Journal Pre-proofs

Research paper

Gene expression patterns and protein cellular localization suggest a novel role for DAZL in developing Tibetan sheep testes

Taotao Li, Xia Wang, Hongyu Zhang, Haolin Chen, Ningbo Liu, Ruilin Xue, Xingxu Zhao, Youji Ma

PII: S0378-1119(20)30004-4
DOI: https://doi.org/10.1016/j.gene.2020.144335
Reference: GENE 144335

To appear in: *Gene Gene*

Received Date: 23 October 2019
Revised Date: 16 December 2019
Accepted Date: 6 January 2020

Please cite this article as: T. Li, X. Wang, H. Zhang, H. Chen, N. Liu, R. Xue, X. Zhao, Y. Ma, Gene expression patterns and protein cellular localization suggest a novel role for DAZL in developing Tibetan sheep testes, *Gene Gene* (2020), doi: https://doi.org/10.1016/j.gene.2020.144335

Gene expression patterns and protein cellular localization suggest a novel role for DAZL in developing Tibetan sheep testes

Taotao Li[a,b], Xia Wang[a], Hongyu Zhang[a], Haolin Chen[a], Ningbo Liu[a], Ruilin Xue[a], Xingxu Zhao[c], Youji Ma[a,b,*]

[a] *College of Animal Science and Technology, Gansu Agricultural University, Lanzhou 730030, Gansu, China*

[b] *Sheep Breeding Biotechnology Engineering Laboratory of Gansu Province, Minqin 733300, Gansu, China*

[c] *College of Veterinary Medicine, Gansu Agricultural University, Lanzhou 730070, Gansu, China*

* Corresponding author.

College of Animal Science and Technology, Gansu Agricultural University, Lanzhou 730030, Gansu, China

E-mail address: yjma@gsau.edu.cn (Y. Ma)

Abstract: Deleted in azoospermia-like (*DAZL*) is essential for mammalian spermatogenesis as it regulates proliferation, development, maturation and functional maintenance of male germ cells. Its expression and regulation vary with different species or in the same animal at different developmental stages, and despite its importance, very little is known about its roles in sheep, especially Tibetan sheep. To investigate the expression patterns and regulatory roles of *DZAL* in Tibetan sheep testis, testicular tissue was isolated from sheep at three crucial development stages: 3 months

old, 1 year old and 3 years old. Using quantitative real-time PCR and Western blot, we found that *DAZL* mRNA first decreased and then increased with advancing age, while DAZL protein exhibited an opposite expression pattern, with first increased and subsequently decreased levels. Immunohistochemistry and immunofluorescence revealed that DAZL protein was located predominantly in the cytoplasm of Leydig cells and in both the cytoplasm and nucleus of spermatids. ELISA indicated that testosterone content within developing testes was first enhanced and then declined. Our results, taken together, demonstrate, for the first time, that *DAZL* gene is involved in Tibetan sheep spermatogenesis by regulating the development of spermatids in post-pubertal rams, along with a novel role in functional maintenance of Leydig cells in postnatal rams.

Key words: Tibetan sheep; DAZL; Leydig cells; spermatogenesis

1. Introduction

The testis is a vital male reproductive and endocrine organ, whose principal function is to produce sperm and secrete androgen. Spermatogenesis within the seminiferous epithelium is a highly intricate biological process that involves a series of cell proliferation and differentiation, such as mitotic division of spermatogonia, meiotic division of spermatocytes, and formation of functional sperm (Macleod and Varmuza, 2014). This process is controlled by numerous genes whose expression has been examined at both transcription and translation levels. Deleted in azoospermia-like (*DAZL*) gene is a crucial member of the *DAZ* gene family that is composed of three members, *BOLL* and *DAZL*, located on autosomes, and *DAZ*, located on the Y

chromosome (Rosario et al., 2016; Zhang et al., 2016). *DAZL*, previously shown to be detectable exclusively in germ cells of male or female mammals, plays multiple roles in regulation early embryo development, differentiation of pluripotent stem cells into functional gametes, and gametogenesis (Fu et al., 2015; Smorag et al., 2014). In females, the *DAZL* gene is involved in meiosis of oocytes during oogenesis (Chen et al., 2011; Rosario et al., 2016; Sousa Martins et al., 2016). Yu et al (2009) have documented that overexpressed *DAZL* in mouse embryos can promote the differentiation of embryonic stem cells into germ cells. Instead, a lack of *DAZL* seriously hinders the development of germ cells (Lin and Page, 2005; Yu et al., 2009). In males, *DAZL* is essential for the formation and differentiation of primordial germ cells (Fu et al., 2015; Kee et al., 2009) as well as meiosis of spermatocytes (Fu et al., 2015; Kee et al., 2009) during testicular development. DAZL post-transcriptionally plays a positive role in regulating the expression of genes associated with germ cell proliferation and survival during murine spermatogenesis (Zagore et al., 2018). Additionally, transgenic human *DAZL* or *DAZ* can partly rescue the gonad phenotypes from *DAZL* null mice (Vogel et al., 2002). These indicate that *DAZL* gene is functionally important for male fertility. A previous study in men documented that *DAZL* is also closely associated with multiple semen parameters such as sperm counts, motility and morphology (Hsu et al., 2010).

