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Novel Way to Isolate Adipose Derive Mesenchymal Stem Cells & Its Future Clinical Applications in Obesity

Eliza Chakraborty[1*] and Neelam R. Shaikh[2]
[1,2:] *Dept. Of Stem Cell Research and Regenerative Medicine, Vidyapratishthans School of Biotechnology (VSBT, University of Pune), Baramati Dist-Pune , Pin -413133India.o*

***Abstract*:** *Adipose-derived stem cells (ADSCs), were isolated from discarded human fat tissue, obtained from c-section with our recently modified methods, in Stem Cell & Regenerative Medicine Lab, VSBT. Here we develop two methods to* i*solate Adipose derived mesenchymal stem cells with enzyme digestion and use of phosphatidylcholine and deoxycholate. Surface protein expression was analyzed by flow cytometry to characterize the cell phenotype. The multi-lineage potential of ADSCs was testified by differentiating cells with adipogenic inducer. ADSCs can be cultured in vitro for up to one month without passage. Also, the flow cytometry analysis showed that ADSCs expressed high levels of stem cell related surface marker CD105. ADSCs have strong proliferation ability, maintain their phenotypes, and have stronger multi-differentiation potential. The molecular basis of ADSC differentiation was studied using bioinformatics tools with an aim to identify the key proteins involved in differentiation, such that they could be used as potential targets for drug development for the treatment of obesity. The key proteins involved were found to be PPARG and C/EBPα. The structures of the proteins were retrieved from MMDB (Molecular Modelling Database) and PDB (Protein Data Bank) respectively.*

Key Words: *Adipose-derived stem cells, Mesenchymal stem cells, Enzyme digestion, Phosphatidylcholine, Deoxycholate, PPARG,* C/*EBPα,* etc.

I. Introduction:

A major source of stem cells is provided by adult bone marrow-derived mesenchymal stem cells (MSCs), present within the bone marrow stroma. The retrieval of stem cells from the bone marrow, however, is highly invasive and provides only low numbers of stem cells. (Casteilla et al., 2004, Michelle Locke et al., 2008) Therefore, alternative sources for MSCs have been investigated. Only recently, adipose tissue was defined as a new source of MSCs. Like the bone marrow, adipose tissue contains a stromal fraction, which contains a population of adipose tissue-derived stem cells (ASCs) (Zuk et al., 2001). An advantage of using adipose tissue as the source of stem cells is that the harvesting of adipose tissue is minimally invasive and can be done under local anesthesia, thus resulting in minimal patient discomfort and low patient risk. (Michelle Locke et al., 2008) Furthermore, adipose tissue provides large numbers of stem cells as compared to bone marrow. Adipose-derived MSC are capable of proliferation in monolayer culture and multi lineage differentiation in response to inductive conditions, and thus have potential clinical application (Bailey et al., 2010; Fraser et al., 2008; Rigotti et al., 2009).

The conventional method of isolating MSC from adipose tissue is using lipoaspirate that consists of at least four main steps: digestion, washing, centrifugation and red blood cell lysis. Adipose tissue from lipoaspirate samples is incubated with collagenase for up to 1 hour. Then, the digests are washed, and centrifuged to separate the floating adipocytes from the pelleted stromal cells. The pelleted stromal cells are finally incubated with red blood cell lysis solution and centrifuged one more time. This procedure generates tissue fragments that should be removed before cell plating through a 100–150 μm nylon mesh. (Zuk et al., 2001) Irrespective of the source of tissue, this method is time consuming and expensive, especially when applied to large volumes of tissue (Baptista et al., 2009); decreased cell viability due to lytic activity is also a problem with this method (Ishige et al., 2009).

We have described a novel method of isolating MSC from whole adipose tissue. The conventional methods are focused only on the use of lipoaspirate, but lipoaspirate is sometimes not available for stem cell isolation and it is also required for reconstructive lipotransfer (R. A. Soriano et al., 2013). Furthermore whole adipose tissue, in some surgical procedure, like caesarian section etc., has to be removed and discarded as medical waste. Such adipose tissue obtained after a surgery can be utilized as a source of autologous stem cells. Our protocol hence is focused on utilization of discarded adipose tissue; it is based on enzymatic tissue digestion and use of phosphoidylcholine and deoxycholate as fat solubilising agents, so as to prepar lipoaspirate in-vitro. Despite the major differences between both methods, similar populations of MSC have been isolated. The populations of cells derived from process were positive for mesenchymal surface marker CD105. They also

were able to accumulate lipid droplets under specific stimuli for each differentiation event. Their proprieties support their use for diverse therapeutic applications. Techniques used on these assays will be detailed below.

