Journal of Morphological Sciences
J Morphol Sci
Brazilian Society of Anatomy

Original Article
J Morphol Sci 2022;39:453-461

Technique of Isolation and Culture of Bone Marrow Mesenchymal Stem Cell in an Animal Model

Julie Christy Amalraj[1], G. Sumathy[2],Saraswathi Perumal[3]

[1]PhD Scholar ,Department of Anatomy BIHER Bharath Institute of higher education Seliyur, Tambaram Chennai
[2]Professor and head Balaji Dental College and Research Institute, Pallikaranai Chennai
[3]Professor Emeritus Tamil Nadu Dr. MGR Medical UNIVERSITY ,Guindy Chennai

Disclose and conflicts of interest: none to be declared by all authors

ABSTRACT

Introduction: the Bone Marrow Mesenchymal Stem cells are known for its regenerative properties. Mesenchymal progenitor Cells or progenitor cells are known for their special characteristics like pluripotency, plasticity, and stemness. It is also known for its self-renewal, immunomodulation, differentiation for various clinical problems. Flexibility of the mesenchymal progenitor cell to change into various lineages which is termed as plasticity. Stem cell plasticity has created great interest because of its potential therapeutic application in degenerative diseases, trauma and inherited diseases. Lineage priming is a molecular model of mesenchymal stem cell which has been studied extensively. This cell can be isolated from the animal as well as human bone marrow. This article clearly explains the requirements, method and cell line maintenance of the bone marrow mesenchymal stem cells and its differentiation into osteogenic progenitor cells

Keywords: Bone Marrow Mesenchymal Stem cells; Culture; Wistar albino rat.

Introduction

Bone marrow stromal contains haematopoietic precursors and non-haematopoietic cells that include the fibroblasts, endothelial cells and mesenchymal stem cells or mesenchymal progenitor cells. mesenchymal stem cells or progenitor cells are known for their special characteristics like pluripotency, plasticity, and stemness. Flexibility of the mesenchymal stem cell to change into various lineages is termed as plasticity. Stem cell plasticity has created great interest because of its potential therapeutic application in degenerative diseases, trauma and inherited diseases. Lineage priming is a molecular model of mesenchymal stem cell which has been studied extensively. Proliferating mesenchymal stem cells has been known to differentiate into various lineages like muscle, liver, nerve, endothelium, epithelium, bone, cartilage, tendons and so on. Specific lineage differentiation requires some transducers, Trans-activator proteins which give the incipient clone proliferation potential.

Stem cells in the presence of some of the effectors like cytokinin, morphogens and hormones bind to the activated receptor site enabling number of signalling pathways, key transcription factors, trans activating the differentiation-associated genes[1]. These genes include structural (cytoskeletal and extracellular matrix [ECM] proteins) and regulatory molecules enzymes, channels, transporters in turn activating the required differentiation.

Osteogenic differentiation of bone marrow mesenchymal stem cells has been well known entity. In vivo differentiation has been proved in various orthopaedic uses. This study involves the in vitro differentiation of bone marrow stromal cells into osteoblasts and its role in closure of cleft palate which is one of the most commonly encountered congenital maxillofacial deformities[4].

Rat as an Animal Model

Animals serve as experimental models for various diseases. They are often selected based on their similarity with the human genome pattern. The Wistar albino rats are best suited with many similarities to human genome. There is often a striking resemblance between cleft palate produced occurring in human beings and in rats by teratogenicity[2] They are animals exactly depicting the human palatogenesis and maxillofacial complex development. Its therefore ideal animal model for congenital maxillofacial growth disorders and deformities[3].

Animal selection: 160 -180 g weighing rat may be selected. The 6-8-week-old rats are preferable. Younger animals are utilised for collection of potent mesenchymal stem cells rather than senile animals. There was not much significant difference between both the sexes.

Methodology

The procedure was performed at the Stem Cell Culture Laboratory, Department of Biotechnology, Indian Institute of Technology, Madras after getting Animal Ethical Clearance (Institutional Ethical Committee and Animal Ethical Committee clearance obtained).

DOI: 10.51929/jms.39.453.2022
ISSN 2177-0298

Table 1. Materials Required for culture and cell line tagging

Materials for isolation of Bone Marrow Mesenchymal Stem Cells	Materials required for Culture of Bone Marrow Mesenchymal Stem Cells
Wistar Albino rats weighing 160-180g weight Ketamine and Xylocaine cocktail anesthetic drug Betadine for sterlising the surgical site BP Blade (11 size) Laminar flow to ensure sterile conditions DMEM (Dulbeco's Minimum Essential Medium F12, Invitrogen, USA) FBS (Fetal Bovine Serum, Invitrogen, USA) DPBS (Dulbaco's Phosphated buffer Solution, Invitrogen, USA)	High speed Cetrifuge Micropipette Co2 Incubator Phase contrast microscope Penstrip Non-essential Amino acid, L-Glutamine Sodium Pyruvate solution EDTA 0.25% Neubauer chamber Tryphan Blue test Basic fibroblastic growth factor

Table 2. List of Disposables and plasticwares

Plastic wares and Disposables
1. T25 Flask 2. T75 Flask 3. 5ml pipette 4. 10ml pipette 5. 200 microlitre tip 6. 35mm dish, 90mm dish 7. 15ml, 50ml centrifuge tubes 8. Cell scrapers 9. Cell strainers

Antibody panel CD 44, CD90, CD 29 positive and CD 45 negative cells.

