM (Gibco-Invitrogen) containing 10 % FBS (Invitrogen), 0.1 μ m dexamethasone (Sigma), 10 mm glyceraldehyde 3-phosphate (Sigma) and 2 mm Ascorbic acid (Sigma). The control cells were treated only with complete medium. After 21 days, the experimental and control cells were stained with von Kossa and Alizarin-Red stain so as to visualize the mineralization of the aggregated cells as dense refractile Ca²⁺ deposits. Similarly, the cells were cultured in adipogenic induction medium (1 μ m dexamethasone, 0.5 mm isobutyl methyl xanthine, 10 μ g insulin, 200 μ m indomethacin). After 18 days, the cells were stained with Oil Red O stain in order to visualise the accumulation of fat droplets in the cells. The confirmation staining protocol for both osteogenic and adipogenic differentiation was carried out according to our previously published protocol (Dhanasekaran et al. 2012a, b). The level of differentiation capacity was determined using the percentage positive cell ratio method.

Statistical analysis

All data obtained from SVF and lineage depleted cell culture samples ($n = 3$) were represented as mean $\pm$ standard error mean (SEM). The data were analysed using Student *t* test and the *p*-values were calculated to determine the statistically significant variations. Results were considered statistically significant when $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$.

Results

Isolation and culture of stromal vascular fraction

Isolated fractions were subjected to subsequent culturing, phenotypic characterization and differentiation into mesodermal lineages. The sorted lin[−] and lin⁺

fractions of SVF were culture expanded till passage 3. Lin[−] and lin⁺ fractions exhibited a good yield in culture, but lin[−] fractions showed comparatively higher number of cells than lin⁺ fractions (Fig. 1). The cells appeared initially as an epithelial contour and later became fibroblastic and mesenchymal in origin. There were morphological variations between the lin[−] and lin⁺ cell cultures. The lin[−] cultures were more of mesenchymal origin with an elongated fibroblastic phenotype in comparison with the morphological appearance of the lin⁺ cultures (Fig. 2).

Flowcytometry characterization

The culture expanded lin[−] and lin⁺ cell fractions and the freshly isolated SVF were investigated using FACS for expression of markers including CD90, CD105, CD73, CD34, CD45, CD31, HLADR, CD54, CD166, CD117, CD49d and CD140b. The expression profile was categorized as sparse (0–10 %), low (11–39 %), moderate (40–74 %), high (75–89 %) and remarkable (90–100 %) according to our previously published paper (Dhanasekaran et al. 2012b) (Table 1). The comparative expression profiles of cell surface markers were comprehended in the form of mean $\pm$ SEM (Table 2) and statistically analysed (Table 3). The dot plots of flowcytometric analysis of markers for lin⁺ and lin[−] (Fig. 3a, b) and SVF (Fig. 4) were illustrated. The expression of MSC and cell adhesion molecule specific markers CD90, CD105, CD73, CD54 and CD166 were comparable and similar in cultures of both the fractions as compared to the lesser expressions identified in the freshly isolated SVF (Fig. 4). On the other hand, the endothelial progenitor markers and perivascular markers, CD34, CD105, CD117 and CD140b, responsible for hematopoiesis, transendothelial migration and angiogenesis were identified to be highly expressed in lin⁺ cell fraction when compared with the lin[−] fractions. Surprisingly, the lin[−] fractions were demonstrated to possess homogenous mesenchymal stem cell and cell adhesion molecules such as CD34[−]/CD45[−]/HLADR[−]/CD49d[−]/CD140b[−]/CD31[−]/CD90⁺/CD105⁺/CD73⁺/CD54⁺/CD166⁺/CD117[−]. Whereas, the lin⁺ fractions were identified to be composed of a heterogenous population consisting of MSCs (characterized by the expression of cell adhesion molecules and perivascular markers), endothelial progenitor cells as well as hematopoietic stem cells (HSCs) such as CD34⁺/CD45⁺/HLADR[−]/CD49d[−]

◀ **Fig. 3** Flowcytometric characterization of lineage depleted cultures. Cell surface marker expression profiles of lineage depleted cell cultures: lin+ (a) and lin− (b) cells

/CD140b+/CD31−/CD90+/CD105+/CD73+/CD54+/CD166+/CD117+ cells (Fig. 5). In addition, sparse expression of CD45, CD31 and HLA-DR was obtained in both lin− and lin+ population in contrary to the higher expression identified in SVF. This demonstrates the fact that both fractions are devoid of committed progenies upon culture.

