ISOLATION OF ADIPOSE STEM CELLS FROM HUMAN SKIN ADIPOSE TISSUE

by

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CERTIFICATE

This is to certify that the dissertation entitled "Isolation of Adipose Stem Cells from Human Skin Adipose Tissue" is the bonafide record of research work done by Ms Mohaini binti Mohamed during the period from July 2008 to October 2008 under my supervision.

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In the name of Allah, The Most Gracious and Most Merciful. All praise is due to Allah, for giving me inspiration and stoutheartedness along this journey.

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LIST OF ABBREVIATIONS AND SYMBOLS

ASCs -Adipose -derived stem cells

MSC-Mesenchymal stem cell

DPBS- Dulbecco's Phosphate Buffered Saline

DMEM-Dulbecco's Modified Eagle Medium

SVF-Stromal Vascular Fraction

ASMA-smooth muscle cell-specific alpha actin

HLA- human leukocyte antigen

GVHD- graft-versus-host disease

rpm- rotations per minute

FBS- Fetal Bovine Serum

PenStrep-Penicillin /Streptomycin

™-Trade Mark

®-Registered

CO_2-Carbon Dioxide

ml- milliliter

µl- microlitre

mg- milligram

i.e- id est

µm-micrometre

ABSTRACT

Traditionally, stem cells are isolated from bone marrow. These cells have the potency to develop into various types of cells. Thus, mesenchymal stem cells from bone marrow can be used in treating the diseases that need cells therapies or regenerative of cells. Much of the procedures that involve the aspiration of stem cells from bone marrow were invasive and painful. Thus, many researches try to find the alternative source of stem cells. Adipose tissue which is ubiquitous in human body and can be easily isolated promise as a novel source for tissue engineering and regenerative medicine. When compared with bone marrow stem cells, adipose-derived stem cells may develop into adipocytes, chondrocytes, osteocytes and myocytes in vitro. In this research project, adipose tissues from humans were collected and isolated in vitro. The isolation procedures involved centrifugation, filtration and culturing with DMEM: F-12 (1:1), FBS and PenStrep. Adipose stem cells were isolated from human subcutaneous adipose tissue, and appeared as fibroblast-like morphology observed under inverted phase-contrast microscope.

ABSTRACT

Traditionally, stem cells are isolated from bone marrow. These cells have the potency to develop into various types of cells. Thus, mesenchymal stem cells from bone marrow can be used in treating the diseases that need cells therapies or regenerative of cells. Much of the procedures that involve the aspiration of stem cells from bone marrow were invasive and painful. Thus, many researches try to find the alternative source of stem cells. Adipose tissue which is ubiquitous in human body and can be easily isolated promise as a novel source for tissue engineering and regenerative medicine. When compared with bone marrow stem cells, adipose-derived stem cells may develop into adipocytes, chondrocytes, osteocytes and myocytes in vitro. In this research project, adipose tissues from humans were collected and isolated in vitro. The isolation procedures involved centrifugation, filtration and culturing with DMEM: F-12 (1:1), FBS and PenStrep. Adipose stem cells were isolated from human subcutaneous adipose tissue, and appeared as fibroblast-like morphology observed under inverted phase-contrast microscope.

ABSTRAK

Stem sel selalunya diambil dari sum-sum tulang. Stem sel dari sumsum ini mempunyai potensi untuk membiak menjadi pelbagai jenis sel. Jadi sel meselkimal ini digunakan secara meluas dalam merawat penyakit yang memerlukan sel terapi dan perubatan regenerasi sel. Kebanyakan prosedur untuk mengambil stem sel dari sum-sum tulang adalah menyakitkan dan invasive. Jadi, banyak kajian yang telah dilakukan untuk mencari alternatif sumber stem sel. Tisu lemak didapati dikebanyakan organ dalam badan manusia dan senang untuk diasingkan dan ia merupakan satu sumber baru untuk kejuruteraan tisu dan perubatan regenerasi. Apabila dibandingkan dengan stem sel dari sum-sum tulang, stem sel dari tisu lemak juga boleh membiak menjadi sel adipos,kondrosite, osteosite and myosite in vitro. Dalam kajian ini, tisu lemak dari manusia akan diambil dan diasingkan untuk mendapatkan stem sel. Proses isolasi melibatkan sentifugasi, menapis and kultur dengan kultur medium yang sesuai iaitu DMEM: F-12 (1:1), FBS dan PenStrep. Stem sel dari tisu lemak diasingkan dari subkutin tisu lemak manusia menyerupai bentuk sel fibroblas apabila diperhatikan melalui mikroskop kontra fasa.

