行政院國家科學委員會專題研究計畫 期中進度報告

血液幹細胞和眼角膜幹細胞關係之研究(2/3)

計畫類別：個別型計畫
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執行單位：國立臺灣大學醫學院眼科

計畫主持人：胡芳蓉

報告類型：精簡報告

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中 華 民 國 92 年 6 月 2 日

血液幹細胞和眼角膜幹細胞關係之研究(2/3)
九十一年度計畫執行進度報告

一、本年度計畫研究方法

In the last year, we successfully isolated and cultured the human mesenchymal stem cells (hMSCs) from Bone Marrow. Induced by various culture conditions along with adding certain growth factors or cytokines, pictures of in-*vitro* differentiation of the cultured human mesenchymal stem cells to corneal endothelium-like cells in morphology were shown in previous report. In the second year, we have performed flow cytometry analysis and RT-PCR experiments, in order to identify the specific markers of the differentiated cells.

Flow cytometry analysis

For flow cytometry analysis, 10^6 cultured cells were washed in ice-cold PBS containing 1% BSA, fixed in freshly prepared 4% paraformaldehyde solution at room temperature, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate solution on ice. Resuspended cells were incubated with TUNEL reaction mixture or label solution as negative control for 60 minutes at 37, and then analyzed on a FACS Calibur (Becton Dickinson Immunocytometry Systems, Ontario, Canada). Data were collected from 10 000 cells per sample after background fluorescence and forward-and side-scatter parameter were set.

Identification of Specific markers in Cultured Cells: Keratin 12 (Epithelium), Keratocan (keratocytes), and Type VIII collagen Markers (Endothelium)

mRNA of specific markers, including collagen VIII as a cell marker for corneal endothelium ; keratin 12, a marker for corneal epithelium; and keratocan, a marker for stromal fibroblasts cells are detected by RT-PCR.

Total RNA from cells is prepared using the acidic phenol/chloroform phase separation method.

For RT-PCR studies, total RNA is prepared from confluent cultures of endothelial cells and stromal fibroblasts and from frozen corneal epithelium using reagent according to the manufacturer's directions (Trizol; Gibco). cDNA is prepared from 1 μg total RNA by reverse transcription in a volume of 20 μl using reagents from a commercially available kit (Promega, Pittsburgh, PA). Primers specific for

each cell marker are designed using software (MacVector 5.0; Oxford Molecular Group, Oxford, UK). The primer pairs used are: collagen VIII: upstream sequence, 5'-CCAAAGGAGAAGGTGGAGTTG-3', downstream sequence, 5'-ACAATTCCTGGGATACCTGGGG-3'; keratin 12: upstream sequence, 5'- GAG TGG ATC TCA CCA AGG -3', downstream sequence, 5'- CAA TCT CTA GGT TCT GCA -3'; and keratocan: upstream sequence, 5'- TTC AGC AAT CTG GAG AAC CTG -3', downstream sequence, 5'- GTT AGA TTG TTG TGT TGT CAT GC -3'. PCR is performed in a reaction mixture containing 1μg cDNA and 2.5 μM each of the upstream and downstream primers, plus reagents from a commercially available kit (Gibco). PCR is performed for 40 cycles in a thermal cycle. Cycle conditions included denaturation at 95°C for 1 minute, annealing at the required temperature for 1 minute, and extension at 72°C for 2 minutes. A 5-minute extension is added at the end of the 40 cycles of PCR. Annealing temperatures are as follows: collagen VIII, 60.0°C; keratin 12, 50°C; and keratocan, 51.0°C. PCR products and 100-bp DNA ladder molecular weight markers are electrophoresed in 1.5% agarose gels containing 0.5 μg/ml ethidium bromide and photographed. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH; Clontech, Palo Alto, CA) acted as a positive control for the PCR. Negative control samples consisted of the PCR reaction mixture, including primers, but without cDNA. To ensure that the total RNA samples are not contaminated with genomic DNA, a negative control using 1μg total RNA is substituted for cDNA in the PCR reaction mixture, along with 2.5 μM each of upstream and downstream G3PDH primers.

二、本年度計畫研究成果

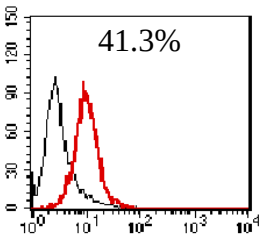
Bone marrow stromal fibroblasts can easily be isolated from other types of cells, including hematopoietic or other stromal cells, because they tend to adhere to tissue culture plastic and grow extensively. They are spindle shape with small nuclei and little cytoplasm. Expanded MSCs then were cultured by different supporting matrix and medium with or without adding different cytokines and growth factors in order to induce differentiation to corneal endothelium cell-like precursors.

