

Allogeneic human dermal fibroblasts are viable in peripheral blood mononuclear co-culture

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ABSTRACT

BACKGROUND

Transplanted allogeneic dermal fibroblasts retain stem cell subpopulations, and are easily isolated, expanded and stored using standard techniques. Their potential for regenerative therapy of chronic wounds should be evaluated. The aim of this study was to determine allogeneic fibroblast viability in the presence of peripheral blood mononuclear cells (PBMC).

METHODS

In this experimental study, fibroblasts were isolated from foreskin explants, expanded in the presence of serum, and stored using slow-freezing. We used one intervention group of allogeneic fibroblasts co-cultured with PBMC and 2 control groups of separate fibroblast and PBMC cultures. Fibroblasts were characterized by their collagen secretion and octamer-binding transcription factor 4 (OCT4) expression. Viability was evaluated using water soluble tetrazolium-1 (WST-1) proliferation assay. Absorbances were measured at 450 nm. Data analysis was performed by student's paired t-test.

RESULTS

Dermal fibroblasts were shown to secrete collagen, express OCT4, be recoverable after cryopreservation, and become attached to the culture dish in a co-culture with PBMC. Co-cultured and control fibroblasts had no significantly different cell viabilities ($p > 0.05$). Calculated viable cell numbers increased 1.8 and 5.1-fold, respectively, at days 2 and 4 in vitro. Both groups showed comparable doubling times at days 2 and 4 in vitro. PBMC did not interfere with allogeneic fibroblast viability and proliferative capacity

CONCLUSIONS

Allogeneic fibroblasts remain viable and proliferate in the presence of host PBMC. Future research should evaluate allogeneic human dermal fibroblast competency in clinical settings. Dermal fibroblasts are a potential source for cell therapy in chronic wound management.

Key words: Fibroblast, PBMC, co-culture, foreskin

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Univ Med 2014;33:91-9

Fibroblas dermis manusia alogenic viabel dalam sistem ko-kultur bersama sel mononuklear darah tepi

ABSTRAK

LATAR BELAKANG

Dermis pada kulit memiliki subpopulasi sel punca. Fibroblas asal dermis dapat diperoleh, diperbanyak, dan disimpan dengan mudah. Potensi fibroblas sebagai salah satu alternatif strategi regeneratif untuk penyakit luka kronik perlu dievaluasi lebih lanjut. Studi ini bertujuan untuk menentukan viabilitas fibroblas asal dermis yang di kultur bersama dengan sel mononuklear asal darah tepi individu yang berbeda.

METODE

Studi ini menggunakan desain eksperimental. Fibroblas diisolasi menggunakan teknik eksplan. Kultur perbanyakan menggunakan serum serta disimpan dengan metode slow-freezing. Studi ini menggunakan satu kelompok perlakuan berupa ko-kultur antara fibroblas dan sel mononuklear darah tepi dan dua kelompok kontrol terdiri atas kultur tersendiri fibroblas dan sel mononuklear darah tepi. Fibroblas dikarakterisasi dengan kemampuan sekresi kolagen dan ekspresi octamer-binding transcription factor 4 (OCT4). Viabilitas dievaluasi menggunakan uji proliferasi kolorimetrik water soluble tetrazolium-1 (WST-1). Absorbansi diukur pada panjang gelombang 450 nm. Analisis data dilakukan menggunakan paired student t-test.

HASIL

Fibroblas mensekresikan kolagen, mengekspresikan OCT4 serta dapat dipulihkan dari kriopreservasi. Tidak ada perbedaan signifikan viabilitas antar kelompok kontrol dan kelompok perlakuan ($p > 0,05$). Jumlah sel mengalami peningkatan 1,8 dan 5,1 kali lipat pada hari kedua dan keempat eksperimen. Kedua kelompok memperlihatkan doubling time yang sebanding pada hari kedua dan keempat in vitro. Sel mononuklear darah tepi tidak mengganggu viabilitas dan proliferasi fibroblas dari individu yang berbeda.

KESIMPULAN

Fibroblas dapat mempertahankan viabilitas dan proliferasinya dalam ko-kultur bersama sel mononuklear darah tepi dari individu yang berbeda. Penelitian selanjutnya dapat menilai kompetensi fibroblas pada situasi klinis. Fibroblas asal dermis adalah sumber potensial untuk terapi sel pada luka kronis.