Thus, *DAZL* plays an important role in regulating spermatogenesis, especially germ cell development in mammals, but its regulatory roles vary among species or in the same animals during different development periods. Previous studies on *DAZL* gene

function during spermatogenesis mainly focused on mammals, including humans (Nailwal and Chauhan, 2017; Zhang et al., 2016), rats (Rocchietti-March et al., 2000) and mice (Gonzalez et al., 2015; Zhang et al., 2016), but little is known about it in sheep, as an economically important livestock animal.

Tibetan sheep (*Ovis aries*), distributed mainly in the alpine pasturing area with an altitude of above 3000 m is one of the top three primitive sheep species in China (Ruan et al., 2004), representing the largest number of domestic animals providing meat and income for local farmers and herdsmen. Under long-term grazing without supplementary feeding conditions, however, the reproductive process of Tibetan sheep is characterized by late sexual maturity (1 year old) (Yuan et al., 2019) and low fertility, including seasonal oestrus, breeding once within one year and having only one lamb at a time, initial mating age at 2.5 years old, and an estrus cycle averaging 18 days (Wang et al., 2016). Till date, there are no reports regarding the expression and regulation of *DAZL* gene in Tibetan sheep testes. For better understanding the potential roles of *DAZL* gene during Tibetan sheep spermatogenesis, in this work, we therefore investigate for the first time its expression patterns and cellular localization in testes of Tibetan sheep at three different reproductive stages including 3 months old (3M, sexual immaturity), 1 year old (1Y, sexual maturity) and 3 years old (3Y, adult).

2. Materials and methods

2.1. Experimental animals and design

A total of 24 healthy Tibetan sheep rams with healthy testes (same male parent, different female parents) at 3 ages, —3M (n = 8), 1Y (n = 8) and 3Y (n = 8), —were

provided by the Ganjia Tibetan Sheep Breeding Cooperative (Xiahe, Gansu, China). Eight animals of each age had similar body weight (3M: 9.53–10.20 kg; 1Y: 36.25–38.63 kg; 3Y: 59.90–62.05 kg). The sheep grazed on natural alpine meadow pastures at an altitude of 3200–3500 m. After rams were sacrificed, samples from right testes were washed with phosphate-buffered saline (PBS) to remove blood, and then collected; a part of the samples was stored at −80 °C to be used for RNA and protein extraction and testosterone assay, and the rest was fixed with 4% paraformaldehyde to be used for making 5 μm paraffin sections. Animal care and experimental procedure guidelines of all animals used in the current study were in strict accordance with the Administration Regulations of Experimental Animals promulgated by the Ministry of Science and Technology of the People's Republic of China (Approval No. 2006-398), and this study was approved by the Animal Care Committee of Gansu Agricultural University.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was extracted with a TRIGene RNA isolation kit (GenStar, Beijing, China), according to the manufacturer′s instructions. The purity and concentration of extracted RNA were detected using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Niederelbert, Germany), and its integrity was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesized from each 500 ng RNA sample using a StarScript II Green Fast Two-Step qRT-PCR synthesis kit (GenStar, Beijing, China) according to the manufacturer′s instructions.

2.3. Quantitative real-time PCR (qPCR) assay

The qPCR experiment was performed on a LightCycler 96 Real-Time System (Roche, Switzerland). The thermal cycling conditions were as follows: pre-denaturation at 94 °C for 30 s; 40 cycles of denaturation at 94 °C for 5 s; and annealing at 60 °C for 30 s. Each final volume of 20 μL qPCR mixture comprised 0.8 μL of cDNA, 0.4 μL of each primer (Table S1), 10 μL of 2×RealStar Green Fast Mixture (GenStar, Beijing, China), and 8.4 μL of ddH_2O. *β-actin* was used as a housekeeping gene for normalization of *DAZL* mRNA abundance. The relative abundance of *DAZL* mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.4. Western blot analysis

