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

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LIGAMENT TISSUE ENGINEERING USING BONE MARROW STEM CELLS AND PLLA MEMBRANES

ABSTRACT

Poly (lactic acid) is a biodegradable biomedical material that has been used for bone and cartilage repair. Biomaterials coated with various ECM have been shown to promote cell adhesion, proliferation, and differentiation. The purpose of this study is to develop scaffolds that are suitable with ligament cells attachment and to use ligament cells derived from bone marrow stem cells to induce tissue regeneration. The first thing of this project is to examine the cellular behaviors of ligament on various ECM coated on three kinds of PLLA membranes. 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 α- 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

Poly ($_{L}$-Lactic acid) (PLLA), one of the few synthetic degradable polymers approved by the Food and Drug Administration (FDA) for certain human clinical applications, is a suitable substrate for osteoblasts culturesand shows fracture healing results comparable to those of metallic implants. Generally, most of PLLA substrates for cell culture have dense and smooth surface morphology. Since the biocompatibility of a substrate is also influenced by its surface morphology, salt-leaching technique has been used to generate porous morphology of biodegradable polymers to improve cell growth and new bone tissue formation.

In Taiwan, basketball is a popular sport. During this game, it is easy to cause anterior cruciate ligament (ACL) injury due to sudden stop motion. In addition, motorcycle is a popular transportation. Therefore, the number of rupture ACL caused by accident in Taiwan is much higher than that in other countries. In the United States, at least 120,000 patients undergo tendon and ligament repairs each year.

ACL is an intra-articular ligament and located in the knee. Fibrin clot does not form in the intra-articular milieu due to the presence of fibrinolytic enzymes in synovial fluid. Hense, the ACL could not form a fibrin clot to bridge the fracture end by itself unlike the Medialcollateral ligament (MCL).

So far, the therapeutic options for reparing torn ligaments involve tissue reconstruction using synthetic prosthesis. Unfortunately, none of these surgical alternatives provides a long-term adequate solution. On the other hand, today the bone-petella-bone autologous grafts have some well-regonized drawbacks. Rehabilitation has to be slow tp protect the transplant during the first two to three months needed for revascularization and collagenistation.

Musculoskeletal soft tissue injuries are quite common, but it is difficult to treat extensive ruptures of supraspinatus tendon of the rotator cuff, the Achilles tendon, and

the cruciate ligament and collateral ligament of knees. The ruptured ends are irreparable due to high tension on approximation. In this era of rapid development of biomaterials and cell therapy, it is promising to use the tissue engineering for ligament and tendon repair.

MATERIALS AND METHODS

PLLA membrane fabrication

Poly (L-Lactide)(PLLA) was supplied by Sigma. The weight average molecular weight (M_W) of PLLA was 85000-160000 as determined by gel permeation chromatography. The PLLA membranes were prepared by the dry process and wet process of the phase inversion method. Polymer solution was cast on a glass plate in a uniform thickness of 300 μm by an autocoater(KCC303，RK print-coat Instruments，UK) at 25℃ for the preparation membranes. In this work，three types of PLLA membranes were prepared by changing the phase inversion process. The casting solution was evaporated at 25℃ for 1 day to obtained dense membrane . Particulate membrane and porous membrane were prepared by immersing the casting solution immediately into the Ethanol bath for 30 minutes. The polymer concentration of porous was 5% and 20% of particulate membrane. After the evaporation or precipitation was completed，the formed membranes were soaked in water to remove the residual solvent and freeze-dried to remove the water.

Cell culture

Human anterior cruciate ligament (ACL) cells and human mesenchymal stem cell (MSC) isolated from human ligament and marrow were used in this study. 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). Ligament cells and marrow were trypsinized using 0.1% trypsin and 0.1% ethylenediaminetetraacetic acid for 5 min, centrifuged at 400 *g* for 5 min, and resuspended in the medium. For determination of the cell adhesion and cell growth, the prepared PLLA membranes with 15 mm in diameter were placed in 24-welled tissue culture polystyrene plates (Costar, U.S.A.). A silicon rubber ring 15-mm diameter was placed on each of the tested membranes in the wells to prevent them from floating. Membranes and silicon rubber rings were sterilized in 70 % alcohol overnight and rinsed extensively with phosphate buffered saline (PBS), followed by treatment under ultraviolet light overnight. Subsequently, 1-ml medium of cell suspension at a density of 1×10^5 cells / ml was added to each well and maintained in a humidified atmosphere with 5% CO_2 at 37°C.

