SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Studying cell surface proteins and the clusterin connectome in chondrocytes

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 05. 06. 2026. 13:00

1. INTRODUCTION

Degenerative diseases affecting the musculoskeletal system and supportive connective tissues represent one of the most significant, yet at the same time one of the most neglected challenges of modern society and healthcare systems. These progressively worsening conditions may lead to chronic, quality-of-life-limiting states, such as inflammatory joint cartilage degeneration, commonly referred to as osteoarthritis (OA).

OA is a heterogeneous disease of multifactorial aetiology that affects not only the cartilage tissue but the entire joint as an organ, and is frequently associated with pain. It is a leading cause of reduced physical performance and early work disability. Epidemiological estimates indicate that in 1990, 4.8% of the global population—approximately 256 million individuals—were affected by OA. This number has increased substantially, as by 2020 OA affected 7.6% of the world's population, corresponding to nearly 595 million people.

OA remains an incurable condition to date. Current therapeutic approaches, including pharmacological and surgical interventions, primarily focus on symptom management, aiming to alleviate pain and improve joint function in order to facilitate daily activities. The development of stem cell-based therapies and the surgical implantation of three-dimensional bio-printed tissues represent promising and exciting new directions. However, the precise characterisation of cartilage progenitor cells, their cost-effective isolation from patients, and their subsequent autologous or allogeneic transplantation are not yet sufficiently efficient. In parallel with therapeutic limitations, disease diagnosis constitutes another major bottleneck, as it still relies predominantly on clinical symptoms and physical examination. In the

knee joint, pain and joint stiffness are among the earliest symptoms. Conventional radiography remains the standard and currently recommended imaging modality for assessing the classical structural deformities associated with OA; however, by the time these changes become detectable, the optimal therapeutic window has often already been missed.

Early diagnosis of OA, as well as the isolation and sorting of high-quality chondrocytes for stem cell-based and cartilage tissue engineering approaches, requires a comprehensive, system-level understanding of cartilage tissue biology and the process of chondrogenic differentiation. Furthermore, there is a critical need for reliable and readily detectable biomarkers that may subsequently be utilised for accurate diagnostic purposes or cell-based therapeutic strategies.

The experimental work forming the basis of this dissertation was guided by two major research directions that closely align with current international trends. Our investigations focused on characterising dynamic changes in the cell surface protein landscape during chondrogenesis and identifying diagnostically and prognostically valuable biomarkers emerging from these alterations. In addition, we mapped the connectome of clusterin (CLU), a chaperone protein that ensures the proper physiological conformation of extracellular (EC) proteins and plays a role in the regulation of cell survival, using bioinformatic databases.

2. AIMS

The primary objective of my doctoral dissertation is to investigate changes in surfaceome expression patterns during *in vitro* chondrogenesis and to identify novel cell surface biomarkers characteristic of specific stages of cartilage development. In addition, the dissertation aims to explore the interaction partners of CLU, a chaperone of cell surface and EC proteins, using network biology–based approaches. Through these investigations, a more detailed understanding of the surfaceome "fingerprint" of chondrocytes at distinct differentiation stages may be achieved, and the biomarkers identified may serve as potential targets for the early diagnosis of degenerative cartilage diseases and for the development of novel therapeutic strategies.

More specifically, during *in vitro* chondrogenic differentiation and by utilising various bioinformatic databases, we sought to address the following questions:

- **What is the qualitative and quantitative composition of the surfaceome during *in vitro* cartilage development?** Which proteins are expressed on the cell surface at different stages of chondrogenesis, and to what extent? What are the major functional categories of cell surface proteins, and do their relative proportions change during differentiation?
- **Are there cell surface proteins that are specific to particular stages of cartilage development?** Can surfaceome proteins be identified that are expressed exclusively during early or late chondrogenesis? Alternatively, are there cell surface proteins that are present throughout the entire differentiation process but exhibit

significant changes in expression levels as chondrogenesis progresses?

- **What potential roles might the identified cell surface biomarkers play in the life cycle and function of chondrocytes?**
- **Finally, what specific functions might CLU fulfil in cartilage physiology and in the pathomechanism of OA?** Which proteins may serve as CLU targets in cartilage tissue, and what putative interactions between these proteins can be inferred based on bioinformatic network analyses?