Kata kunci: *Fibroblas, PBMC, ko-kultur, preputium*

INTRODUCTION

Recently, adult dermal fibroblasts were found to retain stem cells subpopulations. These cells expressed stage-specific embryonic antigen 3 (SSEA-3) and octamer-binding transcription factor 4 (OCT4), and co-expressed with CD105, a well-known marker for marrow and adiposederived mesenchymal stem cells. Human dermal fibroblasts (HDFs) can differentiate into epidermal melanocytes, neurons, adipocytes,

hepatocytes and islet-like cell clusters.⁽¹⁻³⁾ This ability suggests that human dermal fibroblasts have multipotent stem cell subpopulations.

Human dermal fibroblasts are easily isolated from foreskin. The compulsory Moslem practice of male circumcision in Indonesia constitutes an abundant and accessible source of adult stem cells for cell therapy. Human dermal stem cells secrete high levels of growth factors, such as basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), vascular

endothelial growth factor (VEGF), and keratinocyte growth factor (KGF), in addition to extracellular proteins, such as collagen, which is important in dermal and epidermal regeneration.⁽³⁻⁵⁾ Potential applications of these cells include regenerative therapy for chronic wounds, such as diabetic foot, decubitus, and burns. Diabetic foot is reported to cause one million amputations annually.⁽⁶⁾ This report has led to an interest to develop novel rehabilitation efforts using regenerative technologies.

Regenerative strategies for wound treatment using cell therapy requires evaluation of cell functionality, storage ability, and compatibility, particularly in allogeneic settings. The risk of host tissue rejection needs to be evaluated before starting clinical trials on humans. Tissue rejection is an immune reaction caused by cellular immunity in the form of skin graft-versus-host disease (GVHD), marked by increased C-X-C motif chemokine 10 (CXCL10) and Langerhans cells as antigen presenting cells (APCs). Receptors for CXCL10 are present in T cells,⁽⁷⁾ and donor T cells presented to skin with intact Langerhans cells will trigger GVHD.

A recent study on mouse dermal fibroblasts found similarities in multipotent capacity between dermal fibroblasts and mesenchymal stem cells. Further investigations revealed that allogeneic mouse dermal fibroblasts retain immunosuppressive properties at days 3-6 in co-culture with mononuclear cells in a mixed lymphocyte reaction (MLR) experimental set up.⁽⁸⁾ However, another study using induced pluripotent stem cells (iPSCs) derived from pigs showed that autologous major histocompatibility complex (MHC)-matched iPSCs were still susceptible to innate immunity, natural killer (NK) cells, and serum complement cytotoxic reactions.⁽⁹⁾

The present study was designed to evaluate the viability of allogeneic human dermal fibroblasts in the presence of blood mononuclear cells. This study did not use heat-inactivated fetal bovine serum that could inhibit serum complement reactions.

METHODS

Research design

The present study using an experimental design was conducted from April 2013 to March 2014 at the Integrated Laboratory of YARSI University.

Cell culture

Cell cultures and proliferation assays were carried out at the Cell Culture Laboratory, YARSI University, Jakarta. One healthy human foreskin sample (from an 8-year old boy) was obtained after a circumcision procedure conducted at the Indonesia House of Circumcision (IHC) in accordance with a manual provided by WHO, with informed consent given by the parents.⁽¹⁰⁾ Fibroblasts were cultured in a modification of minimal essential medium (α -MEM, Sigma) supplemented with fetal bovine serum (FBS, Sigma) and glutamine (Sigma). Antibiotics and antimycotics used were penicillin-streptomycin (Sigma) and amphotericin-B (Sigma). The same medium was used for co-culture with peripheral blood mononuclear cells (PBMC).

Sample preparation

The collected foreskin sample was refrigerated for 60 minutes before decontamination using povidone iodine and 70% ethyl alcohol (EtOH). The technique for separation of the dermis from the epidermis has been described elsewhere.⁽¹¹⁾ The skin sample was minced and placed in a 35-mm diameter culture dish with α -MEM and incubated overnight at 37° C with 5% CO₂. The medium was increased to working volume the next day and supplemented with 20% FBS for expansion culture. Fibroblast outgrowths appeared within 7 days (Figure 1a) and were subcultured twice before being used for the experiment. Excess cells were cryopreserved using a slow-freezing method. Approximately one million viable cells were resuspended in cryomedium [Dulbecco's modified Eagle's medium (DMEM) 20% FBS,

FBS and dimethyl sulfoxide (DMSO) in a 7:2:1 ratio], incubated overnight at -80°C and transferred directly to liquid nitrogen cryofreezer the next morning.⁽¹²⁾

Fibroblast characterization

Cultured fibroblasts were characterized functionally by quantifying collagen 1 and 3 secretion. Conditioned medium from cultured fibroblasts at 1-3 days *in vitro* were collected, centrifuged at 1500 rpm for 10 minutes, and the resulting supernatant used to quantify collagen 1 and 3 using COL1A1 and COL3A1 ELISA kits (Uscn). To find out whether the dermal fibroblasts retained a multipotent subpopulation, we calculated relative OCT4 mRNA expression between passages 4-6.

