GROWTH CHARACTERISTICS AND EXPRESSION OF CD73 AND CD146 IN THE CELLS CULTURED FROM DENTAL PULP - A FLOW CYTOMETRY AND IMMUNOCYTOCHEMICAL STUDY

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CERTIFICATE

This is to certify that this dissertation titled "**GROWTH CHARACTERISTICS AND EXPRESSION OF CD73 AND CD146 IN THE CELLS CULTURED FROM DENTAL PULP - A FLOW CYTOMETRY AND IMMUNOCYTOCHEMICAL STUDY**" is a bonafide dissertation performed by **VAISHNAVI SIVASANKAR** under our guidance during the postgraduate period 2010-2013.

This dissertation is submitted to **THE TAMILNADU DR. M.G.R MEDICAL UNIVERSITY,** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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Vidyārambham karishyāmi, siddhirbhavatu mé sadhā ‖

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Cell culture is an amazing phenomenon. Watching life in its most simplistic form fuelled in me a passion for which I am forever grateful.

"Every experiment is a conversation with a prior experiment, every new theory a refutation of the old"- Siddhartha Mukherjee, *The Emperor of All Maladies*

CONTENTS

ABSTRACT

Background: Dental Pulp Stem Cells (DPSCs) in permanent teeth and Stem Cells from Human Exfoliated Deciduous (SHED) teeth are a source of adult mesenchymal stem cells**.** Very little is known of the similarities and differences between DPSCs and SHED during their early passage in cultures.

Aims and objectives: To culture stem cells from the pulp of deciduous and permanent teeth and compare their growth characteristics, morphology and immuno-phenotype using mesenchymal stem cell markers: CD146 and CD73 in their 1st, 3rd and 5th passage of culture.

Materials and Methods: 15 teeth were obtained for isolation of dental pulp of which 12 were permanent teeth and 3 were deciduous teeth. Growth characteristics, morphology and colony forming efficiency were assessed for the deciduous and permanent samples. Immunocytochemistry and flow cytometry using CD146 and CD73 was performed in deciduous and permanent samples in the 1st, 3rd and 5th passage of culture. Data was analyzed using SPSS ^{TM} software (version 17.0.0).

Results: Seven of the fifteen teeth cultured yielded sufficient cells for characterization. The time taken to reach confluency and the population doubling time was lower in SHED compared to DPSCs, and the colony forming unit efficiency was higher in SHED compared to DPSCs but the results were not statistically significant. The seeding efficiency was significantly higher in SHED compared to DPSCs (P=0.046). There was a decrease in mitotic phenotype and increase in post-mitotic phenotype in both deciduous and permanent samples from day 1 to day 8 of culture (P< 0.001). Immunocytochemistry using CD73 and CD146 did not show consistent results. Flow cytometry analysis using CD73 and CD146 showed varied expression between passages.

Conclusion: Cells isolated from the pulp of deciduous teeth and permanent teeth are a viable source of stem cells.

Keywords: stem cells, immunocytochemistry, flow cytometry, deciduous teeth, permanent teeth, CD73, CD146, phenotype

Introduction

Stem cells in the dental pulp originate from cells of the neural crest and display plasticity, multipotentiality and are a source of cells for the formation of mineralized dental tissue. They are called Dental Pulp Stem Cells (DPSCs) in permanent teeth and Stem Cells from Human Exfoliated Deciduous (SHED) in deciduous teeth. Many studies have established their self-renewal capability and also their ability to differentiate into neurogenic, osteogenic, dentinogenic, and myogenic cell lineages when grown in specific inductive media [1, 2, 3].

Stem cells (SCs) are identified based on the expression of surface proteins (markers). However, the ability of self-renewal is the ultimate way to show "stemness" [4]. Since there is a lack of a specific stem cell surface marker, the identification of Dental Pulp Stem Cells (DPSCs) relies on a panel of markers and biological features that include small cell volume, high proliferation potency, high clonogenicity, self-renewal, and potential to differentiate into multiple lineages. Dental pulp and bone marrow stem cell populations share similar putative stem cell surface markers: CD44, CD106, CD146, 3G5, and Stro-1 [5, 6]. It is generally accepted that cells that express CD44, CD90, CD73, CD105, STRO-1, and CD146 represent a mesenchymal stem cell population [7-9].

CD146 (MUC18 / Melanoma Cell Adhesion Molecule / MCAM) is a marker for mesenchymal stem cells isolated from multiple adult and fetal organs, and its expression is linked to multipotency. Mesenchymal stem cells with greater differentiation potential express higher levels of CD146 on the cell surface [10].

CD73 (Ecto-5'-nucleotidase) is a glycosyl phosphatidylinositol (GPI) - linked, membrane-bound glycoprotein expressed on different cell types, including vascular

endothelial cells and certain subtypes of lymphocytes. CD73 (recognized by the Monoclonal Ab SH3 and SH4), along with CD90 and CD105 is used to characterize mesenchymal stem cells. CD73 has been reported to be expressed in SHED and DPSCs [7, 11, 12].

Characterization of DPSCs and SHED is a vital step in establishing their role in tissue regeneration. The aim of this study was (1) to compare the growth characteristics of DPSCs and SHED and (2) to compare the morphological phenotype and (3) immuno-phenotype of DPSCs and SHED using CD73 and CD146 in the 1st, 3rd, and 5th passage of cell culture.

Aims and Objectives

AIM

To culture stem cells from the pulp of deciduous and permanent teeth and compare their growth characteristics, morphology and immuno-phenotype using mesenchymal stem cell markers: CD146 and CD73 in their 1^{st}, 3^{rd} and 5^{th} passages.

OBJECTIVES

- To isolate stem cells from the pulp of deciduous and permanent teeth by enzymatic disaggregation technique and culture them in α-MEM media.
- To compare the growth characteristics and population doubling time of stem cells isolated from the pulp of deciduous and permanent teeth.
- To assess the proliferative capacity of stem cells isolated from pulp of deciduous and permanent teeth using colony forming unit assay (CFU).
- To characterize the morphology of stem cells isolated from the pulp of deciduous and permanent teeth.
- To characterize the immuno-phenotype of stem cells isolated from the pulp of deciduous and permanent teeth using mesenchymal stem cell markers - CD73 and CD146 in the 1^{st}, 3^{rd} and 5^{th} passage of subculture using flow cytometry.

HYPOTHESIS

Cells isolated from the pulp of deciduous teeth and permanent teeth are a viable source of stem cells.

Materials and Methods

MATERIALS

Materials for tissue culture

Reagents:

1. Growth medium: α- modified minimal essential medium (*α*-MEM)
2. Fetal Bovine Serum (Invitrogen ™)
3. Antibiotics:

    -Penicillin-100 IU/ml.

    -Streptomycin-100μg/ml.
4. Dulbecco's- Phosphate Buffered Saline (D-PBS) (Potassium chloride-0.2g/l, Potassium phosphate monobasic- 0.2g/l,Sodium chloride-8g/l, Sodium phosphate dibasic-1.15g/l)
5. Distilled water
6. De-ionized water
7. Collagenase (type I, filtered) (CLS-1- Worthington Biochemical Corporation ™)
8. Dispase (neutral protease, grade II) (Roche ™)
9. Trypsin 1:125 (Tissue culture grade, Hi media ™)
10. Ethylene-di-amine-tetra-acetic acid (Hi Media ™)

Equipment:

1. Culture dishes (Tarsons™)
2. 24-well plates (Cell star™)
3. Disposable pipettes and pipette tips
4. Glass pipettes

5. BP blade no. 15
6. Centrifuge tubes
7. Leak-proof screw-cap vials
8. Scott Duran bottles
9. Laminar flow cabinet
10. Carbon dioxide incubator. (Thermo electron Corporation. Forma series II water jacketed-HEPA class 100)
11. Phase contrast microscope. (Olympus CKX41™)
12. Digital camera. (Kodak AF3X, 8.2 mega pixels, 3x optical zoom)
13. Improved Neubauer counting chamber
14. Laboratory centrifuge (R-86 Remi™)
15. Cyclomixer (C101 Remi ™)
16. Electronic balance (Dhona 200D ™)
17. Prabivac vacuum pump
18. Cellulose acetate filter (pore size 0.2µm)
19. Autoclave
20. Hot air oven
21. Micromotor (Marathon ™)
22. Contra-angled Hand piece (NSK ™)
23. Carborundum discs
24. Chisel
25. Mallet

Materials for immunocytochemistry and flow cytometry

Reagents

1. Antibodies (Abcam ™)
    a. Mouse monoclonal [4G4] to CD73 **[Annexure – I]**
    b. Mouse monoclonal [P1H12] to CD146 **[Annexure – II]**
    c. Rabbit polyclonal secondary antibody to mouse IgG – H&L (HRP) **[Annexure – III]**
2. SuperSensitive™ One-step Polymer-HRP Detection System (Biogenex)
3. Sodium azide (Loba chemie ™)
4. Bovine Serum Albumin (BSA) (Hi media ™)
5. Glycerol for molecular biology (SRL ™)
6. Phosphate buffered saline (sodium chloride 7.714g/l, dipotassium hydrogen ortho phosphate 1.496g/l , potassium dihydrogen orthophosphate .204g/l)
7. Acetone (Merck ™)
8. APES (3-aminopropyl-triethoxy-silane)
9. Paraformaldehyde (chenchems)
10. Hydrochloric acid (Merck ™)
11. Sodium hydroxide
12. DPX (distrene , dibutyl phthalate, xylene)

Equipments

1. Glass slides
2. Micro centrifuge tubes (Tarsons™)

3. Cryo boxes (Tarsons^{TM})
4. Couplin jars
5. Humidified chamber
6. Electronic timer
7. Cover slips
8. Light microscope
9. Flow Cytometer (BD Bioscience)

METHODOLOGY

SPECIMEN COLLECTION

Extracted third molars, exfoliating/extracted deciduous teeth, and teeth extracted for orthodontic treatment were obtained from the patients who reported to Ragas Dental College and Hospital, Chennai. 15 teeth were obtained for isolation of dental pulp of which 12 were permanent teeth and 3 were deciduous teeth. Informed consent was obtained from patients above 18 years and from the parents of children for the collection of extracted teeth **[Annexure IV]**.

INSTITUTIONAL REVIEW BOARD APPROVAL

The study was approved by the institutional review board (IRB) of Ragas Dental College and Hospitals **[Annexure V]**. The protocol has been submitted to the IRB **[Annexure VI]**.

Inclusion criteria:

- Extracted third molars, exfoliating/extracted deciduous teeth, and teeth extracted for orthodontic treatment, trauma or periodontal disease
- Freshly extracted teeth immediately transferred to transport medium until pulp extirpation.
- Teeth with vital pulpal tissue

Exclusion criteria:

- Teeth with evidence of decay or pulpal necrosis.
- Extracted/exfoliated teeth that have not been transferred to transport media within 15 minutes of extraction.

SPECIMEN TRANSPORTATION

Teeth extracted under sterile condition were rinsed in saline and transferred to serum-free α-Minimal Essential Medium(α-MEM), with 5X antibiotics (Penicillin-100 IU, Streptomycin-100µg/ml), at a pH of 7.2 to 7.4 and maintained at 4°C with the help of ice-packs. They were transported in leak-proof, sterilized culture vials.

ISOLATION AND PROCESSING OF TISSUE

a. Tooth surface was cleaned well by immersing the tooth in povidone iodine solution for 30 seconds and washing thrice with Dulbecco's Phosphate Buffered

Saline (D-PBS). Deciduous teeth were not subjected to povidone iodine decontamination as the root was resorbed and the pulp was exposed.

b. Grooves were placed around the cemento-enamel junction with a carborundum disc and ice-cold D-PBS irrigation to avoid heating while cutting.
c. Tooth was split with chisel and mallet to expose the pulp chamber.
d. The pulp tissue was obtained from the pulp chamber with the help of forceps and spoon excavator and put into 2ml of α-Minimal Essential Medium(α-MEM) on a Petri dish (60mm diameter) to prevent the tissue from drying.

PRIMARY CULTURE OF DENTAL PULP CELLS

a. The dental pulp tissue was minced into tiny pieces (approx. $1mm^3$ in size) with a surgical blade. Pulp tissue from deciduous teeth was directly taken for enzyme disaggregation.
b. The tissue was immersed into 1 ml of α-Minimal Essential Medium (α-MEM) containing collagenase (2mg) and dispase (1mg).
c. It was incubated at 37^0C and 5% CO_2 for up to 4 hours for permanent teeth and one hour for deciduous teeth. Mechanical tapping facilitated enzymatic disaggregation.
d. Cells were centrifuged at 2400 rpm for 5 minutes.
e. The supernatant was removed and the pellet re-suspended in α-Minimal Essential Medium (α-MEM) containing 15% Fetal Bovine Serum (FBS) and 1X antibiotics and plated in a 60mm petri dish.

f. The cells were maintained at 37^0C and 5 % CO_2 in the incubator.

g. Forty-eight hours after the cell isolation, the culture media was discarded and fresh media added to the Petri dish. Media change was repeated every third day until cell confluence was reached.

SUBCULTURE

Five to seven days after the cell isolation, colonies were identified in the culture plates, where the cells had a typical spindle / fibroblastoid shape. The cells were sub-cultured after they reached 70% confluency on a 60mm culture grade petri dish. The number of days taken for the primary culture to reach confluency was recorded for each sample. The plating density was ~12 x 10^5 cells/plate.

a. The culture was examined carefully for signs of deterioration or contamination.

b. The media was discarded from the petri dish.

c. Two washes with 2ml D-PBS was done to remove any residual serum.

d. 1ml trypsin 0.25% with EDTA 0.05% was added to the Petri dish (60mm diameter).

e. The monolayer was checked under the microscope to check for detachment and rounding-up of the cells.

f. The plate was tapped at the bottom until all the cells were detached.

g. Cells suspended in trypsin was collected in a centrifuge tube and centrifuged at 2400 rpm for 3 minutes.

h. Supernatant obtained after centrifugation was discarded. To the remaining cell pellet, the medium was added and cells were dispersed by repeated pipetting.

i. The cells were counted in a haemocytometer.

j. The cell suspension was diluted to appropriate seeding concentration by adding adequate volume of medium.

k. Split ratios for subculture were 1:2. In passage 5, the split ratio was set to 1:3.

l. The petri dish was closed and returned to the incubator.

PHENOTYPIC CHARACTERIZATION

f I, f II, f III, f IV, f V, f VI and fVII (mitotic and post-mitotic) phenotypes [13,14]

a. Cell lines from the fourth passage were plated on three 60 mm tissue culture Petri dishes at a concentration of 0.5×10^4 cells /ml.

b. Using a phase contrast microscope (20x magnification), 30 cells from each dish (90 cells in total) were observed and counted for eight consecutive days and classified morphologically as mitotic (f I, f II, f III) and post-mitotic (f IV, f V, f VI, f VII) phenotypes as described by Bayreuther et al.[13] The F1 (spindle shaped, diploid), F2 (epitheloid, diploid) and F3 (stellate, tetraploid) described by Mollenhauer and Bayreuther (1986) [14] was also assessed.

FLOW CYTOMETRY

a. Cells in the 1^{st}, 3^{rd}, and 5^{th} passage were trypsinized, counted and resuspended to approximately $1\text{-}5 \times 10^6$ cells/ml in ice cold PBS, 10% FBS and 1% sodium azide.

b. 100 µl of cell suspension was added to each tube along with 0.1-10 µg/ml of the primary antibody (CD73 or CD146).
c. The tubes were incubated overnight at 4^{o}C in the dark.
d. The cells were washed 3-times by centrifugation at 1500 rpm for 5 min and resuspended in ice cold PBS.
e. The fluorochrome-labeled secondary antibody (FITC conjugated IgG) in 3% BSA/PBS was used to resuspend the cells.
f. The tubes were incubated for 2 hrs at room temperature in the dark.
g. The cells were washed 3-times by centrifugation at 1500 rpm for 5 min and resuspended in ice cold PBS, 3% BSA and 1% sodium azide.
h. Analysis: The cells were analyzed on the flow cytometer (BD Bioscience). Histograms of **10,000 events** (cells analyzed) each were obtained from flow cytometric analysis of unstained, isotype control (secondary antibody only) and stained samples. The control was gated using FlowJo software ™ and the same gate was applied to the corresponding stained sample for determining the percentage of positives.

IMMUNOCYTOCHEMISTRY

Cells were fixed on APES (3-aminopropyl-triethoxy-silane) coated slides using methanol and immunologically stained for CD 146 and CD73.

Protocol for growing and fixing of cells on APES coated slides

a. Slides soaked in soap-water for 2 hours

b. Slides washed thrice in tap water

c. Soaked overnight in 1/10 N hydrochloric acid

d. Slides washed thrice in distilled water

e. Slides baked in hot-air oven for 4 hours at 60ºC

f. Slides dipped in 50ml acetone for 2 minutes

g. In 2% APES for 5 minutes

h. Two dips in distilled water

i. Slides autoclaved

Growing cells on a slide

a. Autoclaved APES coated slides were transferred to a 10mm petri dish.

b. Cells in the 1^{st}, 3^{rd} and 5^{th} passage were trypsinized, resuspended and plated.

c. Cells were allowed to grow to confluence with the addition of fresh media.

Fixation

a. The cells were washed thoroughly (5x2 min) in PBS and fixed with methanol for 5-10 minutes.

b. The slides were rinsed (3x5 min) in PBS and stored at -4°C.

Endogenous Peroxidase Blocking Step

The endogenous peroxidase was blocked by incubating in 3% H_2O_2 in PBS for 10-30 minutes (This step is required only if an HRP conjugated secondary antibody is used.)

The slides were rinsed (3x5min) in PBS.

Blocking of Non-specific Binding

Protein block was done with 2-5% normal serum in PBS for 1 hour. Normal serum should

be the same species from which the secondary antibody was raised. (Alternatively, 5% BSA is sometimes used as blocking agent.)

Primary antibody incubation

The primary antibody was diluted to the recommended concentration in 1% normal serum and PBS.

The primary antibody was added to each well and incubated overnight at 4°C.

The primary antibody solution was removed and the slides were rinsed (3 x 5 min) in PBS.

Secondary Antibody Incubation

The HRP – conjugated secondary antibody was diluted in 1% BSA diluent.

Excess fluid was removed from the slide. Secondary antibody solution was added to each slide and incubated for 1 hour at room temperature.

The slides were rinsed (3 x5 min) in PBS and the excess fluid was removed.

Color Development

The chromogen, 3, 3'-Diaminobenzidine (DAB) solution was added to each slide. Once the cells started turning brown (this can be observed under a microscope), the slides were washed (2 x 5 min) in PBS.

Counter-stain

The slides were dipped into a staining dish of hematoxylin for 30 seconds.

The slides were removed and placed into an acid bath (200ml distilled water and 1-3 drops of acetic acid).

The slides were rinsed with distilled water.

Cover Slips

Cover slips were added to the slides for examination under the microscope.

ESTIMATION OF GROWTH CURVE AND ITS DERIVATIVES

a. Cells were inoculated at 1.2×10^4 cells /ml/well on 24-well plates
b. After overnight attachment, cells from 3 randomly selected wells were trypsinized and counted using a haemocytometer.
c. The medium was changed on the 3^{rd} and 6^{th} day.
d. The count was repeated every 24 hours for 8 days.
e. Cells from each well were counted thrice to avoid error.
f. The averages of daily cell counts of each well were used to plot the growth curve.
g. The seeding cell count and cell count on the first day (i.e. 12 hours of seeding) was used to estimate the seeding efficiency in percentage by using the equation

$$\frac{\text{Cell count/well/ml after 12 hours} \times 100}{\text{Seeding cell count/well/ml}}$$

h. The growth curve was plotted and population doubling time (PDT) derived from the exponential growth phase.

CLONAL ASSAY AND ESTIMATION OF COLONY FORMING EFFICIENCY

a. Fifth passage cells were seeded at 40 cells/40mm petri dish in triplicate.
b. The cells were cultured for 14 days with fresh media being added after 7 days.
c. The plates were then washed with PBS and stained with 3% Crystal Violet at room temperature for 30 minutes.
d. All colonies greater than 2 mm (with >50 cells) in diameter were counted.
e. The CFU assay was performed in triplicate for each donor.
f. Colony forming efficiency =

$$\frac{\text{Total no. of colonies}}{\text{Initial no. of cells seeded}} \times 100$$

STATISTICAL ANALYSIS

Data analysis was done using SPSS ™ (statistical package for social science) version 17.0.0.

Linear regression analysis was used to derive the slope from growth curves of each cell populations for determination of the population doubling time.

Mann-Whitney Test was done to compare the colony forming unit efficiency, seeding efficiency and the population doubling time between the permanent and deciduous tooth derived cell populations.

Correlation coefficient was used to compare the morphological phenotypes between the permanent and deciduous tooth derived cell populations.

Review of Literature

STEM CELLS: DEFINITIONS

Potten and Loeffler in 1990 defined *stem cells as undifferentiated cells capable of, (a) proliferation, (b) self maintenance, (c) the production of a large number of differentiated, functional progeny, (d) regenerating the tissue after injury, and (e) a flexibility in the use of these options.*[15]An amended definition was later introduced in view of changing concepts and data obtained in the field of stem cell research. According to this *Stem cells of a particular tissue are: (1) a potentially heterogeneous population of functionally undifferentiated cells, capable of: (2) homing to an appropriate growth environment; (3) proliferation; (4) production of a large number of differentiated progeny; (5) self-renewing or self-maintaining their population; (6) regenerating the functional tissue after injury with (7) flexibility and reversibility in the use of these options*.[16] The amended definition of tissue stem cells gave the following features a greater emphasis than previous definitions: a shift from the cellular view to a system view; emphasizing stemness as a capability rather than as a cellular property; including the growth environment; emphasizing within-tissue plasticity; considering functionality of the tissue stem cells and the tissue; extension of self-maintenance to self-renewing capability [16].

This is in contrast to *Maturing cells*, which can be defined as cells with full expression of functional differentiation markers, no capability of proliferation, no capability of self-renewal or self-maintenance, and hence, no ability to regenerate tissue after injury. *Transit cells* can be defined as a cell stage which is intermediate between

stem cells and maturing cells. Loeffler and Roeder in 2002 defined transit cells by the following criteria: they are characterized by the onset of differentiation marker expression during their development which is, however, not mandatory, they are capable of proliferation, and they do not self-maintain or self-renew [16].

One cell type stems from the other and hence the term "stem cell." The word "stem" originated from botany, from the same terminology as the stems of plants, where stem cells were demonstrated in the apical root and shoot meristems that were responsible for the regenerative potential of plants [17].

PROPERTIES OF STEM CELLS: A BASIS FOR CLASSIFICATION

A stem cell is defined by three basic properties: 1. the ability to self-renew while being maintained in a state of undifferentiation and 2. The capacity to generate functionally differentiated progeny and 3. *In vivo* functional reconstitution of a given tissue.[18] Stem cells in any tissue are a self-renewing population, achieving this by asymmetric division. Each stem cell division gives rise to one replacement stem cell and one transit amplifying cell (TAC) which eventually differentiates into a committed cell of a specific lineage, thereby maintaining the stem cell pool.

Multi-lineage differentiation refers to the capacity of a single population of stem cells to differentiate into at least two distinctively different cell types.[19] Based on their differentiation capability, stem cells are classified as: (1) pluripotent stem cells, capable of differentiating into any of the three germ layers comprising an organism i.e. ectoderm, endoderm and mesoderm, and (2) multipotent stem cells, capable of differentiating in to

cells of one tissue or germ layer[18]. The multipotency of stem cells is reduced over time due to progressive gene silencing. Genes active in earlier progenitors are gradually silenced at developmentally later stages, and subsets of cell type-specific genes are turned on eventually leading to lineage commitment [20].

The stem cell microenvironment

The success of stem cells culture is governed by its micro-environmental niche. The concept of a niche was introduced by Schofield (1978) as a physiologically limited microenvironment that houses stem cells[21]. The ultrastructure of each niche is essentially composed of cells, extracellular matrix, and the 3-dimensional spaces they form along with paracrine regulation by secreted proteins and other non-cellular components which regulate metabolism. The constraints of this architectural space, physical proximity of the neighboring cell membranes and extracellular matrix through tethering molecules, signal interactions at the niche interface, paracrine and endocrine signals, neural regulation and metabolic activity form the key elements of the niche microenvironment[22].

SOURCES OF STEM CELLS

The origin of the stem cell is the basis for classification into 4 types- stem cells from embryos; stem cells from the fetus; stem cells from the umbilical cord; and stem cells from the adult.[17] Stem cells can also be classified broadly into embryonic/fetal and adult stem cells. Prior to birth, the embryonic stem cells play a crucial role in organogenesis, development and growth. After birth, adult stem cells support tissue regeneration by replenishing cells lost naturally (apoptosis) or in injury, and form an

important component of tissue homeostasis. Adult derived stem cells or precursor cells are divided into three categories based on their potential for differentiation. These three categories of precursor cells are epiblast-like stem cells, germ layer lineage stem cells, and progenitor cells [23].

Embryonic stem cells

Human embryonic stem (ES) cells are undifferentiated cells derived from the inner cell mass of blastocyst stage embryos. They are unique in their capacity to self-renew indefinitely in culture, while maintaining a normal phenotype, and remaining pluripotent, namely, harboring the capacity to differentiate into multiple cell types of the three germ layers. Three kinds of mammalian pluripotent stem cell types have been described: embryonic stem (ES) cells, embryonic germ (EG) cells derived from primordial germ cells, and embryonal carcinoma (EC) cells derived from teratocarcinomas. Embryonic stem cells have unlimited self-renewal and differentiation potential. However, the use of human embryonic stem cells poses ethical issues and risk of teratoma formation[18].**Embryonic germ cells** are primordial germ cells or diploid germ cell precursors that transiently exist in the embryo, before they closely associate with somatic cells of the gonads and then become committed as germ cells. These stem cells are pluripotent and are able to produce cells of all three germ layers. **Fetal stem cells** are primitive cell types found in the organs of fetuses. Fetal blood, placenta and umbilical cord are rich sources of fetal hematopoietic stem cells. **Umbilical cord stem cells** are circulating hematopoietic stem cells. The frequency of umbilical cord blood hematopoietic stem cells equals or exceeds that of bone marrow and they are known to produce large

colonies *in vitro*, have long telomeres and can be expanded in long term culture. Cord blood shows decreased graft versus host reaction compared with bone marrow. Cord blood stem cells have been shown to be multipotent.

Adult stem cells

Adult stem cells also satisfy the criteria of a stem cell, but their self-renewal and differentiation potential is more restricted. They can differentiate into specific cell lineages i.e. they are multipotent. They have also been found to have the capacity to differentiate into a lineage different from which the cells are derived, a property called stem cell plasticity. A population of pluripotent stem cells, termed multipotent adult progenitor cells has been isolated from the bone marrow and more recently in other adult tissue as well.

Hematopoietic stem cells

Bone marrow possesses stem cells that are hematopoietic and mesenchymal in origin. Bone marrow stem cells are very plastic and versatile because they are multipotent and can be differentiated into many cell types both *in vitro* and *in vivo*.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are found postnatally in the non-hematopoietic bone marrow stroma. Apart from bone marrow stroma, MSCs can also be derived from periosteum, fat and skin. MSCs are multipotent cells that are capable of differentiating into cartilage, bone, muscle, tendon, ligament and fat.[17]

MESENCHYMAL STEM CELLS

S. Sethe *et al.* in 2006 defined Mesenchymal Stem Cells (MSCs) as post-embryonic, bone-marrow derived cells, naturally capable of multipotent differentiation into connective tissue of nonhaematopoietic lineage; in particular bone, ligaments, tendons, fibers, cartilage, and adipose tissue.[22] MSC was initially identified in bone marrow, but later have also been isolated from several other tissues such as adipose tissue, periosteum, tendon, synovial fluid, skin, amniotic fluid, umbilical cord, umbilical cord blood, brain, spleen, liver, kidney, lungs, muscle, thymus and pancreas, menstrual blood, and testes.[24]

Virtually all craniofacial structures such as cartilage, bone, ligaments, cranial sutures, musculature, tendons, the periodontium, and the teeth are derivatives of mesenchymal cells. Once migrated, Mesenchymal Cells and mesodermal cells, both derivatives of embryonic stem cells, work synergistically in the morphogenesis of craniofacial structures. Mesenchymal cells undergo asymmetric division, with one offspring cell differentiating toward an end-stage cell, while the other replicates into an offspring mesenchymal cell. Residual offspring of mesenchymal cells, upon the completion of morphogenesis, continue to reside in various craniofacial tissues, and retain their status as stem cells. After birth, mesenchymal cells are called 'mesenchymal stem cells' (MSCs). In the adult, MSCs maintain physiologically necessary tissue turnover and, upon injury or disease, differentiate to partake in tissue regeneration.[25]

STEM CELLS FROM CRANIOFACIAL TISSUES: DENTAL PULP AS A SOURCE

Craniofacial stem cells resemble bone marrow MSCs, especially in terms of their differentiation capacities. Mesenchymal stem cell populations have been identified in the dental pulp of adult teeth, exfoliated deciduous teeth, periodontal ligament, dental follicle and apical papilla[4].

Dental Pulp Stem Cells (DPSCs)

In the year 2000, Gronthos and co-workers isolated stem cells from the human dental pulp (DPSCs). Dental pulp stem cells (DPSCs) have been isolated from extracted human third molars and are morphologically and phenotypically similar to mesenchymal stem cells of the bone marrow, capable of self-renewal and multipotential differentiation. MSCs In the dental pulp are thought to reside in a perivascular niche.[1-4]

Stem Cells from Exfoliated Deciduous Teeth (SHED)

Stem cells from human exfoliated deciduous teeth (SHED) have been identified as a population of postnatal stem cells capable of differentiating into osteogenic, odontogenic, adipogenic cells, and neural cells. *In vivo* SHED cells can induce bone or dentin formation but, in contrast to dental pulp, DPSC failed to produce a dentin-pulp complex.[4]

Periodontal ligament stem cells (PDLSC)

The PDL is a specialized tissue located between the cementum and the alveolar bone and has as a role the maintenance and support of the teeth. Its continuous regeneration requires maintenance by progenitor cells thought to arise from the dental

follicle. PDL contains STRO-1 positive cells that maintain certain plasticity and can differentiate into adipogenic, osteogenic and chondrogenic phenotypes *in vitro*. It is thus obvious that PDL itself contains progenitors, which can be activated to self-renew and regenerate other tissues such as cementum and alveolar bone.

Stem cells from the dental follicle (DFSC)

DFSC have been isolated from follicle of human third molars and express the stem cell markers Notch1, STRO-1 and nestin and have been maintained in culture for up to 15 passages. STRO-1 positive DFSC can differentiate into cementoblasts *in vitro* and are able to form cementum *in vivo*.

Stem cells from the apical part of the papilla (SCAP)

Stem cells from the apical part of the human dental papilla (SCAP) exhibit a higher proliferative rate and appear more effective than PDLSC for tooth formation. Importantly, SCAP are easily accessible since they can be isolated from human third molars.[4]

DENTAL PULP STEM CELLS (DPSCs)

In 2000, Gronthos and co-workers isolated a clonogenic, rapidly proliferative population of cells from adult human dental pulp and compared them with human bone marrow stromal cells (BMSCs), known precursors of osteoblasts. Both cell types were found to share a similar immunophenotype *in vitro*.[1] Postnatal human DPSC have the ability to form a dentin pulp-like complex.

Shi *et al.* characterized the self-renewal capability, multi-lineage differentiation capacity, and clonogenic efficiency of human dental pulp stem cells (DPSCs). DPSCs were capable of forming ectopic dentin and associated pulp tissue *in vivo*. When retransplanted into immunocompromised mice, they could generate a dentin-pulp-like tissue, demonstrating their self-renewal capability. DPSCs were also found to be capable of differentiating into adipocytes and neural-like cells.[3]

Dental pulp stem cells have been isolated from the pulp tissues of exfoliated deciduous teeth, primary incisors, permanent third molar teeth, natal teeth, supernumerary teeth, teeth with complicated crown fracture and inflamed pulp tissue. Successful isolation of human dental pulp stem cells (hDPSCs) has been achieved even 120 hours after tooth extraction [26].

Human dental pulp stem/stromal cells (hDPSCs) have been isolated from the pulp tissues of complicated crown-fractured teeth requiring root canal therapy, without tooth extraction. The hDPSCs derived from complicated crown-fractured teeth were found to differentiate into adipogenic, chondrogenic, and osteogenic lineages and also expressed stem cells markers and differentiation markers along with high expression for bone marrow stem cell markers including CD29, CD90, and CD105 and exhibited very low expression of markers specific for hematopoietic cells such as CD14, CD34, and CD45[27].