MTT assay

After cell culturing for 1, and 4 days, the viability of ligament cells was determined by MTT assay. The method of Mosmann was modified and used in this study. The 100 μl of MTT (SIGMA; USA) solution was added to each well. After 3 hours incubation at 37°C, 200 μl of dimethyl sulfoxide (Nacalai Tesque, Japan) was added to dissolve the formazan crystals. The dissolvable solution was jogged homogeneously about 15 minutes by the shaker. The optical density of the formazan solution was read on an ELISA plate reader (ELx 800, BIO-TEK,) at 570 nm.

Cell morphology

Cells adhering to the membranes were washed with phosphate buffered saline (PBS) after 4 and 24 h incubation. Subsequently, the cells were fixed with 2.5%

glutaraldehyde in PBS for 1 h at 4°C. The specimens, after being thoroughly washed with PBS, were dehydrated using graded ethanol changes, critical point dried, gold splattered in vacuum, and examined using SEM.

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 1×10^6 cells/ml. One hundred microliters of cells preparation (1×10^5) 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.

Results and Discussion

Cell and membrane morphology

In this article PLLA membranes with dense, porous and particulate morphologies were easily prepared by the phase inversion method and the behavior of ligament cells on these PLLA membranes were compared. Figure1 depict the optical photographs of the mesenchymal stem cells (MSC) and ACL cells which take from patients. The figure showed that the MSC cells were large and spindle-shaped. Some of previous reports also call the kinds of MSC cells were fibroblast-like cells. Fig 2 were the top surface structure of three kinds of PLLA membranes：particulate, porous, and dense.

MTT assay

We used the PLLA particles to prepare three kinds of PLLA membranes which with different kinds of morphologies and seeded the ACL cells on the membranes to know the cell biocompatibility. The results in figure3, figure4, and figure 5 depict the MTT conversion of cells attached to three types of membranes without and with coating fibronectin and collagen after 1,4 and 7 days in culture. Figure3 depict the OD value without obvious difference between three types of PLLA membranes. Figure4 depict after 7 days incubation, particulate membrane coating fibronectin showed the highest OD value. Figure5 depict particulate membrane coating collagen showed the highest OD value after 4h incubation. We consider that collagen can help cell adhesion of ligament cells, and fibronectin can help the cell proliferation. Because of the particulate membrane with more surface area that could adsorb more ECM, therefore, after coating with ECM, particulate membrane had the best biocompatibility.

Flow cytometric analysis

The MSC cells were screened by immunochemistry coupled with flow cytometry for the presence of CD105, CD34, CD29, CD44, and CD71. Ten thousand cells per sample were examined in gated populations designated. The resulted reveal that MSC cells exhibited positive staining for CD105 (53.8%), CD29 (71.7%), CD44 (67.9%), CD71 (42.9%) and negative staining for CD34 (5.4%). Because bone marrow contains at least two kinds of stem cells, hematopoietic stem cells and stem cells for nonhematopoietic tissues. The hematopoietic stem cells exhibited positive staining for CD34 and mesenchymal stem cells exhibited negative staining for CD34. Therefore, in our research, cells surface marker analysis revealed that more than 53.8% of the cells were positive for CD105, a putative mesenchymal stem cell marker, and negative for CD34, a marker for hematopoietic cells.

In figure 7, the result 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.

Electrophoretic analysis

Figure9 reveal that the cell mobility of three kinds of cells was different and mesenchymal stem cell was the most fast. 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.

Smooth muscle actin

Figure10 and Figure11 depict the result of the smooth muscle actin staining, we investigated that both ACL cells and MSC were positive. We will try to use other types of actins to discriminate them.