A total of one million viable cells were collected for total RNA isolation using High Pure RNA Isolation kit (Roche), quantified using a Qubit Fluorometer (Invitrogen). Real-time polymerase chain reaction (RT-PCR, Roche) was performed using Lightcycler RNA Master SYBR Green I (Roche). Forward and reverse primers for OCT4 were 5'-AGGTGTTTCAGCCAAACGACC-3' and 5'-TGATCGTTTGCCCTTCTGGC-3', respectively. Beta-Actin was used as house keeping gene with the following primer sequences: forward 5'-AGAGCTACGAGCTGCCTGAC-3' and reverse 5'-AGCAC TGTGTTGGCGTACAG-3'. Both primer sequences had been confirmed to be specific using the Nucleotide Blast facility from NCBI.

Peripheral blood monocyte isolation

The peripheral blood samples collected from one healthy human volunteer was treated with EDTA and cooled in a refrigerator for 60 minutes. PBMC isolation was conducted by centrifugation for 30 minutes at 2000 rpm using Ficoll Hypaque (1.077 g/dL; Sigma) with a 1:1:1 ratio of blood, phosphate buffer saline (PBS, Gibco) and Ficoll.⁽¹²⁾ The thin buffy coat layer visible between serum and Ficoll layer was collected and washed three times with PBS at 1500 rpm for 10 minutes.

Co-culture

The experiments were conducted on 96-well culture plates. A total of 3×10^3 viable fibroblasts were seeded with 1.5×10^5 PBMC in 100 μl α -MEM supplemented with 10% FBS per well. The experimental groups used in this study were the intervention group consisting of a co-culture of fibroblast and PBMC, and two control groups consisting of fibroblast control group and PBMC control group. This set up was arranged on two plates and repeated in triplicate per plate. Both plate were incubated at 37°C with 5% CO_2 and assessed at two time point, one for each plate at 2 and 4 day *in vitro*, respectively.

We used separate fibroblast and PBMC cultures, each in their own 35 mm culture dishes, seeded at the same density as used for the experiment. This separate setup was for evaluating fibroblast confluence and PBMC attachment in standard culture. Mitochondrial activity was assessed by colorimetric method using water soluble tetrazolium-1 (WST-1) proliferation assay (Roche) on a 96-well culture plate. A microplate reader with 450 nm filter was used to read the absorbance.

Data Analysis

The collected data were processed on an Excel spreadsheet (version 2007; Microsoft). Blank absorbance values were subtracted from the original data. Absorbance values were used to calculate viable cells with an equation derived from the linear chart shown in Figure 1. Calculated viable cell numbers were further analyzed to derive population doubling, doubling time, and simulated growth curve. OCT4 expression data were presented as ratios, using data from passage 6 for comparison. Statistical significance was determined using paired Student's t-test.

RESULTS

The separate dermal fibroblast control culture in the 35-mm culture dish at the same

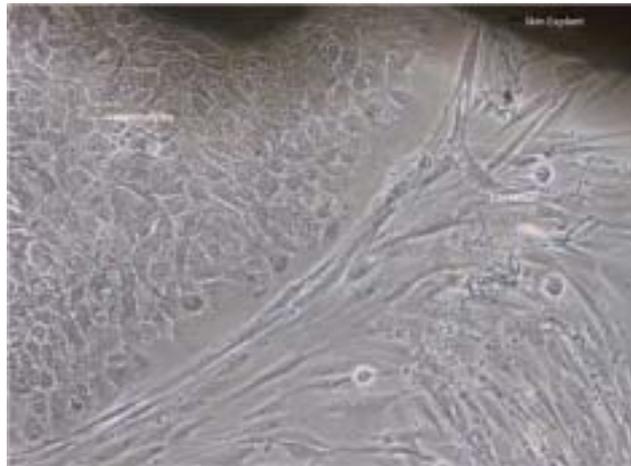


Figure 1a. Outgrowth of primary human dermal fibroblasts and keratinocytes from skin explants seen as spindle-shaped and cobble-stone cells, respectively. DIV 6, 20x Nikon (DIV = Day In Vitro)