Karaöz *et al.* isolated and characterized stem cells derived from human natal dental pulp (hNDP) using gene expression profiles and their properties were compared with that of mesenchymal stem cells (MSCs) from bone marrow (BM). hNDP-

SCs and hBM-MSCs expressed CD13, CD44, CD90, CD146 and CD166, but not CD3, CD8, CD11b, CD14, CD15, CD19, CD33, CD34, CD45, CD117, and HLA-DR. They also expressed some adipogenic (leptin, adipophilin and PPARgamma), myogenic (desmin, myogenin, myosinIIa, and alpha-SMA), neurogenic (gamma-enolase, MAP2a,b, c-fos, nestin, NF-H, NF-L, GFAP and betaIII tubulin), osteogenic (osteonectin, osteocalcin, osteopontin, Runx-2, and type I collagen) and chondrogenic (type II collagen, SOX9) markers without any stimulation towards differentiation under basal conditions. Embryonic stem cell markers Oct4, Rex-1, FoxD-3, Sox2, and Nanog were also identified[28].

Suchanek *et al.* isolated dental pulp stem cells from impacted third molars and cultivated them in various media. They found that ITS supplement in the cultivation media greatly increased the proliferative activity of DPSCs. The viability of DPSCs in the 9th passage was over 90%. Phenotypical analysis was highly positivity for CD29, CD44, CD90 and HLA I, and negative for CD34, CD45, CD71, HLA II[29].

hDPCs cultured in the presence of bFGF irrespective of the presence or absence of the bovine serum are rich in mesenchymal stem cells or progenitor cells and useful for cell-based therapies to treat dental diseases. Morito et al. isolated adherent fibroblastic cells after collagenase and dispase treatment of human dental pulp. When human dental pulp cells (hDPCs) were cultured in the presence of basic fibroblast growth factor (bFGF), the ratio of hDPCs expressing STRO-1 as a marker of stem cell populations increased approximately eightfold in the presence of bFGF as opposed to that in the absence of bFGF. When cultured with the medium containing serum and bFGF, they were highly

proliferative and capable of differentiating in vitro into osteoblasts, chondrocytes, and adipocytes and which was confirmed at both the protein and gene expression levels. Transplantation of hDPCs expanded ex vivo in the presence of bFGF into immunocompromised mice revealed the formation of bone, cartilage, and adipose tissue. When cultured with a serum-free medium containing bFGF, the hDPCs strongly expressed STRO-1 immunoreactive products and sustained self-renewal, and thus were almost identical in differentiation potential and proliferation activity to hDPCs cultured with the medium containing serum and bFGF[30].

STEM CELLS FROM EXFOLIATED DECIDUOUS TEETH (SHED)

Stem cells from human exfoliated deciduous teeth (SHED) are highly proliferative, clonogenic and multipotent stem cells with a neural crest cell origin. Additionally, they can be collected with minimal invasiveness in comparison with other sources of mesenchymal stem cells (MSCs). SHED could be a desirable option for potential therapeutic applications.

In 2003, Miura *et al.* found that exfoliated human deciduous tooth contains multipotent stem cells. SHED are not only derived from a very accessible tissue resource but are also capable of providing enough cells for potential clinical application. SHED were identified to be a population of highly proliferative, clonogenic cells capable of differentiating into a variety of cell types including neural cells, adipocytes, and odontoblasts. After *in vivo* transplantation, SHED were found to be able to induce bone

formation, generate dentin, and survive in mouse brain along with expression of neural markers.[2]

SHEDs isolated from deciduous dental pulp of 6 to 9 year-old children have typical fibroblastoid morphology and express antigens characteristic of MSCs such as STRO1, CD146, CD45, CD90, CD106 and CD166, but not the hematopoietic and endothelial markers, CD34 and CD31. SHEDs also have a strong potential to differentiate into osteogenic and adipogenic lineages and can also differentiate into neural cells, thus being potential candidates for the autologous transplantation of a wide variety of neurological diseases and neurotraumatic injuries[31].

SHED are an accessible and feasible mesenchymal stem cell source for treating immune disorders like SLE. Yamaza *et al.* compared the mesenchymal stem cell properties of SHED in comparison to human bone marrow mesenchymal stem cells (BMMSCs) and found that SHED were capable of differentiating into osteogenic and adipogenic cells, expressing mesenchymal surface molecules (STRO-1, CD146, SSEA4, CD73, CD105, and CD166), and activating multiple signaling pathways, including TGFbeta, ERK, Akt, Wnt, and PDGF. They also compared the immunomodulatory properties of SHED with BMMSCs and found that SHED had significant effects on inhibiting T helper 17 (Th17) cells in vitro. SHED transplantation was capable of effectively reversing SLE-associated disorders in MRL/lpr mice. At the cellular level, SHED transplantation elevated the ratio of regulatory T cells (Tregs) via Th17 cells[32].

CULTURE DETAILS: BIOLOGY OF CULTURED STEM CELLS AND IDENTIFICATION

As stated by Robey PG, "we have learned to recognize stem cells, not necessarily by what they do in their dependent organism, but rather by what we can do with them in the laboratory"[33].

The culture of stem cells aims to accomplish 3 objectives: sustaining self-renewal properties, maintaining capacity for differentiation and enabling cryopreservation for maintaining the established cell line. Stem cells cannot be identified with certainty in any tissue: scientists rely on indirect properties such as the expression of a repertoire of surface proteins, slow cell cycle, clonogenicity, or an undifferentiated state. However, none of these criteria are specific. The evaluation of self renewal is the ultimate way to show "stemness", which relies on the isolation and transplantation of a putative stem cell (clonal analysis) followed by its serial transplantation and long-term reconstitution of a tissue.[4] To sustain capacity for self-regeneration, it is important to identify markers associated with self-renewal.

MSCs are likely to represent a restricted progeny of putative pluripotent stem cells selected on the basis of their rapid plastic adherence and high proliferation potential in 10% fetal calf serum[34].

MSCs were first recognized and have been primarily characterized in vitro. Characteristics of MSCs differ among laboratories and species, and there is no specific marker or combination of markers that identify MSCs either in vivo or in vitro. In addition, there are no quantitative assays to assess the presence of MSCs in any given

population. Therefore, MSCs are currently defined by a combination of physical, morphologic, phenotypic, and functional properties. MSCs from other sources in general, share most or all of the in vitro characteristics of MSCs, including plastic adherence, fibroblast-like morphology, CFU-F content, phenotypic characteristics, and tridifferentiation potential in appropriate inductive conditions[33].

In unstimulated cultures, MSCs appear as fusiform fibroblasts. In vitro aged MSC are reportedly bigger than their young counterparts; they exhibit more podia and spread further. Increase in cell size is often associated with senescence. MSC from older patients show no spindle morphology in culture, whereas MSC from young donors exhibit the spindle-type morphology in very early cultivation and a gradual loss of these features over cultivation time [34, 35].

To differentiate mesenchymal stem cells from fibroblasts, Halfon *et al.* evaluated the expression of different markers and found that markers that currently define a mesenchymal stem cell population like CD105, CD166, CD90, CD44, CD29, CD73 and CD9 are expressed on both mesenchymal stem cells and on human skin or lung fibroblasts. The level of expression of CD166 and, a new marker, CD106 was significantly higher and CD9 was lower in mesenchymal stem cells. CD146 was expressed only on mesenchymal stem cells. The expression of these markers were down-regulated in passage 6 [36].

MSC divide with a donor-dependent average initial doubling time of 12–24 h, dependent on initial plating density. Different MSC populations demonstrate varying

propensities toward senescence. Human MSCs senesce after approximately 40 population doublings [25].

In a study comparing properties of multipotent mesenchymal stromal cells from different human tissues with that of CD146+ pericytes and fibroblasts, Covas *et al.* found that human MSC and pericytes are similar cells located in the wall of the vasculature where they function as cell sources for repair and tissue maintenance, whereas fibroblasts are more differentiated cells with more restricted differentiation potential.[10]

Suchánek *et al.* established a protocol for isolation and cultivation of DPSCs either from adult or exfoliated tooth, and to compared these cells with mesenchymal progenitor cell (MPCs) from human bone marrow in culture. They cultivated undifferentiated DPSCs for over 60 population doublings in cultivation media designed for bone marrow MPCs. After reaching Hayflick's limit, they still had normal karyotype. Initial doubling time of our cultures was from 12 to 50 hours for first 40 population doublings, after reaching 50 population doublings, doubling time had increased to 60-90 hours. In comparison with bone marrow MPCs, DPSCs shared similar biological characteristics and stem cell properties. DPSCs from adult and exfoliated teeth were found to differ in morphology[37, 38].

Suchanek *et al.* were able to cultivate DPSCs in all tested cultivation media over 40 population doublings[29].

Another important property, but not defining feature, of MSC populations in vitro is their ability to form colonies after low-density plating or single-cell sorting. Colony forming unit (CFU) is used to describe a colony originated from a single cell. During

aging, total CFU numbers from MSC is found to decrease in certain populations. In addition to decreases in total CFU numbers, there is also evidence that the average colony size decreases in aged MSC. Big colonies tend to be composed of spindle-shaped cells while small colonies often consist of broad, flattened (senescent) cells. In cultures of dental pulp stem cells, approximately 40 single-colony clusters can be retrieved from 10,000 cells in culture [1].

Comparison of Dental pulp stem cells (DPSCs) to bone marrow-derived mesenchymal stem cells (BMMSCs) yields conflicting results, possibly due to donor-associated variability. Alge *et al.* sought to address this problem using a donor-matched experimental design compare the biological properties of DPSCs and BMMSCs using adult Sprague-Dawley rat. They showed that DPSCs and BMMSCs had similar morphologies and flow cytometry profiles, were capable of forming colonies in vitro and were capable of osteogenic, chondrogenic and adipogenic differentiation. However, quantitative comparisons revealed that DPSCs had a faster population doubling time and a higher percentage of stem/progenitor cells in the population, as determined by clonogenic assays. Furthermore, while both cell populations formed mineral in vitro, DPSCs had significantly higher alkaline phosphatase activity than BMMSCs after 3 weeks in osteogenic medium[39].

Govindasamy *et al.* studied the effects of culture niche on long-term expansion of dental pulp stem cells in terms of cell morphology, growth kinetics, senescence pattern, cell surface marker expression differentiation capacity, and seeding plating density of dental pulp stem cells in four different, widely used media composition. Among the various basal media tested, α-minimum essential media and knock out-minimum essential

media supplemented with 10% fetal bovine serum were found to be the most optimal media composition in preserving the phenotypic characteristics and differentiation potential for prolonged periods as compared with DMEM-F12 and DMEM-LG. Plating density was shown to affect overall yield[25].

Dental pulp stem cells (DPSCs) can be isolated and cultured in low-serum containing medium supplemented with growth factors while exhibiting multipotency and immature phenotypic characteristics. In a study to assess the potential to differentiate towards osteogenic lineages using various culture conditions it was found that certain environmental cues can enhance differentiation process of DPSCs[40].

SHED can be cultivated to over 45 population doublings. Under same conditions as DPSC, Suchánek *et al.* found that SHED had longer average population doubling time (41.3 hrs for SHED vs. 24.5 hrs for DPSC) and phenotypically, showed differential expression of CD29, CD44, CD71, CD117 and CD 166. These results indicated that in comparison to DPSC, proliferation rate was about 50% slower for SHED, and these cells also showed a slightly different phenotype Also, during long-term cultivation, SHED did not showed any signs of degeneration or spontaneous differentiation[37].

DPSCs from adult and exfoliated teeth were found to differ in morphology[41]. Eslaminejad *et al.* compared stem cells from deciduous and permanent human teeth in terms of their growth kinetics and culture requirements. They found that stem cells from both sources appeared as fibroblastic cells capable of differentiating into osteoblastic, odontoblastic, adipocytic and chondrocytic cell lineages. In contrast to stem cells from third molars, those from the deciduous incisor tooth expressed neurogenic markers, ßIII

Tubulin and Tau. The cells from permanent teeth tended to have a lower PDT value (20.79, SD=2.8 versus 25.55, SD=2.9 hours), higher clonogenic activity and better growth curve than those from the deciduous teeth. Both cells exhibited high expansion rate when being plated in a medium with 20% phosphate buffer solution at a density of 100 cells/cm^2 [42].

Substantial quantities of stem cells of an excellent quality and at early (2-5) passages are necessary for clinical use, which currently is a problem for use of adult stem cells. Dental Pulps were cultured generating stem cells at least during six months through multiple mechanical transfers into a new culture dish every 3-4 days. Lizier *et al.* compared stem cells isolated from the same DP before (early population, EP) and six months after several mechanical transfers (late population, LP). No changes, in both EP and LP, were observed in morphology, expression of stem cells markers (nestin, vimentin, fibronectin, SH2, SH3 and Oct3/4), chondrogenic and myogenic differentiation potential, even after cryopreservation[43].

Characteristics of cells in long term culture

Despite their large proliferative capacity, stable viability, phenotype, and genotype over prolonged cultivation, Mokry *et al.* found that excessive ex vivo expansion of human dental pulp stem cells leads to progressive telomere shortening. They found that relative telomere length (T/S) was inversely correlated with cumulative doubling time and suggested that ex vivo expansion of adult stem cells should be kept to a minimum to avoid detrimental effects on telomere maintenance and measurement of telomere length should

become a standard when certificating the status and replicative age of stem cells prior to therapeutic applications[44].

Yu *et al.* studied the biological features of STRO-1+ DPSCs at the 1st and 9th passages. They found that during long-term passage, the proliferation ability of human STRO-1+ DPSCs was downregulated as indicated by the growth kinetics. The differentiation capacity of DPSCs changes during cell passaging, and DPSCs at the 9th passage restricted their differentiation potential to the osteoblast lineage in vivo. In view of these findings, they suggested that STRO-1+ DPSCs consist of several interrelated subpopulations which can spontaneously differentiate into odontoblasts, osteoblasts, and chondrocytes, but this multipotency decreases with passaging[45].

Cryopreservation

Human dental pulp stem cells (hDPSCs) from the pulp of third molars can show multilineage differentiation after cryopreservation. Following recovery from liquid nitrogen, hDPSCs could be maintained for at least 25 passages and were capable to advance into all 5 differentiation pathways (neurogenic, osteogenic/odontogenic, adipogenic, myogenic, and chondrogenic)[46].

Efficient recovery of DPSC from cryopreserved intact teeth and second-passage DPSC cultures have been achieved. Perry *et al.* found that DPSC isolation is feasible for at least 5 days after tooth extraction, and processing immediately after extraction may not be required for successful banking of DPSC[26].

In a study to determine optimal cryopreservation conditions for dental pulp stem cells, Woods *et al.* found that Me^2SO at a concentration between 1 and 1.5M was the ideal

cryopreservative for DPSCs. It was also determined that DPSC viability after cryopreservation is not limited by the concentration of cells frozen, at least up to $2x10^6$cells/mL. It was further established that DPSC can be stored at -85 degrees C or -196 degrees C for at least six months without loss of functionality. The optimal results with the least manipulation were achieved by isolating and cryopreserving the tooth pulp tissues, with digestion and culture performed post-thaw[47].

Viable hDPSCs have been isolated chiefly from cryopreserved healthy molar teeth. hDPSCs has been isolated from both healthy and diseased, but vital teeth of various tooth types when the intact tooth or their undigested dental pulp tissue when cryopreserved in liquid nitrogen. Higher success rates of hDPSC isolation were achieved from cryopreserved dental pulp tissue than from cryopreserved intact teeth. In a study comparing isolation of hDPSCs from diseased but vital teeth, 100% hDPSC isolation was achieved from freshly isolated dental pulp tissue and cryopreserved dental pulp tissue, but only 20% success rates for isolation from cryopreserved intact teeth. All groups demonstrated self-renewal properties and similar multipotent potential characteristics of adipogenic, chondrogenic and osteogenic differentiation[26, 48].

Immuno-phenotype

MSC populations are heterogeneous, with individual cells capable of varying differentiation potential and expansion capacity. They thus show variable expression of CD90 (Thy1.1), CD117 (c-kit), SH2 (CD105 or endoglin), SH3 or SH4 (CD73), and STRO-1. MSCs lack expression of hematopoietic markers such as CD45, CD14, CD11 and CD34 [49].

Markers for dental pulp stem cells

MSC from dental pulp have been extensively characterized *in vitro* by the expression of markers such as STRO-1, CD146 or CD44. STRO-1 is a cell surface antigen used to identify osteogenic precursors in bone marrow, CD146 a pericyte marker, and CD44 a mesenchymal stem cell marker.[4] Expression of various perivascular markers such as STRO-1, VCAM-1, MUC-18, and smooth-muscle actin (SMA) provides clues that DPSCs are a heterogeneous population of MSCs and likely located in the perivascular niche in the pulp [1, 3].

Ex vivo expanded SHED expressed STRO-1 and CD146 (MUC18), two early cell-surface markers for bone-marrow-derived MSCs. In addition, SHED expressed a variety of osteoblast/odontoblastic markers.

Dental pulp stem cells express mesenchymal cell markers STRO-1, vimentin, CD29, CD44, CD73, CD90, CD166, and stem cell markers Sox2, nestin, and nucleostemin[11].

Alongi *et al.* characterized normal pulps (NPs) and inflamed pulps (IPs) in vitro and showed that IPs expressed higher levels of mesenchymal stem cell markers STRO-1, CD90, CD105 and CD146 compared with NPs. Flow cytometry analysis showed that DPSCs from both NPs and IPs expressed moderate to high levels of CD146, stage-specific embryonic antigen-4, CD73 and CD166. [12]

Alipour *et al.* compared the expression of stem cell surface markers on two populations of mesenchymal stem cells, one derived from human exfoliated deciduous teeth and the other derived from human adipose tissue. They found that both different cell populations expressed CD44, CD90 and CD13 (stem cell markers) with similar intensity.

They did not express hematopoietic markers (CD11b, CD19 and CD34), and lymphocyte or leukocyte antigens CD3, CD7, CD20, CD14, CD45, CCR5 (CD195), CD11b and CD10 on their surfaces. Two different cell types demonstrated different levels of expression in CD56 and CD146. Mesenchymal stem cells from human exfoliated deciduous teeth were positive for CD105 and were negative for CCR3 and CCR4 expression[50].

Nourbakhsh *et al.* characterized Stem cells from human exfoliated deciduous teeth (SHED). They had typical fibroblastoid morphology and expressed antigens characteristic of MSCs, STRO1, CD146, CD45, CD90, CD106 and CD166, but not the hematopoietic and endothelial markers, CD34 and CD31, as assessed by FACS analysis[51].

Mesenchymal stem cells from the dental pulp can find applications in prevention and reversal of many human diseases such as type-1 diabetes and prevention of liver fibrotic process.

Alipour *et al.* compared mesenchymal stem cells derived from human exfoliated deciduous teeth and from human adipose tissue and were found to express the stem cell markers CD44, CD90 and CD13 with similar intensity. Different levels of expression were seen in CD56 and CD146 and mesenchymal stem cells from human exfoliated deciduous teeth were positive for CD105[50].

Ferro et al. demonstrated that Oct4, Nanog, Klf4 and c-Myc are expressed in adult stem cells and, with the exception of c-Myc, they are significantly down-regulated following differentiation. Cell differentiation was also associated with a significant reduction in the fraction of DPSC expressing the stem cell markers CD10, CD29 and CD117[52].

Karaöz *et al.* isolated and characterized stem cells derived from human natal dental pulp (hNDP) and identified the expression of embryonic stem cell markers Oct4, Rex-1, FoxD-3, Sox2, and Nanog[53].

A study demonstrated the presence of stem cell populations with embryonic phenotypes in human dental pulp from the third molar. The dental pulp tissue was cultured in media with the presence of LIF, EGF, and PDGF. The new population of pluripotent stem cells isolated from dental pulp (DPPSC) were SSEA-4(+), Oct4(+), Nanog(+), FLK-1(+), HNF3beta(+), Nestin(+), Sox2(+), Lin28(+), c-Myc(+), CD13(+), CD105(+), CD3(-), CD45(-), CD90(low), CD29(+), CD73(low), STRO-1(low) and CD146(-). The capacity of DPPSCs to differentiate in vitro into tissues that have similar characteristics to embryonic mesoderm and endoderm layers support the use of these cells, which are derived from an easily accessible source and can be used in future regeneration protocols for many tissue types that differentiate from the three embryonic layers[54].

Differentiation

The typical default pathway for most MSCs, in culture, is osteogenesis. Within a given MSC population there is a low frequency of cells capable of tripotential differentiation with most of the cells on clonal analysis having bipotential or even unipotential capacity. There are also generally only a few clones capable of extensive expansion.

When STRO-1(+) (stromal precursor cell marker) DPSCs (dental pulp stem cells) and BMSSCs (bone marrow stromal stem cells) were isolated from rat dental pulp and

bone marrow respectively and compared, DPSCs presented more striking odontogenic capability than BMSSCs.[5]

Human adult dental pulp stem cells (DPSCs) reside within the perivascular niche of dental pulp and are thought to originate from migrating cranial neural crest (CNC) cells. During embryonic development, CNC cells differentiate into a wide variety of cell types, including neurons of the peripheral nervous system.

DPSCs exposed to the appropriate environmental cues differentiate into functionally active neurons, acquiring a neuronal morphology, expressing neuronal-specific markers at both the gene and protein levels and also exhibited the capacity to produce a sodium current consistent with functional neuronal cells when exposed to neuronal inductive media. Furthermore, DPSCs expressed neuronal markers and acquired a neuronal morphology in vivo in an avian xenotransplantation assay[55].

Dental pulp stem cells (DPSCs) are multipotent stem cells derived from neural crest and mesenchyme and have the capacity to differentiate into multiple cell lineages. Studies have demonstrated that DPSCs can differentiate into melanocyte-like cells when cultivated in a specific melanocyte differentiating medium they have also recently been found to spontaneously differentiate into mature melanocytes, with the expression of melanocyte specific markers. The spontaneous differentiating potential of these cells strongly suggests their possible applications in regenerative medicine [41].

Adipogenesis can be induced in dental pulp derived stem cells by adding insulin, 3-isobutyl-1-methylxanthine, and dexamethasone. Cells containing lipid droplets can be observed after induction. A study using rat dental pulp-derived cells demonstrated the

potential to differentiate into adipocytes in vitro. Pluripotent markers Oct-3/4 and Sox2 were found to be down-regulated during differentiation, whereas the expression of Nanog was not significantly changed during differentiation[31].

Karaöz *et al*. found that hDP-SCs were more developed and metabolically active cells could not only differentiate into adipogenic, osteogenic, and chondrogenic lineage, but also expressed neural stem cell and epithelial markers implying that under defined conditions, hDP-SCs can differentiate into both neural and vascular endothelial cells in vitro[53].

Ishkitiev N *et al*. demonstrated the ability of mesenchymal cell cultures from deciduous and permanent tooth pulp to undergo endodermal differentiation and acquire morphological and functional characteristics of hepatocytes [56].

Stem cells in inflamed dental pulp

Wang *et al*. isolated pulp cells from healthy teeth and teeth with clinically diagnosed irreversible pulpitis. On comparing cell proliferation, stem cell marker STRO-1 expression, and cell odonto-osteogenic differentiation competence, they found that cells from the diseased group demonstrated decreased colony formation capacity and a slightly decreased cell proliferation rate. These cells, however, had similar STRO-1 expression and exhibited a similar percentage of positive ex vivo osteogenic induction and dentin sialophosphoprotein expression to stem cells isolated from healthy teeth[57].

Alongi *et al*. compared the characteristics of DPSCs from freshly collected normal pulps (NPs) and inflamed pulps (IPs) in vitro and tested their tissue regeneration potential in vivo using a study model. They found that IPs expressed higher levels of mesenchymal

stem cell markers STRO-1, CD90, CD105 and CD146 compared with NPs. Furthermore, DPSCs from both NPs and IPs expressed moderate to high levels of CD146, stage-specific embryonic antigen-4, CD73 and CD166. Total population doubling of DPSCs-IPs (44.6 ± 2.9) was lower than that of DPSCs-NPs (58.9 ± 2.5), and DPSCs-IPs appeared to have a decreased osteo/dentinogenic potential compared with DPSCs-NPs based on the mineral deposition in cultures. However, DPSCs-IPs were able to form pulp/dentin complexes similar to DPSCs-NPs when transplanted into immuno-compromised mice [58].

USES OF STEM CELLS- Pre-clinical and clinical studies

When DPSCs were transplanted into immunocompromised mice, they generated a dentin-like structure lined with human odontoblast-like cells that surrounded a pulp-like interstitial tissue.[1-3]

Kumabe and co-workers used an alginate scaffold to transplant subcultured human dental pulp cells subcutaneously into the backs of nude mice. They found an increase in alkaline phosphatase, which is an early marker for odontoblast differentiation and at 6 weeks after implantation, subcutaneous formation of radio-opaque calcified bodies was observed in situ. They concluded that subcultured dental pulp cells actively differentiate into odontoblast-like cells and induce calcification in an alginate scaffold[59].

Human dental pulp contains precursor cells termed dental pulp stem cells (DPSC) that show self-renewal and multilineage differentiation and also secrete multiple proangiogenic and antiapoptotic factors. These cells have shown therapeutic potential in

the repair of myocardial infarction (MI), when injected intramyocardially into nude rats seven days after induction of myocardial infarction by coronary artery ligation [60].

When critical-size calvarial defects were induced in immunocompromised mice and SHED was transplanted with hydroxyapatite/tricalcium phosphate as a carrier into the defect areas, SHED was able to repair the defects with substantial bone formation. Furthermore, SHED were found to co-express mesenchymal stem cell marker, CC9/MUC18/CD146, with an array of growth factor receptors[61].

A study evaluated the capacity of human dental pulp stem cells (hDPSC), isolated from deciduous teeth, to reconstruct large sized cranial bone defects in nonimmunosuppressed (NIS) rats. The hDPSC lineage was positive for the four mesenchymal cell markers tested and showed osteogenic, adipogenic, and myogenic in vitro differentiation. The use of hDPSC in NIS rats, also, did not cause any graft rejection[62].

A pre-clinical study in a large-animal model, specifically swine, allows for testing of a stem cells/scaffold construct in the restoration of orofacial skeletal defects. Zheng *et al*. found that stem cells from miniature pig deciduous teeth, were able to engraft and regenerate bone to repair critical-size mandibular defects at 6 months post-surgical reconstruction[63].

A clinical study utilized biocomplex constructed from dental pulp stem/progenitor cells (DPCs) and a collagen sponge scaffold for oro-maxillo-facial (OMF) bone tissue repair in patients requiring extraction of their third molars. Three months after autologous DPC grafting, alveolar bone of patients had optimal vertical repair and complete

restoration of periodontal tissue back to the second molars, as assessed by clinical probing and X-rays. Histological observations clearly demonstrated the complete regeneration of bone at the injury site. Optimal bone regeneration was still evident one year after grafting demonstrating that a DPC/collagen sponge biocomplex can completely restore human mandible bone defects and that this cell population could be used for the repair and/or regeneration of tissues and organs[64].

Huang *et al*. tested the possibility of regenerating vascularized human dental pulp in emptied root canal space and producing new dentin on existing dentinal walls using a stem/progenitor cell-mediated approach with a human root fragment and an immunocompromised mouse model. They found that the root canal space was filled entirely by a pulp-like tissue with well-established vascularity and a continuous layer of dentin-like tissue was deposited onto the canal dentinal wall. This study provides the first evidence showing that pulp-like tissue can be regenerated de novo in emptied root canal space by stem cells from apical papilla and dental pulp stem cells that give rise to odontoblast-like cells producing dentin-like tissue on existing dentinal walls[65].

Tissue-engineered cell sheet composed of human undifferentiated immature dental pulp stem cells (hIDPSC) has been successful in reconstructing corneal epithelium in an animal model of total limbal stem cell deficiency (LSCD) [66].

Human dermal fibroblasts are currently the most accessible and feasible cell source for iPS generation. Cells of ectomesenchymal origin, such as progenitor cells from the dental pulp can also be used as an alternative source for generating iPS cells. Yan *et al*. used the 4 factors Lin28/Nanog/Oct4/Sox2 or c Myc/Klf4/Oct4/Sox2 carried by viral

vectors to reprogram 3 different dental stem/progenitor cells: stem cells from exfoliated deciduous teeth (SHED), stem cells from apical papilla (SCAP), and dental pulp stem cells (DPSCs). They showed that all 3 can be reprogrammed into iPS cells and appeared to be at a higher rate than fibroblasts. They exhibited morphology indistinguishable from human embryonic stem (hES) cells in cultures and expressed hES cell markers SSEA-4, TRA-1-60, TRA-1-80, TRA-2-49, Nanog, Oct4, and Sox2. They formed embryoid bodies in vitro and teratomas in vivo containing tissues of all 3 germ layers[67].

Regenerative surgical procedures utilizing hDPSCs can be used in pathologies and traumas with critical size bone defects. In a study by Riccio *et al*. DPSCs were differentiated toward osteogenic lineage on 2D surface by using an osteogenic medium and expressed specific bone proteins like Runx-2, Osx, OPN and OCN and produce extracellular calcium deposits. When cultured on two bioscaffolds, Matrigel™ and Collagen sponge, osteogenic differentiation and mineralized extracellular matrix production significantly improved [68].

Three-dimensional (3D) calcium phosphate (CaP) porous granules in combination with hDPSCs been has shown to provide favorable 3D substrate conditions for cell growth and odontogenic development, making it ideal for tissue engineering purposes [69].

Stem cells from human exfoliated deciduous teeth (SHED) might offer a unique stem cell resource and the possibility of novel cell therapies for wound healing in the future. Stem cells from human exfoliated deciduous teeth (SHED) and human mesenchymal stromal cells (hMSCs) accelerated wound healing compared with human fibroblasts (hFibro) and a control (phosphate-buffered saline; PBS) in a mouse model [70].

When human Dental Pulp Stem cells (DPSCs) were seeded onto a three-dimensional (3D) Biocoral scaffold, which is a porous natural hydroxyapatite, DPSCs differentiated into osteoblasts, forming a biocomplex made of Biocoral, secreted extracellular matrix and differentiated cells[71].

Results

Successful cultures were obtained from 2 of 3 deciduous teeth, and 5 of 12 permanent teeth, making it 7 of the 15 teeth collected. Deciduous tooth samples were obtained from retained deciduous mandibular canines and mandibular incisor. Permanent tooth samples were obtained from impacted maxillary/mandibular molars from patients who were scheduled for extraction. The details described below are tabulated in *Table. 1*. *Graph 1* summarizes the tooth samples cultured, the days taken to reach confluency and the number of passages up to which the cells were subcultured in each sample.

In the cultured SHED, growth characteristics and morphology was assessed for both the successful deciduous samples (1dp B, 2dp). Colony forming unit efficiency was assessed for one deciduous sample (2dp). Flow cytometry and immunocytochemistry was done in passages 1, 3 and 5 of one deciduous sample (1dp B) and only for passage 3 in the other deciduous sample (2dp).

In the cultured DPSCs, growth characteristics and morphology was assessed for two samples (2ap, 12ap). Colony forming unit efficiency was assessed for one permanent sample (12ap). Flow cytometry and immunocytochemistry was done in passages 1 for 2 samples (1ap, 8ap), passage 3 for 1 sample (2ap) and in passage 5 for one sample (11ap).

Culture of dental pulp stem cells from deciduous teeth

Deciduous samples 1 and 2 were obtained from a 12 year old male with retained deciduous canines (tooth no. 73 and 83). *Sample 1 (1dp A)* did not have sufficient tissue to establish a culture. *Sample 2 (1dp B)* had ~1mm^3 of tissue and the primary culture reached

confluence in 26 days. Nuclear and cytoplasmic inclusions were noted and debris was seen in the media. Following media change, the cells and media remained clear. Cells obtained were cultured up to the 5^{th} passage.

Growth characteristics *(Graph. 2, Table. 2)*: A decrease in cell count was noted, following seeding in the first 24 hrs. Log phase began at day 3 and the cell count increased steadily thereafter. The seeding efficiency was calculated to be 87% and the population doubling time was 34.96 hours.

Morphological phenotype *(Graph. 3-6, Table. 3-6)*: There was a decrease in mitotic phenotype and increase in the post-mitotic phenotype from day 1 to day 8 of culture. The F2 subpopulation was the highest in the first few days of culture. Day 4 showed an increase in the F3 subpopulation. On the 8^{th} day, the F3 subpopulation was the highest. The epitheloid cell type was increased in comparison to the fibroblast-like cells in all 8 days.

Immunocytochemistry *(Graph. 7, Table. 7)*: No expression (grade 0) of CD73 was seen in passage 1, intense staining (grade 3) was seen in passage 3 and moderate staining (grade 2) was seen in passage 5. CD146 expression was moderate (grade 2) in passage 1 and passage 3 and intense staining (grade 3) was seen in passage 5.

