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Quantitative proteomics analysis of deer antlerogenic periosteal cells reveals potential bioactive factors in velvet antlers

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ABSTRACT

Velvet antlers (VA) have been used as medicines and nutraceuticals for over 2000 years. Meanwhile, deer antlers are the only mammalian organs that can fully regenerate after annual shedding. The antler formation and regeneration rely on the stem cells resident in antlerogenic periosteum (AP), transplantation of which can induce ectopic antler formation. Here, a comprehensive quantitative proteomic analysis of antlerogenic periosteal cells (AP cells), compared with the adjacent facial periosteal cells (FP cells), was carried out, from both extracellular and intracellular perspectives. In this study, the stable isotope labeling by amino acids in cell culture (SILAC) was applied to ensure the precision of quantification. Then, the protein equalization strategy and reverse-phase liquid chromatography (RPLC) separation in high pH were utilized to improve the depth of proteome profiling. Proteomics analysis of the conditioned media (CM) from AP and FP cells showed that significantly over-expressed extracellular proteins in AP cells were involved in cell proliferation, angiogenesis and neurogenesis. Combining the extracellular and intracellular proteomes, we found several potential secreted proteins might regulate antler formation and regeneration, such as SFRP4 and LUM. These results provide new insight into the underlying mechanism of antler formation and regeneration.

1. Introduction

Velvet antlers (VA) is a precious traditional Chinese medicine and has been widely used for over 20 centuries. Many studies show that the extracts of velvet antlers have various pharmacological activities, such as immune system modulation, anti-fatigue and anti-inflammation [1,2]. The tip section of a VA is generally considered more medicinally valuable compared with the base section. Presumably, this is attributed to the antler stem cells, which presents in the growing tip of VA and is believed to be responsible for the annual full regeneration and fast growth of deer antlers [3]. Antler stem cells derive from antlerogenic periosteum (AP), which retains embryonic stem cell capabilities, i.e. pluripotency. We have successfully stimulated the differentiation of AP cells into chondrocytes, adipocytes and possible neural cells in vitro. Interestingly, we found that the AP cells can secret instructive factors to regulate the velvet antler development and regeneration [4].

Recently, few studies have investigated the proteins in antler stem cell populations using two-dimensional difference gel electrophoresis (2D-DIGE) [4,5]. Meanwhile, several growth factors and cytokines have been identified in the CM of antler stem cells [6–8]. However, no proteomic studies have focused on the extracellular proteins of antler stem cells. Comprehensive quantitative proteome profiling of the AP cells compared with the adjacent facial periosteal cells (FP cells), from both extracellular and intracellular perspectives, will result in a better understanding in antler development and regeneration and help us identify active regulators involved in these processes.

It is extremely difficult to analyze the extracellular proteome of cells in serum-containing CM for the low abundance of the secreted proteins relative to the complex background of highly abundant serum proteins. Meanwhile, it is also a great challenge to map the entire cell proteome because great efforts still should be made to improve the identification of membrane proteins, which are low abundance and high hydrophobicity. Recently, we

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developed a MLEFF (Metabolic Labeling, protein Equalization, protein Fractionation, and Filter-aided sample preparation) strategy to encounter the challenge of proteomic analysis on serum-containing CM and introduce an ionic liquid for membrane protein extraction and large-scale proteome analysis [9,10]. In this study, these techniques were applied to investigate the extracellular and intracellular comparative proteomics of the AP cells and FP cells by adopting a shotgun proteomic approach. The extracellular proteome analysis demonstrated that the proteins that involve in cell proliferation, angiogenesis and neurogenesis were highly expressed in the CM of the AP cells, as well as the low-expressed proteins related to cell adhesion and extracellular matrix (ECM) organization. The results will improve our understanding of antler formation and regeneration mechanism.

2. Materials and methods

2.1. Cell culture and metabolic labeling

AP and FP cells were labeled using stable isotope labeling with amino acids in cell culture (SILAC) media (Thermo, MA) at 37 °C with 5% CO_2. For the "medium" labeling media, L-lysine- and L-arginine-depleted SILAC DMEM media were supplemented with [4,4,5,5-D_4] L-lysine (100 μg mL^{-1}) and L-arginine (100 μg mL^{-1}), 10% dialyzed fetal bovine serum (FBS), and a 1% penicillin/streptomycin mixture. For the "heavy" labeling media, only [4,4,5,5-D_4] L-lysine was replaced with [$^{13}C_6$, $^{15}N_2$] L-lysine. The AP and FP cells were grown in the "medium" and "heavy" media, respectively. After 36 h, the cells and CM were both harvested and were mixed based on equal number of each cell type.

2.2. Western blotting analysis

AP or FP cells (5×10^6) were washed with PBS and extracted with 4% SDS containing 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich, MA). After centrifugation at 15 000 rpm at 4 °C for 20 min, the supernatant was collected and the protein concentration was determined by bicinchoninic acid (BCA) assay (Beyotime, China). 20 μg of proteins were separated on 12% SDS-PAGE, and then transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad, CA). The PVDF membrane was blocked in Tris-buffered saline containing 5% milk for 1 h at room temperature, followed by incubation with the antibodies including SFEP4 and CD9 (Genetex, CA) overnight at 4 °C. After incubation with anti-HRP conjugate (Thermo, MA), bands were visualized with a chemiluminescence reagent (Thermo, MA) and detected using the ChemiDoc system (Bio-Rad, CA).

