RESEARCH ARTICLE

Label-free proteomic analysis of the hydrophobic membrane protein complement in articular chondrocytes: a technique for identification of membrane biomarkers

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Abstract
Context: There is insufficient knowledge about the chondrocyte membranome and its molecular composition.
Objective: To develop a Triton X-114 based separation technique using nanoLC-MS/MS combined with shotgun proteomics to identify chondrocyte membrane proteins.
Materials and methods: Articular chondrocytes from equine metacarpophalangeal joints were separated into hydrophobic and hydrophilic fractions; trypsin-digested proteins were analysed by nanoLC-MS/MS.
Results: A total of 315 proteins were identified. The phase extraction method yielded a high proportion of membrane proteins (56%) including CD276, S100-A6 and three VDAC isoforms.
Discussion: Defining the chondrocyte membranome is likely to reveal new biomarker targets for conventional and biological drug discovery.

Introduction

Proteins that are embedded in or associated with biological membranes play critically important roles in a wide range of vital cellular functions including transport, cell–cell communication and signalling processes. As the plasma membrane (PM) acts as the first barrier to the extracellular environment, PM proteins enable cells to sense and respond to external stimuli in a specific manner – they include receptors; cell recognition, cell–cell or cell–matrix adhesion sites; enzymes; as well as channels, pores and transporters for ions, small molecules and nutrients (Cordwell & Thingholm, 2010). Based on domain predictions by different methods, membrane proteins comprise approx. 15–30% of the human proteome (Almen et al., 2009; Kabbani, 2008), highlighting the fundamental importance of membrane-associated physiological processes. PM proteins are also the primary targets of many of the drugs that are currently in our pharmaceutical arsenal; indeed, the majority (over 70%) of currently marketed drugs act on PM proteins (Almen et al., 2009; Rabilloud, 2003). The qualitative and quantitative composition of the PM proteome is known to be significantly altered during cellular differentiation and disease. Membrane proteins have the potential to be selective and sensitive biomarkers for disease progression and prognosis. Furthermore, membrane proteins that exhibit altered expression in disease states could be suitable candidates for the development of sensitive receptor-targeted imaging agents for non-invasive monitoring of biological and inflammatory processes (Dissoki et al., 2015; Samkoe et al., 2014; Sega & Low, 2008). Therefore, there is a critical need for the development of tools and technologies for identification and characterisation of membrane proteins to complement physiological methods for elucidating their functions. This combined approach will promote the discovery...
of new and better drugs, and the development of novel treatment strategies of diseases.

Integral membrane proteins have an amphiphilic structure; apart from hydrophilic domains located on the external cytosolic or organellar surfaces, they also contain hydrophobic (membrane-spanning) regions that directly interact with the lipid bilayer of the membranes in which they are embedded. High-resolution and high-throughput proteomic techniques have been widely applied to study the PM proteome of various cell types [for a review please see Cordwell & Thingholm (2010)]. However, there are serious (mainly technical) limitations that currently hinder advances in this field. In addition to their very low relative abundance, their amphiphilic nature and poor solubility makes membrane proteins challenging to purify, identify and characterise on a proteomic scale. The use of non-ionic detergents (e.g. the Triton X series in which the number of hydrophilic oxyethylene units attached to the hydrophobic octylphenyl residue determines the specific physicochemical properties) has enabled the solubilisation and characterisation of these proteins. Their use is based on the principle that water-soluble proteins, unlike amphiphilic membrane proteins, show little or no interaction with these compounds; consequently, only integral membrane proteins form mixed micelles with non-ionic detergents (Bordier, 1981). The cloud point, the temperature at which phase separation occurs between the detergent and the aqueous phase, is at approximately 20 °C for Triton X-114, which makes its application particularly convenient in studies aimed at analysing integral membrane proteins (Bordier, 1981; English et al., 2012; Mathias et al., 2011).

In addition to the application of non-ionic detergents, a number of other approaches have been developed over the past decades for the selective enrichment of membrane proteins including precipitation and gradient centrifugation, biotinylation and affinity enrichment or the application of glycoproteomics [reviewed in Cordwell & Thingholm (2010)]. The main technical challenge remaining in the analysis of integral membrane subproteomes, however, is the ability to obtain high purity membrane protein samples without the presence of high abundance contaminating proteins from the cytoplasm or other intracellular organelles. Comprehensive analyses of the membrane protein complement (also known as the membranome) of distinct cell types are relatively scarce; this can at least partially be attributed to the challenges and limitations described above. It is particularly true for chondrocytes, the single cell type in articular cartilage that serves as a specialised load-bearing tissue with unique tribological properties such as a low-friction gliding surface and peculiar rheology in synovial joints. The extracellular matrix (ECM) of hyaline cartilage, in which chondrocytes are embedded, primarily consists of a meshwork of type II collagen fibres and other minor collagens (types VI, IX and XI); large aggregating proteoglycans (e.g. aggrecan) and their constituent glycosaminoglycans (GAGs); as well as high quantities of osmotically bound water (approx. 70% of the net weight of ECM) and counteracting cations attracted by the net negative charge of GAGs (Archer & Francis-West, 2003). Because of its avascular nature and the inability of mature chondrocytes to divide in situ, once damaged, articular cartilage seldom regenerates on its own. Therefore, lesions due to either osteoarthritis (OA) or traumatic injuries are associated with progressive degeneration of articular cartilage, pain and disability. OA is still an unresolved clinical problem, and developing novel therapies or drug targets poses a major challenge (Mobasheri, 2013).

In order to identify proteins involved in pathological processes affecting the structure and function of articular cartilage such as OA, it is first necessary to characterise the normal protein complement of chondrocytes in healthy tissues. For proteomic studies, cartilage is very challenging as the chondrocyte, its sole cell type, forms only 1–2% of the volume of the tissue (Lambrecht et al., 2010). Although the proteome of healthy (Lambrecht et al., 2010; Ruiz-Romero et al., 2005) and OA-affected chondrocytes (Lambrecht et al., 2008; Ruiz-Romero et al., 2008; Tsolis et al., 2015), as well as the secretory profile (secretome) of a cartilage tissue explant model of OA (Williams et al., 2013) has been published, the “hidden” proteome, i.e. low-abundance membrane proteins or other poorly soluble proteins may have remained undiscovered in those studies. Here, we report a technique for profiling integral membrane proteins in primary equine articular chondrocytes using an optimised Triton X-114 phase partitioning technique and LC-MS/MS analysis for protein identification. To the best of our knowledge, this work represents the first and most comprehensive analysis of the integral membrane subproteome in chondrocytes reported. This technique allowed us to establish CD276, S100-A6 (calcyclin) and three VDAC isoforms as key components of the chondrocyte membranome.

Materials and methods

Isolation and culture of primary equine articular chondrocytes

Articular chondrocytes were isolated from equine articular cartilage. The animal used in this study was euthanized in a UK-based abattoir for research-unrelated purposes, and stunned before slaughter in accordance with Welfare of Animals (Slaughter or Killing) Regulations 1995. Ethical approval for the use of abattoir-derived animal tissues was obtained from the Ethics Committee of the School of Veterinary Science and Medicine, University of Nottingham, with input from members of the University of Nottingham Animal Welfare and Ethical Review Body (AWERB). After opening the metacarpal phalangeal joint cavity under aseptic conditions and rinsing the articular cartilage surface with sterile physiological saline, articular cartilage shavings were taken from the distal end of the metacarpal bone using a sterile surgical blade and placed in serum-free DMEM (Thermo Fisher Scientific, Inc., Waltham, MA) supplemented with 4% Penicillin/Streptomycin solution (P/S, Sigma-Aldrich, St. Louis, MO) pre-warmed to 37 °C as described previously (Williams et al., 2013). The shavings (~100µm thick, ~5mm in diameter) were taken from the superficial part of macroscopically normal cartilage areas without any visible signs of degeneration, including discolouration, fibrillation and surface irregularities, to avoid the deep (calcified) layers of articular cartilage or the cartilage–bone interface. The surface of articular cartilage did not receive treatment prior to sampling to preserve the lamina...
splendens (the uppermost surface layer of articular cartilage) (Dunham et al., 1988).

Cartilage shavings were washed three times with sterile PBS containing 10% P/S. Articular chondrocytes were isolated by overnight incubation with 0.1% type II collagenase (from Clostridium histolyticum; Invitrogen, Carlsbad, CA) dissolved in serum-free DMEM containing 4% P/S solution at 37°C. Following dissociation of cartilage shavings by trituration the solution was filtered through a 70-μm nylon mesh filter to yield a single cell suspension, and centrifuged at 800 × g for 5 min at room temperature. After washing twice in serum-free DMEM, cells were resuspended in DMEM containing 10% foetal calf serum (FCS; Invitrogen) and 2% P/S solution, seeded into tissue culture flasks (Nunc; Thermo Fisher Scientific), and cultured in a CO₂ incubator at 37°C. Cells were subcultured when they reached approx. 80% confluency. The media was changed on every second day. Cells from the second passage were used for further experiments. A schematic overview of the experimental design is shown in Figure 1.

Sample preparation, phase partitioning using triton X-114, and methanol/chloroform extraction

Approximately 80% confluent cultures of primary equine articular chondrocytes from passage 2 were washed with PBS, then 2 mL of PBS containing 80 μL of protease inhibitor cocktail (25×, Sigma-Aldrich) was added to the flasks. The flasks were placed on ice, and cells were liberated using a cell scraper (Greiner, Stonehouse, UK). The solution was centrifuged (at 850 × g for 2 min, room temperature), and the pellet was resuspended in 600 μL of PBS containing 24 μL of 25 × protease inhibitor cocktail. After incubating on ice for 15 min, the suspension was transferred into a glass homogeniser and the cells were lysed.

Following the addition of Triton X-114 (Sigma-Aldrich) at a final concentration of 0.75%, the lysate was incubated on ice
for 30 min with vortexing every 5 min. After centrifugation (30 min, 10,000 g, 4°C) the supernatant was retained and incubated at 37°C for 5 min, and then on ice for 15 min. The sample was centrifuged again (30 min, 10,000 g, 4°C) and the supernatant was incubated at 37°C for 5 min. Following centrifugation for 3 min (1000 × g, room temperature), two layers appeared. The upper layer (aqueous phase) contained the hydrophilic proteins, the lower layer (detergent phase) contained the hydrophobic proteins. To maximise the recovery of membrane proteins, the upper layer was extracted further by adding Triton X-114 at a final concentration of 0.75% and the phase partitioning procedure was repeated. Finally, the two lower layers were combined together to constitute the hydrophobic fraction, and the upper layer was treated as the hydrophilic fraction.

