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Proteomic analysis of engineered cartilage

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Summary

Tissue engineering holds promise for the treatment of damaged and diseased tissues, especially for those tissues that do not undergo repair and regeneration readily *in situ*. Many techniques are available for cell and tissue culturing and differentiation of chondrocytes using a variety of cell types, differentiation methods, and scaffolds. In each case, it is critical to demonstrate the cellular phenotype and tissue composition, with particular attention to the extracellular matrix molecules that play a structural role and that contribute to the mechanical properties of the resulting tissue construct. Mass spectrometry provides an ideal analytical method with which to characterize the full spectrum of proteins produced by tissue engineered cartilage. Using normal cartilage tissue as a standard, tissue engineered cartilage can be optimized according to the entire proteome. Proteomic analysis is a complementary approach to biochemical, immunohistochemical, and mechanical testing of cartilage constructs. Proteomics is applicable as an analysis approach to most cartilage constructs generated from a variety of cellular sources including primary chondrocytes, mesenchymal stem cells from bone marrow, adipose tissue, induced pluripotent stem cells, and embryonic stem cells. Additionally, proteomics can be used to optimize novel scaffolds and bioreactor applications, yielding cartilage tissue with the proteomic profile of natural cartilage.

Keywords

proteomics; mass spectrometry; cartilage; SDS-electrophoresis; extracellular matrix; chondrocyte

1. Introduction

Proteins of the extracellular matrix play an essential role in determining the mechanical properties of tissues. Cartilage is an avascular tissue, rich in extracellular matrix molecules. Collagens and proteoglycans work together to create a structure that supports the function of cartilage as a resilient tissue that is able to withstand forces. Injury to the cartilage can result in irreversible damage and tissue engineering holds promise for the treatment of damaged and diseased cartilage. It is critical to recapitulate the normal composition and organization of the extracellular molecules that play a structural role in tissue engineered cartilage.

Mass spectrometry (MS) and proteomics provide an economical and efficient method to monitor the complete profile of extracellular matrix molecules from a sample of cartilage

tissue. By comparing to native cartilage, it is possible to assess how closely the tissue-engineered cartilage resembles the native tissue.

Several laboratories have developed methods of extraction and separation of cartilage proteins and subsequent analysis by mass spectrometry to evaluate the cartilage proteome. While efforts to generate laboratory grown cartilage have been carried out for more than 20 years, the application of mass spectrometry for proteomic analysis has seen a steady increase over the past ten years. In 2005 and 2006, methods to characterize the proteome of human normal articular chondrocytes were developed and applied to osteoarthritis (1–5). Osteoarthritis continues to be one of the primary foci for proteomic analysis (6), along with questions regarding the proteome of growth plate cartilage and changes in cartilage proteome during development (7, 8). Recently, matrix-assisted laser desorption/ionization (MALDI) was used to characterize the proteome of osteoarthritic cartilage (9).

Quantitative proteomics has been applied to cartilage studies utilizing two-dimensional gel electrophoresis for the separation of extracted proteins prior to mass spectrometry (10, 11), isobaric tag for relative and absolute quantitation (iTRAQ) labeling in conjunction with two-dimensional gel techniques (12, 13), and stable isotope labeling by amino acids in cell culture (SILAC) (14). Label-free techniques for quantitative proteomics of cartilage have also been developed (15). Extraction methods have been optimized for proteomic analysis of cartilage. Wilson and Bateman applied sequential extraction and fractionation with two-dimensional electrophoresis of cartilage proteins to improve the data obtained (16).