2.3. MS sample preparation

The CM were processed according to our previously reported MLEFF strategy with minor modifications. In brief, the collected CM were centrifuged at 500 g for 5 min and 4000 g for 20 min to remove cells and cell debris, respectively. The supernatant was supplemented with 1% (v/v) protease inhibitor cocktail additive and filtrated through a 0.22 μm filter unit (Merck, Germany). Then the CM was concentrated and desalted with water via Amicon 3 kDa filter devices (Merck, Germany), increasing the protein concentration to approximately 50 mg mL^{-1}. The concentrated CM was processed with a protein equalization beads (ProteoMiner, Bio-Rad, CA) using published protocols. Then, the "equalized" protein sample was separated on a GELFrEE 8100 Fractionation System (Expedeon, CA) according to the manufacturer's protocol with minor modifications. 10 fractions were collected and processed by the filter-aided sample preparation (FASP) method and the digested peptides were obtained through centrifugation and dried in a Speed Vac Concentrator (Thermo, MA). All of the samples were stored at −80 °C for further analysis.

The collected cells were extracted with 4% 1-dodecyl-3-methylimidazolium chloride ([C_{12}-mim]Cl) (m/v, containing 1% (v/v) protease inhibitor cocktail) and ultrasonicated on ice for 180 s in total (10 s intervals every 10 s), followed by centrifugation at 15 000 g for 15 min. The supernatants were collected, and the protein concentration was determined by a BCA assay (Beyotime, China). Proteins were reduced in 20 mM dithiothreitol (DTT, Merck, Germany) at 95 °C for 5 min, and then alkylated in 40 mM iodoacetamide (IAA, Merck, Germany) at room temperature in the dark for 30 min. Next, the proteins were transferred to 10 kDa filter devices (Sartorius AG, Germany) and washed with 300 μL of 8 M urea in 25 mM Ammonia bicarbonate (ABC, pH 8.5) by centrifugation (14 000 g) three times. The concentrates were washed with 300 μL of 25 mM ABC three times. After that, the concentrates were diluted with 100 μL of 25 mM ABC containing 1 μg of trypsin (Promega, WI), and incubated at 37 °C for 16 h. Subsequently, peptides were separated and collected by an Agilent 2100 HPLC system (Agilent, CA) with a high pH-stable RP column (4.6 mm × 250 mm, 5 μm, 100 Å, Durashell, China) at a flow rate of 0.5 mL min^{-1} using a gradient from 5 to 45% solvent B over 55 min (solvent A: 30 mM ammonium acetate, pH 10; solvent B: acetonitrile, 30 mM ammonium acetate, pH 10). A total of 50 fractions (0.5 mL) were collected from 6 min to 55 min and the fractions with equal collection time intervals (10 min) were pooled. Finally, 10 pooled fractions were lyophilized in a Speed Vac Concentrator (Thermo, MA). All the samples were stored at −80 °C for further analysis.

2.4. LC-MS/MS analysis

The peptides were analyzed with a nano-RPLC-MS/MS on a Q-Exactive MS (Thermo, CA) coupled with an Ultimate 3000 (Dionex, Germany) nano-LC system. The mobile phases were: buffers A (2% acetonitrile, 98% water, and 0.1% formic acid); B (98% acetonitrile, 2% water, and 0.1% formic acid). Fused-silica capillaries were purchased from Sino Sumtech (Handan, China). Both trap column (75 μm i.d. × 15 cm) and separation column (150 μm i.d. × 5 cm) were homemade and packed with Daiso C_{18} particles (5 μm, 100 Å, Osaka, Japan). A 110 min gradient was applied, comprised of 90 min of 6%−22% buffer B, followed by a 20 min of 22%−35% buffer B. The spray voltage was set to 2.5 kV, and temperature of the ion transfer capillary was set to 275 °C. The Q-Exactive MS was operated in positive ion mode, and the 10 most intense ions were subjected to HCD fragmentation with normalized collision energy at 28%. The MS scans were performed at a resolution of 70 000 from m/z 300 to 1800 (automatic gain control (AGC) value, 1 E^6; maximum injection time, 100 ms), and the data were acquired in profile mode. The MS/MS scans were performed at a resolution of 17 500 (AGC, 1 E^5; maximum injection time, 60 ms), and the data were acquired in centroid mode using a 20 s exclusion window. The unassigned ions or those with a charge of +1 and >+7 were rejected. One microscan was acquired for each MS and MS/MS scan.

