

Identification of proteins that mediate the role of androgens in antler regeneration using label free proteomics in sika deer (*Cervus nippon*)

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ABSTRACT

Deer antlers offer a unique model to study organ regeneration in mammals. Antler regeneration relies on the pedicle periosteum (PP) cells and is triggered by a decrease in circulating testosterone (T). The molecular mechanism for antler regeneration is however, unclear. Label-free liquid chromatography-mass spectrometry (LC-MS/MS) was used to identify differentially-expressed proteins (DEPs) in the regeneration-potential PP (under low T environment) over the non-regeneration-potential PP (under high T environment). Out of total 273 DEPs, 189 were significantly up-regulated and 84 were down-regulated from these comparisons: after castration vs before castration, natural T vs before castration, and exogenous T vs before castration. We focused on the analysis only of those DEPs that were present in fully permissive environment to antler regeneration (low T). Nine transduction pathways were identified through the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, including the estrogen signaling pathway. A total of 639 gene ontology terms were found to be significantly enriched in regeneration-potential PP (low T) from the DEPs. Reliability of the label free LC-MS/MS was determined by qRT-PCR to estimate the expression level of selected genes. The results suggest that up-regulated heat shock proteins (HSP90AB1, HSP90B1), peptidyl-prolyl cis-trans isomerase 4 (FKBP4), mitogen-activated protein kinase 3 (MAPK3) and calreticulin (CALR) and down-regulated SHC-transforming protein 1 (SHC1), heat shock protein family A member 1A (HSPA1A) and proto-oncogene tyrosine-protein kinase (SRC) may be associated directly or indirectly with antler regeneration. Further studies are required to investigate the roles of these proteins in regeneration using appropriate *in vivo* models.

1. Introduction

Historically, regeneration research has been compromised due to a lack of tools to address mechanistic questions in most classical animal models capable of replacing lost tissues. Development of molecular and genomic tools in the last two decades has ushered in a renaissance in regeneration research (Li and Chu, 2016; Echeverri and Zayas, 2018). Research in stem cells and regenerative biology using classical and emerging models is expanding and opening new horizons for application of comparative genomics (Chen and Poss, 2017; Wang et al., 2019). This research is focused on fundamental similarities in the context of cell differentiation, morphogenesis and tissue patterning of regeneration underlying organismal development (King and Newmark, 2012). Moreover, collective efforts of regeneration scientists are revealing the

molecular differences that confer regenerative abilities in postnatal organisms, such as scarless wound healing, adult stem cell regulation and the formation of the regeneration blastema (Echeverri and Zayas, 2018). The exploration of different aspects of regeneration of deer antlers, as a remarkable example of regeneration among mammals (Wislocki et al., 1947; Jaczewski et al., 1976; Li and Suttie, 2000; Li and Chu, 2016; Wang et al., 2019), is progressing. Regeneration of antlers has remained largely overlooked as a research model to achieve the “Holy Grail” of growing a new limb on a mammal. However, it has become possible to explore the development of the pedicle (the permanent bony protuberance, from which antlers are cast and regenerate) and the antler and reveal cellular mechanisms related to regeneration (Li and Suttie, 2000; Haines and Suttie, 2001; Kierdorf and Kierdorf, 2002; Li, 2010). The onset of antler regeneration, through multiple

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regulatory levels, is associated with (Li, 2003 #48) the activation of the pedicle periosteal cells (PPCs), which are known as antler stem cells. The mesenchymal-epithelial interactions between the pedicle periosteum (PP), particularly the distal potentiated PP (Li et al., 2009) and the enveloping skin are vital for providing the niche for antler stem cells (Li et al., 2007). Deer antlers are male secondary sexual characters, and their growth is under the control of androgen hormones (Goss, 1968; Goss, 1983; Bubenik, 1990; Lincoln, 1992; Kolle et al., 1993; Suttie et al., 1995; Li et al., 2003; Gaspar-López et al., 2010), although other growth factors are also pivotal in antler development.

The role of androgens is well-defined, although the underlying mechanisms involving cellular interactions, androgen control of pedicle and antler development *in vivo* (Li et al., 2001) are complex and some aspects have not been satisfactorily explained in antler biology (Goss, 1968; Goss, 1983; Bubenik, 1990; Lincoln, 1992; Kolle et al., 1993; Suttie et al., 1995; Li et al., 2003; Gaspar-López et al., 2010; Li and Chu, 2016). Pedicle growth is under the influence of increasing and elevated plasma testosterone (T) levels; conversely, declining T levels result in the transformation of the first antler from a fully-formed pedicle (Li et al., 2003). Female deer (excluding the Rangifer species), although possess the tissue to form pedicles and antlers, do not develop these structures due to lack of sufficient levels of androgen hormone for stimulation (Li et al., 2003). Castrated deer do not shed velvet (Wislocki et al., 1947; Goss, 1968), and castrated roe deer can even develop velvet antlers into tumor-like overgrowth, called peruke (Kierdorf et al., 2004).

Increasing or high levels of insulin-like growth factor 1 (IGF1) are stimulatory of antler growth (Suttie et al., 1989), while low circulating T levels are associated with casting of the hard antler, the maintenance of these low levels is necessary for normal antler growth (Li and Chu, 2016). Both direct and indirect pathways mediate the role of local factors (environment and nutrition) under the influence of endocrine factors and consequently, proliferation of the potentiated PP cells results in formation of the antler blastema and subsequent antler regeneration (Li and Chu, 2016).

The involvement of different signaling pathways in developmental processes can be determined by proteomic (Bartos et al., 2012) and transcriptomic methods including mass spectrometry and bioinformatics tools (Altelaar et al., 2013). Such techniques can be applied to understanding the expression of molecules involved in development of the deer antlers, and, in this respect, the investigation of differential expression of molecules in the PP compared with the deer facial periosteum (FP, the periosteum adjacent to the PP) was reported using 2-dimensional gel electrophoresis (Li et al., 2012). The isobaric tags for the relative and absolute quantification (iTRAQ) labeling of the peptides coupled with 2-D LC-MS/MS were employed (Dong et al., 2016) to compare proteome profiles of the potentiated and dormant PP cells. The plasma membrane proteins of antler stem cells have also been investigated using label-free LC-MS/MS by our group (Wang et al., 2018). The RNA-Seq method (Yao et al., 2018) was used to define gene expression patterns in the different zones of the sika deer antler growth center (skin, mesenchyme, pre-cartilage, and cartilage) during the stage of rapid antler growth.

