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Proteomics and Bioinformatics Analysis of Cartilage in Post-Traumatic Osteoarthritis in a Mini-Pig Model of Anterior Cruciate Ligament Repair

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Although osteoarthritis (OA) is a degenerative disease that is increasingly common with age, the pathogenesis of post-traumatic OA (PTOA) is poorly understood. This study aimed to undertake proteomics and bioinformatics analysis of cartilage in PTOA in a mini-pig model of anterior cruciate ligament repair (ACLR).

Material/Methods: The mini-pig model of PTOA involved autologous orthotopic ACLR. Screening and identification of differentially expressed proteins in the knee joint cartilage in the OA cartilage group were compared with the control group using tandem mass tag (TMT)-labeling liquid chromatography with tandem mass spectrometry (LC-MS-MS). A protein expression level >1.2 fold-change represented protein upregulation and <0.83 fold-change represented protein down-regulation Bioinformatics analysis included Gene Ontology (GO) functional annotation and the Kyoto Encyclopedia of Genes and Genome (KEGG) pathway enrichment analysis to determine the biological functions and pathways of proteins showing altered expression profiles associated with OA.

Results: There were 2,950 proteins screened from the knee cartilage tissues of the OA model group using quantitative TMT-labeling LC-MS-MS. There were 491 proteins identified with altered expression profiles, 198 proteins were upregulated and 293 proteins were down-regulated in the OA cartilage group. GO function and KEGG pathway enrichment analysis of the 491 proteins identified their functions in cellular processes, metabolic processes, and biological regulation.

Conclusions: Proteomics and bioinformatics analysis of cartilage in PTOA in a mini-pig model of ACLR identified OA-related proteins.

MeSH Keywords: Anterior Cruciate Ligament Reconstruction • Osteoarthritis, Knee • Proteomics • Swine, Miniature

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Background

Although osteoarthritis (OA) is a common degenerative disease that is increasingly common with age, the pathogenesis of post-traumatic OA (PTOA) is poorly understood [1]. PTOA is characterized by pathological changes that include degeneration of the articular cartilage, and secondary hyperplasia and ossification of the joint cartilage. Clinical symptoms of PTOA include joint pain and dysfunction. However, the pathogenesis and etiology of PTOA are poorly understood.

Current animal models of OA models involve the destruction of joint structural stability and damage to the joint articular surface created by surgical methods that mimic human PTOA. However, some patients develop long-term PTOA despite having undergone reconstruction of their cruciate ligament and having a relatively stable joint structure. An experimental sheep model of PTOA has previously been established using an autologous ligament autograft reconstruction method following anterior cruciate ligament (ACL) [1]. In this sheep model of PTOA, increased matrix metalloproteinases, and interleukin-like inflammatory mediators were measured in the joint fluid [1]. This type of animal model holds promise for use in research studies on the pathogenesis of PTOA. However, the thin joint cartilage of the sheep poses technical challenges during ACL reconstruction surgery. Therefore, a mini-pig model was developed that included *in situ* ACL autograft reconstruction to simulate the pathophysiological process of OA cartilage degeneration after ACL reconstruction.

Mini-pigs have previously been used as animal models for studies on cardiovascular, gastrointestinal, and urinary disease, and for studies on skin burns, stomatology, and pharmacological toxicology, as well as in preclinical orthopedic studies [2]. Unlike the OA mini-pig model, existing OA animal models have an issue of PTOA development due to inherent joint instability [3]. Therefore, the mini-pig is an ideal animal model to study the mechanisms underlying inflammation and early degenerative changes in PTOA, as well as therapeutic studies. The bone tissues of the mini-pig closely resemble human bone tissue in terms of morphology, bone composition, microstructure, and remodeling characteristics [4].

Therefore, this study aimed to undertake proteomics and bioinformatics analysis of cartilage in post-traumatic osteoarthritis (OA) in a mini-pig model of anterior cruciate ligament repair (ACLR). The OA mini-pig model was established, followed by proteomics screening of the knee joint articular cartilage using tandem mass tag (TMT)-labeling liquid chromatography with tandem mass spectrometry (LC-MS-MS) to screen for and identify differentially expressed proteins. Bioinformatics analysis was used to determine the biological functions and pathways of the differentially expressed OA-related proteins.

Material and Methods

The mini-pig model of post-traumatic osteoarthritis (PTOA) using anterior cruciate ligament repair (ACLR)

Female Bama mini-pigs, 16–18 months of age (Beijing Shichuang Century Mini-Pig Breeding Base, Beijing, China) who had achieved bone maturity were used. The limbs of the mini-pigs were studied in two groups: the group that underwent anterior cruciate ligament repair (ACLR) surgery for PTOA (the OA cartilage group) of the right hind limb, and the control group that included the non-treated left hind limbs. The study design and conduct, including the animal surgery, preoperative feeding, and postoperative care were approved by the Ethics Committee of the Second Hospital of Shanxi Medical University, China, and were performed in accordance with current animal protection agreements and regulations.

Each mini-pig was first anesthetized using an intramuscular injection of 125 mg of Zoletil 50 (25 mg/ml + 25 mg/ml lyophilisate and solvent) (Virbac Group, Carros, France) before ACLR surgery, as previously described [5]. The lateral position was located, and the right hind limb incision was performed with the humerus as the central reference point. The skin, subcutaneous tissue, and joint capsule were then incised in layers. The knee joint was dislocated and flexed, the ACL and the lateral side of the lateral femoral condyle were exposed. A guide needle (Arthrex Inc, Naples, FL, USA) was placed along the ACL reconstruction at a 45° angle between the guide and the longitudinal axis of the femur. A hollow drill created a tunnel about 0.8 cm in diameter from the lateral condyle of the lateral femur to the inner lateral condyle attached by the ACL along the direction of the guide needle. A thin-walled annular bone chisel of 1 mm diameter was used to prevent the cartilage from splitting inside the femoral condyle. The point of attachment of the ACL was removed completely along with the bone segment in the tunnel. A reference mark was made before removal to prevent the bone segment from rotating during restoration. The tendon and bone segment were pushed out of the tunnel. After confirming the integrity of the ACL point of attachment, the tendon segment was pushed back into the tunnel without rotation of the bone. Two crossed Kirschner needles were used to fix the bone in position, the suture was trimmed, the patella was reduced, and the incision was sutured in layers.