In cartilage tissue engineering, proteomics has been applied to the study of cartilage tissue explants as well as chondrocytes maintained in serum-containing and serum-free culture (14, 17). To better understand the mechanobiology of cartilage tissue, proteomics has been used to characterize the effect of mechanical compression injury as well as the effect of stretching on cartilage (18, 19). Various sources of cells for tissue engineering of cartilage have been characterized using proteomics including primary chondrocytes, bone marrow-derived chondrogenic precursor cells (20, 21), adipose-derived chondrogenic stem cells (22), and human mesenchymal stem cells used for chondrogenic differentiation and cartilage tissue engineering (23). Application of proteomics to the field of cartilage biology and tissue engineering includes the analysis of posttranslational modifications (18), degradation of cartilage matrix molecules (24), explant models of inflammation (25, 26), and for questions of molecular interactions within the extracellular matrix of cartilage, or the ‘interactome’ (27, 28).

Here we outline a versatile method of extraction, separation, mass spectrometry, and proteomic analysis for the evaluation of the extracellular matrix generated by primary chondrocytes maintained in 3-dimensional culture using native cartilage as a standard.

2. Materials

Materials for cell isolation, expansion and differentiation, seeding on scaffolds and bioreactor use are provided in accompanying chapters and will not be covered here. Materials described are for analysis of proteins from chondrocytes maintained in 3-dimensional culture compared to proteins extracted from native cartilage. Nanopure water

(18.2 M Ω) is used to prepare all reagent solutions. All reagents are of analytical grade unless otherwise specified.

2.1. Protein extraction from native cartilage and chondrocytes

1. Cartilage tissue, e.g. we obtained cartilage from the femoral head and condyles of an early third trimester fetal calf.
2. Tissue homogenizer (Polytron).
3. Low salt cartilage extraction buffer: 0.1 M NaCl containing 0.05 M Tris-HCl, 0.01 M EDTA, and protease inhibitors 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF, 1 mM), Pepstatin A (10 μ M), E-64 (150 μ M), Bestatin (50 μ M), Leupeptin (20 μ M), and aprotinin (0.8 μ M), pH 7.0.
4. High salt cartilage extraction buffer: 1 M NaCl containing 0.05 M Tris-HCl, 0.01 M EDTA, and protease inhibitors AEBSF, (1 mM), Pepstatin A (10 μ M), E-64 (150 μ M), Bestatin (50 μ M), Leupeptin (20 μ M), and aprotinin (0.8 μ M), pH 7.0.
5. PBS: 0.1 M phosphate buffered saline, pH 7.4.
6. Cell lysis buffer: 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and protease inhibitors AEBSF, (1 mM), Pepstatin A (10 μ M), E-64 (150 μ M), Bestatin (50 μ M), Leupeptin (20 μ M), and aprotinin (0.8 μ M).

2.2. Extract treatment and protein quantification

1. 50% Cibacron Blue agarose resin slurry.
2. Spin columns with column volume of 1000 μ l.
3. Albumin binding/wash buffer: 25 mM Tris, 25 mM NaCl, pH 7.5.
4. Bicinchoninic acid (BCA) protein assay kit.
5. Acetone solution: 80% acetone in Nanopure water.

2.3. SDS polyacrylamide gel electrophoresis

1. Electrophoresis System.
2. 4–12% Bis-Tris mini gel.
3. Sample loading buffer: 10% glycerol, 0.14 M Tris Base, 0.1 M Tris-HCl, 2% lithium dodecyl sulfate (LDS), 0.5mM EDTA, 0.02% Serva Blue G250; 0.006% phenol red, 1.25% 2-mercaptoethanol, pH 8.5. Running buffer: 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.2
4. Coomassie Blue stain.

2.4. In-gel tryptic protein digestion

1. Gel washing solution: 50 mM NH₄HCO₃, prepared freshly.

2. Coomassie Blue destaining solution: 40% MS-grade acetonitrile, 50 mM NH_4HCO_3 , prepared freshly.
3. Trypsin solution: 20 $\mu\text{g}/\text{mL}$ of proteomic-grade trypsin prepared just before use and kept on ice.
4. Reducing solution: 10 mM dithiothreitol (DTT), prepared freshly.
5. Alkylating solution: 55 mM iodoacetamide (IAA), prepared freshly.
6. Acetonitrile/formic acid solution: 60% MS-grade acetonitrile in water containing 5% formic acid.
7. Peptide reconstitution solution: 5% MS-grade acetonitrile, 0.1% MS-grade formic acid.