1.0 INTRODUCTION

1.1 Research Background

Stem cells have the ability to renew and differentiate into many specific tissue lineages. Many research regarding stem cells had been done worldwide since stem cells promises a great advantage in cell and tissue engineering applications. It was originally thought that these cells can be found exclusively in bone marrow. Apart from that, embryonic and placenta were also common stem cells source. Recently, it was found that stem cells also reside in other tissues such as adipose tissue, periosteum, synovial membrane, muscle, dermis, pericytes, blood and trabecular bone (Tuan *et al.,* 2003). Adipose tissue which is highly abundant in human body and easily accessible by simple surgical procedures with little discomfort makes this tissue most attractive source of stem cells. In addition, adipose tissue can be obtained repeatedly in large quantities with uncomplicated enzyme-based isolation procedure. Thus, it represent as an alternative source of stem cells as bone marrow procurement was extremely painful and high cost needed. Adipose-derived stem cells have equal potential compared to bone marrow stem cells to differentiate into many specific tissue lineages. They have the potential to differentiate into fat, bone, cartilage, tendons and skeletal muscle when cultivated under appropriate lineage specific conditions. Multipotent properties of these cells can be used in the management of human diseases, such as inherited, traumatic or degenerative bone, joint and soft tissue defects (skeletal regeneration and cartilage repair) (Schäffler and Büchler, 2007).

Adipose-derived stem cells are obtained primarily from subcutaneous fat from surgery. The isolation of cells from adipose tissue was pioneered by Rodbell and colleagues in the 1960s (Bunnell *et al.*, 2008) where they minced fats, washed extensively to remove contaminating hematopoietic cells, incubated tissue fragments with collagenase and centrifuged the digest, thereby separating the floating population of the mature adipocytes from the pelleted stromal vascular fraction (SVF). SVF which contain heterogenous cells including "preadipocytes" (Bunnell *et al.*, 2008). "Adherent cells can be expanded and used in a variety of assays" (Bunnell *et al.*, 2008).

There are terms had been used to refer to the adherent cells isolated from adipose tissue. The following terms have been used to identify the same adipose tissue cell population: adipose derived stem/stromal cells (ASCs), adipose – derived adult stem (ADAS) cells, adipose-derived adult stromal cells, adipose-derived stromal cells (ADSC), adipose stromal cells (ASCs), adipose mesenchymal stem cells (AdMSCs), lipoblast, pericyte, pre-adipocyte, processed lipoaspirate (PLA) cells. The International Fat Applied Technology Society reached a consensus to adopt the term "adipose-derived stem cells" (ASCs) to identify the isolated, plastic-adherent, multipotent cell population (Mitchell *et al.*, 2006; Gimble *et al.*, 2007; Schäffler and Büchler, 2007; Bunnell *et al.*, 2008). From now on, we will refer to these cells as ASCs.

There were many factors that influence the establishment of cells such as donor age, site of tissue taken, and methods of isolation and expansion (Schäffler

and Büchler, 2007). The establishment of adipose cells from human skin involving manipulation of some protocols that were used in previous research.

2.0 OBJECTIVE

The objective is to establish primary adipose stem cells from human skin adipose tissues in vitro.

3.0 LITERATURE REVIEW

3.1 Characteristics of stem cells

Stem cells are known as a potential source for cells and tissue regeneration for many years. Mainly they were found in the bone marrow and embryo. There are three characteristics of stem cells that distinguish them from other cell types. Stem cells have the ability to self-renew, which is able to undergo self division over long periods of time, thus increase the cell number. A number of stem cells will become up to millions of cells if they proliferate themselves in the laboratory. Stem cells are not specialized and they are not immature, meaning that they have no tissue specificity. Stem cells also have the capacity to differentiate into at least one type of specific tissue when induced or under normal physiologic condition (Gomillion *et al.*, 2006). Stem cells for regenerative medicinal applications should has these criteria; i) should be found in abundant quantities (millions to billions of cells); (ii) can be collected and harvested by a minimally invasive procedure; (iii) can be differentiated along multiple cell lineage pathways in a reproducible manner; (iv) can be safely and effectively transplanted to either an autologous or allogeneic host (Gimble, 2003).

