PROTEOMIC ANALYSIS OF THE CHONDROCYTE CELL SURFACE

Research Project

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ABSTRACT

Osteoarthritis, which is characterized by the progressive degradation of articular cartilage, is one of the major challenges for medical science because of its prevalence and lack of effective therapy. Currently, many scientists are interested in the biology of chondrocytes in health and disease because they play key roles in cartilage degradation. Membrane proteins are responsible for many important biological processes but they are poorly understood. Thus, relevant analysis of membrane proteins is still deficient. Thus, this project focuses on how to extract membrane proteins from chondrocytes and identifying these membrane proteins with the help of proteomics techniques.

The main aims of this project were to develop a detergent-based separation method (Triton X-114) to extract membrane proteins from equine articular chondrocytes and use nanoLC-MS/MS with two proteomics methodologies (shotgun and gel-based methods) to identify membrane proteins and evaluate the efficacy of the extraction method.

As a result, approximately 50% of the proteins identified in the hydrophobic fraction were membrane proteins, which support the efficacy of the Triton X-114 extraction method. In total, 52 membrane proteins were identified in this project, some of them play important roles in chondrocyte cell biology and even in osteoarthritis, such as integrin beta-1, CD44 and CD151.

CONTENTS

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This project gave me a memorable experience and it is very beneficial for my future study. Here, I am sincerely and heartily grateful to my supervisors, Ali Mobasheri (School of Veterinary Medicine and Science) and Susan Liddell (School of Biosciences), for the guidance they showed me throughout this project. Also, special thanks to Julia Smith (Bruker UK), for her kind help of MS equipment. In addition, I would like to thank my module leader, Chungui Lu, who gave me so much help during this year. Thank all the staffs in Proteomics Lab and Vet School who gave me many helps. Finally, I would like to thank my families and my girl friend for the endless support.

ABBREVIATIONS

AMBIC	ammonium bicarbonate
CD	cluster of designation
DTT	dithiothreitol
IAA	iodoacetamide
MCF-7	Michigan Cancer Foundation–7
OA	osteoarthritis
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
STZ	the superficial zone

Chapter1

INTRODUCTION

1.1 Articular cartilage

Articular cartilage is a connective tissue which covers the articular ends of joints (Figure 1). This smooth, white tissue has important and specialized biological functions: facilitating load transmission and decreasing friction of movement (Ratcliffe *et al*., 1991).

Compared with other types of tissue, articular cartilage is aneural, avascular and alymphatic (Mobasheri *et al*., 2011). Thus, this kind of tissue depends on synovial fluid to receive nutrients and cannot repair spontaneously. If lesions or aging related changes happen, it is more likely to lead to degeneration. Macroscopically, as a kind of hyaline cartilage, articular cartilage is glistening white tissue and about 2 to 4 mm thick. Microscopically, it is composed of an extracellular matrix (ECM) and unique resident cells which are called chondrocytes (Figure 2). The ECM consists principally of water, proteoglycans, and collagens. Other elements such as glycoproteins and lipids are also present, although these account for smaller proportion of the whole tissue (Buckwalter *et al*., 1988; Buckwalter *et al*., 1997). Each component has different functions. For example, as predominant structural protein, collagens provide a supportive framework for the entrapment of hydrated glycoproteins and proteoglycans, which allow cartilage to swell and resist compressive forces. Most collagens are responsible for tensile strength, stiffness and resilience. More specifically, in extracellular matrix, type Ⅱ and Ⅺ collagens form a fibrillar network in order to provide the tensile strength (Mobasheri *et al*., 2011). Type Ⅵ collagens surround the

chondrocytes and are responsible for transmitting biochemical and biomechanical signals (Guilak *et al*., 2006; Roughley *et al*., 1994). Proteoglycans are involved in the compressive pliability and stiffness of cartilage (Inerot *et al*., 1978). Overall, due to the chondrocytes and matrix constituting this complicated system, articular cartilage is endowed with the characteristic functions of distributing, absorbing and sharing the stress from joint activities (Poole, 1997).

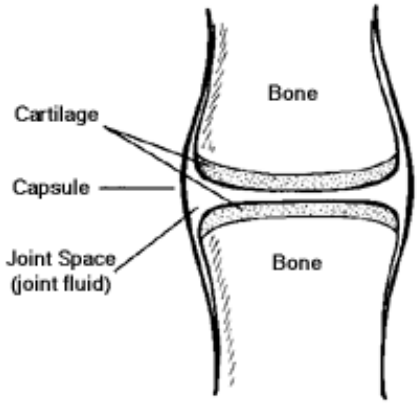

Figure 1: The location of articular cartilage [1]

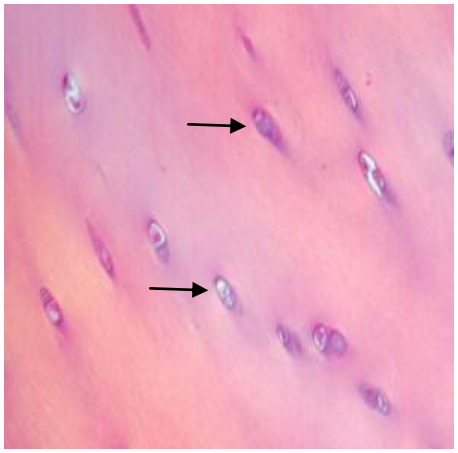

Figure 2: Chondrocytes (arrows) in articular cartilage and metrix around them [2]

Articular cartilage is divided into 4 horizontal zones: the superficial zone, the middle zone, the deep zone and the calcified zone (Figure 3).

(1) The superficial zone: This zone makes up about 10% to 20% of the thickness (Sophia *et al*., 2009). The collagen fibers, which are mainly type Ⅱ and Ⅸ collagens, are arranged compactly and parallel to the articular surface. Chondrocytes are flattened in this zone and there are a small number of proteoglycans. In addition, maintaining the integrity of this zone is quite important for the preservation and protection of deeper layers, but there is limited potential of repair because of low metabolic activity. Also, this zone connects with synovial fluid and it is mainly in charge of the tensile stress of

[1] http://www.cascadewellnessclinic.com/
[2] http://www.osteoarthritisblog.com

cartilage.

(2) The middle zone: It accounts for most of the cartilage volume, approximately 40% to 60% (Sophia *et al*., 2009). The arrangement of collagen in this layer is oblique. Chondrocytes are ball-shaped but present at lower density than other zones. The function of this zone is resisting compressive force.

(3) The deep zone: It represents 30% of volume (Sophia *et al*., 2009). The collagen fibers here, which have the largest diameter of all the zones, are positioned vertical to the surface. Chondrocytes are also arranged vertically (Sophia *et al*., 2009). One characteristic of the deep zone is that proteoglycans are very abundant but the amount of water is the lowest. This zone plays a core role in resistance to compressive forces.

(4) The calcified zone: This zone is separated from deep zone by the tidemark. The distribution of chondrocytes is deficient and cells are hypertrophic (Sophia *et al*., 2009). Additionally, this can be treated as a transitional area between cartilage and subchondral bone. As the final layer of cartilage, it anchors the collagen fibers of the deep zone to ensure cartilage is fixed to the bone.

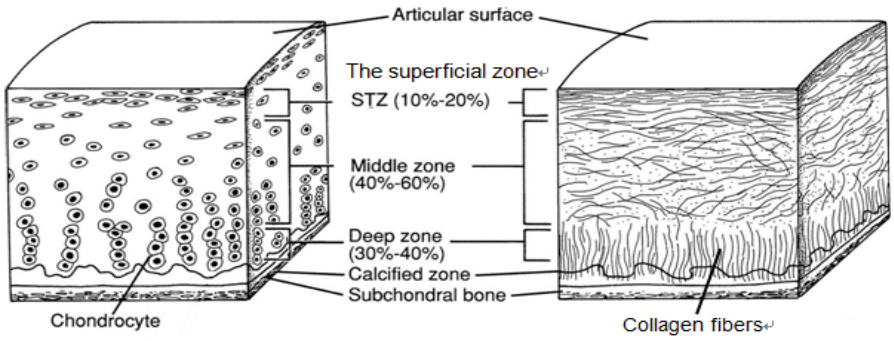

Figure 3: The zones of articular cartilage [3]

[3] http://www.grin.com

1.11 Repair of articular cartilage

In terms of the repair of cartilage, the following factors seriously restrict this capability. First, because chondrocytes are limited by the lacunae, they fail to move to the damaged area. Also, the matrix forms very slowly and the blood supply of cartilage is insufficient. Consequently, impaired cartilage is usually replaced by fibrocartilage scar tissues.

Intriguingly, different degree of lesion may arouse some repair activities. If the injury does not penetrate the subchondral bone, the repair is only expressed as matrix remodeling. But when deeper lesions happen, the well-vascularized subchondral bone will provide a blood supply (Hunziker, 2002). At that time, fibrocytes and stem cells in the blood can form fibro-chondrocytes (Jackson *et al*., 2001; Wei *et al*., 1999). Besides these, the bone also releases growth factors when it receives relevant stimuli, and these exert a significant influence on repair response. However, such a repair system is not perfect. The newly produced tissue usually lacks all of its biomechanical properties and fails to perform normal function as before (Hunziker, 2002). In addition, spontaneous healing is also related to the age, physiological condition and genetic characters of the individual (Melero-Martin *et al*., 2007).

1.12 Degeneration of articular cartilage

Throughout our lives, articular cartilage continuously undergoes a remodeling process

which is also called degradation. According to studies, excessive loading can promote the deterioration of chondrocytes (Mobasheri *et al*., 2002). The serious lack of usage of the joint also results in the loss of normal tissue structures. This kind of impairment, however, is mainly due to the change of components in the extracellular matrix rather than the alteration of chondrocytes (Millward-Sadler *et al*., 2004). In addition, aging is also a factor that can cause the change of the extracellular matrix and influence the relevant activities of chondrocytes (Hudelmaier *et al*., 2001). Generally speaking, all these conditions are able to accelerate the procedure of degradation. Currently, scientists place more emphasis on the interactions between the chondrocytes and the extracellular matrix. Such interactions may provide us a better understanding of pathophysiological processes of articular cartilage degradation (Mobasheri *et al*., 2011).

1.2 Chondrocytes

The chondrocyte is the only cell type present in the cartilage. As a kind of specialized cell, it is responsible for the production, maintenance and repair of the extracellular matrix (ECM). In other words, chondrocytes produce the crucial components of articular cartilage, such as proteoglycans, collagen, other proteins and lipids. The ancestor of the chondrocyte is called mesenchymal stem cell (MSC) which can differentiate into two different modalities, chondrocyte and osteoblast. Generally, the direction of the differentiation depends on the condition of the environment. In vascularized areas, such as bone, MSCs will develop into osteoblasts. However, if the differentiation happens in a non-vascularized area, as well as articular cartilage, MSCs

tend to yield chondrocytes.

Chondrocytes account for only 2% of the total volume of articular cartilage (Alford *et al*., 2005). Articular cartilage has several different zones, in which the morphology of chondrocyte varies from region to region (Sophia *et al*., 2009). For example, in the superficial zone, chondrocytes are smaller, flatter and denser than in other zones.

The absent of vasculature means that these cells exist in a hypoxic environment, but chondrocytes are glycolytic cells which can overcome the deficiency of oxygen and glucose (Mobasheri *et al*., 2005; Mobasheri *et al*., 2008). Also, chondrocytes locate in the lacunae which is scattered throughout articular cartilage. This condition restricts the migration of chondrocytes. Usually, chondrocytes exchange metabolites, transducer signals or respond to any stimuli through the connection between chondrocytes and ECM.

Based on the unique extracellular environment of chondrocytes, cartilage has a poor healing capability. Moreover, since the optimal chemical environment is prerequisite for the survival of chondrocyte, changes of ECM are prone to trigger the changes of their metabolic processes and even apoptosis. Such negative changes of chondrocytes have direct relationships with some joint diseases. Currently, many scientists are interested in the biology of chondrocytes in health and disease because they play important roles in cartilage degradation in osteoarthritis.

1.3 Osteoarthritis

Osteoarthritis (OA) is the most common type of arthritis in elderly people (Buckwalter *et al*., 2006). According to the study of WHO, about 9.6% of men and 18.0% of women aged over 60 years have symptomatic osteoarthritis [5]. The major hallmark of osteoarthritis is the progressive destruction of joint cartilage, which results in mechanical instability. Clinically, corresponding symptoms include pain, stiffness, limited range of joint motion, swelling, cracking and crepitation [6]. Studies have shown these symptoms of OA are caused by joint degeneration which includes increasing loss of articular cartilage accompanied with remodeling of subchondral bone and osteophyte formation (Buckwalter *et al*., 2006). With the advance of degradation, the bone surfaces can no longer be protected by cartilage so subchondral bones rub against each other and are damaged and destroyed (Figure 5). Currently, it is well known that increasing age and over-weight joint loading will raise the risk of cartilage degradation. However, the explicit pathophysiologic progress and how to diagnose it in early stage are still poorly understood.

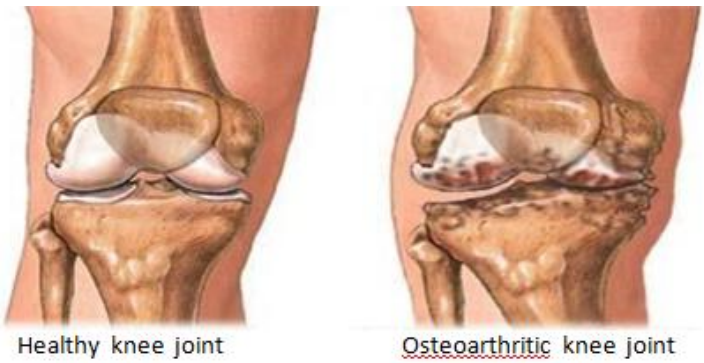

Figure 5: Comparison of healthy joint and osteoarthritic joint [6]. The cartilage disappeared and the bone was damaged in the osteoarthritic joint.

[5] http://www.who.int/chp/topics/rheumatic/en/

[6] http://www.nlm.nih.gov

In terms of high risk factors, osteoarthritis may correlates with the following:

- ✓ ***Aging***

According to epidemiological surveys, there is a close relationship between increasing age and the morbidity of OA. First of all, aging leads to some negative changes of articular cartilage. For example, aging results in the structure alteration of proteoglycan, which influence the resilience of cartilage (Inerot *et al*., 1978). Such environmental changes make the susceptible chondrocytes alter their biological process, so the degradation happens inevitably. Furthermore, evidence indicates that the age-related changes in joint cartilage, like morphologic changes, decrease the capability of chondrocytes to synthesize some proteins so they accelerate the development of OA (Buckwalter *et al*., 2000; Buckwalter *et al*., 2005). Additionally, studies showed that aging is responsible for the apoptosis of chondrocytes (Todd Allen *et al*., 2004; Robertson *et al*., 2006).

- ✓ ***Cartilage trauma and associated inflammation***

Cartilage injury leads to many biological changes. It can activate chondrocytes and synovial fibroblasts. As a result, some pro-inflammatory cytokines and mediators are released, like tumor necrosis factor-α (TNF- α), interleaukin-1β (IL-1 β), VEGF (Pufe *et al*., 2004). So there is no doubt that inflammation will be aggravated. Also, these cytokines lead to some metabolic responses in cartilage. For example, the synthesis of extracellular matrix will be inhibited and the generation of many inflammatory mediators and enzymes which advance the degradation of extracellular matrix such as

matrix metalloproteinases (MMPs). Additionally, these cytokines induce chondrocyte apoptosis (Borelli, 2006; Borrelli *et al*., 2004).

- ✓ ***Obesity***

Obesity is a major risk factor not only because of creating excess weight on joints, but also as a result of elevated levels of systemic and joint localized inflammatory cytokines (Abramson *et al*., 2009).

- ✓ ***Genetic background***

The hereditary character of OA was first mentioned by Kellgren (Kellgren *et al*., 1963). It was evident that there is an extremely high concordance for osteoarthritis between monozygotic twins (Zhai *et al*., 2007). Moreover, there are multiple genetic disorders involved in OA. For example, such disorders exert a negative influence on the architecture of subchondral bone through investigations in mouse models (Dabovic *et al*., 2002).

Today, people pay more attention to osteoarthritis because of its prevalence and the lack of therapy. Limited self-repair of articular cartilage further increase the challenges. More than 40 million people suffer from this disease in America (Gobezie *et al*., 2007). Currently, there is no effective treatment to prevent or remedy osteoarthritis. As a progressive disease, people who have osteoarthritis must suffer an aggravating pain constantly and may develop disability. Thus, patients endure a huge health and economic burden (Buckwalter *et al*., 2006).

Because the degradation of articular cartilage and the alternation of extracellular matrix (ECM) are the main features of OA (Calamia *et al*., 2011), an increasing number of articles focus on the chondrocyte which is the only cell type in articular cartilage and responsible for the biosynthesis of ECM. It is hope that such studies will reveal the underlying pathophysiologic progresses in OA and also help discover new biomarkers or other molecules that are useful for monitoring responses to therapy.

1.4 Membrane proteins

The cell membrane not only provides a physical barrier between intracellular and extracellular environments, but also has prolific membrane proteins which play a critical role in many cellular biological processes, such as signal transduction, transportation of metabolites and ions, cell adhesion, endocytosis (Santoni *et al*., 2000). With the help of membrane proteins, cell-cell and cell-matrix connections can be established. In terms of structure, many membrane proteins have hydrophobic domains which span the plasma membrane and hydrophilic domains which are located on the external or internal sides of plasma membrane (Cordwell *et al*., 2010). Also, due to the phospholipid bilayer structure of the plasma membrane, membrane proteins are divided into integral membrane proteins, which have one or more transmembrane-spanning regions, and peripheral membrane proteins, which are associated with the face of bilayer but have no transmembrane-spanning region (Cordwell *et al*., 2010).

There are many membrane proteins. According to genomic and statistical analyses, membrane proteins account for about 30% of total cellular proteins (Santoni *et al*., 2000). However, thorough and penetrating analyses of them are quite deficient (Wu *et al*., 2003). Although proteomics is a fast moving field, two major challenges for membrane protein profiling still cannot be overcome entirely. First of all, membrane proteins are very low in abundance. Current techniques lack such high sensitivity and protein loss during the experiments significantly affects membrane protein identification. Secondly, poor solubility is another limitation. The existence of

hydrophobic domains brings many difficulties for purification, which directly leads to inferior results (Cordwell *et al*., 2010).

Due to the fact that membrane proteins perform pivotal functions in many biological processes, it is therefore valuable to analyze them, not only for clarification of their functions, but also for applications in medical science. For example, membrane proteins are often involved in the impaired cellular signaling which is obvious in some kinds of cancer (Dhillon *et al*., 2007; Lallet Daher *et al*., 2009). Some membrane proteins can be applied as biomarkers and play a key role in cancer diagnosis, such as mucins (e.g. CA19.9, CA125) (Leth-Larsen *et al*., 2010). In addition, due to the function of cell signaling and transportation, membrane proteins make a notable contribution to drug design. Studies showed they represent approximately 70% of the known protein targets for drugs (Rabilloud, 2003). Thus, such features demonstrate the potential value of identification and characterization of membrane proteins.

1.5 Phase separation *(Detergent based membrane protein extraction)*

The value and potential of membrane proteomics are gradually recognized and understood by scientists. Therefore, there are an increasing number of research articles that focus on this area.

Due to the low abundance of membrane proteins and the complexity of protein

samples extracted from complete cells, establishing methodologies to extract such proteins becomes an essential first step in order to isolate and enrich membrane proteins. The development of simple protocols will also help to increase the accuracy of analysis. Currently, there are several methods which can achieve this purpose, such as sequential protein extraction, centrifugal protein extraction, affinity based methods and detergent-based protein extraction. Detergent-based membrane protein extraction, which is so-called phase separation, can obtain a satisfactory amount of membrane proteins and show stable performance and it is suitable for limited amount of sample material (Lehner *et al*., 2003; Ramsby *et al*., 1994). In addition, using phase separation to extract membrane proteins is easy to control even in very large sample volumes. So it has been applied in industrial processes (Selber *et al*., 2004).

Phase separation is a straightforward, economical and efficient method for membrane protein extraction. Generally, membrane proteins are associated with the so-called hydrophobic fraction so they do not solubilise easily in water, but often require the presence of detergents for solubilisation. Using this character, membrane proteins can be separated from the complex mixtures of total cellular proteins.

- **Comparison of advantages and disadvantages of phase separation**

Merits:

(1) The method is easy to use without any expensive and complicated equipment. Also, it can easily be applied to large volumes (Arnold *et al*., 2007).

(2) The efficiency of extraction is satisfactory.

(3) Many membrane proteins can be enriched simultaneously (Arnold *et al*., 2007).

Drawbacks:

(1) The high concentration of detergent included in the solution is detrimental to downstream applications, especially electrospray mass spectrometry. So it is essential to use a treatment which can remove detergent before analysis.

(2) It is difficult to extract entirely pure membrane proteins (Lehner *et al*., 2003). For example, cytoplasmic proteins still exist.

- **Theory of detergents**

Detergents are composed of amphiphilic molecules which have a polar head group and a hydrophobic hydrocarbon chain. Usually, the molecules are soluble in water. However, when the detergent concentration increases above a point called the critical micelle concentration (CMC), the molecules of detergent aggregate together and form micelles. The size of micelles produced is different for each detergent. Also, micelle size and CMC are related to the temperature and ionic strength. In terms of ionic detergents, the CMC will decrease when the salt concentration increases. But the CMC of nonionic detergents mainly depends on the increase of temperature (Helenius *et al*., 1975).

The cloud point, which means the micelle solution becomes cloudy at this temperature, can be attained by the variation of temperature or salt concentration. At this point, micelles will form large aggregates and cannot mix with water. So they separate from water and the solution becomes turbid. Then after centrifugation, the detergent-rich

phase may lie on top or below the aqueous phase (this can be also called the detergent-poor phase). Hydrophobic proteins are retained in the detergent phase and hydrophilic proteins are remained in the aqueous phase. Thus, through collecting different phases separately, we can obtain the hydrophobic fraction (detergent phase) which has the membrane proteins and achieve the purpose of enrichment.

Currently, there are various detergents available for phase separation studies. In this project, the detergent Triton X-114 was used. TX-114 is a classic detergent for membrane protein extraction. It was first described by Bordier in 1981 (Bordier, 1981). It is widely used because there is one phase when the solution is on ice and the cloud point can be easily reached at room temperature. This means we can solubilize the sample whilst on ice and then increase the temperature to separate the detergent-rich phase. Studies suggested that TX-114 is suitable for the enrichment of membrane proteins from various sample types (Pryde *et al*., 1986; Shetty et al., 2001; Elortza *et al*., 2003).

1.6 Proteomics

Proteomics is the large-scale study of protein structure, function, expression and diversity within a cell or tissue. The term “proteome” was first described by Marc Wilkins in the mid 1990’s (Wilkins, *et al*., 1996) and since then it has become one of the most advanced research areas in biological and biomedical disciplines. It defined as the total complement of proteins which are expressed by a genome (Bayés *et al*., 2009).

As a post-genomic realm, proteomics enable to improve our comprehension of biological processes substantially (Wilkins *et al*., 1997). Because proteins directly participate in biological processes, the analysis of proteome can provide us with a clear view of biological or pathophysiological changes and help us to address some current scientific bottlenecks.

- **Challenges of Proteomics**

Proteomics has become a large and complex scientific discipline. Within this area there are a number of unresolved and challenging technical problems. First, unlike the genome, in the proteome, the quantity and variety of proteins are highly variable and complex. For example, there are between 20,000 and 25,000 genes in the human genome [4]. These may encode a significantly more complex variety of proteins. Also, gene splicing and post-translational modifications can give rise to many more proteins. However, many proteins encoded by genes are poorly studied and understood (Shen *et al*., 2002). Moreover, the dynamic character of proteome, which means entire protein composition is affected by environmental stimuli, makes such samples highly variable. For instance, disease will lead to abnormal expression of some proteins. In addition, protein mixtures are complex and highly labile, which make them hard to deal with. At every step of experimentation in proteomics there is the risk of sample loss (Shen *et al*., 2002). Although problems exist, there is no doubt that proteomics has potential for helping us to achieve a better and more penetrating understanding of physiological conditions. For example, some proteins are “biomarkers” of certain diseases, which contributes to diagnosis and predicting responses to therapy (Rifai *et al*., 2006).

[4] International Human Genome Sequencing Consortium, Finishing the euchromatic sequence of the human genome, 2004, Nature, 431, 931-945

1.61 NanoLC-MS/MS

NanoLC tandem MS possesses many cutting edge techniques and it is quite popular in recent years.

Compared with other mass spectrometry techniques, nanoLC-MS/MS has unrivalled sensitivity. The ionization source is known as nanoESI (low flow electrospray ionization). More specifically, the spray needle is extremely small and closer to the entrance of mass spectrometer and the flow rate is very low (normally flow rates are controlled at tens to hundreds of nanoliters per minute). This design dramatically enhances the efficiency of ionization and thereby decreases the amount of sample needed. Moreover, nanoESI is more tolerant of impurities than higher flow rate ESI (Siuzdak , 2006).

Another important process is tandem MS (MS/MS). The character of this is that selected peptides are effectively analyzed twice in the mass analyzer. The mass of the peptides are firstly measured. Then, specific ions are selected for fragmentation. These ions are dissociated by a range of processes. A commonly used method is called collision-induced dissociation (CID). For each of the selected peptide, not only the mass can be measured, but also the precursor and fragment ions can yield structural information and peptide sequence. Even if two peptides have identical amino acid components, their different sequences will result in the different fragmentation patterns. Consequently, tandem MS is more precise than other techniques which only

measure the mass of a peptide.

In addition, typical biological samples are too complex to analyze directly, even in the current highly sensitive and sophisticated instruments. So prior to MS analysis, the molecules in these complex samples need to be fractionated. A separation technique called liquid chromatography (LC) can be combined with mass spectrometer. Other separation techniques such as gel electrophoresis can also be used to separate complex mixtures of proteins (Manade, 1999). However, LC has many benefits and it is often used together with other techniques. More specifically, first of all, unlike 2D gel, LC does not affect the detection of membrane proteins. Secondly, LC is easy to control and effectively simplify the complexity of samples. Also, it reduces the interference of impurities.

Generally speaking, nanoLC-MS/MS has many merits and can ensure higher sensitivity and accuracy. According to this project, many low abundant membrane proteins are major targets, so nanoLC-MS/MS is a suitable approach.

1.62 Protein mapping

The methodology called protein mapping relies on the application of mass spectrometry since this can measure the mass of peptides and peptide fragments, even the sequence of peptides can be determined. This means that all the measured

information can be compared with the information of the known protein sequences present in the public databases. A variety of database tools are used to search databases with MS data. Bioinformatics will generate a score list and indicate which protein in the database is the most similar to the experimental results. So we can identify the protein in the sample. Indeed, it is only a statistical result and may have errors if the matched peptides are too limited. However, it is a useful and popular technique that can compare differences of the proteome. In this project, all the protein results are obtained after NCBInr and UniProt database searching.

1.63 Gel-based method & Shotgun method

With the advance of modern analytical techniques, especially the application of mass spectrometry, the area of proteomics is brought onto a new stage. Currently, there are two major methodologies. One is the gel-based approach; the other is the so-called shotgun method.

