iTRAQ-Based Sperm Proteomic Analyses Reveals Candidate Proteins Affecting the Quality of Spermatozoa From Boar on Plateaus

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Research

Keywords: boar, comparative proteomics, iTRAQ, sperm quality, plateaus

Posted Date: November 6th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-102654/v1

Version of Record: A version of this preprint was published at Proteome Science on July 30th, 2021. See the published version at https://doi.org/10.1186/s12953-021-00177-9.

Abstract

Background: Tibetan pigs (TP) exhibit heritable adaptations to their hypoxic environments as a result of natural selection. Whereas, what candidate proteins affecting the sperm quality of boar on plateaus has not been clearly investigated yet.

Methods: In this study, to reveal the candidate proteins affecting the quality of spermatozoa from boar on plateaus, we analyzed the sperm quality by Computer-assisted semen analysis (CASA) system and Reactive oxygen species (ROS) levels, compared the sperm proteomes between TP and Yorkshire pigs (YP) raised at high altitudes using the isobaric tags for relative and absolute quantitation (iTRAQ) in combination with the liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomic method, and confirmed the relative expression levels of the four proteins by western blot.

Results: The sperm quality of the TP was superior to that of the YP on plateaus. Of 1,555 quantified proteins, 318 differentially expressed proteins (DEPs) were identified. Gene ontology (GO) analysis revealed that the DEPs were predominantly associated with the sorbitol metabolic process, removal of superoxide radicals, cellular response to superoxide, response to superoxide and regulation of the mitotic spindle assembly. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were mainly enriched in pathways involved in the regulation of the actin cytoskeleton, glutathione metabolism, oxidative phosphorylation, and estrogen signaling. And based on the protein-protein interaction (PPI) network analysis, we identified 8 candidate proteins (FN1, EGF, HSP90B1, CFL1, GPX4, NDUFA6, VDAC2, and CP) that might play important roles that affect the sperm quality of boar on plateaus. Moreover, the relative expression levels of the proteins (CFL1, EGF, FN1, and GPX4) were confirmed by western blot.

Conclusions: Our results reveal 8 candidate proteins (FN1, EGF, HSP90B1, CFL1, GPX4, NDUFA6, VDAC2, and CP) affecting the sperm quality of boar on plateaus, providing a reference for studies on improving sperm quality and the molecular breeding of boar on plateaus.

Background

On plateaus, the reproductive system and antioxidants involved in oxidative stress in male boars are affected by hypobaric hypoxia [1]. The Tibetan pig (TP) is a unique and geographically isolated pig breed that inhabits high-altitude regions on the Qinghai-Tibetan plateau. These animals exhibit heritable adaptations to their hypoxic environments as a result of natural selection [2]. Both the Yorkshire pig (YP), which migrated from the lowland to the plateaus, and the TP are able to produce offspring normally on plateaus, providing a reference for studies on improving sperm quality and the molecular breeding of boar on plateaus to reveal candidate proteins affecting the sperm quality of these animals. Reactive oxygen species (ROS) have been recognized as problems for sperm survival and fertility when its production exceeds formation and membrane lipid peroxidation, the reduction in motility may have been due to a ROS-induced lesion in ATP utilization or in the contractile apparatus of the flagellum [3]. So, it was necessary that sperm ROS levels should also be evaluated during sperm quality analysis besides the

evaluation of sperm motility and morphology. Previous studies have shown that boar spermatozoa are very susceptible to ROS, which may stimulate the acrosome reaction in boar sperm through membrane lipid peroxidation and phospholipase A activation [4]. But the sperm quality analysis of TP and YP raised at high altitudes have not yet been conduced. Proteomic data enable a better understanding of sperm biochemistry [5]. In-depth proteomic analysis of boar spermatozoa was implemented using shotgun and gel-based methods [6]. The proteome of pig spermatozoa is remodeled during ejaculation [7]. The comparative proteomes of prenatal muscle tissues among TP, Wujin pig and large White pig were analyzed using the isobaric tag for relative and absolute quantification (iTRAQ) [8]. Comparative proteomics of TP spermatozoa at high and low altitudes were analyzed to elucidate the mechanisms underlying the high-altitude tolerance of TP spermatozoa, which provides new insights into how the reproductive mechanisms of TP have adapted to tolerate high-altitude environments [9]. However, the sperm proteomes of TP and YP raised at high altitudes have not yet been compared.

iTRAQ is a powerful technique for the quantitative analysis of proteomes [10] that has the advantages of high sensitivity, good repeatability, the ability to label almost all enzymatic peptides, and the ability to label 8 samples simultaneously [11]. In this study, to reveal candidate proteins affecting the sperm quality of boar on plateaus, we analyzed the sperm quality, compared the sperm proteomes between TP and YP raised at high altitudes using iTRAQ, and confirmed the relative expression levels of the four proteins by western blotting. The present results provide a reference for studies investigating the improvement of sperm quality and the molecular breeding of boar on plateaus.

Methods

Animal samples

Experiments were performed using pigs from two different populations: Tibetan pigs living in highlands (Linzhi, 3,000 m, TP), Yorkshire pigs that migrated from lowland (Beijing, 100 m) to highland (Linzhi, 3,000 m, YP) about 3 yr ago. TP and YP used were respectively raised in the Tibet Agricultural and Animal Husbandry University Farm (Linzhi, 3,000 m) and Tibet Linzhi ga ma breeding co. Ltd (Linzhi, 3,000 m), ten boars (1.5 years old, normal fertility and nutrition level) from each population were used in this study.