The sampling of cartilage in the mini-pig model of PTOA using ACLR

All animals were euthanized four months after surgery. At this time, significant morphological changes of OA were found in the knee joint of the surgical site of the mini-pig. After the left and right hind limbs of the mini-pig were severed from the hip joint, cartilage tissue was removed from the medial and lateral

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tibial plateau, the medial and lateral femoral condyle, and the intercondylar fossa of the knee joint with a small sharp knife. All necessary precautions were taken to prevent samples of subchondral bone from mixing with the cartilage tissue. The excised tissue samples were stored at –80°C for further study.

Protein preparation

Protein was extracted from mini-pig knee articular cartilage tissue samples by lysis using SDT buffer consisting of 4% (w/v) sodium dodecyl sulfate (SDS), 100 mM Tris-HCl (pH 7.6), and 0.1 M dithiothreitol (DTT) (Sigma-Aldrich, St. Louis MO, USA). The protein concentration was detected using the bicinchoninic acid (BCA) assay (Sigma-Aldrich, St. Louis MO, USA). The filter-aided proteome preparation (FASP) method was used for each sample containing an appropriate amount of protein, and the samples underwent trypsin digestion. The OD_{280} value (peptide content) was determined using the NanoDrop™ 2000 microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The quality of the total protein extracted from mini-pig knee cartilage tissues was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sigma-Aldrich, St. Louis MO, USA) and tandem mass tag (TMT)-labeling liquid chromatography with tandem mass spectrometry (LC-MS-MS) (Thermo Fisher Scientific, USA). Each sample of 100 μg of the peptide was tagged using the Tandem Mass Tag™ 6-plex kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

Reverse-phase peptide grading

Each group of tagged peptides was mixed in equal amounts, and the tagged peptides were graded using a high-pH reverse-phase peptide grading kit (Thermo Fisher Scientific, Waltham, MA, USA). The centrifuge column was first equilibrated with acetonitrile and 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis MO, USA) before loading the sample and the mixed tagged peptide samples for desalination. The column-bound peptides underwent gradient elution using increasing concentrations of a high-pH acetonitrile solution. The eluted peptides were vacuum-dried and then re-dissolved and lyophilized with 12 μl 0.1% formic acid (Sigma-Aldrich, St. Louis MO, USA). The peptide concentration (OD_{280} value) was determined using an ultramicrospectrophotometer.

LC-MS-MS data acquisition

Each graded sample was separated using a Thermo Scientific EASY-nLC 1200 high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Waltham, MA, USA). The HPLC buffers included buffer A (0.1% formic acid aqueous solution) and buffer B (0.1% formic acid acetonitrile aqueous solution). The sample was loaded from the autosampler to the Acclaim™ PepMap™ 100 C18 HPLC (100 μm×2 cm) nanoViper column (Thermo Fisher Scientific, Waltham, MA, USA) and was separated using a Thermo Scientific EASY-Column™ capillary column (10 cm; 75 μm ID; 3 μm C18-A2) (Thermo Fisher Scientific, Waltham, MA, USA) at a flow rate of 300 ml/min.

Mass spectrometry (MS) analysis was performed using the positive ion detection method with a Thermo Scientific™ Q Exactive™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The following parameters were used: parent ion scanning range, 300–1800 m/z; first-stage mass spectrometry resolution of 70,000 at 200 m/z; automatic gain control (AGC) of 1×10^6; IT maximum of 50 ms; and 60 s dynamic exclusion time. The mass to charge ratios of polypeptides and their fragments were as follows: 20 fragment maps (MS2 scan) were collected after each full scan; higher-energy collisional dissociation (HCD) MS2 activation type; 2 m/z isolation window; second-stage mass spectral resolution of 17,500 at 200 m/z; normalized collision energy of 30 eV; and 0.1% underfill. The mass spectrometry raw data were identified and quantitatively analyzed using Mascot version 2.2 (Matrix Science, London, UK) and Proteome Discoverer version 1.4 (Thermo Fisher Scientific, Waltham, MA, USA). Differentially expressed proteins were screened based on the criteria that upregulated proteins ($P<0.05$) had a protein expression fold change >1.2, and down-regulated proteins ($P<0.05$) had a protein expression fold change <0.83 (Table 1). Qualitative analysis of the LC-MS-MS data used a false discovery rate (FDR) <0.01 as the screening standard. The MS map data were analyzed in combination with the Mascot version 2.2 analysis tool to obtain the score of each MS2 map.

Bioinformatics analysis

Clustering analysis was performed to classify the data based on similarity for exploratory data analysis [6]. Application of the hierarchical clustering K-mean algorithm was used to differentiate data with highly similar patterns from those with low similarity while taking into account the two dimensions of samples and variables [7,8].

Gene Ontology (GO) (*http://www.geneontology.org/*), a standardized functional classification system was used. GO provided a set of dynamically updated standardized vocabulary, and described the properties of genes and gene products in terms of involved biological process (BP), molecular function (MF), and cellular components (CC) [9]. GO functional annotation of target protein sets was performed using Blast2GO software (BioBam Bioinformatics, Valencia, Spain), using the four steps of blast mapping, GO annotation, and supplemental annotation. GO function analysis was based on function entry for

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Table 1. Tandem mass tag (TMT)-labeling liquid chromatography with tandem mass spectrometry (LC-MS-MS) parameters used for protein identification and analysis.

