Stem Cell Reports, Volume *2*

Supplemental Information

Identification of Specific Cell-Surface Markers of Adipose-Derived Stem Cells from Subcutaneous and Visceral Fat Depots

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Inventory of Supplemental Information

- Figure S1: Preceeding experiments done before high content screening (HCS) experiments in Figure 1
- Figure S2: This is the additional 2nd stage analysis for Figure 2
- Figure S3: A is investigation of different species (mouse) from human subjects in Figure 3; B and C are complementary to Figure 3
- Figure S4: A is complementary to Figure 4A. B and C are complementary to Figure 4C and 4D, respectively. D and E are also complementary to Figure 4C and 4D, respectively.
- Table S1: Donor information
- Table S2: Sequence of qPCR primers, related to Figure 2, Figure 4 and Figure S3
- Table S3: Excel spreadsheet describing signal intensities estimated by raw fluorescence intensity values (AU) or by manual assessment through visual inspection.
- Table S4: Excel spreadsheet showing detailed selection / exclusion criteria results for each cell surface marker.
- Stage Selection Criteria and Statistical Consideration: This explains details of the 3 stage selection criteria, method to generate the null distribution for computing p-value, and statistical result for the 3-stage approach.

SUPPLEMENTAL INFORMATION

Supplemental Experimental Procedures

Isolation and culture of ASCs

WAT was isolated from the subcutaneous (abdominal region) and visceral (omental region) depots from a cohort of 12 human obese volunteers (S1 to S12), with body mass index (BMI) of more than 30, undergoing bariatric surgery (Table S1). This was done with informed consent from all subjects and approved by the NHG Domain Specific Review Board (DSRB) at National Healthcare Group, Singapore. ASCs were then isolated from the WAT by collagenase digestion and cultured, up to passage 10, in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum, non-essential amino acid, penicillin streptomycin and 5 ng/ml basic human fibroblast growth factor (bFGF). All cells were maintained at 37°C in a 5% CO_2 humidified incubator. The medium was changed every 2-3 days and passaged at 80% confluency. ASCs were checked for their multipotent capacity by osteogenesis (STEMPRO Osteogenesis Differentiation Kit) and chondrogenesis (STEMPRO Chondrogenesis Differentiation Kit) assays, followed by Alizarin Red S and Alcian Blue stainings, respectively, in addition to adipogenesis described below.

Adipogenesis and Oil Red O staining

Cells were seeded in 12- or 24-well plates and adipogenesis was induced on D_0, 2 days after the cells reaching confluence state, with adipogenic cocktail containing 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX) and 167 nM insulin plus 100 μM indomethacin in the absence of bFGF. On D_4, cells were switched to medium with 167 nM insulin and maintained till at least D_{12}. The cells were then fixed in 3.7% formaldehyde in PBS for 1 h, washed with 60% isopropanol, air-dried and stained with Oil Red O solution (in 60% isopropanol) for 1 h followed by repeated washing with water. Stained cells were

imaged on Nikon TS100 microscopes. After air-dried, stained lipids were extracted by using isopropanol for lipid content measurements. The absorbance of extracted solution was measured at 500 nm wavelength.

High content screening

High content screening assay of 242 human cell surface markers using BD Lyoplate Human Cell Surface Marker Screening Panel (BD Biosciences) was performed on SC- and VS-ASCs from S1, commercial SC-ASC (Lonza PT-5006), BM-MSC (Lonza PT-2501) and HFF-1 (ATCC SCRC-1041) according to the manufacturer's manual. The immunofluorescence cell images of four regions per well were acquired using automated fluorescence microscope system (ImageXpress Micro, Molecular Device). Fluorescence intensity was analyzed by using MetaXpress cellular image analysis software and visual confirmation. Marker candidates were selected based on combination of two methods. First, signal intensities estimated by the software are considered. In addition, manual visual identification was performed for those whose signal intensities are too weak to give significant values due to different efficiencies of staining by individual antibodies.