In terms of gel-based methods, it means gel electrophoresis techniques are applied to separate complex protein mixtures before MS analysis. The target gel bands or entire gel lanes are cut into slices and digested by enzyme. The peptides are released from the gel slices into the buffer and it is delivered to the MS equipment. Typically, 2-D gel is often used because of its better separation ability. However, SDS-PAGE is more suitable for membrane protein than conventional two-dimensional gel electrophoresis. First of all, transmembrane proteins have hydrophobic regions which cannot be

resolved easily by isoelectric focusing even when detergents are included in the buffer (Wu *et al*., 2003). Secondly, proteins tend to precipitate at their isoelectric point. These drawbacks are disastrous for membrane proteins which are nearly always in lower abundance (Nilsson *et al*., 2000). Although improved 2-D gel methods for membrane protein separations have been developed, they are not so widely used (Brookes *et al*., 2002; Devreese *et al*., 2002). Consequently, due to the superior solubilizing power of detergent (SDS) and effective separation by molecular weight, the methodology of SDS-PAGE (1-D gel) is a preferred approach. However, some limits still exist. It is obvious that protein loss and contaminants cannot be ignored. Moreover, it is difficult to extract all peptides from the gel slices, which can affect protein identification.

The shotgun method (Nesvizhskii *et al*., 2005), provides another effective and powerful strategy. Without separating protein mixture by gel electrophoresis, samples are digested by protease directly and then analyzed by LC-MS/MS. These mixtures are extremely complex and sometimes it is hard to realize high-sequence coverage. So it is necessary to require more sensitive instrumentation like nanoLC-MS/MS. In addition, some researches use multidimensional LC separation to overcome the problem of high sample complexity (van Ling *et al*., 2007). Nevertheless, shotgun method has many obvious advantages and it cannot be replaced by others. The major benefits of this approach are high throughput and automatic control and it is widely used in most large scale proteomic studies.

These two research methodologies are commonly used and both of them have their own benefits, but neither of them can detect the whole proteome with the restriction

of current techniques (Figure 4). In this project, these two methods were used separately to analyze the samples. A more detailed interpretation of these two methods is found in the discussion section of this thesis.

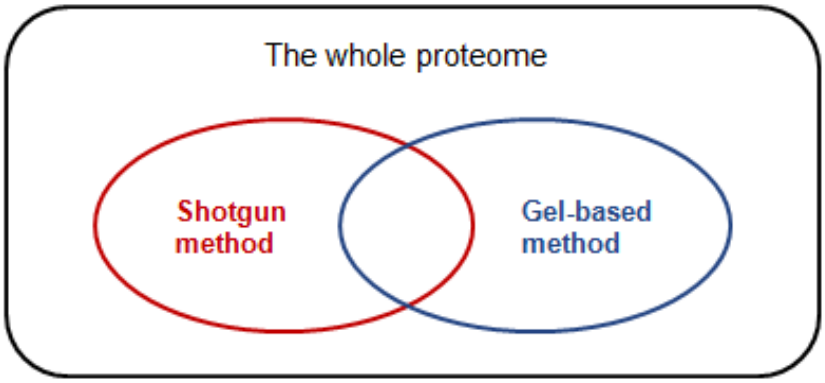

Figure 4: The relationship of the two methodologies

1.7 The aim of this study

There were three aims in this study: (i) to use detergent-based separation techniques to enrich membrane proteins from equine articular chondrocytes, (ii) through applying cutting edge proteomic techniques, such as nanoLC-MS/MS, and combining the shotgun and gel-based methods to identify the proteins in the extracted samples and then evaluate the efficacy of this extraction method, (iii) to identify the membrane proteins of the chondrocyte cell surface.

In addition, two kinds of cells were mentioned in this project, the equine chondrocytes and MCF-7 cells. As a kind of non-cartilaginous cells, the MCF-7 cells were used as a control group to test the ability of TX-114 extraction method.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.11 Buffers:

(1) Cell culture medium

- Liquid medium: Dulbecco's Modified Eagle's Medium (DMEM)/ LOW GLUCOSE (with 4mM L-Glutamine, 1000 mg/L Glucose, 110mg/L Sodium Pyruvate), *HyClone*
- Fetal Calf Serum (FCS): *Gibco*
- Cell culture medium: DMEM (above) supplemented with 10% FCS and 2% antibiotics (Penicillin and Streptomycin, *Gibco*)

(2) PBS: 1X Phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH2PO4) made up in dH_2O

(3) 1.5M Tris-HCL Buffer pH 8.8, *Bio-Rad*

(4) 0.5M Tris-HCL Buffer pH 6.8, *Bio-Rad*

(5) 10X TGS buffer (Tris/Glycine/SDS) (Electrophoresis grade, *Geneflow*): diluted to 1X in dH_2O when used

(6) Laemmli buffer (4X): 125mM Tris-HCL pH 6.8 (*Bio-Rad*), 4% SDS (*Bio-Rad*), 40% Glycerol (Analytical grade, *Fisher Scientific*), 0.02% Bromophenol Blue (*BDH*)

(7) Sample resuspension buffer: 4% SDS (*Bio-Rad*), 0.2M Tris pH 7.4 (*Bio-Rad*), 0.15M NaOH (Analytical grade, *Fisher Scientific*)

(8) PBS-T: 1X PBS which included 1% Tween 20 (*Fisher Scientific*)

(9) Transfer buffer: 10% (v/v) 10X TG buffer (Tris/Glycine, *Bio-Rad*) + 20% (v/v) Methanol +70% (v/v) dH_2O

2.12 Cultured cells

(1) Chondrocytes: primary cell cultures

Chondrocytes were isolated from articular cartilage from a 20-year-old female horse. The reason for euthanasia is unknown but the horse did not have osteoarthritis. The cell solutions were cryopreserved until used. Cryopreserved vials of equine chondrocytes were a gift from Miss J. Penny (School of Veterinary Medicine and Science).

(2) MCF-7 cells: a cell line

MCF-7 is the acronym of Michigan Cancer Foundation–7 (Soule *et al*., 1973). This cell line was developed from a human breast cancer tissue.

2.13 Antibodies:

Primary antibodies:

1. Anti-mouse ABCG2 antibody (BXP-21), *Thermo Scientific*

2. Anti-mouse Na, K-ATPase antibody (α6F), *DSHB*

Secondary antibody:

Anti-mouse/anti-rabbit labelled polymer HRP, *DakoCytomation*

2.2 Methods

The whole processes of this project were showed in Figure 6.

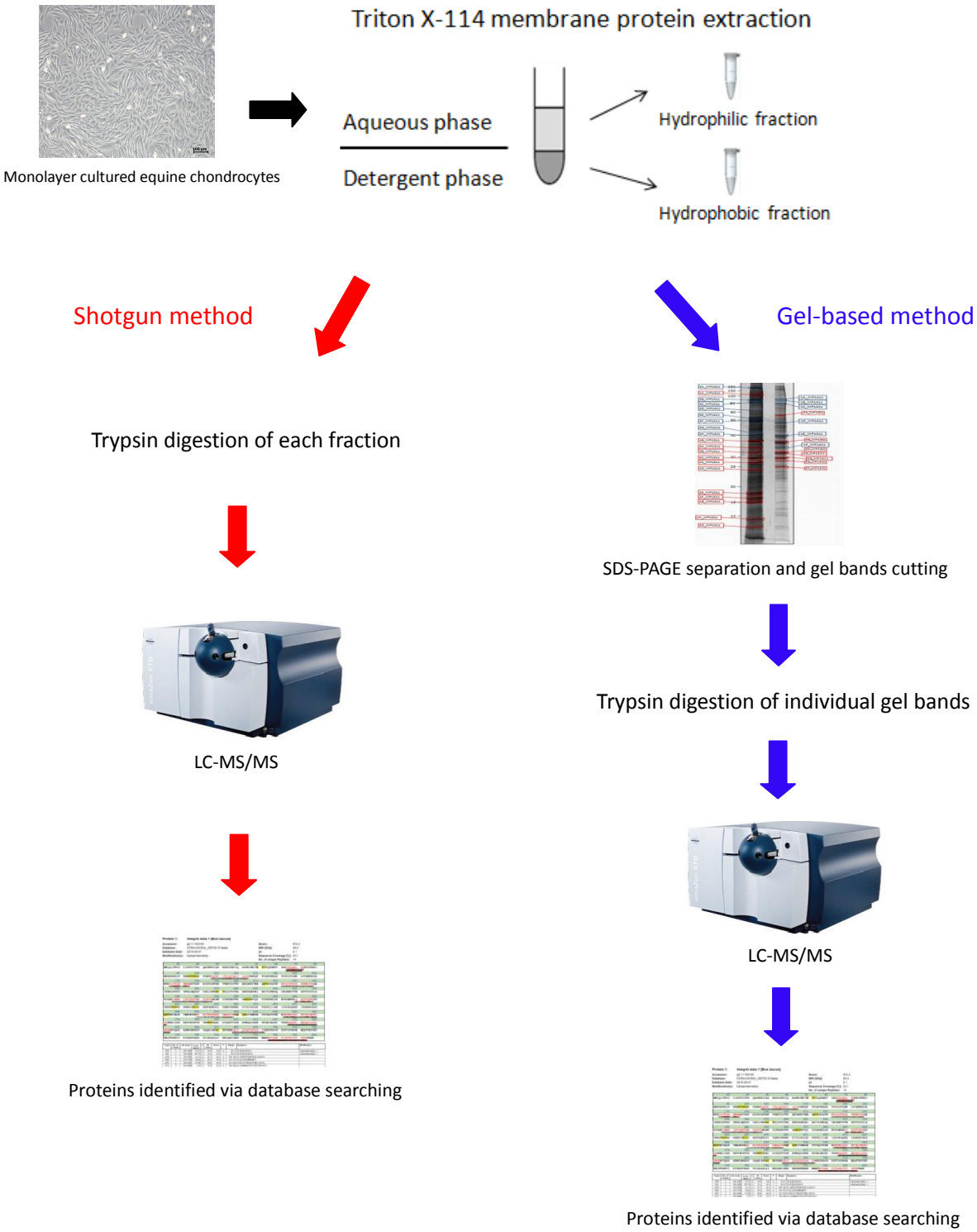

Figure 6: Schematic overview of the project

● Cell culture

Chondrocytes were cultured in DMEM medium (*HyClone*) supplemented with 10% FCS and 2% antibiotics. Most of the steps were performed in the hood except centrifugation and the water bath. The cells were first revived from freezer stocks and then passaged in T175 flasks. The cells were harvested when they had nearly reached confluence.

For cell revival, the vial was thawed in a 37°C water bath. The contents of the vial were removed to a 50 ml sterile falcon tube. 15 ml of pre-warmed media was added to the falcon tube. The cell sample was centrifuged at 1000 rpm (revolutions per minute) at room temperature for 5 minutes. The supernatant was discarded and the pellet was suspended in 15 ml of fresh medium. This cell suspension was dispensed into a T75 flask. The flask was kept in a 37°C/ 5% CO_2 incubator with a media change (10 ml) every two days. Typically, the cells were ready after 5 days.

The next step was cell passage. The medium was discarded and the flask was washed with 5ml of sterile PBS. After PBS was removed, 3 ml of Trypsin (diluted from 10X Trypsin, *Gibco*) was added. The flask was placed back into the incubator for 10 minutes. The trypsin suspension was pipetted up and down to wash the adhered surface 3 times and then removed to a new 50 ml sterile falcon tube. The tube was centrifuged at 1000 rpm for 10 minutes at room temperature after filled up to 50 ml with sterile PBS. The final pellet was suspended in 9 ml medium. Aliquots of 3 ml of this solution were

dispensed into three pre-warmed T175 flasks which contain 20 ml media in each one. Finally, the T175 flasks were incubated in the 37°C/ 5% CO_2 incubator with a media change (20ml each) each 2 days. Typically, the cells were ready after about 1.5 weeks.

● Triton X-114 extraction

Before protein extraction the chondrocytes were first washed in PBS, then 2 ml PBS containing 80 µl protease inhibitor cocktail (25X, *Sigma*) was added to each flask. The flask was placed on ice and a cell scrape (*Greiner bio-one*) was used to liberate the cells from the flask surface. The entire solution was collected and centrifuged (850 x g, 2 minutes, room temperature). The pellet was suspended in 600 µl PBS which included 24 µl of protease inhibitor cocktail (25X, *Sigma*). After incubating on ice for 15 minutes, the suspension was placed into the glass homogenizer and the cells were lysed. Triton X-114 (*Fluka*) was added to the solution to a final concentration of 0.75% and then the homogenizer was incubated on ice for 30 minutes with vortexing every 5 minutes. After centrifugation (30 minutes, 10000 x g, 4 °C) the supernatant was retained and incubated at 37 °C for 5 minutes and then on ice for 15 minutes. The sample was centrifuged (30 minutes, 10000 x g, 4 °C) again and the supernatant was incubated at 37 °C for 5 minutes. After centrifugation for 3 minutes (1000 x g, room temperature), two layers were appeared. The upper layer (aqueous phase) contained the hydrophilic proteins. The lower layer (detergent phase) contained hydrophobic proteins. To maximize the recovery of membrane proteins, the upper layer was extracted further. To do this, the upper layer was separated and added Triton X-114 to a final

concentration of 0.75%. After incubating for 30 minutes on ice with vortex every 5 minutes, the sample was centrifuged for 3 minutes (1000 x g, room temperature). After incubating at 37°C for 5 minutes, two layers were present again. Finally, the two bottom layers were combined together to constitute the hydrophobic fraction and the upper layer was treated as hydrophilic fraction. HPLC grade water was used to make two fractions have the same volume.

● Methanol and chloroform clean-up Method

The TX-114 detergent needed to be removed before the fractions could be used in the downstream analytical methods.

First, 4 times the sample volume of methanol (Analytical grade, *Fisher Chemical*) was added. For example, 400 µl methanol were added to 100 µl sample. After centrifugation (maximum speed, 10 seconds, room temperature), 2 times the original sample volume of chloroform (*Fluka*) were added. The mixture was centrifuged the same as before. After that, 3 times the sample volume of HPLC grade water were added and then it was centrifuged for 5 minutes (14000 rpm, room temperature). During centrifugation, two solution layers formed and the proteins were located at the interface between them. The upper layer was removed carefully and then 3 times the sample volume of methanol were added. The mixture was centrifuged for 5 minutes (14000 rpm, 4°C). The pellet was retained and air-dried for a few minutes.

● Protein assay

The Bio-Rad DC Protein Assay Kits was used to estimate the protein concentration in the sample. Protein standards were prepared using dilutions of BSA (2 µg/µl) which were prepared from a stock of Albumin solution at 2 mg/ml (*Thermo Scientific*). A series of dilutions of BSA (0.6 µg/µl, 0.3 µg/µl , 0.15 µg/µl , 0.075 µg/µl , 0.0375 µg/µl , 0.01875 µg/µl) were created using sample resuspension buffer and these dilutions were used to make the standard curve.

After clean up, the pellets were dissolved in sample resuspension buffer. Next, 5 µl of each standard or sample was placed into separate wells of a 96 well microtiter plate. Some reagents of Kits were added in each well. The plate was incubated at room temperature for 15 minutes. The plate was placed in a Bio-Rad Benchmark Microplate Reader. Using relevant software (Microplate Manager 5.2), the absorbance of the assayed samples at 655 nm was read. Using the series BSA concentrations and their absorbance readings, the standard curve was created by plotting the concentration of the protein (x-axis) against the absorbance readings (y-axis). Thus, the sample protein concentrations can be estimated.

● SDS-PAGE

(1) Mini-gel Format

Gel solutions were prepared according to Table 1, stock solution of 30% (w/v) acrylamide solution, which included 0.8% bis-acrylamide was used (*Geneflow*).

Materials	5% stacking gel	12% resolving gel
HPLC water	4.1 ml	4.9 ml
30% acrylamide solution	1.0 ml	6.0 ml
Tris-HCL, 1.5M, pH 8.8	-------	3.8 ml
Tris-HCL, 0.5M, pH 6.8	750 µl	-------
10% SDS	60 µl	150 µl

Table 1: Materials for preparing gel solutions for SDS-PAGE (Bio-Rad protean III Kit). These volumes were prepared for 2 mini-gels.

The Bio-Rad Protean III Kit was used. 10% Ammonium persulphate (APS) (electrophoresis grade, *Sigma*) was prepared freshly. After adding 150 µl 10% APS and 8 µl Tetramethylethylene-diamine (TEMED) (electrophoresis grade, *Sigma*), the resolving gel solution was injected between the glass plate sandwiches. When the solution was fully polymerized, 60 µl 10% APS and 8 µl TEMED ware added into stacking gel solution. Then stacking gel solution was poured up to the top of the resolving gel layer and combs to form the sample wells were inserted. The combs were left until gels had set.

After clean up, each pellet was dissolved in sample buffer (18 µl sample resuspension buffer + 4.8 µl Laemmli buffer (4X) + 1.2 µl 3M Dithiothreitol (DTT) (*Bio-Rad*)). After the gels were fully polymerized, the gel plate sandwiches were assembled in the gel running tank. The tank and every gel well were filled with 1X TGS buffer (see materials). Samples (24µl each) were loaded into wells. Each gel was run at 130 constant volts for approximately 80 minutes. The power was stopped when the bromophenol blue dye front arrived at the end of gel.

(2) Large Format Gels

This time the Bio-Rad Protean XI Kit was used. Gel solutions were prepared according to Table 2. 30% (w/v) acrylamide solution, which included 0.8% bis-acrylamide, was provided by Geneflow.

Materials	5% stacking gel	12% resolving gel
HPLC grade water	8.2 ml	13.12 ml
30% acrylamide solution	2 ml	16.68 ml
Tris-HCL, 1.5M, pH 8.8	-------	10 ml
Tris-HCL, 0.5M, pH 6.8	1.5 ml	-------
10% SDS	120 µl	200 µl

Table 2: Materials for preparing gel solutions for SDS-PAGE (Bio-Rad Protean XI Kit). These volumes were prepared for 2 gels.

200µl of freshly made 10% (w/v) APS and 13.2 µl TEMED were added to the resolving gel solution which was injected into the glass plate sandwich. When the gels were fully

polymerized, 120 µl fresh 10% APS and 10 µl TEMED were added to the stacking gel solution and it was poured up to the top of the resolving gel. Finally, the comb was inserted and left until the gel was polymerized.

After clean up, each protein pellet was dissolved in sample buffer (75 µl sample resuspension buffer + 20 µl Laemmli buffer (4X) + 5 µl 3M DTT). Once the gel polymerization was completed, gel sandwich assemblies were inserted into the electrode assembly and then placed into the gel running tank. The gel chambers and tank were filled with 1X TGS buffer subsequently. Samples (total volume was 100 µl each) were loaded into chosen wells. Gels were run initially at 32 mA constant current. When the dye front reached the bottom of the stacking gel, the current was increased to 45 mA. The gel running was continued approximately 3 hours. The power was switched off when the blue dye front reached the bottom of gel.

- Coomassie blue staining

Each gel was washed in a clean tray with enough distilled water to completely cover it for 3 × 5 minutes. Then, the tray was filled with enough stain solution (Imperial™ Protein Stain, *Thermo Scientific*) to cover the gel and left on the shaker overnight. Gels were rinsed with distilled water until the background was clear.

● Silver staining

(1) Mini-gels

When the gel finished running, it is put into the silver stain machine. The Hoefer Processor Plus automated gel stainer (*Amersham Pharmacia Biotech*) was used. The solutions and procedure followed a mass spectrometry compatible silver staining procedure (Electrophoresis 2000: 21, 3666-3672). All the solutions were made freshly. The chemicals and running time of each step are described in Table 3.

Step	Function	Time (mins)	Composition of Solution (each solution is made up to a final volume of 225 ml with 18 megaohm water)	Final concentration
1	Fix	15	90 ml Methanol 22 ml Acetic Acid	40% Methanol 10% Acetic Acid
2	Fix	15	90 ml Methanol 22 ml Acetic Acid	40% Methanol 10% Acetic Acid
3	Sensitise	30	9 ml 5% STS* 15.3 g Sodium Acetate 67.5 ml Methanol	0.2% (w/v) STS* 68g/L Sodium Acetate 30% Methanol
4-6	wash	3×5	18 megaohm water	------
7	Silver	20	562 mg $AgNO_3$	0.25% $AgNO_3$, 2.5 g/L
8-9	wash	2×1	18 megaohm water	------
10	Develop	Variable	5.6 g Na_2CO_3	25 g/L Na_2CO_3

		(Typically 2 min)	12.1 µl 5% STS* 90 µl Formaldehyde[£]	0.00027% w/v STS 0.014% (v/v) Formaldehyde[£]
11	Stop	10	3.3 g EDTA	14.6 g/L EDTA
12-13	Wash	3×5	18 megaohm water	------

Table 3: Silver Staining solutions and incubation times. 18 megaohm water was used for all solutions. *STS: 5% (w/v) STS is made by dissolving 2 g sodium thiosulphate ($Na_2S_2O_3$. $5H_2O$) in 40 ml water (18 megaohm water). [£]The standard solution of formaldehyde (HCHO) is 36%.

(2) Large Gels

The same solutions and procedure were used as for mini-gels, but the larger volumes (425 ml) were required.

- Preparation and trypsin digestion of hydrophobic and hydrophilic solution samples for LC-MS/MS analysis

(1) In-solution Digestion, for Shotgun Method

20 µg protein of each fraction (hydrophobic and hydrophilic) were used. The pellets from the methanol & chloroform clean-up method were suspended in a solution of 50 mM ammonium bicarbonate (AMBIC) (*Sigma*) and 10mM Dithiothreitol (DTT) (*Bio-Rad*) and incubated at 37°C for 30 minutes. During this time the samples were vortexed every 10 minutes. Iodoacetamide (IAA) (*Bio-Rad*) was added in the supernatant to a

final concentration of 55 mM. Samples were incubated at 37°C for a further 45 minutes in dark environment. Following this, 1.2 ml of acetone (chilled at -20°C) was added to each sample. After mixing well, the samples were incubated at 4°C overnight.

Protein precipitates were pelleted by centrifugation at 15000 x g for 5 minutes at 4°C. Pellets were air-dried for 1 minute. Pellets were suspended in 20 µl trypsin buffer, which included 50 mM AMBIC and 10 ng/µl Trypsin Gold (MS Grade, *Promega*). Samples were vortexed until the pellets were fully suspended and then incubated at 37°C for 16 hours. Finally, 1 µl formic acid (1%) was added to each sample to stop the reaction. The samples were stored at -80°C until use. The final protein concentration of the samples was 1 µg/µl.

(2) In-gel Digestion, for Gel-based method

In this project, the shotgun method was applied as the major procedure. Gel-based method acted as a complementary method, so only clear bands were sliced from the gel instead of cutting the entire gel. Protein bands were excised from a large format 1D gel. Each band was cut into small pieces and each sample was placed into an individual well of a microtitre plate. According to the stain intensity, every selected band was given a rank. “SS” stood for super strong level of staining, “S” stood for strong level, “M” stood for medium level, “L” meant low level. These descriptions were recorded in order to ensure low abundant samples could be injected more into the LC-MS/MS. The samples in the plate were processed using the MassPREP STATION Robotic Protein Handling System.

In brief, the steps were as follows. The Coomassie blue stained gel pieces were incubated in a de-stain solution of 50 mM AMBIC and 50% (v/v) acetonitrile. The silver stained gel pieces were incubated in a solution of potassium ferricyanide/ sodium thiosulphate. After removal of de-stain solutions, the samples were incubated in 50 µl acetonitrile for 5 minutes in order to cause dehydration. The acetonitrile was removed and gel pieces were dried for 10 minutes. 50 µl reducing solution (10 mM DTT, 100 mM AMBIC) was injected into each well and samples were incubated in this solution for 30 minutes. In the alkylation step, the gel pieces were incubated in a solution of 55 mM IAA and 100mM AMBIC. After that, samples were washed in 50 µl 100 mM AMBIC for 10 minutes and then 50 µl acetonitrile for 5 minutes. For the digestion step, 25 µl trypsin digestion buffer (10 ng/µl trypsin gold (*Promega*) in 50 mM AMBIC) was added into each well. Samples were incubated at 6°C for 30 minutes and then at 40°C for 5 hours. Finally, samples were incubated in 30 µl of 0.1% (v/v) Formic acid for 30 minutes. After that, the liquid which contained the peptides was removed into a new eppendorf and frozen until MS analysis.

● LC-MS/MS analysis

(1) Shotgun samples

Digested samples were defrosted at room temperature. The sample was injected onto a 15 cm C18 Pepmap column using a Bruker Easy-nanoLC chromatography platform with a flow rate of 300nl/min. Each sample was made up to 0.1% Formic acid and loaded. With the help of an auto-sampler, 3 µl of each sample was injected into the HPLC column. After peptide binding and washing processes on the column, the

complex peptides mixture was separated and eluted by a gradient of solution B (100% ACN, 0.1% Formic acid) over 115 minutes, followed by column washing and re-equilibration.

The peptides were delivered to a Bruker amaZon ETD ion trap instrument. Selected peptides were analyzed in two steps by MS/MS. The top 5 most abundant peptides from each MS scan were collected for fragmentation. Processed data were compiled into files that were submitted to the MASCOT search engine and compared to entries in the NCBInr databases. Some data searching parameters were: mammals, trypsin, and 2 missed cleavages.

(2) Gel samples

Different steps were as follows. The samples were diluted 1: 5 with 0.1% formic acid. According to the descriptions of stain intensity, different volumes were injected into the column. The one which described “SS” was 1 µl, “S” was injected 2 µl, “M” was 3µl, and “L” was 5 µl. Peptides were eluted by a gradient of solution B for 30 minutes. A top 3 MS/MS method was used in these samples. All other steps were the same as shotgun samples.

● Western blotting validation of selected membrane proteins

Hydrophobic and hydrophilic protein samples were loaded onto mini gels. After the gel running finished, it was equilibrated in transfer buffer. The PVDF membrane (Immun-

Blot™ PVDF Membrane, Bio-Rad) was pre-soaked in methanol and transfer buffer (see materials). Similarly, filter papers (BBS 50 Filter Blotter Paper, Web scientific) and fiber pads were also pre-soaked in transfer buffer. The Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) was used. To make up a "blotting sandwich", fiber pads, filter papers, membrane and gel were assembled in gel holder cassette in a specific order so that the proteins migrated from the gel onto the membrane. The sandwich was placed into the gel tank. The tank was filled with transfer buffer and then was run at a constant voltage (80 V) for two hours.

Once transfer was completed, the membrane was incubated in a protein blocking solution (PBS-Tween which included 5% nonfat dried milk powder (*Marvel*)) for one hour at room temperature. Membranes were incubated with primary antibodies (anti-mouse ABCG2 was diluted 1:2000, anti-mouse Na, K-ATPase was diluted 1:100) in blocking solution at 4°C overnight, with gentle rotation. Membrane was incubated with secondary antibody (anti-mouse & anti-rabbit labelled polymer HRP, *DakoCytomation*, 1:1000 dilution) in blocking solution at room temperature for one hour.

Chemiluminescence was used to detect the target protein with ECL plus Western Blotting Detection System (*Amersham Biosciences*). The membrane was covered with substrate solution and incubated for 5 minutes. In the dark room, the auto-radiographic film (Hyperfilm, *Amersham Biosciences*) was laid on the membrane for appropriate time. Film was developed, rinsed and fixed. An image of the film was acquired by scanning on a calibrated densitometer (*Bio-Rad* GS800) which was operated using Quanitity One-4.4.1 software (*Bio-Rad*).

Chapter 3

RESULTS

3.1 Cell culture

Chondrocytes reached confluent growth in T175 flasks after approximately 1-1.5 weeks (Figure 7). Each flask contained 5.25-8.75 x 10^6 cells when the cultures became confluent.

