

Secretome proteins regulate comparative osteogenic and adipogenic potential in bone marrow and dental stem cells

Ajay Kumar^{1,5}, Vinod Kumar², Vidya Rattan³, Vivekananda Jha^{2,4}, Shalmoli Bhattacharyya^{1*}

1. Department of Biophysics, PGIMER, Chandigarh, India
2. Department of Nephrology, PGIMER, Chandigarh, India
3. Unit of Oral and Maxillofacial surgery, Oral health science centre, PGIMER, Chandigarh, India
4. The George Institute for Global Health, India.
5. Department of Ophthalmology, University of Pittsburgh, USA.

*Correspondence address:

Dr. Shalmoli Bhattacharyya, PhD, FISBT, MAMS, Professor,
Department of Biophysics, PGIMER, Chandigarh, 160012, India,
Tel: 91-9876186816, Fax: 91-172-2744401, E-mail: shalmoli2007@yahoo.co.in

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Abstract

Introduction: Dental stem cells (DMSC) have been studied extensively since their early discovery. However, the data regarding osteogenic potential of DMSC with other cell types is sparse and the secretome proteins underlying these differences have not been explored. In this study, we have compared the osteogenic and adipogenic potential of DMSC with Bone Marrow Stem cells (BMSC) and reported the contribution of secretome proteins in controlling their differentiation.

Methods: Osteogenic potential of these stem cells was compared by mineralization assay, alkaline phosphatase (ALP) assay, immunofluorescence of dentine sialo phosphoprotein (DSPP) & qPCR for osteogenic genes. Adipogenic potential was compared by Oil Red O staining and qPCR for PPAR- γ , leptin & adiponin. Proteomic analysis of secretome was performed by employing WATERS nano Lc-MS/MS system.

Results: We observed a higher osteogenic potential in DMSC, especially dental pulp stem cells (DPSC) as compared to BMSC population but adipogenic potential was found to be better in BMSC as compared to DMSC. Deeper investigations into secretome of these cells by Lc-MS/MS revealed the presence of proteins pertaining to osteogenic and adipogenic lineage. Presence of some important proteins regulating osteogenic (DSPP, BMP7, DDR2, USP9X) and adipogenic differentiation (NCOA2, PEG10, LPA) in secretome of BMSC and DMSC reflected the role of paracrine factors during differentiation.

Conclusion: Our study provides first evidence regarding regulation of osteogenic/adipogenic potential by secretome proteins in DMSC and BMSC. DMSC especially **DPSC and its secretome show an inherent tendency for higher osteogenic differentiation and lower adipogenic differentiation, these may be potential candidates for effective future therapy in osteoporosis where disturbance of osteocyte/adipocyte homeostasis is reported.**

1. Introduction:

The main aim of regenerative medicine is the repair of diseased organs due to aging, trauma or any congenital defects and to generate biocompatible organ replacement in future. Dental tissue has emerged as a potential source of adult stem cells which can be useful for treatment of organ specific debilitating diseases. It is now well established that stem cells show preference in their differentiation for a particular lineage depending on the tissue from which they originate. For example, adipose derived stem cells (ADSC) has poor tendency for differentiation into osteocytes as compared to BMSC which has been nicely demonstrated in a recent study which involved donor matched comparison of ADSC and BMSC (1, 2). Similarly, BMSC were found to have a higher osteogenic and adipogenic tendency than peripheral blood stem cells (PB-MSC), Umbilical cord stem cells (UCMSC), placenta derived stem cell (PDSC) and periodontal ligament stem cells (PDLSCs) (3-6). In terms of osteogenic potential, a hierarchical pattern was observed where fetal MSC showed higher osteogenic potential than adult BMSC which in turn showed more osteogenic potential than liver stem cells (7). DMSC have been demonstrated to differentiate into neurons, cementoblasts, osteocytes (8), and hepatocytes (9) etc. thus generating cell lineages of all three germ layers. However, very limited information is available about the detailed osteogenic and adipogenic comparison of DMSC and BMSC. By comparing the differentiation potential of DMSC versus BMSC into osteocytes, we may be able to evaluate therapeutic advantage of one stem cell type over to be used for different bone related disorders. The balance between the adipose and osteogenic differentiation maintains an important homeostasis between bone and adipose tissue turnover. The loss of this balance result into osteoporosis which results in brittle and weak bones which are prone to breakage. The gradual replacement of bone with adipose results in osteoporosis, common debilitating condition, so any approach which can increase the osteogenic differentiation and decrease adipogenic differentiation may emerge as a potential treatment for osteoporosis (10, 11).