Item	Value
Enzyme	Trypsin
Max. missed cleavages	2
Fixed modifications	Carbamidomethyl (C), TMT 6/10plex (N-term), TMT 6/10plex (K)
Variable modifications	Oxidation (M), TMT 6/10plex (Y)
Peptide mass tolerance	±20 ppm
Fragment mass tolerance	0.1 Da
Database	uniprot_Sus_scrofa_50051_20180611.fasta
Database pattern	Decoy
Peptide false discovery rate (FDR)	≤0.01
Protein quantification	The protein ratios were calculated as the median of only unique peptides of the protein
Experimental bias	All peptide ratios normalized by the median

the unit, and the results directly showed the overall function of enrichment characteristics of differentially expressed proteins that were significantly enriched in the GO function. This analysis differed from ordinary protein function annotation as it allowed the enrichment of differentially expressed proteins.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) (*http://www.kegg.jp/*) was used, containing pathway information regarding metabolism, genetic information processing, environmental information processing, cell processes, biological systems, human diseases, and drug development [10]. KEGG pathway analysis enabled the systematic and comprehensive understanding of biological processes, disease occurrence mechanisms, or drug action mechanisms [11]. Similar to the GO functional enrichment analysis method, KEGG pathway analysis was treated as a unit and the identified total protein. The KEGG automatic annotation server tool software (KASS) was used for the KEGG pathway annotation of target protein sets.

Statistical analysis

Fisher's exact test was used to compare the distribution of each GO classification or KEGG pathway in the target protein set and the overall protein set for differences in protein enrichment in each pathway. Enrichment analysis of GO function or KEGG pathway was performed on all protein sets. The quantitative information of the target protein set was first normalized (to the –1 to 1 interval) before two-dimensional (2D) classification of the sample and protein expressions (distance algorithm, Euclidean connection method, average linkage) using the ComplexHeatmap R package version 3.4 (Bioconductor, Boston, MA, USA) with generation of the hierarchical clustering heatmap. Interactions between target proteins were determined using the STRING database (*http://string-db.org/*), and the protein interaction network was generated and analyzed using Cytoscape version 3.2.1 software.

Table 2. The proteins identified from tandem mass tag (TMT)-labeling liquid chromatography with tandem mass spectrometry (LC-MS-MS).

Item	Value
Database	uniprot_Sus_scrofa
Spectra (PSM)	61056
Peptides	19161
Unique peptides	14405
Protein groups	2950

Results

Tandem mass tag (TMT)-labeling liquid chromatography with tandem mass spectrometry (LC-MS-MS) of proteins from knee joint cartilage in the mini-pig model

Quantitative TMT-labeling LC-MS-MS of proteins from knee joint cartilage in the mini-pig model of post-traumatic OA (PTOA) following anterior cruciate ligament repair (ACLR) identified 2,950 proteins following extraction and separation of total proteins (Table 2). There were 491 differentially expressed proteins from the osteoarthritis (OA) cartilage group compared with the control (or normal) cartilage group (Table 3, Figure 1), with 198 upregulated proteins and 293 down-regulated proteins in the OA cartilage group.

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Table 3. Proteins with altered expression profiles in the osteoarthritis (OA) and control groups in the post-traumatic osteoarthritis (PTOA) mini-pig model of anterior cruciate ligament repair (ACLR).

Item	Value
Comparison	**OA group versus Control group**
Upregulated proteins	198
Down-regulated proteins	293
All proteins	491

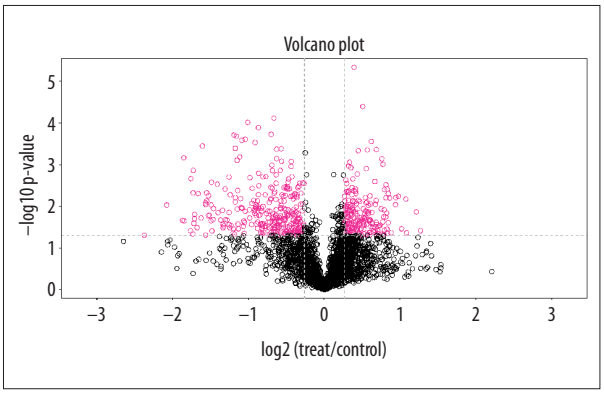

Figure 1. The volcano plot of the osteoarthritis (OA) cartilage group compared with the normal cartilage group in the post-traumatic osteoarthritis (PTOA) mini-pig model of anterior cruciate ligament repair (ACLR). The red circles indicate upregulated proteins with a fold change >1.2 times (P<0.05). The black circles indicate proteins with no change in expression between the two study groups.

Bioinformatics analysis

Hierarchical clustering, presented in a tree-shaped heat map in Figure 2, followed by Gene Ontology (GO) function enrichment analysis (Figure 3), enabled the identification of proteins with altered expression profiles in the OA cartilage group compared with the control group. Clustering analysis confirmed the association between the differentially expressed proteins and OA in this animal model. Also, clustering of proteins with similar expression profiles facilitated the analysis of potentially overlapping biological functions or regulatory pathways, including metabolic and signaling pathways [12]. GO function enrichment analysis [13] enabled the comparison of differentially expressed proteins against all proteins identified in the study (P<0.05). The functions in the GO database were not completely aligned, and represented approximate hierarchical relationships, as shown by the tree-shaped structure in Figure 2, where the higher the level number, the higher the accuracy of the description of protein properties and functions.