To remove Triton X-114 from the samples, four times the sample volume of methanol (Thermo Fisher Scientific) was added to both fractions. After centrifugation at 15,000 × g for 10 s at room temperature, two times the original sample volume of chloroform (Sigma-Aldrich) was added. The mixture was centrifuged again, and after adding three times the original sample volume of HPLC grade water, the sample was centrifuged for 5 min (15,000 × g, room temperature). The proteins accumulated at the interface between the two layers formed during the last centrifugation step. Following removal of the upper layer, three times the sample volume of methanol was added, and after spinning for 5 min (15,000 × g, 4°C), the pellet containing the proteins was retained and air-dried.

Quantification of proteins

After methanol/chloroform extraction, the pellets were dissolved in sample resuspension buffer containing 4% SDS (Bio-Rad Laboratories, Hercules, CA), 0.2 M Tris pH 7.4 (Bio-Rad) and 0.15 M NaOH (Thermo Fisher Scientific). Protein concentration in the samples was determined using the Bio-Rad DC Protein Assay Kit according to the manufacturer’s protocol (Bio-Rad). The absorbance of the assayed samples at 655 nm was read using a Bio-Rad Benchmark Microplate Reader.

Polyacrylamide gel electrophoresis (SDS–PAGE)

Loading buffer containing 4 × Laemmli buffer and 3 M dithiothreitol (DTT; Bio-Rad) was added to each sample (typically, 4.8 μL 4 × Laemmli buffer and 1.2 μL 3M DTT was added to 18 μL sample resuspension buffer), and then proteins were fractionated by SDS–PAGE on a 12% polyacrylamide gel. Proteins were initially run at 32 mA constant current, and once the dye front reached the bottom of the stacking gel, the current was increased to 45 mA. Protein bands were visualised by silver staining using a Hoefer Processor Plus automated gel stainer (Amersham, GE Healthcare Life Sciences, UK). The protocol for silver staining was performed as described previously (Yan et al., 2000).

Preparation and trypsin digestion of proteins for LC-MS/MS analysis: in-solution digestion

The protein pellets from the methanol/chloroform extraction step were resuspended in a solution of 50 mM ammonium bicarbonate (AMBIC) (Sigma-Aldrich) and 10 mM DTT (Bio-Rad), and incubated at 37°C for 30 min, vortexing every 10 min. Following the addition of iodoacetamide (IAA, Bio-Rad) at a final concentration of 55 mM, samples were incubated at 37°C for 45 min in dark. Then, 1.2 mL of −20°C acetone was added to each sample, and after mixing, the samples were incubated at 4°C overnight. Protein precipitates were pelleted by centrifugation at 15,000 × g for 5 min at 4°C. Pellets were air-dried for 1 min, and then resuspended in 20 μL of trypsin buffer including 50 mM AMBIC and 10 ng/μL Trypsin Gold (Promega, Madison, WA). Samples were vortexed until the pellets were fully dissolved and then incubated at 37°C for 16 h. Finally, 1 μL of formic acid (1%) was added to each sample to stop the reaction. Samples were stored at −80°C until analysis.

LC-MS/MS analysis

Samples were injected into a 15 cm C18 Pepmap column using a Bruker (Coventry, UK) Easy-nanoLC UltiMate® (Bruker, Coventry, UK) 3000 RSLCnano chromatography platform with a flow rate of 300 nL/min to separate peptides. Three microlitres of each sample was injected into the HPLC column. After peptide binding and washing processes on the column, the complex peptide mixture was separated and eluted by a gradient of solution A (100% water + 0.1% formic acid) and solution B (100% ACN + 0.1% formic acid) over 115 min, followed by column washing and re-equilibration. The peptides were delivered to a Bruker (Coventry, UK) amaZon ETD ion trap instrument (Bruker, Coventry, UK). The top five most intense ions from each MS scan were selected for fragmentation. The nanoLC-MS/MS analysis was performed three times on the samples (all triplicates).

Peptide and protein identification, data analysis and bioinformatics

Processed data were compiled into *.MGF files and submitted to the Mascot search engine (version: 2.4.1) and compared to mammalian entries in the SwissProt and NCBInr databases. The data search parameters were as follows: two missed trypsin cleavage sites; peptide tolerance, ±0.3 Da; number of C13; peptide charge, 1+, 2+ and 3+ ions. Carbamidomethyl cysteine was specified as a fixed modification, and oxidised methionine and deamidated asparagine and glutamine residues were specified as variable modifications. Individual ions Mascot scores above 50 indicated identity or extensive homology. Only protein identifications with probability-based protein family Mascot MOWSE scores above the significant threshold of >50 (p < 0.05) were accepted. After mass spectrometric identification, 315 proteins were classified manually using the UniProt (http://www.uniprot.org/) database, considering homologous proteins and further literature information. For many proteins, assigning a definitive cellular compartment and/or function was a difficult task because of the limitations in accurate predictions and lack of experimental evidence. Also, many proteins may actually reside in multiple cellular compartments. To assign identified proteins to specific organelles, the references to subcellular localisations in the
UniProt database, as well as gene ontology (GO) annotations were used.

Validation of selected membrane proteins by western blotting

Hydrophobic and hydrophilic protein samples were loaded onto Mini-Protean 3 gels. Approximately 20 µg protein per lane was separated by 7.5% SDS–PAGE gel for immunological detection of selected proteins. Proteins were transferred to PVDF membranes (Immun-Blot™ PVDF Membrane, Bio-Rad). After blocking in 5% non-fat dry milk in PBST, membranes were incubated with the anti- Na⁺, K⁺-ATPase primary antibody (diluted 1:100) in blocking solution at 4°C overnight, with gentle rotation. Membranes were then incubated with the secondary antibody (anti-mouse labelled polymer HRP, DakoCytomation, 1:1000 dilution) in blocking solution at room temperature for 1 h. Membranes were developed by enhanced chemiluminescence reaction (Amersham) according to the instructions of the manufacturer and using auto-radiographic films (Hyperfilm, Amersham). Films were scanned on a calibrated densitometer (Bio-Rad GS800) operated by Quantity One version 4.4.1 software (Bio-Rad). Optical density of bands was determined using ImageJ version 1.47 (ImageJ, Bethesda, MD; http://imagej.nih.gov/ij); data were normalised to the value detectable in the hydrophilic fraction.

Results

Triton X-114 phase separation efficiently enriches membrane proteins from primary chondrocyte cultures

To confirm whether the Triton X-114 phase separation method was able to efficiently extract and enrich lipid-soluble membrane proteins from primary articular chondrocytes cultured in vitro, equal amounts of proteins (25 µg) from the hydrophobic and the hydrophilic fractions were loaded onto polyacrylamide gels. Following SDS–PAGE and silver staining, protein bands with clearly different patterns appeared in the gels with several strong bands present in the hydrophobic fraction only (Figure 2A). To validate the effectiveness of the Triton X-114 extraction method, western blot experiments were performed on both fractions to probe for the presence and relative abundance of a membrane-bound Na⁺, K⁺-ATPase. As seen in Figure 2B, the band for this protein in the hydrophobic pool was more than 2.6-fold stronger than that in the hydrophilic pool, demonstrating that lipid-soluble proteins were extracted and enriched in the hydrophobic fraction.

To investigate the protein content of the two fractions, trypsin-digested protein fractions were analysed by nanoLC-MS/MS using Bruker Easy-nanoLC chromatography and a Bruker amaZon ion trap instrument with shotgun proteomics methodologies. A total of 315 unique proteins were reliably (p < 0.05) identified in this study; 208 proteins were detected in the hydrophobic fraction and 192 proteins in the hydrophilic fraction, with 73 (23%) proteins present in both fractions. According to the subcellular localisation data in the UniProt database entries and gene ontology (GO) annotations, in the hydrophobic pool 115 proteins (55%) were membrane proteins and only the remaining 93 proteins (45%) were non-membrane proteins. In contrast, only 38 proteins (20%) were listed as membrane proteins in the hydrophilic fraction, and the other 154 proteins (80%) were non-membrane proteins (Figure 2C). Based on the distribution of membrane versus non-membrane proteins in the two fractions, using the Triton X-114 phase separation method, we successfully extracted and enriched membrane proteins in lysates of primary articular chondrocytes.

Further analysis of the hydrophobic pool reveals various types of membrane proteins

Proteins identified in the hydrophobic fraction were further analysed according to subcellular localisation based on gene ontology (GO) annotation data in the UniProt database entries (Figure 3). Of the 115 membrane proteins in this pool, PM localisation was indicated for 64 proteins (56%), and the other 51 proteins (44%) were localised in organelar membranes. The PM proteins were further subdivided according to their main functions (Table 1). Eighteen proteins (28%) were transporters or involved in membrane/vesicle traffic; 11 and 10 proteins (17 and 16%) were adhesion molecules and proteins with enzyme functions, respectively; 15 proteins (23%) were receptors, and the remaining 10 PM proteins (16%) could not be assigned to any of the previous groups or their function was unknown.

The membrane proteins with other organelar localisations were also subdivided according to their subcellular localisations (Table 2). The majority (23 proteins; 45%) were localised in the membrane of the Golgi complex or the endoplasmic reticulum; 13 proteins (25%) were localised to exosome/lysosome/endosome/other vesicular membranes; another big portion (11 proteins; 22%) were mitochondrial membrane proteins; two proteins (4%) were nuclear membrane proteins; and the remaining two proteins (4%) were ambiguous in terms of specific subcellular localisation.

The majority of the non-membrane proteins in the hydrophobic pool were cytoplasmic/cytoskeletal proteins (46 proteins; 50%) and secreted (extracellular) proteins (19 entries; 20%). Other subcellular localisations included the lysosome/endosome (4 proteins; 4%), the mitochondrion (1 protein; 1%), the Golgi complex or the endoplasmic reticulum lumen (8 proteins; 9%), the nucleus (10 proteins; 11%), and the remaining five proteins were either contaminants or their subcellular localisation was ambiguous (5%; Figure 4 and Table 3).