Mouse studies

39 week old male C57BL6/J mice were fed either with normal chow or high fat diet for 27 weeks (5 mice each). Inguinal (subcutaneous), epididymal and mesenteric fat depots were harvested, and ASCs were isolated as described above. mRNA was isolated from the cultured ASCs, then used for the qPCR analysis. This animal work was approved by the Institutional Animal Care and Use Committee of Biological Resource Centre, Singapore.

Real time qPCR

Total RNA from the cultured cells was extracted using Trizol reagent (Invitrogen) and treated with DNase I to remove genomic DNA. cDNA conversion was performed using the RevertAid H minus first strand cDNA synthesis kit (Fermentas, USA) with oligo d(T) 18 primer according to manufacturer's instructions. For qPCR, cDNA samples were analysed in triplicates using the SYBR® Green PCR Master Mix reagent kit (Applied Biosystems) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using the primer pairs shown in Table S2. Relative mRNA levels were calculated and normalized to that of *Gapdh*.

Immunoblot analysis

Cell lysates were prepared Nonidet-P40 (NP-40) buffer containing protease inhibitors. Protein quantifications were performed using the Bradford Protein Assay (BioRad). Equal amounts of protein (20 μg) were loaded into 10% SDS-PAGE mini gels and blotted onto a nitrocellulose membrane by using the iBlot Dry Blotting System (Invitrogen). Membranes were blocked in 3% BSA for 1 h at room temperature. They were then probed with primary antibodies overnight at 4°C, followed by HRP-conjugated secondary antibodies for 1 h at room temperature. Primary antibodies used were anti-CD10 (Leica Microsystmes CD10-270-L-CE) and anti-β-Actin (Santa Cruz).

Flow cytometry and cell sorting

For immunophenotypic characterization, ASCs (passage number > 2) were tripsinized and a total of 2 x 10^5 cells were suspended in 0.2 ml staining buffer (DMEM without phenol red with 2% FBS) for immunostaining. The cells were incubated for 30 min on ice, either directly with a fluorochrome-conjugated antibody or with an unconjugated primary antibody followed by an appropriate secondary antibody conjugated with fluorochrome. Antibodies against

CD10, CD73, CD90, CD105 and CD200 were of the same clones as those provided in the BD Lyoplate (BD Biosciences, USA). The stained cells were washed and suspended in sorting buffer (PBS with 0.5% BSA and 2 mM EDTA) before analysis by flow cytometry (LSRII, BD Biosciences). Cells for sorting were processed in a similar manner before sorted by Moflo XDP Cell Sorter (Beckman Coulter).

Statistical analysis

Results are presented as means ± SEM. The Student's *t*-test or Wilcoxon signed-rank test was used where appropriate to determine differences in the mean or median between two groups. A p-value < 0.05 was considered significant. Statistical consideration for the 3-stage approach is described in Supplemental Information.