2.5. Database searching

The raw data were uploaded into Proteome Discoverer (PD, version 1.4.1.14) with Mascot (2.3.2) and were searched against the UniProtKB Bovine complete proteome sequence database (release 2017_06, 24,148 entries). The reverse sequences were appended for an FDR evaluation. The mass tolerances were set at 0.5 Da for the parent ions and at 10 ppm for the fragments. The peptides were searched using tryptic cleavage constraints, and a maximum of two missed cleavages were allowed. The minimal peptide length was six amino acids. Carbamidomethylation (C, +57.0215 Da) was used

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as the fixed modification. Oxidation (M, +15.9949 Da) and acetylation (protein N-termini, +42.0106 Da) were searched as variable modifications. Two SILAC-based labels, (Lys 4, +4.0251 Da) and (Lys 8, +8.0142 Da), were used as variable modification. The peptide and protein identifications were filtered by PD to keep the FDR ≤1%. At least one unique peptide was required for each protein identification.

2.6. Bioinformatic analysis

A protein was defined as a classical secreted protein when it was annotated to contain a "signal peptide" or had the keyword "secreted" in UniProtKB (http://www.uniprot.org), or it was predicted by the Signal P 4.1 server to had a "signal peptide". Those proteins that did not contain a "signal peptide" and were predicted by Secretome P 2.0 with an NN score >0.5, were defined as non-classical secreted proteins. The exosomal proteins were matched by the ExoCarta database (http://exocarta.org). The Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analysis were performed using DAVID 6.8 (https://david.ncifcrf.gov). ECM-related proteins were matched by the matrisome database (http://matrixdb.univ-lyon1.fr).

3. Results

3.1. Principle of the research strategy

SILAC labeled AP cells (medium labelled) and FP cells (heavy labelled) were used to ensure the quantitative accuracy. Meanwhile, ionic liquid based protein preparation method was applied to improve the extraction of membrane proteins, combining with high-pH RP-RPLC separation to deepen the coverage of the intracellular proteome. For extracellular proteomic analysis, CM was subjected to a MLEFF strategy, which combines metabolic labeling, protein "equalization", protein fractionation, and filter-aided sample preparation (FASP), allowing an in-depth extracellular proteome profile. (Fig. 1). All experiment were performed in three biological replicates.

3.2. Comparative extracellular proteomics analysis of the AP and FP cells

In total, 256 proteins were quantified (Fig. 2A), including 9 growth factors, 27 proteases, 26 protease inhibitors and 11 cell adhesion molecules (Table S-1). Among them, 165 proteins (64.5%) were identified as classical secreted proteins. 10 proteins were

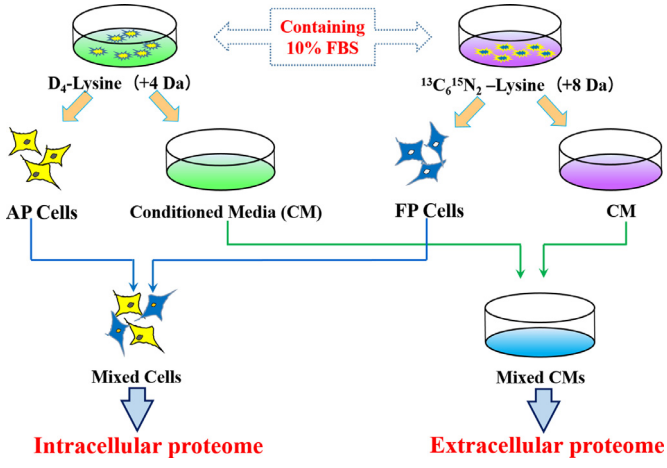

Fig. 1. Flow chat of the intracellular and extracellular proteome analysis of the AP cells compared with the FP cells.

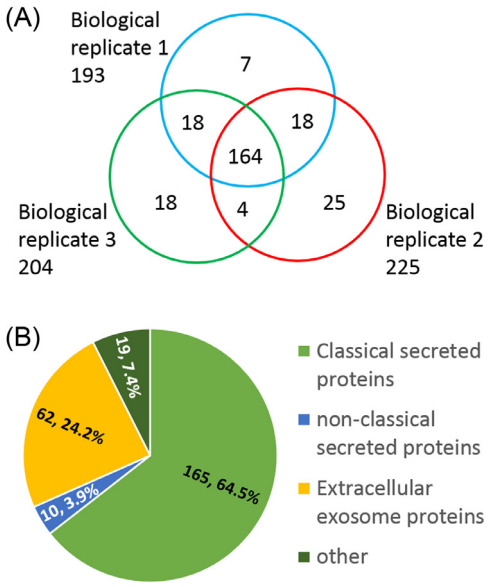

Fig. 2. (A) Number of proteins identified in the extracellular proteome. The Venn diagram shows the overlap of the three biological replicates with the total number of proteins identified per replicate. (B) Components of the extracellular proteome. Vast majority of proteins (237, 92.6%) were identified as classical secreted protein, non-classical secreted protein, and extracellular exosome protein. The remaining proteins (19, 7.4%) were unassigned.

predicted as non-classical secreted proteins by Secretome P 2.0, and 62 proteins were recorded in the ExoCarta exosome database (Fig. 2B). Collectively, the extracellular-region located proteins were accounted for 92.6% of all quantified proteins, demonstrating an in-depth proteome reflecting the real profile of cell secretion in the presence of even 10% (v/v) FBS in cell culture media.