Flow cytometry *(Graph. 8a, 8b, Table. 8)*: CD73 expression was 52.9% in passage 1, 15.5% in passage 3 and 88.7% in passage 5. CD146 expression was 96.3% in passage 1, 17.2% in passage 3 and 76.1% in passage 5.

Deciduous sample 3 (2dp) was obtained from an 8 year old female with a retained deciduous incisor (tooth no. 81). The sample had <1mm^3 of tissue and the primary culture reached confluence in 12 days. Cells obtained were cultured up to the 3rd passage.

Growth characteristics *(Graph. 9, Table. 9)*: There was no decrease in cell count following seeding. Log phase began at day 4 and the cell count increased steadily thereafter. The seeding efficiency was calculated to be 112% and the population doubling time was 27.60 hours.

Colony forming unit efficiency *(Graph. 10a, b, Table. 10)*: 19 colonies were seen in plate 1, 31 colonies were seen in plate 2 and 28 colonies were seen in plate 3. The overall colony forming efficiency was 65%.

Morphological phenotype *(Graph. 11-14, Table. 11-14)*: There was a decrease in mitotic phenotype and increase in the post-mitotic phenotype from day 1 to day 8 of culture. The F2 subpopulation was the highest in the first few days of culture. At day 5, there was an increase in the F3 subpopulation. At day 8, the F3 subpopulation was the highest. The population of cells that exhibited epitheloid morphology was more than the population that had spindle cell morphology from day 1 to day 8.

Immunocytochemistry: No staining was seen with CD73 and CD146 in the 3rd passage of culture.

Flow cytometry *(Graph. 15a, 15b, Table. 15)*: Cells from passage 3 were taken for flow cytometry analysis. CD73 expression in passage 3 was 88.7% and CD146 expression was 76.1%.

Culture of dental pulp stem cells from permanent teeth

Permanent sample 1 (1ap) was obtained from a 38 year old female with pericoronitis (tooth no. 38). Cells in the primary culture appeared to grow outwards from a central bit of tissue. After 12 days of culture the cells appeared larger, with prominent intracytoplasmic filaments. The cells were more polygonal in shape with cellular processes. Refractile material was seen in the media. Cell growth was slow and the cells took 54 days to reach 70 % confluence for passaging. Cells from passage 1 were grown on slides for immunocytochemical analysis and the remaining cells were taken for flow cytometry analysis.

Immunocytochemistry *(Graph. 26, Table. 26)*: Moderate staining (grade 2) was seen with CD73 in passage 1. CD146 expression was mild (grade 1) in passage 1.

Flow cytometry *(Graph. 27, Table. 27)*: The percentage of cells in passage 1 that expressed CD73 positivity was 26.4%. The percentage of cells in passage 1 that expressed CD146 positivity was 24.8%.

Permanent sample 2 (2ap) was obtained from a 26 year old female with pericoronitis (tooth no. 38). Cells in primary culture appeared clustered together, joined by their cell processes. After 20 days the cells appeared to grow larger and assumed a fibroblast-like and stellate morphology. The cells appeared to proliferate in sheets. Cell growth was slow and the cells took 54 days to reach confluence. Cells were grown up to the 3rd passage.

Growth characteristics *(Graph. 16, Table. 16)*: A decrease in cell count was noted following seeding in the first 24 hrs. Log phase began at day 3 and the cell count increased steadily thereafter. The seeding efficacy was calculated to be 44% and the population doubling time was 40.07 hours.

Morphological phenotype *(Graph. 17-20, Table. 17-20)*: There was a decrease in mitotic phenotype and increase in the post-mitotic phenotype from day 1 to day 8 of culture. The F2 subpopulation was the highest in the first few days of culture. At day 5, there was an increase in the F3 subpopulation. At day 8, the F3 subpopulation was the highest. The population of cells that exhibited epitheloid morphology was more than the population that had spindle cell morphology from day 1 to day 8.

Immunocytochemistry *(Graph. 26, Table. 26)*: Mild staining (grade 1) was seen with CD73 and no staining (grade 0) was seen with CD146 in passage 3.

Flow cytometry *(Graph. 27, Table. 27)*: Cells from passage 3 were analyzed. The percentage of cells that expressed CD73 positivity was 93.4%. The percentage of cells that expressed CD146 positivity was 51.1%.

Permanent sample 3 was obtained from a 21 year old female with pericoronitis (tooth no. 28). A few colonies of cells and single fibroblast-like cells were seen after 48 hours in primary culture. Debris was seen in the media and the media was changed. After 12 days of culture, bacterial colonies were seen throughout the culture plate and the sample was discarded.

Permanent samples 4 and 5 were obtained from a 22 and 27 year old patient respectively with pericoronitis (tooth no. 18 and 48). The samples were incubated with

only dispase for enzyme disaggregation. The tissue did not disaggregate after 18 hours and when plated, an explants-like culture was obtained. However, no cell growth was seen even after 72 hours. The sample was discarded.

Permanent sample 6 was obtained from a 28 year old male with pericoronitis (tooth no. 48). However, when the cells were observed after 48 hours, the media appeared turbid and fungal hyphae could be seen floating in the media, microscopically. The sample was discarded. The media was smeared on a slide and stained with PAS. Fungal bodies were observed.

Permanent sample 7 was obtained from a 25 year old female with pericoronitis (tooth no. 48). Large colonies of cells were seen in the primary culture 48 hours after plating. The cells were small and appeared to be growing outwards from bits of refractile tissue. The cells reached confluence in 22 days and were grown till the 2^{nd} passage. Following trypsinization and re-seeding, cell growth was very slow. Media became turbid. The sample was suspected to be contaminated with mycoplasma and discarded immediately.

Permanent sample 8 was obtained from a 31 year old male with pericoronitis (tooth no. 38). The cells were predominantly stellate shaped and appeared to be joined by their processes initially. They reached confluency in 28 days and all cells from the 1^{st} passage were taken for flow cytometry analysis.

Immunocytochemistry: No staining was seen with CD73 and CD146 in the 1^{st} passage.

Flow cytometry *(Graph. 27, Table. 27)*: Cells from passage 1 were analyzed. The percentage of cells that expressed CD73 positivity was 94%. The percentage of cells that expressed CD146 positivity was 90.3%.

Permanent sample 9 was obtained from a 26 year old male with pericoronitis (tooth no. 18). The pulp tissue appeared yellow and necrotic so only the root pulp which was white in color was taken for culture. The tissue was washed thoroughly with 5X PBS prior to enzyme disaggregation. No cells were seen after 1 week in culture. The sample was discarded.

Permanent sample 10 was obtained from a 24 year old male with pericoronitis (tooth no. 38). Most of the pulp chamber was calcified and a very small bit of tissue was isolated. A few cells were cells were seen after 72 hours of culture but the cells did not proliferate. After 1 week, no cells were seen in culture and the media contained debris in spite of media change every third day. The sample was discarded.

Permanent sample 11 was obtained from a 22 year old female with pericoronitis (tooth no. 38). The samples grew as clusters of cells which were predominantly stellate in appearance. The cells grew in sheets and reached confluence in 29 days. Cells were grown up to the 5^{th} passage. All cells were taken for flow cytometry analysis.

Immunocytochemistry *(Graph. 26, Table. 26)*: Mild staining (grade 1) was seen with CD73 and no staining (grade 0) was seen with CD146 in passage 5.

Flow cytometry *(Graph. 27, Table. 27)*: Cells from passage 5 were analyzed. The percentage of cells that expressed CD73 positivity was 10.3%. The percentage of cells that expressed CD146 positivity was 21.4%.

Permanent sample 12 was obtained from a 32 year old female with pericoronitis (tooth no. 48). Cells were seen growing in clusters and were small and stellate in appearance. The cells reached confluence in 28 days and were grown up to the 4^{th} passage. Cells in the later passages appeared larger, polyhedral and contained intra-cytoplasmic filaments.

Growth characteristics *(Graph. 21, Table. 21)*: A decrease in cell count was noted following seeding in the first 24 hrs. Log phase began at day 3 and the cell count increased steadily thereafter. The seeding efficacy was calculated to be 41.67% and the population doubling time was 33.76 hours.

Colony forming unit efficiency *(Graph. 10, Table 10)*: 17 colonies were seen in plate 1, 9 colonies were seen in plate 2 and 19 colonies were seen in plate 3. The overall colony forming efficiency was 37.5%.

Morphological phenotype *(Graph. 22-25, Table. 22-25)*: There was a decrease in mitotic phenotype and increase in the post-mitotic phenotype from day 1 to day 8 of culture. The F2 subpopulation was the highest in the first few days of culture. At day 8, the F1 subpopulation was the highest. The population of cells that exhibited epitheloid morphology was more than the population that had spindle cell morphology from day 1 to day 8.

Tables and Graphs

Table 1
Demographics

Sample no.	Sample type	Age/ Gender	Source	Disease	Time to reach confluence (days)	Final passage no.	Outcome	Morphology	Growth characteristics	Flow cytometry	ICC	CFU-F
1dpA	deciduous	12/m	73	retained deciduous	-	-						
1dpB	deciduous	12/m	83	retained deciduous	26	5	Successful	Yes	Yes	Yes (passage 1,3,5)	Yes	
2dp	deciduous	8/f	81	retained deciduous	12	3	Successful	Yes	Yes	Yes (passage 3)	Yes	Yes
1ap	permanent	38/f	38	pericoronitis	54	1	Successful			Yes (passage 1)	Yes	
2ap	permanent	26/f	38	pericoronitis	54	3	Successful	Yes	Yes	Yes (passage 3)	Yes	
3ap	permanent	21/f	28	pericoronitis	-	-	Bacterial contamination					
4ap	permanent	22/f	18	pericoronitis	-	-	no cells					
5ap	permanent	27/f	48	pericoronitis	-	-	no cells					
6ap	permanent	28/m	48	pericoronitis	-	-	fungal contamination					
7ap	permanent	25/f	48	pericoronitis	22	2	slow growth					
8ap	permanent	31/m	38	pericoronitis	28	1	Successful			Yes (passage 1)	Yes	
9ap	permanent	26/m	18	pericoronitis	-	-	no cells					
10ap	permanent	24/m	38	pericoronitis	-	-	slow growth					
11ap	permanent	22/f	38	pericoronitis	29	5	Successful			Yes (passage 5)	Yes	
12ap	permanent	32/f	48	pericoronitis	28	4	Successful	Yes	Yes			Yes

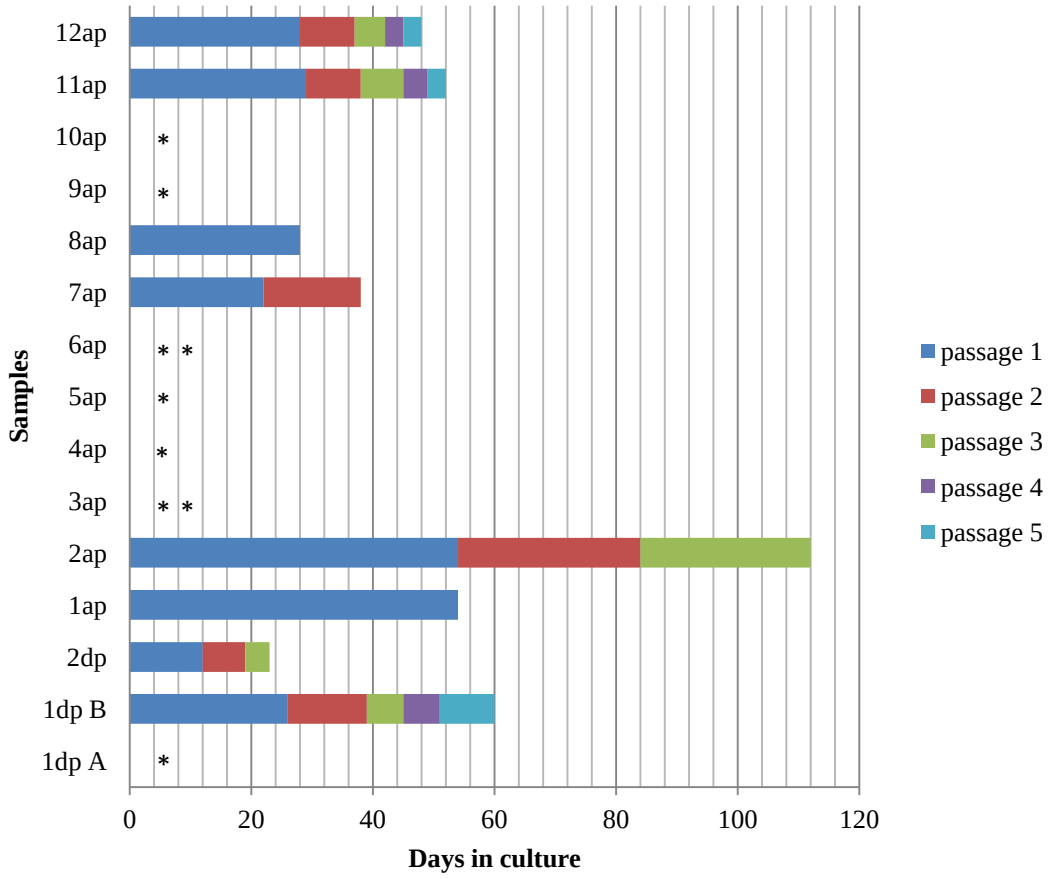
ICC- Immunocytochemistry, CFU-F- Colony forming unit-Fibroblast, dp- deciduous pulp, ap- adult pulp

Successful cultures of deciduous samples (SHED)

Successful cultures of adult samples (DPSCs)

Samples, days in culture and number of passages

Graph 1
Days in culture and no. of passages for each sample (n=15)



* No pulp tissue during primary isolation/ no cells seen after 1 week of plating
** Contamination

Growth characteristics of deciduous sample 1 (1dp B)

Graph 2
Growth curve of deciduous sample 1 (1dp B)

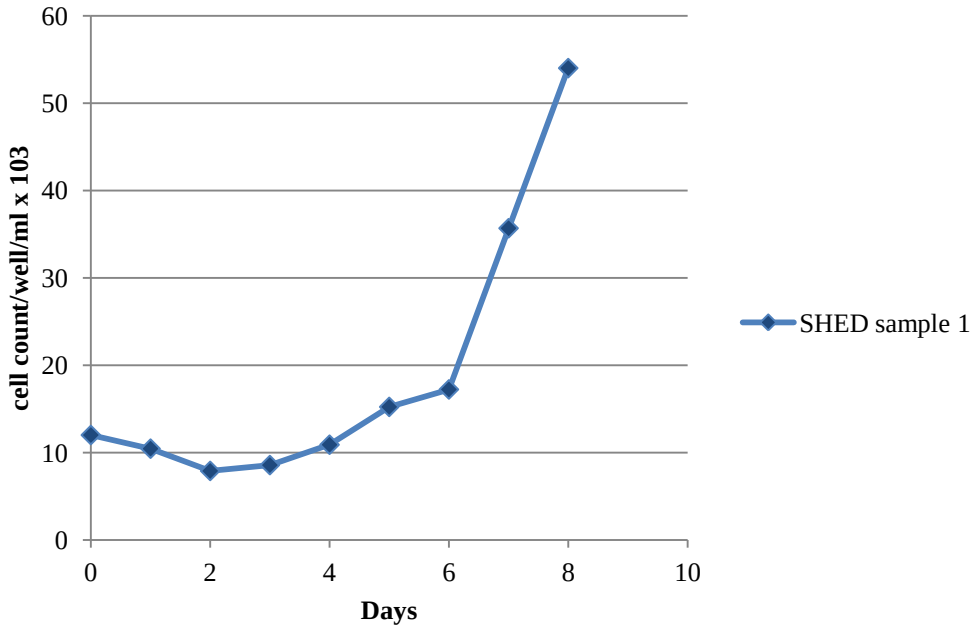


Table 2
Growth curve derivatives of deciduous sample 1 (1dp B)

Day	Cell count/well/ml x 10^3	Slope	Standard deviation (SD)	Population doubling time (hours)	Seeding efficiency (%)
0	12	4.4835	1.1649	34.96	87
1	10.44				
2	7.89				
3	8.56				
4	10.89				
5	15.22				
6	17.22				
7	35.67				
8	54				

Subpopulation analysis of deciduous sample 1 (1dp B)

Graph 3
f I-f VII subpopulation of deciduous sample 1 (1dp B)

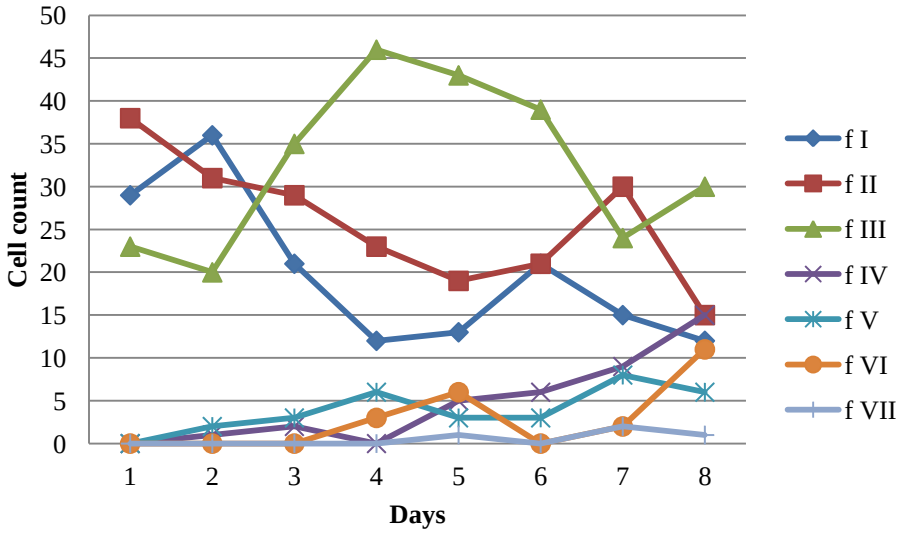


Table 3
f I-f VII subpopulation of deciduous sample 1 (1dp B)

Day	f I	f II	f III	f IV	f V	f VI	f VII
1	29	38	23	0	0	0	0
2	36	31	20	1	2	0	0
3	21	29	35	2	3	0	0
4	12	23	46	0	6	3	0
5	13	19	43	5	3	6	1
6	21	21	39	6	3	0	0
7	15	30	24	9	8	2	2
8	12	15	30	15	6	11	1

Subpopulation analysis of deciduous sample 1 (1dp B)

Graph 4
Mitotic and post-mitotic phenotypes in deciduous sample 1 (1dp B)

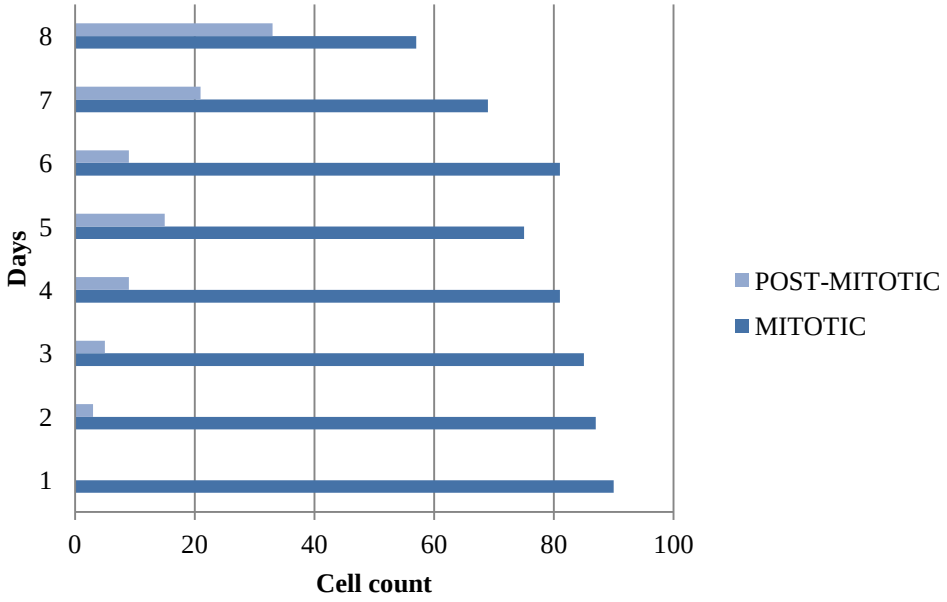


Table 4
Mitotic and post-mitotic phenotypes in deciduous sample 1 (1dp B)

Day	MITOTIC	POST-MITOTIC
1	90	0
2	87	3
3	85	5
4	81	9
5	75	15
6	81	9
7	69	21
8	57	33

Subpopulation analysis of deciduous sample 1 (1dp B)

Graph 5
F1-F3 subpopulation of deciduous sample 1 (1dp B)

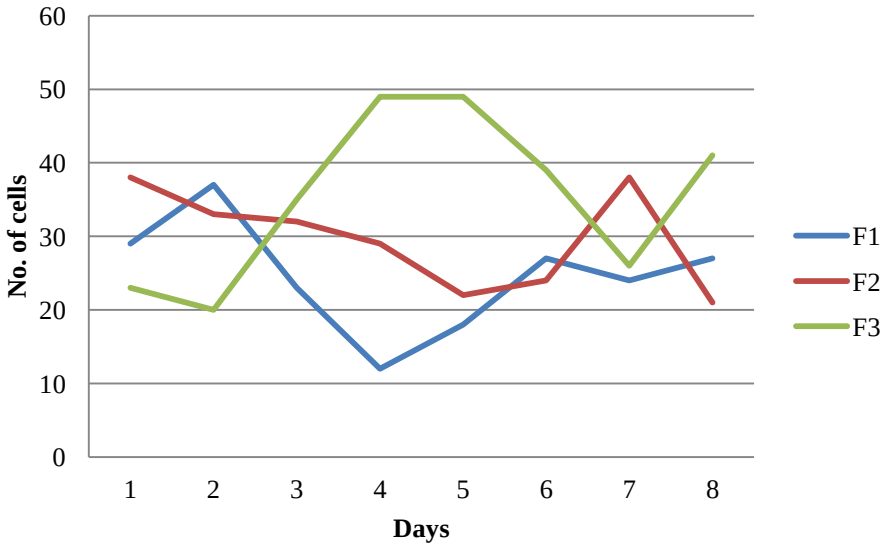


Table 5
F1-F3 subpopulation of deciduous sample 1 (1dp B)

Day	F1	F2	F3
1	29	38	23
2	37	33	20
3	23	32	35
4	12	29	49
5	18	22	49
6	27	24	39
7	24	38	26
8	27	21	41

Subpopulation analysis of deciduous sample 1 (1dp B)

Graph 6

Fibroblast-like and epitheloid phenotypes in deciduous sample 1(1dp B)

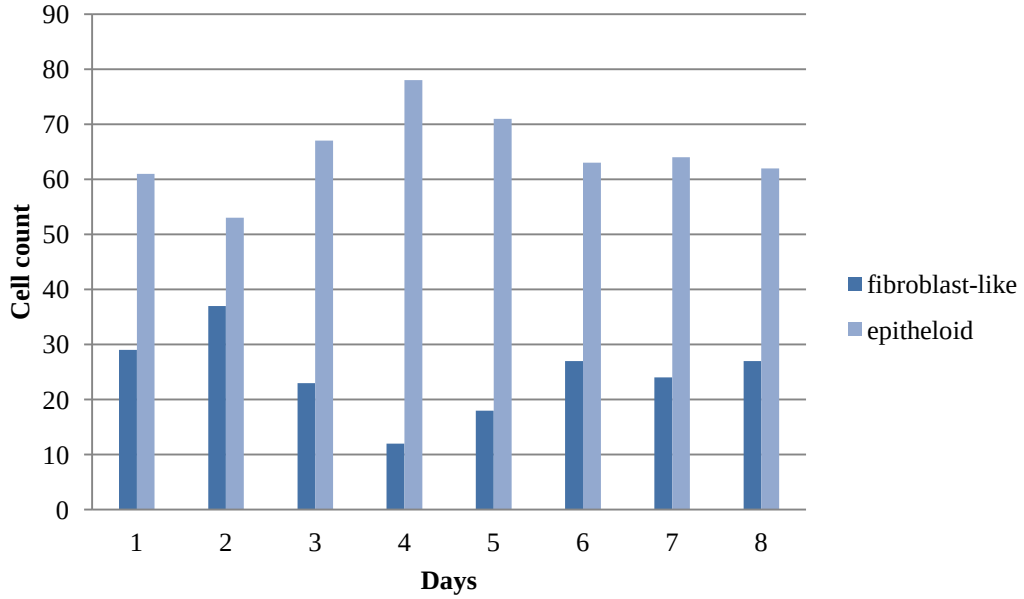


Table 6

Fibroblast-like and epitheloid phenotypes in deciduous sample 1(1dp B)

Day	fibroblast-like	epitheloid
1	29	61
2	37	53
3	23	67
4	12	78
5	18	71
6	27	63
7	24	64
8	27	62

Immunocytochemical analysis of CD73 and CD146 in deciduous sample 1 (1dp B) in the 1st, 3rd and 5th passage of culture

Graph 7

Immunocytochemical expression in deciduous sample 1 (1dp B)

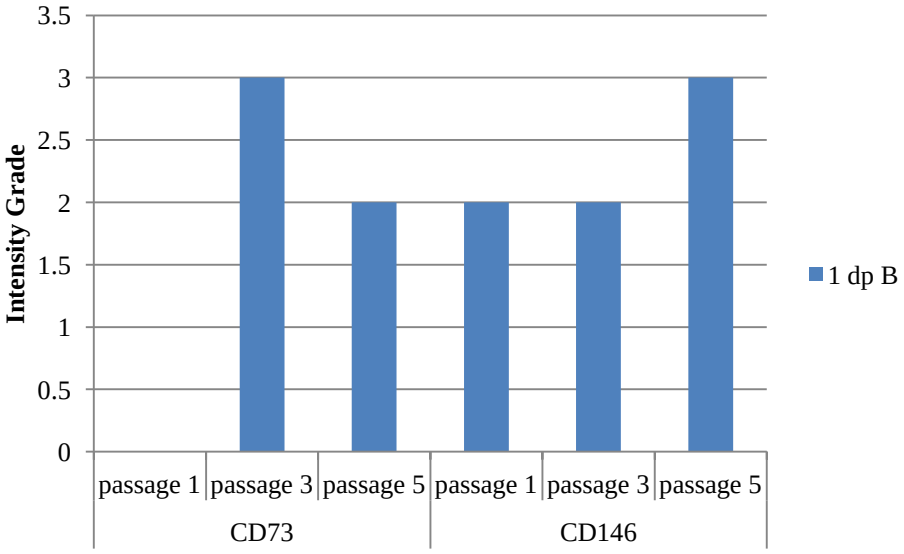


Table 7

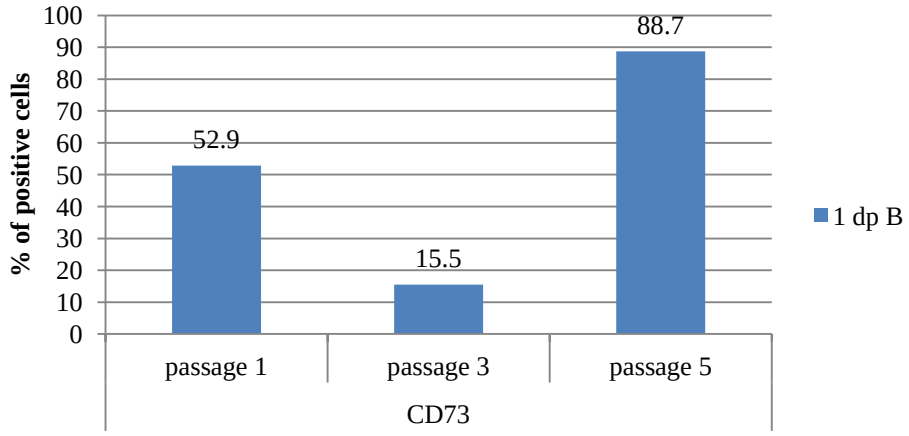
Immunocytochemical in deciduous sample 1 (1dp B)

Deciduous samples	passage 1		passage 3		passage 5	
	CD73	**CD146**	**CD73**	**CD146**	**CD73**	**CD146**
Staining intensity	0	2	3	2	2	3

0- no staining
1- Mild staining
2- Moderate staining
3- Intense staining

Flow cytometry analysis of deciduous sample 1 (1dp B) in the 1st, 3rd and 5th passage of culture

Graph 8a
Flow cytometry analysis of CD73 in deciduous sample 1 (1 dp B)



Graph 8b
Flow cytometry analysis of CD146 in deciduous sample 1 (1 dp B)

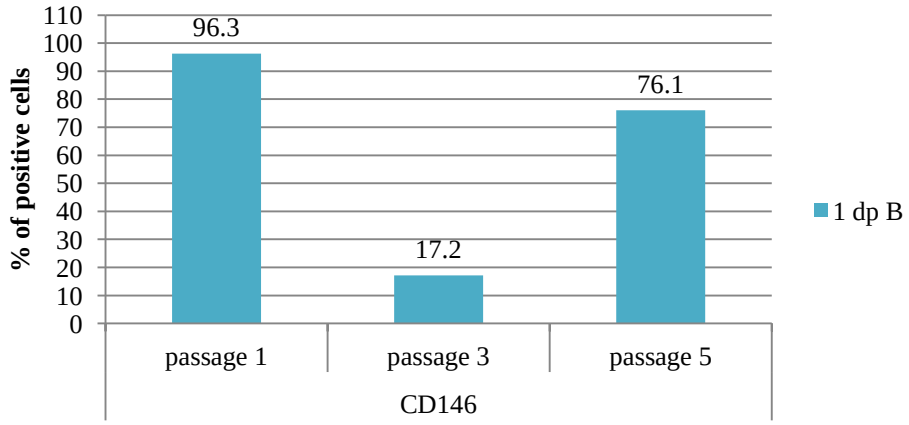


Table 8
Flow cytometry analysis of CD73 and CD146 in deciduous sample 1 (1 dp B)

	CD73 (%)			**CD146 (%)**		
	passage 1	**passage 3**	**passage 5**	**passage 1**	**passage 3**	**passage 5**
1 dp B	52.9	15.5	88.7	96.3	17.2	76.1

Growth characteristics of deciduous sample 2 (2dp)

Graph 9
Growth curve of Deciduous sample 2 (2dp)

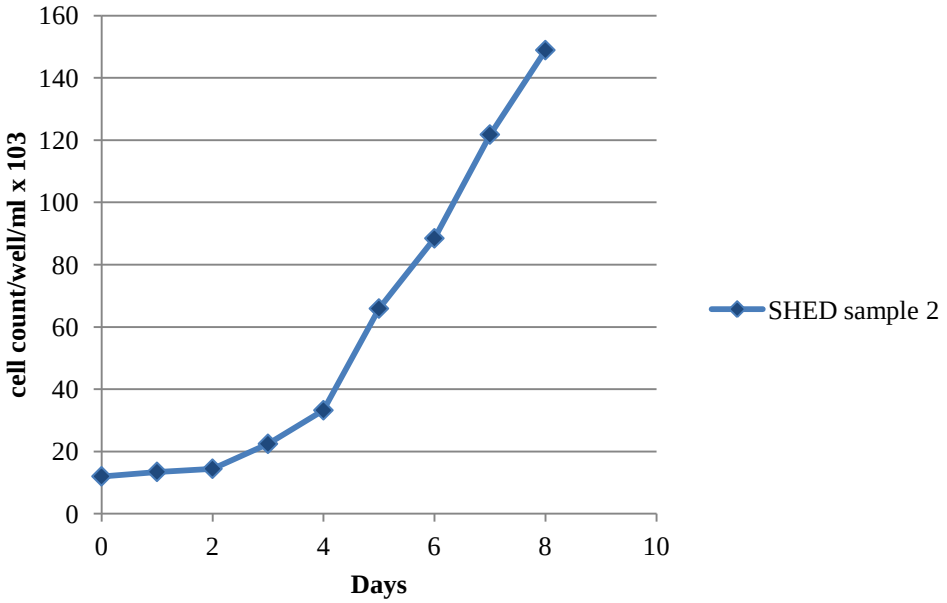


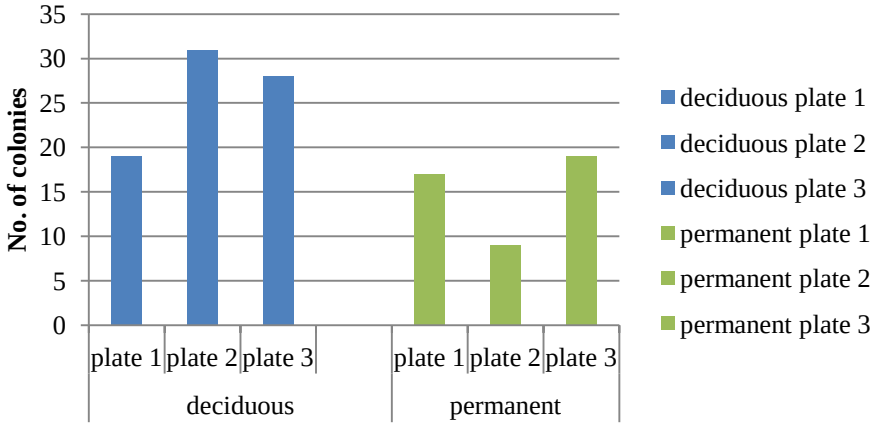
Table 9
Growth curve derivatives of Deciduous sample 2 (2dp)

