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Isolation, culture and characterization of mesenchymal stem cells from mouse bone

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Abstract
The bone marrow contains a rare population of cells termed mesenchymal stem cells having the ability to differentiate into cells of the osteogenic, chondrogenic and adipogenic lineages. There is considerable interest in utilizing MSCs in a broad repertoire of cell-based therapies for the treatment of various diseases. Therefore, rapid and simple method for culture of MSCs is required. Here we describe method for the isolation and *in vitro* expansion of a highly enriched population of MSC from mouse cortical/compact bone. The procedure includes flushing bone marrow out of the long bones in complete α-MEM medium. This was followed by two times washing in complete α-MEM medium and finally plating in tissue culture flasks. After 2 days of primary culture, non adherent cells were removed by changing the medium at every third day. Once the cells reach 70-90% confluence, the cells were passaged further to augment cell population. Identification assays confirmed that these cells were MSCs, which may be used for therapeutic purpose.

Keywords: Bone marrow, complete α-MEM medium, isolation, In-*vitro* expansion, identification

Introduction
Stem cells have been a focus of intense research and publicity for the last decade. They are reconstructing our understanding of development, physiology and pathophysiology of diseases (Joseph & Morrison, 2005; Clarke & Fuller, 2006) [23, 6]. Stem cells are considered today as the most assuring cells, because they can divide without limit to replenish other type of cells in the body as a backup for the repair system. Better understanding of the mechanisms regulating the development of specialized cells from stem cells help scientists to study the malfunctions that occur during the process of development (Odorico *et al*., 2001) [37]. These are undifferentiated cells found in the embryonic, fetal, and adult stages of life give rise to differentiated cells that are building blocks of tissue and organs. The three major characteristics of stem cells are: (a) self-renewal (proliferation), (b) clonality (usually arising from a single cell), and (c) potency (differentiation ability) (Kolios and Moodley, 2013) [26]. Stem cells can be of four types depending on their origin: ESCs, fetal and adult stem cells, and iPSCs (Ilic & Polak, 2011; Bongso & Richards, 2004) [22, 5]. Two stem cell populations with distinct progenies are housed within adult BM, hematopoietic stem cells and MSCs (Pittenger *et al*., 1999) [41]. Mesenchymal stem cells are fibroblastic cell type that have the potential to give rise to clonal colonies with bone and tissue generating properties and was first discovered by Friedenstein from mouse and guinea pig bone marrow (Friedenstein *et al*., 1966; Friedenstein *et al*., 1970) [15, 13]. Mesenchymal stem cells (MSCs) have also been referred by other names such as colony – forming fibroblasts cells (Friedenstein *et al*., 1976), BM stromal stem cells (Bianco *et al*., 2001) [4], mesenchymal progenitor cells (Prockop, 1997). Source of MSCs origin is not only restricted to fetal stage but also found throughout the other organs of adult body. The most commonly studied source of MSC, the bone marrow has been extensively reported (Wang *et al*., 2013) [54]. Moreover, MSCs have also been isolated successfully from other sources like umbilical cord blood (Kumar *et al*., 2007) [27], Wharton's jelly (Zhang *et al*., 2013) [58], peripheral blood (Faast *et al*., 2006) [11], amniotic fluid (Sartore *et al*., 2005) [45], endometrium (Subbarao *et al*., 2015) [50], neural progenitors (Yin *et al*., 2011) [55], retinal progenitors (Klassen *et al*., 2007) [24], skin (Lermen *et al*., 2010) [29], synovial fluid (Moriguchi *et al*., 2013) [33], aorta tunica media (Zaniboni *et al*., 2014), dental tissues (Marrelli *et al*., 2015) [30] and adipose tissue (Zuk *et al*., 2002) [59].
Effectiveness of MSCs in hematopoietic recovery, bone regeneration and in the treatment of patients with osteogenesis imperfecta, infracted myocardium or joint diseases has been well

