Regulation by HSP70/90 in the different tissues and testis

development of male cattle (Cattle-yak and Yak)

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Abstract

HSP70/90 play important role in testis develop and spermatozoa regulation, but the contact of HSP70/90 with infertility in cattle is unclear. Here, we focus on male cattle-yak and yak, which to investigate the expression and localization of HSP70/90 in different tissues, and explore the influence of HSP70/90 to infertility. In our study, a total of 54 cattle (24 cattle-yak and 30 yak) were examined. The HSP90 mRNA of cattle-yak was cloned first and found amino acid variation in HSP90, which led to difference at protein spatial structure compare with yak. To investigate whether the expression of HSP70/90 mRNA and protein are different in cattle-yak and yak, we used real-time quantitative PCR (qRT-PCR) and Western blot (WB) to examine them. We found that the expression level of HSP70/90 mRNA and protein are disparity in different tissues and testis development stages, and obviously high

28 expression was observed in testicle during juvenile and adult, Moreover,
 29 it's interestingly in which the HSP70 expression is significant high in yak
 30 whereas HSP90 in cattle-yak ($P<0.01$). On this bases, we detect the
 31 location of HSP70/90 in testis by immunohistochemical (IHC) and
 32 immunofluorescence (IF), the results demonstrate that HSP70/90 were
 33 located in the epithelial cells, spermatogenic cells and mesenchymal cells.
 34 In summary, our study proved the expression of HSP70/90 are different in
 35 tissues, and the expression of HSP90 is obviously high in testis of
 36 cattle-yak, which propose that the infertility of cattle-yak may cause from
 37 up-regulating of HSP90.

38

39 **Key words :** HSP70/90, expression, localization, regulation, yak

40

41 **Introduction**

42 Cattle-yak (*Bos cattle-yak*) and yak (*Bos grunniens*) are important
 43 animals for the local herdsman in the Qinghai-Tibet Plateau in China.
 44 Cattle-yak as a hybrid exhibit obvious heterosis of yak and cattle, such as
 45 tall variety, robustness, fast growth speed, drought tolerance, and disease
 46 resistance (Wiener et al. 2003). Very strong resistance to cold and high
 47 altitude is necessary for this species to adapt to the cold environment.
 48 Heat shock protein 70/90(HSP70/90) plays key roles in protecting the
 49 body to avoid irritation. In this study, we determined the relationship

50 between HSP70/90 and tissue specificities of cattle and the regulation of
51 testicular development and sperm production.

52 Recent studies have shown that HSP70/90 could protect cells
53 from damage, organ failure, and body shock, among others. HSP70/90
54 not only directly or indirectly participates in the tumorigenesis,
55 autoimmunity, wound healing, cell migration and transshipment for
56 growth and development but also protects reproductive cell activity and
57 regulates protein metabolism of organisms (Carper et al. 1987). Previous
58 studies demonstrated high concentrations of HSPs in developing tissues
59 and tumor cells, such as in stomach cancer, colon cancer, liver cancer,
60 breast cancer, and lung cancer (Manjili et al. 2002; Weng et al. (2014)
61 showed that the HSPA2 expression levels significantly differed in the
62 various tissues of male yak, especially in the testes, followed by the brain,
63 kidney, heart, lung, and liver, and its weakest expression was found in the
64 spleen (Weng et al. 2014). That showed HSP70/90 play important role in
65 tissue specificities of female yaks as well as lactating function (Liu et al.
66 2017). This study was performed to investigate whether HSP70/90 was
67 differentially expressed in the various tissues of cattle-yak and yak.

68 The testis is one of the most important male reproductive organs,
69 and the place of its cell proliferation and development is important. Many
70 factors are responsible for the infertility of male cattle-yak. These factors
71 include testicular dysplasia, hindered spermatogenesis, metamorphosis

72 phase, and sustentacular cells assisting the germ cell differentiation in the
73 seminiferous tubule (Lu and Zi 2014). Thus, studies on the fertility of
74 male cattle-yak and male yak have focused on distinguishing histologic
75 structural development, the effect of gene expression genes, and the
76 intensity of protein involved in the production and control of sperms
77 (Shao et al. 2016).