In this data set, 167 proteins were quantified in all three replicates. Among them, proteins with ratios more than 1.5 or less than 0.67 (Log_2 ratio (AP CM /FP CM) >0.585 or <−0.585) and with *t*-test *p*-value less than 0.05 ($n=3$) were considered as significantly changed proteins (Table S2). On this basis, 35 proteins were significantly highly expressed, whereas 36 were lowly expressed in the AP cell CM (Fig. 3A). Using the software DAVID, these significantly changed proteins were categorized into cellular component, molecular function and biological process (*p*-value <0.01), as shown in Fig. 4. As expected, most of these proteins located at the extracellular space (Fig. 4A) with 38 proteins associated with ECM in cellular component categories (Table S3). Among the enriched 8 GO terms in the molecular function category, the mostly enriched categories were binding and ECM structural constituent (Fig. 4B). Biological function clustering revealed 11 GO terms, showing the most abundant categories related to angiogenesis and axonogenesis. Notably, antler blood vessel and nerves have been shown to elongate at a very fast rate during antler growth [3,11,12] (Fig. 4C).

3.3. Comparative proteomics analysis of the AP and FP cells

In total, 6074 proteins were quantified from the AP and FP cells (Table S4). Only 516 proteins (8.5%) were identified as classical secreted proteins, far less than the corresponding rate in the extracellular proteome. 4270 proteins were reliably quantified in three replicates, Among them, 546 proteins with ratios more than 1.5 or less than 0.67 (Log_2 ratio (AP cells / FP cells) >0.585 or <−0.585) and *t*-test *p*-value less than 0.05 ($n=3$) were considered to be significantly changed (Fig. 3B, Table S5). On this basis, we found that 242 and 304 proteins were elevated and decreased in the AP cells compared with those of FP cells, respectively.

The systematic GO analysis of 546 significantly changed proteins was performed using the DAVID (*p*-value <0.01), as shown

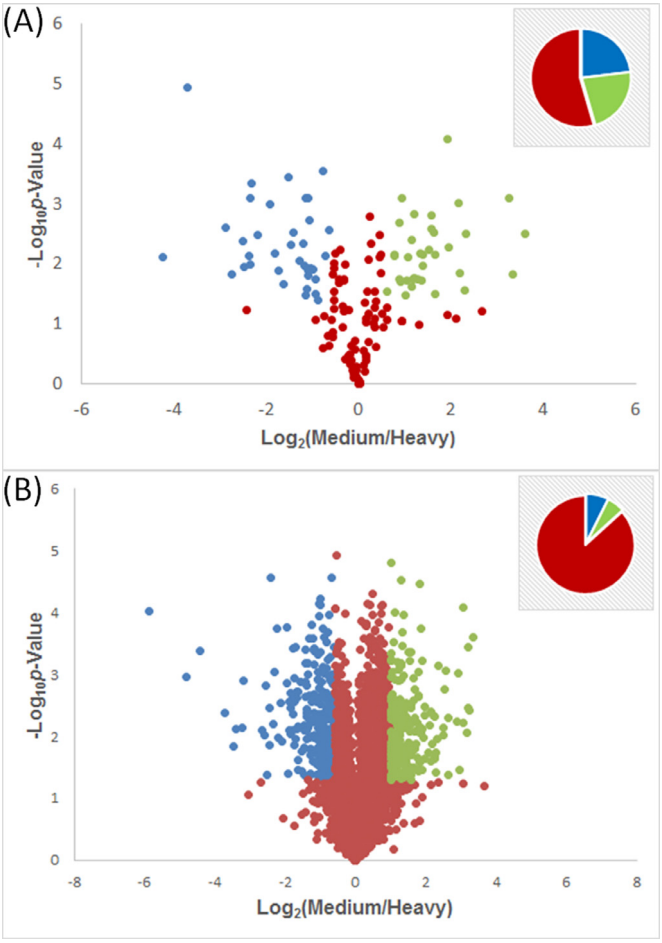

Fig. 3. Volcano plot of the quantified proteins from 3 biological replicates. The P value was determined using a two-sided Student's *t*-test. Significant expression of the proteins was defined as log2 (AP cells/FP cells) > 0.585 or < −0.585 where $p < 0.05$. The unchanged, highly-expressed, and lowly-expressed proteins are shown in red, green and blue, respectively. The components of each type of protein are shown in the pie chart. (A) Extracellular proteome, (B) Intracellular proteome. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in Fig. S1. The cellular component analysis revealed that the most abundant category is extracellular exosome, in which 140 proteins were included (Fig. S1A). Next, 128 proteins belonging to the cytoplasm category showed significant differences between both AP and FP cells. Meanwhile, the proteins associated with extracellular space, focal adhesion, actin cytoskeleton and membrane raft were found all to take part in regulating of cell-ECM interactions. The molecular function analysis displayed 8 enriched GO terms (Fig. S1B), with most of them associated with binding, such as calcium ion binding, magnesium ion binding, actin binding, pyridoxal phosphate binding and extracellular matrix binding. Finally, Oxidation-reduction and carbohydrate metabolic process were the two most dominant GO biological process categories. Besides, several cell growth related processes, such as negative regulation of transforming growth factor beta (TGFb) receptor signaling pathway, endothelial cell proliferation and p38MAPK cascade were also enriched (Fig. S1C).