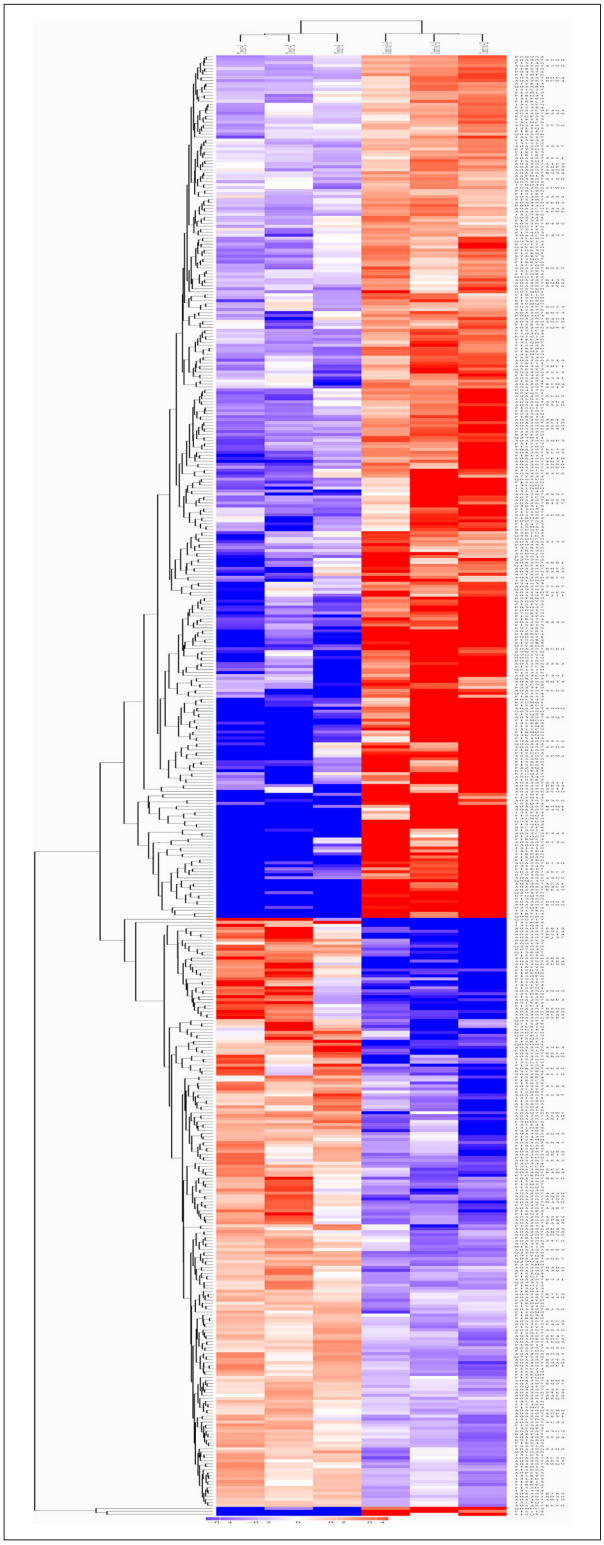

Figure 2. The clustering analysis heatmap shows proteins that were differentially expressed in the osteoarthritis (OA) cartilage group compared with the normal cartilage group in the post-traumatic osteoarthritis (PTOA) mini-pig model of anterior cruciate ligament repair (ACLR).

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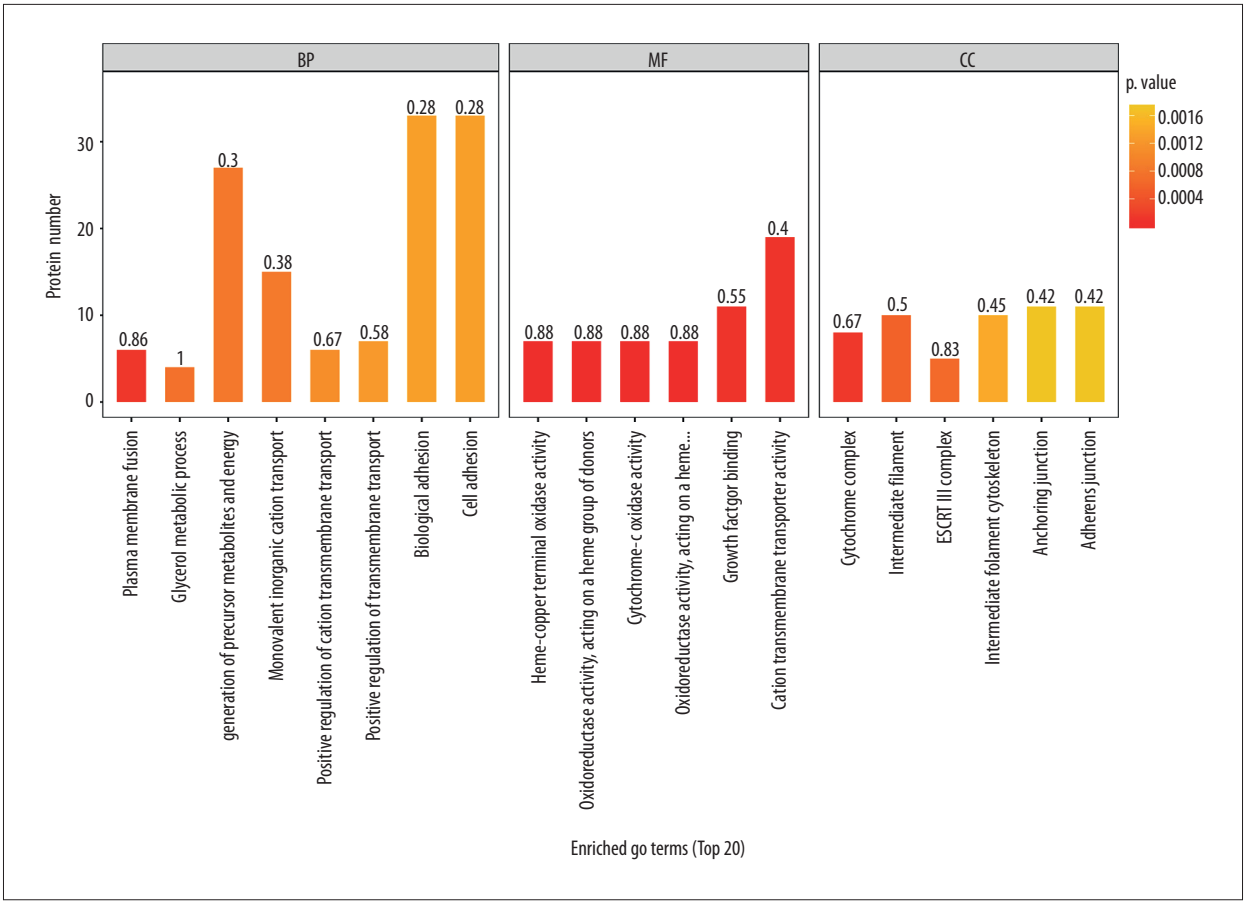

Figure 3. Gene Ontology (GO) function enrichment analysis of the osteoarthritis (OA) cartilage group compared with the normal cartilage group in the post-traumatic osteoarthritis (PTOA) mini-pig model of anterior cruciate ligament repair (ACLR).