In previous reports, we mentioned that the differentiated MSCs turned out to be round shape and had abundant cytoplasm which looked like the morphology of the endothelial cells.

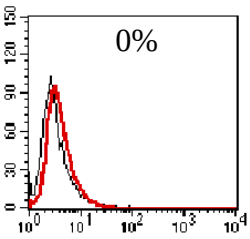
Flow cytometry analysis

Mensenchymal stem cells cultured for 8-12 passages were labeled with FITC-coupled antibodies against integrin αV, integrin α1, integrin α2, integrin α3, integrin β1, integrin β3, CD 34, CD44, CD45, CD 54, CD56, CD58, CD71, CD90, CD105, CD106, CD117, CD166, L-selectin and ICAM-2.Cells were analyzed using FACS-Calibur. In the following figures, black line for control immunoglobulin; red line, specific antibody.

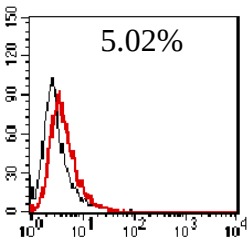
Integrin αV



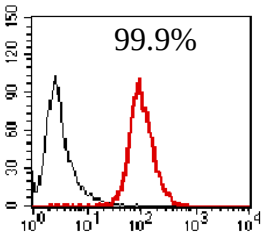
Integrin β3



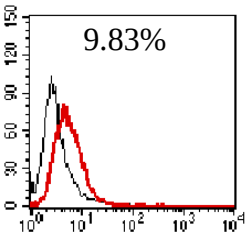
