# 行政院國家科學委員會專題研究計畫　成果報告

## 利用骨髓幹細胞及聚乳酸幫助受損肌腱及韌帶組織之修補（II）

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# HOW TO DISCRIMINATE LIGAMENT CELLS AND BONE MARROW STEM CELLS-THE APPLICATION OF ELETROPHORESIS

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## ABSTRACT

Before the proceeding of the co-culture system of mesenchymal stem cell (MSC) and ligament cells, we develop some method to differentiate BMSC from ligament cells. First we used the flow cytometry to determine a lot of surface antigens of mesenchymal stem cell like CD105, CD29, CD44, CD71, and CD34. The differentiated fibroblasts are examined by scanning electron microscopy, immunostaining of $\alpha$- smooth muscle antibody. Furthermore, we develop a novel electrophoretic analysis for characterization. Since there are no definite markers for fibroblasts in the literature, our unique study will provide important information for further characterization of ligament or tendon fibroblasts.

## INTRODUCTION

The anterior cruciate ligament (ACL), an intraarticular ligament of the knee, is an important joint stabilizer that is frequently injured in sports and exercise. Severe injury to the ACL can cause knee instability, meniscal damage, and osteoarthritis (ref). Because of a poor intrinsic healing capacity, operative intervention is often necessary and more than 60,000 ACL reconstructions are performed each year in the United States alone(ref). Patellar tendon (PT) autografts and allografts are widely used but are not ideally suited for this purpose. Problems associated with PT autografts include lengthy rehabilitation and persistent patellar pain. PT allografts carry the risk of diease transmission. Both autografts and allografts may become necrotic and weak shortly after implantation, and the knee must be protected from high mechanical loads while the graft gradually gains strength. Synthetic ACL prostheses may perform satisfactorily in the short time, but tend to break down and fail in the long term. Although a variety of biological grafts and synthetic polymers have been investigated as replacement materials for the anterior cruciate ligament, an ideal graft or device has not been developed.

In addition, cell-seeded, resorbable materials have been tried as a means a to accelerate the healing process for tendons and ligaments (ref). Another promising approach, advocated by bruder et al. has utilized noncommitted progenitor cells of musculoskeletal tissues to regenerate soft and hard tissues. These cells, termed mesenchymal stem cells, were isolated from a small volume of bone marrow aspirate and culture-expanded without undergoing differentiation to more committed cell types. Even in older individuals, bone marrow stroma is relatively easily harvested, contains biosynthetically active precursors and multipotent cells, and thus can serve as a basis for tissue rejection. In addition, mesenchymal stem cells demonstrated the ability to contract collagen matrices and showed some promise when applied to a tendon defect

site (ref). However, there were no methods, which could discriminate mesenchycal stem cells and ACL cells, has been illustrate in previous paper. How to identify the two types of cells is very important for ACL research. Therefore, in this study, we develop the novel electrophoretic analysis for characterization. Since there are no definite markers for fibroblasts in the literature, our unique study will provide important information for further characterization of ligament or tendon fibroblasts.

## MATERIALS AND METHODS

### Cell culture

### Isolation and Growth of MSCs

To isolate human MSCs, bone marrow aspirates of 10-20ml were taken from the iliac crest of normal patients ranging in age from 40 to 60 years old. Nucleated cells were isolated with a density gradient [Ficoll/paque (Pharmacia)] and resuspended in complete culture medium. The culture medium used was modified Eagle's medium (MEM) supplemented with 10% fetal calf serum (Gibco-RBL Life Technologies, Paisley, UK) and antibiotic/antimycotic (penicillin G sodium 100 U/ml, streptomycin 100 μg/ml, amphotericin B 0.25 μg/ml, Gibco-BRL Life Technologies, Paisley, UK). All of the cells were plated in 15 ml of medium in a 75-$cm^2$ culture flask (Cony) and maintained in a humidified atmosphere with 5% $CO_2$ at 37°C. After 24h, nonadherent cells were discarded, and adhernent cells were thoroughly washed twice with PBS. After the cultures reached confluency, the cells were harvested with 0.2% trypsin and 1mM EDTA for 5 min at 37°C, subsequently, 1-ml medium of cell suspension at a density of 1 x $10^5$ cells / ml was added to each well and maintained in a humidified atmosphere with 5% $CO_2$ at 37°C.

### Isolation and Growth of ACLs

To isolate human ACL cells, tissue pieces were taken from the normal patients ranging in age from 30 to 50 years old. Samples of human ACL were obtained, cut into 1-2 mm pieces, and placed in polystyrene tissue-culture plates (Cony). The culture medium used in the procedure was the same with the MSCs, as described previously. After approximately 21 days, the outgrowth cells were removed with 0.2 % trypsin-EDTA for subculture. Cells were maintained in culture using standard procedures and used at passage 5.