(Invitrogen Antibodies with flow cytometry application, Monoclonal antibodies with different fluorochrome
(Catalogue numbers of Invitrogen antibodies used A14798 ALEXAFLOUR MR6404, CD45RA A14887 CD29 HAMSTER ANTI-MOUSE)

The wistar albino rats were anesthetized with Xylozaine and Ketamine cocktail as anaesthetic drug. 0.8ml of diluted drug cocktail and can be used for a 180 g weighing rat. The entire procedure is done at sterile conditions. Laminar flow with UV light sterilization is mandatory to avoid contamination from the surrounding. The surgical area was cleaned using betadine and incision was placed on the posterior aspect of the thigh and in the upper extremities. The femur and tibiae were removed and muscles, connective tissue were cleaned. The cut bones were rinsed in the phosphate buffer solution. DMEM (Dulbaco's minimum essential medium along with Fetal Bovine Serum). The culture medium is flushed into the medullary cavity of the long bones the flushed medium is collected in 15ml Eppendorf's tube. The flushed medium is centrifuged at a speed of 1250 rpm. The supernatant solution is discarded and the adherent cells are pipetted out into T75 and T25 flasks. The cells were retrieved and passaged. The first passage of the cells was done 21 days after the retrieval. The growths of the cells were observed day to day. The culture medium will be changed when the growth retards.

The Dulbaco's minimum essential medium (sigma Aldrich pvt ltd) was used in T25 and T 75 flasks. The cell growth was faster in T 75 due to available surface area. 10% fetal bovine serum was added along with 100mg/ml streptomycin to avoid microbial contamination in the culture medium. Foetal bovine serum is a know enrichment medium for active cell growth. The culture plates were incubated in Co2 incubator at 37 degree Celsius. Prior to every differentiation the cells were seeded at 15,000 cells/$cm^{2.}$

Based on the confluence of the cell growth they were split and grown in different flasks and allowed to differentiate. The osteoblastic differentiation was induced by the 10nm dexamethasone (cat no:H0888-14 sigma Aldrich), 200µm ascorbic acid(cat no A4403 sigma Aldrich) and 10 mM of glycerol 2-phosphate. The medium was changed twice a week.

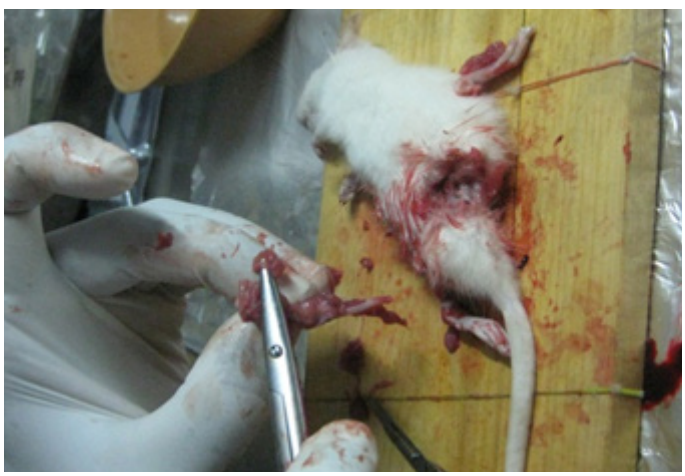


Figure 1. Incision on the lower limbs to remove the femur and tibia.

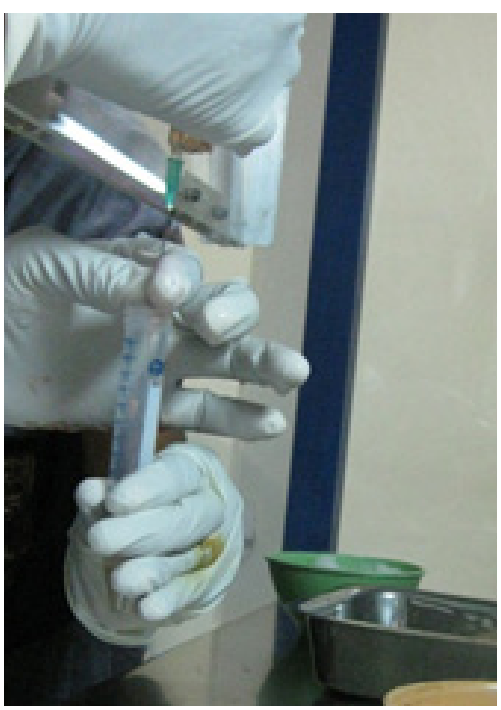


Figure 2. Flushing of the bone marrow from the long bones femur and tibia of the wistar albino rats.

