# APPENDIX

# RECONSTITUTION OF CELL COMMUNITIES AND TISSUES OF NORMAL PERIPHERAL BLOOD STEM CELLS IN CONVENTIONAL AND 3D CULTURE SYSTEM

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**Abstract**

Reconstitution of cell communities and tissues of peripheral blood stem cells (PBSCs) is of important for understanding stem cell biology and clinical translations. This study rigorously investigated the pluripotency and differentiation of PBSCs in conventional and in 3D-PVDF nanofibrous scaffold culture systems. The PBSCs was found concentrated about 1% of total PBMCs obtained from the ficoll gradient centrifugation. The PBSCs isolated divided into 3 groups depending on their size and granularity; the smallest size and lowest granularity preserves the self-renewal property while the bigger and higher granularity undergo differentiation or re-entering to the cells cycle. When the cell were let in continuous culture for 24 days, a variety of cellular morphology including irregular, spindle, rod, cone, round, neuron-network were found. PBSCs in culture have the homeostasis process for controlling the number of cells which is the characteristic of normal stem cells. PBSCs are able to generate variety of cell communities and tissues in the appropriate microenviroments particularly by using 3D- PVDF nanofibrous scaffolds of both non-woven and alignment matrices that can hang the cells in space and restrict controlled the microenviroments.

**Keywords:** Peripheral blood stem cells (PBSCs), peripheral blood mononuclear cell (PBMCs), homeostasis, 3D-PVDF nanofibrous scaffolds, SEM, EDX

**Introduction**

Mobilized peripheral blood stem cells (PBSCs) are of important for stem cell therapy particular the use to reconstitute hematopoiesis after high dose chemotherapy for treatment of hematopoietic cancers and solid cancers [1-4]. *Ex vivo* expansion, following stem cell transplantation currently use to reduce time for hematopoietic recovery and to overcome limited availability of PBSC or poor mobilization [2-3]. Because PBSCs were found only 0.1 % in the blood circulation of normal subject (~5 $\times 10^4$ cell/mL) [4], thus the efficiently isolation technics that allow to obtain high number of PBSCs enough for initiating their ex vivo expansion are needed to be develop. For resolving the limited number of PBSCs in blood circulation, researchers applied the chemotherapy-containing mobilization regimen such as cyclosphamide, cytosine-arabiniside, etoposide etc. to stimulate an increase in mobilized PBSCs before apheresis [5-6]. The immunological analysis of $CD34^+$ cells is currently considered to reflect the best surrogate measure of hematopoietic stem and progenitor cells and also the most precise marker to guide timing of peripheral blood stem cell (PBSC) apheresis [4, 7]. The *ex vivo* expansion of PBSCs was developed in the presence of cytokines, mesenchymal stem cells, and combination of these conditions. However, these conditions of *ex vivo* expansion are not exactly physiological-like conditions due to the use of cytotoxic agents and cytokines might later responsible to cellular biochemistry changes.

In fact the PBSCs were found concentrate in the peripheral blood mononuclear cells (PBMCs) fraction obtained from ficoll gradient centrifugation. Such as a very small amount of whole blood of 5 mL collected from normal subjects without treatment of chemotherapy-containing mobilization regimen was sufficient to provide high enough amounts of cells for initiating the PBSCs expansion in classical culture conditions that later presented the colonies of PBSCs. The *ex vivo* PBSCs expansion in these situations might be mimic physiological conditions and is of prime important for our understanding of stem cell biology.

The behaviors and cell-cell communication when the stem cells were supported and hung in the space using 3D-nanofibrous scaffolds seem to be the next step of studying the stem cell biology. However, unfortunately the convenience and easily achieve of PBSCs as a source of stem cells, rare studies were studied the PBSCs behaviors in 3D-scaffold. Therefore, this study preliminarily questioned on the pluripotency of PBSCs when submitted to an *ex-vivo* expansion under conventional culture conditions without any addition of mitogens or cytokines. How the PBSCs behave and form a variety of cell community and tissues in a simple ways of creation specific microenvironments by using 3D-nanofibrous scaffolds merged in the same culture medium use in the conventional culture conditions.

Recently, our research groups have designed and fabricated poly-vinylidene fluoride (PVDF) nanofibrous tissues by electro-spun technique owing to its excellent chemical resistance and good thermal stability. The 3D-PVDF nanofibrous tissues were mixed morphology of

non-woven and aligned form (thickness varied from 10 μm to 100 μm) and are able to absorb and retain a large amount of liquid electrolyte. Due to differences in morphologies, the mixed of non-woven and aligned tissues can themselves generate specific microenvironments even they were merged in the homogeneous solution. For answering the first question we rigorously isolated and cultured the PBSCs from blood donors in conventional conditions and characterized the ability of self-renewal and differentiation to be a variety of specific cell types. For the second question, the 3D-PVDF nanofibrous scaffolds were in house made and used to generate microenvironments and extracellular matrix support and hang the cells in the 3D-space.

The results reported here in show that the initial density of PBMCs play important role on the number of colony and differentiation of PBSCs; such as when $4 \times 10^6$ cell/mL PBMCs were inoculated in the culture system for 11 days, the colonies of PBSCs was increase in number and size without adhesion onto the bottom surface of culture flasks. While the initial density of PBSCs was varied between $10^5$ to $10^6$ cells/mL, a mixture of cell types were observed, as adherent cells such as neurons, endothelials, fibroblasts, myocytes etc…, and as suspension cells including colonies of PBSCs and leucocytes. It was clearly demonstrated that the PBSCs preserved the renewal and differentiation to specific cell properties. This study demonstrated for the first time that the mixed morphology including non-woven and alignment 3D-nanofibrous scaffold was biocompatible and can be used as extracellular matrix that generates the specific microenvironments of stem cell, especially for long term stem cell culture that allow to establish the growth pattern and specific tissue organization.

**Materials and methods**

**Collection of human peripheral blood mononucleated cells (PBMCs)**: Human PBMCs were obtained from blood donor and were isolated from buffy coats using ficoll solutions. The PBMCs ($4\times10^6$ cells/mL) were cultured in RPMI-1640 supplemented with 10 % fetal calf serum (Gibco Biocult Ltd.) and 1 % penicillin /streptomycin (BioMedia) at 37 °C in 5 % $CO_2$ atmosphere for 24 h before assays.

Samples were imaged and analyzed by scanning electron microscope (SEM) (JSM-5410LV JOEL, Tokyo, Japan) and energy dispersion X-ray fluorescence spectrometer (EDX) (INCAPentaFETx3, Oxford, UK) combined with INCA oxford instrument software.

**Enumeration of *CD34*$^+$ cells from PBMCs cultures**: cells ($10^6$) were centrifuged at 7,000 rpm for 1 minute and washed once using phosphate buffer pH 7.4 at 25°C. The successive addition of 10 μL anti-*CD34*$^+$ - FITC (USbiological, Swampscott, Massachusetts, catalog no. c2386-24A) following 100 μL PBS pH 7.4 at 37 °C was added into the cell pellets. The suspension of cells was incubated in the dark at 37 °C for 15 minutes. Finally, 400 μL PBS was added and the cells were analyzed by using both flow cytometer (Coulter Epics XL-MCL; Coultronics France SA) and fluorescence microscope (Leica, Germany, DMI 4000B).

