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Identification of differentially expressed proteins in the testes of normal yaks and sterile hybrids by two-dimensional electrophoresis and mass spectrometry

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ABSTRACT

The study was conducted to compare differentially expressed proteins in the testes of adult yaks (*Bos grunniens*) and sterile cattle-yaks, in order to elucidate mechanisms of hybrid male sterility. Total proteins were extracted from testes and subject to 2-dimensional electrophoresis and mass spectrometry identification. A total of 19 differentially expressed proteins were identified, of which 13 were downregulated and 4 upregulated in cattle-yak testes. Interestingly, two chaperones (T-complex protein 1 and peptidyl-prolyl *cis-trans* isomerase) with decreased expressions in the testes of cattle-yaks were revealed, however, their association with the hybrid sterility is unclear. Protein phosphatase methylesterase-1 was decreased by over 8-folds in the testes of cattle-yaks than yaks, and may be associated with cell cycle arrest during spermatogenesis. Several proteins involved in male fertility and/or energy metabolism were also identified, such as mitochondrial peroxiredoxin-5 and sorbitol dehydrogenase. In addition, N (G), N (G)-dimethylarginine dimethylaminohydrolase (DDAH), which can increase the production of nitric oxide and affect normal spermatogenesis in testis, was upregulated in cattle-yak testes. The increased expression of DDAH is probably associated with male sterility of cattle-yaks. The results of this study suggest that the mechanisms of male sterility of cattle-yaks might be associated with many proteins of diverse functions, and some proteins such as chaperones and testis-specific proteins may play important roles.

Key words: *Bos grunniens*, Hybrid, Male sterility, Testis, Two-dimensional electrophoresis

Yak (*Bos grunniens*) is a unique bovine species adapted to the hypoxic environment of Qinghai-Tibetan plateau. Cattle-yak is a hybrid of male cattle (*Bos taurus*) with female yak, exhibiting F1 to F3 male sterility but normal females. The genetic cause of male sterility of cattle-yaks is still unknown. Hybrid sterility and other hybrid incompatibilities have been a focus of genetic studies of speciation (McDermott and Noor 2010), and might involve multiple mechanisms such as X-linked incompatibilities (Good *et al*. 2008) and failure to execute meiosis correctly (Matzuk and Lamb 2008, Thomsen *et al*. 2011). Experiments showed that few germ cells developed further than the stage of pachytene spermatocytes and decreased expressions of several genes were detected in the testes of sterile cattle-yaks (Zhang *et al.* 2009, Huang *et al.* 2012). However, these studies have not yet provided convincing evidences for elucidating the mechanism of male sterility of cattle-yaks.

Proteomics is an important discipline in post-genomic era. Two-dimensional gel electrophoresis and mass spectrometry have been employed to assay proteome profile changes during testis development. Testicular proteome was reported in pigs, mice and rats (Huang *et al.* 2005, Paz *et al.* 2006, Shi *et al.* 2010). However, such study has not been conducted in yaks. The hypothesis of current study was that the hybrid male sterility of cattle-yaks might involve dysfunctions of many genes and corresponding proteins. Therefore, in this experiment total proteins were extracted from the testes of normal adult yaks and sterile cattle-yaks, and then compared by two-dimensional electrophoresis and mass spectrometry identification, aiming at identifying differentially expressed testicular proteins and shedding light on the molecular mechanism of hybrid sterility of cattle-yaks.

MATERIALS AND METHODS

Sample collection: The experimental animals included 4 male yaks (*Bos grunniens*) and 3 male cattle-yaks (hybrid of male cattle with female yak, showing F1 through F3 male sterility), 4 to 6 year-old. At a slaughterhouse, the testes and cauda epididymides of yaks and cattle-yaks were

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collected immediately after slaughter and cut into several parts, one portion was transferred to laboratory using dry ice and stored at –80°C for protein extraction, other portions were fixed in Bouin stationary liquid for histology study. This experiment was conducted according to the guidelines of Chinese government for the use of experimental animals including animal welfare and conditions.