Integrin α2



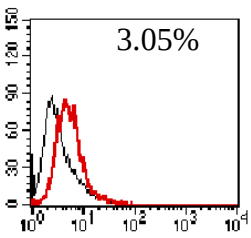
CD44



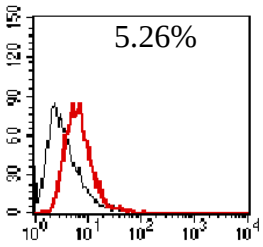
CD54



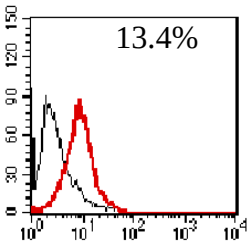
L-Selectin



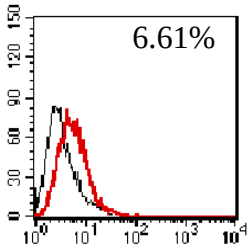
Integrin α5



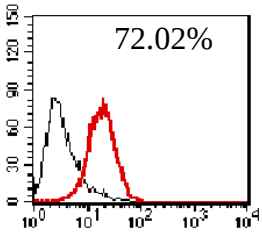
ICAM-2



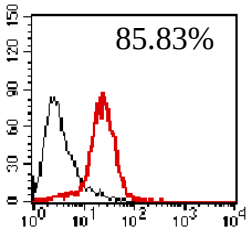
Integrin α1



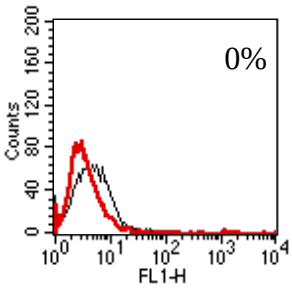
CD166

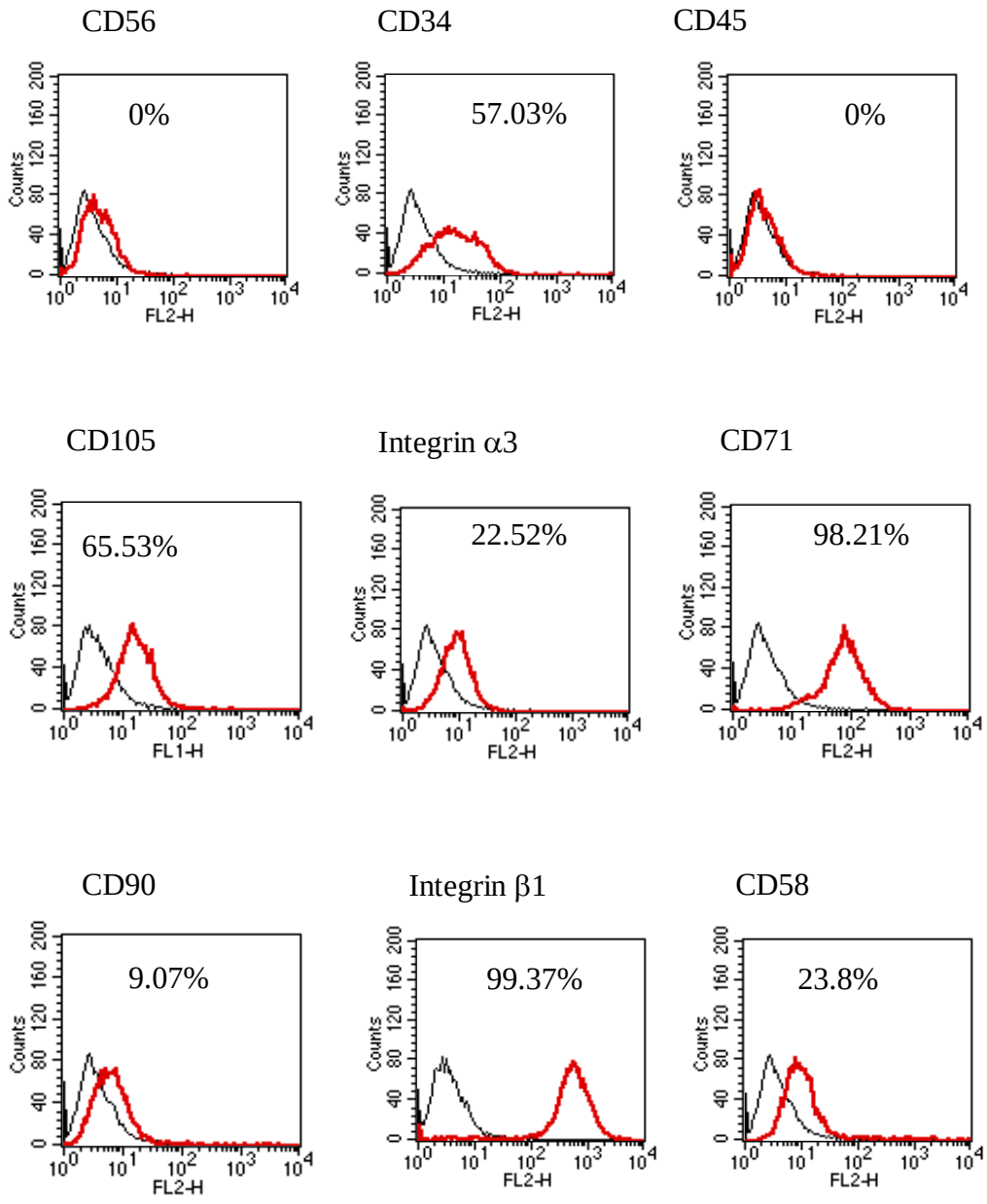


CD117



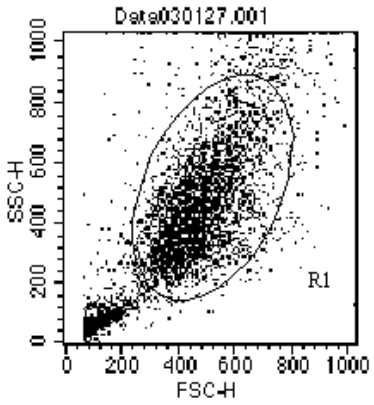
CD106

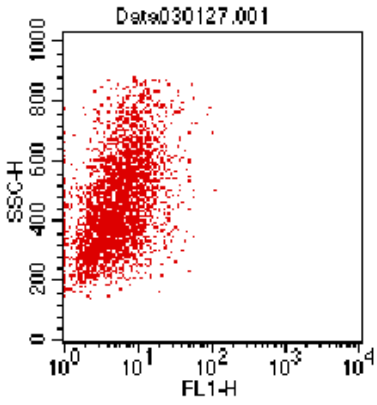


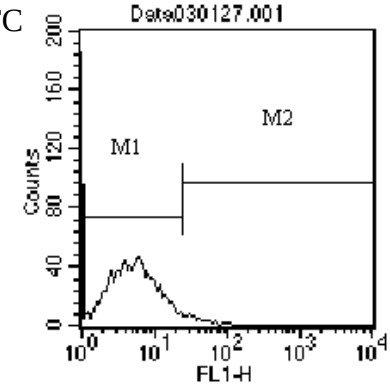


For type VIII collagen, we use CD34 as control surface antibody; we found there is no Collagen type VIII on the surface of cultured Macs. It is better to choose the 1: 50 dilute collagen type VIII antibody.