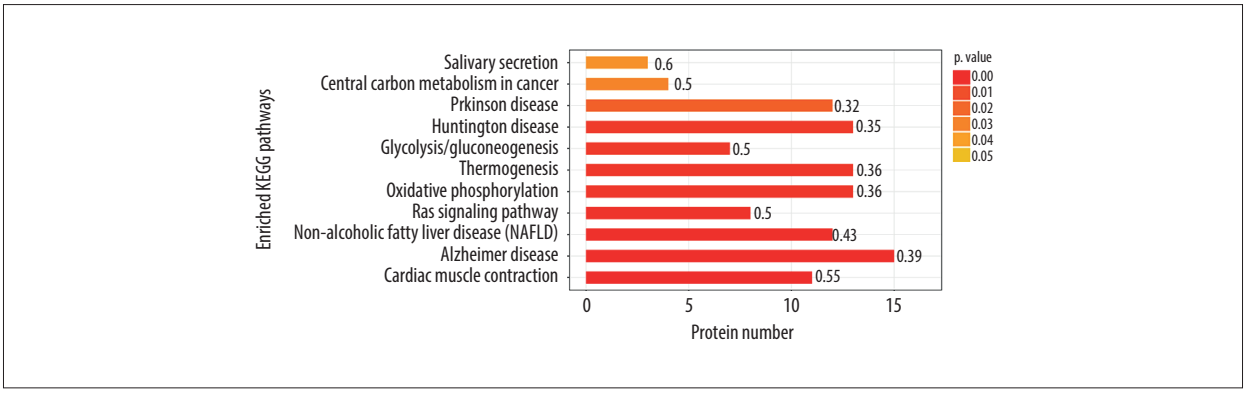

Figure 4. The Kyoto Encyclopedia of Genes and Genome (KEGG) pathway enrichment analysis of the osteoarthritis (OA) cartilage group compared with the normal cartilage group in the post-traumatic osteoarthritis (PTOA) mini-pig model of anterior cruciate ligament repair (ACLR).

In the mini-pig model of PTOA, proteins showing altered expression patterns that were associated with a variety of important biological processes (Figure 3). The list of functions included plasma membrane fusion, glycerol metabolism, the generation of precursor metabolites and energy, monovalent inorganic cation transport, positive regulation of cationic transmembrane transport, enzymatic functions of heme and copper terminal oxidase activity, oxidoreductase activity, and cytochrome-C oxidase activity, redox function, and growth factor binding.

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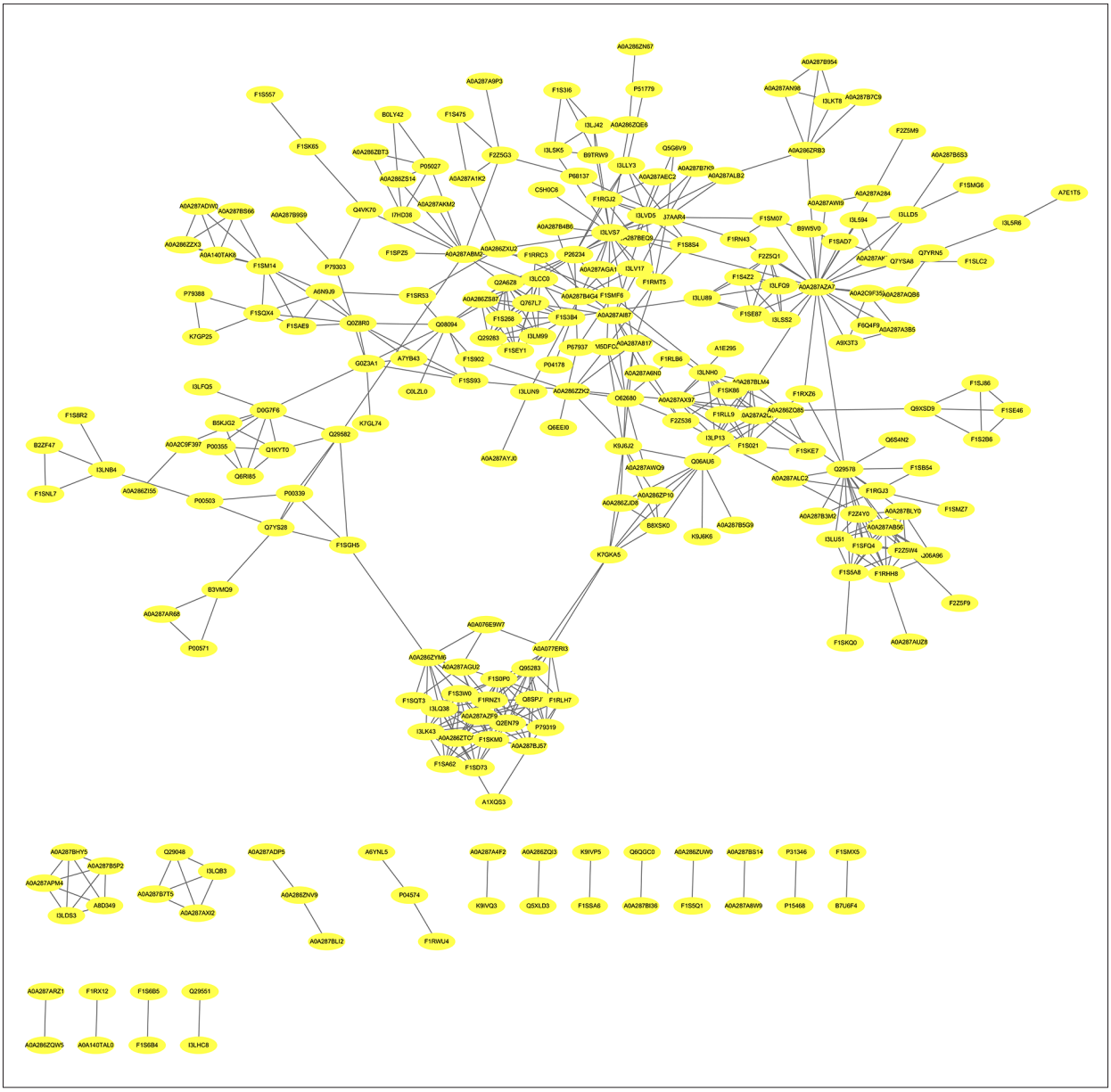