Testis protein extraction: The testis aliquots of equal weights from yaks (4, control group) or cattle-yaks (3, experiment group) were pooled respectively before processing. Minced testis tissues were homogenized with electric homogenizer in 5-fold volumes of pre-cooled lysis buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 65 mmol/L dithiothreitol, 0.2% ampholyte and 1 mmol/L phenylmethylsulfonyl fluoride. The homogenates were then treated by ultrasonic in ice water for 2 min and incubated for 1 h, followed by centrifugation at 15,000 g and 4°C for 30 min. The supernatant was desalted with 4–fold volumes of ice-cold acetone containing 0.07% of ß-mercaptoethanol and precipitated overnight at –20°C, and the resulting pellet was washed with ice-cold acetone followed by additional centrifugation for 10 min. The pellet was dried for 1 h at room temperature. Lysis buffer was added to resuspend the proteins, and the protein content was determined with the modified Bradford method using BSA as a standard and stored at –80°C.

Two-dimensional electrophoresis: Two-dimensional gel electrophoresis (2-DE) was performed to compare the expression differences of testicular proteins between yaks and cattle-yaks. Isoelectric focusing electrophoresis (IEF) was conducted using an IEF cell system. 17-cm pH 3–10 nonlinear immobilized pH gradient (IPG) strips were rehydrated for 14 h at 20°C in a rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 65 mmol/L dithiothreitol, 0.2% ampholyte, 0.001% bromophenol blue, 300 µl for each IPG strip) containing 1,500 µg of testicular proteins. The strips were then focused at 20°C using the following voltage program: 250 V for 3 h (slow), 500 V for 1 h (slow); 1000 V for 1 h (slow); 5000 V for 3 h (rapid); 10000 V until a total of 60000 Vh. Each sample was analyzed in triplicate to ensure reproducibility and reliability of the quantitative change in protein expression.

After focusing, IPG strips were equilibrated with gentle shaking for 20 min in equilibration buffer (6 mol/L urea, 2% SDS, 0.375 mol/L Tris-HCl, pH 8.8, 20% glycerol) containing 2% dithiothreitol, followed by a further equilibration for 15 min with the same buffer but containing 2.5% iodoacetamide instead. The IPG strips were run in the second dimension on 13% polyacrylamide gels at 200 V for 5 h at 20°C using a protein II electrophoresis unit. Gels were stained by Coomassie brilliant blue R250 and stored in 7% acetic acid.

Protein identification: The gel images were captured with a gel imaging system and analyzed with PDQuest v7.0.1 software. Protein quantities were defined as the total optical density of the spots. The proteins with at least 2-fold expression differences between yaks and cattle-yaks were manually excised, followed by identification with mass spectrometry.

Histology comparison of testes and epididymis of yaks and cattle-yaks: The testes and cauda epididymis were fixed in Bouin's solution overnight, and then dehydrated and embedded in paraffin wax, finally sectioned at 4 µm. The sections were stained with hematoxylin-eosin (HE) using standard techniques and observed under a light microscope.

Statistical analysis: Normalized spot volumes of individual proteins were expressed as means±SD, Statistical significance was estimated by the unpaired student's t-test. The level of significance of differences was set at $P<0.05$.

RESULTS AND DISCUSSION

Two-dimensional electrophoresis of testicular proteins of yaks and cattle-yaks: Total proteins extracted from pooled testes of yaks or cattle-yaks were separated by 2-DE with good resolution and reproducibility (Fig. 1). A total of 837 and 805 spots that were consistently detected in 3 gels were revealed from yaks and cattle-yaks, respectively. Twenty protein spots that showed at least 2-fold expression changes between yaks and cattle-yaks (Fig. 2) and appeared in all 3 gels were analyzed by mass spectrometry. In addition, two protein spots (spots A11 and A21) showed similar expression levels were also assayed.

