EKSPRESI PROTEIN CD73/CD90/CD105/CD34/CD45/CD11b/CD19/HLA-DR PADA SEL PUNCA ASAL JARINGAN LEMAK MANUSIA MENGGUNAKAN *FLOW CYTOMETRY*

EXPRESSION OF PROTEIN CD73/CD90/CD105/CD34/CD45/CD11b/CD19/HLA-DR ON STEM CELLS FROM HUMAN FAT TISSUE, USING CYTOMETRY FLOW

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Abstrak

Sel punca merupakan sel yang dapat membelah dan berdiferensiasi menjadi sel jenis lainnya. Sel punca asal jaringan lemak potensial dikembangkan sebagai salah satu alternatif sel punca yang bersumber dari limbah sedot lemak manusia. Sel punca asal jaringan lemak akan mengekspresikan protein spesifik penanda permukaan CD73, CD90, CD105 dalam persentase yang tinggi dan CD34/CD45/CD11b/CD19/HLA-DR dalam persentase yang rendah. Studi ini bertujuan untuk memanfaatkan limbah sedot lemak manusia dengan melakukan isolasi sel punca asal jaringan lemak dan menguji protein penanda permukaan spesifik sel punca. Beberapa tahapan dalam studi ini adalah isolasi *stromal vascular fraction* (SVF) dan kultur sel punca asal jaringan lemak manusia, *population doubling time* (PDT) serta analisis protein penanda permukaan CD7Ee3, CD90, CD105, dan CD34/CD45/CD11b/CD19/HLA-DR pada pasase ke-1 dari 3 donor. Hasil dari studi ini menunjukkan bahwa sel dari jaringan lemak berhasil dikultur dengan durasi pembelahan sel adalah 3,3 hari. Sel mengekspresikan CD73 (99,79%), CD90 (94,17%), CD105 (48,75%), dan CD34/CD45/CD11b/CD19/HLA-DR (kurang dari 2%). Ekspresi CD105 yang rendah dari ketiga donor diduga berkaitan dengan tingkatan pasase sel yang digunakan. Berdasarkan hasil tersebut dapat disimpulkan bahwa sel punca asal jaringan lemak pasase ke-1 telah mengekspresikan ketiga marker protein penanda permukaan sel punca, yaitu CD73, CD90 dan CD105.

Kata kunci: CD73; CD90; CD105; Jaringan lemak; Protein permukaan; Sel punca

Abstract

Stem cells are cells that can divide into other different types of similar cells. Stem cells from fat tissue potential have been developed as an alternative stem cell from human liposuction. Stem cells from fat tissue will express high protein-specific markers on CD73, CD90, CD105 and CD34/CD45/CD11b/CD19/HLA-DR in a low percentage. This study aims to utilize human liposuction waste by isolating stem cells from fat tissue and testing protein-specific stem cell surface markers. Some stages in this study are isolation of stromal vascular fraction (SVF) and stem cell culture from human fat tissue, population doubling time (PDT) and protein analysis of surface markers CD73, CD90, CD105, and CD34/CD45/CD11b/CD19/HLA-DR on the 1st passage of 3 donors. The results of this study showed that cells from fat tissue were successfully cultured with cell division duration of 3.3 days. Cells expressed CD73 (99.79%), CD90 (94.17%), CD105 (48.75%), and CD34/CD45/CD11b/CD19/HLA-DR (less than 2%). The low expression of CD105 from all three donors is thought to be related to the level of cell passage used. Based on these results, it can be concluded that the stem cells from first passage fat tissue have expressed the three protein markers of stem cell surface markers, namely CD73, CD90 and CD105.