Semen collection and quality analysis

Twenty fresh semen samples, one per boar were respectively obtained from 10 TP and 10 YP by using the gloved-hand technique. After semen collection, there was no adverse effect noted on the health and growth of the pigs. Sperm quality were meautured by sperm motility parameters and ROS levels. Computer-assisted semen analysis (CASA) system (Hamilton Thorne Research, Beverly, MA, USA) was used to measure the sperm concentration, motility, VAP, and abnormality rate according to the manufacturer's instructions. In short, put the semen to incubation at 37 ℃ for 10 min, then 3 μL semen was dropped into the preheated (37 ℃) Makler sperm count board, sperm motility, etc. were assessed by using CASA system. Look at at least 3 visual fields to get the average. According to the manufacturer's protocol of ROS assay kit (S0033M, Shanghai Beyotime Biotechnology Co. Ltd, Shanghai, China), sperm

ROS level was evaluated by using the probe 2', 7'-dichlorodi-hydrofluorescein diacetate (DCFH-DA). Breifly, the semen samples were washed with PBS three times, resuspended and incubated with 10 μM DCFH-DA at 37 °C in the dark for 25 min. For intracellular DCFH-DA was deesterified to dichlorodihydrofluorescein which is oxidized by ROS to produce dichlorofluorescein with strong fluorescence, the fluorescence intensity could be conveniently monitored using a fluorescent microplate reader (Biotek Synergy, SynergyH4,USA) at an excitation wavelength of 488 nm and at an emission wavelength of 525 nm.

Protein preparation

To remove seminal plasma and contamination (e.g., extender components and somatic cells such as leukocytes and testicular cells), semen samples were centrifuged at 500 × g for 20 min with a discontinuous (70% [v/v] and 35% [v/v]) Percoll gradient (Sigma, St Louis, MO, USA), and then the sperm pellets were washed 3 times with cold phosphate-buffered saline (PBS). For protein extraction, each sperm sample (3×10^8 spermatozoa) was resuspended in lysis buffer (8 M urea, 4% CHAPS, 50 mM DTT and protease inhibitor, pH 8.0) at 4°C. The lysates were centrifuged at 10,000 *g* for 30 min to remove insoluble material, and the supernatants were collected for further analysis. The protein content was measured with the Bradford protein assay kit (P0006C, Beyotime Institute of Biotechnology, Nanjing, China).

Protein labeling and LC-MS/MS

The proteins of the sperm samples and then digested using the filter-assisted sample preparation (FASP) as previously described [12]. The resulting TP and YP peptides were labeled 116 (TP1), 121 (TP2), 113 (YP1) and 119 (YP2), according to the instructions supplied with the iTRAQ® Reagent-8PLEX Multiplex Kit (4381663, AB SCIEX, USA). The labeled samples were then loaded onto a high-pH reverse-phase liquid chromatography (RPLC) XBridge C18 column (Waters, Milford, MA, USA) connected to a liquid chromatography system (e2695, Waters, Milford, MA, USA). The column was eluted with a 51 min gradient of 0 ~ 5% buffer B (98% acetonitrile, pH 10.0) for 5 min, 5 ~ 35% buffer B for 45 min, and 35 ~ 50% buffer B for 10 min at a flow rate of 1 mL/min. The fractionated peptides were analyzed by LC-MS/MS using a nano-LC (Easy nLC 1000, Thermo Fisher Scientific, Odense, Demark) in tandem with an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). MS/MS scans in the range from *m/z* 350 to 1800 were recorded with a mass resolution of 70,000 at *m/z* 400. The LC-MS/MS data were acquired in a data-dependent mode, in which the ten most intense precursor ions were isolated and fragmented by collision-induced dissociation (CID) with 32% normalized collision energy. Dynamic exclusion was enabled (exclusion list size: 500, exclusion duration: 40 s).

Database search and bioinformatics

The MS/MS data were searched against the NCBI Sus_refesq_20180716.fasta (63,695 sequences) Fasta database for peptide identification and quantification using Mascot 2.5.1 and Proteome Discoverer 1.4 (Thermo). The search parameters were specified as follows: one missed enzymatic cleavage site was allowed, the mass tolerance was set at 10 ppm for precursor ions and ± 0.05 Da for fragment ions,

carbamidomethylation was set as a fixed modification, oxidation and iTRAQ-4plex were set as variable modifications. The false-positive detection rate (FDR) was calculated using a decoy database search, with FDR < 1.0%, identifying each protein to at least 1 specific polypeptide, normalized by the median of the data. We compared the expression levels of all identified proteins between the TP and the YP groups to identify the proteins involved in reproduction traits in boars on plateaus. The Student's t-test was used to compare differences in protein expression between the TP and YP groups and to calculate p values. $p < 0.05$ and a fold change ≥ 1.5 or ≤ 0.67 were set as the threshold to identify differentially expressed proteins (DEPs). The average of six labeled sample mixtures was used as reference (ref) based on the weighted average of the intensity of reported ions in each identified peptide. The final ratios of proteins were normalized according to the median average protein ratio for the mixtures of different labeled samples (TP1/ref, TP2/ref, YP1/ref, and YP2/ref).