The hydrophilic pool contains proteins with different solubility and subcellular distribution

The Triton X-114 phase separation technique effectively extracted and enriched lipid-soluble membrane proteins in the hydrophobic phase, and left comparably few membrane proteins (only 20%) in the hydrophilic fraction (Figure 2C). As in the case of the hydrophobic fraction, the majority of the 38 lipid-soluble membrane proteins were localised in the PM (25 proteins; 66%), whilst the others were localised in various
organellar membranes (Golgi complex/endoplasmic reticulum membrane, 16%; mitochondrial membrane, 8%; nuclear membrane, 2%; Figure 5 and Table 4).

Taken together, we have identified 78 unique PM proteins in equine articular chondrocytes in this work. Among them, 32 proteins possessed receptor/adhesion functions; the most important ones are the cluster of differentiation (CD) proteins and integrins. Furthermore, 21 PM proteins with transporter functions were detected in articular chondrocytes (Tables 1 and 4).

The non-membrane protein complement in the hydrophilic fraction comprised 154 proteins, the majority of which (77 proteins; 50%) were cytoplasmic/cytoskeletal proteins. Also in good correlation with the non-membrane protein distribution observed in the hydrophobic samples, the secreted (extracellular) and the nuclear proteins were the second and third largest groups in this fraction (23 proteins, 15%; and 22 proteins, 14%, respectively). Other subcellular localisations included the lysosome/endosome (5 proteins; 3%), the mitochondrion (6 proteins; 4%), the Golgi complex or the endoplasmic reticulum lumen (13 proteins; 9%), and the remaining eight proteins were either contaminants or their subcellular localisation was not determined (5%; Figure 5 and Table 5).

Discussion

The application of mass spectrometry (MS) has recently become an important tool in cartilage biology as it offers numerous advantages over more conventional biochemical approaches such as western blotting. To date, a number of proteomic studies have been performed on cartilage tissue and on chondrocytes, confirming that this analytical tool is particularly suitable for high-throughput and large-scale analysis of the protein complement in health and disease [reviewed in Hsueh et al. (2014) and Williams et al. (2011)]. The first proteomic study carried out on normal human knee articular chondrocyte cultures aimed at creating a two-dimensional gel electrophoresis (2-DE) reference map and generated 93 unique protein identities (Ruiz-Romero et al., 2005). A 1-D SDS–PAGE approach combined with MS/MS resulted in the identification of over 100 different proteins...
from human knee cartilage supernatants (Garcia et al., 2006). In the first large-scale MS analysis of human articular cartilage, which was designed to extract both extracellular and intracellular proteins from samples depleted of highly abundant ECM proteins such as collagens and aggrecan to allow the detection of less abundant proteins, a total number of 814 distinct proteins were identified (Wu et al., 2007). In a more recent study, the proteome of articular chondrocytes from healthy and OA patients using high resolution label-free MS was analysed, leading to the identification of 2400 proteins (Tsolis et al., 2015).

Despite these studies and the impressive number of proteins identified, our knowledge about the proteome of cartilage and its resident cell, the chondrocyte, can still be improved. One significant drawback is that at least in some of the studies the identified proteins have not been properly analysed in terms of subcellular locations and/or functions. In addition, the majority of proteins identified in these studies were located in the ECM because of their high abundance relative to cellular proteins in chondrocytes. More specifically, the “hidden” proteome, which comprises low abundance proteins and/or is not accessible by standard methods, is still poorly characterised. In a study that combined extensive pre-fractionation followed by electrospray ionisation mass spectrometry (ESI-MS/MS), 779 unique proteins expressed by cultured chondrocytes were identified, of which 203 were annotated to the membrane (Lambrecht et al., 2010). However, the authors did not carry out a detailed analysis with respect to specific subcellular location and/or function of the identified membrane proteins, making further data interpretation attempts challenging.

Therefore, the aim of this study was to extend the current knowledge of the chondrocyte proteome by using the Triton X-114 phase separation technique to discover the membrane
Table 1. Functional classification of PM proteins in the hydrophobic fraction identified in equine articular chondrocytes based on GO annotations.

| # | Name                                                   | Accession   | Mascot score | Seq. coverage (%) | No. of matched peptides |
|---|--------------------------------------------------------|-------------|--------------|-------------------|-------------------------|
| 1 | Transporters, membrane/vesicle traffic               |             |              |                   |                         |
| 2 | Voltage-dependent anion-selective channel protein 1  | VDAC1       | 383          | 36.7              | 8                       |
| 3 | Voltage-dependent anion-selective channel protein 2  | VDAC2       | 313          | 25.9              | 6                       |
| 4 | Voltage-dependent anion-selective channel protein 3  | VDAC3       | 139          | 12.0              | 3                       |
| 5 | Ras-related protein Rab-5B                           | RAB5B       | 134          | 15.8              | 3                       |
| 6 | Ras-related protein Rab-5C                           | RAB5C       | 164          | 16.2              | 3                       |
| 7 | Ras-related protein Rab-8A                           | RAB8A       | 135          | 17.4              | 3                       |
| 8 | Ras-related protein Rab-8B                           | RAB8B       | 145          | 17.4              | 3                       |
| 9 | Membrane-associated progesterone receptor component 2| PGR2C       | 178          | 17.0              | 4                       |
| 10| Annexin A1                                            | ANXA1       | 125          | 9.2               | 2                       |
| 11 | Dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48 kDa subunit | OST48       | 86           | 4.6               | 2                       |
| 12| Solute carrier family 2, facilitated glucose transporter member 1 | GTR1       | 118          | 6.3               | 3                       |
| 13| Solute carrier family 2, facilitated glucose transporter member 3 | GTR3       | 74           | 4.0               | 3                       |
| 14| Caveolin-1                                             | CAV1        | 69           | 11.8              | 1                       |
| 15| Monocarboxylate transporter 1                         | MOT1        | 75           | 2.4               | 1                       |
| 16| Predicted: Melanotransferrin (CD228 antigen)         | MFI2        | 64           | 4.5               | 2                       |
| 17| Predicted: Equilibrative nucleoside transporter 1 isoform X1 | SCL29A1   | 67           | 5.5               | 2                       |
| 18| Predicted: Synaptosomal-associated protein 23         | SNAP23      | 57           | 11.4              | 1                       |
| 19| Adhesion molecules                                     |             |              |                   |                         |
| 20| Integrin alpha-5 (CD49e antigen, Fibronectin receptor alpha subunit; fragment) | ITA5       | 97           | 6.5               | 2                       |
| 21| Integrin alpha-V (CD51 antigen, Vitronectin receptor alpha subunit) | ITAV       | 84           | 3.2               | 3                       |
| 22| Integrin beta-1 (CD29 antigen, Fibronectin receptor beta subunit) | ITB1       | 753          | 22.4              | 16                      |
| 23| Thrombospondin-1                                      | TSP1        | 435          | 9.7               | 9                       |
| 24| RA175 (Cell adhesion molecule 1)                      | CADM1       | 58           | 5.5               | 1                       |
| 25| CD151 antigen (Tetraspanin-24)                        | CD151       | 79           | 5.9               | 2                       |
| 26| CD166 antigen (ALCAM, Fragment)                       | CD166       | 125          | 8.4               | 3                       |
| 27| CD107 antigen (Lysosome-associated membrane glycoprotein) | LAMP2      | 53           | 2.0               | 2                       |
| 28| Predicted: CD9 antigen (tetranspin-29)                | CD9         | 88           | 10.4              | 2                       |
| 29| Predicted: integrin alpha-3 isoform 2 (CD49c antigen) | ITA3        | 60           | 1.2               | 1                       |
| 30| Predicted: integrin beta-3 (CD61 antigen)             | ITB3        | 57           | 0.8               | 1                       |
| 31| Receptors                                             |             |              |                   |                         |
| 32| Protein S100-A6 (Calcyclin)                           | S10A6       | 277          | 53.3              | 4                       |
| 33| CD44 antigen (Hyaluronan receptor)                    | CD44        | 260          | 15.3              | 5                       |
| 34| CD63 antigen (Tetraspanin-30)                         | CD63        | 55           | 2.5               | 1                       |
| 35| CD81 antigen (Tetraspanin-28)                         | CD81        | 54           | 8.5               | 1                       |
| 36| Coflin-1                                              | COF1        | 234          | 39.8              | 5                       |
| 37| Myristoylated alanine-rich C-kinase substrate          | MARCS       | 172          | 9.0               | 3                       |
| 38| Cell division control protein 42 homologue            | CDC42       | 154          | 6.5               | 3                       |
| 39| CD71 antigen (Transferin receptor protein 1)           | TFR1        | 64           | 1.7               | 1                       |
| 40| Predicted: Thy-1 membrane glycoprotein (CD90 antigen) | THY1        | 152          | 21.1              | 3                       |
| 41| Basigin (CD147 antigen) precursor                     | BSG         | 100          | 13.7              | 3                       |
| 42| Predicted: disintegrin and metalloproteinase domain-containing protein 9 isoform 1 | ADAM9 | 83 | 1.7 | 1 |
| 43| P48 (Cytokine receptor-like factor 3)                 | CRLF3       | 80           | 6.8               | 2                       |
| 44| Membrane steroid binding protein                      | gi|7689365 | 191 | 29.2 | 4 |
| 45| Mannose receptor, C type 2                            | MRC2        | 69           | 0.9               | 1                       |
| 46| Lactadherin                                           | MPGM        | 66           | 2.7               | 1                       |
| 47| Enzymes                                               |             |              |                   |                         |
| 48| Protein disulfide-isomerase                            | PDIA1       | 320          | 11.8              | 5                       |
| 49| Alpha-1-enolase                                        | ENOA        | 295          | 24.4              | 7                       |
| 50| CD73 antigen (5'–nucleotidase)                         | SNTD        | 160          | 12.0              | 5                       |
| 51| Ras-related protein Rap-1b                            | RAP1B       | 146          | 19.0              | 3                       |
| 52| Prolyl endopeptidase FAP                              | SEPR        | 130          | 5.3               | 3                       |
| 53| Transforming protein RhoA                              | RHOA        | 146          | 24.9              | 4                       |
| 54| TRAF2 and NCK-interacting protein kinase               | TNK         | 61           | 0.6               | 1                       |
| 55| Thioricin-related transmembrane protein 1              | TMX1        | 67           | 4.3               | 1                       |
| 56| Rho GTPase-activating protein 21                       | RHG21       | 56           | 0.4               | 1                       |
| 57| Predicted: adipocyte plasma membrane-associated protein isoform X1 | APMAP | 62 | 3.0 | 1 |
| 58| Miscellaneous                                          |             |              |                   |                         |
| 59| Brain acid soluble protein 1                          | BASP1       | 223          | 21.1              | 4                       |
| 60| Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12 | GGB12 | 98 | 56.9 | 3 |
| 61| Receptor expression-enhancing protein 5                | REEP5       | 86           | 11.6              | 2                       |
| 62| CD276 antigen                                          | CD276       | 55           | 10.8              | 2                       |
| 63| Annexin A5                                            | ANXA5       | 54           | 3.8               | 1                       |
| 64| Tuberin                                                | TSC2        | 54           | 0.4               | 1                       |
| 65| Predicted: Tetraspanin-6 isoform X1                    | TSPAN6      | 105          | 7.8               | 2                       |
| 66| Predicted: Matrix-remodeling-associated protein 7      | MXRA7       | 79           | 20.3              | 1                       |
| 67| Predicted: Protein lifeguard 3                         | TMBIM1      | 77           | 4.5               | 1                       |
| 68| Predicted: Proteolipid protein 2                       | PLP2        | 72           | 18.4              | 2                       |