2.5. Nanoscale liquid chromatography (LC) and nano electrospray - tandem mass spectrometry

1. Nanoscale LC system.
2. C18 desalting column: 2 cm, ID 100 μm , 5 μm .
3. C18 analytical column: 10 cm, ID 75 μm , 3 μm .
4. Mobile phase A: 99.9% water, 0.1% MS-grade formic acid.
5. Mobile phase B: 99.9% MS-grade acetonitrile, 0.1% MS-grade formic acid.
6. Nano electrospray ionization (ESI) source, e.g. Flex II nano-ESI source (Thermo Scientific).
7. Mass spectrometer, e.g. Velos Pro Dual-Pressure Linear Ion Trap mass spectrometer (Thermo Scientific).

2.6. Peptide and protein identification by database searching

1. Database search software, e.g. Proteome Discoverer 1.4 (Thermo Scientific).
2. Database search engine, e.g. Sequest HT (Thermo Scientific) and Mascot 2.4 (Matrix Science).
3. Protein sequence database, e.g. National Center for Biotechnology Information (NCBI) non-redundant protein database (in this case, we used the non-redundant protein database for *Bos taurus* downloaded on June 16th, 2014).

3. Methods

Methods for cell isolation, expansion and differentiation, seeding on scaffolds and bioreactor use are provided in accompanying chapters and will not be covered here. In the method described here, proteins from chondrocytes maintained in 3-dimensional culture are compared to proteins extracted from native cartilage. A commonly used gel-LC-MS/MS proteomic profiling workflow is described in detail (Figure 1).

Keratin contamination is a significant challenge in MS-based bottom-up proteomics (29). Large amounts of keratin in a sample can mask proteins of interest that are less abundant. Thus, efforts need to be taken to minimize keratin contamination in all steps prior to tryptic digestion (*see* Note 1).

3.1. Cartilage tissue preparation and extraction

1. Remove perichondrium and adhering tissues from primary cartilage material.
2. Mince and homogenize the cartilage using a tissue homogenizer.
3. Extract cartilage proteins in low salt cartilage extraction buffer at 4°C with stirring for 4 hours.
4. Centrifuge sample at $100,000 \times g$ at 4°C for 1 hour to pellet insoluble material.
5. Carefully transfer the supernatant to a new tube.
6. Extract cartilage proteins from resulting pellet in high salt cartilage extraction buffer at 4°C with stirring for 4 hours.
7. Centrifuge sample at $100,000 \times g$ at 4°C for 1 hour to pellet insoluble material.
8. Carefully transfer the supernatant to a new tube.
9. Take an aliquot of each supernatant to determine the protein concentration using the bicinchoninic acid (BCA) assay.
10. Store the rest of the supernatant at -80°C for further analysis.

3.2. Protein extraction from primary chondrocytes and chondrocytes in 3-dimensional cultures

1. After maintaining the chondrocytes in 3-dimensional culture for the desired period of time, carefully transfer the medium and the 3-dimensional scaffold carrying the chondrocytes to a clean 15-mL conical tube and centrifuge at $2500 \times g$ for 5 minutes to pellet the 3-dimensional scaffold carrying the cells. Save the supernatant for secreted protein analysis.
2. Wash the pellet three times in cold PBS. Centrifuge at $2500 \times g$ for 5 minutes.
3. Carefully decant the supernatant.
4. Add 0.5 ml of cell lysis buffer to the pellet and gently mix.
5. Incubate on ice for 30 minutes. Gently mix every 10 minutes.
6. Centrifuge at $14,000 \times g$ for 15 minutes at 4°C.
7. Carefully transfer the supernatant to a new tube.