Conclusion

1. We developed a new particulate membrane that could supply more surface area to adsorb more extracellular matrix.
2. We found collagen could help cell adhesion and fibronectin could facilitate cell growth. This outcome could be applied to the regeneration of ruptures ligament.
3. We consider that to change the Grain size and pore size of PLLA membrane will affect the cell adhesion and growth.
4. The result of flow cytometry revealed that the surface marker of CD105 could differentiate MSC and ligament.
5. We develop a novel electrophoretic analysis for characterization the cell mobility of BMSC and ligament cell.
6. 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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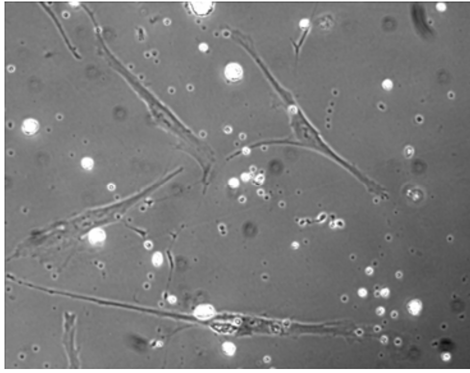
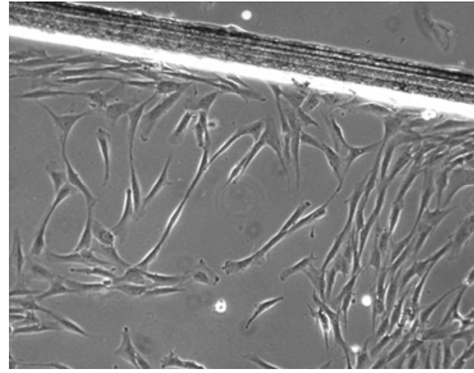
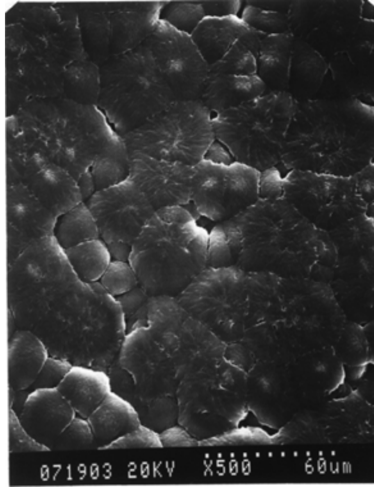


(a) MSC

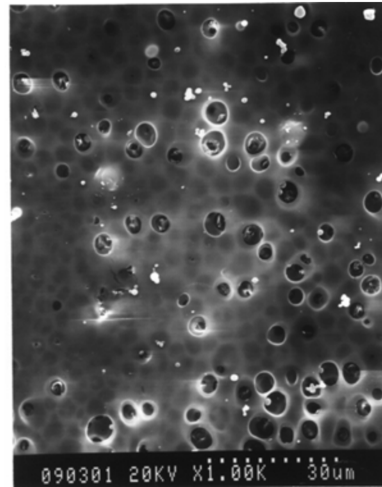


(b) ACL cells

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



(a) Particulate



(b)Porous

(c) Dense

Fig2: SEM photographs of the top surface of PLLA membrane (a) particulate (b) porous (c) dense