The DEP data were analyzed using bioinformatics, while the UniProt IDs of the DEPs were converted to mouse UniProt IDs due to the small number of studies on gene function in pigs. Gene ontology (GO) annotation and enrichment of DEPs were analyzed using the GO consortium database for GO assignment (http://geneontology.org/). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and protein-protein interaction (PPI) analyses were performed using STRING online software (https://string-db.org/). The results of the GO analysis were mapped into a senior bubble map using the OmicShare tool, a free online platform for data analysis (http://www.omicshare.com/tools), which was also used to map the volcano figure and heatmap using the OmicShare tool. PPI networks were visualized and analyzed using Cytoscape 3.2.1 software [13].

Validation of DEPs by western blot

Cofilin-1 (CFL1), pro-epidermal growth factor (EGF), fibronectin 1 (FN1), and glutathione peroxidase 4 (GPX4) expression levels were determined by western blot analysis, using beta actin (β-actin) as a loading control. The bar line charts were created using Sigmaplot 10.0 (Systat Software, San Jose, CA, USA). In brief, denatured sperm proteins (30 μg) from TP and YP were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 4% stacking gel and 12% separating gel) and transferred to polyvinylidene fluoride (PVDF) membranes using a Hoefer TE22 blotting instrument (Hoefer, Holliston, MA, USA). The membranes were blocked overnight in blocking buffer (P0071, Shanghai Beyotime Biotechnology Co. Ltd, Shanghai, China), incubated with the appropriate primary antibody (1:1000, ab42824, ab231103, ab32419, ab231174 or ab8227, Abcam, Cambridge, UK) and gently shaken at room temperature for 2 h. After three washes with phosphate-buffered saline containing 0.1% Tween 20 (PBST), the membranes were incubated with the appropriate secondary antibody (1:1000, A0208, Beyotime Ltd., Shanghai, China) for 1 h. After three washes in Tris-buffered saline with Tween 20 for 30 min, the immune complexes on the membranes were visualized using BeyoECL Plus (P0018S, A0216, Beyotime Ltd., Shanghai, China) following the manufacturer's instructions. To determine the expression levels of CFL1, EGF, FN1 and GPX4 relative to β-actin, the gray value of the bands was analyzed using ImageJ 1.44 (NIH, Bethesda, MA, USA).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics v17.0 (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc.). Graphs were prepared using SigmaPlot 10.0 (Systat Software, San Jose, CA, USA). One-way analysis of variance (ANOVA) was used to determine the significance of differences between the two groups. All quantitative data are presented as the mean ± standard deviation (S.D.). We considered $P < 0.05$ (*) as statistically significant and $P < 0.01$ (**) as extremely statistically significant.

Results

Comparison of sperm quality in the TP and the YP

Sperm concentration and motility were statistically higher but abnormalities were statistically lower in TP compared with those in YP, although there were no significant difference in the average path velocity (VAP) and ROS fluorescence Unit (RFU, which represent the level of intracellular ROS) of spermatozoa between the TP and YP (Table 1). Obviously, the sperm quality of the TP was superior to that of the YP on plateaus.

Table 1 Sperm quality analysis of TP and YP

Semen	Concentration (10^8/mL)	Motility (%)	VAP (μm/s)	Abnormality (%)	RFU
TP	3.81 ± 0.22	86.00 ± 1.63	21.59 ± 0.52	8.61 ± 0.24	17.73 ± 3.81
YP	2.91± 0.13**	73.00 ± 3.00**	20.03 ± 0.54	9.40 ± 0.17*	25.40 ±4.42

* Note: Each bar represents the mean ± standard deviation (SD); * significant difference ($p < 0.05$), ** extremely significant difference ($p < 0.01$). VAP = Average path velocity; RFU = Reactive oxygen species (ROS) fluorescence Unit, which represent sperm ROS level; TP = Tibetan pig (n = 10); YP = Yorkshire pig (n = 10).

DEP identification

Overall, 33,093 spectra were obtained from the LC-MS/MS analysis. A total of 1,555 proteins were detected from 6,375 unique peptides by quantitative proteomic analysis (Additional file 1: Table S1). Among the proteins detected, 318 DEPs were detected from two biological duplicates ($P < 0.05$ and a fold change ≥1.5 or ≤ 0.67). Of the DEPs identified, 186 proteins were upregulated and 132 proteins were downregulated in the TP compared with those in the control group of YP (Fig. 1a and additional file 2: Table S2). The cluster analysis based on the protein abundance data of the 318 DEPs, which showed that

the two biological duplications in each breed were clustered into one group and that TP1, and TP2 as well as YP1, and YP2 were clustered together (Fig. 1b and Additional file 3: Table S3).

GO and KEGG enrichment analysis of DEPs

The DEPs were classified by gene ontology annotation based on three categories: biological process (BP), molecular function (MF), and cellular component (CC) (Additional file 2: Table S4, displaying only results for FDR $P < 0.05$). Of the BP terms, sorbitol metabolic process, removal of superoxide radicals, cellular response to superoxide, cellular response to oxygen radical, regulation of protein folding, response to superoxide and regulation of mitotic spindle assembly were enriched for DEPs (Fig. 2a). Of the MF terms, S-100 protein binding, unfolded protein binding, heat shock protein binding, nucleoside binding, ribonucleoside binding, and signaling receptor activity were enriched for DEPs (Fig. 2b). Of the CC terms, mitochondrial envelope, mitochondrial inner membrane, acrosomal vesicle, and sperm part were enriched for DEPs (Fig. 2c).