NS IgG-FITC





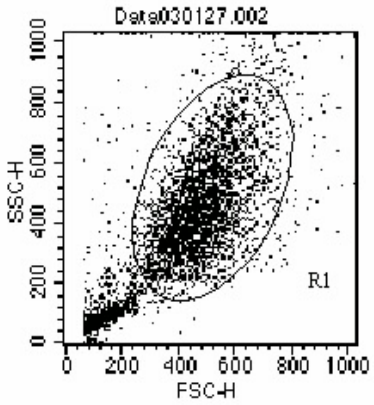


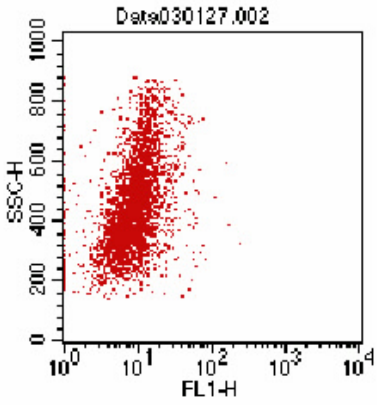
Histogram Statistics

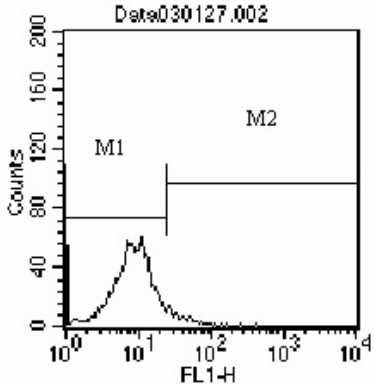
File: Data030127.001 Gate: G1
Gated Events: 6741 Total Events: 10000
X Parameter: FL1-H (Log)

Marker	Events	% Gated	% Total
All	6741	100.00	67.41
M1	6549	97.15	65.49
M2	194	2.88	1.94

Collagen type VIII 1:50







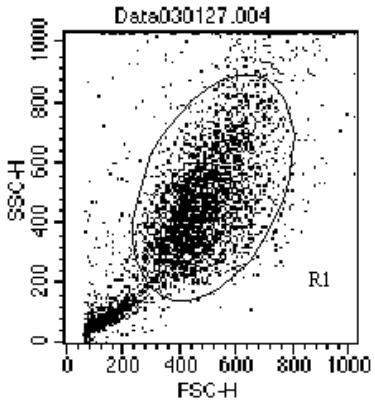
Histogram Statistics

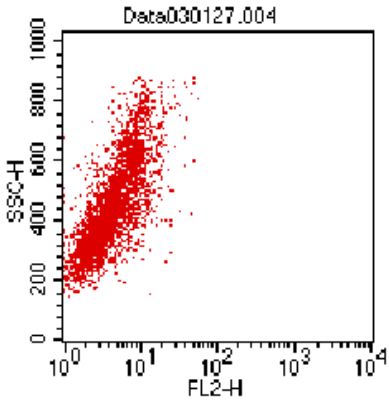
File: Data030127.002 Gate: G1
Gated Events: 6553 Total Events: 10000
X Parameter: FL1-H (Log)

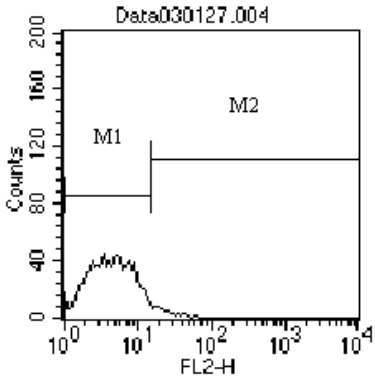
Marker	Events	% Gated	% Total
All	6553	100.00	65.53
M1	6272	95.71	62.72
M2	294	4.49	2.94