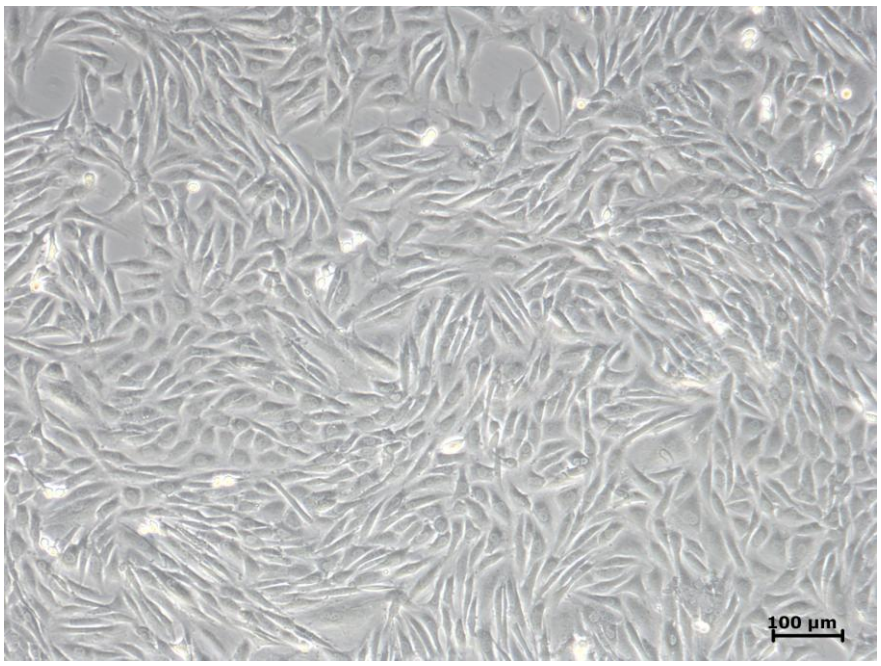

Figure 7: A monolayer of cultured chondrocytes in a T175 flask. After about 1 week of culture these cells were near confluent. These cells stood for those used in Triton X-114 extraction which means all the proteins were separated into hydrophobic and hydrophilic fractions.

3.2 Assessment of Triton X-114 capability

- **SDS-PAGE**

The purpose of separating the protein preparations on SDS-PAGE was to test the ability of TX114 method. After protein assay, the concentrations of hydrophobic and hydrophilic fraction were about 0.18 µg/µl and 0.84 µg/µl separately. According to

these concentrations, the hydrophilic sample contained about 5 times as much protein as the hydrophobic fraction. Therefore, the volumes of samples used in this gel experiment were 150 µl and 30 µl (hydrophobic/hydrophilic) (Figure 8). The purpose was to make sure each gel lane had similar amount of protein (about 25-27 µg here) so that the overall protein profiles could be compared easily. Also, the high sensitivity of silver stain was the reason that it was used instead of Coomassie stain. In the resultant gel (Figure 8) the red arrows indicated the obvious bands which were present uniquely in the hydrophobic fraction. Therefore such bands in hydrophobic lane might contain many membrane proteins. Additionally, each band did not stand for only one kinds of protein. In all, this result was satisfactory and membrane proteins were separated well with the TX114 method.

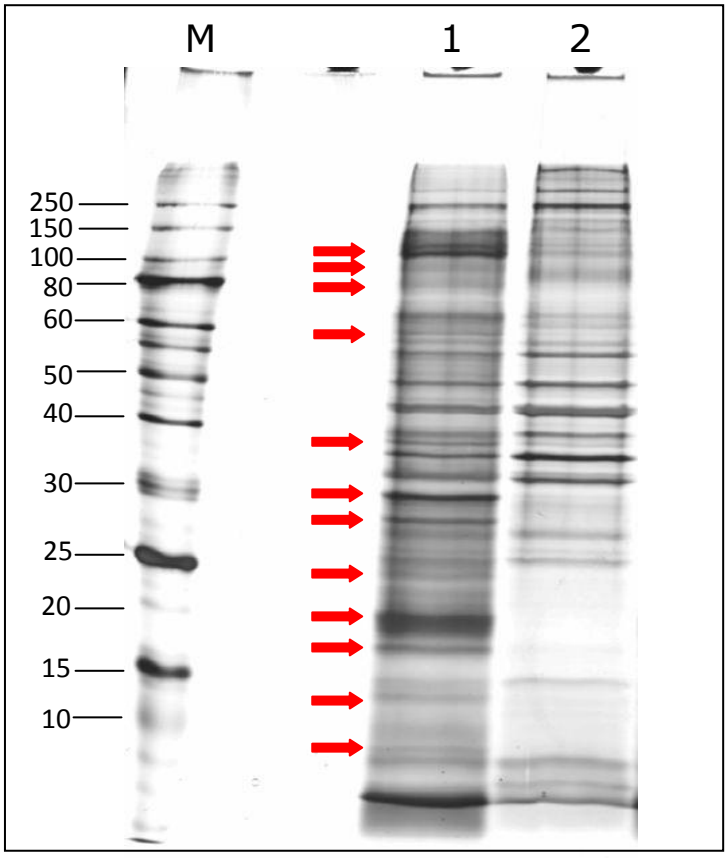

Figure 8: SDS-PAGE mini-gel analysis for two fractions from TX-114 extraction. M is the unstained molecular weight marker. 1 is hydrophobic fraction (about 27 µg proteins). 2 is hydrophilic fraction (about 25.2 µg proteins). Silver stain was used and the red arrows show the obvious hydrophobic bands which were different with hydrophilic ones.

- **Western blotting**

Two western blotting experiments were used to confirm the effectiveness of TX114 extraction through detecting specific membrane proteins. Two different antibodies and two kinds of cells (MCF-7 cells and primary chondrocytes) were used. Here, the MCF-7 cells are non-cartilaginous cells and they were used as a control group to test the ability of TX-114 extraction method.

(1) MCF-7 cells

MCF-7 cells (Michigan Cancer Foundation – 7), is a human breast cancer cell line, which has a high level of a 70 kDa membrane transporter called ABCG2 in the cell membrane. Similar amount proteins of hydrophobic and hydrophilic fractions were probed with an antibody to ABCG2. The resulting blot was showed in Figure 9. There was an extremely strong signal between 60 and 80 kDa in size in the hydrophobic lane. Nevertheless, the corresponding signal in hydrophilic fraction was very weak. Thus, the majority of the ABCG2 protein was extracted into the hydrophobic fraction.

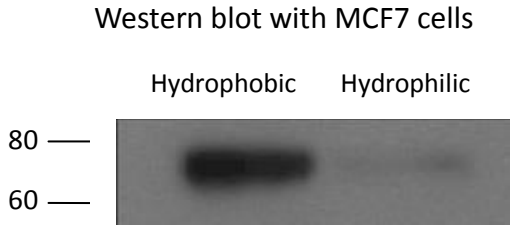

Figure 9: Western blot for two fractions of MCF7 cells using TX114 extraction. Hydrophobic fraction (100µl samples, about 25 µg proteins) and hydrophilic fraction (25µl sample, about 26 µg proteins) were compared. Primary antibody (anti-ABCG2) was diluted 1:2000 and secondary antibody was in 1:1000 dilutions. ECL plus kit was used and the film was developed in dark room for 15 minutes.

(2) Chondrocytes

Chondrocyte hydrophobic and hydrophilic fractions were probed in a separate experiment. In this case, the target protein was Na-K ATPase, which is a highly abundant membrane protein. In Figure 10, the signal in hydrophobic lane was much stronger than that in hydrophilic lane, which also demonstrated that the majority of this membrane protein was extracted into the hydrophobic fraction.

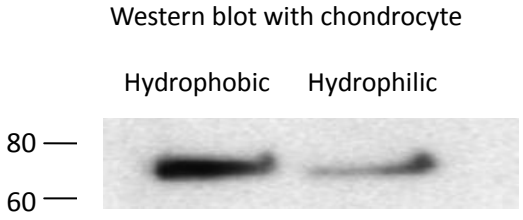

Figure 10: Western blot for two fractions of chondrocytes using TX114 extraction. Hydrophobic fraction (150 µl samples, about 7.2 µg proteins) and hydrophilic fraction (30µl sample, about 7.8 µg proteins) were compared. Primary antibody (anti-Na,K-ATPase) was diluted 1 :100. Second antibody was in 1:1000 dilutions. ECL plus kit was used and the film was developed 20 minutes.

3.3 Excising protein bands for gel-based method

A large format gel was used which loaded 200 µl sample of each fraction. As a result, hydrophobic lane was too light to select bands after Coomassie stain (Figure 11), because the sample amount were not enough. Then the left part of the gel which included hydrophobic lane and marker lane was cut off and it was re-stained with silver. It was evident that more protein bands were visualized in large gel than mini one (Figure 12). Selected protein bands were excised for trypsin digestion (Figure 13).

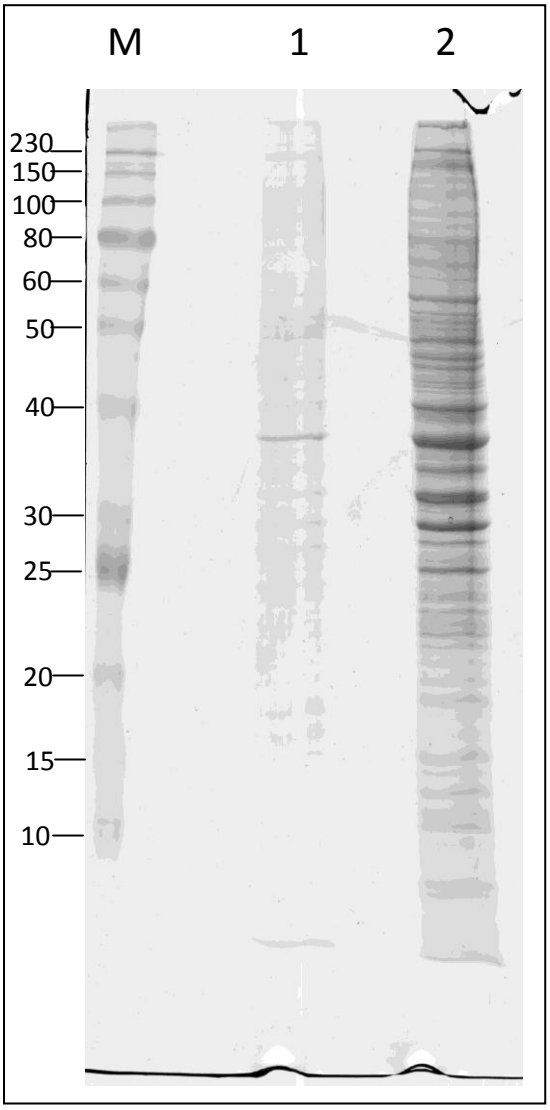

Figure 11: Large gel analysis for two fractions of chondrocyte TX114 extraction stained with Coomassie blue. Lane M was marker. Lane 1 was hydrophobic fraction. Lane 2 was hydrophilic fraction. Each of them was loaded with 200µl sample.

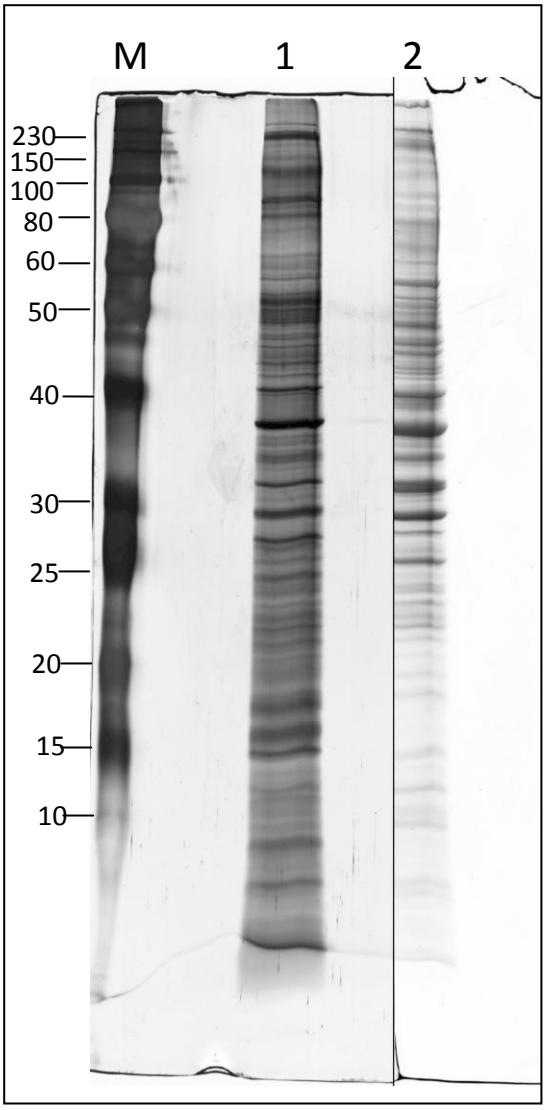

Figure 12: The same gel as shown in figure 10 was shown after silver staining of lanes M and 1 to visualize the hydrophobic proteins more clearly. Lane M was marker. Lane 1 was hydrophobic fraction. Lane 2 was hydrophilic fraction (Coomassie blue stained only). Both hydrophobic and hydrophilic lanes were loaded with 200µl sample

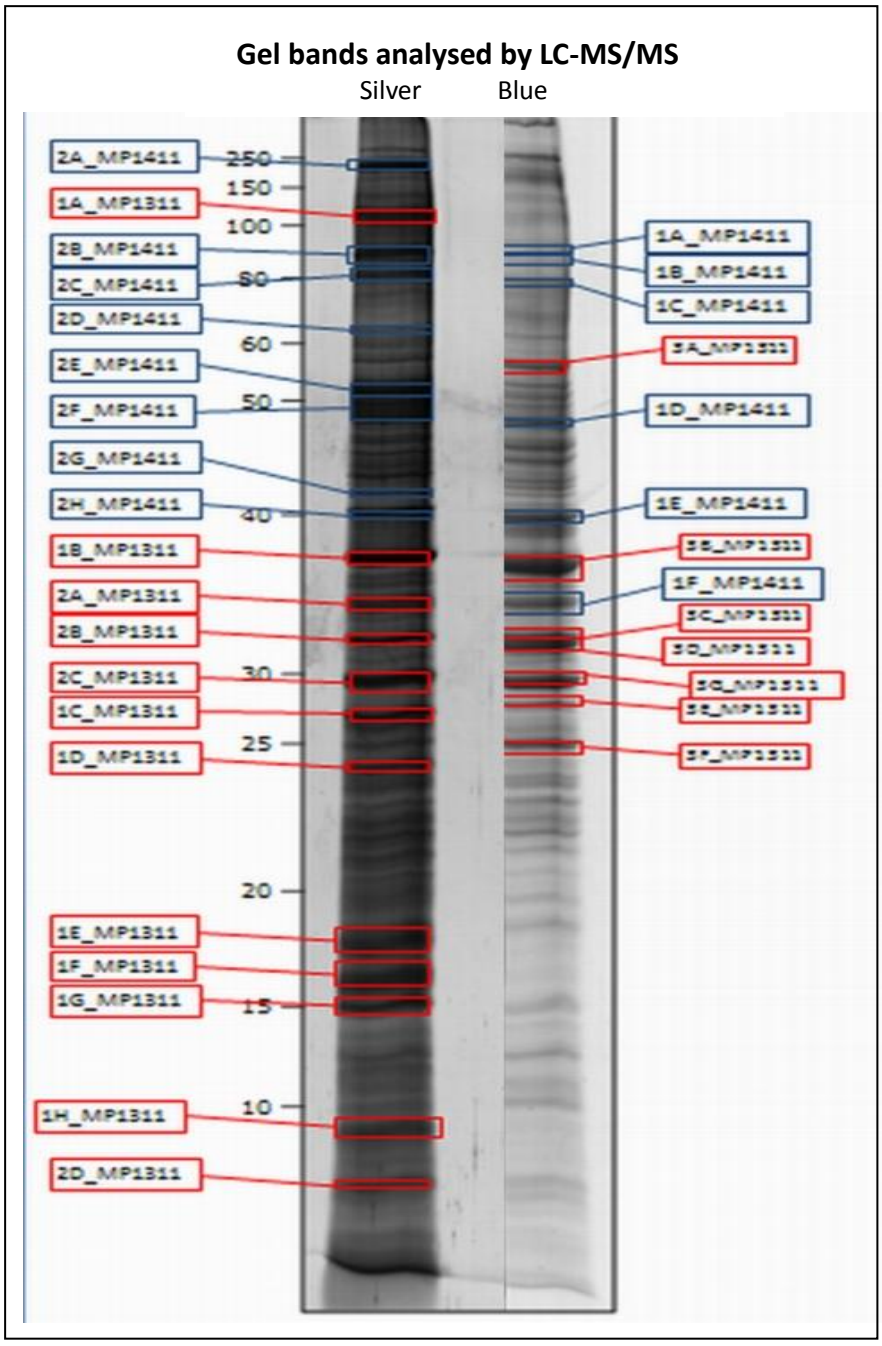

Figure 13: The protein bands excised are shown. Different colours of the text boxes presented two microtitre plates. The red one was MP1311 and blue one is MP1411. Each band was named individually according to the name of wells in plate.

3.4 Protein identification results

(1) LC-MS/MS results of in-solution digestion samples (shotgun method)

After NCBInr and UniProt database searching, the details of identified proteins are shown in the tables 4 & 5 in appendix 1. Table 4 lists all the proteins which were identified in the Triton-X114 hydrophobic fraction. Table 5 displays the proteins in hydrophilic fraction. Admittedly, some proteins were not identified well. For example, some were unnamed proteins. So these data were excluded in all the tables.

(2) LC-MS/MS results of in-gel digestion samples (gel-based method)

After NCBInr and UniProt database searching, the details of identified proteins are shown in Tables 6 & 7 in appendix 2. Table 6 details the proteins from the hydrophobic bands which were cut from silver stained lane. However, B2, C2, D2 bands in MP1311 plate and A2, B2, C2, E2, F2, H2 bands in MP1411 plate did not have any identified proteins. May be the peptides were not efficiently extracted from the gel, or insufficient samples was loaded for LC-MS/MS. Table 7 shows the identified proteins from hydrophilic bands which cut from the Coomassie blue stained lane. Also, there were also some proteins which were not identified well, like unnamed protein. Although the number was much less than shotgun method, such data were not included in tables.

3.5 Summary of the cellular distributions of hydrophobic and hydrophilic fraction proteins

In the shotgun method, a total of 72 proteins were indentified in the hydrophobic fraction and 90 proteins in the hydrophilic fraction. More specifically, 35 proteins were membrane proteins in hydrophobic fraction, which accounted for about 49%. But this percentage in hydrophilic fraction was only about 13% (12 proteins). On the other hand, fewer proteins were identified in the gel-based method. In total, 26 and 70 proteins were indentified in hydrophobic and hydrophilic bands respectively. However, the percentage of membrane proteins was similar to that found using the shotgun method. About 42% (11 proteins) of the proteins identified from the hydrophobic bands were membrane proteins and this proportion was about 21% (15 proteins) in hydrophilic ones. To evaluate the TX-114 extraction, it is necessary to compare the proportion of membrane proteins in each fraction. Figures 14 & 15 show the distribution of hydrophobic and hydrophilic proteins identified in the shotgun method. Figures 16 & 17 show the distribution of two fraction proteins identified in the gel-based method.

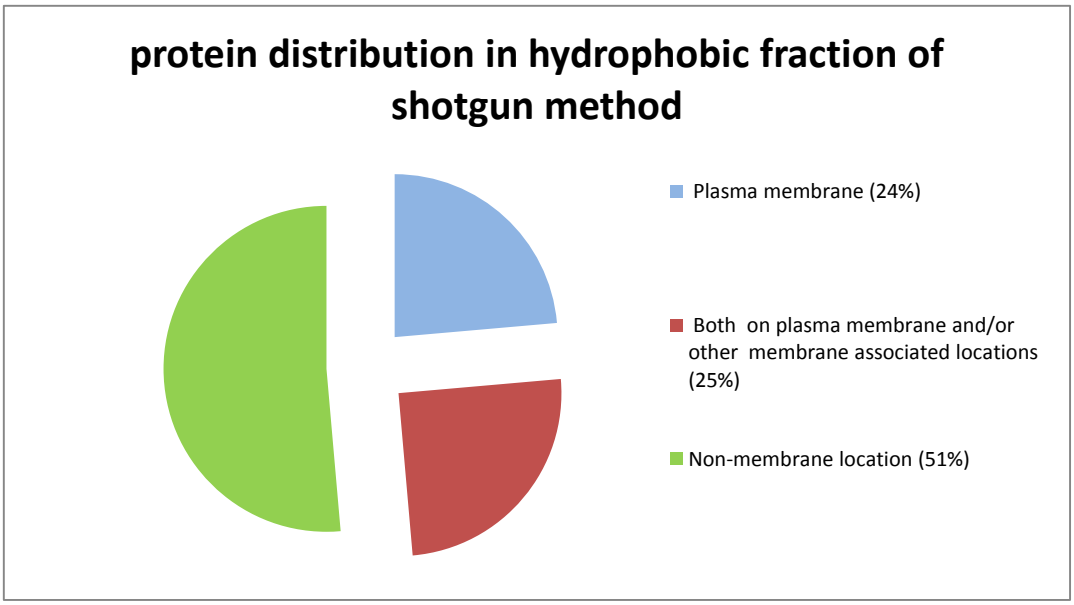

Figure 14: The distribution of protein locations in hydrophobic fraction in the shotgun method is shown. In total 72 proteins, there were 17 proteins which are known to be located only on the plasma membrane. 18 proteins were found that are known to be located both on the plasma membrane and/or other membrane associated locations. 37 proteins are non-membrane locations.

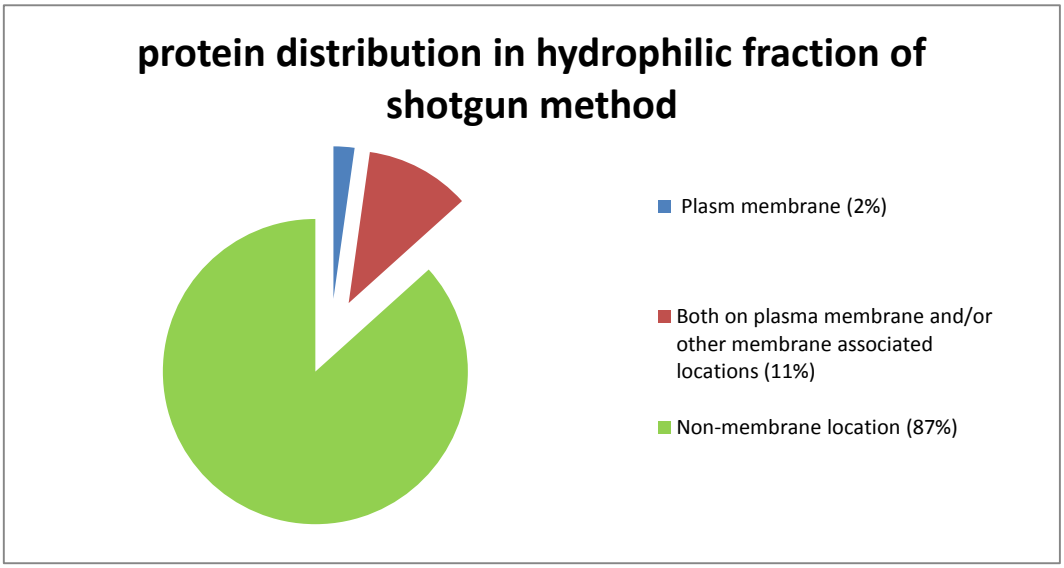

Figure 15: The distribution of protein locations in hydrophilic fraction in the shotgun method is shown. In total 90 proteins were detected, there are 2 proteins which are only located on the plasma membrane. 10 proteins are located both on the plasma membrane and/or other membrane associated locations. The majority of the hydrophilic proteins, 78 proteins, were found in non-membrane locations.

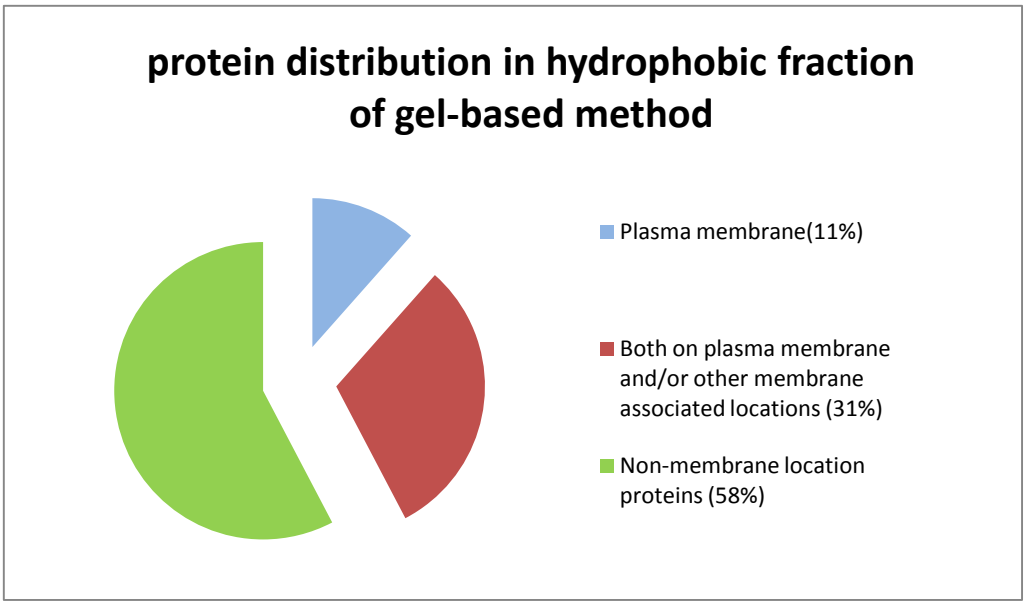

Figure 16: The distribution of protein locations in hydrophobic fraction in gel-based method is showed. In total 26 proteins, there are 3 proteins which are only located on the plasma membrane. 8 proteins are located both on the plasma membrane and/or other membrane associated locations. 15 proteins are non-membrane locations.

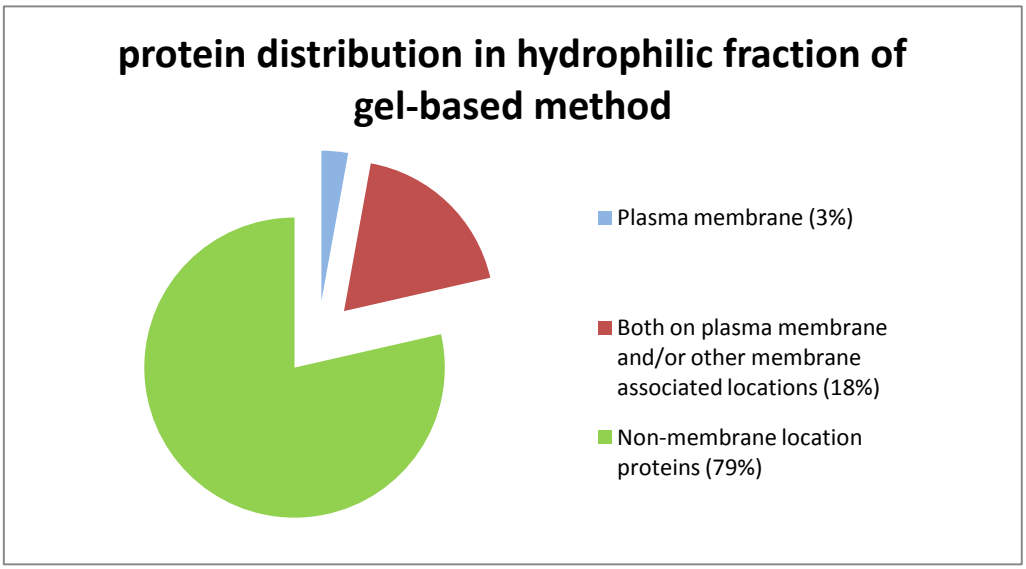

Figure 17: The distribution of protein locations in hydrophilic fraction in gel-based method is shown. In total 70 proteins were identified, there are 2 proteins which are only located on the plasma membrane. 13 proteins are located both on the plasma membrane and/or other membrane associated locations. 55 proteins are non-membrane location.