To determine potential pathways of the DEPs, KEGG pathway analysis was performed. Forty-one of 318 DEPs were successfully mapped to 13 term IDs (false discovery rate < 0.05, Additional file 5: Table S5), and some important pathways are shown in Table 2. These important pathways were regulation of actin cytoskeleton, glutathione metabolism, oxidative phosphorylation, and estrogen signaling pathway, among others.

Table 2 Important Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways

Term ID	Term description	Protein counts	Matching proteins in your network (labels)
mmu04216	Ferroptosis	5	Acsl6, Cp, Gpx4, Ica, Vdac2
mmu04810	Regulation of actin cytoskeleton	9	Arpc5l,Baiap2, Cfl1, Egf, Ezr, Fn1, Nras, Rras, Scin
mmu00480	Glutathione metabolism	5	Anpep, Gpx4, Gsta2, Gstm5, Gsto2
mmu04612	Antigen processing and presentation	5	Canx, Hspa1a, Hspa1l, Lgmn, Pdia3
mmu00190	Oxidative phosphorylation	6	Cox5b, Cox6b2, Cox7a2, Ndufa6, Ndufv3, mt-Atp8
mmu00790	Folate biosynthesis	3	Alpl, Cbr1, Ggh
mmu04714	Thermogenesis	8	Acsl6, Cox5b, Cox6b2, Cox7a2, Ndufa6, Ndufv3, Nras, mt-Atp8
mmu04915	Estrogen signaling pathway	6	Hsp90b1, Hspa1a, Hspa1l, Krt18, Krt19, Nras

Protein–protein interaction (PPI) network construction and analysis

To further identify candidate proteins among DEPs involved in important pathways, we constructed a PPI network using STRING and Cytoscape 3.2.1 software (Additional file 6: Table S6 - PPI relationships and scores). The PPI network consisted of 28 nodes and 57 edges (Fig. 2d). The details are presented in Additional file 7: Table S7. Based on the protein–protein interaction (PPI) network analysis, we identified 8 candidate proteins (FN1, EGF, HSP90B1, CFL1, GPX4, NDUFA6, VDAC2, CP) that might have important roles affecting sperm quality of boar on plateaus (Table 3).

Table 3 Candidate proteins affecting sperm quality of boar on plateaus

Protein description	Accession No.	Symbol	MW [kDa]	Fold Change*	Regulation*
Fibronectin	XP_003133690.2	FN1	289	0.55	Down
Pro-epidermal growth factor precursor	NP_999185.1 (+2)	EGF	134	1.7	Up
Endoplasmin precursor	Q9DCX2	HSP90B1	93	1.66	Up
Cofilin-1	NP_001004043.1	CFL1	19	1.71	Up
Phospholipid hydroperoxide glutathione peroxidase precursor	NP_999572.1	GPX4	22	0.47	Down
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6	NP_001172107.1	NDUFA6	15	1.66	Up
Voltage-dependent anion-selective channel protein 2	NP_999534.1 (+1)	VDAC2	32	0.37	Down
Ceruloplasmin precursor	NP_001254623.2 (+2)	CP	122	0.38	Down

Western blot validation

The results of the western blot analysis of the four proteins are presented in Fig. 3a. The gray value for each lane in the western blot was consistent with the iTRAQ quantification levels (Fig. 3b).

Discussion

Oxidative stress has been established as one of the main causes of male infertility. It results from high concentrations of free radicals and suppression of antioxidant potential, which may alter protein expression in spermatozoa [14]. The positive effects of Astragalus polysaccharide on boar sperm quality were mainly due to the elimination of excessive mitochondrial ROS, the improvement of antioxidant capacities and the enhancement of ATP levels [15]. Feeding diet with 500 mg/kg oregano essential oil may resulted in decrease in sperm ROS [16]. Tibetan pigs have survived at high altitudes for thousands of years and have adapted to tolerate the hypoxic environment. In our previous studies, we found that although the sperm VAP of Tibetan pigs was significantly lower in high-altitude plateau areas compared with low-altitude pigs, their sperm counts, motility and abnormality were equivalent [9]. In the present study, we found that the TP had superior sperm quality compared to the YP on plateaus. These results suggested that the male reproductive system of the TP was more adaptive to high-altitude conditions compared with that of other migrated pig breeds such as the YP. To reveal candidate proteins affecting sperm quality of boar on plateaus, we identified and analyzed 318 DEPs between spermatozoa of the TP and YP. We found that the DEPs were predominantly associated with sorbitol metabolic process, removal of superoxide radicals, cellular response to superoxide, response to superoxide and mitotic spindle

assembly regulation, among others. KEGG pathways were mainly enriched in the regulation of actin cytoskeleton, glutathione metabolism, oxidative phosphorylation, and estrogen signaling pathway. Additionally, based on the PPI network analysis, we identified 8 candidate proteins (FN1, EGF, HSP90B1, CFL1, GPX4, NDUFA6, VDAC2, CP) that could have important roles affecting sperm quality of boar on plateaus.