Keywords: CD73; CD90; CD105; Fat tissue; Stem cell; Surface protein

INTRODUCTION

Stem cells are cells that can renew themselves and differentiate into other types of cells (Nicoletti, De Francesco, D'Andrea, & Ferraro, 2015). Stem cells were first discovered in the 19th century grouped into two based on their sources, which is sourced from embryonic (embryonic stem cells) and adult tissue (adult stem cells) (Zuk, 2010). Stem cells from embryonic are pluripotent, which can differentiate into many types of cells, whereas stem cells from adult tissue are multipotent/unipotent, which can differentiate into three or one cell types only. Embryonic stem cells have weaknesses related to ethics, so stem cells from adult tissue are more commonly used as cell sources in research (Robertson, 2010; Kern, Eichler, Stoeve, Klüter, & Bieback, 2006). The ability and manipulation techniques of stem cells from adult tissues are still being developed, researched and studied. Some sources of stem cells from adult tissues that have been developed recently are stem cells from muscles, nerves, umbilical cord, blood, bone marrow and fat tissue (Zuk, 2010; Mennan *et al.*, 2013).

Stem cells from fat tissue (adipose-derived stem cells or ADSCs) are a source of stem cells that are widely used because they are thought to originate from liposuction surgery waste (Zuk, 2010). The number of ADSCs is also 5 to 50 times more than bone marrow stem cells (bone marrow mesenchymal stem cells or BMSCs). The characteristics of ADSCs are almost the same as bone marrow stem cells, but the advantages of ADSCs compared to bone marrow stem cells include that stem cells can be obtained more with more accessible procedures and lower risk. The comparison of the number of BMSCs with ADSCs is 100–1,000 cells with ~5,000 cells. Both sources of stem cells can be grown (cultured) on Dulbecco's Modified Eagle's (DMEM) medium (Gibco) which generally contains 10% fetal bovine serum (FBS) (Zhu et al., 2008; Pawitan, 2009).

BMSCs express the certain surface marker proteins (surface markers) that are characteristic of mesenchymal stem cells (Mennan *et al*., 2013; Zhang, Meng, Zhang, Chen, & Wang, 2017). Stem cell surface markers from fat tissue do not yet have a certain standard consensus. The International Society for Cellular Therapy (ISCT) sets the standard for BMSCs surface markers, namely hematopoietic markers. CD34/CD45/CD11b/CD19/HLA-DR must be negative or less than 2%, while maximal markers CD73, CD90, CD105 must be more than 95% (Dominici et al., 2006). Surface marker proteins of CD73, CD90, CD105 have been confirmed that these proteins are also expressed in stem cells from fatty tissue origin by Zhang et al. (2017). Therefore, in this study, an analysis of human ADSCs surface marker protein expression analyze was conducted to study and prove the level of surface protein expression of CD73, CD90, and CD105. Stem cells are obtained from a stromal vascular fraction (SVF) which is enzymatically isolated in fat tissue and cultured until the 1st passage. The duration needed for cells to divide is also analyzed using population doubling time (PDT) techniques.

MATERIALS AND METHODS
Isolation of Stromal Vascular Fraction (SVF)

Stromal vascular fraction (SVF) cells were isolated from 3 donors of lipoaspirate fat tissue under a woman's skin. Isolation of human ADSCs refers to the protocol of Remelia, Rosadi, Sobariah, Rosliana, and Karina (2016), which performs the process of fat tissue digestion in the solution of the recombinant H-Remedy enzyme. As many as 10% of the recombinant enzyme was added to the fat tissue samples by incubation at a temperature of 37 °C, 300 rpm, for 1 hour. The enzyme was then activated by adding Dulbecco's Modified Eagle's (DMEM) low glucose (LG) media (1 g/L) containing L-glutamine (4 mM) (Gibco, USA) and centrifuged at 600 xg for 5 minutes. The supernatant formed in the upper layer then discarded, and re-centrifuged at 600 xg for 10 minutes. SVF cell pellets containing ADSCs cultured at 37 °C, 5% CO_2.