**Expansion of adult PBSCs in conventional culture:** cells ($10^6$/mL)

were cultured in 25 $cm^2$ cultured flask or 24-well plates with RPMI 1640 (Gibco, USA) or MEM (Gibco, USA) media supplemented with 1% penicillin-streptomycin (Gibco, USA) and 10% fetal bovine serum (PAA, Austria) at 37 °C in a humidified incubator of 95% humidified. The cell morphology were observed and micrograph under inverted light microscope at 24 hr of interval time. The cells were counted using haemocytometer.

**Evaluation of adult PBSCs differentiation using *CD34*$^+$ and *CD11b*$^+$**: The surface protein including *CD34*$^+$ and *CD11b*$^+$ were used for characterizing the PBSCs and their differentiation. Cells ($10^6$) were centrifuged at 7000 rpm for 1 minute and washed once using phosphate buffer pH 7.4 at 25°C. The successive addition of 10 µL anti-*CD34*$^+$ - FITC or 20 µL *CD11b*$^+$-FITC (USbiological, Swampscott, Massachusetts, catalog no. c2386-24A) following 100 µL PBS pH 7.4 at 37 °C was added into the cell pellets. The suspension of cells was incubated in the dark at 37 °C for 15 minutes. Finally, 400 µL PBS was added and the cells were analyzed by using both flow cytometer and fluorescence microscope.

**Flow cytometric analysis of cellular DNA contents**: cells ($10^6$/mL) were cultured in 24-well plates with RPMI 1640 (Gibco, USA) or MEM (Gibco, USA) media supplemented with 1% penicillin-streptomycin (Gibco, USA) and 10% fetal bovine serum (PAA, Austria) at 37 °C in a humidified incubator of 95% humidified. The cell morphology were observed and micrograph under inverted light microscope at 24 hr of interval time. A total of cells were centrifuged and washed once using PBS. The cell pellets were resuspended in 300 mL PBS then 700 mL ice-cold ethanol was added, dropwise, with gentle agitation and future incubation at 4 °C overnight. The cells were centrifuged at 14,000*g*. Aspirate the supernatant and resuspend cells in 500 µl of PBS containing 0.2 mg/ml RNase A and 0.1% Triton X-100 then let incubated at room temperature for 30 minutes. Add 500 µl of PBS containg 10 mg/mL propidium iodide (USbiological, USA) vortex briefly, and analyze in a flow cytometer.

**Electrospun of PVDF 3D-nanofibrous scaffold:** The in house made 3D-nanofibrous PVDF membrane was fabricated using electrospinning technique as extensive described by Chanunpanich [9]. Poly(vinylidene fluoride) the so-called PVDF (Solvay Co. , France) of 19 wt.% was dissolved in *N,N*-Dimethylacetamide (DMAA) (Fluka) and acetone. Syringe having metal needle (G22) was used as the solution reservoir. A drum shaped counter electrode was located opposite to the reservoir. The fibers were collected on manmade plastic plate at the winding drum of 52 m/s. The distance between the capillary tip and the collector (tip-to-collector distance, TCD) was 10 cm and the applied voltage was 10 kV. A thin polymer fiber is deposited on the collector. The thickness of the fibers was expressed as the spinning time such as 1, 2 and 3 hrs. Electrospun fibers are collected as mix morphology of a non-woven and aligned form. Morphology of the electrospun PVDF nanofiber was observed with scanning electron microscope (SEM) of JEOL model JSM-5410LV. The circular and square 3D-nanofibrous PVDF scaffolds with varied thickness were

constructed by fixing the membrane on the structure of polyethylene plastic.

**PBSCs growth and differentiation on PVDF 3D-nanofibrous scaffold:** The 3D-nanofibrous PVDF scaffolds were disinfected by purging in 70% alcohol for 30 minutes and were washed using sterile phosphate buffer pH 7.4 at room temperature in biohazard cabinet. The scaffolds were UV-C irradiated for 1 hr then immersing into sterile RPMI-1640 medium and let incubated at 37 °C for 24 hrs in a $CO_2$-incubator. The scaffolds were transferred to 6-well plates. PBMCs (50 µL of $3\times10^6$ cells) were seeded onto the scaffolds and further incubation at 37 °C for 24 hrs in a $CO_2$-incubator, then 4 mL of fresh RPMI 1640 medium was added into the wells and further incubation for 72 hrs. Then the scaffolds were transferred into new 6-well plates and completed with 4 mL of RPMI 1640, incubated at 37 °C in a $CO_2$-incubator. The culture was maintained by changing the culture medium once a week.

**Scanning electron microscopy and EDX sample preparation:** The scaffolds were firstly fixed by immersing 2.5% glutaraldehyde in 0.1M Cacodylate buffer, pH 7.4 for 2 hours at room temperature or at 4° C (in refrigerator) overnight. They were washed 3 times in 0.1 M cacodylate buffer pH 7.4; each 5 minute duration. Then the second fixation was performed by immersing the scaffolds in 1% osmium tetroxide (aqueous) pH 7.4 for 1 hour at room temperature and in a light tight container. The scaffolds were again washed 3 times in 0.1 M cacodylate buffer pH 7.4; each 5 minute duration. The scaffolds were dehydrated by sequentially immersing as follows: 1 x 10 min. in 25% ethanol, 1 x 10 min. in 50% ethanol, 1 x 10 min. in 70% ethanol, 1 x 10 min. in 85% ethanol, 1 x 10 min. in 95% ethanol, 2 x 10 min. in 100% ethanol, 1 x 10 min. in 100% ethanol (EM grade). The scaffolds were then submitted to perform critical point dry which is an automated process takes approximately 40 minutes. The scaffolds were mounted onto metal stub with double sided carbon tape. Finally, a thin layer of gold and palladium were coated over the scaffolds using an automated sputter coater.

**Results**

**Enumeration of *CD34*$^+$ cells from PBMCs cultures**

The PBMCs were isolated using ficoll gradient centrifugation technique. Once suspension in RPMI 1640 or MEM medium, PBMCs are round shape and have the cellular diameter about 8 ± 2 µm (Figure 1a). The stem cells found in the PBMCs isolated were identified by positively staining with anti-*CD34*$^+$-FITC (Figure 1b). The enumeration of the anti-*CD34*$^+$-FITC positive cells was analyzed by flow cytometry (Figure 1c). By using our isolation technique, 0.1 % PBMCs was identified as PBSCs.

**Expansion of adult PBSCs in conventional culture**

At high density such as $1\times10^7$ cells/mL, PBMCs could be maintained in RPMI 1640 and MEM medium at least 6 days without observation of any changes in cellular morphology. While the differentiation of cells and colonies of PBSCs were observed when the cultures were performed at lower density of PBMCs (varied from $10^5$ to $10^6$ cell/mL).