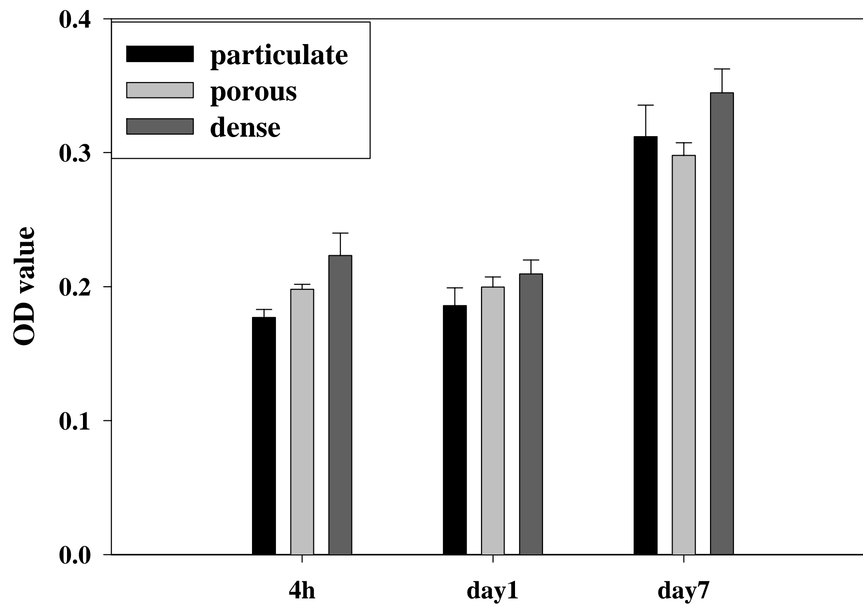


Fig 3: The MTT result of ligament cell culture on three kinds of PLLA membranes

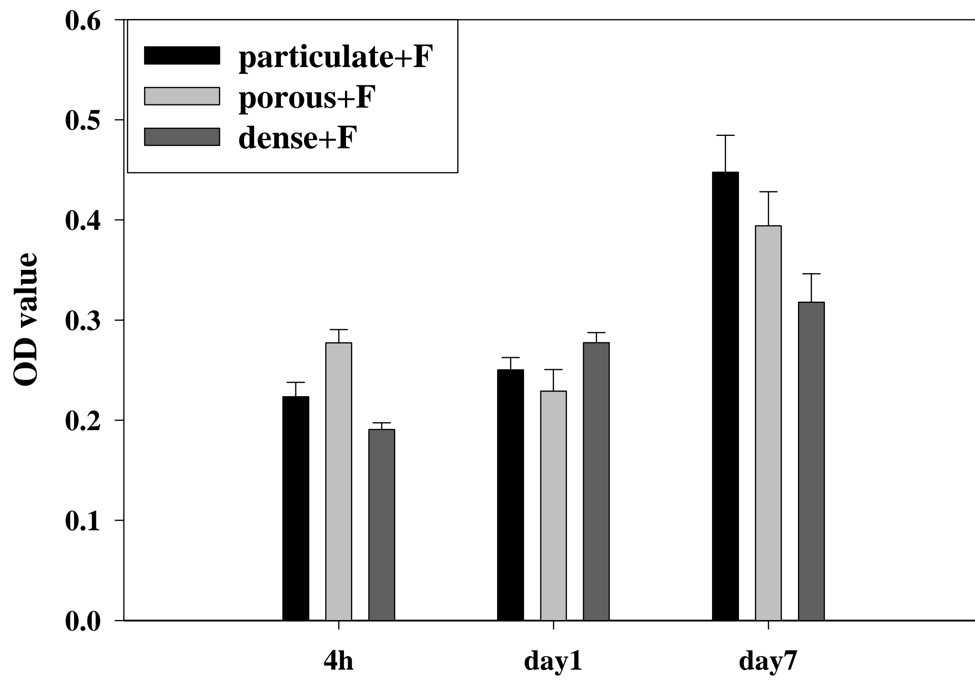


Fig 4: The MTT result of ligament cell culture on three kinds of PLLA membranes coated Fibronectin

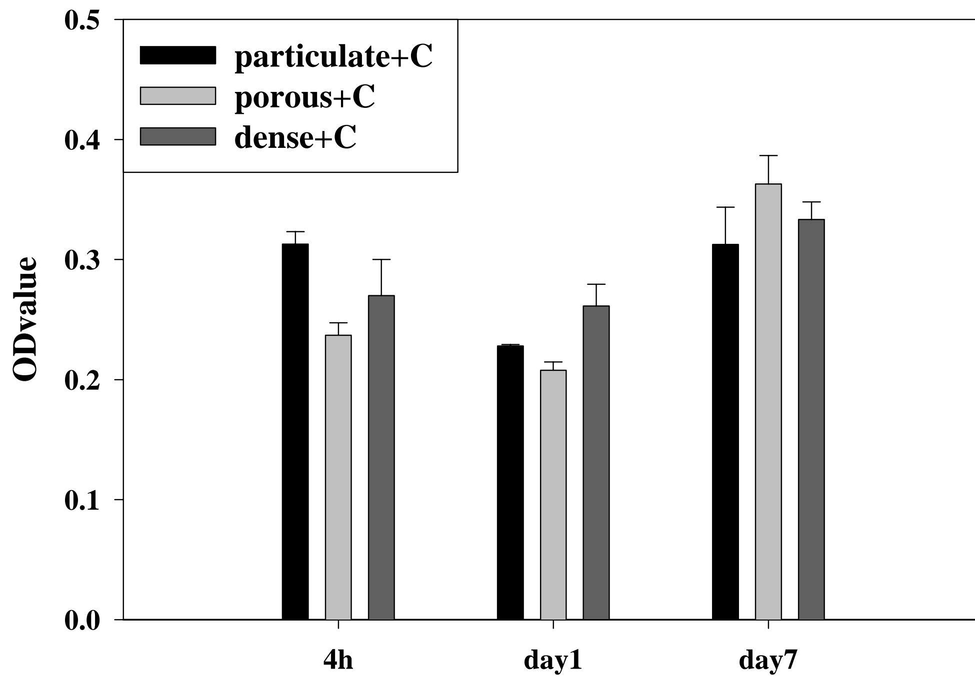
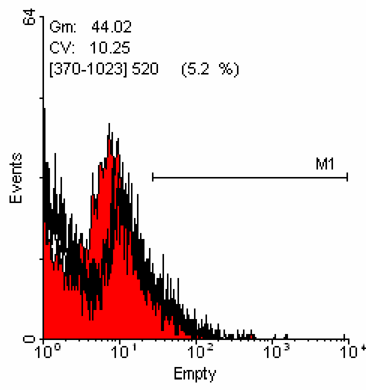
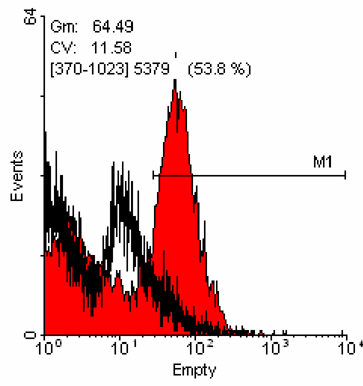