In some organs, such as the gut and bone marrow, stem cells regularly divide to repair and replace worn out or damaged tissues. In other organs, however, such as the pancreas and the heart, stem cells only divide under special conditions. Stem cells capacity to differentiate into different cell phenotypes will be defined in several terms. The cells are either totipotent, pluripotent, multipotent or

unipotent. Totipotent stem cells are able to give rise to all embryonic somatic cells and germ cells. In other words, they can build a whole animal. The zygote and a few early cells of the morula are totipotent. Pluripotent stem cell is the cells that can give rise to cells derived from all three embryonic layers—mesoderm, endoderm, and ectoderm. These three germ layers are the embryonic source of all cells of the body. All of the many different kinds of specialized cells that make up the body are derived from one of these germ layers. Pluripotent cells have the potential to differentiate into all cells, excluding placental cells (Jennifer and Atala, 2008). In 1981, pluripotent cells were found in the inner cell mass of the mouse embryo. Multipotent stem cells produce cells of a particular lineage or closely related family. Unipotent stem cell, a term that is usually applied to a cell in adult organisms, which is the cells are capable of differentiating along only one lineage. In addition, it may be that the adult stem cells in many differentiated, undamaged tissues are typically unipotent and give rise to just one cell type under normal conditions. This process would allow a steady state of self-renewal.

3.2 Sources of stem cells

Bone marrow and embryonic stem cells were thought the only sources of stem cells in regenerative medicine. Recently, several sources of stem cells are recognized. There were two kinds of stem cells from animal and human that scientists worked with; embryonic and non –embryonic "somatic "or "adult" stem cells (Snow NE, 2003).

3.2.1 Embryonic stem cells

In 1981, scientists discovered ways to derive embryonic stem cells from early mouse embryos and the term "embryonic stem cell" was introduced. Then, in 1998, scientists discovered a method to derive stem cells from human embryos and grow the cells in the laboratory and the cells called as human embryonic stem cells. The embryonic stem cell is defined by its origin that is from one of the earliest stages of the development of the embryo, called the blastocyst. Embryonic stem cells harvested from inner cell mass of the pre-implantation blastocyst (3 to 5 days old embryo), and have been obtained from mice, non-human primates and human (Gomillion *et al.,* 2006). Human embryonic stem cells can be isolated from blastocyst using immunological technique. Blastocyst incubated with antibodies specific to the trophectoderm and complement to trophectoderm resulted in lysis of trophectoderm. Thus, the only surviving cells were inner cell mass (Jennifer, Anthony, 2008). The in vitro isolation of human embryonic stem cells involves transferring the inner cell mass of the blastocyst to a culture medium that is supplemented with mouse embryonic skin cells that have been treated that

prevents cellular differentiation of mouse embryonic skin cells (Atala, 2004). Mouse embryonic skin cells coated at the inner surface of culture dish and known as feeder layer. Feeder layer supply nutrients to inner cell mass and provide sticky surface for cells to attach.

Embryonic stem cells can be self-renewal; the cells can divide to make copies of them without differentiating and pluripotent; can give rise to all three embryonic germ layers even after being grown in culture for a long time. Ectoderm gives rise to brain, spinal cord, nerve cells, hair, skin, teeth, sensory cells of eyes, ears, nose, mouth and pigment cells. Mesoderm gives rise to muscles, blood, blood vessels, connective tissues, and the heart. Endoderm gives rise to the gut such as pancreas, stomach, liver, lungs, bladder, and germ cells (eggs or sperm). However, the use of embryonic stem cells was controversial and unethical because the act of taking blastocyst by destroying embryo was considered equivalent to destroying human life (Snow NE, 2003).