3. MATERIALS AND METHODS

3.1. Experimental model: chondrogenic micromass primary cell culture derived from chicken embryonic limb buds

The process of *in vitro* chondrogenesis was investigated using chondroprogenitor cells isolated from the limb buds of chicken embryos, which were differentiated in high-density micromass cultures. For the experiments, fertilised eggs (typically 100–150 eggs per biological replicate) were incubated in a hatchery for 4.5 days. At the end of the incubation period, the embryos reached developmental stages 23–24 according to the Hamburger–Hamilton (HH) staging system. Subsequently, under a microscope and using sterile forceps, the distal one-third of the forelimb and hindlimb buds—containing predominantly chondroprogenitor cells—was excised in a laminar flow cabinet. The tissue fragments were digested with 0.25% trypsin–EDTA solution for one hour at 37 °C in a cell culture incubator maintained at 5% CO_2 and 90% relative humidity, after which the digestion was terminated by the addition of foetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Following filtration of the cell suspension, cell density was determined using a Luna automated cell counter, and the suspension was adjusted to a concentration of 1.5×10^7 cells/mL. Depending on the experimental requirements, 30–100 µL droplets of the cell suspension (1–30 droplets per culture vessel) were applied to appropriate culture plates. Cells were allowed to adhere to the bottom surface of the culture vessels for two hours, after which the cultures were supplemented with complete Ham's F12 medium (Sigma-Aldrich, St. Louis, Missouri, USA), which was replaced every two days.

For experimental analyses, cultures were harvested at defined time points corresponding to key stages of chondrogenesis: chondroprogenitor cells on day 1, chondroblasts on day 3, mature chondrocytes on day 6, pre-hypertrophic chondrocytes on day 10, and hypertrophic chondrocytes on day 15.

The use of chicken embryos for research purposes does not require an ethical approval from the Ethics Committee of the University of Debrecen.

3.2. Application of histological staining

During the experiments, changes in cartilage-specific extracellular matrix (ECM) production during cartilage development were monitored using acidic dimethyl-methylene blue (aDMMB) histological staining (Sigma-Aldrich). The orthochromatic colour of the dye is blue; however, upon the onset of chondrogenesis, a metachromatic purple staining becomes apparent, which is caused by the high abundance of proteoglycans and glycosaminoglycans in the forming cartilage matrix. Images of aDMMB-stained cultures were acquired using an Olympus BX53 microscope (Olympus Corporation) with a 4× objective. From the metachromatic pixels visible in the digital images, a metachromatic index was calculated using the MatLab software package (The MathWorks Inc., Natick, MA, USA) based on the applied RGB code (R190, G10, B174), enabling quantitative and qualitative assessment of cartilage matrix composition.

3.3. Isolation and enrichment of cell surface proteins using the aminooxy-biotin technique

The application of cell surface protein enrichment methods is necessary due to the relatively low abundance of plasma membrane (PM) proteins compared to the total cellular proteome. As a result, mass spectrometry-based analyses of whole-cell lysates are often dominated by highly abundant cytoplasmic and intracellular proteins, which can mask the detection of lower-abundance surface-associated proteins.

Prior to labelling, serum-derived proteins were removed from chondrocyte cultures at different developmental stages by thorough washing. Cell surface glycoproteins were then oxidised using 1 mM sodium metaperiodate (Thermo Fisher Scientific) and subsequently labelled with 100 mM aminooxy-biotin (Biotium, Fremont, CA, USA) for one hour. The labelling reaction was terminated by the addition of glycerol (Sigma-Aldrich) to a final concentration of 1 mM, after which cells were lysed using lysis buffer. Cellular debris was removed by successive centrifugation steps (2,800 × g for 5 min, followed by 16,000 × g for 15 min twice, at 4 °C), and the protein-containing supernatant was loaded onto Snap Cap columns containing NeutrAvidin agarose beads (Thermo Fisher Scientific). Protein samples were subjected to sequential washing steps—3 × 15 and 5 × 10 washes, respectively—using the following solutions in order: lysis buffer; PBS containing 0.5% (w/v) sodium dodecyl sulphate (SDS; Amresco); UC buffer (6 M urea [Invitrogen, Waltham, MA, USA], 100 mM Tris–HCl [pH 8.5]); then UC buffer again; 5 M NaCl; 100 mM Na_2CO_3 (Sigma-Aldrich); PBS; and finally HPLC-grade water (Molar Chemicals Kft.).

Following the washing steps, NeutrAvidin-bound cell surface proteins were digested into peptides using a solution containing 50 mM NH_4HCO_3 (Sigma-Aldrich) and 5 µg Pierce Trypsin Protease (Thermo Fisher Scientific), and the resulting peptides were collected. Finally, the digested peptides were lyophilised and submitted for mass spectrometry analysis.

3.4. Isolation of the total proteome of chondrocytes

In parallel with surfaceome isolation, total proteome (whole protein complement) samples were collected from micromass cultures on the same culture days (days 1, 3, 6, 10, and 15). Chondrocytes were lysed in 100 µL RIPA buffer (SERVA Electrophoresis GmbH, Heidelberg, Germany) and subsequently subjected to ultrasonication (3 × 10-second pulses) to ensure efficient solubilisation of various membrane-associated proteins.

