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RESEARCH ARTICLE

IDENTIFICATION AND DIFFERENTIAL EXPRESSION OF MESENCHYMAL STEM CELLS FROM HEMATOPOIETIC STEM CELLS.

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ABSTRACT

Objective

The main objective is to differentiate mesenchymal stem cells from human bone marrow into osteoblast using osteoblast differentiation media.

Method

Mononuclear cells isolated from bone marrow using Ficoll-paque were cultured in DMEM, 10% FBS and 1% antibiotics pen strep (Penicillin and streptomycin). Cell viability was checked by Trypan blue method and 95% cell viability was achieved at every consecutive passage. Further, cells were grown in Osteoblast differentiation media (0.1% dexamethasone, 10mM beta Glycerophosphate, 0.2mM ascorbic acid with 10% FBS and 1% Penicillin (10000U/ml) and streptomycin(10000µg/ml) GIBCO, Invitrogen, NY USA) . Differentiated osteoblast cells were stained with von-kossa for calcium phosphate deposits. MSC cells at each passage P1 and P2 were checked for FACS analysis using CD29, CD90, CD105, CD34, CD45, CD133 markers compared with controls.

Results

Mesenchymal stem cell markers, CD29+ positive cells are highly expressed in hMNC (97.2%) when compared to CD90+ (36.5%) and CD 105+ (56.6%). With subsequent passages CD29+, CD90+ and CD105+ expression increased which almost was 99% in P3. CD133+ is expressed in very high fraction of cells in the fresh sample (77%) and decreases in subsequent passages. This is an expected phenomenon as CD133+ is a pure HSC marker. Therefore CD29+, CD90, CD90+, correlates with pure hMSC cells from HSC cells and can be used for differentiation.

Conclusion

Bone marrow is the major source of hMSCs contributing circulating bone marrow mono-nuclear cells, Therefore differentiation of MSC from HSC can be of great importance in regenerative medicine.

FBS(fetal bovine serum), DMEM(Dulbecco modified eagles media), FACS(Fluorescence activated cell sorting), ACS(acceleration),MSC (Mesenchymal stem cells), P1(Passaging), MNC(Mononuclear cells),BM(Bone marrow), PE(phycoerythrin), APC(allophycocyanin), CTR(Control), SPL(sample) , PBS(Phosphate buffered saline), HSC(Hematopoietic stem cell), Abs(antibiotics), hMSC-Human mesenchymal stem cell

INTRODUCTION

Human bone marrow consists of a group of cells such as endothelial cells, fibroblasts, adipocytes and osteogenic cells. The bone marrow stroma helps hematopoiesis to occur as well as growth and acts as precursor of various myeloid, lymphoid, pluripotent cells like CFU-L, CFU-E, CFU-GM, CFU-ME, CFU-B, CFU-E0 etc. The typical bone marrow consists of two types of cells, hematopoietic stem cells and hMSCs. hMSCs consist of heterogeneous population of adherent fibroblast, which in culture are able to renew and differentiate into bone, adipose tissues and cartilage.

hMSCs derived from human bone marrow, because of its ability to differentiate into osteoblast cells based on its specific markers such as CD29+, CD90+, CD 105+. This study highlights the markers of hMSCs from hematopoietic stem cells by flow cytometry method.

The purpose of this study is to differentiate hMSC into osteoblast cells from hHSC specifically based on markers. Further various clinical applications of these osteoblast in regenerative medicine can be evaluated as osteoblast can help in regeneration of bone, cartilages, tendons and definitely requires further study explore its use in healing.

MATERIALS AND METHODS

METHODOLOGY

A Total of one healthy individual was taken for bone marrow aspiration under aseptic conditions with proper consent informing the patient about the outcome and complication of the procedure. From the above bone marrow of the person Mononuclear cells were isolated using ficoll paque and was taken as control, further hMSCs were isolated using the

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DMEM media and was taken as cases. Later from the cases differentiation of osteoblast cells were done using osteoblast differential media and stained with Von Kossa stain specific for osteoblast and the comparison between the cases and control was done.