Passaging of the Cells

Passaging is changing the Medium in the flask to eliminate contamination and unwanted cell growth. The medium was pipetted out and the flask was washed with DPBS (Dulbecco's phosphate Buffer Solution) to remove the remnants of the medium in the flask. Trypsin EDTA 0.25% 500 micro lire was added to the flask for de- attachment of the mesenchymal stem cells in the flask. Then the flask was incubated in Co_2 incubator for 1 minute at 37^0 C to enhance its activation. After tryptinisation the morphology of the cells turns round and motile. When viewed under the microscope. Then the cells are split into 1:5 ratios. The cells are allowed to grow in the flask.

Second passage was done at an interval of 25 days and 3rd passage at 11 days interval. After 85% confluence is reached. 4th passage was done at 6 days interval the time duration between the second and fourth passage was shorter than the time duration taken for 1st passage. This indicates that the cells take a longer time to adapt to the in vitro conditions. They required growth factors to fasten its growth. Basic FGF (Fibroblastic Growth Factor) was added to the medium to enhance the growth of the cells in vitro medium.

Chart 1. Chart showing the duration between the passages.

Passage number	Duration	Confluence of cells
Retrival	0	0
1st passage	12	70%
Second passage	25	80%
3-5	15	85%
6th	3	90%

Figure 3. showing passaging of the cells under laminar flow.

Figure 4. spent medium being discarded and fresh medium being added.

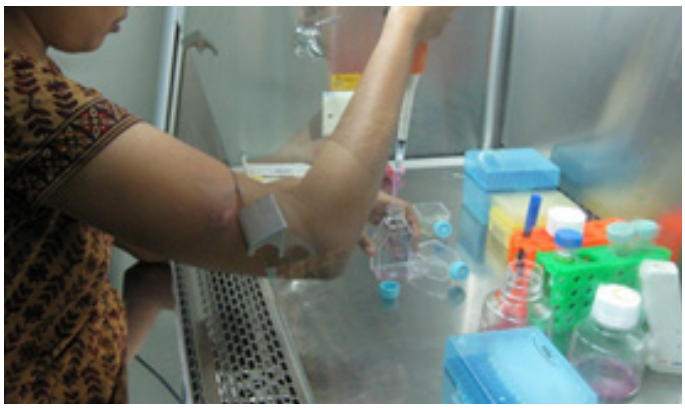


Figure 5. Fresh Medium being added to the flask.

Figure 6. Cells being carefully viewed to assess the growth.

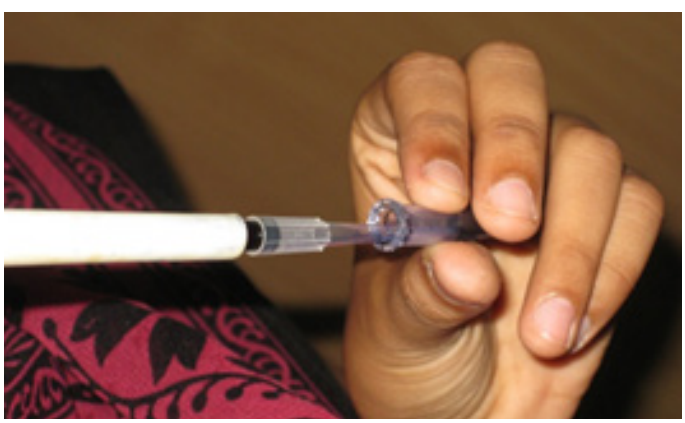


Figure 7. Tryphan blue staining to show the vitality.

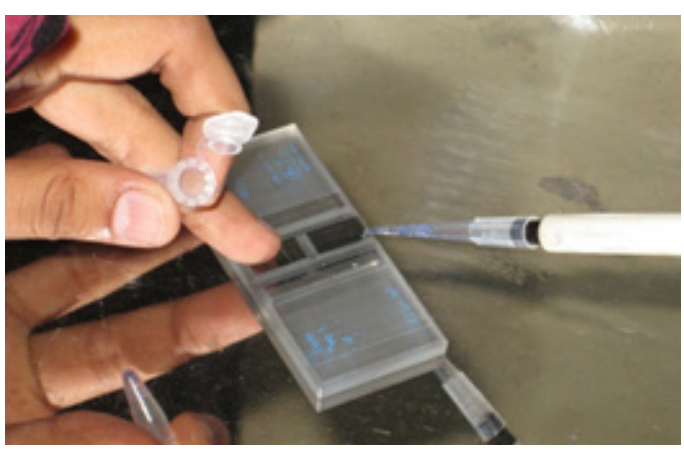


Figure 8. Neubauer chamber used to count the number of cells per ml.

Figure 9. Poly lactic co-glycolic acid bio scaffold International Singapore.

Flow Cytometry analysis

The 6th passage cells were analysed by flow cytometry which showed 100% positivity for CD 44 antibody. 90% confluent cells were centrifuged at 1250 rpm and the vitality was tested using Tryphan blue staining. The cell count was done using Neubauer's chamber. There were 18 million cells in 1ml of the centrifuged content.

Figure 10. Centrifuged Cells.