¹Keratin contamination is a significant challenge in MS-based shotgun proteomics (29). The main source of keratin contamination is dust that contains dead skin cells from humans. Although keratin contamination cannot be completely avoided, it is important to take every effort to minimize the contamination. A key factor in avoiding keratin is to avoid the contamination of samples, supplies, and reagents with dust particles. Thus, efforts should be taken to keep the workplace free of dust. Wear a clean lab coat and nitrile gloves at all times during sample handling. Perform sample preparation in a HEPA-filtered laminar-airflow hood when possible. Always use analytical grade or higher reagents and store all the reagents and samples properly to minimize their exposure to dust.

8. Take an aliquot of the supernatant to determine the protein concentration using the BCA assay.
9. Store the supernatant at -80°C for further analysis.

3.3. Bovine serum albumin depletion and protein quantification

Serum albumin may be removed from culture medium samples to improve the detection of lower abundance proteins (*see* Note 2).

1. Centrifuge the medium collected in Section 3.2, Item 1 at $3000 \times g$ to eliminate cell debris.
2. Transfer 5 ml of medium into dialysis tubing with a molecular cut-off of 3,000 Da. Dialyze the medium against 1 liter of albumin binding/wash buffer for 24 hours at 4°C .
3. Replace the buffer and continue the dialysis for 24 hours at 4°C .
4. Place the spin column into a 2.0-mL collection tube. Shake the Cibacron Blue agarose resin bottle to resuspend the resin. Using a wide-bore micropipette tip, transfer 400 μL of the slurry (corresponding to 200 μL of settled resin volume) into the spin column and loosely cap the column.
5. Centrifuge at $12,000 \times g$ for 1 minute to remove excess liquid. Discard flow-through and place the spin column back into the same collection tube.
6. Add 200 μL of albumin binding/wash buffer to the spin column.
7. Centrifuge at $12,000 \times g$ for 1 minute. Discard flow-through and place the spin column into a 1.5-ml collection tube.
8. Apply 50 μL of dialyzed medium sample to the resin and incubate for 1–2 minutes at room temperature.
9. Centrifuge at $12,000 \times g$ for 1 minute. Reapply the flow-through to the spin column and incubate for 1–2 minutes at room temperature to ensure maximal albumin binding.
10. Centrifuge at $12,000 \times g$ for 1 minute. Retain the flow-through. Place the spin column in a new collection tube.
11. Wash the resin to release unbound proteins by adding 50 μL of albumin binding/wash buffer.
12. Centrifuge at $12,000 \times g$ for 1 minute. Retain the flow-through. Place the spin column in a new collection tube.
13. Repeat Steps 11 and 12 four additional times.

²The high concentration of albumin in the fetal bovine serum commonly used in cell culture may obscure the detection of many secreted proteins in culture media. Thus, the depletion of albumin is necessary. There are different methods available to deplete albumin in cell culture media. We found that agarose resin of affinity ligand Cibacron Blue dye gives satisfactory results as evidenced by both SDS-PAGE and mass spectrometry analysis (Figure 4).

14. Combine all the fractions. Concentrate the depleted medium by a factor of 20 using a protein ultrafiltration filter with a cutoff of 3 kDa.
15. Take an aliquot of the concentrated medium to determine the protein concentration using the BCA assay.
16. Store the rest of the medium at -80°C for further analysis.

3.4. Acetone protein precipitation

Precipitate protein using acetone to concentrate proteins and eliminate compounds that may interfere with SDS-PAGE and LC-MS analysis.

1. Chill acetone and 80% acetone solution to -20°C .
2. Place chondrocyte lysate solution containing 100 μg of total protein into a clean 1.5-ml microcentrifuge tube.
3. Add four times the sample volume of cold (-20°C) acetone to the tube.
4. Mix the sample and incubate overnight at -20°C .
5. Centrifuge for 15 minutes at $14,000 \times g$ at 4°C .
6. Carefully decant the supernatant without dislodging the protein pellet.
7. Add 1 mL of cold (-20°C) 80% acetone solution and resuspend the protein pellet.
8. Centrifuge for 15 minutes at $14,000 \times g$ at 4°C .
9. Carefully decant the supernatant without dislodging the protein pellet. Pipette off the residual washing solution using a gel-loading tip.
10. Air-dry the protein pellet for 5 minutes at room temperature (*see* Note 3).