3.5. LC–MS/MS-based mass spectrometry analysis of surfaceome and total proteome peptide samples and evaluation of raw data

Lyophilised peptide samples were shipped under cooled conditions to our collaborative partner, David J. Boocock, at the Nottingham Trent University, UK. Mass spectrometry analysis of the samples, as well as evaluation of the raw data, was performed by his research group.

3.6. Bioinformatic analysis of expression data derived from quantitative mass spectrometry

3.6.1. Mapping protein expression patterns using expression clustering and heatmap analysis

Expression clusters, heatmaps generated from quantitative proteomic data, and subsequent Gene Ontology (GO) and Principal Component Analysis (PCA) were performed by our collaborative partner, Péter Brázda, at Utrecht University, the Netherlands.

3.6.2. Surfaceome-based filtering of proteins and functional classification using GO terms, with qualitative comparison by Venn diagram analysis

The first objective was to evaluate the efficiency of cell surface protein labelling using annotation data obtained from the UniProt database. To this end, the "GO cellular component" and "GO biological process" columns were examined, and filtering was performed using selected GO codes characteristic of cell surface localisation.

The filtered protein list was then subjected to an additional search based on four major functional categories: receptors, transporters, enzymes, and adhesion proteins. To broaden the scope of the analysis, two additional annotation columns—"GO biological process" and "GO molecular function"—were considered. In this case, keyword-based searches were applied instead of GO codes.

Finally, surface-enriched protein lists derived from different culture days were qualitatively compared using Venn diagram analysis.

3.7. Validation of the expression of newly identified proteins by western blot analysis

The expression of proteins identified by Venn diagram that had not previously been detected in chondrocytes was validated by western blot analysis using three biological replicates. Protein concentrations of samples obtained by RIPA buffer lysis and ultrasonication were determined using a BCA assay (Thermo Fisher Scientific). Samples were diluted to a uniform concentration of 1 mg/mL using 4× Laemmli buffer (Thermo Fisher Scientific) and PBS, and the resulting mixtures were boiled for 10 minutes at 95 °C in sealed Eppendorf tubes using a preheated incubator (Bioer Technology, Hangzhou, Zhejiang, China).

Under reducing conditions, denatured proteins were separated based on charge and molecular weight by polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Hercules, California, USA), followed by transfer onto nitrocellulose membranes (Bio-Rad Laboratories) using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). Membranes were then incubated overnight with the appropriate primary antibodies diluted in milk–PBS solutions: anti-PODXL polyclonal antibody (1:500 dilution; catalogue number: 18150-1-AP; Proteintech, Rosemont, Illinois, USA), anti-CNTFR polyclonal antibody (1:500 dilution; catalogue number: ab127425; Abcam, Cambridge, UK), and anti-β-actin monoclonal antibody (1:5000 dilution; catalogue number: A5441; Sigma-Aldrich).

Following washing with PBS containing Tween-20 (PBST; Amresco, Solon, OH, USA), membranes were incubated for one hour with horseradish peroxidase-conjugated secondary antibodies: anti-rabbit (catalogue number: 170-6515; Bio-Rad Laboratories) and anti-mouse (catalogue number: 170-6516; Bio-Rad Laboratories). Excess secondary

antibodies were removed by PBST washes, and chemiluminescent signals generated by the secondary antibodies were detected using a gel documentation system (ChemiDoc MP Imaging System, Bio-Rad Laboratories).

3.8. Validation of the cellular localisation of newly identified proteins by immunocytochemistry and confocal microscopy

For immunocytochemical labelling, chondrogenic progenitor cells isolated from chicken embryos were cultured at a density of 15 million cells/mL, similarly to the procedures described above. However, once the cell cultures reached the required culture days (days 1, 3, 6, 10, and 15), micromass cultures were enzymatically detached from the bottom of the culture vessels using 0.25% type I collagenase solution (Sigma-Aldrich), which was also applied during surfaceome isolation. Digestion was carried out for 5–20 minutes, depending on the amount of ECM present.

Subsequently, 500 µL of cell suspension was pipetted into each well of a 24-well plate (Eppendorf) containing 12 mm glass coverslips, thereby establishing monolayer cultures. Cells were allowed to adhere to the bottom of the culture plate for 2 hours. Following formalin fixation, non-specific binding sites were blocked using a solution containing 3% BSA (Amresco) and 10% goat serum (Invitrogen). Primary anti-CNTFR and anti-PODXL antibodies were applied at a dilution of 1:500 in PBS containing 1% BSA and 3% goat serum. This was followed by incubation with an Alexa Fluor 555-conjugated anti-rabbit secondary antibody (Invitrogen) at a dilution of 1:1000. Finally, coverslips containing the labelled cells were mounted onto microscope slides using Vectashield mounting medium containing 2 µL DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories Inc., Burlingame, CA, USA).