78 Cattle-yak infertility is not only reflected histologically, but studies
79 also showed that the differential expression of related gene and protein
80 generated by sperms is another factor for infertility. HSP is mainly
81 involved in biological processes, such as sperm movement, energy
82 metabolism, protein processing, and oxidative stress. Asthenozoospermia
83 creatures have low content of eight differential proteins containing
84 triosephosphate isomerase, glycerol kinase, and HSP (Siva et al. 2010;
85 Sönmez et al. 2017). HSP70 plays an important role in sperm meiosis,
86 sperm maturation, and sperm-egg recognition. In the sperm of human
87 beings, the expression of HSPA2 in the elongated sperm and the sperm
88 cells is significantly higher than that of the primary spermatocyte (Motiei
89 et al. 2013). In addition, temperature plays an important role in
90 spermatogenesis. Hyperthermy induce cells to express HSP, which
91 improves heat tolerance, protects protein structure from injuries, and
92 repairs the injury of the protein structure and functional integrity. High
93 temperature triggers cell apoptosis and causes the redistribution of HSP in

the cells. For example, the cytoplasm moves to the nucleus and nuclear membrane to protect cells from damage. Different degrees of damage result in diverse consequences. Several damages stop cell proliferation and activate cell repair mechanisms simultaneously, while some damages directly initiate cell death (Chen et al. 2013). Heating the testis for 10 min at 43 °C does not evidently change the morphology of the seminiferous tubule. Spermatocyte and circular sperm cells are sensitive to heat shock, and apoptosis occurs with heating (LI et al. 2016). Sudden change in temperature during animal breeding stage can cause HSP60/70/90 to rapidly respond and become highly expressed in the gene level to improve the ability to protect gap-associated protein from disturbance (Mikulski et al. 2011). However, it is still a blank that the correlation between male sterile and HSP70/90 of mules and cattle-yak. Therefore, this study will provide theoretical basis for hybrid sterility.

Results

1. Analysis physical and chemical properties of HSP90

The cDNA sequence of HSP90 was cloned and submitted to Genbank with accession number KF690731.

The nucleotide sequence and predicted amino acid sequence of HSP90 were presented. HSP90 nucleic acids were 3064 bp long. Results from the analysis of the contig showed that the predicted HSP90 cDNA of 3064 bp contained an ORF of 2202 bp from 146 bp to 2347 bp. The small

116 three ORFs (ORF1, ORF2, and ORF3) coded for a nonfunctional protein
 117 was composed of 123 and 171 amino acids. Analysis of the HSP90 cDNA
 118 sequence in our study confirmed this conclusion. To verify this result,
 119 three primers H90-1, H90-2, and H90-3 were used to clone the full-length
 120 ORF of HSP90. RT-PCR results show the successful isolation of a cDNA
 121 fragment of 2202 bp from the cattle-yak total RNA of the heart. Finally,
 122 this confirmed cDNA sequence was deposited in the GenBank under
 123 accession number KF690731. To obtain the genomic DNA of HSP90, the
 124 publicly available cow genome database at the NCBI Bovine Genome
 125 Resources (<http://www.ncbi.nlm.nih.gov/projects/genome/guide/cow/>)
 126 was screened using the HSP90 cDNA sequence as a query. A cow (*Bos*
 127 *taurus*) contig (GenBank Accession No. NM_001012670) which
 128 encompasses the entire HSP90 gene was identified by BLASTGen
 129 analysis.

130 The basic physical and chemical properties of HSP90 were
 131 analyzed. The atomic number of the protein coding region is 11710.
 132 HSP90 has a molecular formula of $C_{3733}H_{5950}N_{986}O_{1202}S_{27}$, molecular
 133 weight of 847408.84 Da, and theoretical isoelectric point of 4.841.
 134 HSP90 has a half-life of approximately 30 h, instability index of 42.77,
 135 fat soluble index of 79.80, and average hydrophobicity index of -0.744.

136 A molecular phylogenetic tree was constructed to analyze the
 137 evolutionary relationship of HSP90 nucleotide sequences (**Fig.1**).

138 Analysis of the gene family tree showed that the cattle-yak HSP90
139 evolutionarily shared a higher sequence identity with *B. grunniens*, *B.*
140 *taurus*, *Capra hircus*, *Ailuropoda melanoleuca*, *Mustela putorius furo*,
141 *Sorex araneus*, *Equus caballus*, and *Cavia porcellus*.

142 **2. Analysis structure specificity with proteins of HSP90**

143 The predicted amino acid sequence of HSP90 was aligned with
144 known *B. grunniens* sequences through BLASTp. The cattle-yak HSP90
145 protein sequence shared a low percentage of similarity to other known
146 HSP90 protein sequences. This result indicated that the HSP90 proteins
147 were different from other members in the HSP family. The amino acid
148 sequence of HSP90 was approximately 99.56%, 99.33%, 96.91%,
149 87.83%, and 86.90% identical to those of HSPs from *B. grunniens*, *B.*
150 *taurus*, *C. hircus*, *E. caballus*, and *Mus musculus*. The highest level of
151 similarity appeared near the C-terminal, while the very low similarity was
152 found in the N-terminal and in the middle of the amino acid sequence.