Day	Cell count/well/ml x 10^3	Slope	Standard deviation (SD)	Population doubling time (hours)	Seeding efficiency (%)
0	12	17.734	13.098	27.603	112
1	13.44				
2	14.44				
3	22.44				
4	33.22				
5	65.89				
6	88.44				
7	121.78				
8	148.89				

Colony forming efficiency of deciduous and permanent samples

Graph 10a

No. of colonies in deciduous and permanent samples



Graph 10b

Colony forming unit efficacy between deciduous and permanent samples

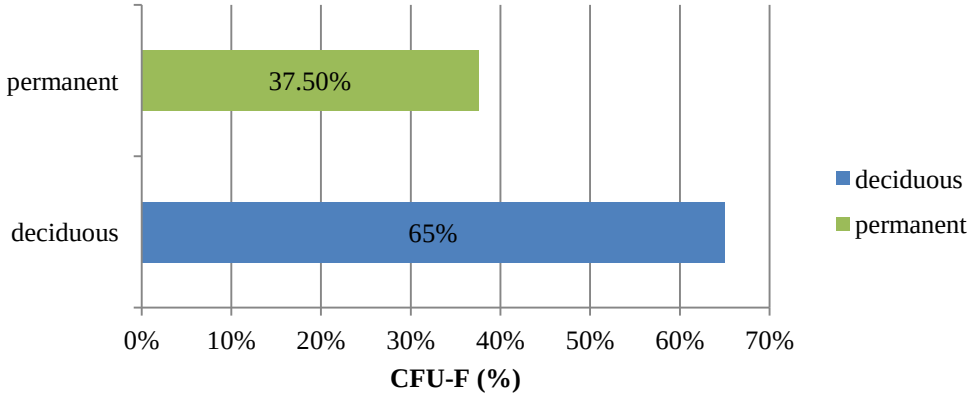


Table 10

Comparison of Colony forming efficiency between deciduous and permanent samples

	sample type	n	Mean	Std. Deviation	P value
CFU-F (%)	deciduous	3	65.0000	15.61249	0.080
	permanent	3	37.5000	13.22876	

CFU-F: Colony forming Unit-Fibroblast

Subpopulation analysis of deciduous sample 2 (2dp)

Graph 11
f I-f VII subpopulation of deciduous sample 2(2dp)

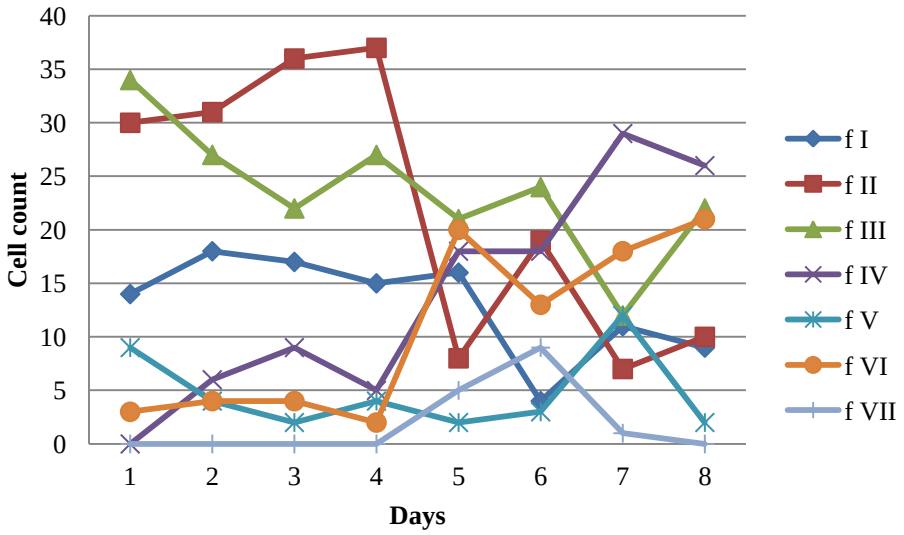


Table 11
f I-f VII subpopulation of deciduous sample 2(2dp)

day	f I	f II	f III	f IV	f V	f VI	f VII
1	22	41	26	0	0	0	1
2	16	36	31	4	2	0	1
3	14	32	34	4	5	1	0
4	11	36	24	12	6	1	0
5	7	12	30	6	17	16	2
6	5	9	12	8	21	34	1
7	9	0	5	21	22	33	0
8	3	2	12	22	29	21	1

Subpopulation analysis of deciduous sample 2 (2dp)

Graph 12
Mitotic and post-mitotic phenotypes in deciduous sample 2 (2dp)

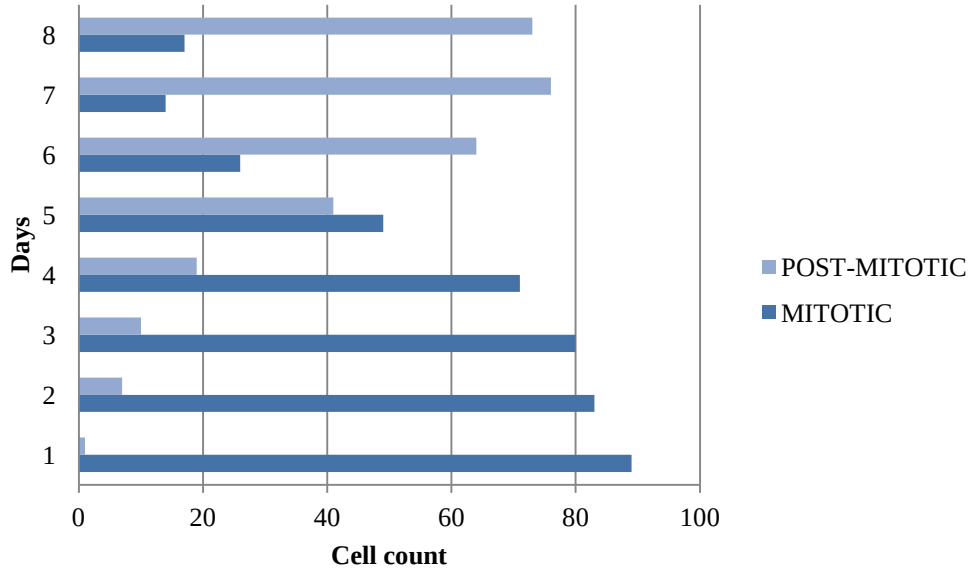


Table 12
Mitotic and post-mitotic phenotypes in deciduous sample 2 (2dp)

Day	MITOTIC	POST-MITOTIC
1	89	1
2	83	7
3	80	10
4	71	19
5	49	41
6	26	64
7	14	76
8	17	73

Subpopulation analysis of deciduous sample 2 (2dp)

Graph 13
F1-F3 subpopulation of deciduous sample 2 (2dp)

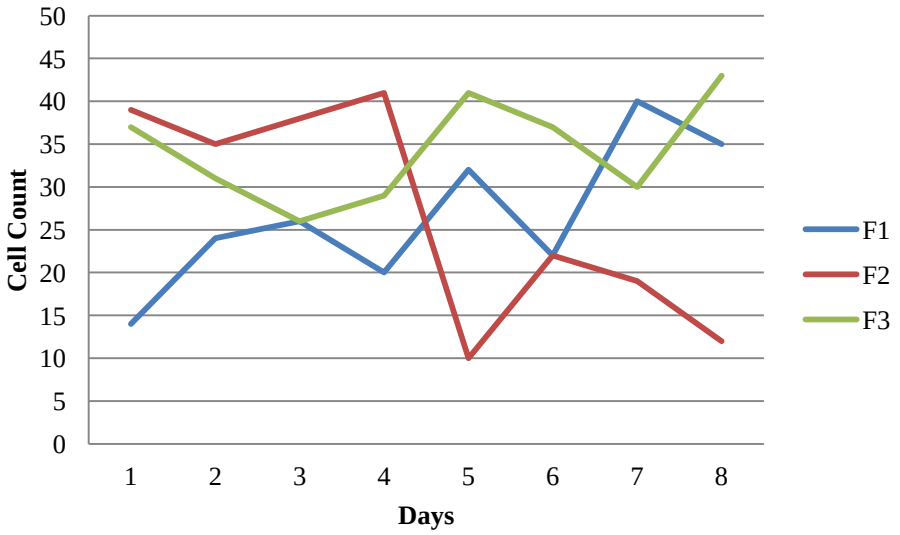


Table 13
F1-F3 subpopulation of deciduous sample 2 (2dp)

Day	F1	F2	F3
1	22	41	26
2	20	38	31
3	18	37	35
4	23	42	25
5	13	29	46
6	13	30	46
7	30	22	38
8	25	31	33

Subpopulation analysis of deciduous sample 2 (2dp)

Graph 14
Fibroblast-like and epitheloid phenotypes in deciduous sample 2 (2dp)

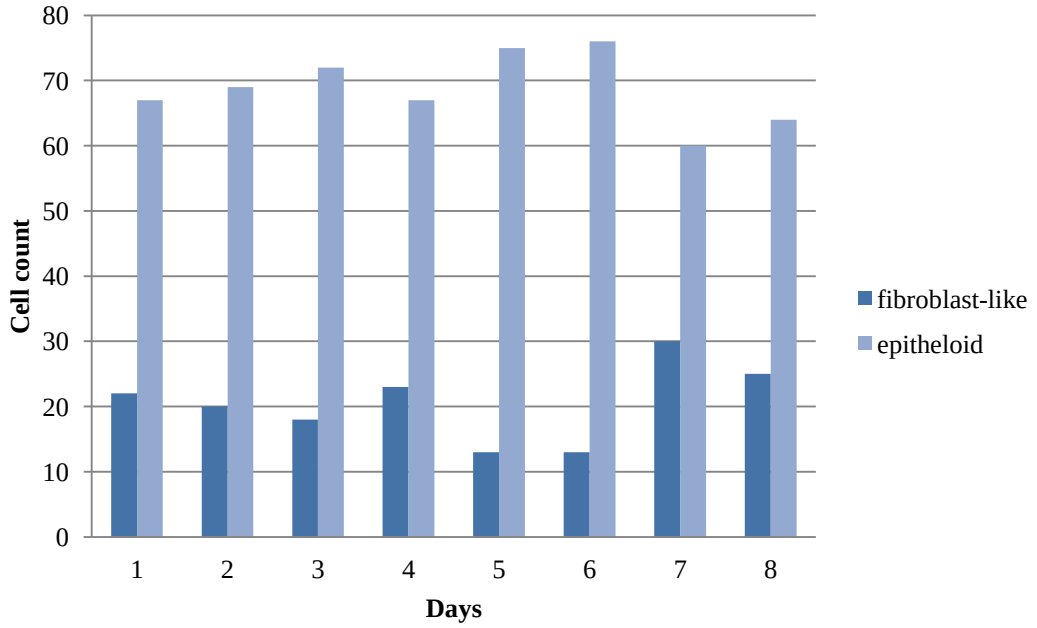
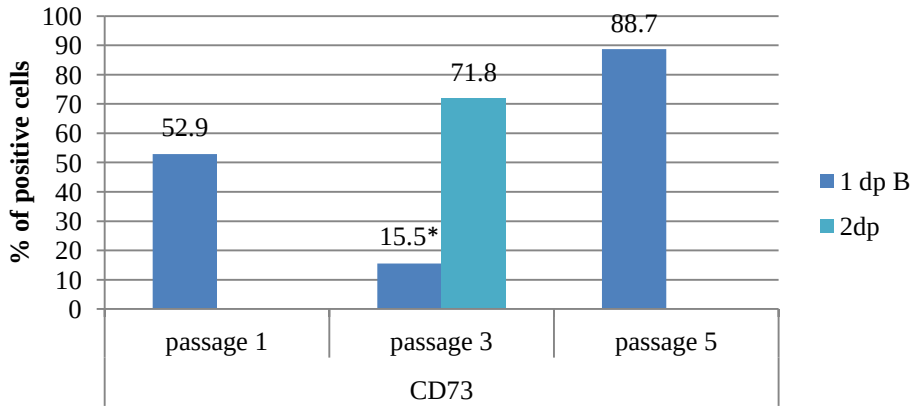


Table 14
Fibroblast-like and epitheloid phenotypes in deciduous sample 2 (2dp)

Day	Fibroblast-like	Epitheloid
1	22	67
2	20	69
3	18	72
4	23	67
5	13	75
6	13	76
7	30	60
8	25	64

Flow cytometry analysis of deciduous samples in the 1st, 3rd and 5th passage of culture

Graph 15a
Flow cytometry analysis of CD73 in deciduous samples



Graph 15b
Flow cytometry analysis of CD146 in deciduous samples

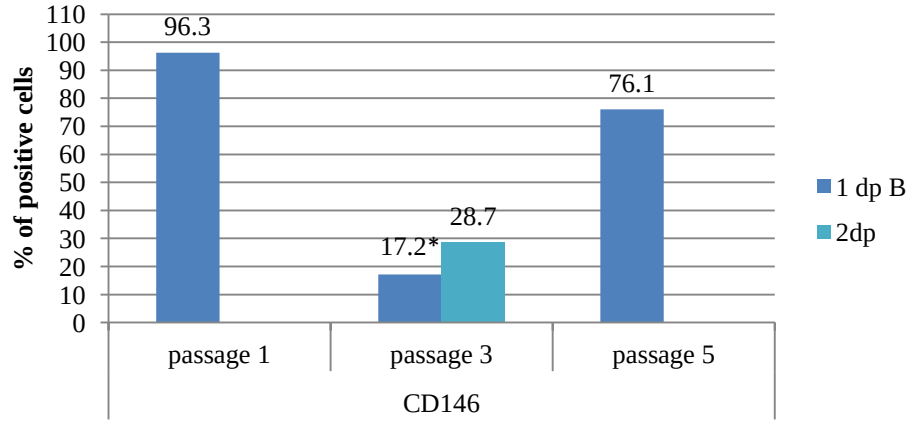


Table 15
Flow cytometry analysis of CD73 and CD146 in deciduous samples

Deciduous samples	**passage 1**		**passage 3**		**passage 5**	
	CD73	**CD146**	**CD73**	**CD146**	**CD73**	**CD146**
1dp B	52.9	96.3	15.5*	17.2*	88.7	76.1
2dp			71.8	28.7		

* samples analyzed twice

Growth characteristics of permanent sample 1(2ap)

Graph 16
Growth curve of Permanent sample 1 (2ap)

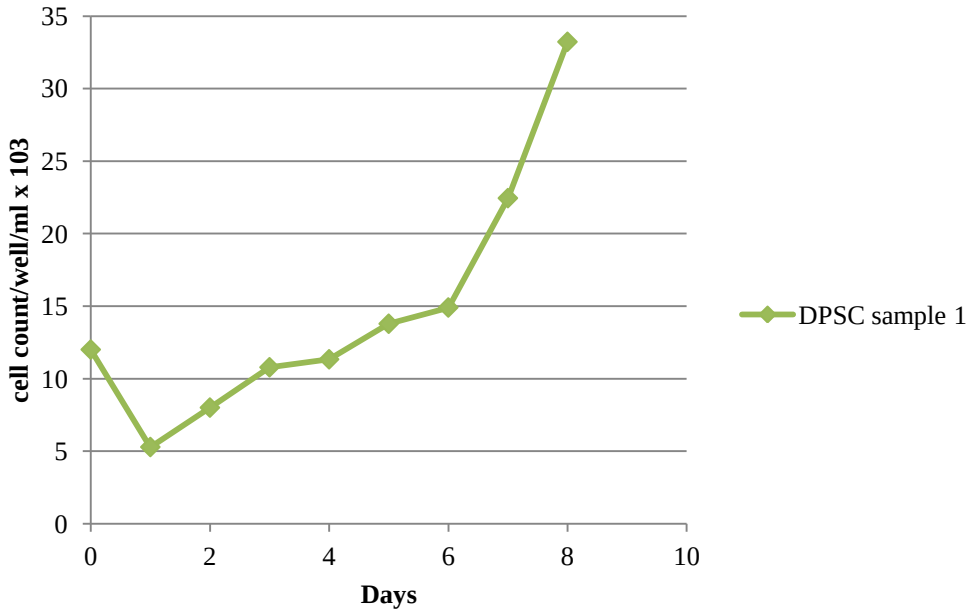


Table 16
Growth curve derivatives of permanent sample 1(2ap)

Day	Cell count/well/ml x 10^3	Slope	Standard deviation (SD)	Population doubling time (hours)	Seeding efficiency (%)
0	12	2.5527	4.426	40.07	44
1	5.28				
2	8				
3	10.78				
4	11.33				
5	13.78				
6	14.9				
7	22.44				
8	33.22				

Subpopulation analysis of permanent sample 1(2ap)

Graph 17
f I-f VII subpopulation of permanent sample 1 (2ap)

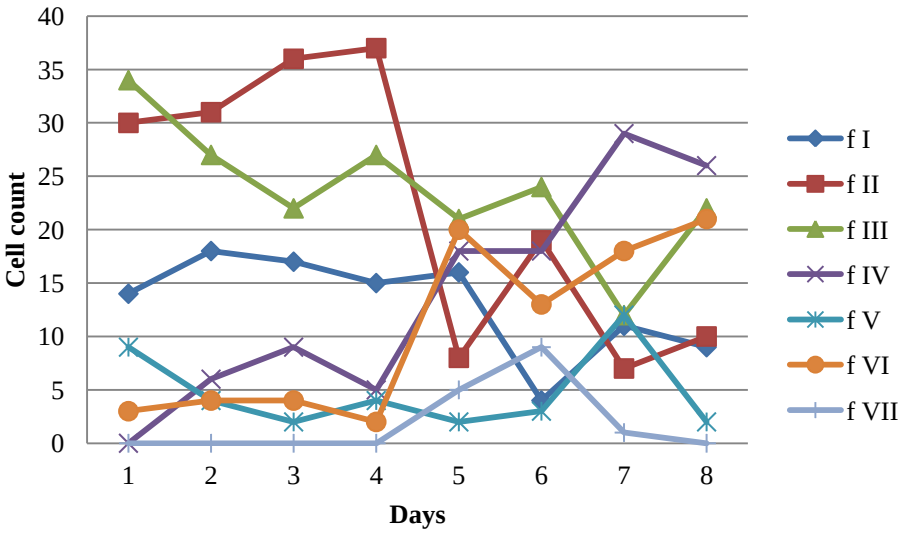


Table 17
f I-f VII subpopulation of permanent sample 1 (2ap)

day	f I	f II	f III	f IV	f V	f VI	f VII
1	14	30	34	0	9	3	0
2	18	31	27	6	4	4	0
3	17	36	22	9	2	4	0
4	15	37	27	5	4	2	0
5	16	8	21	18	2	20	5
6	4	19	24	18	3	13	9
7	11	7	12	29	12	18	1
8	9	10	22	26	2	21	0

Subpopulation analysis of permanent sample 1(2ap)

Graph 18
Mitotic and post-mitotic phenotypes in permanent sample 1 (2ap)

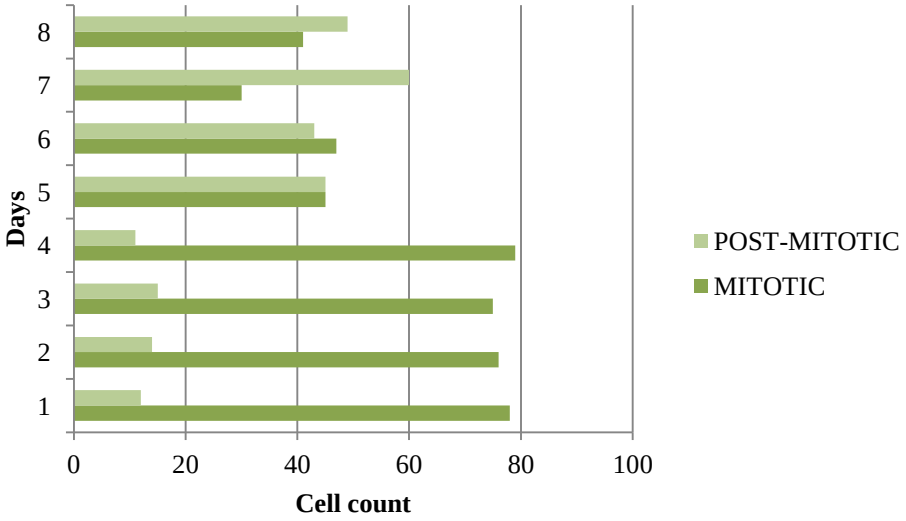


Table 18
Mitotic and post-mitotic phenotypes in permanent sample 1 (2ap)

Day	MITOTIC	POST-MITOTIC
1	78	12
2	76	14
3	75	15
4	79	11
5	45	45
6	47	43
7	30	60
8	41	49

Subpopulation analysis of permanent sample 1(2ap)

Graph 19
F1-F3 subpopulation of permanent sample 1(2ap)

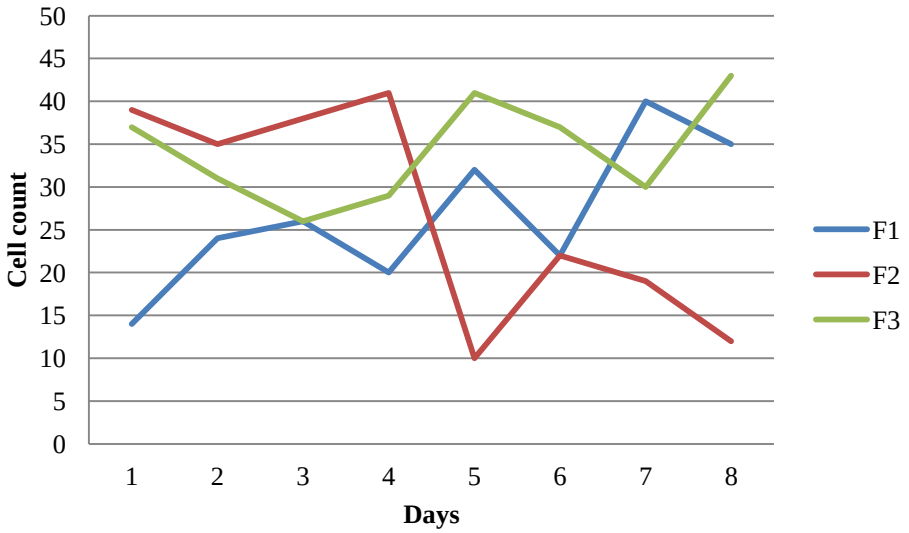


Table 19
F1-F3 subpopulation of permanent sample 1(2ap)

Day	F1	F2	F3
1	14	39	37
2	24	35	31
3	26	38	26
4	20	41	29
5	32	10	41
6	22	22	37
7	40	19	30
8	35	12	43

Subpopulation analysis of permanent sample 1(2ap)

Graph 20
Fibroblast-like and epitheloid phenotypes in permanent sample 1(2ap)

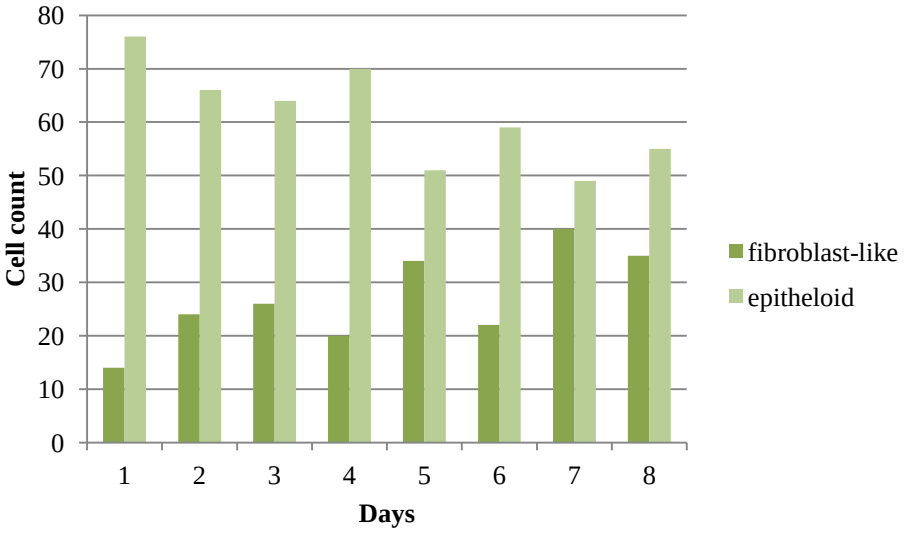


Table 20
Fibroblast-like and epitheloid phenotypes in permanent sample 1(2ap)

Day	Fibroblast-like	Epitheloid
1	14	76
2	24	66
3	26	64
4	20	70
5	34	51
6	22	59
7	40	49
8	35	55

Growth characteristics of permanent sample 2 (12ap)

Graph 21
Growth curve of Permanent sample 2 (12ap)

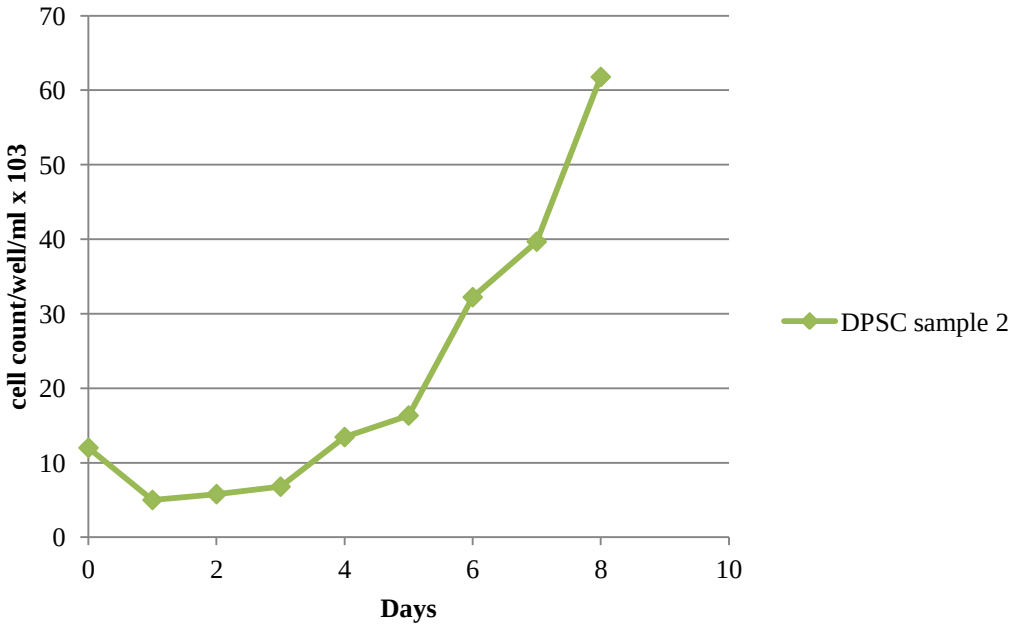


Table 21
Growth curve derivatives of permanent sample 2 (12ap)

Day	Cell count/well/ml x 10^3	Slope	Standard deviation (SD)	Population doubling time (hours)	Seeding efficiency (%)
0	12	6.0927	2.9262	33.76	41.67
1	5				
2	5.78				
3	6.78				
4	13.44				
5	16.33				
6	32.22				
7	39.67				
8	61.78				

Subpopulation analysis of permanent sample 2 (12ap)

Graph 22
f I-f VII subpopulation of permanent sample 2 (12ap)

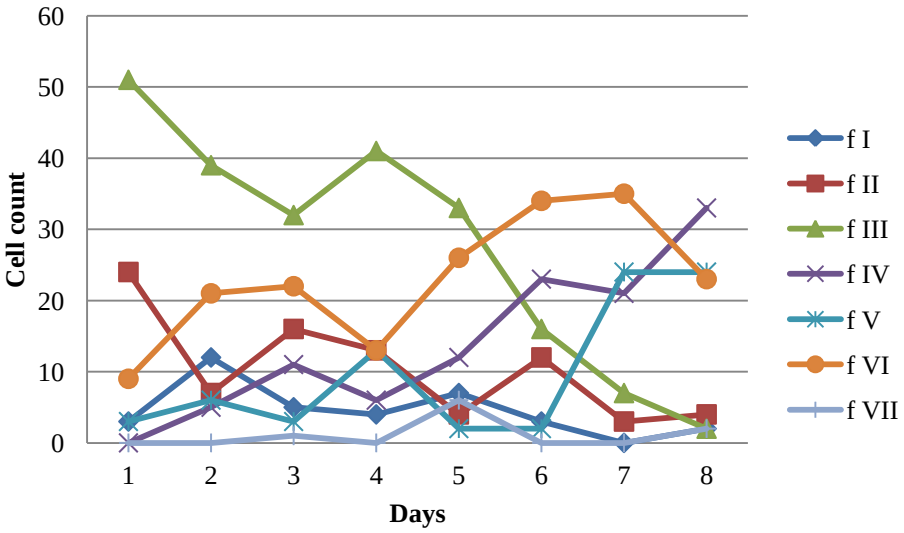


Table 22
f I-f VII subpopulation of permanent sample 2 (12ap)

day	f I	f II	f III	f IV	f V	f VI	f VII
1	3	24	51	0	3	9	0
2	12	7	39	5	6	21	0
3	5	16	32	11	3	22	1
4	4	13	41	6	13	13	0
5	7	4	33	12	2	26	6
6	3	12	16	23	2	34	0
7	0	3	7	21	24	35	0
8	2	4	2	33	24	23	2

Subpopulation analysis of permanent sample 2 (12ap)

Graph 23
Mitotic and post-mitotic phenotypes in permanent sample 2 (12ap)

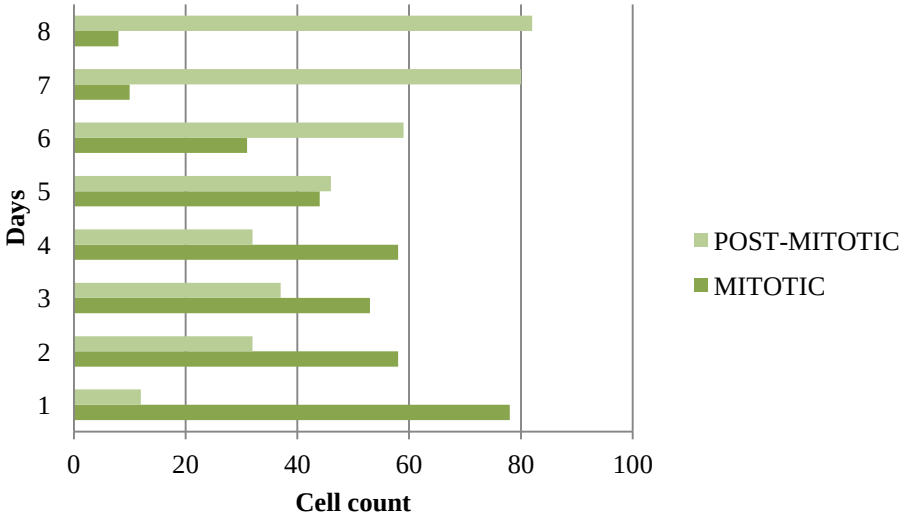


Table 23
Mitotic and post-mitotic phenotypes in permanent sample 2 (12ap)

Day	MITOTIC	POST-MITOTIC
1	78	12
2	58	32
3	53	37
4	58	32
5	44	46
6	31	59
7	10	80
8	8	82

Subpopulation analysis of permanent sample 2 (12ap)

Graph 24
F1-F3 subpopulation of permanent sample 2 (12ap)