Furthermore, lineage switches can be changed by minor alterations in the niche/microenvironment by mechanical or chemical means (12). The paracrine factors secreted by particular type of MSCs can influence the differentiation towards a particular lineage. The entire spectrum of trophic growth factors and cytokines secreted by a cell is collectively known as secretome. The stem cell secretome contain extracellular vesicles (exosomes, macrovesicles)

as well as soluble growth factors and small peptides (13-16). There has been a plethora of studies regarding improved regeneration by application of secretome for various disease conditions emphasizing its emerging importance in the field of regenerative medicine (17). There are a few studies reporting the secretome obtained from BMSC, DPSC, SCAP and DFSC in context of protein profiling and regeneration induced in disease models (18-21). The proteins present in secretome also include important differentiation factors which can enhance lineage differentiation of stem cells by different biological mechanism (22, 23). However, its role in context of osteogenic and adipogenic differentiation has not been explored much. Further investigations into secretome of MSCs from different origin may pave a way towards better understanding and applications of stem cell based and stem “cell free” therapy in regenerative medicine.

2. Material & Methods:

2.1. Primary culture: DMSC culture was established from dental tissues extracted from patients visiting to our tertiary care hospital as described previously (22, 24, 25) and BMSC were used from already available cultures in stem cell facility of the institute. At least 3-5 patient samples for each cell type were used for the study with written informed consent. All ethical guidelines were followed for sample collection as per Helsinki declaration and study was approved by Institutional Committee on Stem Cell Research and Institutional Ethics Committee (IEC No. 9195/PG-12 ITRG/2571-72). The following criteria were used to recruit patients in the study.

Inclusion criteria

1. Derivation of dental tissue coming to PGIMER OPD for orthodontic treatment.
2. Age between 6 to 25 years.
3. Healthy, non-decayed tooth

Exclusion criteria

1. Decayed or tooth with any disease like dental caries, fluorosis etc.
2. Patient age >25 years.

The tissue samples obtained from the consenting donors were transported to tissue culture laboratory and dental stem cell isolation was done by explant culture method. BMSCs were obtained from already established cultures in stem cell facility of the institute. No separate extraction was performed for this study for BMSCs. Cells were cultured in minimal essential

media (MEM α) supplemented with non-essential amino acids, L-glutamine, 10% foetal bovine serum (US origin, Sigma Aldrich) and antibiotics penicillin and streptomycin (Himedia) in a humidified CO₂ (5%) incubator maintained at 37°C. The cell lines were designated based on their anatomical location in the dental tissue, cells obtained from dental pulp were DPSC (Dental Pulp Stem Cells), those obtained from apical papilla were SCAP (Stem Cells from Apical Papilla) and tooth follicle derived stem cells were DFSC (Dental Follicle Stem Cells).

2.2. Flow cytometry: Flow cytometry (BD FACS Aria) was used to assess the expression of stem cell surface markers. Briefly, cells were trypsinised upon reaching 70-80% confluence using 0.05% trypsin-EDTA solution (Sigma Aldrich). Cells were centrifuged for repeated washing with phosphate buffered saline (PBS) at 2000 rpm for 5 minutes. After washing, nonspecific antibody binding sites were blocked with 1% bovine serum albumin (BSA) treatment for 30 minutes. Cells were then incubated with different antibodies for 30 minutes in dark. Antibodies used for the study included CD90-FITC, CD73-FITC/PE, CD105-FITC, CD34-FITC and CD45-PE (BD Bioscience, San Jose, CA). Minimum 5×10^3 events were acquired for each sample in triplicates. FACSDIVA and FloJo software were used for further analysis.