153 The cattle-yak HSP90 protein sequence showed that the mutational
154 nucleotide caused the change of amino acids, such as the GLY into GLU,
155 FGI into LEF, GLN into ARG, and MET into ILE (**Fig. 2**). The most
156 important result is the change in the protein spatial structure and
157 differences in protein function, such as the H-key number (397), spiral
158 number (30), link number (23) and corner number(89). In addition,
159 compare with yak, the initiating terminal and terminal of the cattle-yak

are longer. The results from the SDS-PAGE analysis indicated that the fusion protein HSP70 has a molecular weight of approximately 70 kDa, whereas the fusion protein HSP90 was approximately 84.7 kDa.

3. Expression and distribution of HSP70/90 in the non-reproductive system

The HSP70/90 mRNA in the tissues of the cattle-yak and yak were investigated through real-time RT-PCR (**Fig. 3-I**), with the total RNA isolated from cattle-yak and yak tissues as a template. As shown in Fig. 3-II, we examined six tissues in cattle-yak and yak, the expression of HSP70/90 are widely distributed in all of them. According from our data, HSP70/90 are both reduce in cattle-yak, the trend from lung, cerebellum, kidney, liver, heart to spleen. There is no significant difference about HSP70 in these tissue, but interestingly the expression levels of HSP90 were consistently higher than HSP70 in almost all the tested tissues ($P<0.01$). At the meantime, although the expression of HSP70/90 are both higher in cerebellum, kidney and heart in yak, the expression of HSP70/90 in different tissues and level are irregular. Taken together our data suggest that the expression of HSP90 was consistently higher than HSP70 in all the tested tissues ($P<0.01$) in cattle-yak but irregular in yak.

The HSP70/90 was mainly observed in the kidney tubules, cardiac muscle cells, hepatocytes, Purkinje cells, and cerebellar medulla (**Fig. 4-I**

181 **and II**). Positioning analysis indicated that the HSP70/90 protein was
182 mainly concentrated in the connective tissue and epithelia. Thus, the
183 proteins were mainly concentrated in the cell membrane and in the
184 cytoplasm but not in the nucleus. Furthermore, the protein expression
185 levels of HSP90 were markedly lower than those of HSP70 in almost all
186 the tested tissues ($P < 0.01$), except in the lung (**Fig. 4-III**).

187 **4. Expression and distribution of HSP70/90 at the different** 188 **development stages of the testis tissue**

189 As we found the difference of HSP70/90 between cattle-yak and yak
190 in six non-reproductive tissue above, we examined testis further. As
191 shown in **Fig. 3-III**, HSP70/90 gene expression levels showed significant
192 differences in the cattle-yak and yak testicular tissue at different
193 development stages ($P < 0.01$). In cattle-yak, the expression of HSP70
194 showed obviously tend from newborn to adult, it increased to highest in
195 juvenile and then tend to decreased, at the same time, HSP90 keep
196 increasing until adult. It is worth notice that the expression of HSP90 was
197 significant higher than HSP70 in every develop stage of testis. Whereas
198 in yak, the expression HSP70 reach top at adult and HSP90 at juvenile,
199 different with cattle-yak, it's HSP70 keep higher during every develop
200 stage of testis (**Fig. 3-III**).

201 As shown in Fig 5, the protein expression levels of HSP70/90 were
202 detected in the testicular tissues at different development stages of

203 cattle-yak and yak. The results showed that the protein expression levels
204 of HSP90 were significantly higher than those of HSP70 in almost all the
205 developmental stages of cattle-yak ($P < 0.01$). By contrast, the protein
206 expression levels of HSP70 were significantly higher than those of
207 HSP90 in almost all the developmental stages of yak ($P < 0.01$), except in
208 the newborn (**Fig. 5-II, Fig. 6-III**).

209 The testicular seminiferous tubule of the newborn cattle-yak and
210 yak was thin and sparse (**Fig. 6-I and 6-II**). As the cattle-yak aged, the
211 number of spermatogenic cell at the different development stages
212 decreased, and few sperm and sperm cells appeared. HSP90 was highly
213 expressed in all levels of spermatocyte in the cattle-yak testicular tissue,
214 whereas the expression levels in the basement-membrane and myogenic
215 cells were the weakest. By contrast, the HSP70 expression levels in
216 spermatocyte were the weakest. We are interested in what was showed, as
217 the organism developed, seminiferous tubules became closely packed,
218 luminals became enlargement, and different amounts of sperm cells
219 appeared during the development of sperms, with the largest count
220 occurring during the adult stage. For the elderly yak, the amount of
221 spermatogenic cells in the seminiferous tubule decreased significantly.
222 The HSP90 expression was strongest in the yak testis in primary
223 spermatocyte, followed by that in the secondary spermatocyte. Sperm
224 cells also had expression, contrary to the spermatogonium cells. The

HSP70 expression levels in the mesenchymal cells were strong, while the expression levels in the basement-membrane and myogenic cells were the weakest.