Figure 1 shows a progressive change of PBMCs. After seeding $10^6$

cell/mL PBMCs into a culture at 24 hr, the cells were individually very rare

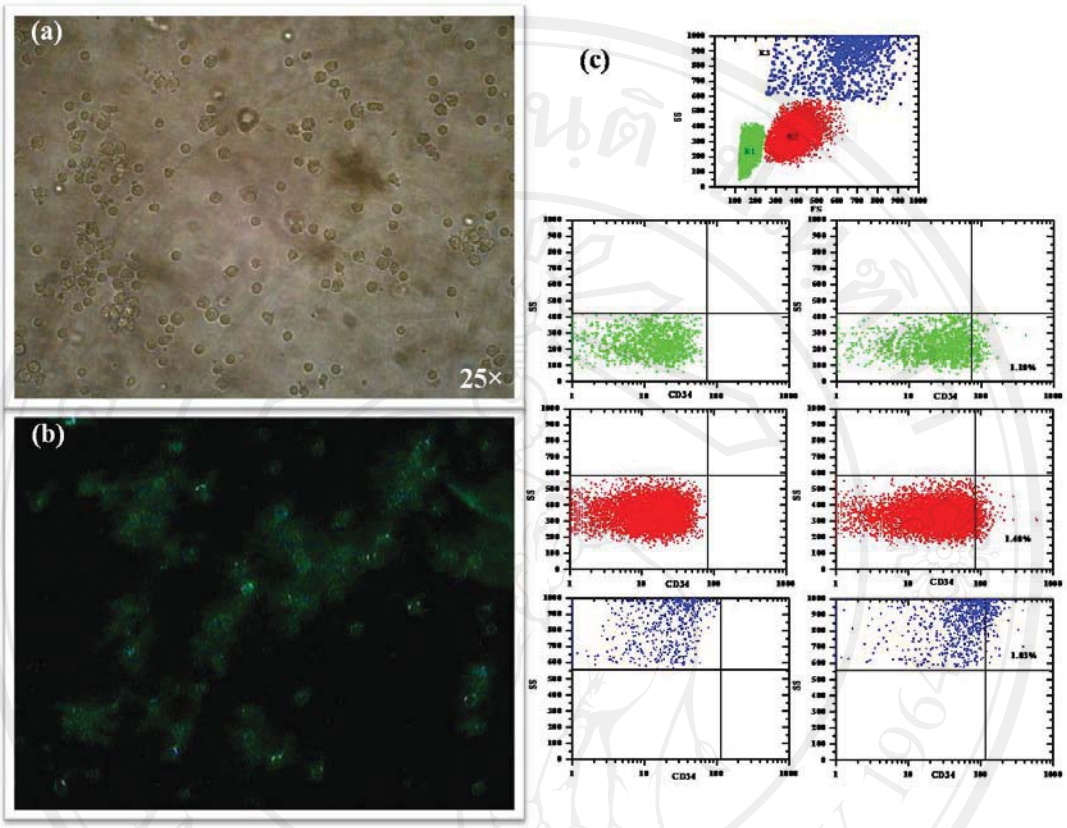



**Figure 1** Characterization of PBSCs by (a) light micrograph, (b) fluorescence micrograph and (c) flow cytometry.

aggregated (2-4 cells) distributed throughout the culture plates. The majority (80%) of PBMCs were found in oval shape (Figure 1a). Small colonies of cells about 3±2 colonies per well were observed. These cells were positively stained with anti-$CD34^{+}$-FITC corresponding to the so-called PBSCs (Figure 1b, 1c). The PBSCs colonies were grown up as a function of culture time. Figure 2b shows an increase in colonies size and number after seeding the PBMCs in the culture for 6 days. As can be seen in Figure 2c some cells still suspended in the medium and some were found to attach onto the bottom surface of wells and the characteristic of PBSCs colonies defined as round cells at core and spindloid cells at the periphery were easily observed at day 8 after culture (Figure 3c). When the cells continuous culture for 24 days, the variety of cellular morphology were observed including spindle cells, rod and cone cells, foam cell, endothelial cells neuron-fiber etc. (Figure 2d). It should be noted that the differentiated cells were observed only for the attached cells.

As previously mentioned the PBMCs were cultured in RPMI 1640 or MEM supplemented with 1 % penicillin-streptomycin and 10% fetal calf serum without any mitogens. The results signify that our conditions of experiments might be suitable for growing and promoting differentiation of the PBSCs. In fact, the PBMCs obtained in this study contain some amounts of platelets. We do a

hypothesis that both platelets and PBMCs should act as extracellular matrix of the PBSCs.

heamocytometer. Figure 3 shows that when the cells were grown in RPMI 1640 and MEM medium the numbers

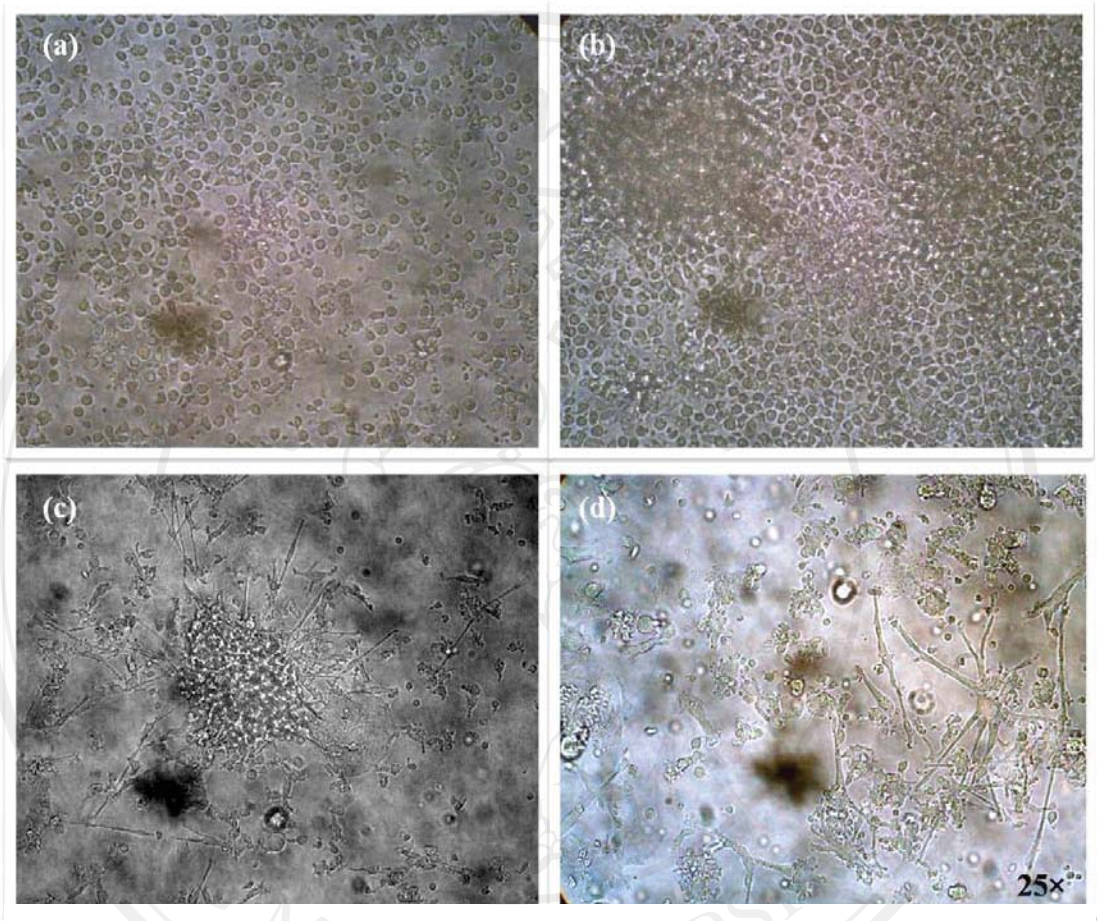



**Figure 2** Micrographs of PBSCs at day 1 (a), day 6 (b), day 15 (c) and day 20 (c) after seeded into the conventional culture system.