3.5. SDS polyacrylamide gel electrophoresis

1. Prepare samples (i.e., air-dried protein pellet obtained from chondrocytes, cartilage extract, and culture medium) in SDS sample buffer. Use $\sim 100 \mu\text{g}$ protein for each sample.
2. Heat the sample at 70°C for 10 minutes.
3. Load the entire volume of sample onto a 4–12% Bis-Tris mini gel.
4. Run the gel at 200 V for 35 minutes.
5. At the end of the electrophoresis, wash the gel in deionized water three times.
6. Stain the gel with Coomassie Blue stain for 1 hour.
7. Wash the gel with deionized water extensively until the water is clear.
8. Record the gel image before proceeding with in-gel tryptic digestion.

³Care should be taken to prevent over drying of the protein pellet as this will make resolubilization difficult.

3.6. In-gel tryptic protein digestion

3.6.1. Gel band excision

1. Cut the entire gel lane into a number of slices of equal length or excise protein bands of interest from the stained gel using a clean razor blade.
2. Cut each gel slice into small pieces (~1 mm³) and transfer the pieces to a 1.5-mL low protein binding microcentrifuge tube pre-rinsed with HPLC-grade acetonitrile.

3.6.2. Destaining of gel pieces

1. Wash the gel pieces with 0.5 mL of gel washing solution. Shake for 15 minutes then discard the washing solution.
2. Add 200 µL of Coomassie Blue destaining solution and shake for 15 minutes (*see* Note 4). Discard the destaining solution.
3. Repeat the destaining step until the gel pieces are completely destained.
4. Add 200 µL of MS-grade acetonitrile and shake for 15 minutes to dehydrate the gel pieces.
5. Spin the tubes briefly and discard the supernatant.
6. Completely dry the gel pieces in a vacuum evaporator.

3.6.3. Reduction and alkylation

1. Add 100 µL of reducing solution to cover the gel pieces, and incubate for 1 hour at 56°C with shaking.
2. Cool the gel pieces to room temperature. Discard the reducing solution.
3. Add 100 µL of alkylating solution to the gel pieces.
4. Incubate for 45 minutes at room temperature in the dark. Mix occasionally.
5. Centrifuge the tubes briefly and discard the alkylating solution.
6. Wash the gel pieces with 200 µL of gel washing solution on a shaker for 15 minutes. Discard the washing solution.
7. Repeat the washing step. Centrifuge briefly, discarding the liquid phase.
8. Add 200 µL of MS-grade acetonitrile and shake for 15 minutes to dehydrate the gel pieces.
9. Centrifuge briefly and discard the liquid phase.
10. Dry the gel pieces completely in a vacuum evaporator.

3.6.4. Tryptic digestion

1. Pre-cool the gel pieces and trypsin solution on ice.

⁴50% acetonitrile ammonium bicarbonate buffer is commonly used to destain Coomassie Blue stained gel pieces (30). We found that 40% acetonitrile ammonium bicarbonate is a more efficient destaining buffer.

2. Add enough trypsin solution (30 – 50 μ L) to the gel pieces to cover.
3. Incubate in ice for 60 minutes.
4. Centrifuge the tube briefly. Pipette off any remaining trypsin solution (*see* Note 5).
5. Add 20 μ L of gel washing solution to cover the gel pieces.
6. Incubate in a shaker at 30°C overnight.