Supplemental Figures

Figure S1

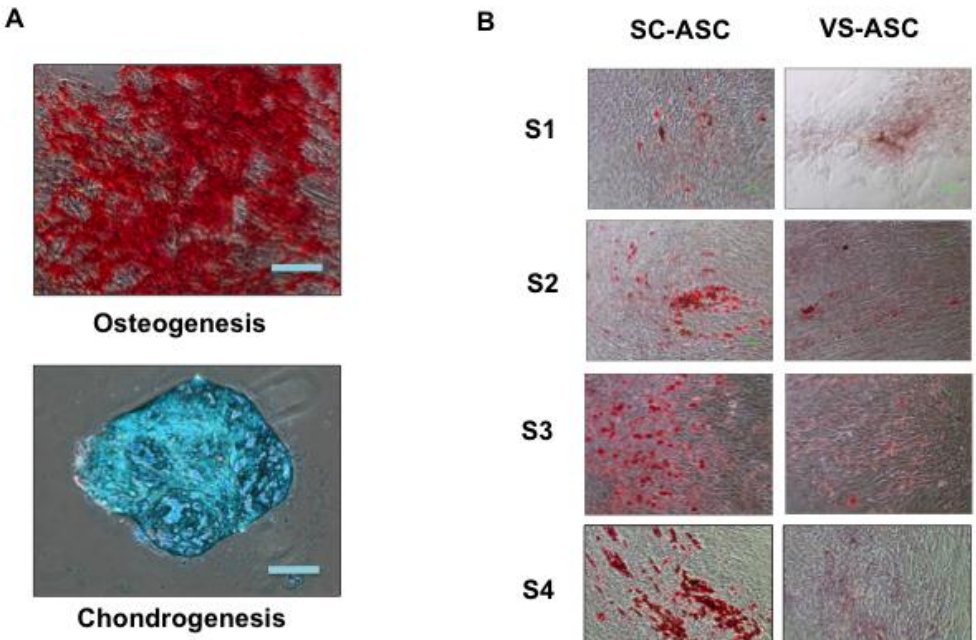


Figure S2

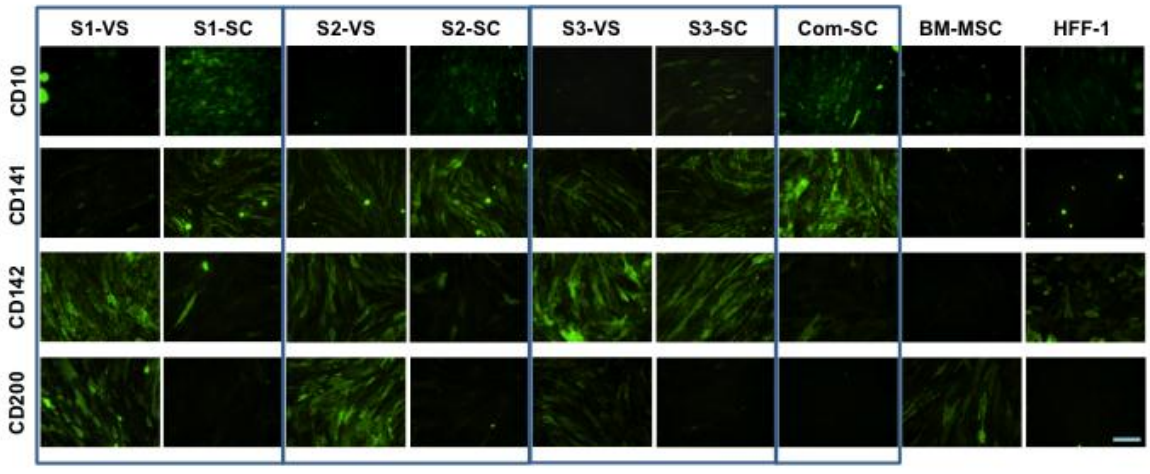


