

Original Article

Establishment and Molecular Characterization of Mesenchymal Stem Cell Lines Derived From Human Visceral & Subcutaneous Adipose Tissues

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Abstract

Mesenchymal stem cells (MSCs), are multipotent stem cells that can differentiate into osteoblasts, chondrocytes, myocytes and adipocytes. We utilized adipose tissue as our primary source, since it is a rich source of MSCs as well as it can be harvested using a minimally invasive surgical procedure. Both visceral and subcutaneous adipose tissue (VSAT, SCAT respectively) samples were cultured using growth medium without using any substratum for their attachment. We observed growth of mesenchymal like cells within 15 days of culturing. In spite of the absence of any substratum, the cells adhered to the bottom of the petri dish, and spread out within 2 hours. Presently VSAT cells have reached at passage 10 whereas; SCAT cells have reached at passage 14. Morphologically MSCs obtained from visceral adipose tissue were larger in shape than subcutaneous adipose tissue. We checked these cells for presence or absence of specific stem cell molecular markers. We found that VSAT and SCAT cells confirmed their MSC phenotype by expression of specific MSC markers CD 105 and CD13 and absence of CD34 and CD 45 markers which are specific for haematopoietic stem cells. These cells also expressed SOX2 gene confirming their ability of self-renewal as well as expressed OCT4, LIF and NANOG for their properties for pluripotency & plasticity. Overall, it was shown that adipose tissue is a good source of mesenchymal stem cells. It was also shown that MSCs, isolated from adipose tissue are multipotent stem cells that can differentiate into osteoblasts, chondrocytes, cardiomyocytes, adipocytes and liver cells which may open a new era for cell based regenerative therapies for bone, cardiac and liver disorders.

Key words; stem cell, mesenchymal stem cells, adipose tissue, and molecular markers

Introduction

Stem cells are undifferentiated cells, which have the ability to produce cells of varied lineages. Characteristics of stem cells include ability of self-renewal, pluripotency, plasticity and controlled differentiation^[1]. Stem cell biology is based on the principle, that any tissue may contain cells that possess the potential for both self-renewal and differentiation into one or more cell types^[2]. Mesenchymal stem cells have been a popular subject of research for many years. They arise from Mesodermal progenitors and can be differentiated into bone cells, cartilage, fat cells and skeletal muscle cells, although recent research has shown that they can also be differentiated into other cell lineages such as liver cells^[3]. Although bone marrow (BM) has been the de facto source for the isolation of multipotent MSCs, the harvest of BM is a highly invasive procedure and the number, differentiation potential, and maximal life span of MSCs from BM decline with increasing age^[4-6]. Umbilical cord blood has also been used as the source for MSCs that can be isolated without any harm to the mother or infant and with minimal invasive procedures. But there is controversy about the multipotent nature of MSCs isolated from umbilical cord blood^[7].

Adipose tissue (AT) is another alternative source that can be obtained by a less invasive method and in larger quantities than BM^[8]. It has been demonstrated that AT contains stem cells similar to BM-MSCs, which are termed as Processed Lipo Aspirate (PLA) cells^[9]. Adipose tissue which is located beneath the skin is called "Subcutaneous fat" and around internal organs is called "Visceral fat". Adipose tissue contains several cell types having a major percentage being adipocytes, which contain fat droplets. Other cell types include fibroblasts, macrophages, and endothelial cells. In 1964, Rodbell first described the isolation of a population of progenitor cells

from adipose tissue^[10]. This procedure for cell isolation was adapted in later years to isolate progenitor cells from human adipose tissue^[11-13].

The study of gene expression profiles is extremely valuable for identification of candidate stem cell genes and markers of different differentiation lineages. Recent research comparing the transcriptomes of multiple human embryonic stem (hES) cell lines has identified a set of approximately 100 genes that are highly expressed in undifferentiated hES cells^[14]. Pluripotency is clearly a key measure of stem cell line performance in which the expected outcomes may vary depending on the cell types such as hES, MSCs etc. There are a number of ways of identifying pluripotency such as Germ line competence, Teratoma formation, generation of embryoid bodies, but it has been shown that the best and accurate way of identification of this phenotype is by using specific molecular markers in these cells. Recent studies have shown that expression of OCT4, NANOG and SOX2 indicate pluripotent nature of stem cells^[15-17].