It is known that antler regeneration is triggered by a decline in testosterone (T) to a very low level (i.e. low T represents a permissive environment for antler regeneration), but is inhibited by high T, whether natural or induced artificially (i.e. high T represents a non-permissive environment for antler regeneration) (Bubenik et al., 1982; Suttie et al., 1995; Gaspar-López et al., 2010). The antler stem cells (ASCs), resident in the pedicle periosteum (i.e. PP cells), provide the sole source of cells for regeneration of the antler. We, therefore, hypothesized that factors expressed by the ASCs around the time of initiation of antler regeneration are factors that are likely to be involved in that process. Differentially-expressed proteins (DEPs) are such candidates; in order to test this hypothesis and identify such proteins, we created the appropriate environments. The experiment was designed to

manipulate the environment by removing or adding the androgen influence – this modified the permissive state for antler regeneration; the target of our interest was in what proteins were differentially expressed in the low T (permissive) vs the high T (non-permissive) environments.

This is the first LC-MS/MS label-free proteomic study for investigation of antler regeneration through detecting critical DEPs in the regeneration-potentiated PP tissues (low T environment) over non-regeneration-potentiated PP tissues (high T environment).

2. Materials and methods

2.1. Tissue sampling and blood collection

Four adult sika deer (*Cervus nippon*) were used. Two were castrated and two were left intact; the T level of one of the intact deer was not manipulated (so experiencing the natural T cycle) and the other was treated with T to inhibit potentiation and activation of the PP tissues.

Deer 1 and Deer 2: PP tissue from the right-side was collected before castration (BC) on 24 April; PP tissue from the left-side was collected when the deer cast their previous (hard) antlers (9 May or 24 May), which were defined as the AC (after castration) samples. Deer 3: PP tissues from both sides (no treatment) were collected on 22 June after the last blood sampling (on 21 June) at the time of natural casting of the hard antler - defined as NT (natural T) samples. PP tissues of both sides of Deer 4 that was treated with exogenous T (T undecanoate at 250 mg/deer) bi-weekly on three occasions were collected 34 days after the last T injection (on 14 August) - defined as ET (exogenous T) samples (Table 1).

A blood sample (5 ml in pre-heparinized tubes) was collected weekly from each deer via jugular vein from 24 April until the PP tissues were collected. Blood samples were centrifuged immediately at 4000 rpm and plasma taken and stored at -20°C until required for analysis. A general anesthetic (Xylazine Hydrochloride, Dunhua Shengda Animal Medicine Co. Ltd., China) was used to sedate the deer.

PP tissues were collected in the surgical operation room on the deer farm by expert surgeons. The tissue collection area was cleaned and shaved thoroughly before sampling to minimize risk of contamination. PP tissues were collected according to Li and Suttie (2003). After collection, the PP tissue strips were cut into two equal parts for further analysis: for label-free proteomics analysis the PP tissue strips were stored in liquid nitrogen in RNA-free tubes and then transferred to storage at -80°C ; for qPCR analysis; the strips were stored in RNA Stabilization Solution (AM7020, Thermo). CAAS Animal Ethics

Table 1
Plasma concentrations (ng/mL) of T in the four experimental deer.

Date	Intervention	Castrated		Intact Not treated	Intact T-treated
		Deer 1	Deer 2	Deer 3	Deer 4
24 April	Deer 1 & 2 castrated	1.25	1.02		
2 May		0.02	0.03	1.24	
9 May	Deer 1 cast antler	0.02	0.02	0.03	
16 May			0.02	0.03	
24 May	Deer 2 cast antler		0.04	0.02	
14 June	Deer 4 received T			0.01	0.04
21 June				0.02	1.28
27 June	Deer 4 received T				0.74
6 July					1.57
11 July	Deer 4 received T				0.53
17 July					1.18
26 July					0.85
1 August					0.70
14 August					0.33

Tissue sampling: 24 April and 9 May (Deer 1), 24 April and 24 May (Deer 2), 22 June (Deer 3) and 14 August (Deer 4).

Committee approval (CAAS2017046C) was received before the collection of the PP tissues and blood samples.

2.2. Plasma testosterone assay

The concentration of T was measured via Enzyme linked immune sorbent assay (ELISA) using commercially-available kits (Amgenix, Inc. USA) as described by Akhtar et al. (2017). The kit contains 96 goat Anti-rabbit IgG-coated microtiter wells. The sensitivity of the T assay was 0.05 ng/ml. The intra-assay and inter-assay coefficients of variation were 5.5% and 12%, respectively. The T reference standards were 0, 0.1, 0.5, 2, 6, and 18 ng/ml. The labelled wells were filled with standards, samples, and controls containing 10 µl each; approximately 100 µl of T-Horseradish Peroxidase (HRP) Conjugate and 50 µl Rabbit anti-T mixture were added into each of the selected wells. Later, the solutions were mixed meticulously for 30 sec and incubated for 90 min at 37 °C. Rinsing of micro-wells was performed using deionized water and flipped at least five times. After this, 100 µl Tetramethylbenzidine (TMB) reagent was added into each well and evenly mixed for 10 sec. The solutions were incubated for 20 min at room temperature (18 to 25 °C). The reaction was stopped by adding approximately 100 µl of stop solution (1 N HCL). This solution was gently mixed in the wells for 30 s until a color transformation of blue to yellow was observed. Absorbance was recorded within 10 min using a microtiter well reader (AMP Diagnostics Platos R 496, Austria).

2.3. Protein extraction and sample preparation

Each PP tissue from liquid nitrogen was ground in a china crucible to make a fine powder with ample supply of liquid nitrogen. The powder was re-suspended in 2 ml of lysis buffer (7 M Urea, 2 M Thiourea, 4% 3-[(3-Cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate (CHAPS), 1% protease inhibitor cocktail, and 200 U/ml Benzonase), and homogenized for 50 min at 25 °C. Supernatants were collected after centrifugation at 15,000 g for 10 min at 4 °C. High-abundance proteins (especially albumin and IgG) in the protein samples from the PP tissues were separated and removed using an Albumin/IgG removal Kit (89875, Thermo) according to the manufacturer's protocol. The proteins were then precipitated and dissolved in 30 mM Tris-HCl, pH 8.8, 7 M Urea, 2 M Thiourea, 4% CHAPS using the 2-D Clean-up Kit, which was designed to improve the protein quality of the samples by clearing interfering substances. The protein samples were then frozen at –80 °C after addition of protease inhibitor cocktail.