Previous research has indicated that alterations in the proteins involved in chromatin assembly and metabolism may result in epigenetic errors during spermatogenesis, leading to inaccurate sperm epigenetic signatures, which could ultimately prevent embryonic development [17]. BAG6 and HIST1H2BA are potential candidates for male infertility biomarkers [18]. CMTM4 is associated with spermatogenesis and sperm quality [19]. Sperm mitochondrial dysfunction and oxidative stress are possible causes of isolated asthenozoospermia [20]. Regionally distinct expression and localization of CETN1 and CSPP1 are strongly related to spermiogenesis and sperm morphology maintenance. Obesity is associated with declines in CETN1 and CSPP1 abundance and compromise of both sperm morphology in mice and relevant clinical samples. The parallelism between altered protein expression in mice and humans suggests that these effects may contribute to poor sperm quality, including increased deformity [21]. Both intensified and relaxed sperm competition can have a pronounced impact on the molecular composition of the male gamete [22]. SPAG6, ACR, LDHC, CALM, ACE and ENO1, which are positively related to a large litter size, are more abundant in Meishan than Duroc spermatozoa. Moreover, APOA1, NDUFS2 and RAB2A, which are negatively related to farrowing rates, are less abundant in Meishan than Duroc spermatozoa. Interestingly, enzymes that are essential in glycolysis/gluconeogenesis, such as HK1, ALDH2, LDHA and LDHC, are markedly upregulated in Meishan compared with Duroc spermatozoa [23]. The sperm proteins that are expressed in greater abundance in high- compared with low-fertility bulls were found to be HSP90, ZFP34, IFNRF4, BCL62, NADHD, TUBB3 and histone H1 [24]. Enolase-1 (ENO1) was found to be overexpressed in the high-fertility and Binder of SPerm-1(BSP1) in the low-fertility group [25]. Sperm triosephosphate isomerase (TPI) content and amounts of epididymal secretory glutathione peroxidase (GPX5) in seminal plasma may be used as quality markers of boar sperm [26]. ATP citrate lyase is overexpressed in liquid-stored sperm, while cytosolic nonspecific dipeptidase is overexpressed in fresh boar sperm samples [27]. These results differ somewhat from our research findings, potentially due to differences in species and environment.

Among the 8 candidate proteins identified, we found that EGF, HSP90B1, CFL1, and NDUFA6 were upregulated in TP spermatozoa and were enriched in the regulation of the actin cytoskeleton, glutathione metabolism, oxidative phosphorylation, and the estrogen signaling pathway（Table 2）. Research has shown that EGF promotes the proliferation and differentiation of mouse spermatogenic cells [28]. HSP90B1 is involved in protein folding and in the targeting of misfolded proteins for endoplasmic reticulum-associated degradation, in addition to participating in calcium storage, which is required for normal functions of spermatozoa [29], and it was found to be upregulated in the normozoospermic group but downregulated in the asthenozoospermic group in comparison to the control group [30]. PKA-dependent phosphorylation of LIMK1 and cofilin is essential for mouse sperm acrosomal exocytosis [31]. NDUFA6 of mitochondrial complex I anchors an acyl carrier protein and is essential for catalytic activity

[32]; downregulation of the NDUFA6 subunit has been linked to the inactivation of complex I, leading to the induction of apoptosis [33]. Consequently, sperm quality is supported by the expression of four proteins (EGF, HSP90B1, CFL1 and NDUFA6) that were upregulated in TP spermatozoa.

Among the 8 candidate proteins, we found that the expression levels of four proteins (FN1, GPX4, VDAC2 and CP) were downregulated in TP spermatozoa, which were enriched in the regulation of the actin cytoskeleton, ferroptosis, and glutathione metabolism (Table 2). Previous studies have found that fibronectin 1 (FN1) is downregulated in spermatozoa of Tibetan pigs living at high versus low altitudes[9]. Levels of FN1 in fresh seminal plasma from boar semen may be used as a sperm freezability marker, thereby facilitating the use of frozen-thawed boar spermatozoa [34]. Glutathione peroxidase 4 (Gpx4) is essential for spermatogenesis; heterozygous expression of a catalytically inactive mutant form of Gpx4 impairs spermatogenesis, and well balanced expression of functional Gpx4 has emerged as prerequisite for complete male fertility [35]. Mitochondrial GPx4 forms the mitochondrial sheath of spermatozoa and thus guarantees male fertility[36]. VDAC2 downregulation inhibits spermatogenesis via the JNK/P53 cascade[37]. The fertility-related protein markers ENO1, ATP5B, VDAC2, GPX4, and UQCRC2 were identified from 20 individual bull semen samples [38]. CP is a Cu-containing protein and an oxidase with high antioxidation capacity, which is involved in scavenging oxygen free radicals and protecting various organs from lipid peroxidation and other types of oxidative attack in the extracellular space [39]. Dietary addition of Mo indirectly increases oxidative stress to exacerbate Cd toxicity by reducing the expression of MT and CP, which protect testicles from lipoperoxidation and other types of oxidative stress [40]. Thus, the downregulated expression of the four proteins (FN1, GPX4, VDAC2 and CP) in TP spermatozoa supported sperm quality.