The novelty of our research lies in the fact that we aim to develop MSCs lines from visceral as well as subcutaneous adipose tissues. Characterisation of these developed cell lines will be carried out using molecular markers CD105 & CD13 for mesenchymal lineage, CD45 & CD 34 for haematopoietic lineage and LIF, OCT4, SOX2 & NANOG for self renewal ability, pluripotency and plasticity of these MSCs. We are also interested in studying differential expression of MSC isolated from subcutaneous and visceral adipose tissues to understand the different roles of these cells in the process of adipogenesis. As subcutaneous cells protect the body from heat shock and visceral plays role in protection from mechanical shock, the identification of these different roles may be useful to understand the mechanism

of obesity and these cell types can be used as a model system for therapies directed to

Materials & Methods:

Adipose Tissue Cell Culture

Adipose tissue samples were collected in sterile containers from obese patients and sent to the stem cell laboratory. An informed consent of the patients was obtained as per the guidelines of Ethical Committee of Jaslok Hospital and Research Centre. The tissue samples were washed with sterile PBS, minced and were digested using 0.25% Trypsin at 37°C for 30 minutes. Trypsinised cells were cultured in a 24 well culture plate in Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine supplemented with 10% fetal bovine serum (HiMedia). Penicillin-streptomycin and Nystatin were added to the media to prevent any bacterial and fungal contaminations. Growing cells were regularly monitored by observing under a phase contrast microscope. They were fed with fresh growth media twice a week. After reaching confluency, cells were passaged to a 65mm culture plate, and on further increase in the number of cells transferred to a culture flask (Nunc). Passaging was done by incubating the cultures with 0.25% Trypsin at 37°C for 5-10 minutes and then transferring the cells to a new flask. All the culture plates were incubated at 37°C in the presence of 5% CO₂ with 90% humidity.

Cryopreservation and Revival of MSCs

Semi-confluent cultures of Mesenchymal stem cells were trypsinised with 0.25% Trypsin- EDTA for 5-10 minutes,

reduce obesity and its related diseases.

centrifuged and washed with PBS. The pellet containing 1x10⁶ cells were suspended in ready-made freezing medium from HiMedia Biosciences, India. The cell viability was checked with 0.1% Erythrocyte B and was found to be 99%. The cells were frozen in liquid Nitrogen. The cells were revived at different passages and were found to have a viability of more than 90% and maintained an appreciable growth rate.

Characterisation of MSCs

Morphological observations

The live cells were observed at different passages for their morphological features under Zeiss phase contrast microscope and photographed at different passages. The cells were also fixed with 50% chilled methanol, stained with Geimsa stain and observed under light microscope for their morphology.

Molecular markers expression

Cells from cultures were trypsinised using 0.25% Trypsin-EDTA (Himedia, India) for 5 minutes at various passages and RNA was extracted from these cells by using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was then transcribed to cDNA by using Applied Biosystems high capacity cDNA kit. (Applied Biosystem, USA). Table 1 & Table 2 show the primers sequences used for various stem cell genes expression and their respective PCR conditions for each gene. The PCR products were checked for their respective amplification on 2% agarose gel.

Table 1: Shows sequences of primers used for respective molecular markers

Sr. No.	Molecular marker	Forward primer (5'-3')	Reverse primer (5'-3')
1	CD105	TGTCTCACTTCATGCCTCCAGCT	AGGCTGTCCATGTTGAGGCAGT
2	CD13	GTCTACTGCAACGCTATCGC	GATGGACACATGTGGGCACCTTG
3	CD34	GCAAGCCACCAGAGCTATTC	GGTCCCAGGTCCTGAGCTAT
4	CD45	ACCAGGGGTTGAAAAGTTTCAG	GGGATTCCAGGTAATTACTCC
5	LIF	GGCCCGGACACCCATAGACG	CCACGCGCCATCCAGGTAATA
6	SOX2	GCCGAGTGGAAACTTTTGTGTC	GTTCATGTGCGCGTAACTGT
7	Oct-4	GAGCAAAAACCCGGAGGAGT	TTCTCTTTCGGGCCTGCAC
8	NANOG	GCTTGCCTTGCTTTGAAGCA	TTCTTGACCGGGACCTTGTC
9	Keratin18	GAGATCGAGGCTCTCAAGGA	CAAGCTGGCCTTCAGATTTTC
10	β - Actin	AACCCCAAGGCCAACCGCGAGAAGATGACC	GGTGATGACCTGGCCGTCAGGCAGCTCGTA