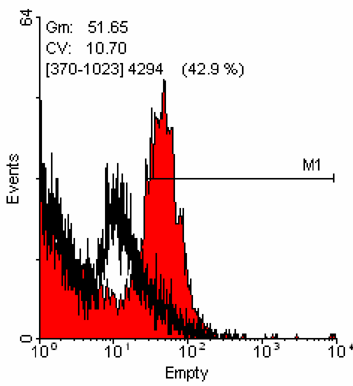
Fig 5: The MTT result of ligament cell culture on three kinds of PLLA membranes coated collagen



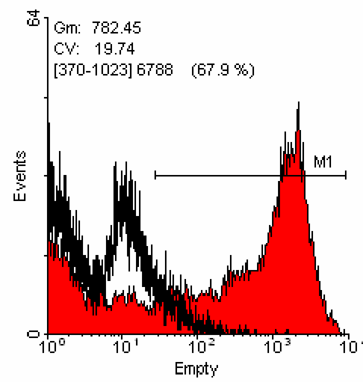
CD34



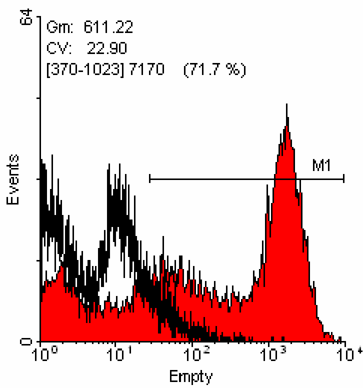
CD105



CD71

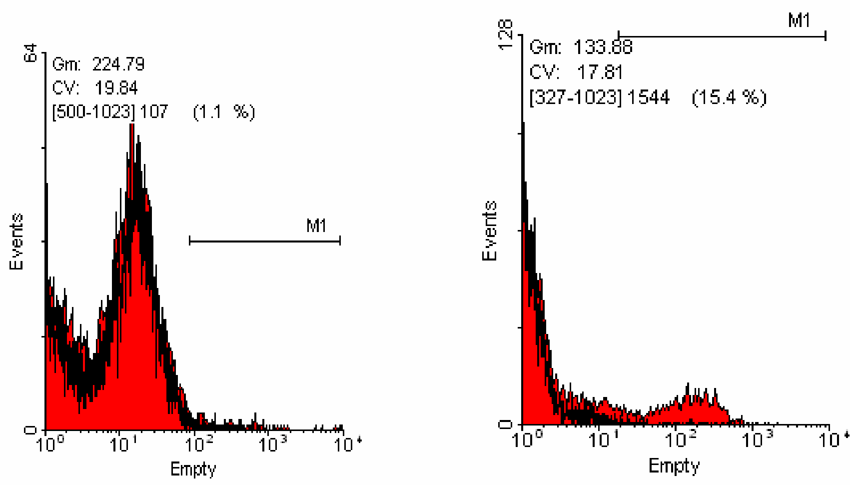


CD44



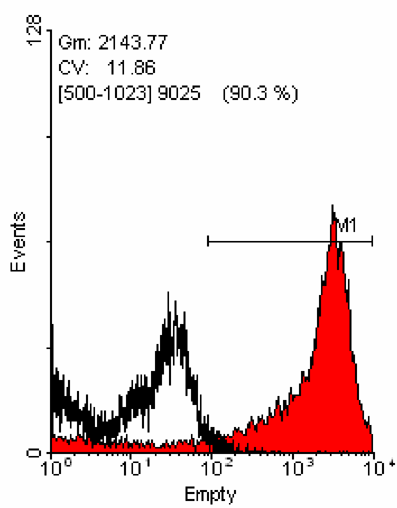
CD29

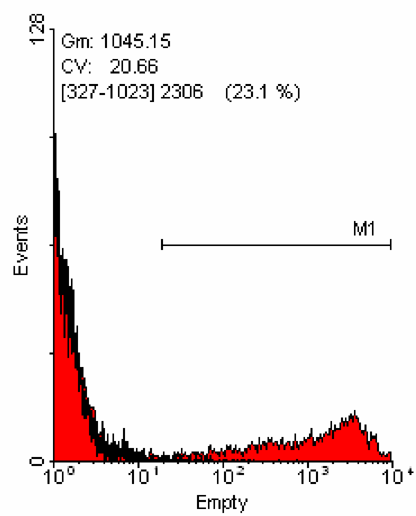
Fig 6 Flow cytometric analysis of adult stem cell of CD34, CD105 , CD29 , CD44 , CD71.