documented (Baksh *et al*., 2004; Horwitz *et al*., 2002; Koc *et al*., 2000; Petite *et al*., 2000; Quarto *et al*., 2001; Grinnemo *et al*., 2004; Barry, 2003) [2, 21, 25, 39, 43, 18, 3]; for this reason, isolation of MSCs from different species such as mouse and extensive preclinical studies is required. BM-MSCs are usually isolated and purified through their physical adherence to plastic cell culture plate (Nadri *et al*., 2002) [35, 36]. A number of techniques have been developed to fulfill this purpose (Peister *et al*., 2004; Phinney *et al*., 1999; Baddoo *et al*., 2003; Tropel *et al*., 2004; Eslaminejad *et al*., 2006; Nadri and Soleimani, 2007; Nadri *et al*., 2002) [38, 40, 1, 51, 10, 34, 35, 36], such as exposure of cultures to cytotoxic materials (Modderman *et al*., 1994; Falla *et al*., 1993) [32, 12], cell sorting (Van Vlasselaer *et al*., 1994) [52], low- and high-density culture (Peister *et al*., 2004, Eslaminejad *et al*., 2006; Wang *et al*., 1990) [38, 10, 53] and negative and positive selection (Peister *et al*., 2004; Baddoo *et al*., 2003; Tropel *et al*., 2004; Nadri and Soleimani, 2007) [38, 1, 51, 35, 36], which permits the isolation of mouse mesenchymal stem cells (mMSCs) from mouse BM. However, these methods have inconvenient effects on the biological properties of stromal cells such as decline in proliferation and differentiation potential of mMSCs. Therefore, an easy and effective protocol for isolation of mouse BM-MSCs is needed.

Materials and method

Glasswares and plasticwares

All the glasswares were of high-grade pyrogen free glass and were procured from Borosil (USA) whereas all the plasticwares of cell culture grade were procured from Millipore Corporation (USA), Bioscience (Germany), Nunc International (Naperville) and Corning Incorporated Life Sciences (Lowell, MA, USA) to be used in present study.

Equipments

Inverted microscope

Inverted microscopes (Olympus – 1X51S8F3,Japan)and ZEISS fluorescent mmicroscope were used for cell culture examination. The inverted microscopes were also having UV florescence and differential interference contrast (DIC) attachment, for capturing cell culture images. Zeiss microscope was equipped with 488 and 405 nm wavelength under FITC and DAPI filters respectively.

CO_2incubator

The culture of murine bone marrow derived MSCs were carried out in a Nuaire US auto flow CO_2 incubator (USA) at 37^0 C.

XP cycler

A thermal cycler (XP Cycler, BIOER, China) was used for synthesizing cDNA from mRNA of murine bone marrow derived MSCs.

Electrophoresis unit

The electrophoresis unit (GENEI^{TM}H06, USA) was used for agarose gel electrophoresis of cDNA and PCR products.

Gel documentation

For analyzing the PCR products and capturing the images, Gel Doc^{TM}XR + (Bio- Rad, USA) documentation system was used.

Chemicals and media

All media and chemicals used were of high quality cell culture grade and were procured from M/S Sigma chemicals Co. (St. Louis, MO, USA), M/S Invitrogen, USA, M/S Gibco, USA and M/S Himedia, India. 0.22 µm, 0.45µm filters from Millipore corporation, Bed ford, MA, USA disposable Petri dish, tissue culture flask (T-25, T-75, 6 well) and 15 ml graduated tube of Nunc brand Nalge (Nunc International Naperville) were used. All the procedures were carried out in highly sterile condition under laminar air flow cabinet to avoid any bacterial and fungal contamination. Water used for media preparation was from reverse osmosis milli Q water system. All the prepared media except DPBS was kept atleast 3-4 hours in CO_2incubator at 37^0 C and 5% CO_2, 90% relative humidity before use.

Sterilization procedures

The glasswares and micropipette tips were sterilized by autoclaving at 121^0 C and 15 psi for 15 min. The culture plates, culture bottles, petridishes, tissue culture flasks, centrifuge tubes and syringes were exposed to UV light for atleast 15 min before use.0.22µm filters were used to sterilize all the media.

Experimental animals

Apparently healthy adult Swiss albino mice of either sex were procured from the Laboratory Animal Resource section(LAR), Indian Veterinary Research Institute. Experimental mice were acclimatized for 5days in the Experimental Animals Sheds, Division of Pharmacology, IVRI, and maintaining under standard management conditions through providing adlibitum feed and water, daily light cycle rotated between 12h of light and 12h of darkness. The animal experiment proposal (IAEC/19.09.2020/S14) was approved by the Institutional Animal Ethics Committee of IVRI, Izatnagar (Approval No-F.No.26-1/2020-21/JDR)/IAEC dated 30/09/2000).

