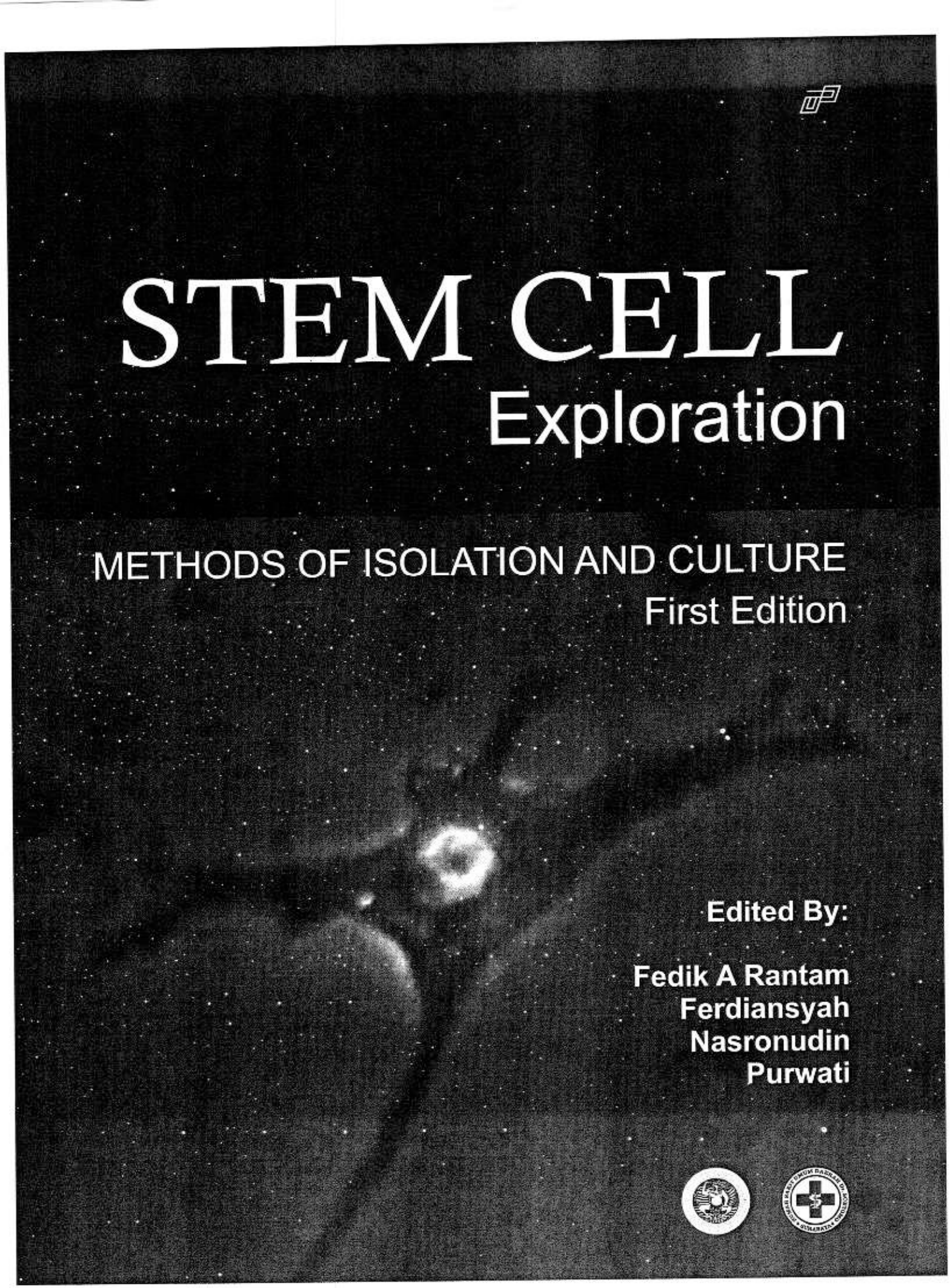
STEM CELL
Exploration

METHODS OF ISOLATION AND CULTURE
First Edition



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PREFACE

In this century are so many progress on developing therapeutic models round on the world. Stem cells are the last generation of therapy model base on the biological nature and differentiation potential of plastic adherent cells from bone marrow. These efforts revealed much information about their cell surface phenotype, proliferative and differentiation potential and culminated in the demonstration that clonally derived rabbit and human populations were multipotent, capable of differentiating into osteoblasts, adipocytes, chondrocytes, and hematopoiesis-supporting stromal cells. The existence of a stem cell in marrow capable of generating most connective tissue cell types had confirmed and capable to develop therapy model in different disease.

Recently, a committee from the International Society of Cell Therapy has adopted the term multipotent mesenchymal stromal cells (MSCs) to define these cells owing to the fact that a definitive description of the bona fide mesenchymal stem cell and the molecular mechanisms that regulate its self-renewal versus differentiation remain forthcoming. Marrow stromal cell, mesenchymal stem cell, and multipotent mesenchymal stromal cell are deemed equivalent and herein will refer to the plastic adherent, fibroblastoid cells from marrow that are defined functionally by their capacity to undergo multi-lineage differentiation into connective tissue cell lineages. The application of stem cells by human In recent years MSCs have still much attention owing to their broad therapeutic efficacy. Initially, Although in the fact round the world were found to have a significant positive impact by reducing the severity of the disease. For example as promising results were subsequently reported using MSCs or related cells from bone marrow in the treatment of Hurler's syndrome, metachromatic leukodystrophy, graft versus/host disease and to enhance engraftment of heterologous bone marrow transplants. Most recently, MSCs have been shown to afford a therapeutic benefit in the treatment like myocardial infarction, stroke, lung diseases, spinal cord injury, tropical and infectious diseases (malaria, HIV, TBC) and other neurological disorders.

At the Institute of Tropical Disease Airlangga University Surabaya Indonesia have been developed many kinds progenitors stem cells from different resource like bone marrow, adipose, umbilical cord blood, peripheral blood mononuclear cells, These results, together with the fact that MSCs can be readily isolated from small volume bone marrow aspirates, expanded to large numbers in vitro. In recent years have been intensified preparing and application design of stem cells in some therapy for different disease, and at the same time have been made standardization of methods and application as well as on clinical trial. In this compendium are providing mostly isolation, culture methods of different resource of stem cells.

In eddition, the pictures are resullting from are researches such as haemopoietics mesenchymal stem cell from human bone marrow, mesenchymaol stem cell from adipose, haemopoietics stem cell from human PBMCs, mesenchymal stem cell from UMCB, mesenchymal stem cell from brain.

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MINISTER OF HEALTH
OF THE REPUBLIC OF INDONESIA

REMARKS
THE MINISTER OF HEALTH, REPUBLIC OF INDONESIA

In the globalization era, the development of technology in medicine needs to follow the sovereignity of its nation in developing innovation. Therefore innovations in highly precision therapy need guidance in order to achieve competitiveness in medical research.

This can be achieved in three phases. First, the genomic approach that focuses on individual polymorphism in genetic molecular level. Secondly, the proteomic approach that focuses on epitope and paratope in cellular level. Thirdly, stem cell approach that focuses on cell differentiation originated from stromal cell.

Stem cell research will develop the highest level in therapy of disease, whether regenerative medicine or tissue cell therapy that may caused by injury elicited by infection or non infection. I, as the Minister of Health of The Republic of Indonesia would like congratulate the Institute of Tropical Disease stem cell team (ITD) and the Faculty of Medicine of Airlangga University Dr. Soetomo Hospital, Surabaya, in developing this remarkable book. This may become the milestone of the development of medical technology in Indonesia.

In such a manner, the transfer of the latest medical technology based on stem cell so it can be applied immediately in hospitals/teaching hospitals in Indonesia. This is highly relevant to the needs of our nation, which during the last decade had showed tendency of increased in malignancy, degenerative, infection, immunodeficiency, and other orthopedics cases of musculoskeletal impairment in the disease pattern.

Finally I would like to congratulate the Surabaya's stem cell team that has work very hard to make the stem cell technology applied for treating Indonesian, solving the health problem. Hopefully this book will inspire other medical centers to develop the technology that is aknowlegde at the global level.

Surabaya, October 2009

Minister of Health of the Republic of Indonesia

Dr.dr. Siti Fadilah Supari, Sp.JP(K)

REMARKS FROM THE DIRECTOR OF DR. SOETOMO TEACHING HOSPITAL SURABAYA

With grateful words to Allah SWT, I am very proud and I would like to welcome the publishing of this book, Stem Cell Exploration, Methods of Isolation and Culture, First Edition.

In accordance with the development of the latest therapy in medicine, stem cell therapy is a promising hope for today and the future time. It is necessary for us to develop stem cell therapy especially in Dr. Soetomo Hospital and also other Medical Centers. With this book being published, we now have guidance in studying and developing the stem cell science.

As we all understand that a successful health development needs the role of health institutions especially hospitals, and also educational institutions as the supplier of a good quality of human resources in health services. In this issue, Tissue Bank Centre Dr. Soetomo Hospital in collaboration with Institute of Tropical Diseases (ITD) Airlangga University as a research institution will assist the performance of health services.

Hopefully this book will be of advantage for our progression.

Surabaya, June 16th 2009

Director of Dr. Soetomo Teaching Hospital

Dr. Slamet R. Yuwono, dr., DTM&H., MARS

REMARKS FROM THE DIRECTOR OF INSTITUTE OF TROPICAL DISEASES

Institute of Tropical Diseases (ITD) is a research institution that studied on tropical diseases infection. In the early phase of development, this institution only focused on infectious diseases whether the basic level or the molecular level, and consisted of a few study groups such as malaria, diarrhea, dengue hemorrhagic fever and hepatitis.

In order to meet the competitive demands, ITD Airlangga University has developed several study groups such as Avian Influenza (AI), stem cell, human genetic, retrovirus (HIV) with the latest facility, in such a way deep exploration can be performed at the molecular level. In this decade, the demands of cellular therapy that focused on regenerative medicine is increasing. Therefore, ITD with the collaboration with Tissue Bank dr. Soetomo Hospital, Faculty of Medicine Airlangga University have been developing stem cells in order to fulfill the needs.

In the future time, stem cell laboratory at ITD will supply many kinds of stem cells that can be inducted into progenitor cells. Therefore can be applied in conformity with the type of disorders found in the community. Also to facilitate therapies intended to Tropical Infection Hospital and Teaching Hospital Airlangga University.

Hopefully in the future, ITD will become a center of stem cell development in Indonesia.

Surabaya, June 17th 2009

Director of ITD

Dr. Nasronudin, dr., Sp.PD., K-PTI

ACKNOWLEDGMENTS

It is with great pleasure and gratitude that we acknowledge many of the people who helped make this book possible. We would also like to thank all of our stem cells team (Budi S Pikir, Heri S, Suwarno, David P, Rahmad, Thaha, Bambang SL, Yetti) and residents (Andri R.W., Andri W, Sulis, Pandji), past and present, as they encourage us to continue learning each and every day. Last but certainly not least, we are extremely lucky to have excellent technician (Helen Susilowati, Eryk Hendrianto).

ABBREVIATIONS

Abbreviation	Definition
α-MEM	α-modified minimal essential medium
ASC	adipose-derived stem cell
BM	bone marrow
BM-HSC	Bone marrow Haematopoietics
BM-MSC	bone marrow mesenchymal stem cell
BMP	bone morphogenetic protein
BSA	bovine serum albumin
BSC	biological safety cabinet
CBE	Cord Blood Derived Embryonic Like Stem Cells
CBMP C	Cord Blood Multipotent Progenitor Cells
CBMSC	Cord Blood Mesenchymal Stem Cells
CDM	Chondrocyte Differentiation Medium
CFU	colony-forming unit
CFU-BL	colony-forming unit-blast
CFU-F	colony forming unit-fibroblast
CMF-Saline G	calcium- and magnesium-free Hanks' Saline G
DMEM	Dulbecco's modification of Eagle's medium
DMEM/F-12	Dulbecco's modified Eagle's medium/nutrient mixture F-12, 1:1
DMSO	dimethyl sulfoxide
D-PBS	Dulbecco's phosphate-buffered saline complete with Ca2+ and Mg2+
EDTA	ethylenediaminetetraacetic acid
ESC	embryonic stem cell
EtOH	ethanol
FBS	fetal bovine serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	Hanks' balanced salt solution
HCEC	human corneal endothelial cell

hECC	human embryonal carcinoma cell
hEGC	human embryonic germ cell
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
hES	human embryonal stem (cell)
hESC	human embryonic stem cell
HSC	haematopoietic stem cell
HUCPV	human umbilical cord perivascular
ICC	immunocytochemistry
MNC	mononuclear cell
MSC	mesenchymal stem cell
NOD/SCID	nonobese diabetic/severe combined immunodeficient
NS	neural stem cell
PBS	phosphate-buffered saline
PBSA	Dulbecco's phosphate-buffered saline, solution A (no Ca2+ or Mg2+)
PBSC	Dulbecco's phosphate-buffered saline with 1 mM CaCl2 and 1 mM
UC	umbilical cord
UCB	umbilical cord blood

CONTENTS

CHAPTER 1
INTRODUCTION

Stem cells have a greater potential to form many differentiated cell types and have potentially useful to regeneration of tissue damaged by disease or injury. Regenerating the cells of the tissue or organ and thereby treating a patient suffering from a medical condition. This idea of "regenerative medicine" holds great hope for millions of patients with degenerative diseases and injuries. Its mean regeneration of tissue damage cause by disease and injury by normal cell. Repair of damaged organs and tissues using stem cells could potentially address the needs of these patients, encompassing most of the leading causes of death in the industrialized nations

The characterize of stem cells are First a continued ability to proliferate so that a pool of cells is always available for further use. Second the ability to respond to appropriate signals by differentiating into one or more differentiated cell types. The embryonic stem cells, taken from the inner cell mass of young (5 to 7 day post-conception) embryos, but adult stem cells, taken to mean any postnatal tissue source and including umbilical cord blood and the solid umbilical cord matrix, placental tissue, and most or all body tissues.

STEM CELL

A stem cell is a cell from the embryo, fetus, or adult that has, under certain conditions, the ability to reproduce itself for long periods or, in the case of adult stem cells, throughout the life of the organism. It also can give rise to specialized cells that make up the tissues and organs of the body. Much basic understanding about embryonic stem cells has come from animal research. In the laboratory, this type of stem cell can proliferate indefinitely, a property that is not shared by adult stem cells.

EMBRYONIC STEM CELL

An embryonic stem cell is derived from a group of cells called the inner cell mass, which is part of the early (4- to 5-day) embryo called the blastocyst. Once removed from the blastocyst, the cells of the inner cell mass can be cultured into embryonic stem cells. These embryonic stem cells are not themselves embryos. In fact, evidence is emerging that these cells do not behave in the laboratory as they would in the developing embryo—that is, the conditions in which these cells develop in culture are likely to differ from those in the developing embryo.

The differentiation of stem cells is call "plasticity" of a stem cell, that is, its ability to form differentiated cell types namely unipotent is limited to forming only one differentiated cell type. Multipotent is with the ability to form multiple cell types but the pluripotent stem cell has the ability to give rise to types of cells that develop from the three germ layers (mesoderm, endoderm, and ectoderm) from which all the cells of the body arise. The only known sources of human pluripotent stem cells are those isolated and cultured from early human embryos and from fetal tissue that was destined to be part of the gonads. The totipotent is with the ability to form all postnatal tissues as well as extra embryonic tissues such as placenta, essentially able to regenerate a complete new embryo and full organism.

Embryonic Stem Cel are derived from the inner cell mass/epiblast of the Blastocyst and capable of undergoing an unlimited number of symmetrical divisions without differentiation give rise to endoderm, ectoderm, mesoderm. ES is capable of colonizing the germline and giving rise to egg or sperm cells transgenic mouse and clonogenic - derived from a single ES cell.

ADULT STEM CELL

An adult stem cell is an undifferentiated cell that occurs in a differentiated tissue, renews itself [self-renewal], and becomes specialized to yield all of the mature cell types of the tissue from which it originated. Sources of adult stem cells include bone marrow, blood, the cornea and the retina of the eye, brain, skeletal muscle, dental pulp, liver, skin, the lining of the gastrointestinal tract, and pancreas.

Adult (Somatic) Stem Cells are found in many adult tissues. Replenish and repair a specific tissue; maintain homeostasis and with more limited differentiation and proliferation potentials than ES cells. may have hidden potential: research within past 6 years had suggested broad developmental plasticity of tissue-specific stem cells, but much remains controversial.

How are Adult and Embryonic Stem Cells Similar? Both are capable of self-renewal and can give rise to specialized cells of the heart, brain, bone, etc. adult stem cells can exist in some tissues for the life of the Individual. Both can engraft, proliferate and differentiate into specialized cells following transplantation.

How are Adult and Embryonic Stem Cells Different? Embryonic stem cells as such may not exist in the embryo existence of adult stem cells has been confirmed for several lineages (generally quiescent). Embryonic stem cells are pluripotent whereas adult stem cells are generally thought of as multipotent, oligopotent, unipotent. Embryonic stem cells can be expanded in the laboratory whereas many adult stem cells generally fail to proliferate or do so to a limited extent. Embryonic stem cells may differentiate preferentially by symmetric cell division. Embryonic stem cells can form teratocarcinomas *in vivo* – can include hair, teeth, bone, etc.

What are the sources of Adult Stem Cells these cells? Can they be manipulated to expand in vitro without differentiation? Can they be manipulated after *in vitro* expansion to differentiate *in vitro* or *in vivo* into functional mature cell types to be used for tissue repair or replacement? Bone marrow (haematopoietics, mesenchymal), Brain (neural), Gut, Skin, Hair follicles (epithelial), Liver (hepatic) Pancreas, Blood vessels (endothelial), Muscle.

The goal to understand the mechanisms regulating haematopoietics stem cell self-renewal and lineage-specific differentiation and then can also develop like *Ex vivo* expansion to provide sufficient quantities of haematopoietics stem cells from cord blood collections for transplantation into adults. Large scale 'manufacturing' of mature blood cells (red cells, platelets) for transfusion. Attractive target cell population for gene therapy Better understanding of leukemia and other blood diseases.

DEFINITIONS AND GENERAL CONCEPTS ABOUT STEM CELLS

Plasticity

Plasticity refers to the ability of an adult stem cell from one tissue to generate the specialized cell type(s) of another tissue. Purported examples include the generation of pancreatic islets of Langerhans, the contribution to several cell types in the brain (microglia, astrocytes and neurons), and the formation of new blood vessels in the portal veins of the liver and in the eye from hematopoietic stem cells.

Stem Cell Plasticity

Do adult stem cells exhibit plasticity as a normal event *in vivo*? If so, is this true of all adult stem cells? What are the signals that regulate the proliferation and differentiation of stem cells that demonstrate plasticity? HSC become muscle, liver, brain, epithelial, endothelial. Muscle become blood (controversial). NSC become blood, embryo. MSC become cartilage, fat, tendon, bone. Fat become muscle (great idea).

Haematopoietics Stem Cell-Based Gene Therapy Applications like Inherited Blood Diseases Acquired Diseases, Immunodeficiency (SCID, adenosine deaminase deficiency, purine nucleoside phosphorylase deficiency). Disorders of phagocytic cells (chronic granulomatous, disease, leukocyte adhesion deficiency). Globin deficiencies (thalassemias, sickle cell anemia). Hemophilia A (factor VIII), Cancer and Infectious diseases (AIDS).

The Laboratory of Stem Cell at the Institute of tropical Disease Airlangga University has collaborative with Biomaterial Center – Tissue Bank Tissue Dr. Soetomo Teaching Hospital are together developing Tissue Engineering using biomaterial some kinds of Stem Cells from different sources like bone marrow (BM), peripheral blood mononuclear cells (PBMCs),adipose and brain. These cells will be developed to become progenitor cells like chondroblast, chondrocyte, cardyomiocyte etc. The goals these experiment to repair of bone fracture, defect tissue organ like liver, pancreas, heart, defect of tendon, skin abration, defect of haematopoietics od immunodeficiency like AIDS. Strategy of stem cells development is as below.

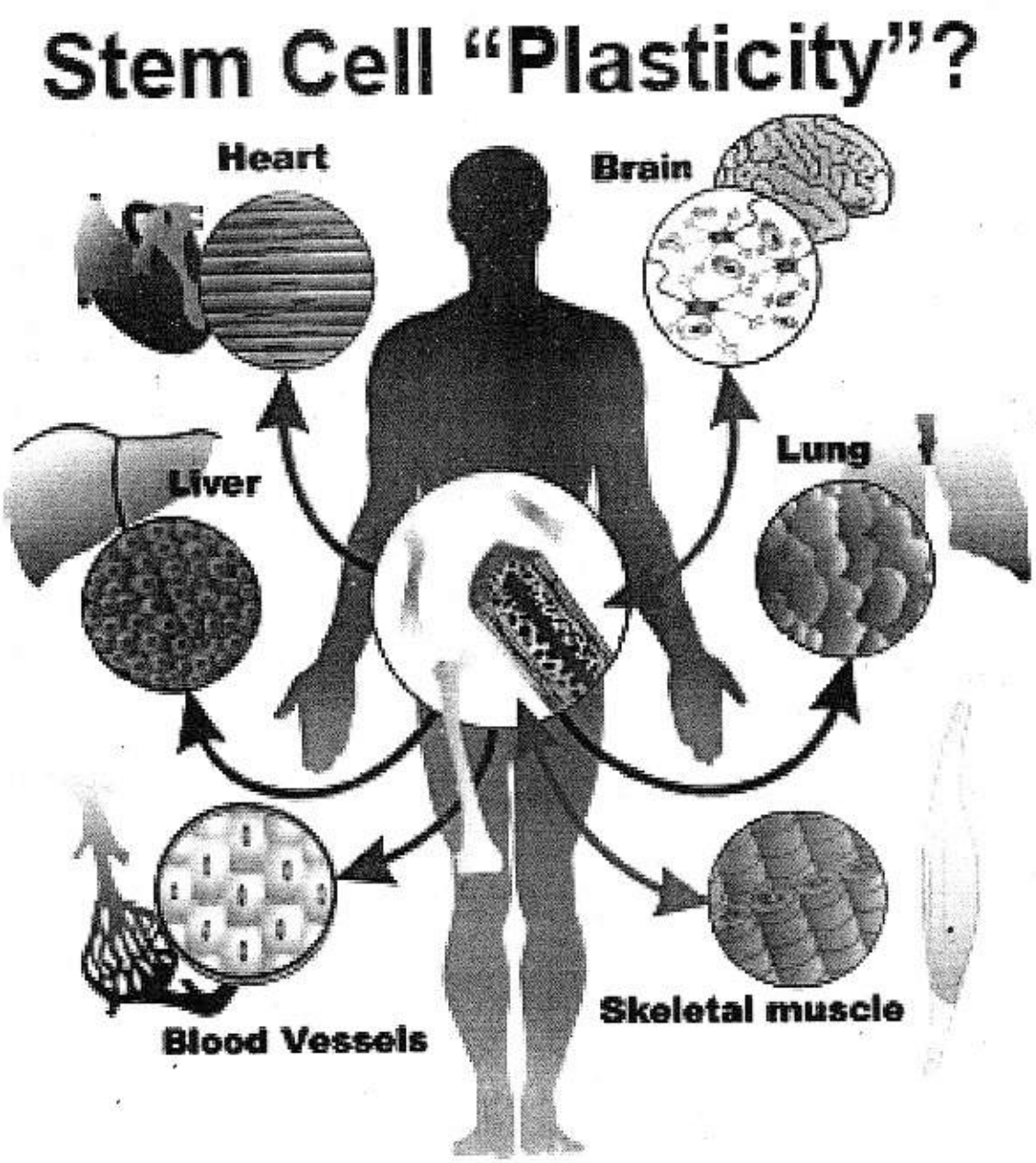


Figure 1. Adult stem cells resourches from different organ (Bunting and Hawley. Scientific World Journal, 2002)

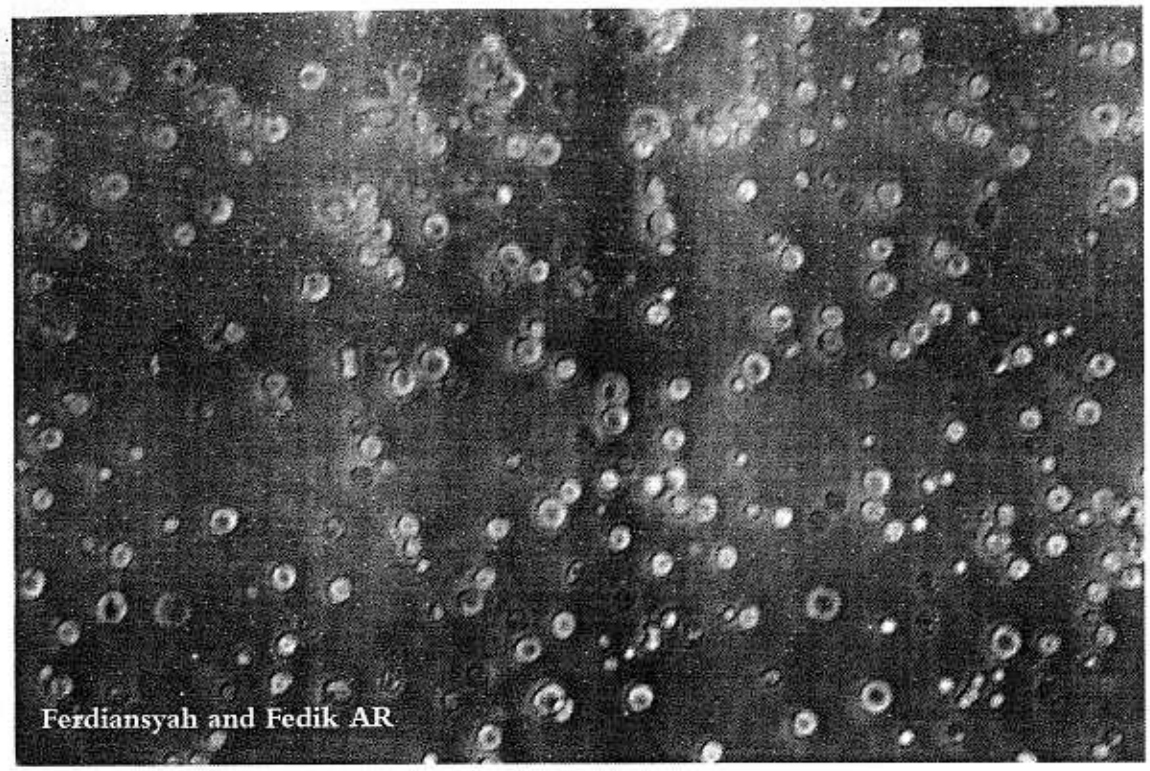
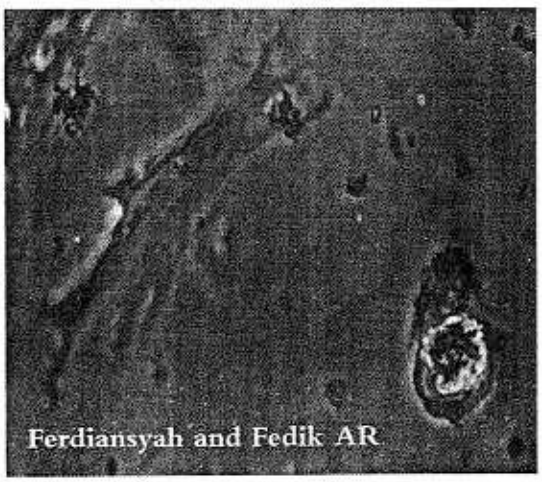


Figure 2. Isolation of stem cells, first form like mononucleated cells aspirate from human adult bone marrow. These cells were isolated using Ficoll histopaque 1,077 after isolation and analyzed under inverted microscope 10x



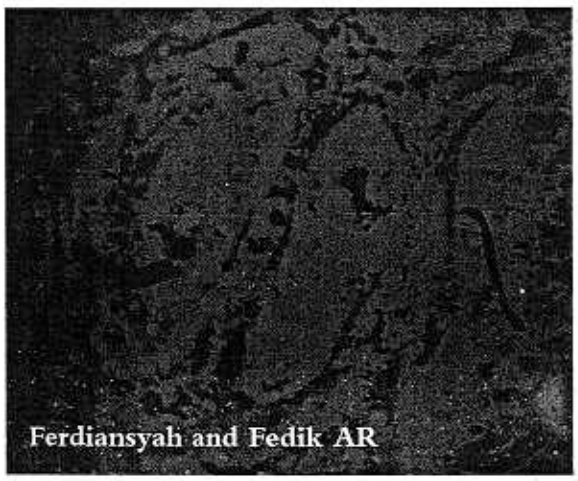


Figure 3. Mesenchymal stem cells like cardyomiocyte cells from adult bone marrow. These cells are growing 3 day after culture using complete medium. It were analyzed under inverted microscope 40x

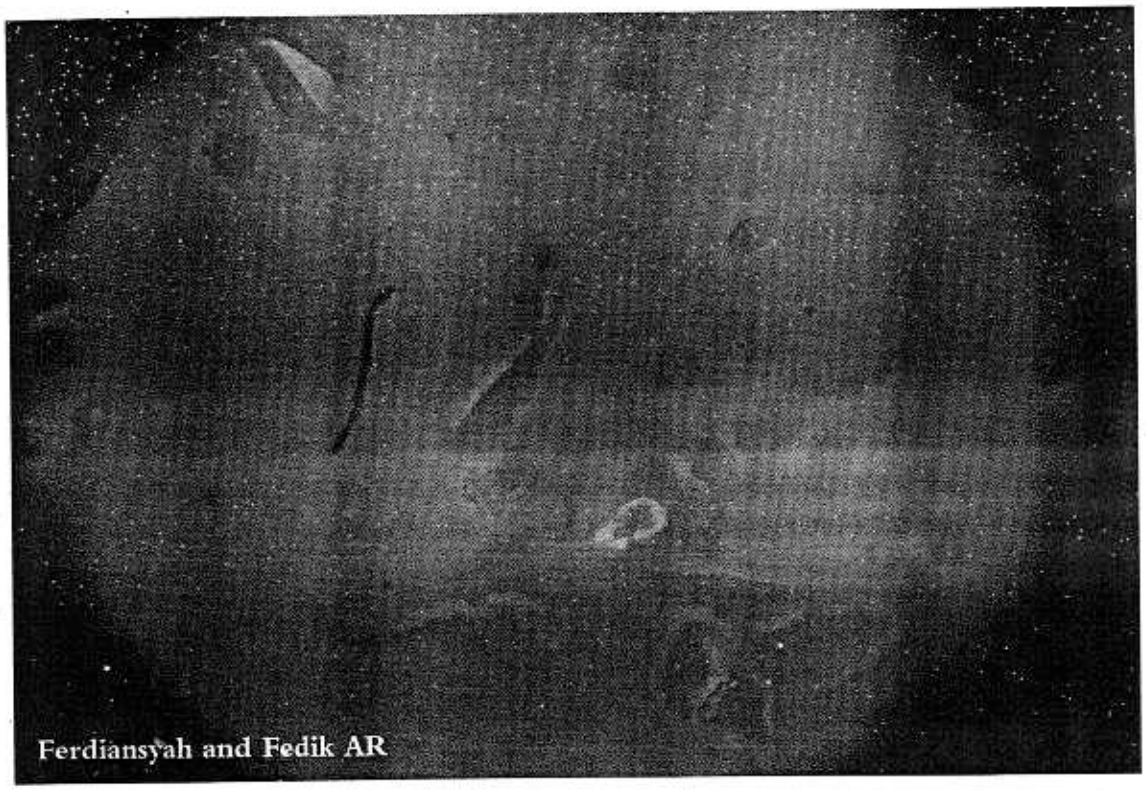


Figure 4. Colonies of mesenchymal cells like fibroblast cells from rabbit adult bone marrow 3 days after culture in dish using complete grow medium analyzed under inverted microscope 40x.