Reagents

DMEM (GIBCO, Invitrogen, USA) with10% FBS (Invitrogen, USA), 2mM L-glutamax (Invitrogen, USA) and 1% Pen-strep (Penicillin 10,000 U/ml) and streptomycin (10,000µg/ml), Ficoll-paque (Sigma-Aldrich), Trypsin-EDTA 0.5% (Sigma-Aldrich) and Von-kossa stain purchased from Abcam, markers (CD 29,CD90,CD105,CD34,CD45,CD133) from Santacruz, USA. Trypan blue (Sigma-Aldrich)

Pbmc Processing

To 20ml of bone marrow sample, 0.36ml of heparin is added to prevent coagulation and was mixed well. In a conical bottom sterile test tube, 10ml of Ficoll gradient solution was added. The sample was then dissolved in PBS in 1:2 ratios carefully and loaded onto the density gradient solution such that mixing does not occur. Centrifugation is done for about half an hour at 400 G. Acceleration and de acceleration were minimized to zero so as to avoid any layer disturbance. Temperature was maintained at 21 degrees Celsius throughout the procedure. After centrifugation, four distinct layers was observed. The top most layer was plasma, second layer was buffy coat, third layer was ficoll and fourth layer was RBC. The visible buffy coat layer was collected by removing plasma. Volume of PBS was added to the buffy coat in a centrifuge tube and was centrifuged at 450G for 10 minutes. 0.7% of ammonium chloride (pH-7.4) was added to the pellet to lyse RBC for 2 minutes. **[4].** Further, 20ml of sodium chloride of 0.9 % was added at 4 degrees temperature to arrest the activity of ACS so that the required lymphocytes do not get lysed. Centrifugation at 4 degree and 300G for ten minutes was done for removal of cleaved RBC's as settled pellets and to retrieve supernatant fluid. It was made up to 40ml using PBS .Before addition of PBS another wash was done to remove more erythrocyte. **[4].** Now isolated lymphocytes was used for characterization and for further analysis and processing. Isolated lymphocytes are subjected to culture and characterized for study **[4].**

Characterization And Analysis Of Mesenchymal, Hematopoietic And Side Population Cells By Facs

After checking cell viability, one million cells was characterized in BD FACS Aria by hematopoietic markers (CD34+,CD45+, CD133+) , mesenchymal markers (CD29+, CD90+, CD105+) and side population cells (CD117+, ABCG2+).

Flowcytometric Protocol For Characterization

One million cells were incubated with antibodies coupled with flourochromes in tube containing 20µl of CD34-PE, 5 µl of CD90-PER CP CY5, 20 µl of CD105-APC, 5 µl of CD117-PE CY7, 20 µl of ABCG2-PE. Now tubes was incubated in dark for 20 minutes. Then the cells was washed with PBS. Then to the pellet 500 µl of PBS was added. Further analysis was carried out by DIVA software in Beckon, Dickson FACS Aria. Argon Ion laser (480nm) and red laser (632nm). The number of cells stained positively is proportional to percentage of cells with 10,000 events recorded **[1, 6]**

Culturing Of Cells

Primary Culture

The cells was counted and seeded onto the culture flask with a minimum density of 3-4X10^4 cells /cm^2. The split ratio which means splitting off cells into the flasks was calculated according to the count obtained. The cells was re-suspended in DMEM, 10% FBS and 1% Abs. The cell containing the media was then transferred to tissue culture flask of T_{25} or T_{75} according to the cells obtained. The tissue culture flask was then incubated in CO_2 incubator containing 37°C, 5% CO_2 and 95% humidity with a pressure of 60psi**.[10].**

Trypsinization

The media change was done twice a week or once in two days until the cells reached 80-90% confluence. Once the cells have reached confluence, the cells were then called as P_0 cells. These are named so as they are obtained from primary culture and not from passage. These P_0 cells were taken for trypsinization. The media was removed from the culture flask by keeping plate at 45° angle. Small volume of sterile PBS was added to wells and allows remaining for 2-3minutes. PBS was replaced with 0.5% trypsin-EDTA at 37°C incubation for five minutes in CO_2 incubator. After 5minutes, cells was viewed under microscope to verify removal of cells from plate. The cells were triturated firmly using Pasteur pipette or 2ml pipette gently. To re-ensure that the cells have removed from the plate 500µl of 10% FBS or culture media was added to neutralize the trypsin reaction. The media containing suspended cells was transferred from culture flask to 2ml tube. **[18,10]**

Harvesting The Cells

The cells were collected in a centrifuge tube and centrifugation at 300G for 5minutes. The supernatant was discarded and pellet was re suspended in PBS. The cells was counted using hemo-cytometer and further re-seeded cells for P_1 according to count. P_0 cells were characterized for stem cell population. The above steps were repeated for P_1 and P_2