Collection, isolation and culture of murine bone marrow MSCs

Mice of 6 – 8 weeks old were terminated by cervical dislocation for collecting bone marrow. Then the whole body was soaked in 70% (v/v) ethanol for 2 min. All the four claws were dissected at ankle and carpal joints, and the incision was given around the connection between hindlimbs and trunk, forelimbs and trunk. Whole skin was removed from the limbs and then the muscles, ligaments and tendons were dissected out carefully from the tibias, femurs and humeri using surgical scissors and scalpel. Bones were taken out onto sterile guaze and the residual soft tissue around the bones was removed by scrubbing. Finally the bones were transferred to a sterile culture dish containing about 10 ml complete Minimum Essential Medium Eagle (Alpha Modification) prepared from stock Minimum Essential Medium Eagle (Alpha Modification) (Himedia, cat#.AL080A).

In laminar air flow, bones were washed twice with PBS having 1% antibiotics (penicillin and streptomycin) to flush away the residual soft tissue and blood cells. Then the bones were transferred into a new 90 mm sterile culture dish containing 10 ml complete α-MEM medium. The two ends of the bone were excised just below the bone marrow using micro dissecting scissors. Marrow was taken out slowly by drawing complete α-MEM medium using 23-guage needle

attached to a 5 ml syringe. Thereafter, the flushed out marrow was mixed properly in complete α-MEM medium. This marrow sample in complete α-MEM medium was centrifuged at 1000 rpm for 5 min. and then the supernatant was decanted and the cell pellet was again washed with complete α-MEM medium and centrifuged at 1000 rpm for 5 min. Supernatant was decanted and cell pellet resuspended in small volume of complete α-MEM medium and then cells were plated in 25 mm^2 tissue culture flasks. Cells were maintained in the culture medium with antibiotics (100 units/ml penicillin, 100µg/ml of streptomycin, Gibco,cat.#15140-122) in an atmosphere of 5% CO_2, 95% humidity at 37 ^{0} C for 48 hours. After 2 days of primary culture, non-adherent cells were removed by changing the medium at every third day.

Expansion and characterization of MSCs

Once the cells reach 70-90% confluence, the cells were passaged further to augment cell population. The medium of culture was aspirated and cell layers were washed twice with PBS and then digested with 2.5 ml of 0.5% trypsin-EDTA (cat.#T4174) and incubated at 37 ^{0} C for 5 min. or until the cells detach. Then double the volume of culture medium was added to flask to neutralize or stop the activity of trypsin and gently mixed with strokes, delivered into 15 ml centrifuge tube and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in culture medium and centrifuged again. 1ml culture medium was added after decanting the supernatant, properly mix the cell pellet and cells were transferred into 25 mm^2 tissue culture flask containing culture medium at a split ratio of 1:3. Flasks were kept in an atmosphere of 5% CO_2, 95% humidity at 37 ^{0} C. Cells were examined regularly for their viability, confluence and morphological features under microscope. Within 3 passages, the MSC was characterized by molecular method and immunocytochemistry for the expression of CD73, CD90, CD 105, CD45.

Molecular characterization: gene expression studies were done using Real Time PCR.

RNA extraction and reverse transcription

For gene expression analysis, cells were isolated by using Trypsin-EDTA and washed with 1X PBS. Then the cells were transferred in 2 ml centrifuge tube for isolating RNA. The total RNA was isolated using Quick-RNA^{TM} MicroPrep, Zymo Research, Cat.# R1005. The cDNA was synthesized using I Script select cDNA Kit (cat# 1708891). Optical density of cDNA was taken and used for real time PCR study.

Real time PCR

Relative quantification was performed by using a real time polymerase chain reaction method. Briefly, a Biorad CFX Manager^{TM} Software and Dynamo color flash QSYBR Green Q-PCR Master Mix (cat.#F416L), as a double stranded DNA-specific fluorescent dye was used. For the amplification of gene, the desired primers were designed in beacon software. The transcript level of all genes were quantified using the relative quantification method based on comparative threshold cycles values (Ct). The abundance of gene was determined relative to the abundance of the housekeeping gene. After that the data analysis was done.

Immunocytochemistry of mesenchymal stem cells

The immunocytochemistry of MSCs was done for the MSC markers, CD 73, CD 90, CD 105, CD45, as per the method followed by the National Institute of Health(NIH), 2009 resources for stem cell research, using fluorescent isothiocynate (FITC) conjugated antibody.