Figure S3

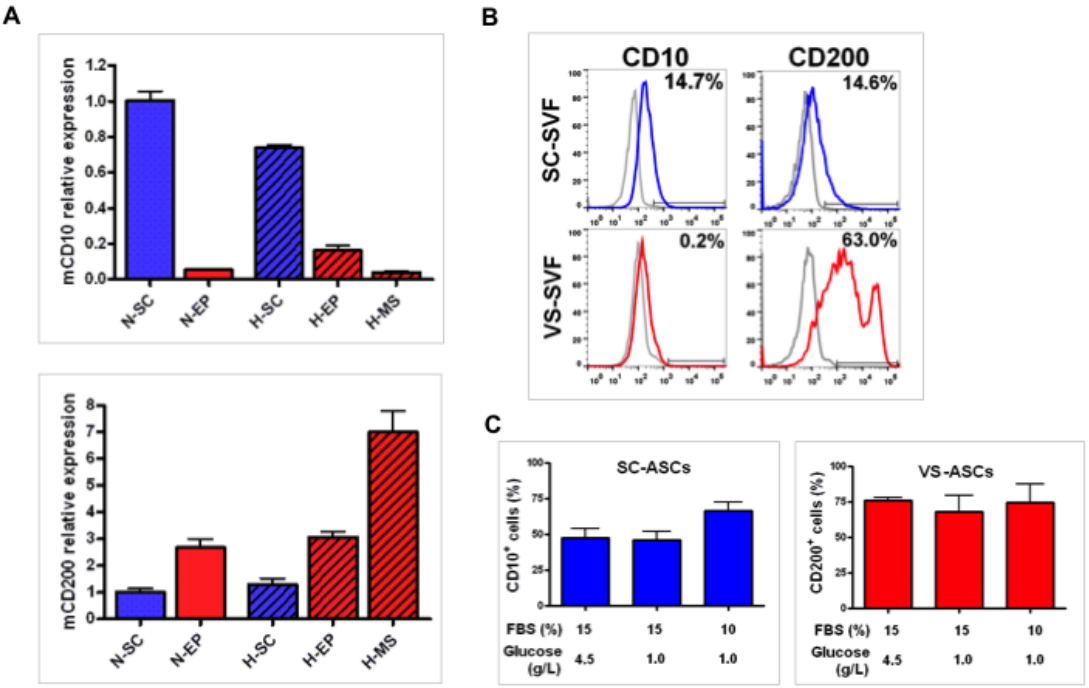
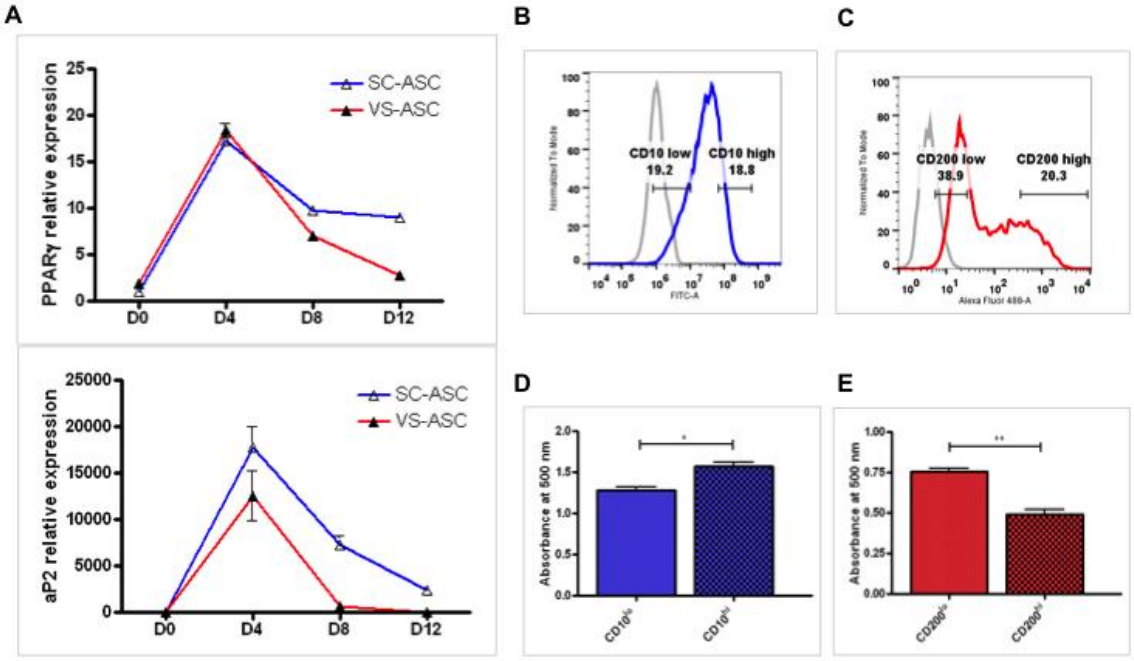