(a) ACL cell *(b) MSC*

Fig 7: The result of flow cytometry of CD105 surface marker. (a)ACL cell (b) MSC





(a) ACL cell *(b) MSC*

Fig 8: The result of flow cytometry of CD44 surface marker. (a) ACL cell (b) MSC

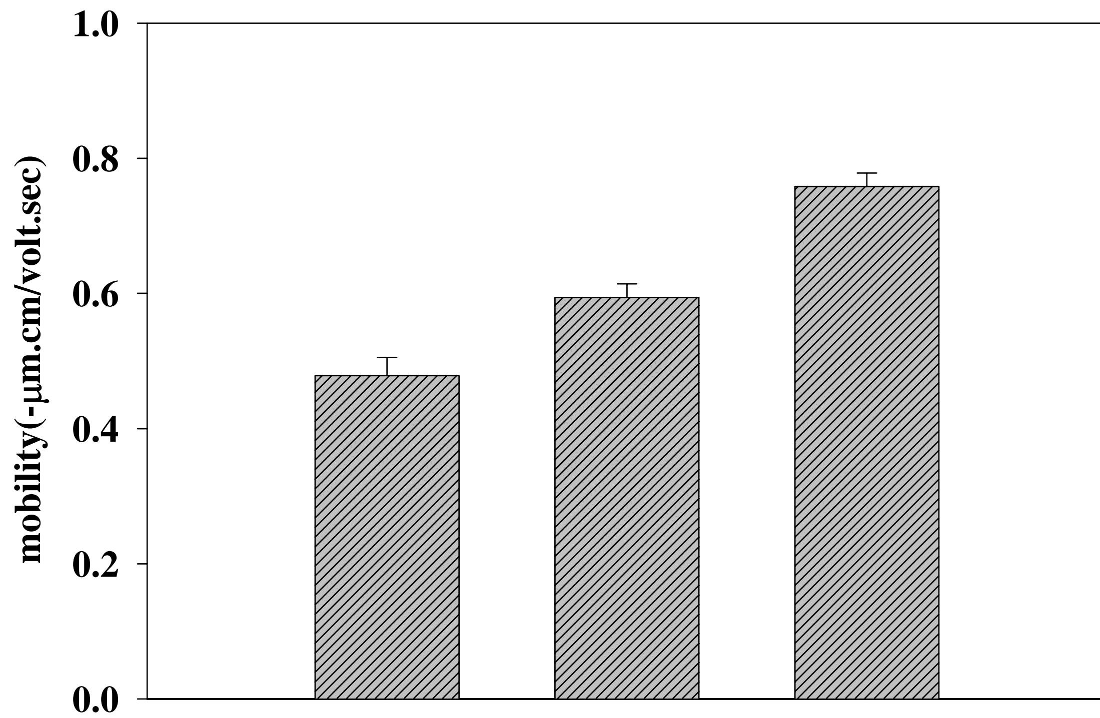


Fig 9: The electrophoretic mobility of anterior cruciate ligament (ACL), medial collateral ligamen t(MCL) ,and mesenchymal stem cell.