3.4. Comparative analysis between the extracellular and intracellular proteomes

To compare the extracellular and intracellular proteomes, 105 proteins overlapped in both proteomes were subjected to further analysis (Table S6). Among them, 66 proteins were defined as classical secreted by using UniproKB and Signal P 4.1. The remaining 39 proteins all matched the ExoCarta exosome database, suggesting all these 105 proteins existed in both extracellular and intracellular spaces under normal physiological conditions.

The quantitative ratios of each protein in the extracellular and intracellular proteome showed obvious differences (Fig. 5A). Only 25 proteins showed the consistent expression pattern (lowly-expressed, highly-expressed or unchanged) in two proteomes (Fig. 5B). This revealed that, as for secreted proteins, there may be a considerable difference in expression pattern between the extracellular and intracellular proteomes, and the extracellular one may be more pertinent to their biological functions.

3.5. Validation of overexpression of SFRP4 and LUM in the AP cells

To validate the differential expression of SFRP4 and LUM proteins in the AP and FP cells, expression levels of these proteins were analyzed via Western blot analysis. Due to the high abundance of interference proteins derived from FBS, SFRP4 and LUM proteins cannot be detected directly from the CM. However, significant overexpression of both SFRP4 and LUM were detected in AP cells compared to the FP cells (Fig. 6), which is consistent with the MS quantification.

4. Discussion

4.1. The extracellular proteomics of the AP and FP cells

The ECM is a collection of extracellular molecules secreted by cell and provides structural and biochemical support to themselves and the surrounding cells. As expected, a large number of ECM-related proteins were identified in the CM. Notably, the majority (38 proteins) of all significantly changed proteins (63 proteins) were ECM-related ones. Among them, 4 collagens (COL6A2, COL6A1, COL1A2, and COL5A1) were all lowly expressed in the AP cells, showing the collagenous ECM components of the AP cells may be significantly lower than that of the FP cells. As for 11 ECM glycoproteins, some members of small Leucine-rich repeat proteoglycans (SLRPs), Lumican (LUM), Osteoglycin (OGN), Podocan (PODN), Fibromodulin (FMOD), and Proline-/arginine-rich end Leucine-rich repeat protein (PRELP), were highly expressed in AP cell CM. This suggests that the AP cells were more prone to secret proteoglycans to form ECM compared to the FP cells. Notably, SLRPs were shown to have the ability to influence various cellular functions through regulating several signaling pathways, such as Transforming growth factor-beta (TGFb), Epidermal growth factor (EGF), IGF and FGF [13–15]. The highly expressed ECM glycoprotein in the AP cell CM was Insulin-like growth factor binding protein 4 (IGFBP4) and IGFBP5. Both of them have regulatory functions on cell proliferation and differentiation through lengthening the half-life of Insulin-like growth factors (IGFs) [16,17], which proved to be critical in antler development and regeneration [18–20].

Apart from the structural components of ECM mentioned above, 18 proteins classified as ECM regulator, ECM-affiliated proteins and secreted factors, were also showed to have regulatory roles in tissue development. Many of them were involved in the process of multicellular organismal development, especially angiogenesis and neurogenesis. Some important endogenous angiogenesis inhibitor, such as Thrombospondins 1 (THBS1), THBS2, Plasminogen activator inhibitor-1 (SERPINE1), secreted protein acidic and rich in cysteine (SPARC) and Fibulin-2 (FBLN2), were significantly lowly expressed in the AP cell CM [21–25]. However, two pro-angiogenic proteins were also up-regulated in CM from the AP cells. Vascular endothelial growth factor C (VEGFC) is a major angiogenic growth