Identification of differentially expressed proteins in testes of yaks and cattle-yaks: Among the 22 protein spots assayed by mass spectrometry, 19 spots were identified successfully (Table 1), thirteen of which showed downregulation in cattle-yak testes (Fig. 1A; Table 2) and 4 proteins upregulated (Fig. 1B; Table 3). The identified proteins include 3 chaperones, a signaling molecule (calmodulin), several enzymes (such as ubiquitin-conjugating enzyme E2 N, L-asparagine amidohydrolase, glutathione S-transferase Mu 1, sorbitol dehydrogenase, and protein phosphatase methylesterase 1), and other proteins. Protein phosphatase methylesterase 1 were more than 8 times downregulated in the testes of cattle-yaks than yaks. The 2 constantly expressed proteins identified were apolipoprotein A-I and phosphatidylethanolamine binding protein 1.

Histology comparison of testes and epididymides of yaks

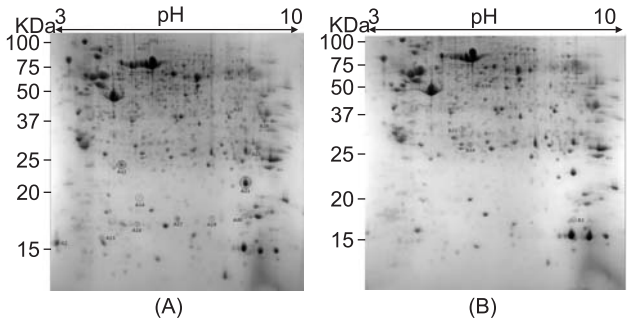

Fig. 1. Two-dimensional electrophoresis of total testis proteins of yaks (A) and cattle-yaks (B). 1500 µg of soluble testicular protein extracts were subject to two-dimensional electrophoresis. Gels were stained with Coomassie brilliant blue R250. The identified proteins were labeled.

Table 1. Identification of differentially expressed proteins in the testes of yaks and cattle-yaks

Spot	Protein ID	Mass (Da)	Isoelectric point	Score	Coverage rate (%)	Protein name
A2	gi|440906158|gb|ELR56459.1|	16695.79	3.84	230	34.46	Calmodulin
A17	ENSBTAP00000011403	23550.37	8.22	579	53.88	Peroxiredoxin-5, mitochondrial
A29	ENSBTAP00000016954	59036.66	6.72	523	61.94	T-complex protein 1 subunit eta
A40	gi|440909238|gb|ELR59169.1|	17600.62	8.76	269	55.90	Peptidyl-prolyl cis-trans isomerase
A23	gi|440909671|gb|ELR59556.1|	27494.1	6.94	421	66.24	Glutathione S-transferase Mu 1
A14	gi|440901470|gb|ELR52405.1|	17699.58	5.78	355	49.66	Ferritin
B22	ENSBTAP00000017991	30317.67	5.61	243	62.15	N(G), N(G)-dimethylarginine dimethylaminohydrolase 2
A11	ENSBTAP00000002914	17734.31	4.99	258	56.21	Apolipoprotein A-I
B19	ENSBTAP00000022763	71288.22	6.09	581	37.73	Serum albumin
A27	ENSBTAP00000035716	40878.98	7.71	294	55.73	Sorbitol dehydrogenase
B2	ENSP00000297258	15349.68	7.83	324	81.48	Epidermal-type fatty acid-binding protein
A34	ENSP00000381461	43760.83	5.81	231	40.25	Protein phosphatase methylesterase 1
A21	ENSBTAP00000024107	20829.57	8.61	402	70.97	Phosphatidylethanolamine-binding protein 1
B24	ENSBTAP00000022763	71288.22	6.09	454	27.51	Serum albumin
A26	ENSBTAP00000028757	35780.81	9.12	591	66.37	Uncharacterized protein
A38	ENSBTAP00000003662	60697.79	5.96	579	60.07	T-complex protein 1 subunit alpha
A16	ENSBTAP00000052296	17184	6.53	177	73.68	Ubiquitin-conjugating enzyme E2 N
A19	ENSBTAP00000009073	34695.5	7.08	408	28.53	L-asparagine amidohydrolase
A33	ENSBTAP00000023778	60128.02	5.43	291	43.49	Chaperonin containing TCP1, subunit 5

