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Comparative analysis on mRNA expression and DNA methylation status of *SYCE1* gene in testes of yaks and cattleyaks*

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In mammals, cases of male infertility have been associated with failure to develop the synaptonemal complex (SC). *SYCE1* (Synaptonemal complex central element protein 1) plays a role in the assembly of SC structures essential for meiosis and reproduction. The absence of *SYCE1* will lead to meiotic arrest, spermatogenetic failure and ultimately male infertility. However, molecular regulation of *SYCE1* during spermatogenesis has never been reported and its function in the bovine testicular tissue remains unclear. To investigate the underlying regulatory mechanism of *SYCE1* expression in cattleyaks, *SYCE1* mRNA expression and *SYCE1* gene methylation patterns in testes of yaks and cattleyaks were detected utilizing quantitative real-time PCR and bisulfite sequencing. As may have been expected, a remarkable decrease of mRNA level was detected in the testes of cattleyaks compared to that of yaks ($P<0.0001$). A significant increase in the methylation level of the *SYCE1* gene promoter in testes was also found in the cattleyaks (78.125%) with male sterility compared with normal spermatogenesis in yaks (28.125%) ($P<0.01$). These results indicate that the methylation status of the *SYCE1* gene promoter can regulate mRNA expression. Taken together, our results demonstrated that the *SYCE1* promoter is differentially methylated in testes; together with

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the functional promoter analysis this suggests that methylation of this promoter may contribute to cattleyak male infertility. Our findings highlighted the different expression of *SYCE1* potentially influencing the assembly of SC structures in cattleyaks and provided an important framework for future researches, which may elucidate the mechanism caused by abnormal chromosomal synapsis in cattleyak meiosis and fertility.

KEYWORDS: DNA methylation/cattleyak/promoter/ SYCE1/male infertility

Yaks (*Bosmutus*) live mainly in Qinghai, Tibet, Sichuan, Gansu and Yunnan Provinces and are well-known both for their excellent adaptation to harsh environment and high-quality meat and milk. The cattleyak hybrid is derived from two bovid species (cattle×yak), which display equivalent adaptability to harsh surroundings, such as those of the Qinghai-Tibetan Plateau, and exhibit apparent heterosis such as early maturity and high meat production [Yu *et al.* 2016; Cai *et al.* 2017]. However, the F1 males of cattleyaks are infertile due to spermatogenetic arrest, which has greatly restricted their hybrid vigor in yak breeding. In the past several years, a large amount of work has been done to investigate the mechanisms underlining male sterility of cattleyaks, with most studies being based on morphological anatomy, histological observations, endocrinology and biochemistry, etc. Additionally, the study on genital organs of cattleyak F1-F3 generations (treated) compared with male yaks (the control) revealed that there was low secretion of gonadotropin to the epithelial basement in cattleyak testes and abnormal development of interstitial cells and seminiferous tubules, which might be responsible for the underdevelopment and lower number of spermatogonia in cattleyaks [Zhang *et al.* 2001].

RNA-sequencing of the transcriptome profilesings between testies tissue of cattleyaks and yaks identified 2960 differentially expressed genes (DEGs) (679 up-regulated and 2281 down-regulated) in cattleyaks and suggested spermatogenic arrest may aggregate during mitosis of spermatogonia differentiation and only some of themfewer are successfully transformedtransfer into spermatocytes [Cai *et al.* 2017]. Over the past years, molecular research concerningabout candidate genes involved in spermatogenesis opened a new window for future research on theabout mechanism involved in male cattleyak infertility. It was reported that the expression levels of the following genes IGF2 [Liu *et al.* 2009], DAZL [Liu *et al*. 2011], SYCP3 [Wang *et al.* 2012], Prdm9 [Lou *et al.* 2014], PIWIL1 [Gu *et al*. 2013] and, FKBP6 [Li *et al*. 2016], were down- regulated in cattleyak testeis as compared with the yak. T, and these investigations have presented valuable information for future studiesy concerningfor expression and regulation of candidate genes involved in spermatogenetic arrest of cattleyaks. AdditionallyMeantime, in recent years, some researchers have attempted to explore the mechanistic basis of infertility by means of mouse intersubspecific hybrids. They observed a spermatogenetic block and apoptosis of primary spermatocytes at the pachytene stage and suggestedpurposed that the asynapsis may represent a universal mechanistic basis forof F1 hybrid sterility manifested by pachytene arrest [Bhattacharyya *et al*. 2013]. Additionally, several studies are in favor of the Dobzhansky-Muller model, which claimed the incompatibility between

X and autosomes can be produced by various mechanisms, such as interactions of sex chromosomes and epigenetic mechanisms [Matsuda *et al.* 1992, Brown *et al.* 2012, Luo *et al*. 2013, Wang *et al.* 2016]. However, the specific mechanism of F1 male cattleyak infertility still remains unknown and is thought to be caused by anthe interaction of multiple factors.