Total protein of testicular tissues from different age groups was extracted using a radio immunoprecipitation assay (RIPA) protein extraction kit (Solarbio, Beijing, China), and the concentration was examined with a bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). The denatured proteins with equal concentrations (15 μg) were separated by 12% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto 0.45 μm polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfer Membrane, Merck Millipore, Tullagreen, Ireland) for Western blot. Transferred blots were blocked in 5% (g/mL) skim milk powder for 2 h at room temperature. The blot was incubated with either rabbit anti-DAZL polyclonal antibody (1:500; Bioss, Beijing, China) or anti-beta-actin polyclonal antibody (1:1500; Bioss, Beijing, China) overnight at 4 °C, followed by incubation with goat anti-rabbit horseradish peroxidase (HRP) IgG antibody (1:5000; Bioss, Beijing, China). The signals were visualized by an enhanced chemiluminescence

(ECL) kit (NCM Biotech, Suzhou, China) in an x-ray room. The band signals were quantified with AlphaEaseFC software (Protein Simple, Santa Clara, CA, USA).

2.5. Immunohistochemistry

Immunohistochemistry was performed as previously described (González-Arto et al., 2017) with some modifications. In brief, after dewaxing, hydration, and microwave antigen retrieval, endogenous peroxidase activity from paraffin sections was eliminated by 3% H_2O_2. Sections were then incubated with polyclonal rabbit anti-DAZL primary antibody (1:100; Bioss, Beijing, China) overnight at 4 °C. Negative controls were generated with PBS in place of primary antibody. Positive DAZL cells in sections were visualized with a diaminobenzidine (DAB) kit (Bioss, Beijing, China), and representative images were acquired by a biological microscope (Sunny EX31, Ningbo, China).

2.6. Immunofluorescence staining

Paraffin sections from testes of different age groups were prepared using the same procedure as immunohistochemistry. After treatment with autofluorescence quencher (Servicebio, Wuhan, China) for 5 min at room temperature, sections were blocked with 5% bovine serum albumin (BSA) in PBS for 30 min. Sections were incubated with polyclonal rabbit anti-DAZL primary antibody (1:150; Bioss, Beijing, China) overnight at 4 °C. Negative controls were generated with 5% BSA in place of primary antibody. Afterward, sections were incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (1:200; Servicebio, Wuhan, China) for 1 h at room temperature in the dark. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI;

Servicebio, Wuhan, China) for 10 min at room temperature in the dark. After being mounted with anti-fade mounting reagent, sections were observed under a fluorescence microscope (Nikon, Eclipse C1, Tokyo, Japan) and representative images were acquired by CaseViewer software (3DHISTECH, Budapest, Hungary).

2.7. Determination of testosterone concentration

Testis homogenates were harvested and testosterone concentration was determined by a commercial enzyme-linked immunosorbent assay (ELISA) kit (MEIMIAN, Jiangsu, China) following the manufacturer's recommendations. Optical density was measured at 450 nm by a microplate reader (BioTek, Winooski, VT, USA).

2.8. Statistical analysis

All experiments were repeated independently at least 3 times. The data were statistically analyzed by one-way ANOVA with least significant difference (LSD) in SPSS 21.0 (IBM Corp., Armonk, NY, USA) and expressed as mean ± SD. *$p < 0.05$ was considered statistically significant, and **$p < 0.01$ was considered extremely significant.

3. Results

3.1. Expression patterns of DAZL mRNA in developing Tibetan sheep testes

Significantly downregulated expression at the *DAZL* mRNA transcript level was detectable in testes from 3M to 1Y group ($p < 0.01$) (Fig. 1). Intriguingly, *DAZL* mRNA level was significantly upregulated in the 3 Y group as compared to 1Y group ($p < 0.01$) (Fig. 1).

3.2. Expression patterns of DAZL protein in developing Tibetan sheep testes

DAZL protein was detectable in Tibetan sheep testes throughout the developing stages (Fig. 2A). Compared to the 3M group, the expression level of DAZL protein was significantly up-regulated in the 1Y group ($p < 0.01$), while it was significantly down-regulated in the 3Y group ($p < 0.01$) (Fig. 2B).

3.3. Cellular localization of DAZL protein in developing Tibetan sheep testes

In the 3M group, DAZL protein with intense signals was observed in Leydig cells (Fig. 3a1). In the 1Y and 3Y groups, positive DAZL protein signals were observed in Leydig cells, along with spermatids within seminiferous tubule (Fig. 3a2, a3).