2.3. Spheroid/colony formation efficiency: Both BMSCs and DMSCs were seeded in six well plate as 1×10^3 cells per well using adherent plates for colony formation and 1×10^3 cells were also seeded in ultra-low attachment plates for spheroid formation for seven days. After completion of seven days, colonies were stained with crystal violet (Himedia) and photographed under microscope (Nikon Eclipse TS200). Temporal photography was done to assess spheroid forming potential of each cell type.

2.4. Multilineage differentiation: Cells were induced to differentiate into distinct lineages by providing the appropriate differentiation media as reported before (22, 24). Briefly, hepatic lineage was induced using induction media containing HGF (hepatocyte growth factor)/EGF (epidermal growth factor) (both 20ng/ml) (Invitrogen, Carlsbad, CA), dexamethasone (0.5mM) (Sigma Aldrich), and insulin-transferrin-selenous acid (ITS) (Gibco, Life Technologies, Gaithersburg, MD). Second media (maturation media) contained OSM (20ng/mL) (Sigma Aldrich), ITS premix (50mg/mL), and dexamethasone (0.5 μ M). The cells were culture 14 days each in induction and maturation media. Hepatogenic potential was assessed using functional low-density lipoprotein (LDL) detection kit from Abcam and staining was done following manufacturer's instructions. For neural differentiation cells were differentiated using

supplement B27 (1%), 20 ng/ml basic fibroblast growth factor, EGF (20ng/ml), N2 supplement (0.2%) and G5 supplement (0.5%) (Invitrogen, Carlsbad, CA) in neurobasal media for 30-41 days (22, 24-26). Neurofilament antibody (Sigma Aldrich, 1:100) was used to confirm the neural differentiation tendency of cells.

α -MEM containing 0.01mM dexamethasone, 1.8mM monopotassium phosphate (KH_2PO_4) (Sigma Aldrich) and 5mM β -glycerophosphate (Sigma Aldrich) for 21 days were used for **Osteogenic** differentiation. Mineralized nodules were visualized by alizarin red (Sigma Aldrich) staining for calcium granules. For quantitative analysis, alizarin was extracted from stained cells by addition of cetylpyridinium chloride (CPC) buffer (pH 7). **Adipogenic** differentiation, was done using α MEM containing 0.5mM isobutyl-methyl xanthine (IBMX) (Sigma Aldrich), 1 μ M dexamethasone, 10 μ M insulin, and 200 μ M indomethacin (Sigma Aldrich) (27) for 21 days. Post differentiation oil droplets were characterized using Oil Red O staining (Himedia). Stain was eluted by 100% isopropanol.

2.5. qPCR analysis: Trizol (Roche, Basel, Switzerland) was used to extract RNA which was then reverse transcribed to cDNA (Applied Biosystems, Foster city, CA). Human-specific primer sets for osteogenic and adipogenic genes were used to characterize particular differentiation. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin were used as internal control. StepOne plus 7700 PCR (Applied Biosystems, Foster city, CA) machine was used for real time PCR. Fold change was calculated after subtracting the Ct values housekeeping control from genes of interest using formula $2^{-\Delta\Delta\text{Ct}}$. The bar diagram represents the final fold gene expression change of each differentiated stem cells after subtraction from its undifferentiated counterpart.

2.6. Measurement of alkaline phosphatase (ALP) activity: ALP converts colourless p-nitro phenyl phosphate to yellow coloured p-nitro phenol by cleaving it's phosphate at pH 10.4 which can be read by spectrophotometer at 410nm wavelength (28). For detection of ALP activity, 5×10^5 cells were cultured per well of 96 well plate. Cells were induced to osteogenic differentiation as described above and added with AMP buffer for lysis post differentiation. Cell lysates were taken and measured for ALP activity by standard method. Briefly, 160 μ l of the AMP buffer was added per well of 96 well plate and mixed with 20 μ l of p-NPP (p-nitrophenoyl phosphate solution). Basal ALP activity was measured by reading absorbance at 405nm. Cell extract was then added (20 μ l) to this solution and again read at 405nm till 1 hour. Basal activity was then subtracted from this value to determine the final ALP activity in the control and

differentiated samples. Proper ALP Standards were taken into consideration. Total protein concentration for each sample was determined by Bradford assay and these values were normalized to calculate the final ALP activity.