3.6.5. Peptide extraction

1. At the end of tryptic digestion, add 50 μ L of gel washing solution to the gel pieces.
2. Shake vigorously for 10 minutes.
3. Centrifuge the tubes briefly. Sonicate in a water bath for 5 minutes.
4. Centrifuge briefly and transfer the extract to a new clean microcentrifuge tube using a long narrow gel-loading tip.
5. Add 50 μ L of acetonitrile/formic acid solution to the gel pieces. Repeat steps 2–3.
6. Centrifuge briefly. Transfer the extract to the tube in step 4 using a gel-loading tip.
7. Dry the extracted digests in a vacuum evaporator.
8. Add 30 μ L of peptide reconstitution solution to each tube. Mix for 10 minutes and sonicate for 5 minutes.
9. Centrifuge the samples for 10 minutes at 15,000 $\times g$.
10. Transfer the reconstituted peptide solution into a standard 2-mL HPLC sample vial with a 100- μ L glass low-volume insert. Samples are now ready for LC-MS analysis (*see* Note 6).

3.7. Reverse phase nano LC separation of peptides

The resulting peptide mixtures are separated by reverse phase nano LC using a two-column setup. All LC mobile phases are degassed with continuous helium sparging (*see* Note 7). Figure 2 shows a typical base peak chromatogram of peptide mixtures.

1. House samples in a refrigerated autosampler during the entire of period of analysis. Inject 8- μ L samples onto a C18 desalting column, desalting with 20 μ L of mobile phase A.
2. Elute desalted peptide mixtures from the desalting column onto a C18 analytical column and separate using a linear gradient with two mobile phases (A and B) at a

⁵It is important to aspirate off excessive trypsin as the abundant peptides from trypsin autolysis may interfere with LC-MS analysis.

⁶The resulting peptide samples should be analyzed immediately if possible. Storing peptides at room temperature or 4°C may result in loss of some peptides. If samples cannot be analyzed immediately, they should be stored at -70°C to minimize the loss of peptides.

⁷It is well known that gases dissolved in the LC mobile phase can cause a variety of problems in liquid chromatography. Air bubbles can also severely interrupt the nanospray. Thus, mobile phase degassing is very important in LC-Nanospray. For instruments that are not equipped with an in-line degasser such as the Thermo Scientific Easy nLC II, it is critically important to properly degas the mobile phase. We found that sonication is not sufficient since the mobile phase will be resaturated with air during the extended period of analysis. We recommend using continuous helium sparging to degas the mobile phases. We found this method provided optimal results.

flow rate of 250 nL/min. Begin the gradient at 0% B, increase linearly to 40% B over 60 minutes and then to 80% B over 16 minutes. Maintain the mobile phases at this percentage for a further 14 minutes as a washing step.

3.8. Nano electrospray - tandem mass spectrometry

1. Use a fused silica emitter (10 μ L ID) directly attached to the analytical column through a zero dead volume union to elute peptides sprayed at a voltage of 2.2 kV.
2. Collect MS/MS data in data-dependent acquisition mode. Use Collision-Induced Dissociation (CID) with a normalized collision energy of 35% to fragment the precursor ions. Collect MS/MS data for the 10 most abundant precursor ions selected from the proceeding full MS scan over the m/z range of 300 – 2000.

3.9. Peptide and protein identification by database searching

Peptide spectral matching and protein identification are achieved by database searching using specific algorithms, e.g. Mascot 2.4.0 (Matrix Science) and Sequest HT algorithms (Thermo Scientific) (*see* Note 8). MS/MS spectra of peptides may be used to search against the NCBI non-redundant protein database (*Bos taurus* in this example), which can be obtained from the NCBI website. The main search parameters for Mascot 2.4 and Sequest HT include: semi trypsin, maximum missed cleavage site of two, precursor mass tolerance of 1.5 Da, fragment mass tolerance of 0.8 Da, fixed modification of carbamidomethyl cysteine (+57.021 Da), and variable modification of oxidized methionine (+15.995 Da). A decoy database search is performed to calculate the false discovery rate (FDR). Proteins containing at least two high confidence peptides (FDR = 0.01) are considered positively identified and are reported. Figure 3 shows a typical peptide ion fragment spectrum. Prevalent detectable proteins are presented in Table 1.