3.6 A summary of all the membrane proteins identified in the two analysis methods

The main purpose of this project was to identify the membrane proteins of chondrocyte. Combining the two methods, 52 membrane proteins were identified. They are divided into two groups. One group is the membrane proteins which are only located on the plasma membrane (18 proteins). The other is the membrane proteins which are located both on the plasma membrane and/or other membrane associated locations, like organelle membrane (34 proteins). Table 9 lists these two groups of proteins. In addition, Figure 18 describes a functional summarization of these 52 membrane proteins.

In terms of transmembrane proteins, there are 6 multi-pass membrane proteins, which account for about 11.5% of all 52 membrane proteins. Also, there are 12 single-pass membrane proteins, which account for about 23% of all identified membrane proteins. For other membrane proteins, they are peripheral membrane proteins or other membrane associated proteins. Table 8 lists the names of these transmembrane proteins.

Multi-pass membrane proteins	CD151 CD9 GP106 CD81 Ribophorin-2 CD63
Single-pass membrane proteins	integrin beta 1, CD44, integral membrane serine protease Seprase, calnexin, mannose-6-phosphate receptor, vesicle-associated membrane protein 3, LAMP 2, Thioredoxin-related transmembrane protein, caveolin 1, 21 kDa transmembrane-trafficking protein , ribophorin I Translocon-associated protein subunit delta,

Table 8: The names of all transmembrane proteins

name	Mascot Score	Accession	Sequence Coverage [%]	No. of matched peptides	location	Molecular function	Biological process
Membrane proteins are only located on the plasma membrane							
heat shock 70kD protein 5	569.6	gi|25742763	22.3	12	Plasma membrane	1.ATP binding 2.ribosome binding	1.ER overload response 2.negative regulation of transforming growth factor beta receptor signaling pathway
keratin 1	395.1	gi|119395750	14.9	8	Plasma membrane	1. protein binding 2. receptor activity 3. structural constituent of cytoskeleton 4. sugar binding	1. complement activation, lectin pathway 2. epidermis development 3. fibrinolysis 4. regulation of angiogenesis 5. response to oxidative stress
integrin beta 1 (CD29)	365.8	gi|114326428	18.9	1	Plasma membrane	1. identical protein binding 2. protein heterodimerization activity 3. receptor activity	1. Cell adhesion 2. Host-virus interaction
ras-related GTP-binding protein RAB10	162.2	gi|33695095	16.5	1	Plasma membrane	1. GTP binding 2. protein binding	1. protein transport 2. small GTPase mediated signal transduction
Chain E, Structure Of Bovine Heart Cytochrome C Oxidase At The Fully Oxidized State	156.3	gi|1942990	43.1	4	Plasma membrane	electron carrier activity	Oxidation reduction
5' nucleotidase, ecto isoform 2	155.2	gi|109071913	11.5	4	Plasma membrane	1. 5'-nucleotidase activity 2. nucleotide binding	1. AMP catabolic process 2. adenosine biosynthetic process 3. negative regulation of inflammatory response
sperm-membrane associated protein P47	145.6	gi|3421015	11.6	4	Plasma membrane		cell adhesion
CD44 antigen (Phagocytic glycoprotein I)	144.2	gi|461713	11.7	4	Plasma membrane	1. hyaluronic acid binding 2. receptor activity	cell adhesion
CD73, isoform CRA_b	137.4	gi|119569020	11.5	4	Plasma membrane	1. 5'-nucleotidase activity 2. nucleotide binding	1. DNA metabolic process 2. purine base metabolic process 3. pyrimidine base metabolic process 4. pyrimidine nucleoside catabolic process
rap1a protein	120.1	gi|56118668	20.7	3	Plasma membrane	1. GTP binding 2. GTPase activity	small GTPase mediated signal transduction
CD151 antigen	117.6	gi|74136205	12.6	4	Plasma membrane	protein binding	1. cell adhesion 2. hemidesmosome assembly
alpha-soluble NSF attachment protein - rat	108.7	gi|2143586	12.9	3	Plasma membrane		1.cellular membrane fusion 2.intra-Golgi vesicle-mediated transport 3.post-Golgi vesicle-mediated transport
Brain acid soluble protein 1 （BASP1）	98.0	gi|109076755	11.9	2	Plasma membrane	1. protein domain specific binding 2. transcription corepressor activity	1. glomerular visceral epithelial cell differentiation 2. negative regulation of transcription, DNA-dependent

CD9 antigen (p24) (Leukocyte antigen MIC3) (Motility-related protein) (MRP-1) (Tetraspanin-29)	77.9	gi|73997761	15.5	2	Plasma membrane	1. platelet activation and aggregation. 2. regulates paranodal junction formation. 3. cell adhesion, cell motility and tumor metastasis. 4. sperm-egg fusion	1. Cell adhesion 2. Fertilization
MGC81440 protein	76.9	gi|46249590	11.5	2	Plasma membrane		
integral membrane serine protease Seprase	70.6	gi|119887629	4.2	2	Plasma membrane	1. Hydrolase 2. Protease 3. Serine protease	1. endothelial cell migration 2. negative regulation of extracellular matrix disassembly 3. proteolysis
Receptor expression-enhancing protein 5 (Polyposis locus protein 1 homolog) (TB2 protein homolog) (GP106)	45.5	gi|2498308	4.9	1	Plasma membrane	protein binding	
CD81 antigen	43.2	gi|13399775	22.2	1	Plasma membrane	protein binding	Host-virus interaction
Membrane proteins are located both on the plasma membrane and/or other membrane associated locations							
vinculin	2012.6	gi|47522618	47.6	47	1. Plasma membrane 2.Cytoplasm	1.actin binding 2.structural molecule activity	1.lamellipodium assembly 2.regulation of cell migration
actinin alpha 1 isoform b	1102.4	gi|94982457	35.2	27	1. Plasma membrane 2.Cytoplasm	1.actin binding 2.calcium ion binding 3.integrin binding 4.vinculin binding	1.focal adhesion assembly 2.negative regulation of cellular component movement 3.platelet activation 4.platelet degranulation 5.regulation of apoptosis

Aspartate aminotransferase, mitochondrial (Transaminase A)	983.3	gi|112982	51.6	19	1. Plasma membrane 2.Mitochondrion	1.L-aspartate:2-oxoglutarate aminotransferase activity 2.protein binding 3.pyridoxal phosphate binding	1.Lipid transport 2.Transport
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	612.4	gi|1169794	37.8	16	1. Plasma membrane 2.Cytoplasm 3.Nucleus	1.Oxidoreductase 2.Transferase	1.Apoptosis 2.Glycolysis
mitochondrial ATP synthase, H+ transporting F1 complex beta subunit (ATP synthase subunit beta, mitochondrial)	579.5	gi|89574051	39.1	13	Mitochondrion membrane	1.ATP binding 2.MHC class I protein binding 3.eukaryotic cell surface binding 4.hydrogen ion transporting ATP synthase activity, rotational mechanism 5.hydrogen-exporting ATPase activity, phosphorylative mechanism 6.proton-transporting ATPase activity, rotational mechanism	1.ATP hydrolysis coupled proton transport 2.angiogenesis 3.regulation of intracellular pH 4.respiratory electron transport chain
hexokinase 1 isoform 2	503.9	gi|114630829	12.8	13	Mitochondrion membrane	1.ATP binding 2.glucokinase activity	1.glucose transport 2.glycolysis 3.transmembrane transport
prolyl 4-hydroxylase, beta subunit	424.3	gi|73964749	19.6	1	1.Plasma membrane 2.Endoplasmic reticulum	1.electron carrier activity 2.procollagen-proline 4-dioxygenase activity 3.protein disulfide isomerase activity 4.protein disulfide oxidoreductase activity	1.cell redox homeostasis 2.glycerol ether metabolic process 3.lipid metabolic process 4.lipoprotein metabolic process 5.peptidyl-proline hydroxylation to 4-hydroxy-L-proline
Transgelin-2 (KIAA0120)	453.6	gi|40788953	47.5	9	1.Plasma membrane 2.Nuclear membrane	protein binding	Muscle organ development

Voltage-dependent anion-selective channel protein 1 (VDAC-1)	428.2	gi|4507879	32.9	8	1.Mitochondrion outer membrane 2.Plasma membrane	1. porin activity 2. protein binding 3. voltage-gated anion channel activity	1. Apoptosis 2. Host-virus interaction 3. Ion transport 4. Transport
calnexin	409.8	gi|109080088	18.1	10	Endoplasmi reticulum membrane	1.calcium ion binding 2.sugar binding 3.unfolded protein binding	1.post-translational protein modification 2.protein N-linked glycosylation via asparagine 3.protein folding 4.protein secretion
thrombospondin 1	301.4	gi|62089410	7.8	8	1.External side of plasma membrane 2. Extracellular matrix 3. Fibrinogen complex 4. Platelet alpha granule lumen	1. mediates cell-to-cell and cell-to-matrix interactions. 2. binds heparin. 3. dentinogenesis and/or maintenance of dentin and dental pulp 4. Ligand for CD36 mediating antiangiogenic properties.	Cell adhesion
gamma enolase	247.8	gi|182118	17.4	4	1. Plasma membrane 2.Cytoplasm	1.magnesium ion binding 2.phosphopyruvate hydratase activity	1.gluconeogenesis 2.glycolysis
enolase 1	225.9	gi|87196501	22.1	6	1. Cytoplasm 2. Plasma membrane	1. magnesium ion binding 2. phosphopyruvate hydratase activity	1. Glycolysis 2. Plasminogen activation
transgelin 2	223.5	gi|55960374	33.7	5	1. Nuclear membrane 2. Plasma membrane	protein binding	muscle organ development
Annexin A1 (Annexin I) (Lipocortin I) (Calpactin II)	215.4	gi|38604884	30.3	6	1.Cilium, 2.Cytoplasm, 3.Plasma membrane 4.Nucleus,	1.calcium ion binding 2.calcium-dependent phospholipid binding 3.phospholipase A2 inhibitor activity 4.protein binding, bridging 5.receptor binding 6.structural molecule activity	1.alpha-beta T cell differentiation 2.anti-apoptosis 3.cell surface receptor linked signaling pathway 4.cellular component movement 5.inflammatory response 6.keratinocyte differentiation 7.lipid metabolic process 8.peptide cross-linking 9.positive regulation of vesicle fusion

Ribophorin-2	208.8	gi|73991922	13.4	5	Endoplasmic reticulum membrane	1.dolichyl-diphosphooligosaccharide-protein glycotransferase activity 2.protein binding	1.post-translational protein modification 2.protein N-linked glycosylation via asparagine
moesin, isoform CRA_b	204.7	gi|119625804	12.9	6	1.Cytoplasm 2.Cytoskeleton, 3.Plasma membrane	1.cell adhesion molecule binding 2.receptor binding 3.structural constituent of cytoskeleton	1.leukocyte cell-cell adhesion 2.leukocyte migration 3.membrane to membrane docking
Protein S100-A6 (S100 calcium-binding protein A6) (Calcyclin)	165.3	gi|6094218	26.1	2	1.Plasma membrane 2.Cytoplasm 3. Nucleus	1.S100 beta binding 2.calcium ion binding 3.calcium-dependent protein binding 4.protein homodimerization activity 5.tropomyosin binding	1.axonogenesis 2.positive regulation of fibroblast proliferation 3.signal transduction
Ras-related protein Rap-1b	132.5	gi|7661678	32.1	4	1.Plasma membrane 2.Cytoplasm	1. GDP binding 2. GTP binding 3. GTPase activity 4. protein binding	1. blood coagulation 2. energy reserve metabolic process 3. regulation of establishment of cell polarity 4. regulation of insulin secretion
Myristoylated alanine-rich C-kinase substrate (MARCKS)	126.6	gi|73974023	9.8	2	1. Cytoplasm 2. Cytoskeleton 3. Plasma membrane	1. actin filament binding 2. calmodulin binding	1. energy reserve metabolic process 2. regulation of insulin secretion
mannose-6-phosphate receptor (cation dependent) isoform 3	108.5	gi|109095548	31.4	3	1. Lysosome membrane 2. Plasma membrane	1. mannose binding 2. mannose transmembrane transporter activity 3. transmembrane receptor activity	1. endosome to lysosome transport 2. receptor-mediated endocytosis
Protein disulfide-isomerase A6, isoform CRA_a	107.0	gi|119621354	2	15.1	1.Plasma membrane 2.Endoplasmic reticulum	1.electron carrier activity 2.isomerase activity 3.protein disulfide oxidoreductase activity	1.cell redox homeostasis 2.glycerol ether metabolic process

vesicle-associated membrane protein 3	100.7	gi|4759300	33.0	2	1. Cell junction 2. Plasma membrane 3.Synapse 4. Synaptosome	protein binding	1. Protein transport 2. Transport
lysosomal-associated membrane protein 2 （LAMP 2）	90.5	gi|77736087	3.9	2	1.Plasma membrane 2.Endosome 3.Lysosome	1. protection 2. cell adhesion 3.Intracellular signal transduction.	1. platelet activation 2. platelet degranulation
Thioredoxin domain containing protein 1 (Thioredoxin-related transmembrane protein)	87.6	gi|73963782	5.4	2	Endoplasmic reticulum membrane	1. arsenate reductase (thioredoxin) activity 2. disulfide oxidoreductase activity	1. Electron transport 2. Transport
CD63 antigen	77.5	gi|57619037	5.9	2	1.Plasma membrane 2.late endosome membrane 3.lysosomal membrane	play some role in signal transduction pathways	signaling
ATP5A1 protein	75.6	gi|13938339	8.3	2	1.Mitochondrion inner membrane 2.Plasma membrane	1. ATP binding 2. MHC class I protein binding 3. eukaryotic cell surface binding 4. hydrogen ion transporting ATP synthase activity, rotational mechanism 5. proton-transporting ATPase activity, rotational mechanism	1. ATP synthesis 2. Hydrogen ion transport 3. Ion transport 4. Transport
PKM2 protein	68.8	gi|34782802	9.3	2	1.Cytoplasm 2.Nucleus 3.plasma membrane	1.ATP binding 2.magnesium ion binding 3.potassium ion binding 4.protein binding 5.pyruvate kinase activity	1.glycolysis 2.programmed cell death
transmembrane trafficking protein **(**21 kDa transmembrane-trafficking protein)	66.1	gi|73963665	10.5	2	Golgi apparatus membrane	protein binding	1.protein transport 2.regulated secretory pathway 3.vesicle targeting, to, from or within Golgi
caveolin 1 isoform beta	64.8	gi|4972627	18.6	1	1.Plasma membrane 2.Golgi apparatus membrane	1. cholesterol binding 2. nitric-oxide synthase binding 3. peptidase activator activity 4. protein binding 5. protein complex scaffold 6. receptor binding	Host-virus interaction

ENO1 protein	61.1	gi|39644728	9.1	2	1. Plasma membrane 2.Cytoplasm 3.Nucleus	1.Lyase 2.Repressor	1.Glycolysis 2.Plasminogen activation 3.Transcription 4.Transcription regulation
Translocon-associated protein subunit delta	60.1	gi|119593214	10.0	1	Endoplasmic reticulum membrane	1. calcium ion binding 2. protein binding	intracellular protein transport
ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	50.4	gi|21536220	8.3	1	Mitochondrion inner membrane	1. hydrogen ion transporting ATP synthase activity, rotational mechanism 2. proton-transporting ATPase activity, rotational mechanism	ATP synthesis coupled proton transport
ribophorin I	44.4	gi|14124942	2.1	1	.Endoplasmic reticulum membrane	1.dolichyl-diphosphooligosaccharide-protein glycotransferase activity 2.protein binding	1.post-translational protein modification 2.protein N-linked glycosylation via asparagine

Table 9: All membrane proteins are shown. They are divided into two groups. One group is membrane proteins which are only found on the plasma membrane (18 proteins). The other group is the membrane proteins are found both on the plasma membrane and/or other membrane associated locations (34 proteins).

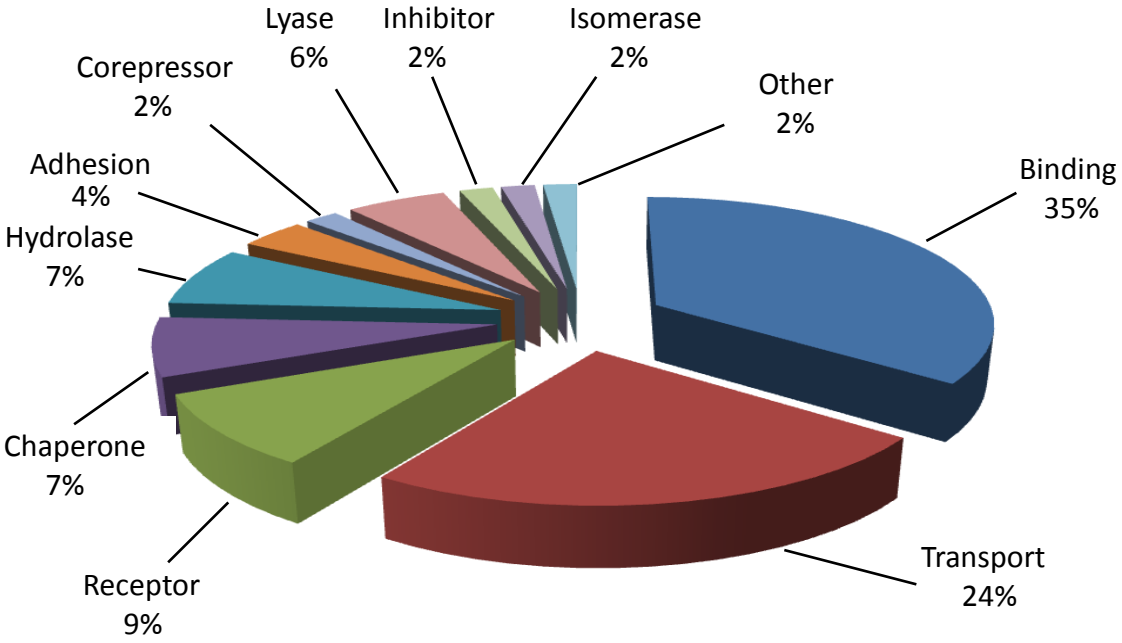

Figure 18: The functional distribution of all identified membrane proteins.

Chapter 4

DISCUSSION

The goals of this project were (i) to find an effective procedure that can extract the membrane proteins of chondrocytes, (ii) use shotgun and gel-based methods to identify the proteins in the extracted samples and evaluate the efficacy of this extraction method, (iii) to identify the membrane proteins of the chondrocyte cell surface. This project aims to establish the foundation for further studies which include clarifying the biology of chondrocytes, exploring potential biomarker and developing therapies for osteoarthritis. In my opinion, the results achieved the original objectives.

4.1 Evaluation of Triton X-114 extraction

With mass spectrometry techniques advancing rapidly over the past two or three decades, the higher sensitivity levels now obtainable facilitates large scale proteomics studies and improves the number of proteins identified dramatically. However, there are still so many challenges which exist in every step. For instance, in order to achieve the cell membrane proteomics profiling, membrane protein enrichment and purification should be seen as the pivotal step of the whole experiment. To some extent, through the extraction of membrane proteins, the complexity of sample decreases substantially and facilitates the mass spectrometric detection of target proteins. However, because the characteristics of membrane proteins include very low abundance and insolubility, these studies are challenging. Also, it is impossible to completely avoid the contamination of cytoplasmic proteins. Although a highly purified membrane protein fraction is difficult to obtain, extraction

procedures can be applied as enrichment methods and they perform satisfactorily in proteomics researches during recent years (Bordier, 1981; Elortza *et al*., 2003; Cordwell *et al*., 2010).

To enrich for membrane proteins, detergent-based phase separation, which is able to divide the sample mixture into hydrophobic and hydrophilic parts, is widely used. This method relies on quite straight-forward manipulations and is also relatively economical. In particular, some strong non-ionic detergents, such as Triton X-114 which was first described by Bordier (Bordier, 1981), are capable of extracting membrane proteins efficiently. The solution of Triton X-114 is homogeneous at 0°C, but when the temperature is raised above 20°C the solution becomes turbid. After centrifugation there are two phases, the detergent and aqueous phases. It is easy to collect these two parts separately. TX-114 has been used for many years with stable acceptable performance (Elortza *et al*., 2003; Shetty *et al*., 2001). Also, a previous study showed it can also be used for glycosylphosphatidylinositol-anchored membrane proteins (Elortza *et al*., 2003). In addition, several other extraction methods could be applied. For example, membrane proteins of human A549 cells were obtained using different solubilizing solutions (*e.g.* urea, thiourea, *etc*) to resolve different proteins according to their hydrophobicity (Lehner *et al*., 2003). Collecting the pellets from sequential centrifuging steps after solubilising in several buffers, a membrane protein rich pellet can be obtained. This is so-called sequential protein extraction (Molloy *et al*., 1998). Another procedure which applies homogenization and increasing force of centrifugation to remove other components is called centrifugal protein extraction or differential centrifugation (Borlakoglu *et al*.,

1990). These two methods have been compared with detergent extraction and in general it seems that detergent-based separation can obtain the greatest amounts of membrane proteins but the sequential extraction provides fractions of higher purity (Lehner *et al*., 2003). Another article suggested detergent-based method was especially suitable for samples with limited material availability (Ramsby *et al*., 1994). Recently, some new methods exhibit encouraging prospects. It was reported that using sucrose density gradient centrifugation to extract the membrane protein of mouse liver tissue, about 50% of proteins identified in the membrane fraction were membrane proteins (Zhang *et al*., 2005). This percentage is similar to the proportion of membrane proteins in the Triton X-114 hydrophobic fraction in this study. Moreover, a more advanced affinity enrichment technique was applied in 2004. After biotinylation of membrane proteins, streptavidin beads were used to enrich the membrane protein fraction and then samples were washed by high-salt and high-pH buffer. This produced a fraction in which 67.5% of detected proteins were classified as integral plasma membrane proteins (Zhao *et al*., 2004). However, this strategy can only be used in cultured cells and has the additional drawback of producing low yields (Zhang *et al*., 2007). In summary, all the procedures have their own drawbacks and contain a significant proportion of contaminants (usually account for 50% of the fraction or more). Thus, these methods all require optimization in order to enhance their accuracy and recovery levels.

In terms of the evaluation of extraction in this project, TX-114 phase partitioning is applied mainly because its prevalence. Although whether it is the most optimal strategy cannot be verified, it is suitable for the purpose of this project. After

combining shotgun and gel-based analyses, 52 membrane proteins were identified. According to the pie charts shown in Figures 14, 15, 16 and 17, approximately 50% of the proteins identified in the Triton X-114 hydrophobic fraction were membrane proteins. This percentage represents an acceptable level which is similar to that found in other studies (Zhang *et al*., 2005; Cordwell *et al*., 2010). On the other hand, the percentages of membrane associated proteins in the Triton X-114 hydrophilic fraction were only about 13% and 21% (shotgun and gel-based separately). Comparing hydrophobic and hydrophilic data, it was apparent that many membrane proteins were extracted into hydrophobic fraction. Moreover, the western blotting experiments also confirmed this conclusion (Figures 9 & 10). Therefore, to some extent, the extraction achieved the original expectation. Admittedly, detergent phase separation cannot produce a highly purified membrane protein fraction. The pie charts clearly showed membrane proteins were not totally extracted into the hydrophobic fraction, which are also confirmed by previous studies and the western blotting results (Figures 9 & 10) (Lehner *et al*., 2003**)**. However, this procedure could be optimized further and the quality of the results (number and proportion of membrane proteins) can be improved. First of all, as far as the author's knowledge, there is no published paper following a similar approach for equine chondrocytes, so all the steps of extraction and other treatments were developed via referral of other relevant articles and preliminary experience. Without a standard procedure of TX-114 extraction, inevitably, some steps may need to be further optimized. For example, some details, such as the time and speed of centrifugation, incubation temperature and the final concentration of TX-114 of the sample, vary from paper to paper (Elortza *et al*., 2003; Shetty *et al*., 2001; Hemphill, 1996). To develop a standard

protocol for equine chondrocytes, some alternations of some technical details may need to be test. One test, which I did during the project, showed small changes of these details affected the extraction result to some extent. For example, it was clear that differences existed when the final concentration of TX-114 was increased from 0.75% to 2% and the incubation time at 37°C water was prolonged to 20 minutes (Figure 19). Such modifications brought a negative result, which meant some gel bands disappeared or were weaker than those found using the previous protocol. Although all the cells which used for this test were from one flask in order to avoid the diversity of sampling, protein loss during manipulation may be another consideration. However, it is worth repeating such tests in order to further improve the results and also to provide a standard for other researchers. Secondly, the process of phase separation can be done several times for one sample. For the extractions in this thesis, after the first separation only the hydrophilic fraction experienced a 2^{nd} extraction and then the two resultant hydrophobic fractions were combined. Possibly, we can extract more times for both fractions to test the result in future. Also, some modifications of LC-MS/MS analysis will be helpful to improve the performance of this separation method and identify more membrane proteins. For example, in this thesis, the LC-MS/MS used a 15 cm pepmap column with 30 minutes gradient wash in gel-based sample and 115 minutes wash in shotgun sample and the top 3 and top 5 MS/MS method were used in gel-based sample and shotgun sample respectively. These procedures are fairly standard. But membrane proteins are special because of the very low abundance. Thus, it is worth to separate peptides on a longer column and increasing the gradient time and combine these alterations with more top ions be analyzed. Moreover, multidimensional LC is also a powerful

technique and worth to try. Study showed that 2-dimensional LC, which combines the strong cation exchange (SCX) and reversed-phase (RP) chromatography, can decrease the complexity much better and improve the identification of tandem MS (Peng *et al*., 2003). With such treatments, more low abundant proteins can be detected. In addition, at each step of a proteomics experiment some protein loss will occur, especially for such low abundant proteins. Some technical details in Triton X-114 partitioning, such as removing the supernatant, separating two phases, and so on, should be dealt extremely carefully so that the sample loss can be minimized. Generally speaking, this project is just a beginning and a new attempt and there is room for further improvements.

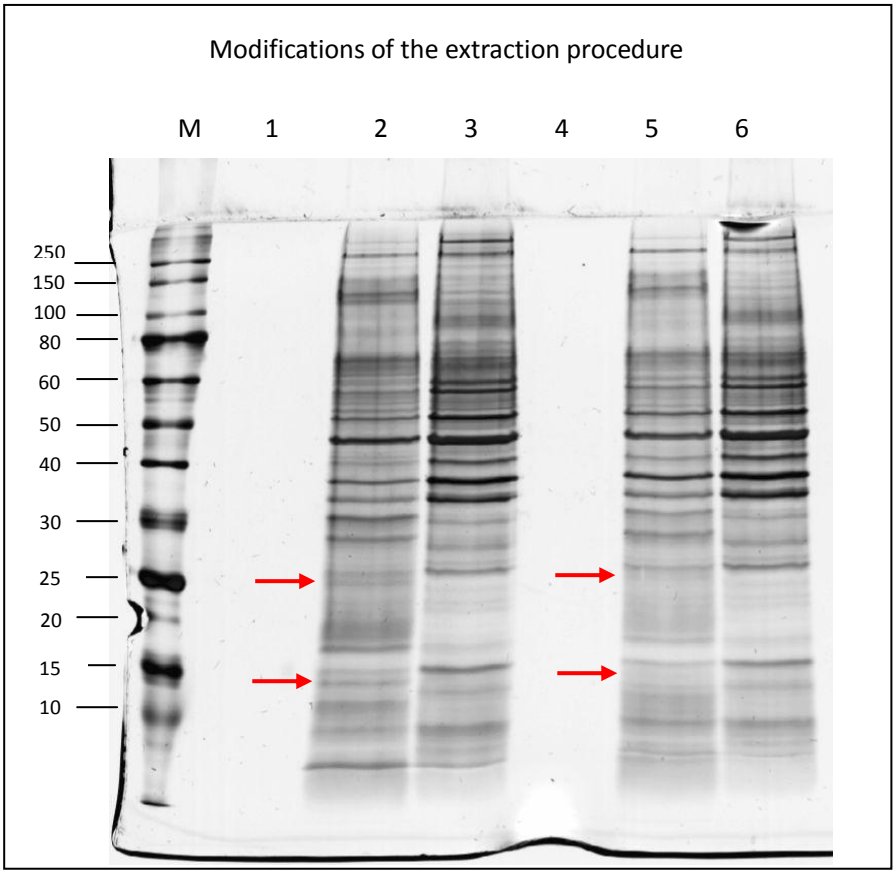

Figure 19: Comparing the outcomes of some modifications of the TX114 procedures. Lane M is unstained marker. Lanes 1 and 4 are blank. Lanes 2 and 5 are hydrophobic fraction. Lanes 3 and 6 are hydrophilic fraction. Lanes 2 and 3 used the standard steps as described in Method section. Lane 5 and 6 increased the final concentration of TX114 from 0.75% to 2% and increased the incubation time at 37°C form 5 min to 20 min. Red arrows showed some differences between two hydrophobic lanes. Two visual bands were lost in the modified protocol (lane 5).