**Characteristic stem cell growth and division**

The observation of cell morphology under an inverted light microscope provides evidence of colony forming units of stem cells in the culture system but it was difficult to enumeration of the stem cells. For the purposes of characterizing the growth pattern of these stem cells, two series with the same conditions of experiments were performed. One series the cells were observed under inverted microscope before trypsinization and the number of PBMCs was counted by

by the days of culture and the maximum numbers were found at day 6 of culture following a decrease. It should be noted that at day 6 of culture, an increased in attached cell numbers and variety of morphology seems to be equilibrate with the suspended cell fraction. This might cause an error of number cell counts.

The other series of experiments, the cells were stained with anti-*CD34*$^{+}$-FITC and anti-*CD11b*-FITC as a function of culture time prior to analysis by flow cytometry. As can be seen in Figure 1a

the cells obtained after letting in

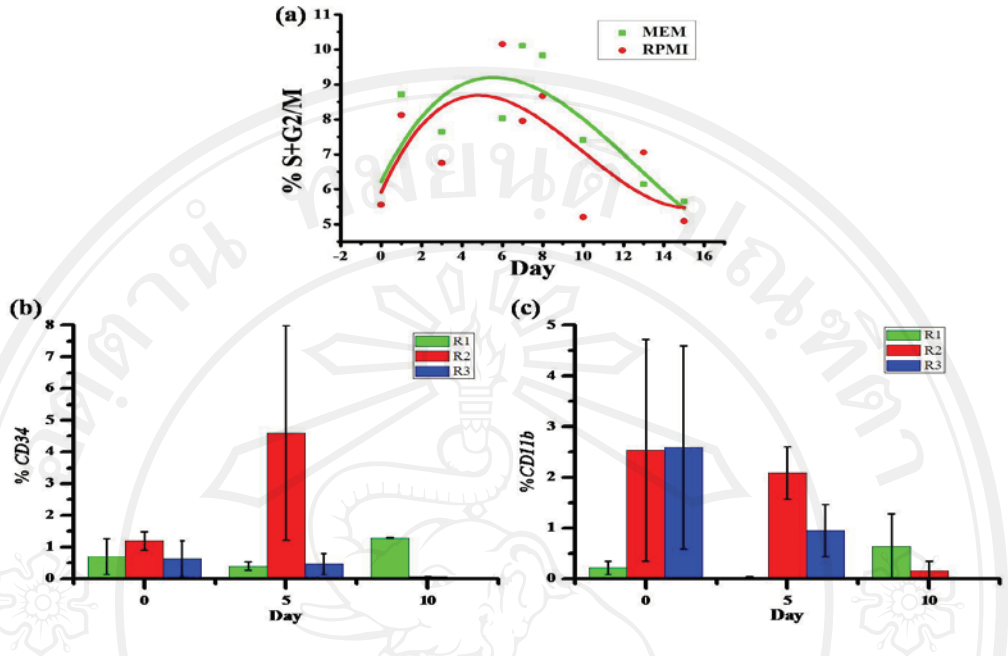



**Figure 3** Growth and differentiation of PBSCs: growth pattern(a), variation of percentage of $CD34^{+}$(b) and $CD11^{+}$(c) cells as a function of culture time.

culture system divided in 3 subgroups assigned as R1, R2 and R3 region. Figure 3b showed that the $CD34^{+}$ cells which corresponding to PBSCs were initially found almost the same percentage in the three regions. At day

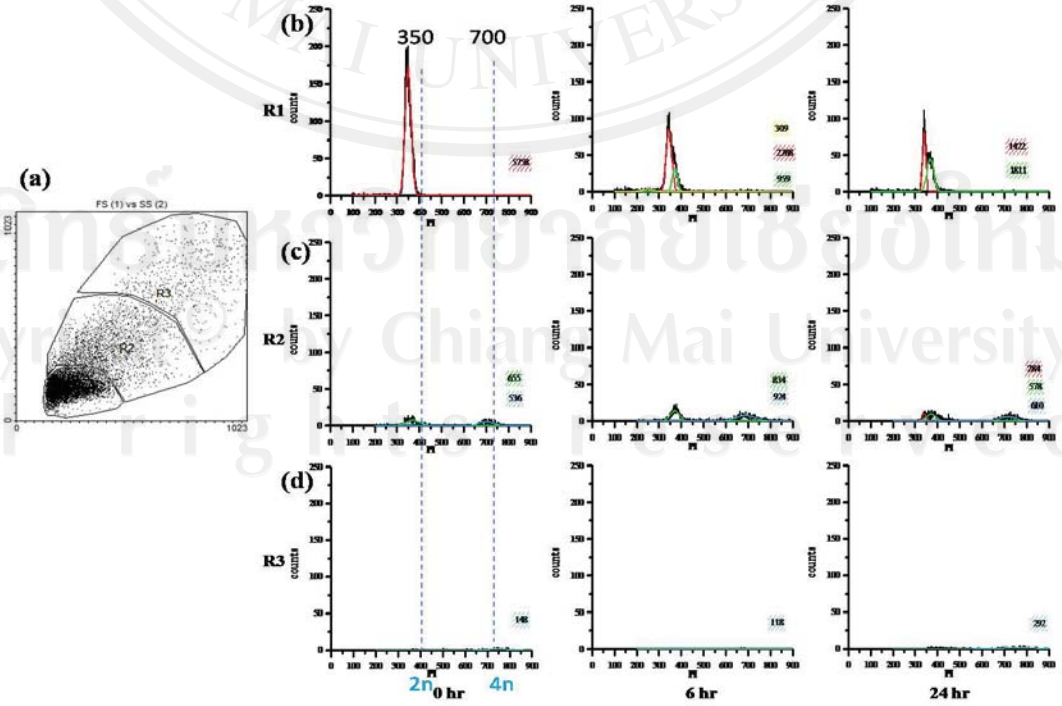



**Figure 4** Flow cytometric analyses of DNA contents: light scattering characteristic of cells (a) and histogram of Cell bound PI fluorescence intensity of R1 (b), R2 (c) and R3 (d) region as a function of culture time.

5 of culture the PBSCs in R2 was increased by 4 times of the initial condition then dramatically decreased at day 10. Contrary the PBSCs in R1 decreased by 50% at day 5 and increased at day 5. It should be noted that the PBSCs in R3 decreased and disappeared at day 10. Figure 3c demonstrated that the *CD11b* cells which corresponding to monocytes and dentritic cells represent the mature cells were initially found in R2 and R3 region, these cells were decreased as a function of culture time and did not find in the culture at day 10. These results strongly suggested that there is an existing of kinetic status and homeostasis of PBSCs in the culture system and the life-span of mature cells should be shorter than 10 days.