Conclusions

In summary, The sperm quality of the TP is better than that of the YP on plateaus, which indicated that the male reproductive system of the TP is more adaptive to high-altitude conditions than that of the YP. The proteins identified by comparative proteomic analyses of spermatozoa between the TP and YP raised at high altitudes were mainly enriched in the regulation of the actin cytoskeleton, glutathione metabolism, oxidative phosphorylation, and the estrogen signaling pathways. We revealed 8 candidate proteins (FN1, EGF, HSP90B1, CFL1, GPX4, NDUFA6, VDAC2, CP) that might have important roles affecting sperm quality in boar on plateaus, which elucidated candidate proteins affecting the sperm quality of boar on plateaus, providing a reference for studies on improving sperm quality and the molecular breeding of boar on plateaus.

Abbreviations

HT: high-altitude **LT:** low-altitude C**ASA:** computer-assisted semen analysis

VAP: average path velocity **LC-MS/MS:** liquid chromatography-tandem mass spectrometry

iTRAQ: isobaric tag for relative and absolute quantification

FASP: filter-assisted sample preparation **DEPs:** differentially expressed proteins

GO: gene ontology B**P:** biological process M**F:** molecular function C**C:** cellular component

KEGG: Kyoto Encyclopedia of Genes and Genomes **PPI:** protein-protein interaction

PVDF: polyvinylidene fluoride **SDS-PAGE:** sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Declarations

Acknowledgements

We thank American Journal Experts for editing this manuscript.

Funding

This study was jointly supported by the two grants from the National Natural Science Foundation of China (No. 31760669 and 31660658).

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Authors' contributions

Conceptualization, Y.Z. and Z.R.; Data curation, Y.W.; Formal analysis, Y.Z.; Investigation, Y.Z., Y.W., F.G., B.L., J.L., and J.W.; Writing-orinal draft, Y.Z.; Writing-review & editing, Y.Z. and Z.R.. All authors read and approved the fifinal manuscript.

Ethics approval and consent to participate

All the methods used in this study comply with the standards of the institutional guideline for ethics in animal experimentation (Rule number 86/609/EEC-24/11/86), and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Tibet Agricultural and Animal Husbandry University. The institutional certification number is 12540000MB0P013721.

Consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interest.

Additional information

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Supplementary information

Supplementary materials can be found at http://link.springer.com. Table S1: A list of all identified proteins, Table S2: A list of the volcano plots, Table S3: A list of the heatmap, Table S4: Go analysis of DEPs, Table S5: KEGG analysis of DEPs, Table S6: The PPI relationships and scores, Table S7: The details of PPI network.