Immunostaining protocol

- During different passages, murine MSCs were cultured on 0.1% gelatin coated cover slips in 6 well plate and were incubated in CO_2incubator.
- After reaching 70-80% confluency, MSCs were fixed using 4% paraformaldehyde (PFA) in 1 X PBS for 20 min.
- MSCs were washed 3 times with PBS-(0.1%) BSA solution at room temperature.
- For intracellular epitopes, cells were incubated for 20 min. in a saturation / permeabilization PBS 1X solution containing 0.3% TritonX- 100 and 4% bovine serum albumin (BSA).
- MSCs were washed 3 times with PBS- BSA and non specific sites were blocked with blocking solution (10% goat serum in PBS-BSA) for 40 min. at room temperature.
- MSCs were washed 3 times with PBS-BSA at room temperature.
- Cells were treated with Primary antibodies against CD105 (Santa Cruiz#sc-19793), CD90 (#sc-9162) rabbit polyclonal IgG, CD73(#sc-25603) rabbit polyclonal IgG, CD 45(#sc-25590) rabbit polyclonal IgG at 1:200 dilution for each markers for overnight at 4^0 C. MSC monolayer was washed 3 times with PBS-BSA at room temperature.
- The appropriate secondary antibodies were diluted in a secondary antibody diluting solution (1:800) and incubated for 2 hours at room temperature in dark room.
- MSC monolayer was washed 2 times with PBS-BSA solution and the other 2 times with only PBS 1X at room temperature.
- MSC monolayer was incubated with Hoechst dye (DAPI) for 10 min. in dark room.
- MSC monolayer was washed 3 times with PBS at room temperature.
- The stained clone was covered with pro-Long gold antifade agent (catalog #. P36930) Invitrogen molecular Probe^{TM} and observed under fluorescent Ziess microscope immediately.

Results and Discussion

Murine mesenchymal stem cells isolation and culture

MSCs were harvested from the bone marrow (tibia, femur and humeri) of the mice using Minimum Essential Medium Eagle (Alpha Modification) (Himedia, cat#.AL080A), which was subsequently centrifuged in complete α-MEM medium twice,

and cell pellet was resuspended in small volume of complete α-MEM medium. Finally, cells were plated in 25 mm^2 tissue culture flasks which were maintained in an atmosphere of 5% CO_2, 95% humidity at 37 ^{0} C. The cells were properly adhered within 2-6 days and proliferated. Non adherent cells were removed by decanting the used media and replacing it with fresh media after every 24h for upto 96 h. Cells were having triangle to spindle shaped morphology within 3-7 days. It took about 10-15days for cells to reach 70-80% confluency. Cells were propagated upto3rd passage, which were used for their characterization. Mesenchymal stem cells at different time interval and passages (upto3) are shown in fig.1.

Mesenchymal stem cells characterization

Molecular characterization

Mesenchymal stem cells were characterized by the expression level of MSC specific genes using RT PCR. To analyse relative abundance of marker genes, GAPDH gene expression was taken as housekeeping gene. Characterization results revealed that cells were found to be positive for CD105, CD73, CD90 (MSC specific markers), whereas negative for CD45 (hematopoitic cell surface marker). Dissociation and melting curve for MSC specific genes are presented in fig.2.Results of agarose gel electrophoresis of PCR products showed positive expression for MSC characterstic markers whereas expression for hematopoietic stem cells markers was not present as depicted in fig.3.

Immunocytochemistry

To further characterize MSCs, immunostaining of the cultured monolayer of murine bone marrow MSCs with MSC specific markers CD105, CD73 CD90 were performed. Immuno-staining results showed that cells were found to be positive for these MSC specific markers, fig.4 (a-i); however, they were found to be negative for CD45, fig.4 (j-l).

These cells attained the characteristic spindle shape after few days of primary culture (fig.1 d & e), similar with the previous report of Lennon *et al*., 2001 and Godsan, 1983 who showed that cells got their fibroblast cells like shape after several days of primary culture. For the characterization of mBM-MSCs, an array of cell molecules was utilized. It is widely accepted that bone marrow derived MSCs (BM-MSC) express SH2 (CD105), SH3/SH4 (CD73), integrin b1(CD29), CD44, Thy-1 (CD90), CD71, activated leukocyte adhesion molecule (CD166),vascular adhesion molecule-1 (CD106), Stro -1, GD2 and melanoma cell adhesion molecule (CD146) (Conget *et al*., 1999; Galmiche *et al*., 1993; Haynesworth *et al*., 1992; Martinez *et al*., 2007; Sacchetti *et al*., 2007; Shi and Gronthos., 2003; Shi *et al*., 2002; Sordi *et al*., 2005; Gronthos *et al*., 2003; Simmons and Torok- Storb, 1991) [7, 16, 20, 31, 44, 19, 46, 47, 49, 48] and believed that BM-MSCs lack the expression of haematopoietic surface molecules including CD45, CD34, CD14 Or CD11b, CD79a or CD19 and HLA-DR (Dominici *et al*., 2006) [9]. In agreement with general MSCs description, this study demonstrated that murine bone marrow derived cells were found positive for CD73, CD105 and CD 90 while negative for CD45. This results was in line with the ISCT (International Society for Cytotherapy) criteria, where the cells were analyzed by the expression of cell surface markers which includes CD44, CD73, CD90 and CD105 (Dominici *et al*., 2006; Yu *et al*., 2007; De Macedo Braga *et al*., 2008) [9, 56, 8].