2.7. Immunofluorescence staining: Cells were fixed in neutral buffered formalin for 30 minutes and permeabilized using 0.1% triton X for 15 minutes. The cells were then incubated with 1% BSA for 1 hour to block non-specific antibody binding sites and then incubated with mouse anti human DSPP primary antibody (1:100) (Sigma Aldrich) overnight at 4°C. Next day, cells were incubated with goat anti-mouse-FITC labelled secondary antibody (1:200) for 2 hrs. Fluorescent cells were examined under inverted microscope (Nikon Eclipse TS100) using 488nm excitation wavelength.

2.8. Secretome collection and protein isolation: All four cell types were grown in the above described culture conditions till 60-70% confluence. Cells were incubated for secretome in log phase of cell growth. For that, cells were given 4X PBS wash (5 minutes each). Cells were then incubated with only α MEM media lacking any additional FBS, growth supplements or antibiotics. Cells were incubated in culture for 48 hours. After that, conditioned media (secretome) from cells was immediately transferred to ice, centrifuged at 3000 rpm for five minutes to remove any cell debris and filtered through 0.2 μ syringe filter. Cells used for secretome harvesting were between 3rd-7th passages. Secretome was then concentrated using protein concentrator (Centricon, Millipore Sigma) and preserved at -80°C until further use. Protein isolation was done by phenol chloroform method. Secretome from at least three donors per cell type was pooled together and used for protein isolation. Protein concentration was determined by BCA method. Proteins samples from each secretome were subjected to one-dimensional SDS-PAGE to assess the complete profile of proteins.

2.9. LC-MS/MS profiling: LC-MS/MS analysis was performed only after a good profile was obtained from each stem cell type. Protein profiling was done using nano LC-MS/MS (WATERS). The samples (100 μ g) were digested using Trypsin In-Solution method. Samples were then dissolved in Formic Acid after proper drying. 1 μ l of sample was separated on the Nano Acquity BEH C18 column. Separated peptides obtained after nano LC were analysed using PLGS (Protein Lynx Global Server). Bioinformatics analysis was done to identify proteins using m/z ratio by mascot search in protein data bank. GO enrichment analysis was carried out

according to false discovery rates of different proteins and only significantly expressed proteins were taken into account. Proteins were classified according to osteogenic/adipogenic lineage specificity. Interaction was studied using STRING software (evidence [view](#)). Pathway analysis was done to observe the role of discovered proteins in various signalling mechanism.

2.10. Statistical analysis:

Data was reported as means \pm SD. Statistical analyses was done by employing SPSS statistical software. Data was analysed using one-way ANOVA and paired t test.

3. Results:

3.1. Primary culture of stem cells

Primary culture of the cells was done as reported previously (22, 24, 25). [Stem cells were derived from at least five patients for each cell type \(Table S1\)](#). Typical spindle shape morphology was observed in primary stem cells after few days of primary culture (**Figure 1a**). Characterization of these cells by flow cytometry indicated the positivity for Stemness markers (CD73, CD90 and CD105) while negativity for endothelial and hematopoietic markers, CD34 & CD45 respectively (**Figure 1b-c**). These cells were successfully induced for hepatic differentiation and showed uptake of LDL with positive expression of LDL receptor on their surface (**Figure 1d**). All four types of primary cells showed the tendency to differentiate into neural cells as shown by positive staining for neurofilament (**Figure 1d**).

Colony formation assay demonstrated that though all the stem cells were able to form colonies under appropriate conditions but the tendency of BMSC to form the colonies was found to be significantly lower than all three DMSC (**Figure 2a-b**). Spheroid formation assay showed almost equal tendency of all stem cells to form spheroids with no significant difference in spheroid forming tendency at day seven (**Figure 2c-d**).