Discussion

This study is the first to isolate, sequence, and characterize cDNA clones that encode HSP90 from cattle-yak. Previous studies found that the protein space structure difference leads to function different of HSP90 (Agarwal et al. 2016). Using our reference sequence, our analytical results showed that three ORFs in the HSP90 sequence illustrated the frame-shifting property of the sequence. We also present evidence that susceptibility of trees to cattle-yak HSP90 associated with *yak*, *taurus*, *goats* were very high, and that cattle-yak evolution with the above animals had very close genetic relationship. In eukaryotic cells and the different HSP gene promoters upstream of TATA, heat shock element (HSE) with size of approximately 20 bp was also necessary in the transcription of specific nucleotide sequence (C-GAA-TTC-G) (Pelham and Bienz. 1982; Jenkins et al. 2016). The amino acid sequence of the various protein spatial structures differed. In humans, HSP90 vary according to their richness in glutamine (Yeyati and Heyningen. 2008). Results showed (Fig 2) that the cattle-yak amino acids happened multipoint mutation relative to yak. So, the molecular data show that HSP90 gene could be used as a single nucleotide polymorphism marker

247 to evaluate the loss of genetic diversity caused by hatchery selection or
248 internal control gene in cattle-yak. Thus, we believed that the amino acid
249 mutation is an important factor for functional differences of HSP90.

250 The gene and protein expression of HSP70/90 was significant not
251 only in the different species but also in the different tissues of the same
252 species. Interestingly, compared with the males, the females had
253 significantly lower post-exercise HSP72 levels which were not influenced
254 by estradiol supplementation. This characteristic is mainly displayed in
255 the heart, liver, lung and red and white vastus muscle (Paroo et al. 1999).
256 In addition, HSPs under cold stress induced oxidative stress in spleen and
257 influenced immune function in chicks (Zhao et al. 2014). It was hard to
258 believe these data also verify the controversies regarding the appropriate
259 physiological function of different tissues. We found that gene and
260 protein expression of HSP70 was higher in the kidney, heart, and
261 cerebellum of cattle. However, HSP90 had higher expression in the lung,
262 cerebellum, and liver of cattle. Studies have shown that the apoptosis
263 were reduced when HSP90 expression by inhibition (Hu et al. 2018).
264 Luckily for us, considering the high expression in the cerebellum and
265 heart of cattle-yak and yak, we speculated that HSP70/90 could protect
266 cells and tissue from the environmental stimulus.

267 Previous studies demonstrated that HSP90 can protect lung tissue
268 from damage caused by cold air. Different stimuli could induce HSPs in

the proximal with significant differential expression in the bronchioles and respiratory bronchiole epithelial cells and smooth muscle cells (Manjili et al. 2002; Li et al. 2015). Gene and protein expression levels of HSP70/90 were significantly upregulated when the porcine fetal fibroblast and oviduct epithelial cells were stimulated by cold (LI et al. 2012). Studies have shown that HSP70/90 played a regulatory role in the adaptation of japonicus to high temperature and low salinity (Wang et al. 2014). This study demonstrated the localization of HSP70/90 in the cell membrane and cytoplasm, not in the nuclei, of organs. It is undeniable that, HSP70/90 plays an active function in the tissue physiological adaptation in cattle-yak and yak.

We performed studies to examine which expression was altered by gene and protein in cattle-yak to elucidate the mechanism by which HSP70/90 affect the development of testicular tissue. Encouragingly, HSP90 expression was generally higher than that of HSP70 in the cattle-yak testicular tissue, especially during childhood, juvenile, and adulthood. Spinaci reported that the expression level of HSP90 was gradually reduced when the pig sperm was frozen, and the protein content was reduced before sperm quality declined (Spinaci et al. 2016). Meanwhile, the expression in the cattle-yak remained at a higher level from juvenile and adulthood. This result is consistent with the results obtained by Abd El-Fatta, who showed that the high expression of HSP90

291 could inhibit testicular dysfunction caused by DEHP (El-Fattah et al.
292 2016). For the male cattle-yak infertility, the expression of HSFY2 was
293 delayed, not absent. The fertility of cattle-yak was restored, as the HSFY2
294 was expressed (Chai et al. 2014; Sarge and Cullen. 1997). In addition, the
295 HSP70/90 lower expression led sperm quality to low of boar (Zannoni et
296 al. 2017). Hsp70 expression may have been upregulated as a protective
297 mechanism against apoptosis in spermatozoa of infertile men (Erata et al.
298 2008). Thus, as the Fig 7, we speculated that the high expression of
299 HSP90 in the testicular tissue of male cattle activate offspring male
300 cattle-yak to restore normal fertility.

