目錄

中文摘要

角膜是眼球外表之透明組織，它具有凸透鏡的功能，可以將光線和影像聚焦在視網膜上形成清楚之影像。如果角膜失去透明性，則光線和影像無法穿過，將無法在網膜上清楚成像。角膜主要有三種細胞層:位於最外層的層狀鱗狀上皮細胞，中間的角膜基質含有角膜細胞，以及最內層特化的單層角膜內皮細胞。角膜組織的透明需要正常眼角膜各層細胞的功能來維持，例如正常健康的輪部幹細胞可以維持角膜上皮細胞的表現型、結膜新生血管才不會長入透明的角膜組織，角膜才能維持透明而能有正常的視覺功能。此外，位於最內的單層角膜內皮細胞扮演著一個平衡滲透壓的屏障之角色，限制水分與各種游離物進入角膜，並主動地排水以保持正常的含水量(依重量約 78%)。而人類角膜內皮細胞由於不能再生，如果因受到疾病或外傷的傷害，使正常的細胞運作超過剩餘角膜內皮細胞的負荷，則角膜組織的水分無法有效排除，角膜就會水腫，使光線無法正確聚焦而影響視力；嚴重的角膜水腫甚至會導致角膜水疱性病變，造成病人的極度不適並增加感染的機會。角膜內皮細胞退化性疾病或外傷、手術性損害，目前都可以以角膜移植手術方式來治療。但現今國人器官捐贈風氣未開，角膜組織來源本不充裕，復加上近年來雷射近視手術盛行，在可預見的將來，角膜移植手術的所需之角膜組織來源的短缺，即將是臨床醫師所要面對的困境。因此，如何尋找一個角膜組織替代來源，是目前的一個重要課題。

角膜不含任何血管，享有特殊的免疫特權(immunological privilege)，此特點也讓它成為理想的組織工程或移植醫學的對象。角膜上皮細胞在胚胎形成時是由胚胎外胚層細胞 (surface ectoderm)所形成，角膜間質細胞(keratocyte)和角膜內皮細胞(endothelial cells)是由神經脊細胞(neural crest cell)所形成。而胚胎時期的間質組織，也是衍生自神經脊。角膜上皮細胞是由其幹細胞，也就是所謂的輪部上皮幹細胞增生分化而來。但是角膜間質細胞和角膜內皮細胞之幹細胞所在目前不清楚。

在當今極受矚目的細胞組織再生醫學 (regenerative medicine)中，骨髓中的造血幹細胞(hematopoeitic stem cell)和骨髓間質幹細胞(bone marrow mesenchymal stem cell , or marrow stromal stem cell, MSC) 是各種組織細胞幹細胞常用的研究題材之一。它們屬於成體幹細胞，沒有胚胎幹細胞的道德包袱和免疫性爭議。已有許多科學家利用這些細胞，添加不同的成長因子、細胞激素和培養基質刺激，成功地使其分化形成各種需要的組織，如骨骼細胞、軟骨細胞、脂肪細胞、肌肉細胞等；更有學者提出誘導其分化為各種胚層細胞的報告。而人體角膜內皮細胞之幹細胞所在目前並不清楚。雖然目前並無成功將這些幹細胞誘導分化為角膜內皮細胞的前例，但是骨髓間質幹細胞在分化上的彈性(plasticity)以及多重潛能 (multipotentiality)，使在胚胎發育上同樣是 mesenchyma 來源的角膜內皮細胞有其潛能可以在適當的誘導下自這些幹細胞誘導分化而來。如果可以利用這些幹細胞來供給我們用以合成人工角膜之組織來源，則是對未來角膜移植手術可產生巨大之影響。

在本研究中，我們首先成功地分離並培養人類骨髓幹細胞，並使用流式細胞儀分析其表面抗原表現型。接着利用了不同的細胞培養基質與添加細胞激素與生長因子，藉以引導這些幹細胞的生長和分化。由初步的實驗中，可在與角膜細胞共同培養的系統下，誘使骨髓幹細胞分化為與人體角膜內皮細胞型態近似的細胞，因此本研究後續專心致力於確立與證實這些分化。首先以 RT-PCR 與免疫螢光染色初步證實這些由骨髓幹細胞分化而來，與人體角膜內皮細胞型態近似的細胞，表現了人體角膜內皮細胞專一性的第八型膠原蛋白。但是因骨髓幹細胞的生長和分化的多重潛能性、共同培養系統的潛在高變因性，復加上骨髓幹細胞的生長速率較慢，細胞數目不易累積，都增加了本實驗的困難度。雖此實驗結果並無法在每次重複實驗中得到一致的結果，但經努力已提高了實驗結果的再現性。未來的努力方向，應是誘導骨髓幹細胞能表現更具角膜內皮細胞功能性的標記。

關鍵詞：人類骨髓間質幹細胞，成體幹細胞，角膜內皮細胞，再生醫學。

英文摘要

The cornea comprises three major cellular layers: an outermost stratified, non-keratinized squamous epithelium, a stroma with keratocytes, and an innermost monolayer of specialized endothelial cells. The structure of the cornea allows it to serve as a barrier to the outside environment and as a major element in the optical pathway of the eye. The cornea is transparent, avascular, and immunologically privileged, making it an excellent candidate for tissue engineering for transplantation. The corneal epithelium is maintained by stem cells, which reside in the basal layer of the limbus. Depletion of the limbal stem-cell pool results in an abnormal corneal surface, which cannot be normalized without the introduction of a new source of stem cells. Corneal diseases as a result of endothelial cell dysfunction may cause epithelial and stromal edema and penetrating keratoplasty is the surgical choice to improve vision. However, due to the shortage of cornea donor, especially with the universal of refractive surgery, to investigate the potential of adult stem cell therapy in corneal diseases is imperative.

Adult stem cells have several advantages as compared with embryonic stem cells (ES cells) which have ethical burden and immunologic concerns. Recent data suggest that adult stem cells generate differentiated cells beyond their own tissue boundaries, a process termed “developmental plasticity. This finding has made the adult stem cells being a practical source of cell therapy for tissue regeneration after trauma, disease, or aging. Not restricted in *in-vitro* studies, both preclinical and clinical trials of various adult stem cells for tissue repair or replacement have been applied in many disease categories. At present, the hempatopoietic stem cells (HSCs), and the stem-like cells for nonhematopoietic tissues which are currently referred to as ***mesenchymal stem cells*** (MSCs), are the most potential sources of adult stem cells. Their multipotentiality to differentiate into various cells, such as bone, cartilage, adipocytes and myocyte are proved by many researchers. Strategy using human MSCs for the treatment of the children with osteogenesis imperfecta had aroused us to investigate the possibility of using similar strategy on treating corneal diseases. In our present study, we focused on the MSCs due to encouraging initial results.

The purposes of our project are threefolds:

(1) To culture and propagate the human mesenchymal stem cells (hMSC) *in vitro*, to maintain the undifferentiated status of these cells, and identified the cells by various surface markers proving by flow cytometry.

(2)To induce the transdifferentiation of cultured human mesenchymal stem cells(hMSC) in the co-culture system with adding different growth factors and cytokines, to simulate the microenvironment, or so-called niche, of stem cell differentiation.

(3)To prove the transdifferentiated phenotype of these induced human mesenchymal stem cells by RT-PCR on the RNA level and by immunofluorescent staining on the protein level.