3.2.2 Adult Stem Cells

Adult stem cells also referred as somatic stem cells or mesenchymal stem cells are undifferentiated cells, found in a specific tissue or organ (Gomillion *et al.*, 2006). Somatic refers to cells of the body except germ cells-sperms and ovum. These cells are self-renewing and are able to differentiate into major specialized cell types that serve to maintain the function and repair the tissues in which they

are found (Vats *et al.*, 2002). These adult stem cells typically include hematopoietic stem cells, neural stem cells, bone marrow stem cells, dermal stem cells and fetal cord blood stem cells (Gomillion *et al.*, 2006). It was discovered that bone marrow contains at least two kinds of stem cells. They are hematopoietic stem cells and bone marrow stem cells. Hematopoietic stem cells form all the types of blood cells in the body, whereas bone marrow stromal stem cells were discovered a few years later. Stem cells from bone marrow are multipotent and able to differentiate into variety of cell lineages such as adipocytes, osteocytes, myocytes, tenocytes and neural cells (Ballas *et al.*, 2002; Zandstra and Nagy, 2001). (Friedenstein 1991) developed in vitro culture methods for differentiation of bone marrow cells. Bone marrow stem cells originally referred to as fibroblast colony forming units, then in the hematological literature as marrow stromal, subsequently as mesenchymal stem cells, and most recently as multipotent mesenchymal stromal cells (MSCs) (Bunnell *et al.*,2008; Kassem and Abdullah, 2008).

These adult stem cells also found to reside in a diverse host of tissues throughout the adult organism and possess the ability to 'regenerate' cell type's specific for these tissues. Examples of these tissues include adipose tissue, periosteum, synovial membrane, muscle, dermis, pericytes, blood and trabecular bone.

3.2.3 ASCs

Adipose tissue is a highly complex tissue which consists of mature adipocytes, preadipocytes, fibroblasts, vascular smooth muscle, endothelial cell, macrophages (Weisberg *et al.*, 2003; Xu *et al.*, 2003) and lymphocytes (Caspar-Bauguil *et al.*, 2005). It is derived from mesenchyme and contains supportive stroma that is easily isolated (Izadpanah *et al.*,2006; Zuk *et al.*,2002). Three criteria have been proposed to define mesenchymal stem cells: a)adherence to plastic; b) multipotent differentiation potential to osteoblasts, adipocytes and chondroblasts; and c) specific surface antigen expression. Stem cells isolated from adipose tissue cultured and seeded on scaffold that will support growth and proliferation. The scaffold material was degraded and new mass of tissue remained on the site of transplanted tissue (Gomillion *et al.*, 2006).

3.3 Function, Anatomy and Physiology of Adipose Tissue

3.3.1 Types of adipose tissue

There are two main forms of adipose tissue; brown and white. Brown adipose tissue named because of its colour and it has high vascularity. Brown adipose tissue is found in the newborn human as a thermogenic organ. Located around the major organs and the brown adipose tissue expresses a unique "uncoupling protein" that allows energy produced in the mitochondria to be converted directly into heat rather than adenosine triphosphate (ATP). This protects the individual during infancy (Sanz-Ruiz *et al.*, 2008). With advancing age, it can be replaced by white adipose tissue that functions as energy source. The

other types of adipose tissue are mammary that function in lactation, mechanical that can be found at palms of the hands, the soles of the feet and infrapatellar and retro-orbital fat pads. Other type that is usually overlooked is bone marrow fat. The function of the marrow adipocyte remains controversial. Some have postulated that bone marrow adipocytes serve a passive role, simply occupying space that is no longer required for hematopoiesis. Other hypothesis said that it functions in energy metabolism, hematopoiesis and osteogenic events (Gimble *et al.*, 2004). Adipose secretion of adiponectin, leptin, resistin and other adipokines axerts systemic physiological and pathological effects (Gimble *et al.*, 2004).