In order to get further insight the cellular kinetic status, the DNA contents of the cells were analysized as a function of culture time by using PI in a flow cytometer. The histogram of DNA contents of cells was reported in Figure 4. As can be seen, at initial time of culture almost of cells were found in R1 where almost the cells were found in G0/G1 phase (94%) and the cells in

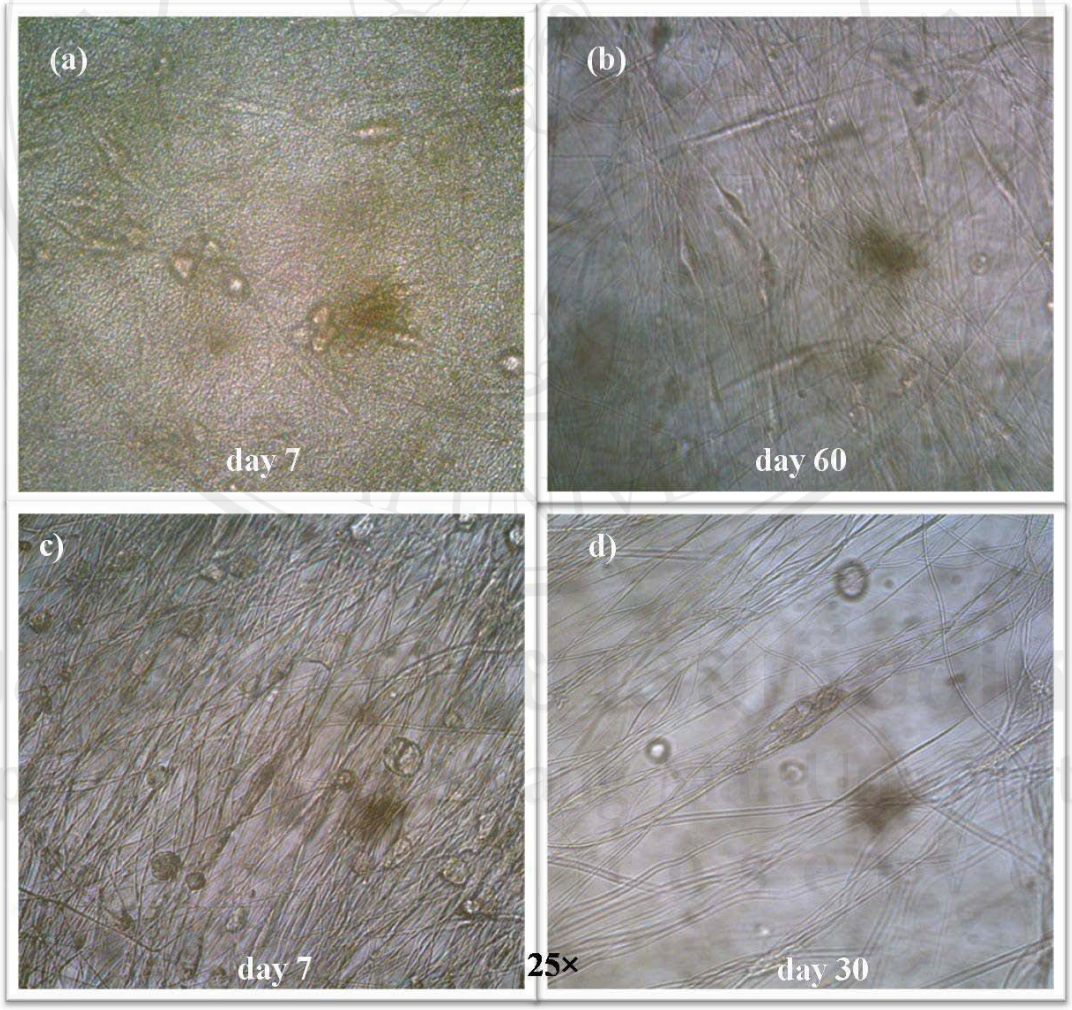



**Figure 5** Micrographs of cells attached onto non-woven zone at day 7 (a) and day 60 (b) and aligned zone at day 7 (c) and day 30 (d) after seeded onto the top surface of 3D-PVDF nanofibrous scaffolds.

R2 and R3 were found in S, G2 and M phase (5.4%). The content of DNA of cells in R1 was found as Gaussian distribution and perfectly fitted using Gaussian equation (mode of PI fluorescence intensity = 350 au and

maintain themselves or in other words, the number of PBSCs at steady state should be stable in our system.