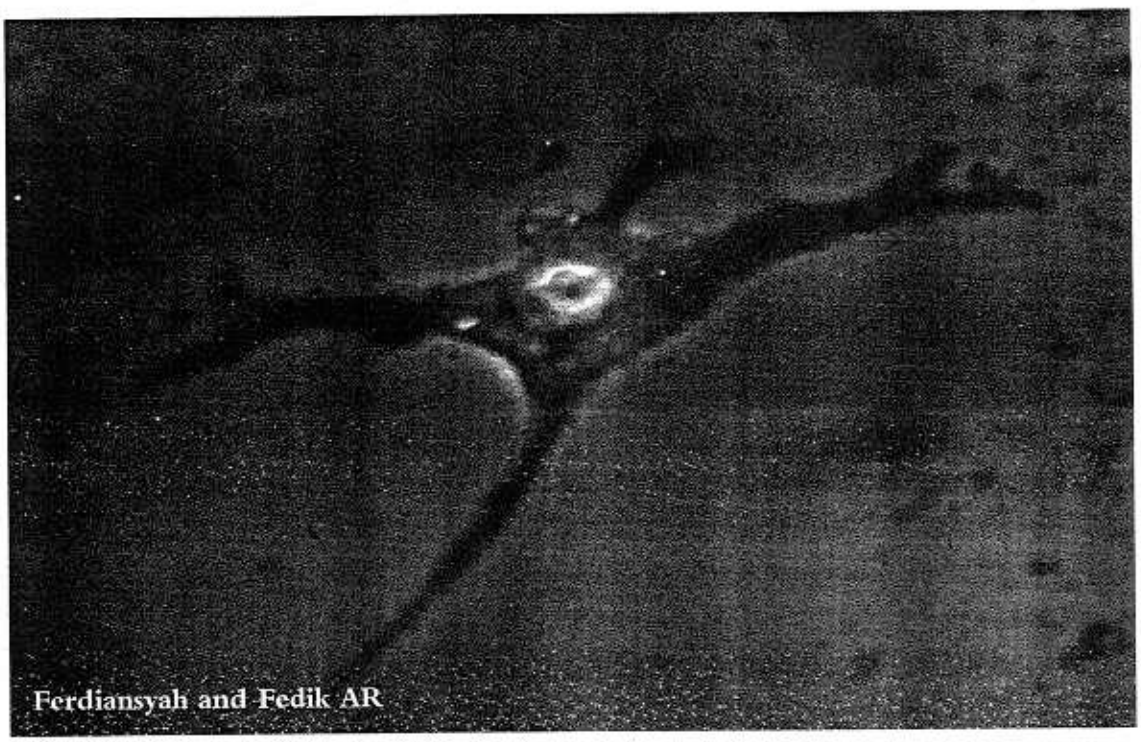


Figure 5. Mesenchymal cell like neuron cells from human adult bone marrow 3 days after culture in dish using complete grow medium. Analysis under inverted microscope 40x

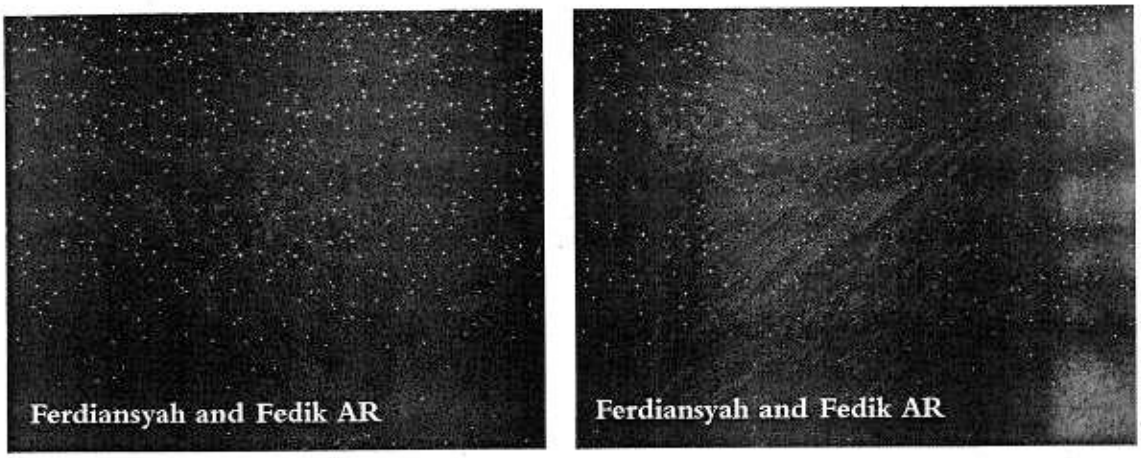


Figure 6. Mesenchymal cell like epithelial cells from adult rabbit bone marrow 3 days after culture in dish using complete grow medium. Analysis under inverted microscope 40x

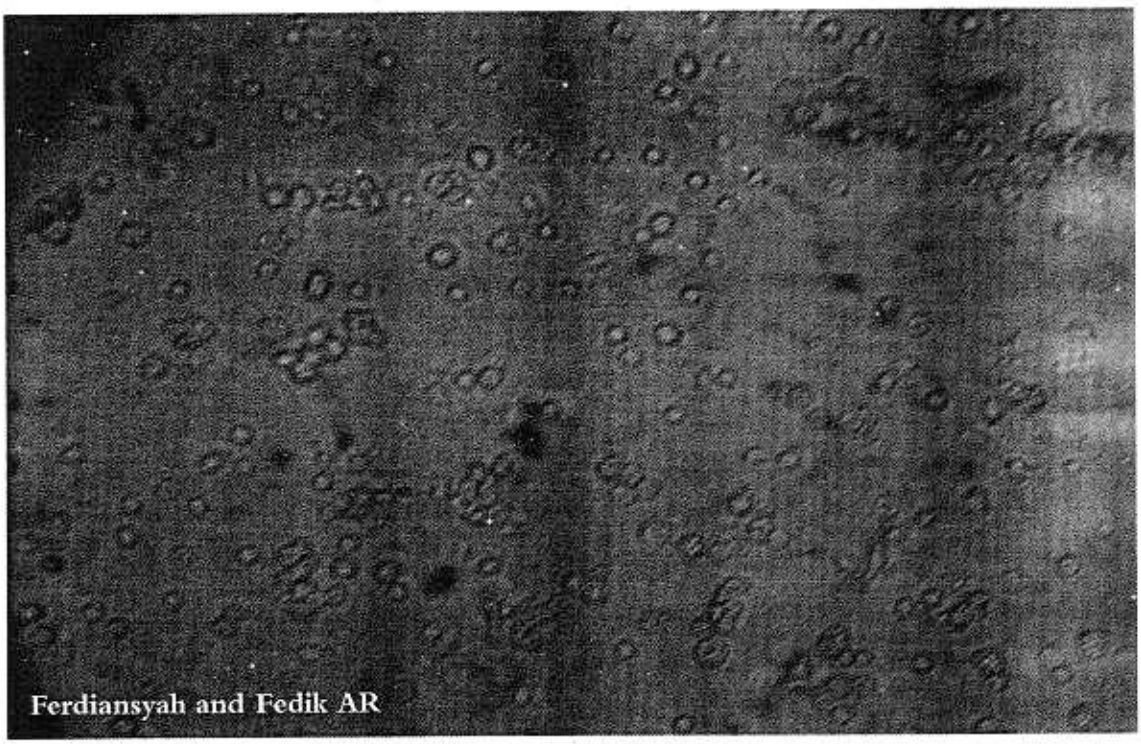


Figure 7. Mesenchymal cell like fibroblast cells from human whole peripheral blood mononuclear cells 3 days after culture in dish using complete grow medium. Analysis under inverted microscope 20x

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CHAPTER 2

ISOLATION AND CULTURE OF STEM CELLS FROM HUMAN BONE MARROW

ISOLATION OF STEM CELLS FROM HUMAN BONE MARROW

Human mesenchymal stem cells (hMSCs) are readily isolated from bone marrow by their adherence to tissue culture plastic and can be expanded through multiple passages in medium containing high concentrations of fetal calf serum (FCS) However, the proliferation rates and other properties of the cells gradually change during expansion, and therefore it is advisable to not expand them beyond 4 or 5 passages. The cells are readily cloned as single-cell derived colonies, but both the colonies and the cells within a colony are heterogeneous in morphology, rates of proliferation, and efficacy with which they differentiate. Two morphologically distinct cell phenotypes are seen in early passage, low density cultures: small, spindle-shaped cells that are rapidly self-renewing (RS cells) and large, flat cells that replicate slowly and appear more mature. The proportion of RS cells remains high for several passages if the cultures are maintained at low density, but the larger cells predominate in later passages. Cultures enriched for RS cells are obtained

if early passage cultures expanded to about 70% are lifted and replated at low density. However, if the cultures are allowed to expand to confluence, RS cells are not recovered on replating at low density. MSCs differentiate into osteoblasts, chondrocytes, adipocytes, myocytes, cardyomiocyte, and other cell types. They can be differentiated in vitro into skeletal muscle and cardyomiocyte.

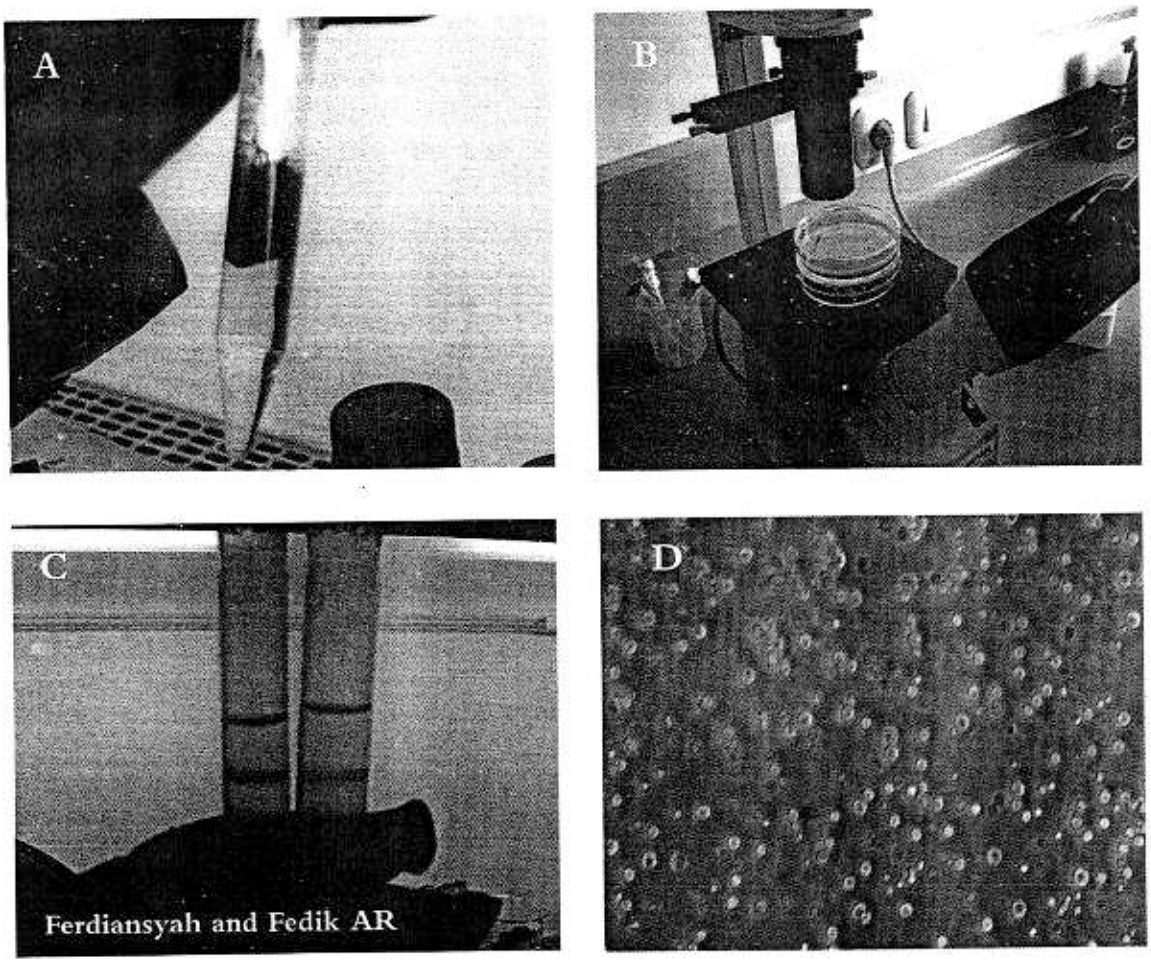

Figure 1. Isolation and culture mesenchymal stem cells from human bone marrow. **A.** Collecting sample bone marrow and isolation bone marrow then using ficoll histopaque for isolation of stem cell isolation. **B.** Bone marrow stem cells isolate ready to culture. **C.** Bone marrow stem cells already cultured in dish 10 cm. **D.** Bone marrow stem cells 30 min after cultured in dish 10 cm

Several investigators identified distinctive surface epitopes on hMSCs, but none have been shown to distinguish early progenitors from more mature cells in cultures of hMSCs. The first step is to isolate mononucleated cells from a marrow aspirate by centrifuging the sample on a density gradient and then recovering the cells that adhere to tissue culture plastic (passage zero cells). The passage zero cells are then expanded by plating them at a low density in a multilayered tissue culture

flask (Cell Factory; Nunc). The expansion of the cultures at low density enhances their content of spindle-shaped and rapidly expanding cells (rapidly self-renewing or RS cells) that are replaced by large, flat and mature hMSCs if the cells are plated at higher density or the cultures are passaged more than 4–6 times. The mature hMSCs expand slowly and have less potential for differentiation than RS but retain the ability to differentiate into mineralizing cells and secrete factors that enhance growth of haematopoietics stem cells and perhaps other cells.

Reagents

1. α-MEM with l-glutamine, without ribonucleosides or deoxyribonucleosides (Sigma, Cat. M0894).
2. Fetal Bovine Serum (FBS) (Biowest, Cat. S1650).
3. l-Glutamine, 200 mM (Invitrogen).
4. Penicillin G (10,000 units/mL), and streptomycin sulfate (10,000 μg/mL) in solution of 0.85% NaCl (Sigma, Cat. P0781).
5. Ficoll-Paque (Amersham Biosciences, catalog # 17-440-02) or similar (Sigma).
6. Phosphate buffered saline (PBS), without Ca++ or Mg++, pH 7.4 (Merc).
7. Trypsin (0.25%)-EDTA.4 NA (0.38 g/L) (Merck).

Equipment

1. Common Equipment

1. Biological Safety Cabinet, Class III (BSC).
2. Centrifuge with swinging bucket rotor, capable of holding various tube sizes up to 200 mL.
3. Incubator, water jacketed and humidified with 5% CO_2, maintained at 37 °C.
4. Microscope, inverted, phase with a super long working distance condenser (SLWDC).
5. 37 °C Waterbath.

2. Equipment for Closed System Only

1. TSCD Sterile Tubing Welder.
2. Hand-held Tube Sealer.

Supplies

Common Supplies

1. Electric or manual pipet filler/dispenser for mouth-free pipeting of solutions (0.1 to 10 mL).
2. Single channel pipetors, air displacement, capable of accurately measuring from 10 µL to 1000 µL, i.e., Eppendorf Research Series 2100 or similar.
3. Sterile aerosol barrier pipet tips, 10, 20, 200, and 1000 µL.
4. Hemocytometer with cover slip.
5. 15, 50, and 200 mL sterile plastic disposable conical centrifuge tubes.
6. Plastic disposable snap-cap centrifuge tubes: 1.5 mL.
7. 1 L Nalgene bottles or other 1 L containers that are sterile or can be sterilized. They are used to collect cell harvest from Cell Factory.
8. Sterile serological pipets: 1, 2, 5, 10 mL.
9. Vacutainer tubes w/sodium heparin.
10. 175 cm^2 tissue culture flasks.
11. Sterile plastic Pasteur transfer pipets.
12. Sterile pipets for aspiration source.
13. Sterile 250 mL filter units 0.22 µm pore size (Millipore).
14. Sterile 500 mL filter units, 0.22 µm pores (Millipore).
15. Sterile 1000 mL filter units, 0.22 µm pores (Millipore).
16. 10-tray Cell Factory, 6,320 cm^2 culture area).

Working Solutions

Complete Culture Medium (CCM): 500 mL α-MEM, 100 mL FBS (final conc. ~16.5%), 6 mL l-Glutamine (final conc. 2 mM), and 6 mL Penicillin G and streptomycin sulfate (final conc. 100 units/mL penicillin and100 µg/mL streptomycin - *Optional).* Filter medium through 0.22 µm sterile filter unit. Divide into aliquots and store at 4 °C for up to 2 wk. Before an experiment, warm the aliquot to 37 °C.

Procedures Isolation

All supplies and reagents are sterile and the culture procedure should be performed in a tissue culture hood using aseptic techniques. All materials to be used in the BSC should be wiped down with 70% Ethanol before bringing it in to the BSC.

Lab coats and gloves should be worn. Culture media and buffers should be pre-warmed to 37 °C before use.

1. Under local anesthetic, bone marrow aspirates are collected from the iliac crest and placed in 15 mL Heparin tubes prefilled with 3 mL α-MEM. The tubes and samples are kept on ice until transported to the laboratory and processed.
2. Each aspirate is transferred into a sterile 15 mL blue cap tube and diluted with sterile 1× Phosphat Buffer Saline (PBS). QS for a total volume of 8 mL.
3. Each tube is then rinsed twice with an additional 5 mL of 1× PBS and the contents combined with the diluted aspirate.
4. For each aspirate, place 5 mL of room temperature Ficoll into a separate 15 mL tube.
5. Gently overlay each aspirate onto the Ficoll.
6. Centrifuge at 1,600 rpm for 15 min at room temperature in a swinging bucket rotor with BRAKE OFF.
7. After centrifugation, collect the "buffy coat" located at the Ficoll-PBS interface with a sterile Pasteur transfer pipet and place the cells into a clean 15 ml tube.
8. Dilute each sample with 1× PBS. QS for a total volume of 15 mL. Invert the tube 3–5 times to mix.
9. Centrifuge at 1,600 rpm for 10 min in a swinging bucket rotor with the BRAKE ON.
10. Remove the supernatant by aspiration and resuspend the cells with 6 mL of prewarmed CCM.
11. Plate the cells in a 10 cm^2 plate or 5 cm^2 plate.
12. Incubate the cells at 37 °C with 5% humidified CO_2 for 18–24 hours to allow the adherent cells to attach.
13. Approximately 24 h later, remove the media and nonadherent cells.
14. Add 5 mL of prewarmed 1× PBS to the culture, rock gently to cover the entire surface area, and then remove the 1× PBS. Repeat the wash 2 additional times.
15. Add 10 mL of fresh CCM to the dish and return to the incubator. Incubate the cells at 37 °C with 5% humidified CO_2 for 5–10 d. Examine daily by inverted microscopy.

    Every third day, remove the media, rinse the cells with 10 mL or 5 ml prewarmed 1 × PBS, remove the PBS, and feed with a fresh 10 mL of CCM. Continue until the cells are between 60 and 80% confluent.

16. If the cells are going to be expanded in a Cell Factory, the Cell Factory must be equilibrated inside a humidified, 5% CO_2, 37 °C incubator for 48 h before use.

CULTURE OF MESENCHYMAL CELLS FROM HUMAN BONE MARROW

Human bone marrow have some kind of stem cells, from this resource can be cultured and getting some different cells like mesenchymal cell. Cells cultured after was isolated using ficol histopaque 0,177 (Sigma) To culture this cell were used growth medium as well as α-modified essential medium (α-MEM) (Sigma), with added fetal bovine serum 20% (Biowest), penicillin and streptomycin (Sigma) and amphotericyne (Sigma). Cells was good growing after washing at 24 hour incubated in incubator using temperature 37 °C with 5% CO_2.

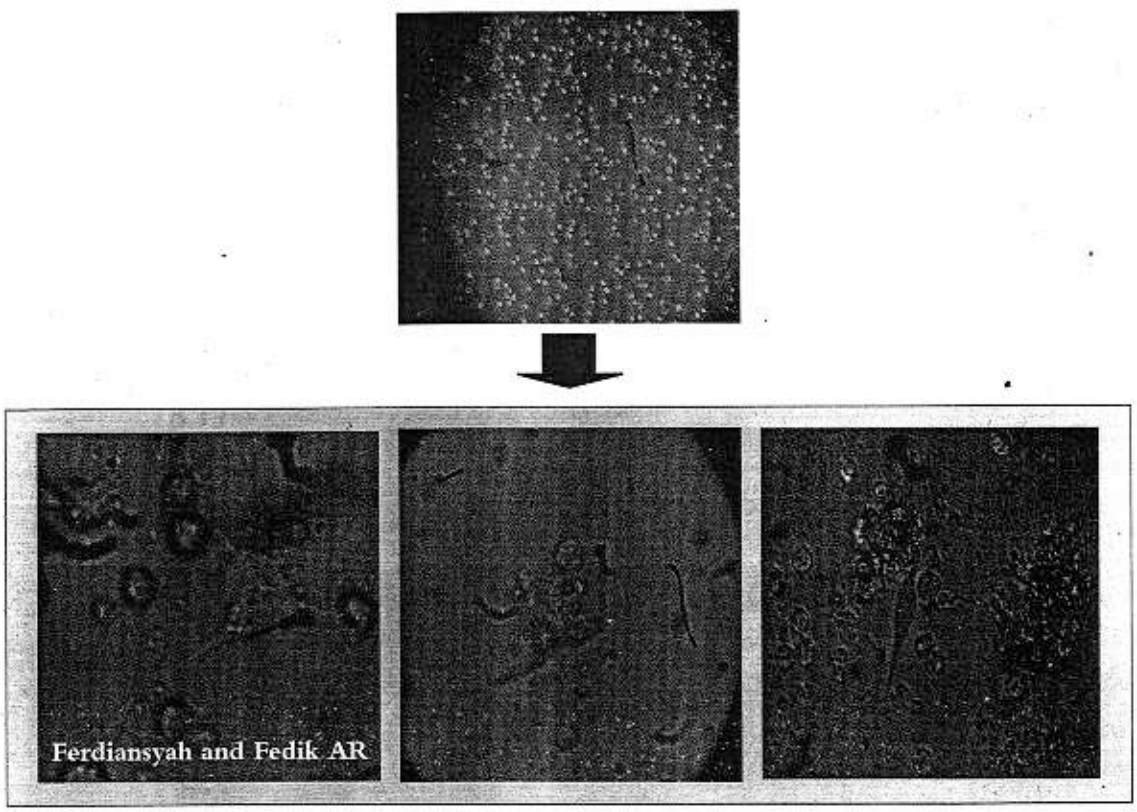

Figure. 2. Multiplicity of adult bone marrow stem cells 2 days after culture. Analyzed under inverted microscope 40x

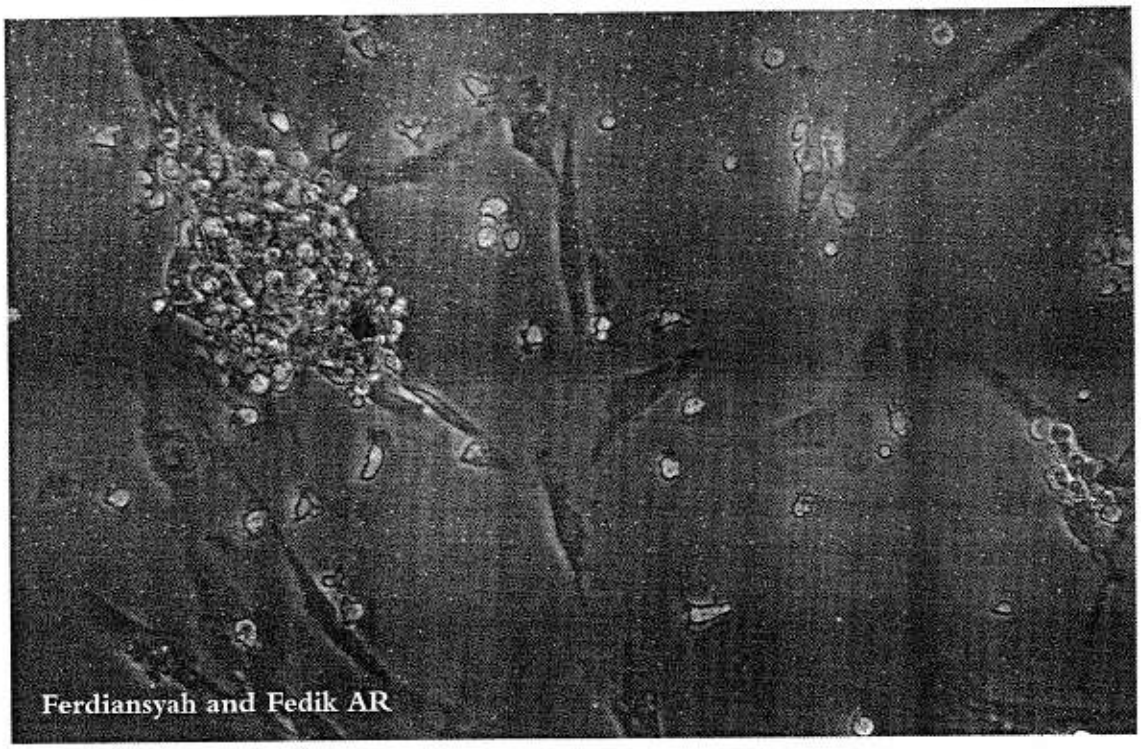

Figure 3. Mesenchymal cells like fibroblast cells from bone marrow, 4 days after culture, analyzed under inverted microscope 40x

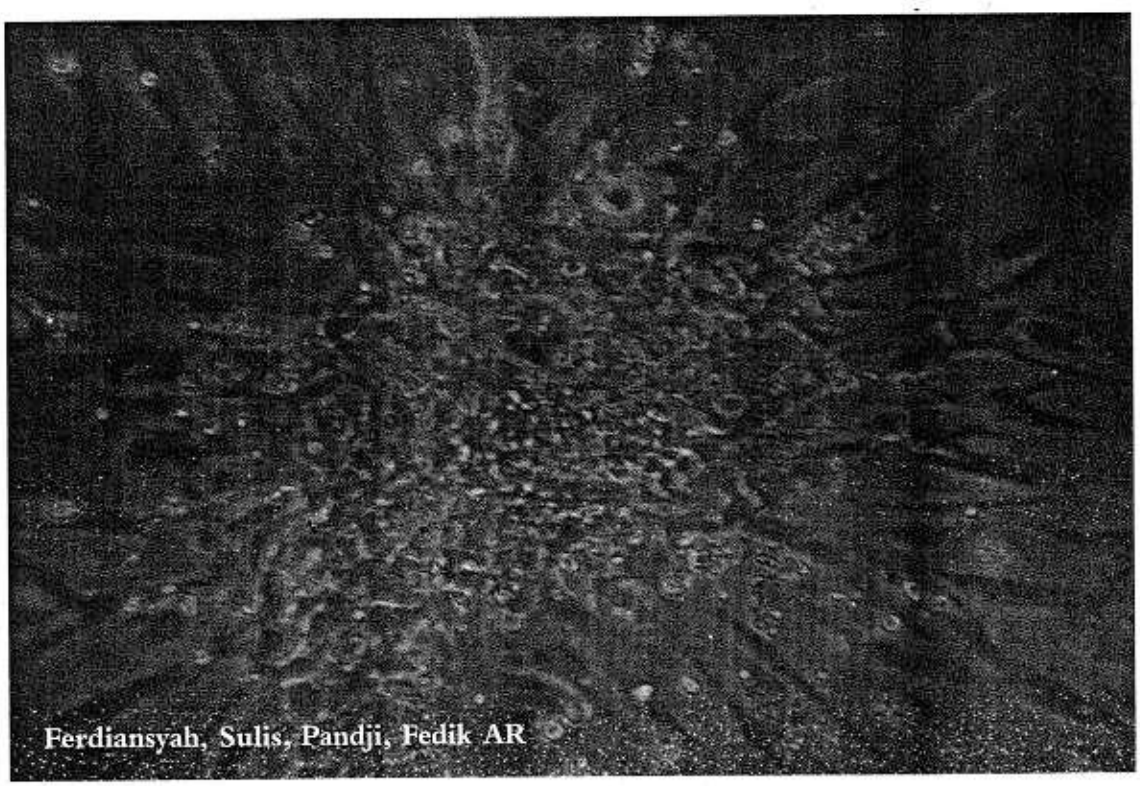

Figure 4. Mesenchymal cells like fibroblast cells from bone marrow, 5 days after culture, analyzed under inverted microscope 40x

The other hand Many different formulations of growth media can be used to isolate and grow MSCs. We have followed the recommendations of Caplan as found in Haynesworth et al. 1992 and use Dulbecco's Modified Eagles media (DMEM) containing 1.0 g/L glucose as opposed to the formulation with 4.5 g/L glucose, which is used in the Mesenchymal Stem Cells from Adult Bone Marrow chondrogenic differentiation media below. Other media that have been used successfully to propagate human MSCs include BGJb, Alpha MEM, DMEM:F12, McCoy's 5A, and RPMI 1640. Although some of these media are richer in certain components than DMEM, they have not proven superior to the original formulation reports and many other works using DMEM. The other major component of MSC isolation and growth media is fetal bovine serum (FBS). Most media formulations usually use 10% fetal calf serum to provide a mixture of undefined growth factors.