ADSC Culture

Stem cells were cultured in the DMEM standard medium (Gibco, USA) which had been given antibiotic-antimycotic concen-

tration 100x (Gibco, USA). Culture medium was changed every three days to maintain cell growth. Cells that have experienced 80% confluence were then harvested. Cells that will be harvested were washed first using Hank's Balanced Salt Solution (HBSS) (Gibco, USA). Then added Triple Select (Gibco, USA) and incubated for 5−10 minutes in the incubator CO_2 37 °C. Stem cells that have been separated (detach), then added DMEM growth medium containing 10% fetal bovine serum (FBS) (Gibco, USA). The cell was then centrifuged at 600 xg for 5 minutes. The number of cells was calculated using a haemocytometer with 0.4% trypan blue solution (Sigma-Aldrich). The number of cells was then cultured for multiplication to get a passage 1 (P1). They were then analyzed by population doubling time (PDT) and specific-protein stem cell surface marker.

Population Doubling Time (PDT)

PDT is calculated based on the number of Stem cells from passage 0 human fat tissue implanted in the flask with the required duration of time. To be calculated until the cell undergoes confluence. The average number of cells cultured for PDT is 5000/cm² cells. This test is used to find out how long a cell divides from one into two cells. PDT is analyzed using the following formula:

$$PDT = \frac{\log 2 \; x \; \Delta t}{\log(NH) - \log(NI)},$$

where Δt= time from planting to harvest cells, NH= total cells were harvested, NI= total cells that were grown. The average PDT from various stem cell donors was then tabulated and analyzed.

Analysis of Protein Expressions CD73, CD90, CD105 and CD34/CD45/CD11b/CD19/HLA-DR

The first passage cell was harvested and washed with HBBS then centrifuged 1,200 rpm for 10 minutes. Then the cell was resuspended in MACSQuant *running buffer* (130−092−747) 1 mL. A total of 15 µL cell suspensions were used to calculate the number of cells and the remainder was used for *flow cytometry* checks. The kit for checking ADSCs mesenchymal characters consists of several antibody *cocktails* listed on the *BD Stemflow ™ hMSC Analysis Kit*. Each tube was labelled, and 2 µL reagents were added, as in the provisions in Table 1.

Table 1. Description label and contents of *flow cytometry* inspection tubes for ADSCs characterization

Tube No.	Content	Purpose
1	Cell + *FITC Mouse Anti-human CD90*	Compensation
2	Cell + *PE Mouse Anti-human CD44*	Compensation
3	Cell + *PerCP-Cy^{TM}5.5 Anti-human Mouse CD105*	Compensation
4	Cell + *APC Mouse anti-human CD73*	Compensation
5	Cell	Instrument setting
6	Cell + *PE hMSC Negative Isotype Control Cocktail + PE hMSC Negative Isotype Control Cocktail*	Gatting
7	Cell + *hMSC Positive Cocktail + PE hMSC Negative Cocktail*	Sample analysis

Cells that have been added by reactor are vortexed, then incubated for 30 minutes in a dark room (covered with aluminium foil and put in a shelf). Then the cell suspension is centrifuged for 10 minutes, 1,200 rpm. Then the cell pellets are washed with 500 µL BD FacsFlow and centrifuged (1,200 rpm, 5 minutes). Cell washing was conducted twice. Then the cell is resuspended in a 300 µL MACSQuant running buffer and read with a flow cytometer. The sample readings start sequentially with the following conditions: (1) Tube 5 which contains only cells, the result of reading is used to determine the cell population that will be analyzed (gatting); (2) Tube 6, which contains cells and mixture of isotype control for positive and negative cocktail, the result of reading is also used for determining cells population that will be further analyzed; this step was conducted to get rid of the non specific binding of Fc receptor with antibody or other cellular protein interaction that might occur, so that the specific the specific antibody primer reagen and antigen on cell can be

confirmed; (3) Tube 1 containing cells and FITC Mouse anti human CD90; this phase was conducted to compensate the overlap fluorochrome PE and FITC; (4) Tube 2 which contains cells and PE anti human mouse CD44; this phase was conducted to compensate the overlap fluorochrome PE and FITC; (5) Tube 3 containing cels and PerCP-Cy^{TM}5.5 Mouse anti human CD105; this step was conducted to compensate for overlapping fluochromespectra PerCP-Cy^{TM}5.5 and APC; (6) Tube 4 containing cells and pc Mouse Anti Human Cd73; this step was conducted to compensate for overlapping fluorodromes-pectra PerCP-Cy^{TM}5.5 and APC; (7) Tube 6 containing cells and mixture of isotype control for positive and negative cocktail for reconfirmation of gatting; (8) Tube 7 which contains cells and mixture of positive and negative coctails for reading real sample.