**Reconstitute cell communities and tissues of PBSCs on PVDF 3D-nanofibrous scaffold**

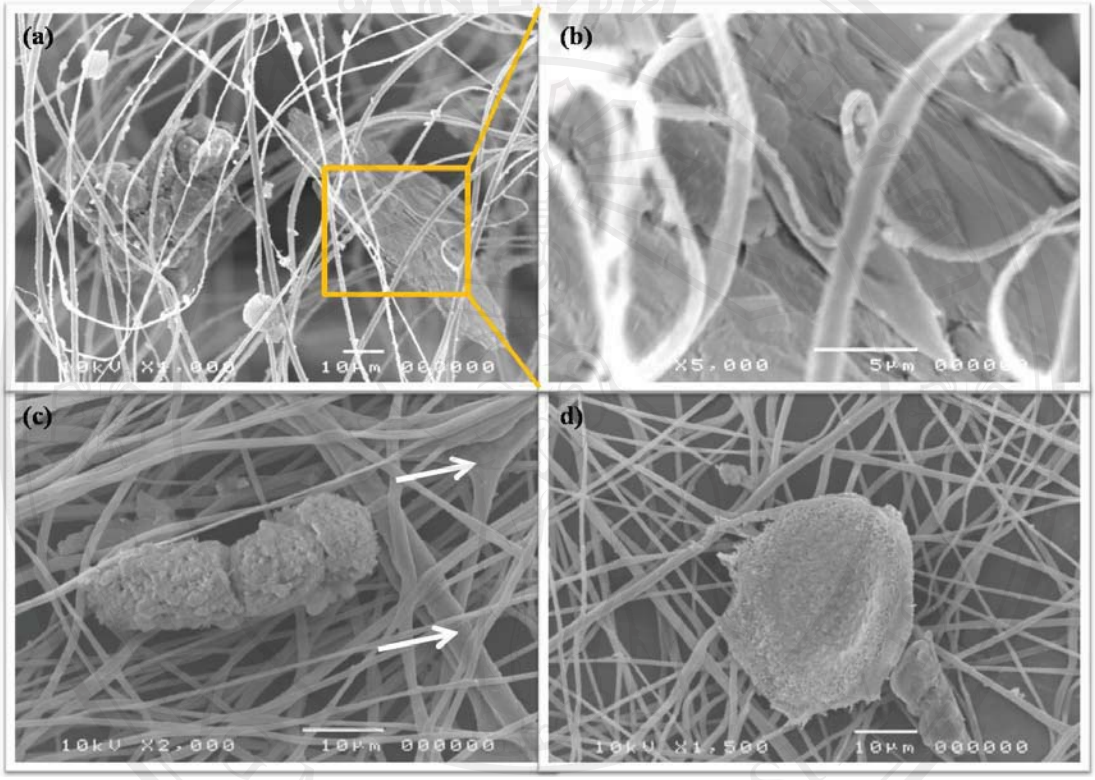



**Figure 6** SEM Micrographs of cells attached onto the 3D-PVDF nanofibrous scaffolds at day 60.

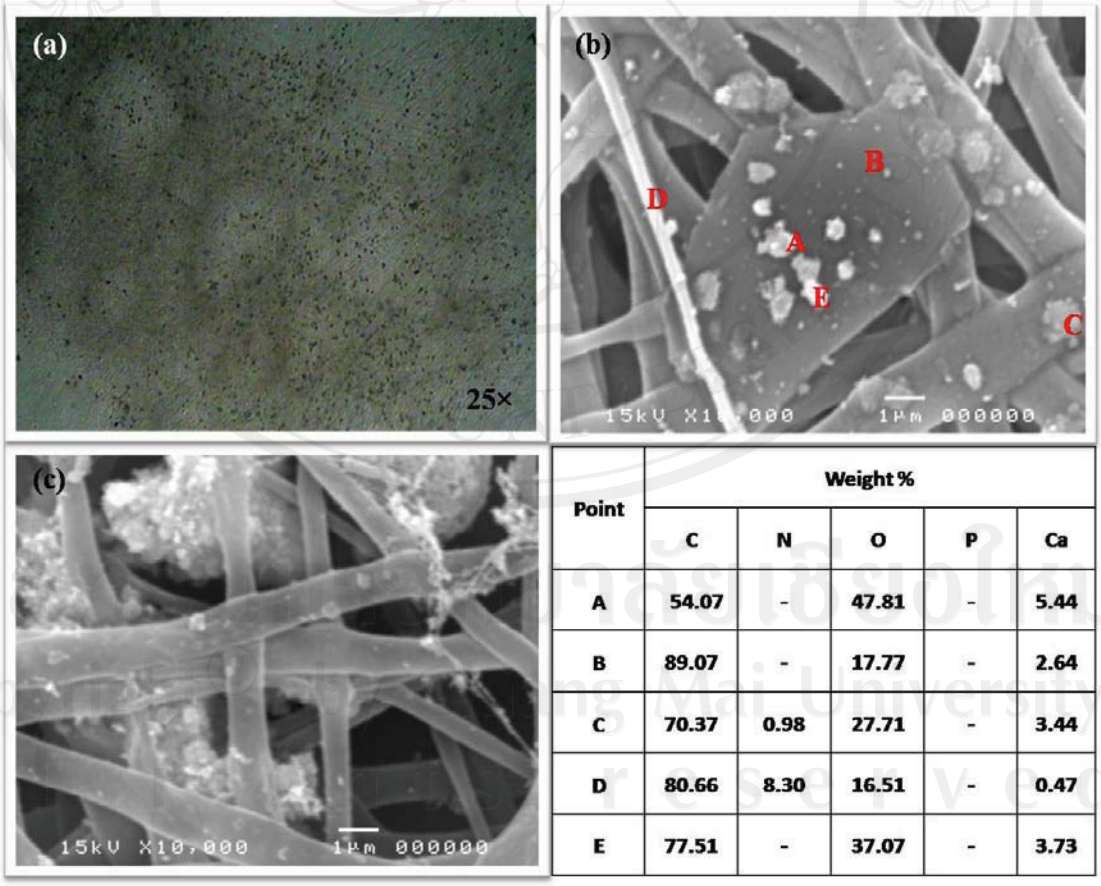
half peak of maximum = 40 au). After 6 hours of culture, two new Gaussian peaks were appeared. One peak, the mode of PI fluorescence intensity shift to right hand of x-axis. The amplitude and shoulder of new peak was increased as a function of time. The results suggested that the cells leave from G1 and are entering to S phase of cell cycle. The other peak, the mode of PI fluorescence intensity shift to left hand of x-axis indicating the cells in sub G0 phase corresponding to apoptotic cells by which obviously determined at 3 days after culture. This signified that PBSCs always

In this series of experiments, the in house made of disc-like scaffolds with 250 mm diameter and 0.5 mm thickness, were used. It was verified that $3\times 10^6$ cells in 50 µL of RPMI 1640 was the most appropriated conditions that can allow the cells to spread throughout on the scaffold surface. After letting the cells contacted to the scaffolds for 24 hours at 37°C in a $CO_2$-incubator, the scaffolds were transferred to new 6-well plates and 3 mL fresh RPMI 1640 medium were added and the cultures were further incubation for at least 90 days and the culture medium was

changed at 4 days interval. It should be noted that the suspended colonies and attached cells outside the scaffolds always present. The attached cells on the well bottom under grew differentiation in similar way that observed in conventional culture technique.

Figure 5 demonstrated that the cells attached to the scaffold in both non-woven and alignment zone but different in morphology (Figure 5a and 5b). Almost the attached cells on the alignment zone were found to be differentiated in a variety of cellular morphologies when the cultures were maintained for 30 days and 60 days (Figure 5c and 5d). In order to get further insight the interaction between the fibers and cells, the scaffolds were analyzed using SEM and EDX. The micrographs also showed that the fibers were coated with small vesicles probably containing proteins (Figure 6a). The most important data obtained from the micrographs were the irregular forms of cell organizations; vascular system and muscle bundle-like used the fibers as support for hanging themselves in the space (Figure 6). These suggested that the cells need the 3 dimensions for organizing their own communities.

Indeed, the non-woven zone of the scaffolds was highly porous matrices and the diameter of pore site




| Point | Weight % | | | | |
|---|---|---|---|---|---|
| | C | N | O | P | Ca |
| A | 54.07 | - | 47.81 | - | 5.44 |
| B | 89.07 | - | 17.77 | - | 2.64 |
| C | 70.37 | 0.98 | 27.71 | - | 3.44 |
| D | 80.66 | 8.30 | 16.51 | - | 0.47 |
| E | 77.51 | - | 37.07 | - | 3.73 |

**Figure 7** Characterization of bone tissue formation found in the non-woven zone of 3D-PVDF nanofibrous scaffolds at day 60 after cultured: (a) photo micrographs of cells, (b and c) high magnification SEM micrographs of the same sample of Figure 7a and (d) elements analysis of the indicated points of Figure 7b.

was average in micrometer range. These should be influenced on the attachment and growth pattern of the cells. As demonstrated in Figure 7 the photo micrographs of revealed that a small black spots spread throughout the scaffold (Figure 7a). These black spots were further characterized by

(Figure 8a and b). Most of the cells were positive stained with anti-*CD34*$^{+}$-FITC (Figure 8c and d). In the non-woven zone of the scaffolds, there were colonies of cells which have dark-blue color inside known as giant cells and mesenchymal stromal cells (Figure 9a). Figure 9b and c showed