3.4. Subcellular localization of DAZL protein in developing Tibetan sheep testes

To verify the results of DAZL protein distribution as analyzed by immunohistochemistry and further determine its subcellular localization, we performed immunofluorescence with paraffin sections. Our results showed that DAZL protein was dominantly observed in the cytoplasm of Leydig cells from testes throughout development stages (Fig. 4a2–f2), and in both the cytoplasm and nucleus of spermatids within seminiferous tubule from 1Y and 3Y testes (Fig. 4c2–f2).

3.5. Changes of testosterone levels in developing Tibetan sheep testes

Changes of testosterone levels in developing Tibetan sheep testes are shown in Fig. 5. Compared with the 3M group, testosterone levels increased significantly in testes from the 1Y group ($p < 0.01$). Subsequently, testosterone levels reduced significantly in testes from the 3Y group ($p < 0.01$).

4. Discussion

DAZL is required for male fertility to regulate the development of germ cells during spermatogenesis (Liu et al., 2011; Williams et al., 2016). The loss of *DAZL* expression and function can lead to multiple defects in murine spermatogenesis, such as mitotic irregularity (Schrans-Stassen et al., 2001) and meiotic arrest (Ruggiu et al., 1997; Saunders et al., 2003). Differential expression patterns of *DAZL* have been reported in many male mammals (Lee et al., 2017; Li et al., 2017; Rocchietti-March et al., 2000). Tibetan sheep are a unique domestic species residing in the Qinghai–Tibet Plateau of China. Although it is inseparable from the daily life of local farmers and herdsmen, subfertility has remained a significant problem. To understand the roles of the *DAZL* gene in Tibetan sheep testis, in this study, we first detected the expression of *DAZL* at the mRNA transcript levels in testes from Tibetan sheep at different developing stages. As a result, *DAZL* mRNA expression was obviously decreased in testes from pre-puberty (3M) to puberty (1Y) and then increased in adult (3Y) testes. Our results were similar to expression patterns of *DAZL* mRNA transcript in developmental Small Tail Han sheep testes at different stages as assessed by RNA sequencing (Bai et al., 2017), indicating *DAZL* is implicated in sheep testicular development, and maybe have different roles in Tibetan sheep testes at different reproductive stages.

We subsequently performed Western blot analysis to evaluate whether expression trends for DAZL at the protein level are similar to those at the mRNA level. On the contrary, DAZL protein expression increased in 1Y testis than that in 3M testis, whereas its expression decreased in 3Y testis. The expressional diversity of DAZL protein may

be caused by different cell compositions and numbers in ram testes at different stages (Bai et al., 2017; Yang et al., 2018). Upregulated DAZL protein patterns from 3M to 1Y Tibetan sheep testes were consistent with the results reported by Li et al. (2017), documenting that DAZL protein increased gradually in goat testes before adulthood with progressing age. The differences regarding *DAZL* expression patterns between mRNA and protein levels may be explained by the following reasons. For one thing, this difference may be caused by post-transcriptional (Chick et al., 2016) and post-translational (Liao et al., 2018) events. There are multiple post-transcriptional regulation events, such as alternative splicing, microRNAs, and polyadenylation (reviewed in (Legrand and Hobbs, 2018; Licatalosi, 2016)), as well as post-translational modifications, such as phosphorylation, ubiquitination, and acetylation (reviewed in (Richburg et al., 2014; Vigodner, 2011)), during mammalian testis development and spermatogenesis that regulate mainly meiosis and sperm maturation. Regarding DAZL, Williams et al. (2016) reported in Drosophila that its expression was subjected to phosphorylation modification by MAPKAP kinase 2 (MK2) to regulate spermatogenesis. For another thing, the expression differences in both mRNA and protein levels may also be related to the stability of mRNA and protein (Rojas-Ríos and Simonelig, 2018). In addition, a probable cause of the expression difference at the mRNA and protein levels can be explained by different methylation patterns of *DAZL*, which has been reported in bovine (cattle, yak, and cattle-yak) testes (Liu et al., 2011). Which reason leads to the result still needs further investigation.