According to the WHO Factsheet (2014), worldwide obesity has nearly doubled since 1980. In 2008 more than 1.4 billion adults were found to be overweight. Of these over 200 million were male and nearly 300 million females. 65% of world population lives in countries where overweight and obesity kills more people than underweight. More than 40 million children under age of 5 were overweight or obese in 2012. Childhood obesity is associated with a high chance of obesity, premature death and disability in adulthood and also increased future risk of breathing disabilities, hypertension early markers of cardiovascular disease, insulin resistance and psychological effects.

Overweight and obesity are leading risks for global deaths. Around 3.4 million adults die each year as a result of being overweight and obese. In addition, 44% of diabetes burden, 23% of ischemic heart disease burden and between 7% and 41% of certain cancer burdens are attributable to obesity.

Considering these facts we tried to analyze the key proteins involved in differentiation of stem cells to adipocytes using bioinformatics approach, so as to find a new target for treating obesity. LifeMap Discovery™ database was used along with KEGG pathway database, MMDB and PDB databases to study the differentiation pathways and protein structure.

The detailed study of databases revealed that two pathways were mainly responsible in stem cell differentiation viz. insulin pathway and Wnt pathway. It was observed that insulin pathway enhances the differentiation and wnt pathway inhibits the differentiation. On further analysis the key proteins involved in differentiation were identified to be PPARG and **C/**EBPα. C/EBPα is a pleiotropic transcriptional activator of adipocyte-specific genes. Promoters from numerous adipocyte genes contain C/EBP regulatory consensus sequences and are trans-activated by C/EBPα. Forced expression of this isoform in 3T3-L1 preadipocytes stimulates adipogenesis in the absence of any hormonal induction. In addition, blocking its expression wit antisense C/EBPα RNA inhibits adipogenesis. Furthermore, C/EBPα(−/−) mice have defects in lipid accumulation. These observations establish the requirement for C/EBPα in preadipocyte differentiation. (Tamara C. Otto et. al., 2014).

PPARG is a central regulator during adipogenesis. The transcription factor is induced during differentiation and is responsible for activating a number of genes involved in fatty acid binding, uptake and storage, including 422/aP2, lipoprotein lipase, acyl coenzyme A synthase and phosphoenol pyruvate carboxykinase. The importance of PPARG in adipogenesis has been demonstrated in several ways. Over-expression of PPARG in non adipogenic fibroblasts stimulates adipogenesis. PPARG (−/−) mice die in utero secondary to a placental defect, but those that survive to term through tetraploid rescue are deficient in brown fat. PPARG is transcriptionally activated by C/EBPβ and C/EBPδ, and once expressed; PPARG and C/EBPα positively regulate each other's expression. (Tamara C. Otto et.al. 2014)

LifeMap Discovery™ provides investigators with an integrated database of embryonic development, stem cell biology and regenerative medicine. The hand-curated reconstruction of cell ontology with stem cell biology; including molecular, cellular, anatomical and disease-related information, provides efficient and easy-to-use, searchable research tools. The database collates in vivo and in vitro gene expression and guides translation from in vitro data to the clinical utility, and thus can be utilized as a powerful tool for research and discovery in stem cell biology, developmental biology, disease mechanisms and therapeutic discovery. LifeMap Discovery is freely available to academic nonprofit institutions at http://discovery.lifemapsc.com

II. Human adipose tissue mesenchymal stem cells: Chakraborty E. et al's protocol

Human abdominal subcutaneous adipose tissue was surgically obtained from females after Cesarean delivery using sterile techniques, with Prior consent of the donors. The sample was first washed in normal saline and then collected in (1X) PBS containing antibiotic antimycotic (Penicillin and Streptomycin) solution 1ml/100ml and then processed within 5 hours after collection. After transferring under sterile conditions, the tissue was washed twice in (1X) PBS containing antibiotic antimycotic solution; to remove blood. The tissue was then cut into two pieces: one of which underwent enzymatic treatment and another was processed for in vitro lipoaspirate production.

