CHAPTER II

Materials and Methods

2.1 PBSCs donors

The project was approved from the Human Research Ethics Committee of Faculty of Associated Medical Sciences, Chiang Mai University (ref. no.361/2556). Twelve healthy donors who have blood group O were recruited and inclusion into the study was based on a questionnaire. The stem cell donors were divided in two age groups. First group was the adult age that comprised male and female who have age between 15 to 40 years and the other was elderly age that also comprise male and female with age between 50 to 70 years.

2.2 Collection of human peripheral blood mononucleated cells (PBMCs) from donors

Whole bloods (100 mL) were collected in the presence of Heparinized (1430 USP units) after the donors agreed and signed the consent form. Whole bloods were centrifuged at 1500 rpm for 30 min and the buffy coat was separated. The total volume of buffy coat 4 mL was transferred to a new 15 mL sterile tube then completed with 4 mL modified-DPBS (MD-DPBS) and gentle mixed. The Ficoll-hypauqe (4 mL) was carefully injected at the bottom of the tube prior to centrifugation at 1500 rpm for 30 min. The PBMCs that consists the PBSCs were isolated and washed once using sterile MD-DPBS. The PBMCs fraction was twice re-suspended in RBC lysing solution for 5 minutes. After that the cells were washed twice using sterile MD-DPBS then were resuspended in RPMI1640 w/o L-glutamine and phenol red, supplemented with 10 % human serum group O or fetal bovine serum and 1 % penicillin /streptomycin (BioMedia) and placed at 37 °C in 5 % CO_2 and 95%humidity in an incubator.

2.3Enumeration of PBMCs and $CD34^+$ cells

The $CD34^+$ cell counts were performed by anti-$CD34^+$-FITC staining and analyzed by flow cytometer. Briefly, cells (10^6) were centrifuged at 7,000 rpm for 1 minute and washed once using MD-DPBS pH 7.4 at 25°C. Afterward, 1 mL ice-cold ethanol (70% v/v) was added drop wise, with gentle agitation and further incubation at 4 °C overnight. The cells were centrifuged at 7,000 rpm for 1 minute. The pellets of cells were discarded then 5 µl Triton x-100 (0.1% v/v), 50 µl RNase (0.2 mg/mL) and 10 µl anti-$CD34^+$-FITC was added prior to further incubation in the dark at room temperature for 30 minutes. Add 440 µl MD-DPBS vortex briefly, analyzed in a flow cytometer and fluorescence microscope (Leica, Germany).

2.4 Flow cytometric analysis of cellular DNA contents

Cells (3×10^5) were centrifuged at 7,000 rpm for 1 minute and washed once using MD-DPBS pH 7.4 at 25°C. Afterward, 1 mL ice-cold ethanol (70% v/v) was added drop wise, with gentle agitation and further incubation at 4 °C overnight. The cells were centrifuged at 7,000 rpm for 1 minute. The pellets of cells were discarded then 5 µl Triton x-100 (0.1% v/v), 50 µl RNase (0.2 mg/mL) and 5 µL propidium iodide (1 mg/ml; US biological, USA) were added prior to further incubation in the dark at room temperature for 30 minutes. Add 440 µl MD-DPBS vortex briefly, analyzed in a flow cytometer and fluorescence microscope (Leica, Germany).

2.5 H&E staining

H&E staining was performed for the adherent cell fraction. Briefly, the medium and suspension cells were removed and the cells were washed twice using modified-DPBS pH 7.4 at 25°C. The cells were fixed on the culture plates using 500µL formalin (4% v/v) and let incubated at room temperature for 15minutes then washed once using MD-DPBS. The membranes of cells were permeabilized using 500 mL Triton-x100 (0.1% v/v) at room temperature for 10 minutes. The cells were washed with MD-PBS following a dehydration process by adding 500 µL of ethanol with sequential incremental percentage of 70%, 95%, and absolute ethanol. Afterward, 500 mL

hymatoxylin was added and placed at room temperature for 30 minutes, washed once using tab water. Add 500 µl ethanol (80% v/v) containing 1% HCl. Add 500 µl eosin and incubated at room temperature for 5 minutes and dehydration in 500 µl 70%, 95%, absolute ethanol. The cell morphology was observed under high resolution microscope.

2.6 Expansion of adult PBSCs in conventional culture

Cells (10^6 cell/mL) were cultured in 24-well plates with RPMI-1640 w/o L-glutamine and phenol red supplemented with 10 % human serum group O or fetal bovine serum and 1 % penicillin /streptomycin (BioMedia) at 37 °C in 5 % $CO_{2(g)}$ atmosphere in a humidified incubator at 95% humidity. The cell morphology was examined under an inverted light microscope every 24 hours. The cells were counted using a flow cytometer.

2.7 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 (60 µL of 10^7 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.

2.8 Scanning electron microscopy sample preparation

The scaffolds were firstly fixed by immersing 2.5% glutaraldehyde in 0.1Mcacodylate buffer, pH 7.4 for 1 hours at room temperature or at 4° C (in refrigerator) overnight. They were washed 2 times in 0.1 M cacodylate buffer pH 7.4; each 10 minute duration. Then the second fixation was performed by immersing the scaffolds in 1% osmium

tetroxide (aqueous) pH 7.4 for 2 hour at room temperature and in a light tight container. The scaffolds were again washed 2 times in 0.1 M cacodylate buffer pH 7.4; each 10 minute duration. The scaffolds were dehydrated by sequentially immersing as follows: 1 x 10 min. in 30% ethanol, 1 x 10 min. in 50% ethanol, 1 x 10 min. in 70% ethanol, 1 x 10 min. in 80% ethanol, 1 x 10 min. in 90% ethanol, 1 x 10 min. in 100% ethanol. 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.