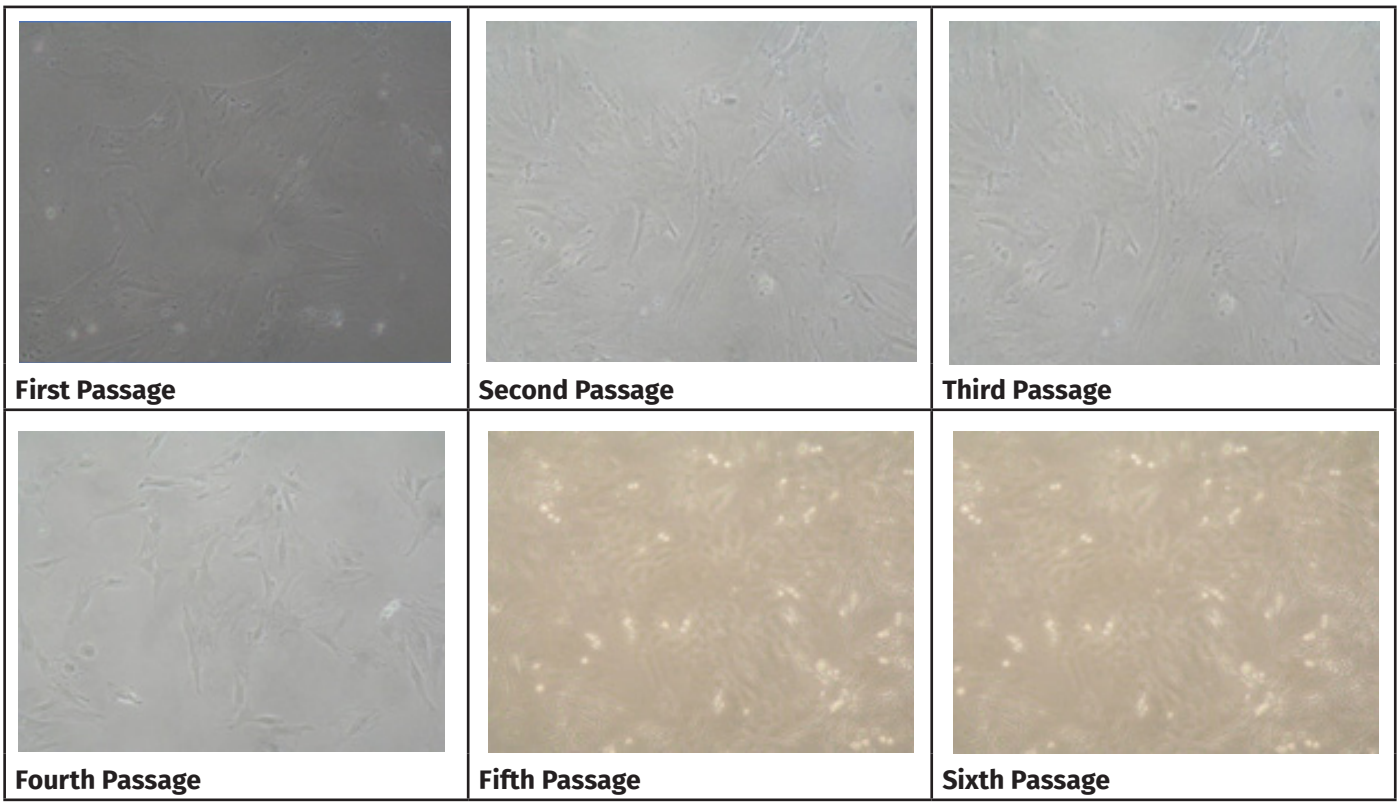


Figure 11. showing 1st to 6th passage (from left to right).

First Passage

The mesenchymal stem cells were fibroblasts like appearance. There cells were round in shape and mixed population. The ratio between the mesenchymal cells and the mixed population was small. The mesenchymal cells showed adherent to the flask. The mesenchymal stem cell had eccentric nuclei and process.

Second Passage

The second passage was done 20 days after the first passage. The growth was seen faster in T25 flask. The mesenchymal stem cells had begun to grow faster. This would have been due to the space available in the T 75 flask when compared to T 25 flask.

Third Passage

The cells were typical of adherent spindle and fibroblast-lik. However, under differentiation, these spindle-like cells growing as adherent cultures can be induced to grow in an attachment-independent fashion, forming spherical type with confluence. The morphology of cell clusters look like islet-like structure.

Fifth Passage

After being induced for 28 days, at least 40% of the MSCs showed remarkable morphological changes(Fig.29D), from having a typical fibroblast-like spindle appearance to having a round or polygonal shape as the same as epithelial cells morphology. The cells demonstrated growth characteristics resembling a monolayer culture of epithelial cells. However, after 14 days of being cultured, an average of 10% of the MSCs showed morphological changes. Non-induced MSCs cultured for 28 days in multiple layers were commonly observed in the control group; these retained the typical fibroblast-like spindle appearance.

Sixth Passage

The Cells grew faster and they appeared homogenous when compared to the earlier passages. The time duration between the 5^{th} and 6^{th} passage was only 3 days and 90% confluent cells showed 100% positivity for CD 44 monoclonal antibody when analysed by flow cytometry.