In our study, we had successfully isolated, cultured and propagated the hMSC and kept the undifferentiated status in a prolonged period. We had also induced the transdifferentiation of hMSC into cells with the morphology similar to the normal human corneal endothelial cells in a co-culture system with adding various growth factors and cytokines such as human leukemia inhibitory factor (hLIF), TGF-β1, TGFβ2, human epidermal growth factor (hEGF), and human fibroblast growth factor (hFGF) at different time point. We also induced the expression of a relative specific marker, type VIII collagen, of corneal endothelial cells and proved the gene expression by RT-PCR on the RNA level and by immunofluorescent staining study on the protein level. However, due to the highly variable co-culture condition and possible heterogeneity of the hMSC, our results cannot be reproduced in every repeated experiment. Besides, the key factor to determine this transdifferentiation is still not defined from our study due to the highly complexity of co-culture system.

In the future, further effort has to be made to define the key factor that determines the transdifferentiation, to induce other specific functional marker like Na^+-K^+ ATPase of corneal endothelial cells to make the clinical application feasible. Besides, to culture a mesenchymal stem cell clone with incorporated marker such as EGFP (enhanced green fluorescent protein) will make the establishment of the in vivo model of MSC transplantation easier.

Key words:
Human mesenchymal stem cells, adult stem cells, corneal endothelial cells, regenerative medicine.

一、前言 (Introduction)

The Cornea provides the eye with protection and the refractive properties essential for visual acuity. Corneal diseases such as aphakic and pseudophakic bullous keratopathy, graft failure, and Fuchs's dystrophy may cause epithelial and stromal edema as a result of endothelial cell dysfunction.

On the other hand, the transparent epithelium is highly specialized with basal and stratified squamous cells that are renewed throughout life from a stem cell population. The corneal epithelium is maintained by stem cells, which reside in the basal layer of the limbus (Sun et al., 1985). Depletion of the limbal stem-cell pool results in an abnormal corneal surface, which cannot be normalized without the introduction of a new source of stem cells.

At present, penetrating keratoplasty is the surgical choice for improving vision. The cornea is transparent, avascular, and immunologically privileged, making it an excellent candidate for tissue engineering for transplantation. However, due to the shortage of cornea donor, especially with the universal of refractive surgery, to investigate the potential of adult stem cell therapy in corneal diseases is imperative.

Since the findings of Thomson's group (Thomson et al. 1998) and Gearhart's group (Shamblott et al. 1998) in 1998 that the pluripotent stem cell have been cultured from human fetal tissue and have shown the ability to give rise to a variety of differentiated cell types found in embryonic germ layers, this exciting achievement opened the way to the clinical application of stem cell therapy. However, embryonic stem cells are not the only candidates for stem cell generation. Adult human stem cells, which are intrinsic to various tissues, have been described and characterized, including hematopoietic stem cell (HSCs), neural stem cell, mesenchymal stem cell (MSC), and endothelial progenitor cell,etc. These cells are capable of maintaining, generating, and replacing terminally differentiated cells within their own specific tissue as a consequence of physiologic cell turnover or tissue damage due to injury (Slack 2000). Recent data suggest that adult stem cells generate differentiated cells beyond their own tissue boundaries, a process termed "developmental plasticity. Unlike the ES cells, which have ethical burden and immunologic concerns, these adult stem cells have several advantages as compared with embryonic stem cells (ES cells) as their practical therapeutic application for tissue regeneration after trauma, disease, or aging. Not restricted in *in-vitro* studies, both preclinical and clinical trials of various adult stem cells for tissue repair or replacement have been applied in many disease categories.

In our present study, we focused on the MSCs due to encouraging initial results. We firstly isolated, cultured and propagated the hMSC and kept the undifferentiated status in a prolonged period. Then we use various surface markers in flow cytometry to identify the phenotype of the cultured hMSCs. In the following experiments, using a co-culture system with adding various growth factors and cytokines such as human leukemia inhibitory factor

(hLIF), TGF-β1, TGFβ2, human epidermal growth factor (hEGF), and human fibroblast growth factor (hFGF) at different time point, we tried to induced the transdifferentiation of hMSC into cells with the morphology similar to the normal human corneal cells. After the transdifferentiation, we then tried to prove the gene expression by RT-PCR on the RNA level and by immunofluorescent staining study on the protein level.

二、研究目的

Various researchers have attempted to fabricate artificial corneas or parts of corneas in vitro(Minami et al. 1993; Zieske et al. 1994), but there have been no reports of successfully reconstructed human corneas that mimic the anatomy and physiology of the human cornea and use the cells without tumorigenesis to generate an artificial cornea. From the study of Griffith *et al*. (Griffith et al. 1999) human corneal equivalents comprising the three main layers of the cornea (epithelium, stroma, and endothelium) were constructed. Each cellular layer was fabricated from immortalized human corneal cells that were screened for use on the basis of morphological, biochemical, and electrophysiological similarity to their natural counterparts. The resulting corneal equivalents mimicked human corneas in key physical and physiological functions, including morphology, biochemical marker expression, transparency, ion and fluid transport, and gene expression. These morphological and functional equivalents to human corneas that can be produced in vitro can be immediately applied in toxicity and drug efficacy testing, and form the basis for future development of implantable tissues. Unfortunately, this corneal equivalent was criticized by its tumorigenecity, and was not implantable in human beings. Therefore, it is a very important issue to find cells which do not cause tumorigenecity and can be used to reconstruct a cornea equivalent. Among the adult stem cells, **bone marrow mesenchymal stem cells (MSCs)**, which have close embryogenic origin to the corneal cells (including keratocytes and corneal endothelial cells), may have potential being the stem cell resource.

In all research regarding the “developmental plasticity” of stem cells, it is imperative to identify and **confirm the stem cell phenotype** at first. Then we have to **build up an appropriate microenvironment**, or so called “niche” for stem cells to induce the transdifferentiation. Finally, immunohistochemistry, immunofluorescence, or cell-type–specific functional assays can be used **to identify and characterize the differentiated cells**.

The purposes of our study are as followed:

1. To culture and propagate the human mesenchymal stem cells (hMSC) *in vitro*, to maintain the undifferentiated status of these cells, and identified and characterized the stem cells using various surface markers by flow cytometry.

2. To simulate the microenvironment, or so-called niche, of cultured human mesenchymal stem cells(hMSC) differentiation. To induce the transdifferentiation of hMSC in the co-culture system with adding different growth factors and cytokines,

3. To characterize the transdifferentiated phenotype of these induced human mesenchymal stem cells by RT-PCR on the RNA level and by immunofluorescent staining on the protein level

三、文獻探討

Stem cells

Stem cells are defined as cells that have clonogenic and self-renewing capabilities and that differentiate into multiple cell lineages(Weissman 2000). Stem cells not only offer scientists a tool to study the early molecular events in organ development, they also offer hope for tissue repair and regeneration to patients suffering from a spectrum of degenerative diseases. The potential use of stem cells as agents of repair in human disease makes them the subject of high-profile studies. Since the findings of Thomson's group(Thomson et al. 1998) and Gearhart's group (Shamblott et al. 1998) in 1998 that the pluripotent stem cell have been cultured from human fetal tissue and have shown the ability to give rise to a variety of differentiated cell types found in embryonic germ layers, this exciting achievement opened the way to the clinical application of stem cell therapy.

The so-called **embryonic stem (ES) cells**, which are derived from the inner mass of blastocysts or primordial germ cells, can be maintained continuously in an undifferentiated pluripotent state. However, ES cells are not the only candidates for stem cell generation. **Adult human stem cells**, which are intrinsic to various tissues, have been described and characterized, including hematopoietic stem cell (HSCs), neural stem cell, mesenchymal stem cell (MSC), and endothelial progenitor cell, etc. These cells are capable of maintaining, generating, and replacing terminally differentiated cells within their own specific tissue as a consequence of physiologic cell turnover or tissue damage due to injury (Slack 2000). Recent data suggest that adult stem cells generate differentiated cells beyond their own tissue boundaries, a process termed "developmental plasticity. Unlike the ES cells, which have ethical burden(Frankel 2000) and immunologic concerns, these adult stem cells have several advantages as compared with embryonic stem cells (ES cells) as their practical therapeutic application for tissue regeneration after trauma, disease, or aging.