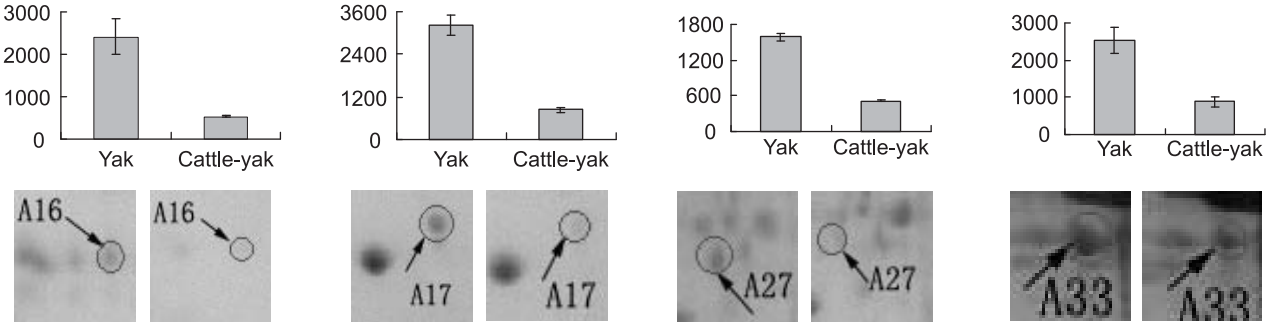

Fig. 2. Comparison of partial proteins differentially expressed in the testes of yaks and cattle-yaks. Panel A, four proteins expressed in yak testes; Panal B, corresponding 4 proteins with reduced expression levels in cattle-yak testes. The number corresponds to intensity of spots on 2-DE gel of Fig. 1.

Table 3. Expression of testis proteins upregulated in cattle-yaks compared with yaks

Spot	Protein definition	Normalized quantity		Fold change
		Yak testis	Cattle-yak testis	
B2	Epidermal-type fatty acid-binding protein	1036±353	2698±553	2.60
B24	Serum albumin	1011±57	3060±667	3.03
B19	Serum albumin	671±50	3392±320	5.05
B22	N(G), N(G)-dimethylarginine dimethylaminohydrolase 2	931±184	2811±401	3.02

The values show the mean±SD of the normalized quantity for each spot.

and cattle-yaks: Compared with normal adult yaks, no mature spermatozoa were present in the lumen of seminiferous tubules and cauda epdidymides of cattle-yaks (Fig. 3). Furthermore, many vacuoles were observed in testis sections of cattle-yaks, and epdimymides of cattle-yaks seemed not as developed as those of yaks. These results have provided direct evidences for the sterility of cattle-yaks.

Hybrid sterility might involve multiple and complex mechanisms. The genetic cause of male sterility of cattle-yak is still unclear. It is well known that spermatogenesis evokes thousands of genes and proteins, many of which are testis specific. Some genes including testis specific genes were reported to be downregulated in the testes of sterile male hybrid cattle-yaks compared to that of adult yaks (Zhang *et al.* 2009, Huang *et al.* 2012). The present study identified 19 differentially expressed testicular proteins, and most of which (13/19) were downregulated in cattle-yaks,

Table 2. Expression of testis proteins downregulated in cattle-yaks compared with yaks