Sexual reproduction occurs in eukaryotes through meiosis, a process by which a diploid germ-line cell divides and gives rise to the formation of haploid cells. This process involves one round of DNA replication and two sequential waves of cell division, i.e. meiosis Ⅰ and Ⅱ. Before the first division in the crucial steps of meiosis two homologous chromosomes pair up and a synaptonemal complex (SC) is formed between them [Chen *et al.* 2018b]. Moreover, the tripartite structure of the SC is highly conserved in evolution from yeast to mammals in terms of its prominent function during meiosis [Schramm *et al.* 2011]. The synapsis of the homologous chromosome mediated by the complete SC ensures normal spermatogenesis in mammalian species. The proper assembly of SC is essential for developing crossover recombination and chromosome exchange. The SC is a zipper-like structure consisting of three components: two lateral elements [Barau *et al.* 2016], the central elements (CEs) and transverse filaments (TFs). These structures consist of meiosis-specific proteins. Assembly of SC is mediated via integration of opposing TFs into a CE, a process that is fully understood. More recently, four CE proteins have been identified: SYCE1, SYCE2, SYCE3, and TEX12 (testis-expressed protein 12), all of which localise exclusively to the central element of the SC and contain predicated alpha-helical domains [Li *et al.* 2018]. To date, the targeted mutagenesis of known SC components in mouse indicated that a complete CE is required in female and male reproduction [Bolcun-Filas *et al.* 2007, Bolcun-Filas *et al.* 2009].

SYCE1 is a specialised gene, which is expressed from the leptotene to the zygotene stage in spermatogenesis and encodes a central component of SC, involved in synapsis, crossover recombination and segregation of meiotic chromosomes [Costa *et al.* 2005, Wang *et al.* 2018b, Zhou *et al.* 2018]. The importance of *SYCE1* in SC assembly and synapsis initiation could also be investigated in greater detail after the corresponding gene knockout mouse became available. The null mutation of *SYCE1* in the mouse resulted in spermatogenic arrest during pachynema due to synapsis failure. More specifically, infertility was caused by disruption of meiosis as a result of the inability of an *SYCE1*$^{-/-}$ mouse to assemble the central element of SC, resulting in a disruption during meiosis followed by apoptosis and a lack of post-meiotic cells in the testis. Primary spermatocytes were the most common germ cell type indicating a spermatogenic arrest at prophase I [Bolcun-Filas *et al.* 2009]. Additionally, the null *SYCE1*$^{-/-}$ mutant testes were only 1/5-1/3 of the size of those from wild-type mice. Interestingly, a previous study reported that, although cattleyaks had the same chromosome number as both parents (2n=60) [Tan *et al.* 1990], the SC of autosome abnormity could be morphologically observed only in several primary spermatocytes and the size of the cattleyak testes was significantly smaller than that of yaks, which is consistent with the phenotypes of

SYCE1$^{-/-}$ mice. Thus, a close association existed between the failure of SC assembly caused by the lack of *SYCE1* and male cattleyak infertility.

In this study we were the first to clone and identify the sequence of the 5'-region of *SYCE1* from the testes of yaks and cattleyaks. To investigate the involvement of *SYCE1* in the regulatory mechanism of cattleyak male sterility, we analyzed the expression pattern and methylation status of *SYCE1* in normal adult yaks and sterile cattleyak testes. We hope that our work could provide a novel basis for further studies of *SYCE1* functioning in the currently unclear mechanism of male infertility in cattleyaks.

Material and methods

Tissue collection and preparation of DNA and RNA

Male yaks (*Bos mutus*) (n=3) and cattleyak hybrids (n=3) were obtained from a Maiwa yak population fed on a pasture in the Hongyuan county, Sichuan province, China. The testes of each animal were sampled via a veterinary surgical operation and the caudal epididymis was firstly resected. All crosscut slices were snap frozen in liquid nitrogen (-196°C), transported to the laboratory and stored at -80°C until RNA and DNA extraction. All experiments were undertaken in accordance with the guidelines of the regional Animals Ethics Committee and all protocols were approved by the Institutional Animal Care and Use Committee of the Southwest University of Science and Technology.