Fig.1: Chopped human adipose tissue

Fig.2: Enzyme digested adipose tissue

Fig.3: PC & DC treated adipose tissue

1. Enzymatic Digestion Method:

Adipose tissue was minced in (1X) PBS using sterile surgical blades and forceps. It was then digested with same volume of PBS containing 1% BSA and 0.2% Collagenase type IA in a water bath for 60 mins or more until the solution appeared clear. The solution was then filtered using cell strainer (100µm) to remove undigested tissue. The filtrate was centrifuged at 1800 RPM for 5 mins. The pellet was resuspended in the same volume of RBC lysis buffer (160 mM/L NH_4Cl in PBS) and incubated for 20 mins at room temperature. The solution was again centrifuged at 1800 RPM for 5 mins. After centrifugation, the supernatant was discarded and the ADSC pellet was resuspended in 1ml complete media (DMEM containing 4.5 g/ml glucose, L-glutamine, sodium pyruvate) with 10% FBS, 1% antibiotic antimycotic solution. Cells were seeded in T25 culture flasks. 3ml of complete medium was added to the flasks. The flasks were incubated in CO_2 incubator at 5% CO_2, at 37^{o} C; for 24 to 48 hours. After 48 hours, 1ml of complete media was added to each flask.

2. In vitro preparation of human lipoaspirate for ADSCs Isolation:

The adipose tissue was minced into small pieces in (1X) PBS using sterile surgical blades and forceps. The tissue was then incubated with 25mg/ml Phosphatidylcholine(Sigma) and 21mg/ml Deoxycholate(Sigma). In vitro lipoaspirate was prepared by mixing it by frequent pipetting and then incubating it for 40-60 mins. It was then digested with same volume of (1X) PBS containing 1% BSA and 0.1% collagenase type IA in a water bath for 60 mins or more until the solution appeared clear. The solution was then filtered using cell strainer (100µm) to remove undigested tissue. The filtrate was centrifuged at 1800 RPM for 5 mins. The pellet was resuspended in the same volume of RBC lysis buffer (160 mM/l NH_4Cl in PBS) and incubated for 20 mins at room temperature. The solution was again centrifuged at 1800 RPM for 5 mins. After centrifugation, the supernatant was discarded and the ADSC pellet was resuspended in 4ml complete media (DMEM containing 4.5 g/ml glucose, L-glutamine, sodium pyruvate) with 10% FBS, 1% antibiotic antimycotic solution. Cells were seeded in T25 culture flasks. 3ml of complete medium was added to the flasks. The flasks were incubated in CO_2 incubator at 5% CO_2, at 37^{o}C; for 24 to 48 hours. After 48 hours, 1ml of complete media was added to each flask.

The change of medium was carried out at the third day, but only half of the medium was replaced. After 3–5 days full medium change was performed. Next medium changes took place twice a week. The passaging was performed using 0.25% trypsin-EDTA solution after confluent growth was seen.

3. Adipogenic Differentiation of ADSCs:

Prior to induction, cultured ADSCs were harvested and plated into the desired tissue culture dish, to attain approximately 80% confluency. The cells were cultured in 2.5ml AdipoXL basal medium (HiMedia) specific for adipogenic differentiation. Cells were cultured for a minimum of 14 days with appropriate changes of the medium every 3-4 days. Highly adipogenic ADSCs populations may show signs of lipid vacuole formation as early as 7 days induction. ADSCs undergoing adipogenic differentiation will develop multiple lipid vacuoles that may be easily visualized under the light microscope. In addition, adipogenic differentiation was confirmed using an Oil Red 'O' staining. Cells were observed under (10X) inverted microscope. Immunophenotyping of ADSCs by Flow Cytometry (FACS Calibur, BD BioScience), using PE tagged CD105 antibody.

Bioinformatics based study for regulation of adipogenesis was carried out using LifeMap Discovery™ database (http://discovery.LifeMapsc.com/).

Open the Embryonic Development page.

i. From the drop down select the Organs/Tissues.
ii. Once you are on the page in the search box type Adipose.
iii. Once you track the adipose tissue, click on the tissue card to open the details.
iv. From the options given on the page select the hyperlink to Signals.
v. Developmental signals related to the organ/tissue are listed with their relevant outcomes, developmental paths and associated cells are given in a tabular format on the page. Signals are listed according to their order of development from the first ancestor(s).
vi. Select the appropriate signalling pathways responsible for differentiation and study the pathway accordingly using the links given along.
vii. Analyse and record the key genes responsible for differentiation of the MSCs to adipocytes.
viii. Retrieve the structure of proteins encoded by those genes using the available databases like NCBI or PDB or MMDB.
ix. Once the structure is retrieved try to find out the ligands that can be used as potential anti obesity drugs.
x. Observations and conclusions were recorded.