Corneal structure, function, diseases and stem cells

The cornea comprises three major cellular layers: an outermost stratified, non-keratinized squamous epithelium, a stroma with keratocytes, and an innermost monolayer of specialized endothelial cells. The structure of the cornea allows it to serve as a barrier to the outside environment and as a major element in the optical pathway of the eye. The cornea is transparent, avascular, and immunologically privileged, making it an excellent candidate for tissue engineering for transplantation.

Embryologically, corneal epithelial cells are derived from surface ectoderm, whereas keratocytes and endothelial cells are from neural crest cell。Embryonic mesenchyma is also derived from the neural crest.

The corneal epithelium is maintained by stem cells, which reside in the basal layer of

the limbus (Sun et al., 1985). However, the stem cells for keratocytes and corneal endothelial cells has not identified at present.

The cornea transparency are maintained by healthy cell layers, for example, healthy limbal stem cells have key role in maintaining the corneal epithelial phenotype, to prevent neovascularization of conjunctival vessels and therefore keep the cornea clear. Once the limbal stem cells diseased or injured, the condition so called limbal insufficiency (eg. partial limbal insufficiency in long-termed contact lens wearers or patients having pterygium; total limbal insufficiency in chemical burn, ocular cicatricial pemphigoid or Stevens-Johnson syndrome), the transparency of the cornea will decreased. The present strategy treating limbal insufficiency is limbal transplantation. Besieds, the innermost single layer coreal endothelial cells, with hexagonal shape and function as a barrier, maintain the proper hydration of the cornea by persistent pumping out the water through the Na^+-K^+ ATPase pump. The human corneal endothelial cells have extremely low regeneration ability, in case of various corneal diseases or injuries that results in the endothelial decompensation, the corneal stromal and epithelial edema ensued. Corneal edema then results in decreased vision, pain, tearing and vulnerable to various infection. At present, penetrating keratoplasty is the surgical choice for improving vision; selective transplantation of only the posterior corneal tissue is an alternative option(Melles et al. 1999).However, due to the shortage of cornea donor, especially with the universal of refractive surgery, to investigate the potential of adult stem cell therapy in corneal diseases is imperative.

The current status of corneal equivalents

As mentioned above, various researchers have attempted to fabricate artificial corneas or parts of corneas in vitro(Minami et al. 1993; Zieske et al. 1994), but there have been no reports of successfully reconstructed human corneas that mimic the anatomy and physiology of the human cornea and use the cells without tumorigenesis to generate an artificial cornea. The present corneal equivalent was criticized by its tumorigenecity, and was not implantable in human beings. Therefore, it is a very important issue to find cells which do not cause tumorigenecity and can be used to reconstruct a cornea equivalent.

Multipotentiality of the Mesenchymal stem cells (MSCs)

Among the adult stem cells, bone marrow mesenchymal stem cells (MSCs), which have close embryogenic origin to the corneal cells (including keratocytes and corneal endothelial cells), may have potential being the stem cell resource. Therefore, we use hMSCs in our present research.

The human adult bone marrow contains hematopoietic lineage cells as well as a heterogeneous population of cells making up the marrow stroma. The bone marrow stromal

cells include adipocytes, reticular cells, endothelial cells, and fibroblast cells. They are in direct contact with hematopoietic cells and compose the bone marrow microenvironment that provides for the maintenance, expansion, and differentiation of hematopoietic progenitor cells. Definitive evidence that bone marrow contains cells that can differentiate into fibroblasts as well as other mesenchymal cells has been available since the pioneering work of Friedenstein, beginning in the mid-1970s (Friedenstein et al. 1976). His initial observations were extended by a number of investigators during the 1980s, particularly by Pieresma and coworkers (Piersma et al. 1983; Piersma et al. 1985) and by Owen and coworkers(Howlett et al. 1986; Mardon et al. 1987; Beresford et al. 1992). These and other studies established that the MSCs isolated by the relatively crude procedure of Friedenstein were multipotential and readily differentiated into osteoblasts (Haynesworth et al. 1992), chondroblasts(Mackay et al. 1998; Yoo et al. 1998), adipocytes (Pittenger et al. 1999), and even tenogenic cells(Young et al. 1998) and myoblasts(Wakitani et al. 1995) under appropriate induction. These previous experiments showed that the isolated expanded hMSC culture would differentiate, in a controlled manner, to multiple lineages. Conditions for differentiating the cells are somewhat species-dependent and are influenced by incompletely defined variables, such as the lot of fetal calf serum used. (Prockop 1997) Various conditions have been proposed. Results from some study groups showed that MSCs from mouse, rat, rabbit, and human readily differentiate into colonies of osteoblasts (depositing mineral in the form of hydroxyapatite), chondrocytes (synthesizing cartilage matrix), and adipocytes in response to dexamethasone, 1,25-dihydroxyvitamin D_3, or cytokines such as BMP-2 (Prockop 1997) . In response to 5-azacytidine and amphotericin B or amphotericin B alone, the MSCs differentiate into myoblasts that fuse into rhythmically beating myotubes. (Wakitani et al. 1995) From Pittenger's experiments, they induced adipogenic differentiation in the expanded hMSC by treatment with 1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin. (Pittenger et al. 1999) For induction of chondrogenic differentiation, culturing without serum and with TGF-β3 have been mentioned. (Mackay et al. 1998; Yoo et al. 1998) The osteogenic differentiation induction could be manipulated by adding dexamethasone, β-glycerol phosphate, and ascorbate and in the presence of 10% fetal bovine serum. The isolated mesenchymal cell will form aggregates or nodules and increased their expression of alkaline phosphatase. The stem-like cells for nonhematopoietic tissues are currently referred to either as ***mesenchymal stem cell*** (MSCs), because of their ability to differentiate into cells that can roughly be defined as mesenchymal origin, such as bone, cartilage, adipocytes; or as ***marrow stroma cells*** (MSCs), because they appear to arise form the complex array of supporting structures found in marrow.

Characteristics of cultured mesenchymal stem cells

Mesenchymal stem cells (MSCs) derived in different laboratories using different techniques share 2 features: growth in culture as adherent cells with a finite life span and

ability to differentiate into osteoblasts, chondroblasts, and adipocytes in response to appropriate stimuli. One of the main hindrances to our understanding of the full potential of MSCs has been confusion in the literature regarding what specifically defines an MSC and how it should be isolated and grown in vitro. A wide array of cytokines (eg, fibroblast growth factor 2 [FGF2], FGF4, platelet-derived growth factor-BB [PDGF-BB], leukemia inhibitory factor) and isolation techniques (eg, immunomagnetic and physical) have been used to identify and expand MSCs. Furthermore, no specific constellation of surface markers has been agreed on for these cells. Some of the surface antigens reported to be on these cells are Stro 1 (Simmons and Torok-Storb 1991; Simmons et al. 1994; Gronthos et al. 2003), CD13, α-integrins (CD49a and CD49b), β1-integrins (CD29), CD44(hyaluronate), CD71 (transferrin), CD90 (thy-1), CD106 (vascular cell adhesion molecule-1 [VCAM-1]), and CD124 (interleukin-4[IL-4] receptor (Pittenger et al. 1999)). MSCs uniformly lack antigens such as CD45 that typically identify hematopoietic cells. Variations in the isolation techniques and culture media used to grow MSCs in different laboratories has led to variable findings regarding the differentiation potential of these cells. For example, some report that MSCs an differentiate in vitro into neuronal-type cells, whereas others cannot obtain this phenotype(Azizi et al. 1998). To resolve these inconsistencies regarding MSCs, laboratories must collaborate to compare and contrast findings using uniform cell populations. Efforts are under way to disperse MSC populations that have been isolated to researchers outside each laboratory.