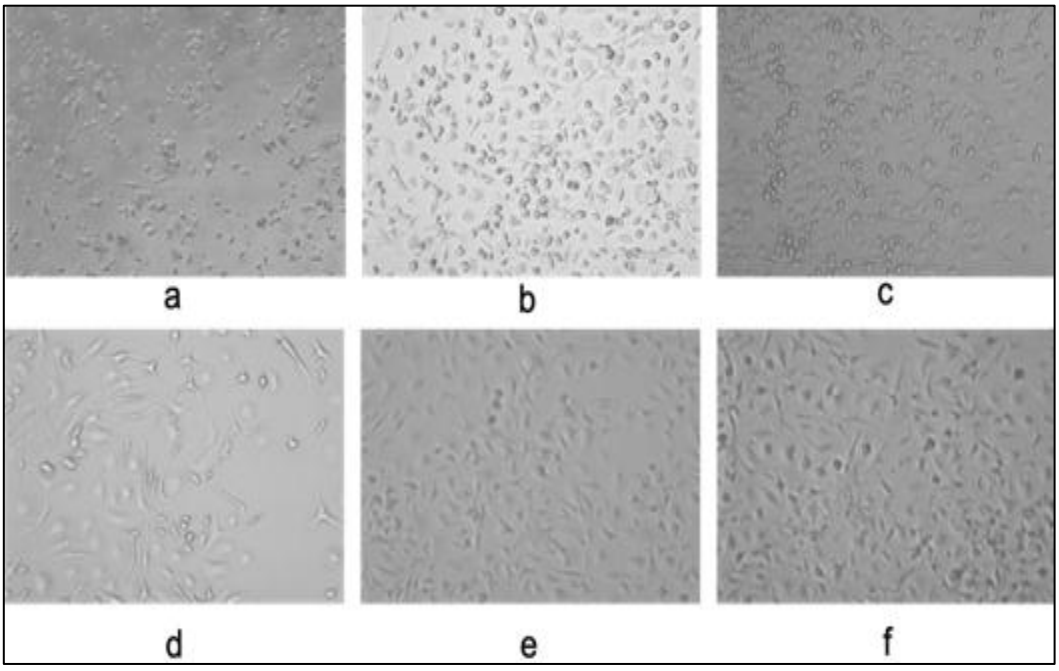

Fig 1: Establishment of murine bone marrow derived mesenchymal stem cells
(a) Primary culture of mBM- MSCon day 0 of culture (10x), (b) Increase in cell confluency on day 6-7 of culture (10x), (C) Cells reached 80% confluency within 10-11 days (10x), (d) Cells maintained their characteristic spindle shape in subsequent passages (P1, 10x), (e) Cells of passage two (P2, 10x), (f) Cells of passage three (P3, 10x)