Spot	Protein definition	Normalized quantity		Fold change
		Yak testis	Cattle-yak testis	
A2	Calmodulin	8981±838	3011±380	2.98
A16	Ubiquitin-conjugating enzyme E2 N	2419±418	536±30	4.51
A14	Ferritin	965±74	425±93	2.27
A17	Peroxiredoxin-5, mitochondrial	3221±304	833±57	3.87
A19	L-asparagine amidohydrolase	1200±41	418±52	2.87
A40	Peptidyl-prolyl cis-trans isomerase	1052±100	325±88	3.24
A23	Glutathione S-transferase Mu 1	3224±219	1368±92	2.36
A26	Uncharacterized protein	1584±12	738±39	2.15
A27	Sorbitol dehydrogenase	1586±57	515±12	3.08
A29	T-complex protein 1 subunit eta	1546±109	540±67	2.86
A34	Protein phosphatase methylesterase 1	716±29	87±16	8.26
A33	Chaperonin containing TCP1, subunit 5	2543±353	884±144	2.88
A38	T-complex protein 1 subunit alpha	1868±102	710±75	2.63

The values show the mean±SD of the normalized quantity for each spot.

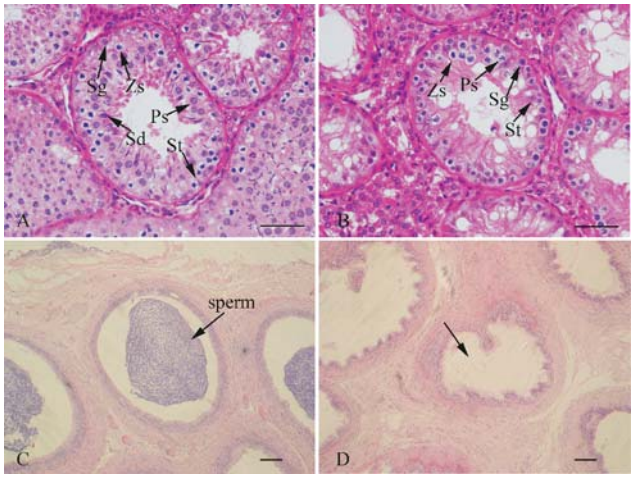

Fig. 3. Sections of HE-stained testis and epididymis of yak and cattle-yak. A, Testis section of adult yak. The seminiferous tubules of normal yak contain multiple germ cells: Sertoli cells (St), spermatogonia (Sg), primary spermatocytes including zygotened spermatocytes (Zs) and pachytene spermatocytes (Ps), secondary spermatocytesÿspermatids (Sd); B, Testis section of cattle-yak. Some seminiferous tubules of cattle-yak contain meiotic-arrested spermatocytes (Ps), Sertoli cells (St), spermatogonia (Sg); C, Section of yak epididymis (sperm, arrow); D, Section of cattle-yak epididymis (no sperm, arrow). Scale bars: 50 μm for A and B, 25μm for C and D.

indicating that the male sterility of cattle-yaks might be associated with multiple proteins downregulated.

Among the 13 testicular proteins downregulated in cattle-yaks, 2 chaperones were revealed. T-complex protein 1 (TCP1) is known to play a role in the folding of actin and tubulin, it can also regulate the process of sperm and egg fusion (Dun *et al.* 2011); Peptidyl-prolyl *cis-trans* isomerase (PPI), which is a widely expressed enzyme in various tissues, catalyzes the peptidyl-prolyl *cis-trans* isomerization and participates in protein folding, signal transduction, trafficking and cell cycle regulation (Göthel and Marahiel 1999). Both proteins have chaperone activity and are downregulated in cattle-yak testes, and further investigation is required to elucidate their association with the male sterility of cattle-yaks. Interestingly, our previous study also identified 6 chaperones (also include TCP1 and PPI) from total protein extracts of adult yak testes, and these chaperones showed decreased expression in the testis of a sterile bisexual yak (our unpublished data). Therefore, we speculate that chaperones are of significance for normal testis functions. High levels of chaperones might involve in the folding of testicular proteins during active spermatogenesis.