四、研究方法與材料

1. Isolation and culture of the human mesenchymal stem cells (hMSCs) from Bone Marrow

Extra samples (3-5ml) from the specimen taken in the routine diagnostic bone marrow aspiration via a routine iliac crest approach were used in these experiments. Informed consents for participants in this study were obtained. Cells were washed in phosphate-buffered saline (PBS) and then incubated in lysis buffer containing 0.8% NH_4Cl and 10uM ethylenediamine-tetraacetic acid (EDTA) for 10 minutes to lyse red blood cells. The pellets of the remaining nucleated cells were resuspended in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD,USA) supplemented with 10 fetal bovine serum (HyClone, Logan, UT, USA), 10 units/mL penicillin G, and 10ug/mL streptomycin (Life Technologies). This medium was referred to as regular medium. The cell suspensions were then cultured in 25-cm^2 flask at 37℃ in a humidified atmosphere containing 5% CO_2. Culture medium was changed every 3 to 4 days. Confluent cells ere detached with trypsin-EDTA (Life Technologies), subcultured into 150-cm^2 flasks, and denoted passage-1 cells.

Before induction, add 10ng/ml human leukemia inhibitory factor(hLIF) (Chemicon,CA,USA) in experiment group.

2. Induce the cultured human mesenchymal stem cells transdifferentiation using in vitro co-culture system

Expanded MSCs (passage 8-12) were subcultured into 24-well plates at 3 x 10^4 cells/well. Supporting matrices were prepared in advance including (1) collagen gel, prepared with Vitrogen®100 (3mg/mL) diluted with DMEM to 1mg/mL level and adjusted with sodium bicarbonate to let the PH value approaching 7.0 and the collagen would become gel form; (2) extracellular matrix secreted by corneal endothelial cells, with Bovine endothelial cells (ATCC CRL-2048) cultured with DMEM with 4mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 1.0mM sodium pyruvate, 90%; FBS,10% for 2 days and treated with trypsin-EDTA (Life Technologies) for 5 minutes and then wash by PBS to remove the adherent endothelial cells;(3) with collagen gel in the bottom, cultured bovine endothelial cells with the condition mentioned above for 2 days then treated with trypsin-EDTA for 5 minutes to remove the adherenet endothelial cells. Then use Transwell® (Corning Costar Cooperation) co-culture system (0.4μm pore size) with tissue culture treated polyester membrane，co-culture the keratocytes in the inserts for induction of the transdifferentiation.

In the above co-culture system, we add various growth factors, for example：add the forementioned LIF(10ng/ml) on the first 2 days, after the cells adhere and propagate, change

medium with TGF-β1(5ng/ml) and TGF-β2 (5ng/ml)(R&D system)， and add 20ng/ml hEGF(human epidermal growth factor) or 20ng/ml hFGF(human fibroblast growth factor) on the 5th days.

Observe the morphology of the cultured human MSCs in the bottom of the co-culture system daily using inverted phase-contrast light microscope(Zeiss, German).

3. Flow cytometry. Cells were trypsinized, collected and incubated for 30 min at 4°C with phycoerythrin (PE) conjugated antibodies against human CD45,CD34; adhesion molecules including CD166(ALCAM),CD44(HCAM) , CD58(LFA-3) ,CD102(ICAM-2) , CD54 (ICAM-1) ,L-selectin, CD56(NCAM) ,CD106(VCAM-1)；integrin including Integrin β1, Integrin αV, Integrin α3, Integrin α1, Integrin α2, Integrin α5, Integrin β3；growth factor receptors including CD71(transferrin receptor) , CD117(c-kit) ,and CD90(Thy-1) (Pharmingen, San Diego, CA, USA). Excess antibody was removed by washing. Detection of PE-labelling was accomplished on a FACS calibur cytometer equipped with 488 nm argon laser (Becton Dickinson, San Diego, CA, USA) using CellQuest software. At least 10,000 events were collected. WinMDI 2.8 software was used to create the histograms.

4. Identify the transdifferentiated cell phenotype by corneal endothelial cell-specific marker, Type VIII collagen

RT-PCR (RNA level)

Total RNA is prepared from confluent cultures of transformed MSCs with different inductive conditions using reagent according to the manufacturer's directions (TRIzol; Gibco). cDNA is prepared from 1 μg total RNA by RT 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 primers used are as followed:

Type VIII collagen: upstream: 5'- GCTTACCATTTCCCTGAGTTCC-3'；
downstream: 5'-CCAATTTCCCCTTTCTGTCC-3'

Keratin 12: upstream：5'-GAGTGGATCTCACCAAGG-3'；
downstream：5'-CAATCTCTAGGTTCTGCA-3'

Keratocan: upstream：5'-TTCAGCAATCTGGAGAACCTG-3'；
downstream：5'-GTTAGATTGTTGTGTTGTCATGC-3'

PCR is then performed in a reaction mixture containing certain amount of cDNA (β-actin as the adjustment) 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 cycler. 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, 57.8°C; keratin 12, 58.4°C; and keratocan, 53.2°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. β-actin (Clontech, Palo Alto, CA) acted as a positive control for the PCR. The amount of cDNA added is adjusted according to the OD measuremet. 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 1 μg total RNA is substituted for cDNA in the PCR reaction mixture, along with 2.5 μM each of upstream and downstream β-actin primers.

Immunofluorescence study (Protein level)

Place glass coverslips at the bottom of the co-culture system before culture. After transdifferentiation induction completed (about 60%~70% confluent)，suction out the culture medium and wash twice with PBS. Then take out the glass coverslips and put into 6-well dish, parafilm is placed first at the bottom for easy manipulation. Then add 30μl 3% paraformaldehyde (Sigma) for fixation, wash with Ca/Mg –PBS 3 times. For permeabilization，add 30μl 1% Triton PBS, wait for 10 minutes. Followed by 0.02% Tween 20 PBS for 5 minutes。Then add 30μl PBS containing primary antibody: 1:100 mouse anti-Type VIII collagen mAb (Seikagaku America, MA,USA)；1:100 anti-vWF mAb (Santa Cruz Biotechnology, CA, USA); use 1:100 anti-β-actin mAb as positive control (Santa Cruz Biotechnology, CA, USA)，water bath in 37°C for 45 minutes (water bath)。 Then wash three times for 5 minutes each time with 0.02% Tween 20 PBS, add 30μl PBS containing 1:50 FITC-conjugated anti-mouse IgG (Sigma Chemical Co.), followed by water bath in 37°C for 45 minutes. Then wash with 0.02% Tween 20 for 5 minutes for three times， then PBS wash for 5 minutes. Dry in 37°C for about 45 minutes. Before observation, add 10~15μl Mounting media (mix PBS, 50-60% glycerol, 2.5% 1,4 diazobicyclo(2,2,2)-octane = Dabco Quencher), seal the coverslips with transparent nail polish.

Observe the coverslips with fluorescent microscope. The wavelength for FITC excitation is 488nm； for emission is 515nm。Image captured by Nikon DX100 digital camera.