The cellular localisation of proteins labelled by immunocytochemistry was examined using an Olympus FV3000 confocal microscope (Olympus Corporation, Shinjuku, Tokyo, Japan). Image acquisition was performed using a 60× PlanApo N oil-immersion objective (NA: 1.42). Visualisation and digital image acquisition were carried out using FV31S-SW software (Olympus Corporation). In all cases, the laser neutral density (ND) filter was set between 4–7%, laser voltage between 400–700 V, gain at 1×, and offset at 0%. Images were generated by averaging three linearly acquired frames at the selected focal plane. Scale bars were embedded into the final images, which were saved in TIFF format for data storage.

3.9. Statistical analysis

All quantitative experimental data presented in the thesis were derived from three independent biological replicates. Bar graphs shown in the dissertation represent mean values calculated from individual replicates, with error bars indicating standard deviation (SD). For mass spectrometry analysis of peptide samples derived from cell surface proteins, normalisation was performed based on the global expression pattern of all detected proteins.

Numerical data analysis and visualisation were performed using Microsoft Excel (version 2507). Statistical evaluation of data obtained from aDMMB histological staining and determination of statistical significance were carried out using Student's two-sample t-test. Comparisons were made between defined culture days of *in vitro* chicken chondrogenesis (days 1, 3, 6, 10, and 15). Differences were considered statistically significant at $p < 0.05$.

3.10. Identification of CLU interaction partners using *in silico* network biology approaches

3.10.1. Mapping CLU interaction partners using the STRING database and Cytoscape software

In our *in silico* studies, the STRING database (version 11.5; www.stringdb.org) and Cytoscape software (version 3.0.0) were initially used to map and visualise the CLU interaction network. Known protein–protein interactions (PPIs) of CLU and their associated GO annotations were retrieved from the STRING database. Subsequently, the PubMed query function integrated within Cytoscape was applied to import the 50 most probable CLU PPI targets based on published literature (confidence threshold: 0.4; network type: full STRING network; query terms: "clusterin" or "clusterin osteoarthritis").

3.10.2. Analysis of CLU interaction partners and their role in OA using Qiagen Ingenuity Pathway Analysis (IPA)

Interaction partners of CLU were further analysed using the IPA database by our collaborative partner. Statistical significance was calculated using Fisher's exact test with a threshold of $p \leq 0.05$ and Benjamini–Hochberg correction. Activation or inhibition of canonical signalling pathways, diseases and disorders, molecular and cellular functions, as well as physiological embryonic developmental processes, were predicted using the IPA *Z*-score algorithm. These predictions were compared against ideal activation or inhibition patterns for each signalling pathway, disease/disorder, or biological function.

Within IPA, the Molecular Activity Predictor (MAP) tool was employed to assess the predicted effects of CLU activation or inhibition on OA-associated signalling pathways.

4. RESULTS

4.1. Verification of the physiological progression of chondrogenic differentiation using aDMMB histological staining

The high-density micromass cell culture generated from chicken embryonic limb buds used in our experiments contains chondrogenic progenitor cells on day 1 of culture, which produce only limited amounts of ECM. By day 3 of chondrogenesis, the cells differentiate into chondroblasts and actively begin to synthesise cartilage matrix components, including glycosaminoglycans (GAGs), which display metachromasia upon aDMMB staining. With the appearance of mature chondrocytes, the amount of ECM increases significantly by culture days 6 and 10, which is clearly reflected both in histological images and in the calculated metachromasia index values derived from these images. Between days 10 and 15, chondrocytes undergo hypertrophic differentiation, during which only a modest further increase in cartilage matrix size is observed.

4.2. Comparison of mass spectrometry results obtained from total proteome and isolated surfaceome samples derived from differentiating chondrocytes at different stages

High-throughput LC–MS/MS analysis was performed on total cell lysates derived from chondrogenic cell cultures, as well as on peptide samples obtained from aminooxy-biotin (AOB)-based cell surface protein isolation. Samples were collected on culture days 1, 3, 6, 10, and 15 (N = 3 biological replicates), corresponding to key stages of chondrogenic differentiation.

4.2.1. PCA-based analysis of total proteome and AOB-enriched surfaceome protein lists

A total of 5,207 proteins or protein groups were identified in the total proteome analysis, whereas 522 proteins or protein groups were detected in the surfaceome-enriched samples ($p < 0.05$). Principal component analysis (PCA) revealed clearly distinct proteomic profiles among the total proteome samples, with biological replicates clustering tightly according to culture day. The first principal component (PC1), explaining 42% of the variance, reflected temporal progression and clearly separated undifferentiated cultures (day 1) from mature cultures (days 10 and 15). The second principal component (PC2; 19% variance) distinguished intermediate stages of cartilage development (days 3–6) from both early and late time points.