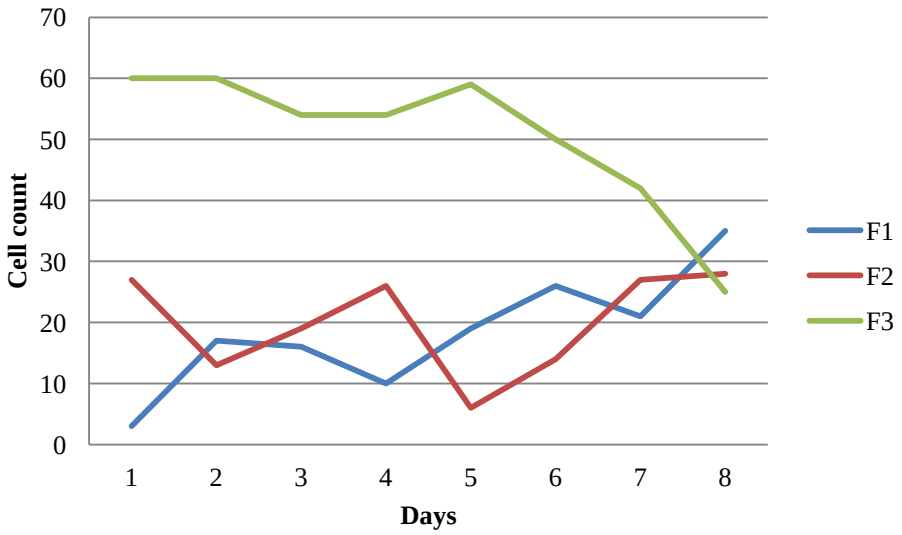


Table 24
F1-F3 subpopulation of permanent sample 2 (12ap)

Day	F1	F2	F3
1	3	27	60
2	17	13	60
3	16	19	54
4	10	26	54
5	19	6	59
6	26	14	50
7	21	27	42
8	35	28	25

Subpopulation analysis of permanent sample 2 (12ap)

Graph 25
Fibroblast-like and epitheloid phenotypes in permanent sample 2 (12ap)

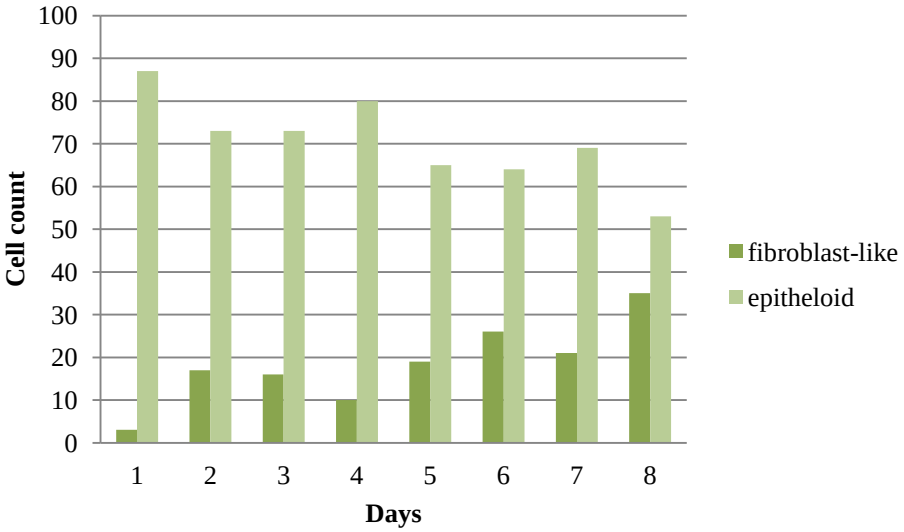


Table 25
Fibroblast-like and epitheloid phenotypes in permanent sample 2 (12ap)

Day	fibroblast-like	epitheloid
1	3	87
2	17	73
3	16	73
4	10	80
5	19	65
6	26	64
7	21	69
8	35	53

Immunocytochemical analysis of CD73 and CD146 in permanent samples in the 1st, 3rd and 5th passage of culture

Graph 26
Immunocytochemical expression in permanent samples

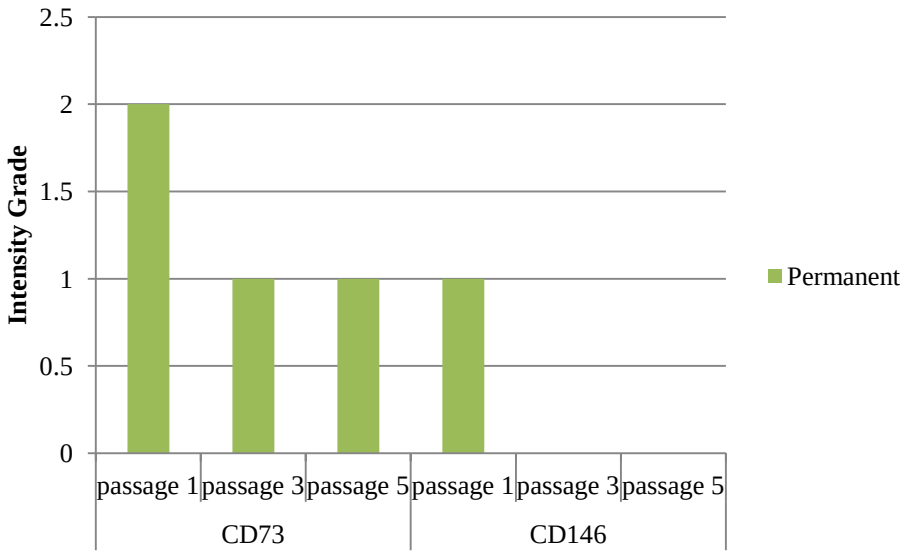


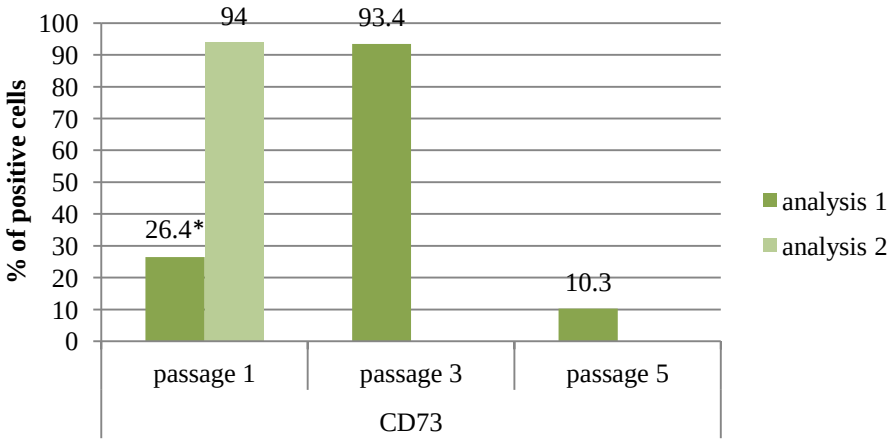
Table 26
Immunocytochemical in permanent samples

Permanent samples	CD73			CD146		
	passage 1	**passage 3**	**passage 5**	**passage 1**	**passage 3**	**passage 5**
Staining intensity	2	1	1	1	0	0

0- no staining
1- Mild staining
2- Moderate staining
3- Intense staining

Flow cytometry analysis of permanent samples in the 1st, 3rd and 5th passage of culture

Graph 27a
Flow cytometry analysis of CD73 in permanent samples



Graph 27b
Flow cytometry analysis of CD146 in permanent samples

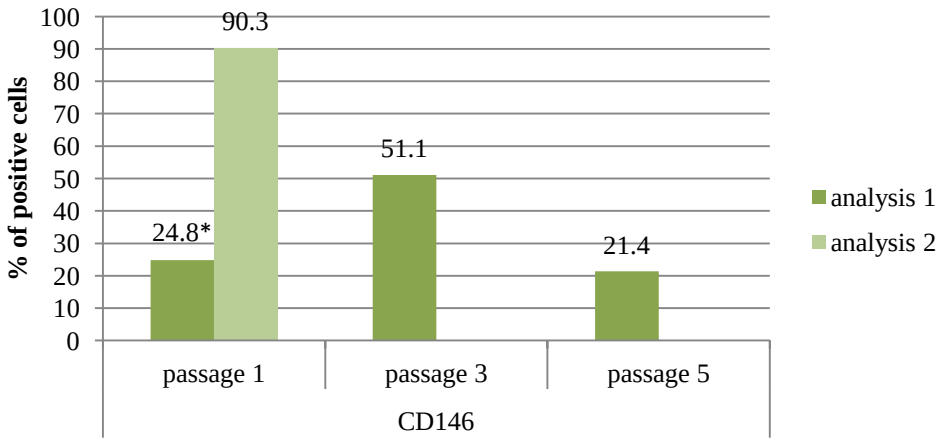


Table 27
Flow cytometry analysis of CD73 and CD146 in permanent samples

permanent samples	CD73			CD146		
	passage 1	**passage 3**	**passage 5**	**passage 1**	**passage 3**	**passage 5**
	26.4	93.4	10.3	24.8	51.1	21.4
	94			90.3		

* Samples analyzed twice

Table 28

Comparison of days to reach confluence between deciduous and permanent samples

	Sample	n	Mean	Std. Deviation	P value
Time taken to reach confluence (days)	Deciduous	2	19.00	9.899	0.139
	Permanent	5	38.60	14.064	

Table 29

Comparison of population doubling time between deciduous and permanent samples

	sample type	n	Mean	Std. Deviation	P value
Population doubling time (hrs)	deciduous	2	31.2815	5.20218	0.365
	permanent	2	36.9150	4.46184	

Table 30

Comparison of seeding efficiency between deciduous and permanent samples

	sample type	n	Mean	Std. Deviation	P value
Seeding efficiency (%)	deciduous	2	99.5000	17.67767	**0.046***
	permanent	2	42.8350	1.64756	

* Statistically significant at 5% level, $p < 0.05$

Table 31
Comparison of f I-f VII phenotypes in deciduous and permanent samples

	Sample	n	Mean	Std. Deviation	P value
f I	Deciduous	16	15.38	8.717	**0.018***
	Permanent	16	8.75	5.994	
f II	Deciduous	16	23.38	12.873	0.116
	Permanent	16	16.31	11.768	
f III	Deciduous	16	27.13	11.331	0.728
	Permanent	16	25.63	12.811	
f IV	Deciduous	16	7.19	7.054	**0.041***
	Permanent	16	13.88	10.366	
f V	Deciduous	16	8.31	8.875	0.701
	Permanent	16	7.19	7.458	
f VI	Deciduous	16	8.00	11.804	**0.034***
	Permanent	16	16.75	10.491	
f VII	Deciduous	16	.63	.719	0.225
	Permanent	16	1.50	2.733	

* Statistically significant at 5% level, $p < 0.05$

Table 32

Comparison of mitotic and post-mitotic phenotypes in deciduous and permanent samples

	Specimen	n	Mean	Std. Deviation	P value
Mitotic	Deciduous	16	65.88	25.861	0.091
	Permanent	16	50.69	23.260	
Post - Mitotic	Deciduous	16	24.13	25.861	0.091
	Permanent	16	39.31	23.260	

Table 33

Comparison of F1, F2 and F3 phenotypes in deciduous and permanent samples

	Specimen	n	Mean	Std. Deviation	P value
F1	Deciduous	16	22.56	6.811	0.983
	Permanent	16	22.50	9.791	
F2	Deciduous	16	31.69	6.935	**0.017***
	Permanent	16	23.50	10.967	
F3	Deciduous	16	35.13	9.472	0.073
	Permanent	16	42.38	12.430	

* Statistically significant at 5% level, $p < 0.05$

Figures

Reagents for cell culture and immunocytochemistry

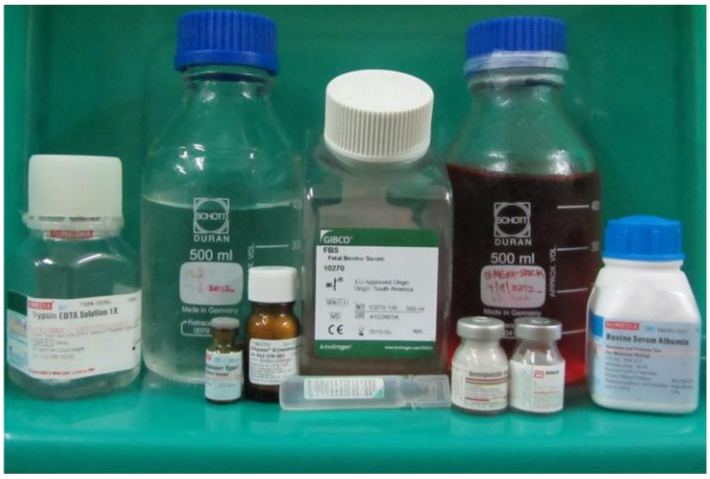


Figure.1: Reagents used for cell culture and immunocytochemistry

Figure.2: Antibodies used for immunocytochemistry

Equipment for cell culture

Figure.3: CO_2 incubator

Figure.4: Inverted phase-contrast microscope

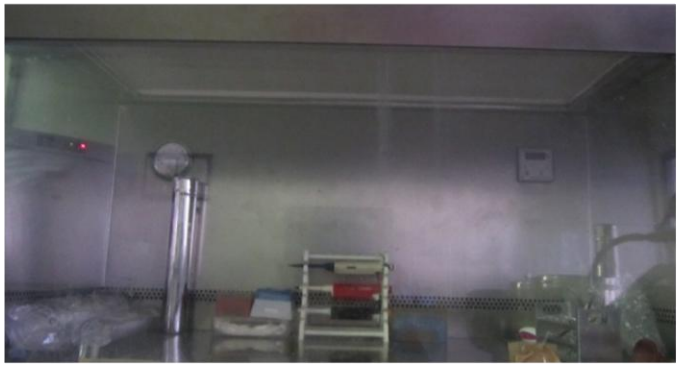


Figure.5: Laminar air flow chamber

Equipment for flow cytometry

Figure.6:Flow cytometer
(BD Bioscience ™)

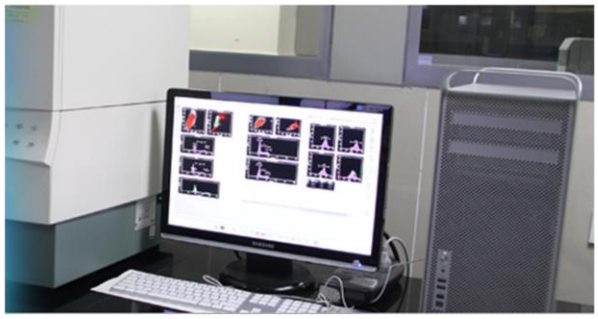


Figure.7:Software for analysis- FlowJo ™

Armamentarium and procedure for isolation of dental pulp tissue

Figure.8: Instruments for isolation of dental pulp tissue

Figure.9: Reagents for isolation of dental pulp tissue

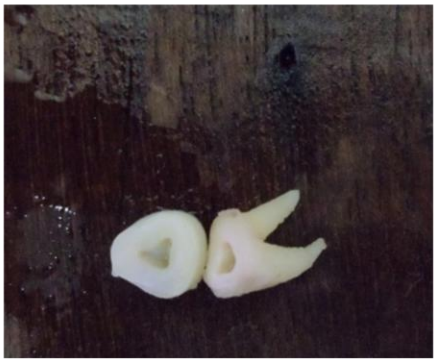


Figure.10:Tooth sectioned at the cemento-enamel junction

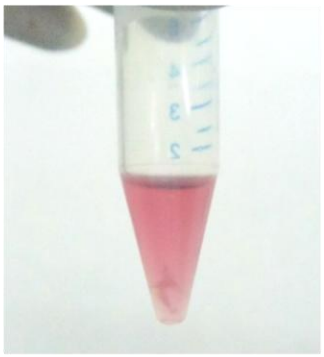
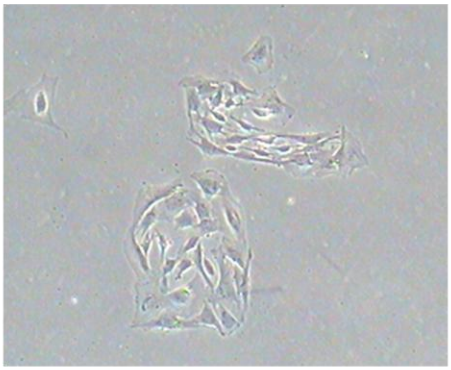


Figure.11: Tissue taken for enzyme disaggregation

DECIDUOUS TOOTH CULTURE (SHED)



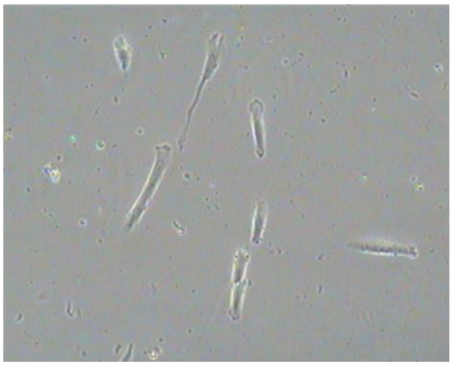


Figure. 12,13:Primary culture

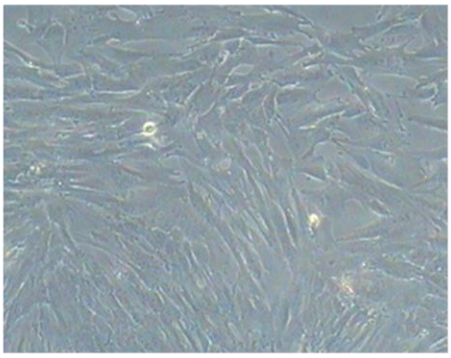


Figure.14: 12 days after plating

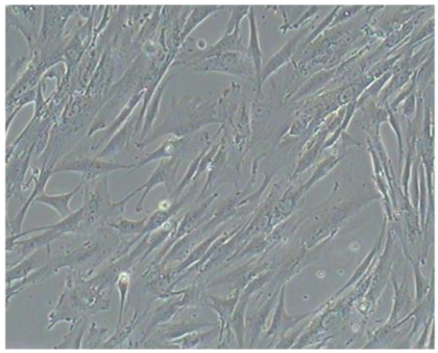


Figure.15: Passage 1

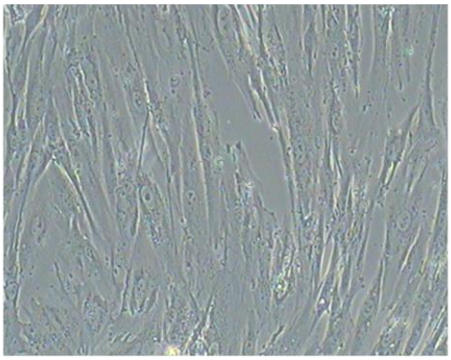


Figure.16: Passage 3

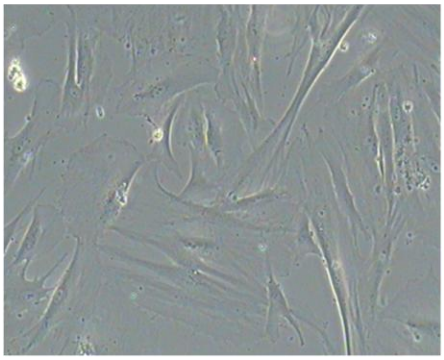
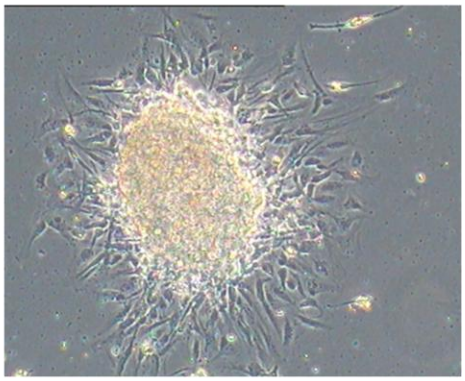


Figure.17: Passage 5

PERMANENT TOOTH CULTURE (DPSC)



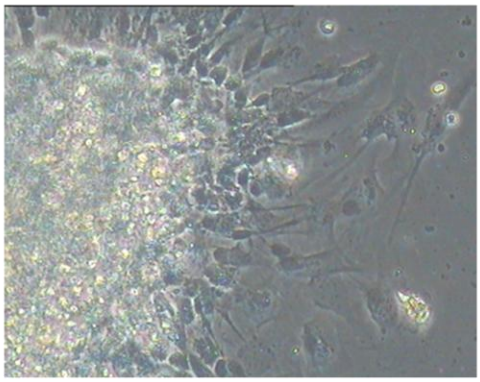


Figure.18,19: Primary culture

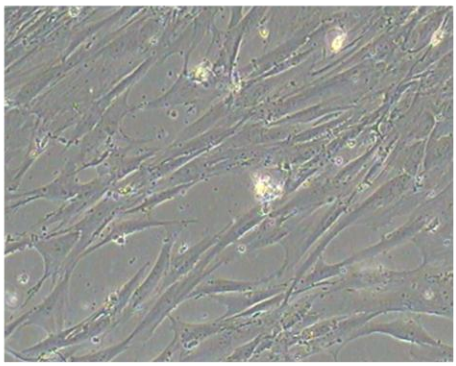


Figure.20: 12 days after plating

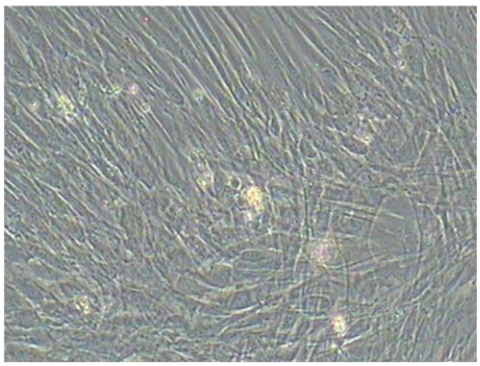


Figure.21: Passage 1

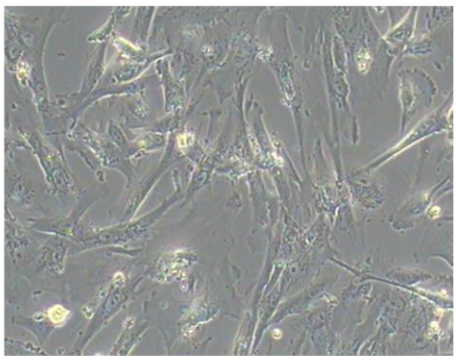


Figure.22: Passage 3

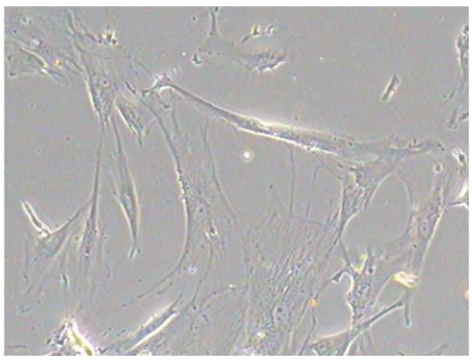


Figure. 23: Passage 5

Trypsinisation and contamination of cultures

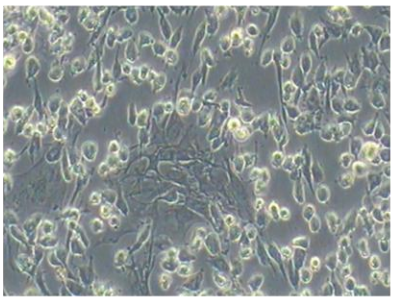


Figure.24: Trypsinisation of cells in culture

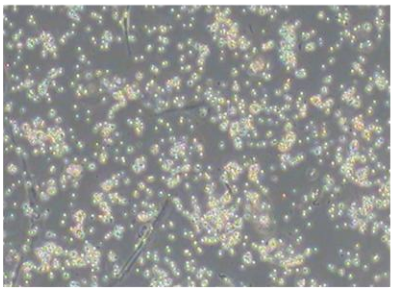


Figure.25: Bacterial contamination

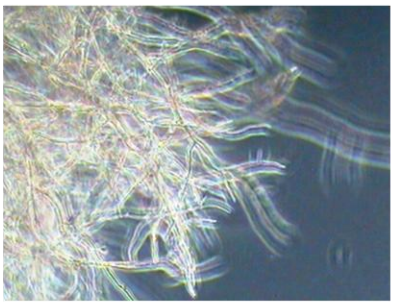


Figure.26: Fungal contamination

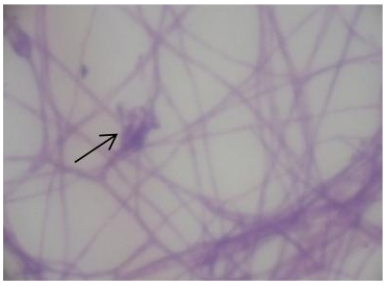


Figure.27: H&E stained smear of fungal contamination

Colony Forming Unit (CFU-F)

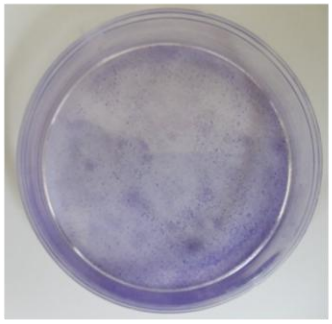


Figure.28: Colonies seen in the culture dish

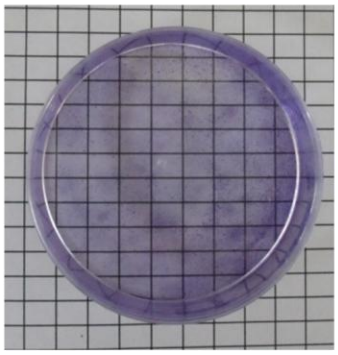


Figure.29: Grid used to count the colonies

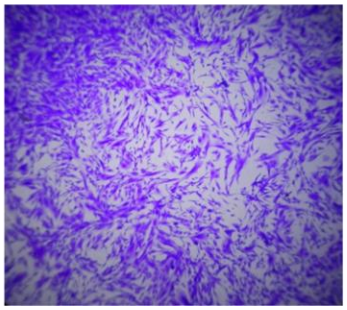


Figure.30: Colonies stained with crystal violet (10x)

Morphology (f I-f VII)
Mitotic and post-mitotic phenotypes
(Bayreuther et al. classification)

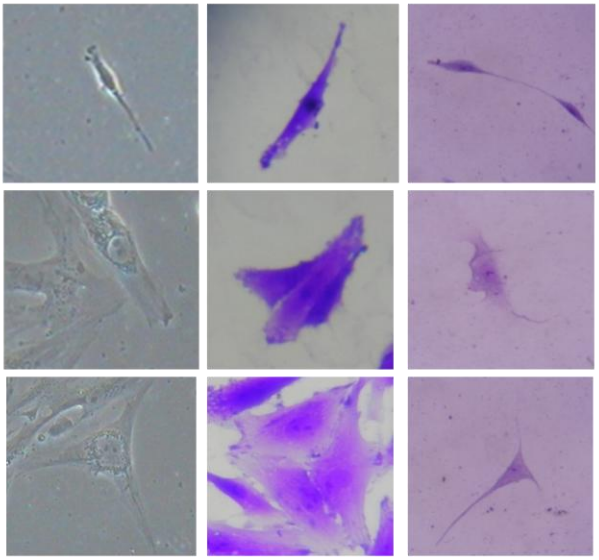


Figure.31-33: **f I mitotic phenotype**
(phase contrast , crystal violet and giemsa staining)

Figure.34-36: **f II mitotic phenotype**
(phase contrast, crystal violet and giemsa staining)

Figure.37-39: **f III mitotic phenotype**
(phase contrast, crystal violet and giemsa staining)

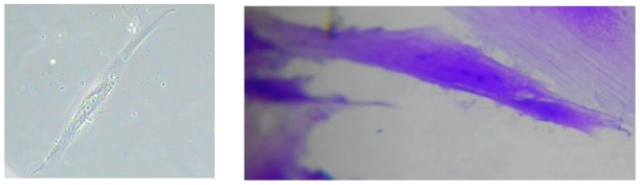


Figure. 40,41: **f IV post-mitotic phenotype**
(phase contrast and crystal violet staining)

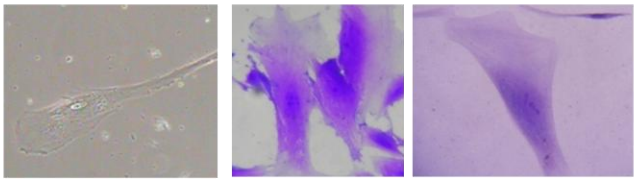


Figure.42-44: **f V post-mitotic phenotype**
(phase contrast, crystal violet and giemsa staining)

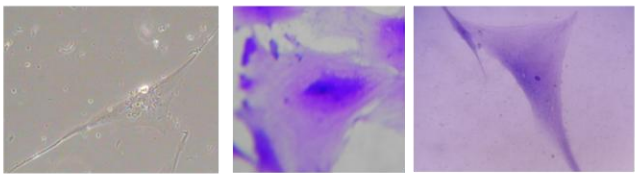


Figure.45-47: **f VI post-mitotic phenotype**
(phase contrast, crystal violet and giemsa staining)

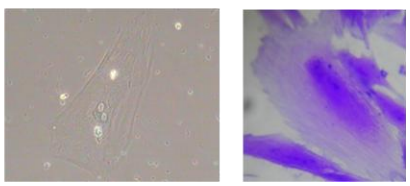


Figure.48,49: **f VII degenerating cell**
(phase contrast and crystal violet staining)