五、結果

1. Isolation and culture of the human mesenchymal stem cells (hMSCs) from Bone Marrow

In our study, we had successfully isolated, cultured and propagated the hMSC and kept the undifferentiated status in a prolonged period (named MSC-19). Fig.1 (upper)showed the morphology of cultured hMSC with fibroblast-like appearance. Cultrue bovine endothelial cells are used in our study, the appearance was showed in Fig.1 (lower). The extracellular matrix secreted by these cells is used as the supporting matrix in some co-culture condition. Fig.2 showed the phenotype of MSC-19 analyzed by flow cytometry (Lee et al. 2002) .The expressed phenotype of the MSC-19 was listed as followed: $CD45^-$,$CD34^-$; adhesion molecules: $CD166(ALCAM)^+$ 、$CD44(HCAM)^+$ ，$CD58(LFA-3)^+$ 、$CD102(ICAM-2)^+$ 、$CD54(ICAM-1)^{Low}$、$L\text{-}selectin^{Low}$、$CD56(NCAM)^-$、$CD106(VCAM-1)^-$； integrin expression are $Integrin\beta1^+$ 、$Integrin\alpha V^+$ 、Integrin $\alpha 3+$ 、$Integrin\alpha 1^{Low}$ 、 $Integrin\alpha 2^{Low}$ 、$Integrin\alpha 5^{Low}$、and Integrin $\beta 3-$；growth factor receptors expression are CD71(transferrin receptor)$^+$ 、$CD117(c\text{-}kit)^+$ and $CD90(Thy\text{-}1)^{Low}$. The results are showed in Fig.2.。

2. Induce the cultured human mesenchymal stem cells transdifferentiation using in vitro co-culture system

Fig 3-5 showed that in a co-culture system with adding various growth factors and cytokines such as human leukemia inhibitory factor (hLIF), TGF-β1, TGFβ2, human epidermal growth factor (hEGF), and human fibroblast growth factor (hFGF) at different time point, we had induced the transdifferentiation of hMSC into cells with the morphology similar to the normal human corneal endothelial cells. Among the various conditions in Fig.3, condition (4) are most close to the in vivo microenvironment, and the morphology seemed to be most alike with the real human corneal endothelial cells.

We also induced the expression of a relative specific marker, type VIII collagen, of corneal endothelial cells and proved the gene expression by RT-PCR on the RNA level and by immunofluorescent staining study on the protein level. However, due to the highly variable co-culture condition and possible heterogeneity of the hMSC, our results cannot be reproduced in every repeated experiment. Besides, the key factor to determine this transdifferentiation is still not defined from our study due to the highly complexity of co-culture system. The morphology of those transdifferentiated cells was more close the real one in Fig.4, when adding TGF-β1(5ng/ml) in co-culture system(1)~(4). At last, the morphology most alike to the real one was in Fig.5, when we added 10ng/ml hLIF in the initial 3 days in the co-culture system，followed by adding 5ng /ml TGF-β1and 20ng/ml hEGF.

In fact, we have tried to use many co-culture system condition except the forementioned ones, for example, we have used the amniontic membrane as supporting matrix; we also used other different cytokines and growth factors like TGF-β2 and hFGF (human fibroblast growth factor)。After repeated experiment, we have found most potential co-culture condition listed in Fig.3-5, especially in Fig.5.

3. Identify the transdifferentiated cell phenotype by corneal endothelial cell-specific marker, Type VIII collagen

In our present study, we had successfully induced the expression of a relative specific marker, type VIII collagen, of corneal endothelial cells in appropriate co-culture conditions and proved the gene expression by RT-PCR on the RNA level and by immunofluorescent staining study on the protein level. Fig.6 showed the expression of Type VIII collagen after induction (condition #1~#3) as compared to the cells without induction (condition #4). Adding TGF-β1(condition #2) or TGF-β2 (condition #3), especially in the co-culture condition which most close to the in vivo condition, the expression of the Type VIII collagen seemed to be up-regulated. Fig.7 showed that the hMSC after induction did not express the corneal epithelium-specific marker, keratin 12 and keratocyte-specific marker, keratocan. However, due to the highly variable co-culture condition and possible heterogeneity of the hMSC, our results cannot be reproduced in every repeated experiment. Besides, the key factor to determine this transdifferentiation is still not defined from our study due to the highly complexity of co-culture system.

六、討論與建議

Mesenchymal stem cells (MSCs) derived in different laboratories using different techniques share 2 features: growth in culture as adherent cells with a finite life span and ability to differentiate into osteoblasts, chondroblasts, and adipocytes in response to appropriate stimuli. One of the main hindrances to our understanding of the full potential of MSCs has been confusion in the literature regarding what specifically defines an MSC and how it should be isolated and grown in vitro. Variations in the isolation techniques and culture media used to grow MSCs in different laboratories have led to variable findings regarding the differentiation potential of these cells. For example, some report that MSCs can differentiate in vitro into neuronal-type cells, whereas others cannot obtain this phenotype (Azizi et al. 1998). To resolve these inconsistencies regarding MSCs, laboratories must collaborate to compare and contrast findings using uniform cell populations. Efforts are under way to disperse MSC populations that have been isolated to researchers outside each laboratory.

In our present study, we had successfully isolated, cultured and propagated the hMSC and kept then in undifferentiated status in a prolonged period. We also characterized the phenotypes of the cultured hMSC by various surface markers using flow cytometry. We had also induced the transdifferentiation of hMSC into cells with the morphology similar to the normal human corneal endothelial cells in a specialized co-culture system. We also induced the expression of a relative specific marker, type VIII collagen, of corneal endothelial cells and proved the gene expression by RT-PCR on the RNA level and by immunofluorescent staining study on the protein level. However, due to the highly variable co-culture condition and possible heterogeneity of the hMSC, our results cannot be reproduced in every repeated experiment. Besides, the key factor to determine this transdifferentiation is still not defined from our study due to the highly complexity of co-culture system.

In the future, further effort has to be made to define the key factor that determines the transdifferentiation, to induce other specific functional marker like Na^{+}-K^{+} ATPase of corneal endothelial cells to make the clinical application feasible. Besides, to culture a mesenchymal stem cell clone with incorporated marker such as EGFP (enhanced green fluorescent protein) will make the establishment of the in vivo model of MSC transplantation easier.

七、計畫成果自評

The success induction of the transdifferentiation of the cultured hMSC to the human corneal endothelial cells has never been reported in the literature. Although the type VIII collagen is only a relative specific marker of human corneal endothelial cells, these findings have already gave us tremendous encouragement and inspiration!!

As mentioned above, the cornea is transparent, avascular, and immunologically privileged, making it an excellent candidate for tissue engineering for transplantation. However, due to the shortage of cornea donor, especially with the universal of refractive surgery, to investigate the potential of adult stem cell therapy in corneal diseases is imperative.

The findings of our results have told us that the idea of induction of the hMSC transdifferentiation to corneal endothelial cell is possible!! Further work has to be done to make the dream come true!! At that time, we don’t have to worry about the shortage of cornea donor anymore.