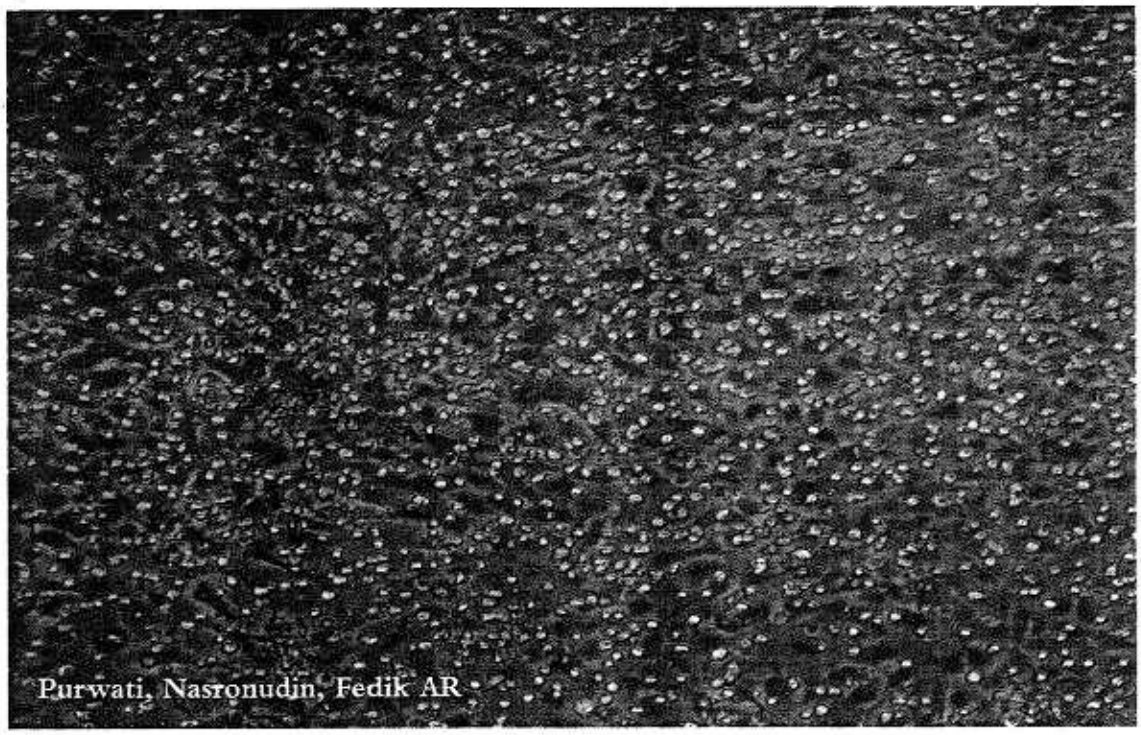

Figure 5. Mesenchymal stem cell from bone marrow are growing together with Haematopoietics cells, 3 days after culture

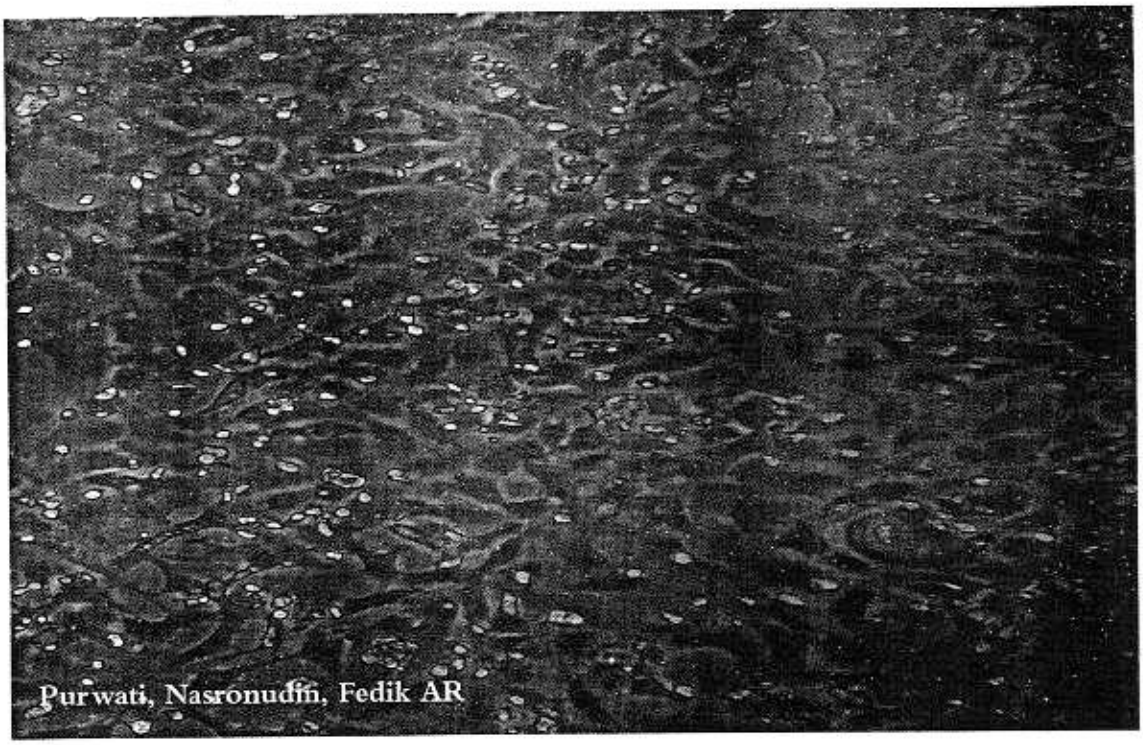

Figure 6. Mesenchymal stem cells from bone marrow, 3 days after cultured and after washing using serum free medium. Under inverted microscope 40x

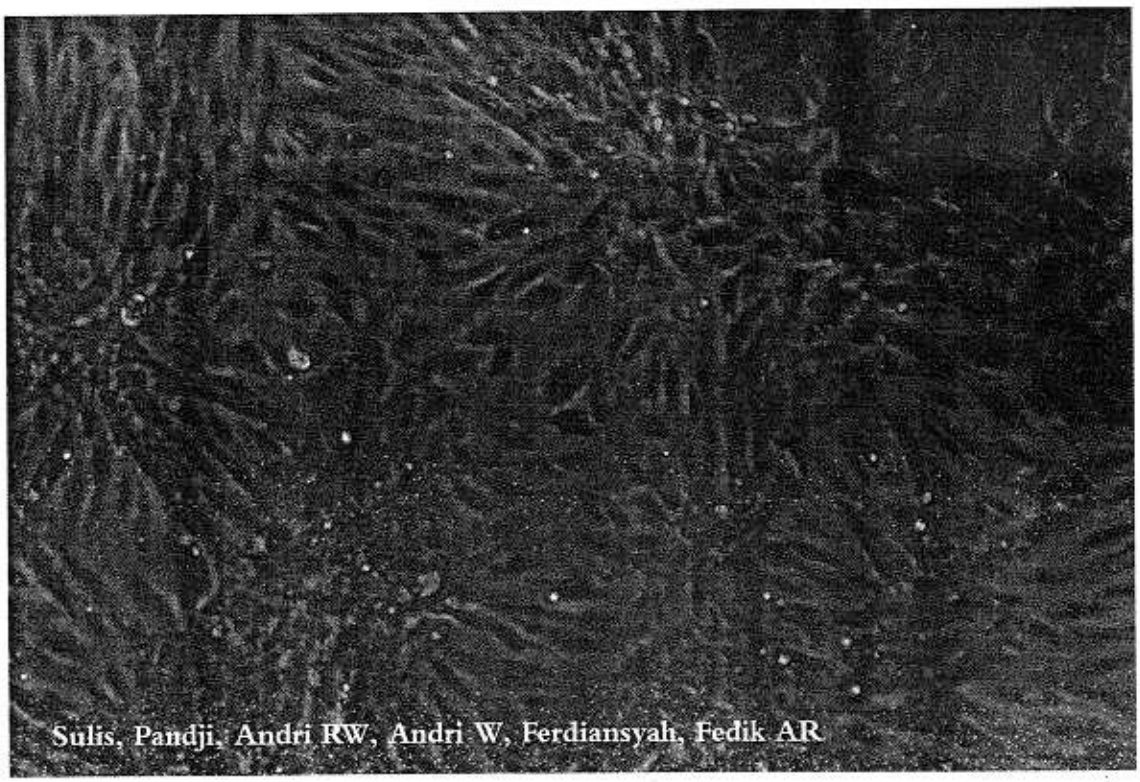

Figure 7. Mesenchymal stem cells from bone marrow, 5 days after cultured. It is growing about 90% confluence. Under inverted microscope 40x

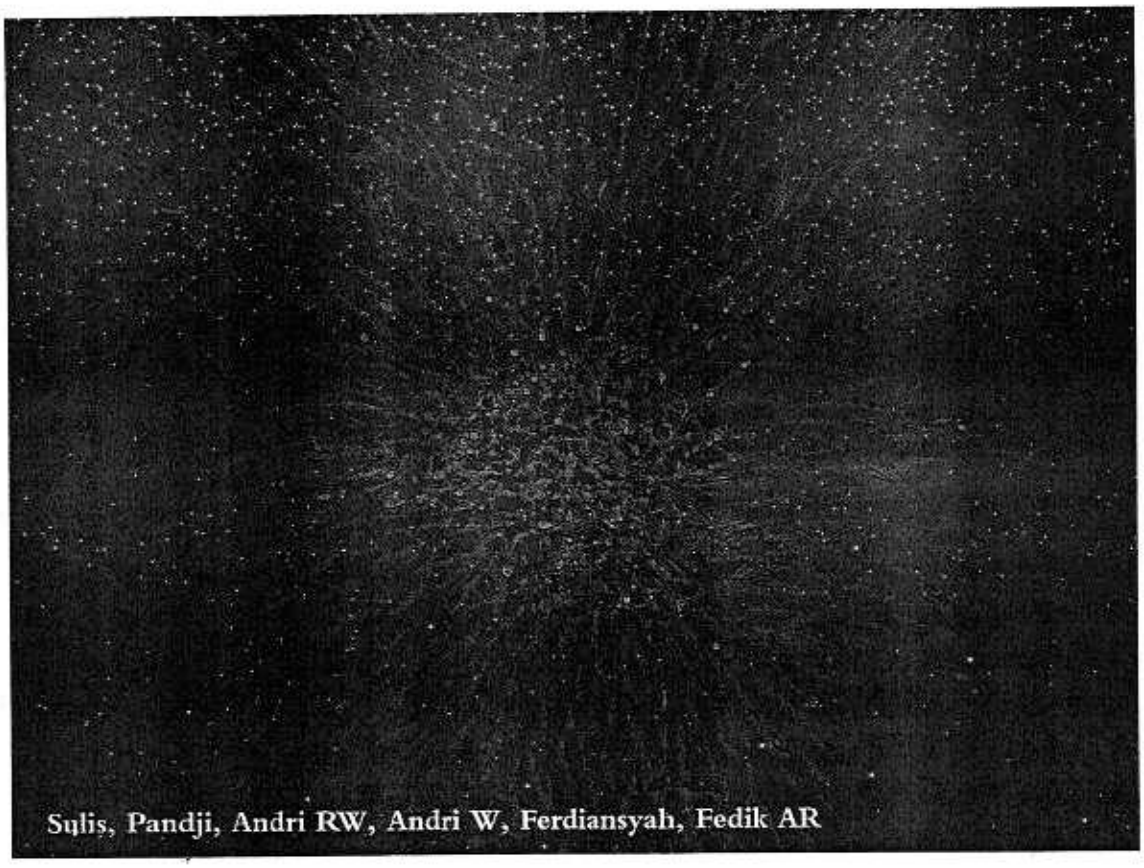

Figure 8. Mesenchymal stem cells from bone marrow, 6 days after cultured. It is growing about 90% confluence. Under inverted microscope 40x

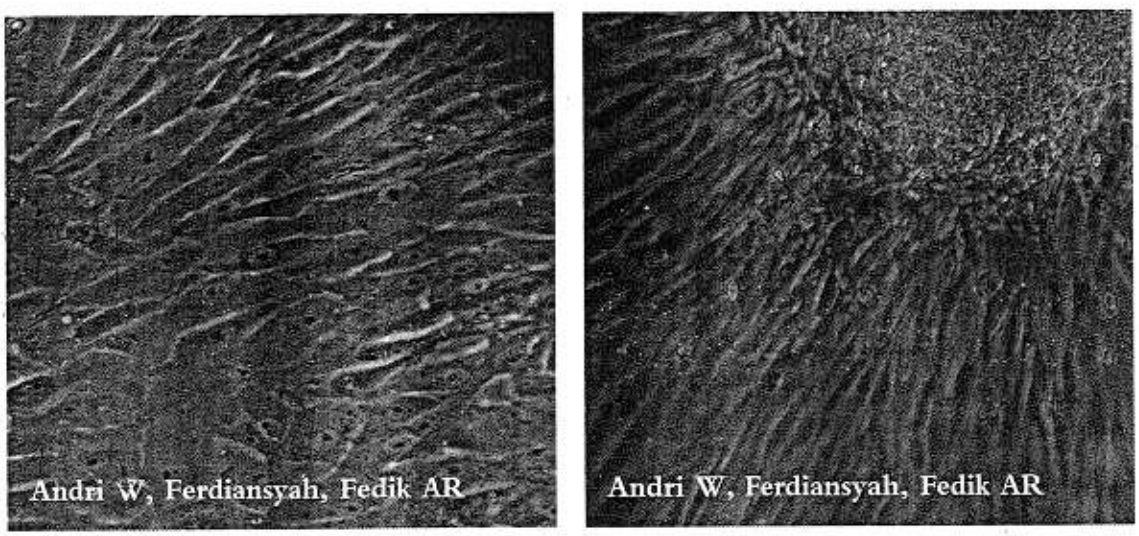

Figure 9. Mesenchymal stem cells from bone marrow, 7 days after cultured. It is growing about 100% confluence. Under inverted microscope 40x.

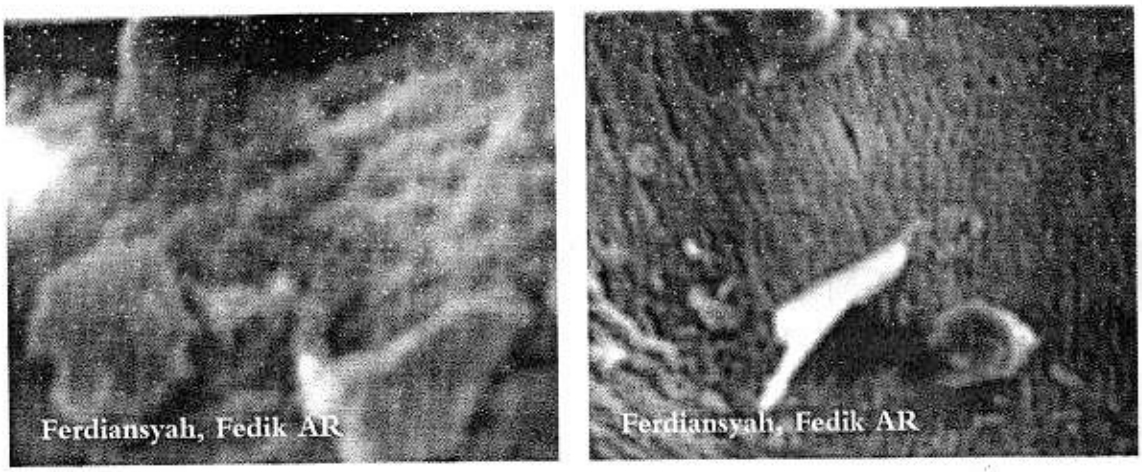

Figure 10. Mesenchymal stem cells from human bone marrow area growing on scaffold. Under electron microscope 750x.

Culture Protocols

Reagents

1. α-MEM low glucose (Sigma, Cat. M0894).
2. 50 mL FCS selected for MSCs (Gibco/Invtirogen) or alternatives Mesenchymal Stem Cell Stimulatory Supplement (Stem Cell Technologies) or Bullet Kit for MSCs.
3. 5 mL Antibiotic/Antimycotic mixture (Sigma, Cat. A2942).
4. Mix solutions, filter sterilize and store at 4 °C in the dark for up to 2 wk of use.

1. Additional Solutions and Items

A number of additional solutions common in tissue culture studies will also be used.

1. Trypsin/EDTA: 0.05% trypsin/0.23 mM EDTA (Merck).
2. Phosphate Buffered Saline (PBS) (Merck).

2. Plastic Tissue Culture Wares

MSCs can be grown on a variety of disposable plastic tissue culture containers. We have routinely used products from NUNC and Corning with good success.

Procedures

1. After bone marrow sample is diluted with 3 equal volumes of MSC growth medium and distributed equally across several dishes.
2. Each dish 10-cm culture dish receives 10 mL of diluted aspirate.
3. The containers are returned to the 5% CO_2 incubator and cultured undisturbed at 37 °C for 4–5 d.
4. The old medium is aspirated away, without concern for removing the red cells that have settled. Thereafter, the medium is changed every 3–4 d, and contaminating red cells and other nonreplicating and nonattaching cells are eventually diluted and rinsed away.
5. Small MSC colonies of attached fibroblastic cells are visible at 5–7 d. These continue to divide and grow whereas some colonies may not propagate and eventually senesce.
6. After 12–14 d the small colonies are easily found. At this point, the cells are rinsed with serum-free α-MEM and subculture. 5 mL of 0.05% trypsin/0.23 m*M* EDTA is added and after several minutes, the cells begin to detach from the substrate. This is observed under the microscope.
7. The excess trypsin/EDTA solution can be carefully aspirated away as long as the cells have not yet fully detached. (If the cells have begun to lift from the substrate, fresh growth medium containing FBS is added and neutralizes the trypsin activity).
8. The MSCs are rinsed from the surface with growth medium and a pipet and divided in to 2 dishes as at this stage they are at a low concentration but will propagate rapidly. Each dish should contain about 10 mL of cell suspension and are returned to the 5% CO_2 incubator.
9. If needed, the MSCs can be concentrated by centrifugation at 1600 rpm for 15 min at 10 °C in a swinging bucket tabletop centrifuge. The supernatant is aspirated off, leaving less than 0.5 mL over the cell pellet, and new growth medium is added to resuspend the MSCs. The cells are counted using a hemocytometer and placed into flasks at a final concentration of approximately.
10. The human MSCs will continue to grow and approximately every 7 d (4–5 d for other species), they can be sub cultured 1:3 with trypsin/EDTA when the cell density is approx 75–80% confluent. Other cell types such as macrophages and fibroblasts either senesce or do not continue to divide in the culture conditions and become diluted out by the propagating MSCs.

The MSCs should not be allowed to become confluent as they will become contact inhibited and cease dividing. If they do become confluent, they will begin to divide again when sub cultured, but this may change some properties of the cells.

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CHAPTER 3

ISOLATION AND CULTURE OF HUMAN ADULT HAEMATOPOIETICS STEM CELLS

Haematopoietics stem cells from adult bone marrow can be developed together wit mesenchymal stem cell. The strategy of culture of haematopoietics bone marrow can be separate one day after culture and using complete growth medium when incubate in incubator ell can be develop CO_2 at 37 °C. These are gradually growing to different forms like macrophage, monocyte, megagranulocyte, polymorphonucleic, dendritic

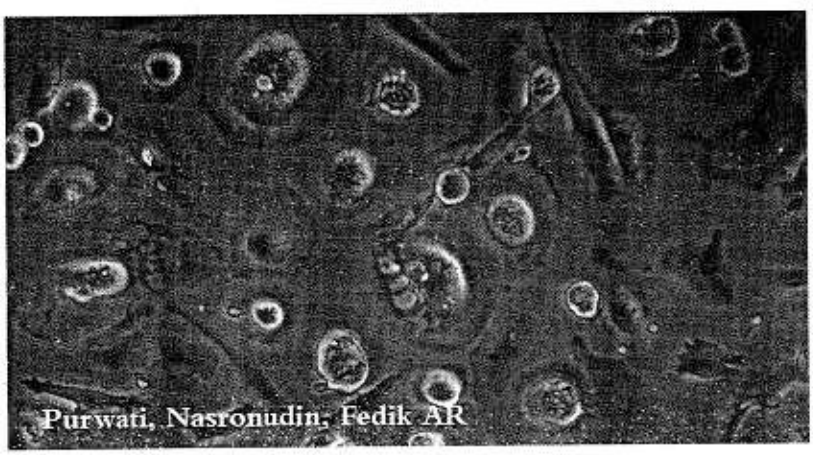

Figure 1. Haematopoietics stem cells from bone marrow 4 days after separation from mesenchymal stem cell of bone marrow. These cells can grow very well and differentiation to other form likes macrophage, monocyte, lymphocyte etc. Analyzed under inverted microscope 400x

The other side these cells can be developed becoming lymphocyte cells and can use for co-cultivation with PBM cells to characterize of biological properties. Cells. Although these cells are still needed to molecular characterize as well as novel antibodies recognizing antigens present on the cell surface of BMHSCs, that are correspondingly not reactive with haematopoietics progenitors, has led to an increased understanding of the biological properties of BMHSCs using antibodies including reactivity with different antigens like STRO-1, CD18, CD49a/CD29, NGF-R, PDGF-R, EGR-R, IGF-R, CD106, CD146, and HOP-26, These approach should be use to determine with varying of efficiencies, to positively select for BMSC from aspirates of human bone marrow.

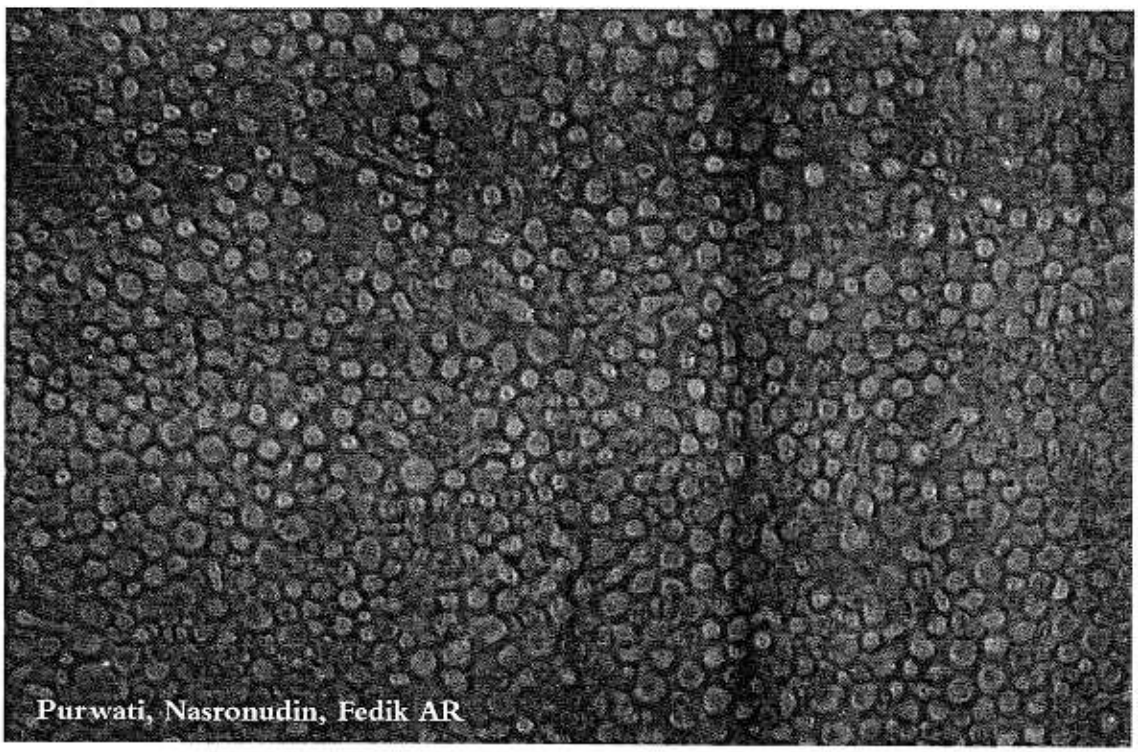

Figure 2. Haematopoietics human adult stem cells, 2 days after separation from mesenchymal stem cells, analyzed under inverted microscope 400x

Further development of stromal specific monoclonal reagents that identify discrete developmental stages may provide essential reagents to enable further characterization of the cellular properties and functions of the BMHSC population. The present chapter describes a method for culture and separation of BMHSC from human BM. The complete protocol to isolate of haematopoietics stem cells like below

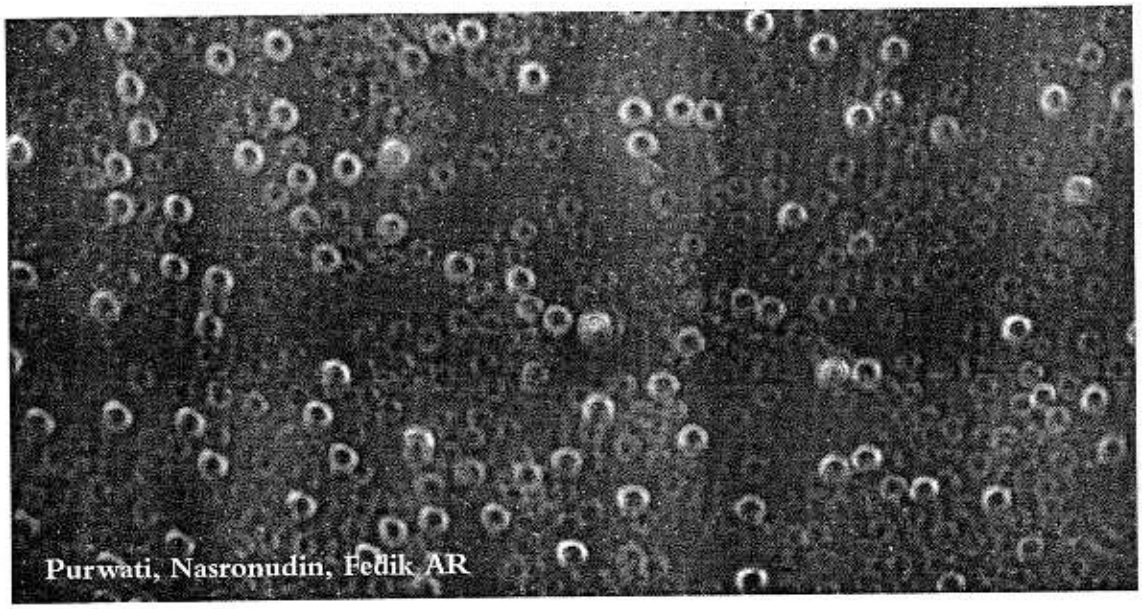

Figure 3. Haematopoietics human adult stem cells from bone marrow, 4 days after culture. Analyzed under inverted microscope 400x