Table 2: Shows all the PCR programs used for respective molecular markers. 40 cycles were carried out for denaturation, annealing and extension in each PCR program.

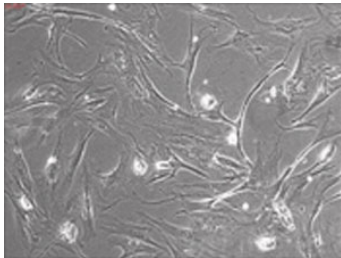
Sr No	Gene	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Forever
	Time	5 mins	30 sec	30 sec	1 min	10 mins	
1	CD105	95°C	94°C	60°C	72°C	72°C	4°C
2	CD13	95°C	94°C	60°C	72°C	72°C	4°C
3	CD45	95°C	94°C	60°C	72°C	72°C	4°C
4	CD34	95°C	94°C	57°C	72°C	72°C	4°C
5	NANOG	95°C	94°C	57°C	72°C	72°C	4°C
6	Oct-4	95°C	94°C	57°C	72°C	72°C	4°C
7	SOX2	95°C	94°C	57°C	72°C	72°C	4°C
8	LIF	95°C	94°C	57°C	72°C	72°C	4°C
9	keratin 18	95°C	94°C	57°C	72°C	72°C	4°C
10	Actin	95°C	94°C	57°C	72°C	72°C	4°C

Results:

Isolation and Culture of MSCs from adipose tissue

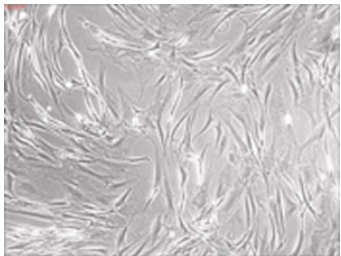
After mincing adipose tissue, cells were grown in growth medium containing DMEM having L-Glutamine + 10% FBS + Penicillin & Streptomycin and incubated in CO₂ incubator at 37°C. Mesenchymal like adherent cells appeared on the 7th days of the culture for both visceral as well as subcutaneous tissue. These cells adhered to the surface of petridishes without any substratum. Both these cell types reached confluency at around 15-20 days. Passaging was done when cells reached 70-80% confluency. Presently VSAT cells are at passage 10 whereas; SCAT cells are at passage 14. Figures 1 & 2 show the phase contrast images of VSAT and SCAT cells, respectively. SCAT cells showed presence of colony like cell aggregation at passage 8 as shown in Figure 3.

Figure 1



Shows visceral adipose tissue mesenchymal stem cells (VSAT) having large nucleus and extended cytoplasm

Figure 2

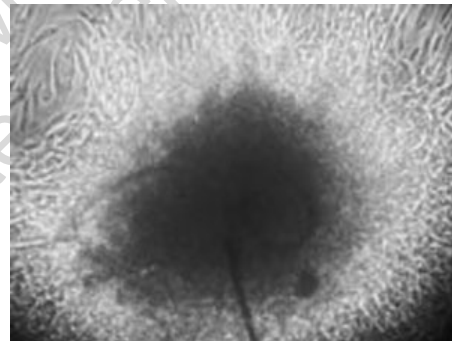


Shows subcutaneous adipose tissue mesenchymal stem cells (SCAT), which are more elongated morphology with nucleus and cytoplasm

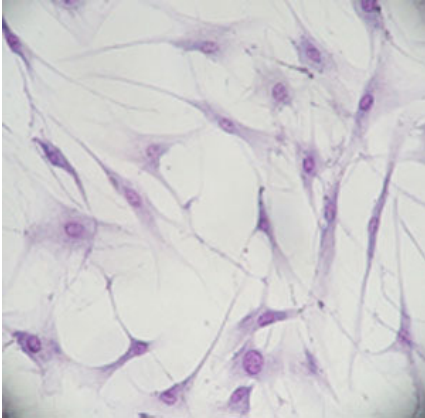
Phase contrast Microscopy of VSAT & SCAT cells.