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九、附圖

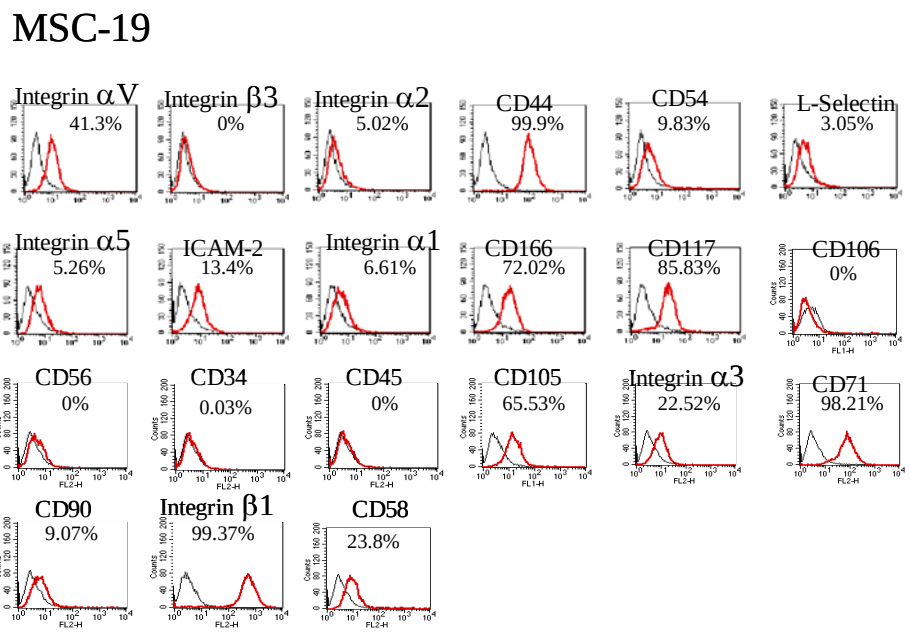


Fig.1：流式細胞儀骨髓間質幹細胞 MSC-19 細胞株各種細胞標記的表現。

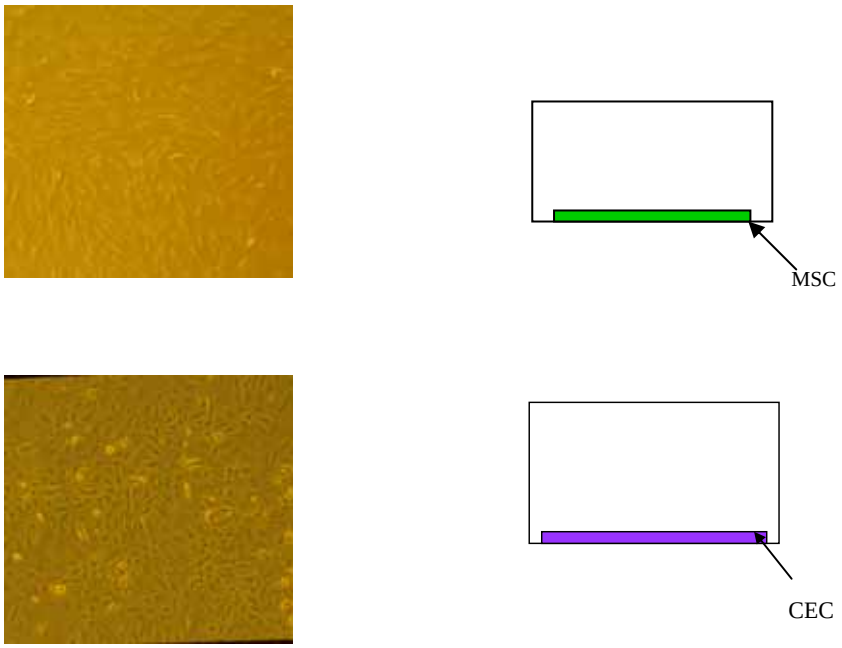
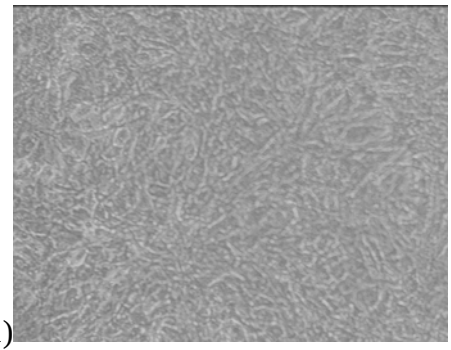
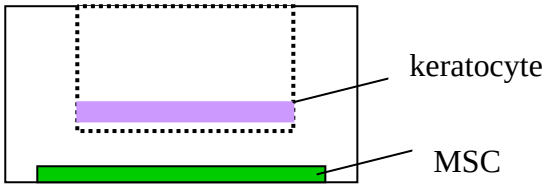


Fig.2：(上) 體外培養的人類骨髓幹細胞 (passage 8)，此處簡稱 MSC；
(下) 體外培養的牛角膜內皮細胞，此處簡稱 CEC。

(1)





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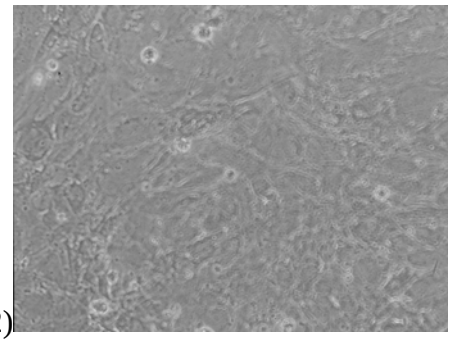

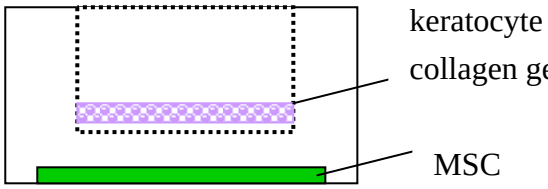

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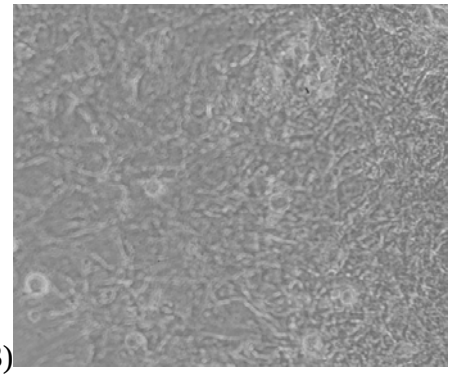
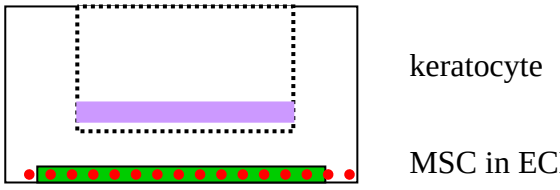
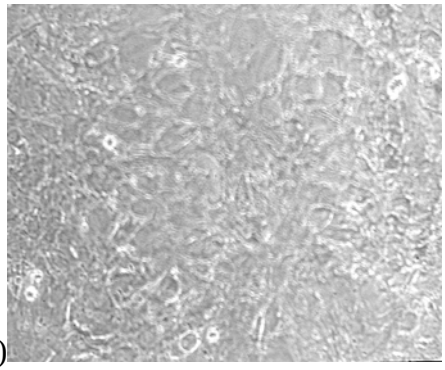




(4)



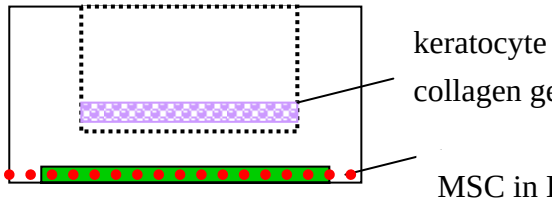


Fig3: 人類骨髓間質幹細胞於體外共同培養系統中經過誘導後形態上的改變情形。(1)~(4)分別為各種的共同培養狀態，每個圖片左方列有各種狀態的示意簡圖。其中 MSC: 骨髓幹細胞；CEC: 角膜內皮細胞；ECM: extracellular matrix。其中以圖(4)最接近活體內的狀態，其改變後的形態，似乎也最接近真實的角膜內皮細胞。