Mesodermal differentiation

The therapeutic efficacy of cultured lin− and lin+ population were further substantiated by its ability to differentiate into mesodermal lineages. The differentiation of cultured lin− and lin+ fractions sorted from SVF were subjected to osteoblast and adipocyte differentiation at P3. Both fractions displayed their ability to

differentiate into both osteoblastic and adipogenic lineages. The osteoblast differentiations of these cells were detected by the deposition of mineralized calcium (Ca^{2+}) deposits over a period of 21 days using von Kossa and Alizarin red (Fig. 6a–f). However, a lesser differentiation capacity was detected in lin+ fractions when compared to that of lin− fractions; evident from its reduced staining and the reduced positive cell ratio obtained (Fig. 7). Adipogenic differentiation was detected within 18 days by visualising the fat droplet accumulation using Oil Red O staining (Fig. 6g–i). Similar to osteogenic differentiation, adipogenic differentiation capacity of lin+ fraction was found to be low as compared to lin− fractions (Fig. 7).

Discussion

Adipose tissue emerged as a redundant, less invasive and ideal source of stem cells in recent years due to its

Fig. 4 Flowcytometric characterization of stromal vascular fraction. Cell surface marker expression profiles of SVF derived from adipose tissue

Fig. 5 Expression profile of cell surface markers. Comparative expression profile of cell surface markers in SVF and lineage depleted (lin– and lin+) cell cultures at P1 and P3

Fig. 6 Conformational staining of mesodermal differentiation. Confirmation of mesodermal differentiation derived from lin– cells and lin+ cells at P3. Osteogenic differentiation using Alizarin red staining: control (a), lin– (b), lin+ (c) and Von

Kossa staining: Control (d), lin– (e), lin+ (f); Adipogenic differentiation using Oil O Red staining: control (g), lin– (h), lin+ (i). scale bar = 20 μ m

Fig. 7 Positive cell ratio analysis for differentiation. Percentage positive cell ratio for osteoblast and adipocyte differentiation confirmation via Von Kossa, Alizarine red and Oil-O-Red staining in comparison to undifferentiated control

colossal abundance and applicability (Zuk et al. 2001, 2002; Gimble 2003; Gimble et al. 2007; Zhu et al. 2008). Identification of the cellular determinants of adipose tissue revealed its heterogeneity that comprises of stem cell and non-stem cell components (Zuk et al. 2001; Gimble et al. 2007). Thus, a prior knowledge about the wide range of cell-specific markers is a pre-requisite to understand this heterogeneity. However, these markers may or may not be evident at primitive stages or may get lost with expansion in vitro or in vivo (Roda et al. 2009), thus identity of these inherent populations for therapeutic interventions becomes a strenuous task. To reduce this heterogeneity and to enhance the therapeutic implications of stem cells, cell enrichment and purification method based on FACS and MACS has become the major goal of many investigators.

In lieu of this, our present study focused on a novel, alternative method of obtaining adipose derived stem cells for therapeutic implications by cell enrichment method. This involves isolation, culturing, phenotypic characterization and differentiation of the lineage depleted cells of SVF obtained from human adipose tissue. Although lin[−] and lin⁺ culture fractions exhibited a higher yield of cells, comparatively better yield was obtained in lin[−] population, thus indicating the fact that lin[−] fractions are more homogenous. However, both culture populations exhibited a higher positivity for a wide range of markers of uncommitted progenies when compared to the heterogenous non-sorted SVF. This substantiates the fact that lin[−] and lin⁺ population might be of potential candidates for its

use in tissue repair and regeneration. These results thus become highly imperative as it makes wider utilization of both depleted culture fractions for its implications in therapeutic applications unlike the existing uncertainties of SVF as indicated in the current literature (Kern et al. 2006; Monaco et al. 2012). The rationale behind the applicability could be unravelled by the analysis of its marker expression profiles obtained from cultured lin[−] and lin⁺ population as well as by their differentiation ability.

Interestingly, the significance of this study in therapeutic interventions is further substantiated from the fact that lin[−] population are non-hematopoietic and enriched with more of homogenous MSC and Cell adhesion molecule population in contrary to the heterogenous lin⁺ cells. Thus, the efficacy of lin[−] population might be correlated with its use in specific treatment of mesodermal related disorders such as bone and cartilage dysfunctions, soft tissue augmentation, muscular disorders and so on (Vieira et al. 2008; Liu et al. 2012; Lendeckel et al. 2004). On the other hand, the efficacy of lin⁺ population, being more of hematopoietic, vascularized and trans-endothelial nature, might be correlated with its use in specific hematological and vascular disorders and obtain prime importance in replacing damaged tissue elements as demonstrated in the literature (Zimmerlin et al. 2010; Robert W Alexander 2012).

In conclusion, significance of this research pursuit on lineage depletion of SVF isolated from human adipose tissue is demonstrated from the comparative efficacy on the identity of individual components such as lin[−] and lin⁺ fractions and their innate capabilities. The study further highlights on the regenerative applicability of the cultured lin[−] and lin⁺ fractions towards treatment of multitude of disorders. Although, both cell populations were identified as a key for biocellular therapies in the near future, further additional research will serve to explore the potentials of human adipose tissue derived stem cells in cell based therapies.

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