*UniProt IDs are shown where available. In other cases, NCBI accession numbers are shown.*
subproteome (the membranome). We have chosen to use equine articular chondrocytes in this study, for the horse is widely involved in occupational/sports activities and considered as an excellent animal model for human joint diseases (Aigner et al., 2010) and yet, current knowledge is limited and relates to only the protein complement of equine chondrocytes. The unique features of our study are as follows. First, to the best of our knowledge, this is the first application of LC-MS/MS proteomics to study the membrane protein complement of cultured articular chondrocytes. Second, a large proportion (133 proteins; 42%) of the 315 proteins identified in this work consisted of membrane

| # | Name | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|---|------|----------------|--------------|------------------|------------------------|
| **Exosome/lysosome/endosome/vesicle membrane** | | | | | |
| 1 | Ras-related protein Rab-7a | RAB7A | 538 | 60.9 | 10 |
| 2 | Vesicle-associated membrane protein 3 | VAMP3 | 239 | 38.5 | 3 |
| 3 | Ras-related protein Rab-10 | RAB10 | 164 | 22.5 | 4 |
| 4 | 14-3-3 protein theta | 1433T | 201 | 22.0 | 5 |
| 5 | Cation-dependent mannose-6-phosphate receptor | MPD | 150 | 13.3 | 3 |
| 6 | Syntaxin-7 | STX7 | 144 | 19.9 | 5 |
| 7 | Ras-related protein Rab-11B | RAB11B | 176 | 14.2 | 3 |
| 8 | Ras-related protein Rab-14 | RAB14 | 128 | 15.3 | 3 |
| 9 | Vesicle-associated membrane protein 5 | VAMP5 | 54 | 11.2 | 1 |
| 10 | Lysosome membrane protein 2 | SCRB2 | 115 | 2.7 | 1 |
| 11 | Charged multisvesicular body protein 6 | CHMP6 | 51 | 6.5 | 1 |
| 12 | PRA1 family protein 2 | PRAF2 | 89 | 16.3 | 2 |
| 13 | PREDICTED: glucosylceramidase isoform X2 | GLCM | 88 | 4.9 | 2 |
| **Golgi/ER membrane** | | | | | |
| 1 | Ras-related protein Rab-1A | RAB1A | 301 | 35.6 | 6 |
| 2 | Ras-related protein Rab-1B | RAB1B | 266 | 25.9 | 5 |
| 3 | Ras-related protein Rab-2A | RAB2A | 213 | 24.5 | 4 |
| 4 | Transmembrane emp24 domain-containing protein 2 (Fragment) | TMED2 | 83 | 10.7 | 2 |
| 5 | Transmembrane emp24 domain-containing protein 5 | TMED5 | 57 | 5.3 | 1 |
| 6 | Transmembrane emp24 domain-containing protein 9 | TMED9 | 116 | 14.9 | 3 |
| 7 | Transmembrane emp24 domain-containing protein 10 | TMEDA | 228 | 22.4 | 5 |
| 8 | Membrane-associated progesterone receptor component 1 | PGR1 | 149 | 11.8 | 2 |
| 9 | Membrane-associated progesterone receptor component 2 | PGR2 | 178 | 17.0 | 4 |
| 10 | Transmembrane emp24 domain-containing protein 4 | TMED4 | 120 | 12.3 | 2 |
| 11 | 78 kDa glucose-regulated protein | GRP78 | 155 | 5.5 | 3 |
| 12 | Translocon-associated protein subunit delta | SSRE | 110 | 17.3 | 2 |
| 13 | Surfeit locus protein 4 | SURF4 | 93 | 8.2 | 2 |
| 14 | Vesicular integral-membrane protein VIP36 | LMN2 | 58 | 3.4 | 1 |
| 15 | Calnexin | CALX | 78 | 2.2 | 1 |
| 16 | B-cell receptor-associated protein 31 | BAP31 | 85 | 8.1 | 2 |
| 17 | Eukaryotic translation initiation factor 5A-1 | IF5A1 | 53 | 10.4 | 2 |
| 18 | Vesicle-associated membrane protein-associated protein A | VAPA | 83 | 4.8 | 1 |
| 19 | Vesicle-trafficking protein SEC22b | SEC22B | 53 | 5.6 | 1 |
| 20 | PREDICTED: PRA1 family protein 3 | PRAF3 | 89 | 16.0 | 3 |
| 21 | lanosterol 14-alpha demethylase | CP51A | 72 | 2.4 | 1 |
| 22 | PREDICTED: golgin subfamily B member 1 | GOGA1 | 69 | 0.4 | 1 |
| 23 | PREDICTED: NADH-cytochrome b5 reductase 3-like isoform 1 | gi| 62 | 4.7 | 1 |
| **Mitochondrial membrane** | | | | | |
| 1 | Cytochrome c oxidase subunit 5A, mitochondrial | COX5A | 197 | 30.9 | 4 |
| 2 | ATP synthase subunit beta, mitochondrial | ATPB | 235 | 16.7 | 7 |
| 3 | ATP synthase subunit alpha, mitochondrial | ATPA | 144 | 6.3 | 3 |
| 4 | ATP synthase subunit delta, mitochondrial | ATPD | 90 | 8.3 | 1 |
| 5 | Apoptosis regulator BAX | BAX | 70 | 5.7 | 1 |
| 6 | Cytochrome c oxidase subunit 4 isoform 1, mitochondrial (Fragment) | COX4I | 59 | 10.3 | 1 |
| 7 | Carbamoyl-phosphate synthase [ammonia], mitochondrial | CPSM | 65 | 1.2 | 1 |
| 8 | prohibitin-2 | PHB2 | 63 | 4.0 | 1 |
| 9 | Cytochrome c oxidase subunit 5B, mitochondrial | COX5B | 61 | 3.3 | 2 |
| 10 | cytochrome oxidase subunit 2 | COX2 | 74 | 3.1 | 1 |
| 11 | PREDICTED: mitochondrial fission 1 protein-like | gi| 58 | 10.5 | 1 |
| **Nuclear membrane** | | | | | |
| 1 | PREDICTED: nesprin-1 | SYNE1 | 73 | 0.1 | 1 |
| 2 | PREDICTED: transmembrane protein 109 isoform X1 | TMEM109 | 60 | 4.9 | 1 |
| **Miscellaneous** | | | | | |
| 1 | Peptidyl-prolyl cis-trans isomerase A | PPIA | 241 | 43.3 | 5 |
| 2 | PREDICTED: cell cycle progression protein 1 isoform X1 | CCPG1 | 65 | 1.6 | 2 |

*UniProt IDs are shown where available. In other cases, NCBInr accession numbers are shown.
proteins, and for some of these only ambiguous data were available in chondrocytes [e.g. CD276, S100-A6 (calcyclin), VDACs]. The proportion of membrane proteins was even higher in the hydrophobic phase (55%). As far as the proportion of membrane proteins is concerned, our results are in good agreement with those reported elsewhere (Hansson et al., 2010). The aim of that study was to characterise the human pancreatic islet membrane proteome by evaluating five different extraction procedures; while the proportion of membrane proteins in the total extracts was 35%, a considerably higher proportion (61%) of membrane proteins was identified following the use of membrane protein-enriching methods. It is also worth noting that the choice of method for extraction of membrane proteins had a strong influence on the number and identity of proteins detected in that analysis, and the hydrophobic phase of Triton X-114 phase separation was found to be the most efficient extraction method (Hansson et al., 2010). These results also underpin that appropriate sample preparation and pre-fractionation methods can increase the amount of identified proteins with specific properties in the MS/MS analysis of proteins in complex biological samples. It was for that reason that we used the Triton X-114 phase separation method in this study.

Although a detailed description of the proteins identified in the chondrocyte membranome is beyond the scope of this article, a few important protein classes merit comment due to their potential involvement in chondrocyte homeostasis. We therefore restrict discussing our results to certain protein groups localised in the PM.

**CD antigens and integrins**

Cluster of differentiation (CD) proteins are four hydrophobic domain-containing cell surface membrane glycoproteins that mediate a range of cellular processes including development, differentiation, activation, growth and motility. Composed of alpha and beta subunits, integrins are integral PM receptors that mediate attachment between a cell and its surroundings. They transduce information from the ECM to the cell and integrin-mediated signalling pathways influence cell shape, mobility, differentiation and the cell cycle. The integrins identified in this study (integrin beta-1 [CD29], integrin alpha-5 [CD49e], integrin alpha-V [CD51], integrin alpha-3 [CD49c] and integrin beta-3 [CD61]) are in complete agreement with what has been published earlier (Mobasheri et al., 2002a; Shakibaei et al., 2008; Woods et al., 1994). Our data also confirm reports on CD antigen expression in articular chondrocytes (Diaz-Romero et al., 2005). We found that equine articular chondrocytes express tetraspanins (CD9 [tetraspanin-19], CD63 [tetraspanin-30], CD81 [tetraspanin-28] and CD151 [tetraspanin-24]); CD44 (hyaluronan receptor); CD71 (transferrin receptor); CD90 (Thy-1); and CD166 (ALCAM). CD73, an ecto-5'-nucleotidase, which plays a crucial role in extracellular adenosine generation, has been reported to be involved in mechanotransduction pathways following cyclic compressive stimulation (Ode et al., 2013). CD107 (LAMP) expression has been recently reported in murine growth plate cartilage and cartilaginous nodules in embryonic limb bud-derived micromass cultures (Hatakeyama et al., 2014). CD147 (basigin; also known as extracellular matrix metalloproteinase inducer) is known to be extensively expressed by chondrocytes both in normal and OA cartilage (Orazizadeh & Salter, 2008). CD228 (melanotransferrin) has also long been known to facilitate the differentiation of prechondrogenic cells (Suardita et al., 2002). While CD276 has not been identified earlier in chondrocytes, it is known to be expressed in undifferentiated mesenchymal stem cells derived from Wharton’s jelly (WJ-MSCs), even after osteogenic, adipogenic and chondrogenic differentiation (La Rocca et al., 2013).