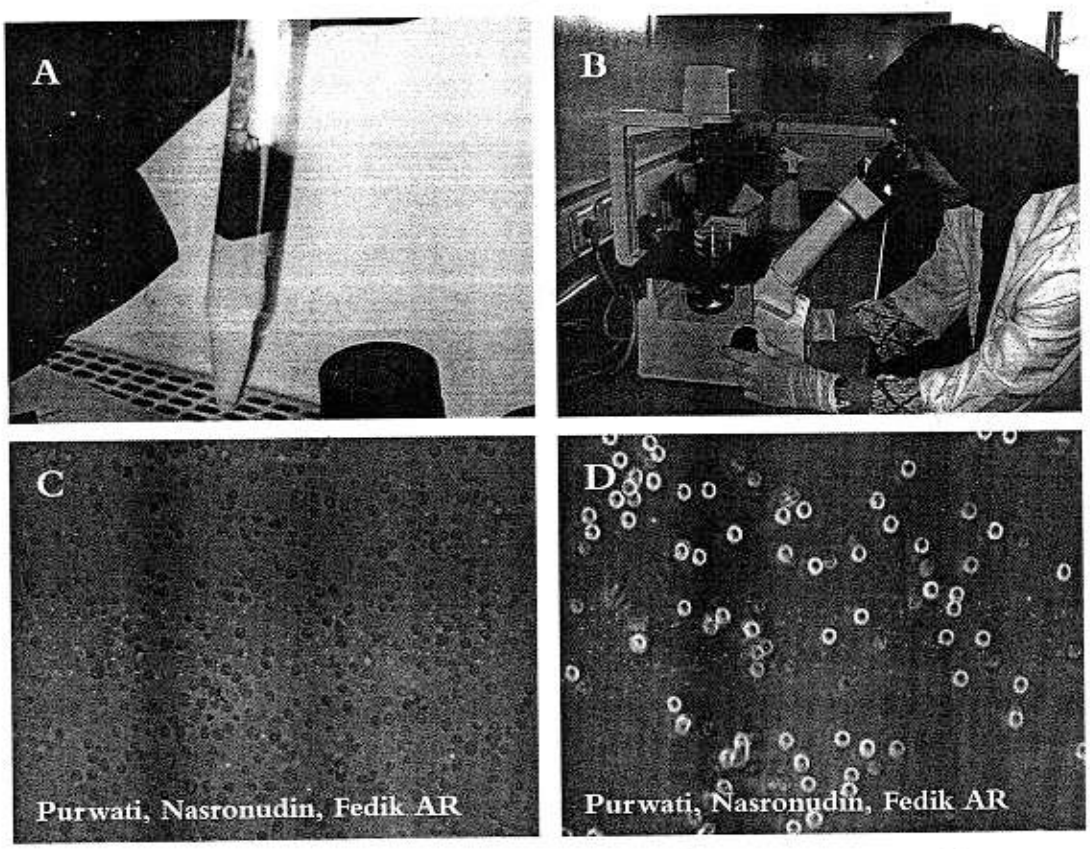

Figure 4. Isolation process of human adult bone marrow stem cells. A. Aspirated bone marrow was coated on the surface of Ficoll histopaque 1,077. B. Analyze of mononucleated cells under inverted microscope. C. Isolate of nucleated bone marrow stem cells are direct cultured in dish. D. nucleated stem cells, after 30 min cultured in dish in incubator CO_2 at 37 °C. Analyzed under inverted microscope 400x

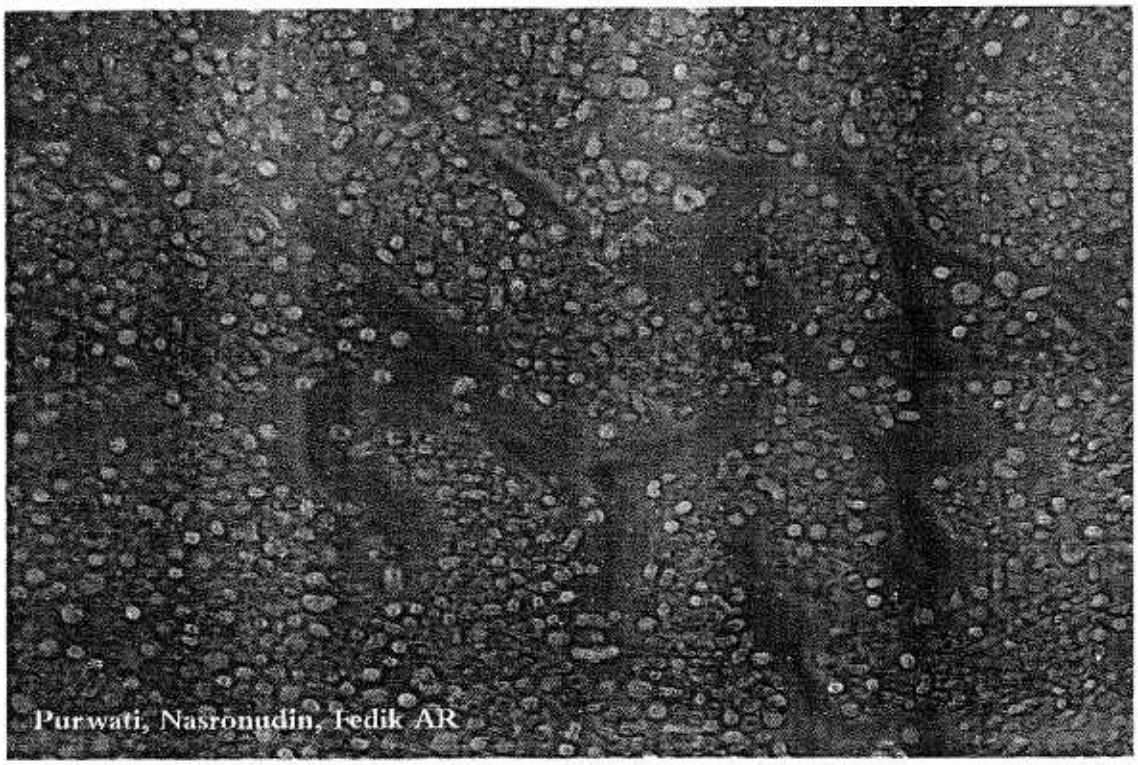

Figure 5. Haematopoietics human adult stem cells. Mesenchymal stem cells growing between haematopoietics cell. 4 days after cultured. Analyzed under inverted microscope 400x

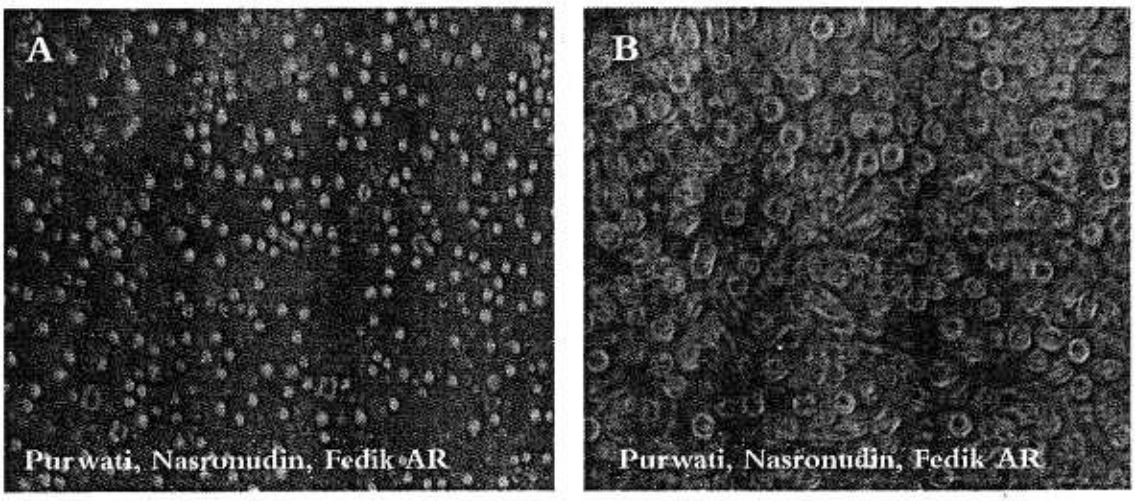

Figure 6. Haematopoietics human adult stem cells. A. Haematopoietics stem cells growing after separation from mesenchymal cells. B. Haematopoietics stem cells growing 5 days after cultured. Analyzed under inverted microscope 400x

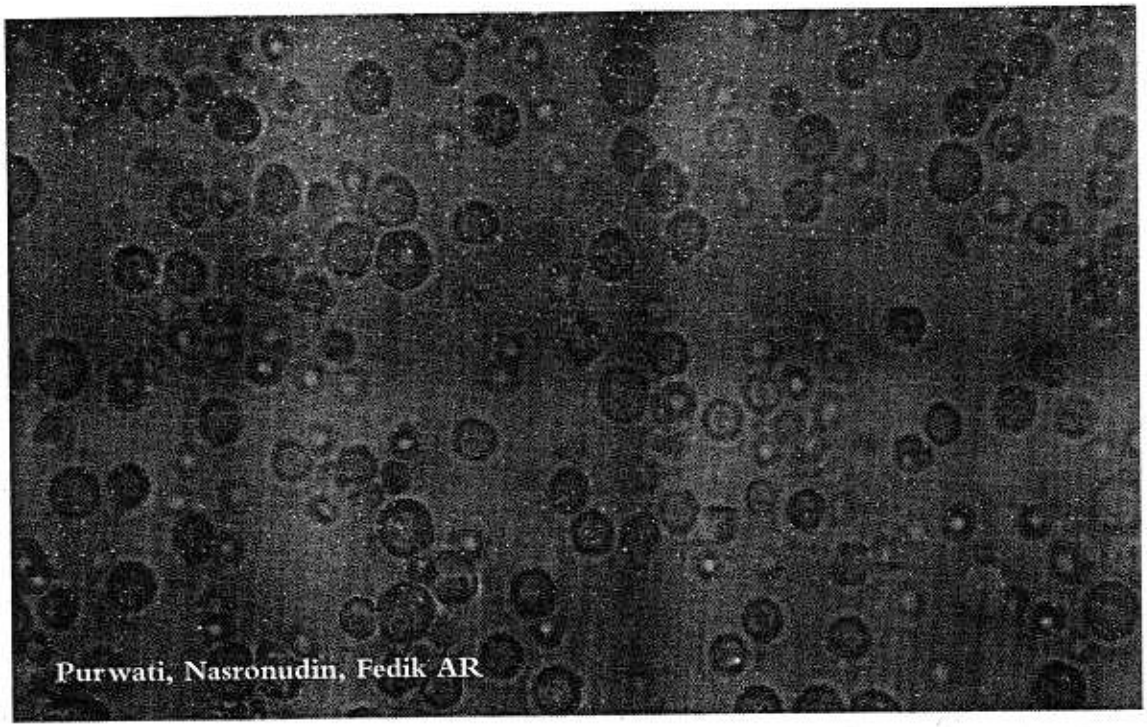

Figure 7. Haematopoietics human adult stem cells. 6 days after culture and ready for co-cultivation with PBMCs from HIV patients.

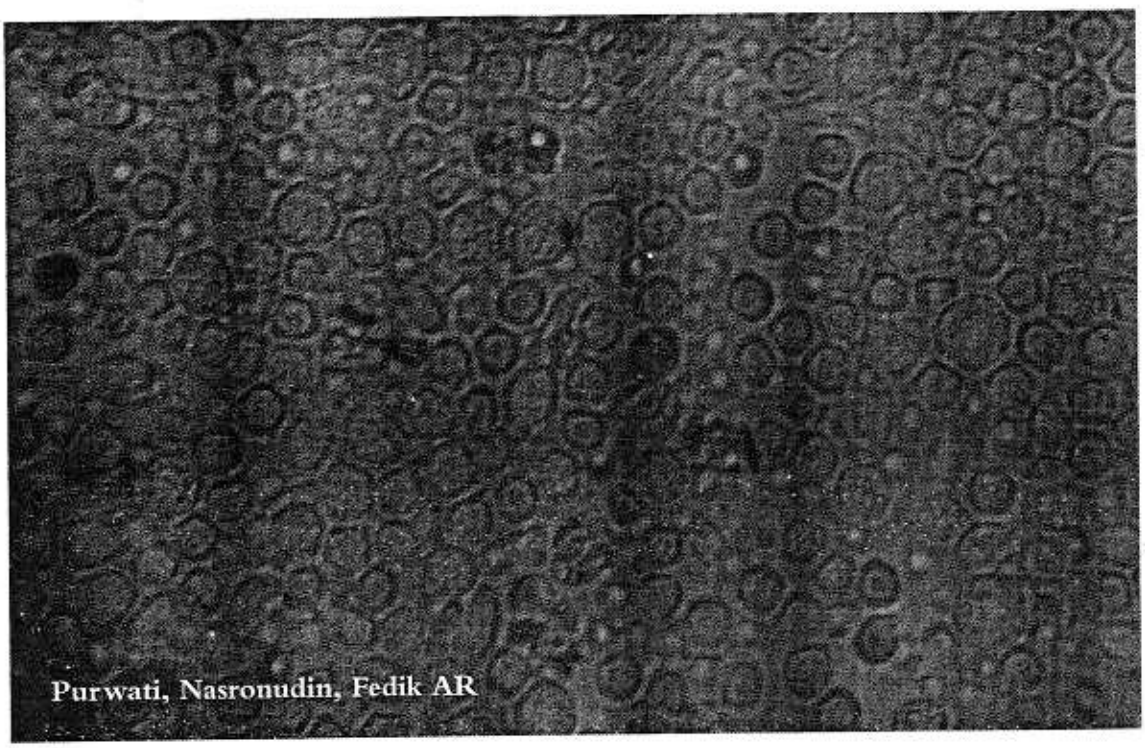

Figure 8. Haematopoietics human adult stem cells. 6 days after co-cultivation with PBMCs from HIV patients and culture, under inverted microscope 400x

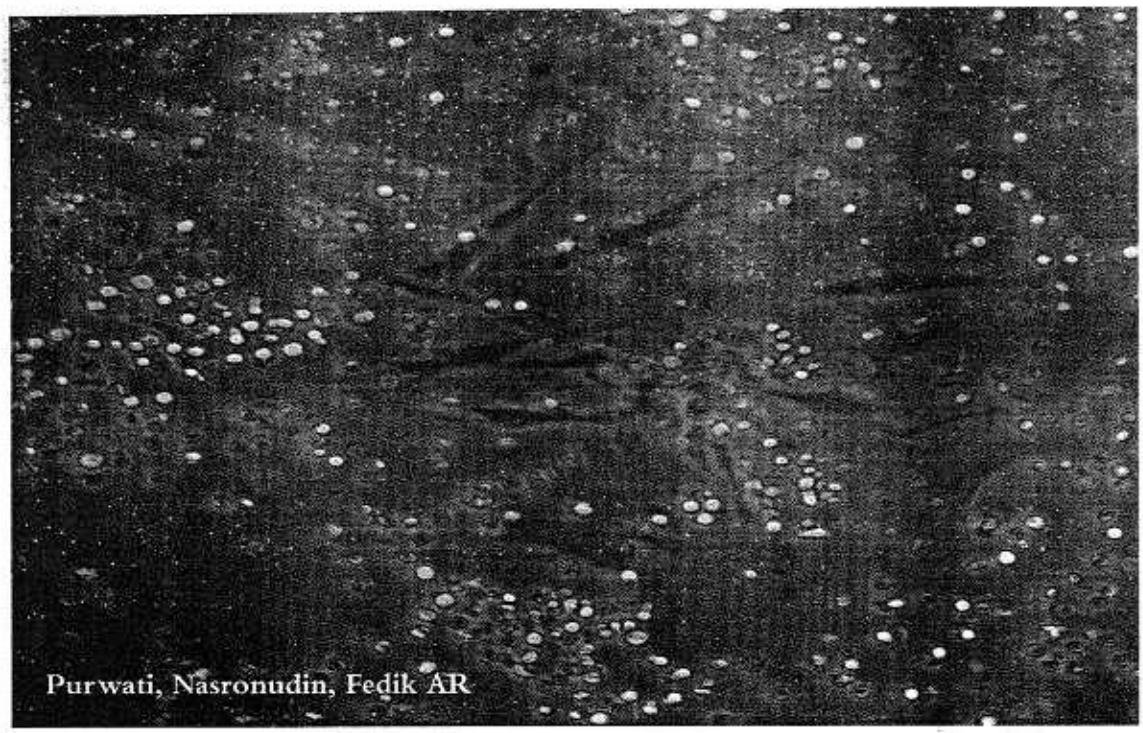

Figure 9. Colonies of mesenchymal cells growing between haematopoietics cells 4 days after culture in dish 10 cm. Analyzed under inverted microscope 40x

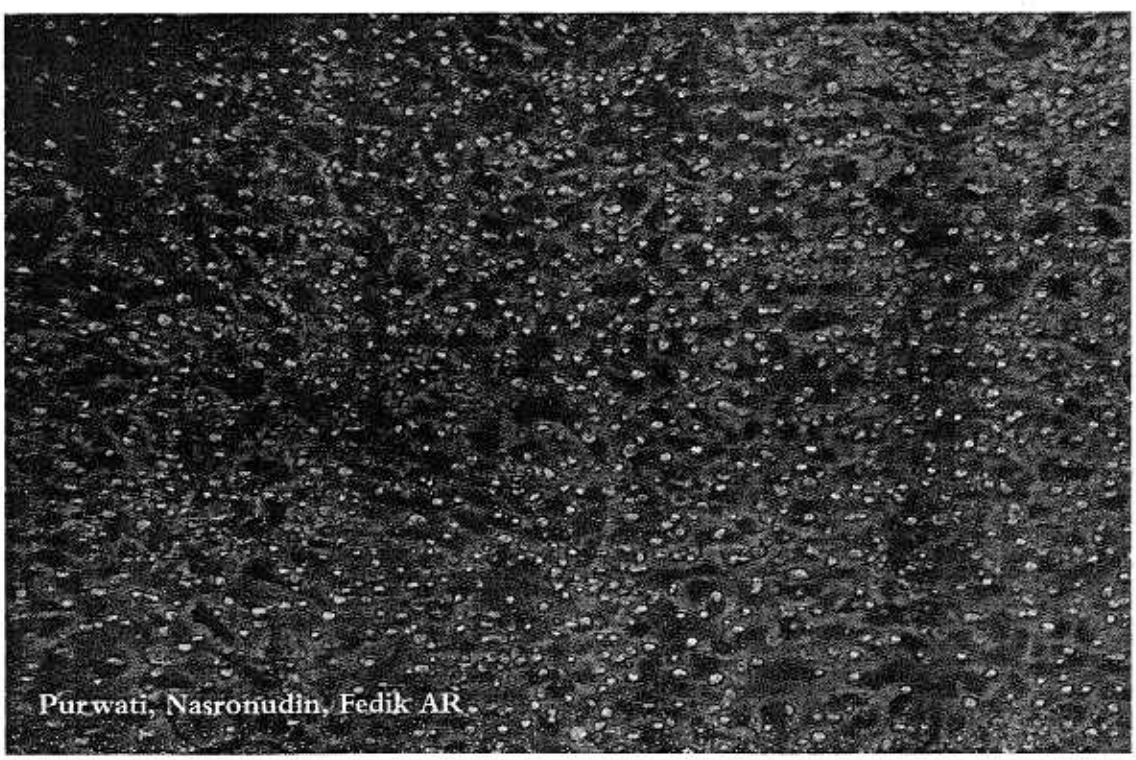

Figure 10. Mesenchymal cells growing together with haematopoietics cells before separation in to other dish as haematopoietics cells. Cells are 5 days after culture Analyzed under inverted microscope 400x

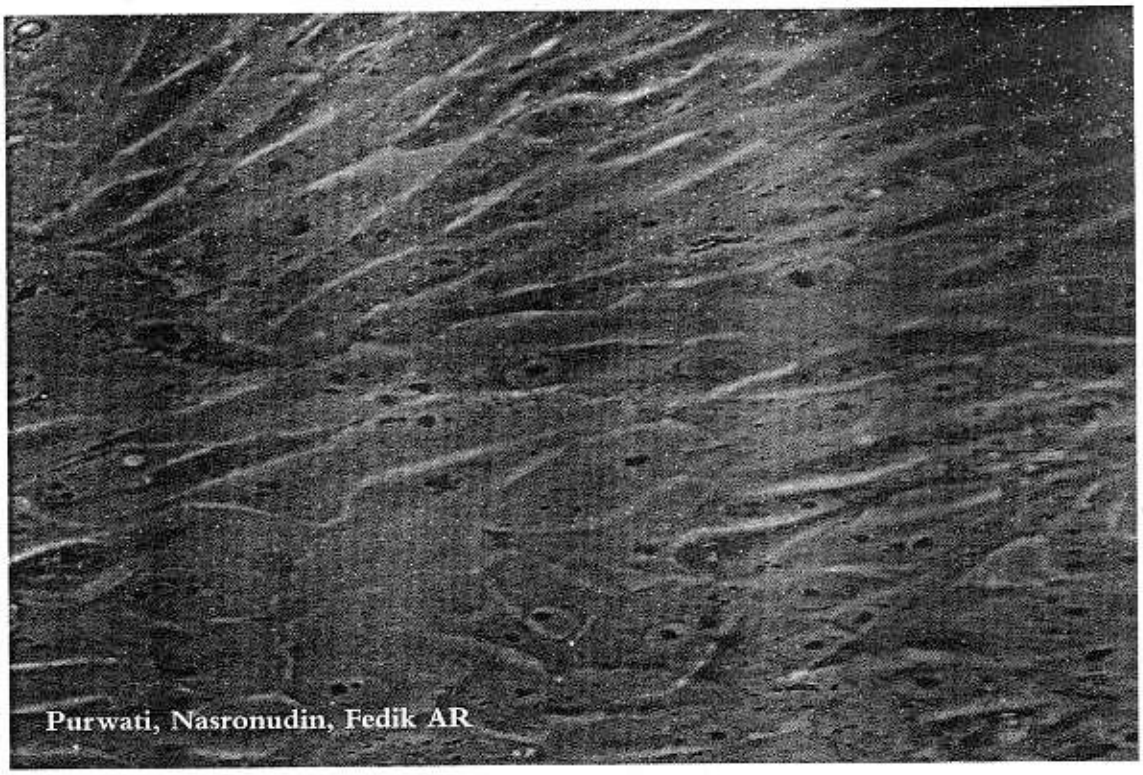

Figure 11. Mesenchymal cells growing confluent 100%, These cells are ready to subculture. Analyzed under inverted microscope 400x

Further culture of human haematopoietics stem cells can develop like in Figure 13 using procedures like below. Stem cells development are very important to explore of early detection to human disease as well as x linkage-chromosome, immunodeficiency syndrome, leucosis, response immune disorder like hypo responsive. The complete protocol as below;

REAGENTS

Processing of Bone Marrow Mononuclear Cells (BMMNC)

1. Sodium heparin (Sigma)
2. Ficoll-Hypaque, Lymphoprep, 1.077 g/dL (Sigma)
3. PBS, phosphate buffered saline solution, pH 7.4 (Merck)
4. Fetal bovine serum (FBS) (Biowest)
5. HBSS supplemented with 20 m*M* HEPES, pH 7.35 and 5% (v/v) FBS (Biowest)
6. 70-µm Falcon cell strainer (Falcon)
7. 50-mL Falcon tube (Falcon)
8. 14-mL polystyrene Falcon tubes (Falcon)

PROCEDURE AND CULTURE

The methods described below outline (1) the isolation and preparation of the bone marrow mononuclear cells (BMMNC), (2) the magnetic activated cell separation (MACS) of clonogenic BMSSC or CFU-F, (3) methods to enumerate the efficiency of the MACS enrichment, (4) methods to ex vivo culture, expand and cryopreserve human BMHSC grown under serum or serum-deprived conditions.

Collecting and Preparation

1. Following informed consent, collect approx 5 mL of human bone marrow (BM) from health human adult volunteers (40 years) by aspiration from the posterior iliac crest (hip bone). BM should be placed immediately into a preservative-free, sodium heparin-containing 15-mL tube.
2. Add of PBS with equal volume and centrifugation in 1600 rpm 10 min at 20 °C.
3. Remove of supernatant suspension and take 5 ml for mononucleated isolation.
4. Dispense 5 mL of Ficoll-Hypaque (Lymphoprep) solution in the bottom of approx 12 round bottom 14-mL polystyrene Falcon tubes and carefully overlay with 7.5 mL of diluted BM.
5. Centrifuge tubes at 1600 rpm for 15 min at room temperature. *Keep centrifuge brake off.*
6. Using a disposable plastic Pasteur pipet, recover the leukocyte band from all tubes and pool into 4 × 14 mL polypropylene tubes.
7. Dilute cells with HHF wash buffer and pellet the BMMNC by centrifugation of the sample at 1600 rpm for 15 min at 10 °C. *Keep centrifuge break on.*
8. Aspirate the buffer and repeat step 7 until all cells are pooled into one tube.

Procedure of Culture

1. After bone marrow sample is diluted with 3 equal volumes of MSC growth medium and distributed equally across several dishes.
2. Each dish 10 -cm culture dish receives 10 mL of diluted aspirate.
3. The containers are returned to the 5% CO_2 incubator and cultured undisturbed at 37 °C for 4–5 d.

4. The old medium is aspirated away, without concern for removing the red cells that have settled. Thereafter, the medium is changed every 2–3 d, and contaminating red cells and other no replicating and no attaching cells are eventually diluted and rinsed away.
5. Supernatant from mesenchymal cells direct culture in to new dish 10 ml and incubated at 37 °C for 4 d. finally like at Figure 6.
6. Small MSC colonies of attached fibroblastic cells are visible at 5–7 d. These continue to divide and grow whereas some colonies may not propagate and eventually senesce. The result is like above at Figure 9.
7. After 12–14 d the small colonies are easily found. At this point, the cells are rinsed with serum-free α-MEM and subculture. 5 mL of 0.05 % trypsin/0.23 m*M* EDTA is added and after several minutes, the cells begin to detach from the substrate. This is observed under the microscope.
8. The excess trypsin/EDTA solution can be carefully aspirated away as long as the cells have not yet fully detached. (If the cells have begun to lift from the substrate, fresh growth medium containing FBS is added and neutralizes the trypsin activity).
9. The MSCs are rinsed from the surface with growth medium and a pipet and divided in to 2 dishes as at this stage they are at a low concentration but will propagate rapidly. Each dish should contain about 10 mL of cell suspension and are returned to the 5% CO_2 incubator.
10. If needed, the MSCs can be concentrated by centrifugation at 1600 rpm for 15 min at 10 °C in a swinging bucket tabletop centrifuge. The supernatant is aspirated off, leaving less than 0.5 mL over the cell pellet, and new growth medium is added to resuspend the MSCs. The cells are counted using a hemocytometer and placed into dishes at a final concentration of approximately.
11. The human MSCs will continue to grow and approximately every 7 d (4–5 d for other species), they can be subculture 1:3 with trypsin/EDTA when the cell density is approx 75–80% confluent. Other cell types such as macrophages and fibroblasts either senesce or do not continue to divide in the culture conditions and become diluted out by the propagating MSCs. The MSCs should not be allowed to become confluent as they will become contact inhibited and cease dividing. If they do become confluent, they will begin to divide again when subculture, but this may change some properties of the cells The results are like above at Figure 10 and 11.

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CHAPTER 4
ISOLATION AND CULTURE MESENCHYM CELL FROM ADIPOSE

The relative frequency of clonogenic cells is one reason for interest in adipose tissue as a stem cell source. Another is the relative ease and low morbidity with which adipose tissue can be harvested. The American Society for Aesthetic Plastic Surgery reported that 478,251 people underwent cosmetic liposuction in the USA during 2004. This represents an enormous volume of immediately available tissue that can be obtained following a very simple informed consent process, without risk to the donor provided that due attention is paid to protecting privacy. The purpose of the present chapter is to describe the methods by which this material can be processed to yield ADSC for research purposes. The results of isolation and culture adipose stem cells as below.