3.3.2 Subcutaneous Adipose Tissue

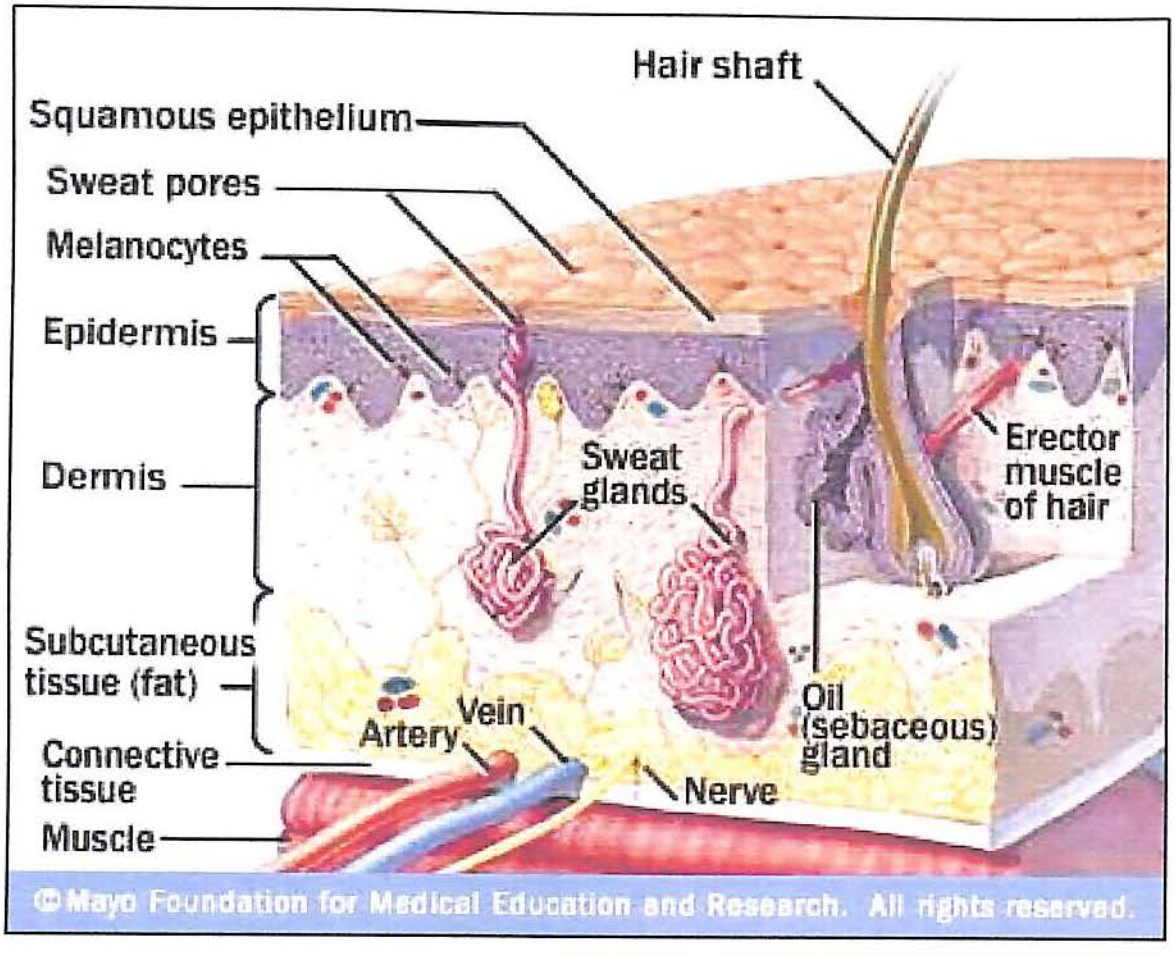


Figure 3.1: Anatomy of the skin

Skin consists of outer layer called epidermis and inner layer called dermis. Subcutaneous fat is found just beneath the skin as opposed to visceral fat which is found in the peritoneal cavity. The subcutaneous tissue may also be called the superficial fascia. It was made of areolar connective tissue and adipose tissue, the superficial fascia connects the dermis to the underlying muscles. Areolar connective tissue or loose connective tissue contains collagen and elastin fibers

and many white blood cells that have left capillaries to wander around in the tissue fluid between skin and muscles. Subcutaneous adipose tissue is a particularly attractive reservoir of progenitor cells, because it is easily accessible, abundant, and self-replenishing. It is derived from the mesodermal germ layer and contains a supportive stromal vascular fraction (SVF) that can be readily isolated (Gronthos *et al.,* 2003; Zuk *et al,* 2001). SVF are cells that were obtained after collagenase digestion (San-Ruiz *et al.*, 2008). SVF has not been further separated based on adherence to tissue culture plastic (Katz *et al.*, 2005).