**Flow Cytometry**

Aliquots of anterior cruciate ligament (ACL) cells and human mesenchymal stem cells were seeded at $10^5$ cells/T-75 flasks, and allowed to expend at at 37°C and 5% $CO_2$ humidified environment. After expansion, cells were released with trypsin and resuspended in medium. The cells were then centrifuged and resuspended in stain buffer at a concentration of $1\times10^6$ cells/ml. One hundred microliters of cells preparation ($1\times10^5$) were stained with saturating concentrations of flurescein isothiocyanate-(FITC), phycoerythrin-(PE) conjugated CD105, CD29, CD44, CD71, and CD34. Analysis was performed using Cellquest software. The presence or absence of staining was determined by comparison with the appropriate isotype control. Gated events were scored for the presence of staining for a CD marker if more than 25% staining was above its isotype control.

**Assessment of cell electrophoretic mobility**

Before the mobility measurement, the cultured cells were detached from the culture well. Subsequently, the cell suspension was centrifuged and the collected cells were redistributed in the medium containing 10m*M*Tris-HCl and 291 m*M*glucose (pH 7.3 to 7.4). The electrophoretic behavior of MSCs and ACL cells were observed using

an inverted microscope attached with a CCD camera, as described previously. Briefly, cell suspension was introduced into a rectangular glass electrophoresis chamber similar to that used by Mironov and Dolgaya. Agar bridges (2% agar) were placed in the ends of the chamber and connected to the DC supply (200 V) with two compartments containing Pt electrodes in saturated KCl solution. The hole for introducing cell suspension was located in the upper side of the chamber and was close to the agar bridge. Current passing through the chamber ranged from 1 to 4 mA. All measurements were conducted at 22±2°C and their duration did not exceed 30 min, to avoid the temperature increase of the chamber because of the heating effect of the microscope illumination. The magnitude of the velocity of MSCs and ACL cells $u$ was measured by recording the time needed for them to pass a fixed number of ratitude divisions (ca. 400 $\mu$m). The electrophoretic mobility was calculated by $\mu = ugS/I$, where $S$ is the crosssectional area of the observation chamber, $I$ is the current,and $g$ is the conductivity of medium measured separately. Each reported mobility was based on 10 readings and expressed as mean±SEM.

**Results and Discussion**

Fig 1 showed the photographs of the ACL cells and MSCs. The photographs reveal that the morphology of the two kinds of cells were almost the same, the phenotype were all typical fibroblast-like cells. Although the sizes were different, it's not an obvious character which can be used to discriminate MSCs and ACL cells. Fig2 were the typical markers of MSCs, including CD29, CD44, CD71, CD34, and CD105. Expect CD34, others markers all displayed positive. In Fig 3, the results of CD105 antibody reveal that the MSC is positive and the ligament cell is negative. CD105 is a very important surface marker to identify MSC. By the result, we also could use CD105 and CD44 to discriminate MSC from ligament cells. Fig 5 depict the result of

the smooth muscle actin staining, we investigated that both ACL cells and MSC were positive.

Fig6 reveal that the cell mobility of three kinds of cells was different and mesenchymal stem cell was the most fast. Fig 6(b) also showed the cell mobility results which isolated from rabbit, the results also reveal the clear difference between MSCs and ACL cells. The results from human and rabbit reveal that electrophoretic analysis is a new method that could analyze the cell mobility. Cell mobility is also a kind of characteristic that could be used to identify cell. Besides MSCs and ACL cells, electrophoretic analysis also could be used in different kinds of cells. If compare with flow cytometry and real time RT-PCR, electrophoretic analysis is a believable method which could take exact data conveniently. In addition, electrophoretic analysis is also a method which can cost down.

**Conclusion**

1. The result of flow cytometry revealed that the surface marker of CD105 could differentiate MSC and ligament.
2. We develop a novel electrophoretic analysis for characterization the cell mobility of BMSC and ligament cell.
3. Through the development of scaffolds that are compatible with ligament or tendon attachment and using fibroblasts derived from bone marrow stem cells, further *in vivo* animal study will be conducted to induce tissue regeneration in large defect in tendon or create a viable ligament analog for reconstruction.