3.2. DMSC displayed higher osteogenic potential as compared to BMSC

BMSC and DMSC were able to differentiate into osteocytes as observed by deposition of calcium granules and mineralized nodules in the differentiated cells. These calcium granules were positive for alizarin red indicating the osteogenic differentiation (**Figure-3a**). Quantitation of alizarin red stain by cetyl pyridinium chloride (CPC) buffer showed that DPSC showed significantly higher accumulation of calcium while BMSC, SCAP and DFSC showed similar tendency for mineralization (**Figure-3b**).

Dentine Sialo phosphoprotein (DSPP) is an important protein marker for mineralization and dentine formation. It is secreted uniquely by odontoblasts, the cells which form tooth dentine. DSPP mutations can result into defects in dentine/bone formation (29). Immunofluorescence analysis for DSPP showed positive staining for antibodies in differentiated DMSC while no positivity could be detected for corresponding BMSC (**Figure-4a-b**). **There was a significant increase in DSPP expression in all three DMSC as compared to BMSC after osteogenic differentiation.**

Alkaline phosphatase was firstly identified as a key player in mineralization and hard tissue formation (30). ALP has become a marker of choice for evaluation of phenotypic and developmental maturity of mineralized tissue as it increases the local accumulation of inorganic phosphate. Measurement of ALP activity revealed a significant increase in the ALP levels in all cells upon osteogenic differentiation as compared to their undifferentiated counterparts. It may be noted that baseline expression of ALP was almost similar in their undifferentiated state as shown in **Figure-4b**. DFSC showed significantly higher expression of ALP as compared to BMSC after differentiation while no significant difference in ALP activity was observed in other cell types (DPSC and SCAP).

Analysis of the expression of osteocalcein (OCN), and RUNX2 (RUNX family of transcription factors) in differentiated osteocytes was performed by qPCR (**primer details in supplementary table S2**). Differentiated DMSC showed a significant higher increase in expression of OCN as compared to BMSC with highest upregulation in DFSC as shown in **Figure-4c**. RUNX2 was observed to be highly upregulated in DPSC as compared to other cells (**Figure 4d**). Thus an overall analysis of osteogenic differentiation showed a higher tendency of DMSC towards osteogenic differentiation as compared to BMSC. Overall comparison of differentiation potential between four type of stem cells based on different parameters is given in supplementary **Figure S1**.

3.3. BMSC had higher adipogenic potential compared to DMSC

Analysis of adipogenic potential was done using oil red O staining and qPCR expression for genes adipisin, leptin & PPAR- γ . As shown in **Figure 5a**, all cells showed accumulation of lipid droplets after adipogenic differentiation which was confirmed by oil red O staining. Quantitation

of oil red O uptake by isopropanol extraction showed significantly higher adipogenic potential of BMSC as compared to DPSC (**Figure 5b**). **Analysis** of adipogenic differentiation by real time PCR analysis showed highest upregulation of Leptin and PPAR γ in differentiated BMSC as compared to DMSC (**Figure 5c-d**). However, DPSC and DFSC showed higher adipsin expression post differentiation as compared to differentiated BMSC (**Figure 5e**).

3.4. Secretome analysis identified the osteogenic and adipogenic lineage related proteins secreted by BMSC and DMSC

Proteomic analysis of secretome showed the presence of total twenty osteogenic lineage related proteins in secretome of BMSC and DMSC. Osteogenic lineage related proteins were found to be higher in number in DMSC secretome (SCAP-14, DPSC-7, DFSC-6) as compared to BMSC (5 proteins) secretome. The detailed description of proteins identified in secretome of these cells with reported function in development and differentiation of osteogenic lineages has been provided in **Table-1**. It may be worth to note that BMSC shared four osteogenic proteins with SCAP except BMPR1A. Amongst these, fibrillin 1 play important role during osteoblast maturation, ZNF423 activates transcription of bone morphogenic protein (BMP) target genes while DDR2 is involved in differentiation and development of bone osteocytes. However, DPSC secretome showed the secretion of important proteins involved in osteogenic differentiation like BMP7 (bone formation) and DSPP which play important role in osteogenic differentiation by facilitating mineralization. Important proteins regulating bone turnover (WISP2), mineralization (enamelin) and endochondral ossification (MINPP1) were found in DFSC secretome. The interactome analysis showed the presence of interaction between BMP7 and DSPP in DPSC secretome while SCAP secretome showed interaction among BMP7, FBN1 and ITGB3 (**Figure 6a**). The secretome proteins from BMSC and DMSC showed no interaction. Pathway analysis showed the presence of many curated pathways in SCAP secretome like elastic fibre formation (ITGB3/FBN1), cytokine-cytokine receptor interaction (INHBA/BMP7) etc. as shown in **Table 2**.