Immunocytochemistry staining with CD73 and CD146

SHED Passage 1

CD73

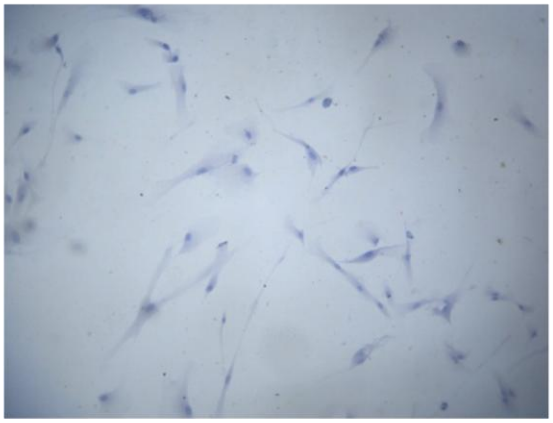


Figure.50: (10x view) intensity grade 0

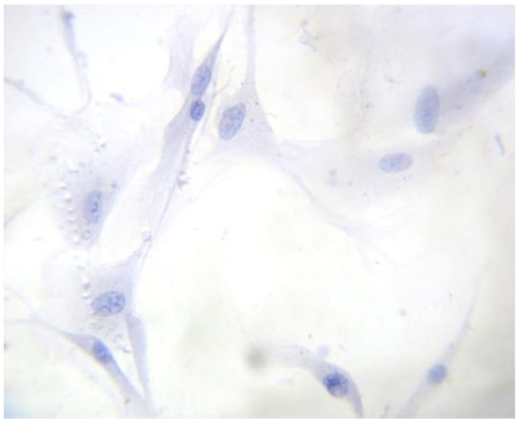


Figure.51: (40x view) intensity grade 0

CD146

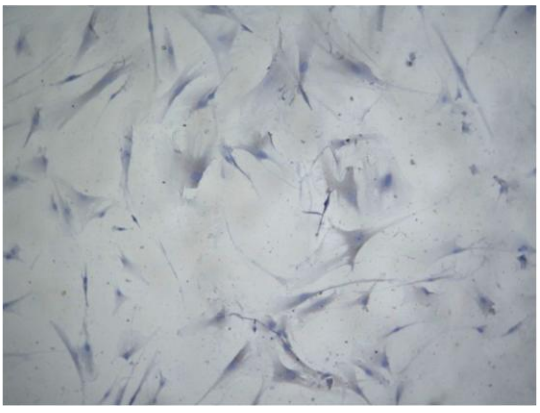


Figure.52: (10x view) intensity grade 2

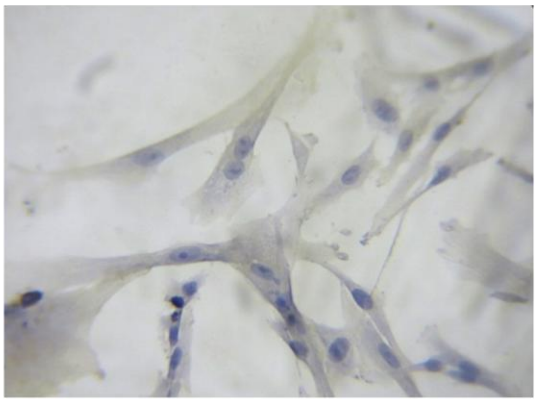


Figure.53: (40x view) intensity grade 2

SHED passage 3

CD73

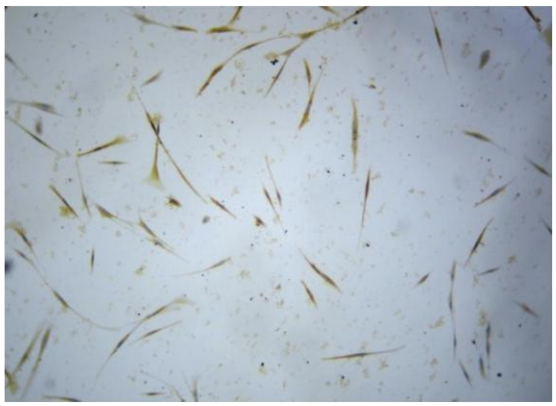


Figure.54: (10x view) intensity grade 3

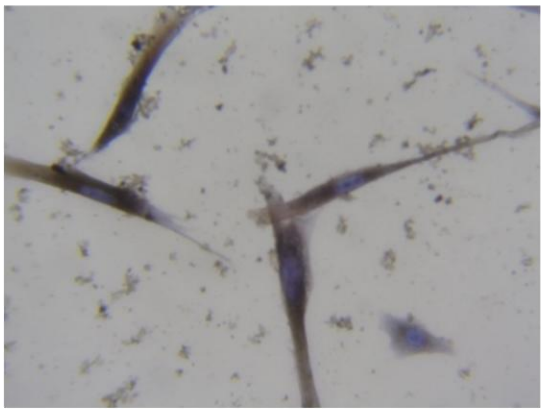


Figure.55: (40x view) intensity grade 3

CD146

Figure.56: (10x view) intensity grade 2

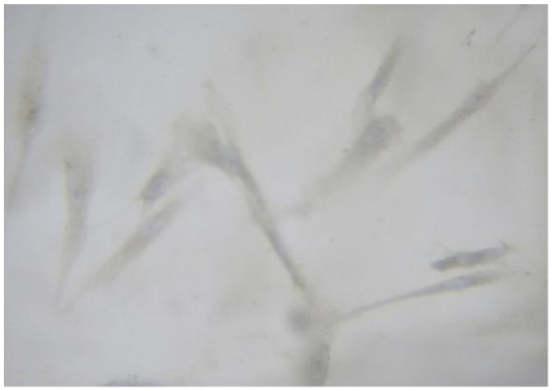


Figure.57: (40x view) intensity grade 2

SHED passage 5

CD73

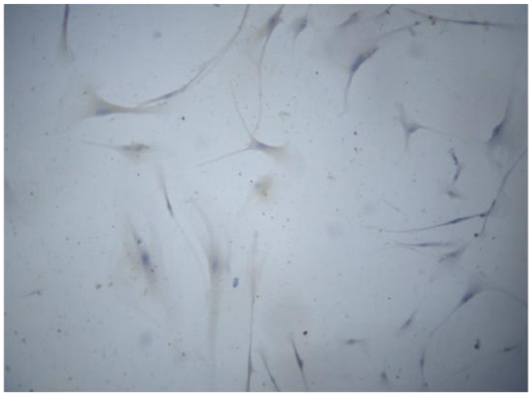


Figure.58: (10x view) intensity grade 2

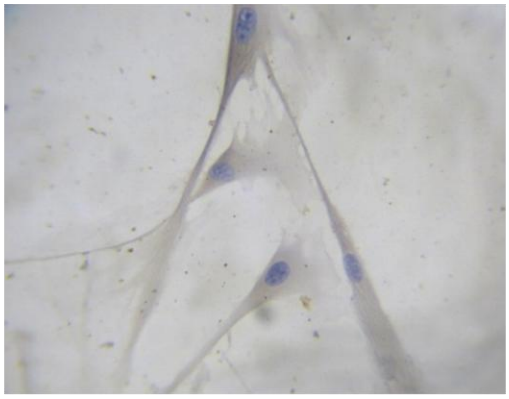


Figure.59: (40x view) intensity grade 2

CD146

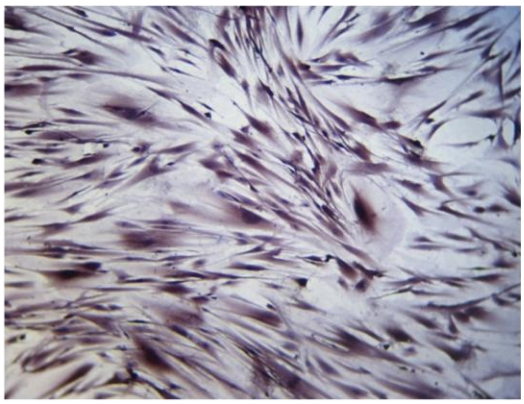


Figure.60: (10x view) intensity grade 3

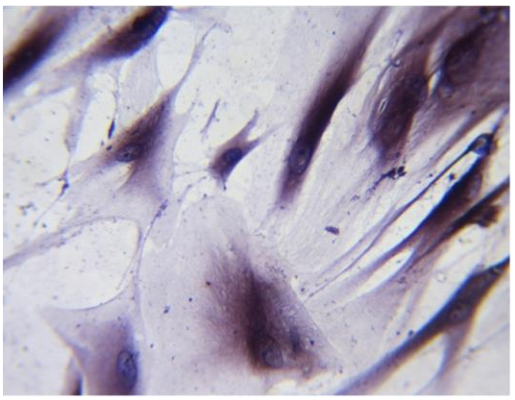


Figure.61: (40x view) intensity grade 3

DPSC passage 1

CD73

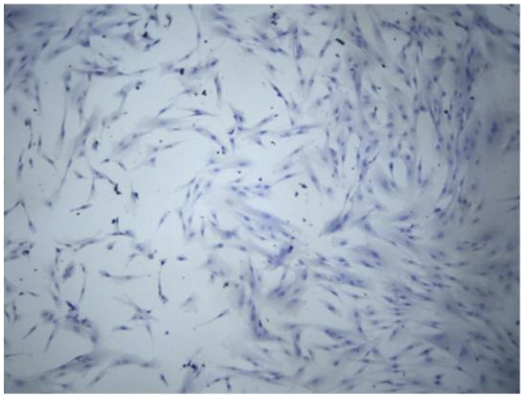


Figure.62: (10x view) intensity grade 2

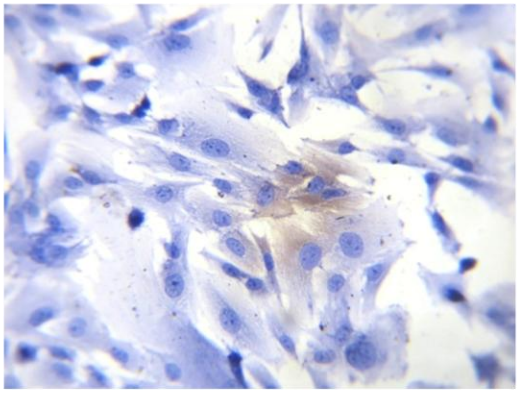


Figure.63: (40x view) intensity grade 2

CD146

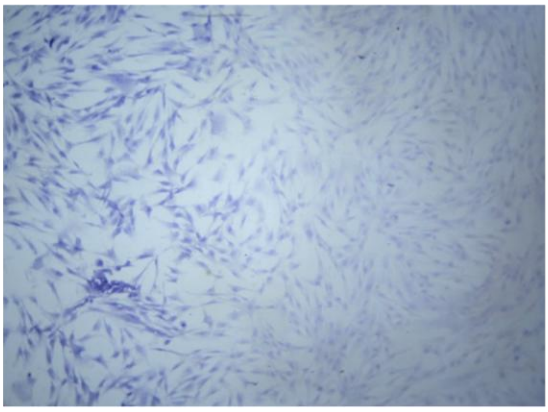


Figure.64: (10x view) intensity grade 1

DPSC Passage 3

CD73

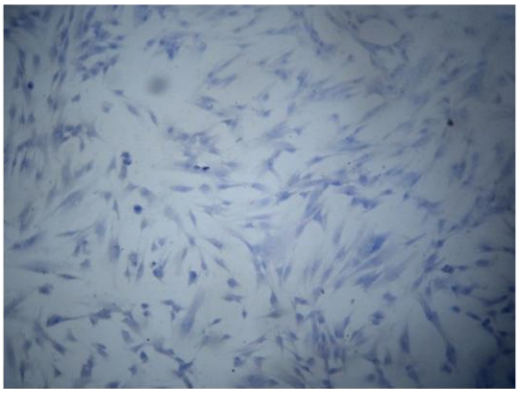


Figure.65: (10x view) intensity grade 1

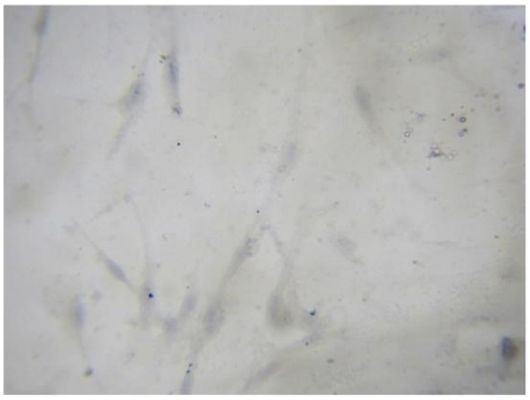


Figure.66: (40x view) intensity grade 1

CD146

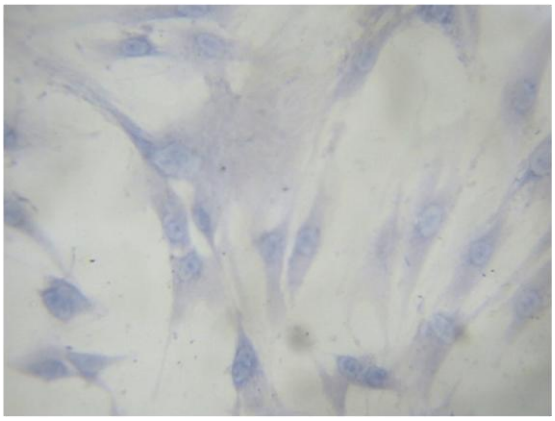


Figure.67: (40x view) intensity grade 0

DPSC passage 5

CD73

Figure.68: (10x view) intensity grade 1

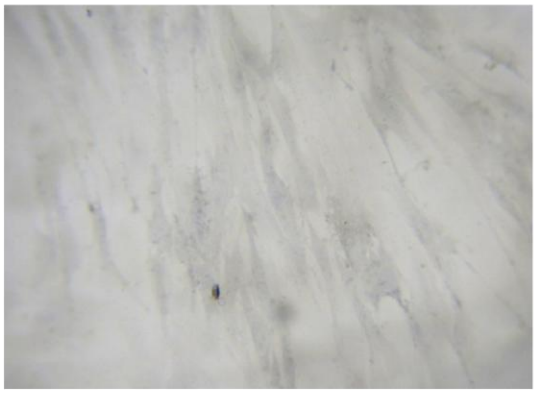


Figure.69: (40x view) intensity grade 1

CD146

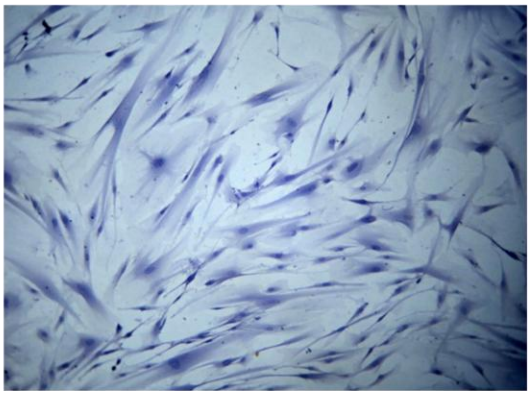


Figure.70: (10x view) intensity grade 0

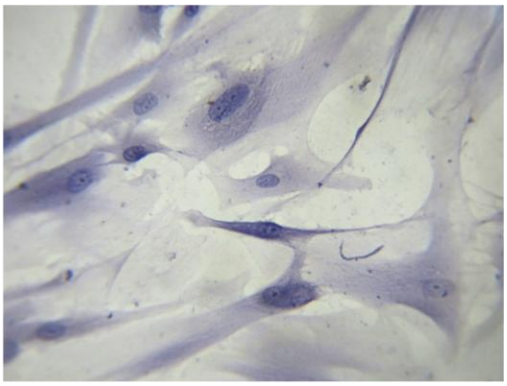


Figure.71: (40x view) intensity grade 0

Discussion

The study was done to compare the growth characteristics and morphology between stem cells isolated from deciduous and permanent teeth and also study their immunophenotype between the 1st, 3rd and 5th passage of culture using mesenchymal stem cell markers, CD73 and CD146.

Stem cells from the dental pulp possess mesenchymal stem cell-like properties. Few studies have compared the characteristics of SHED and DPSCs to find the better source of stem cells and have found differences in growth characteristics and cell surface antigen profiles [12, 42]. SHED and DPSCs have the potential to differentiate into classic lineages such as osteogenic, adipogenic and chondrogenic [12] as well as into certain specialized differentiation pathways such as neuronal and hepatic [56]. These cells, in culture, can also undergo spontaneous differentiation and the default pathway is osteogenic [45]. However, little is known about when the transformation to lineage-committed cells occurs in culture. Furthermore, loss of differential potential with increase in passage number may be due to contamination of mesenchymal stem cell (MSC) cultures by mature cells like fibroblasts. One of our objectives was to evaluate the cell surface antigen profiles at different passages so as to identify when the transformation of cells to differentiated phenotypes occurs. This can help in deriving purified cultures with optimal yield for clinical applications and also prevent the potential for tumor formation following transplantation [36].

Culture characteristics

Cultures of cells from the oral cavity are fraught with difficulties, the major problem being contamination. Cells in the culture environment are extremely sensitive and oral microbial flora, which are commensals in the oral cavity, can become detrimental to these cells in culture. Cells from the dental pulp are, however, physically encased in a protected niche and relatively free of contamination unless the tooth is decayed or injured. Transport of the sample and isolation of the pulp needs sterile conditions. In our study, in spite of taking the necessary precautions, 2 of the 15 samples cultured, succumbed to contamination. No cell growth was seen after plating in 5 of the 15 samples cultured. This was due to excessive washing of the tissues with PBS containing 5X antibiotics, small amounts of tissue obtained from the pulp or using only dispase (without collagenase) for enzyme disaggregation. In studies by Perry *et al.*, 7 of 40 cultures were contaminated with yeast and 2 of 40 cultures showed no cell growth [26]. We found that preliminary decontamination with povidone iodine similar to that done by Perry *et al.* decreased the amount of contamination from tooth samples and only one sample succumbed to bacterial contamination and one sample, to fungal contamination. This may be due to donor-related factors such as oral health and flora [26].

In our study, alpha-MEM supplemented with antibiotics was used as the transport medium and all samples were processed immediately. Perry *et al.* suggested that if extracted teeth are to be processed immediately, PBS may be an acceptable, inexpensive, and widely available collection/transport medium. They also found that viable stem cells can be isolated from teeth after storage for up to 120 hours and extensive tissue processing

immediately after extraction may not be a limiting factor for successful culture of DPSC. Following cryopreservation, they found that DPSC could be recovered from 100% of early passage cultures and from 70% of whole intact teeth frozen for at least 1 month [26].

Growth characteristics

We observed that very small bits of tissue from exfoliating deciduous teeth was sufficient to establish cultures and cells in primary culture reached confluency very fast (19 days ±9.89 SD). In permanent tooth samples, even with extirpation of the entire pulp, cells took longer to grow (38.6days ±14.06 SD) *(Table. 28).* In a study by Perry *et al.*, 70 % confluence was reached in 14 days [26]

Seeding efficiency determines the percentage of cells getting attached to the plate after 12 hours of plating the cells [72]. In our study, the seeding efficiency was significantly higher in deciduous samples (mean 99.5% ± 7.68 SD) than permanent samples (mean 42.84% ± 1.65 SD) ($P<0.05$). Deciduous samples (mean 31.28 hrs ± 5.20 SD) had a lower population doubling time than permanent samples (mean 36.92 hrs ± 4.46 SD) *(Table. 29, 30).*

In a previous study done in our department [73], the seeding efficiency of SHED ranged from 42.6 to 119.4% and that of DPSCs ranged from 37 to 112%. SHED had a doubling time ranging from 13 hours to 246 hours and the doubling time of DPSCs ranged from 37 hours to 144 hours. In another study [74], the seeding efficiency of the cell lines was 84.25% for SHED and 88.9% and 91.7% for DPSCs. The population doubling time for DPSCs was 26 hours and 27 hours and for SHED, it was 22 hours.

Our results were similar to that of Miura *et al.* where SHED had more population doublings that DPSCs and BMMSCs (>140 population doublings) [2]. Govindasamy *et al.* (2010) found that the population doubling time varied between 27 hours (P1) to 29 hours (P5) for SHED, and 28 hours (P1) and 29 hours (P5) in DPSCs [12]. Suchanek *et al.* found the SHED had a higher average doubling time (60.8 hrs) compared to DPSCs (24.5 hrs). The differences in Population Doubling Time in our study and studies by other authors could be due to the fact that the PDT is an average value, representing a population of cells which includes dividing cells, non-dividing cells and dying cells [72]. Elevated proliferation capacity of SHED has been attributed to increased telomerase activity in SHED [32]. Low proliferation in DPSCs can also be due to their function as reserve cells in the dental pulp of adult teeth. Stimulation is brought about by minor inflammation, but pro-inflammatory cytokines may also cause DPSCs to senesce early [58]. In SHED, it is possible that a small amount of inflammation is present due to root resorption. This, along with the age of the individual and the possibly the presence of local chemokines and growth factors, make stem cells from SHED highly proliferative and cultures can be established from very small quantities of tissue.

Colony forming efficiency

Each colony of cells originates from a single progenitor cell, the colony forming unit-fibroblast (CFU-F). The colony forming efficiency denotes the clonogenic potential of the cell population and can be derived from the number of colonies formed by seeding the cells at low density.

In our study, the number of colonies formed from the deciduous samples was higher (mean 26 ± 6.25 SD) than those formed by the permanent sample (mean 15 ± 5.29 SD). The colonies were also visibly larger in the deciduous sample than in the permanent sample. The colony forming unit efficiency was higher in the deciduous samples (mean 65 ± 15.61 SD) when compared to the permanent samples (mean 37.50 ± 13.23 SD) *(Graph. 14, Table. 14).*

In previous studies done in our department [74], the average colony forming efficiency of deciduous tooth pulp was 17.1%. The lower percentage obtained may be due to the loss of attachment of the initially seeded cells and the formation of colonies less than 2mm in diameter, that were not enumerated in the colony-count used to calculate the colony forming efficiency. Gronthos *et al.* found that the frequency of colony forming cells from dental pulp (22-70 colonies/10^4 cells plated) was higher than that of bone marrow derived cells [1]. Govindasamy *et al.* (2010) found that the CFUs were higher in SCD (151.67±10.5) as compared with DPSCs (133±17.62). The number and size of colonies were also more in SCD than in DPSCs, indicating a higher proliferation rate [12].

The higher number of colonies in DPSCs compared to bone marrow is thought to be due to a difference in the composition of the two connective tissues [1]. Similarly SHED could be more clonogenic when compared to DPSCs due to the increased number of blood vessels which has been established as niches in the dental pulp. The biological changes during exfoliation can also induce local factors that increase the proliferative capacity of SHED.

Morphology

Bayreuther *et al.* classified the fibroblast subpopulation as mitotic subtypes (f I – small spindle shaped cells ,f II – small epitheloid cells and f III – larger pleomorphic epitheloid cells) and postmitotic subtypes (f 1V – large spindle shaped cells, f V- larger epitheloid cells, f VI - largest epitheloid cells and f VII - degenerating fibroblasts)[13]. Mollenhauer and Bayreuther (1986) also subtyped the cells as F1 – spindle shaped cells, diploid with higher proliferation potential, F2 – epitheloid cells, diploid with less proliferation and F3 – stellate, tetraploid with least proliferation ability[14].

In our study, in the deciduous population, the mitotic population (mean=65.86 ± 25.86 SD) was significantly higher than the post-mitotic population (mean=24.13 ± 25.86 SD). There was a significant decrease in the mitotic population and an increase in post-mitotic population from day 1 to day 8 of culture ($P<0.001$). In the permanent population, the mitotic population (mean=50.69± 23.26 SD) was significantly higher than the post-mitotic population (mean=39.31 ± 23.26 SD). There was a significant decrease in the mitotic population and an increase in post-mitotic population from day 1 to day 8 of culture ($P<0.001$). The f I mitotic population was significantly higher in deciduous samples ($P<0.05$) and the f IV and f VI post-mitotic populations were significantly higher in the permanent samples ($P<0.05$). There was no significant difference between the total mitotic and post-mitotic subpopulations between deciduous and permanent samples. *(Table. 31, 32)*

In our study, in the deciduous population, the F3 population was the highest (35.13 ± 9.47 SD), followed by F2 population (31.69±6.94 SD) and F1 population (22.56±6.81

SD). In the permanent population, the F3 population is the highest (42.38 ± 12.43 SD), followed by F2 population (23.5 ± 10.97 SD) and F1 population (22.56 ± 9.79 SD).There was a significantly higher F2 subpopulation in the deciduous samples when compared with the permanent samples ($P<0.05$). *(Table. 33)*

In previous studies done in our department [73, 74], there were few F1 phenotypes that had faster proliferation rate, the major proportion of the cells we observed were F2 and F3 that had lesser proliferation rate compared to that of F1. These F2 and F3 cells were differentiating cells with less proliferation.

Studies on the isolation and characterization of stem cells from extracted deciduous (SCD) and dental pulp stem cells from permanent teeth (DPSCs) found that both populations had a fibroblast-like morphology resembling that of BM-MSCs [12]. Our results were similar to that obtained by Suchanek *et al.* who found that SHED generated rounded cells without long processes [37].

Immunophenotype

Cell surface antigen profiles reflect the phenotype of cells and expression of mesenchymal stem cell markers, CD73 and CD146 were used in our study to characterize SHED and DPSCs as mesenchymal stem cells. The expression of these markers in the 1st, 3rd and 5th passage of culture was evaluated to assess the change in phenotypes with passaging. Immunocytochemistry was done to evaluate the presence or absence of the marker and flow cytometry was used to quantify the percentage of expression.

Immunocytochemistry

Immunocytochemistry using antibodies to CD73 and CD146 was done to evaluate the expression of these mesenchymal markers on the surface of the cells cultured from deciduous and permanent tooth pulp. In our study positive staining was seen in cells of both deciduous and permanent samples but the results were not consistent and could not be reproduced for different samples. Staining quality could be affected by the type of fixatives used. In our study we used methanol. Previous studies done in our department used paraformaldehyde, but did not achieve positive staining [73]. However, different antibodies were used in their study and negative staining cannot be attributed to any one cause. To overcome this variability, flow cytometry was done.

Flow cytometry

In our study, both SHED and DPSCs expressed mesenchymal stem cell markers, CD73 and CD146. In SHED, the expression of CD73 increased with passaging and the expression of CD146 decreased with passaging. In DPSCs, the expression of CD73 decreased with passaging and the expression of CD146 also decreased with passaging. There was a decreased expression in SHED (passage 3) and DPSCs (passage 1), and so the cells from these passages were analyzed twice and the higher value was considered. The low values in the first analysis could be due to a decreased number of cells available for analysis *(Graph. 12, Table. 12).*

The increase in CD73 from passages 1 through 5 in SHED is contradictory to the trend seen in DPSCs, where there is a decrease in expression with passaging. Since only

one cell line was analyzed, and reading taken twice for the 3rd passage, these results need to be re-analyzed to confirm the trend. The expression of CD146 decreased consistently with increase in passage number, indicating a change from a primitive phenotype to a more differentiated phenotype with gradual loss of expression of mesenchymal stem cell markers.

Our findings are similar to a study by Karaöz *et al.* who found expression of both CD73 and CD146 in dental pulp stem cells from natal teeth [28]. Previous studies of the cell surface antigen profiles using flow cytometry revealed high expression of CD73 in SCD (99.88±3.1) and DPSCs (99.45±4.1) [12]. Perry *et al.* found >95% expression of CD73 in DPSCs [26]. Suchanek *et al.* found that SHED showed high positivity for CD73 (82.3%) and was negative for CD146 (1.7%). DPSCs showed similar expression of both the markers [37]. In contrast, Gronthos *et al.* found high positivity of DPSCs for CD146, similar to that seen by BMSCs.

CD73 and CD146 (MUC18) are early mesenchymal stem-cell markers, previously found to be present in BMSSCs, DPSCs and SHED. Immunohistochemical staining studies have revealed that CD146-positive cells were located around blood vessels of the remnant pulp, implying that SHED may originate from a perivascular microenvironment [2, 58]. Gronthos *et al.* suggested that the expression of endothelial markers on DPSCs could indicate that these stem cells originate from developing blood vessels and there is evidence that osteoprogenitors are associated with the outer surface of vasculature [1]. The variation in marker expression among different studies is thought to be due to the heterogenous nature of the cell population, with each subset, expressing a different set of

markers [1]. The difference in expression could also be due to different composition of media used for cultivation. Suchanek *et al.* used alpha-MEM media supplemented with 2% FCS [37]. In our study and also in that performed by Gronthos *et al.* (2000) and Miura *et al.* (2003) a higher concentration of serum was used [1, 2, 75].

Deciduous and permanent tooth pulp are both good sources of stem cells. In our study, we found that SHED take lesser time to reach confluency, have a better seeding efficiency, lower population doubling time and have a better clonogenic capacity in comparison to DPSCs indicating that deciduous tooth pulp is a better source of stem cells. Cells from both deciduous and permanent tooth pulp express markers suggestive of a mesenchymal stem cell population. In our study, expression of mesenchymal stem cell markers, CD73 and CD146 varied with passaging in both SHED and DPSCs. There was a consistent increase in CD73 expression in deciduous samples, and a decrease in expression of CD73 in permanent samples with increase in passage number. There was a consistent decrease in CD146 expression with increase in passage number in both deciduous and permanent samples, indicating a change in phenotype from immature progenitors to more mature differentiated forms. In our study, immunocytochemistry for both markers was also done but the results were not reproducible. We thus conclude that due to the versatility of flow cytometry, it is a better method of immunophenotyping.

Our findings indicate that a sufficiently large population representative of viable mesenchymal stem cells can be obtained from the dental pulp which can be used for translational research and clinical applications.

Summary and Conclusion

- Cells were cultured from 2 deciduous and 5 permanent samples out of 15 samples obtained.
- The time taken to reach confluency was lower in SHED compared to DPSCs but the results were not statistically significant.
- The population doubling time was lower in SHED compared DPSCs but the results were not statistically significant.
- The seeding efficiency was significantly higher in SHED than in DPSCs ($P< 0.05$).
- The colony forming unit efficiency was higher in SHED compared to DPSCs but the results were not statistically significant.
- There was a decrease in mitotic phenotype and increase in post-mitotic phenotype in both SHED and DPSCs from day 1 to day 8 of culture ($P<0.001$). The SHED had a significantly higher f I mitotic population ($P<0.05$) and the DPSCs had a significantly higher f IV and f VI post-mitotic population ($P<0.05$).
- There was a significantly higher overall F2 population in SHED, compared to DPSCs ($P<0.05$). The F3 population was increased in the DPSCs compared to SHED but was not statistically significant. This indicated a progression from fibroblast-like population to an epitheloid cell population.
- In our study, immunocytochemistry using CD73 and CD146 did not show consistent results.
- Flow cytometry analysis of SHED showed an increased expression of CD73 from passage 1 through passage 3 to passage 5 (52.9%, 71.8% and 88.7% respectively).

In DPSCs, the CD73 expression decreased from passage 1 through passage 3 and 5 (94%, 93.4% and 10.3% respectively).

- Flow cytometry analysis of SHED showed a decrease in CD146 expression from passage 1 (96.3%) to passage 3 (28.7%). The expression increased in passage 5 (76.1%), but still remained less than that of passage 1. In DPSCs, the CD146 expression decreased from passage 1 through passage 3 and 5 (90.3%, 51.1% and 21.4% respectively).

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Annexures

ANNEXURE I

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Anti-CD73 antibody [4G4] (ab81720)

5 References 2 Images URL for this product: http://www.abcam.com/CD73-antibody-4G4-ab81720.html

Product overview

Description	Mouse monoclonal [4G4] to CD73
Host species	Mouse
Tested applications	WB, IP, Flow Cyt, IHC-Fr, Functional Studies, ICC/IF
Cross reactivity	**Reacts with** Human
Immunogen	Tissue / cell preparation : human endothelial cell preparation from inflamed synovial stroma from rheumatoid arthritis patients.
Positive control	HUVEC and fresh lymphocytes.

Target

Function	Hydrolyzes extracellular nucleotides into membrane permeable nucleosides.
Involvement in disease	Defects in NT5E are the cause of calcification of joints and arteries (CAJA) [MIM:211800]. A condition characterized by adult-onset calcification of the lower extremity arteries, including the iliac, femoral and tibial arteries, and hand and foot capsule joints. Age of onset has been reported as early as the second decade of life, usually involving intense joint pain or calcification in the hands.
Sequence similarities	Belongs to the 5'-nucleotidase family.
Cellular localization	Cell membrane.

Target information above from: UniProt accessionP21589 *The UniProt Consortium*
The Universal Protein Resource (UniProt) in 2010
Nucleic Acids Res. 38:D142-D148 (2010).

Alternative names	5' NT antibody 5' nucleotidase (CD73) antibody 5' nucleotidase precursor antibody 5' nucleotidase, ecto antibody 5' nucleotidase, ecto (CD73) antibody 5'-NT antibody 5'-nucleotidase antibody 5NTD_HUMAN antibody CD73 antibody CD73 antigen antibody E5NT antibody Ecto 5' nucleotidase antibody Ecto-5'-nucleotidase antibody eN antibody eNT antibody

NT antibody
NT5 antibody
NT5E antibody
NTE antibody
Purine 5 Prime Nucleotidase antibody

Properties

Form	Liquid
Storage instructions	Shipped at 4°C. Upon delivery aliquot and store at -20°C. Avoid freeze / thaw cycles.

UK office:
Abcam plc
330 Cambridge Science Park Cambridge CB4 0FL UK
Tel: +44 (0)1223 696000
Fax: +44 (0)1223 215 215
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US office:
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Tel: (888) 77-ABCAM (22226)
Fax: (877) 774-8286
These numbers are toll free in the US/Canada
Email: us.orders@abcam.com

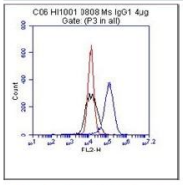
Last updated on
December 21, 2012

Storage buffer	Preservative: None Constituents: 0.1% BSA, PBS See the website for more SDS information for this product.
Purity	Protein G purified
Purification notes	ab81720 is purified and 0.2µM filtered.
Primary antibody notes	ab81720 causes a reduction in CD73 expression on lymphocytes.
Clonality	Monoclonal
Clone number	4G4
Isotype	IgG1

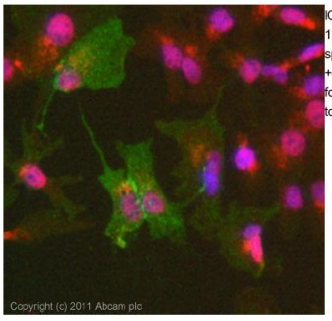
Applications

WB	WB: Use at an assay dependent dilution. Predicted molecular weight: 63 kDa.
IP	IP: 1/50.
Flow Cyt	Flow Cyt: 1/50.
IHC-Fr	IHC-Fr: 1/50. Tissue sections were fixed in acetone.
Functional Studies	FuncS: Use at an assay dependent dilution. Antibody 4G4 functions as an inhibitor of lymphocyte binding to HUVEC. The antibody was functionally tested by incubating the lymphocytes with the antibody before adding the lymphocytes to an EC monolayer. Furthermore the monoclonal antibody 4G4 causes a reduction in CD73 expression on lymphocytes and reduces enzyme activity.
ICC/IF	ICC/IF: 1/50.

Images (See the website for higher resolution images of this product)



ab81720 at 4 µg/ml detecting CD73 in HUVEC by Flow cytometric analysis. Blue histogram represents detection of CD73. Red and black histograms represent cells without antibody and isotype control respectively.



ICC/IF image of ab81720 stained HepG2 cells. The cells were 4% formaldehyde (10 min) and then incubated in 1%BSA / 10% normal goat serum / 0.3M glycine in 0.1% PBS-Tween for 1h to permeabilise the cells and block non-specific protein-protein interactions. The cells were then incubated with the antibody (ab81720, 5µg/ml) overnight at +4°C. The secondary antibody (green) was ab96879 Dylight 488 goat anti-mouse IgG (H+L) used at a 1/250 dilution for 1h. Alexa Fluor® 594 WGA was used to label plasma membranes (red) at a 1/200 dilution for 1h. DAPI was used to stain the cell nuclei (blue) at a concentration of 1.43µM.