The morphology of VSAT & SCAT cultured cells was regularly monitored using a phase contrast microscope. These cultured cells exhibited various kinds of morphologies such as elongated- fibroblastic cell, spread cells with enlarged cytoplasm, neuron-like cells and cells having cytoplasmic vacuolation. Figure 4 shows the SCAT cells stained with Geimsa stain. It was observed that VSAT cells are much larger in morphology than SCAT cells.

Figure 3



Shows colony formation having aggregation of cells observed in subcutaneous adipose tissue mesenchymal stem cell culture at passage 8

Figure 4

Shows Giemsa stained subcutaneous adipose tissue mesenchymal stem cells having large nucleus and cytoplasm

Molecular marker analysis

Selection of Positive Controls: - Our previous work has shown that CD 105, CD13, CD45, CD34 and SOX2 genes were upregulated in patients with Chronic Myeloid Leukaemia (CML). PCR for each of the above mentioned genes was carried out using CML blood samples available in our laboratory. Amplified PCR products were sequenced for confirmation of required gene sequences using specific primers. The sequenced obtained for each gene was Blast for confirmation of the gene in NCBI database [data not shown]. We found 100% match for these specific genes reported here. Similar approach was used for OCT4, NANOG, LIF and Cytokeratin 18, which were expressed in Normal Blood Cells (NBS). On the basis of these results, positive controls were selected for above genes.

Comparative molecular marker analysis was done for cells from various sources viz., peripheral blood, umbilical cord blood, umbilical cord tissue, adipose tissue, aphaeresis blood and cultured MSCs from adipose tissue. Results obtained were as shown in table 3.

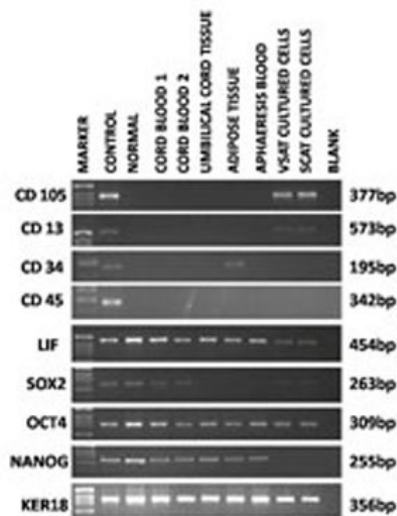
Figure 5 shows composite expression of all the above genes in given samples. CD 105 and CD 13 are positive markers for MSCs. They were expressed only in cultured VSAT and SCAT cells. CD 34 was expressed in only adipose tissue whereas CD 45 being a haematopoietic marker was not expressed in any of the samples. SOX2 being a self-renewal marker was expressed in normal, cord blood and cultured VSAT and SCAT cells. OCT4, NANOG and LIF being pluripotency markers were expressed in all the samples. Cytokeratin 18 was also expressed in all the samples.

Cultured cells from adipose tissue were also characterised using 9 molecular stem cell markers at various passages. Results obtained were as shown in table 4 for VSAT and SCAT cell lines. CD 105 and CD 13 being positive markers for MSCs, were expressed in all the VSAT as well as SCAT cell lines at all passages except VSAT at passage 7 showed down regulation of CD 13. CD 45 and CD 34 being haematopoietic markers and negative markers for MSCs were expressed in earlier passages of both the cell lines and down regulated at later passages indicating confirmation of MSCs Phenotypes of VSAT and SCAT cell lines. Pluripotency markers OCT4 and NANOG and self renewal marker SOX2, were expressed in both VSAT and SCAT cell lines at all passages except VSAT at passage 7 where cells may have differentiated. Leukaemia Inhibitory Factor (LIF), which is an important factor for stem cells for maintaining their undifferentiated stage, was expressed in both VSAT and SCAT at all passages. Even both these cell lines expressed Cytokeratin 18 at all passages indicating these cell were MSCs and quite different from fibroblast cells.