4.2 The complementarity of gel-based and shotgun methods

The gel-based method combined one dimensional gel separation with LC tandem MS. Typically, gel-based method often utilize 2D gel mainly because of better separation which can simplify complex mixture significantly. But for membrane proteins which solubilise poorly and are difficult to obtain in 2D gel, the 1D gel separation is a suitable alternative and also has some merits of the gel-based method. First of all, the complex protein mixture is separated by molecular mass, which leads to a decrease in the sample complexity. Secondly, this linear gel facilitates concentration of proteins into specific mass ranges (Stasyk *et al*., 2004). Also, with the gel stain system, it is possible to separate high abundant and low abundant proteins according to the stain intensity. It is very useful not only because low abundant samples can be controlled to inject more into the LC-MS/MS after gel slices digestion, more importantly, low abundant proteins are analyzed directly so that they can avoid their signals being masked by high abundant ones. Thus, this method could improve the identification of low abundant proteins to some extent. Moreover, the technique of 1D gel is much easier than 2D gel which should avoid some errors of operation. In addition, liquid chromatographic method is used to further decrease the complexity and MS/MS performs with high sensitivity. Consequently, the results may have increased reliability because more peptides can be analyzed and matched per protein. However, the limits are also obvious. In addition to the lack of automation, another disadvantage is that the detectable range of stain intensity is incapable of reflecting the whole range of protein concentrations which can cover a 1,000,000-fold range

(Delahunty *et al*., 2005). Unless the entire gel lane is cut and analyzed, low abundant proteins which are not visualized well will be ignored inevitably. Moreover, the protein loss during the tedious experimental steps is a problem continuously in every method.

On the other hand, the shotgun method which only uses the LC-MS/MS technique to analyze digested sample contains many other characteristics. Firstly, total automation and simple treatment before LC-MS/MS not only save on labor, but also avoid sample loss to some extent. More importantly, the shotgun method can achieve high throughput and identify large numbers of proteins nowadays with the help of well-developed equipment and powerful data analysis. In addition, the detection of low abundance proteins is improved considerably with cutting-edged techniques which have higher sensitivity, such as nano LC. Consequently, shotgun analysis is widely used in recent years, especially in large scale studies. However, one drawback is that highly complex samples leads to less peptide matched per protein. Sometimes the results from such limited data may not reliable. For example, if only one peptide is detected and it can match several proteins, it is difficult to determine which one is correct.

Due to different technical characteristics, the identified proteins of two methods are not totally the same. But with the restriction of current techniques, neither of them can cover the whole dynamic range of the proteome. In order to identify more proteins, combining the results of these two methods is a better way.

In this project, the shotgun method was applied as the major procedure. As a complementary procedure, nearly all the obvious gel bands were cut off. According to my identification results, although some proteins are same, there are still a number of different ones. Combining all the results, 52 membrane proteins were identified (Table 9). In addition, it is obvious that some gel-based results had very high scores, even higher than 1000. This is reasonable because the gel-based method decreased the complexity significantly and more peptides could be matched per protein. Also, the shotgun method identified many more proteins than the gel-based method. There may be several reasons. First of all, due to more treatments need to be done prior to LC-MS/MS analysis in gel-based method, protein loss is common and easy to happen. It may appear in every processing step, like adsorption in tips and any other containers (Granvogl *et al.*, 2007). Another factor is many peptides cannot be extracted from the gel which leads to 15%-50% losing (Stewart *et al.*, 2001). Moreover, as a complementary method, only clear bands were sliced from the gel instead of cutting the entire gel, which limited the number of identified proteins to a large extent.

4.3 Membrane proteins on the chondrocyte surface

Membrane protein analysis is a big challenge in proteomics. According to a previous study, membrane proteins roughly account for 30% of cellular proteins (Wu *et al*., 2003). Currently, research studies still have a long distance to cover in order to

achieve analysis of this number. Of all the membrane proteins I found, there were 18 proteins are found only on the plasma membrane. 34 proteins are found both on the plasma membrane and/or other membrane associated locations. In all these 52 membrane proteins, 18 transmembrane proteins were identified. This group of proteins accounts for about 35% of total identified membrane proteins. This percentage should be increased in future study. First of all, Uniprot database was only used in searching transmembrane domains. Some proteins did not have such detailed information. Other powerful database should be used together. Secondly, if we can modify some steps and optimize this experiment, like what I have discussed above, more proteins should be identified and the number of transmembrane protein will increase at the same time. In addition, in the summarization of their functions in Figure 18, it is clear that most of these membrane proteins are responsible for binding and transportation. Their percentages are 35% and 24% respectively. Thus, it is obvious that chondrocytes have a close interaction with extracellular matrix. Many membrane proteins can bind with other molecules to perform biological functions, such as mediating metabolic reactions. Another major group of these membrane proteins are used to transport metabolites and signals. From above percentages, we can understand the fact that the environmental changes always lead to the abnormality of chondrocytes and the pathological chondrocytes can also aggravate the alternation of matrix as well. In addition, the third important function group is receptors which accounts for 9%, they are also play a key role in physiological processes. Besides these, other classes include chaperone (7%), hydrolase (7%), cell adhesion (4%), lyase (6%), corepressor (2%), inhibitor (2%), isomerase (2%) and other functions (2%).

More specifically, some plasma membrane proteins identified in this study are particularly important for chondrocytes and are known to be altered in osteoarthritis. Take the following three proteins for example:

- **Beta-1 Integrin (CD29 or VLA)**

Integrin is a heterodimetric glycoprotein which has two different chains, the alpha and beta subunits. As a transmembrane protein, every subunit has extracellular and cytoplasmic domains. Both of these domains are able to bind relevant biological molecules so that its special functions can be performed. There are various types of alpha and beta subunits and different composition of these two subunits make up different integrins, such as $\alpha_1\beta_1$ integrin. The β1 integrins are a group of integrins which are mainly used for binding extracellular matrix components (Mobasheri *et al*., 2002). They all have β1 subunit, although the α subunits are different (Mobasheri *et al*., 2002). Studies showed chondrocytes express β1 and many other integrins (Salter *et al*., 1992; Shakibaei *et al*., 1995). Additionally, any form of chondrocyte cell culture, either as cell monolayers or cells suspended in alginate matrix, induces high expression of β1 integrin (Loeser *et al*., 1995). Because chondrocytes grow in cartilage which provides the extracellular matrix environment, a repair response will happen when the matrix is removed. This response results in an increase of β1 integrin expression accompanying with a raise of ECM proteins.

In terms of the biological function, it is well known that integrins are cell surface

receptors which are responsible for cell attachment to extracellular matrix components (Mobasheri *et al*., 2002). Also, integrins mediate many signal pathways across the plasma membrane in both inside-out and outside-in directions (Hynes, 2002). β1 integrin, which has a very high abundance on chondrocyte, is mainly responsible for adhesion mediation to matrix proteins. Through the adhesion to relevant matrix proteins, a lot of focal adhesion associated proteins, like tensin and paxillin, and signaling proteins can be activated (Mobasheri *et al*., 2005). In chondrocytes, integrins are involved in many biological processes, not only cell-matrix interaction and signal transduction, but also cartilage remodelling and chondrogenesis (Mobasheri *et al*., 2005). For instance, the cooperation of β1 integrin and IGF-IR (insulin-like grown factor- I receptor) controls the interaction with the cytoskeleton and mediates mitogen-activated protein kinase signaling pathways (Mobasheri *et al*., 2005). Moreover, it is a fact that β1 integrin expresses at a higher level in osteoarthritic cartilage than that in normal one (Loeser *et al*., 1995). There are a number of relevant studies. Through particular treatment, like ECM perturbation or TGF-β stimulus, the expression of β1 integrin produces significant change. Thus, β1 integrin plays a very critical role in the chondrocyte-matrix interactions. Also, the increase of β1 integrin in culture process, which is mentioned above, suggests that β1 integrin is closely associated with cartilage repair. Recently, some articles showed that β1 integrin had important research value for apoptosis (Cao et al., 1999; Goggsa et al., 2003). It is well known that the core changes of cartilage in joint disease especially osteoarthritis is the progressive degradation and destruction. At the cytological level, the whole pathological process of cartilage degradation accompanied with chondrocyte apoptosis (Goggsa *et al*., 2003).

Surprisingly, integrins play a key role in providing the survival signals because they maintain a constant link with extracellular matrix (Mobasheri *et al*., 2002). More specifically, as a surface molecule of chondrocytes, β1 integrin is able to bind the collagen (Enomoto *et al*., 1993) and through some experiments, which apply collagenase or anti-integrin β1 antibody treatment. Scientists proved that the interactions between collagen and β1 integrin could leads to aggregation of chondrocytes and it is important for chondrocyte survival (Cao *et al*., 1999).

- **CD 44 antigen**

CD44 is a single-pass glycoprotein which is expressed by many cell types including chondrocyte (Knudson *et al*., 1996; Chow *et al*., 1995). As a transmembrane receptor, it is located in the plasma membrane and is responsible for numerous biological processes including cell attachment, cell adhesion, cell migration, lymphocyte activation, tissue remodeling (Carsten *et al*., 2005). In chondrocytes, CD44 is capable of binding to extracellular matrix components, such as collagen, fibronectin and hyaluronan (HA). Importantly, it is regarded as the principal cell surface receptor for HA (Aruffo *et al*., 1990). Previous studies showed that through making HA combine with chondrocyte, CD44 play a regulatory role in cartilage function. More specifically, the chondrocyte-HA adhesion via CD44 induced the expression of c-myc, a proto-oncogenes related to the proliferation and maturation of chondrocytes (Iwamoto *et al*., 1993; Farquharson *et al*., 1992). In addition, such adhesion also regulates the expression level of TGF-β which acts as a growth factor to mediate chondrocyte differentiation and assemble matrix components such as proteoglycans and collagens.

Thus, it is clear that the CD44 mediated adhesion between chondrocyte and HA regulates the proliferation and maturation of chondrocyte and also the synthesis of extracellular matrix ingredients (Ishida *et al*., 1997). Such proliferation regulated by CD44 close relates to disease states like OA and RA because normal chondrocytes multiply slowly.

In addition, the expression of CD44 increases during the development of osteoarthritis and has a relationship with matrix degradation (Carsten *et al*., 2005). It has been suggested that CD44 is able to mediate chondrocyte endocytosis of HA which reduces the number of extracellular HA molecules (Hua *et al*., 1993). To some extent, this change disrupts the integrity of extracellular matrix which is quite essential for matrix function (Hua *et al*., 1993).

Maintaining the homeostasis of cartilage is another important function of CD44 (McDonald, 1989; Lin *et al*., 1993). Either limiting the expression of CD44 or disturbing the CD44-related chondrocyte-matrix interactions influenced the metabolism of cartilage in a negative way (Knudson *et al*., 2002). For example, due to lack of relevant interactions, matrix could be stimulated to some remodeling activities such as matrix biosynthesis and chondrolysis (Knudson *et al*., 2002).

- **CD 151**

CD151 is a membrane protein which belongs to the tetraspanin family (Lotus *et al*., 2002). Tetraspanin proteins are given this name because they have 4 membrane spanning domains. The cytoplasmic and extracellular domains allow tetraspanins

interact with many other molecules. As one of the important functions, tetrapanin affects the coupling of signaling pathways positively through combining with other specific proteins (Lotus *et al*., 2002). Thus, it was shown that CD151 is able to generate complexes which are involved in many cell-cell and cell-matrix interactions (Grogan *et al*., 2007). CD151 combines with laminin-binding integrins like $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$ integrins and co-distributes with them (Grogan *et al*., 2007). Due to $\alpha_6\beta_1$ being expressed in chondrocytes (Brakebusch *et al*., 2005), detecting CD151 is not too difficult. Also, with the help of flow cytometry, researchers found monolayer cultured chondrocytes experience some changes in phenotype and gene expression which includes an up-regulation of CD151 expression (Gaillard *et al*., 2005).

CD151 is involved in a great number of biological processes, such as cell adhesion, cell migration, signaling, cell polarization (Lotus *et al*., 2002). Recent reports showed that CD151 is overexpressed in articular cartilage of osteoarthritis (Fujita *et al*., 2006). More specifically, it is found that CD151 is co-expressed with the pro-enzyme of matrix metalloproteinase 7 (proMMP-7) in OA cartilage (Fujita *et al*., 2006). Both of these molecules are over-expressed. Previous studies found MMP-7 performs high specific activity against a lot of extracellular matrix molecules (Fujita *et al*., 2006). The activation of proMMP-7 is closely related with the degradation of cartilage. Through experiments, although the detailed mechanism still requires further study, it has been confirmed that CD151 can mediate the activation of proMMP-7, which plays a significant role in the destruction of cartilage in OA (Fujita *et al*., 2006).

4.4 Other small problems during the study

A phenomenon I noted was the cell shape changing during monolayer culture. We can observe that the shape of some cells had elongated and they were not circle type any more. Comparison showed that the morphology of multiply passaged cells altered from a round polygonal to an elongated fibroblast-like phenotype (Figure 20). This process is termed dedifferentiation (Darling *et al*., 2005; Diaz-Romero *et al*., 2008). Some proteins of chondrocytes alter their expressions during monolayer culture. For example, dramatic loss of collagen II and chondromodulin expression was found in cultured cells (Schnabel *et al*., 2002). Dedifferentiation also occurs in other kinds of chondrocyte cell culture methods and in vivo natural processes (e.g. environmental changes due to biochemical or physical damage), but it develops rapidly in monolayer culture (Darling *et al*., 2005). It is possible that the lack of cell-cell and cell-matrix interactions may arouse such altered expression of chondrocytes.

Comparison of cell shape change

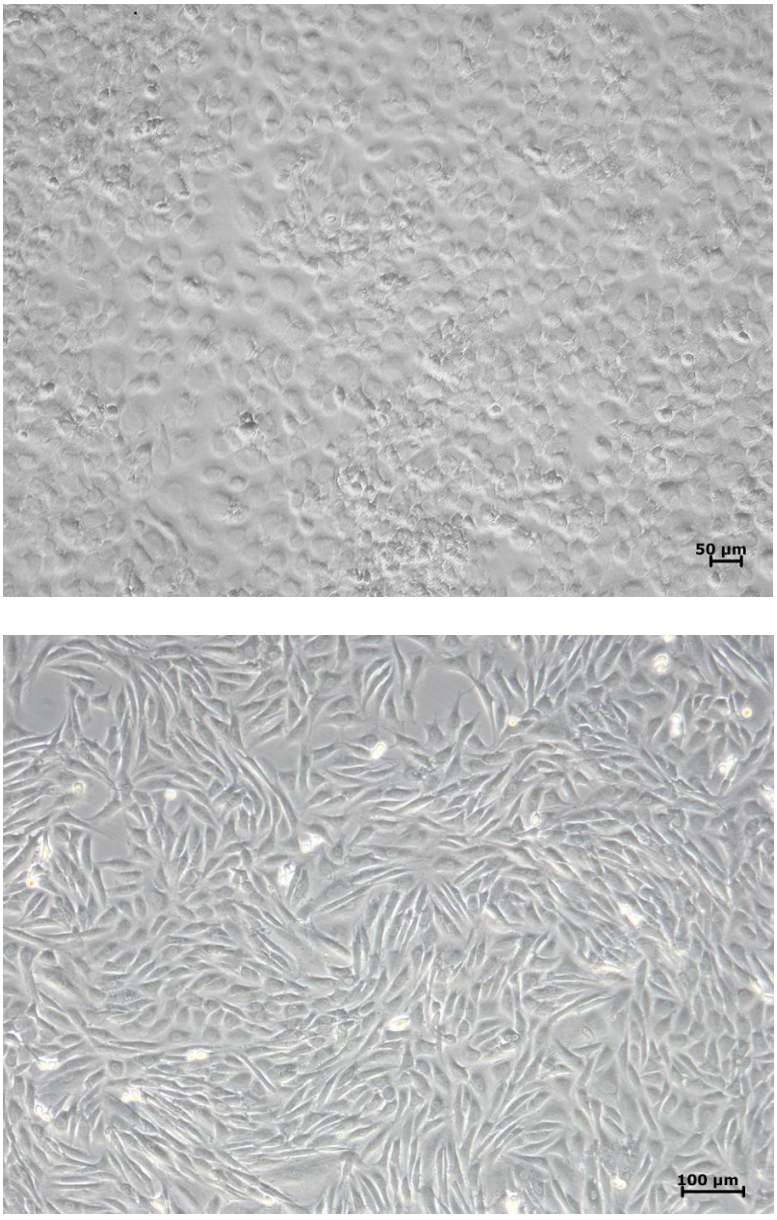

Figure 20: Morphological changes in chondrocytes during monolayer culture. The upper image shows cells which had been passaged in culture only once. The lower image shows cells which had been passaged 2 times. The shape change is obvious.

5. Conclusion

In this project, the use of Triton X-114 phase separation to extract membrane proteins of chondrocyte was verified as a successful and satisfactory method for preliminary studies. Many membrane proteins were extracted into the hydrophobic fraction. To identify proteins, shotgun and gel-based methods using nanoLC-MS/MS were applied. The two methods have different characters. The shotgun method has high automation, high throughput and it is easy to discover large numbers of proteins. But highly complex mixtures result in fewer peptides matched per protein, which makes the reliability of identification is not as good as gel-based one, at least in some cases. In comparison, the gel-based method easily leads to the sample loss during the tedious steps, but more peptides can be matched per protein because the sample complexity is decreased dramatically. The proteins identified in two methods are not entirely the same. Thus, combining the two methods to perform the proteomics analysis is recommended. Finally, a total of 52 membrane proteins were identified in this project, 18 of them are located only on the plasma membrane, and 34 proteins are located both on plasma membrane and/or other membrane associated locations. Also, these membrane proteins include 18 transmembrane proteins. In addition, most of these membrane proteins are responsible for binding, transport and receptor activities. Some of the identified membrane proteins play important roles in chondrocyte cell biology and a possible relationship with osteoarthritis, such as integrin beta-1, CD44 and CD151.

6. Future work

In this project, we found that Triton X-114 method can extract membrane proteins efficiently. Also, combining shotgun and gel-based methods can identify more proteins with the help of nanoLC-MS/MS. In future, we can apply this methodology to compare the differences in the membrane proteome between healthy and pathological samples. In terms of cartilage degradation which is stimulated by aging, we can also analyze the condition of the membrane proteome of chondrocytes from old and young animals. If we find any important protein, western blotting can be used to test their expression changes and functional assays can be carried out for some identified proteins. In addition, some modifications could optimize this proteome profiling. For example, using multiple dimensional LC or a longer column can decrease the complexity of the samples and improve the protein identification. If we want to detect more proteins in the gel-based method, slicing the entire gel into small pieces is a good choice. Consequently, by following these procedures and expanding this study, we will have a better understanding of the physiology of chondrocytes and may discover some biomarkers or key proteins of osteoarthritis and cartilage degradation.

7. References

Abramson S.B., Attur M., 2009. Developments in the scientific understanding of osteoarthritis. *Arthritis Research & Therapy*, 11, 227

Alford J.W., Cole B.J., 2005. Cartilage restoration, part I: basic science, historical perspective, patient evaluation and treatment options. *Am J Sports Med*. 33, 295-306.

Arnold T., Linke D., 2007. Phase separation in the isolation and purification of membrane proteins, *BioTechniques*, 43, 427-440

Aruffo A., Stamenkovic I., Melnick M., Underhill C.B., Seed B., 1990. CD44 is the principal cell surface receptor for hyaluronate. *Cell*, 61, 1303–13.

Bayés A., Grant S.G.N., 2009. Neuroproteomics: understanding the molecular organization and complexity of the brain. *Nature Reviews Neuroscience,* 10, 635-646

Bordier C., 1981. Phase Separation of Integral Membrane Proteins in Triton X-114 Solution, *The journal of biological chemistry*, 256, 4, 1604-1607.

Borelli J.J., 2006. Chondrocyte apoptosis and post-traumatic arthrosis. *J. Orthop. Trauma,* 20, 726–731.

Borlakoglu J.T., Drew M.G.B., Wilkins J.P.G., Dils R.R., 1990. Effects of molecular substitution patterns on the cytochrome P450-dependent metabolism of 2,2′,3,5,5′,6- and 2,2′,3,4,4′,6-hexachlorobiphenyl by rat liver microsomal monooxygenases. *Biochim. biophys. Acta.* 1036, 167–175

Borrelli J.J., Ricci W.M., 2004. Acuteeffectsof cartilage impact. *Clin. Orthop. Relat. Res.* 423, 33–39.

Brakebusch C., F¨assler, R., 2005. β1 integrin function in vivo: Adhesion, migration and more. *Cancer and Metastasis Reviews,* 24, 403–411

Brookes P.S., Pinner A., Ramachandran A., Coward L., Barnes S., Kim H., Darley-Usmar V.M., 2002. High throughput two-dimensional blue-native electrophoresis: a tool for functional proteomics of mitochondria and signaling complexes. *Proteomics*, 2, 969-977.

Buckwalter J.A., Hunzinker E., Rosenberg L., 1988. Articular cartilage: composition and structure. In: Woo SLY, Buckwalter JA, eds. Injury and Repair of the Musculoskeletal Soft Tissues. Park Ridge, IL: American Academy of Orthopaedic Surgeons. 405-425.

Buckwalter J.A., Mankin H.J., 1997. Articular cartilage, part 1: tissue design and chondrocyte-matrix interaction. *J Bone Joint Surg Am*. 79, 600-611.

Buckwalter J.A., Mankin H.J., Grodzinsky A.J., 2005. Articular cartilage and osteoarthritis. *Instructional Course Lectures*, 54, 465–480.

Buckwalter J.A., Martin J., Mankin H.J., 2000. Synovial joint degeneration and the syndrome of osteoarthritis. *Instructional Course Lectures*, 49, 481–489.

Buckwalter J.A., Martin J.A., 2006. Osteoarthritis. *Advanced Drug Delivery Reviews*, 58, 150-167

Calamia V., Rocha B., usMateos J., Andez-Puente P.F., Ruiz-Romero C., Blanco F.J., 2011. Metabolic Labeling of Chondrocytes for the Quantitative Analysis of the Interleukin-1-beta-mediated Modulation of Their Intracellular and Extracellular Proteomes. *J. Proteome Res.* 10, 3701–3711

Cao L., Lee W., Adams M.E., Kiani C., Zhang, Y., Hu W., Yang B.B., 1999. β1-integrin-collagen interaction reduces chondrocyte apoptosis. *Matrix Biology*, 18, 343-355.

Chem. 268,9645–9652.

Chow G., Knudson C.B., Homandberg G., Knudson W., 1995. Increased CD44 expression in bovine articular chondrocytes by catabolic cellular mediators. *J. Biol. Chem.* 270, 27734–27741

Cordwell S.J., Thingholm T.E., 2010. Technologies for plasma membrane proteomics. *Proteomics*, 10, 611-627

Dabovic B., Chen Y., Colarossi C., Zambuto L., Obata H., Rifkin D.B., 2002. Bone defects in latent TGF-beta binding protein (Ltbp)-3 null mice; a role for Ltbp in TGF-beta presentation. *J Endocrinol.* 175, 129-141

Darling E.M., Athanasiou K.A., 2005. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *Journal of Orthopaedic Research,* 23, 425-432

Delahunty C, Yates J.R., 2005. Proteomics: A Shotgun Approach without Two-dimensional Gels. *Encyclopedia of life sciences,* 1-6.

Devreese B., Vanrobaeys F., Smet J., Van Beeumen J., Van Coster R., 2002. Mass spectrometric identification of mitochondrial oxidative phosphorylation subunits separated by two-dimensional blue-native polyacrylamide gel electrophoresis. *Electrophoresis*, 23, 2525–2533.

Dhillon A.S., Hagan S., Rath, O., Kolch W., 2007. "MAPkinasesignaling pathwaysincancer", *Oncogene,* 26, 3279-3290.

Diaz-Romero J., Nesic D., Grogan S.P., Heini P., Mainil-Varlet P., 2008. Immunophenotypic Changes of Human Articular Chondrocytes During Monolayer Culture Reflect Bona Fide Dedifferentiation Rather Than Amplification of Progenitor Cells*. J. Cell. Physiol*. 214, 75–83.

Elortza F., Nu¨ hse T.S., Foster L.J., Stensballe A., Peck S.C., Jensen O.N., 2003.

Proteomic Analysis of Glycosylphosphatidylinositol-anchored Membrane Proteins. *Molecular & Cellular Proteomics*, 2, 12, 1261–1270.

Elortza F., Nühse T.S., Foster L.J., Stensballe A., Peck S.C., Jensen O.N., 2003. Proteomic analysis of glycosylphosphatidylinositol-anchored membrane proteins. *Molecular & cellular proteomics*, 2, 12, 1261-70.

Enomoto M., Leboy P.S., Menko A.S., Boettiger D., 1993. ß1 Integrins mediate chondrocyte interaction with type I collagen, type II collagen, and fibronectin. *Exp. Cell Res.* 205, 276-285.

Farquharson C., Hesketh J.E., Loveridge N., 1992. The protooncogene c-myc is involved in cell differentiation as well as cell proliferation: Studies on growth plate chondrocytes in situ. *J Cell Physiol.* 152, 135–144.

Fujita Y., Shiomi T., Yanagimoto S., Matsumoto H., Toyama Y., Okada Y., 2006. Tetraspanin CD151 is Expressed in Osteoarthritic Cartilage and is Involved in Pericellular Activation of Pro–Matrix Metalloproteinase 7 in Osteoarthritic Chondrocytes. *ARTHRITIS & RHEUMATISM*, 54, 10, 3233–3243.

Gaillard J.P., Diaz-Romero J., Grogan S.P., Nesic D., Trub T., Mainil-Varlet P., 2005. Immunophenotypic Analysis of Human Articular Chondrocytes: Changes in Surface Markers Associated With Cell Expansion in Monolayer Culture. *JOURNAL OF CELLULAR PHYSIOLOGY,* 202,731–742.

Gobezie, R., Kho A., Krastins B., Sarracino D.A., Thornhill T.S., Chase M., Millett P.J., Lee D.M., 2007. High abundance synovial fluid proteome: distinct profiles in health and osteoarthritis. *Arthritis Research & Therapy*, 9

Goggsa R., Cartera S.D., Schulze-Tanzilb G., Shakibaeib M., Mobasheri A., 2003. Apoptosisand the loss of chondrocyte survival signals contribute to articular cartilage degradation in osteoarthritis. *The Veterinary Journal*, 166, 2, 140-158

Granvogl B., Gruber P., Eichacker L.A., 2007. Standardisation of rapid in-gel digestion by mass spectrometry. *Proteomics*, 7, 642–654.