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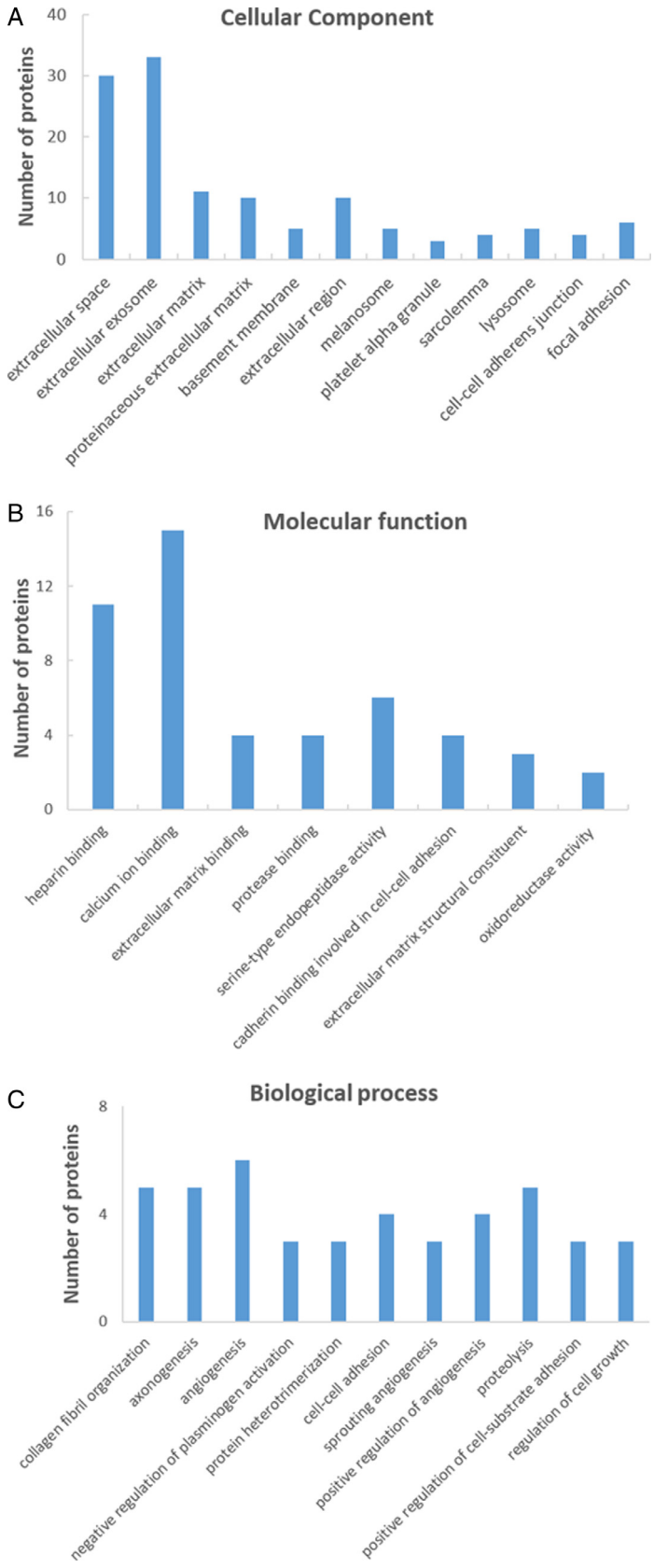

Fig. 4. Gene Ontology (GO) analysis of the extracellular proteome using DAVID. Only are the terms that had p-value $\leq$ 0.01 shown. (A) cell component, (B) molecular function, (C) biological process.

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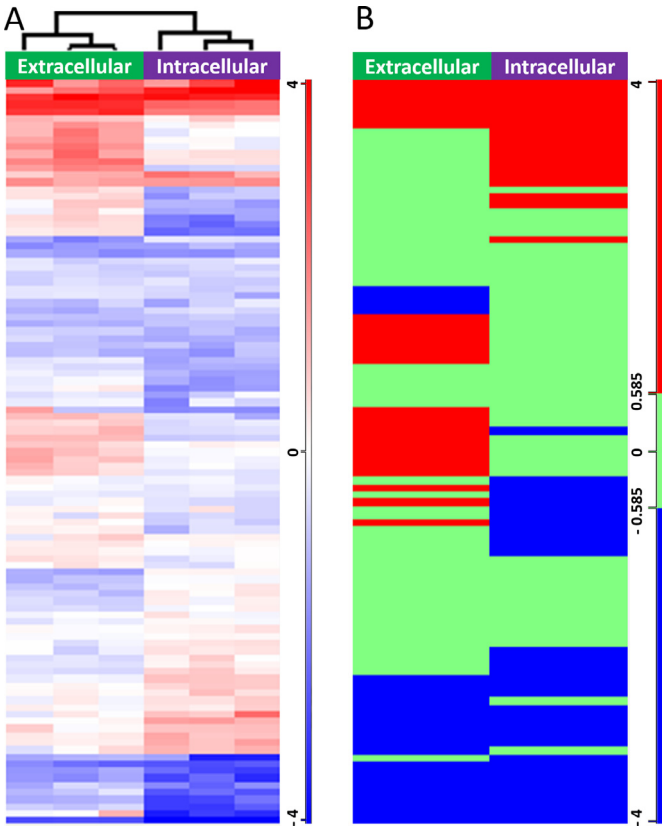

Fig. 5. Heat map showing the expression of 105 proteins reproducibly quantified from the extracellular and intracellular proteomes. (A) proteins with Log2 of quantitative ratio (AP / FP) = 0, > 0 or < 0 are color coded in white, red and blue, respectively. (B) Significantly changed proteins in expression level was defined as log2 (AP / FP) > 0.585 or < −0.585. The unchanged, highly-expressed, and lowly-expressed proteins are shown in green, red, and blue, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

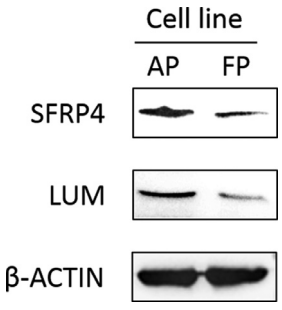

Fig. 6. Western blot analysis of SFRP4 and LUM expression in the AP and FP cells.

factor that is essential for blood vessel formation during embryonic development [26]. Pro-cathepsin H (CTSH) is involved in ECM remodeling and then promotes the development of blood vessel [27]. Several regulators of neurogenesis, such as VEGFC, Amyloid beta (A4) protein (APP) and Macrophage colony-stimulating factor 1 (CSF1, 4.3-fold, with p-value <0.05) were also found to be up-regulated in AP Cell CM. VEGFC is reported to promote neuroproliferation besides angiogenesis via its receptors VEGFR3. APP plays an important role in neural growth and maturation during brain development, while CSF1 is an essential factor in the expansion of microglia in vitro [28,29]. These proteins may contribute to the rapid vasculature and concomitant neurogenesis presented in antler during its rapid growth. The increased understanding of the neurovascular niche also suggests that the development of nervous system and vasculature is coordinated.