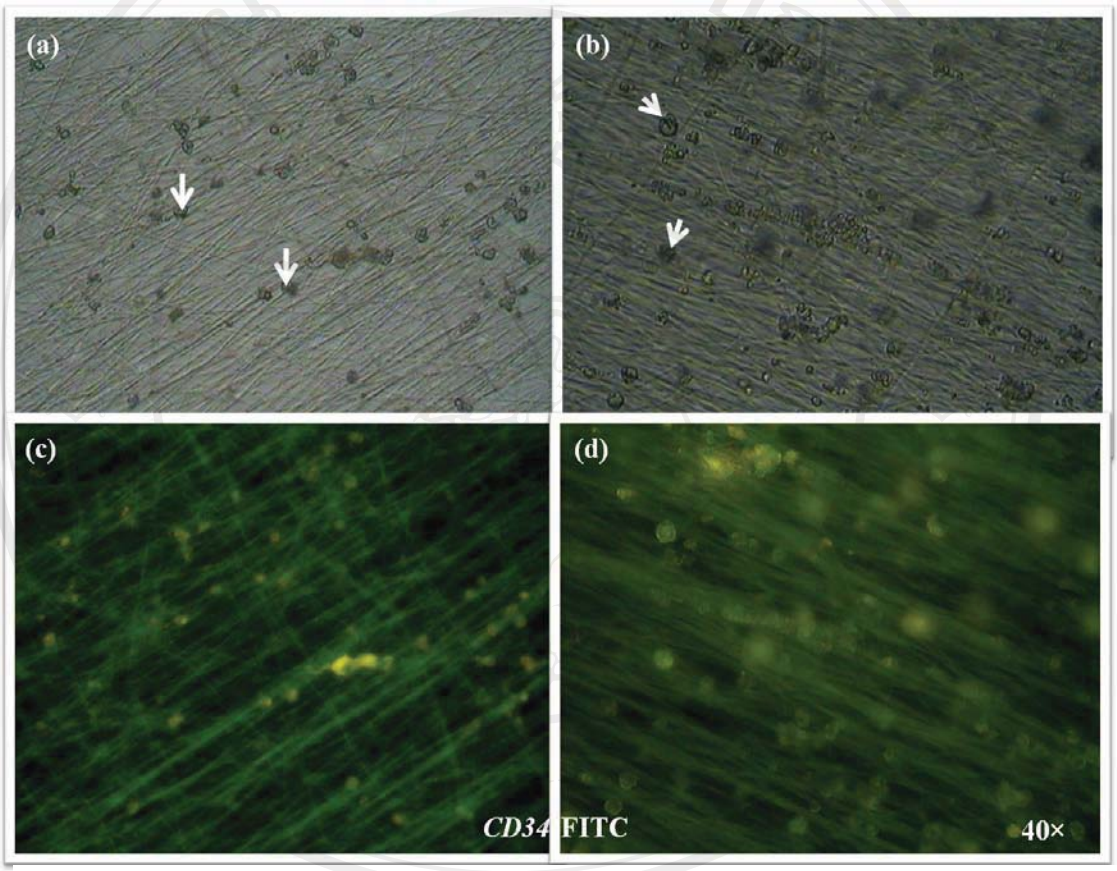



**Figure 8** Micrographs of cells stained with anti-*CD34*$^{+}$-FITC: (a and b) bright field of photo micrographs and (c and d) fluorescence micrographs of the same microscopic field at day 60.

SEM and EDX as indicated in Figure 7 b, c and d. Using high magnification of 10,000×, the spots consist of cells, extracellular matrix such as and protein fibers connect to micro vesicles containing proteins and complexes of calcium. The cells found in the non-woven zone were irregular in shape and sites. The giant cells contain small fine dark granules concentrated in the perinuclear zone were found distributed throughout the scaffold

small cells of varying shape, irregular, polygonal, oval, and round with small nucleus adhere to the surface of the complex of giant and mesenchymal stromal cells. These cells were recognized as hematopoietic stem cells. This signified that most of them should preserve the properties of stem cells. The results were confirmed by SEM and EDX experiments.

**Discussion and conclusion**

Adult peripheral blood stem cells (PBSCs) are found in normal subject's blood circulation about 0.1% (of total white blood cells). This study makes evidence that PBSCs is one of important source of stem cells which were very easy to achieve and expand in culture without any addition neither mitogens nor specific growth factors. Contrary to huge reports accumulated since the last decade that researchers were focused on cytokines, stromal cells and combined these two studied fields to optimize the experimental conditions for hematopoietic stem cell expansion [9-13]. The PBSCs can be concentrated in the PMCs fraction of ficoll gradient centrifugation and were characterized by using the immunological analysis of *CD 34*$^{+}$ marker which is currently considered to reflect the best surrogate measure of hematopoietic stem and progenitor cells and also the most precise marker to guide timing of peripheral blood stem cell (PBSC) apheresis [4,8]. The results clearly showed by flow cytometric analysis revealed that the *CD34*$^{+}$ cells were found throughout the R1, R2 and R3 region but only those found in R1 region that have smallest and lowest granularity can preserve re-newal characteristic. The *CD34*$^{+}$ cells found in R2 and R3 which have bigger and higher granularity than

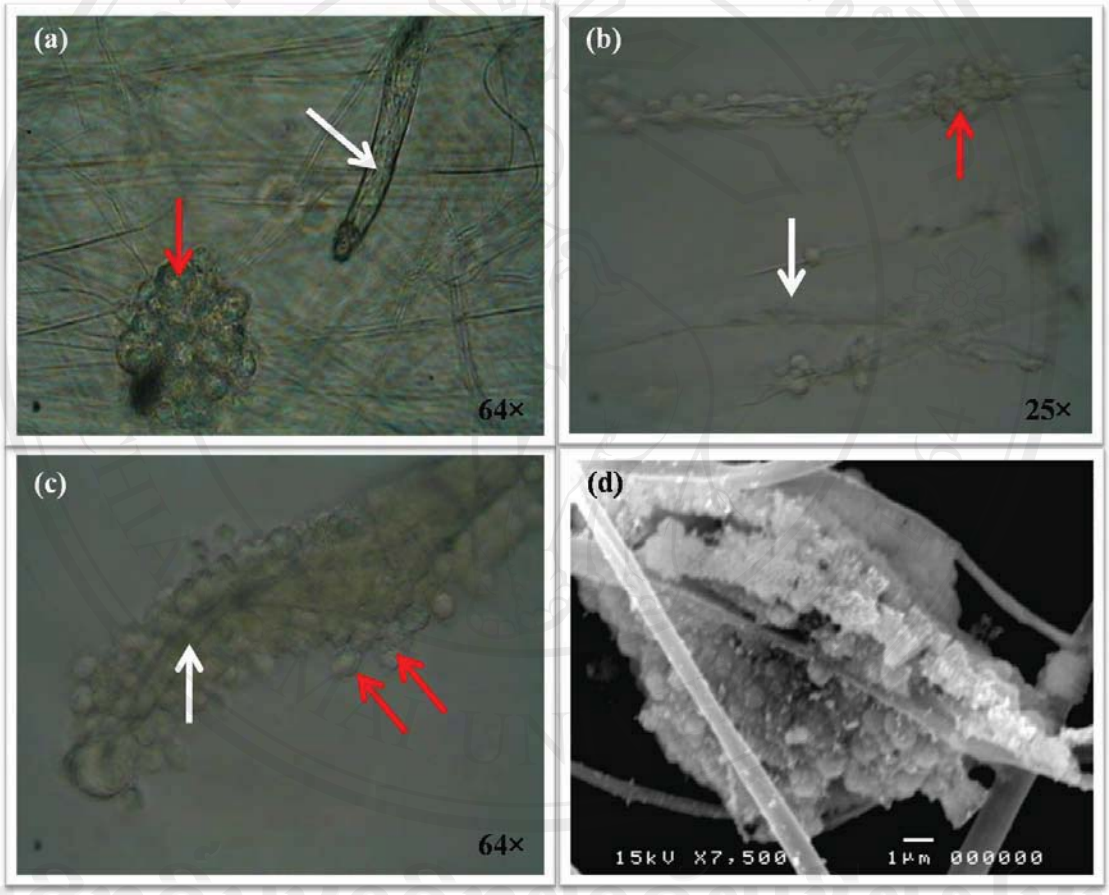



**Figure 9** Micrographs of cells which cultured on the 3d-PVDF nanofibrous scaffold at day 60: (a, b and c) bright field photo micrographs of giant (red arrow) and stromal (white arrow) cells and (d) SEM micrograph of hematopoietic tissue.

those found R1 were differentiated to specific cell types. In fact, the smallest size of PBSCs preserve the self-renewal characteristic. The results signified that differentiation to a variety of specific cell types of PBSCs should be along with an increase in biomass of cells. The similar findings of self renewal and differentiation to a variety of specific cell types were reported in the human when PBSCs were used for autologous transplantation. Transplantation using PBSCs has several advantages including more rapid neutrophil and platelet recovery, and reduced platelet and red blood cell transfusion requirements [4].