Fluroscent Activated Cell Sorting (FACS)

FACS analysis is the latest application used to sort the heterogeneous cell populations of bone marrow stroma. The 6^{th} passage cell with 90% confluency is tagged with fluorochrome antibodies. This staining is done by primary and secondary antibodies. The primary antibody is stained by a fluorochrome has specific wavelength. The laser beam in the flow cytometer detects the fluorochrome and separates the cells which carry the fluorochrome tagging in the cells. Based on the wavelength the colours differ.

The results of Flow Cytometry are expressed as a graph drawn with X axis as Forward Scatter Channel and Y Axis representing the Side Scattered Channel

Light that is scattered in the forward direction, typically up to 20^{o} offset from the laser beams axis, is collected by a lens known as the Forward Scatter Channel (FSC). The FSC intensity roughly equates to the particle's size and can also be used to distinguish between cellular debris and living cells. Light measured approximately at a 90^{o} angle to the excitation line is called side scatter. The side scatter channel (SSC) provides information about the granular content within a particle. Both FSC and SSC are unique for every particle, and a combination of the two may be used to differentiate different cell types in a heterogeneous sample. Based on the wavelength of the fluorochrome that is tagged the flowcytometry analyses the cell and separates it from the unstained cells. There is control isotype control added to the panel for comparison.

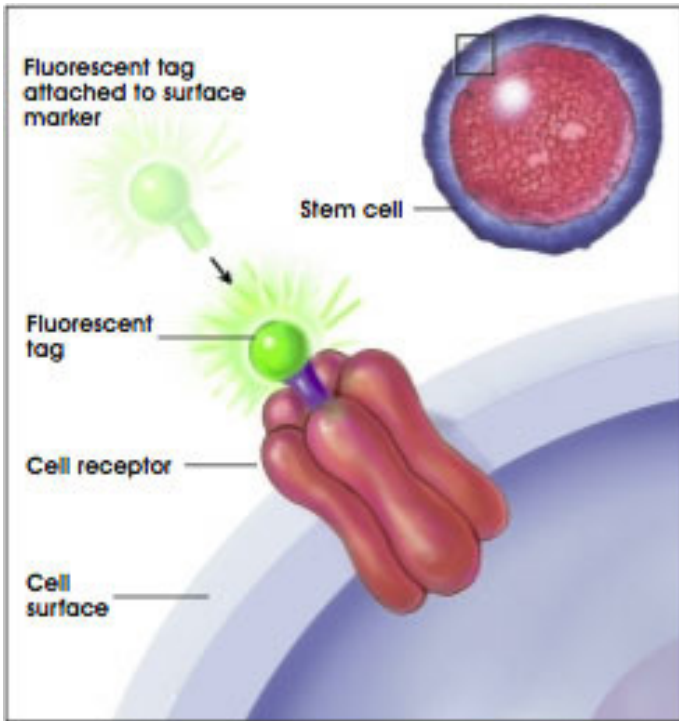


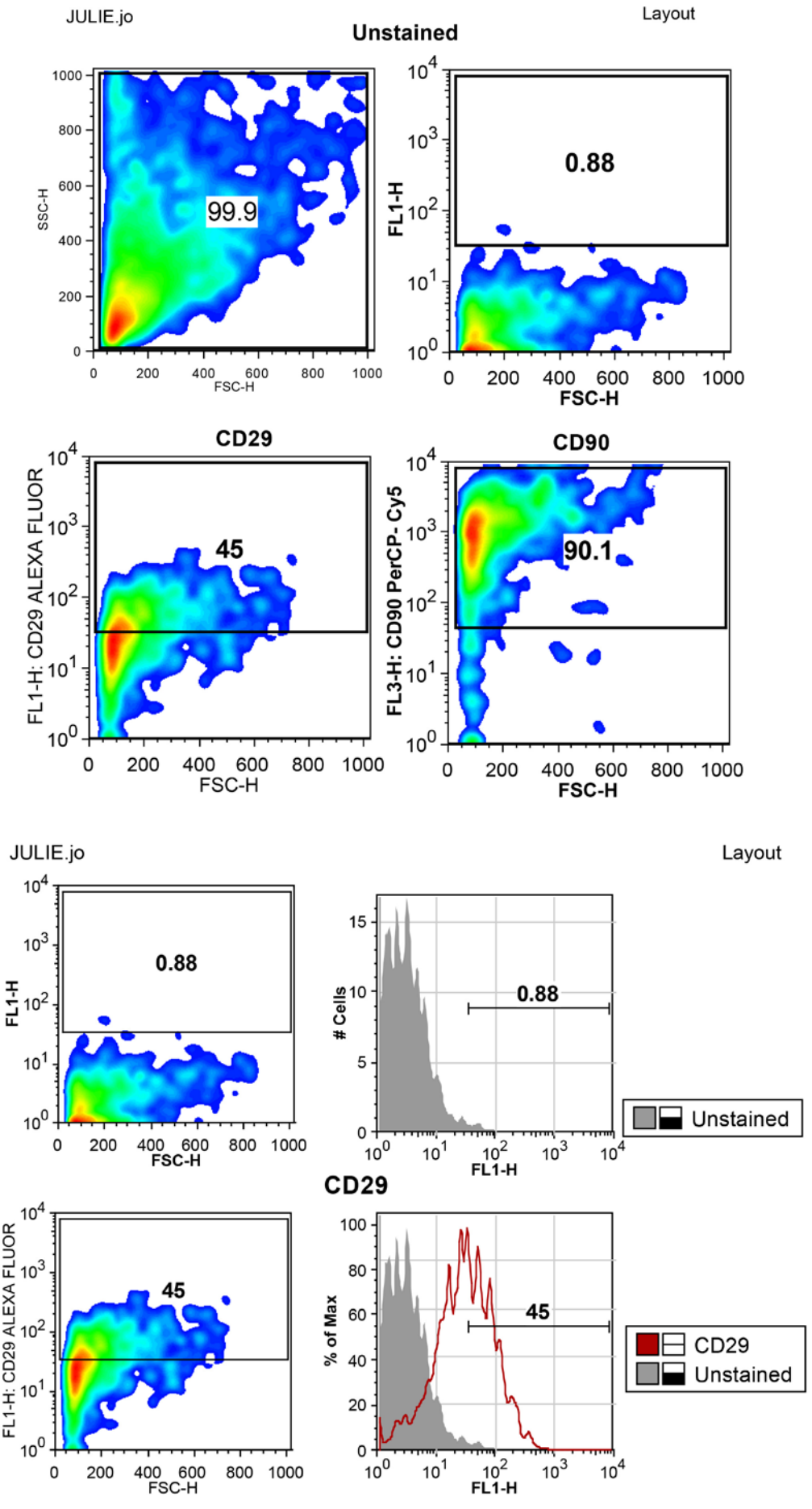
Figure 12. Image showing fluorochrome tagged antibody.