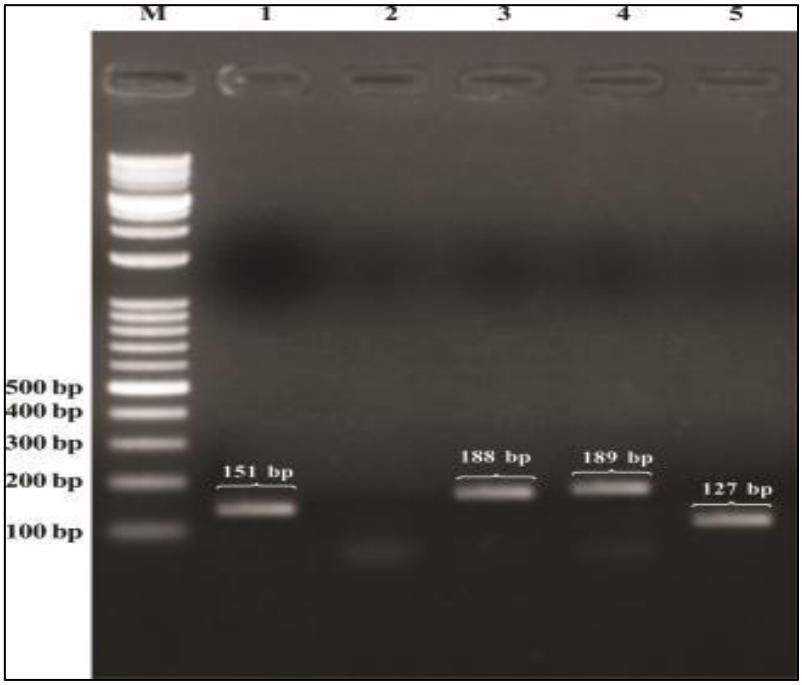

Fig 2a: Gel electrophoresis of PCR amplified products of mesenchymal stem cells of passage 3 in 2% agarose gel
Lane M: 100bp DNA ladder, Lane 1: GAPDH (151 bp), Lane 2: CD45(143 bp), Lane 3 : ENG (188 bp), Lane 4: THY 1 (189 bp), Lane 5: CD73 (127 bp)

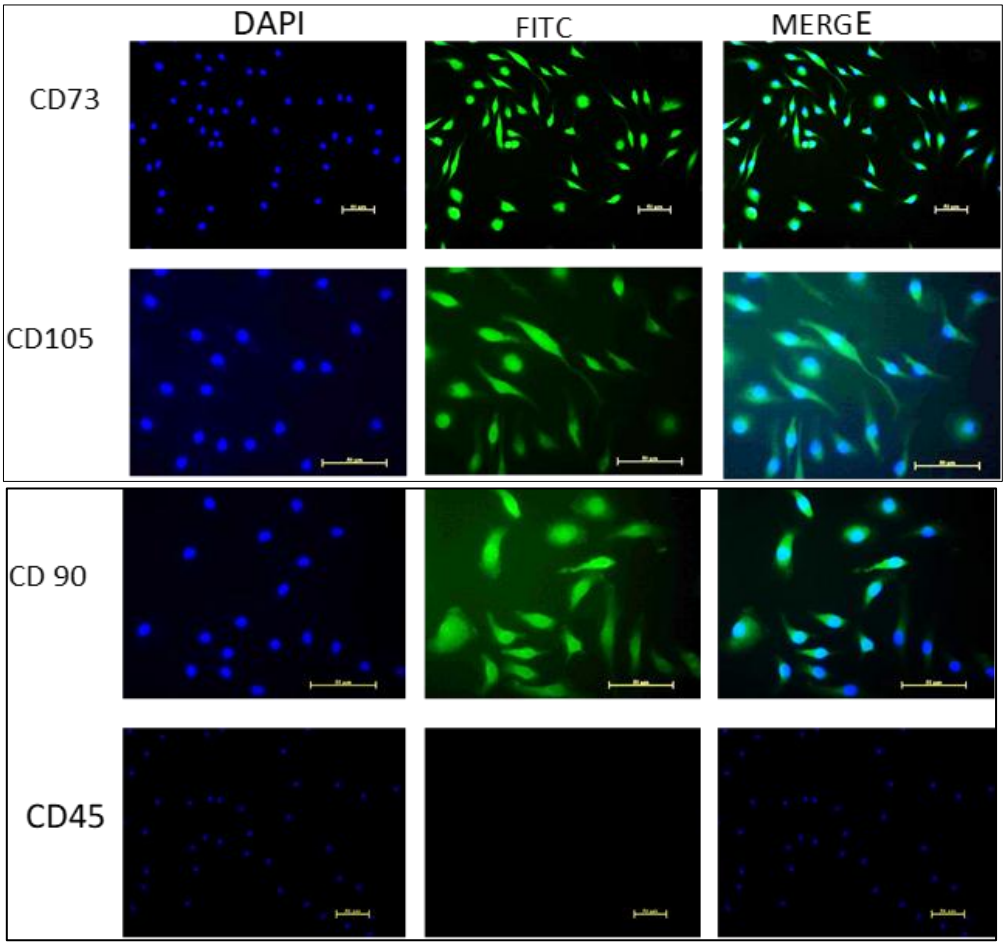

Fig 2b: Immunolocalization of surface antigens associated markers in bone marrow derived cells. Cells were stained with primary antibodies directed against CD73 (a-c), CD105 (d-f), CD90 (g-i) and CD45 (j-l) and further stained by FITC conjugated secondary antibodies (Scale bar 50 µm)

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Conflict of Interest

There is no conflict of interest among the authors.

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