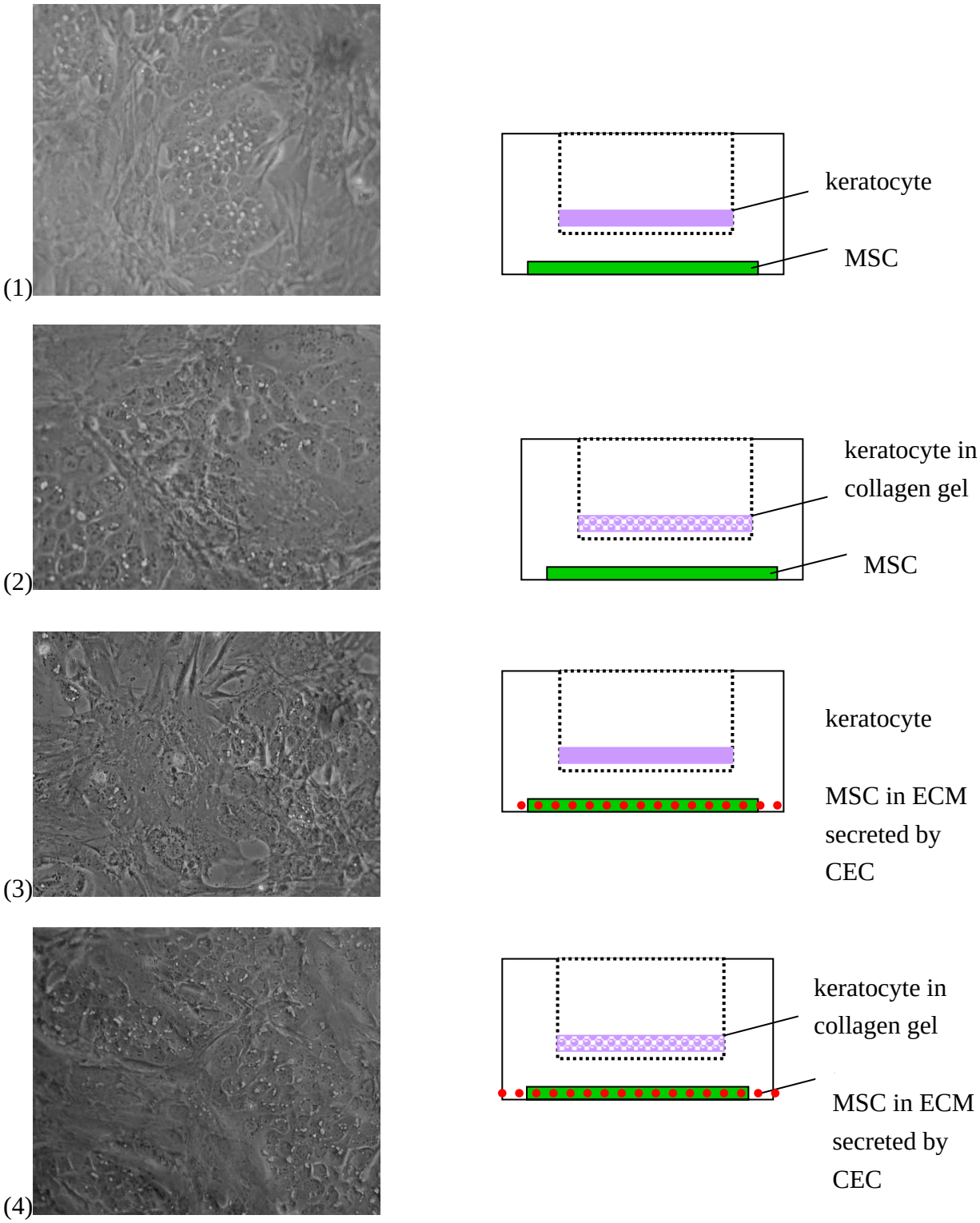
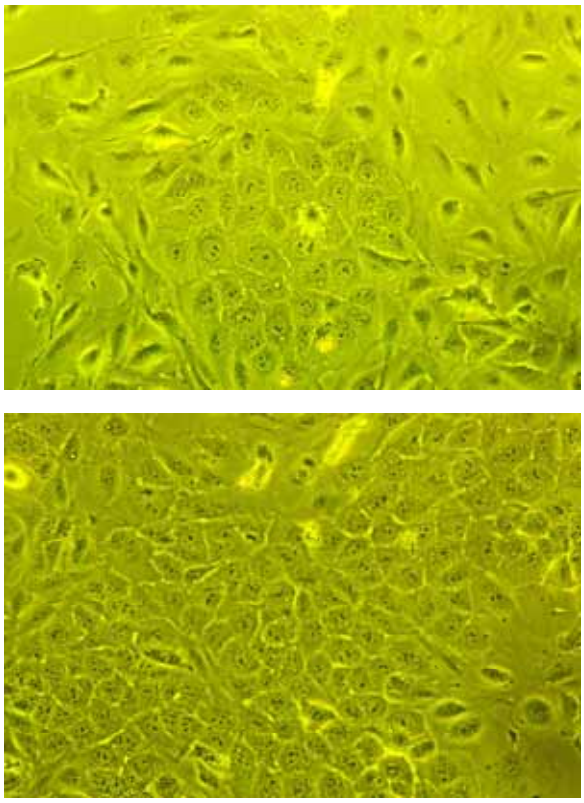


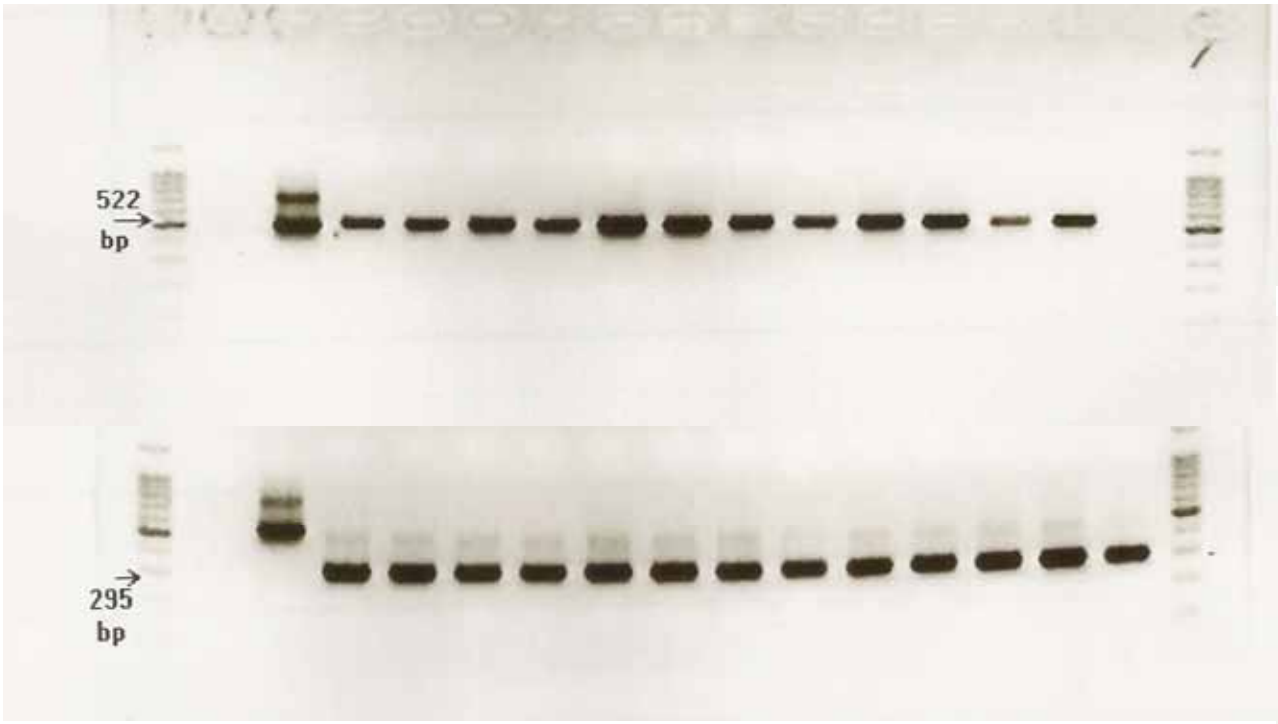
Fig4：人類骨髓間質幹細胞於體外共同培養系統中經過誘導後形態上的改變情形，惟此處於培養基中加入 TGF-β1(5ng/ml)。圖(1)~(4)似乎比圖二的誘導更接近真實人類角膜內皮細胞的外觀