2.4. LC-MS/MS analysis for protein identification

LC-MS/MS analysis was performed as described by Cholewa et al. (2014) and Wang et al. (2018). In brief, 100 µg protein of each sample was digested with 3 µg trypsin (Promega, Fitchburg, MA, USA) in 40 µl 25 mM NH₄HCO₃ at 37 °C overnight. The concentration of peptides was calculated by MicroBCA assay (Thermo, Rockford, IL, USA). UltiMate 3000RSLCnano high-performance liquid chromatography (HPLC) system using a C18 BEH column and C18 Acclaim PepMap RSLC column (Thermo) was used to detect different peptides in the samples. Peptides were then eluted in a linear gradient of acetonitrile (from 4% to 35% with 0.1% formic acid) over 140 min and 35–45% for about 10 min. At 160 min, the acetonitrile gradient was elevated to 95% and was held for 10 min. Thermo Scientific Q Exactive Orbitrap mass spectrometer (Thermo) was used for further analysis of separated peptides by MS/MS. Resolutions of MS scans were set at a 70,000 and 17,500 for the data-dependent MS/MS scans. The MS scan range was set from 300 to 2200 m/z. Separation of peptides mapping across multiple LC-MS measurements was performed by using their coordinates on the mass-to-charge and retention-time dimensions. The total ion current of the peptide signal was then measured and used as a quantitative measurement of the original peptide concentration. MaxQuant software

Table 2

Maxquant identification and quantitative parameters in the label-free analysis.

Item	Value
Enzyme	Trypsin
Max Missed Cleavages	2
Main search	6 ppm
First search	20 ppm
MS/MS Tolerance	20 ppm
Fixed modifications	Carbamidomethyl (C)
Variable modifications	Oxidation (M), Acetyl (Protein N-term)
Database	NCBI_Cervidae_87693_20171106_modified.fasta
Database pattern	Target-Reverse
Peptide FDR	≤ 0.01
Protein FDR	≤ 0.01
Time window (match between runs)	2 min
Protein quantification	Used razor and unique peptides
LFQ	True
LFQ min. Ratio count	1

(v1.5.3.17; Max Planck Institute of Biochemistry, Martinsried, Germany) was used to analyze the MS data according to the set parameters (Table 2). Statistical analysis (t-test) was performed using SAS (Statistical Analysis System; v9.0). Significant levels were taken as the fold change of > 1.5 and p < 0.05.

2.5. Bioinformatics analysis

Gene ontology (GO) annotation and enriched pathways containing the identified DEPs were identified online using the David database (v6.8; <http://david.abcc.ncifcrf.gov/>). The interactive network containing the identified DEPs was identified using the online STRING database (v10.5; <https://string-db.org>). Key pathways related to androgens were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. KEGG enriched pathways were found after mapping the identified DEPs to the KEGG database (<https://www.kegg.jp/kegg/pathway.html>).

2.6. Validation through qRT-PCR

Validation of the three groups of DEPs was performed using qRT-PCR. Total RNA from tissues was extracted using Trizol (15596026, Invitrogen) as per the manufacturer's protocol: 5 µg of RNA/sample was used to do reverse transcription using a PrimeScriptTM II First-Strand cDNA Synthesis Kit (6210A TaKaRa, Japan) following the manufacturer's instructions. Primer Premier 6.0 (PREMIER Biosoft International, USA) was used to design gene-specific primers. Light Cycler 480 II PCR with FastStart Universal SYBR Green Master (ROX) (4913850001, Roche, Switzerland) was used to conduct qRT-PCR. The PCR cycling profile was carried out under the following conditions: 5 min at 95 °C, followed by 22 cycles of 30 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C with a final extension of 10 min at 72 °C. The 2^{–ΔΔCt} method was used to analyze qRT-PCR data and results were shown as mean ± SD. Statistical significance was performed using one-way ANOVA followed by post-hoc Tukey's test using graph pad prism 7 (v7.04). The p-value of < 0.05 was considered statistically significant. All samples were measured in triplicate, and the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used for normalization.

3. Results

3.1. Plasma T concentrations in deer with different treatments

Plasma T concentrations (ng/ml) in the four deer are presented in Table 1. The concentrations of T in the castrated deer (Deer 1 and Deer 2) were lower than in the intact deer (Deer 3), whereas, T concentration

Table 3

DEPs in three comparisons identified from label-free LC-MS/MS.

Comparison	T status	Identified (Supplementary Table number)	Up-regulated	Down-regulated	Total up- and down- regulated
AC vs BC	Low T vs High T	1028 (1)	142	71	213
NT vs BC	Low T vs High T	994 (2)	80	10	90
ET vs BC	High T vs High T	934 (3)	21	17	38
Total		2956	243	98	341

AC, after castration; BC, before castration; NT, natural T; ET, exogenous T.

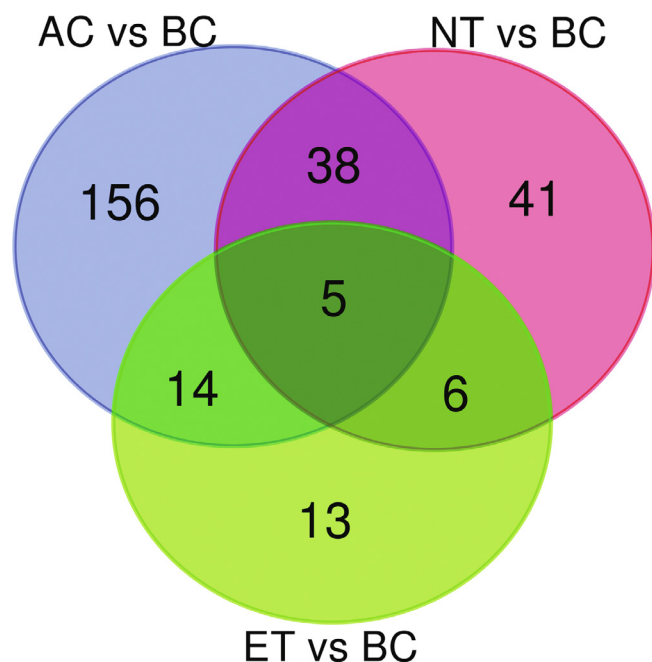


Fig. 1. Venn diagram showing the number of differentially-expressed proteins (DEPs) in comparisons between the tissues in different T environments. The overlapping areas show the number of shared DEPs. Of 273 DEPs from all three comparisons, those of particular interest are the 38 that are differentially-expressed between the low T and high T environments common to the AC vs BC and the NT vs BC comparisons. The others of interest in the low T vs high T environments are the 156 DEPs (AC vs BC) and 41 DEPs (NT vs BC). AC, after castration; BC, before castration; NT, natural T; ET, exogenous T.

in the T-treated deer (Deer 4) was higher than that of Deer 3.