Differentiation Protocol

After trypsinization, the cells was re-suspended with media (Low glucose) and left undisturbed for 24 hours. After 24 hrs, the cells was treated with (0.1µm Dexamethasone 10mM β-Glycerophosphate, 0.2mM Ascorbic Acid.) with 10% FBS and 1% Pen-step (Abs). The media was changed twice a week and the cells were observed every day for morphological changes. Once the morphological changes of osteogenesis are observed, to confirm the differentiation potency, the cells were stained for confirmation using Von Kossa stain**. [16,17].**

Von Kossa Staining

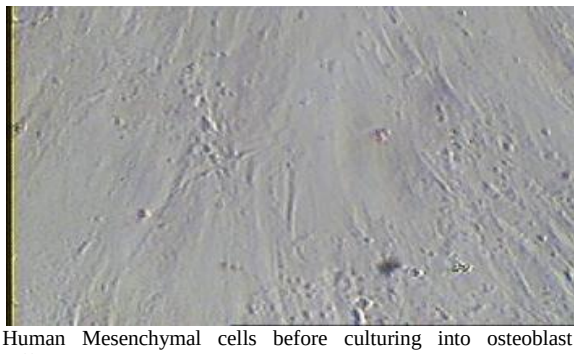
This staining method is to detect Calcium phosphate deposits present in osteoblast cells **[2,3].** After washing with PBS for three times, the cells was fixed with 10% formalin at Room temperature for 1hr. Once cells washed with PBS, 1ml of 5% silver nitrate added to the cells and left for three minutes and exposed to UV for 45 minutes **[3].** 5% sodium thio-phosphate was added and further washing was done with distilled water for 3 times. The cells stained with nuclear fast and was left for 1 minute and again washing was done with distilled water. Deposits of Calcium phosphate stained black, other tissue and nuclei stained pink **[21,22].**

Table1: Expression of markers at different passages from bonemarrow derived stemcells

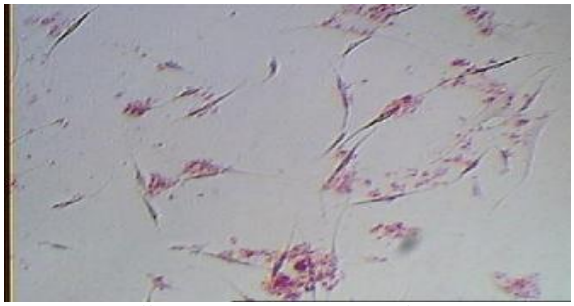
MARKERS	MNC	P0	P1	P2
CD29+	97.2	99.8	99.7	99.8
CD90+	96.5	99.6	98.4	99.7
CD105+	96.6	99.7	99.7	99.9
CD34+	15.8	7.5	4.2	3.3
CD45+	92.9	29.6	6.9	4.3
CD133+	77.0	9.9	2.8	1.7

Control

Undifferentiated osteo blastic cells



Human Mesenchymal cells before culturing into osteoblast differential media



On day 13 differentiated osteoblast cells from hMSC were confirmed by deposit of calcium crystals using Von-Kossa staining

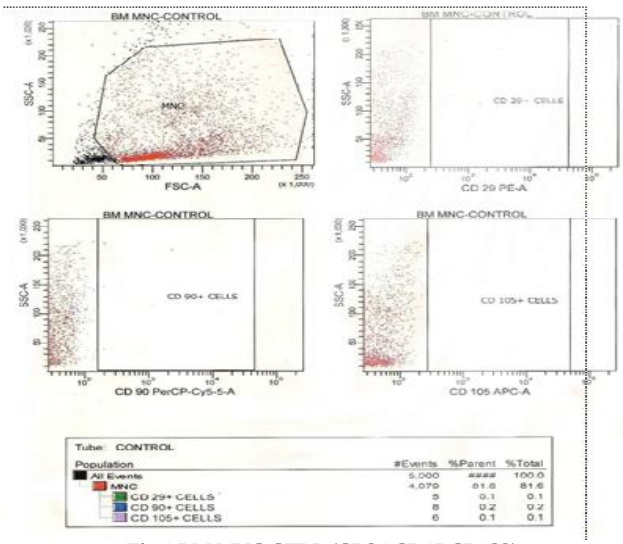


Fig 1 BM MNC CTRL (CD34,CD45,CD133)