Although DAZL is essential for testicular development and spermatogenesis in male mammals, its distribution and roles in testis vary with the species and development stage. In donkeys, DAZL protein is present in the cytoplasm of spermatogonia for pre-pubertal testes, whereas it is present in spermatogonia and primary spermatocytes for post-pubertal testes (Lee et al., 2017). In goats, DAZL protein is expressed in the cytoplasm of spermatogonia and primary spermatocytes for pre-pubertal testes, while it is expressed strongly in spermatogonia and primary spermatocytes as well as secondary spermatocytes and spermatids for post-pubertal testes (Li et al., 2017). In rats, DAZL protein is observed in spermatogonia, primary spermatocytes, and spermatids (Rocchietti-March et al., 2000). In mice, DAZL protein is expressed in spermatogonia for adult testes (Gonzalez et al., 2015). To further clarify the potential role of DAZL during testicular development of Tibetan sheep, distribution of positive DAZL protein in testes was visualized by immunohistochemistry and immunofluorescence. Similar to previous reports on post-pubertal goats (Li et al., 2017) and rats (Rocchietti-March et al., 2000), our results indicate that DAZL protein was located in both the cytoplasm and nucleus of spermatids in post-pubertal (1Y and 3Y) Tibetan sheep testes. Combined with previous studies, these findings demonstrate that the role of *DAZL* gene in the development of spermatids during spermatogenesis of post-pubertal Tibetan sheep testes.

Unlike prior studies in other species, however, our results documented that the DAZL protein was distributed in the cytoplasm of Leydig cells presenting in the interstitial spaces between the seminiferous tubules of Tibetan sheep testes throughout

the development stages. This might be caused by different breeds and living environments. For Tibetan sheep, the high–altitude hypoxia and poor nutrition on the Qinghai–Tibetan Plateau affect ovine reproductive performance–related gene expression (Jing et al., 2017). One can speculate that *DAZL* participates in regulating the development and functional maintenance of Leydig cells during ram spermatogenesis. The principle function of Leydig cells serving as steroidogenic cell in the testes is to produce androgens, the main and representative component of which is testosterone (Yu et al., 2018). Testosterone secreted by Leydig cells plays an important role in meiosis during spermatogenesis through its effects on spermatids, which is reviewed by Zhou et al. (2019) and Stanton et al. (2012). With a lack of testosterone, spermatogenesis arrests at the stage of meiosis or even the preceding stage (Stanton et al., 2012). These reports suggest that testosterone plays indispensable role in stimulating spermatogenesis and promoting sperm maturation. We therefore speculate that *DAZL* gene that is expressed in ram Leydig cells may be involved in the regulation of testosterone secretion. In light of this, we estimated testosterone levels within Tibetan sheep testes at different development stages. Testosterone levels were first elevated and then subsided with advancing age, which was consistent with change trends of plasma testosterone levels in Hu sheep at different development stages, as reported by Yang et al. (2018). As expected, the change trend of intratesticular testosterone concentration was similar with the expression trend of DAZL protein in ram testes at different development stages. Protein, as a product coded by genes, is a carrier of biological activities and a direct executors of biological functions. Therefore,

our findings suggest that *DAZL* is involved in the function of Leydig cells to induce secretion of testosterone during Tibetan sheep testis development, thereby regulating spermatogenesis (Fig. 6). *BOULE*, a homologous gene of *DAZL*, is previously reported to regulate the development of germ cells during murine spermatogenesis through a testosterone-independent pathway (Gonzalez et al., 2015). However, so far, there are no reports in other species regarding *DAZL* gene on whether and how it engages in regulating testosterone secretion from Leydig cells, which thus needs to be continued in-depth investigation and verification.

5. Conclusions

In conclusion, this is the first report investigating the expression and localization patterns of the *DAZL* gene in Tibetan sheep testes. Our results document that *DAZL* is expressed in the Leydig cells of Tibetan sheep testes throughout the development stages in addition to spermatids in post-puberty, but has an opposite pattern of expression at mRNA and protein levels, with the lowest mRNA level and the highest protein level during sexual maturity. These findings demonstrate that ovine *DAZL* gene plays a crucial role in the development of spermatids and in the development and functional maintenance of Leydig cells by modulating testosterone secretion.

Author contributions

YM and TL conceived and designed the experiments; XW, HC, NL, and RX. collected samples; TL, HZ, and XW performed the experiments and analyzed the data; TL wrote the paper; YM and XZ revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant number: 31960662); the National Key R&D Program of China (grant number: 2018YFD0502103); and the Fostering Foundation for the Excellent Ph.D. Dissertation of Gansu Agricultural University (grant number: YB2018001).

Conflict of interest

The authors declare no conflict of interest.