The DNA content analysis of cells revealed that the number of PBSCs in resting phase G0 at steady state was constant in our system. One fraction of cells in G0 undergo differentiation to a variety of specific cell types while the other fraction of cells leave from G0 entering to G1, S, G2 and M phase of cell cycle or the proliferating cells. These proliferating cells might undergo apoptotic cell death by which controlled the number of cells in the system the so-called “the cellular homeostasis”. In this study PBSCs can be expanded and the maximum of PBSCs which preserve the renewal property were about 4 times within 6 days compared with those at the initial situation. In conventional cell culture conditions, a variety of specific cells types such as endothelial cells, neuron cells, chondrocytes, myocytes, lymphocytes, etc. were observed.

The pluripotency of PBSCs can also be demonstrated in the 3D-PVDF nanofibrous scaffold culture system. The 3D-PVDF nanofibrous scaffolds were in house fabricated by electro spun technique. The nanofibers were designed to have mixed morphology of non-woven and alignment form called the egg-net scaffold. Indeed, the mixed morphology of non-woven and alignment scaffolds has high porosity. The oval shape of egg form consists of the nanofibers lined in non-woven form generating opaque and highly porous matrices, while the net shape consist of aligned nanofibers resulting in translucent loose tissue. By using the egg-net scaffolds in the culture system, they themselves generate the specific microenviroments that influenced on the stem cell attachment, growth following cell community and tissue formations. This study show that the applications of the in house made of disc-like scaffolds with 250 mm diameter and 0.5 mm thickness and $3\times 10^6$ cells in 50 μL of RPMI 1640 was the most appropriated conditions that can allow the cells to spread throughout on the scaffold surface. During cell culture, beside the cells grew on the scaffold, the suspended colonies and attached cells outside the scaffolds always present and underwent differentiation in similar way that observed in conventional culture technique. These results strongly suggested that the 3D-PVDF nanofibrous scaffolds are biocompatible and did not disturb the growth and differentiation patterns of cells. The scaffold should act as extracellular matrix supports and hangs the attached cells in the space, later develop communication and create a specific cell communities and specific tissues (Figure 6, 7, 8 and 9). These signified that the culture system used in this study should provide an appropriate microenvironments such as complex network of cytokines (stem cell factor (SCF), Flt-3 ligand (FL),

thrombopoietin (TPO), leukemia-inhibiting factor (LIF), interleukin; IL-6, IL-7, IL-8, IL-11, IL-12, IL-14, and IL-15 [14, 15]), adhesion molecules, and extracellular matrix proteins that regulate survival, proliferation, growth and differentiation of PBSCs [16-17].

The overall results suggest that the PBMCs fraction is an important of source of pluripotency stem cells which particularly have the homeostasis process for controlling the number of cells which is the characteristic of normal stem cells. These PBSCs are able to generate cell communities and tissues in the appropriate microenviroments. The results also clearly shown that the 3D-PVDF nanofibrous scaffold of both non-woven and alignment matrices were very good supporters that can hang the cells in space and restrictly controlled the micro enviroments allow the cells organizing the 3D tissues.

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**References**

1. Devine, S. M. (2002). "Mesenchymal stem cells: will they have a role in the clinic?" J Cell Biochem Suppl **38**: 73-9.
2. Corso, A., M. Varettoni, et al. (2005). "Bone marrow $CD34^{+}$ cell count is predictive for adequate peripheral progenitor cell collection." Leuk Res **29**(2): 159-63.
3. Sagar, J., B. Chaib, et al. (2007). "Role of stem cells in cancer therapy and cancer stem cells: a review." Cancer Cell Int **7**: 9.
4. Rowley, S. D., J. Yu, et al. (2001). "Trafficking of CD34+ cells into the peripheral circulation during collection of peripheral blood stem cells by apheresis." Bone Marrow Transplant **28**(7): 649-56.
5. Gutensohn, K., Magens, M. M., et al. (2001). "Monitoring the timing of peripheral blood stem cell apheresis: Application of the hematopoietic progenitor cell analysis." Infusionstherapie und Transfusionsmedizin **28**(5): 271-276.
6. Fontao-Wendel, R., A. Lazar, et al. (1999). "The absolute number of circulating CD34+ cells as the best predictor of peripheral hematopoietic stem cell yield." Journal of Hematotherapy **8**(3): 255-262.
7. Noronha, J. F. A., I. G. H. Lorand-Metze, et al. (2006). "Hematopoietic progenitor cells (HPC) and immature reticulocytes evaluations in mobilization process: New parameters measured by conventional blood cell counter." Journal of Clinical Laboratory Analysis **20**(4): 149-153.
8. Choi, S.-S., Lee, Y. S., et al. (2004). "Electrospun PVDF nanofiber web as polymer electrolyte or separator." Electrochimica Acta **50**(2-3): 339-343.
9. Harvey, K. and E. Dzierzak (2004). "Cell-cell contact and anatomical compatibility in stromal cell-mediated HSC support during development." Stem Cells **22**(3): 253-8.
10. Kadereit, S., L. S. Deeds, et al. (2002). "Expansion of LTC-ICs and maintenance of p21 and BCL-2 expression in cord blood

CD34(+)/CD38(-) early progenitors cultured over human MSCs as a feeder layer." Stem Cells **20**(6): 573-82.
11. Koh, S. H., H. S. Choi, et al. (2005). "Co-culture of human CD34+ cells with mesenchymal stem cells increases the survival of CD34+ cells against the 5-aza-deoxycytidine- or trichostatin A-induced cell death." Biochem Biophys Res Commun **329**(3): 1039-45.
12. Fukushima, N. and H. Ohkawa (1995). "Hematopoietic stem cells and microenvironment: the proliferation and differentiation of stromal cells." Crit Rev Oncol Hematol **20**(3): 255-70.
13. Cheng, L., P. Qasba, et al. (2000). "Human mesenchymal stem cells support megakaryocyte and pro-platelet formation from CD34(+) hematopoietic progenitor cells." J Cell Physiol **184**(1): 58-69.
14. Jang, Y. K., D. H. Jung, et al. (2006). "Mesenchymal stem cells feeder layer from human umbilical cord blood for ex vivo expanded growth and proliferation of hematopoietic progenitor cells." Annals of Hematology **85**(4): 212-225.
15. Placzek, M. R., I. M. Chung, et al. (2009). "Stem cell bioprocessing: Fundamentals and principles." Journal of the Royal Society Interface **6**(32): 209-232.
16. Janowska-Wieczorek, A., L. A. Marquez, et al. (1999). "Growth factors and cytokines upregulate gelatinase expression in bone marrow CD34+ cells and their transmigration through reconstituted basement membrane." Blood **93**(10): 3379-3390.

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