Grogan S.P., Barbero A., Diaz-Romero J., Cleton-Jansen A.M., Soeder S., Whiteside R., Hogendoorn P.C., Farhadi J., Aigner T., Martin I., Mainil-Varlet P., 2007. Identification of Markers to Characterize and Sort Human Articular Chondrocytes With Enhanced In Vitro Chondrogenic Capacity. *ARTHRITIS & RHEUMATISM*, 56, 2, 586–595.

Guilak F., Alexopoulos L.G., Upton M.L., Youn I., Choi J.B., Cao L., Setton L.A., Haider M.A., 2006. The pericellular matrix as a transducer of biomechanical and biochemical signals in articular cartilage. *Ann N Y Acad Sci.,* 1068, 498-512.

Helenius A., Simons K., 1975. Solubilization of membranes by detergents. *Biochim. Biophys. Acta*, 415, 29-79.

Hemphill A., 1996. Subcellular Localization and Functional Characterization of Nc-p43, a Major Neospora caninum Tachyzoite Surface Protein. *Infection and immunity*, 64, 10, 4279–4287.

Hua Q., Knudson C.B., Knudson W., 1993. Internalization of hyaluronan by chondrocytes occurs via receptor-mediated endocytosis. *J. Cell Sci.* 106, 365–75.

Hudelmaier M., Glaser C., Hohe J., Englmeier K.H., Reiser M., Putz R., Eckstein F., 2001. Age-related changes in the morphology and deformational behavior of knee joint cartilage. *Arthritis Rheum*, 44, 11, 2556-2561.

Hunziker E.B., 2002. Articular cartilage repair: basic science and clinical progress. *A review of the current status and prospects*, 10, 432-463

Hynes R.O., 2002. Integrins: Bidirectional, Allosteric Signaling Machines, *Cell*, 110, 673–687.

Inerot S., Heinegard D., lars Audell L., Olsson S.E., 1978. Articular-Cartilage Proteoglycans in Aging and Osteoarthritis. *Biochem. J.* 169, 143-156

Ishida O., Tanaka Y., Morimoto I., Takigawa M., Eto S., 1997. Chondrocytes Are Regulated by Cellular Adhesion Through CD44 and Hyaluronic Acid Pathway. *JOURNAL OF BONE AND MINERAL RESEARCH*, 12, 10.

Iwamoto M., Yagami K., Lu V.P., Olsen B.R., Petropoulos C.J., Ewert D.L., Pacifici M., 1993. Expression and role of c-myc in chondrocytes undergoing endochondral ossification. *J Biol Chem.* 268, 13, 9645-52

Jackson D.W., Lalor P.A., Aberman H.M., Simon T.M., 2001. Spontaneous repair of full-thickness defects of articular cartilage in a goat model: A preliminary study. *J Bone Joint Surg Am*. 83-A, 53-64.

Kellgren J.H., Lawrence J.S., Bier F., 1963. Genetic factors in generalized osteoarthrosis. *Ann Rheum Dis.* 22, 237-255.

Knudson W., Aguiar D. J., Hua Q., Knudson C.B., 1996. CD44-anchored hyaluronan-rich pericellular matrices: an ultrastructural and biochemical analysis. *Exp. Cell Res.* 228, 216–228.

Knudson W., Loeser R.F., 2002. CD44 and integrin matrix receptors participate in cartilage homeostasis. *Cell. Mol. Life Sci*. 59, 36–44.

Lallet-Daher H., Roudbaraki M., Bavencoffe A., Mariot P., Gackière F., Bidaux G., Urbain R., Gosset P., Delcourt P., Fleurisse L., Slomianny C., Dewailly E., Mauroy B., Bonnal J.L., Skryma R., Prevarskaya N., 2009. Intermediate conductance $Ca2^{++}$activated K^{+} channels (IKCa1) regulate human prostate cancer cell proliferation through a close control of calcium entry. *Oncogene,* 28, 1792-1806.

Lehner I., Niehof M., Borlak J., 2003. An optimized method for the isolation and identification of membrane proteins. *Electrophoresis,* 24, 1795–1808

Lehner I., Niehof M., Borlak J., 2003. An optimized method for the isolation and identification of membrane proteins. *Electrophoresis*, 24, 1795–1808

Leth-Larsen R., Lund R.R., Ditzel H.J., 2010. Plasma Membrane Proteomics and Its Application in Clinical Cancer Biomarker Discovery. *Molecular & Cellular Proteomics*, 9, 1369-1382.

Lin C.Q., Bissell M.J., 1993. Multi-faceted regulation of cell differentiation by extracellular matrix. *FASEB J.* 7, 737–743.

Loeser R.F., Carlson C.S., Mcgee M.P., 1995. Expression of β1 integrins by Cultured Articular Chondrocytes and in Osteoarthritic Cartilage, *EXPERIMENTAL CELL RESEARCH*, 217, 248-257.

Lotus M.T., Cecile A.W., Geuijen José G., van den Berg, Claessen N., Weening J.J., Sonnenberg A., 2002. Association of the tetraspanin CD151 with the lamininbinding integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$ $\alpha_6\beta_4$ and $\alpha_7\beta_1$ in cells in culture and in vivo. *Journal of Cell Science,* 115, 1161-1173.

Manade T., 1999. Capillary electrophoresis of proteins for proteomic studies,

Electrophoresis, 20, 3116-3121

McDonald J.A., 1989. Receptors for extracellular matrix components. *Am. J. Physiol.* 257, 331–337.

Melero-Martin J.M., Al-Rubeai M., 2007. In Vitro Expansion of Chondrocytes. *Topics in Tissue Engineering*, Vol. 3

Millward-Sadler S.J., Salter D.M., 2004. Integrin-dependent signal cascades in chondrocyte mechanotransduction. *Ann Biomed Eng*., 32, 3, 435-46.

Mobasheri A., Barrett-Jolley R., Carter S.D., Martín-Vasallo P., Schulze-Tanzil G., Shakibaei M., 2005. Functional Roles of Mechanosensitive Ion Channels, β1 integrins and Kinase Cascades in Chondrocyte Mechanotransduction. *Mechanosensitivity in Cells and Tissues*.

Mobasheri A., Bondy C.A., Moley K., Mendes A.F., Rosa S.C., Richardson S.M., Hoyland J.A., Barrett-Jolley R., Shakibaei M., 2008. Facilitative glucose transporters in articular chondrocytes. Expression, distribution and functional regulation of GLUT isoforms by hypoxia, hypoxia mimetics, growth factors and pro-inflammatory cytokines. *Adv Anat Embryol Cell Biol.* 200, 1-84

Mobasheri A., Carter S.D., Martin-Vasallo P., Shakibaei M., 2002. Integrins and stretch activated ion channels: Putative components of functional cell surface

mechanoreceptors in articular chondrocytes. *Cell Biol Int*. 26, 1, 1-18.

Mobasheri A., Henrotin Y., 2011. Biomarkers of Osteoarthritis: A Review of Recent Research Progress on Soluble Biochemical Markers Published Patents and Areas for Future Development. *Recent Patents on Biomarkers,* 1, 1-18

Mobasheri A., Richardson S., Mobasheri R., Shakibaei M., Hoyland J.A., 2005. Hypoxia inducible factor-1 and facilitative glucose transporters GLUT1 and GLUT3: Putative molecular components of the oxygen and glucose sensing apparatus in articular chondrocytes. *Histol Histopathol,* 20, 4, 1327-38

Mobasheri A., Shakibaei M., 2011. Applications of Mesenchymal Stem Cells in Cartilage Tissue Engineering. *Recent Patents on Regenerative Medicine*, 1, 1-12

Molloy M.P., Herbert B.R., Walsh B.J., Eler M.I., Traini M., Sanchez J.C., Hochstrasser D.F., Williams K.L., Gooley A.A., 1998. Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis. *Electrophoresis*, 19, 837-844.

Nesvizhskii A.I., Aebersold R., 2005. Interpretation of Shotgun Preteomic Data, *Molecular & Cellular Proteomics*, 4, 1419-1440.

Nilsson C.L., Davidsson P., 2000. NEW SEPARATION TOOLS FOR COMPREHENSIVE STUDIES OF PROTEIN EXPRESSION BY MASS SPECTROMETRY. *Mass Spectrometry*

Reviews, 19, 390– 397.

Poole C.A., 1997. Articular cartilage chondrons: form, function and failure. *J. Anat*. 191, 1-13

Pryde J.G., Phillips J.H., 1986. Fractionation of membrane proteins by temperature-induced phase separation in Triton X-114. *Biochem. J.* 233, 525-533

Peng J., Elias J.E., Thoreen C.C., Licklider L.J., Gygi S.P., 2003. Evaluation of Multidimensional Chromatography Coupled with Tandem Mass Spectrometry (LC/LC-MS/MS) for Large-Scale Protein Analysis: The Yeast Proteome. *Journal of Proteome Research*, 2, 43-50

Pufe T., Lemke A., Kurz B., Petersen W., Tillmann B., Grodzinsky A.J., Mentlein R., 2004. Mechanical overload induces VEGF in cartilage discs via hypoxia- inducible factor. *Am.J.Pathol.* 164, 1, 185–192.

Rabilloud T., 2003. Membrane proteins ride shotgun. *Nat Biotechnol*, 21, 508-510.

Ramsby M.L., Makowski G.S., Khairallah E.A., 1994. Differential detergent fractionation of isolated hepatocytes: Biochemical, immunochemical and two-dimensional gel electrophoresis characterization of cytoskeletal and noncytoskeletal Compartments. Electrophoresis, 15, 265–277.

Ratcliffe, A., Mow, V.C., 1991. The structure, function and biologic repair of articular cartilage. In Bone and cartilage allograft (Friedlaender, G. E., and Goldberg, V. M., eds) pp. 123-154, American Academy of Orthopaedic Surgeons, Park Ridge, IL, USA

Rifai N., Gillette M.A., Carr S.A., 2006. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *NATURE BIOTECHNOLOGY,* 4, 8, 971-983

Robertson C.M., Pennock A.T., Harwood F.L., Pomerleau A.C., Allen R.T., Amiel D., 2006. Characterization of pro-apoptotic and matrix-degradative gene expression following induction of osteoarthritis in mature and aged rabbits. *Osteoarthritis Cartilage*, 14, 471-476.

Roughley P.J., Lee E.R., 1994. Cartilage proteoglycans: Structure and potential functions. *Microsc Res Tech*., 28, 5, 385-97.

Salter D.M., Hughes D.E., Simpson R., Gardner D.L., 1992. Integrin expression by human articular chondrocytes. *Br J Rheumatol*, 31, 231–234.

Santoni V., Molloy M., Rabilloud T., 2000. Membrane proteins and proteomics: Un amour impossible? *Electrophoresis*, 21, 1054-1070

Schnabel M., Marlovits S., Eckhoff G., Fichtel I., Gotzen L., Ve´csei V., Schlegel J., 2002. Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. *Osteoarthritis and Cartilage*, 10, 62–70

Selber K., Tjerneld F., Collen A., Hyytia T., Nakari-Setala T., Bailey M., Fagerstrom R., Kan J., *et al.* 2004. Large-scale separation and production of engineered proteins, designed for facilitated recovery in detergent-based aqueous two-phase extraction systems. *Process Biochem.* 39, 889-896.

Shakibaei M., 1995. Integrin expression on epiphyseal mouse chondrocytes in monolayer culture. *Histol Histopathol*, 10, 339–349.

Shen Y., Smith R.D., 2002. Proteomics based on high-efficiency capillary separations, *Electrophoresis,* 23, 3106–3124

Shetty J., Diekman A.B., Jayes F.C.L., Sherman N.E., Naaby-Hansen S., Flickinger C.J., Herr J.C., 2001. Differential extraction and enrichment of human sperm surface proteins in a proteome: Identification of immunocontraceptive candidates. *Electrophoresis*, 22, 3053–3066.

Shetty J., Diekman A.B., Jayes F.C.L., Sherman N.E., Naaby-Hansen S., Flickinger C.J., Herr J.C., 2001. Differential extraction and enrichment of human sperm surface proteins in a proteome: Identification of immunocontraceptive candidates. *Electrophoresis*, 22, 3053–3066

Siuzdak G., 2006. The Expanding Role of Mass Spectrometry in Biotechnology, Second Edition

Sophia A.J., Bedi A., Rodeo S.A., 2009. The basic science of Articular Cartilage: Structure, Composition, and Function. *Sports Health: A Multidisciplinary Approach*, 1, 461-468

Soule H.D., Vazguez J., Long A., Albert S., Brennan M., 1973. A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst.* 51, 5, 1409-16.

Stasyk T, Huber L.A., 2004. Zooming in: Fractionation strategies in proteomics. *Proteomics*, 4, 3704–3716.

Stewart I.I., Figeys T.T.D., 2001. O^{18} labeling: a tool for proteomics. *Rapid Commun. Mass Spectrom*. 15, 2456–2465.

Tibesku C.O., Szuwart T., Ocken S.A., Skwara A., Fuchs S., 2005. Increase in the Expression of the Transmembrane Surface Receptor CD44v6 on Chondrocytes in Animals with Osteoarthritis. *ARTHRITIS & RHEUMATISM*, 52, 3, 810–817.

Todd-Allen R., Robertson C.M., Harwood F.L., Sasho T., Williams S.K., Pomerleau A.C., Amiel D., 2004. Characterization of mature vs aged rabbit articular cartilage: analysis of cell density, apoptosis-related gene expression and mechanisms controlling chondrocyte apoptosis. *Osteoarthritis Cartilage*, 12:917-923.

Van Ling R., Dolman B., Sneekes E., Swart R., Van Gils M., 2007. Fully Automated Off-Line Multidimensional LC Methods in Proteomics. *J Biomol Tech.* 18, 1, 21

Wei X., Messner K., 1999. Maturation-dependent durability of spontaneous cartilage repair in rabbit knee joint. *J Biomed Mater Res*. 46, 539-548.

Wilkins M.R., et al. 1996. From Proteins to Proteomes: Large Scale Protein Identification by Two-Dimensional Electrophoresis and Amino Acid Analysis, *Nature Biotechnology,* 14, 1, 61–65

Wilkins M.R., Williams K.L., Appel R.D., Hochstrasser D.F., 1997. Proteome Research: New Frontiers in Functional Genomics, Springer, Berlin, Germany

Wu C.C., Yates J.R., 2003. The application of mass spectrometry to membrane proteomics. Nature Biotechnology, 21, 262 – 267.

Zhai G., Hart D.J., Kato B.S., MacGregor A., Spector T.D., 2007. Genetic influence on the progression of radiographic knee osteoarthritis: a longitudinal twin study. *Osteoarthritis Cartilage*, 15, 222-225.

Zhang L., Xie J., Wang X., Liu X., Tang X, Cao R., Hu W., Nie S., Fan C., Liang S., 2005. Proteomic analysis of mouse liver plasma membrane: Use of differential extraction to enrich hydrophobic membrane proteins. *Proteomics*, 5, 4510–4524.

Zhang, L., Wang, X., Peng, X., Wei, Y., Cao R., Liu Z., Xiong J., Ying X., Chen P., Liang S., 2007. Immunoaffinity purification of plasma membrane with secondary antibody superparamagnetic beads for proteomic analysis. *J. Proteome Res.* 6, 34–43.

Zhao Y., Zhang W., Kho Y., Zhao Y., 2004. Proteomic Analysis of Integral Plasma Membrane Proteins. Anal. Chem. 76, 1817-1823

http://www.cascadewellnessclinic.com/

http://www.grin.com

http://www.who.int/chp/topics/rheumatic/en/

http://www.nlm.nih.gov

http://www.osteoarthritisblog.com

8. Appendics

- Appendix 1 : All the proteins identified in the shotgun method

Proteins identified in hydrophobic fraction using the shotgun method

name	Mascot Score	Accession	Sequence Coverage [%]	No. of matched peptides	location	Molecular function	Biological process
Chain A, Structure Of Bovine Beta-Actin-Profilin Complex With Actin Bound Atp Phosphates Solvent Accessible	619.4	gi|2624850	48.8	14	cytoplasm	Nucleotide binding	Cytoskeleton organization
actin 5	600.0	gi|67782283	47.6	1	1.Cytoplasm 2.Cytoskeleton	ATP binding	
beta-actin	569.5	gi|33526989	46.9	1	1.Cytoplasm 2.Cytoskeleton	ATP binding	
alpha-2-macroglobulin isoform 1	563.0	gi|119893035	14.7	16	Secreted	serine-type endopeptidase inhibitor activity	
muscle actin（Actin, alpha skeletal muscle）	544.8	gi|797290	42.4	1	1.Cytoplasm 2.Cytoskeleton.	1. ADP binding 2. ATP binding 3. myosin binding 4. structural constituent of cytoskeleton	1. muscle filament sliding 2. skeletal muscle fiber development 3. skeletal muscle thin filament assembly
KIAA0120	453.6	gi|40788953	47.5	9	1. Nuclear 2. Plasma membrane	protein binding	Muscle organ development
voltage-dependent anion channel 1	428.2	gi|4507879	32.9	8	1.Mitochondrion outer membrane 2.Plasma membrane	1. porin activity 2. protein binding 3. voltage-gated anion channel activity	1. Apoptosis 2. Host-virus interaction 3. Ion transport 4. Transport
tropomyosin 2 (beta) isoform 2 isoform 1	412.9	gi|73971272	33.1	10	1. Cytoplasm 2. Cytoskeleton.	1. actin binding 2. structural constituent of muscle	1. muscle filament sliding 2. regulation of ATPase activity
keratin 1	395.1	gi|119395750	14.9	8	Plasma membrane	1. protein binding 2. receptor activity	1. complement activation, lectin pathway 2. epidermis development

						3. structural constituent of cytoskeleton 4. sugar binding	3. fibrinolysis 4. regulation of angiogenesis 5. response to oxidative stress
integrin beta 1	365.8	gi|114326428	18.9	1	Plasma membrane	1. identical protein binding 2. protein heterodimerization activity 3. receptor activity	1. Cell adhesion 2. Host-virus interaction
tropomyosin 4	350.2	gi|4507651	34.3	4	1. Cytoplasm 2. Cytoskeleton	1. actin binding 2. calcium ion binding 3. structural constituent of muscle	1. cellular component movement 2.muscle filament sliding 3. response to oxidative stress
GTP-binding protein, 23K (Ras-related protein Rab-7a)	343.3	gi|92022	60.7	10	1.Cytoplasmic vesicle 2. Endosome 3. Lysosome	1. GDP binding 2. GTP binding 3. GTPase activity 4. Rac GTPase binding	1. Protein transport 2. Transport
RAB1, member RAS oncogene family isoform 3 (RAB1, member RAS oncogene family)	335.9	gi|109103158	47.1	9	Golgi apparatus	GTP binding	1. intracellular protein transport 2. small GTPase mediated signal transduction
ribosomal protein P2	309.2	gi|4506671	53.0	4	ribosome	1. RNA binding 2. structural constituent of ribosome	1. endocrine pancreas development 2. translational elongation 3.translational termination 4. viral transcription
thrombospondin 1	301.4	gi|62089410	7.8	8	1. plasma membrane 2. extracellular matrix 3. fibrinogen complex 4. platelet alpha granule lumen	1. mediates cell-to-cell and cell-to-matrix interactions. 2. binds heparin. 3. dentinogenesis and/or maintenance of dentin and dental pulp 4. Ligand for CD36 mediating antiangiogenic properties.	Cell adhesion
tropomyosin 1 alpha chain isoform 4	299.1	gi|63252900	30.3	8	1. Cytoplasm 2. Cytoskeleton	1. actin binding 2. structural constituent of cytoskeleton 3. structural constituent of muscle	1. cardiac muscle contraction 2. cellular component movement 3. cellular response to reactive oxygen species 4. muscle filament sliding 5. cell migration 6. ATPase activity 7. cell adhesion

							8. heart rate by epinephrine 9. stress fiber assembly 10. muscle contraction 11. ruffle organization 12. sarcomere organization 13. ventricular cardiac muscle tissue morphogenesis 14. wound healing
myosin catalytic light chain LC17b	283.3	gi|89246	42.0	6	myosin complex	1. actin-dependent ATPase activity 2. calcium ion binding 3. structural constituent of muscle	
voltage-dependent anion channel 2	259.3	gi|55664661	24.8	7	Mitochondrion outer membrane	1. nucleotide binding 2. porin activity 3. protein binding 4. voltage-gated anion channel activity	1. Ion transport 2. Transport
MYL6 protein	244.7	gi|113812151	44.7	6	1.cytosol 2.unconventional myosin complex	1. actin-dependent ATPase activity 2. calcium ion binding 3. motor activity 4. structural constituent of muscle	1. axon guidance 2. muscle filament sliding 3.skeletal muscle tissue development
cathepsin D	232.7	gi|13637914	22.1	6	1. Lysosome. 2. Melanosome	aspartic-type endopeptidase activity	proteolysis
smooth muscle protein SM22 homolog	231.0	gi|543113	19.3	6	cytoplasm	actin binding	muscle organ development
enolase 1	225.9	gi|87196501	22.1	6	1. Cytoplasm 2. Plasma membrane	1. magnesium ion binding 2. phosphopyruvate hydratase activity	1. Glycolysis 2. Plasminogen activation
transgelin 2	223.5	gi|55960374	33.7	5	1. nuclear membrane 2. plasma membrane	protein binding	muscle organ development
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	195.9	gi|6166169	15.8	4	1. Cytoplasm 2. Plasma membrane 3. Nucleus	1. NAD binding 2.glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity 3. peptidyl-cysteine S-nitrosylase activity 4. protein binding	1. Apoptosis 2. Glycolysis
prosaposin	188.3	gi|120419464	6.5	4	lysosome		sphingolipid metabolic process

peptidylprolyl isomerase A (cyclophilin A)	182.7	gi|28189246	29.3	4	Cytoplasm	1. peptide binding 2. peptidyl-prolyl cis-trans isomerase activity	1. protein folding 2. regulation of viral genome replication
keratin 10 isoform 3	178.2	gi|109115262	10.7	5	keratin filament (cell component)	1. protein binding 2. structural molecule activity	1. cellular response to calcium ion 2. epithelial cell differentiation
Protein S100-A6 (S100 calcium-binding protein A6) (Calcyclin)	165.3	gi|6094218	26.1	2	1.Plasma membrane 2. Cytoplasm 3. Nucleus	1. calcium ion binding	
Far upstream element binding protein 1 (FUSE binding protein 1) (FBP) (DNA helicase V) (HDH V) isoform 17	163.9	gi|73959784	10.0	6	Nucleus	1. RNA binding 2. protein binding 3. sequence-specific DNA binding transcription factor activity 4. single-stranded DNA binding	1. Transcription 2. Transcription regulation
ras-related GTP-binding protein RAB10	162.2	gi|33695095	16.5	1	Plasma membrane	1. GTP binding 2. protein binding	1. protein transport 2. small GTPase mediated signal transduction
Chain E, Structure Of Bovine Heart Cytochrome C Oxidase At The Fully Oxidized State	156.3	gi|1942990	43.1	4	Plasma membrane	1. electron carrier activity	1. oxidation reduction
5' nucleotidase, ecto isoform 2	155.2	gi|109071913	11.5	4	Plasma membrane	1. 5'-nucleotidase activity 2. nucleotide binding	1. AMP catabolic process 2. adenosine biosynthetic process 3. negative regulation of inflammatory response
ALB protein (BSA)	154.9	gi|74267962	8.9	5	Secreted	1. DNA binding 2. drug binding 3. fatty acid binding 4. metal ion binding 5. pyridoxal phosphate binding 6. toxin binding	1. cellular response to starvation 2. hemolysis by symbiont of host erythrocytes 3. maintenance of mitochondrion location 4. negative regulation of apoptosis 5. transport
sperm-membrane associated protein P47	145.6	gi|3421015	11.6	4	Plasma membrane		cell adhesion
CD44 antigen (Phagocytic glycoprotein I) (PGP-1) (HUTCH-I) (Extracellular matrix receptor-III)	144.2	gi|461713	11.7	4	Plasma membrane	1. hyaluronic acid binding 2. receptor activity	cell adhesion

CD73, isoform CRA_b	137.4	gi|119569020	11.5	4	Plasma membrane	1. 5'-nucleotidase activity 2. nucleotide binding	1. DNA metabolic process 2. purine base metabolic process 3. pyrimidine base metabolic process 4. pyrimidine nucleoside catabolic process
reticulocalbin 3, EF-hand calcium binding domain	135.3	gi|73947965	13.1	4	Endoplasmic reticulum lumen	1. calcium ion binding 2. protein binding	
Ras-related protein Rap-1b	132.5	gi|7661678	32.1	4	1. Plasma membrane 2. Cytoplasm	1. GDP binding 2. GTP binding 3. GTPase activity 4. protein binding	1. blood coagulation 2. energy reserve metabolic process 3. regulation of establishment of cell polarity 4. regulation of insulin secretion
Myristoylated alanine-rich C-kinase substrate (MARCKS) (ACAMP-81)	129.8	gi|585447	11.4	4	1. Plasma membrane 2.Cytoplasm	1. actin binding 2. calmodulin binding	
Chain B, Heterotrimeric Complex Of PhosducinTRANSDUCIN BETA-Gamma	124.5	gi|4139469	14.4	3	Photoreceptor inner segment	signal transducer activity	response to stimulus
Keratin, type I cytoskeletal 14 (Cytokeratin-14) (CK-14) (Keratin-14) (K14)	123.8	gi|73974023	9.8	2	1. Cytoplasm. 2. Nucleus	1. protein binding 2. structural constituent of cytoskeleton	1. epidermis development 2. hemidesmosome assembly 3. intermediate filament bundle assembly
rap1a protein	120.1	gi|56118668	20.7	3	Plasma membrane	1. GTP binding 2. GTPase activity	small GTPase mediated signal transduction
cofilin 1 (non-muscle)	119.2	gi|119594857	28.5	3	1. Nucleus matrix 2. Cytoplasm 3. cytoskeleton.	Actin binding	1. Rho protein signal transduction 2. actin cytoskeleton organization 3. anti-apoptosis 4. axon guidance 5. platelet activation 6. platelet degranulation 7. response to virus
CD151 antigen	117.6	gi|74136205	12.6	4	Plasma membrane	protein binding	1. cell adhesion 2. hemidesmosome assembly
ATP synthase beta subunit	109.5	gi|71370874	10.4	3	Plasma membrane	1. ATP binding 2. hydrogen ion transporting ATP synthase activity, rotational mechanism 3. hydrogen-exporting ATPase activity, phosphorylative mechanism	1. ATP synthesis 2. Hydrogen ion transport 3. Ion transport 4. Transport