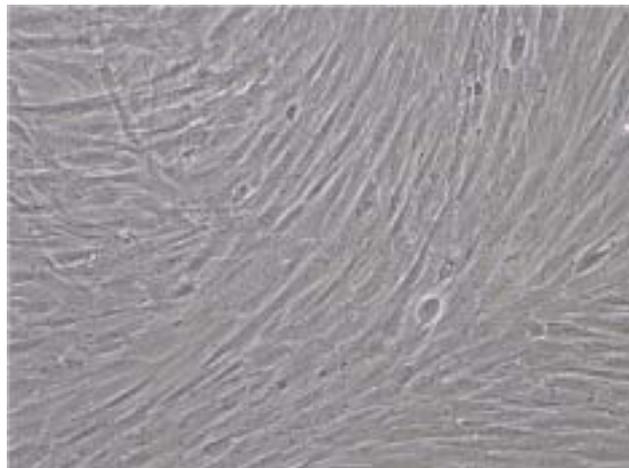


Figure 1b. Fibroblast confluence >80% (DIV4, 20x Nikon)

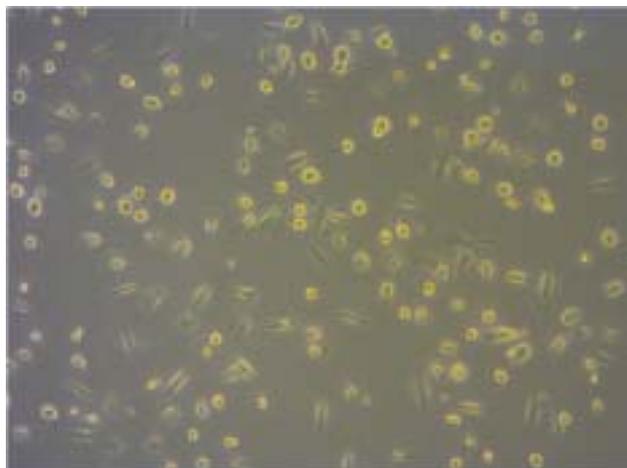


Figure 1c. Plastic-attached, spindle-shaped, fibroblast-like cells from PBMCs known as mesenchymal progenitor cells (MPCs) or fibrocytes (DIV18, 10x Axiocam; DIV = Day In Vitro)

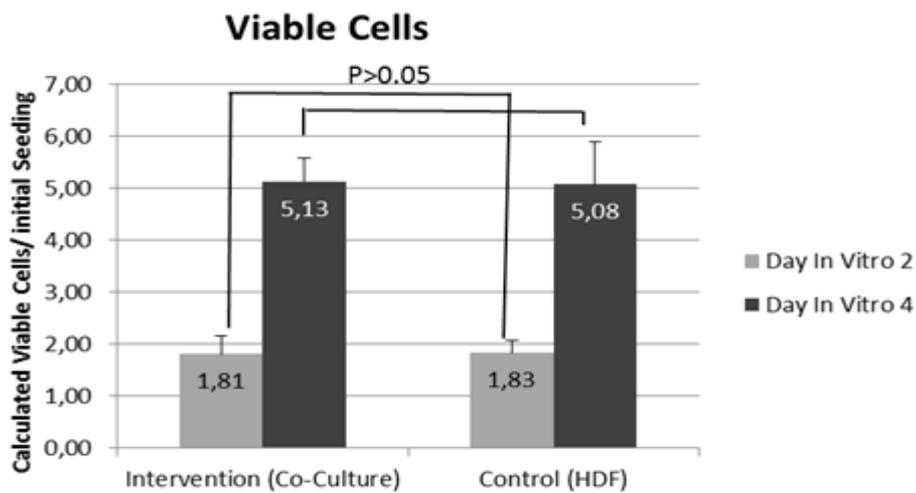


Figure 2. No significant difference in calculated viable cell number after 2 days (DIV2) and 4 days (DIV4) of culture ($p > 0.05$) (Ratio of viable cells calculated from absorbance reading/initial seed number)

seed density as the co-culture, reached confluence in 4 days in vitro (Figure 1b), while the PBMC control culture in their own 35 mm culture dish showed plastic-attached, spindle-shaped cell colonies after 5 days in vitro (Figure 1c).