ISOLATION OF NUCLEATED CELL

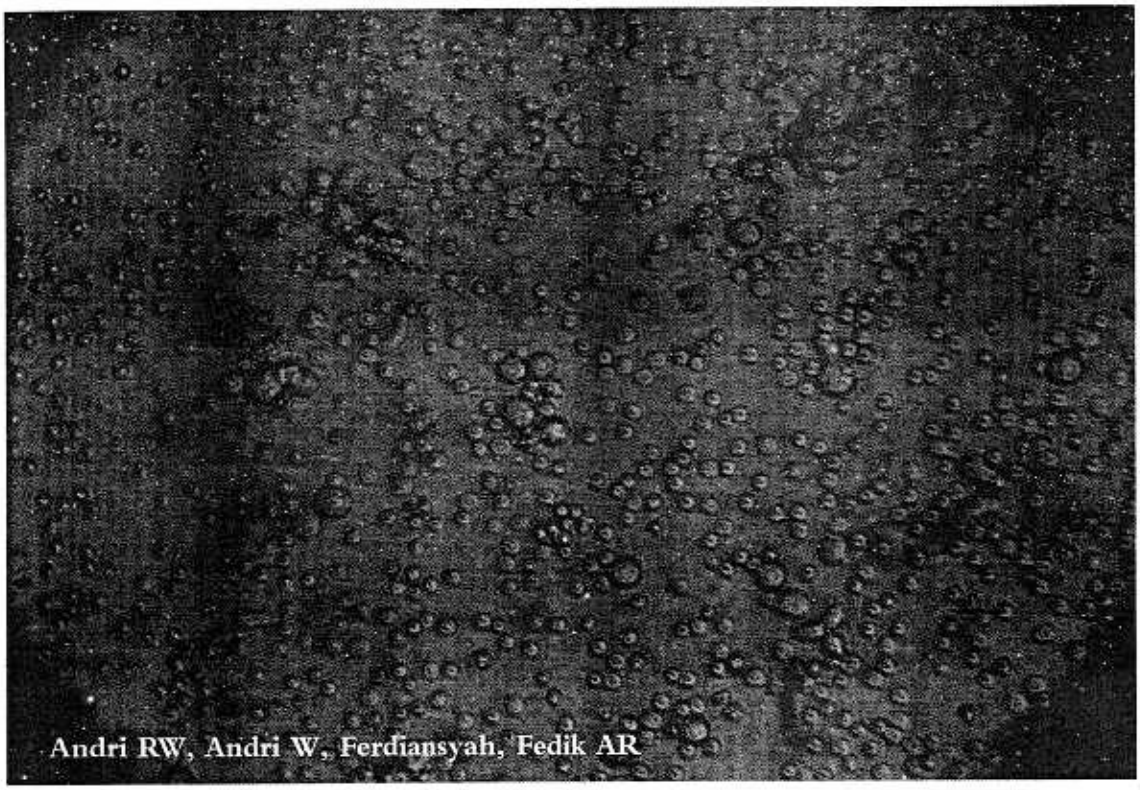

Figure 1. Isolation of human adult adipose stem cells. 24 h after culture. in dish and analyzed under inverted microscope 400x

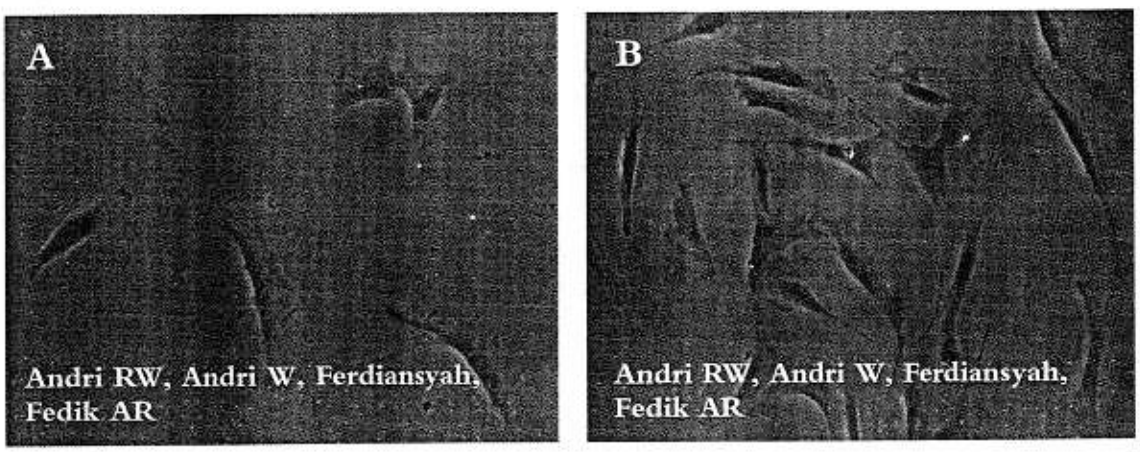

Figure 2. Mesenchymal stem cells like fibroblast cells from human adult adipose. A. 4 days after cultured.in dish. B. 6 days after cultured, and analized under inverted microscope 400x

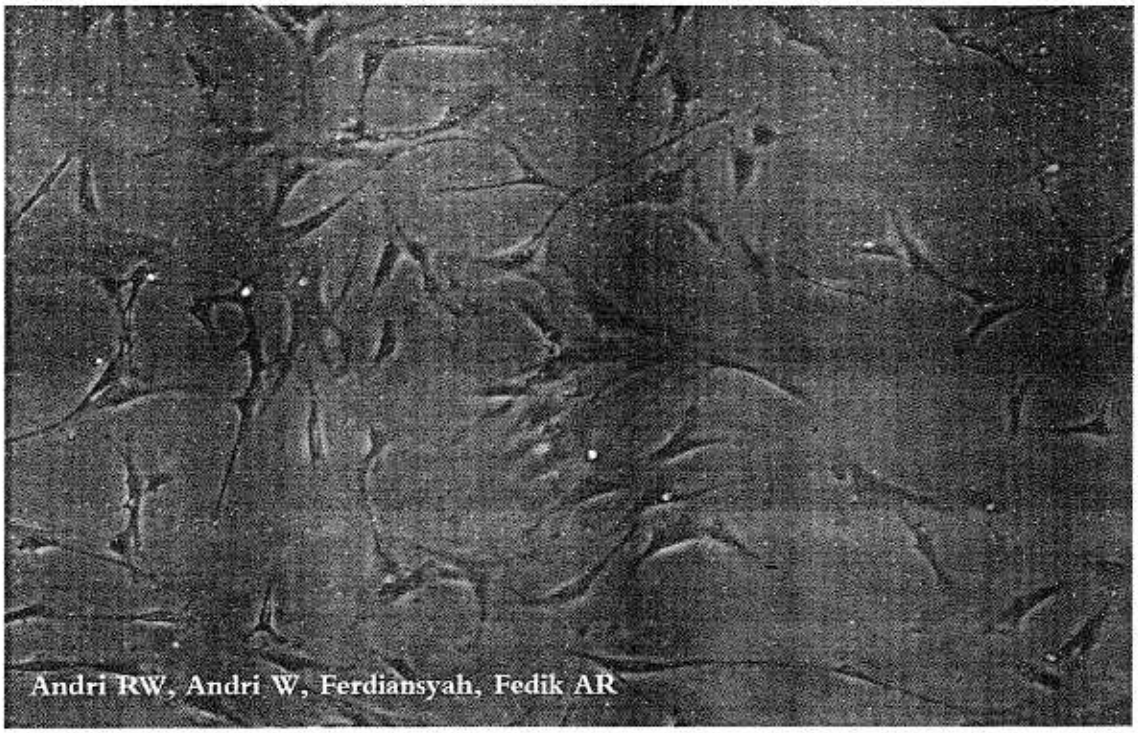

Figure 3. Mesenchymal stem cells like fibroblast cells from human adult adipose. 8 days after cultured and cells growing in dish 30% and analyzed under inverted microscope 400x

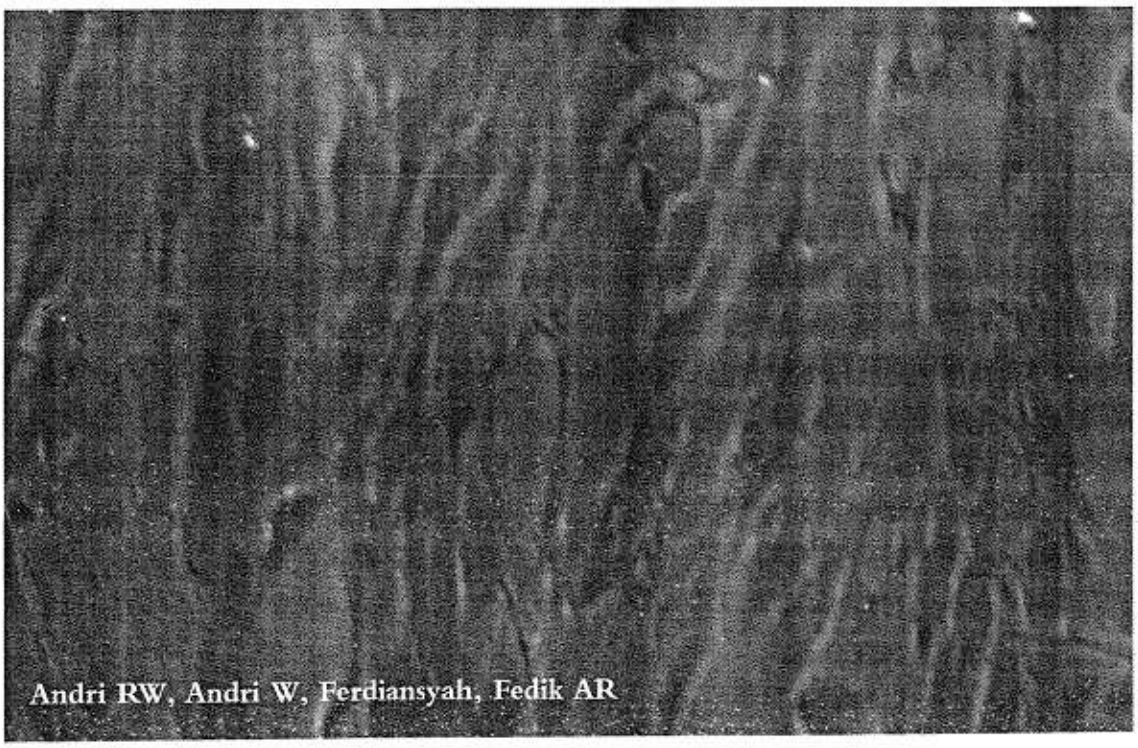

Figure 4. Mesenchymal stem cells like fibroblast cells from human adult adipose. 10 days after cultured and cells growing in dish 80%. These cells are ready to made progenitors cells, and analyzed under inverted microscope 400x

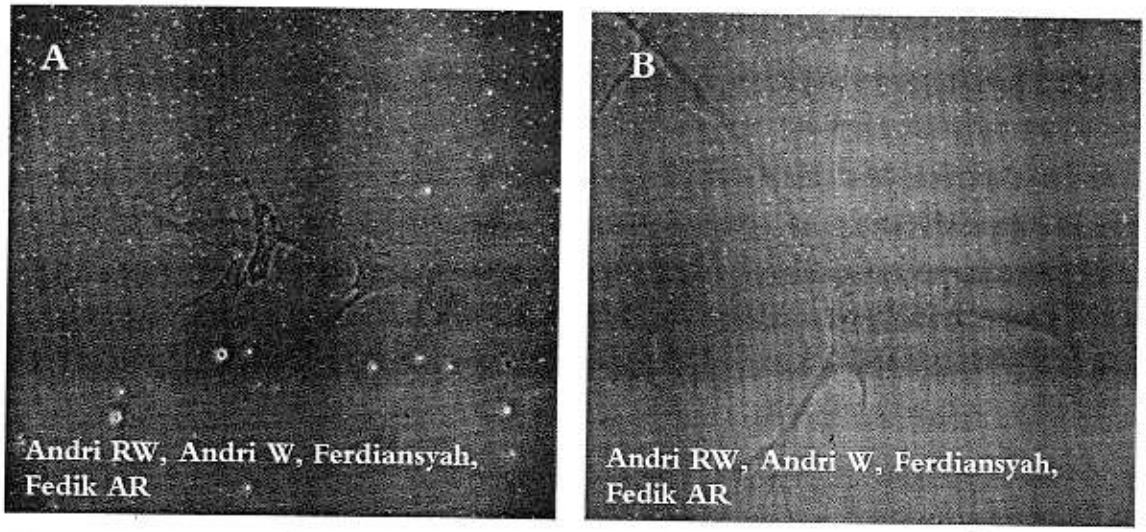

Figure 5. A. and B. Mesenchymal stem cells like neuron cells from human adult adipose. 10 days after cultured and cells growing in dish, analyzed under inverted microscope 400x

Reagents

1. Sterile saline for tissue washing.
2. Dulbecco's phosphate buffered saline supplemented with 25 mM HEPES, and 2% human serum albumin or fetal calf serum (Gibco).
3. Type IA Collagenase (Sigma) or Other commercially available collagenase preparations such as Roche's Blendzyme family also provide satisfactory results.
4. Sterile separatory funnels with stop cock.
5. Shaking water bath or incubator.
6. α-MEM/F-12 50/50,1× with L-glutamine (Sigma, Cat. M0894).
7. Antibiotic/Antimycotic 100× solution (ABAM), sterile, contains 10,000 units Penicillin-G/mL;10,000 mcg Streptomycin/Ml (Sigma, Cat. PO781) 25 mcg Amphotericin B (Sigma, Cat. M0894).
8. Complete culture medium: α-MEM/F-12 based commercially prepared cell culture media in which 10% fetal bovine sera (FBS) and 1% v/v antibiotic/ antimycotic have been previously added.
9. Falcon 3046 Multiwell 6-well tissue culture plate, Becton Dickinson.
10. 10% formalin.
11. Hematoxylin (Gill III formula).

Tissue

Adipose tissue samples obtained from liposuction aspirates and needle biopsy from adult volunteer.

Supplies

1. 200-mL plastic centrifugation bottles (Nalgene).
2. 0.2-μm filter units.
3. 50-mL conical tubes.
4. 2-mL tubes.
5. Scissors.
6. Hemocytometer.
7. Freezing apparatus (alcohol container).

Equipment

1. Inverted microscope.
2. Shaking water bath.
3. Centrifuge.
4. Biosafety Hood Class III.
5. CO_2 Incubator.

Tissue Processing

Aspirated tissue is generally collected into a sealed nonsterile container that is disposed of as biohazardous medical waste. The material collected is a mixture of tumescent solution, blood, free lipid released from lysed adipocytes, and aspirated tissue fragments the precise proportions of which are largely determined by physician practice. Following informed consent this material should be transported to the laboratory for processing as quickly as possible; we have noted that the yield of ADSC, as measured by the fibroblast colony-forming unit (CFU-F) assay, falls by approx 50% for 24 h of storage before initiation of processing. Because the common collection containers are not designed for transport, precautions must be taken to avoid contamination and spills during transfer from the surgical facility to the processing laboratory. We suggest bagging in a sealed, spill-proof pouch, placing this bag within a rupture-proof secondary container such as a Tyvek® bag, and finally use of a crush-resistant outer container bearing labeling consistent with local and federal regulations.

Human lipoaspirate is frequently obtained without the researchers being aware of the infectious disease status of the donor; that is, without available results of serologic testing for biohazardous agents such as human immunodeficiency virus (HIV) and hepatitis B. For this reason all procedures involving manipulation of tissue should be performed in a protective environment such as a biological safety cabinet and operators should wear appropriate protective clothing and equipment at all times during tissue processing.

Tissue Washing

The buoyancy of adipose tissue is such that processing can be performed using approaches similar to those used in organic chemistry. Specifically, we apply sterile (autoclaved) separator funnels in tissue processing to separate buoyant tissue fragments from tumescent solution and blood. Washing may be repeated until the buoyant fraction is a vivid orange color and the infranatant is clear. Use of large volume funnels allows maximization of the ratio of saline:aspirate and more efficient washing. Alternatively, washing may be performed in beakers and the infranatant removed by aspiration.

1. Place stopcock to the closed position and decant lipoaspirate into the sterile separatory funnel.
2. Add sterile saline, prewarmed to 37 °C, and invert the funnel 4–5 times with the cap in place. Return to the upright position and allow 3–5 min for phase separation.
3. Remove the cap, open the stopcock and let blood-saline mixture flow into a liquid pathological waste container. Close the stopcock before the fat blood/saline interface.
4. Repeat steps 2 and 3 until the infranatant is clear or residual opacity no longer declines substantially with additional wash cycles.

Tissue Digestion

A number of different enzymes and enzyme combinations have been described for digestion of human adipose tissue. We have developed a proprietary mixture of enzymes that optimizes processing. However, off-the-shelf enzymes such as the collagenase preparations listed above will yield satisfactory results.

1. Estimate the volume of washed fat (volume of fat after the last wash).
2. Prepare an equal volume of warm, sterile buffered saline containing 500 CDU/mL (equivalent to 0.5 Wünsch units/mL) collagenase.

3. Pour washed fat from the separatory funnel into a 600 mL, 1,000 mL, or 2,000 mL sterile bottle, depending on the estimated volume of washed fat (container volume should be at least 4 times that of the aspirate).
4. Add the buffered saline/collagenase mixture, seal the container and place on a thermal shaker, prewarmed to 35–38 °C for 20 ± 5 min. Initiate shaking. The frequency and amplitude of shaking should be set such that it is just sufficient to prevent separation of the buoyant tissue from the collagenase solution. Excessive amplitude or frequency can cause loss of cell recovery.
5. Inspect the digestion frequently after the first 15 min to ensure that overdigestion does not occur. Digestion time will vary with different tissue donors and physicians. For example, a larger cannula may generate larger fragments of tissue that may take longer to digest. The digestion may be halted when the quantity of residual fragments of adipose tissue is approx 5% of the initial amount.
6. On completion of digestion transfer the digestate to a fresh sterile glass separatory funnel. Allow the solution to sit for 5–10 min for phase separation to occur. Undigested and partially-digested adipose tissue, free adipocytes, and free lipid will float.The speed of phase separation may be increased by adding additional warm, sterile buffered saline to the funnel.
7. Open the stopcock and transfer the nonbuoyant fraction through a sterile 265 mm filter and into a sterile beaker.
8. Add warm, buffered sterile saline to the separatory funnel and invert the funnel 4–5 times with the cap in place. Return to the upright position and allow 3–5 min for phase separation.
9. Open the stopcock and transfer the nonbuoyant fraction through a sterile 265 mm filter into the material collected in step 7.
10. Aliquot the nonbuoyant solution collected in the beaker into multiple 50-mL centrifuge tubes.
11. Centrifuge at 400 g for 5 min at room temperature with a low-medium brake speed.
12. Gently pour off or aspirate the supernatant (top layer) into a liquid pathological waste container without disturbing the cell pellet.
13. Resuspend the pellets in buffered saline and combine the pelleted cells.
14. Repeat wash/centrifugation twice more to remove residual collagenase
15. Pass the cell suspension through a 100 mm cell strainer and collect into a new, sterile 50-mL centrifuge tube.

16. Perform cell counting using fluorescent live/dead dyes such as 7 amino actinomycin D or propidium iodide in combination with a nuclear counterstain such as Acridine Orange or systems that use esterase substrates. Simple vital dye exclusion systems (for example Trypan Blue) that do not detect cell activity or the presence of a nucleus can be confounded by residual small lipid droplets. In general this procedure yields a heterogeneous mixture of vascular cells, preadipocytes, lymphoid cells, blood cells, and ADSC. The process typically yields 2 × 104 nucleated cells per milliliter of human adipose tissue processed.

CULTURE PROCEDURES OF MESENCHYMAL CELLS FROM ADIPOSE

After transportation to the laboratory, the liposuction sample can be kept at room temperature for no more than 24 h before use. Before performing the experiment, warm up the water bath to 37 °C. All the following procedures are performed in biosafety hoods. Investigators should be trained in the handling of human tissues and human pathogens before initiation of any studies.

Small Volumes of Adipose Tissue from Needle Biopsy

1. Warm up buffer (PBS + 1% antibiotic).
2. Warm up freshly prepared *collagenase solution* in the 37 °C water bath.
3. Prepare PBS (or KRB) solution with 1% BSA, filter the solution and warm it in the 37 °C water bath.
4. Prepare stromal medium: *cf Media stock solution*. This should have been done in advance of the procedure.
5. Tissue washes:
   A small amount of tissue (up to 150 mg) is placed in a 2-mL tube containing 500 µl of warm PBS with 1% antibiotic solution. The sample is then washed with 300 µl of warm PBS containing 1% antibiotic solution. The washing step is repeated until all blood vessels and connective tissues appear to have been liberated (usually 2 washes). To facilitate the subsequent tissue digestion, mince the adipose tissue sample into small pieces using sterilized scissors.
6. Tissue digestion:
   If the volume of adipose tissue sample is small (150–250 mg), it is recommended that you increase the volume of collagenase solution (up to 400 µl for an equivalent of 150–250 mg) to improve the efficiency of the tissue digestion. Wrap the tube(s) with parafilm and place into the 37 °C shaking water bath

at ~75 rpm for 60 min until the tissue appears smooth on visual inspection. Because over digestion can damage the cells and reduce the final yield, you should inspect the reaction visually during the incubation and stop the reaction, as soon as the tissue appears smooth and fully digested.

7. Isolation stromal vascular fraction (SVF):
   After digestion, spin the samples at 300 g in an appropriate centrifuge for 5 min at room temperature. Take the samples out of the centrifuge and shake them vigorously to thoroughly disrupt the pellet and to mix the cells. This is to complete the separation of the stromal cells from the primary adipocytes. Repeat the centrifugation step.
8. Pellet clean-up:
   After spinning, aspirate all the collagenase solution above the pellet without disturbing the cells. Add to each tube a volume of 200 µl of warm PBS solution containing 1% BSA. Centrifuge the cells at 300 g in an appropriate centrifuge for 5 minutes at room temperature. Re-suspend the cells with 200 µl of stromal medium. Centrifuge the cells at 300 g in an appropriate centrifuge for 5 min at room temperature.
9. Plate the cells:
   After spinning, aspirate the supernatant and suspend the cells in 100 µl of stromal medium Innoculate the cells in a single well of a 12-well plate for an amount of about 500 mg of adipose tissue or in a single well of a 24-well plate for an amount of 250–150 mg of adipose tissue. Then add a volume of stromal medium according to the well capacity of the culture plate
   Table for plating
   - Plate Area per plate Cells per plate Cells per well Media per well
   - 6-well plate 60 cm2 1.8 × 106 30 × 104 2.5 mL
   - 24-well plate 48 cm2 1.44 × 106 6 × 104 1 mL
   - 96-well plate 31 cm2 0.93 × 106 104 200 µL
   - 5 Isolation of Human Adipose-derived Stem Cells 73
   - The samples are then maintained in a humidified incubator at 37 °C with 5% CO_2.
10. Change stromal medium:
    Seventy-two hours after plating, aspirate the entire medium from the wells. Wash the cells with prewarmed PBS (1% antibiotic can be added to the solution) by pipeting up-and-down to clean the cells thoroughly from any

tissue fragments and/or blood cells. Add a volume of fresh stromal medium according to the well capacity of the culture plate.

Critical steps

The medium is then changed every 2–3 d until the cells achieve 80–90% confluence. Þ Troubleshooting

11. Harvesting cells:

When the cells reach 80–90% confluence there are 2 options: either harvest the cells or directly induce the adipocyte differentiation. To harvest the cells, the following procedure can be used. Remove medium from wells and save the sterile "conditioned media" in a sterile tube for future cell culture application (this should be sterile filtered before use). Add a small volume (250–500 µl) of sterile warm PBS to the wells and allow PBS to remain on cells for 2 min. Replace the PBS with 500 µl of Trypsin/EDTA solution (0.5%). Incubate in incubator for 5–10 min. Verify under microscope that more than 90% of the cells have detached and then add 500 µl of stromal medium to allow the serum contained in the solution to neutralize the trypsin reaction.

Critical steps

Transfer the medium containing the suspended cells from the well to a sterile 2 mL tube. Centrifuge at 300 g for 5 min. Aspirate the supernatant and suspend the cells in a small volume of stromal medium (~250 µl). Proceed to cell counting by taking an aliquot of cells diluted in trypan blue (for a 1:8 dilution: add 12.5 µl of suspended cells to 87.5 µl of trypan blue). Count cells using the hematocytometer. After counting, cells can then be replated according to the well capacity in adequate cell culture plates.

12. Adipocyte differentiation:

When the cells reach between 80 and 90% confluence (before or after harvesting the cells), the preadipocytes are induced to differentiate. Aspirate the medium, add a small volume (about 1.5 mL for a 6-well plate) of prewarmed PBS + 1% antibiotic to wash the cells, and then remove the PBS by aspiration. Next, add the differentiation medium. The cells will be maintained in the differentiation medium for 3 d.

13. Day +3 differentiation:

Aspirate the differentiation medium and wash the cells with prewarmed PBS + 1% antibiotic. Then add a volume (2.5–3 mL for a 6 well plate) of adipocyte medium. The adipocyte medium will be changed every 3 d until mature adipocytes are obtained (Day +9–+12 differentiation).

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CHAPTER 5

ISOLATION AND CULTURE STEM CELLS FROM HUMAN PBMCs

Adult stem cells have the potential of self-renewal and terminal differentiation to replace peripheral mature cells continuously lost because of normal tissue turnover. Although numerous articles have identified adult stem cells in tissues such as skin, fat, muscle, blood vessels, and brain, among others, the haematopoietics tissue (from bone marrow and peripheral blood) represents one of the most extensively studied in terms of its cell dynamism and heterogeneity. For instance, in experiment that 20 to 100 haematopoietics stem cells have the capacity to replace the whole lymphohematopoietic system in adult mice after lethal doses of radiation.

The percentage of donor cell engraftment can also be assessed in skin, esophagus, stomach, small bowel, large bowel, and bronchi. Other studies have reported similar results in kidney epithelium, pancreas, myocardium, skeletal muscle, and central nervous system neurons. Bone marrow-derived cells have been reported as transit-amplifying cells at the injured tissue, where they differentiate into keratinocytes.

Clinically, in a study of archival specimens from patients who received transplantation of peripheral-blood stem cells, Korbling and colleagues demonstrated that those cells could differentiate into mature epithelial cells in skin, lungs, gastrointestinal tract, and liver. In addition, other authors have described myocardial regeneration, and neuron renewal. In similar experiments involving skin damage,

the application of $CD34^+$ peripheral blood mononuclear cells (PBMCs) accelerated the neovascularization and epidermal healing in a model of chronic full-thickness skin wounds in diabetic mice but in human still unclear.

