

## **Proteomic Analysis of Differential Protein Expression in Fibroblast-like Synoviocytes of Pig**

Baatarsogt. O · Hyun-Kyung So · In-Hee Cho · Jeong-Soo Lee · Hee-Kyung Lim ·  
Jong-Ha Lee<sup>1</sup> · Eun-Kuk Kim<sup>1</sup> · Kung-Duk Choi

*School of Applied Life Science, Hankyong National University,*

*<sup>1</sup>Medical Center of Kyung Hee University*

### **Abstract**

The innermost structures of synovium consist of one to three layers of cells generally identified as synovial lining cells (SLC). The present studies were initiated to determine the protein expression patterns of fibroblast-like synovial (FLS) cells derived from the synovia of rheumatoid arthritis. Post-traumatic arthritis (PTA) is one of the most common causes of secondary osteoarthritis, and usually affects younger people. The proteins were separated by two-dimensional polyacrylamide gel electrophoresis and RNA expression investigated by RT-PCR. Proteome analyses led to the identification of more than 1,500 protein spots and of 11 differently expressed protein spots among them. Six proteins were down-regulated, and five proteins were up-regulated in ACL-transected synovial tissue. Among these, spots 3 and 8 were identified as cofilin-1 and smooth muscle protein 22- $\alpha$ , respectively. Therefore, the proteome analysis of synovial tissue is a useful approach to investigate a joint after an injury and can be used to understand the pathogenesis of PTA.

**Key words:** fibroblast-like synovial cell, 2-DE, proteomics, down-regulated, up-regulated.

### **Introduction**

The synovial membrane is a thin lining layer within the joint cavity that is responsible for maintaining normal joint function and homeostasis. Within the synovial membrane, the cells most closely associated with this homeostatic function are the normally highly synthetic fibroblast-like synovial (FLS) cells. These are the primary source of articular hyaluronic acid and other glycoproteins such as lubricin. In chronic inflammatory disorders such as

rheumatoid arthritis (RA), the synovial membrane becomes the target of a persistent inflammatory process that leads to fundamental changes in the phenotype and function of FLS cells. Although the pathogenesis of this phenotypic change remains uncertain, available data suggest that this may involve the acquisition of a combination of increased proliferative potential and resistance to apoptosis [3]. We are investigated protein expression of synthetic fibroblast-like synovial (FLS) cells by 2-DE polyacrylamide gel electrophoresis or MALDI-TOF/MS and RNA expression by RT-PCR.

## **Materials and Methods**

### **1. Isolation and Primary Culture of Pig Articular Synovial Membranes**

Pig articular synovial joints were released from of synovial membranes 5-week-old pig by enzymatic digestion. FLS were isolated by digesting with in 2.5% collagenase type II (400 units/mg) (Gibco BRL) in Dulbecco's modified Eagle's medium, and with 3% heatinactivated FCS for 2 h at 37°C, centrifuged at 1,500 g for 10 min, washed twice in 1xPBS and cultured in Dulbecco's modified Eagle's medium containing 4.00 mM/L L-glutamine, 4,500 ug/L glucose and supplemented with 10% (v/v) fetal bovine calf serum, and were then plated on culture dishes at a density of  $2 \times 10^4$  cells/ml. The cell cultured was 10% heat-inactivated FCS at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### **2. Protein Sample Pereparation**

All reagents used in 2-DE were obtained from Amersham Biosciences (USA). FLS were of sample buffer (8 M urea, 2% CHAPS, 30 mM Tris-HCl, 20 mM DTT, 0.5% IPGphore buffer) was added and mixed thoroughly.

### **3. Two-dimensional Gel Electrophoresis**

2-DE was performed in a horizontal apparatus (IPGphor and Hoefer 600 SE; Amersham Biosciences, Uppsala, Sweden). For the analytical gels, 250 g of protein was applied onto immobilized pH gradient (IPG) strips (18 cm, pH 3-10NL) 8M Urea, CHAPS, according to the method of Principles et al. After iso-electric focusing, the strips were applied to the top of SDS-PAGE gels (13%), and the proteins were separated according to their molecular mass.