Figure S4



Supplemental Figure Legends

Figure S1: Multipotency of cultured ASCs. (A) Osteogenesis and chondrogenesis assays of isolated ASCs. Photos are shown by Alizarin Red S (osteoblast dye) and Alcian Blue (chondrocyte dye) staining of ASCs. (B) Representative images showing higher adipogenic potential of SC-ASCs in comparison with respective VS-ASCs isolated from 4 subjects. ASCs were subjected to standard adipogenic stimuli, and stained with Oil Red O. Scale bar = 200 μm.

Figure S2: Fluorescence image montage highlighting the consistent depot-dependent expression of CD10 and CD200 across S1 to S3 in the follow-up high content screening, which is part of stage 2 analyses together with those shown in Figure 2. In contrast, other cell surface markers represented by CD141 and CD142 were not consistent across individuals.

Figure S3: (A) The relative expression level of mouse *Cd10* and *Cd200* by qPCR between SC-ASCs from inguinal region (expression level defined as 1) and VS-ASCs from epididymal (EP) and mesenteric (MS) regions. This is the additional investigation to Figure 3. These ASCs were isolated from two pools of five mice, fed with normal chow (N) or high-fed diet (H). Each value is the mean ± SEM from three technical replicates from a qPCR reaction. (B) Histograms showing flow cytometry analysis of SC- and VS-SVF populations from S12 after 6 days in culture (passage number=0), related to Figure 3. (C) Effects of different FBS and glucose concentrations on expression of CD10 and CD200. SC- or VS-ASCs from S9 were passaged and cultured for 10 days in medium with lower glucose (1.0g/L) or FBS (10%) concentrations than used in Figure 3 (4.5g/L glucose and 15% FBS). Percentage of $CD10^+$ SC-ASCs and $CD200^+$ VS-ASCs in each culture condition was

assessed by flow cytometry analyses. Each value is the mean ± SEM from two separate wells of cells cultured under the same specified condition.

Figure S4: (A) mRNA expression level of adipogenic markers (*PPARG* and *aP2*) by qPCR during the course of adipogenesis, related to Figure 4A. Adipogenesis of ASCs were induced with the standard adipogenic cocktail including indomethacin for D_0-D_4, followed by maintenance medium with insulin alone (D_4-D_{12}). The expression level of *PPARG* and *aP2* positively correlated with *CD10* expression but negatively correlated with that of *CD200*. Each value is the mean ± SEM from three technical replicates from a representative qPCR reaction. (B) Cell sorting of SC-ASCs into $CD10^{hi}$ and $CD10^{lo}$ populations (data represented by S4), related to Figure 4C, and (C) Sorting of VS-ASCs into $CD200^{hi}$ and $CD200^{lo}$ populations (represented by S2), related to Figure 4D. Relative cell count is indicated in Y-axis and fluorescence intensity is indicated in X-axis, grey line represents "unstained control" and the percentage of positively-stained cells is as indicated. (D) Similar to Figure 4C, $CD10^{hi}$ cells sorted from S6 SC-ASCs differentiated better than their $CD10^{lo}$ counterparts. (E) Similar to Figure 4D, $CD200^{lo}$ cells sorted from S6 VS-ASCs differentiated better than the $CD200^{hi}$ counterparts. Each value is the mean ± SEM from three independent wells from one cell preparation. Statistical significance was assessed by using Student's t-test: $**p < 0.01$, $*p < 0.05$.

Table S1: Donor information and relative adipogenic potential of isolated SC- and VS-ASCs from the donors.