						4. proton-transporting ATPase activity, rotational mechanism	
mannose-6-phosphate receptor (cation dependent) isoform 3	108.5	gi|109095548	31.4	3	1. Lysosome 2. Plasma membrane	1. mannose binding 2. mannose transmembrane transporter activity 3. transmembrane receptor activity	1. endosome to lysosome transport 2. receptor-mediated endocytosis
Glucosidase II beta subunit precursor (Protein kinase C substrate, 60.1 kDa protein, heavy chain) (PKCSH) (80K-H protein)	103.7	gi|73986886	3.7	2	endoplasmic reticulum lumen	1. calcium ion binding 2. protein kinase C binding	1. innate immune response 2. intracellular protein kinase cascade 3. post-translational protein modification 4. protein N-linked glycosylation via asparagine 5. protein folding
Thioredoxin (Trx)	103.3	gi|20140452	21.0	2	1. Nucleus. 2. Cytoplasm 3. Secreted	1. electron carrier activity 2. protein binding 3. protein disulfide oxidoreductase activity	1. Electron transport 2. Transcription 3. Transcription regulation 4. Transport
vesicle-associated membrane protein 3	100.7	gi|4759300	33.0	2	1. Cell junction 2. Plasma membrane 3.Synapse 4. Synaptosome	protein binding	1. Protein transport 2. Transport
brain abundant, membrane attached signal protein 1 （BASP1）	98.0	gi|109076755	11.9	2	Plasma membrane	1. protein domain specific binding 2. transcription corepressor activity	1. glomerular visceral epithelial cell differentiation 2. negative regulation of transcription, DNA-dependent
lysosomal-associated membrane protein 2 （LAMP 2）	90.5	gi|77736087	3.9	2	1. Plasma membrane 2.Endosome 3.Lysosome	1. protection 2. cell adhesion 3.Intracellular signal transduction.	1. platelet activation 2. platelet degranulation
protein disulfide-isomerase (EC 5.3.4.1) ER60	90.3	gi|1083063	7.2	3	1. endoplasmic reticulum lumen 2. melanosome	1. electron carrier activity 2. protein disulfide isomerase activity 3. protein disulfide oxidoreductase activity	1. cell redox homeostasis 2. glycerol ether metabolic process 3. positive regulation of apoptosis
Thioredoxin domain containing protein 1 (Transmembrane Trx-related protein) (Thioredoxin-related transmembrane protein)	87.6	gi|73963782	5.4	2	1. Endoplasmic reticulum 2. Plasma membrane	1. arsenate reductase (thioredoxin) activity 2. disulfide oxidoreductase activity	1. Electron transport 2. Transport

S100 calcium binding protein A11	80.1	gi|45384028	10.9	1	1. cytoplasm 2. nucleus	calcium ion binding	S100 calcium binding protein A11
CD9 antigen (p24) (Leukocyte antigen MIC3) (Motility-related protein) (MRP-1) (Tetraspanin-29) (Tspan-29)	77.9	gi|73997761	15.5	2	Plasma membrane	1. platelet activation and aggregation. 2. Regulates paranodal junction formation. 3. cell adhesion, cell motility and tumour metastasis. 4. sperm-egg fusion	1. Cell adhesion 2. Fertilization
CD63 antigen	77.5	gi|57619037	5.9	2	1.Plasma membrane 2.late endosome membrane 3.lysosomal membrane	play some role in signal transduction pathways	
MGC81440 protein	76.9	gi|46249590	11.5	2	Plasma membrane		
ATP5A1 protein	75.6	gi|13938339	8.3	2	1.Mitochondrion inner membrane 2. Plasma membrane	1. ATP binding 2. MHC class I protein binding 3. eukaryotic cell surface binding 4. hydrogen ion transporting ATP synthase activity, rotational mechanism 5. proton-transporting ATPase activity, rotational mechanism	1. ATP synthesis 2. Hydrogen ion transport 3. Ion transport 4. Transport
Alpha-1-antiproteinase	73.1	gi|57526646	4.3	2	Secreted	1. Protease inhibitor 2. Serine protease inhibitor	
integral membrane serine protease Seprase	70.6	gi|119887629	4.2	2	Plasma membrane	1. Hydrolase 2. Protease 3. Serine protease	1. endothelial cell migration 2. negative regulation of extracellular matrix disassembly 3. proteolysis
myosin:SUBUNIT=regulatory light chain isoform 2	66.3	gi|76651842	24.8	2	myosin complex	calcium ion binding	
caveolin 1 isoform beta	64.8	gi|4972627	18.6	1	1. Plasma membrane 2.Golgi apparatus membrane	1. cholesterol binding 2. nitric-oxide synthase binding 3. peptidase activator activity 4. protein binding 5. protein complex scaffold 6. receptor binding	Host-virus interaction
phosphatidylethanolamine-binding	61.5	gi|90969257	20.5	2	Cytoplasm	1. ATP binding	

protein						2.phosphatidylethanolamine binding 3. serine-type endopeptidase inhibitor activity	
Translocon-associated protein subunit delta	60.1	gi|119593214	10.0	1	1.Endoplasmic reticulum membrane	1. calcium ion binding 2. protein binding	intracellular protein transport
ribosomal protein S27a	55.8	gi|17933007	47.1	1	ribosome	structural constituent of ribosome	translation
ribosomal protein P1	57.0	gi|37725082	17.6	1	ribosome	structural constituent of ribosome	translational elongation
S100 calcium-binding protein A1	51.2	gi|57089139	16.0	1	Cytoplasm	1. ATPase binding 2. S100 alpha binding 3. S100 beta binding 4. calcium ion binding 5. protein homodimerization activity	1. intracellular signal transduction 2. regulation of heart contraction
ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	50.4	gi|21536220	8.3	1	.Mitochondrion inner membrane	1. hydrogen ion transporting ATP synthase activity, rotational mechanism 2. proton-transporting ATPase activity, rotational mechanism	ATP synthesis coupled proton transport
Receptor expression-enhancing protein 5 (Polyposis locus protein 1 homolog) (TB2 protein homolog) (GP106)	45.5	gi|2498308	4.9	1	Plasma membrane	protein binding	
RNA binding motif protein 3 isoform b	45.9	gi|63054840	20.0	1	1. Nucleus 2. Cytoplasm	1. RNA binding 2. nucleotide binding 3. ribosomal large subunit binding	1. RNA processing 2.positive regulation of translation 3. production of miRNAs involved in gene silencing by miRNA
alpha-tubulin isoform 1	44.9	gi|28189695	15.2	1	Microtubule	GTP binding	1. microtubule-based process 2. protein polymerization
CD81 antigen	43.2	gi|13399775	22.2	1	Plasma membrane	protein binding	Host-virus interaction

Table 4: All the proteins identified in hydrophobic fraction using the shotgun method are shown.

Proteins identified in hydrophilic fraction using the shotgun method

name	Mascot Score	Accession	No. of matched peptides	Sequence coverage (%)	location	Molecular function	Biological process
alpha-2-macroglobulin isoform 1	1510.8	gi|119893035	36	30.5	Extracellular space	serine-type endopeptidase inhibitor activity	
tropomyosin 4	996.0	gi|4507651	19	52.4	1.Cytoplasm 2.Cytoskeleton.	1.actin binding 2.calcium ion binding 3.structural constituent of muscle	1.cellular component movement 2.muscle filament sliding 3.response to oxidative stress
tropomyosin 2 (beta) isoform 2 isoform 1	953.4	gi|73971272	11	55.3	1.Cytoplasm 2.Cytoskeleton	1.actin binding 2.structural constituent of muscle	1.muscle filament sliding 2.regulation of ATPase activity
tropomyosin 1 alpha chain	882.7	gi|63252900	12	60.2	1.Cytoplasm 2.Cytoskeleton	1.actin binding 2.structural constituent of cytoskeleton 3.structural constituent of muscle	1.cardiac muscle contraction 2.cellular component movement 3.cellular response to reactive oxygen species 4.muscle filament sliding 5.negative regulation of cell migration 6.positive regulation of ATPase activity … etc
tropomyosin 3 isoform 2 isoform 18	779.4	gi|73961101	10	60.8	1.Cytoplasm 2.Cytoskeleton	actin binding	1.cellular component movement 2.muscle filament sliding 3.regulation of muscle contraction
heat shock 70kD protein 5	569.6	gi|25742763	12	22.3	Plasma membrane	1.ATP binding 2.ribosome binding	1.ER overload response 2.negative regulation of transforming growth factor beta receptor signaling pathway
procollagen-proline, 2-oxoglutarate 4-dioxygenase	565.8	gi|27806501	14	33.5	Endoplasmic reticulum lumen	1.L-ascorbic acid binding 2.iron ion binding 3.oxidoreductase activity, 4.procollagen-proline 4-dioxygenase activity	
thrombospondin 1	521.5	gi|109080672	14	11.3	1.external side of plasma membrane 2.extracellular matrix 3.fibrinogen complex 4.platelet alpha granule lumen	1.calcium ion binding 2.heparin binding 3.structural molecule activity	Cell adhesion
Chain A, Structure Of Bovine Beta-Actin-Profilin Complex With Actin Bound Atp Phosphates Solvent Accessible	492.8	gi|2624850	12	39.2	cytoplasm	1.nucleotide binding 2.structural constituent of cytoskeleton 3.ATP binding 4.identical protein binding	1.protein folding 2.sarcomere organization 3.chaperone mediated protein folding independent of cofactor
Collagen-binding protein 2 (Collagen 2) (Rheumatoid arthritis related antigen RA-A47)	479.1	gi|73988139	1	35.9	Endoplasmic reticulum	1.collagen binding 2.serine-type endopeptidase inhibitor activity	1.regulation of proteolysis 2.response to unfolded protein

Pyruvate kinase, isozymes M1/M2 isoform 1 (Pyruvate kinase muscle isozyme) (Cytosolic thyroid hormone-binding protein) (CTHBP) (THBP1)	449.6	gi|74000677	12	31.1	1.Cytoplasm 2.Nucleus	1.ATP binding 2.magnesium ion binding 3.potassium ion binding 4.protein binding 5.pyruvate kinase activity	1.glycolysis 2.programmed cell death
Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	447.0	gi|75061499	10	23.7	Cytoplasm	1.ATP binding 2.ATPase activity, coupled 3.protein binding	1.Host-virus interaction 2.Stress response 3.Transcription
smooth muscle protein SM22 homolog - bovine (fragments)	444.5	gi|543113	9	49.7	Cytoplasm	actin binding	muscle organ development
transgelin 2	440.9	gi|74006321	9	42.8	1.Nuclear membrane 2.Plasma membrane	protein binding	muscle organ development
glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (EC 1.2.1.12)	439.5	gi|65987	9	38.6	1.Cytoplasm, 2.Plasma membrane 3.Nucleus	1.NAD binding 2.glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity 3.peptidyl-cysteine S-nitrosylase activity 4.protein binding	1.gluconeogenesis 2.glycolysis 3.neuron apoptosis 4.peptidyl-cysteine S-trans-nitrosylation 5.protein stabilization
Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1)	431.0	gi|1346343	9	16.1	Plasma membrane	1.protein binding 2.receptor activity 3.structural constituent of cytoskeleton 4.sugar binding	1.complement activation, lectin pathway 2.epidermis development 3.fibrinolysis 4.regulation of angiogenesis 5.response to oxidative stress
prolyl 4-hydroxylase, beta subunit	424.3	gi|73964749	1	19.6	1.Plasma membrane 2.Endoplasmic reticulum	1.electron carrier activity 2.procollagen-proline 4-dioxygenase activity 3.protein disulfide isomerase activity 4.protein disulfide oxidoreductase activity	1.cell redox homeostasis 2.glycerol ether metabolic process 3.lipid metabolic process 4.lipoprotein metabolic process 5.peptidyl-proline hydroxylation to 4-hydroxy-L-proline
Protein disulfide isomerase-associated 3 isoform 2	417.5	gi|109080872	13	31.7	Endoplasmic reticulum	1.electron carrier activity 2.isomerase activity 3.protein disulfide oxidoreductase activity	1.cell redox homeostasis 2.glycerol ether metabolic process
Phosphoglycerate kinase 1	387.5	gi|52783777	9	31.9	Cytoplasm	1.ATP binding 2.phosphoglycerate kinase activity	1.gluconeogenesis 2.glycolysis
enolase 1	368.3	gi|87196501	9	27.2	1.Cytoplasm 2.Plasma membrane	1.magnesium ion binding 2.phosphopyruvate hydratase activity	glycolysis
triosephosphate isomerase 1, isoform CRA_b	366.6	gi|119609128	8	39.5	cytosol	triose-phosphate isomerase activity	1.fatty acid biosynthetic process 2.gluconeogenesis 3.glycolysis 4.pentose-phosphate shunt
protein disulfide-isomerase (EC	366.0	gi|1083063	1	22.1	Endoplasmic reticulum	1.electron carrier activity	1.cell redox homeostasis

5.3.4.1) ER60						2.procollagen-proline 4-dioxygenase activity 3.protein disulfide isomerase activity 4.protein disulfide oxidoreductase activity	2.glycerol ether metabolic process 3.lipid metabolic process 4.lipoprotein metabolic process 5.peptidyl-proline hydroxylation to 4-hydroxy-L-proline
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	360.6	gi|109112233	10	23.2	Endoplasmic reticulum	1.ATP binding 2.ATPase activity 3.protein domain specific binding	
L-lactate dehydrogenase A chain isoform 2 (LDH-A)	318.3	gi|73988675	7	21.7	Cytoplasm	1.L-lactate dehydrogenase activity 2.protein binding	1.glycolysis 2.pyruvate metabolic process
Calreticulin	313.7	gi|11693172	10	34.1	Endoplasmic reticulum lumen	1.calcium ion binding 2.hormone binding 3.iron ion binding 4.mRNA binding 5.peptide binding 6.sugar binding 7.unfolded protein binding	1.cardiac muscle cell differentiation 2.cellular response to lithium ion 3.cellular response to organic substance 4.negative regulation of translation 5.protein folding 6.response to drug 7.response to estradiol stimulus 8.response to testosterone stimulus 9.spermatogenesis
calumenin isoform 2 isoform 1	313.0	gi|109068104	8	31.4	1.Golgi apparatus, 2.Extracellular region, 3.Melanosome, 4.Sarcoplasmic reticulum lumen	1.calcium ion binding 2.protein binding	1.platelet activation 2.platelet degranulation
Chain A, Fructose-1,6-Bisphosphate Aldolase From Rabbit Muscle	310.2	gi|67464529	7	24.0	Cytoplasm	1.fructose-bisphosphate aldolase activity 2.protein binding	1.glycolysis 2.protein homotetramerization
tubulin, beta polypeptide	305.8	gi|57209813	8	28.4	Microtubule	1.GTP binding 2.GTPase activity 3.structural molecule activity	1.microtubule-based movement 2.protein polymerization
upsilon-crystallin	305.0	gi|30844319	1	20.5	Cytoplasm	1.L-lactate dehydrogenase activity 2.binding	glycolysis
peptidylprolyl isomerase A	300.4	gi|47523764	6	41.5	Cytoplasm	1.peptide binding 2.peptidyl-prolyl cis-trans isomerase activity	1.protein folding 2.regulation of viral genome replication
cofilin 1 (non-muscle)	296.9	gi|5031635	6	42.2	1.Cytoplasm, 2.Cytoskeleton, 3.Nuclear matrix	actin binding	1.Rho protein signal transduction 2.actin cytoskeleton organization 3.anti-apoptosis 4.axon guidance 5.platelet activation 6.platelet degranulation 7.response to virus

Peroxiredoxin-4 (Prx-IV) (Thioredoxin-dependent peroxide reductase A0372) (Antioxidant enzyme AOE372)	277.7	gi|109130180	7	32.5	1.Cytoplasm, 2.Secreted	thioredoxin peroxidase activity	1.I-kappaB phosphorylation 2.cell redox homeostasis
14-3-3 protein	265.6	gi|530049	6	31.6	1.Cytoplasm, 2.Melanosome	1.histone deacetylase binding 2.phosphoserine binding	Host-virus interaction
tumor rejection antigen gp96	259.4	gi|58865966	5	9.5	1.Endoplasmic reticulum lumen, 2.Melanosome		tumor rejection antigen gp96
phosphoglycerate mutase 1 (brain) isoform 1	258.7	gi|109100083	7	42.9	Cytosol	1.2,3-bisphospho-D-glycerate 2-phosphohydrolase activity 2.bisphosphoglycerate mutase activity 3.phosphoglycerate mutase activity 4.protein kinase binding	1.gluconeogenesis 2.glycolysis 3.regulation of glycolysis 4.regulation of pentose-phosphate shunt 5.respiratory burst
myosin, heavy polypeptide 9, non-muscle	233.4	gi|12667788	9	7.9	Myosin complex	1.ATP binding 2.actin binding 3.motor activity	
Filamin A (Endothelial actin-binding protein) (ABP-280)	224.9	gi|74008815	7	3.2	1.Cytoplasm, 2.Cytoskeleton	1.Fc-gamma receptor I complex binding 2.GTP-Ral binding 3.Rac GTPase binding …etc	1.actin crosslink formation 2.actin cytoskeleton reorganization 3.cell junction assembly …etc
annexin A2	217.3	gi|54020966	6	24.8	1.Basement membrane, 2.Extracellular matrix, Secreted	1.calcium ion binding 2.calcium-dependent phospholipid binding 3.cytoskeletal protein binding 4.phospholipase inhibitor activity	
tyrosine 3-monooxygenase/tryptopan 5-monooxygenase activation protein epsilon isoform	217.2	gi|55824574	3	30.5	Cytoplasm	1.monooxygenase activity 2.protein domain specific binding	
Annexin A1 (Annexin I) (Lipocortin I) (Calpactin II)	215.4	gi|38604884	6	30.3	1.Cilium, 2.Cytoplasm, 3.Plasma membrane, 4.Nucleus,	1.calcium ion binding 2.calcium-dependent phospholipid binding 3.phospholipase A2 inhibitor activity 4.protein binding, bridging 5.receptor binding 6.structural molecule activity	1.alpha-beta T cell differentiation 2.anti-apoptosis 3.cell surface receptor linked signaling pathway 4.cellular component movement 5.inflammatory response 6.keratinocyte differentiation 7.lipid metabolic process 8.peptide cross-linking 9.positive regulation of vesicle fusion
Erp57	211.3	gi|114793397	1	41.6	Endoplasmic reticulum	Isomerase	
moesin, isoform CRA_b	204.7	gi|119625804	6	12.9	1.Cytoplasm, 2.Cytoskeleton,	1.cell adhesion molecule binding 2.receptor binding	1.leukocyte cell-cell adhesion 2.leukocyte migration

					3.Plasma membrane	3.structural constituent of cytoskeleton	3.membrane to membrane docking
alpha-1 antiproteinase, antitrypsin	201.5	gi|27806941	5	10.1	Secreted	serine-type endopeptidase inhibitor activity	regulation of proteolysis
nucleolin, C23	198.2	gi|387050	7	10.3	1.Cytoplasm, 2.Nucleus	1.RNA binding 2.nucleotide binding 3.protein C-terminus binding 4.telomeric DNA binding	angiogenesis
keratin 10 isoform 2	193.4	gi|114667513	5	12.2	Keratin		
SET	184.3	gi|3953617	3	16.7	1.Cytoplasm, 2.Endoplasmic 3.reticulum, 4.Nucleus	1.Multitasking protein 2.histone binding.	nucleosome assembly
Caldesmon (CDM)	169.6	gi|2498204	5	6.8	1.Cytoplasm 2.Cytoskeleton	1.actin binding 2.calmodulin binding 3.myosin binding 4.tropomyosin binding	1.cellular component movement 2.muscle contraction
reticulocalbin 3, EF-hand calcium binding domain	167.1	gi|73947965	3	11.0	Endoplasmic reticulum lumen	1.calcium ion binding 2.protein binding	
Hemopexin	157.6	gi|77736171	6	13.9	Extracellular region	metal ion binding	transport
ALB protein	157.2	gi|74267962	5	10.5	Secreted		
keratin 9 (epidermolytic palmoplantar keratoderma)	154.7	gi|119581148	5	11.0	Intermediate filament	1.protein binding 2.structural constituent of cytoskeleton	1.intermediate filament organization 2.skin development
Chain A, Structure Of The Phosphatidylethanolamine-Binding Protein From Bovine Brain	148.9	gi|6729706	3	23.1	Cytoplasm	1.ATP binding 2.lipid binding 3.serine-type endopeptidase inhibitor activity	
ACTN4 protein	141.9	gi|33874637	3	6.9	1.Cytoplasm, 2.Nucleus	1.actin filament binding 2.calcium ion binding 3.integrin binding 4.nucleoside binding 5.protein homodimerization activity	1.Protein transport 2.Transport
Lasp-1	141.4	gi|1407651	4	24.9	1.Cytoplasm, 2.Cytoskeleton	1.SH3/SH2 adaptor activity 2.ion transmembrane transporter activity 3.zinc ion binding	1.Ion transport 2.Transport
Glucosidase II beta subunit (Protein kinase C substrate, 60.1 kDa protein, heavy chain) (PKCSH) (80K-H protein)	140.4	gi|73986886	2	5.1	Endoplasmic reticulum lumen	1.calcium ion binding 2.protein kinase C binding	1.innate immune response 2.intracellular protein kinase cascade 3.post-translational protein modification 4.protein N-linked glycosylation via asparagine 5.protein folding
phosphatidylethanolamine binding	137.7	gi|114326321	2	26.2	Cytoplasm	1.ATP binding	

protein 1						2.lipid binding 3.serine-type endopeptidase inhibitor activity	
peroxiredoxin 1	134.9	gi|55959888	3	29.9	Cytoplasm	1.protein binding 2.thioredoxin peroxidase activity	1.cell proliferation 2.cell redox homeostasis 3.hydrogen peroxide catabolic process 4.skeletal system development
profilin 1	127.1	gi|4826898	3	25.7	1.Cytoplasm 2.Cytoskeleton	1.actin binding 2.proline-rich region binding	1.actin cytoskeleton organization 2.platelet activation 3.platelet degranulation
Keratin, type II cytoskeletal 2 epidermal (Cytokeratin-2e) (CK 2e)	126.6	gi|547754	3	4.5	1.Golgi apparatus, 2.Keratin filament	1.protein binding 2.structural constituent of cytoskeleton	1.keratinization 2.keratinocyte activation 3.keratinocyte migration 4.keratinocyte proliferation
lectin, galactoside-binding, soluble, 1 (galectin 1) isoform 1	124.0	gi|109149584	4	32.0	Extracellular matrix	1.galactoside binding 2.signal transducer activity	1.apoptosis 2.positive regulation of I-kappaB kinase/NF-kappaB cascade 3.regulation of apoptosis
Complement C4-A (Acidic complement C4)	121.6	gi|119915491	4	3.9	Secreted	endopeptidase inhibitor activity	1.complement activation, classical pathway 2.inflammatory response 3.innate immune response
heterogeneous nuclear ribonucleoprotein A2/B1 isoform 2 isoform 7	115.2	gi|73976092	3	15.6	1.Cytoplasm, 2.Nucleus	1.RNA binding 2.nucleotide binding 3.protein binding 4.single-stranded telomeric DNA binding	RNA transport
Protein disulfide-isomerase A6, isoform CRA_a	107.0	gi|119621354	2	15.1	1.Plasma membrane, 2.Endoplasmic reticulum	1.electron carrier activity 2.protein binding 3.protein disulfide isomerase activity 4.protein disulfide oxidoreductase activity	1.cell redox homeostasis 2.glycerol ether metabolic process 3.protein folding
Thioredoxin (Trx)	104.2	gi|20140452	2	21.0	1.Nucleus, 2.Cytoplasm, 3.Secreted	1.electron carrier activity 2.protein binding 3.protein disulfide oxidoreductase activity	1.Electron transpot 2.Transcription 3.Transcription regulation 4.Transport
glutathione transferase (EC 2.5.1.18) class pi 2 - pig	100.0	gi|108301	2	15.8	Cytosol	Transferase	
caldesmon 1 isoform 2 isoform 1	96.3	gi|109068315	2	4.0	1.Cytoplasm, 2.Cytoskeleton	1.actin binding 2.calmodulin binding 3.myosin binding 4.tropomyosin binding	1.cellular component movement 2.muscle contraction
connective tissue growth factor	95.9	gi|83016755	3	24.6	Extracellular matrix	insulin-like growth factor binding	regulation of cell growth
HSD48	91.3	gi|56181368	2	15.7	1.Cytoplasm, 2.Nucleus	DNA binding	1.interspecies interaction between organisms 2.protein transport

							3.transcription, DNA-dependent 4.translation
Chain A, Solution Structure Of Calcium-Calmodulin N-Terminal Domain	88.1	gi|16974825	2	39.5	1.Cytoplasm, 2.Cytoskeleton		
hnRNP A/B related protein	81.3	gi|5052976	2	9.3	1.Cytoplasm, 2.Nucleus	1.nucleic acid binding 2.nucleotide binding	1.epithelial to mesenchymal transition 2.positive regulation of transcription, DNA-dependent 3.transcription, DNA-dependent
YBX1 protein	79.0	gi|33875177	2	13.5	1.Cytoplasm, 2.Nucleus, 3.Secreted	1.RNA binding 2.double-stranded DNA binding 3.protein binding 4.sequence-specific DNA binding transcription factor activity 5.single-stranded DNA binding	1.Transcription 2.Transcription regulation 3.mRNA processing 4.mRNA splicing
ubiquitin	75.7	gi|229532	2	33.8	Cytoplasm	1.ATP binding 2.ubiquitin protein ligase binding 3.ubiquitin-ubiquitin ligase activity	1.protein K48-linked ubiquitination 2.ubiquitin-dependent protein catabolic process
acidic (leucine-rich) nuclear phosphoprotein 32 family, member B	75.0	gi|55958589	2	18.6	1.Cytoplasm, 2.Nucleus	Chaperone	
SET translocation	72.0	gi|123295279	2	16.6	nucleus		nucleosome assembly
GDP dissociation inhibitor 2	72.0	gi|56410848	2	14.4	Cytoplasm	Rab GDP-dissociation inhibitor activity	protein transport
Polyubiquitin	71.4	gi|1675359	2	39.0	1.Cytoplasm, 2.Nucleus	structural constituent of ribosome	translation
Plasminogen activator inhibitor 1 RNA-binding protein (PAI-RBP1)	70.4	gi|73956307	2	5.0	1.Cytoplasm 2.Nucleus	1.mRNA 3'-UTR binding 2.protein binding	regulation of mRNA stability
acidic (leucine-rich) nuclear phosphoprotein 32 family, member B isoform 1	68.6	gi|109110852	2	15.5	Nucleus	Chaperone	
lysosomal-associated membrane protein 2	64.3	gi|77736087	1	2.0	1.Lysosome, 2.Plasma membrane		transport
Hnrpa2b1 protein	64.1	gi|37747847	2	12.3	1.Cytoplasm, 2.Nucleus, 3.Spliceosome	1.RNA binding 2.nucleotide binding 3.protein binding 4.single-stranded telomeric DNA binding	1.RNA splicing 2.RNA transport 3.mRNA processing
macrophage migration inhibitory factor	56.3	gi|187181	1	8.5	Cytoplasm	Cytokine Isomerase	1.Immunity 2.Inflammatory response 3.Innate immunity
60S ribosomal protein L22 (Heparin-binding protein HBp15)	55.5	gi|113414714	1	18.1	cytosolic large ribosomal subunit	1.RNA binding 2.heparin binding 3.structural constituent of ribosome	1.endocrine pancreas development 2.translational elongation 3.translational termination

							4.viral transcription
ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	52.1	gi|21536220	1	8.3	Mitochondrion	1.hydrogen ion transporting ATP synthase activity, rotational mechanism 2.proton-transporting ATPase activity, rotational mechanism	ATP synthesis coupled proton transport
RSB-11-77	51.0	gi|30313489	1	2.4	Cytoplasm	mitogen-activated protein kinase binding	1.cell differentiation 2.multicellular organismal development 3.spermatogenesis
myosin light chain	49.9	gi|85812161	1	14.9	Myosin complex	calcium ion binding	
Metallothionein	45.5	gi|55925012	1	35.3	Cytosol	metal ion binding	
40S ribosomal protein S19	43.5	gi|82966156	1	11.9	1.Nucleolus, 2.Cytoplasm	structural constituent of ribosome	1.erythrocyte differentiation 2.gas transport 3.translation
FK506 binding protein12	41.5	gi|95007801	1	35.1	Cytoplasm	1.FK506 binding 2.peptidyl-prolyl cis-trans isomerase activity 3.receptor binding	
putative secreted protein ZSIG9	41.3	gi|6014632	1	19.0	Endoplasmic reticulum	protein binding	

Table 5: All the proteins identified in hydrophilic fraction using the shogun method are shown.