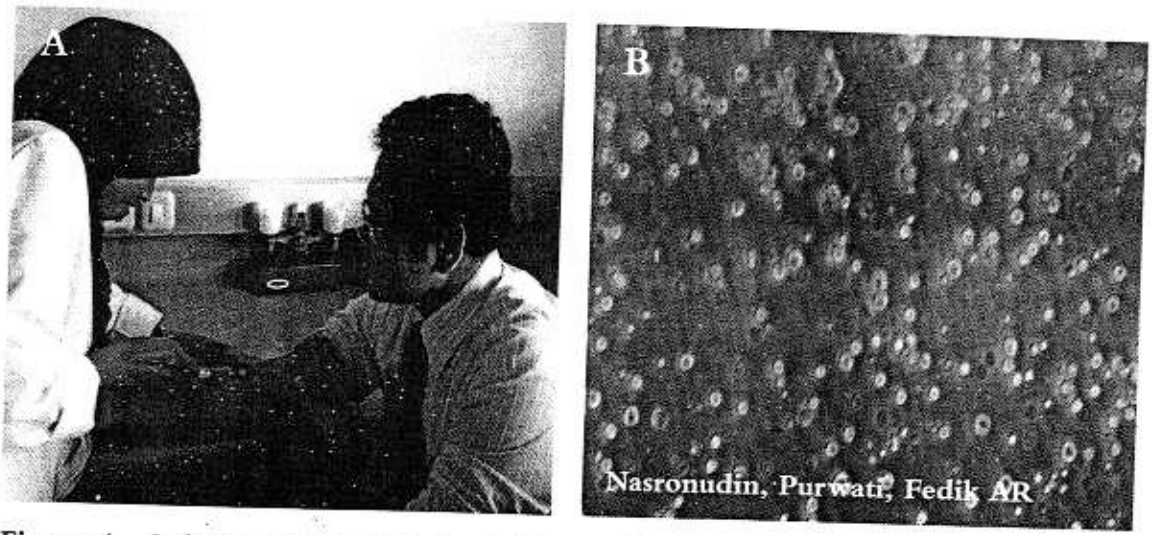

Figure 1. Isolation mononucleated cells from peripheral blood. A. Aspiration of peripheral blood. B. Isolated of mononucleated cells using Ficoll Histopaque 1,077. Cells were cultured in dish. Analyzed under inverted microscope 400x

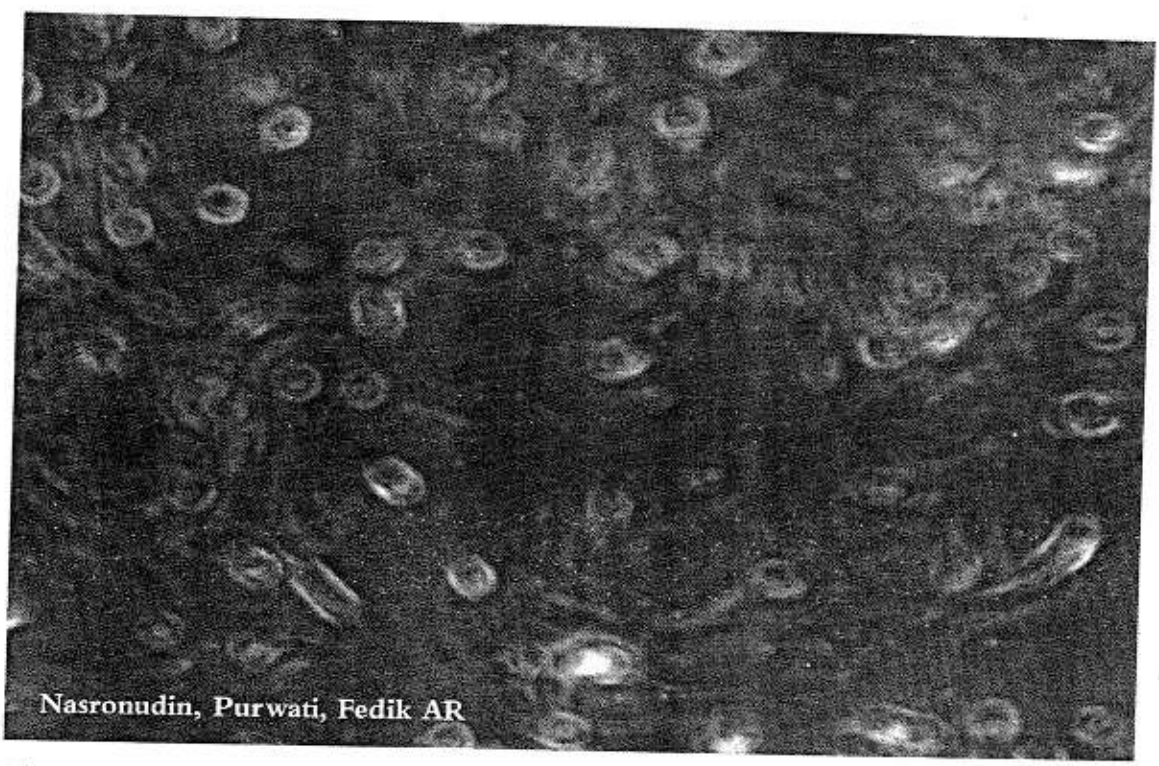

Figure 2. Mesenchymal stem cells from human peripheral blood growing together between mononuclear cells. 3 days after culture in dish and analyzed using inverted microscope 400x

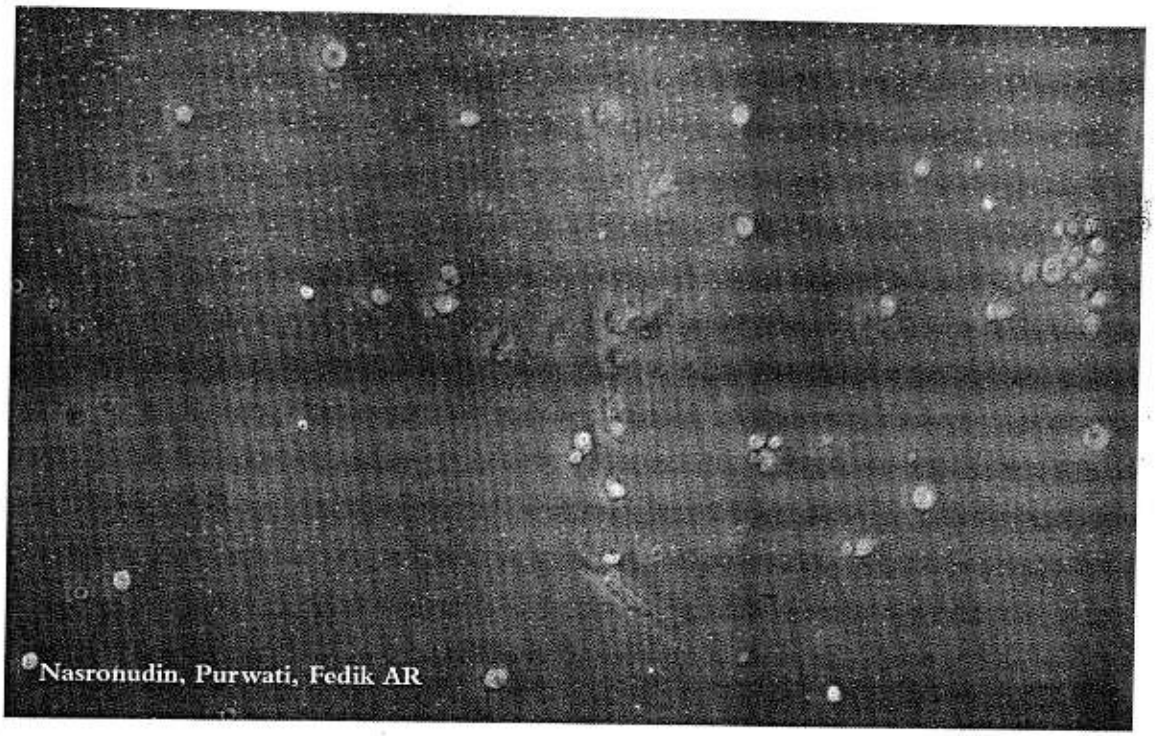

Figure 3. Mesenchymal stem cells from human peripheral blood, 4 days after culture in dish and analyzed using inverted microscope 400x

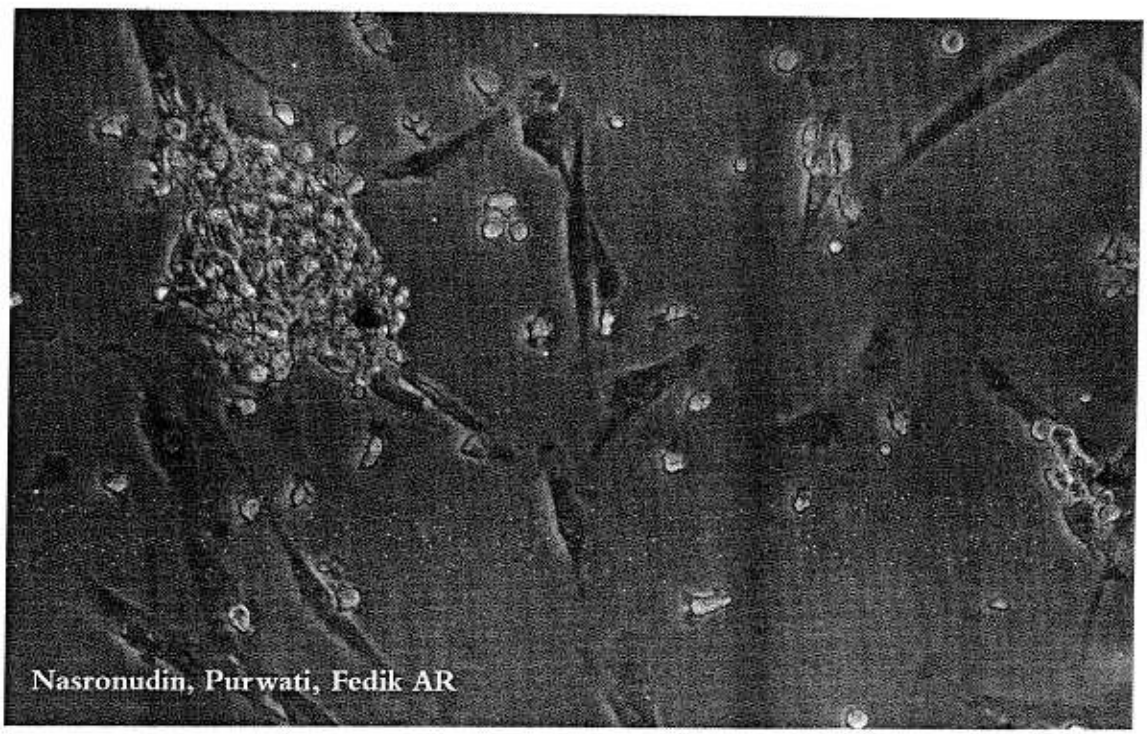

Figure 4. Mesenchymal stem cells from human peripheral blood, 6 days after culture in dish and analyzed using inverted microscope 400x

A similar clinical approach has been performed in chronically wounded patients by using autologous bone marrow-derived cells applied on the wound surface and injected into the wound margins. As a result of this treatment, patients healed completely, and dermis tended to recover its structure. Therefore, the traditional concept of a hierarchical haematopoietics stem cell differentiation with a restrictive, unidirectional, and preprogrammed cell commitment has been changed for a more flexible and reversible system. Thus, adult stem and precursor cells can move through boundaries of cell lineage, tissue, and germ layer to give rise to unexpected non-haematopoietics cells according to the tissue in which they reside, especially after inductive signals such as tissue damage, transplantation, or in *ex vivo* culture. This phenomenon, called cell Tran differentiation, lineage conversion, or stem cell plasticity, constitutes an important characteristic of bone marrow-derived cells to repopulate somatic tissues.

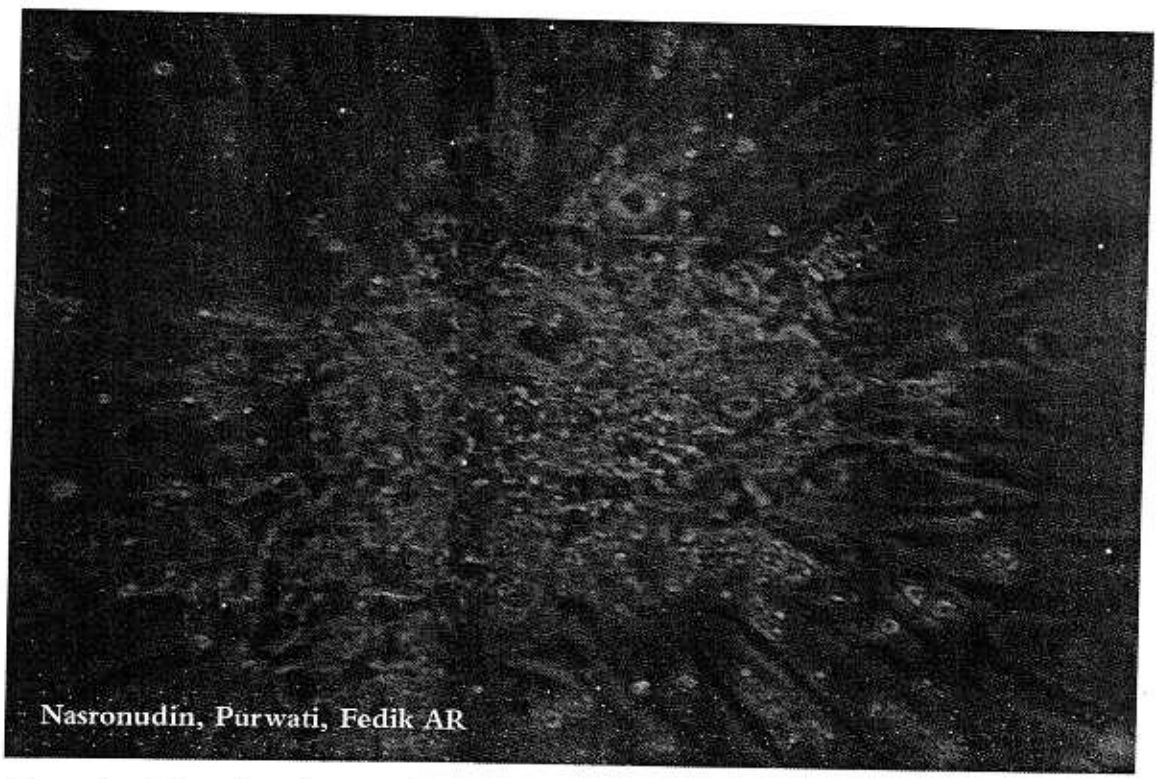

Figure 5. Mesenchymal stem cells from human peripheral blood, 8 days after culture in dish. These cells can be develop to be progenitors cells. Analyzed using inverted microscope 400x

Thus, circulating bone marrow-derived haematopoietics stem cells (also referred to as $CD34^+$ cells) migrate to damaged areas and participate in the local tissue regeneration. Thus, circulating $CD34^+$ bone marrow-derived adult stem cells constitute fibrocytes that rapidly infiltrate the wound bed and settle in particular

locations within the dermis. Subsequently, the exposure to transforming growth factor-ß induces the fibrocyte differentiation into myofibroblasts and could contribute to the tissue contraction. In addition, within resident stem cell niches of skin, located at the bulge region of hair follicles and basal layer of the interfollicular epidermis, $CD34^+$ keratinocyte precursors have been identified.

Even though the local injection of CCL27 seems to accelerate healing process by increasing the $CD34^+$ bone marrow-derived cell migration, the source of these cells as well as their role in both normal cell renewal and tissue repair have yet to be determined. Unfortunately, there is no reliable marker that displays long-term expression for local identification and tracking of cells along the transition process of epithelial cell maturation. In this regard, the protein 14-3-3*s* has been described as a specific marker for epithelial cells. In addition, we have recently described a releasable form of keratinocyte-derived 14-3-3*s* that induces matrix metalloproteinase (MMP)-1 expression in dermal fibroblasts. Thus, because of its distinct expression pattern and anti-fibrotic effects on dermal fibroblasts, 14-3-3*s* serves as a promising protein to elucidate further the functional commitment of PBMCs into epithelial-like cells.

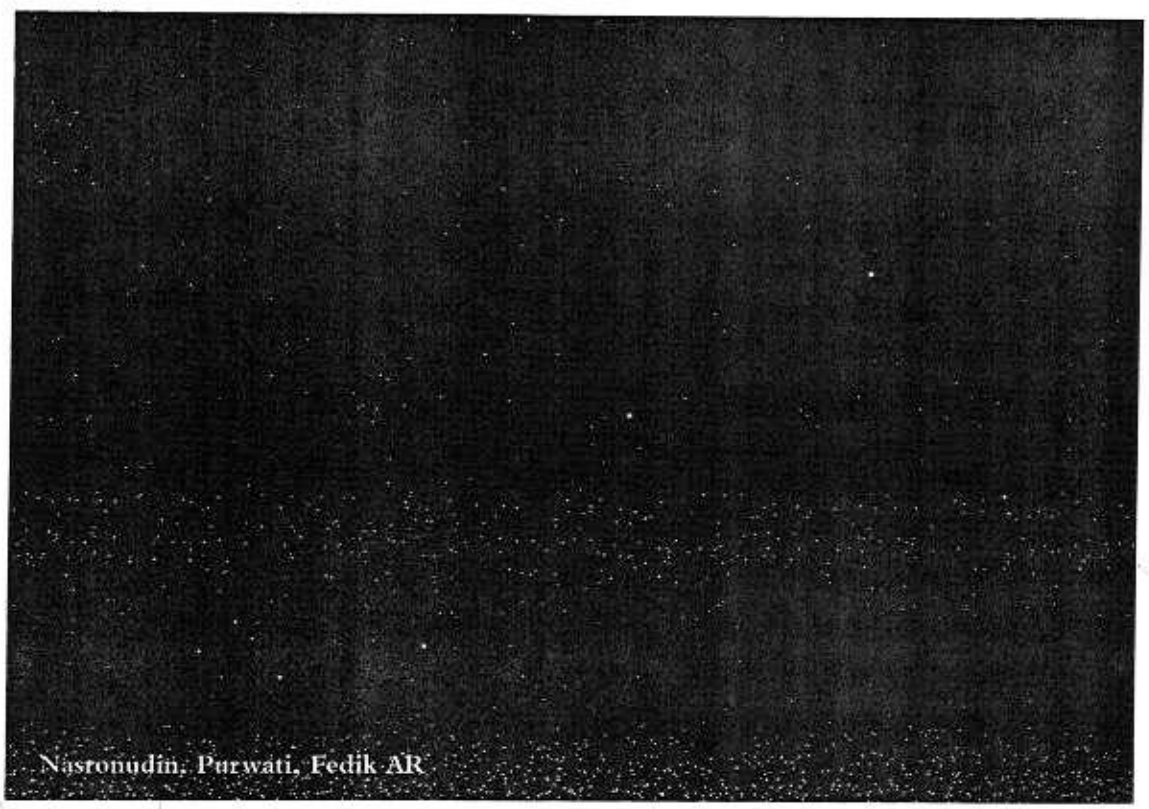

Figure 6. Mesenchymal stem cells from human peripheral blood, 9 days after culture in dish. These cells can be develop to be progenitors cells. Analyzed using inverted microscope 400x

PREPARATION OF PBMCS

PBMCs were isolated from whole blood of volunteers by using Ficoll-Histopaque density gradient centrifugation following the manufacturer's protocol. Blood from donors was carefully layered on Ficoll solution (density 1.077; Sigma). Centrifugation was performed at 3000 rpm for 5 minutes at 10 °C. The mononuclear cell interphase was taken and washed three times in 1× phosphate-buffered saline (PBS) for 10 minutes at 1600 rpm. Cells were counted and suspended in appropriate culture medium (see below) containing 2×10^6 cells/ml so that cell suspensions were added to either chamber slides.

ISOLATION OF HUMAN PBMCS

After PBMCs were isolated, circulating stem/precursor cells were isolated by using the EasySep negative selection human progenitor cell enrichment kit with CD41 depletion (Stem Cell Technology) following the manufacturer's recommendations. Cell suspension at a concentration of 5×10^7 cell/ml were prepared using 1x PBS containing 2% heat-inactivated fetal bovine serum qualified (FBS) Within a 12 x 75-mm polystyrene tube, the EasySep negative selection progenitor cell enrichment cocktail with CD41 depletion was added at 50 µl/ml cell suspension. The mixture was incubated for 15 minutes at room temperature. Then, EasySep magnetic nano particles (StemCell Technologies) were added at 50 µl/ml cell suspension and incubated at room temperature for another 15 minutes under constant rotation. After mixing, the sample tube was placed without cap into the magnet (EasySep Magnet 18000; StemCell Technologies) for 10 minutes. The liquid content with wanted cells was subsequently poured off into a new tube.

CULTURE PROCEDURES OF PBMCS

After the isolation procedure, PBMCs were resuspended in culture medium containing α-MEM, FBS, growth supplements, penicillin G sodium (100 U/ml), and streptomycin sulfate (100 µg/ml), and amphotericin B (0.25 µg/ml). Half of the culture medium was changed every other day. At different time points, cells were harvested by using 0.05% ethylenediaminetetraacetic acid (EDTA) and 0.1% trypsin, in 1x phosphate-buffered saline (PBS) and gentle scraping with a rubber policeman. In case of morphological studies, adherent cells were directly fixed on chamber slides with 4% paraformaldehyde. In parallel experiments, at the same time points, conditioned media from PBMC-derived epithelial-like cells were collected

after a 24-hour incubation with a test media containing 50% DMEM and 50% KSFM without supplement and growth factors. The same procedure described above was used with circulating precursor cells to induce cell Tran differentiation into epithelial-like cells. The complete procedures for culture as below.

1. Dilute the peripheral blood sample 1:1 with PBS.
2. Washing using PBS.
3. Pipette 5 mL of Ficoll-Hypaque into a 15-mL conical centrifuge tube.
4. Slowly layer 30 mL of the mixture of PBSA and sample over the Ficoll histopaque. Do not disturb the Ficoll-Histopaque/sample interface.
5. Centrifuge for 20–30 min at 1600 rpm 10 men at room temperature.
6. After centrifugation, a layer of mononuclear cells should be visible on top of the Ficoll-Histopaque phase, as they have a lower density than the Ficoll-Histopaque solution.
7. Using a Pasteur pipette, transfer the interface layer containing the mononuclear cells to a centrifuge tube.
8. Wash the cells with PBSA and recover the cells by centrifugation for 10 min at 1600 rpm and room temperature.
9. Discard the supernate, resuspend the cell pellet in PBS, and repeat the washing procedure, Step 8.
10. Finally, resuspend the cells in appropriate medium (freezing or culture medium). These cells ready to culture.

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CHAPTER 6

ISOLATION AND CULTURE OF MESENCHYM STEM CELLS FROM UMCB

INTRODUCTION

Umbilical cord blood (UMCB) is the blood remaining in the umbilical cord (UC) and placenta after birth. This had been regarded as medical waste and was discarded routinely in the past. However, in recent years, UMCB is being widely accepted as a rich alternative source of HSCs and other stem cells with practical and ethical advantages.

Since the first UMCB transplantation was performed in 1988 for a child with Fanconi anemia, it has become a safe and accepted mode of HSC transplantation for recipients because of the low viral exposure and reduced incidence of the more severe grades of acute graft-versushost disease (GvHD) when one or two human

leukocyte antigen (HLA)-mismatched unrelated donor transplants are performed, whereas BM transplantation requires strict histocompatibility between donors and recipients. In addition to HSCs, it is known that the UMCB also contains other stem cells such as MSCs, which have the potential to differentiate into various other types of cells and can be used to repair damaged cells and tissues in the human body. The human UC embryologically formed at day 26 of gestation is the lifeline between the fetus and the placenta.

The UC normally contains two umbilical arteries and one umbilical vein, a main reservoir of UMCB. These are embedded within a loose, proteoglycan-rich matrix known as Wharton's jelly (WJ), the jelly has physical properties like a polyurethane pillow, which serves to protect the critical vascular lifeline that connects the placenta and the fetus. Recently, stem cells have been isolated separately from umbilical veins.

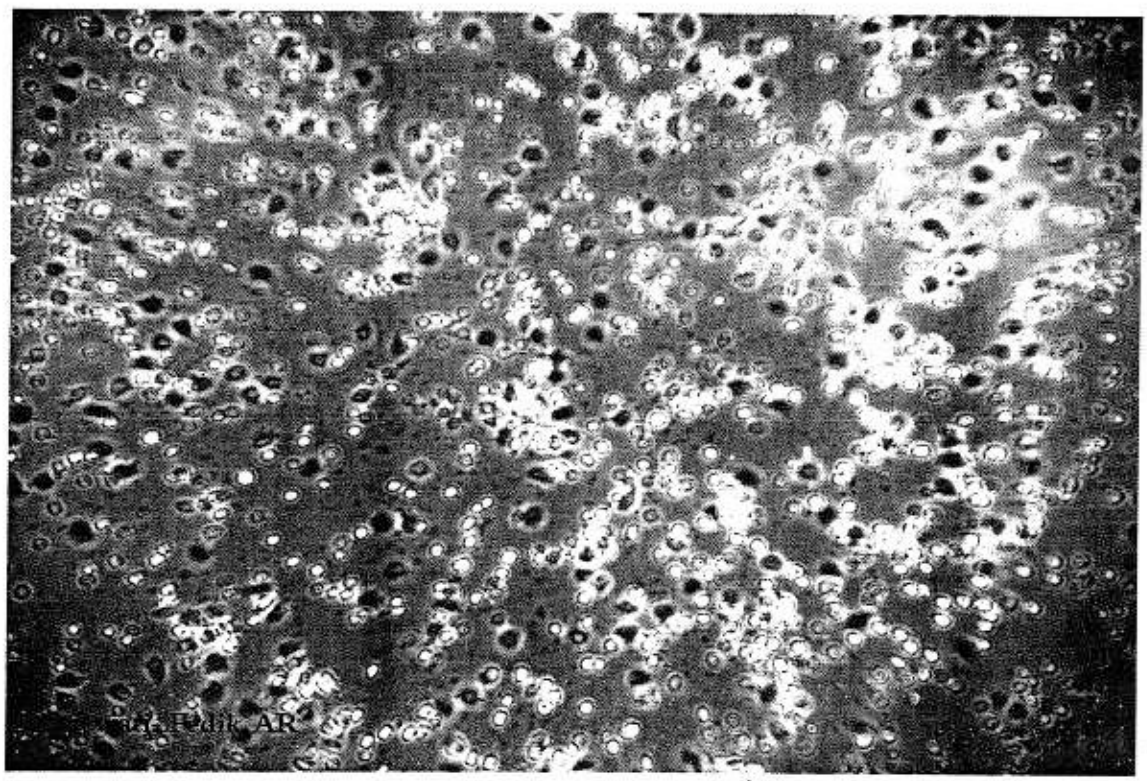

Figure 1. Mononucleated cells separated from umbilical cord. 24 h after isolation and was cultured in dish. Analyzed using inverted microscope 400x

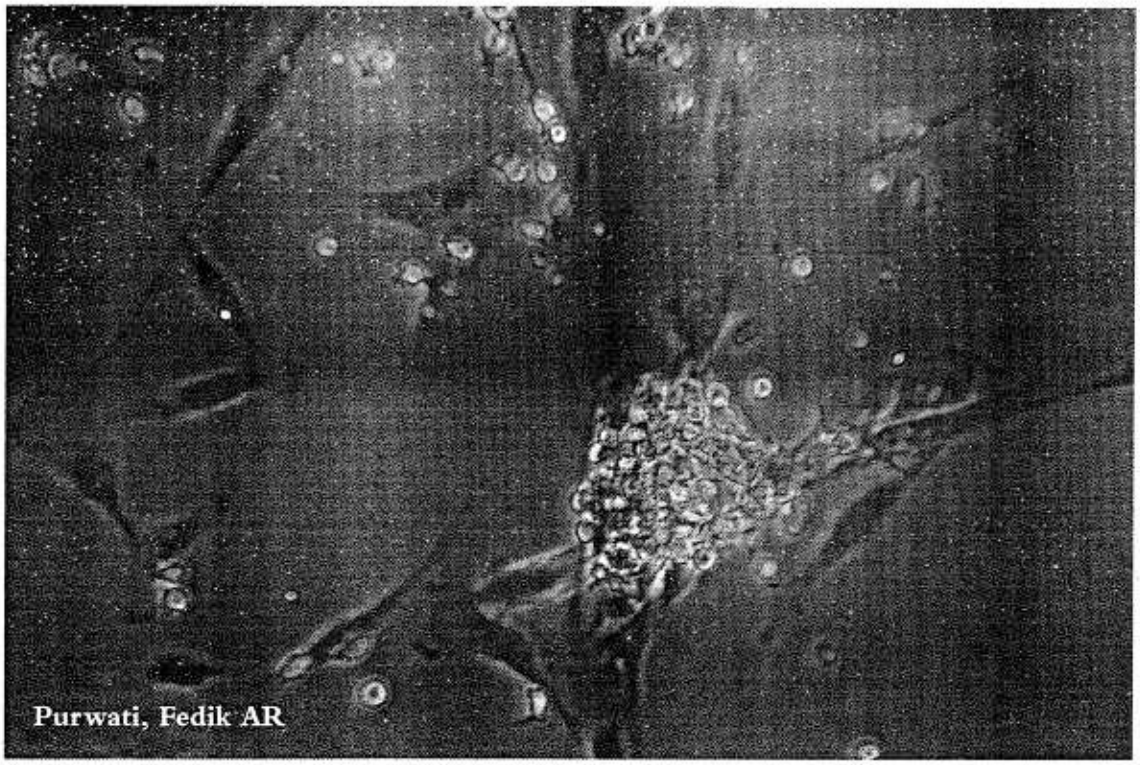

Figure 2. Mesenchymal stem cells from umbilical cord, 4 days growing after cultured in dish. Analyzed using inverted microscope 400x

UMBILICAL CORD-DERIVED STEM CELLS

UC-derived stem cells have been isolated and cultured mainly from umbilical vein. This suggested that MSC-like cells are present in the sub endothelial layer of the human umbilical vein and could be successfully isolated, cultured, and expanded with routine technical approaches. The results of morphological studies and immunophenotyping of cultured MSC like cells from human umbilical vein have shown that these cells closely resemble cultured MSCs obtained from bone marrow and other sources also reported that human umbilical cord perivascular (HUCPV) cells, which were either discarded or not specifically isolated, should contain a subpopulation of cells that would be capable of exhibiting a functional mesenchymal phenotype.

Another potential alternative source of mesenchymal stem cells can be isolated "pre-chondrocytes," using a similar approach, isolated fibroblast-like cells, which could be induced to differentiate into "neural-like" cells. In addition to HSCs in UCB, potential alternative stem cells such as MSCs, unrestricted somatic stem cells (USSCs), cord blood-derived embryonic-like stem cells (CBEs), and cord blood multipotent progenitor cells (CB-MPCs) have been isolated and characterized by

their different growth conditions. Because UCB derived cells have been regarded as progenitors cells.