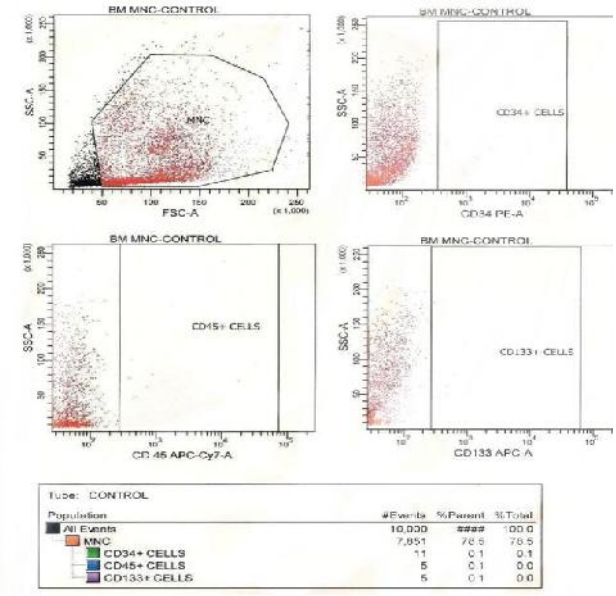


Fig 2 BM MNC CTRL (CD34,CD45,CD133)

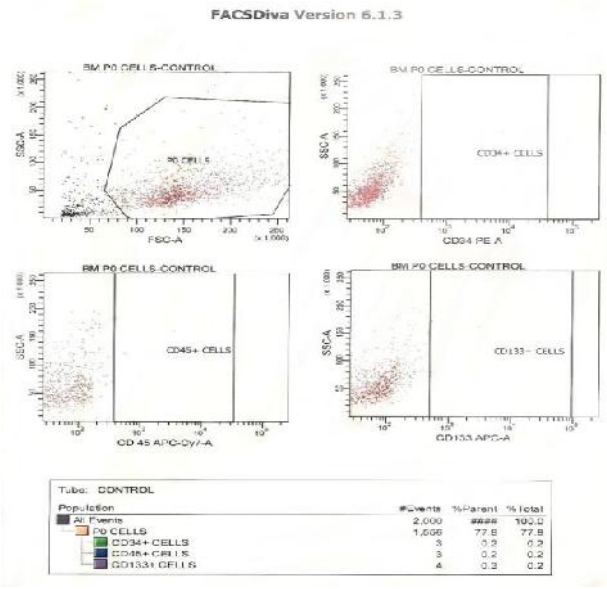


Fig 3 BM MNC CTRL P0 (CD34,CD45,CD133)

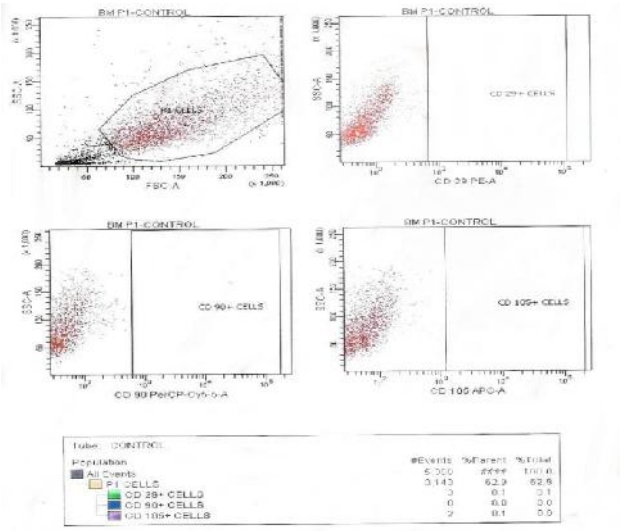


Fig 4 BM MNC CTRL P1(CD29,CD90,CD105)