**S100 proteins**

Of particular interest is protein S100-A6 (calcyclin) expression in equine articular chondrocytes. The S100 family of proteins consists of 24 members, which show cell-specific expression patterns and are involved in a wide range of cellular processes including proliferation, differentiation, apoptosis, Ca²⁺ homeostasis, energy metabolism, inflammation and migration/invagination through interactions with a variety of target proteins ranging from enzymes, cytoskeletal...
Table 3. Subcellular distribution of non-membrane proteins in the hydrophobic pool identified in equine articular chondrocytes based on GO annotations.

| #     | Name                                  | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|-------|---------------------------------------|----------------|--------------|-------------------|-------------------------|
| 1     | Alpha-2-macroglobulin                 | A2MG           | 914          | 15.8              | 19                      |
| 2     | Transgelin-2                          | TAGL2          | 426          | 47.7              | 9                       |
| 3     | Apolipoprotein D                      | APOD           | 188          | 24.3              | 6                       |
| 4     | Alpha-1-antiproteinase (Serpin A1, antitrypsin) | A1AT          | 220          | 9.4               | 5                       |
| 5     | Guanine nucleotide-binding protein G(i)/G(S)/G(T) subunit beta-1 | GBB1          | 90           | 6.2               | 2                       |
| 6     | Annexin A2                            | ANXA2          | 83           | 7.7               | 2                       |
| 7     | Guanine nucleotide-binding protein subunit beta-4 | GBB4          | 119          | 9.7               | 3                       |
| 8     | Galectin-1                            | LEG1           | 119          | 16.3              | 2                       |
| 9     | Pancreatic trypsin inhibitor          | BPT1           | 52           | 13.0              | 1                       |
| 10    | Transhyretin                           | TTHY           | 95           | 19.0              | 2                       |
| 11    | SPARC                                 | SPRC           | 190          | 25.3              | 5                       |
| 12    | Alpha-S1-casein                       | CASA1          | 72           | 10.3              | 2                       |
| 13    | Triosephosphate isomerase             | TPIS           | 214          | 21.3              | 4                       |
| 14    | Hemopexin                             | HEMO           | 55           | 3.5               | 2                       |
| 15    | Apolipoprotein E                      | APOE           | 54           | 6.5               | 2                       |
| 16    | Hypothetical protein PANDA_010395 (lipocalin) | gi|281339160     | 152           | 5.9               | 1                       |
| 17    | PREDICTED: cell growth regulator with EF hand domain protein 1 isoformX1 | gi|149727690     | 111          | 14.1              | 3                       |
| 18    | Complement component C4               | CO4            | 63           | 11.6              | 1                       |
| 19    | PREDICTED: ovostatin-like             | gi|344278152     | 57           | 1.1               | 1                       |

Cytoplasm/cytoskeleton

| #     | Name                                  | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|-------|---------------------------------------|----------------|--------------|-------------------|-------------------------|
| 1     | Actin, cytoplasmic 1                   | ACTB           | 610          | 41.1              | 14                      |
| 2     | Tropomyosin alpha-4 chain              | TPM4           | 538          | 32.7              | 10                      |
| 3     | Myosin light polypeptide 6             | MYL6           | 337          | 52.3              | 8                       |
| 4     | 60S acidic ribosomal protein P2        | RLA2           | 334          | 60.0              | 5                       |
| 5     | Tropomyosin alpha-1 chain              | TPM1           | 339          | 21.5              | 7                       |
| 6     | Tropomyosin beta chain                 | TPM2           | 319          | 18.0              | 6                       |
| 7     | Glyceraldehyde-3-phosphate dehydrogenase | G3P           | 271          | 15.9              | 4                       |
| 8     | Tropomyosin alpha-3 chain              | TPM3           | 298          | 21.4              | 6                       |
| 9     | Tubulin alpha-1B chain                | TBA1B          | 381          | 19.1              | 7                       |
| 10    | Transgelin                            | TAGL           | 136          | 16.4              | 3                       |
| 11    | L-lactate dehydrogenase A chain        | LDHA           | 217          | 13.3              | 4                       |
| 12    | Pyruvate kinase PKM                    | KPYM           | 186          | 12.6              | 5                       |
| 13    | Tubulin alpha-1A chain                | TBA1A          | 168          | 12.2              | 4                       |
| 14    | Heat shock protein beta-1 (hsp25, hsp27) | HSPB1         | 209          | 22.5              | 5                       |
| 15    | Peroxiredoxin-1                       | PRDX1          | 217          | 27.1              | 6                       |
| 16    | 14-3-3 protein zeta/delta             | 1433Z          | 74           | 19.2              | 3                       |
| 17    | Tubulin beta-5 chain                  | TBB5           | 122          | 9.9               | 3                       |
| 18    | Profilin-1                            | PROF1          | 112          | 24.3              | 4                       |
| 19    | Fructose-bisphosphate aldolase A      | ALDOA          | 178          | 13.7              | 3                       |
| 20    | Hsc70-interacting protein             | F10A1          | 63           | 6.8               | 2                       |
| 21    | 14-3-3 protein epsilon                | 1433E          | 57           | 11.4              | 2                       |
| 22    | Phosphoglycerate kinase 1             | PGK1           | 83           | 6.7               | 2                       |
| 23    | Myosin-9                              | MYH9           | 100          | 0.6               | 1                       |
| 24    | Far upstream element-binding protein 2 | FUBP2         | 103          | 4.6               | 3                       |
| 25    | Caldesmon                             | CALD1          | 82           | 3.9               | 2                       |
| 26    | 14-3-3 protein beta/alpha             | 1433B          | 81           | 11.8              | 2                       |
| 27    | Guanine nucleotide-binding protein G(i) subunit alpha-2 | GNAI2         | 77           | 7.9               | 2                       |
| 28    | 60S acidic ribosomal protein P1       | RLA1           | 64           | 14.0              | 1                       |
| 29    | 40S ribosomal protein S12             | RS12           | 63           | 19.7              | 2                       |
| 30    | Nuclease-sensitive element-binding protein 1 | YBOX1         | 61           | 4.6               | 1                       |
| 31    | Calmodulin                            | CALM           | 59           | 22.1              | 2                       |
| 32    | Elongation factor 1-alpha 1           | EF1A           | 56           | 5.0               | 2                       |
| 33    | Heat shock cognate 71 kDa protein     | HSP7C          | 134          | 6.3               | 3                       |
| 34    | Metallothionein-1A                    | MT1A           | 52           | 19.7              | 1                       |
| 35    | Protein S100-A1                       | S10A1          | 50           | 16.0              | 1                       |
| 36    | PREDICTED: protein S100-A11           | gi|149751468     | 160          | 27.0              | 3                       |
| 37    | A-kinase anchor protein 9              | AKAP9          | 82           | 0.3               | 1                       |
| 38    | PREDICTED: plasminogen activator inhibitor 1 RNA-binding protein isoform 1 | gi|345800374     | 81           | 4.1               | 1                       |
| 39    | PREDICTED: peroxiredoxin-6            | gi|149707887     | 80           | 17.9              | 3                       |
| 40    | hsp70A1                               | gi|193983       | 79           | 2.7               | 2                       |
| 41    | 40S ribosomal protein S28             | RPS28          | 79           | 30.4              | 2                       |
| 42    | PREDICTED: phosphorylcholine-lipid binding protein 1 | gi|149720563     | 74           | 18.7              | 3                       |
| 43    | G-protein beta subunit                | gi|51116        | 72           | 17.1              | 2                       |
| 44    | phosphoglycerate mutase               | gi|189868       | 59           | 9.5               | 2                       |
| 45    | Stathmin                              | STMN1          | 59           | 15.1              | 2                       |
| 46    | Tropomyosin 3, gamma isoform 19-like protein | gi|528766928     | 268          | 18.6              | 7                       |

(continued)
subunits, receptors, transcription factors and nucleic acids [reviewed in Donato et al. (2013)]. In particular, S100-A6 may function as a Ca\(^{2+}\) sensor and modulator, contributing to Ca\(^{2+}\) signalling pathways. It is also implicated in cell proliferation and cytoskeletal dynamics, and known to have a potential role in cell responses to different stressors. Calcyclin was reported to be significantly upregulated in serially passaged adipose tissue-derived MSCs (Capra et al., 2012), which may correspond to previous data suggesting that it is frequently upregulated during proliferation and differentiation and it is induced by different growth factors. There are only sporadic data available relating to S100-A6 expression in chondrocytes. Its mRNA transcript has been shown to be downregulated following chondrogenic induction by BMP4 in ATDC5 cells, and it has also been identified in one of the chondrocyte proteome studies discussed earlier (Lambrecht et al., 2010).

Other S100 proteins identified in this study include S100-A1 and S100-A11 (Tables 3 and 5). S100-A1 is localised in the cytoplasm where it is associated with cytoskeletal components and mitochondria. It can influence Ca\(^{2+}\) handling in cultured ventricular cardiomyocytes through interaction with the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase and RyR2; it also modulates Ca\(_v\)1 channel currents in a PKA-dependent manner. S100-A1 also regulates energy metabolism by stimulating fructose-1,6-biphosphate aldolase and inhibiting phosphoglucomutase and glycogen phosphorylase (Donato et al., 2013). Also localised in the cytoplasm, S100-A11 is reported to modulate cell growth via binding either to nucleolin or Rad54B (Donato et al., 2013). In particular, S100-A11 can activate the p38 MAPK pathway to accelerate chondrocyte hypertrophy and ECM catabolism that may promote OA progression (Cecil & Terkeltaub, 2008). Both S100-A1 and S100-A11 have been reported to be expressed and functional in chondrocytes (Donato et al., 2013; Patti et al., 1999), and both proteins were identified in a previous MS study (Lambrecht et al., 2010).