**ACKNOWLEDGMENT**

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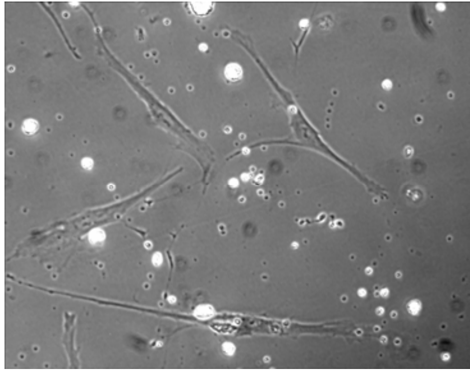
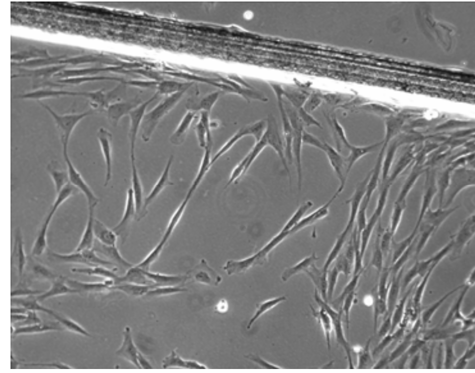
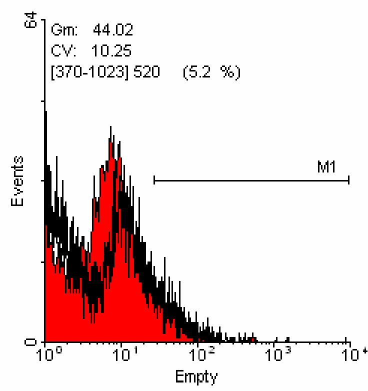


*(a) ACL cells*

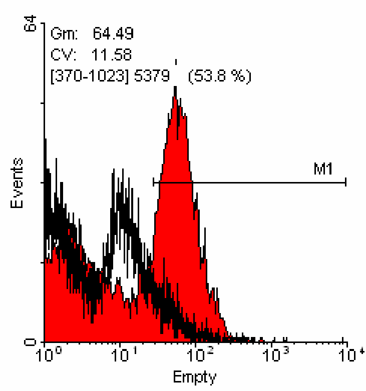


*(b) MSC*

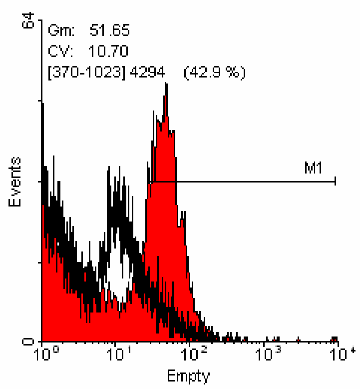
*Fig1: The photographs of the ACL cells and mesenchymal stem cells (MSC)*



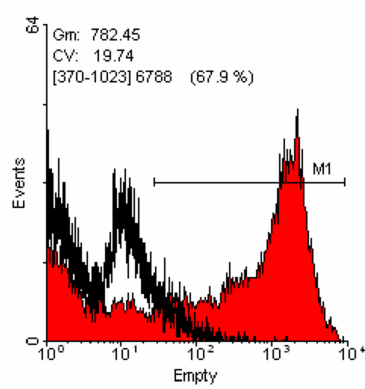

*CD34*



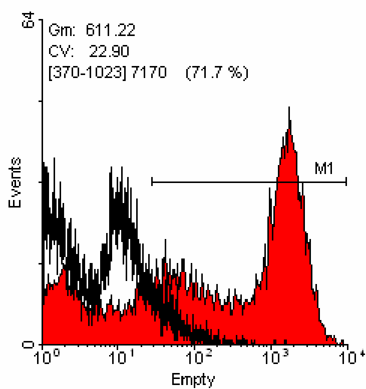

*CD105*




*CD71*

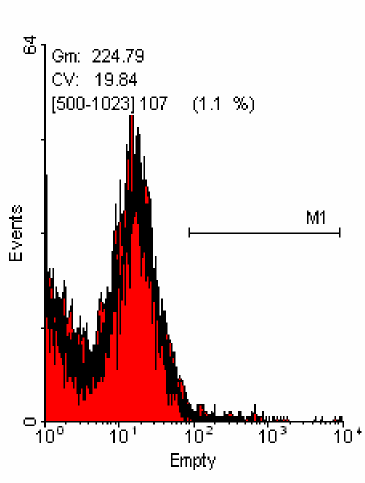



*CD44*

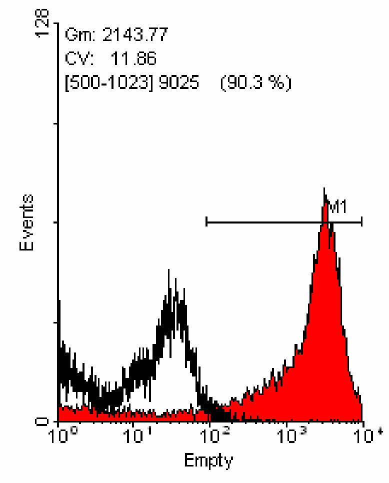



*CD29*

*Fig 2 Flow cytometric analysis of adult stem cell of CD34, CD105 , CD29 , CD44 , CD71.*