3.2. Differentially-expressed proteins (DEPs) identified from label-free LC-MS/MS

DEPs were identified from the three comparisons: AC vs BC, NT vs BC, and ET vs BC (Table 3 and Fig. 1; Supplementary Tables 1–3). These comparisons were principally made to identify the difference between a low and high T environment. The results showed that the greatest number of DEPs were evident in the AC vs BC.

The 235 DEPs (Fig. 1) across the comparisons of AC vs BC (156) and NT vs BC (41) (plus 38 common to both high vs low T comparisons) are the key proteins of interest and likely to be involved in antler regeneration, as they are expressed differentially in the fully-permissive environments compared with the non-permissive environments. In this respect, the comparison of the two high T environments (ET vs BC, Fig. 1) was designed to provide a further filter to remove proteins that are less likely to be involved in antler regeneration, but rather more likely to be involved in seasonal or other changes; 25 proteins (14 + 5 + 6) fitted this category.

3.3. Functional classification of the identified DEPs

A total of 639 GO terms were generated (Supplementary Table 4); of those terms, 450 were found to be involved in “biological process”, 107 in “cellular process” and 82 in “molecular function” ($p < 0.05$). The 10 and 5 predominant GO terms for each biological process, cellular process and molecular function are shown in Fig. 2 and Table 4.

3.4. Interactive network containing identified DEPs

A total of 235 DEPs (Supplementary Table 5) were found to be

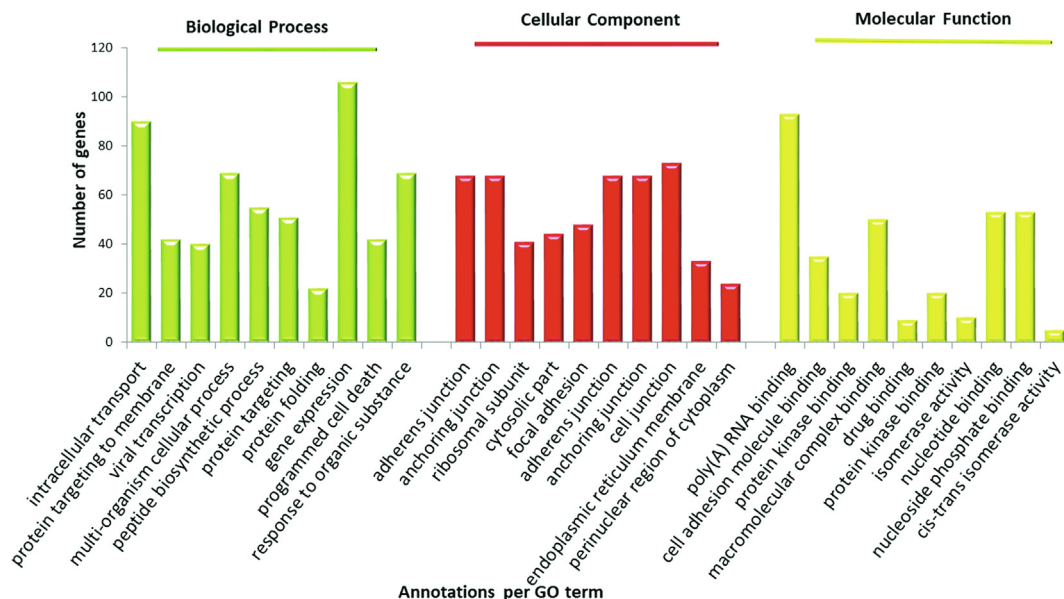


Fig. 2. Predominant GO (Gene ontology) enrichment analysis ($p < 0.05$) of the DEPs from the potentiated (low T) over non-potentiated (high T) PP tissues.

Table 4

Predominant GO terms involved in Biological processes, Cellular component and Molecular function.

GO Term	Biological processes (450)	Cellular component (107)	Molecular function (82)
intracellular transport	4.94×10^{-33}		
protein targeting to membrane	1.52×10^{-37}		
viral transcription	1.08×10^{-35}		
multi-organism cellular process	2.91×10^{-30}		
peptide biosynthetic process	7.31×10^{-27}		
adherens junction		5.63×10^{-35}	
anchoring junction		2.53×10^{-34}	
ribosomal subunit		4.35×10^{-34}	
cytosolic part		1.77×10^{-33}	
focal adhesion		1.92×10^{-28}	
poly(A) RNA binding			5.86×10^{-44}
cell adhesion molecule binding			4.87×10^{-15}
protein kinase binding			2.87×10^{-04}
macromolecular complex binding			4.86×10^{-10}
drug binding			1.46×10^{-04}

GO terms are expressed as p values ($p < 0.05$).

involved in the interactive network (170 up-regulated and 65 down-regulated) with the fold-change of proteins indicated by the gradient color (Fig. 3).

3.5. Enriched signaling pathways containing the identified DEPs

A total of 9 KEGG pathways (Supplementary Table 6) were identified as significantly enriched ($p < 0.05$) from the DEPs (Fig. 4 and Table 5). The most significant pathways relevant to mammals were Ribosome (40, all up-regulated), Protein processing in endoplasmic reticulum proteins (PPER, 17, 15 up-regulated and 2 down-regulated), Arginine and proline metabolism (7, all up-regulated), Legionellosis (7, 6 up-regulated and 1 down-regulated), Complement and coagulation cascades (CCC, 7, 3 up-regulated and 4 down-regulated), beta-Alanine metabolism (5, 4 up-regulated and 1 down-regulated), Biosynthesis of antibiotics (11, all up-regulated), Estrogen signaling pathway (7, 4 up-regulated and 3 down) and Spliceosome (8, 5 up-regulated and 3 down-regulated). In Table 5 ribosomal proteins are not shown as these are structural proteins.