Adding hLIF 10ng/ml for first 3 days, then TGF-β1 5ng/ml and hEGF 20ng/ml

Fig5：(上圖及中圖)以 Fig4 的第(4)種培養狀態，在前 3 天加入 10ng/ml hLIF(human leukemia factor)，之後換加入 5ng/ml TGF-β1 以及 20ng/ml hEGF。此處經誘導後的骨髓幹細胞，其形態與排列與真實人類的角膜內皮細胞(下四圖)極為相似

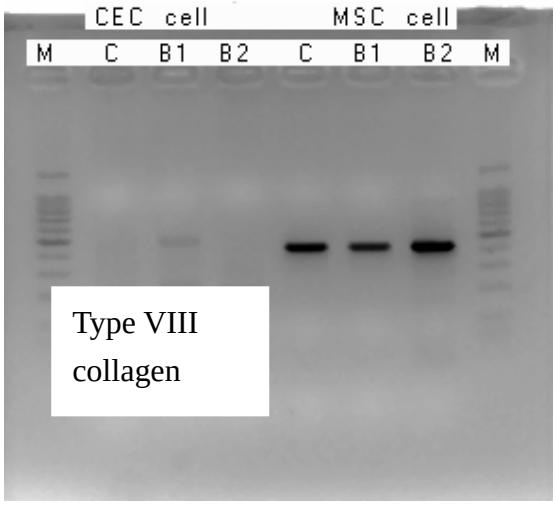
#1: MSC in co-culture
#2: MSC in co-culture + TGFβ1
#3: MSC in co-culture + TGFβ2
#4: MSC only



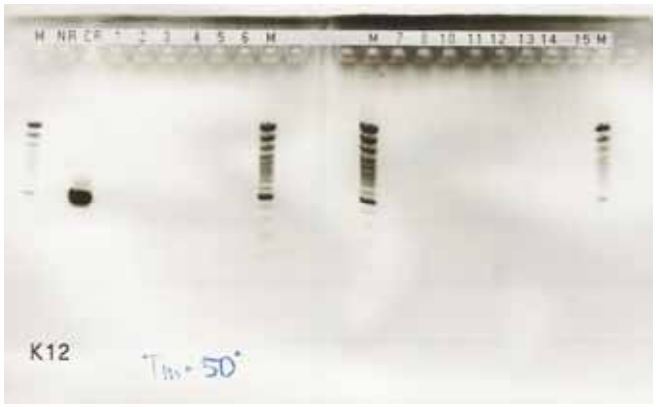
MSC	+	+	+	+	+	+	+	+	+	+	+	+	+
CE ECM	+	+	-	-	+	+	-	-	+	+	-	-	-
Co-cultured Keratocyte	+	+	+	+	+	+	+	+	+	+	+	+	-
Collagen gel	-	+	-	+	-	+	-	+	-	+	-	+	-

Fig6：以角膜內皮細胞特異性標記 Type VIII collagen 定義骨髓幹細胞的分化，其 RT-PCR 的結果。(以β-actin 做為 internal control)

522bp



(1)

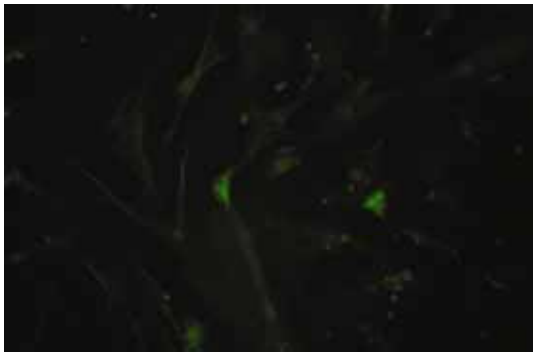


(2)

(3)

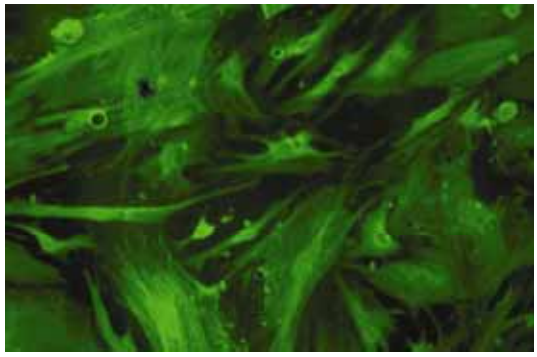
Fig7：RT-PCR 的結果顯示：(1)體外培養系統牛角膜內皮細胞株並未分泌 type VIII collagen，而相對的，經誘導的人類骨髓幹細胞則分化為會分泌 type VIII collagen 的細胞，但卻不分泌 keratin 12 (K12)或 keratocan 圖(2)及圖(3)。

(1)



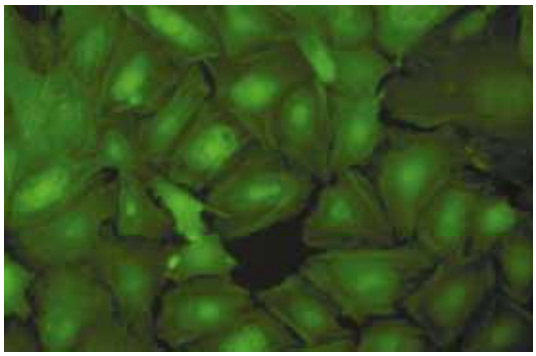
MSC, Type VIII collagen(-)

(2)



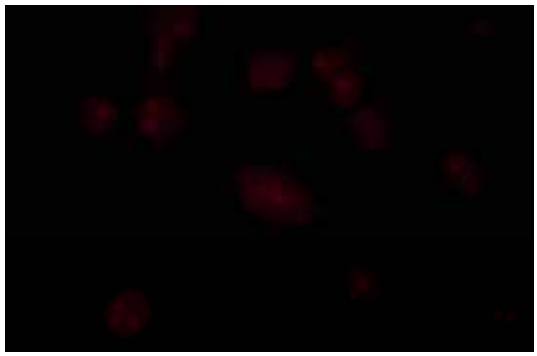
MSC, β-actin(+)

(3)



MSC in co-culture system,
Type VIII collagen (+)

(4)



vWF(-)

Fig8：免疫螢光染色顯示：圖(1)為未經誘導的人類骨髓幹細胞，不表現 type VIII collagen；圖(2)為以β-actin 做為 positive control；圖(3)為人類骨髓幹細胞在經過圖四的共同培養系統誘導後表現 type VIII collagen；但卻不表現血管內皮細胞專一的 vWF (von Willebrand factor)圖(4)。