This SVF from adipose tissue consists of a heterogeneous mixture of cells, including endothelial cells, smooth muscle cells, pericytes, leukocytes, mast cells, and pre-adipocytes (Oedayrajsingh- Varma *et al.*, 2006; Peterson *et al.*, 2005). In addition to these cells, the SVF contains an abundant population of multipotent adipose-tissue-derived stem cells (ASCs) that possess the capacity to differentiate into cells of mesodermal origin in vitro such as adipocytes, chondrocytes, osteoblasts, and (cardio) myocytes (Erickson *et al.*, 2002; Guilak *et al.*, 2004; Halvorsen *et al.*, 2001; Hattori *et al.*, 2004; Planat-Benard *et al.*, 2004; Rangappa *et al.*, 2003; Zuk *et al.*, 2001).

3.4 Factors influencing yield of ASCs

Both the type of surgical procedure and anatomical site of the adipose tissue affect the total number of viable cells that can be obtained from cells suspension. The effect of three different surgical procedures for the harvesting of adipose tissue, i.e., resection, tumescent or conventional liposuction, and

ultrasound-assisted liposuction, on the yield and function of the stem cells had been investigated. The SVF isolates from adipose tissue harvested by ultrasound-assisted liposuction contain fewer stem cells, and the stem cells have a longer population doubling time. So that resection and tumescent liposuction are preferable to ultrasound-assisted liposuction for harvesting adipose tissue, if the cells are to be used for tissue-engineering purposes (Oedayrajsingh-Varma *et al.*, 2006). Adipose tissue from different site of harvesting influence the yield of adipose derived stem cells, but does not influence the abilities of ASCs to differentiate into cell lineages and total amount of nucleated cells per volume (Oedayrajsingh-Varma *et al.*, 2008). The abdomen is preferable than hip/ thigh region for harvesting adipose tissue (Oedayrajsingh-Varma *et al.*, 2008). A research done by Toyoda *et al*, 2009 showed that subcutaneous adipose tissue contained more ASCs than omental adipose tissue, while omental adipose tissue contained more blood-derived cells.

3.5 Differentiation of ASCs

ASCs has the ability to differentiate to cells of adipogenic, chondrogenic, myogenic and osteogenic lineage when cultured with the appropriate lineage specific stimuli as shown in the table below (Gomillion *et al.*, 2006). The multipotentcy has generated interest in their potential therapeutic value. The age of donor can be a factor of differentiation, since some studies suggested that the differentiation capacity was higher in culture from younger subjects compared to older people (Bunnell *et al.*, 2008). Many scientists had used adipose stem cells in repairing the organ and disease management. Mostly done in treating the diseases that involved cardiac, skeletal and bone defect. Table 3.1 summarizes the clinical implications of adipose-derived stem cells (Schäffler and Büchler 2007). This property of ASCs contributes to future perspectives in organ-specific tissue engineering and tissue reconstruction. However, many of these theoretical applications (i.e., cardiomyocytes or neuronal cells) are far from clinical use. Moreover, safety issues concerning the clinical applications might resemble those when using bone marrow-derived mesenchymal stem cell but have not yet been investigated extensively in the human system (Schäffler and Büchler 2007).

Type of differentiation	Clinical implications
Adipogenic	Breast soft tissue reconstruction after tumor surgery for breast cancer, breast asymmetry, and soft tissue and subdermal defects after trauma, surgery, or burn injury
Chondrogenic	Cartilage repair in joint and disc defects, plastic reconstruction of ear and nose defects
Osteogenic	Skeletal regeneration of inherited and tumor- or trauma-induced bone defects
Myogenic	Tissue reconstruction after trauma and surgery, dystrophic muscle disorder
Cardiomyogenic	Heart muscle regeneration, functional improvement after myocardial infarction, heart failure
Vascular/endothelial	Neovascularization, ischemic diseases
Neurogenic	Brain injury, stroke, peripheral nerve injury
Pancreatic/endocrine	Insulin-secreting cells, type 1 diabetes Mellitus
Hepatic	Chronic liver failure, hepatic regeneration, hepatocyte transplantation
Hematopoietic GVHD	Bone marrow support

Table 3.1: Clinical implications of ASCs.