RNA preparation and quantitative real-time PCR (qPCR)

The SYCE1 mRNA isolated from testes was quantitated against the β-actin housekeeping gene using real-time PCR. Total RNA was extracted from six testicular tissue samples using TRIzol (Invitrogen, CA, USA). The quality and concentration of total RNA were assessed using a Quawell micro volume spectrophotometer Q6000 (Quawell, San Jose, CA, USA). RNase-free DNase I (TaKaRa, Dalian, China) was used to remove genomic DNA from the RNA samples. The primers used for qPCR are listed in Table 1. The cDNA was synthesised using the PrimeScript RT Master Mix (Takara,Dalian,China). Real-Time PCR was performed in 96-well Optical Reaction Plates (Applied Biosystems, USA). The 25μL PCR reaction mixture in each well included 12.5 μL of 2×TB Green Premix Ex Taq (Applied Biosystems, USA), 1.0 μl each of the forward and reverse primers (10 μM), 2 μL of cDNA and 8.5 μL ddH2O. PCR reactions were performed in the CFX96 Real-Time PCR Detection System (Applied Biosystems, USA) using the following program: 30 sec at 95°C for nucleic acid denaturation and activation of the Taq Polymerase, followed by 40 cycles of 95°C for 5 sec and 57.5°C for 30 sec and finally a slow heating cycle to obtain a dissociation curve for the products. Individual samples were run in triplicate. The expression levels relative to the β-actin gene were then calculated using the 2-ΔΔCt method.

Bioinformatics analysis

Multiple sequence alignment was performed using the DNASTAR 5.22 software. Genomic organisation and chromosomal locations were analysed based on data from the genome database of NCBI (http://www.ncbi.nlm.nih.gov/mapview/). The core promoter region of the gene was predicted via the online software NNPP (http://www.fruitfly.org/seq_tools/promoter.html). The CpG islands were investigated using an online program (http://www.urogene.org/cgi-bin/methprimer/ methprimer.cgi). The putative promoter region was predicted using the ProScan software (http://www-bimas.cit.nih.gov/molbio/proscan/). The potential transcription factor response elements were analysed using TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html).

DNA preparation and bisulfite treatment

Genomic DNA was extracted from six testicular tissue samples according to the procedure of the TIANamp Genomic DNA Kit (commercial kit) (Tiangen Biotech, Beijing, China). The quantity and quality of DNA were measured using a Quawell micro volume spectrophotometer Q6000 (Quawell, San Jose, CA, USA). For bisulfite sequencing, genomic DNA was treated with an EZ DNA Methylation-Direct Kit (ZYMO Research, USA) according to the manufacturer's instructions. Bisulfite-treated DNA was subjected to PCR amplification.

Cloning and sequencing of yak SYCE1 5' UTR

According to the cattle *SYCE1* gene, primers were designed to amplify the 5'-untranslated region of *SYCE1*. Each cycle of PCR was carried out following the steps as follows: 35 cycles of 30 s at 94 °C, annealing for 40 s at 62°C and extension for 1 min at 72 °C. The PCR was terminated by a final extension step at 72°C for 10 min. Then, PCR products were identified by agarose gel electrophoresis, purified with a Gel DNA Extraction Kit (Takara, Dalian, China), cloned into pMD^{TM}19-T vectors (Takara, Dalian, China) and ultimately transformed into the Escherichia coli Tran*5α* competent cells (TransGen Biotech, Beijing, China). Four or five independent positive clones were sequenced by standard Sanger sequencing using sequencing primers M13F and M13R from both strands.

Table1. Primers used in this study

Accession no.	Gene	Primer sequences (5' to 3')	Length (bp)	Annealing (°C)	Application
NC_037353	*SYCE1*	F:AAGGTCCGTCTAGTCAAGGC R:CATGGGGCTGAAGGTCATTT	1090	62	5'UTR clone
NW_005395378	*SYCE1*	F:ATTTTTTGGAAAAGATTTTGATGTT R:CCCAATTCAATTCAATTCAATC	216	50	Bisulfite sequence PCR
XM_005906893	*SYCE1*	F:TGCAGAAAGTGGGAAGCCTA R:TCCTCTGTGCCTCATTCTCC	249	58	Real-time PCR
DQ838049	*β-actin*	F:CTGGACTTCGAGCAGGAGAT R:TAGTTTCGTGAATGCCGCAG	172	58	Real-time PCR

F – forward primer; R – reverse primer.