Within the surfaceome dataset, 18 of the 522 quantified proteins or protein groups were excluded from PCA because they were detected exclusively on a single culture day, leaving 504 proteins for analysis. PCA revealed low variability among biological replicates and clearly separated samples by culture day along PC1 (40% variance), similarly to the trends observed in the total proteome analysis.

4.2.2. Assessment of overlap between total proteome and AOB-enriched surfaceome protein lists

Comparative analysis revealed an average overlap of only 23% between proteins detected in total cell lysates and those identified in cell surface protein-enriched samples. Based on this observation, it can be inferred that 77% of AOB-isolated proteins—corresponding to 205–238 proteins—were not detectable in total cell lysates. This subset was designated as the “hidden surfaceome”. These findings substantiate the

enhanced sensitivity of the AOB-based enrichment strategy for identifying low-abundance plasma membrane-associated proteins.

4.2.3. In silico GO-based filtering of total proteome and AOB-enriched surfaceome protein lists

To maximise the identification of cell surface proteins, an *in silico* approach was applied to infer surface localisation for each detected protein based on well-established GO annotations. This predictive strategy was first applied to the total cell lysate dataset to define a GO-based cell surface subproteome of chondrocyte cultures. Among the 5,207 proteins identified in this dataset, 885 (17%) were predicted to possess cell surface localisation.

Subsequently, the same GO-based filtering approach was applied to the surfaceome samples. AOB-based enrichment significantly increased the proportion of identified cell surface proteins: across all analysed time points and biological replicates, 55–60% of proteins were annotated as cell surface-localised. These results indicate that the combination of AOB labelling and GO-based filtering represents a substantial methodological advance for achieving a more comprehensive characterisation of the cellular surfaceome.

4.3. DEP and PPI analysis of quantitative mass spectrometry data derived from surfaceome-enriched samples of chondrocytes

Among the 504 analysed proteins, those were considered significantly differentially expressed proteins (DEPs) that exhibited a $\log_2$ fold change ($\log_2$FC) greater than 1.0 and a Benjamini–Hochberg-adjusted *p*-value below 0.05. When comparing consecutive time points, the numbers of up- and downregulated DEPs were as follows: between

days 1 and 3, 72 proteins were up- and 10 were downregulated; between days 3 and 6, 74 proteins were up- and 82 were downregulated; between days 6 and 10, 89 proteins were up- and 31 were downregulated; and between days 10 and 15, 19 proteins were up- and 47 were downregulated.

The identified DEPs were subsequently grouped into four clusters based on their expression profiles, followed by GO enrichment analysis for each cluster. The cluster "A" comprised 96 proteins that displayed progressive upregulation throughout *in vitro* chondrogenesis. This cluster was enriched for GO terms related to ECM organisation (GO:0030198), L-ascorbic acid binding (GO:0031418), peptidyl-proline hydroxylation (GO:0019511) required for collagen alpha-chain synthesis, and bone development (GO:0060348). The cluster "B" consisted of 60 proteins whose expression peaked on day 6 of culture and was characterised by the overrepresentation of GO terms associated with transmembrane ephrin receptor activity (GO:0005005) and fibroblast growth factor binding (GO:0017134). In contrast, the 63 proteins of the cluster "C" exhibited a transient decrease in expression on day 6, followed by renewed upregulation during the late stages of cartilage development in mature cultures. GO terms dominating this cluster included RNA polymerase II phosphorylation (GO:0008353) and cyclin-dependent protein serine/threonine kinase activity (GO:0004693) involved in cell cycle regulation. The cluster "D" contained 88 proteins whose expression steadily declined from day 3 onwards, suggesting a predominant role during early differentiation. Similar to the cluster "B", this group was enriched for ephrin receptor-related (GO:0005005) and fibroblast growth factor-related (GO:0017134) GO terms and also showed evidence of WNT signalling (GO:0042813) involvement. In addition, GO terms associated with muscle and nervous system development (GO:0007517,

GO:0007411, GO:0021953) were detected, potentially reflecting the gradual loss of stem cell-associated plasticity.