3.6. Validation of the three selected DEPs

Three DEPs, CALR, FKBP4 and C4B, were selected for validation of their expression using an independent technique, qRT-PCR. The expression levels of mRNA were normalized based on the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 5; Table 6). Expression levels of CALR, FKBP4 and C4B were found to be up-regulated at a low level of T (AC, NT) and down-regulated at a high level of T (BC, ET). The qRT-PCR results were found to be consistent with those of the label free LC-MS/MS. Moreover, C4B did not show significant expression in the PP tissue with ET treatment.

4. Discussion

Label-free quantification is a simple, gel-free system that allows comparisons of large number of samples in a single run compared to the gel systems currently available (Dephoure and Gygi, 2012). This is the first LC-MS/MS label-free proteomic study to identify differentially-expressed proteins (DEPs) from the potentiated PP (in low T, permissive) over the non-potentiated PP (in high T, non-permissive) environment for antler regeneration, which could be expected to help revealing the underlying molecular mechanism of role of androgen in antler regeneration. In this study, we analyzed those DEPs that up-or down-regulated in the low T, permissive environment for antler regeneration. Both puberty and season influence the episodic secretion of LH, which dictates the changes in activity of the testes and secretion of

T. Development of the pedicle and first antler in the young stags, and the cycles of casting and re-growth of the antlers in the adult stags, are largely controlled by the changes in T concentration (Fennessy et al., 1988; Suttie et al., 1989; Bubenik, 1990; Suttie et al., 1995; Gaspar-López et al., 2010). It is known that castration of an adult male deer at hard antler phase induces casting of the hard antlers normally within two weeks, and triggers regeneration of new velvet antlers (Bubenik et al., 1982; Suttie et al., 1995). These results highlight that androgen signaling controls antler regeneration. In the present study, the concentration of T in the castrated deer (Deer 1 and Deer 2) was lower, but in the exogenous-T-treated deer (Deer 4) was higher than that of the control Deer 3 (intact). The low T level triggered casting of the hard antlers, whereas in the high T environment, the hard antlers were retained. Therefore, in the present study we successfully created permissive and non-permissive environments for antler regeneration through artificially manipulating the availability of endogenous androgen hormones.

Although the effects of androgen hormones on antler development have been functionally confirmed, the underlying molecular mechanism is unclear. It is reported that effects of T on secondary sexual organs can be achieved via T itself, or by its reduced form (dihydrotestosterone by 5 α reductase) or aromatized to estradiol (Davey and Grossmann, 2016). In the case of antler, the indirect effect of T has been hypothesized to be via local conversion to estradiol by aromatase (Suttie et al., 1985; Price and Allen, 2004). In the present study, we found that the DEPs were enriched in the estrogen signalling pathway, suggesting that the estrogen pathway was activated. These enriched DEPs include Heat shock proteins (HSP90AB1, HSP90-beta; HSP90B1, HSP 90 kDa beta member 1; endoplasmic, gp96, grp94, or ERp99); FKBP4 (Peptidyl-prolyl cis-trans isomerase 4); MAPK3 (Mitogen-activated protein kinase 3); SHC1 (SHC-transforming protein 1); SRC (Proto-oncogene tyrosine-protein kinase); HSPA1A (Heat shock protein family A (Hsp70) member 1A). Among these DEPs, HSP90AB1, HSP90B1, FKBP4, and MAPK3 were up-regulated; whereas SHC1, HSPA1A and SRC were down-regulated in estrogen signaling pathway. Therefore, there is reasonable evidence that the effect of T on antler regeneration may be achieved through aromatization to estradiol.

The heat shock proteins (HSPs) act as molecular chaperones to ensure proper protein folding (Young et al., 2004). HSP90 has a role in cell viability and transcription in steroid hormone signaling (Sanchez, 2012) and is involved in conformational maturation of several signaling molecules including steroid receptors (Pratt and Toft, 1997). In the present study, HSP90AB1 (90-beta) was up-regulated in a low T environment. Therefore, the molecule may be involved in regeneration of antlers, a secondary sexual character. Also, this molecule interacts with the protein kinase (e.g. mitogen-activated protein kinase (MAPK) and