The surface markers analyzed were CD73, CD90 and CD105 markers (positive >95%) and CD34/CD45/CD11b/CD19/HLA-DR (positive <2%) markers according to criteria established by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006).

RESULTS

Total SVF cells obtained and cultured from a variety of donors varies with an average of 1.18×10^8 cells SVF. The average SVF cell viability of the three donors was 98.90% (Table 2). Stem cells from SVF cultures have been observed attached to the bottom of the *flask*. Morphological forms of stem cells are like fibroblast cells. The time needed for stem cells in passage 0 to reach the confluence of each donor has a different time span. Cell confluence of more than 80% is presented in Figure 1.

The average confluence of passage 0 (P0) cells was 13.5 days with an average number of stem cells obtained 3.2×10^5 cells. P0 stem cells are reproduced again to passage 1 (P1) with an average cell harvest value of 9.6×10^5 cells and with the duration of time needed for confluent is 7.5 days. PDSC values of ADSCs from all three donors indicate that cells need time to divide into two cells for 3.3 days. Based on these results shows that ADSCs from the three donors have been successfully cultured and propagated for further analysis of specific protein surface stem cell markers.
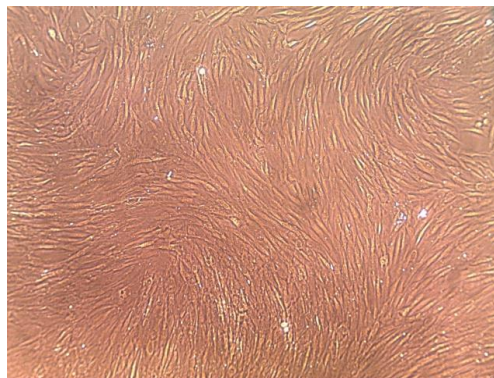
Figure 1. Morphology of stem cells from human fat tissue attached to the bottom of the cup and reaching confluence on day 15, Passage 0, Magnification 10x, Optilab, Nikon

**Table 2.** Number of SVF cells, number of stem cells and population doubling time

Sample	Gender	SVF Cell (cell)	Viability (%)	Passage stem cells (P)				PDT (day)
				P0 (cell)	Duration (days)	P1 (cell)	Duration (days)	
Donor-1	female	63,000,000	98.4	370,000	12	1,013,000	10	3,4
Donor-2	female	150,000,000	98.68	270,000	15	920,000	5	2.8
Donor-3	female	143,550,000	99.62	750,000	17	970,000	11	3,7
Average		118,850,000	98.9	320,000	13.5	966,500	7.5	3,3

Human passage 1 (P1) ADSCs from all three donors expressed the positive protein surface markers CD73, CD90 and CD105 presented in Figure 2. The X-axis on the