Detailed PPI network analysis of the surfaceome protein clusters was performed using the STRING database. In the cluster “A” PPI network, proteins associated with ECM remodelling (MMP2, FN1, VCAN, DCN, PLOD1, CRTAP, P3H2, P4HA1/2), collagen biosynthesis (PLOD1, CRTAP, P3H2, P4HA1/2, P4HB), and protein folding (HSPA5, PDIA3) were prominently represented, alongside components involved in vesicular transport and oxidative stress response (PRDX6). In the cluster “B”, ephrin signalling was dominant, with four ephrin receptors (EPHA7, EPHB1, EPHB2, EPHB3) highlighting their role in the establishment of developmental patterning. Proteins involved in endocytosis (AP2M1, RAB5C) and RAB GTPases (RAB5C, RAB14), recognised as central regulators of dynamic intracellular membrane trafficking, were also present. SRC and FGFR2 provided a functional link between focal adhesions and FGF signalling. The cluster “C” was enriched for proteins associated with cytoskeletal organisation (ACTN1, ACTN4, MYH9, ARF6), calcium signalling (CALM3, S100A6, ANXA1), and cell adhesion pathways (DMD, ARF6, RAP1B). Within this network, focal adhesion and actin cytoskeleton pathways predominated through actinin (ACTN1, ACTN4), myosin (MYH9), and dystrophin (DMD). Calcium signalling components may integrate metabolic and inflammatory responses (CALM3, S100A6), while HMGB1 linked nuclear DNA repair mechanisms with extracellular VEGF-mediated signalling. This cluster also included proteins implicated in endochondral ossification (TIMP3, TGFB2, THBS2, VLDLR). In the cluster “D”, ephrin signalling again predominated, with multiple ephrin receptors (EPHA3, EPHA4, EPHA5, EPHA7, EPHB1, EPHB2, EPHB3) potentially contributing to the

regulation of cell migration during cartilage nodule formation. The Wnt (FZD1, FZD2, FZD7) and Hedgehog (SMO) signalling pathways represented key developmental regulators, while EGFR and FGFR family receptors (EGFR, FGFR1, FGFR2, FGFR3) mediated growth factor-dependent activation of cytoplasmic MAPK signalling.

4.4. Functional classification of AOB-enriched surfaceome proteins

Cell surface proteins selected based on surfaceome-specific GO terms were classified into four main functional categories according to GO "molecular function" and "biological process" annotations: receptors, enzymes, transporters, and cell adhesion and junction proteins. The functional distribution of surfaceome proteins remained largely stable throughout the entire course of chondrogenesis.

4.5. Qualitative comparison of AOB-enriched surfaceome lists derived from different culture days using Venn diagram

Following GO-based filtering of cell surface proteins, qualitative comparisons of protein presence across different culture days were performed to identify potential future biomarker candidates characteristic of specific stages of cartilage differentiation. Only two proteins fulfilled the criteria for early chondrogenic stages (culture days 1–6): ciliary neurotrophic factor receptor (CNTFR) and podocalyxin (PODXL).

4.6. A Validation of CNTFR and PODXL expression and cellular localisation

For both proteins, quantitative mass spectrometry-derived expression data revealed a progressive decrease as differentiation advanced, and neither protein was detectable on culture days 10 and 15.

Expression of CNTFR and PODXL was validated using western blot analysis. In the case of CNTFR, a well-defined band was observed at approximately 43 kDa, corresponding to the predicted molecular weight of the protein. The expression pattern detected by western blot closely mirrored the differentiation-dependent dynamics observed in the quantitative proteomic analysis. Similarly, PODXL appeared as a specific band at approximately 60–70 kDa, and its expression kinetics also followed the quantitative trends obtained from mass spectrometry.

To determine the cellular localisation of CNTFR and PODXL, immunocytochemical staining was performed and analysed by confocal microscopy. During the early stages of differentiation, the fluorescent signal of CNTFR displayed a homogeneous, granular distribution and was present in both the cytoplasm and the nucleus. From day 6 onwards, the signal gradually localised to regions proximal to the plasma membrane, a pattern that remained evident on day 15. In contrast, PODXL exhibited a more focal distribution from day 1 onwards. The intensity of its fluorescent signal progressively decreased during chondrogenesis and was detectable only at very low levels by day 15. Notably, PODXL predominantly localised near the leading edge of cellular protrusions, suggesting a potential role in regulating cell morphology and migration, particularly during the early stages of cartilage development.

4.7. Identification of clusterin interaction partners and their altered expression in OA

4.7.1. Characterisation of the CLU interaction network derived from the STRING database using Cytoscape

According to the STRING database, the CLU protein currently has 25 known interaction partners. Key components of the CLU network

included intracellular chaperones (HSPA5, HSP90B1) as well as aggregation-prone proteins (APP, SNCA, PRNP), findings that are consistent with the well-established multi-level association between CLU and neurodegenerative diseases.