Figure 5. Protein interaction network analysis of differentially expressed proteins in the osteoarthritis (OA) cartilage group compared with the normal cartilage group in the post-traumatic osteoarthritis (PTOA) mini-pig model of anterior cruciate ligament repair (ACLR).

Protein expression profiles

The proteins with altered expression profiles in the OA cartilage group included those belonging to the cytochrome complexes, intermediate filaments, endosomal sorting complexes required for transport (ESCRT)-III complexes, intermediate filament cytoskeleton, and anchoring junctions. The Kyoto Encyclopedia of Genes and Genome (KEGG) pathway enrichment analysis showed altered protein expression in the OA cartilage group, indicating their roles in important pathways including cardiac muscle contraction, Alzheimer's disease, non-alcoholic fatty liver disease (NAFLD), the RAS signaling pathway, and oxidative phosphorylation (Figure 4). Protein interaction network analysis of the OA-related proteins showed altered expression profiles (Figure 5).

Quality assessment of peptides from the experimental and control groups in the mini-pig model of PTOA

The quality of the total protein extracted from mini-pig knee joint cartilage was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which confirmed

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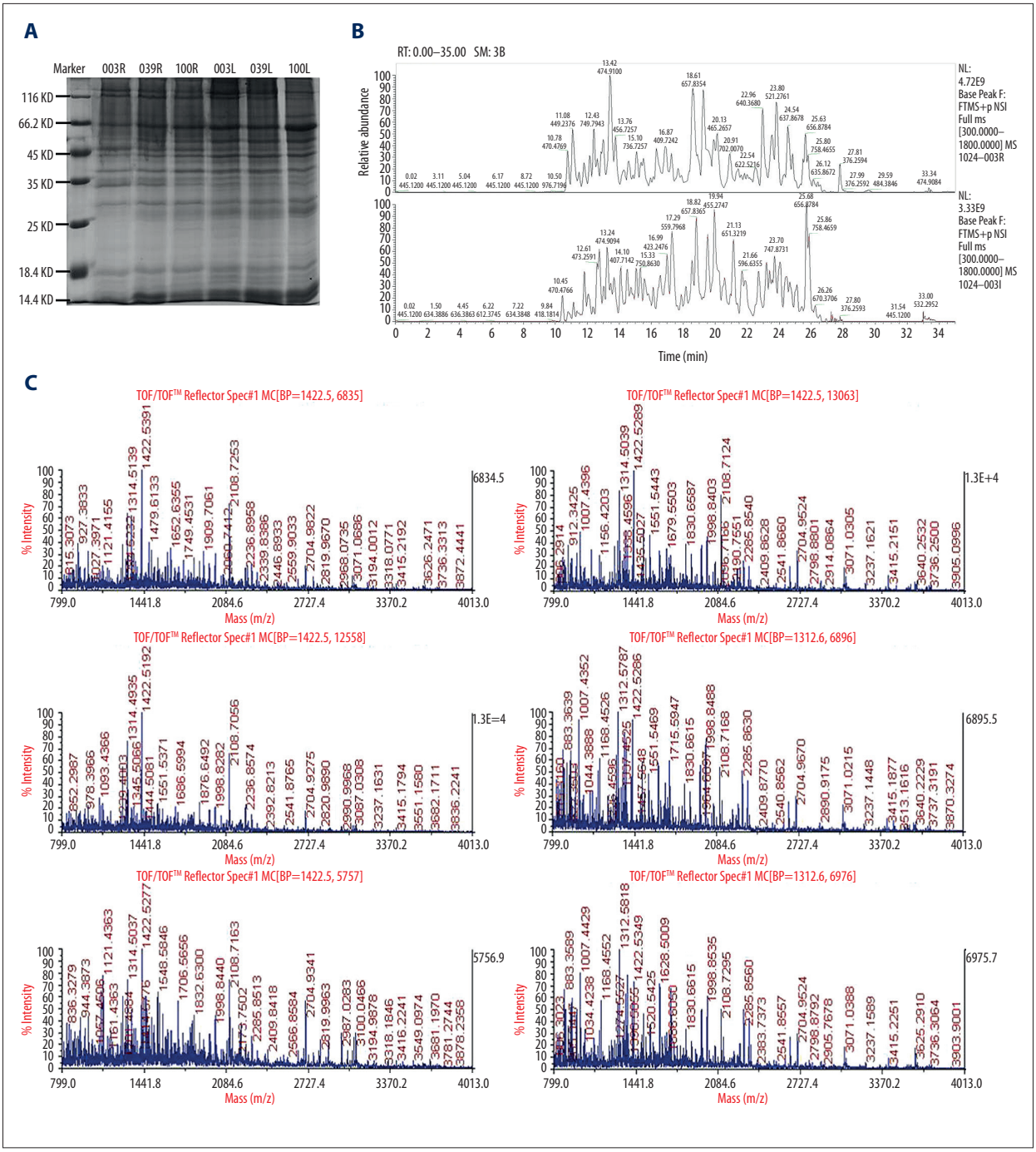

Figure 6. Peptide analysis in the knee joint cartilage in the post-traumatic osteoarthritis (PTOA) mini-pig model of anterior cruciate ligament repair (ACLR). (**A**) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of peptide samples from the OA cartilage group (003R, 039R, 100R) and the control group (003L, 039L, 100L). (**B**) Tandem mass tag (TMT)-labeling liquid chromatography with tandem mass spectrometry (LC-MS-MS) base peak chromatogram. (**C**) Peptide mass fingerprint spectra of all six samples.

the peptide signal intensity, resolution, and consistency between samples (Figure 6A). The chromatogram showed many peaks with different elution times and high relative abundance, indicating that the mini-pig cartilage samples comprised multiple peptides and exhibited higher complexity (Figure 6B). The peptide mass fingerprint spectra showed normal enzymatic hydrolysis for both groups and good consistency between groups (Figure 6C).