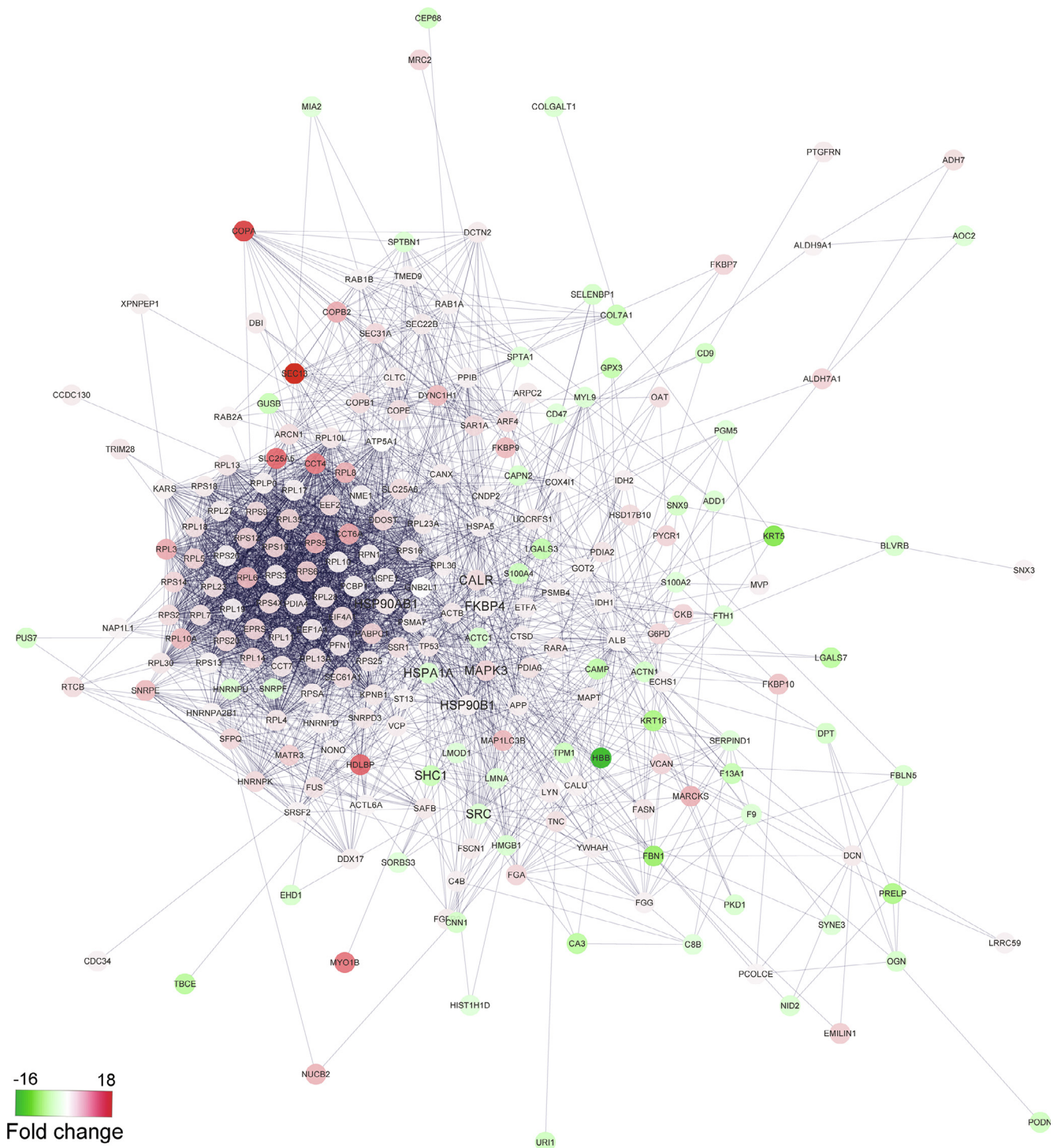


Fig. 3. Interaction network of the DEPs associated directly or indirectly with antler regeneration. The panorama network consisted of 235DEPs, including 170 up- and 65 down-regulated proteins. ●: up-regulated proteins and ●: down-regulated proteins. Bar color: logarithmic scale from -16.00 to 18.00 .

the phosphoinositol-3-kinase (PI3K) pathways (Citri et al., 2006). It has been postulated that it may be directly or indirectly involved in regulation of cell cycle progression, cell proliferation, differentiation, and migration (Zhao et al., 2012). Growth rate of regenerating antlers (up to 2 cm/day; Goss, 1970) is arguably the fastest among mammalian tissues, and therefore any molecule that has the ability to stimulate cell proliferation may be relevant to antler growth. The oncogenic pathways are involved in the rapid regeneration of antler tissue and simultaneously some tumor suppressor genes are under strong selection in deer (Wang et al., 2019).

HSP90B1, another type of HSP protein, is reported to be a key downstream chaperone in the endoplasmic reticulum (ER) that mediates the ER unfolded protein response, the latter being crucial for maintaining protein homeostasis in the ER (Yang and Li, 2005). It is known that cellular stress disrupts the repressive complex and activates heat shock factor 1 (HSF-1), which then induces expression of HSPs to facilitate cell survival in the suboptimal conditions (Vydra et al., 2014). In an axolotl limb stump study, 10 chaperones (five HSPs) were detected that accelerate protein folding in the ER; three of the HSPs (HSPB3, HSP90B1, HSP90AB2P) were up-regulated while two (HSP27,

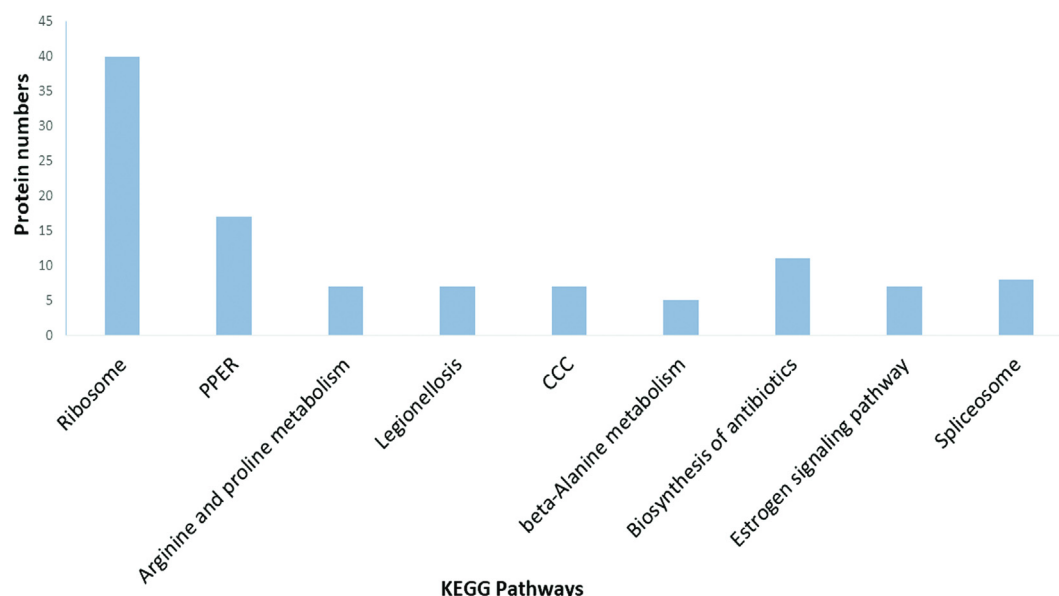


Fig. 4. Distribution of KEGG pathways ($p < 0.05$) of the DEPs of regenerated over non-regenerated PP tissues. KEGG: Kyoto Encyclopedia of Genes and Genomes; PPER: protein processing in endoplasmic reticulum; CCC: complement and coagulation cascade. Number of DEPs (n) in Ribosome (40); PPER (17); Arginine and proline metabolism (7); Legionellosis (7); CCC (7); beta-Alanine metabolism (5); Biosynthesis of antibiotics (11); Estrogen signaling pathway (7) and Spliceosome (8).