NS

IgG1-PE







Histogram Statistics

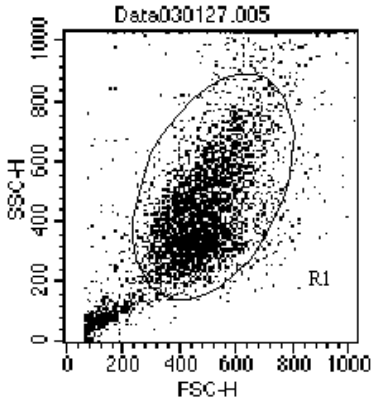
File: Data030127.004 Gate: G1

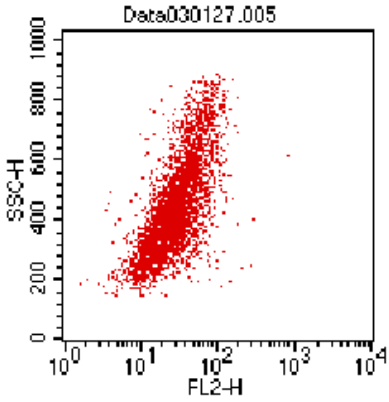
Gated Events: 6456 Total Events: 10000

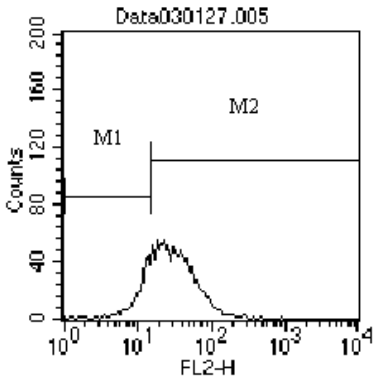
X Parameter: FL2-H (Log)

Marker	Events	% Gated	% Total
All	6456	100.00	64.56
M1	6279	97.26	62.79
M2	181	2.80	1.81

CD34PE







Histogram Statistics

File: Data030127.005 Gate: G1

Gated Events: 7490 Total Events: 10000

X Parameter: FL2-H (Log)

Marker	Events	% Gated	% Total
All	7490	100.00	74.90
M1	1730	23.10	17.30
M2	5801	77.45	58.01

Identification of Specific markers in Cultured Cells: Keratin 12 (Epithelium), Keratocan (keratocytes), and Type VIII collagen Markers (Endothelium)

After induced by various culture conditions along with adding certain growth factors or cytokines, we performed RT-PCR to detect the mRNA of specific markers, including collagen VIII as a cell marker for corneal endothelium ; keratin 12, a marker for corneal epithelium; and keratocan, a marker for stromal fibroblasts cells, was detected by RT-PCR. The initial results will be shown in the following figure.

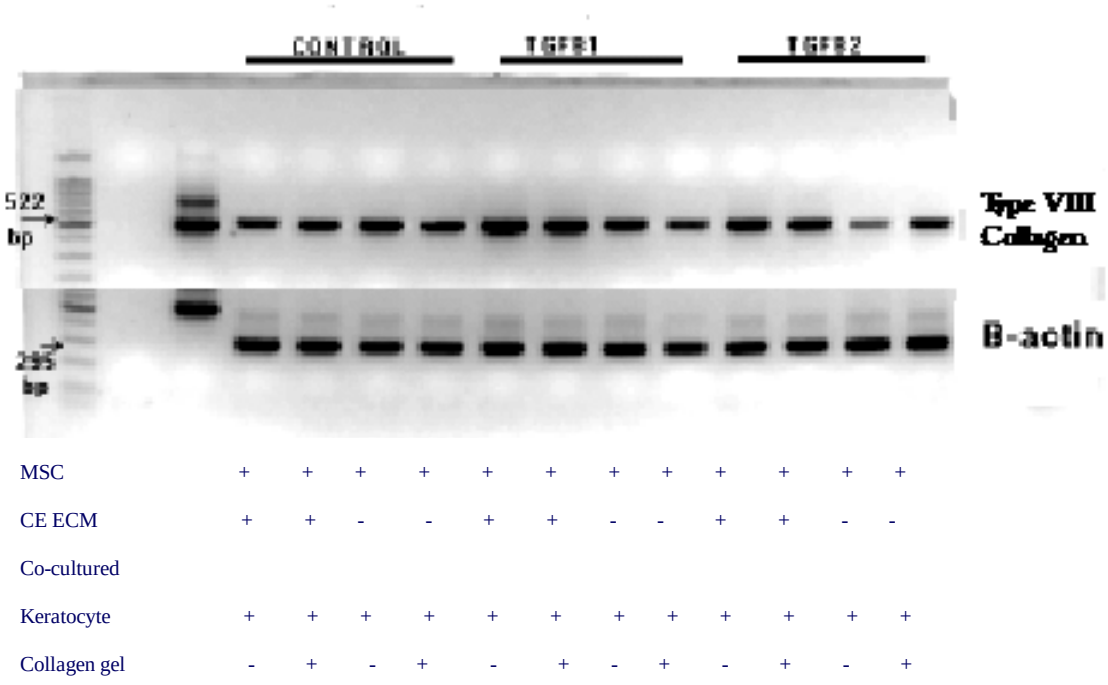


Fig. RT-PCR of mRNA of type VIII collagen and β-actin (as control) revealed up-regulation of the expression of type VIII collagen after certain induction.

In RT-PCR analysis, we found that type VIII collagen have been up-regulated after certain induction. In addition, keratin 12 and keratocan have not expressed after differentiation induction.