In vitro studies showed that inhibition of the canonical Wnt signaling increased apoptosis and also stimulated differentiation of the stem cells in antler growth zone [30]. Notably, secreted frizzled-related protein 4 (SFRP4), the highly-expressed protein in the AP cell CM is an extracellular Wnt antagonist. SFRP4 functions via inactivating the Wnt/beta-catenin signaling pathway [31]. Because constitutive activation of beta-catenin signaling can inhibit embryonic stem cells to differentiate into the three germ layers, SFRP4 may be a critical regulator in differentiation of the AP cells. Besides, another Wnt member, Serpin family E member 2 (SERPINE2) was also found to be highly expressed in the AP cell CM. Its expression is tightly and finely regulated in different organs at distinct stages of embryonic development, especially in the development of vasculature and the nervous system [32].

4.2. The intracellular proteomics of the AP and FP cells

Similar to the extracellular proteome, Wnt/beta-catenin pathway also showed a significant difference between the AP and FP cells in the intracellular proteome. The core molecule of Wnt/beta-catenin pathway, Beta-catenin (CTNNB1) and its downstream targets G1/S-specific cyclin-D1 (CCND1), was over-expressed in the AP cells. It is reported that the enhancement of beta-catenin/CCND1 activities can maintain the stemness of Leukemia-initiating cells [33]. Whereas, SFRP4, the antagonist of Wnt signaling and stimulator of stem cell differentiation, was also over-expressed in the AP cells. Except these highly expressed members of Wnt signaling pathway, CSNK1G1, a member of Casein kinase 1 family, functioning as a regulator of Wnt signaling pathway were lowly expressed in the AP cells, indicating a complicated regulation network in Wnt/beta-catenin pathway in this cell type [34,35].

Phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway is reported to be involved in the proliferation of the antler stem cells via regulating actin cytoskeleton [4,36,37]. Although PI3K was not quantified in this work, we found that AKT3, one AKT isoform, was highly expressed in the AP cells. Inhibition of AKT3 can lead to apoptosis in mouse embryonic stem cells (ESCs) involving the control of p53 activity [38]. In addition, several members of PI3K pathway, such as Neuroblastoma RAS viral oncogene homolog (NRAS), Hosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1) and Inositol polyphosphate-5-phosphatase A (INPP5A), were also highly expressed in the AP cells. It is reported that PIK3R1 depletion can promote the phosphorylation of AKT and enhances proliferation, migration and epithelial-mesenchymal transition (EMT) in renal cancer cells, which gains a stem-like phenotype [39]. NRAS down-regulation can decrease glioma cell migration, invasion, tube formation and slow tumor growth and angiogenesis in vitro and in vivo [40]. INPP5A overexpression significantly inhibits cell proliferation and invasion capacity and enhances cell apoptosis in cervical cancer cells [41]. These proteins may provide a bi-directional regulation on PI3K/AKT pathway in the proliferation of the AP cells.

EGF and its receptor (EGFR) are known to be extensively expressed in almost all cell types in regenerating antler [42]. Although lacking of EGF and EGFR, several proteins of this pathway were found to have significant differences in expression in both cell lines. Among them, only Mitogen-activated protein kinase (MAPK) kinase 3 (MAP2K3) was lowly expressed in the AP cells. Activation of MAP2K3 and its downstream effector p38 MAPK has been proved to regulate lineage determination during myelopoiesis [43]. Among these highly-expressed proteins, Basitas B-cell lymphoma (CBL) and Basitas B-cell lymphoma B (CBLB) belongs to BL-family ubiquitin ligases, which are believed to play a critical role in the maintenance of hematopoietic stem cells (HSC)

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homeostasis, and are required in epithelial stem cell maintenance during organ development and remodeling through modulation of mTOR signaling [44,45]. Besides, a regulatory subunit of Protein phosphatase 2A (PP2A), PP2A regulatory Subunit B', gamma (PPP2R5C) was also found to be highly expressed. The inactivation of PP2A was proved to maintain hESC self-renewal in the absence of basic fibroblast growth factor (bFGF) [46]. In addition, two members of RAS family, Muscle RAS oncogene homolog (MRAS) and Neuroblastoma RAS oncogene homolog (NRAS) were also highly expressed. MRAS functions as a signal transducer for a wide variety of signaling pathways, including those plays crucial role in differentiation of mESCs. It is also reported that MRAS modulates cell fate at early steps of development and during neurogenesis in Xenopus [47]. As for NRAS, it can promote leukemogenesis in mice via expansion of myeloid progenitors and increased long-term reconstitution of bone marrow cells [48].