References

Bai, M., Sun, L., Zhao, J., Xiang, L., Cheng, X., Li, J., Jia, C., Jiang, H., 2017. Histological analysis and identification of spermatogenesis-related genes in 2-, 6-, and 12-month-old sheep testes. Naturwissenschaften 104, 84. doi: 10.1007/s00114-017-1505-1

Chen, J., Melton, C., Suh, N., Oh, J., Horner, K., Xie, F., Sette, C., Blelloch, R., Conti, M., 2011. Genome-wide analysis of translation reveals a critical role for deleted in azoospermia-like (Dazl) at the oocyte-to-zygote transition. Genes Dev. 25, 755-766. doi: 10.1101/gad.2028911

Chick, J.M., Munger, S.C., Simecek, P., Huttlin, E.L., Choi, K., Gatti, D.M., Raghupathy, N., Svenson, K.L., Churchill, G.A., Gygi, S.P., 2016. Defining the consequences of genetic variation on a proteome-wide scale. Nature 534, 500-505. doi: 10.1038/nature18270

Fu, X.F., Cheng, S.F., Wang, L.Q., Yin, S., Felici, M.D., Shen, W., 2015. DAZ family proteins, key players for germ cell development. Int. J. Biol. Sci. 11, 1226-1235. doi: 10.7150/ijbs.11536

González-Arto, M., Aguilar, D., Gaspar-Torrubia, E., Gallego, M., Carvajal-Serna, M., Herrera-Marcos, L.V., Serrano-Blesa, E., Hamilton, T.R.D.S., Pérez-Pé, R., Muiño-Blanco, T., 2017. Melatonin MT_1 and MT_2 receptors in the ram reproductive tract. Int. J. Mol. Sci. 18, 662. doi: 10.3390/ijms18030662

Gonzalez, C.R., Dorfman, V.B., Vitullo, A.D., 2015. IGF1 regulation of BOULE and CDC25A transcripts via a testosterone-independent pathway in spermatogenesis of adult mice. Reprod. Biol. 15, 48-55. doi: 10.1016/j.repbio.2014.10.003

Hsu, C., Kuo, P., Lee, I., Su, M., Tseng, J., Kuo, P., 2010. Quantitative trait analysis suggests human DAZL may be involved in regulating sperm counts and motility. Reprod. Biomed. Online 21, 77-83. doi: 10.1016/j.rbmo.2010.03.026

Jing, X., Peng, Q., Hu, R., Wang, H., Yu, X., Degen, A., Zou, H., Bao, S., Zhao, S., Wang, Z., 2017. Effect of supplements during the cold season on the reproductive system in prepubertal Tibetan sheep ewes. Anim. Sci. J. 88, 1269-1278. doi: 10.1111/asj.12762

Kee, K., Angeles, V.T., Flores, M., Nguyen, H.N., Reijo Pera, R.A., 2009. Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation. Nature 462, 222-225. doi: 10.1038/nature08562

Liao, C.H., Wang, Y.H., Chang, W.W., Yang, B.C., Wu, T.J., Liu, W.L., Yu, A.L., Yu, J., 2018. Leucine-rich repeat neuronal protein 1 regulates differentiation of embryonic stem cells by post-translational modifications of pluripotency factors. Stem Cells 36, 1514-1524. doi: 10.1002/stem.2862

Licatalosi, D.D., 2016. Roles of RNA-binding proteins and post-transcriptional regulation in driving male germ cell development in the mouse. Adv. Exp. Med. Biol. 907, 123-151. doi: 10.1007/978-3-319-29073-7_6

Lin, Y., Page, D., 2005. Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice. Dev. Biol. 288, 309-316. doi: 10.1016/j.ydbio.2005.06.032

Liu, Z., Li, Q., Pan, Z., Qu, X., Zhang, C., Xie, Z., 2011. Comparative analysis on mRNA expression level and methylation status of *DAZL* gene between cattle-yaks and their parents. Anim. Reprod. Sci. 126, 258-264. doi: 10.1016/j.anireprosci.2011.05.013

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25,

402-408. doi: 10.1006/meth.2001.1262

Macleod, G., Varmuza, S., 2014. The application of proteomic approaches to the study of mammalian spermatogenesis and sperm function. FEBS J. 280, 5635-5651. doi: 10.1111/febs.12461

Nailwal, M., Chauhan, J.B., 2017. In silico analysis of non-synonymous single nucleotide polymorphisms in human DAZL gene associated with male infertility. Syst. Biol. Reprod. Med. 63, 248-258. doi: 10.1080/19396368.2017.1305466

Richburg, J.H., Myers, J.L., Bratton, S.B., 2014. The role of E3 ligases in the ubiquitin-dependent regulation of spermatogenesis. Semin. Cell Dev. Biol. 30, 27-35. doi: 10.1016/j.semcdb.2014.03.001