Proteomic analysis of adipogenic lineage related proteins in secretome of BMSC and DMSC identified total ten proteins in all four types of cells. BMSC secreted four such proteins, DPSC/DFSC secreted five each while SCAP was found to secrete only one protein (**supplementary Table S3**). NCOA2 was secreted commonly by all cell types which is required

for energy balance in adipose tissue. PEG10 which is involved in immediate early stage of adipocyte differentiation was secreted by only BMSC and DFSC while absent in DPSC and SCAP. Interactome analysis showed no interaction between the secretome proteins related to adipogenic lineage except NCOA2 and ACSL1 in DPSC secretome (**Figure 6b**). Pathway analysis for these cells showed that ACSL1 and ADIPOR1, expressed in DPSC secretome are important components of adipocytokine signaling pathway (**supplementary Table S4**).

4. Discussion:

Osteocytes play an important role in bone homeostasis which is disturbed during bone defects arising due to trauma, aging or any other pathological manifestation. Osteocytes differentiate from stem cells through a regulated process or pathways like BMP, WNT and many other proteins play an intricate role during this differentiation and bone remodelling. Higher alizarin red staining is an important indicator of osteogenic potential in DMSC as compared to BMSC. Apart from alizarin red, alkaline phosphatase (ALP) also serves an important parameter for indicating osteogenic differentiation. ALP is involved in hard tissue formation and mineralization (30). A higher expression of ALP after osteogenic differentiation in each stem cell type indicated an enhanced osteogenic differentiation in the cells. ALP is an early marker of osteogenic differentiation and it may be noted that besides having almost similar level of ALP in all four types of stem cells at undifferentiated state, DFSC showed a highest increase in ALP levels post differentiation, which might account for the higher osteogenic potential of DFSC.

Dentine sialo phosphoprotein (DSPP) is secreted exclusively by odontoblasts throughout the formation of dentine, and it is cleaved into dentin sialoprotein and dentin phosphoprotein (31). DSPP has been reported to be an important marker for odontogenic differentiation of stem cells (32). In our study, greater DSPP staining in DMSC post differentiation as compared to BMSC indicated a higher osteogenic potential of DMSC.

Another important protein in formation of bone matrix is osteocalcein (OCN) which is the most abundant noncollagenous protein and is solely secreted by osteoblasts (33). It is secreted in medium by differentiating cells at early stages of osteoblastic commitment which increased progressively with time in differentiating cells. OCN is an important regulator of osteogenic differentiation (34) with its interaction with runt related gene 2 (RUNX-2). RUNX2 activates

OCN which is secreted by differentiated osteoblasts and helps in osteocyte maturation and terminal differentiation to osteocytes (35). As OCN is an early marker of osteogenic differentiation, a higher expression of both RUNX-2 and OCN genes after osteogenic differentiation in DPSC compared to other cell types may account for its higher osteogenic potential.

Previous reports by Jensen *et.al.* reported that DPSC has higher osteogenic potential as compared to BMSC based on calcium deposition and alkaline phosphatase activity in pig model (36). We observed similar results in all three DMSCs as compared to BMSC which highlighted the potential role of DMSC in bone tissue engineering. Luddin *et.al.* have reported that there is an inherent expression of osteogenic markers like ALP, Col 1 and OCN in dental stem cells (37) which might account for an inherent tendency of the DMSC to differentiate more effectively towards osteogenic lineage as compared to BMSC. So, a detailed analysis of alizarin red, alkaline phosphatase, DSPP expression and gene expression of RUNX-2 and OCN marked the higher osteogenic potential of DMSC (especially DPSC) as compared to BMSC.