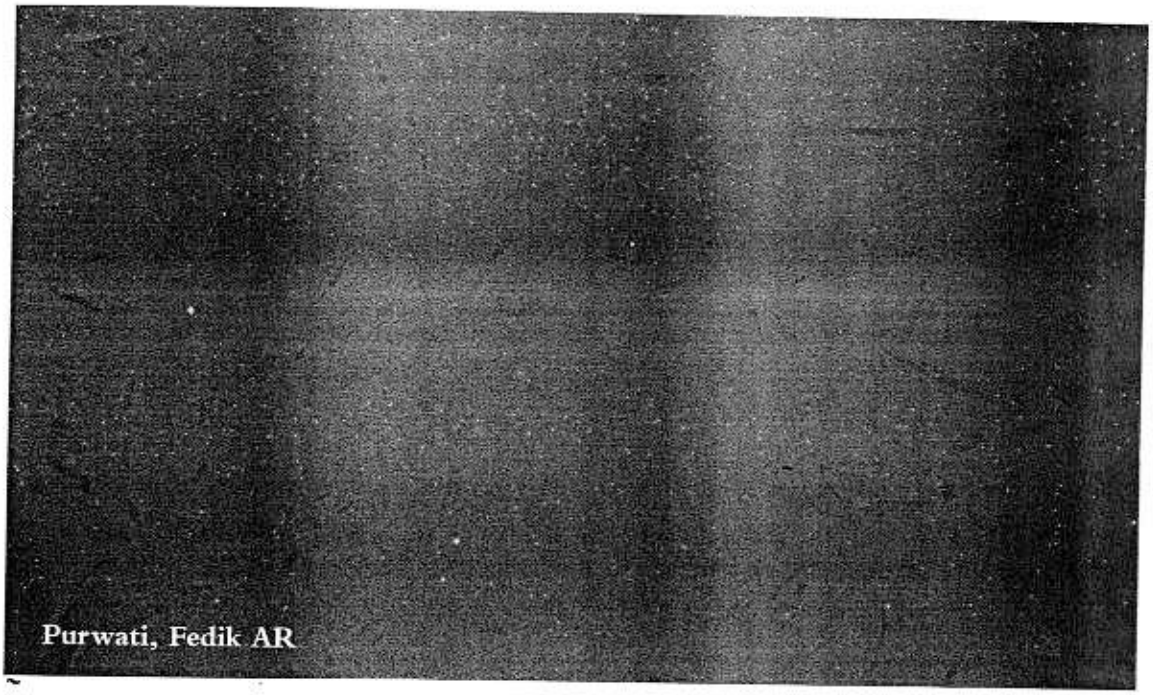

Figure 3. Mesenchymal stem cells from umbilical cord, 5 days growing after cultured in dish. Analyzed using inverted microscope 400x

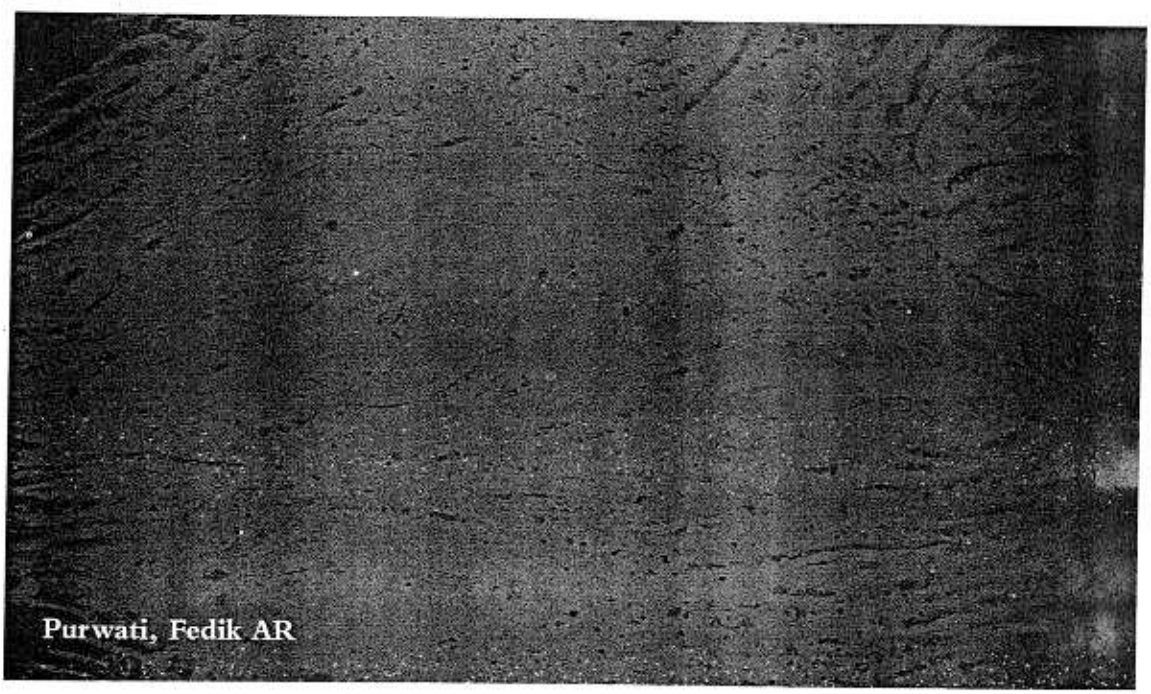

Figure 4. Mesenchymal stem cells from umbilical cord, 8 days growing 90% confluent after cultured in dish. These cells are ready to stimulate to be progenitors cells. Analyzed using inverted microscope 400x

Reagents

Transport Medium for Umbilical Cord

Doebelco modified essential medium (DMEM) supplemented with 300 U/mL penicillin, 300 μg/mL streptomycin (Sigma, Cat. P0781), 150 μg/mL gentamicin, and 1 μg/mL fungizone (Sigma, Cat. A2942).

Reagents and Materials

Sterile

- Transport medium: see above
- Clamps
- Scissors

Procedures

1. After birth, close the umbilical cord with two clamps on the end adjacent to the infant and one on the end adjacent to the placenta.
2. Snip between the two clamps near the infant and cut off the placenta from the other end.
3. Carry the umbilical cord to the laboratory in transport medium and process within 6–12 h.

Preparation of Umbilical Cord Blood: the Bag or Syringe Method

Reagents and Materials

Must be Sterile

- Anticoagulant: CPDA-1
- Betadine or 70% alcohol
- Clamps
- Syringe needle, 18 gauge
- Cord blood collection bag, 175 mL, containing 24.5 mL of CPDA-1
- Syringe, 50 mL, containing 200 I.U. of heparin

Procedure

1. Clamp and cut the cord as close as possible to the infant.
2. Swab the needle insertion site at the fetal end of the cord with Betadine or 70% alcohol.

3. To maximize collection volume, minimize manipulation of the cord.
4. Insert the needle with the attached collection bag or 50-mL syringes at the insertion site of the umbilical vein prepared in Step (2).
5. Keep the bag at a lower level than the insertion site so that cord blood is allowed to fill the container by gravity or slowly aspirate the blood from the umbilical vein with 50-mL syringes. Allow as much blood to collect as possible. The range of volumes obtained will be 60 to 150 mL.
6. If the vein collapses, reinsert the needle farther up the cord after swabbing with Betadine or 70% alcohol.
7. After the blood flow has stopped, activate the needle safety cover by pushing it into locked position. ***Note***: The cord blood should remain at room temperature. Do not refrigerate.
8. For the bag method, clamp the tubing with the attached clasp and tie two secure knots in the tubing as close to the blood bag as possible to prevent leakage, then cut off the needle and discard it in a sharps container.
9. Gently invert the bag or syringe several times to thoroughly mix the cord blood and anticoagulant.

Preparation of Stem Cells from Umbilical Cord

Mononucleated umbilical cord isolated and cultured MSC-like cells from the sub-endothelial layer of umbilical vein containing no endothelium- or leukocyte specific antigens but expressing α-smooth muscle actin and several mesenchymal cell markers. WJ-derived stem cells, one potential alternative source of mesenchymal cells, have been isolated and cultured by the explant method or the enzymatic digestion method.

Isolation of Stem Cells from Umbilical Vein

Reagents and Materials

Sterile

- Culture medium: complete LG-DMEM (Gibco)
- PBSA (Merck)
- Trypsin, 0.05% with EDTA (Merck)
- Collagenase (Sigma)
- Culture flasks (SPL)
- Catheter

Culture procedures

Procedure

1. Collect and process umbilical cord within 6–12 h after normal delivery.
2. Catheterize umbilical vein and wash twice internally with PBSA.
3. Clamp the distal end.
4. Fill the vein with 0.1% collagenase solution.
5. Clamp the proximal end.
6. Incubate the umbilical cord at 37 °C for 20 min.
7. Massage the cord gently, collect the suspension of endothelial and subendothelial cells, and centrifuge at 600 *g* for 10 min.
8. Resuspend the cell pellet in culture medium.
9. After counting, seed cell suspension in 75-cm^2 culture flasks with a density of approximately 1 × 103 cells/cm^2.
10. Remove nonadherent cells after 3 days by changing the medium and keep adherent cells in culture, feeding with fresh medium every 3 days until the outgrowth of fibroblastoid cells about 2 weeks later.
11. At that time, harvest cells with 0.05% trypsin/EDTA and passage into a new dish for further expansion.

Isolation of Mononuclear Cells (MNCs) by Density Separation

Reagents

Must be sterile: Appropriate culture medium or freezing medium

- PBSA (Merck)
- Ficoll-Hypaque (density 1.077 g/L) (Sigma)
- Pasteur pipettes/serological pipette (Pirex)
- Conical centrifuge tubes, 50 mL (Falcon)

Procedure

a. Dilute the cord blood sample 1:1 with PBSA.
b. Pipette 15 mL of Ficoll-Hypaque into a 50-mL conical centrifuge tube.
c. Slowly layer 30 mL of the mixture of PBSA and sample over the Ficoll Hypaque. Do not disturb the Ficoll-Hypaque/sample interface.
d. Centrifuge for 20–30 min at 450 *g* at room temperature.
e. After centrifugation, a layer of mononuclear cells should be visible on top of the Ficoll-Hypaque phase, as they have a lower density than the Ficoll-Hypaque solution.

f. Using a Pasteur pipette, transfer the interface layer containing the mononuclear cells to a centrifuge tube.
g. Wash the cells with PBSA and recover the cells by centrifugation for 10 min at 200 *g* and room temperature.
h. Discard the supernate, resuspend the cell pellet in PBSA, and repeat the washing procedure, Step (g).
i. Finally, resuspend the cells in appropriate medium (freezing or culture medium).

Cryopreservation of CB-MNCs

Reagents and Materials

Must be sterile

- FBS (Biowest)
- Dimethyl sulfoxide (DMSO) (Serva)
- Cryovials (Biologic)
- Slow-freezing container (e.g., Mr. Frosty) or controlled-rate freezer (Biologic)
- LN2-resistant storage box (Biologic)

PREPARATION OF STEM CELLS FROM UMBILICAL CORD BLOOD

Umbilical cord blood (UCB) is well known to be a rich source of haematopoietics stem cells (HSCs) with practical and ethical advantages. HSCs have been defined as primitive, undifferentiated cells that are capable of both self-renewal and differentiation into all blood cell types. The majority of HSCs express the CD34 antigen, an integral membrane glycoprotein of 90–120 kDa that functions as a regulator of haematopoietics cell adhesion to stromal cells of the hematopoietic microenvironment. Thus HSCs have been isolated mostly by using reactivity with anti-CD34 antibody. Several types of stem cells, which contain CB-MSCs, USSCs, CBEs, and CB-MPCs, have been isolated from fresh or cryopreserved UCB, under different growth conditions.

Procedures

1. Prepare the freezing medium: 90% FBS + 10% DMSO; chill on ice or place in 4 °C refrigerator for at least 30 min.
2. Count cell numbers of the CB-MNCs.

3. Resuspend CB-MNCs in cold freezing medium and adjust the cell concentration to 5–10 × 106 viable cells/mL.
4. Dispense 1 mL into cryovials.
5. Immediately place the cryovials in a slow-freezing container and place the
6. container in a –70° C freezer for 4–24 h. Alternatively, place the cryovials into the freezing chamber of a controlled-rate LN2 freezer.
7. After 4–24 h in a –70° C freezer or controlled-rate freezer, transfer the cryovials into a LN2-resistant storage box and place the box into the vapor phase (approximately –135° C) or liquid phase (–196° C) of a liquid nitrogen freezer.

Thawing CB-MNCs

Reagents and Materials

Must be Sterile

1. Appropriate medium (*see* above) supplemented with 10%
2. Fetal bovine serum (FBS) (Biowest)
3. Phosphat buffer saline albumin (PBSA) (Merck)

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CHAPTER 7
MESENCHYMAL STEM CELLS FROM BRAIN

INTRODUCTION

The discovery that undifferentiated proliferative cells could be isolated from the adult mammalian CNS under favorable cell culture conditions, and induced to differentiate along both glial and neuronal lineages, this revolutionizing about the limited ability of the CNS to replace neural cells lost to injury or disease. Stem cells are able to differentiate into all the different types of cells in a given tissue, while maintaining a pool of themselves. During development of the CNS, in analogy with the haematopoietics system, maturation of neural stem cells involves a continuing loss of potential and restriction of commitment before, finally, a lineage of fully differentiated cells is established. Isolation and long-term culturing of multipotent neural stem/progenitor cells that upon differentiation generate the major building blocks of the CNS: neurons, astrocytes, and oligodendrocytes have been advanced by the finding that some mitogenic growth factors have a proliferative effect on these cells.

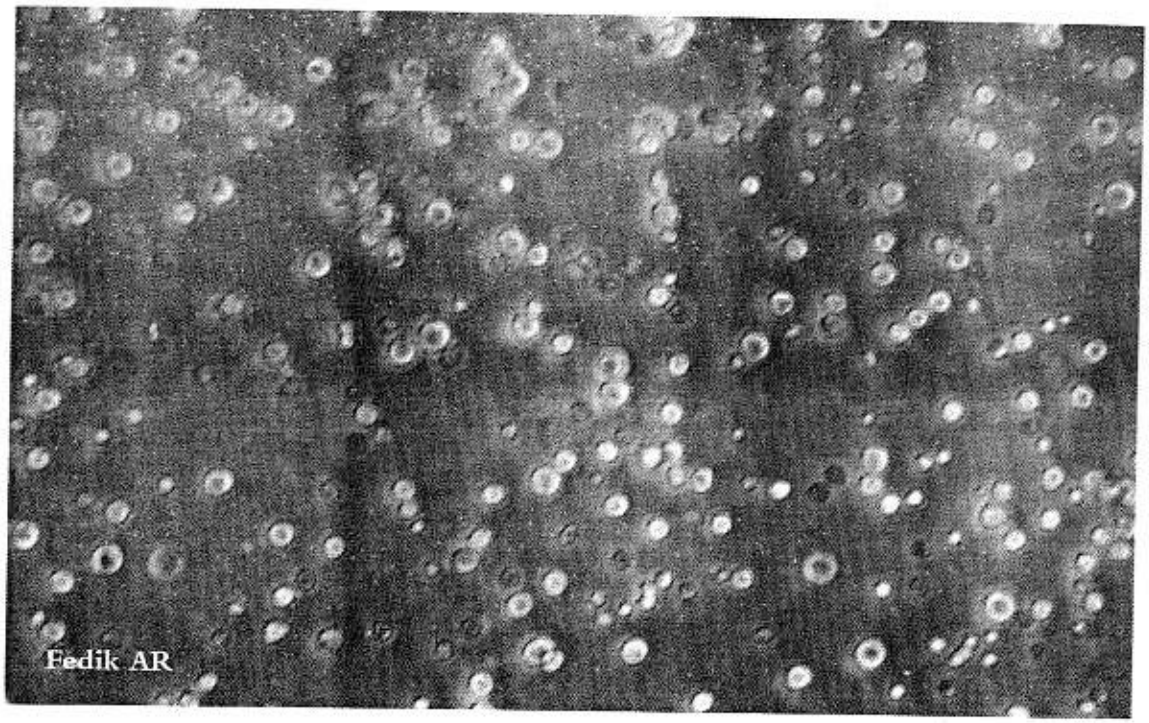

Figure 1. Monucleated cells from rabbit brain one day all. These cells isolated using trypsin 0,025% with EDTA. Analyzed under inverted microscope 400x

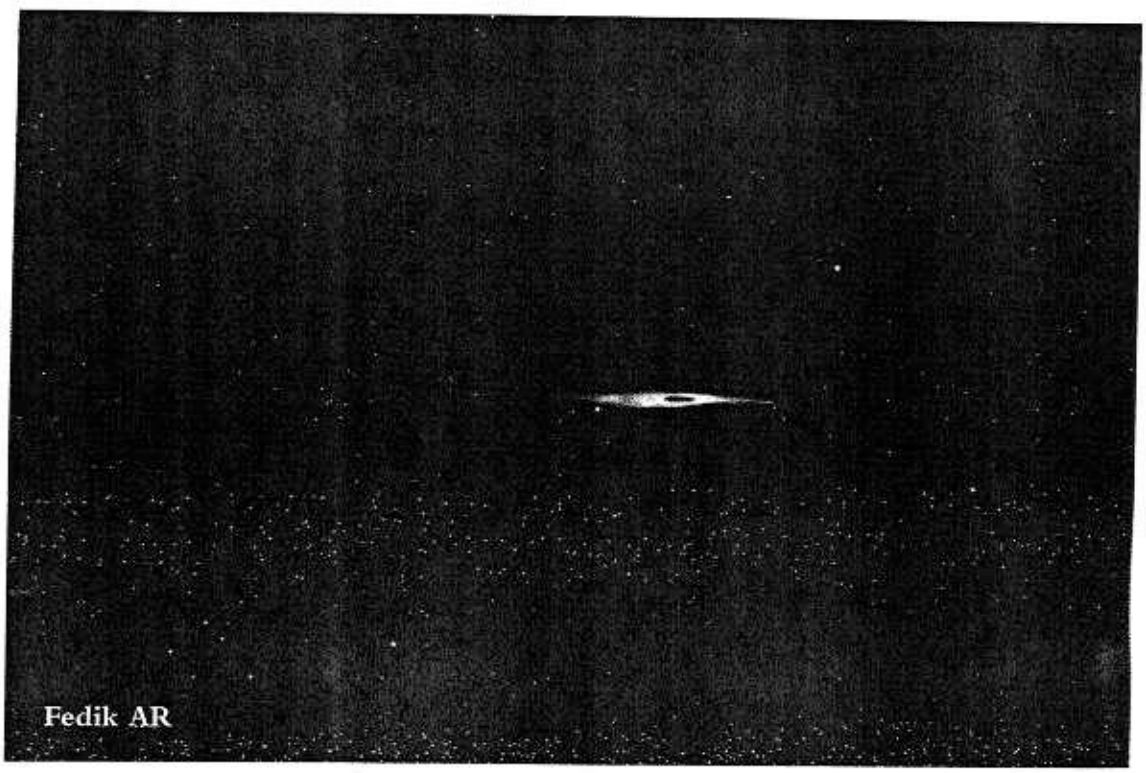

Figure 2. Mesenchymal cells from rabbit brain. 6 days after culture and staining using antibody monoclonal specific membrane protein and labeled with fluorescence isothiocyanat (FITC). Analyzed under fluorescence microscope 400x.

Figure 3. Mesenchymal cells like oligodendrits, astrocyte from rabbit brain. 7 days after culture and staining using antibody monoclonal specific membrane protein and labeled with fluorescence isothiocyanat (FITC). Analyzed under fluorescence microscope 400x.

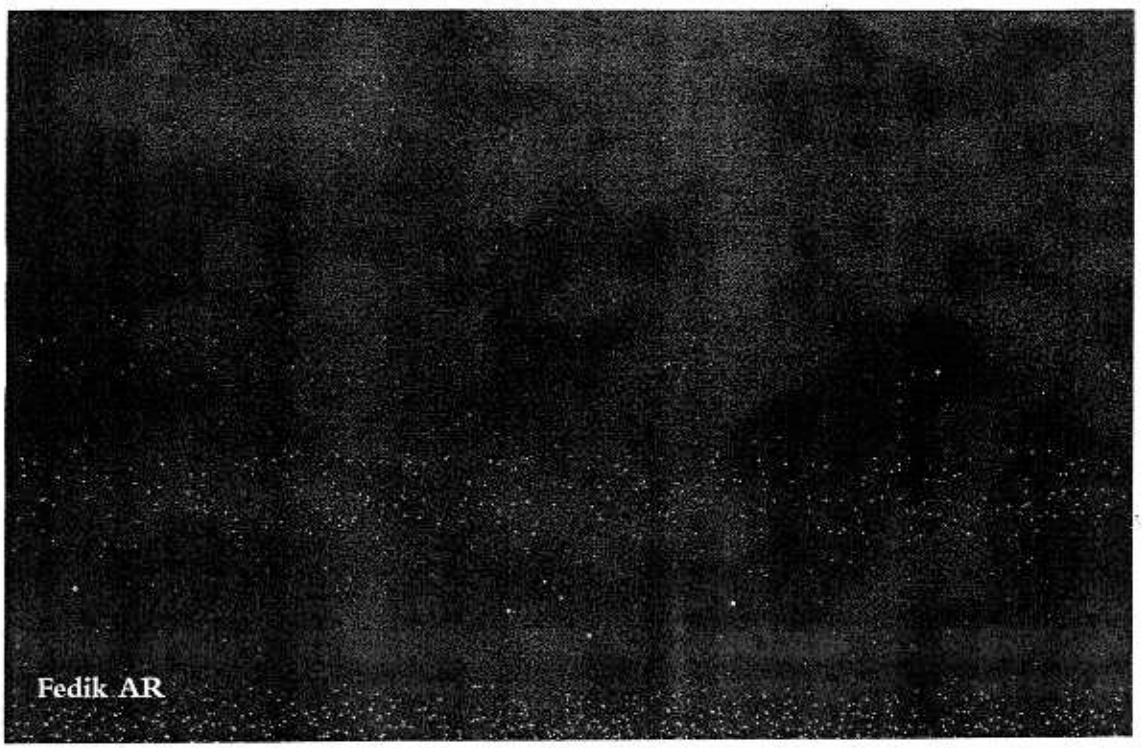

Figure 4. Mesenchymal cells like oligodendrits, astrocyte from rabbit brain. 7 days after culture and staining using antibody monoclonal specific membrane protein and labeled with Rhodamine. Analyzed under fluorescence microscope 400x.

Multipotent stem cells that respond to epidermal growth factor (EGF) or basic fibroblast growth factor (FGF)-2 have been isolated from both neurogenic, and non-neurogenic regions of the adult mammalian CNS by using different culture systems Isolated and cultured stem cells provide an important source of cells for in vitro studies to address issues related to development, as well as in transplantation studies to explore their potential as a source of donor cells for therapeutic purposes. In this regard, neural stem/progenitor cells have aroused a great deal of interest for their therapeutic potential in neurological disorders. After transplantation into the CNS, neural stem/progenitor cells migrate throughout the CNS, differentiate exclusively into neural cell types in a region-specific manner, and survive several months. Adult neural stem/progenitor cells can also be isolated from human postmortem tissue, potentially providing an alternative source of cells for therapeutic applications. Methods for isolation of stem cells from the brain have been described previously, Here, this book describe a method for isolating and culturing cells from rabbit brain cell, the generation of clonal population, and the characterization of these cells.

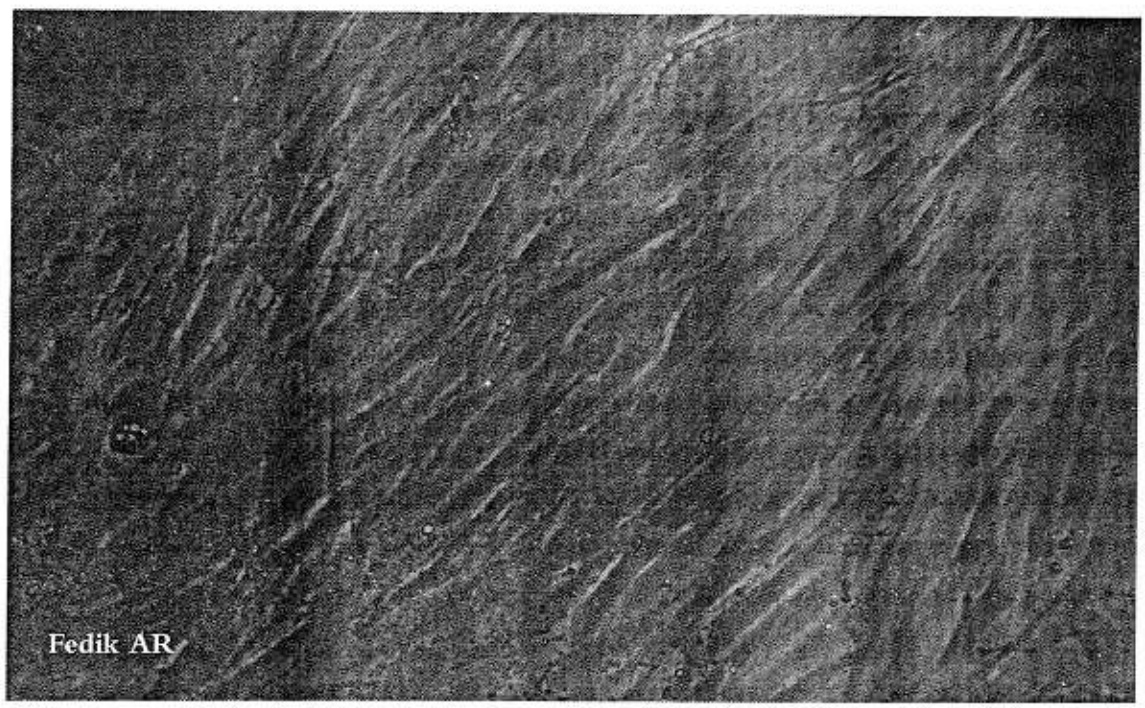

Figure 5. Mesenchymal cells from rabbit brain. 8 days after culture and ready to make for progenitors cells.. Analyzed under fluorescence microscope 400x.

REAGENTS

Reagents for Culture Media

1. Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco)
2. Earle's balanced salts (EBSS) (Sigma)
3. Glutamine: 200 mM (100×) (Invitrogen)
4. Heparin: sodium salt, grade 1A (Sigma)
5. Glucose (Sigma)
6. HEPES (Invitrogen)
7. NaHCO3 (Invitrogen)

PROCEDURES

1. This protocol applies to a single pregnant CD1 mouse, assuming tissue from 10 fetuses is recovered.
2. Place a 15-ml Falcon tube that contains 5 ml of N2 medium with added bFGF on ice. Sacrifice the animal by CO_2 intoxication, harvest the embryos, and place them in a 10-cm dish with PBS. The dissection of the fetal brains can be performed with two forceps and a razor blade under a dissection microscope.
3. Remove the brains from the fetuses and place them in a 10-cm culture dish on ice, with 5 ml of N2 plus bFGF to keep the tissue from drying and to maximize fibroblast growth factor receptor activation. Separate the two hemispheres by using the blade. Using the forceps, remove the cerebral cortices and place them in the Falcon tube containing N2 and bFGF.
4. When all tissue is collected, transport the Falcon tube containing the tissue in a sterile cell culture incubator. Allow the tissue to settle to the bottom of the tube by gravity. Using a hand-held pipette with a 1-ml tip, carefully remove the N2 medium until 1 ml is left. Place the tip of the pipette close to the bottom of the tube and triturate the tissue approximately 12 times, until a homogenous suspension is formed. Allow remaining fragments to sink by gravity for 1 min, and collect the medium leaving the bottom 100 µl.
5. Transfer the mixture to a 50-ml Falcon tube containing 50 ml of cold N2 medium plus bFGF. Mix gently and add 6 ml to each of eight 10-cm dishes that have been coated and place in the low oxygen cell culture incubator overnight.

6. This method should result in approximately one million cells per dish; this can be confirmed by 0.2% trypan blue cytometry. (Expect 70–80% of the cells to exclude trypan blue, indicating that they are alive. Cells should be seen attaching onto the dish within 15 min).

DAILY CARE

Twenty-four hours after plating, prewarm 50 ml of N2 with bFGF added and replace the medium in the culture dishes. The next day, add a bolus of bFGF to the medium within the culture dishes. Continue alternating medium/bFGF changes or bFGF additions for a total of 5 days.

PASSAGING

1. After 5 days in culture, the cells should have formed colonies containing a few hundred cells, with cell-free space between colonies. At this stage, the cultures should be approximately 80% confluent, and they are ready for passaging.
2. To passage, place culture dishes in a cell culture hood and wash twice with 5 ml of prewarmed HBSS (do this one dish at a time to avoid cell loss). Aspirate the HBSS and add another 5 ml of pre warmed HBSS containing bFGF. Place the dishes in the cell culture incubator for 5 min.
3. Return the dishes to the cell culture hood to harvest cells. Using a 5-ml pipette, spray the HBSS several times over the entire surface of the dish to lift the cells off. Collect the HBSS containing bFGF plus cells in a 15-ml Falcon tube and pellet by centrifugation (1,000 × g, 5 min, 4 °C).
4. Resuspend the pellet in the desired volume of N2 containing bFGF and seed new coated plates at the desired cell density. At this point, due to selective expansion of the SC population, the culture contains almost exclusively nestin-positive multipotent precursors.

FREEZE-STORING CELL

1. Alternative to passaging, cells can be frozen in liquid nitrogen for long-term storage. For this, the pellet is reconstituted in 3 ml of cold N2 plus bFGF, and the cells are counted by hemacytometry. The volume of N2 is adjusted to achieve a cell density of 1.1 million cells per 100 µl. Typically, each 10-cm dish may yield 5 million cells before passaging.

2. DMSO is subsequently added to the mixture to a final concentration of 10%. Aliquots (100 μl; 1 million cells) are prepared in cell freezing vials.
3. The vials are immediately placed in a freezing container, precooled to 4 °C, and the container is placed in a –80 °C freezer overnight. The next day, the cell cryofreezing vials are placed in a liquid nitrogen tank, where they can be stored indefinitely.