HSPA1A) were down-regulated (Rao et al., 2014). The significant up-regulation of HSP90B1 in the PP tissue in our study may be due to the rapid division of PP cells in initiation of antler regeneration in the low T permissive environment, which could be expected to generate an ER stress response (Yang and Li, 2005) and the ER unfolded protein response (UPR), (Rao et al., 2014). Consequently, expression of HSP90B1 in the PP tissue may be a response to the ER stress with the UPR occurring during initiation of antler regeneration.

HSPA1A, which encodes cognate HSP70, plays important roles in various cellular metabolic pathways. Overexpression of HSPA1A enhances osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) partly through Wnt/ β -catenin pathway in rats (Zhang et al., 2016). HSF-1 is a transcriptional factor that binds specific cis-acting sequences upstream of the HSP70 gene promoter and regulates HSP70 at the mRNA level (Tang et al., 2016). In a non-stressed state, HSF-1 remains inactive in the cytosol as a complex (Zou et al., 1998), but cellular stress disrupts the repressive complex and activates HSF-1. The activated HSF-1 induces expression of HSPs in order to enable cells to survive in suboptimal conditions (Vydra et al., 2014). These studies highlight that HSPA1A expression has manifold roles in osteogenic and chondrogenic differentiation (Li et al., 2018). In the current study, we found that expression of HSPA1A was down-regulated in the low T permissive environment for antler regeneration. In one of our previous

studies (Li and Chu, 2016), we reported that, at the very initial stage of antler regeneration, a blastema must be formed and thus cell proliferation takes priority over differentiation (irrespective of the lineage), such that differentiation must be effectively restrained. Therefore, down-regulation of HSPA1A expression might be related to formation of blastema at the initial stage of antler regeneration.

The transmission of extracellular signals to intracellular responses is a complex process which often involves one or more mitogen-activated protein kinases (MAPK3, MAPK2 and MAPK). These MAPKs participate in signal transduction pathways that control intracellular events including acute responses to hormones and major developmental changes in organisms. The MAPK groups are major components of pathways controlling cell differentiation, cell proliferation, and cell death (Raman et al., 2007). Proliferation signals from cytoplasm to nucleus transmit via MAPKKK-MAPKK-MAPK pathways (Keshet and Seger, 2010). The MAPK pathway is involved in the maintenance and activation of antler stem cells (i.e. PP cells that have potentiated to regenerate antlers) (Li et al., 2009). In the present study, MAPK3 was significantly up-regulated under low T (permissive environment). Therefore, the MAPK3 pathway probably has a vital role in antler regeneration through regulation of proliferation, maintenance and activation of the potentiated PP cells.

FKBP4 is a part of the androgen receptor (AR) complex required for

Table 5
Important KEGG pathways containing the identified DEPs.

KEGG Pathway	Number of proteins	Up-regulated proteins	Down-regulated proteins
Protein processing in endoplasmic reticulum	17	HSP90AB1, SEC31A, PDIA6, PDIA4, CALR, CANX, SSR1, HSP90B1, VCP, RPN1, SEC13, HSPA5, DDOST, SAR1A, SEC61A1	HSPA1A, CAPN2,
Arginine and proline metabolism	7	GOT2, PYCR1, ALDH7A1, CNDP2, OAT, ALDH9A1, CKB	
Legionellosis	7	EEF1A1, VCP, SEC22B, RAB1B, SAR1A, RAB1A	HSPA1A
Complement and coagulation cascades	7	FGG, FGA, C4B	C8B, F13A1, F9, SERPIND1
beta-Alanine metabolism	5	ALDH7A1, CNDP2, ECHS1, ALDH9A1	AOC2
Biosynthesis of antibiotics	11	GOT2, HSD17B10, PYCR1, ALDH7A1, G6PD, NME1, IDH2, ECHS1, IDH1, OAT, ALDH9A1	
Estrogen signaling pathway	7	HSP90AB1, HSP90B1, FKBP4, MAPK3	SHC1, HSPA1A, SRC
Spliceosome	8	SRSF2, HNRNPK, PCBP1, SNRPD3, SNRPE	HSPA1A, SNRPF, HNRNPU

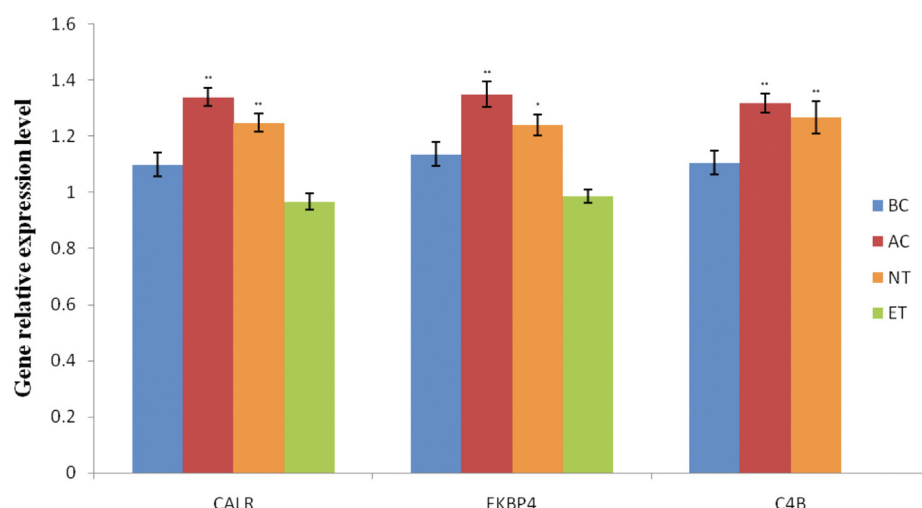


Fig. 5. Verification of relative gene expression levels of *CALR*, *FKBP4* and *C4B* using qRT-PCR analysis. The data are expressed as mean $\pm$ SD; and statistical significance: *P < 0.05 and **P < 0.01. Note that BC served as a control for the comparison of gene expression with natural T, low T and high T. BC (before castration); AC (after castration); NT (natural T); ET (exogenous T).

nuclear translocation of the AR after ligand binding (Federer-Gsponer et al., 2018). The FKBP4 promotes fast nuclear translocation in neurons and FKBP5 inhibits translocation of glucocorticoid (GR) in the absence of the hormone in neurons (Tatro et al., 2009). Together, these molecules regulate an ultrafast feedback loop within the cells. Following knockdown of FKBP4, growth rate of prostate cancer cells was decreased, but cancer cell proliferation increased after over-expression of FKBP4 (Federer-Gsponer et al., 2018). Over-expression of FKBP4 was found to be ineffective in stimulating proliferation in the total absence of androgens, suggesting that FKBP4 function depends on presence of androgens. In fact, FKBP4 may increase androgen receptor affinity and nuclear translocation (Storer et al., 2011), thus maintaining AR function under androgen deprivation therapy (Federer-Gsponer et al., 2018). Our study found that FKBP4 was significantly up-regulated in the low T environment, suggesting that this FKBP4 up-regulation may have a role in stimulating proliferation of PP cells.