This difference in the osteogenic or adipogenic potential of BMSC and DMSC might be due to their tissue origin. We have previously shown that paracrine cues might regulate the differentiation potential of stem cells (22, 24) which led us to investigate for proteins in secretome that might provide additional support for directed differentiation of these cells to osteogenic/adipogenic lineage. Secretome characterization in BMSC led to identification of important proteins like BMPRI1A which is a receptor for BMP2 and BMP4 (38), DDR2 which is involved in osteoblast differentiation (39) and also proteins like FBN1 which control osteoblast maturation (40). These proteins might regulate the osteogenic differentiation of BMSC. Secretome analysis also showed the presence of important BMP pathway proteins like BMP7 in secretome of DPSC and SCAP. BMP pathway regulates the expression of RUNX2 through SMAD proteins (41) which is in correspondence with presence of BMP7 in DPSC and SCAP and resulting higher upregulation of RUNX2 in these cells as compared to BMSC (lack BMP7 in secretome). Secreted DSPP in only DPSC secretome again provide support for higher osteogenic potential of these cells in comparison to other cells. USP9X (42) and ZNF521 (43) play important role in activating BMP signaling. ROR2 is involved in osteocyte differentiation (44). Presence of these proteins in SCAP secretome may explain their higher osteogenic potential as

compared to BMSC. Other important proteins uncovered in only DFSC secretome like enamelin (45) and WISP2 (46) positively regulate mineralization and promote bone turnover respectively. This might explain the high osteogenic potential of DFSC.

We obtained higher oil red O stain uptake and expression of adipogenic markers in BMSC as compared to DPSC which emphasized on higher adipogenic potential of BMSC, making them a suitable candidate for adipose/fat tissue reconstruction related applications. Leptin is another important regulator of metabolism and energy homeostasis in adipocytes which acts in an autocrine or paracrine fashion (47). PPAR- γ is an important nuclear factor primarily involved in development of adipose tissue (48). There have been reports on PPAR- γ crosstalk with insulin signalling which sensitize the differentiating cells to insulin and ultimately enhance adipogenesis (49). Adipsin is an adipocyte differentiation dependent serine protease gene and its level increase with adipogenic differentiation and it is secreted in abundance by differentiating adipocytes in culture (50). Increase of these markers after adipogenic differentiation in BMSC as compared to DMSC might explain their higher adipogenic potential.

Since regulator proteins of adipogenic differentiation were found in BMSC and DMSC, presence of some other key proteins in BMSC secretome might explain higher adipogenic potential of these cells. NCOA2 is an important protein to maintain energy balance between white and brown adipose tissue. It has been reported to upregulate PPAR- γ and hence increasing the tendency for adipogenic differentiation (51). All cells secreted this protein highlighting its importance in mediating adipogenic differentiation. PEG10 expression is generally observed at onset of adipocyte differentiation and secretion of this by BMSC might explain the higher adipogenic tendency of these cells as compared to DPSC and SCAP which lack PEG10 in their secretome. Further MECR which control fatty acid synthesis (52) and lipoprotein A were exclusively present in BMSC secretome. **Interactome studies help to understand the functional fate of paralogs and network interconnectivity in proteins have been a useful tool to determine cellular functions (53). The interaction network involved in osteogenic proteins in DMSC hence might explain the increased tendency of these cells to differentiate to osteocytes.**

Conclusion: Cell based differences in committed differentiation towards osteogenic and adipogenic differentiation might be helpful in designing future stem cell based therapies. Using this knowledge, one can select DMSC for their enhanced osteogenic potential while BMSC for

their adipogenic potential in translational studies. Important proteins found in secretome may serve as basis for use of secretome during regenerative applications for bone healing or fat reconstruction.

Clinical implication of the study: Osteoporosis results from gradual replacement of the bone tissue with fat tissue. A treatment which can increase osteogenic differentiation and decrease adipose differentiation can be a potential therapy for osteoporosis. DPSC with higher osteogenic and low adipogenic potential can be important candidates for cell therapy in osteoporosis. Since secretome from DPSC assisted in increased osteogenic and decreased adipogenic potential of these cells, using DPSC secretome in osteoporosis can be a promising “cell free” therapeutic modality of the future.