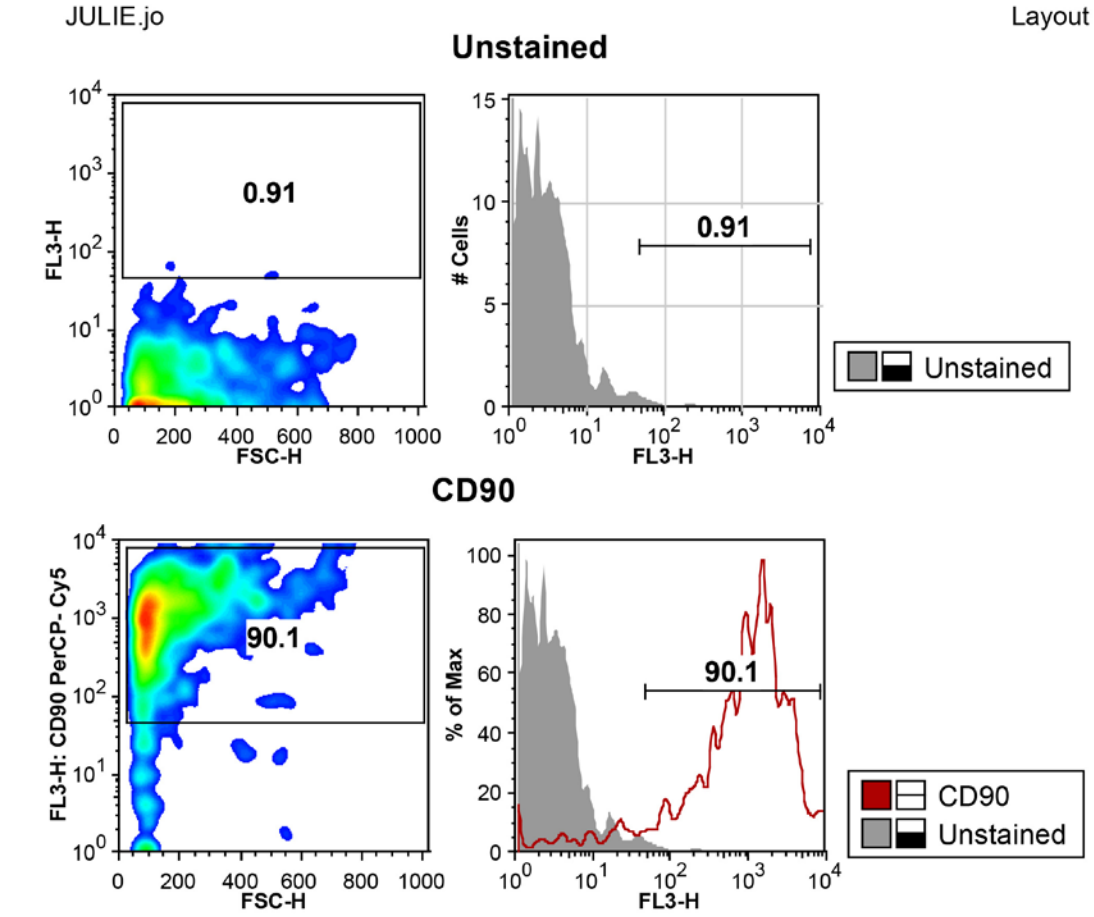
Antibody Selection

The antibody selection for Rodents or rat samples are usually rabbits, human or chicken, sheep, goa can be used. The can be unlabelled antibodies, enzyme conjugates (eg: alkaline phosphatase, Horse radish peroxidase) and Fluorescent conjugates Alexa flour, FITC, Phycoerthyrin, biotin etc)

Flow Cytometry Results

The Bone marrow Mesenchymal stem cell was differentiated into osteogenic cells tagged with antibodies were sorted and transplanted into Non syndromic cleft palate animal model for cleft palate reconstruction. Bone marrow mesenchymal stem cells used along with PLGA (polyglycolic acid) (Bioscaffold international, Singapore) was used. 1ml of the centrifuged cells was used along with scaffold diluted in normal saline and transplanted into the defect.