ANNEXURE II

abcam®
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Anti-CD146 antibody [P1H12] (ab24577)

3 Abreviews | 5 References | 5 Images | URL for this product: http://www.abcam.com/CD146-antibody-P1H12-ab24577.html

Product overview

Description	Mouse monoclonal [P1H12] to CD146
Host species	Mouse
Specificity	We have data to indicate that this antibody may not cross react with Rat. However, this has not been conclusively tested and expression levels may vary in certain cell lines/tissues.
Tested applications	ICC/IF, IHC-P, WB, ELISA, IHC-Fr, IP, Flow Cyt
Cross reactivity	**Reacts with** Mouse, Rabbit, Dog, Human
Immunogen	Tissue / cell preparation: human umbilical vein endothelial cells (HUVECs).
Positive control	HUVEC cells, FFPE human aorta tissue sections

Target

Function	Plays a role in cell adhesion, and in cohesion of the endothelial monolayer at intercellular junctions in vascular tissue. Its expression may allow melanoma cells to interact with cellular elements of the vascular system, thereby enhancing hematogeneous tumor spread. Could be an adhesion molecule active in neural crest cells during embryonic development. Acts as surface receptor that triggers tyrosine phosphorylation of FYN and PTK2, and a transient increase in the intracellular calcium concentration.
Tissue specificity	Detected in endothelial cells in vascular tissue throughout the body. May appear at the surface of neural crest cells during their embryonic migration. Appears to be limited to vascular smooth muscle in normal adult tissues. Associated with tumor progression and the development of metastasis in human malignant melanoma. Expressed most strongly on metastatic lesions and advanced primary tumors and is only rarely detected in benign melanocytic nevi and thin primary melanomas with a low probability of metastasis.
Sequence similarities	Contains 3 Ig-like C2-type (immunoglobulin-like) domains. Contains 2 Ig-like V-type (immunoglobulin-like) domains.
Cellular localization	Membrane.

Target information above from: UniProt accession P43121 *The UniProt Consortium*
The Universal Protein Resource (UniProt) in 2010
Nucleic Acids Res. 38:D142-D148 (2010).

Alternative names	A32 antigen antibody CD 146 antibody CD146 antibody CD146 antigen antibody Cell surface glycoprotein MUC18 antibody Cell surface glycoprotein P1H12 antibody Gicerin antibody Mcam antibody Melanoma adhesion molecule antibody Melanoma associated antigen A32 antibody Melanoma associated antigen MUC18 antibody Melanoma associated glycoprotein MUC18 antibody Melanoma cell adhesion molecule antibody Melanoma-associated antigen A32 antibody Melanoma-associated antigen MUC18 antibody MelCAM antibody MUC 18 antibody MUC18 antibody MUC18_HUMAN antibody

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These numbers are toll free in the US/Canada
Email: us.orders@abcam.com

Last updated on
December 21, 2012

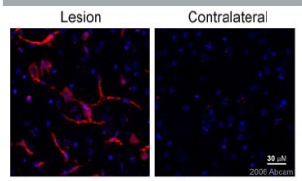
Properties

Form	Liquid
Storage instructions	Shipped at 4°C. Upon delivery aliquot and store at -20°C. Avoid freeze / thaw cycles.
Storage buffer	Preservative: 0.1% Sodium Azide Constituents: 0.25M Sodium chloride, 0.02M PBS, pH 7.6 See the website for more SDS information for this product.
Purity	Immunogen affinity purified
Clonality	Monoclonal
Clone number	P1H12
Isotype	IgG1

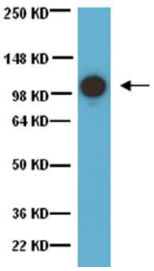
Applications

ICC/IF	ICC/IF: Use at an assay dependent concentration.
IHC-P	IHC-P: Use a concentration of 5 µg/ml. Perform heat mediated antigen retrieval before commencing with IHC staining protocol.
WB	WB: 1/500.
ELISA	ELISA: Use a concentration of 1 - 10 µg/ml.
IHC-Fr	IHC-Fr: 1/1000. See Abreview.
IP	IP: 1/150.
Flow Cyt	Flow Cyt: 1/1000.

Images (See the website for higher resolution images of this product)



ab24577 at 1/1000 dilution staining mouse brain tissue sections by Immunohistochemistry (Frozen sections). Mice were processed by transcardial perfusion first with saline, then 4% PF. After overnight incubation in 4% PF, brains were transfered to sucrose. Upon saturation, brains were frozen, sectioned with a cryostat, and then the sections were immediately mounted on slides. The tissue was incubated with ab24577 for 2 hours and then an Alexa Fluor ® 594 goat anti-mouse IgG was used as the secondary (red). DAPI staining is shown in blue. Images were taken with a confocal microscope in comparable cortex regions of the lesion or contralateral side in the same section. The lesion image shown is from this model of ischemia-hypoxia, with 1 hour of recovery time after injury, when endothelial cell activation is quite robust.



This image is courtesy of an Abreview submitted by Mr Faisal Adhami

Anti-CD146 antibody [P1H12] (ab24577) at 1/500 dilution + HUVEC lysate

Observed band size : 110 kDa (why is the actual band size different from the predicted?)

Proteins were visualized using a goat anti-mouse secondary antibody conjugated to HRP and a chemiluminescence detection system.

ANNEXURE III

abcam®
discover more

Rabbit polyclonal Secondary Antibody to Mouse IgG - H&L (HRP) (ab97046)

1 Reference 2 Images URL for this product: http://www.abcam.com/Rabbit-polyclonal-Secondary-Antibody-to-Mouse-IgG-HL-HRP-ab97046.html

Product overview

Description	Rabbit polyclonal Secondary Antibody to Mouse IgG - H&L (HRP)
Host species	Rabbit
Target species	Mouse
Specificity	By immunoelectrophoresis and ELISA this antibody reacts specifically with mouse IgG and with light chains common to other mouse immunoglobulins. No antibody was detected against non-immunoglobulin serum proteins.
Tested applications	ICC, IHC-P, ELISA, WB

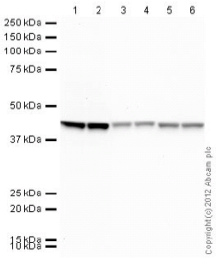
Properties

Form	Liquid
Storage instructions	Store at +4°C.
Storage buffer	Preservative: 0.1% Proclin Constituents: 0.2% BSA, PBS
Purity	Immunogen affinity purified
Purification notes	This antibody was isolated by affinity chromatography using antigen coupled to agarose beads and conjugated to Horse Radish Peroxidase (HRP).
Conjugation notes	Molar enzyme/ antibody protein ratio is 4:1
Clonality	Polyclonal
Isotype	IgG
General notes	Part of the **AbExcel** range.

Applications

ICC	ICC: Use at an assay dependent dilution.
IHC-P	IHC-P: 1/200 - 1/5000.
ELISA	ELISA: 1/10000 - 1/100000. (Primary)
WB	WB: 1/2000 - 1/20000. (Colorimetric). (Chemiluminescent 1/10000 - 1/50000).

Images (See the website for higher resolution images of this product)



All lanes : Anti-beta Actin antibody [mAbcam 8226] - Loading Control (ab8226) at 1 µg/ml

Lanes 1-6 : HeLa (Human epithelial carcinoma cell line) Whole Cell Lysate
Lysates/proteins at 10 µg per lane.

Secondary
Lanes 1 - 2 : Rabbit polyclonal Secondary Antibody to Mouse IgG - H&L (HRP) (ab97046) at 1/2000 dilution
Lanes 3 - 4 : Rabbit polyclonal Secondary Antibody to Mouse IgG - H&L (HRP) (ab97046) at 1/10000 dilution
Lanes 5 - 6 : Rabbit polyclonal Secondary Antibody to Mouse IgG - H&L (HRP) (ab97046) at 1/20000 dilution

Developed using the ECL technique

Performed under reducing conditions.

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Tel: (888) 77-ABCAM (22226)
Fax: (877) 774-8286
These numbers are toll free in the US/Canada
Email: us.orders@abcam.com

Last updated on
December 21, 2012

ANNEXURE IV

INFORMED CONSENT FORM

If you have read the informed consent (or if you have had it explained to you) and understand the information, and you voluntarily agree to take part in this study, please sign your name below.

----------	----------	----------
Volunteer's Name (Typed or printed)	Volunteer's Signature	Date
OR		
----------	----------	-------
Volunteer's Legal Guardian or Representative	Legal Guardian's Signature	Date
----------	----------	-------
Witness's Name	Witness's Signature	Date
----------	----------	-------
Interviewer’s Name	Interviewer’s Signature	Date

ANNEXURE V

INSTITUTIONAL REVIEW BOARD APPROVAL

14/03/2011

From,

Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi, Chennai

The dissertation topic titled **'Growth characteristics and expression of CD73 and CD146 in the cells cultured from dental pulp- a flow cytometry and immunocytochemical study'** submitted by Dr.Vaishnavi Sivasankar has been approved by the Institutional Review Board of Ragas Dental College and Hospital on 14th March 2011.

Dr.K.Ranganathan
Secretary,
Ragas, IRB

Dr.S.Ramachandran
Chairman,
Ragas, IRB

ANNEXURE VI

INSTITUTIONAL REVIEW BOARD PROTOCOL

Title of the proposed research project

Expression of mesenchymal stem cell markers, CD 73 and CD146, in pulp isolated from deciduous and permanent teeth -*an Immunocytochemistry study*

Name and designation of the principal investigator

Vaishnavi Sivasankar

I Year Post graduate student

Department of Oral and Maxillofacial Pathology

Name of HOD & staff in charge

Dr Ranganathan K (Professor and Head)

Dr Umadevi K (Professor)

Dr Elizabeth Joshua (Associate Professor)

Dr Rooban T (Associate Professor)

Department where project is to be carried out

Department of Oral and Maxillofacial Pathology,

Ragas Dental College,

Chennai

Duration of the project-

12 months

Signature of principal investigator _____

Signature of Head of Department _____

Remarks of committee _____

Permission Granted YES / NO

Modifications / comments _____

HYPOTHESIS

Pulp from deciduous teeth forms a better source of mesenchymal stem cells than pulp from permanent teeth and show maximum 'stemness' during early passages of culture.

AIM

- To isolate and grow the pulp stem cells from deciduous and permanent teeth
- Immuno-phenotypic characterization of the deciduous and permanent pulp stem cells during 1^{st}, 3^{rd} and 5^{th} passage.

OBJECTIVE

- To identify the stem cell population within the mesenchymal cells isolated from permanent and deciduous teeth
- Establish the better source of stem cells among deciduous and permanent teeth and the stage at which it demonstrates maximum 'stemness' in culture.

METHODOLOGY

PATIENT SELECTION

Extracted third molars, exfoliating/extracted deciduous teeth, and teeth extracted for orthodontic treatment are to be obtained from the patients who report to Ragas Dental College. Informed consent will be obtained from patients above 18 years and from the parents of children for the collection of extracted teeth.

SAMPLE SIZE

Pulp tissue specimens for culture are taken from 5 freshly extracted deciduous teeth (**Group A**) and 5 freshly extracted permanent teeth (**Group B**) following informed consent.

Inclusion criteria:

- Extracted third molars, exfoliating/extracted deciduous teeth, and teeth extracted for orthodontic treatment, trauma or periodontal disease

- Freshly extracted teeth immediately transferred to transport medium until pulp extirpation.
- Teeth with vital pulpal tissue

Exclusion criteria:

- Teeth with evidence of decay or pulpal necrosis.
- Extracted/exfoliated teeth that have not been transferred to transport media immediately.

SPECIMEN COLLECTION

Deciduous and permanent teeth with no evidence of decay or pulpal necrosis are collected immediately following extraction/ exfoliation.

TRANSPORTATION OF THE SPECIMEN

α-MEM without serum at a pH of 7.2 to 7.4 with 2 x Antibiotics (Penicillin-100 IU, Streptomycin-100µg/ml, Amphotericin B-1µg/ml) maintained at 4°C with ice pack is used to transport tissue specimens. They are transported in leak proof sterilized culture vials and the pulpal tissue is extirpated within 1 hour following collection.

PULP EXTIRPATION

- Tooth surface is cleaned well by washing three times with Phosphate Buffer Saline (PBSA).
- Grooves are placed around the cemento-enamel junction with a tungsten carbide bur. The tooth is soaked intermittently in ice-cold PBSA to avoid heating while cutting.
- Tooth is split with chisel and mallet to reveal the pulp chamber.
- Separation of pulp tissue from pulp chamber is done with small fine forceps and spoon excavator.
- The pulp tissue is put into a on a Petri dish containing 2ml of Mesenchymal Stem Cell (MSC) medium (α-modified minimal essential medium (α-MEM) with 2 mM glutamine and supplemented with 15% fetal bovine serum (FBS), 0.1 mM l-ascorbic acid phosphate, 100 U/ml penicillin, and100 µg/ml streptomycin) to avoid drying.

PRIMARY CULTURE OF DENTAL PULP CELLS:

- The dental pulp tissue is minced into tiny pieces with a surgical blade.

- The tissue is immersed into a mixed collagenase /dispase solution (1:1)
- It is incubated at 37^0C for up to 30-60 minutes and mixed well intermittently.
- After the digestion, the enzymes are inactivated by dilution in sufficient MSC medium.
- Cells centrifuged at 2400 rpm for 6 minutes.
- The supernatant is removed and the pellet re-suspended with MSC medium.
- The cells are counted (using a counting chamber) and then seeded into dishes at 1-10 X $10^3/cm^2$.(6-well plate)
- The cells are cultured in MSC medium at 37^0C and 5% CO_2 in the incubator.
- Attachment of the cells can be observed after 48 hours. Media change is to be done on every third day. Mono layer of cells can be observed within 2-3 weeks time.

SUBCULTURE

Usually around one week after the cell isolation, colonies are identified in the culture plates, where the cells have a typical fibroblast – like spindle shape. Before the cells become 100% confluent (usually after about 2-3 weeks), they are sub-cultured.

CHARACTERIZATION USING IMMUNOCYTOCHEMISTRY

Cells are fixed on APES coated slides using methanol, and immunologically stained for CD 146 and CD73.

ANNEXURE VII

ABBREVIATIONS

2D/3D	2/3 Dimensional
α-MEM	Alpha-Modification Minimal Essential Medium
ALP	Alkaline Phosphatase
APES	3-aminopropyl-triethoxy-silane
BM	Bone Marrow
BMP	Bone Morphogenic Protein
BMSC	Bone Marrow Stromal/Stem Cell
BSA	Bovine Serum Albumin
BSP	Bone Sialoprotein
CD	Cluster of Differentiation
CFE	Colony Forming Efficiency
CFU-F	Colony Forming Unit-Fibroblasts
CO_2	Carbon Dioxide
DAB	3, 3'-Diaminobenzidine
dd.H_2O	Double Distilled Water

DFSC	Dental Follicle Stem Cell
DPSC	Dental Pulp Stem Cell
EC	Embryonal Carcinoma cell
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetate
EG	Embryonic Germ cell
ESC	Embryonic Stem Cell
ET3	Endothelin-3
FACS	Fluorescence Activated Cell Sorting
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
GAG	Glycosaminoglycans
GFAP	Glial Fibrillary Acidic Protein
GSC	Germ-Line Stem Cells
HRP	Horseradish Peroxidase
HSC	Haematopoietic Stem Cell
hDPSC	human Dental Pulp Stem Cell

hNDP	human Natal Dental Pulp
ICC	Immunocytochemistry
IGF-1	Insulin-Like Growth Factor I
IHC	Immunohistochemistry
IL	Interleukin
iPS	Induced Pluripotent Stem Cell
IRB	Institutional Review Board
Klf4	Krüppel-Like Factor 4
KOH	Potassium Hydoxide
LIF	Leukaemia Inhibitory Factor
LPS	Lipopolysaccharide
MACS	Magnetic Activated Cell Sorting
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem/Stromal Cell
Msx1	Msh Homeobox-1
NaOH	Sodium Hydroxide
NCP	Non-Collagenous Protein

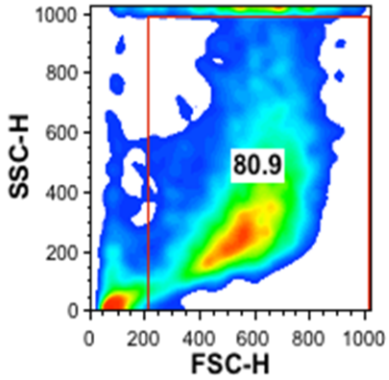
OCN	Osteocalcin
Oct4	Octamer-Binding Transcription Factor 4
ON	Osteonectin
OPN	Osteopontin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDT	Population Doubling Time
PDGF	Platelet Derived Growth Factor
PDL	Periodontal Ligament
PDLSC	Periodontal Ligament Stem Cell
PFA	Paraformaldehyde
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SC	Stem cell(s)
SCAP	Stem Cell of the Apical Papillae
SHED	Stem Cells from the Human Exfoliated Deciduous Teeth
SMA	Smooth Muscle Actin

SPSS	Statistical Package for Social Science
SSEA	Stage-Specific Embryonic Antigen
TAC	Transit Amplifying Cell
TBS	Tris-Buffered Saline
TGF-β	Transforming Growth Factor-Beta
UV	Ultra Violet
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
Wnt	Wingless in Drosophila

ANNEXURE VIII

Flow cytometry results: dot plot and histograms

SHED Passage 1



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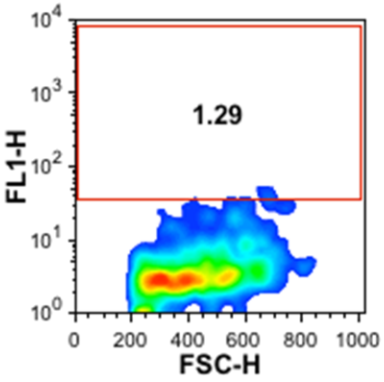

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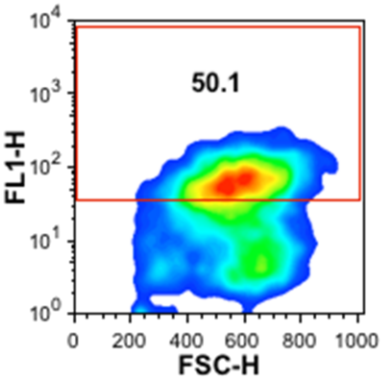
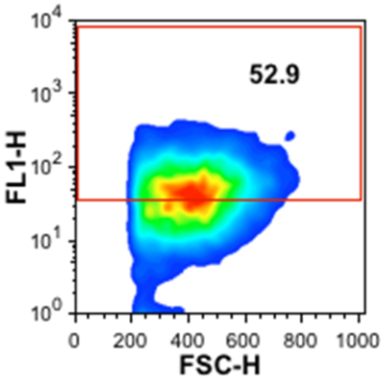


CD 73



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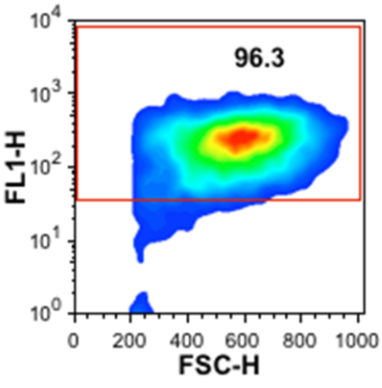

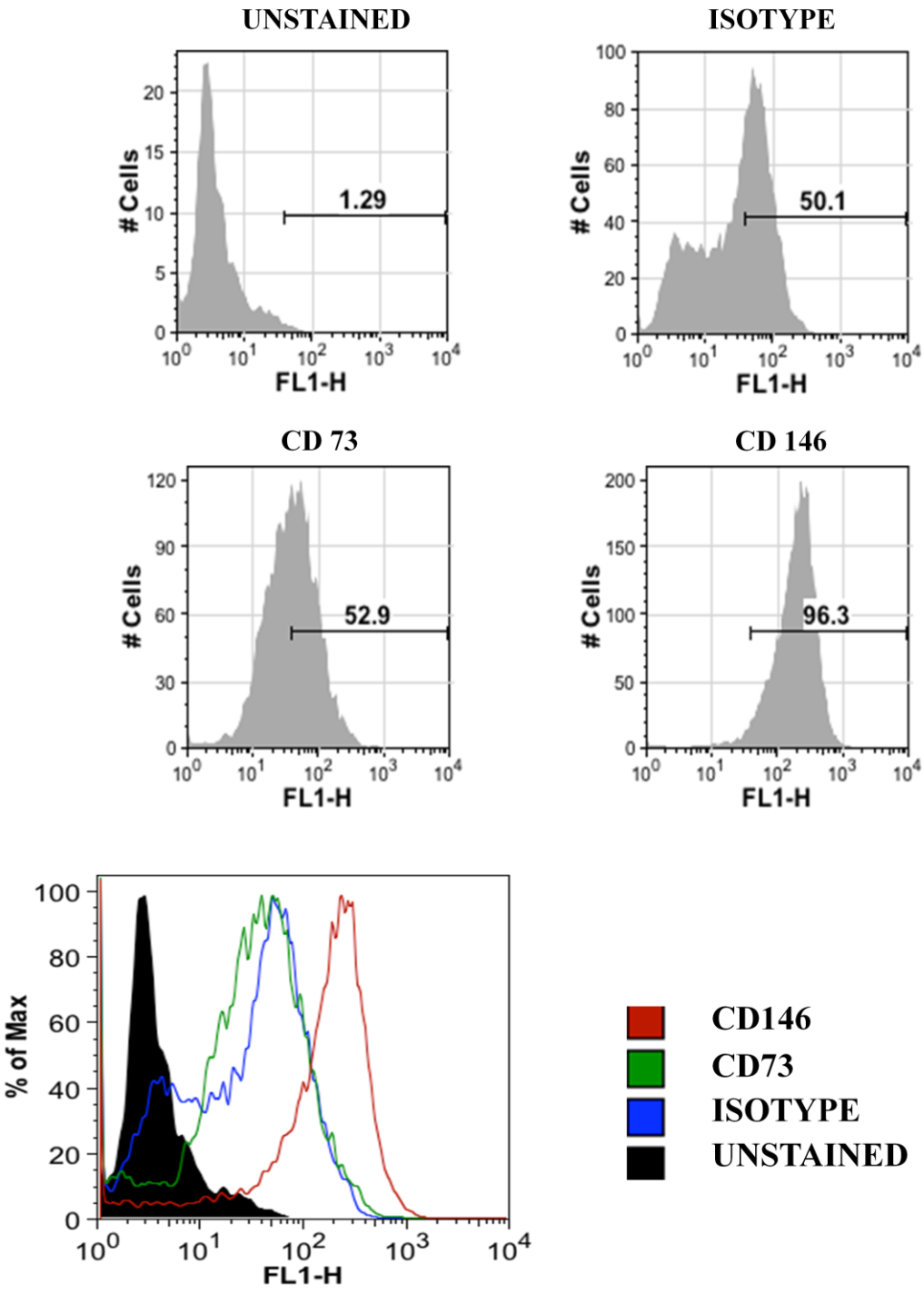
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ISOTYPE
CD 73
CD 146
Cells
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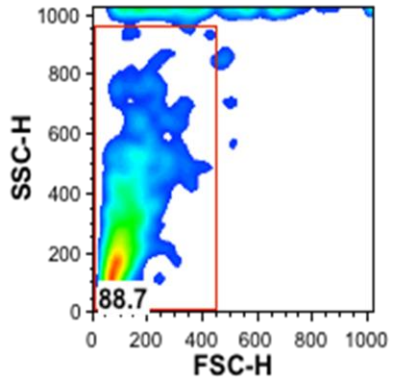
1.29
50.1
52.9
96.3
% of Max
CD146
CD73
ISOTYPE
UNSTAINED

SHED passage 3 analysis 1



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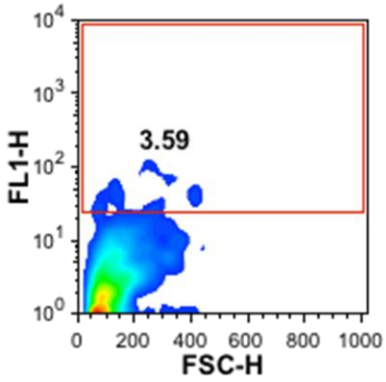

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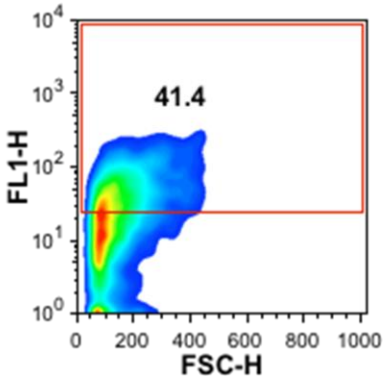

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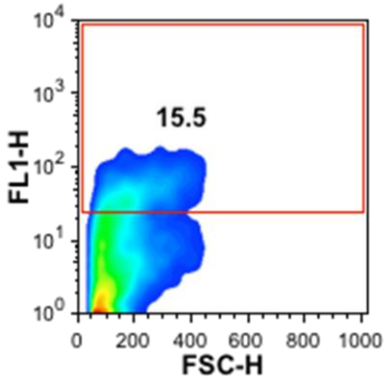

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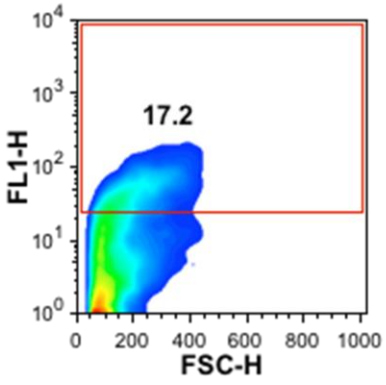
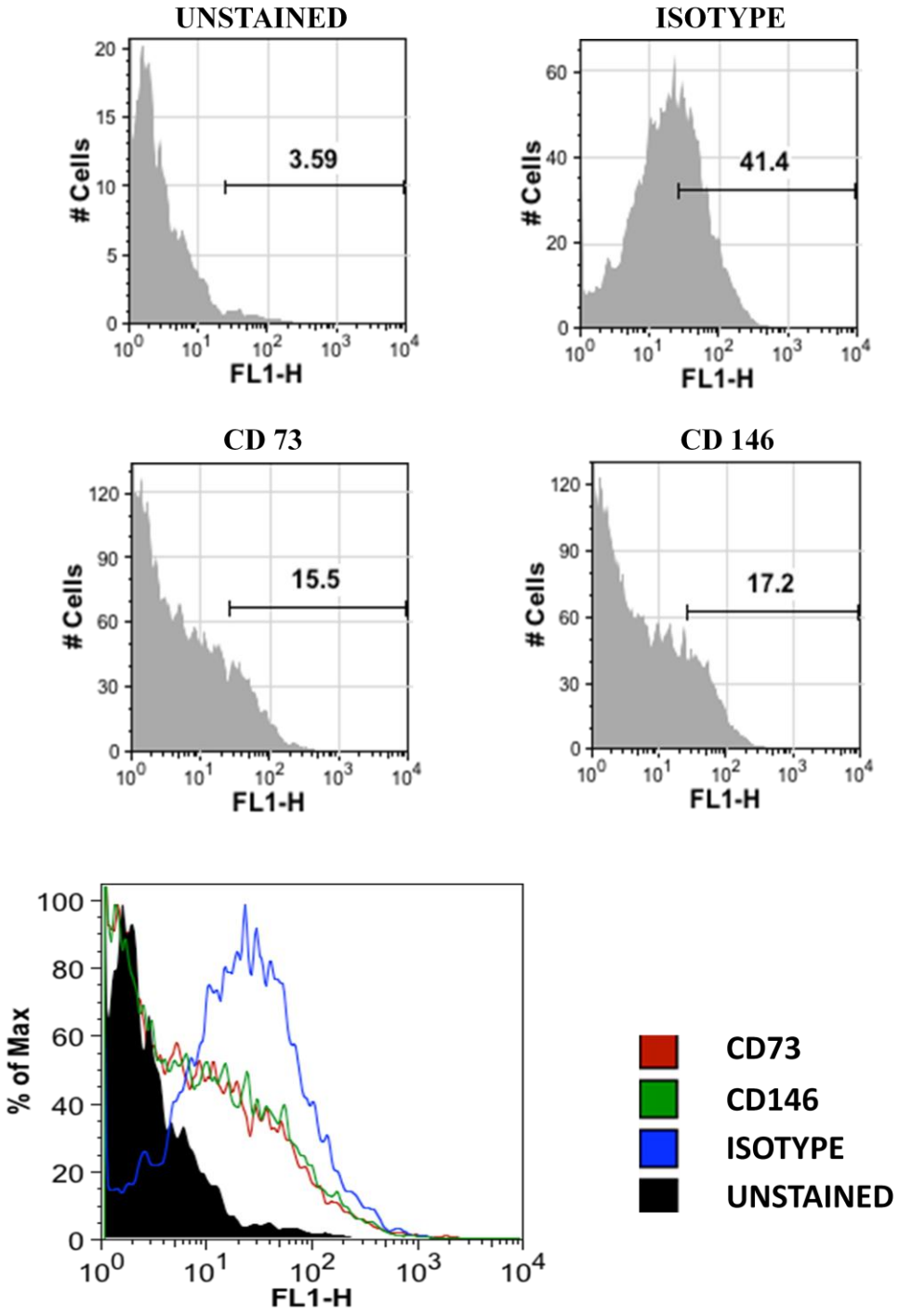
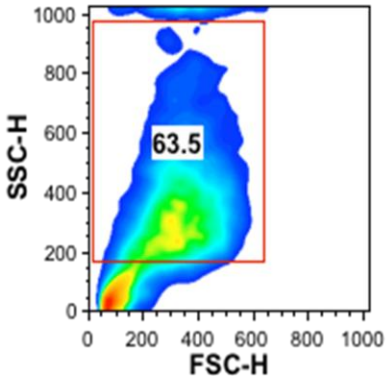



UNSTAINED
ISOTYPE
CD 73
CD 146
Cells
FL1-H
3.59
41.4
15.5
17.2
% of Max
CD73
CD146
ISOTYPE
UNSTAINED

SHED passage 3 analysis 2



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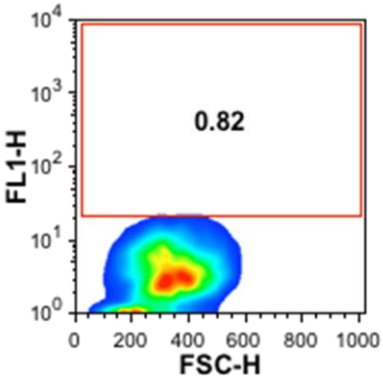

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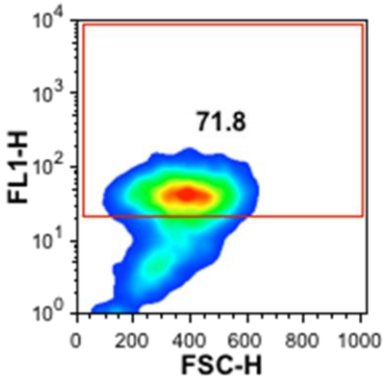

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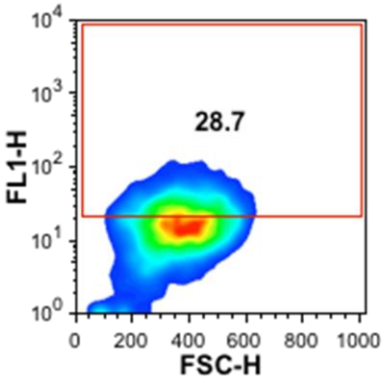

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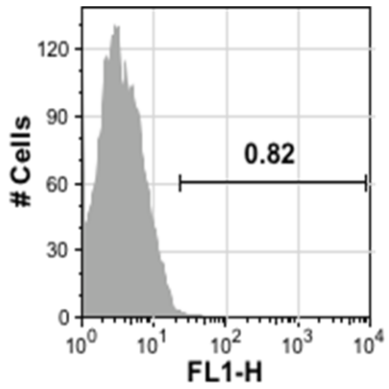

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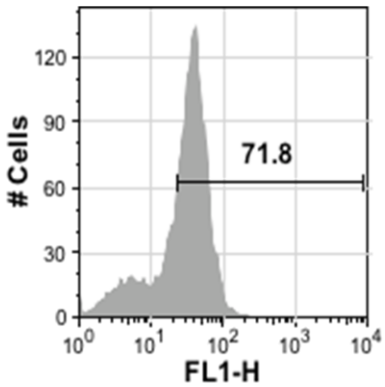