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Figures

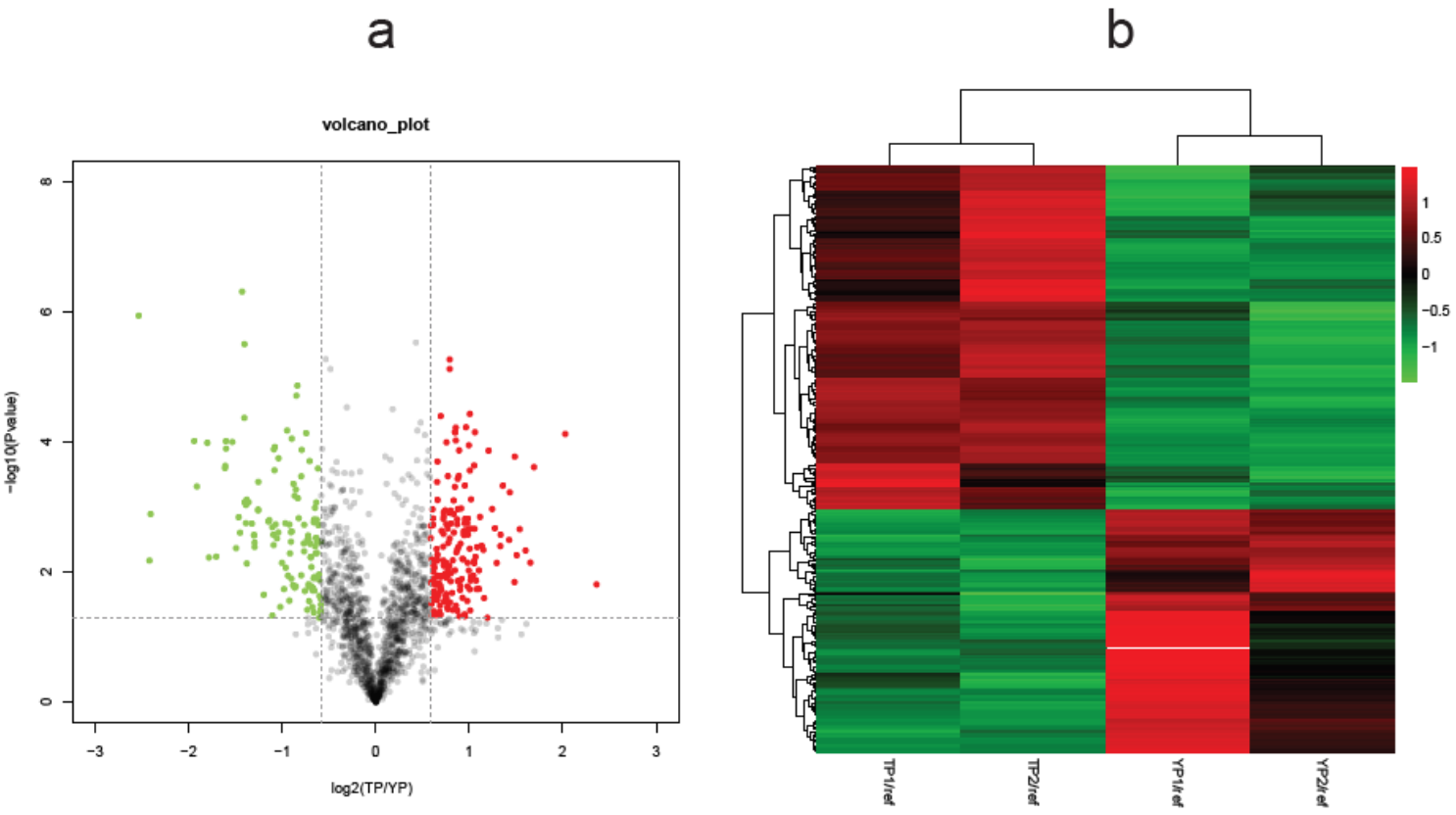

Figure 1

Comparison of differentially expressed proteins (DEPs) from four labeled samples. (a) Volcano plots of DEPs. Each point in the volcanic map represents a protein; the abscissa represents the logarithmic value of the expression of a certain protein in the two samples. The red point represents upregulated DEPs, the green point represents downregulated DEPs, and the black dot represents nondifferentially expressed protein; (b) Hierarchical clustering heat maps of DEPs. The expression of each protein is illustrated in red and green to indicate high and low expression, respectively. TP = Tibetan pig (n = 10); YP = Yorkshire pig (n = 10).