To evaluate the results we subtracted absorbance values of the co-culture group with those of the PBMC control group and compared the resulting data with the fibroblast control group. Mitochondrial cellular dehydrogenase activity was used to quantify viable cells after incubation. This study did not reveal any significant differences in viable cell numbers between the co-culture group and the fibroblast control group ($p > 0.05$) (Figure 2).

The numbers of viable cells in both co-culture and fibroblast control groups after four days in vitro were correspondingly greater than the numbers of viable cells after two days in vitro. Calculated viable cell numbers increased

1.8 and 5.1 times, respectively, at two and four days in vitro. From the population doubling and doubling time values it was apparent that in the first two days of culture, the fibroblasts were in the lag phase (long doubling time, 56 hours) and entered the proliferative phase (shorter doubling time, 32 hours) in the next two days. However, the simulated growth curve derived from the doubling time differs with the actual cell numbers by more than 40% (Table 1). These data reveal that co-culture of fibroblasts with PBMC did not reduce fibroblast proliferation capacity.

The fibroblasts were confirmed to secrete collagen 1 and 3 into the culture media; collagen 1 secretion was however significantly lower than collagen 3 secretion ($p < 0.05$) (Figure 3).

OCT4 mRNA expression levels show a steady decline from passage 4 to 6 (Figure 4). Cryopreserved fibroblasts were thawed after 2 days in liquid nitrogen and evaluated using the trypan-blue exclusion method. Fibroblast

Table 1. Comparison of cell numbers between simulated cell numbers derived from fibroblast doubling time and actual cell numbers calculated using Linear WST-1 curve

Hours	Actual co-culture	Actual fibroblasts	Simulated co-culture	Simulated fibroblasts
0	3,00	3,00	3,00	3,00
40	5,43	5,49	5,49	5,62
80	15,38	15,23	26,91	26,04

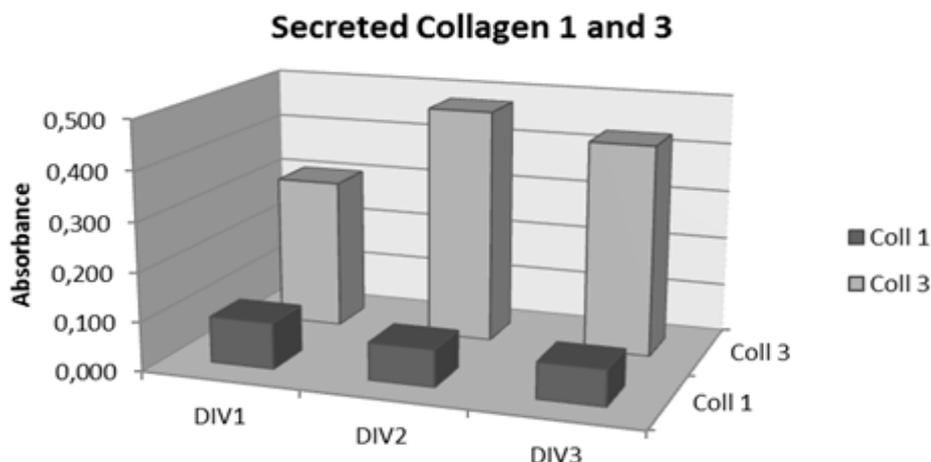


Figure 3. Secreted collagen 1 and 3 on DIV1, DIV2 and DIV3, significantly different ($p < 0.05$); highest difference at DIV2 (DIV: Day In Vitro)

recovery was over 90%; however, we recorded a loss of about 40% of the initial cell population under going cryopreservation.

DISCUSSION

Our findings reveal that co-culture of allogeneic fibroblasts with PBMC is feasible. A mixed lymphocyte reaction study will show different results, because in that case both fibroblasts and PBMC will contain immune-reactive cells, such as T lymphocytes and APCs. Classic study of epidermal cells in a mixed skin cell lymphocyte reaction study (MLSR) requires

the presence of Langerhans cell as APCs to stimulate the blood mononuclear cells. Our study results indicate that the fibroblast culture was not contaminated by Langerhans cells or T lymphocytes, both of them able to act as APCs. The results also show no adverse effects from innate immunity, NK cell activity, and serum complement cytotoxic reactions.