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Figure Legends:

Figure-1. Culture and characterization of primary stem cells. **a.** Differential interference contrast (DIC) pictures of primary culture of stem cells showing BMSCs and DMSCs, **DMSC cultures show migration of the cells from respective tissue explants, b-c.** Flow histograms and bar diagram showing positivity for different stem cell markers in DMSCs, **d.** Upper panel, hepatic differentiation of all types of stem cells as evident by functional uptake of LDL and positive staining for LDL receptor, lower panel, neural differentiation of four type of stem cells shown by immunofluorescence staining of neurofilament antibody. DAPI was used as nuclear control, **scale bar-50 μ m. **P<0.01. n=3 per cell type.**

Figure-2. Assessment of colony formation efficiency. **a.** Colony formation potential of different stem cells shown by colonies stained with crystal violet (CV) stain, **b.** Bar diagram showing comparative colony number formed by different stem cells after seven days of culture, **c.** spheroid assay to show the temporal spheroid formation in various stem cells, **d.** Bar diagram showing comparison of spheroid size among different stem cells. **scale bar-50 μ m. *P<0.05, **P<0.01, ***P<0.0001 (n=3).**

Figure-3. Osteogenic differentiation of BMSC & DMSC. **a.** Stem cells (BMSC & DMSC) stained with alizarin red stain, **post 21 days of osteogenic differentiation.** **b.** Bar diagram showing relative difference in alizarin red staining post differentiation of different stem cells (BMSC & DMSC). **The comparison was made using BMSC as control. scale bar-50 μ m. *P<0.05 (n=3).**

Figure-4. Evaluation of comparative osteogenic potential. **a.** Immunofluorescent staining showing expression of DSPP protein post osteogenic differentiation in different stem cells, **b.** Bar diagram showing quantification of average fluorescence intensity of DSPP by image J. **At least ten cells were quantified per cell type.** **c.** Bar diagram showing comparison of alkaline phosphatase activity between control and differentiated cells (BMSC & DMSC). *indicating comparison between control and differentiated cells of individual stem cell type. \$represents comparison between differentiated BMSC and rest of the DMSC. Scale bar-100 μ m. **d-e.** Expression fold change in Osteocalcein (OCN) and Runt related transcription factor (RUNX2)

respectively after osteogenic differentiation in BMSC & DMSC. **BMSC were used as control for statistical comparison. scale bar-50μm. */#P<0.05, **/##P<0.01, ***/### P<0.001. (n=3).**

Figure-5. Comparative adipogenic differentiation potential of BMSC & DMSC. **a.** All four types of stem cells showing positivity for Oil Red O depicting adipogenic differentiation after 21 days of culture in differentiation media. scale bar-50μm, **b.** Bar diagram showing comparative uptake of Oil Red O by different stem cells post adipogenic differentiation, Scale bar-50μm, **c-e.** Bar diagram showing relative fold expression change in leptin, PPAR-γ and adipsin respectively after adipogenic differentiation in various stem cells. **scale bar-50μm. *P<0.05, ** P<0.01 (n=3).**

Figure:6. Interactome analysis. a-b. Interaction analysis (interactome) of osteogenic (**a**) and adipogenic (**b**) lineage related proteins respectively in secretome of BMSC and DMSCs by STRING software. Solid balls indicate protein with 3D structure inside. Strings indicate interaction between the proteins.

Figure S1. Heatmap showing comparative analysis of osteogenic and adipogenic differentiation potential between various stem cell types based on various parameters.

Table-1. List of osteogenic lineage related proteins in secretome

Table-2. List of adipogenic lineage related proteins in secretome

Table-S1. Details of primary cultures obtained from patients

Table-S2. Primers used for characterization of differentiation

Table-S3. Pathway analysis for osteogenic lineage related secretome proteins

Table-S4. Pathway analysis for adipogenic lineage related secretome proteins