- Appendix 2: All the proteins identified in the gel-based method

Proteins identified in gel-based method

Hydrophobic bands							
MP1311 A1 Mass range: 150-100 kDa							
name	**Mascot Score /MW**	**Accession**	**Sequence Coverage [%]**	**No. of matched peptides**	**location**	**Molecular function**	**Biological process**
Protein S100-A6 (S100 calcium-binding protein A6) (Calcyclin)	117.1 /10.3	gi|6094218	43.5	2	1.plasma membrane 2.Cytoplasm 3.Nucleus	1.S100 beta binding 2.calcium ion binding 3.calcium-dependent protein binding 4.protein homodimerization activity 5.tropomyosin binding	1.axonogenesis 2.positive regulation of fibroblast proliferation 3.signal transduction
alpha-2-macroglobulin isoform 1	72.3 /164.2	gi|119893035	2.9	2	Secreted	1.serine-type endopeptidase inhibitor activity	
beta-actin (Actin, cytoplasmic 1)	68.9 /16.9	gi|84626383	29.1	2	1.Cytoplasm 2.Cytoskeleton	ATP binding	
eukaryotic translation elongation factor 1 alpha 2 isoform 1	63.8 /15.9	gi|74010091	20.3	1	Nucleus	1.GTP binding 2.GTPase activity 3.translation elongation factor activity	Protein biosynthesis
glyceraldehyde-3-phosphate dehydrogenase	51.5 /23.6	gi|89573911	9.5	1	1.plasma membrane 2.Cytoplasm 3.Nucleus	Oxidoreductase Transferase	Apoptosis Glycolysis
ENO3, muscle enolase 3 beta	45.2/ 16.9	gi|8546856	14.3	1	Cytoplasm	1.magnesium ion binding 2.phosphopyruvate hydratase activity	1.gluconeogenesis 2.glycolysis
MP1311 B1 Mass range: 40-30 kDa							
Protein S100-A6 (S100 calcium-binding protein A6) (Calcyclin)	89.4/ 10.3	gi|6094218	43.5	2	1.plasma membrane 2.Cytoplasm 3.Nucleus	1.S100 beta binding 2.calcium ion binding 3.calcium-dependent protein binding 4.protein homodimerization activity 5.tropomyosin binding	1.axonogenesis 2.positive regulation of fibroblast proliferation 3.signal transduction
PKM2 protein	68.8/ 37.3	gi|34782802	9.3	2	1.Cytoplasm 2.Nucleus 3.plasma membrane	1.ATP binding 2.magnesium ion binding 3.potassium ion binding 4.protein binding 5.pyruvate kinase activity	1.glycolysis 2.programmed cell death

beta-actin	48.5/ 8.6	gi|48237757	29.9	1	1.Cytoplasm 2.Cytoskeleton	ATP binding	
lysosomal-associated membrane protein 2	43.6/ 44.5	gi|77736087	9.6	1	1.plasma membrane 2.Lysosome		transport
MP1311 C1 Mass range: 30-25 kDa							
Protein S100-A6 (S100 calcium-binding protein A6) (Calcyclin)	76.1/ 10.3	gi|6094218	43.5	2	1.plasma membrane 2.Cytoplasm 3.Nucleus	1.S100 beta binding 2.calcium ion binding 3.calcium-dependent protein binding 4.protein homodimerization activity 5.tropomyosin binding	1.axonogenesis 2.positive regulation of fibroblast proliferation 3.signal transduction
MP1311 D1 Mass range: 25-20 kDa							
Protein S100-A6 (S100 calcium-binding protein A6) (Calcyclin)	53.0/ 10.3	gi|6094218	16.3	1	1.plasma membrane 2.Cytoplasm 3.Nucleus	1.S100 beta binding 2.calcium ion binding 3.calcium-dependent protein binding 4.protein homodimerization activity 5.tropomyosin binding	1.axonogenesis 2.positive regulation of fibroblast proliferation 3.signal transduction
MP1311 E1 Mass range: 20-15 kDa							
CD73 antigen (Ecto-5-nucleotidase) (5-NT)	221.7/ 58.8	gi|73973882	13.0	8	plasma membrane	1.5'-nucleotidase activity 2.nucleotide binding	1.DNA metabolic process 2.purine base metabolic process 3.pyrimidine base metabolic process 4.pyrimidine nucleoside catabolic process
5' nucleotidase, ecto	173.2/ 62.9	gi|99028963	8.7	1	plasma membrane	1.5'-nucleotidase activity 2.ferrous iron binding 3.nucleotide binding	purine nucleotide biosynthetic process
ribophorin I	44.4/ 64.5	gi|14124942	2.1	1	Endoplasmic reticulum membrane	1.dolichyl-diphosphooligosaccharide-protein glycotransferase activity 2.protein binding	1.post-translational protein modification 2.protein N-linked glycosylation via asparagine
LDH-A - mouse (fragment)	44.4/ 4.4	gi|2117454	47.6	1	Cytoplasm	1.L-lactate dehydrogenase activity 2.protein binding	1.cellular response to extracellular stimulus 2.glycolysis
MP1311 F1 Mass range: 20-15kDa							
keratin 1	283.4/ 66.0	gi|119395750	15.2	9	plasma membrane	1.protein binding 2.receptor activity 3.structural constituent of cytoskeleton 4.sugar binding	1.complement activation, lectin pathway 2.epidermis development 3.fibrinolysis 4.regulation of angiogenesis 5.response to oxidative stress

MP1311 G1 Mass range: ≈15 kDa							
keratin 10 isoform 3	1227.9/ 58.2	gi|114667511	33.1	24	keratin filament	1.protein binding 2.structural molecule activity	1.cellular response to calcium ion 2.epithelial cell differentiation
keratin 1	1214.7/ 66.0	gi|119395750	38.7	25	plasma membrane	1.protein binding 2.receptor activity 3.structural constituent of cytoskeleton 4.sugar binding	1.complement activation, lectin pathway 2.epidermis development 3.fibrinolysis 4.regulation of angiogenesis 5.response to oxidative stress
mitochondrial ATP synthase, H+ transporting F1 complex beta subunit (ATP synthase subunit beta, mitochondrial)	579.5/ 47.1	gi|89574051	39.1	13	Mitochondrion membrane	1.ATP binding 2.MHC class I protein binding 3.eukaryotic cell surface binding 4.hydrogen ion transporting ATP synthase activity, rotational mechanism 5.hydrogen-exporting ATPase activity, phosphorylative mechanism 6.proton-transporting ATPase activity, rotational mechanism	1.ATP hydrolysis coupled proton transport 2.angiogenesis 3.regulation of intracellular pH 4.respiratory electron transport chain
keratin 2 (Keratin-2 epidermis)	555.7/ 65.4	gi|47132620	27.5	11	1.Golgi apparatus 2.keratin filament	1.protein binding 2.structural constituent of cytoskeleton	1.keratinization 2.keratinocyte activation 3.keratinocyte migration 4.keratinocyte proliferation
keratin 5 isoform 9	377.0/ 51.5	gi|109096809	17.9	6	1.cytosol 2.keratin filament	1.protein binding 2.structural constituent of cytoskeleton	1.epidermis development 2.hemidesmosome assembly
RAX protein	321.6/ 40.1	gi|119850798	15.4	3	nucleus	DNA binding	Transcription regulation
MP1311 H1 Mass range: 10-0 kDa							
enolase 1	402.5/ 47.3	gi|87196501	31.6	12	1. plasma membrane 2.Cytoplasm	1.magnesium ion binding 2.phosphopyruvate hydratase activity	glycolysis
HERC2	42.7/ 20.6	gi|27923090	7.0	1	nucleus		
MP1311 A2 Mass range: 40-30 kDa							
L-lactate dehydrogenase A chain (LDH-A) (LDH muscle subunit) (LDH-M) isoform 2	230.5/ 36.6	gi|73988675	20.2	7	Cytoplasm	1.L-lactate dehydrogenase activity 2.protein binding	1.glycolysis 2.pyruvate metabolic process
skeletal muscle tropomyosin	215.7/ 30.4	gi|339956	22.3	7	1.cytosol 2.troponin complex	Muscle protein	
TPM4	143.6/ 27.5	gi|10441386	11.8	1	1.Cytoplasm 2.Cytoskeleton	1.actin binding 2.calcium ion binding	1.cellular component movement 2.muscle filament sliding

						3.structural constituent of muscle	3.response to oxidative stress
MP1411 D2 Mass range: 80-60 kDa							
voltage-dependent anion channel 3 (**VDAC3)**	162.9/30.7	gi|27807415	23.0	5	Mitochondrion outer membrane	1.nucleotide binding 2.porin activity 3.voltage-gated anion channel activity	1.Ion transport 2.Transport
cathepsin D {EC 3.4.23.5} [cattle, Peptide Partial, 346 aa]	110.0/37.7	gi|299522	10.4	4	Lysosome	aspartic-type endopeptidase activity	1.cell death 2.proteolysis
MP1411 G2 Mass range: 50-40 kDa							
transmembrane trafficking protein **(**21 kDa transmembrane-trafficking protein)	66.1/24.8	gi|73963665	10.5	2	Golgi apparatus membrane	protein binding	1.protein transport 2.regulated secretory pathway 3.vesicle targeting, to, from or within Golgi

Table 6: All the proteins identified in hydrophobic bands using gel-based method are shown.

Hydrophilic bands							
MP1311 A3 Mass range: 60-50 kDa							
name	Mascot Score /MW	Accession	Sequence Coverage [%]	No. of matched peptides	location	Molecular function	Biological process
vinculin	2012.6/ 116.8	gi|47522618	47.6	47	1. Plasma membrane 2.Cytoplasm	1.actin binding 2.structural molecule activity	1.lamellipodium assembly 2.regulation of cell migration
lysosomal-associated membrane protein 2	44.9/ 44.5	gi|77736087	2.0	1	1. Plasma membrane 2.Lysosome		transport
keratin 9	44.1/ 57.5	gi|119581148	3.0	1	1.Intermediate filament 2.Keratin	1.protein binding 2.structural constituent of cytoskeleton	1.intermediate filament organization 2.skin development
MP1311 B3 Mass range: 40-30 kDa							
hexokinase 1 isoform 2 [Pan troglodytes]	503.9/ 108.0	gi|114630829	12.8	13	.Mitochondrion membrane	1.ATP binding 2.glucokinase activity	1.glucose transport 2.glycolysis 3.transmembrane transport
lysosomal-associated membrane protein 2	45.7/ 44.5	gi|77736087	2.0	1	1. Plasma membrane 2.Lysosome		transport
MP1311 C3 Mass range: 40-30 kDa							
actinin alpha 1 isoform b	1102.4/ 105.5	gi|94982457	35.2	27	1. Plasma membrane 2.Cytoplasm	1.actin binding 2.calcium ion binding 3.integrin binding 4.vinculin binding	1.focal adhesion assembly 2.negative regulation of cellular component movement 3.platelet activation 4.platelet degranulation 5.regulation of apoptosis
tumor rejection antigen (gp96) 1	924.3/ 92.5	gi|50979166	33.6	25	endoplasmic reticulum	1.ATP binding 2.unfolded protein binding	1.protein folding 2.response to stress
calnexin precursor isoform 8	409.8/ 67.6	gi|109080088	18.1	10	Endoplasmi reticulum membrane	1.calcium ion binding 2.sugar binding 3.unfolded protein binding	1.post-translational protein modification 2.protein N-linked glycosylation via asparagine 3.protein folding 4.protein secretion
alpha glucosidase 2 alpha neutral subunit	341.8/ 109.3	gi|6679891	12.8	11	alpha-glucosidase II complex	1.carbohydrate binding 2.hydrolase activity, hydrolyzing O-glycosyl compounds 3.protein binding	N-glycan processing
MP1311 D3 Mass range: 40-30 kDa							
Calreticulin precursor (CRP55)	499.3/	gi|28558064	32.9	14	1.Cytoplasm	Chaperone	

(Calregulin) (HACBP) (ERp60)	48.2				Endoplasmic reticulum 2.Extracellular matrix 3.Secreted		
keratin 1	215.0/ 66.0	gi|11935049	10.6	6	Plasma membrane	1.protein binding 2.receptor activity 3.structural constituent of cytoskeleton 4.sugar binding	1.complement activation, lectin pathway 2.epidermis development 3.fibrinolysis 4.regulation of angiogenesis 5.response to oxidative stress
Ribophorin-2	208.8/ 65.1	gi|73991922	13.4	5	Endoplasmic reticulum membrane	1.dolichyl-diphosphooligosaccharide-protein glycotransferase activity 2.protein binding	1.post-translational protein modification 2.protein N-linked glycosylation via asparagine
Beta-galactosidase isoform 3	193.2/ 72.7	gi|119372312	9.3	5	1.lysosome 2.cytoplasm	1.beta-galactosidase activity 2.cation binding 3.protein binding	carbohydrate metabolic process
heterogeneous nuclear ribonucleoprotein K isoform 12	95.8/ 44.2	gi|73946451	9.4	3	1.Cytoplasm 2.Nucleus	Ribonucleoprotein	1.Host-virus interaction 2.mRNA processing 3.mRNA splicing
lysosomal-associated membrane protein 2	43.2/ 44.5	gi|77736087	2.0	1	1. Plasma membrane 2.Lysosome		transport
MP1311 E3 Mass range: 30-25 kDa							
enolase 1	888.9/ 47.3	gi|87196501	35.3	17	1. Plasma membrane 2.Cytoplasm	1.magnesium ion binding 2.phosphopyruvate hydratase activity	1.glycolysis
gamma enolase	247.8/ 44.2	gi|182118	17.4	4	1. Plasma membrane 2.Cytoplasm	1.magnesium ion binding 2.phosphopyruvate hydratase activity	1.gluconeogenesis 2.glycolysis
SS-B/La protein	46.2/ 7.2	gi|338497	17.2	1	Nucleus	nucleotide binding	
MP1311 F3 Mass range: ≈25 kDa							
fructose-1,6-bisphosphate aldolase A isoform 2 (**Fructose-bisphosphate aldolase A)**	1047.2/ 51.3	gi|119916947	42.1	19	1.I band 2.actin cytoskeleton 3.cytosol 4.extracellular vesicular exosome 5.platelet alpha granule lumen	Lyase	Glycolysis
Aspartate aminotransferase, mitochondrial (Transaminase A) (Glutamate oxaloacetate transaminase 2)	983.3/ 44.5	gi|112982	51.6	19	1. Plasma membrane 2.Mitochondrion	1.L-aspartate:2-oxoglutarate aminotransferase activity 2.protein binding 3.pyridoxal phosphate binding	1.Lipid transport 2.Transport
tropomyosin 1 alpha chain	701.3/	gi|109081399	49.6	16	1.cytoplasm	actin binding	1.cell adhesion

	32.6				2.cytoskeleton		2.cell migration (etc)
put. beta-actin (aa 27-375) [Mus musculus]	506.3/ 39.2	gi|49868	38.7	11	1.cytoplasm 2.cytoskeleton	ATP binding	
Alpha-actin-2 [Homo sapiens]	452.2/ 42.0	gi|4501883	33.4	4	1.Cytoplasm 2.Cytoskeleton	ATP binding	response to virus
smooth muscle alpha-tropomyosin **(**Alpha-tropomyosin)	428.7/ 14.1	gi|37732143	49.2	1	1.Cytoplasm 2.Cytoskeleton	actin binding	
SET [Mus musculus]	146.0/ 24.3	gi|3953617	27.1	4	1.Cytoplasm 2.Endoplasmic reticulum 3.Nucleus	Multitasking protein	nucleosome assembly
heterogeneous nuclear ribonucleoprotein C isoform 3	106.8/ 10.2	gi|73961866	30.1	2	1.Nucleus 2.Spliceosome	2.RNA binding 1.identical protein binding 3.nucleotide binding	1.mRNA processing 2.mRNA splicing
A+U-rich RNA-binding protein-mouse (fragment)	89.9/ 19.4	gi|2137113	12.4	2	1.Cytoplasm 2.Nucleus	Ribonucleoprotein	1.Transcription 2.Transcription regulation
heterogeneous nuclear ribonucleoprotein A3 isoform 5	78.9/ 21.3	gi|74004686	13.4	2	1.Nucleus 2.Spliceosome	1.RNA binding 2.nucleotide binding 3.protein binding	1.mRNA processing 2.mRNA splicing
ENO1 protein	61.1/ 28.9	gi|39644728	9.1	2	1. Plasma membrane 2.Cytoplasm 3.Nucleus	1.Lyase 2.Repressor	1.Glycolysis 2.Plasminogen activation 3.Transcription 4.Transcription regulation
GTP-binding protein alpha o1 subunit, Go alpha o1 subunit	50.7/ 1.5	gi|233102	73.3	1		1.GTP binding 2.signal transducer activity	G-protein coupled receptor protein signalling pathway
keratin K5	42.7/ 9.4	gi|205055	15.2	1	keratin filament	structural molecule activity	
MP1311 G3 Mass range: 30-25 kDa							
tropomyosin 1, alpha	1264.5/ 32.7	gi|31560030	63.7	28	1.Cytoplasm 2.Cytoskeleton	1.actin binding 2.structural constituent of cytoskeleton	1.cardiac muscle contraction 2.in utero embryonic development 3.positive regulation of heart rate by epinephrine 4.ventricular cardiac muscle tissue morphogenesis
Tropomyosin alpha-4 chain (Tropomyosin-4)	750.8/ 28.5	gi|20178270	39.9	10	1.Cytoplasm 2.Cytoskeleton	1.actin binding 2.calcium ion binding 3.structural constituent of muscle	1.cellular component movement 2.muscle filament sliding 3.response to oxidative stress
mitochondrial malate dehydrogenase 2, NAD	578.4/ 28.4	gi|89574139	53.9	12	Mitochondrion	Oxidoreductase	Tricarboxylic acid cycle
L-lactate dehydrogenase A chain	577.3/	gi|73988675	28.6	11	Cytoplasm	1.L-lactate dehydrogenase activity	1.glycolysis

(LDH-A) (LDH muscle subunit) (LDH-M) (Proliferation-inducing gene 19 protein) isoform 2	36.6					2.protein binding	2.pyruvate metabolic process
upsilon-crystallin	568.2/ 36.4	gi|30844319	29.8	2	cytoplasm	Oxidoreductase	Glycolysis
lactate dehydrogenase B	393.5/ 36.7	gi|116282345	24.3	5	Cytoplasm	Oxidoreductase	Glycolysis
Actin, cytoplasmic 1 (Beta-actin 1)	87.9/ 41.9	gi|57043600	9.0	2	1.Cytoplasm 2.Cytoskeleton	ATP binding	
annexin A8	65.4/ 36.8	gi|27806317	7.6	2		1.calcium ion binding 2.calcium-dependent phospholipid binding	blood coagulation
alpha-1 antiproteinase, antitrypsin	54.2/ 46.0	gi|57526646	2.4	1	Secreted	serine-type endopeptidase inhibitor activity	
pyruvate dehydrogenase beta-subunit	43.2/ 6.5	gi|189758	28.6	1	Mitochondrion	Oxidoreductase	Glycolysis
MP1411 A1 Mass range: 100-80 kDa							
heat-shock 70-kDa protein 5	1970.0/ 66.9	gi|55824560	51.7	32	cytoplasm	ATP binding	
moesin	926.9/ 67.8	gi|4505257	37.6	21	1. Plasma membrane 2.Cytoplasm 3.Cytoskeleton	1.cell adhesion molecule binding 2.receptor binding 3.structural constituent of cytoskeleton	1.leukocyte cell-cell adhesion 2.leukocyte migration 3.membrane to membrane docking
hydroxyacyl dehydrogenase, subunit A isoform 3 (Trifunctional enzyme subunit alpha, mitochondrial)	232.5/ 83.0	gi|109102275	10.1	6	Mitochondrion	1.Lyase 2.Oxidoreductase	1.Fatty acid metabolism 2.Lipid metabolism
keratin 1	144.9/ 64.2	gi|114644564	5.3	3	Plasma membrane	structural molecule activity	
Chain A, Prolyl Oligopeptidase From Porcine Brain, D641a Mutant With Bound Peptide Ligand Suc-Gly-Pro	113.1/ 80.7	gi|27066372	6.5	4	cytoplasm	1.hydrolase activity 2.peptidase activity 3.serine-type peptidase activity 4.serine-type endopeptidase activity 5.prolyl oligopeptidase activity	proteolysis
hemopexin	77.9/ 52.2	gi|77736171	3.1	2	Secreted	metal ion binding	transport
NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (CI-75kD)	59.7/ 79.0	gi|109100713	2.2	1	Mitochondrion	Oxidoreductase	1.Electron transport 2.Respiratory chain 3.Transport
alpha-2-macroglobulin	53.9/	gi|41176597	9.6	1	Secreted		

	15.1						
lysosomal alpha-mannosidase	46.5/ 113.1	gi|18140139	1.3	1	Lysosome	1.Glycosidase 2.Hydrolase	mannose metabolic process
MP1411 B1 Mass range: 100-80 kDa							
Chain A, Structure Of Bovine Beta-Actin-Profilin Complex With Actin Bound Atp Phosphates Solvent Accessible	1296.9/ 41.7	gi|2624850	60.0	24	cytoplasm	1.nucleotide binding 2.protein binding 3.ATP binding	protein folding
phosphoglycerate kinase (EC 2.7.2.3) - horse	1257.3/ 44.5	gi|66893	58.4	22	Cytoplasm	1.ATP binding 2.phosphoglycerate kinase activity	1.glycolysis
alpha-cardiac actin	1046.5/ 41.8	gi|387090	51.5	9	1.Cytoplasm 2.Cytoskeleton	1.ATP binding 2.ATPase activity 3.myosin binding	1.apoptosis 2.cardiac muscle tissue morphogenesis 3.cardiac myofibril assembly 4.muscle filament sliding 5.skeletal muscle thin filament assembly
beta-actin	825.3/ 35.8	gi|2724046	54.9	1	1.Cytoplasm 2.Cytoskeleton	ATP binding	
citrate synthase [Canis familiaris]	189.8/ 51.8	gi|73968367	10.1	5	Mitochondrion	citrate (Si)-synthase activity	1.cellular carbohydrate metabolic process 2.tricarboxylic acid cycle
Chain A, Crystal Structure Of Nadp-Dependent Isocitrate Dehydrogenase From Porcine Heart Mitochondria	51.2/ 46.6	gi|27065480	3.6	1	mitochondrion	oxidoreductase activity	oxidation reduction
MP1411 C1 Mass range: 100-80 kDa							
tropomyosin 2, beta isoform 2 isoform 1	1225.2/ 33.0	gi|73971272	62.0	23	1.Cytoplasm 2.Cytoskeleton	1.actin binding 2.structural constituent of muscle	1.muscle filament sliding 2.regulation of ATPase activity
tropomyosin 1, alpha isoform c	745.2/ 32.8	gi|78000192	49.3	6	1.Cytoplasm 2.Cytoskeleton	actin binding	1.muscle filament sliding 2.negative regulation of cell migration 3.positive regulation of ATPase activity 4.positive regulation of cell adhesion 5.positive regulation of stress fiber assembly 6.ruffle organization 7.wound healing
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	612.4/ 35.8	gi|1169794	37.8	16	1. Plasma membrane 2.Cytoplasm 3.Nucleus	1.Oxidoreductase 2.Transferase	1.Apoptosis 2.Glycolysis
transaldolase 1 isoform 4	440.9/ 37.5	gi|57099501	26.7	10	Cytoplasm	Transferase	1.energy reserve metabolic process 2.xylulose biosynthetic process
beta-actin	112.9/	gi|8809716	25.2	3	1.Cytoplasm	ATP binding	

	25.2				2.Cytoskeleton		
Arginase-2, mitochondrial (Non-hepatic arginase) (Kidney-type arginase)	59.3/ 19.4	gi|73963319	6.6	1	Mitochondrion	1.arginase activity 2.metal ion binding	1.arginine metabolic process 2.nitric oxide biosynthetic process 3.urea cycle
MP1411 D1 Mass range: 50 kDa							
glyceraldehyde-3-phosphate dehydrogenase	531.9/35.8	gi|57163839	34.5	12	1.Cytoplasm 2.Nucleus	1.Oxidoreductase 2.Transferase	1.Apoptosis 2.Glycolysis
Tropomyosin 2, beta isoform 2 isoform 1	438.0/33.0	gi|73971272	48.2	13	1.Cytoplasm 2.Cytoskeleton	1.actin binding 2.structural constituent of muscle	1.muscle filament sliding 2.regulation of ATPase activity
Annexin A1 (Annexin I) (Lipocortin I) (Calpactin II)	410.4/ 38.7	gi|38604884	36.1	9	1.basolateral plasma membrane 2.cilium 3.cytoplasm 4.nucleus	1.calcium ion binding 2.calcium-dependent phospholipid binding 3.phospholipase A2 inhibitor activity	
Nucleophosmin (Nucleolar phosphoprotein B23)	50.8/ 12.6	gi|73953448	17.0	1	Nucleus	Chaperone	Host-virus interaction
MP1411 E1 Mass range: 50-40 kDa							
tropomyosin 1, alpha isoform c	1347.8/32.8	gi|78000192	64.1	29	1.Cytoplasm 2.Cytoskeleton	actin binding	1.muscle filament sliding 2.negative regulation of cell migration 3.positive regulation of ATPase activity 4.positive regulation of cell adhesion 5.positive regulation of stress fiber assembly 6.ruffle organization 7.wound healing
Annexin A5 (Annexin V) (Lipocortin V, Endonexin Ii, Placental Anticoagulant Protein)	469.1/ 35.8	gi|999926	39.5	12	cytoplasm	1.calcium ion binding 2.calcium-dependent phospholipid binding 3.phospholipase inhibitor activity	1.anti-apoptosis 2.blood coagulation 3.negative regulation of coagulation 4.signal transduction
proliferating cell nuclear antigen	117.1/ 28.7	gi|11693142	23.4	4	Nucleus	DNA polymerase processivity factor activity	DNA replication
alpha-soluble NSF attachment protein - rat	108.7/ 33.2	gi|2143586	12.9	3	Plasma membrane		1.cellular membrane fusion 2.intra-Golgi vesicle-mediated transport 3.post-Golgi vesicle-mediated transport
glyceraldehyde-3-phosphate dehydrogenase	42.4/23.6	gi|89573911	9.5	1	cytoplasm	Oxidoreductase	glycolysis
MP1411 F1 Mass range: 40-30 kDa							
Tropomyosin alpha-4 chain (Tropomyosin-4)	1159.6/28.5	gi|20178270	54.0	23	1.Cytoplasm 2.Cytoskeleton	1.actin binding 2.calcium ion binding	1.cellular component movement 2.muscle filament sliding

						3.structural constituent of muscle	3.response to oxidative stress
tropomyosin 3, gamma isoform 2	1156.6/29.0	gi|29336093	60.5	14	1.Cytoplasm 2.Cytoskeleton	actin binding	brain development
Heterogeneous nuclear ribonucleoprotein A1 (Chain , Up1, The Two Rna-Recognition Motif Domain Of Hnrnp A1)	185.7/20.8	gi|2554653	26.9	4	1.Cytoplasm 2.Nucleus	1.nucleotide binding 2.protein bindin 3.single-stranded DNA binding	1.interspecies interaction between organisms 2.mRNA transport 3.nuclear import
CAPZB protein	165.6/21.1	gi|19352984	25.4	5	1.Cytoplasm 2.Cytoskeleton	actin binding	actin cytoskeleton organization
60S ribosomal protein L8 isoform 2	46.2/16.0	gi|73974790	7.5	1	Cytoplasm	1.rRNA binding 2.structural constituent of ribosome	1.endocrine pancreas development 2.translational elongation 3.translational termination 4.viral transcription
SHROOM3 protein	41.2/83.2	gi|13938323	1.2	1	1.Cell junction 2.Cytoplasm 3.Cytoskeleton 4.Microtubule	actin binding	Cell shape

Table 7: All the proteins identified in hydrophilic bands using gel-based method are shown.