THAWING FROZEN CELLS

1. Remove desired number of frozen vials from the liquid nitrogen tank. Place each vial on dry ice, and take the vials to a cell culture hood where they are removed from dry ice and allowed to stand for 30 s at room temperature.
2. Add 1 ml of prechilled (4 °C) N2 plus bFGF and let stand for 30 s. Using a 1-ml pipette, gently resuspend and transfer in a Falcon tube with the appropriate volume of prechilled N2 plus bFGF for further plating.
3. Plate the cells in appropriate precoated culture dishes or plates and place in an incubator. The cells will attach within 20 min, at which point, replace the medium with fresh prewarmed (37 °C) N2 plus bFGF to wash off the DMSO. Take care when handling cryo-vials because the expanding gas during thawing may cause an explosion.

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CHAPTER 8
STEM CELL: HISTORY, FUTURE AND REGULATION

HISTORY OF HUMAN STEM CELL RESEARCH

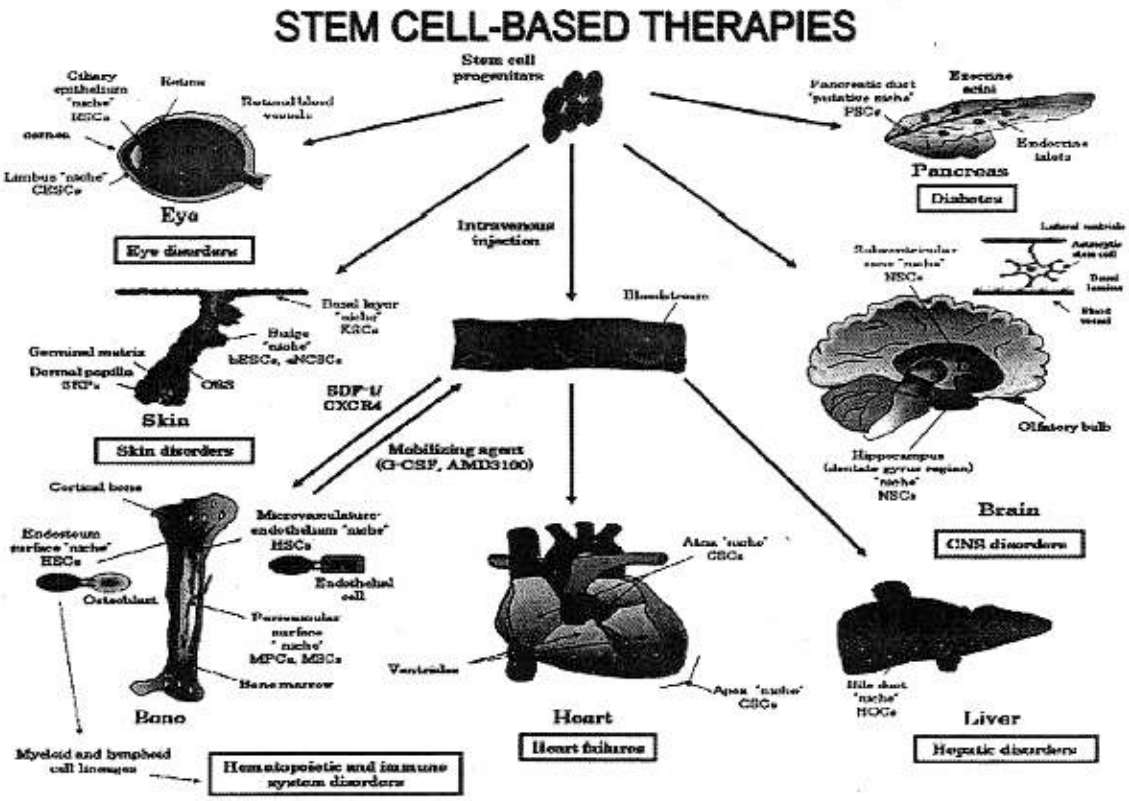
1. **In 1968 In 1968**, the first bone marrow transplant was successfully used in treatment of SCID (Severe Combined Immune Deficiency Disease).
2. **Since the 1970's Since the 1970's**, bone marrow transplants have been, used for treatment of immunodeficiency and leukemia.
3. **In 1998 In 1998** James Thomson was able to develop Human Embryonic stem cells.
4. **In 2007 In 2007** Shinya Yamanaka and James Thomson developed iPS by inserting 4 genes in human developed fibroblast.
5. **In 2009 In 2009** Shinya Yamanaka and James Thomson developed iPS without viral vectors **In 1998, James Thomson (University of Wisconsin-Madison Wisconsin-Madison)** isolated cells from the inner cell mass of the early embryo and developed the first human embryonic stem cell lines.

HISTORY OF HUMAN EMBRYONIC STEM CELL RESEARCH

1. In 1998, John Gearhart (Johns Hopkins University) derived human embryonic germ cells from cells in fetal gonadal tissue (primordial germ cells).
2. Pluripotent stem cell "lines" were developed from both sources.

POSSIBLE USES OF STEM CELL TECHNOLOGY

1. Replaceable tissues/organs (heart, nerves, liver, pancreas, bone, cartilage, skin etc).
2. Repair of defective cell types (genetic disorders).
3. Degenerative diseases of OLD AGE.
4. Delivery of genetic therapies.
5. Delivery chemotherapeutic agents.
6. Drug development and screening.

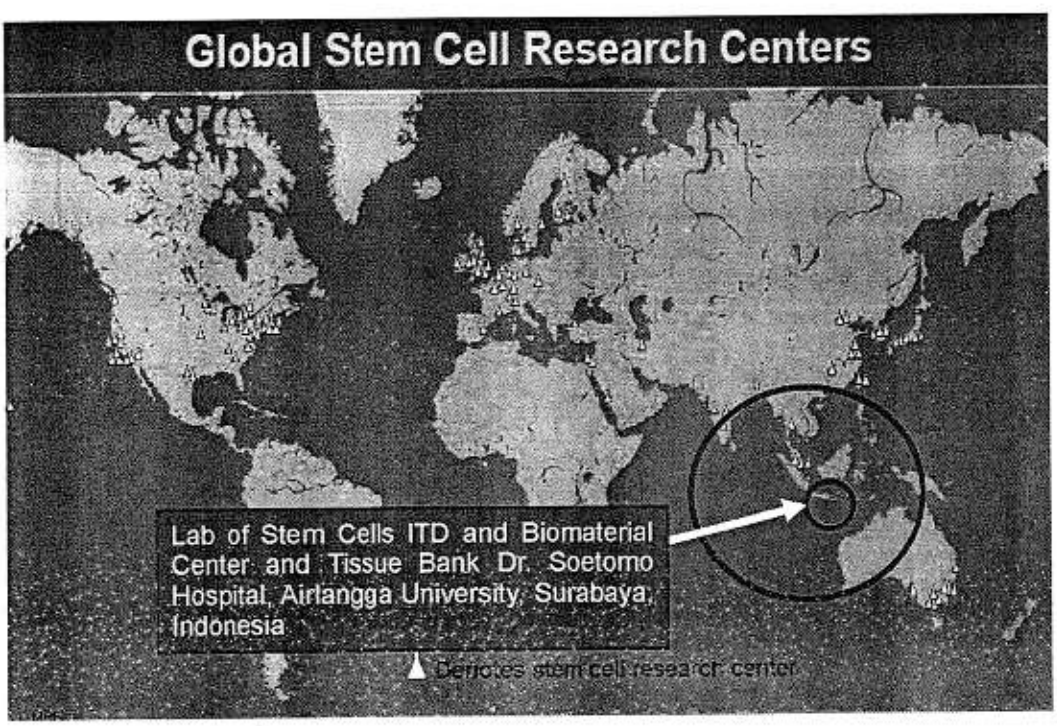


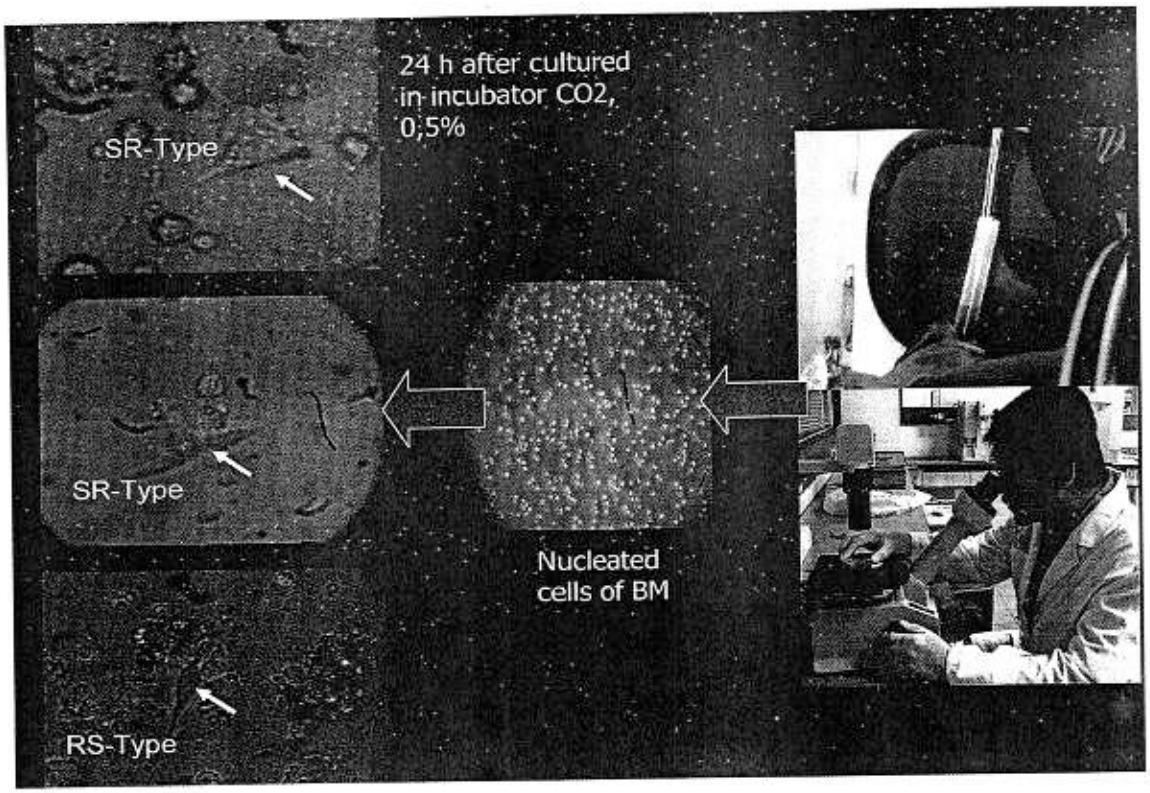
WORLDWIDE STEM CELL REGULATIONS

- 2003 proposal to ban reproductive and research cloning worldwide was was tabled by the United Nations following objections by Great Britain and other countries.
- The proposal will be taken up again in 2004, but dropped 2004.
- Bush in August-2001, prohibited use of federal research funds for hESC.
- Obama in March 2009 lifted up this prohibition and the clinical application of stem cell research will develop very fast.

STEM CELL RESEARCH AT ITD AND Dr. SOETOMO TEACHING HOSPITAL, AIRLANGGA UNIVERSITY–SURABAYA–INDONESIA

The Laboratory of Stem Cell at the Institute of tropical Disease Airlangga University has collaborative with Biomaterial Center – Tissue Bank Dr. Soetomo Teaching Hospital are together developing Tissue Engineering using biomaterial some kinds of Stem Cells from different sources like bone marrow (BM), peripheral blood mononuclear cells (PBMCs), adipose and brain. These cells will be developed to become progenitor cells like chondroblast, chondrocyte, cardyomiocyte etc. The goals these experiment to repair of bone fracture, defect tissue organ like liver, pancreas, heart, defect of tendon, skin abration, defect of haematopoietics od immunodeficiency like AIDS.





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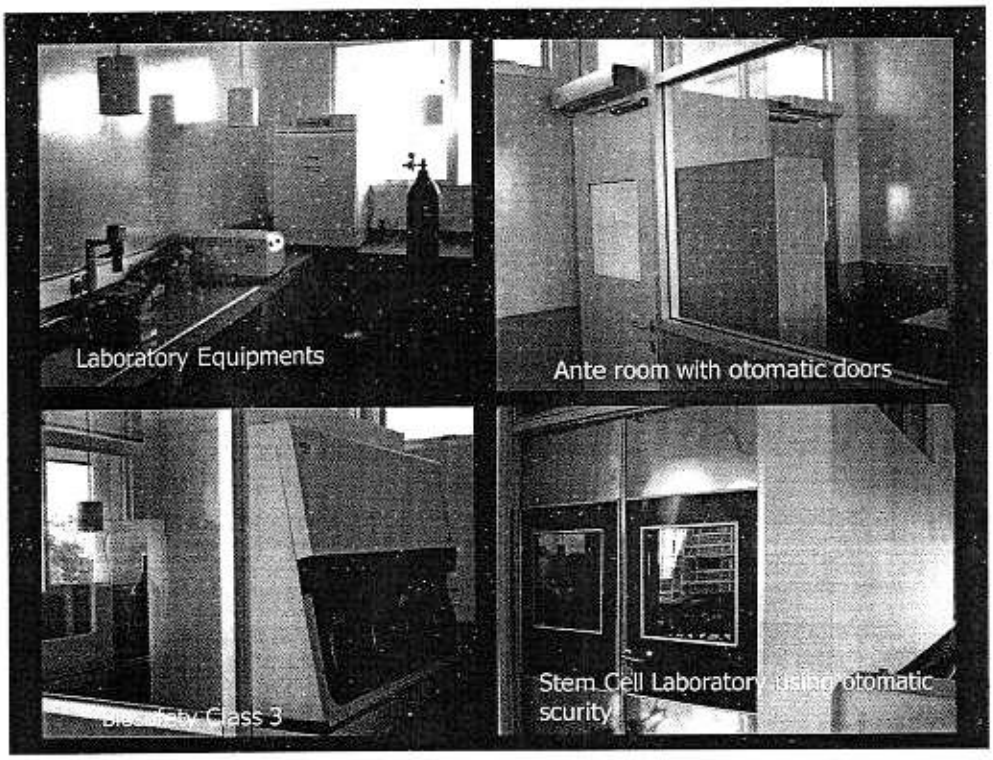

Figure 1. Laboratory of Stem Cell at Institute of Tropical Disease, Airlangga University

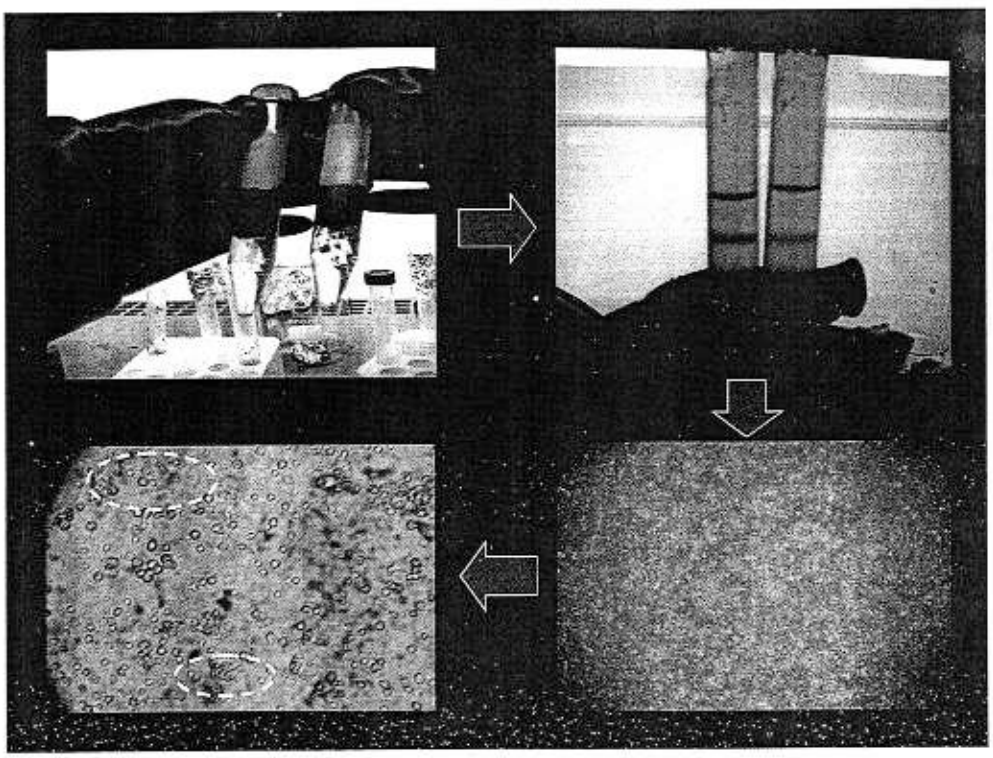

Figure 2. Isolation and Culturing Procedure of Nucleated from Pheripheral Blood

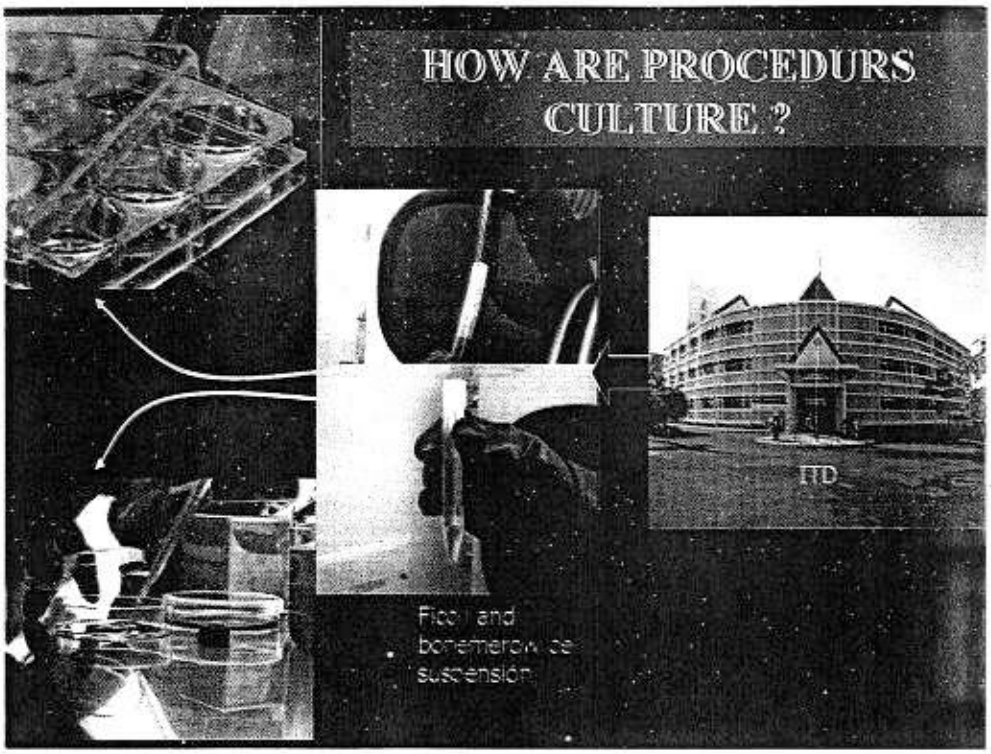

Figure 3. Isolation of Nucleated Cell from Bone Marrow

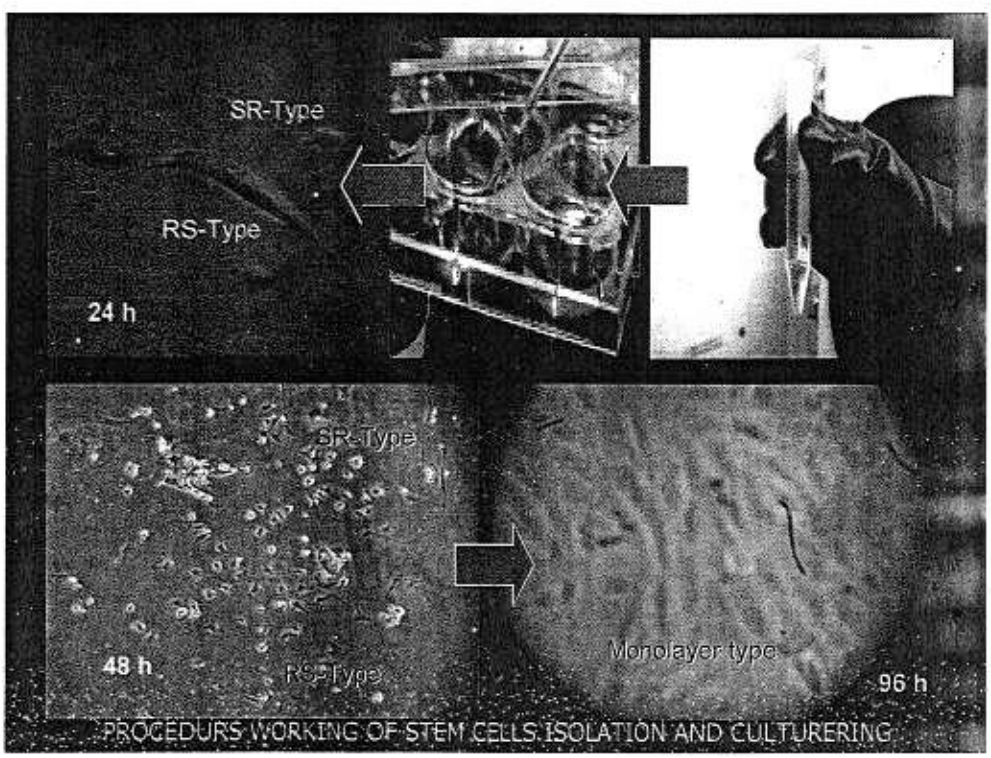

Figure 4. Culturing of Mesenchymal Cell of Nucleated Celll from Bone Marrow

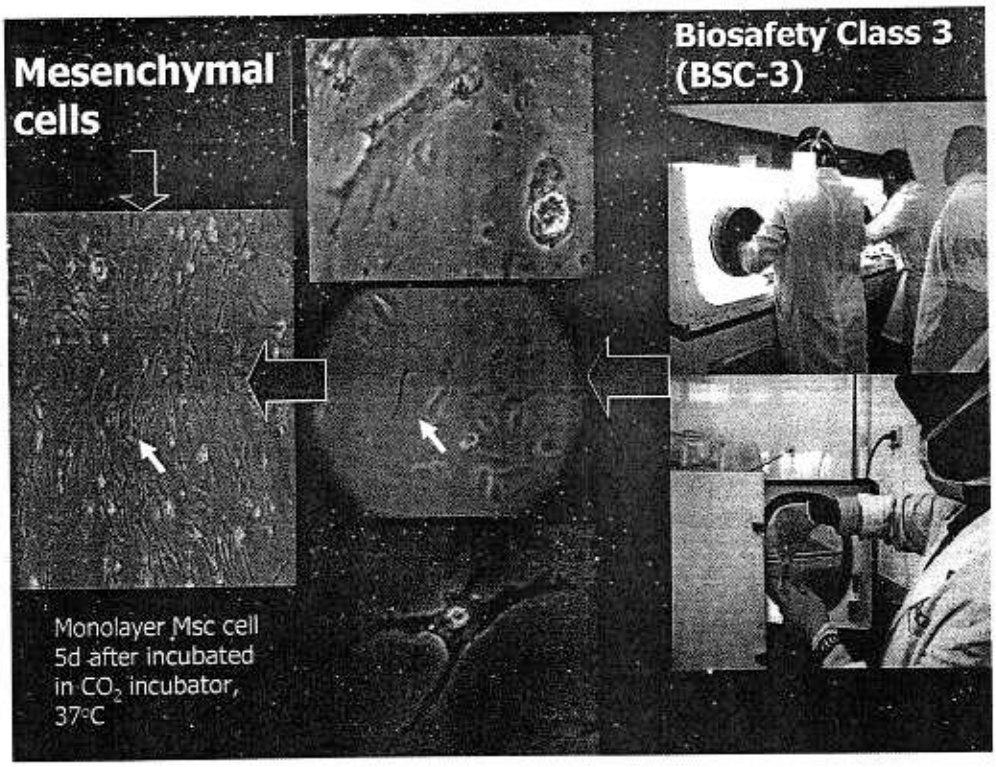

Figure 5. Isolation and Culturing Mesenchymal Cell from Human Bone Marrow

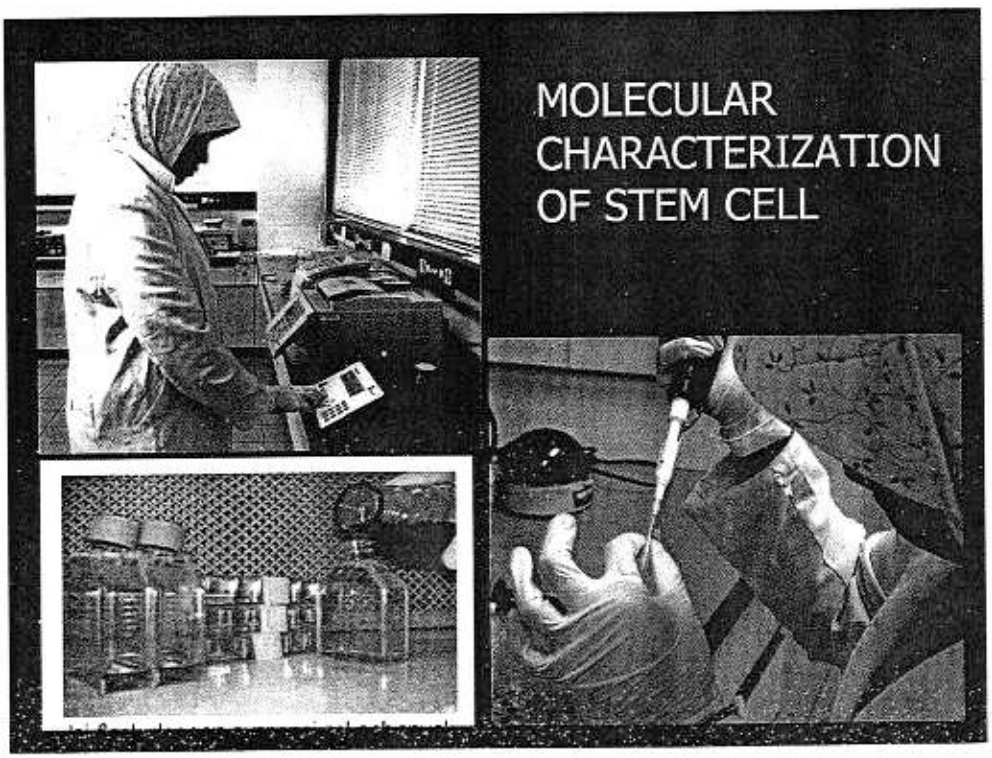

Figure 6. Molecular Characterization of Stem Cell Using Polymerase Chain Reaction (PCR)

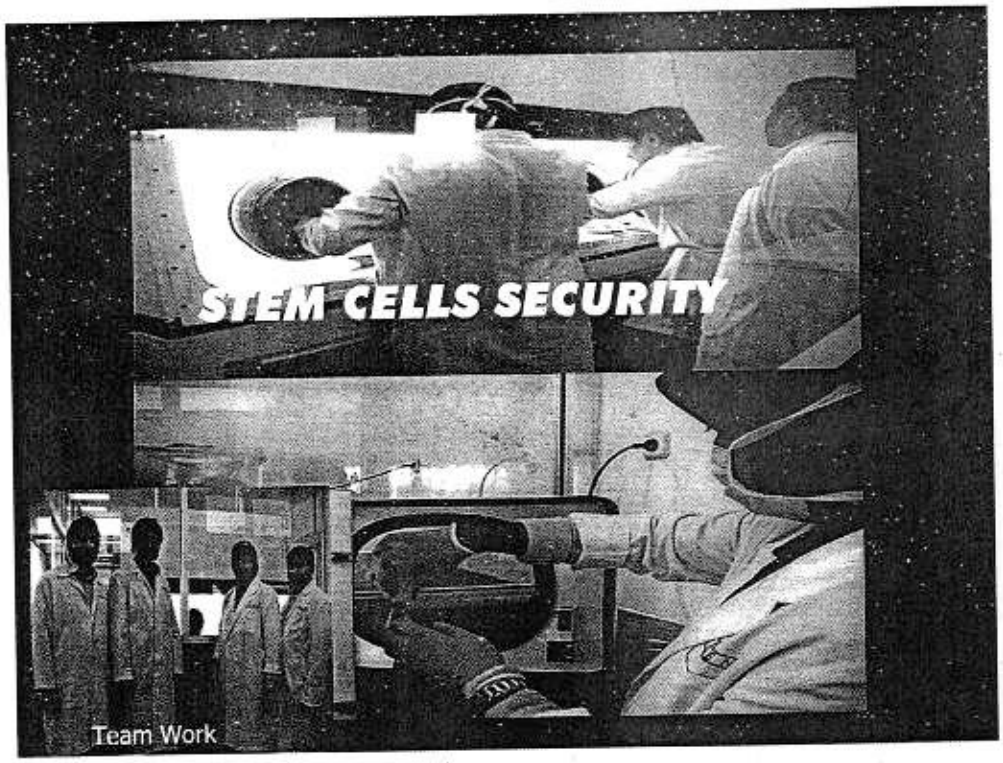

Figure 7. Atmospher of Human Resourches in Laboratory of Stem Cell at Institute of Tropical Disease (ITD) Airlangga University, Surabaya Indonesia