301 We analyzed the HSP70/90 expression in the testicular tissue during
302 the different stages of yak development to establish whether the
303 HSP70/90 content has positive correlations with the reproductive capacity
304 of yak. The results indicated that the highest HSP70-2 gene expression
305 level was in the yak testicular tissue, followed by those of the cattle and
306 cattle-yak (Yl et al. 2016). In addition, HSP 70 could protect against
307 sperm apoptosis when received the stimulation by high temperature
308 (Mann et al. 2002). This result is largely consistent with our experimental
309 result that the HSP70 expression level in the testicular tissue of yak
310 increased gradually with age, and the strongest expression was during the
311 adult stage, then gradually decreased in old age. Dix reported that mutant
312 HSP70-2 could inhibit the coupling of nuclear chromosome coming into

313 meiosis (Dix et al. 1996). Furthermore, the HSP90 firstly increases
314 and then decrease at testicular development process of rabbit (Wu et al.
315 2011). What makes this amusing, as the Fig 7, we speculated that the low
316 expression of HSP90 and high expression of HSP70 were activated the
317 normal reproduction of yak.

318 The immune response of the HSP family protein was mainly
319 concentrated in the epithelial tissue and cell serous. Cedenho found that
320 HSP70-2 was expressed in the normal testis and testicular tissue
321 with disturbance of sperm maturation (Cedenho et al. 2006). However,
322 the expression was higher in the normal testicular tissue and
323 spermatoblast serous, lower in the testicular tissue and spermatoblast
324 serous with disturbance of sperm maturation. No expression was found in
325 the testicular tissue of sustentacular cell syndrome patients. Economou
326 proved stage and cell-specific expression and intracellular localization of
327 the small Hsp27 during oogenesis and spermatogenesis in the
328 Mediterranean fruit fly (Economou et al. 2017). The expression was
329 strongest in the yak testis in the primary spermatocyte, followed by the
330 secondary spermatocyte. Sperm cells also showed expression, contrary to
331 spermatogonium cells. Thus, we demonstrated that the low expression of
332 HSP70 was related to male sterility. HSP90 was highly expressed in all
333 levels of spermatocyte of cattle-yak testicular tissue, especially in the
334 mesenchymal cells. In 2003, Cardozo declared the evident increase in the

intensity of immune response in the testicular tissue with disturbance of sperm production, with the most obvious effect in the myoid cells (Cardozo et al. 2003). In addition, the lack of sperm caused infertility and hastened spermatocyte apoptosis in the spermatogenic epithelium. Even more remarkably, the mule, a classic example of hybrid sterility in mammals also exhibits a similar spermatogenesis breakdown, its failure at the first meiotic stage (Steiner and Ryder. 2013). Thus, the high expression of HSP90 caused the blockage of spermatocyte development. Is most proud of is, this study showed an important breakthrough in the study of male infertility.

Conclusions

The following conclusions can be drawn from this study. The amino acids were changed that the main cause for protein functional differences of HSP90. HSP70/90 has an obvious differential expression in different organs and differences age paragraph testicles of cattle-yak and yak. In addition, HSP70/90 was mainly detected in epithelial of tissue, spermatogenic cell and mesenchymal in the testicular. At the core, HSP70/90 showed the specific expression in yak and cattle-yak testicular tissue, and it has important correlation with cattle-yak infertility.

Materials and Methods

1. Experimental animals

Cattle were sampled from the pasture in the Tibetan plateau in

357 Qinghai, China (Gu et al. 2015). The study was approved by the Animal
358 Ethics Committee of Gansu Agricultural University. All experiments were
359 performed in accordance with relevant guidelines and regulations. A total
360 of 54 healthy cattle (24 cattle-yak and 30 yaks) were included in this
361 study (Table 1), All cattle were kept under the same natural conditions
362 (altitude, approximately 2,300 m; temperature, 2–5 °C; and oxygen
363 content, 14.97%) (Wiener et al. 2003). Male cattle-yak without feeding
364 value for herders were excluded, so no samples for the senile group were
365 included. The cattle with conventional disease were considered healthy
366 individuals.