Bisulfite Sequencing

The BSP primers were designed by a web-tool named the MethPrimer software [Li et al.2002] (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi), and the CpG islands were examinedinvestigated usingwith this online program. The PCR primer sequences arewere listed in Table 1. We used the TaKaRa Taq™ Version 2.0 plus dye (Takara, Dalian, China) for BSP. PCR was performed in 50μL of reaction volume, containing 100 ng genomic DNA, 20 μM of each primer, and 25μL Takara EpiTaq HS (Takara, Dalian, China). The PCR was performed inwith a DNA Engine Thermal Cycler (Bio-Rad, USA) applyingby the following program: 35 cycles of 30 s at 94°C, annealing for 40 s at 50°C and extension for 40 s at 72°C. For sequence analysis the obtained, PCR products were then purified with a Gel DNA Extraction Kit (Takara, Dalian, China) and cloned into pMDTM19-T vectors (Takara, Dalian, China). Individual clones were sequenced by standard Sanger sequencing. Multiple sequence alignment were analyzed by the DNAMAN V5.2.2 software. All of the sequences were further analyszed using the QUMA online methylation tool (http://quma.cdb.riken.jp/) [Kumaki *et al.* 2008].

Statistical analysis

We calculated the total percentage of methylated CpGs in each group using the QUMA software and compared the methylation patterns among different species. The levels of gene expression and DNA methylation status of promoter regions of the *SYCE1* gene for the cattleyak and the yak were tested by the paired t-test as implemented in the Graphpad prism 8.0 [Pfaffl 2001]. software. Data were expressed as means (and standard error) \ from each group and significant differences were set at $P<0.05$ and $P<0.01$, respectively.

Results and discussion

Characterisation of 5'-untranslated region of SYCE1 for yaks

Initially, due to the lack of DNA sequence information on the yak *SYCE1* gene in the GeneBank database we designed primers to amplify the 5'-untranslated region based on the *SYCE1* sequence of cattle comprising the promoter region, exon 1 and partial intron 1 region. The results of the amplification are shown in Fig. 1A. Sequences of the individually amplified PCR products resulted in a 1090-bp fragment. Bioinformatic analysis using BLAST revealed that 98.03% of the 5'-untranslated regions of *SYCE1* for the yaks was homologous to the corresponding sequences of cattle. After promoter prediction analysis (http://www.cbil.upenn.edu /tess/), the core promoter region was determined as located from -515 to -353 nt in the 5'-untranslated regions, which was 163 in length and included multiple transcription factor (TF) binding sites, such as Sp1, SF-1and NF-1 (Fig. 3).

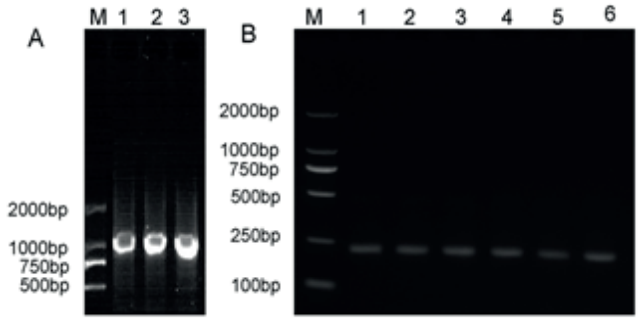

Fig. 1. Representative gel photographs of PCR-amplified fragments. (A) Representative gel photograph of PCR-amplified yak SYCE1 5'region sequences. (B) Representative gel photograph of BSP analyses using bisulfite-treated DNA. 1-3: yaks; 4-6: cattleyaks.

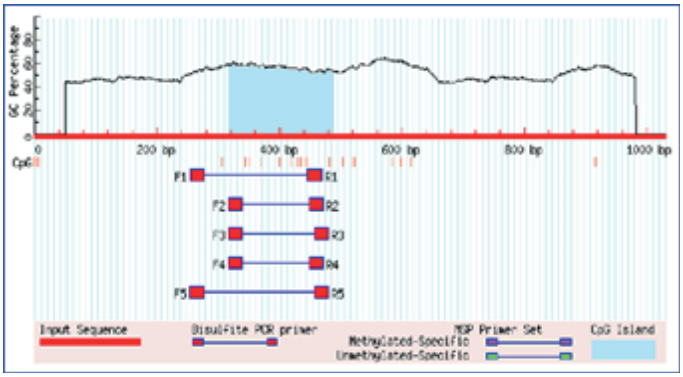

Fig. 2. Prediction of the CpG island in the promoter region of yak SYCE1.

SYCE1 gene expression profile in testes of yaks and cattleyaks

In order to estimate whether the expression of *SYCE1* was correlated with hybrid male sterility, we used the cDNA from six testes of six animals to detect the mRNA expression of *SYCE1*. The relative expression levels of the *SYCE1* gene in the two species, the yak and the cattleyak, are shown in Figure 4. A striking (almost 5-fold) reduction of *SYCE1* expression was found by real-time PCR, which reveals significantly reduced mRNA expression of *SYCE1* in the testes of cattleyaks compared with yaks ($P<0.0001$), suggesting that there may exist a strong correlation between the drastic down-regulated status of the *SYCE1* gene and the spermatogenic arrest of male cattleyaks. However, further research on *SYCE1* functioning is needed.