III. Result and discussion:

Enzyme digestion and in-vitro lipoaspirate production both proved to be efficient methods for isolation of stem cells.

Cells were isolated using two different methods: enzymatic digestion and by PC & DC treatment**.** In enzyme digestion 0.2% Collagenase type IA was used. Collagenase is unique among proteases in its ability to degrade the triplehelical native collagen fibrils commonly found in connective tissue. Enzyme digestion produced a clear solution, from which ADSCs were isolated after further processing. PC & DC treatment of adipose tissue led to formation of three different layers similar to the layers formed in standard lipoaspirate production protocol (Patricia A. Zuk et al., 2002)

1. Morphology of adipose-derived cells:

A majority of cells exhibited multiple extensions from a relatively round cell body, whereas a small number of cells showed a flat and extended cell body with shorter and less obvious extensions.

Fig.4: Observation of ADSCs under (10X) after 14 days.

Fig.5: Observation of ADSCs under (10X) after 28 days.

2. Microscopic observations of stained adipocytes:

ADSCs were differentiated to adipocytes using AdipoXL (HiMedia) basal media. The differentiation of cells was confirmed by oil red staining.

The differentiation was seen after 8-9 days of induction. The differentiated cells appeared bigger and round in shape and showed accumulation of multiple lipid vacuoles that appeared red on oil red staining may be easily visualized under the light microscope.

The differentiation of stem cells into adipocytes confirmed the presence of stem cells in the culture.

Fig.6: Observation of stained Adipocytes under (40X)

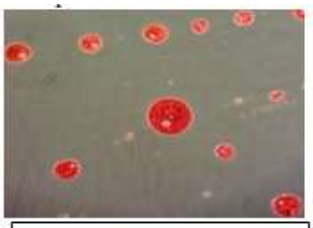
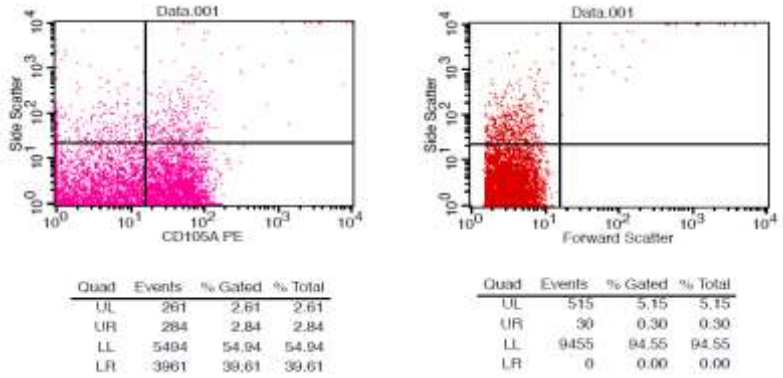


Fig.7: Observation of stained Adipocytes under (10X)

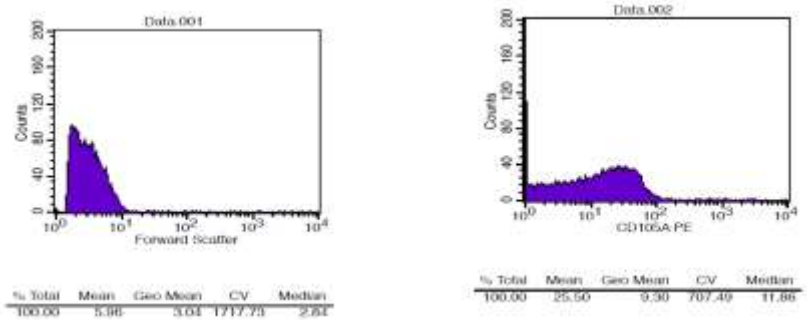
3. Characterisation of ADSCs by FACS analysis:



Quad	Events	% Gated	% Total
UL	261	2.61	2.61
UR	284	2.84	2.84
LL	5494	54.94	54.94
LR	3961	39.61	39.61

Quad	Events	% Gated	% Total
UL	515	5.15	5.15
UR	30	0.30	0.30
LL	9455	94.55	94.55
LR	0	0.00	0.00

Fig.8: Dot-plot of labelled sample (PE tagged CD105) and control sample resp.