### Transporters

Ion channels and transporters are essential components of chondrocytes that control the movement of ions and other small molecules across the PM. An increasing number of studies have reported the presence of an ever-expanding list of

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**Table 3. Continued**

| #   | Name                                      | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|-----|-------------------------------------------|----------------|--------------|------------------|-------------------------|
| 1   | Cathepsin D                               | CATD           | 218          | 15.6             | 7                       |
| 2   | Prosaposin                                | SAP            | 184          | 6.5              | 4                       |
| 3   | Cathepsin K                               | CATK           | 56           | 5.2              | 2                       |
| 4   | CLN2 protein (tripeptidyl peptidase 1)    | TPP1           | 64           | 1.8              | 1                       |
|     | **Mitochondrion**                         |                |              |                  |                         |
| 1   | PREDICTED: Diablo homologue, mitochondrial-like | gi|345323079  | 69            | 4.1              | 1                       |
| 1   | Reticulocalbin-3                          | gi|345323079  | 69            | 4.1              | 1                       |
| 2   | Glucosidase 2 subunit beta                | GLU2B          | 129          | 4.3              | 2                       |
| 3   | Endoplasmic (hsp90beta1)                  | ENPL           | 123          | 2.5              | 2                       |
| 4   | Glucosidase 2 subunit beta                | GLU2B          | 100          | 5.8              | 2                       |
| 5   | Calumenin                                 | CALU           | 73           | 10.2             | 2                       |
| 6   | Serpin H1                                 | SERPH          | 73           | 3.6              | 1                       |
| 7   | Calreticulin                              | CALR           | 65           | 3.1              | 1                       |
| 8   | Endoplasmic reticulum resident protein 29 | ERP29          | 51           | 8.4              | 2                       |
|     | **Nucleus**                               |                |              |                  |                         |
| 1   | Far upstream element-binding protein 1    | FUBP1          | 237          | 14.4             | 7                       |
| 2   | Prothymosin alpha                         | PTMA           | 198          | 21.8             | 4                       |
| 3   | Neuroblast differentiation-associated protein AHNAK | AHNK | 166          | 0.8              | 4                       |
| 4   | Thioredoxin                               | THIO           | 130          | 2.1              | 2                       |
| 5   | Coiled-coil domain-containing protein 57  | CCD357         | 67           | 0.8              | 1                       |
| 6   | Small ubiquitin-related modifier 2        | SUMO2          | 54           | 12.6             | 1                       |
| 7   | PREDICTED: zinc finger protein 764-like   | gi|558191623  | 66            | 2.7              | 1                       |
| 8   | PREDICTED: Fanconi anaemia group C protein | gi|348565316 | 56            | 1.6              | 1                       |
| 9   | PREDICTED: poly [ADP-ribose] polymerase 6 isofromX1 | gi|545216657 | 206          | 4.9              | 2                       |
| 10  | Bromodomain adjacent to zinc finger domain protein 2A | BAZ2A          | 198          | 2.7              | 5                       |
|     | **Contaminants**                          |                |              |                  |                         |
| 1   | Keratin, type II cytoskeletal 1           | K2C1           | 592          | 21.4             | 12                      |
| 2   | Serum albumin                            | ALBU           | 195          | 7.7              | 4                       |
| 3   | Trypsin                                  | TRYP           | 75           | 7.8              | 2                       |
|     | **Miscellaneous**                        |                |              |                  |                         |
| 1   | PREDICTED: leptin receptor gene-related protein-like isofrom X1 | gi|545218045  | 79            | 11.5             | 1                       |
| 2   | Chain C, Ternary Complex Of A Calcineurin A Fragment, Calcineurin B, Fkbp12 And The Immunosuppressant Drug Fk506 (tacrolimus) | gi|1942335  | 65            | 12.1             | 1                       |

*UniProt IDs are shown where available. In other cases, NCBInr accession numbers are shown.
ion channels and transporters in chondrocytes [reviewed in Barrett-Jolley et al. (2010) and Matta et al. (2015)]. Based on GO annotations, 21 proteins with transporter functions were identified in the PM in this study (Tables 1 and 4). Originally described as being localised in the outer mitochondrial membrane (Benz, 1994), voltage-dependent anion-selective channels (VDACs), also known as mitochondrial porins, form the pores that allow the transport of small hydrophilic solutes across the membrane. However, accumulating evidence support that VDACs can also be expressed in the PM (De Pinto et al., 2010), where they exhibit voltage-gated anion channel activity, and its electrophysiological phenotype is that of a maxi-chloride channel (Lewis et al., 2013). Although VDACs have not been unequivocally reported to be expressed and function in chondrocytes, the anion channel identified in some previous studies was the maxi-chloride channel (Lewis et al., 2013). Although VDACs have not been unequivocally reported to be expressed and function in chondrocytes, the anion channel identified in some previous studies was the maxi-chloride channel, which is remarkably similar to the maxi-Cl/VDAC channel (Sugimoto et al., 1996; Tsuga et al., 2002). Although all three VDAC proteins were identified in chondrocytes in our experiments and also by others (Lambrecht et al., 2010), further studies will need to functionally investigate the physiological and pathophysiological roles of these transporters in the chondrocyte PM.

The chloride intracellular channel (CLIC) proteins possess pH-dependent chloride ion channel activity. CLIC1 and CLIC4, in addition to other members of the CLIC family, are often referred to as ‘‘p64-related’’ proteins, and while they may localise to intracellular compartments (e.g. the nucleus), they also appear to be in the PM and could serve a role in secretion (Lewis et al., 2013). Once again, although the CLIC1 protein was identified in chondrocytes in this study and by others (Lambrecht et al., 2010), its presence and function has not been unambiguously demonstrated earlier.

In addition to anion channels, glucose transporter (GLUT) proteins (facilitative glucose transporter 1 and 3; GLUT 1 and GLUT3) were also identified in our study. Glucose is a key metabolite and a structural precursor for articular cartilage and its transport has significant consequences for cartilage development and functional integrity. Our results are in a good agreement with previously published data (Mobasher et al., 2002b), confirming here by proteomic techniques the expression of these two GLUT isoforms in articular chondrocytes.

Conclusion

In summary, studying the membranome profile of equine articular chondrocytes by LC-MS/MS following enrichment using Triton X-114 pre-fractionation has turned out to be an excellent approach to gain insight into proteins involved in a wide range of membrane-bound processes such as signal transduction, adhesion and transport of ions and other molecules. In spite of the significant enrichment of lipid-soluble membrane proteins in the hydrophobic phase, the proteins that are present in an extremely low abundance in chondrocytes such as the majority of ion channels and other transporter molecules in the PM remained undetectable. Although detergent-based phase partitioning enriches PM proteins relative to total soluble proteins, the membrane proteins in the ER, mitochondria and other organelles are also enriched; and the abundance of proteins in the contaminating organelles can interfere with the ability to detect PM proteins (Zhang & Peck, 2011). To mitigate these limitations, a
A combination of the Triton X-114 phase separation method with other membrane protein enrichment techniques could also be considered.

Our study confirms some previous findings and adds further proteins to the proteomic profile of equine articular chondrocytes. Some of the identified proteins including the CD276 antigen, S100-A6 (calcyclin) or VDACs have not been unambiguously reported before to be components of articular chondrocytes. However, there are certain limitations to this work. First and foremost, protein identifications were somewhat aggravated by the fact that our search results listed the same proteins several times but for different species (primarily human or bovine but no horse entries), suggesting that the protein was present in the sample and that the identification was made by virtue of the horse protein sharing homology with several other species. This is one of the disadvantages when “cross species matching” is used to identify proteins. Another possible disadvantage of using equine articular chondrocytes is that there may be subtle differences between the two species and that the entirety of our results may not be directly applicable to human articular chondrocytes.

A more detailed and comprehensive insight into the chondrocyte membranome is likely to make a significant contribution to the development of novel drugs for arthritic diseases. The development and refinement of proteomics-based techniques will enable a better understanding of regulatory proteins and enhance the search for new drug targets. It may also help to discover novel cartilage disease-specific biomarkers. Thus, our data represent a significant addition to the comprehensive cartilage proteome database that is essential for understanding the molecular mechanisms underlying cartilage function and OA.

Table 4. Functional classification of PM proteins and other membrane proteins in the hydrophilic pool identified in equine articular chondrocytes based on GO annotations.

| # | Name | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides | Function of PM protein |
|---|---|---|---|---|---|---|
| 1 | Thrombospondin-1 | TSP1 | 811 | 14.3 | 14 | Adhesion |
| 2 | Protein disulfide-isomerase | PDIA1 | 614 | 28.1 | 13 | Enzyme |
| 3 | Alpha-enolase | ENOA | 557 | 32.5 | 11 | Enzyme |
| 4 | Annexin A1 | ANXA1 | 468 | 39.9 | 10 | Transporter |
| 5 | Cofilin-1 | COF1 | 306 | 38.6 | 5 | Receptor |
| 6 | Moesin | MOES | 303 | 15.1 | 8 | Adhesion |
| 7 | Annexin A5 | ANXA5 | 258 | 19.1 | 6 | Other (receptor) |
| 8 | Integrin beta-1 (CD29 antigen, Fibronectin receptor beta subunit) | ITB1 | 160 | 6.3 | 4 | Adhesion |
| 9 | Protein disulfide-isomerase A6 | PDIA6 | 147 | 14.1 | 4 | Enzyme |
| 10 | Protein disulfide-isomerase A4 | PDIA4 | 109 | 1.9 | 1 | Enzyme |
| 11 | Prolow-density lipoprotein receptor-related protein 1 | LRP1 | 76 | 0.5 | 2 | Receptor |
| 12 | Annexin A4 | ANXA4 | 58 | 5.6 | 2 | Other (receptor) |
| 13 | 40S ribosomal protein SA (Laminin receptor) | RSSA | 57 | 4.4 | 1 | Receptor |
| 14 | Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 | PLOD2 | 53 | 1.9 | 1 | Enzyme |
| 15 | Myristoylated alanine-rich C-kinase substrate | MARCS | 52 | 4.5 | 1 | Other |
| 16 | Cytoskeleton-associated protein 4 | CKAP4 | 99 | 5.0 | 3 | Receptor |
| 17 | Guanine nucleotide-binding protein subunit beta-2-like 1 | GBLP | 66 | 7.3 | 2 | Other |
| 18 | Brain acid soluble protein 1 | BASP1 | 52 | 5.7 | 1 | Other |
| 19 | Talin-1 | TLN1 | 418 | 3.1 | 8 | Other (receptor) |
| 20 | PREDICTED: annexin A8 isoform X1 | gi|545213509 | 116 | 7.3 | 2 | Other |
| 21 | PREDICTED: alpha-2-macroglobulin receptor-associated protein | gi|149732344 | 68 | 12.4 | 2 | Transporter |
| 22 | PREDICTED: ADP/ATP translocase 2-like | gi|558210559 | 64 | 2.7 | 1 | Transporter |
| 23 | PREDICTED: utrophin | gi|507925858 | 57 | 0.4 | 1 | Other |
| 24 | PREDICTED: annexin A8 isoform X1 | gi|466052157 | 62 | 0.3 | 1 | Other |
| 25 | Vacuolar protein sorting-associated protein 35 | VPS35 | 65 | 1.6 | 1 | Transporter |