To further expand the identification of interacting components within the CLU network, the built-in PubMed-based text-mining function of Cytoscape was applied to extend the previously defined connectome. Subsequently, a CLU interaction network specifically associated with OA was also constructed using the Cytoscape PubMed text-mining module with the keywords “clusterin” and “osteoarthritis”. This targeted search yielded a total of 13 interacting entities, of which five molecules exhibited primary, direct interactions (AFM, APOA4, HPX, CLU, C7), while an additional seven proteins showed secondary, indirect interactions (PRG4, MMP3, ORM2, PROC, IGLL5, IGFALS, SERPINA4).

4.7.2. CLU interaction network generated using the IPA knowledge base

From the IPA database, 26 molecules were selected whose expression was reported in the literature to be positively or negatively regulated by CLU and were therefore incorporated into the network analysis. CLU was found to inhibit the expression of 12 proteins, including histone H3, BAX, ATP7B, SREBF1, CIAP, IL-6, prostaglandin E2, cholesterol ester, CDKN1A, CXCL8, CDH1, and ATP7A. In contrast, CLU enhanced the expression of 14 proteins, including BCL2L1, NFKBIB, MMP2, NFKBIA, RAD17, APP, SMAD3, AKT1, SMAD2, HSPA5, TP53, TNF, BCL2, and MMP9.

Comparison with interaction partners identified using the STRING database revealed overlapping associations in both analyses, particularly

involving BAX, ATP7B, BCL2L1, APP, and HSPA5 as CLU-interacting proteins.

4.7.3. Molecular activity-based prediction of CLU network involvement in OA

Molecular activity-based predictive analysis using the IPA knowledge base identified five key regulatory signalling pathways potentially relevant to CLU function in OA pathology. These included the pro-inflammatory cytokine IL-6, the stress response regulator TNF-α, matrix metalloproteinases influencing ECM composition (MMP2, MMP9), the cell cycle inhibitor CDKN2A, and the SMAD2/3 pathways involved in TGF-β signalling.

5. MAJOR RESULTS AND CONCLUSIONS

One of the main objectives of my doctoral dissertation was to map the dynamically changing qualitative and quantitative composition of cell surface proteins (the surfaceome) of chondrocytes during *in vitro* chondrogenesis. A further aim was to investigate the network formed by the CLU protein and its interacting partners, in order to understand how these molecular pathways influence the pathomechanism of joint diseases. The most important novel findings presented in the dissertation are summarized below:

Novel findings related to the characterization of the chondrogenic surfaceome:

- Application of the AOB–based cell surface protein enrichment method enabled the identification of 205–238 low-abundance proteins that were not detectable by proteomic analysis of total cell lysates.
- Among the surface proteins displaying a consistently decreasing expression pattern over time, several members of the ephrin receptor family were identified (EPHA3, EPHA4, EPHA5, EPHA7, EPHB1, EPHB2, EPHB3), suggesting that ephrin signalling may play an important regulatory role not only during early embryonic development but also throughout cartilage development.
- Decreasing expression pattern and proximal plasma membrane localization of CNTFR and PODXL proteins were confirmed. These proteins have not previously been associated with cartilage development and may serve as

potential chondroprogenitor cell markers with relevance for the development of cartilage tissue engineering–based therapeutic strategies.

Novel findings related to the clusterin protein:

- Analysis using the STRING database revealed novel associations involving CLU-related selenium metabolism and the CLU–plexin–semaphorin signalling axis, offering new research directions for a deeper understanding of the pathomechanism of OA.
- Molecular activity–based IPA network analysis identified five CLU-regulated pathways that have previously been associated with OA progression. This observation further supports the potential utility of CLU as a biomarker in inflammatory conditions affecting articular cartilage.

6. LIST OF PUBLICATIONS

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H-4002 Egyetem tér 1, Debrecen
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Registry number: DEENK/104/2026.PL
Subject: PhD Publication List

Candidate: Patrik Kovács
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10080142

List of publications related to the dissertation

1. **Kovács, P.**, Brázda, P., Hajdú, T., Harsányi, B., Juhász, K. Z., Takács, R. Á., Vágó, J., Wang, Z., Coveney, C., Boocock, D. J., Matta, C.: Podocalyxin and ciliary neurotrophic factor receptor are novel components of the surfaceome of chondrogenic cells.
Cell Commun. Signal. 24 (8), 1-20, 2026.
DOI: http://dx.doi.org/10.1186/s12964-025-02552-x
IF: 8.9 (2024)

2. **Kovács, P.**, Pushparaj, P. N., Takács, R. Á., Mobasheri, A., Matta, C.: The clusterin connectome: Emerging players in chondrocyte biology and putative exploratory biomarkers of osteoarthritis.
Front. Immunol. 14, 1-21, 2023.
DOI: http://dx.doi.org/10.3389/fimmu.2023.1103097
IF: 5.7