JULIE.jo
Layout
Unstained
SSC-H
FSC-H
99.9
FL1-H
0.88
CD29
FL1-H: CD29 ALEXA FLUOR
45
CD90
FL3-H: CD90 PerCP- Cy5
90.1
JULIE.jo
Layout
FL1-H
0.88
Cells
0.88
Unstained
CD29
FL1-H: CD29 ALEXA FLUOR
45
% of Max
45
CD29
Unstained



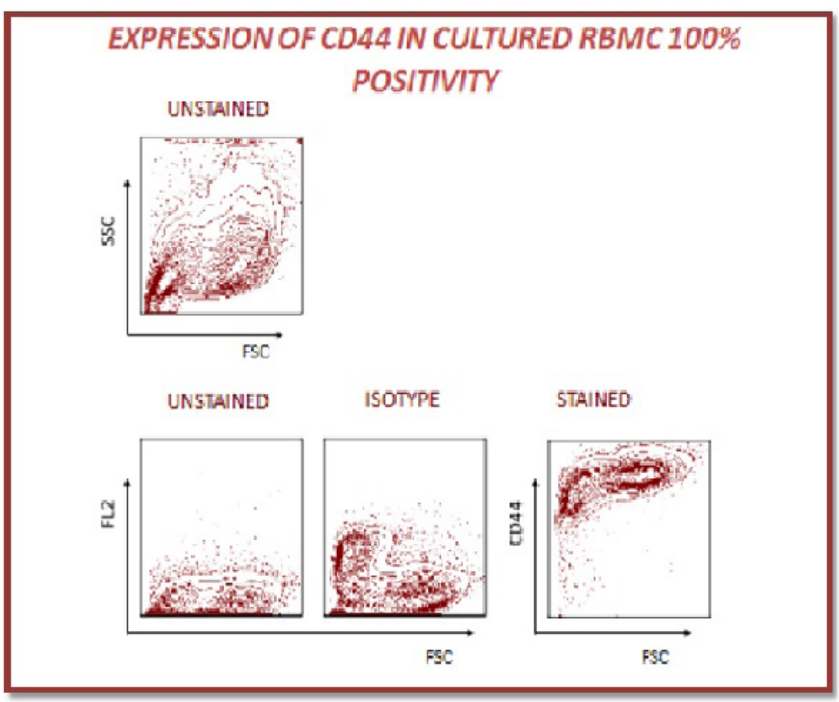


FIGURE 2a: CD 44 EXPRESSION IN CULTURED BONE MARROW MESENCHYMAL STEM CELLS

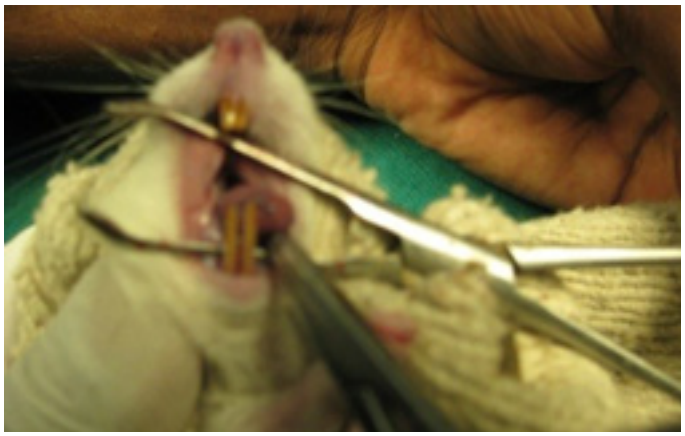


Figure 13. Preoperative palate with cleft defect.

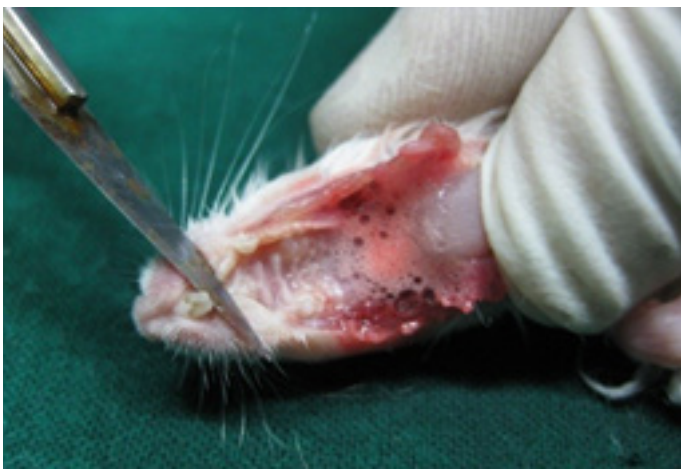


Figure 14. Post-operative picture with palatal cleft closure with bone marrow mesenchymal stem cells.