3.6 Adipose Stem Cell Markers

Subcutaneous adipose tissue contains heterogenous mixture of cells. Stem cells markers are important in order to isolate and recognize stem cells population in adipose tissue for further use. Mesenchymal stem cells expressed surface proteins. These proteins can be used as marker to identify stem cells. Surface proteins can be classified into some categories; adhesion molecules such as integrins (CD29 and CD49e), receptor molecules such as hyaluronate (CD44), cadherins(CD144), surface enzymes (CD73),extracellular matrix proteins (CD90, CD105), intercellular adhesion molecules (CD54), vascular adhesion molecules (CD106), complement regulatory molecules and histocompatibility antigens (Hazel *et al.*, 2009).

According to the International Society for Cellular Therapy (ISCT), the minimal criteria that define mesenchymal stem cells (MSC) as the following; a) Adherence to plastic in standard culture conditions; b) Phenotype positive (≥95 %+):CD105, CD73, CD9 and phenotype negative (≤2 %+):CD45, CD34, CD14 or CD11b, CD79 α or CD19 and human leukocyte antigen (HLA-DR); and c) in vitro differentiation: osteoblast, adipocytes, chondroblast (demonstrated by staining of in vitro cell) (Schäffler and Büchler 2007).

Both PLA (processed lipoaspirate) and mesenchymal stem cells expressed CD29, CD44, CD71, CD90 and CD105/SH2 and SH3, which together with SH2, is considered a marker for MSCs (Zuk *et al*,2002). There was no expression of the hematopoietic lineage markers CD31, CD34 and CD45 was observed in cultures of

PLA and bone marrow mesenchymal stem cells. Two CD marker antigen differ in PLA and bone marrow mesenchymal stem cells populations were CD49d (α4 integrin) and CD106 (VCAM). Specifically, PLA expressed CD49d, whereas this antigen not expressed in bone marrow mesenchymal stem cells. Many papers analyzed cellular markers and genes present on adipose-derived stem cells. The table below summarizes the expression of cellular markers and genes on ASCs according to data derived from the literature (Wagner *et al.*, 2005; Oedayrajsingh-Varma *et al.*, 2006; Gronthos *et al.*, 2001; Oedayrajsingh –Varma *et al.*, 2007; Mitchell *et al.*, 2006). Note that all the gene and surface marker expression profiles apply to in vitro-expanded cells, not primary cells. All attempts to establish both an exact phenotypical definition of mesenchymal stem cells and a clear discrimination between these cells and fibroblasts have been unsuccessful up to now.

Positive cellular markers and genes	Negative cellular markers and genes
CD9	CD11b
CD10	CD14
CD13	CD19
CD29	CD31
CD44	CD34
CD49	CD45
CD54	CD79α
CD55	CD80
CD59	CD117
CD73	CD133
CD90	CD144
CD105	HLA-DR
CD106	c-kit
CD146	Myd88
CD166	STRO-I
HLA-I	Lin
Fibronectin	HLA II
Ebdomucin	
ASMA	
Vimentin	
Collagen –I	

Table 3.2: Summary of cellular markers and genes for ASCs

4.0 MATERIALS AND METHOD

4.1 Materials

4.1.1 Human skin adipose tissue

Samples from surgery; subcutaneous adipose tissue were obtained from elective surgical procedures in the Department of Plastic Surgery, Hospital Universiti Sains Malaysia, Kelantan. Sample processing was done in sterile laminar flow to avoid contamination. Generally, sample was minced, centrifuged and cultured using appropriate medium. The last part was identification of adipose stem cells by surface markers.

4.1.2 Culture materials

1X Dulbecco's Phosphate-buffered saline (GIBCO ®), 0.075% collagenase Type I, 1X Dulbecco's Modified Eagle Medium (GIBCO ®), culture medium -10% FBS in DMEM, Penicillin Streptomycin (GIBCO®), centrifuge, CO_2 Incubator, culture plates, Trypsin (TrypLE Express), 70 µm cell strainer nylon, BD Falcon™, 50ml tubes , T-25 flasks, scissors, scalpels.