367 **2. Preparation of mRNA and proteins**

368 Tissue samples were selected from the healthy cattle subjects. A
369 total of 21 tissues were dissected, collected in frozen liquid nitrogen, and
370 then stored at –80 °C before use. The RNA from the cattle tissues were
371 isolated using a TRIzol kit (R1100, USA), and reverse-transcription
372 polymerase chain reaction (RT-PCR) was conducted to clone the cDNA.

373 Frozen samples were ground in liquid nitrogen and transferred to
374 centrifuge tubes with RIPA/PMSF (Solarbio, China). After fully blending
375 and mixing until a pink color was obtained, the sample tubes were
376 incubated on a spiral oscillator for 2 h (200 r/h) on ice. After centrifuging
377 at 4 °C for 10 min (12000 r/h), the complete divided protein was obtained.
378 In addition, the total protein concentration for each sample was measured.

379 The total protein concentration was then adjusted to the same level, and
380 4× sample buffer at 100 °C was added for 12 mins to completely denature
381 the proteins.

382 **3. Molecular cloning of HSP90**

383 The degenerate primers used to amplify the HSP90 sequence were
384 based on the published partial sequence of HSP90 mRNA in the National
385 Center for Biotechnology Information (NCBI) database (Accession No.
386 NM-001012670.2). Primers for cloning the initial fragment of HSP90
387 mRNA were designed according to the predicted conserved sequences in
388 other *Bos* cattle-yak animals (Table 2). The amplified segments were
389 inserted into the cloning vector pMD-18T and were transfected into
390 *Escherichia coli* JM109 competent cells. The primers for 5' HSP90 and 3'
391 HSP90 were designed using the sequencing data. Then, the segments
392 from 5' to 3' HSP90 from the first-strand cDNA were used for cloning
393 and sequencing.

394 **4. Analysis of HSP90 protein**

395 The open reading frames (ORF) in the complete mRNA sequence of
396 HSP90 were identified using an ORF finder
397 (<http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi>), and then the
398 nucleotide sequences were translated into amino acids using the Vector
399 NTI 11 software (He et al. 2016). The HSP90 sequence was analyzed
400 using the ODC HSP90 finder software, and the codon region and

frameshifting site were identified (Gandre et al. 2003).

Homology searches were performed using BLASTn and BLASTp in NCBI. The Conserved Domain (CD) Search service was used to identify the CDs in the predicted protein sequences (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>). The 3-D structure of the predicted protein was predicted according to the methods described in the website (<http://bioinf.cs.ucl.ac.uk/psipred/>). The deduced amino acid sequence of HSP90 was aligned using the CLC main workbench software (<http://www.clcbio.com>) with known homologous proteins of the HSP90 class obtained from the GenBank. A phylogenetic tree was constructed using the CLC main workbench software through the neighbor-joining method for the amino acid sequences of HSP90 from the SwissProt databank/Genbank.

5. Expression of HSP70/90 gene in the different tissues

The distribution of HSP70/90 in the tissues of cattle-yak and yaks were detected during the development of testicles and in the non-reproductive system (kidney, heart, cerebellum, liver, lung, and spleen). The expression levels of HSP70/90 in the different tissues were detected using quantitative real-time PCR (Invitrogen, USA) with HSP70/90 specific primers. Actin was used as a reference gene to normalize the amount and quality of each cDNA, because this gene is expressed constitutively in the different tissues (Gandre et al. 2003).

423 **6. Protein expression of HSP70/90 in testicle tissues**

424 Protein samples was thawed, mixed and separated into beads using a
425 spin column (Bio-Rad), and then separated on a 5% SDS-PAGE gel for
426 Western blot analysis. After electrophoresis, the proteins were transferred
427 from the gel onto NC membranes (Millipore Corporation, Billerica, MA,
428 USA). The membranes containing protein were blocked with 5% fat-free
429 milk in TBST at room temperature for 2 h and hybridized using
430 HSP70/90 Abcam (1:1000) or rabbit polyclonal Abcam (1:2000) at 4 °C
431 overnight. The membrane was then washed four times with TBST and
432 labeled with HRP-conjugated secondary Ab (1:4000) for 2 h at room
433 temperature. After washing five times with 1× TBST, HSP70/90 was
434 detected on the membrane with an ECL detection kit (Beyotime, China).
435 The intensities of the bands on the blots were measured using a
436 densitometric analysis system (Bio-Rad). The intensities of the β -actin
437 bands were used for normalization (Hu et al. 2016).

438 **7. Immunofluorescence and Immunohistochemical Assays**

439 The main organs and tissues of the cattle-yak and yak were fixed in
440 4% paraformaldehyde solution at room temperature for hebdomad. Tissue
441 pieces were clipped and paraffin-embedded, and the sections were sliced,
442 dried, and stored.