Table 3: Shows expression of molecular markers in the samples of normal blood, cord blood 1, cord blood 2, umbilical cord tissue, aphaeresis blood, cultured VSAT cells and cultured SCAT cells as well as positive control samples used. Key: +: Expressed - : Not Expressed, NC- Not Checked CML: Chronic Myeloid Leukaemia, NBS: Normal Blood Sample

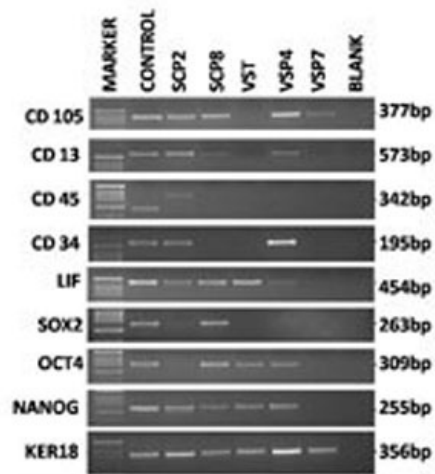
Sample	CD105	CD13	CD45	CD34	LIF	SOX2	4-Oct	NANOG	Keratin 18
Positive sample used	CML +	CML +	CML +	CML +	NBS +	CML +	NBS +	NBS +	NBS +
Normal blood	-	-	-	-	+	+	+	+	+
Cord blood 1	-	-	-	-	+	+	+	+	+
Cord blood 2	-	-	-	-	+	+	+	+	+
Umbilical cord tissue	-	-	-	-	+	-	+	+	+
Adipose tissue	-	-	-	+	+	-	+	+	+
Aphaeresis blood	-	-	-	-	+	-	+	+	+
VSAT	+	+	-	-	+	+	+	NC	+
SCAT	+	+	-	-	+	+	+	NC	+

Figure 5



Shows expression of molecular markers in samples of normal blood, cord blood, umbilical cord tissue, adipose tissue, aphaeresis blood, VSAT cells and SCAT cells.

Figure 6



Shows expression of various molecular markers in VSAT and SCAT cultures at various passages. key: SCP2- SCAT passage 2, SCP8- SCAT passage 8, VST- VSAT tissue, VSP4- VSAT passage 4, VSP7- VSAT passage 7.

Table 4

Molecular marker	Positive Control used	Visceral adipose tissue	VSAT Passage 4	VSAT passage 7	SCAT passage 2	SCAT passage 8
CD105	CML +	-	+	+	+	+
CD13	CML +	-	+	-	+	+
CD34	CML +	-	+	-	+	-
CD45	CML +	-	-	-	+	-
LIF	NBS +	+	+	+	+	+
SOX2	CML +	-	+	-	+	+
Keratin18	NBS +	+	+	+	+	+
Oct-4	NBS +	+	+	-	+	+
NANOG	NBS +	+	+	-	+	+
Actin	NBS +	+	+	+	+	+

Table 4: Shows positive control used and expression of given molecular markers in cultured VSAT and SCAT cells at various passages key- +: Expressed, -: Not Expressed, CML: Chronis Myeloid Leukaemia, NBS: Normal Blood Sample

Discussion

Mesenchymal stem cells, or MSCs, are multipotent stem cells that can differentiate into osteoblasts, chondrocytes, myocytes and adipocytes. Adipose tissue^[9] has been used as one of the sources for MSCs for many years due to its ease of access and ability to generate better mesenchymal stem cell lines. Adipose tissues MSCs have been differentiated in vitro towards the osteogenic, adipogenic, myogenic, and chondrogenic lineages when treated with established lineage- specific factors.^[9]

In the present study, we cultured MSCs from adipose tissue without any substratum and confirmed their mesenchymal nature using a set of stem cell specific molecular markers. The studies also characterized the plasticity, self-renewal ability and Pluripotent nature of stem cells. The purpose of this work was two-fold. First being, isolation and culture of MSCs from adipose tissue and second, their characterization using molecular markers. In our findings, we compared MSCs from visceral adipose tissue and subcutaneous adipose tissue, which is an entirely novel area of research. We were able to culture MSC cell lines from both the sources

successfully and also characterize them using various molecular markers.