histogram with two blue and red peaks indicates the intensity of fluorescence detected while the Y-axis indicates the number of cells. The peak of the histogram in red is the *isotype* of the sample while the peak of the histogram in blue is the luminescence of the sample that has been labelled with specific antibodies namely CD73, CD90, CD105 and CD34/CD45/CD11b/CD19/HLA-DR. The percentage of surface proteins expressed positive stem cells from a population quantified by means of overlap (overlay) between the peak sample histogram and isotype. Positive stem cells expressing surface proteins located on the right, the histogram is blue while the isotype is shown on the histogram in red (Figure 2).
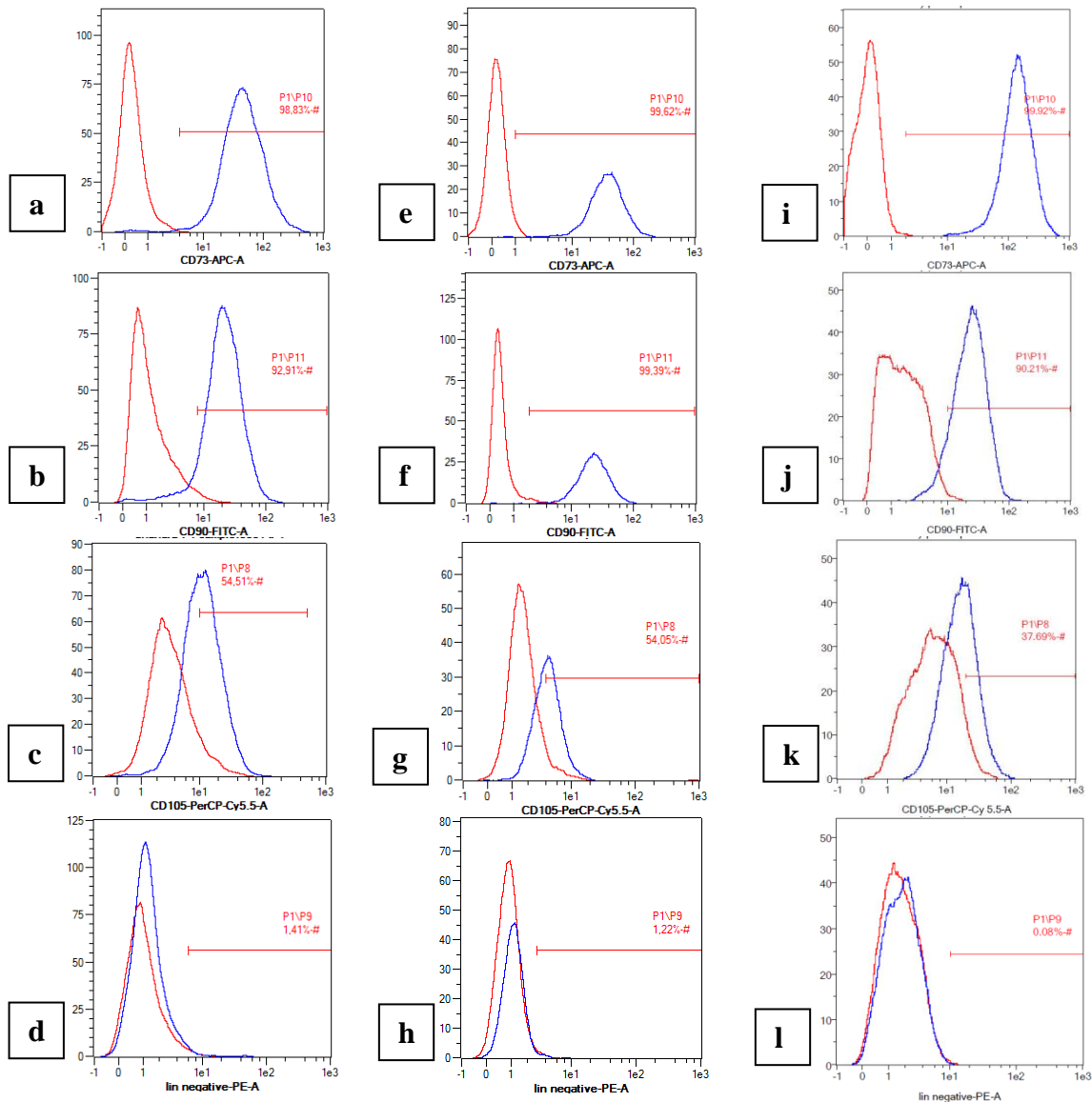