Exosome/lysosome/endosome/vesicle membrane

| # | Name | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides | Function of PM protein |
|---|---|---|---|---|---|---|
| 1 | 78 kDa glucose-regulated protein | GRP78 | 734 | 30.0 | 15 | |
| 2 | Peptidyl-prolyl cis-trans isomerase A | PPIA | 268 | 43.3 | 7 | |
| 3 | Peptidyl-prolyl cis-trans isomerase B | PPIB | 174 | 21.3 | 4 | |
| 4 | ATP synthase subunit beta, mitochondrial | ATPB | 120 | 4.5 | 2 | |
| 5 | ATP synthase subunit alpha, mitochondrial | ATPA | 89 | 6.1 | 2 | |
| 6 | ATP synthase subunit delta, mitochondrial | ATPD | 74 | 8.3 | 1 | |

Nuclear membrane

| # | Name | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides | Function of PM protein |
|---|---|---|---|---|---|---|
| 1 | Nesprin-1 | SYNE1 | 57 | 0.1 | 1 | |

Miscellaneous

| # | Name | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides | Function of PM protein |
|---|---|---|---|---|---|---|
| 1 | DNA primase small subunit | gi|431914029 | 203 | 2.8 | 3 | |
| 2 | PREDICTED: calpastatin isoform X5 | gi|545185308 | 69 | 3.4 | 2 | |
| 3 | CD209 antigen-like protein C | C209C | 54 | 3.4 | 1 | |

*UniProt IDs are shown where available. In other cases, NCBInr accession numbers are shown.
Table 5. Functional classification of non-membrane proteins in the hydrophilic pool identified in equine articular chondrocytes based on GO annotations.

| #  | Name                                      | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|----|-------------------------------------------|----------------|--------------|-------------------|-------------------------|
| 1  | Secreted (extracellular), exosome         |                |              |                   |                         |
| 1  | Alpha-2-macroglobulin                     | A2MG           | 2098         | 37.0              | 45                      |
| 2  | Transgelin-2                               | TAGL2          | 475          | 44.2              | 7                       |
| 3  | Annexin A2                                | ANXA2          | 397          | 27.7              | 8                       |
| 4  | Triosephosphate isomerase                 | TPIS           | 337          | 30.5              | 6                       |
| 5  | Hemopexin                                 | HEMO           | 270          | 17.9              | 7                       |
| 6  | Serotransferrin                           | TRFE           | 268          | 13.1              | 7                       |
| 7  | Alpha-1-antiproteinase                    | A1AT           | 254          | 10.3              | 5                       |
| 8  | Collagen alpha-1(I) chain                 | CO1A1          | 141          | 1.8               | 2                       |
| 9  | Myosin regulatory light chain RLC-A        | MRLCA          | 127          | 22.7              | 3                       |
| 10 | Galectin-1                                | LEG1           | 109          | 22.2              | 3                       |
| 11 | Transthreititin                           | TTHY           | 91           | 19.0              | 2                       |
| 12 | Macrophage migration inhibitory factor    | MIF            | 86           | 17.4              | 2                       |
| 13 | Complement C4 (Fragments)                 | CO4            | 63           | 3.6               | 2                       |
| 14 | Alpha-1-inhibitor 3                       | A1I3           | 59           | 0.8               | 1                       |
| 15 | Pancreatic trypsin inhibitor              | BPT1           | 51           | 13.0              | 1                       |
| 16 | Connective tissue growth factor           | CTGF           | 98           | 2.9               | 1                       |
| 17 | SH3 domain-binding glutamic acid-rich-like protein | SH3L1 | 62 | 7.9 | 1 |
| 18 | Collagen alpha-5(VI) chain                | CO6A5          | 58           | 0.2               | 1                       |
| 19 | Thrombospondin-2                          | TSP2           | 50           | 1.0               | 1                       |
| 20 | PREDICTED: heat shock protein HSP 90-alpha-like, partial | gi|507695623 | 99 | 3.8 | 2 |
| 21 | PREDICTED: glia-derived nexin (Serpin E2) | gi|344268474 | 60 | 3.0 | 1 |
| 22 | AM2 receptor                              | gi|499442 | 60 | 0.4 | 2 |
| 23 | Semaphorin-3G precursor                   | gi|9910362 | 57 | 1.3 | 1 |

Cytoplasm/cytoskeleton

| #  | Name                                      | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|----|-------------------------------------------|----------------|--------------|-------------------|-------------------------|
| 1  | Tropomyosin alpha-4 chain                 | TPM4           | 902          | 47.6              | 15                      |
| 2  | Tropomyosin beta chain                    | TPM2           | 722          | 31.7              | 12                      |
| 3  | Tropomyosin alpha-3 chain                 | TPM3           | 639          | 34.7              | 10                      |
| 4  | Actin, cytoplasmic 1                      | ACTB           | 616          | 43.5              | 13                      |
| 5  | Tropomyosin alpha-1 chain                 | TPM1           | 584          | 32.4              | 11                      |
| 6  | Phosphoglycerate kinase 1                 | PGK1           | 555          | 42.7              | 13                      |
| 7  | L-lactate dehydrogenase A chain           | LDHA           | 492          | 22.6              | 6                       |
| 8  | Heat shock cognate 71 kDa protein         | HSP7C          | 486          | 17.7              | 10                      |
| 9  | Pyruvate kinase PKM                       | KPYM           | 463          | 23.7              | 10                      |
| 10 | 14-3-3 protein epsilon                    | I433E          | 433          | 37.6              | 9                       |
| 11 | Fructose-bisphosphate aldolase A          | ALDOA          | 423          | 27.7              | 9                       |
| 12 | Filamin-A OS = Homo sapiens              | FLNA           | 406          | 4.3               | 8                       |
| 13 | Tubulin alpha-1B chain                   | TBA1B          | 358          | 20.2              | 7                       |
| 14 | Transgelin                                | TAGL           | 357          | 41.3              | 8                       |
| 15 | Myosin-9                                  | MYH9           | 351          | 7.2               | 9                       |
| 16 | Glyceraldehyde-3-phosphate dehydrogenase | G3P            | 335          | 22.8              | 6                       |
| 17 | 14-3-3 protein zeta/delta                | I433Z          | 319          | 24.1              | 5                       |
| 18 | Tubulin beta-5 chain                      | TBB5           | 310          | 20.7              | 7                       |
| 19 | Phosphoglycerate mutase 1                 | PGAM1          | 271          | 27.2              | 5                       |
| 20 | Myosin light polypeptide 6                | MYL6           | 249          | 52.3              | 7                       |
| 21 | Protein SET                               | SET            | 225          | 18.3              | 4                       |
| 22 | Elongation factor 1-alpha 1              | EF1A1          | 222          | 11.3              | 6                       |
| 23 | Nuclease-sensitive element-binding protein 1 | YBOX1   | 211          | 14.8              | 3                       |
| 24 | Glutathione S-transferase P              | GSTP1          | 202          | 20.0              | 3                       |
| 25 | Phosphatidylethanolamine-binding protein 1 | PE8P1    | 196          | 26.2              | 3                       |
| 26 | Peroxiredoxin-1                          | PRDX1          | 195          | 28.1              | 5                       |
| 27 | Profilin-1                                | PROF1          | 195          | 40.7              | 6                       |
| 28 | Alpha-actinin-4                           | ACTN4          | 193          | 6.0               | 5                       |
| 29 | L-lactate dehydrogenase B chain           | LDHB           | 188          | 9.9               | 3                       |
| 30 | Caldesmon                                 | CALD1          | 182          | 5.7               | 5                       |
| 31 | 14-3-3 protein beta/alpha                | I433B          | 174          | 18.3              | 4                       |
| 32 | Statmin                                   | STMN1          | 159          | 37.6              | 5                       |
| 33 | LIM and SH3 domain protein 1             | LASP1          | 153          | 13.7              | 3                       |
| 34 | Elongation factor 2                      | EF2            | 147          | 4.7               | 4                       |
| 35 | L-lactate dehydrogenase C chain           | LDHC           | 144          | 7.2               | 3                       |
| 36 | Heat shock 70 kDa protein 1A             | HS71A          | 138          | 5.3               | 3                       |
| 37 | Heat shock protein HSP 90-beta           | HS90B          | 128          | 4.7               | 3                       |
| 38 | 14-3-3 protein gamma                     | I433G          | 112          | 6.1               | 2                       |
| 39 | Ferritin light chain                     | FRIL           | 109          | 16.0              | 2                       |
| 40 | Vinculin                                  | VINC           | 107          | 2.5               | 2                       |
| 41 | Heat shock protein HSP 90-alpha          | HS90A          | 100          | 4.9               | 3                       |