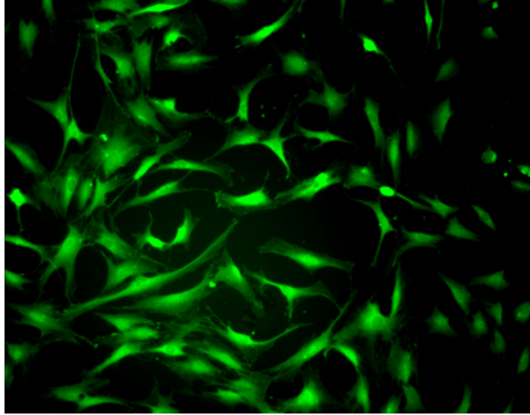



(a) CD105

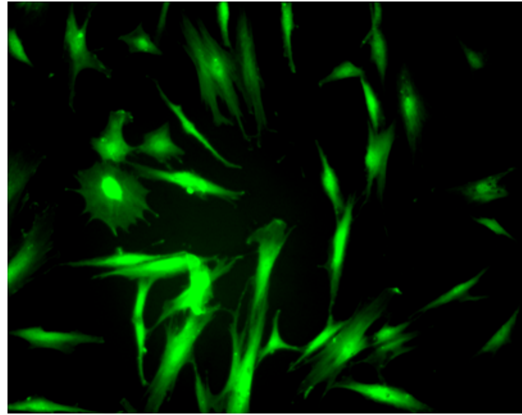



(b) CD44

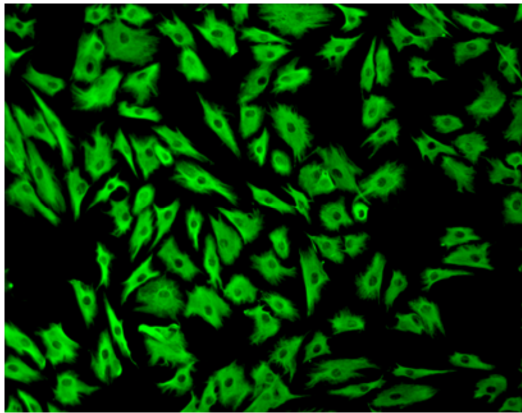
*Fig 3: The result of flow cytometry of ACL cells surface marker. (a) CD105 (b) CD44*



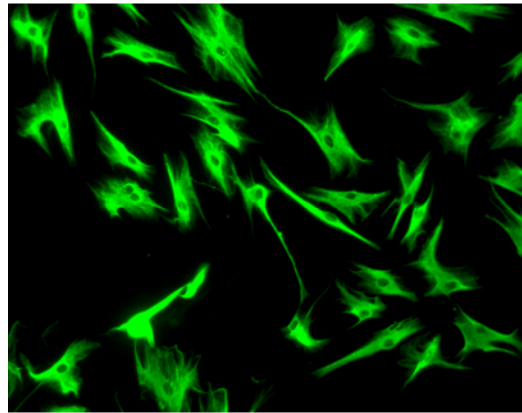
ACL- $\alpha$ -Smooth muscle actin-100X



BMSC- $\alpha$ -Smooth muscle actin-100X

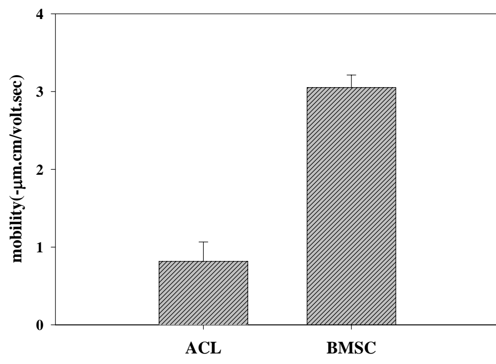


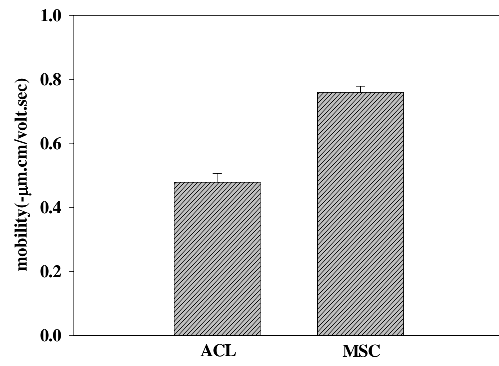
ACL-Vimentin-100X



BMSC-Vimentin-100X

*Fig 5: $\alpha$-smooth muscle actin and Vimentin staining of ACL cells and MSCs.*




(a) (b)

*Fig 6: The electrophoretic mobility of anterior cruciate ligament (ACL) and mesenchymal stem cell. (a) Human (b) Rabbit*