### **4. Image Analysis**

Digitized images of the stained gels were analyzed using the 2-DE gel analysis program Image Master 2D Elite software (Amersham Pharmacia Biotech, Uppsala, Sweden). A comparison report of the qualitative and quantitative differences between the samples for

each data set of was generated.

### **5. In-Gel Digestion and Mass Spectrometric Analysis**

Differentially expressed protein spots were excised from the gels, cut into smaller pieces, and digested with trypsin (Promega, Madison, WI, U.S.A.), as previously described. For MALDI-TOF MS analysis, the tryptic peptides were concentrated on POROS R2 columns (Applied Biosystems, Foster City, CA, U.S.A.).

### **6. Semiquantitative RT-PCR**

Single-stranded cDNA was synthesized from 2 g of total RNA using an oligodeoxythymidylic acid primer and AMV reverse transcriptase (Promega, U.S.A.). Each cDNA was diluted for subsequent PCR amplification. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was amplified as a quantitative control. Each PCR was carried out in a 20 $\mu$ l volume of 1 $\times$  PCR buffer with in a GProgram Temp Control System PC-808.

## **Results**

### **1. Isolation and Characterization of Pig FLS**

FLS were isolated from healthy and pathological pig synovial membranes by using collagenase. A small number of FLS were obtained after digestion. Cells of the fifth passage examined by electron microscopy showed broad processes containing rough endoplasmic reticulum characteristic of fibroblast-like synoviocytes.

### **2. 2-DE Analysis of Pig Articular Synovial Joints from Control and Cruciate Pathological Synovium Cell**

To examine the differential expression of proteins in synovial membran from normal synovium cells and pathological synovium cells, proteomic analysis was performed using high-resolution 2-DE. Fig. 2 is a representative master gel image showing the separation of proteins from normal pig synovium cell. By conducting 2-DE using several types of IPG strips, we found that the 3~10 pH range covers a majority of the proteins of interest in , normal synovium cells and pathological synovium cells was the best choice for initial survey investigations.

### **3. Protein Identification**

The protein spots that revealed statistically significant differences between normal pigs and pathological pigs were excised from the gels, trypsinized, and analyzed by MALDI/TOF MS. Of these, we have been able to identify 11 proteins (Table 1). The number of matching peptides, the percentage of sequence coverage, and the accuracy of mass

estimates were used to evaluate the database search results.

#### 4. Differential Expression of Cofilin-1 and Smooth Muscle Protein 22 Alpha Genes

To determine whether the up and down regulation of SMP-22 alpha, and cofilin-1 expression occurred at the mRNA level, we performed semiquantitative RT-PCR analysis (Fig.3)

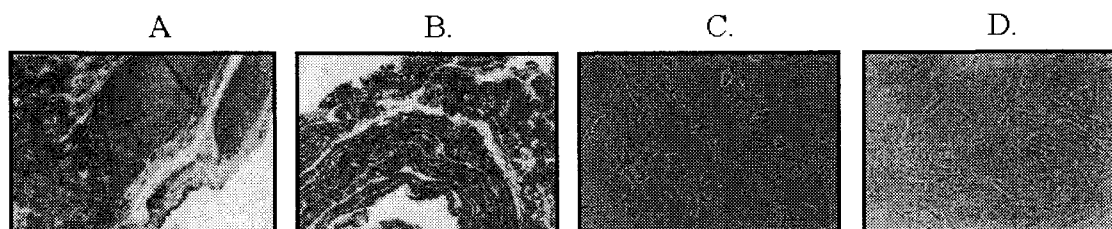
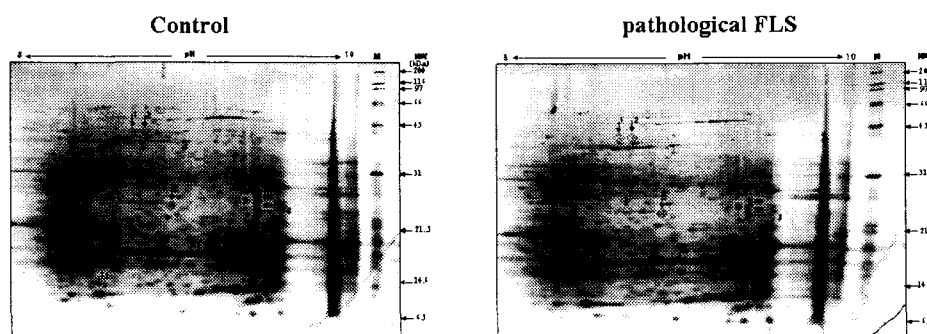