Shc family proteins serve as phosphotyrosine adaptor molecules in various receptor-mediated signaling pathways. Genetic and biological evidence indicates that three mammalian ShcA forms share structural features and regulate functions as diverse as growth (p52/p46Shc), apoptosis (p66Shc) and life span (p66Shc), (Luzi et al., 2000). ShcA is expressed in a number of cell types, while ShcB and ShcC are expressed only in neuronal cells (Sakai et al., 2000). In the present study, we found that ShcA expression was down-regulated in the permissive environment (low T) for antler regeneration. It is reported that the expression of ShcA was down-regulated significantly in the CA/07 virus-infected adenocarcinomic human alveolar basal epithelial cells (A549) and this suppression of ShcA expression in response to CA/07 virus infection may contribute to the immunosuppression phenomenon (Yang et al., 2010). Therefore, it seems plausible that ShcA down-regulation in our study may offer a protective role against pathogen infection of the potentiated PP tissues during formation of antler blastema, as at this stage, there is a large open wound following the casting of the previous hard antler.

SRC (proto-oncogene tyrosine-protein kinase) is known for its diverse functions including signal transduction (Mahnaz et al., 2003), physiological response regulation, cell cycle control, cell proliferation, differentiation, adhesion, migration, and survival (Thomas and Brugge,

1997). AR receptors are present in osteoblasts, osteoclasts and the progenitors of these cells (Kousteni et al., 2001). The level of expression of AR in osteoblasts and osteoclasts is about ten-fold lower than that in reproductive tissues (Eriksen et al., 1988). Alternatively, in the case of steroid stimulation, SRC might trigger membrane-associated metallo-proteases which release heparin-bound-epidermal growth factor (EGF) from the cell surface; as a result, EGF binds to EGF receptor (EGFR) and finally the complex activates the MAPK pathway (Mahnaz et al., 2003). The present study found that SRC was down-regulated in the low T permissive environment. Interestingly, our group (Z Liu et al. unpublished observations) has found that SRC1N1, an inhibitor of SRC, at transcription level was significantly increased in the androgen-activated antler stem cell tissue for the initial formation of pedicles and first antlers, suggesting that SRC also negatively regulates pedicle development. The mechanism underlying regulation of androgen hormones on SRC expression in antler generation and regeneration needs further study.

CALR, a type of molecular chaperone, is involved in a number of cellular functions including chondrogenesis and osteogenesis through regulation of Ca²⁺ homeostasis (Michalak et al., 2009). In the present study, we found that expression of CALR was up-regulated (KEGG pathway: protein processing in endoplasmic reticulum) in the low T environment. A decrease in the T concentration and an increase in IGF-1 trigger the initiation of antler regeneration, which constitutes a permissive environment to allow local factors to activate proliferation and migration of the potentiated PP cells to form the early antler blastema, which provides a pool of progenitor cells for subsequent antler growth (Li and Chu, 2016). Investigation of the potential role of CALR, given its up-regulation as found in this study, may help to determine whether or not CALR acts as a nuclear export factor through localization of steroid receptors (AR), (Nguyen et al., 2009) in antler regeneration.

5. Conclusions

This is the first comprehensive study to detect DEPs of potentiated PP (low T) over non-potentiated PP (high T) tissues for deer antler regeneration using label-free proteomics. The study analyzed only those DEPs that were identified in the comparisons between the low T and

Table 6

Gene and primers used for validation of gene expression through qRT-PCR.

Gene	Gene name	Gene ID	Accession number	Forward primer	Reverse primer	Amplification length
<i>CALR</i>	Calreticulin	P27797	NM_004343.3	5'-CGGTGAAACACAGCAGAAC-3'	5'-CTTGCCCTGTAGTTGAAGATGA-3'	173 bp
<i>FKBP4</i>	Peptidyl-prolyl cis-trans isomerase	Q02790	NM_002014.4	5'-GCTTCTCCTGCTGCCATT-3'	5'-AGCCCGTGCCAAATCAAA-3'	123 bp
<i>C4B</i>	Complement C4-B	P0C0L5	BC_063289.1	5'-ACGCCTGGCTTTCTTACTGAC-3'	5'-GCTGCTGCTTGGTTTTCCTT-3'	210 bp

high T environments, particularly the up-regulated HSP90AB1, HSP90B1, FKBP4, and MAPK3, and down-regulated SHC1, HSPA1A and SRC in the low T environment which is permissive for antler regeneration. CALR, in the low T environment, also showed significant up-regulation in protein processing in endoplasmic reticulum as identified by KEGG pathway. We further identified transduction pathways through the KEGG database. We also analyzed DEPs that may be involved in regeneration, different biological functions, and signal transduction pathways and explored the underlying molecular mechanism of androgen hormones regulating cell proliferation, differentiation and gene expression in antler regeneration. Further studies are required to investigate the roles of these proteins, with the ultimate goal being to reveal the mechanism of activation of antler regeneration by androgen hormones at the molecular level.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

Conceptualization, R.W.A. and C.L.; Formal analysis, R.W.A.; Funding acquisition, C.L.; Investigation, R.W.A.; Methodology, R.W.A., L.Z., D.W., H.B. and C.L.; Project administration, C.L.; Resources, C.L.; Software, R.W.A., D.W. and H.B.; Supervision, C.L.; Validation, R.W.A.; Data Curation & bibliography, S.A.H.S.; Writing – original draft, R.W.A.; Writing – review & editing, C.L. and S.A.H.S.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ygcen.2019.113235>.

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