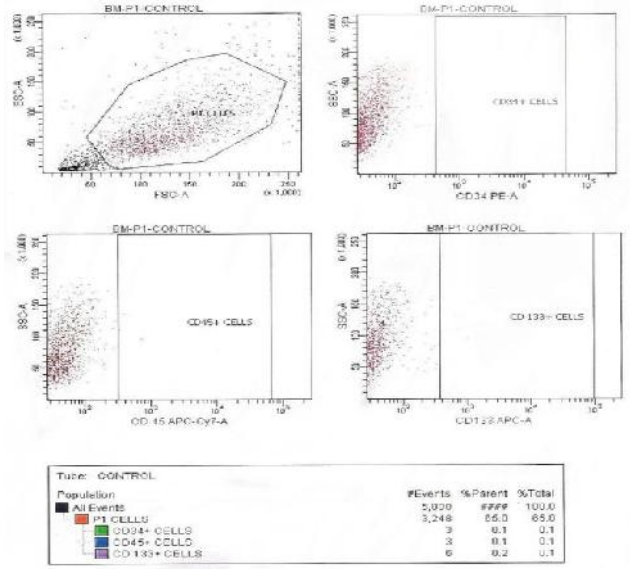


Fig 5 BM MNC P1 CTRL (CD34,45,133)

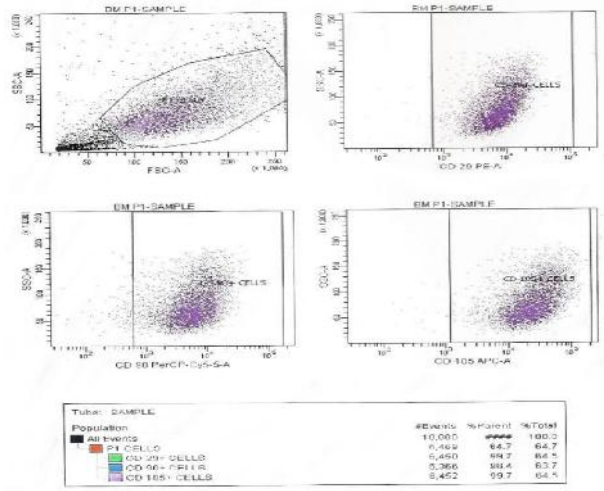


FIG 6 BM MNC SPL P1(CD29,CD90,CD105)

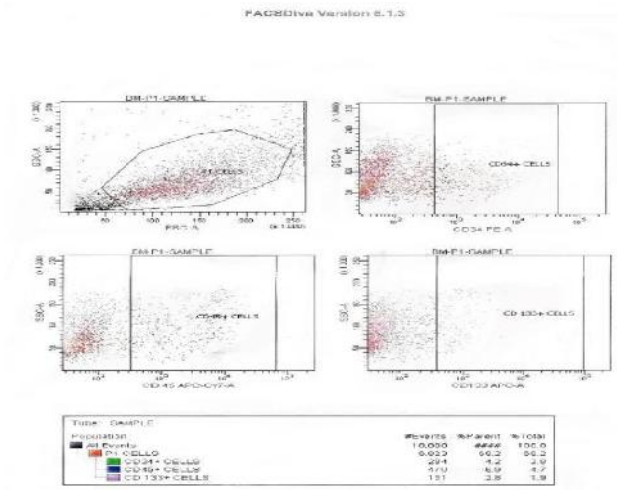


FIG 7 BM MNC SPL P1(CD34,CD45,CD133**)**

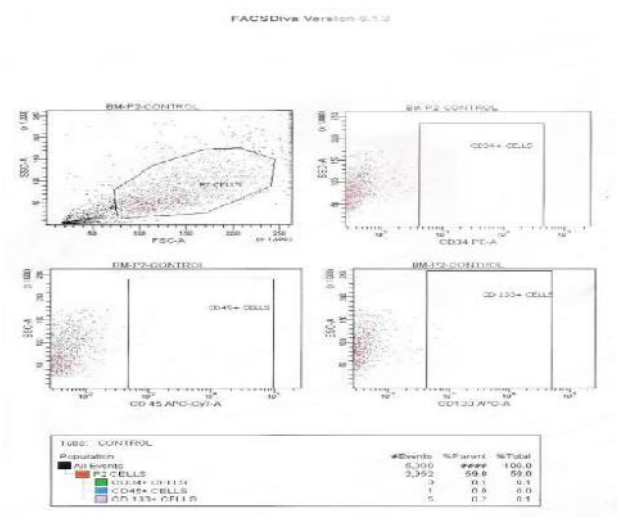


Fig 8 BM MNC P2 CTRL (CD34,CD45,CD133)