% Total	Mean	Geo Mean	CV	Median
100.00	5.06	3.04	1717.73	2.64

% Total	Mean	Geo Mean	CV	Median
100.00	25.50	0.30	707.49	11.86

Fig.9: Histogram obtained from the FACS analysis of PE tagged CD105 labelled sample and control sample

ADSCs were characterized by Flow Cytometry using PE tagged Mouse Anti-human CD105 antibody. The major shift in the plot in comparison to control, after gating, confirmed the presence of stem cells. CD105 is a specific surface marker expressed by Adipose derived mesenchymal stem cells. Hence, the presence of CD105 expressing cells confirmed the presence of stem cells.

4. Study of crucial pathways responsible for stem cell differentiation into adipocytes:

The crucial pathways responsible in differentiation were studied using the LifeMap Discovery database.

After exploring the database and studying the given signalling pathways, it was seen that two pathways play the major role in differentiation of MSCs into adipocytes. The first pathway was, Insulin pathway which induces the differentiation of MSCs to adipocytes whereas, Wnt pathway inhibits the adipogenesis.

Thus, by inhibiting the insulin pathway and inducing the Wnt pathway the differentiation of adipocytes can be directed to formation of muscle cells, than the adipocytes; thus, reducing the obesity and enhancing muscle formation.

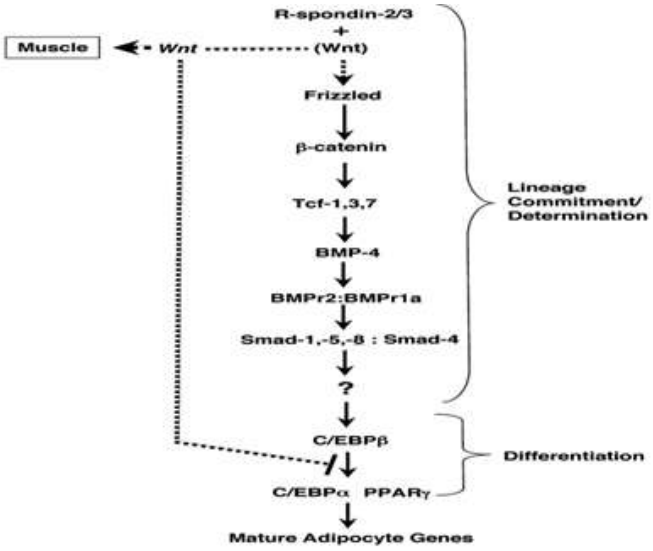


Fig.10: Image showing the key genes responsible for differentiation of ADSCs

The key proteins regulating the differentiation were found to be the **PPARG** (peroxisome proliferator-activated receptor gamma) and **C/EBPα.** (CCAAT/enhancer binding protein alpha)

C/EBPα is a pleiotropic transcriptional activator of adipocyte-specific genes. Promoters from numerous adipocyte genes contain C/EBP regulatory consensus sequences and are trans-activated by C/EBPα.

Forced expression of this isoform in 3T3-L1 preadipocytes stimulates adipogenesis in the absence of any hormonal induction. In addition, blocking its expression wit antisense C/EBPα RNA inhibits adipogenesis. Furthermore, C/EBPα(−/−) mice have defects in lipid accumulation. These observations establish the requirement for C/EBPα in preadipocyte differentiation. (Tamara C. Otto and M. Daniel Lane, 2014)

PPARγ is a central regulator during adipogenesis. The transcription factor is induced during differentiation and is responsible for activating a number of genes involved in fatty acid binding, uptake and storage, including 422/aP2, lipoprotein lipase, acyl coenzyme A synthase and phosphoenol pyruvate carboxykinase. The importance of PPARγ in adipogenesis has been demonstrated in several ways. Over-expression of PPARγ in non adipogenic fibroblasts stimulates adipogenesis. PPARγ (−/−) mice die in utero secondary to a placental defect, but those that survive to term through tetraploid rescue are deficient in brown fat. PPARγ is transcriptionally activated by C/EBPβ and C/EBPδ, and once expressed, PPARγ and C/EBPα positively regulate each other's expression. (Tamara C. Otto and M. Daniel Lane, 2014)