Rocchietti-March, M., Weinbauer, G.F., Page, D.C., Nieschlag, E., Gromoll, J., 2000. Dazl protein expression in adult rat testis is up-regulated at meiosis and not hormonally regulated. Int. J. Androl. 23, 51-56. doi: 10.1046/j.1365-2605.2000.00207.x

Rojas-Ríos, P., Simonelig, M., 2018. piRNAs and PIWI proteins: regulators of gene expression in development and stem cells. Development 145, dev161786. doi: 10.1242/dev.161786

Rosario, R., Adams, I.R., Anderson, R.A., 2016. Is there a role for DAZL in human female fertility? Mol. Human Reprod. 22, 377-383. doi: 10.1093/molehr/gaw024

Rossignol, F., Solares, M., Balanza, E., Coudert, J., Clottes, E., 2003. Expression of lactate dehydrogenase A and B genes in different tissues of rats adapted to chronic hypobaric hypoxia. J. Cell. Biochem. 89, 67-79. doi: 10.1002/jcb.10484

Ruan, Z., Koizumi, T., Sakai, A., Ishizaki, T., Wang, Z., 2004. Endogenous nitric oxide and pulmonary circulation response to hypoxia in high-altitude adapted Tibetan sheep. Eur. J. Appl. Physiol. 93, 190. 10.1007/s00421-004-1197-z

Ruggiu, M., Speed, R., Taggart, M., McKay, S., Kilanowski, F., Saunders, P., Dorin, J., Cooke, H., 1997. The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis. Nature 389, 73-77. doi: 10.1038/37987

Saunders, P.T., Turner, J.M., Ruggiu, M., Taggart, M., Burgoyne, P.S., Elliott, D., Cooke, H.J., 2003. Absence of mDazl produces a final block on germ cell development at meiosis. Reproduction 126, 589-597.

Schrans-Stassen, B., Saunders, P., Cooke, H., de Rooij, D.J., 2001. Nature of the spermatogenic arrest in Dazl -/- mice. Biol. Reprod. 65, 771-776. doi: 10.1095/biolreprod65.3.771

Smorag, L., Xu, X., Engel, W., Pantakani, D., 2014. The roles of DAZL in RNA biology and development. Wiley Interdiscip Rev RNA 5, 527-535. doi: 10.1002/wrna.1228

Sousa Martins, J.P., Liu, X., Oke, A., Arora, R., Franciosi, F., Viville, S., Laird, D.J., Fung, J.C., Conti, M., 2016. DAZL and CPEB1 regulate mRNA translation synergistically during oocyte maturation. J. Cell Sci. 129, 1271-1282. doi: 10.1242/jcs.179218

Stanton, P.G., Sluka, P., Foo, C.F.H., Stephens, A.N., Smith, A.I., Mclachlan, R.I., Aposdonnell, L., 2012. Proteomic changes in rat spermatogenesis in response to in vivo androgen manipulation; impact on meiotic cells. PLoS One 7, e41718. doi: 10.1371/journal.pone.0041718

Vigodner, M., 2011. Roles of Small ubiquitin-related modifiers in male reproductive function. Int. Rev. Cell Mol. Biol. 288, 227-259. doi: 10.1016/B978-0-12-386041-5.00006-6

Vogel, T., Speed, R.M., Ross, A., Cooke, H.J., 2002. Partial rescue of the *Dazl* knockout mouse by the human *DAZL* gene. Mol. Human Reprod. 8, 797-804. doi: 10.1093/molehr/8.9.797

Wang, W., He, Y., Han, Z., Shi, J., Huo, J., Zhu, X., 2016. Expression of *FSHR* gene and *LHR* gene in the ovary of Ganjia Tibetan sheep in different stages of estrous cycle. Chinese Veterinary Science 46, 97-103. doi: 10.16656/j.issn.1673-4696.2016.01.017

Williams, P., Krug, M., McMillan, E., Peake, J., Davis, T., Cocklin, S., Strochlic, T., 2016. Phosphorylation of the RNA-binding protein Dazl by MAPKAP kinase 2 regulates spermatogenesis. Mol. Biol. Cell 27, 2341-2350. doi:

10.1091/mbc.E15-11-0773

Yang, H., Wang, F., Li, F., Ren, C., Pang, J., Wan, Y., Wang, Z., Feng, X., Zhang, Y., 2018. Comprehensive analysis of long non-coding RNA and mRNA expression patterns in sheep testicular maturation. Biol. Reprod. 99, 650-661. doi: 10.1093/biolre/ioy088