List of other publications

3. **Kovács, P.**, Vágó, J., Mobasheri, A., Jenei-Lanzl, Z., Zaucke, F., Madry, H., Csernoch, L., Szekanecz, Z., Szondy, Z., Szöőr, Á., Szatmári, I., Oláh, T., Matta, C.: Emerging Concepts in Osteoarthritis and Musculoskeletal Diseases: insights from the University of Debrecen Musculoskeletal Symposium 2025.
Osteoarthr. Cartil. Open. 8 (1), 1-8, 2026.
DOI: http://dx.doi.org/10.1016/j.ocarto.2025.100728
IF: 2.8 (2024)

4. **Kovács, P.**, Wang, Z., Hajdú, T., Juhász, K. Z., Katona, É., Takács, R. Á., Vágó, J., Zákány, R., Póliska, S., Szentesi, P., Csernoch, L., Matta, C.: Microgravity-induced transcriptional reprogramming in embryonic chicken limb bud-derived chondrogenic cultures.
Front. Cell. Dev. Biol. 14, 1-16, 2026.
DOI: http://dx.doi.org/10.3389/fcell.2026.1746313
IF: 4.3 (2024)

5. **Kovács, P.**, Boocock, D. J., Coveney, C., Mobasheri, A., Matta, C.: Aminooxy Biotin-Based Characterization of the Surfaceome of Chondrogenic Cells.
In: The Surfaceome: Methods and Protocols / Csaba Matta, Ali Mobasheri, Roland Takács, Humana Press Inc, New York, 65-79, 2025, (Methods and Protocols)

6. Hajdú, T., **Kovács, P.**, Katona, É., Nguyen, M. N., Vágó, J., Fillér, C., Zákány, R., Emri, G., Tóth, G., Reglődi, D., Juhász, T.: Exploring the Link Between PACAP Signalling and Hyaluronic Acid Production in Melanoma Progression.
Int. J. Mol. Sci. 26 (24), 1-25, 2025.
DOI: http://dx.doi.org/10.3390/ijms262412049
IF: 4.9 (2024)

7. Juhász, K. Z., Hajdú, T., **Kovács, P.**, Vágó, J., Matta, C., Takács, R. Á.: Hypoxic Conditions Modulate Chondrogenesis through the Circadian Clock: the Role of Hypoxia-Inducible Factor-1α.
Cells. 13 (6), 1-17, 2024.
DOI: http://dx.doi.org/10.3390/cells13060512
IF: 5.2

8. Takács, R. Á., **Kovács, P.**, Ebeid, R. A., Almássy, J., Fodor, J., Ducza, L., Barrett-Jolley, R., Lewis, R., Matta, C.: Ca2+-Activated K+ Channels in Progenitor Cells of Musculoskeletal Tissues: A Narrative Review.
Int. J. Mol. Sci. 24 (7), 6796, 2023.
DOI: http://dx.doi.org/10.3390/ijms24076796
IF: 4.9

9. Vágó, J., Takács, R. Á., **Kovács, P.**, Hajdú, T., Veen, D. v. d., Matta, C.: Combining biomechanical stimulation and chronobiology: a novel approach for augmented chondrogenesis?
Front. Bioeng. Biotechnol. 11, 1-7, 2023.
DOI: http://dx.doi.org/10.3389/fbioe.2023.1232465
IF: 4.3

10. Takács, R. Á., Vágó, J., Póliska, S., Pushparaj, P. N., Ducza, L., **Kovács, P.**, Jin, E. J., Barrett-Jolley, R., Zákány, R., Matta, C.: The temporal transcriptomic signature of cartilage formation.
Nucleic Acids Res. 51 (8), 3590-3617, 2023.
DOI: http://dx.doi.org/10.1093/nar/gkad210
IF: 16.6

11. Vágó, J., Katona, É., Takács, R. Á., Dócs, K., Hajdú, T., **Kovács, P.**, Zákány, R., Veen, D. v. d., Matta, C.: Cyclic uniaxial mechanical load enhances chondrogenesis through entraining the molecular circadian clock.
J. Pineal Res. 73 (4), e12827, 2022.
DOI: http://dx.doi.org/10.1111/jpi.12827
IF: 10.3

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12. Hajdú, T., **Kovács, P.**, Zsigrai, E., Takács, R. Á., Vágó, J., Cho, S., Sasi Szabó, L. A., Becsky, D., Keller-Pintér, A., Emri, G., Rácz, K., Reglődi, D., Zákány, R., Juhász, T.: Pituitary Adenylate Cyclase Activating Polypeptide Has Inhibitory Effects on Melanoma Cell Proliferation and Migration In Vitro.
Front Oncol. 11, 681603, 2021.
DOI: http://dx.doi.org/10.3389/fonc.2021.681603
IF: 5.738

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