The fibroblasts used in this study were subcultured twice before entering the experiment. The cells were considered to be functional because they were able to secrete collagen 1 and 3. The different amounts of collagen secreted by both types is considered

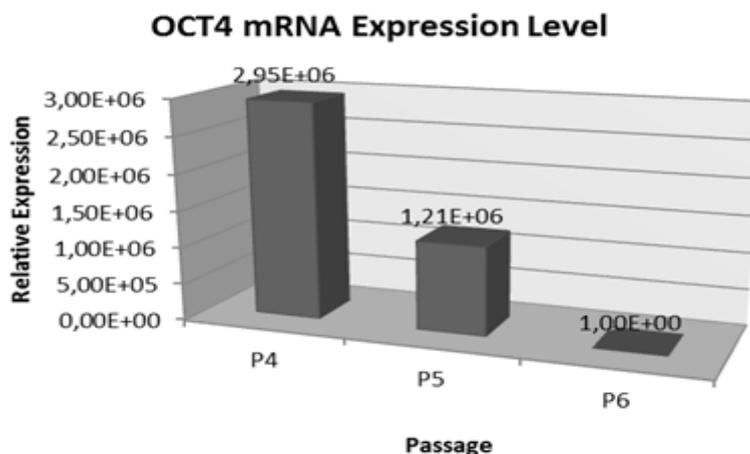


Figure 4. OCT4 mRNA relative expression level compared between passages, 1.0E+06 viable cells used for total mRNA extraction

acceptable because collagen 3 is secreted in larger amounts by younger children.⁽¹³⁾ Passage-two fibroblasts were used because further passaging showed significantly lower OCT4 expression levels. This finding suggested a down regulation of OCT4 which could be attributed to spontaneous differentiation, frequently occurring in stem cells cultured in the presence of serum.

Passaging also sufficiently removed potential contaminants from the cell suspension, such as epidermal Langerhans cells and T-cells, which are common contaminants in fibroblasts isolated from skin explants. The actual fibroblast growth curve in our study shows that both control and intervention fibroblasts remained for the same amount of time in the lag phase and entered the proliferative phase at about the same time. The difference between simulated and actual growth curve may be explained by assuming that space restriction in the culture wells initiated contact inhibition, thus lowering the cell numbers observed.

The results indicate that after allogeneic transplantation, the fibroblasts should be able to integrate with the host tissue. Dermal substitute products such as Transcyte™ and Dermagraft™, that are derived from allogeneic living fibroblasts, are known to have been successfully applied empirically for various types of wound.^(14,15) The results of our experiment promise the achievement of similar results using locally collected sources of fibroblasts. Our study should be followed up by other experiments, such as the generation of induced pluripotent stem cells from fibroblasts^(16–18) or clinical trials that require large numbers of cells demanding a storage solution. Our slow-freezing cryopreservation method at low (10%) DMSO concentration showed a high recovery rate (90%), but with a technical loss of 40% of the initial cell numbers.

This study was limited by the lack of autologous co-culture because of difficulties in obtaining samples. The incubation period was restricted to 4 days, because on the 5th day

PBMC subpopulations known as mesenchymal progenitor cells (MPCs) or fibrocytes exhibiting plastic-attachment ability, started to interfere with the fibroblast viability assessment.^(19,20) This study demonstrated the potential application of locally collected foreskin-derived dermal fibroblasts for cell therapy, particularly the regenerative approach, for chronic wound healing. Successful isolation of dermal fibroblasts that retain multipotent stem cell subpopulations also offers patient-specific fibroblast populations for regenerative and reprogramming purposes.

CONCLUSIONS

Allogeneic human dermal fibroblasts retain their viability and proliferative capacity in PBMC co-culture system. Future research should be directed to evaluate allogeneic human dermal fibroblast competency in clinical settings. Research could also be directed to construct dermal-epidermal composites for the production of bilayered human skin equivalents that can be used in wound therapy, cosmetic toxicological protocols, and drug delivery studies.

ACKNOWLEDGEMENTS

Funding was provided by the *Riset Unggulan Universitas 2013* grant scheme. We thank Intan Razari for laboratory assistance, Ratih Rinendyaputri and Prof. Drh. Arief Boediono, Ph.D for consultation on cryopreservation methods, dr. Linda Julianti Wijayadi, Sp.KK for the ELISA procedure, dr Mahdian Nur Nasution, Sp.BS for the circumcision procedure and Viko and Wiza for their enthusiasm in research. 

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