(continued)
| #  | Name                                           | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|----|------------------------------------------------|---------------|--------------|------------------|-------------------------|
| 42 | Calmodulin                                     | CALM          | 97           | 33.6             | 4                       |
| 43 | Centromere protein F                           | CENPF         | 96           | 0.2              | 1                       |
| 44 | Nascent polypeptide-associated complex subunit alpha, muscle-specific form | NACAM | 95 | 0.7 | 1 |
| 45 | Ubiquitin                                      | UBQ           | 92           | 32.9             | 2                       |
| 46 | Peroxiredoxin-4                                | PRDX4         | 91           | 11.8             | 3                       |
| 47 | Peroxiredoxin-6                                | PRDX6         | 89           | 10.3             | 2                       |
| 48 | Titin                                         | TITIN         | 81           | 0.0              | 2                       |
| 49 | Plastin-3                                      | PLST          | 77           | 4.9              | 2                       |
| 50 | Nucleoside diphosphate kinase B                | NDKB          | 69           | 17.1             | 2                       |
| 51 | 40S ribosomal protein S28                      | RS28          | 69           | 17.4             | 1                       |
| 52 | Eukaryotic translation initiation factor 4B     | IF4B          | 63           | 2.8              | 1                       |
| 53 | Hepatoma-derived growth factor                 | HDGF          | 61           | 4.2              | 1                       |
| 54 | Rho GDP-dissociation inhibitor 1               | GDRI1         | 60           | 15.2             | 2                       |
| 55 | Fucin-3 (Fragment)                             | FUC3          | 59           | 84.6             | 2                       |
| 56 | Peptidyl-prolyl cis-trans isomerase FKB1A       | FKB1A         | 59           | 12.0             | 1                       |
| 57 | Eukaryotic translation initiation factor 4H     | IF4H          | 55           | 5.2              | 1                       |
| 58 | Metallothionein-1A                             | MT1A          | 55           | 19.7             | 1                       |
| 59 | Heterogeneous nuclear ribonucleoprotein Q      | HNRNPQ        | 53           | 5.6              | 2                       |
| 60 | Rab GDP dissociation inhibitor alpha            | GDIA          | 53           | 8.5              | 2                       |
| 61 | 60S ribosomal protein L22                      | RL22          | 52           | 8.6              | 1                       |
| 62 | 40S ribosomal protein S21                      | RS21          | 103          | 12.0             | 1                       |
| 63 | Myosin-10                                      | MYH10         | 95           | 2.3              | 3                       |
| 64 | 40S ribosomal protein S19                      | RS19          | 81           | 6.9              | 1                       |
| 65 | Ran-specific GTPase-activating protein          | RANG          | 65           | 5.3              | 1                       |
| 66 | Peroxiredoxin-2                                | PRDX2         | 57           | 8.1              | 2                       |
| 67 | Plastin-2                                      | PLSL          | 56           | 1.4              | 1                       |
| 68 | Prostaglandin E synthase 3                     | TEBP          | 50           | 8.1              | 1                       |
| 69 | Transitional endoplasmic reticulum ATPase      | TERA          | 50           | 1.6              | 1                       |
| 70 | Tropomyosin 3, gamma isoform 19-like protein    | gi|528766928  | 639          | 27.2             | 12                      |
| 71 | Striated-muscle alpha tropomyosin              | gi|207349   | 590          | 34.2             | 12                      |
| 72 | PREDICTED: protein S100-A11                    | gi|149751468 | 148          | 27.0             | 3                       |
| 73 | PREDICTED: hsc70-interacting protein isoform X1 | gi|149743058 | 113          | 7.3              | 2                       |
| 74 | PREDICTED: ubiquitin-60S ribosomal protein L40 isoform X1 | gi|532055807 | 82          | 19.5             | 2                       |
| 75 | PREDICTED: 60S ribosomal protein L19-like       | gi|532037025 | 63           | 4.7              | 1                       |
| 76 | Ribosomal protein S3, isoform CRA_f            | gi|148684444 | 57           | 5.4              | 1                       |
| 77 | PREDICTED: dynine heavy chain 9, axonemal      | gi|403275402 | 62           | 0.1              | 1                       |

**Lysosome/melanosome/endosome**

| #  | Name                                           | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|----|------------------------------------------------|---------------|--------------|------------------|-------------------------|
| 1  | Myosin-11                                      | MYH11         | 166          | 2.9              | 4                       |
| 2  | Cathepsin K                                    | CATK          | 105          | 11.5             | 3                       |
| 3  | Cathepsin D                                    | CATD          | 91           | 6.8              | 2                       |
| 4  | Prosaposin                                     | SAP           | 81           | 4.6              | 2                       |
| 5  | PREDICTED: cathepsin B isoform X1              | gi|149698064 | 96           | 9.4              | 2                       |

**Mitochondrion**

| #  | Name                                           | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|----|------------------------------------------------|---------------|--------------|------------------|-------------------------|
| 1  | 60 kDa heat shock protein, mitochondrial       | CH60          | 167          | 14.0             | 5                       |
| 2  | Malate dehydrogenase, mitochondrial            | MDHM          | 145          | 14.8             | 4                       |
| 3  | 10 kDa heat shock protein, mitochondrial       | CH10          | 87           | 29.4             | 3                       |
| 4  | Arginase-2, mitochondrial                      | ARG12         | 67           | 3.4              | 1                       |
| 5  | Stress-70 protein, mitochondrial (Fragments)   | GRP75         | 53           | 2.4              | 1                       |
| 6  | Aspartate aminotransferase, mitochondrial      | AATM          | 67           | 2.8              | 1                       |

**Golg/ER lumen**

| #  | Name                                           | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|----|------------------------------------------------|---------------|--------------|------------------|-------------------------|
| 1  | Protein disulfide-isomerase A3                  | PDIA3         | 573          | 27.5             | 14                      |
| 2  | Calumenin                                       | CALU          | 374          | 40.0             | 9                       |
| 3  | Serpin H1                                       | SERPH         | 367          | 27.3             | 9                       |
| 4  | Endoplasm LIN                                   | ENPL          | 344          | 11.1             | 7                       |
| 5  | Calreticulin                                    | CALR          | 257          | 24.7             | 8                       |
| 6  | Reticulocalbin-3                                | RCN3          | 154          | 12.5             | 3                       |
| 7  | Glucosidase 2 subunit beta                      | GLU2B         | 114          | 7.9              | 3                       |
| 8  | Endoplasmic reticulum resident protein 29       | ERP29         | 92           | 11.1             | 3                       |
| 9  | Prolyl 4-hydroxylase subunit alpha-1            | P4HA1         | 78           | 2.6              | 1                       |
| 10 | Proteasome-associated protein ECM29 homologue   | ECM29         | 58           | 0.3              | 1                       |
| 11 | Thioredoxin domain-containing protein 5         | TXN5D         | 56           | 2.3              | 1                       |
| 12 | PREDICTED: reticulocalbin-1-like                | gi|334331754 | 72           | 8.2              | 2                       |
| 13 | PREDICTED: hypoxia up-regulated protein 1       | gi|514466500 | 62           | 2.1              | 1                       |

(continued)
Declaration of interest

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Table 5. Continued

| # | Name | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|---|---|---|---|---|---|
| 1 | Neuroblast differentiation-associated protein AHNAK | AHNK | 404 | 1.3 | 7 |
| 2 | Nucleolin | NUCL | 248 | 6.7 | 4 |
| 3 | Prothymosin alpha | PTMA | 198 | 22.7 | 4 |
| 4 | Heterogeneous nuclear ribonucleoprotein A2/B1 | ROA2 | 163 | 12.6 | 4 |
| 5 | Heterogeneous nuclear ribonucleoprotein A/B | ROAA | 163 | 13.0 | 3 |
| 6 | Heterogeneous nuclear ribonucleoprotein D0 | HNRPD | 157 | 12.4 | 4 |
| 7 | Heterogeneous nuclear ribonucleoprotein A1 | ROA1 | 139 | 12.8 | 3 |
| 8 | Thioredoxin | THIO | 135 | 21.0 | 2 |
| 9 | Plasminogen activator inhibitor 1 RNA-binding protein | PAIRB | 132 | 6.6 | 2 |
| 10 | High mobility group protein B1 | HMGB1 | 129 | 17.7 | 3 |
| 11 | Parathymosin | PTMS | 120 | 22.5 | 3 |
| 12 | Acridine leucine-rich nuclear phosphoprotein 32 family member A | AN32A | 115 | 11.6 | 2 |
| 13 | Peptidyl-prolyl cis-trans isomerase FKB3 | FKB3 | 82 | 9.8 | 2 |
| 14 | Synaptosomal complex protein 1 | SYCP1 | 82 | 1.0 | 1 |
| 15 | RNA-binding protein 3 | RBM3 | 76 | 17.8 | 2 |
| 16 | Lupus La protein homologue | LA | 69 | 2.7 | 1 |
| 17 | Nucleophosmin | NPM | 65 | 4.8 | 1 |
| 18 | Thyroid hormone receptor-associated protein 3 | TR150 | 57 | 1.8 | 1 |
| 19 | DDB1- and CUL4-associated factor 13 | DCA13 | 57 | 1.8 | 1 |
| 20 | DNA-binding protein | gi|189194 | 157 | 13.4 | 4 |
| 21 | Ran/TC4 Binding Protein | gi|431422 | 65 | 5.4 | 1 |
| 22 | PREDICTED: DDB1- and CUL4-associated factor 13 | gi|348588727 | 59 | 1.8 | 1 |

Contaminants

| # | Name | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|---|---|---|---|---|---|
| 1 | Keratin, type II cytoskeletal 1 | K2C1 | 380 | 13.8 | 8 |
| 2 | Serum albumin | ALBU | 168 | 5.8 | 3 |
| 3 | Trypsin | TRYP | 51 | 3.5 | 1 |

Miscellaneous

| # | Name | Accession No.* | Mascot score | Seq. coverage (%) | No. of matched peptides |
|---|---|---|---|---|---|
| 1 | Coiled-coil domain-containing protein 18 | CCD18 | 55 | 1.8 | 2 |
| 2 | Outer dense fibre protein 2-like | ODF2L | 76 | 1.6 | 1 |
| 3 | Chain C, Ternary Complex Of A Calcinurin A Fragment, Calcinurin B, Fkbp12 And The Immunosuppressant Drug Fk506 (tacrolimus) | gi|1942335 | 59 | 12.1 | 1 |
| 4 | PREDICTED: 28 kDa heat- and acid-stable phosphoprotein | gi|149755423 | 59 | 11.0 | 1 |
| 5 | PREDICTED: LOW QUALITY PROTEIN: 60S ribosomal protein L29-like | gi|513000456 | 60 | 5.2 | 1 |

*UniProt IDs are shown where available. In other cases, NCBInr accession numbers are shown.
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