Yu, K., Deng, S., Sun, T., Li, Y., Liu, Y., 2018. Melatonin regulates the synthesis of steroid hormones on male reproduction: a review. Molecules 23, 447. doi: 10.3390/molecules23020447

Yu, Z., Ji, P., Cao, J., Zhu, S., Li, Y., Zheng, L., Chen, X., Feng, L., 2009. Dazl promotes germ cell differentiation from embryonic stem cells. J. Mol. Cell. Biol. 1, 93-103. doi: 10.1093/jmcb/mjp026

Yuan, L., Song, X., Yang, J., Liang, X., Chen, G., 2019. Comparison of distribution characteristics of TGF-β1 and its receptors in the testis of the Tibetan sheep before and after sexual mature. Acta Vet Zootech Sin 50, 750-757. doi: 10.11843/j.issn.0366-6964.2019.04.008

Zagore, L., Sweet, T., Hannigan, M., Weyn-Vanhentenryck, S., 2018. DAZL regulates germ cell survival through a network of PolyA-proximal mRNA interactions. Cell Rep. 25, 1225-1240. doi: 10.1016/j.celrep.2018.10.012

Zhang, C., Xue, P., Gao, L., Chen, X., Lin, K., Yang, X., Dai, Y., Xu, E.Y., 2016. Highly conserved epigenetic regulation of BOULE and DAZL is associated with human fertility. FASEB J. 30, 3424-3440. doi: 10.1096/fj.201500167R

Zhou, R., Wu, J., Liu, B., Jiang, Y., Chen, W., Li, J., He, Q., He, Z., 2019. The roles and mechanisms of Leydig cells and myoid cells in regulating spermatogenesis. Cell. Mol Life Sci. 76, 2681-2695. doi: 10.1007/s00018-019-03101-9

Figure legends

Fig. 1. Relative DAZL mRNA abundance in developing Tibetan sheep testes. Data represent mean ± SD from eight individuals per group. ** $p < 0.01$. 3M, 3 months old; 1Y, 1 year old; 3Y, 3 years old.

Fig. 2. Relative DAZL protein level in Tibetan sheep testes from 3M, 1Y and 3Y groups. (A) Western blot results for DAZL protein. (B) Comparative analysis of relative DAZL protein expression by integrated density value. Data represent mean ± SD from eight individuals per group. ** $p < 0.01$. ns, non-significant; 3M, 3 months old; 1Y, 1 year old; 3Y, 3 years old.

Fig. 3. Immunohistochemical localization of DAZL protein within developing Tibetan sheep testes. (a1–a3) Immunostaining patterns for DAZL protein in 3-month-old, 1-year-old and 3-year-old ram testes, respectively; (b1–b3) substitution of phosphate-buffered saline (PBS) for primary antibody served as negative control. LC, Leydig cell; Sp, spermatid.

Fig. 4. Immunofluorescence staining of DAZL protein in Tibetan sheep testes from 3M, 1Y and 3Y groups. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Immunofluorescence for nuclei (blue in a1, b1, c1, d1, e1, and f1) and DAZL (green in a2, b2, c2, d2, e2, and f2) in (a1-b3) 3M, (c1-d3) 1Y, and (e1-f3) 3Y groups. 3M, 3 months old; 1Y, 1 year old; 3Y, 3 years old. Scale bar, 50 μm.

Fig. 5. Testosterone concentrations in developing Tibetan sheep testes. Data represent the mean ± SD from eight individuals per group. ** $p < 0.01$. 3M, 3 months old; 1Y, 1 year old; 3Y, 3 years old. T, testosterone.

Fig. 6. Proposed schematic illustration of roles of DAZL in regulating spermatogenesis in sheep testis.

Abbreviations:

DAB, diaminobenzidine;

DAZL, deleted in azoospermia-like;

HRP, horseradish peroxidase;

PBS, phosphate-buffered saline;

qPCR, quantitative real-time PCR;

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Author contributions

YM and TL conceived and designed the experiments; XW, HC, NL, and RX. collected samples; TL, HZ, and XW performed the experiments and analyzed the data; TL wrote the paper; YM and XZ revised the manuscript. All authors read and approved the final manuscript.

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

1. We firstly investigated the expression patterns of *DAZL* in Tibetan sheep testes.
2. *DAZL* was detectable in Tibetan sheep testes throughout development stages.
3. Opposite *DAZL* mRNA and protein expression trends were found in developmental testes.
4. It is firstly reported that a potential role for *DAZL* in Tibetan sheep Leydig cells.
5. *DAZL* ~~may be~~is linked to development of Leydig cells and spermatids for Tibetan sheep.