Discussion

The isolation of bone marrow msenchmyal stem cells was done from a 6 week old female wistar albino rat. According to Hui Hong et al in 2008[5] cell passage and age affects the differentiation potential of the cultured rat bone marrow mesenchymal stem cells. Hui Hong also states that significant interaction of donor age and passage reflects the culture from aged Donors[5]. Stem cell from younger rats had higher osteogenic differenciation potential, similarly cells from 4-6th passages had higher osteogenic differentiation potential (Kassis et al 2006)[7] when compared to the initial passages. According to Zhang et al[6] the rat bone marrow mesenchymal stem cells had large expansive potential and typical fibroblasts like appearance uniformly expressed positivity for CD 44 and CD45 Fibronectin and collagen. The cells used for the experiments were tested for viability using tryphan blue test. The vital cells were tagged with fluorochrome antibodies and analysed using flow cytometry. The cells exhibited positivity for CD 44, CD 90, Alexa flour and negativity for CD 45. The cells showing fluorochrome positivity were sorted by flow cytometry. The sorted cells were cultured in separate flasks with additions of basic fibroblastic growth factors. The growing medium was basic dulbaco's minimum essential medium with 10% foetal bovine serum. Addition of 10 mM dexamethasone, 200µm ascorbic acid (cat no A4403 sigma Aldrich) and 10 mM of glycerol 2-phosphate for osteogenic differentiation.

Colony Forming Units

The cells were arranged in colony forming units. There were around 25 cells/cm^2 50 cells/cm^2, and 75 cells/cm^2 were plated in 100-mm cell culture dishes and incubated for 10 days. The cells were stellate in shape and fibroblast like cells. Vitality of the cells were checked using trypan blue, dead cells were eliminated and population doubling time was estimated. The population doubling time between initial passages were longer when compared to the passages done at later phases[8,9].

The Osteogenic potential of mesenchymal stem cells proved its efficacy in faster closure of the cleft palate defect in wistar albino rats[10].

JC et al states the closure of the non syndromic cleft palate defect was rapid with transplantation of bone marrow mesenchymal stem cell when compared to the other techniques.

Conclusion

The bone marrow mesenchymal stem cell has been proved to be a better therapeutic solution for cleft palate in Wister albino rats. More studies in various other animal models and human trials are needed to bring it into current medical practise.

References

1. Omo-Aghoja VW, Omo-Aghoja L O, Antenatal determinants of oro-facial clefts in Southern Nigeria. African Health Sciences 2010; 10(1): 31 – 39
Pathol 55, 2–9.
2. Peters H, Neubuser A, Kratochwil K, Balling R (1998) Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. Genes Dev 12, 2735–2747
PMCID: PMC2825068
3. Schüpbach PM. Experimental induction of an incomplete hard-palate cleft in the rat. Oral Surg Oral Med Oral Pathol. 1983;55:2–9
4. Liu et al cleft palate cells can regenerate a palatal mucosa in vitro: J dent Res.2008 Aug; 87(8):788-792
5. Hui Hong et al (2008) Donor age and cell passage affects differenciation potential of murine bone marrow derived stem cells BMC cell biology 2008,9:60 doi:10.1186/1-471-2121.9-60
6. Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen YP (2002) Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. Development 129, 4135–4146
7. I Kassis, L Zangi, R Rivkin, Isolation of mesenchymal stem cells from G-CSF-mobilized human peripheral blood using fibrin microbeads Bone Marrow Transplantation (2006) 37, 967–976.
8. Roshan Adhikari,1,2 Chongxiao Chen,1,2 Elizabeth Waters,2,3

Franklin D. West,2,3 Isolation and Differentiation of Mesenchymal Stem Cells From Broiler Chicken Compact BonesFront Physiol. 2018; 9: 1892. Published online 2019 Jan 22. doi: 10.3389/fphys.2018.01892
9. Brown C, McKee C, Bakshi S, Walker K, Hakman E, Halassy S, Svinarich D, Dodds R, Govind CK, Chaudhry GR. Mesenchymal stem cells: Cell therapy and regeneration potential. J Tissue Eng Regen Med. 2019 Sep;13(9):1738-1755. doi: 10.1002/term.2914. Epub 2019 Jul 25. PMID: 31216380.
10. Amalraj JC, Gangothri M, Babu H. Reconstruction of Drug-induced Cleft Palate Using Bone Marrow Mesenchymal Stem Cell in Rodents. Ann Maxillofac Surg. 2017 Jan-Jun;7(1):82-88. doi: 10.4103/ams.ams_140_16. PMID: 28713741; PMCID: PMC5502521.

Received: July 10, 2022
Accepted: August 7, 2022

Corresponding author
Julie Christy Amalraj
E-mail: drjuliechristy@gmail.com