443 Samples were dewaxed using dimethyl benzene and then dehydrated
444 with an increasing alcohol gradient for immunohistochemical staining to

investigate the HSP70 and HSP90 expression levels. The endogenous peroxidase was eliminated with 3% deionized H₂O₂ (18–22 min), and the sections were rehydrated and sealed with goat serum (18–22 min). After overnight incubation at 4 °C with the primary rabbit anti-HSP70 and mouse anti-HSP90 monoclonal antibodies (1:300, Abcam, Hong Kong), the sections were then incubated with the secondary antibody. Then, the labeled samples were counterstained with 3,3'-diaminobenzidine, and the nuclei were appeared (Gu et al. 2015). In addition, two antibodies showed different colors in immunofluorescence, and redyeing the nuclei was not necessary.

8. Measurement and statistical analyses

The intensity in the Western blot images and immunofluorescence and immunohistochemical assays were measured using integrated optical density and Image-Pro plus 6.0. Data were analyzed using SPSS 21.0. Spearman correlation of coefficients was analyzed between β -actin and sample protein levels. Other data were analyzed by one-way ANOVA and Duncan's post hoc test. P-values less than 0.05 between groups were considered statistically significant (He et al. 2016).

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472 **Competing interests**

473 The authors have declared that no competing financial interest.

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- 599

600 **Figure legends**

601 **Fig1. The HSP90 phylogenetic tree of cattle-yak.**

602 The phylogenetic tree was constructed with CLC main workbench software using the
603 neighbor-joining method for the amino acid sequences of HSP90 from the Swiss-Prot
604 databank/GenBank.

605

606 **Fig2. Analysis of HSP90 protein structure of cattle-yak and yak.**

607 land. The HSP90 nucleotide and amino acid sequence analysis that the sites and
608 types of mutation in cattle-yak and yak.

609 III. The HSP90 protein structure of cattle-yak. (A) The initiating terminal of the
610 cattle-yak is much more than that of yaks, as follows: MET, PRO, GLU, GLU, THR,
611 GLN, ALA, GLN, ASP, PRO, PRO. (B) (D), (E) Mutation site and amino acids are as
612 follows: 25(GLU), 36(THR), 37(PHE), 38(TYR), 347(ARG), 595(ILE). (C) More
613 than 92 amino acids at the terminal.

614 IV. The HSP90 protein structure of yak. (A), (B), (C), (D) Mutation site and amino
615 acids are as follows: 14(GLY), 25(THR), 26(PHE), 27(TYR), 336(GLN), 584(MET).

616

617 **Fig 3. The gene expression in different tissues of cattle-yak and yak.**

618 □. The results of RT-PCR. (1) Lung; (2) Cerebellum; (3) Kidney; (4) Liver; (5) Heart;
619 (6) Spleen. (A) Newborn; (B) Childhood; (C) Juvenile; (D) Adult; and (E) Senile.

620 II. The gene expression levels of HSP70 and HSP90 in non-reproductive system
621 (cattle-yak and yak)

622 III. The gene expression levels of HSP70 and HSP90 in testicular tissue of male cattle

623 (cattle-yak and yak).

624 Note. The curved lines represent the expression trend of HSP70/90 protein in different
625 organizations and testis development stages. Different colors represent different
626 expressions of HSP70/90 in the organizations and testicular tissue of cattle-yak and
627 yak.

628

629 **Fig4. IHC and IF localization of HSP70/90 in the non-reproductive system.**

630 □. (A) The control sections collected from the kidneys of yak, without
631 immunoreactions (negative control). (B), (D) Positive staining for HSP90 was
632 observed in the terminal bronchioles, distal convoluted tubule and proximal
633 convoluted tubule of the lungs and kidney. (C), (E) Positive staining for HSP70 was
634 observed in the cardiac muscle fibers and red pulp of heart and spleen. (a), (c), (e)
635 Positive staining for HSP70 was observed in the distal convoluted tubule, proximal
636 convoluted tubule, cardiac muscle fibers and hepatocytes in kidney, heart and liver.
637 (b), (d) Positive staining for HSP90 was observed in the terminal bronchioles,
638 cerebellar medulla, granular layer and purkinje cell layer of the lungs and cerebellum.

639 II. (A) The control sections collected from the lung of yak, without immunoreactions
640 (negative control). (B), (D) Positive staining for HSP70 was observed in the terminal
641 bronchioles, cerebellar medulla, granular layer and purkinje cell layer of the lungs and
642 cerebellum. (C), (E) Positive staining for HSP90 was observed in hepatocytes, distal
643 convoluted tubule and proximal convoluted tubule of the liver and kidney. (a), (c), (e)
644 Positive staining for HSP90 was observed in the cardiac muscle fibers, hepatocytes,
645 distal convoluted tubule and proximal convoluted tubule of heart, liver and kidney. (b),
646 (d) Positive staining for HSP70 was observed in the red pulp, cerebellar medulla,
647 granular layer and purkinje cell layer of the spleen and cerebellum.