4.2 Methodology

4.2.1 Preparation of culture medium (DMEM: F12, 1:1)

22 ml of F-12 Nutrient Mixture (HAM), 25 ml of DMEM 1x, 200 µl of PenStrep, 10% of FBS were prepared in a 50 ml tube.

4.2.2 Method of Isolation and Culturing

Sample processed in sterile laminar air flow to avoid contamination. Subcutaneous fat was cut from skin. Fat tissue was washed in ethanol to remove debris and blood by using scalpel and scissor in petri dish. Then, washed extensively with 20ml of (PBS) containing 5% PenStrep. Sample minced into major portion only using scissors. Upon removal of blood and debris, adipose tissue minced in 15 ml of 0.075% collagenase type I with 1 ml P/S using scissors and scalpel into possibly smallest part of tissue. Collagenase used to digest extracellular matrix to release cellular fraction. The sample that has been minced and collagenase solution aspirated using pipette into a 50 ml tube and incubated in the water bath for an hour. Then, tissue filtered through 70 µm cell strainer into a new 50 ml tube and centrifuged at 2000 rpm for 5 minutes to obtain the cells. After centrifugation, supernatant was discarded without disturbing the cells and the cells suspended in 20ml of PBS. The sample pipetted up and down several times to further disintegrate aggregates of adipose tissue. Cells suspension centrifuged once again for 5 minutes at 2000 rpm and supernatant discarded. Cells suspended in 5ml of culture medium which consists of DMEM: F12 with added 10 % of FBS and PenStrep. Cells were inoculated in T-25 culture flask. Cells observed under

inverted phase-contrast microscope and maintained in the culture medium at 37°C with 5% of CO_2.

After two days, the entire medium aspirated from the culture flask. The cells washed with PBS to remove any tissue fragments or blood cells by pipetting the solution over the cells. New culture medium replaced into the flask. The cells maintained in the incubator at 37°C with 5%CO_2. Culture medium replaced for every two days to make sure that the cells have enough supplements in order for the cells to expand and renew themselves. When the cell growth had reach 70-80% confluence, cells were subcultured into new culture plate.

4.2.3 Subculture

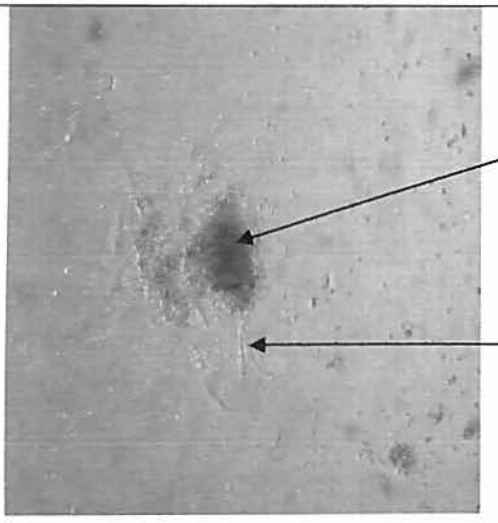
Culture medium was discarded by aspirating the medium with pipette and the flask was washed with 4ml of PBS. Five ml of trypsin (TrypLE Express) added in culture plate and incubated in the incubator for about 5 minutes and observed under microscope whether the cells detached or not. Trypsin was used to detach the cells from culture plate. Eight ml of culture medium added into culture plate to inactivate activity of trypsin because trypsin might cause cell death if was not inactivated. The cells centrifuged in 50ml tube at 2000rpm for 5 minutes. Supernatant discarded. Cells suspended in 5ml of culture medium (DMEM: F12) and plated in a well and the solution suspended to disperse the cells over the culture plate. Cells maintained in the incubator with 5% CO_2 at 37°C. Culture medium replaced every 2 days.

5.0 RESULT

5.1 Culture of ASCs

The morphology of cells was observed with inverted phase-contrast microscope after two days primary cells were cultured with DMEM: F12 medium. Morphology of the adipose-derived stem cells generally resembled fibroblast–like cells namely spindle-shaped cells.

A) Day 3 primary adipose derived stem cells



Magnification: 25x 10x

Description:

Stem cell start to renew themselves. Note that the cells which was fibroblast- like grow out from adipose cells.

Figure 5.1: ASCs at day 3