There were various morphological differences observed in VSAT and SCAT cell lineages which included the cell shape and cell size. VSAT MSCs were larger cells with more spread appearance whereas subcutaneous MSCs were with comparatively smaller size and had varying morphology such as spindle shape, neuron like appearance etc. Visceral MSCs appeared to contain more fat droplets and were seldom granulated. This phenomenon was not observed in subcutaneous MSCs. Presently VSAT cell line is at passage 10 and SCAT cell line is at passage¹⁴.

VSAT and SCAT cultures were characterized with a set of molecular markers. CD105 or Endoglin is a member of the TGF-beta receptor complex that modulates TGF-beta signalling. Among others, Endoglin (CD105) was suggested as a putative Mesenchymal stem cell marker^[1,18]. CD 13 or aminopeptidase N is also considered as a positive marker for MSCs.

^[19]. Both VSAT & SCAT cultures were positive for CD105 [1,18] and CD13 ^[19] [as shown in Table 3 and Table 4] confirming their MSCs nature. CD 34 and CD 45 are positive markers for haematopoietic stem cells as suggested by L Healy et al 1995 ^[20]. Presences of CD34 ^[20] and CD45 ^[21] expression at later passages exhibit the presence of cells of other lineages than MSCs. This also suggests phenomenon of spontaneous differentiation taking place in vitro. The OCT3/4 gene, a POU family transcription factor, has been noted as being specifically expressed in embryonic stem cells and in tumour cells but not in cells of differentiated tissues by Mei-Hui Tai et al 2005 ^[17]. NANOG is regulated by an adjacent pair of highly conserved OCT-4 and SOX-2 binding sites, through an interaction between OCT-3/4 and SOX2. Wenjing Shi et al 2006 have shown that transcription factors Oct-3/4, NANOG, SOX2, and FOXD3 anchor a negative feedback loop to maintain the expression of Pluripotent factors at a steady state. ^[22] Tracy Wilson et al 1988 have shown that the LIF maintains the developmental potential of embryonic stem cells, which can be regarded as a pluripotency marker. ^[23] Both the cell lineages were positive for SOX2, which mainly marks self-renewal ability of undifferentiated stem cells, which is an important factor for development of stem cell lines. Presence of OCT4, NANOG and LIF show pluripotent nature of both Visceral and Subcutaneous Mesenchymal stem cell lines. Human keratin 18 and the homologous mouse Endo B are type I IF protein subunits, the expression of which is restricted in adults to a variety of simple epithelial tissues. ^[24] It is a marker specific for cells of epithelial origin. It was expressed in both VSAT and SCAT cell lines, again suggesting phenomena of spontaneous differentiation. In case of VSAT cell line, at later passages, pluripotency markers like SOX2, OCT4 and NANOG were completely downregulated suggesting that cells might have lost their undifferentiated 'stem cell' nature as well as plasticity and cell renewal ability and become differentiated into other

cell types. CD 13, which is a mesenchymal stem cell marker, was also observed to be downregulated suggesting that cells might be losing their Mesenchymal stem cell nature.

The present paper describes development and characterization of VSAT and SCAT cell lines from adipose tissue. It has also been shown for the first time that both these cell types differ in their characteristics by morphology as well as at molecular level, indicating that both cell types play different roles in the adipogenesis process. It has already been shown that VSAT protects body organs from mechanical shock whereas SCAT protects the body from heat shock. VSAT has been observed to play a key role in metabolic syndromes unlike SCAT and therefore these two developed MSC cell lines become model systems to study obesity and its related disorders like 'Diabetes mellitus'. These cell lines can also be treated with specific differentiating agents to give rise to chondrocytes, cardiomyocytes etc., which can be used in regenerative therapies for treatment of various bone and cardiac disorders.

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