Protein phosphatase methylesterase-1 (PME-1) was dramatically decreased in the testes of cattle-yaks than adult yaks (Table 2). This enzyme catalyzes the demethylation and inactivation of protein phosphatase (PP2A), which is a phosphoserine / threonine protein phosphatase associated with growth inhibition and cell cycle arrest. Failure to inhibit PP2A causes arrest of the cell cycle in G2 phase (Hunt 2013). PP2A can mediate the fine-tuning of phosphorylation during cell growth (Shimada and Nakanishi 2013). PP2A Cdc55 is required for the proper temporal initiation of multiple meiotic events (Nolt *et al*. 2011) and also essential for meiotic cells to attain levels of Cdk activity required for bipolar spindle assembly and for preventing premature exit from meiosis I (Kerr *et al.* 2011). We suggest that the decreased PME-1 level could influence the normal spermatogenesis in cattle-yaks through PP2A; Ferritin can store iron and is highly expressed in mouse testis and other cell types characterized by high metabolic activity and oxygen consumption (Santambrogio *et al*. 2007); Ferritin secretion from Sertoli cells may play an important role in iron acquisition of primary spermatocytes (Leichtmann-Bardoogo *et al*. 2012); Sorbitol dehydrogenase (SDH) participates in the polyol pathway of glucose metabolism and has high expression level in testes and epididymis (Lee *et al*. 1995, Frenette *et al*. 2004); Calmodulin controls a variety of cellular processes mostly related to calcium signaling and stabilized the spindle pole body (SPB) during

meiosis and sporulation in fission yeast (Itadani *et al*. 2010). The abnormal expression of these proteins in cattle-yak testes might be associated with the male sterility of cattle-yaks.

An upregulated testicular protein, N (G), N (G)-dimethylarginine dimethylaminohydrolase (DDAH), was observed in cattle-yaks, this protein has two types (DDAH1 and DDAH2) in animals, DDAH2 is widely distributed in testis and epididymis, it hydrolyzes N (G), N(G)-dimethyl-L-arginine (ADMA) and N(G)-monomethyl-L-arginine (MMA) which act as inhibitors of nitric oxide synthase, and has therefore a role in the regulation of nitric oxide generation (Achan *et al.* 2003). The excess accumulation of MMA and ADMA may regulate nitric oxide production (Leiper and Vallance 2003). Low concentration of nitric oxide could promote the secretion of testosterone, while high concentration of nitric oxide has opposite effect (Valenti *et al.* 1999). It was also reported that excessive nitric oxide decreased sperm viability and survival rate (Rosselli *et al*. 1995). We speculate that the increased expression of DDAH in cattle-yak testes may cause the boost of NOS and hence increase the production of nitric oxide, which can affect normal spermatogenesis.

In this study, we also identified two constantly expressed proteins. Apolipoprotein A-I is the major protein of plasma high density lipoprotein and plays an important role in lipid transport and metabolism, it is the component of sperm activating protein complex (SPAP) regulating sperm capacitation (Akerlöf *et al.* 1991), and also binds bovine sperm albumin specifically (Manjunath *et al*. 1989); Phosphatidylethanolamine-binding protein 1 (PEBP1) is a highly conserved basic protein with diverse biological functions. PEBP1 was reported to be located in mouse spermatozoa and involved in sperm capacitation (Gibbons *et al.* 2005). These results show that not all proteins involved in spermatogenesis or capacitation are changed in the testes of sterile cattle-yaks.

In conclusion, this experiment identified 19 differentially expressed proteins in the testes of normal yaks and sterile cattle-yaks, and most of which were down regulated in cattle-yak testes. The results of this study suggest that the male sterility of cattle-yaks might be associated with many proteins, and proteins such as chaperones and some testis-specific proteins may play crucial roles.

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