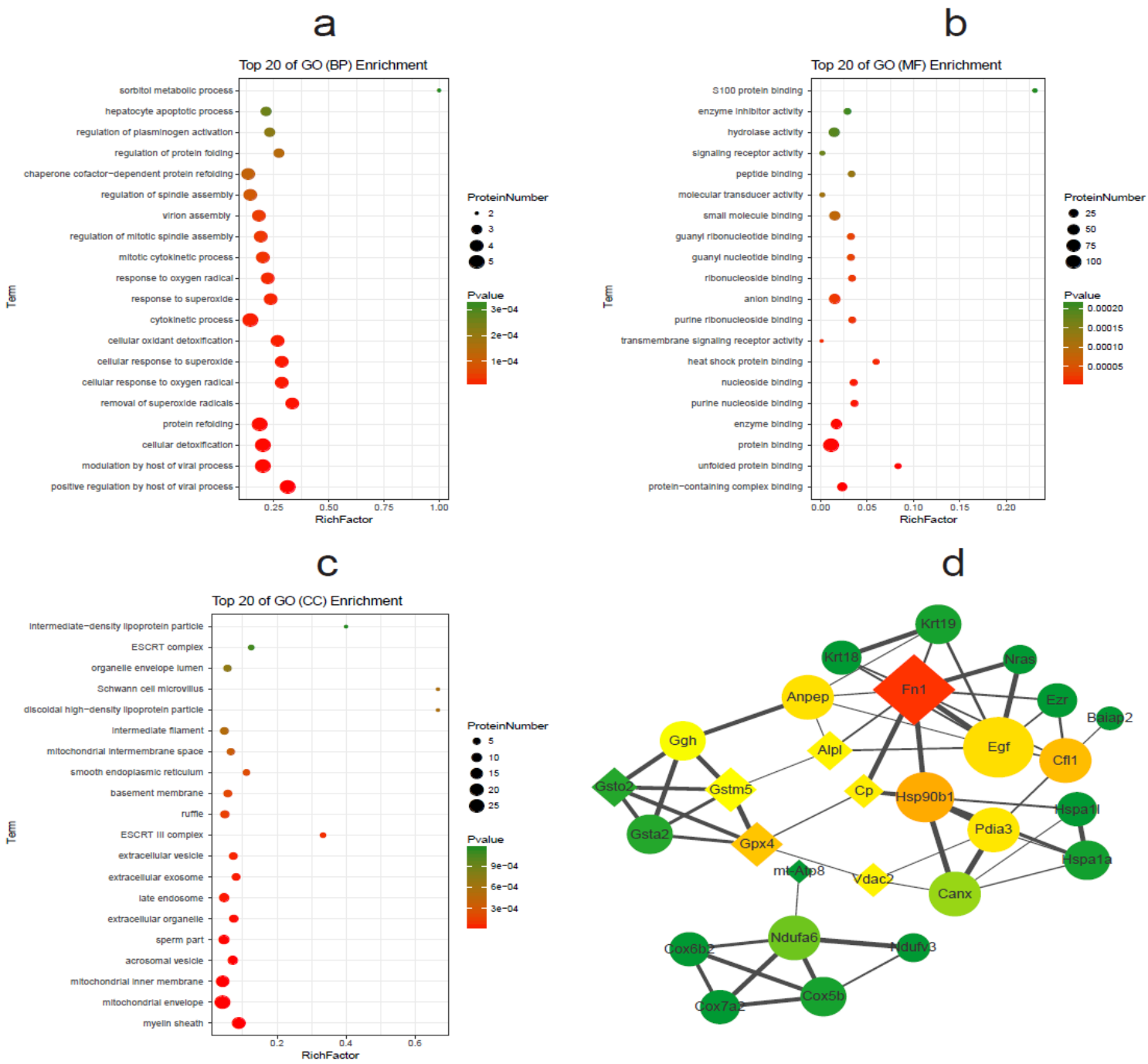

Figure 2

Gene ontology (GO) enrichment analyses and protein-protein interaction (PPI) network construction. (a) The top 20 GO (biological process, BP) enrichment terms for differently expressed proteins (DEPs); (b) The top 20 GO (molecular function, MF) enrichment terms for DEPs; (c) The top 20 GO (cellular component, CC) enrichment terms of DEPs; (d) PPI network of DEPs involved in important pathways. The nodes represent DEPs, and the edges between the nodes indicate interactions between two connecting DEPs. The node colors indicate the betweenness of the node interaction: the more red the color, the larger is the betweenness, indicating a greater influence in the network. The node sizes indicate the degree of interaction between nodes: the larger the size, the higher is the degree, indicating a stronger stability in the

network. The node shapes represent upregulated proteins (ellipse) or downregulated proteins (diamond). The degrees of edge width represents the protein–protein interaction scores.

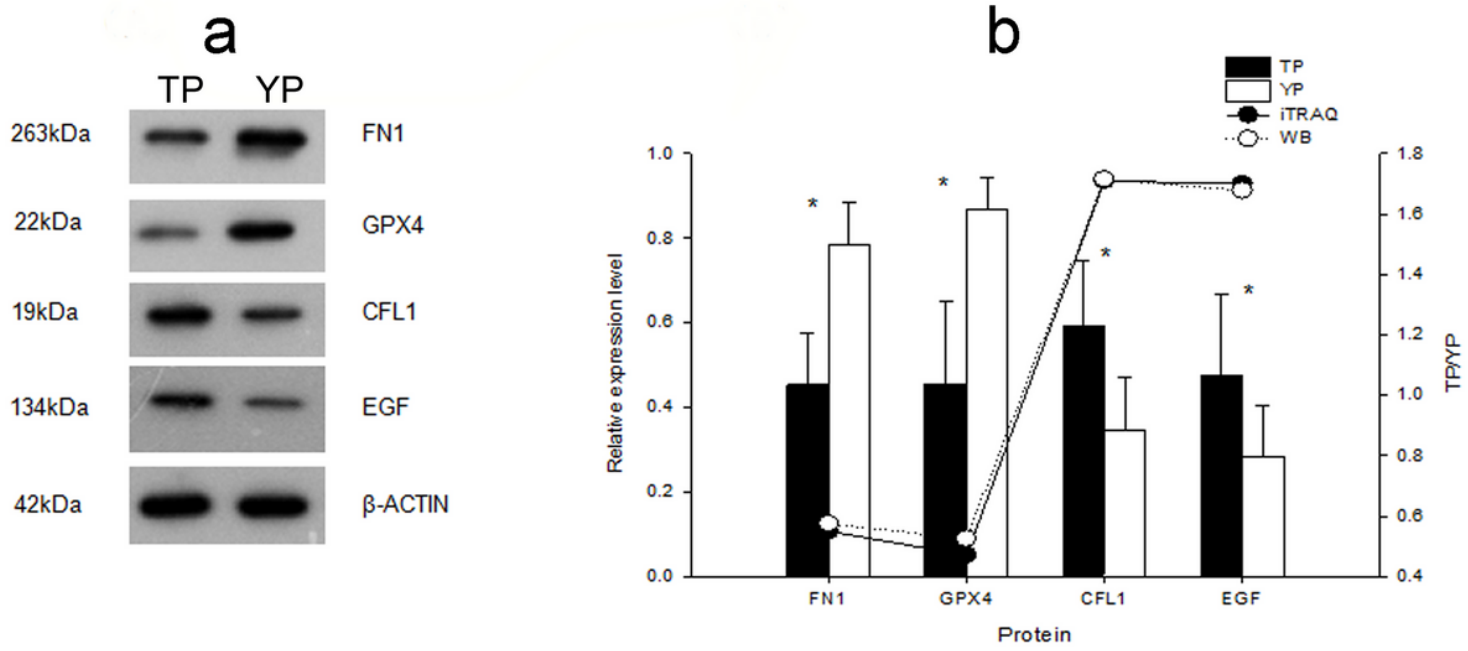

Figure 3

Western blot validation. (a) western blot (WB) analysis of four proteins (CFL1, CYCS, FN1, and GPX4). β-actin was used as a loading control. (b) Statistical results of WB and quantitative comparison of the isobaric tag for relative and absolute quantification (iTRAQ) and WB for the four proteins. The bar chart shows the statistical results of WB. The line chart represents the quantitative comparison of iTRAQ and WB for the four proteins. The data for the WB expression levels are represented as the means ± standard deviation (SD); * Significant difference ($P < 0.05$), ** extremely significant difference ($P < 0.01$). TP = Tibetan pig (n = 10); YP = Yorkshire pig (n = 10).

Supplementary Files

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