648 Note. Terminal bronchiole (TB); hepatocyte (Hc); central vein (CV); cardiac muscle
649 fibers (CMF); distal convoluted tubule (DCT); Purkinje cell layer (PCL); renal
650 glomerulus (RG); biofilm (Bf); trabecula (Tc); red pulp (RP); white pulp (WP);
651 cerebellar medulla (CM); molecular layer (ML); granular layer (GL); and proximal
652 convoluted tubule (PCT).

653 III. The result of optical density analysis value. The curved lines represent the
654 expression trend of HSP protein in different organizations, and different color
655 represents different proteins.

656

657 **Fig5. The protein expression in the testicular tissue of cattle-yak and yak.**

658 I. The schematic diagram labels bands and sizes.

659 II. Different color represents expressions of HSP70/90 in the different testicular tissue
660 of cattle-yak and yak.

661

662 **Fig6. IHC and IF localization of HSP70/90 in the testicular tissue of cattle-yak**

663 **and yak.**

664 I. (A) The control sections collected from the testicular tissue of adult yak, without
 665 immunoreactions (negative control). (B), (D) Positive staining for HSP70 was
 666 observed in the spermatogonium and primary spermatocyte of the newborn and
 667 juvenile testis. (C), (E) Positive staining for HSP90 was observed in mesenchymal,
 668 primary spermatocyte and secondary spermatocyte cells of the childhood and adult
 669 testis. (a) The control sections collected from the testicular tissue of adult yak, without
 670 immunoreactions (negative control). (b), (d) Positive staining for HSP90 was
 671 observed in the mesenchymal and primary spermatocyte of the newborn and adult
 672 testis. (c), (e) Positive staining for HSP70 was observed in the spermatogonium,
 673 sertoli cells and seminiferous tubule of the childhood and adult testis.

674 II. (A), (C), (E) Positive staining for HSP90 was observed in the spermatogonium,
 675 secondary spermatocyte and mesenchymal cells of the newborn, juvenile and senile
 676 testis. (B), (D) Positive staining for HSP70 was observed in primary spermatocyte,
 677 sertoli cells, spermatoblast and spermatogonium of the childhood and adult testis. (a),
 678 (c), (e) Positive staining for HSP70 was observed in the spermatogonium, sperm and
 679 mesenchymal cells of the newborn, juvenile and senile testis. (b), (d) Positive staining
 680 for HSP90 was observed in the mesenchymal cells and sperm of the childhood and
 681 adult testis.

682 Note. Primary spermatocyte (PS), secondary spermatocyte (SS), spermatogonium
 683 (Sg), spermatoblast (Sb), sperm (S),sertoli cells (SC), mesenchymal cells(MC), myoid
 684 cells (MC), seminiferous tubule (ST).

685 III. The result of optical density analysis value. The curved lines represent the
 686 expression trend of HSP protein in different testis tissues, and different color
 687 represents different proteins.

688

689 **Fig7. The HSP70/90 schematic diagram of action mechanism in the testicular**
 690 **tissue.**

691 The higher expressed HSP90 could block the development of the primary
 692 spermatocyte in testis of cattle-yak. The higher expressed HSP70 and the decreased
 693 HSP90 could promote the sustained development from the spermatocyte to mature
 694 sperm in testis of yak.

695

696

697

698

699

700

701

702

703

I

IHC

IF

II

IHC

IF

III

I β -actin

42KDa

Bos cattle-yak Hsp70

70KDa

Bos cattle-yak Hsp90

84.7KDa

Bos grunniens Hsp70

70KDa

Bos grunniens Hsp90

84.7KDa

A**B****C****D****E****II**

Relative expression(Sample/Negative)

12

10

8

6

4

2

0

- *Bos cattle-yak Hsp70*
- *Bos cattle-yak Hsp90*
- *Bos grunniens Hsp70*
- *Bos grunniens Hsp90*

Newborn**Calf****Juvenile****Adult****Senior**

I**IHC** **IF** **II****IHC** **IF** **III**

Testis (Cattle)

Spermatogonium

HSP70 *HSP90*
Inhibitor *Activation*

Primary spermatocyte (Yak)

Primary spermatocyte (Cattle-yak)

Spermoblast (Yak)

Sperm (Yak)

Stop developing

Secondary spermatocyte (Yak)

Next cycle