Subject	Age	Sex	Ethnic Group	SC-ASC Adipogenic Potential	VS-ASC Adipogenic Potential
S1	43	F	Malay	±	- -
S2	36	M	Malay	+	±
S3	54	F	Indian	+ +	±
S4	51	F	Malay	+ +	- -
S5	53	M	Malay	±	- -
S6	35	F	Malay	+ +	±
S7	51	F	Malay	+	-
S8	39	F	Chinese	+ +	-
S9	26	F	Malay	+ +	-
S10	34	F	Chinese	±	- -
S11	37	M	Chinese	+ +	+
S12	25	F	Chinese	+ +	±

Adipogenic potential scoring: very good (++), good (+), moderate (±), poor (-) and very poor (- -).

Table S2: qPCR primer sequences, related to Figure 2, Figure 4 and Figure S3.

Gene	**Forward**	**Reverse**
hCD10	TTGTTGGCACTGATGATAAGAATTC	TCCAGTGCATTCATAGTAATCTCTAGAAG
hCD200	TGTGACCGACTTTAAGCAAACCGTC	TTAGGGCTCTCGGTCCTGATTCC
mCd10	GAACTTTGCCCAGGTGTGGT	GCGGCAATGAAAGGCATCTG
mCd200	GAGCACAGCTCAAGTGGAAGT	GTTTTCTGGGCTCACGGCTT
hPPARG	GACAGGAAAGACAACAGACAAATC	GGGGTGATGTGTTTGAACTTG
haP2	CCTTTAAAAATACTGAGATTTCCTTCA	GGACACCCCCATCTAAGGTT
hGAPDH/ mGapdh	CAAGGTCATCCATGACAACTTTG	GGCCATCCACAGTCTTCTGG

Stage Selection Criteria and Statistical Consideration

Details of the 3 stage selection criteria

1st stage: High throughput screening of 242 markers of 5 cell lines (including two SC-ASC and one VS-ASC)

Criterion: Comparing signal intensities among samples that specifically expressed in either depot ASC, but not in other cells

a. Both S1 and Com in SC groups must have the largest two intensity values (numerically or visually) among the 5 cell lines, or
b. VS (S1) has the largest intensity value (numerically or visually) among the 5 cell lines.

[Note: Only 58 markers had valid readings. Comparison was done numerically or visually. Each marker had one experiment.]

2nd stage: Immunofluorescence analysis to show depot specificity across 3 subjects.

Criterion: 1 analysis (or experiment) to compare brightness of VS vs ASC

a. VS must be brighter than SC (or vice versa) across all 3 samples.

[Note: Comparison was done visually. Each marker had one experiment or one analysis.]

3rd stage: Analysis of depot specificity in human subjects with mRNA expression through qPCR (n = 6 subjects) and protein expression through flow cytometry (n = 12 subjects)

Criterion: Two experiments to compare brightness of VS vs SC.

a. The p-values from the two experiments must be <0.05 for all two experiments.

[Note: Wilcoxon signed-rank test used for mRNA, and T-test used for cytometry. Each marker had two experiments resulting in two p-values.]

Method to generate the null distribution for computing p-value

We generated the null distribution of the number of identified markers from the three stage approach by assuming that all 242 markers do not contain any biological signal. To generate the number of markers with valid fluorescence readings (n_0), we assume each marker independently has a probability of 58/242 in having valid readings. For the first stage, we randomly assigned the rankings of the intensity values to the five cell lines for each of the n_0 markers. We identified the number of markers that satisfy the selection criterion of the first stage (n_1). For the second stage, we randomly assigned the rankings of brightness to the 6 samples (3 VS and 3 SC) for each of the n_1 markers. We identified the number of markers that satisfy the selection criterion of the second stage (n_2). For the third stage, we generated p-values from a uniform distribution (i.e. null scenario) for mRNA (qPCR) and protein (cytometry) expression experiments for each of the n_2 markers. We identified the number of markers that satisfy the selection criterion of the third stage (n_3). We simulated the null scenario 10000 times to compute the p-value (i.e. the number of null scenarios with 2 or more markers identified at the third stage divided by 10000).

Result for the 3-stage approach

The p-value is <0.001 given that two markers were identified from 242 markers from the three stage approach.