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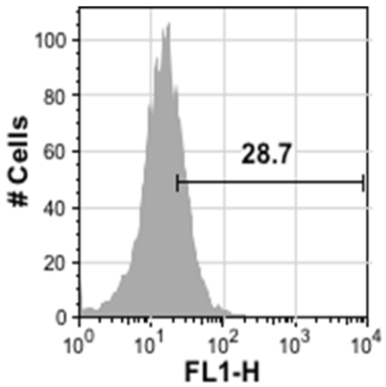
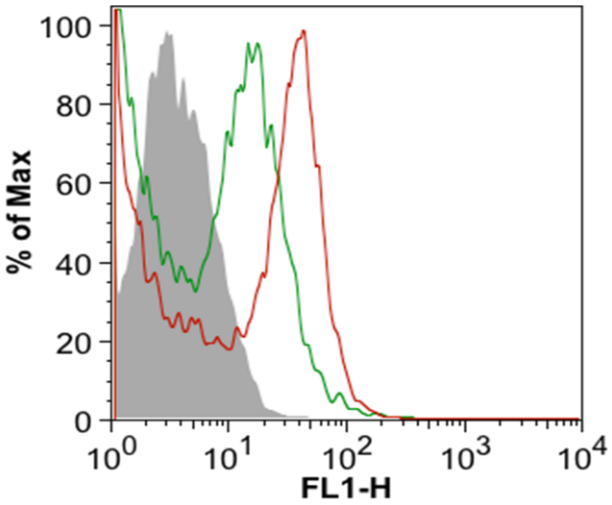


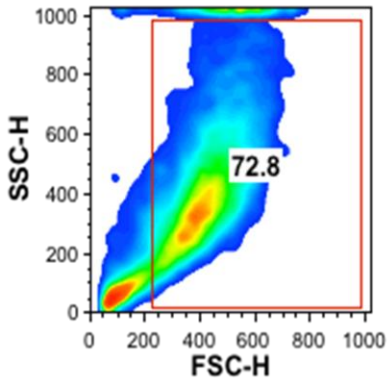


CD73

CD146

UNSTAINED

SHED passage 5



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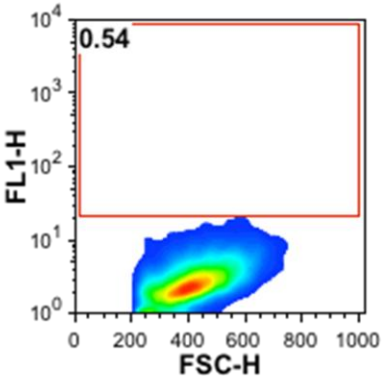

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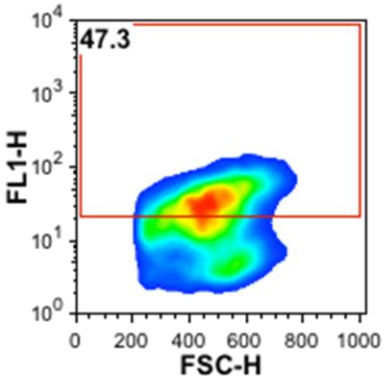
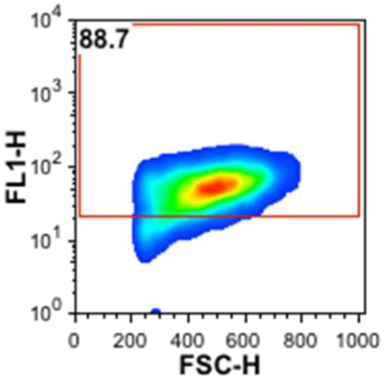
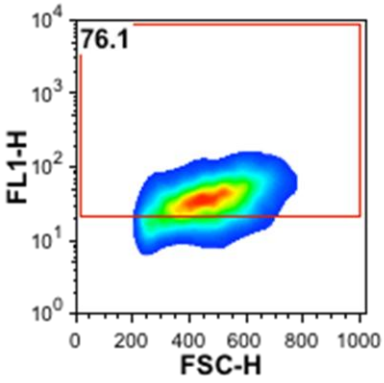
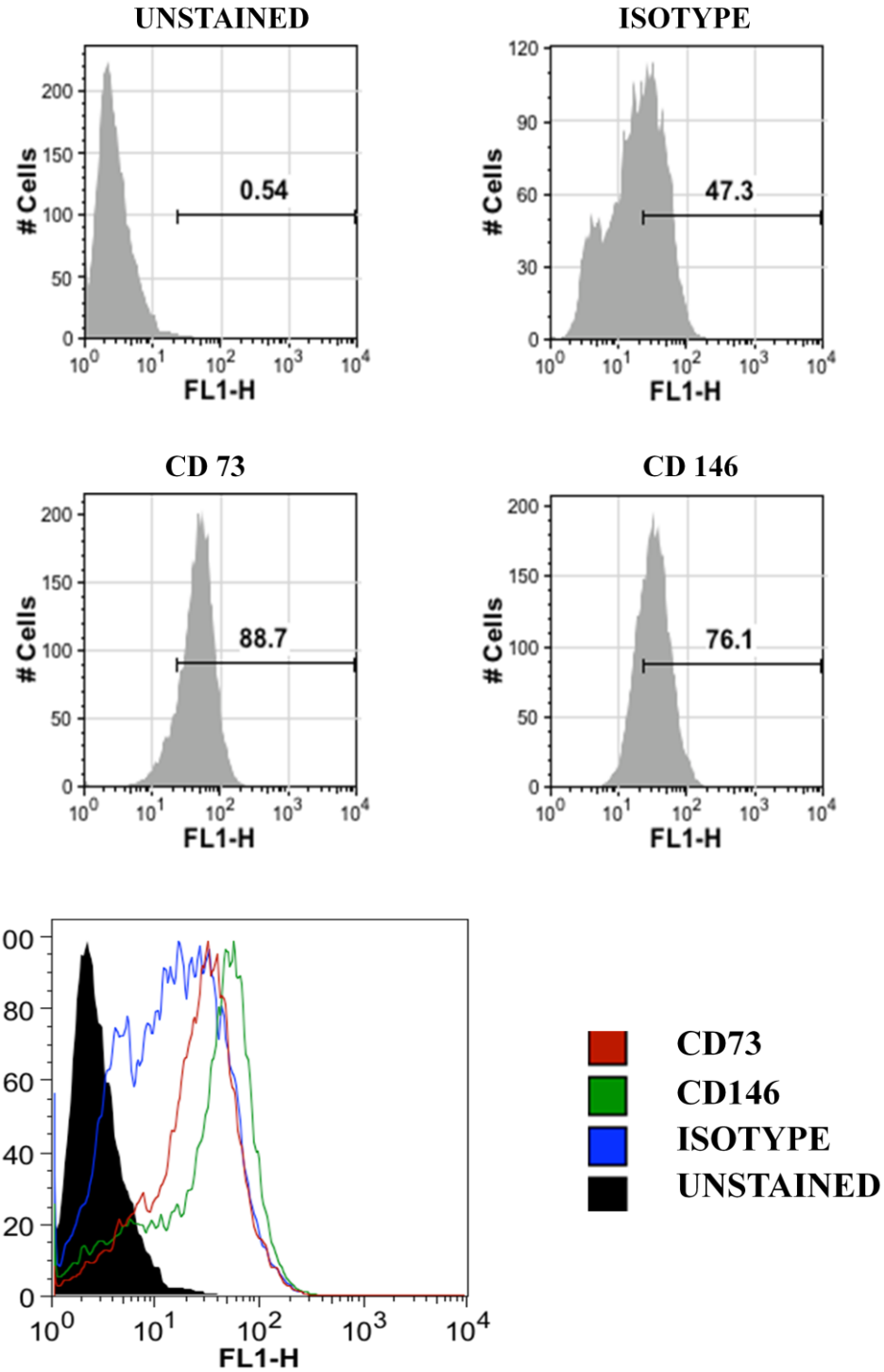


CD 73

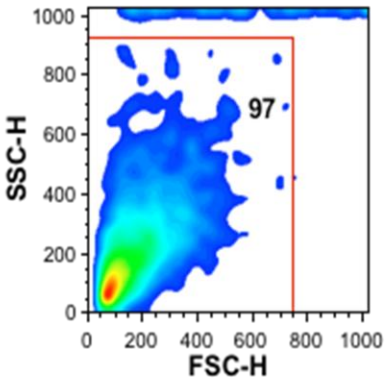


CD 146

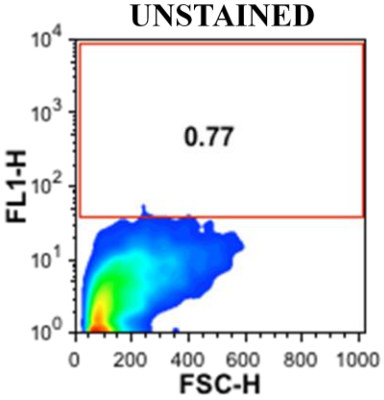



UNSTAINED
ISOTYPE
CD 73
CD 146
Cells
FL1-H
0.54
47.3
88.7
76.1
% of Max
CD73
CD146
ISOTYPE
UNSTAINED

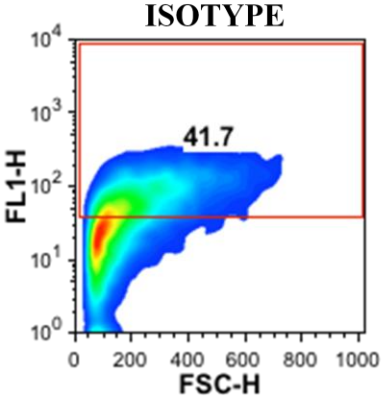
DPSC passage 1 analysis 1



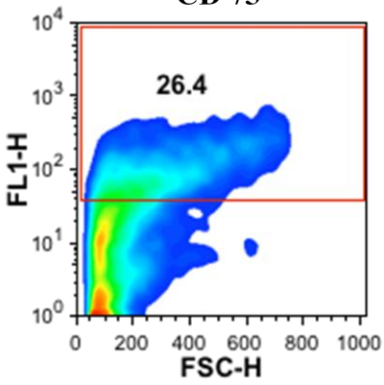
UNSTAINED



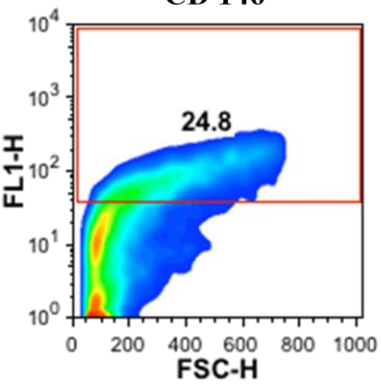
ISOTYPE

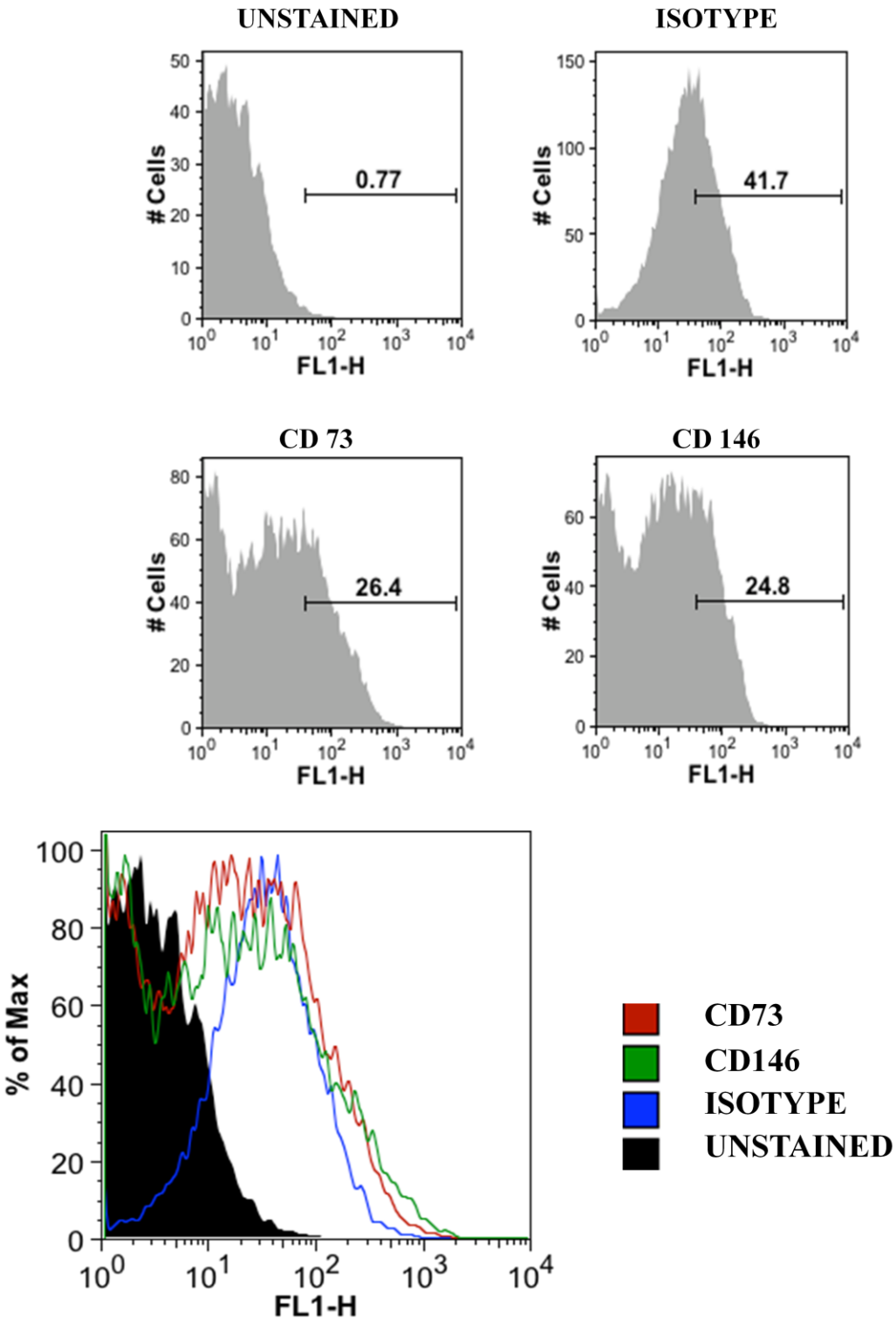


CD 73

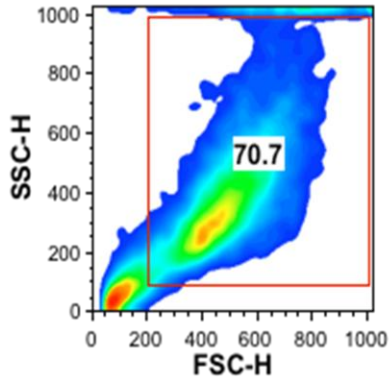


CD 146

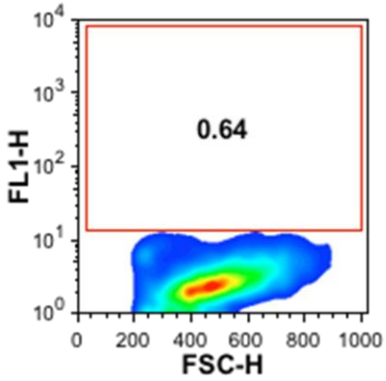



UNSTAINED
ISOTYPE
CD 73
CD 146
Cells
FL1-H
0.77
41.7
26.4
24.8
% of Max
CD73
CD146
ISOTYPE
UNSTAINED

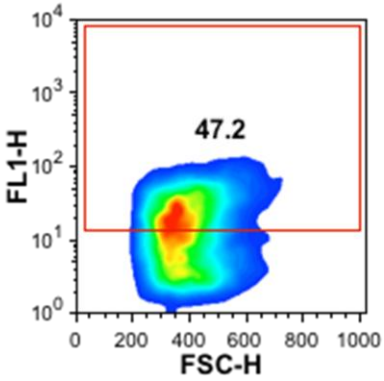
DPSC Passage 1 analysis 2



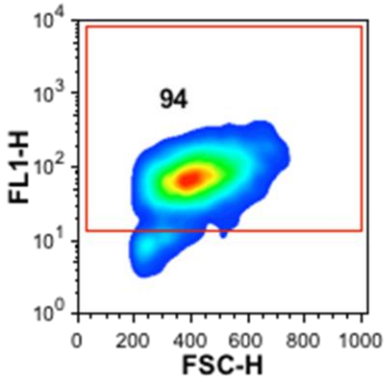
UNSTAINED



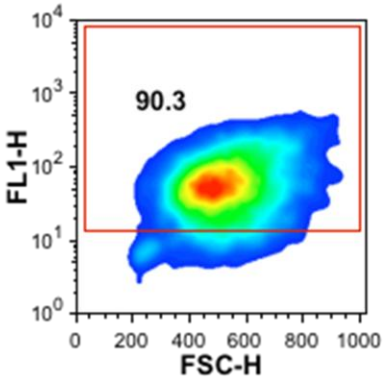
ISOTYPE

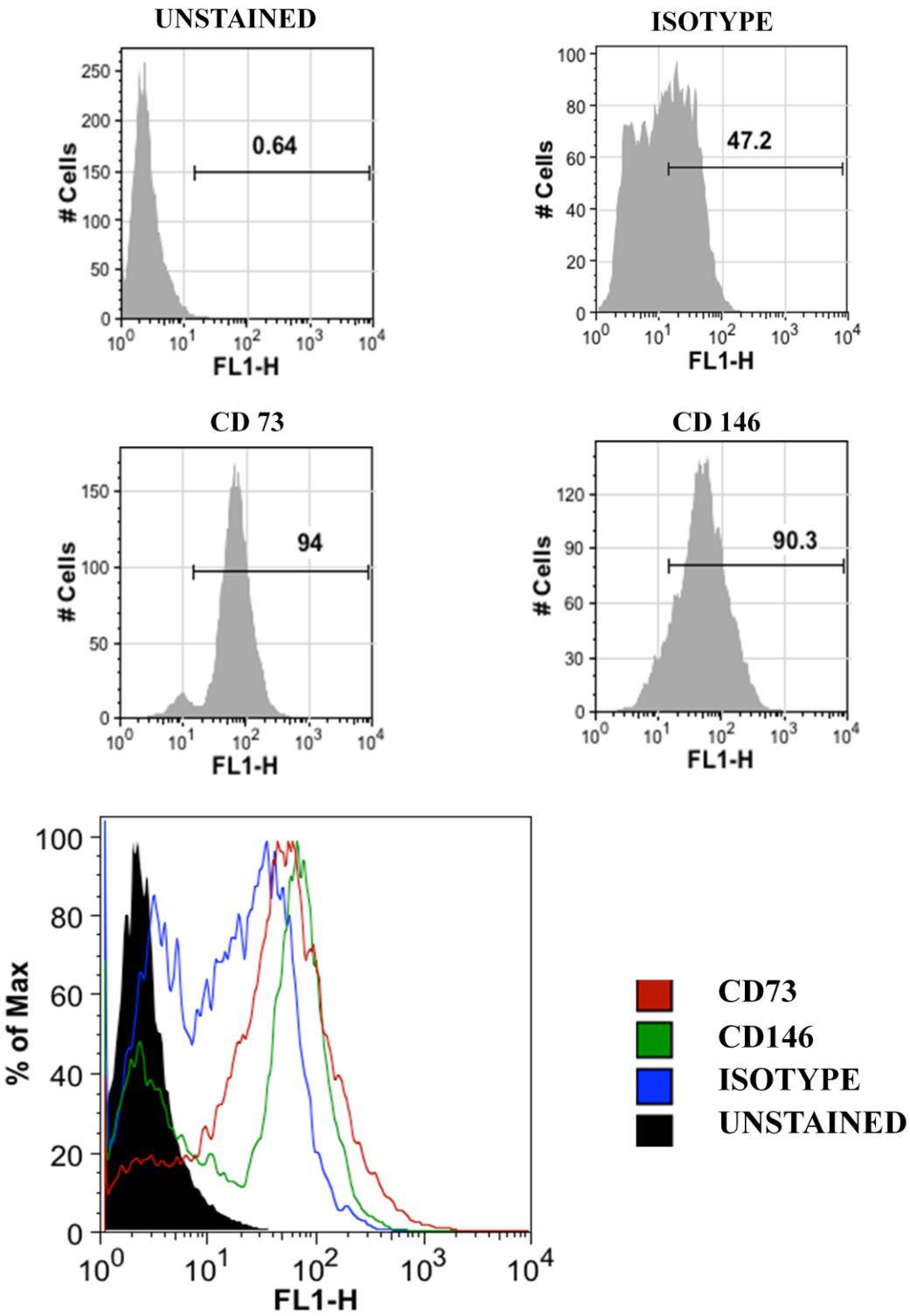


CD 73

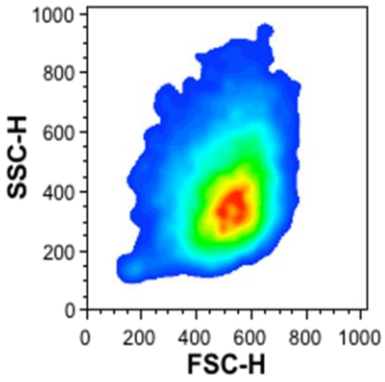


CD 146

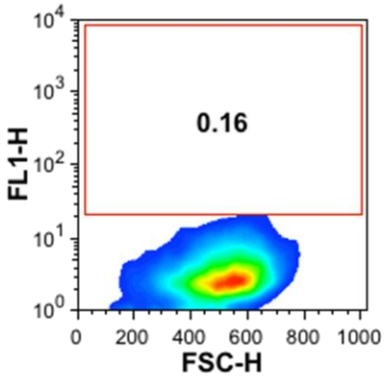



UNSTAINED
0.64
Cells
FL1-H
ISOTYPE
47.2
Cells
FL1-H
CD 73
94
Cells
FL1-H
CD 146
90.3
Cells
FL1-H
% of Max
FL1-H
CD73
CD146
ISOTYPE
UNSTAINED

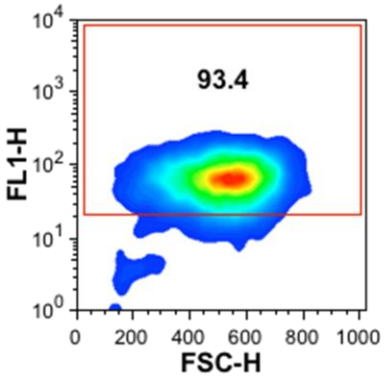
DPSC Passage 3



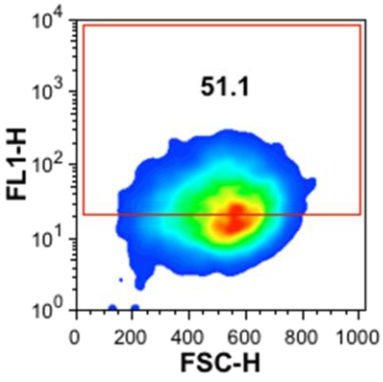
UNSTAINED

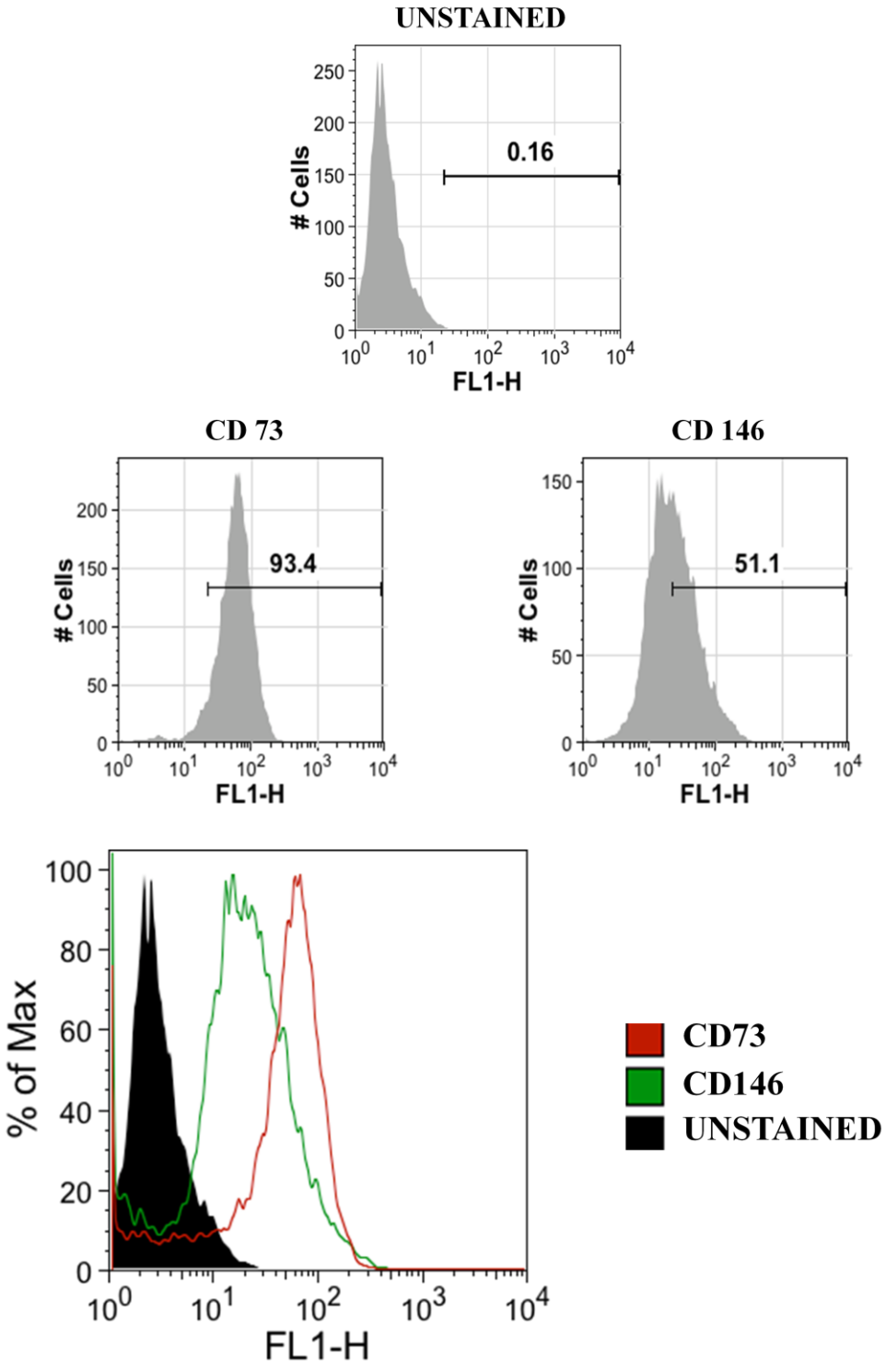


CD 73

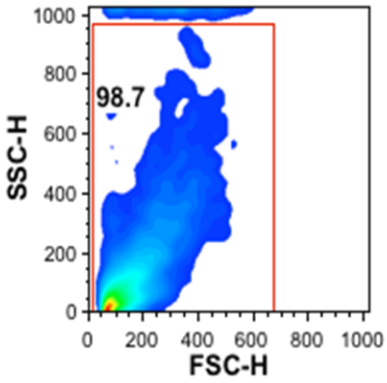


CD 146

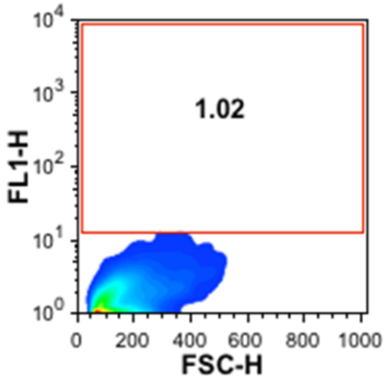



UNSTAINED
Cells
0.16
FL1-H
CD 73
93.4
CD 146
51.1
% of Max
CD73
CD146
UNSTAINED

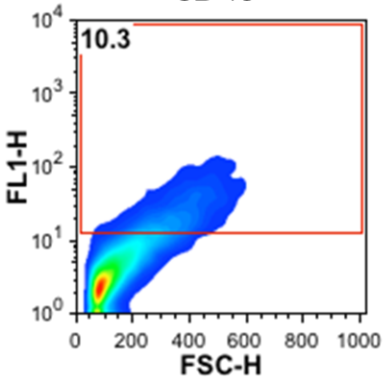
DPSC passage 5



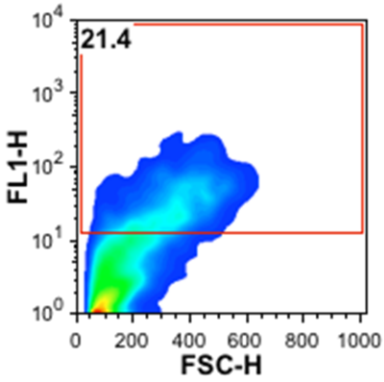
UNSTAINED

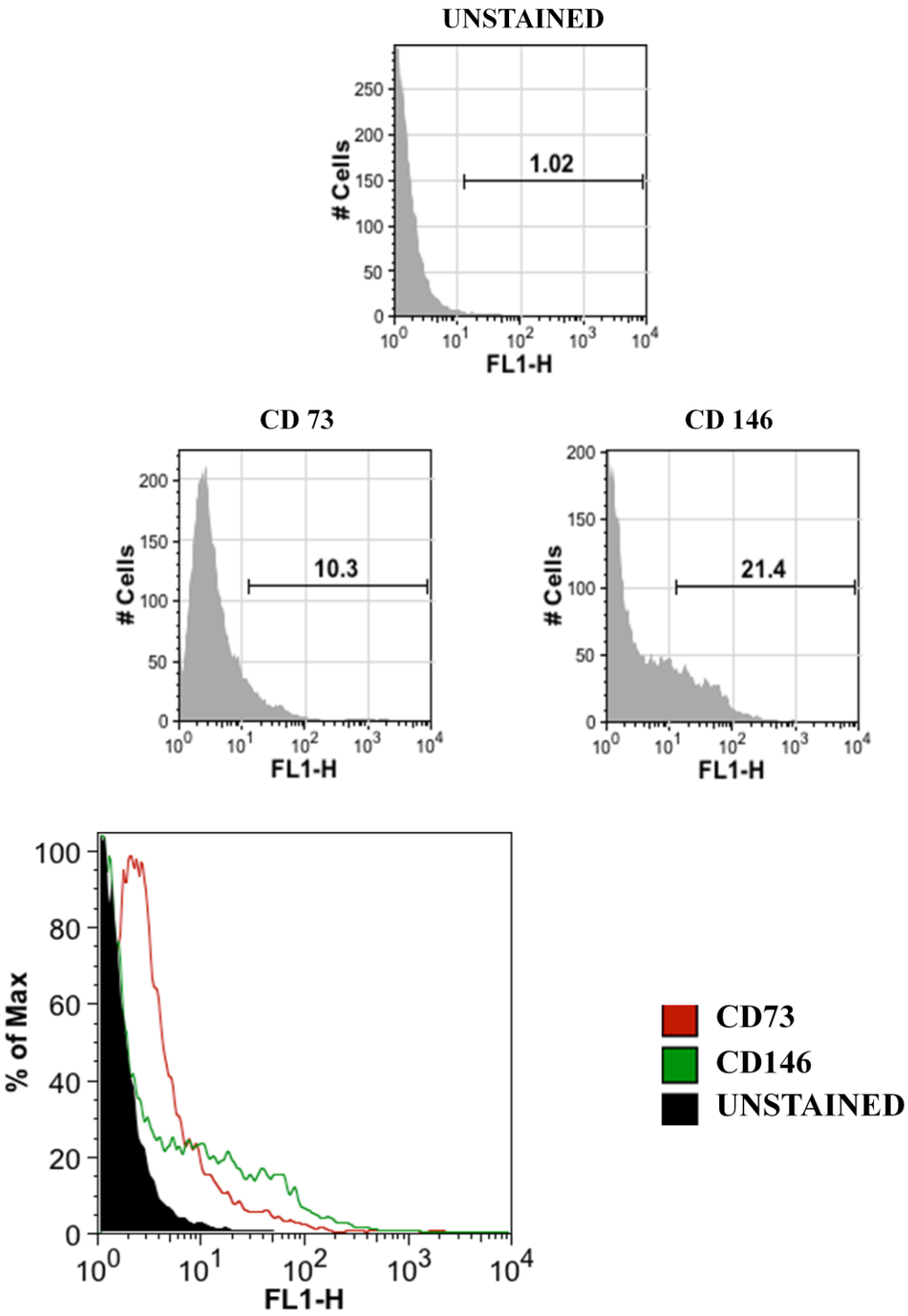


CD 73



CD 146




UNSTAINED
Cells
1.02
FL1-H
CD 73
10.3
CD 146
21.4
% of Max
CD73
CD146
UNSTAINED