Figure 2. The percentage value of passport 1 human ADSCs surface protein from the three donors namely donor 1 (a-d), donor 2 (e-h), and donor 3 (i-l) with the peak of the histogram in blue showing the percentage value of the sample while the peak of the histogram in red shows the isotype. The surface protein expressions of CD73, CD90, and CD105 are shown in figures a, e, i; b, f, j and c, g, k in all three donors. Expression of negative surface marker proteins for stem cells (CD34/CD45/CD11b/CD19/HLA-DR) is shown in figures d, h, l in all three donors. The percentage value of P1 / P9 for each figure (a-l) shows the percentage value of protein expressed by the sample

Based on the results obtained, the CD73 surface marker protein is consistently expressed more than any other protein, which is more than 95%. The percentage values of

donors 1, 2, and 3 for the CD73 surface marker protein, respectively, were 98.83%; 99.62% and 99.92%. The protein expression of the third donor CD90 surface marker is expressed more than 90% with the highest percentage of CD90 expression found in donor 2, which is expressed as much as 99.39%. CD90 expression by cells from donors 1 and 3 was 92.91% and 90.21%. Results with a low percentage of expression consistently occur on CD105 markers in all three donors. The percentage values of the three donors are 54.51%; 54.05% and 37.69%. The negative protein expression of the stem cell surface marker i.e. CD34/CD45/CD11b/CD19/HLA-DR is less than 2% (Figure 3).
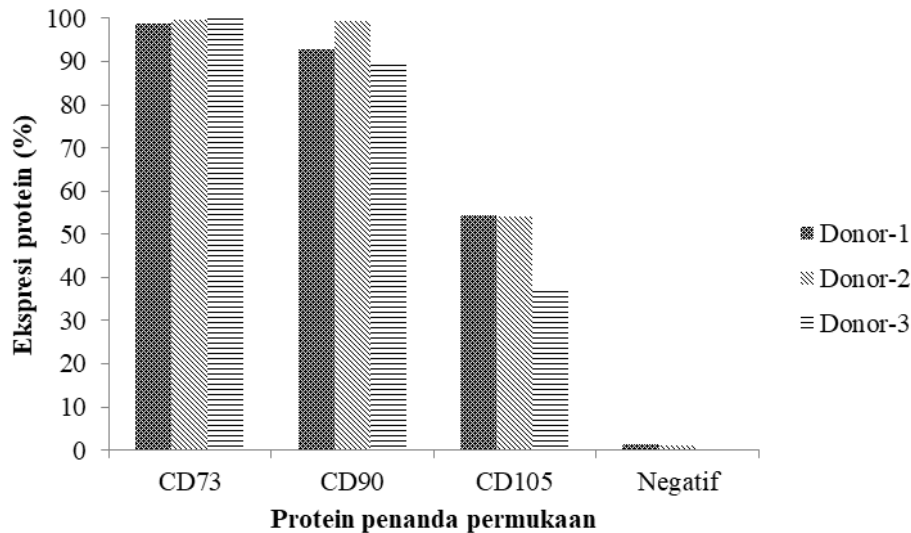
Figure 3. CD73, CD90, CD105 and CD34 / CD45 / CD11b / CD19 / HLA-DR (negative) surface marker protein percentage values of passage 1 human ADSCs

DISCUSSION

Stromal vascular fraction (SVF) consists of diverse (heterogeneous) cell populations such as ADSCs, hematopoietic stem cells, precursor cells, endothelial cells, regulatory T cells, macrophages, and preadipocytes (Nguyen et al., 2016; Bora & Majumdar, 2017). The number of ADSCs is ~2–10%, then hematopoietic stem cells around 0.004%, macrophages around ~10%, T cells range from 5–70%, and endothelial progenitor cells range from 7–30% (Dykstra et al., 2017). The selection of these cells can be made by administering a specific medium for specific cell growth, such as DMEM. Various cells contained in the SVF can be classified into 2, namely cells attached to the base of the flask (adherent) and cells that are not attached or floating (non-adherent). ADSCs have characteristics attached to flask so that it is quite easy to separate them from other non-adherent cell types through periodic media replacement. Stem cells from all three donors have different proliferation rates indicated by the length of time the cell divides in PDT data.