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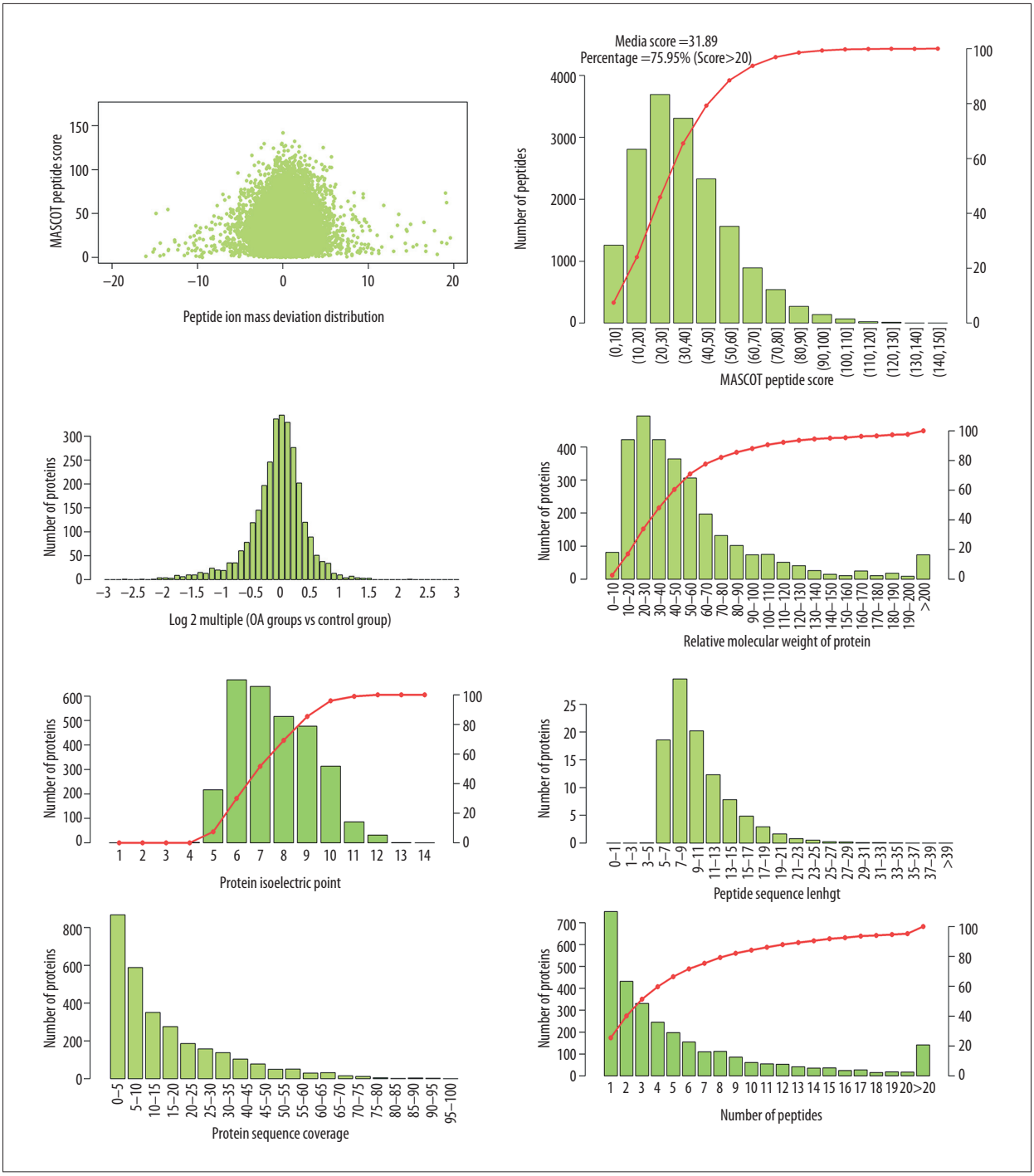

Figure 7. Quality control of the expression profiles of the osteoarthritis (OA) cartilage group compared with the normal cartilage group in the post-traumatic osteoarthritis (PTOA) mini-pig model of anterior cruciate ligament repair (ACLR).

Quality check of protein expression profile analysis

The TMT-labeling LC-MS-MS data acquired using the Thermo Scientific™ Q Exactive™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) were found to be accurate and reliable, as mass deviations of the identified peptides in the MS1 and MS2 spectra were mainly distributed within 10 parts per million (ppm) (Figure 7). All LC-MS-MS data were qualitatively analyzed using a false discovery rate (FDR) <0.01 as a screening standard. The MS map data were analyzed in combination with the Mascot version 2.2 analysis tool to obtain the score of each MS2 map. The Mascot score further confirmed

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the quality of the MS data acquired, with a peptide score of >75.95% equivalent to >20 points, and the median peptide score was 31.89 points.

Discussion

This study used tandem mass tag (TMT)-labeling liquid chromatography with tandem mass spectrometry (LC-MS-MS) of proteins from knee joint cartilage in the mini-pig model of post-traumatic osteoarthritis (PTOA) following of anterior cruciate ligament repair (ACLR). Previous studies on protein expression profiles and the regulatory mechanisms involved offer a direct approach for understanding biological functions [14] and pathogenesis [15–17], as many diseases are associated with altered gene expression [18]. In the present study, a differential expression proteomics approach was chosen rather than the use of functional proteomics [19].