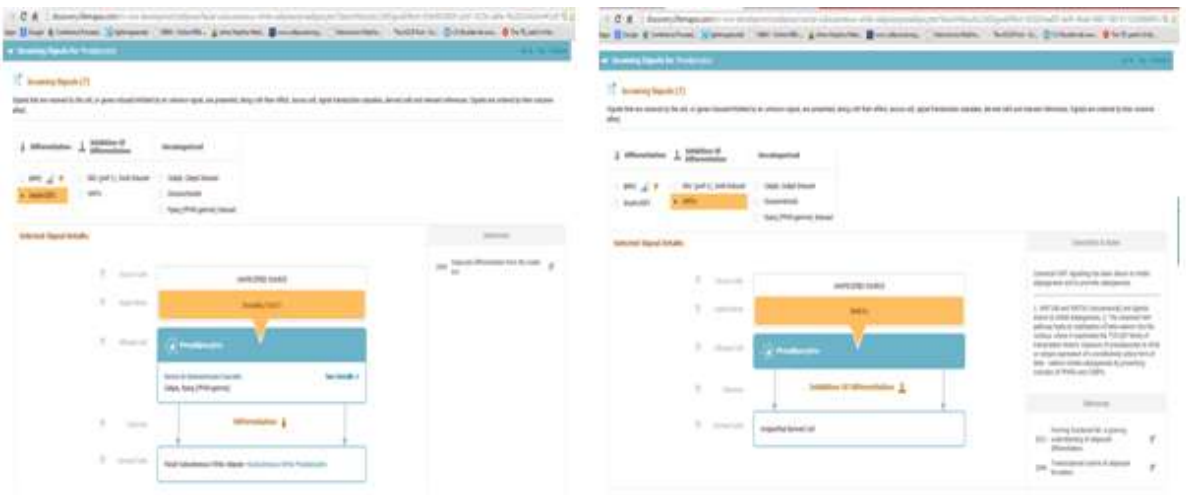


Fig.11: Image showing the role of Insulin pathway in adipocytes differentiation and the key genes involved in it.

Fig.12: Image showing the role of Wnt pathway in adipocytes' differentiation.

Proteins encoded by these two genes hence can be possible target to control the differentiation of MSCs to adipocytes.

The structure of the key proteins PPARG and C/EBPα was retrieved from MMDB and PDB respectively.

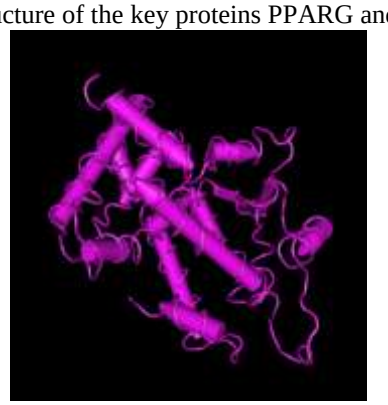


Fig.13: Image showing the structure of PPARG, retrieved from MMDB (MMDB ID-66390)

Fig.14: Image showing the structure of C/EBPα , retrieved from PDB.

The anti-bodies or ligands specific to these proteins or RNAi technology can be used as potential treatment for obesity.

As Obesity is a major health problem resulting from increases in adipocyte size and number, blocking the differentiation of the MSCs into adipocytes can serve to be an applicable treatment for obesity and also to avoid obesity in future.

IV. Conclusion:

Lipoaspirate is sometimes not available or cannot be obtained for isolation of stem cells. In some surgical cases like caesarian section, abdominal surgeries, etc. whole fat tissue is extracted. This tissue is discarded as medical waste and in such cases this discarded whole tissue can be used for stem cell isolation. In contrast to the conventional method that makes use of lipoaspirate, the use of whole adipose tissue can prove to be useful. With our present method we can preserve and banking adult stem cell also from adult person's future use which can help them to treat Diabetes and other diseases by using a very tiny amount of adipose tissue.

The key proteins involved in stem cell differentiation were identified using bioinformatics tools and biological databases, LifeMap Discovery^{TM} database and KEGG pathway database. The key proteins playing a crucial role in differentiation were identified to be PPARG and C/EBPα.

Thus, by blocking these proteins, it would be possible to control the adipogenesis of stem cells. The anti-bodies or ligands raised against these proteins can be used as potential drugs for treating obesity and may also help in avoiding obesity in future. Also, RNAi technology can be used for blocking the translation of the genes coding these proteins.

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