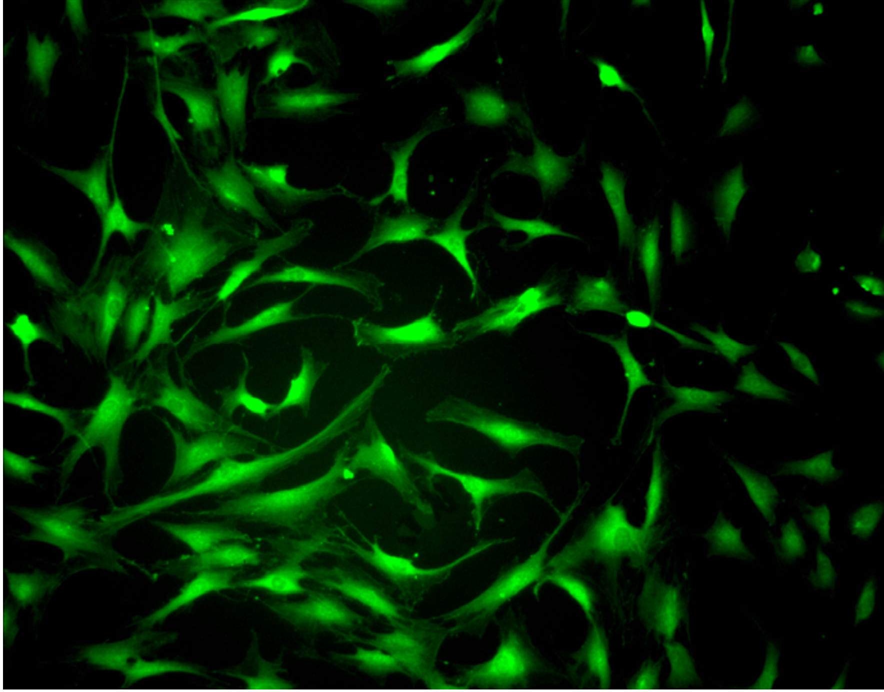


Fig 10: smooth muscle actin staining of ACL cells

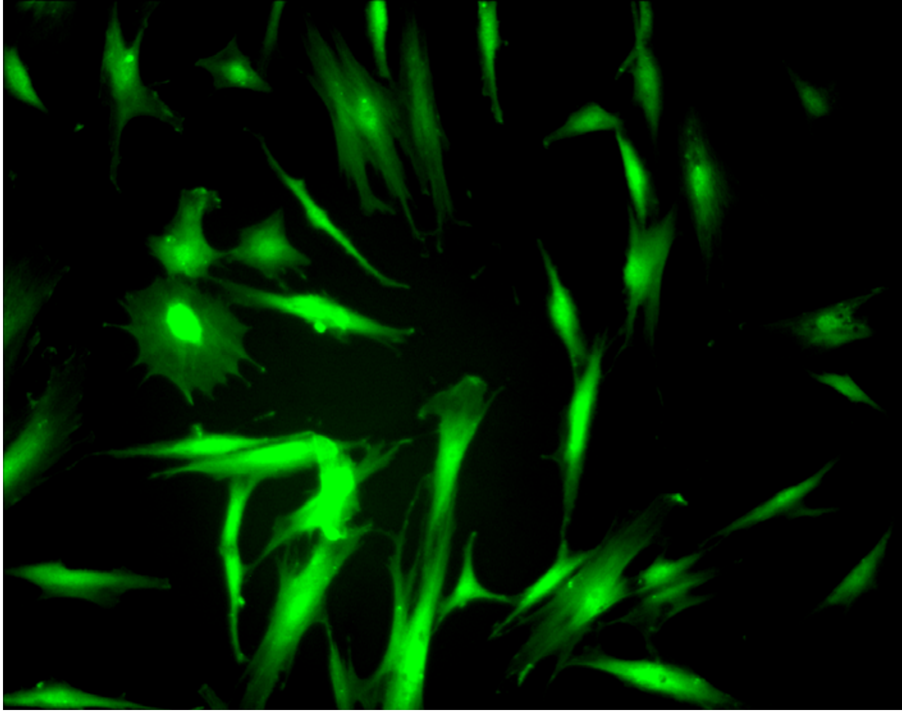


Fig 11: smooth muscle actin staining of MSC cells