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⁸A crucial component of the analysis of shotgun proteomics data is the search engine. There are many different search engines available. Each search engine has its unique searching algorithm. It has been shown that searching the same datasets using multiple search engines and then combining the search results usually improves the analysis and gives better protein coverage (31). We routinely use both Sequest HT and Mascot 2.0 algorithms in database search. We find that this approach gives improved protein and peptide coverage (Figure 5). Thus we recommend using multiple search engines and combining the search results.

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Figure 1.
A Gel-LC-MS/MS workflow for proteomic profiling.

Figure 2.
A typical base peak chromatogram of peptide mixture resulting from in-gel tryptic digestion following gradient elution.

Figure 3.
Ion fragment spectrum of peptide DAEEVISQTIDTITDMIK from collagen alpha-1(VI) (*Bos taurus*).

Figure 4. SDS-PAGE of cell culture medium containing 10% fetal bovine serum before and after bovine serum albumin depletion.

Figure 5.
Number of peptides and proteins identified by Sequest HT and Mascot 2.0.

Table 1Readily detectable proteins in cartilage constructs (*Bos taurus*).

Protein name ^{a)}	Accession number ^{b)}
Actin, β	GENE ID: 280979
Actinin, α 4	GENE ID: 522269
Aggrecan	GENE ID: 280985
Aldolase A, fructose-bisphosphate	GENE ID: 509566
Annexin A1	GENE ID: 327662
Annexin A2	GENE ID: 282689
Annexin A5	GENE ID: 281626
Acidic (leucine-rich) nuclear phosphoprotein 32 family, member B	GENE ID: 509685
Biglycan	GENE ID: 280733
Calreticulin	GENE ID: 281036
Chondroadherin	GENE ID: 281069
Collagen, type I, α 1	GENE ID: 282187
Chondrocalcin-Carboxy propeptide of collagen type II α 1	GENE ID: 407142
Collagen, type II, α 1	GENE ID: 407142
Collagen, type VI, α 1	GENE ID: 511422
Collagen, type IX, α 1	GENE ID: 282195
Collagen, type XI, α 1	GENE ID: 287013
Collagen, type XI, α 2 (PARP)	GENE ID: 515435
Collagen, type XII, α 1	GENE ID: 359712
Collagen, type XIV, α 1	GENE ID: 7373*
Cartilage oligomeric matrix protein	GENE ID: 281088
Decorin	GENE ID: 280760
Epiphygan	GENE ID: 281747
Fibromodulin	GENE ID: 281168
Hyaluronan and proteoglycan link protein 1	GENE ID: 281717
Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	GENEID: 415113
Heparan sulfate proteoglycan 2	GENE ID: 444872
Lactate dehydrogenase A	GENE ID: 281274
Matrilin 1, cartilage matrix protein	GENE ID: 512059
Matrilin 3	GENE ID: 540041
Nidogen 2 (osteonidogen)	GENE ID: 521854
Nucleolin	GENE ID: 497013
Phosphoglycerate kinase 1	GENE ID: 507476
Procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1	GENE ID: 281409
Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	GENE ID: 533642
Protein disulfide isomerase family A, member 3	GENE ID: 281803

Protein name ^{a)}	Accession number ^{b)}
Protein disulfide isomerase family A, member 4	GENEID: 415110
Protein disulfide isomerase family A, member 5	GENE ID: 511603
Prolyl 4-hydroxylase	GENE ID: 281373
Pyruvate kinase, muscle	GENE ID: 512571
Semenogelin I (α -Inhibin)	GENE ID: 281254
Serpin peptidase inhibitor, clade H (collagen binding protein 1)	GENE ID: 510850
Tenascin	GENE ID: 540664
Serotransferrin	GENE ID: 280705
Thrombospondin 1	GENE ID: 281530
Thrombospondin 1, Amino terminal domain	GENE ID: 281530
Tumor rejection antigen (gp96 or HSP90B1)	GENE ID: 282646
Vitrin	GENE ID: 280957

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^{a)} Entrez Gene Full Name Protein Accession number.

^{b)} GENE ID Accession Number. Organism: Bos taurus.

* By homology to human sequence; Homo sapiens.