Fig. 1. (A) Normal FLS tissue of pig, (B) pathological synovium tissue of pig, (C) Normal FLS cells of pig, (D) pathological synovium cells of pig.



**Table 1 . Differentially expressed proteins in the pathological with (Cruciate) –induced FLS of pig**

Spot No.	Protein identity	MW(kDa)	pI	Accession No.	Difference
1	cytoskeletal beta actin	44.76	5.55	AAS55927	↓
2	cytoskeletal beta actin	44.76	5.55	AAS55927	↓
3	cofilin-1 (cofilin, non-muscli isoform)	18.51	8.16	Q6B7M7	↓
4	Destrin, Nmr, Minimized average structure	19.34	8.05	1AK6	↓
5	unnamed protein product	47.21	6.37	CAA32409	↓
6	Rho GDP dissociation inhibitor(GDI) alpha	23.41	5.12	NP_788823	↓
7	alpha-b crystallin	20.17	6.76	JC5971	↑
8	smooth muscle protein 22-alpha	10.1	4.93	AAC08010	↑
9	smooth muscle protein 22-alpha	10.1	4.93	AAC08010	↑
10	cytoskeletal beta actin	44.76	5.55	AAS55927	↑
11	cytoskeletal beta actin	44.76	5.55	AAS55927	↑

\*Sequencing coverage is defined as the percentage of the whole length of the protein sequence which is covered by matched peptides identified by the MALDI-TOF MS analysis, Spots shown in (Fig. 2). In order to preselect proteins exhibiting variations in expression levels, the protein patterns from normal and pathological synovium cells were divided into three classes, and the quantities of all detected spots in both classes were compared by ImageMaster 2-DE gel Elite software. After comparing the 2-DE protein patterns on duplicate gels of FLS from three normal and three pathological pigs. we found 11 protein spots that were significantly different in, normal synovium cells and pathological synovium cells . Among them, five spots were meaningfully increased, and six spots were decreased in the synovium cells of pathological pigs .

using and SMP-22- $\alpha$  and Cofilin-1 primers. The results confirmed that the mRNAs of Smooth muscle protein 22- $\alpha$  and were expressed at higher levels in the pathological synovium cells. These results substantiate the specific up-regulation of SMP-22- $\alpha$ , expression in the normal synovium cells and pathological synovium cells of normal pigs compared with those of pathological pigs.

## Discussion

The intent of these studies was to acquire information regarding the protein expression

patterns of typical RA FLS cells. The present studies provide a preliminary analysis of the synovial proteome. It should also be appreciated that the current analysis describes only the major FLS cellular proteins. Future studies will require the development of enrichment steps for low abundance proteins. Total cell lysates were separated on nonlinear immobilized pH gradient strips (pH 3~10) and fractionated in the second dimension on large-format SDS-PAGE gels. The separated proteins were visualized by staining with colloidal silver stain. The spots were excised, destained and digested in gel with trypsin. The peptides were extracted and analyzed by MALDI mass spectrometry. In excess of 11 spots were detected with silver stain. The cellular proteins were separated by two-dimensional polyacrylamide gel electrophoresis and RNA expression investigated by RT-PCR. A total of 11 spots were examined and 4 (cytoskeletal beta actin) or 2 (smooth muscle protein 22-alpha) identifications were made. These proteome analyses may significantly increase our understanding of the molecular biology related to the pathogenesis and pathophysiology of synovial joint degeneration after injury. In addition, a study of other tissues such as articular chondrocytes, serum, or synovial fluid, under the same condition as that described in this study, may open new avenues for research in the pathogenesis of PTA.

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