In this study, differentially expressed proteins were screened and identified using expression profiling to understand better the underlying molecular mechanisms involved in OA. The mini-pig model of OA in ACLR was developed and used to screen for OA-related proteins using TMT-labeling LC-MS-MS technology. There were 491 differentially expressed proteins identified, and hierarchical clustering and Gene Ontology (GO) function enrichment analysis identified these proteins in several important biological processes that were associated with catalytic activity, structural activity, transporter activity, and regulation of molecular functions. The Kyoto Encyclopedia of Genes and Genome (KEGG) pathway enrichment analysis identified the metabolic and signal transduction pathways that were altered in OA in this animal model. The proteins with altered expression patterns were found to be primarily involved in cellular process, metabolic process, regulation of the biological process, cellular component organization, and other important biological processes. The findings from this preliminary study in the mini-pig model may serve as the foundation for subsequent studies on the functional roles of the identified OA-related proteins as well as validation of these proteins as prognostic or diagnostic markers of PTOA.

Currently, commonly used differential proteomics techniques include bidirectional electrophoresis, isobaric tags for relative and absolute quantitation (iTRAQ), and tandem mass tags and stable isotope labeling in cell culture (TMT-SILAC) hyperplexing [20]. Isotope-tagged tandem mass spectrometry is the most commonly used method in quantitative proteomics research [21,22]. This technique involves the separation, identification, and quantitative analysis of peptides from various sample sources that are pre-attached with stable isotopic affinity tags [23]. There are two types of tagging strategies, which use either *in vivo* markers such as SILAC, or *in vitro* markers, such as iTRAQ [24]. Tandem mass tag (TMT)-labeling high-throughput screening technology [25], which uses isotopic tagging of the N-terminal amino group of the polypeptide and the amino group of lysine side chains. This technique can identify and compare the protein expression profiles of up to ten different samples simultaneously and be coupled with LC-MS for tandem analysis [26]. The TMT reagent consists of three components: the reporting group, the mass balancing group, and the peptide reaction tagging group, which binds to the N-terminal amino group of the polypeptide and the amino group of lysine side chains [27].

In this study, the TMT-labeling LC-MS-MS method was used to screen for differentially expressed proteins. This technique includes high detection flux of qualitative and quantitative information on thousands of proteins, a wide application range that combines LC-MS and tandem mass spectrometry to increase the sensitivity of the identification of low levels of proteins of less than 10 kD or greater than 100 kD [28]. Also, TMT-labeling LC-MS-MS has a practical use, as tagging can be performed for almost any protein sample, and good reproducibility [28].

However, a disadvantage of the LC-MS-MS proteomics technique is that it primarily detects more highly abundant proteins within the biological matrix and is unable to detect or quantify proteins with low abundance adequately. Although antibody-based depletion methods may improve quantification of low expressed proteins through filtering out the highly abundant proteins, proteins with lower abundance may also be filtered out through this method [11]. Therefore, additional in-depth or targeted approaches are necessary to provide accurate quantification of overall protein content in the joint synovial fluid. We previously used a similar MS-based proteomics approach to study the synovial fluid proteome of healthy porcine knees [15]. The substantial overlap between the findings of our previous study and proteins identified in the present study supports the use of an MS-based proteomics approach. However, further studies are required to validate the identified proteins in individual samples compared with the pooled samples in independent cohorts.

In the present study, macroscopic assessment of the cartilage was performed using cross-sectional time points, which was a limitation of the study. A potential future improvement of the study design would be to include longitudinal analysis and to better characterize changes in the synovial fluid proteome in response to joint degeneration over time. The present study identified proteins in the synovial fluid that may contribute to or influence the development of PTOA. Further functional studies should be performed to investigate the mechanisms underlying the elucidated associations from the current study and to determine the potential application of the identified targets as biomarkers of OA progression or as disease modifiers

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for the development of novel treatments. Protein-protein interaction (PPI) network analysis is used to create a network of interacting proteins in an organism [29], thereby facilitating the understanding of complex biological functions of proteins [30]. For example, in an interconnected network, highly aggregated proteins may have the same or similar biological functions. Highly linked proteins may be key nodes that affect the metabolic or signal transduction pathways in an entire system. Therefore, in this study, the results of protein interaction network analysis were combined with the enrichment results of pathway annotation to generate a more comprehensive and systematic model of biological activity at the molecular level. This approach has facilitated the subsequent selection of new target proteins for future research and data mining.

This study successfully established an ACL reconstruction OA model using mini-pigs, which allowed histological examination of degeneration of the meniscus, cartilage, and subchondral bone in the knee joint after ACL reconstruction. Further analysis and characterization of the identified protein hits from the present study may be applied in the early diagnosis and intervention of OA after ACL reconstruction. When conducting high-throughput proteomics research, the goal is to collect all proteins from tissues and cells *in vivo*. Understanding the function of these proteins and the biological pathways involved is the primary goal of proteomics. The selection criteria for novel target proteins that have been identified from the initial screening for subsequent in-depth research and mechanism mining is controversial. Generally, the criteria used to identify protein targets include their relationship to the disease, previously identified proteins, the number of unique peptides, differences in expression, and the similarity between the sample groups. Based on the results of the protein interaction network in the present study (Figure 5), and these criteria, future studies will be undertaken to select new target proteins to be tested.

Conclusions

This study aimed to undertake proteomics and bioinformatics analysis of cartilage in post-traumatic osteoarthritis (PTOA) in a mini-pig model of anterior cruciate ligament repair (ACLR). Proteomics and bioinformatics analysis of cartilage in the knee joint of the animal model identified proteins associated with OA that may have a role in the diagnosis, prognosis, or treatment of OA.

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Conflict of interest

None.

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