Base on Gene ontology (GO) analysis, the highest enriched biological process is the UDP-N-acetylglucosamine metabolic process. Involved proteins, Glutamine-fructose-6-phosphate transaminase 1 (GFPT1), Glutamine-fructose-6-phosphate transaminase 2 (GFPT2), UDP-N-acetylglucosamine pyrophosphorylase 1 (UAP1), Glucosamine-6-phosphate N-acetyltransferase 1 (GNPNAT1), and Bifunctional UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase (GNE), are all highly expressed in the AP cells. GFPT1, the first enzyme of the hexosamine biosynthetic pathway (HBP) showed the highest change ratio. It is reported that mutation in GFPT1 specifically affects melanocyte differentiation at a late stage during regeneration [49]. Glycosylation of UAP1 is proved to be under the control of androgen [50]. Notably, levels of circulating androgen or a low abundance of androgen receptors in the AP cells could lead to poor antler growth [51]. GNPNAT1 is a key enzyme in the pathway toward biosynthesis of UDP-N-acetylglucosamine, an important donor substrate for N-linked glycosylation, and has several important functions such as involved in embryonic development and growth [52]. GFPT2 plays a role in glutamine metabolism and is observed to be highly expressed in mesenchymal cell lines [53]. As the key enzyme of sialic acid biosynthesis, GNE expression level and the cellular sialic acid concentration is directly correlated with proliferation of embryonic stem cells from mice and inactivation of GNE causes early embryonic lethality [54]. In addition, some growth factors were highly expressed in the AP cells. Among them, Fibroblast growth factor (FGF2) were believed to play important roles in regulating cell differentiation [55]. Myeloid-derived growth factor (MYDGF), was strongly expressed in several organs and can promote endothelial cell proliferation, cardiac myocyte survival and angiogenesis [56].

4.3. Quantitative comparison between the extracellular and intracellular proteomes

In previous studies of proteomic analysis on antler tissue or cells, almost all the attention has been directed toward the intracellular proteins. In this study, we combined two aspects of information, the extracellular and intracellular proteomes to gain insight into the mechanism of antler regeneration. As expected, 72.3% quantified proteins in the extracellular proteome were classical secreted proteins or localized at extracellular space, while the corresponding proportion of the intercellular proteome was to 10.8%. The higher percentage of classical secreted proteins in this study could be attributed to the serum-containing media, which enhance the cell survival rate during incubation (36 h) and thus dramatically reduce the release of cytoplasmic and nuclear proteins into CM. Interestingly, the Chaperone occupied a larger proportion in the extracellular proteome. Chaperones are commonly studied for its intracellular functions in assisting the covalent folding/unfolding and the assembly /disassembly of macromolecular. However, recent research indicate that the extracellular space also needs chaperones to safeguard against the stresses that negatively impact the extracellular protein homeostasis [57].

In addition, 105 highly confident proteins were identified in both extracellular and intracellular proteomes. Heat map analysis demonstrated that the expression patterns of the extracellular proteins were different with the intracellular ones. Unexpected, the extracellular expression patterns of some secreted proteins were contrary to the intracellular ones. For example, SPARC was found to be lowly expressed in AP cell CM according to the extracellular proteome, however the intracellular proteome showed that it was a highly-expressed protein in AP cells. Obviously, the extracellular quantitative ratio of this protein is more meaningful in view of SPARC mainly regulating cell growth through interactions with the ECM and cytokines in extracellular space [58]. As for eight quantified collagens, none showed consistent expression patterns between the extracellular and intracellular proteome. However, two showed a reversed expression pattern. Therefore, in order to more accurately describe the proteins' functions, especially for those of secreted proteins, a combination of intracellular with extracellular proteomic analysis is necessary.

More importantly, several proteins showed similar expression patterns in both extracellular and intracellular proteome. Among them, SFRP4 showed the highest overexpression rate in the AP cell CM, which is higher than that in the intracellular proteome. SFRP4, as an antagonist of Wnt signaling, can stimulate embryonic stem cells to differentiate into the three germ layers. Lumican (LUM) was also highly expressed in both extracellular and intracellular proteomes of the AP cells. LUM can inhibit the activity of TGF beta 1, which positively and negatively regulates many other growth factors and can control proliferation, differentiation and other functions in stem cells [59]. The high expression of these two proteins in both extracellular and intracellular proteomes of the AP cells indicate the potential their roles in antler formation and regeneration.

5. Conclusion

In summary, we performed extracellular and intracellular proteomic analysis of the AP and FP cells and demonstrated the differences in the signaling pathway and biological function between these two cell lines. Proteome profiling also showed that several secreted proteins, such as SFRP4 and LUM, were significantly overexpressed in the AP cells and its CM, indicating their potential regulatory roles in antler formation and regeneration. Taken together, the combination of extracellular and intracellular proteomics analysis enables an all-around profiling of differential expression proteins between the AP and FP cells, and aid to discover the critical regulatory factor of antler formation and regeneration presented in CM.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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Supplementary materials

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