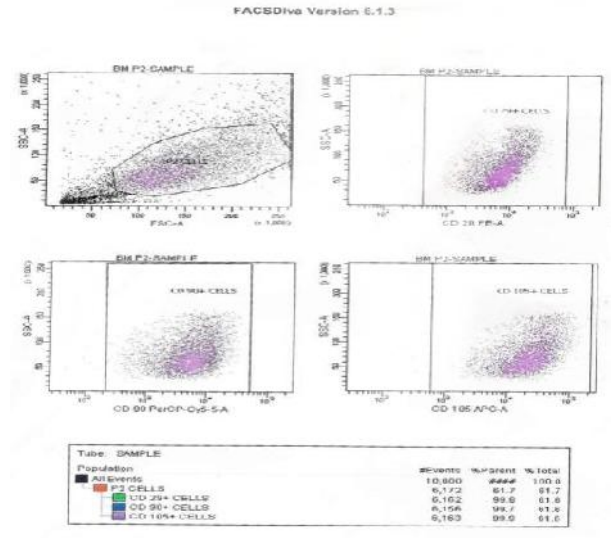


Fig 9 BM MNC P2 SPL (CD29,CD90,CD105)

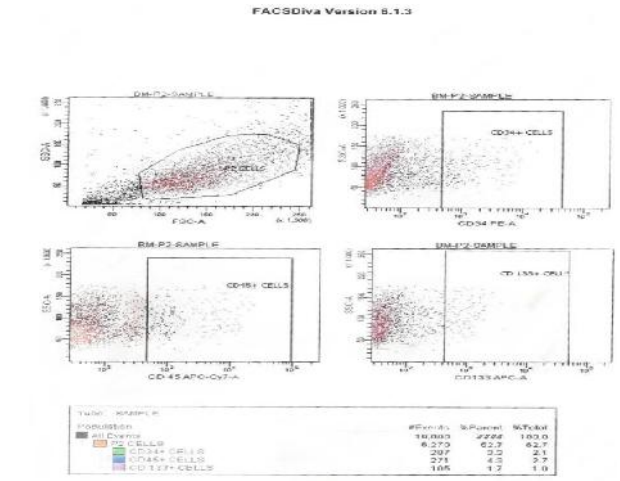


Fig 10 BM MNC P2 Sample (CD34,CD45,CD133)

RESULTS

The following results was inferred from the above study which was represented in the Figures above. The figure 1 and 2 represents the cells before culturing in DMEM media which shows the population of hMSCs and hHSCs. MNCs population were high but hHSCs population was less (0.2%). passage in hMNCs control there is no increase of hHSCs and hMSC population.

Figure 6 and 7 represents the P1 with proportion of hMSCs marker increased (64.5%) than hHSCs population.

Figure 9 and 10 represents P2, indicating the consequtive rise in hMSCs markers to about (61.6%) in comparision the hHSCs markers like CD 34 -2.1%, CD 45 – 2.7 % and CD 133 – 1.1 %.The proportion of hMSCs increased with subsequent passage whereas the hHSCs population decreased from P0-2.

DISCUSSION

Samples of human bone marrow were collected and PBMC was isolated using ficoll paque **[19].** Further, isolated mononuclear cells was grown in DMEM with 10% FBS. Once the cells reached different passage, trypsinization was done and cell viability was checked. After two passages P1 and P2, cells grown in osteoblast differential medium**[20]** containing dexamethasone, glycero-phosphate and ascorbic acid, within few days, morphological changes was observed and to confirm the osteoblast cells Von Kossa staining**[21]** was done.

At each passage P0, P1, P2 the cells was characterized by FACS using hMSCs marker (CD29+, CD90+, CD105+) **[22]** and hHSCs marker (CD34+, CD45+, CD133+)**[23].** The proportion of hMSCs markers has shown more than 95% cell viability increase at P1 and P2 but hHSC group distributed more without any passage. This determines that cells at P1 and P2 are pure hMSCs and cells distributed and analyzed by CD133+ are pure hHSCs**[24].**

The markers of hMSCs such as CD29+, CD90+, CD105+ **[22]** has to be studied further in various diseases and alteration in differentiation to be ascertained. According to above study, it is shown that isolated osteoblast cells has high mode of differentiation capacity and therefore its use in regenerative medicine in various bone disorder should be investigated.

CONCLUSION

Therefore based on this study it is concluded that depending on markers like CD29+,CD90+,CD105+**[22],** osteoblast cells was differentiated which was further confirmed by Von Kossa staining**[21],** these osteoblast further can be used in bone tissue engineering which has to be explored more extensively**[24].**

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