The mean value of PDT is ideal for stem cell growth, according to Mark et al. (2013) is 2.6 to 4 days. These data indicate that the condition of stem cells from fat tissue in this study can divide well. The protein contained in the media influences the rate of stem cell division. Protein fibroblast growth factor (FGF) is a significant factor in the initiation of stem cell division from fat tissue with a medium containing FBS (Wang, Kim, Vunjak-Novakovic, & Kaplan, 2006; Gentile, 2012). Some other growth factor proteins are platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor- β (TGF-β), and macrophage inhibitory factor (MIF) (Kang, Jeon, & Song, 2005; Palumbo, Tsai, & Li, 2014; Atashi, Jaconi, Pittet-Cuenod, & Modarressi, 2014). PDGF, FGF and MIF proteins will bind to their respective receptors to stimulate the AKT signalling. The AKT signalling pathway activates protein synthesis for cell growth and inhibits apoptosis (Atashi et al., 2014). Activation of AKT pathway can increase ADSC proliferation through the expression of cell cycle-related proteins such as cyclin D1 protein to be higher (Atashi, Serre-Beinier, Nayernia, Pittet-Cuénod, & Modarressi, 2015). Cells that experience

growth (proliferation), will also express specific surface proteins.

Protein markers of specific surface cells from fat tissue in uncultured SVF such as CD73, CD90 and CD105 have not been widely expressed. The CD73 marker, also known as ecto-5-nucleotidase, is a protein found in many types of tissue and plays a role in cell interactions with cells and cells with a matrix (Zhang, 2010). CD90 glycoproteins found on cell surfaces are found in stem cells, fibroblast cells, and activated endothelial cells (Kisselbach, Merges, Bossie, & Boyd, 2009). The CD105 endoglin protein is a component of the transforming growth factor-beta receptor (TGF-β) to be able to form the TGFβ receptor complex, but the role of CD105 in interactions with its league is still needed in-depth studies (Pierelli et al., 2001). The expression of stem cell surface marking proteins namely CD73, CD90, and CD105 will increase in the percentage of entry along with the increasing number of subcultures (passage), but there will be a decrease in negative surface marker proteins, namely CD34/CD45/CD11b/CD19/HLA-DR (Varma et al., 2007; Mitchell et al., 2006). Mitchell et al. (2006) reported that SVF cell surface marker proteins for CD73, CD90, CD105, and CD34 were 25% respectively; 54.8%; 4.9% and 60% then when cultured until the first passage, the expression became 85.3%; 90.4%; 52.8% and 21.5%. In the 4th passage, there was an increase in protein CD73, CD90, CD105, namely 94.2%; 97.2%; 70.5% while the CD34 protein decreases to less than 2% which is 1.7%.

Another study reported by Xie et al. (2013) showed that CD105 protein was expressed at a low percentage, i.e. ~60% in stem cells from bone marrow. Results with low CD105 expression values were also reported by De Schauwer et al. (2012), which ranges from 0.1% to 20%. Study of CD73, CD90, and CD105 protein expression with expression values of more than 90% reported by Barberini et al. (2014) in stem cells from the 3rd fat, bone marrow and umbilical cord tissue from the third passage. The results of some of these studies have similarities with the results of this study which indicate the possibility of low

expression of CD105 due to the level of culture that is still small, namely the first passage.

CONCLUSIONS AND SUGGESTIONS

Stem cells from human fat tissue from 3 donors have a mean length of splitting time of 3.3 days and expressing CD73, CD90, CD105 are 99.79% respectively; 94.17%; 48.75% and less than 2% detected for the CD34/CD45/CD11b/CD19/HLA-DR marker. Suggestions for further studies are that analysis needs to be carried out at a higher passage using stem cells from human fat tissue until stable CD105 expression is expressed.

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