DNA methylation analysis by BSP

The CpG island of the promoter region of the yak *SYCE1* gene was predicted applying the MethPrimer online tool at the CpG island prediction website. It was found that there is one CpG island in the 5'-untranslated region *SYCE1*, consisting of a

AAG GTCCGTCTAG TCAAGGCTAT GGTTTTTCCT GTGGTCATGT ATGGATGTGA -841
GAGTTGGACT GAGAGAAGGC TGAGTGCCRA ATAATTGATG CTTTTGAAGT GTGGTGTTGG -781
AGAAGACTCT TGAGAGTCCC TTGGACTGCA AGGAGATCCA ACCAATCCAT TCTGAAGGAG -721
ATGAGCCCTG GGATCTTTTT GAAGGAATGG TGCTAAAGCT CAAACTCCAG TACTTTGGCC -661
ACCTCATGCA AAGAGTTGAC TCATTGGAAA AGACTCTGAT GCTGGGAGGG ATTGGGGGCA -601
GGAGGAGAAG GGGACGACAG AGGAGGAGAT GGCTGGATGG CATCACTGAC TCGATGGACG -541
TGAGTCTGAG TGAACTCCGG GAGTTGGTGA TGCCGCCCGGA GGCTGGCGGT GCTGCAATTC -481
Sp1 NF-1
ATGGGGTCGA AAAGAGTGAC CTTCAGCTGA GCGACTGAAC TGGCTATGGT TTTTCTGGGG -421
SF-1 Core-binding factor
GACAATTTAC GGCCTCCCTC CAGAAAACCA CGCAGAGTCC TTGGGTAGGG CGGCAACCTC -361
Promoter Sp1
TCAACATGTG CAGAGACTCT GCTCCCCACC ACCAGGTTCA GGTGCCCTCC TCGCAAGGTG -301
GGTGACGCCA CTTGGGACAG TCCCTCGTGA GACCTCTGTC ATTAGCAGGT CGCCGGCTCC -241
TCAACTAGCC AATCGCCAGC CTGGAAGACG CGTGCGCGCC CCACACCTGC TCTGCACGGA -181
ATCTGTTCCG GGTGGACTCA AGCGTGCGCA CCGGAGCTTG CCCAACCGCC ACCCTAGCTG -121
CCGGCTGGAA GAAGGGTACG GCGCAGAGGT GCACTGCGCA GGCGTGCTGA GGACGCCCCG -61
CCCCTGGCGC GGGTTCCGTC CAATCCCAGA GGAGGCGCTC AGCCGGTGAA CTGAGAGGAC -1
ATGGCTGGGC GGCCGGGATC GTCGAATGCT GAAGCGGCGG GAGCCGTGGG CCCGACTGAC 60
GAGGCCAGAG GTAGAGCTGG GCCTGGCGGC GGCCCCCAAC CGCGGCTGTT GAAGGCTGGG 120
CGGGCAGGGA GCGCACGCAG ACGCCGCTTC CTCAGAGTCG GGGCGGGCGT GAGGAAAAAA 181
TGACCTTCAG CCCCATGGGA AGGATCTTGG CGGC

Fig. 3. Sequence analysis of the 5' regulatory region sequence of yak SYCE1. Nucleotide numbering is relative to the ATG (+1) translation start site. The solid line indicates the core promoter. Putative binding sites for the transcription factors are bold underlined.

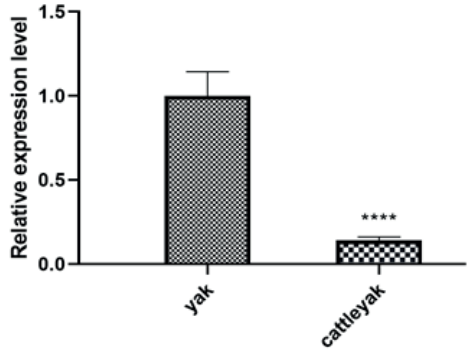

Fig. 4. Transcriptional levels of SYCE1 in testes of yak and cattleyak. ****Significant difference ($p<0.0001$).

fragment of 175 bp (-579 to -404 from ATG), and the results are shown in Figure 2. To assess the methylation status of the promoter region of yak *SYCE1*, Bisulfite sequence PCR was applied to amplify the *SYCE1* promoter (Fig. 1B). We then validated the

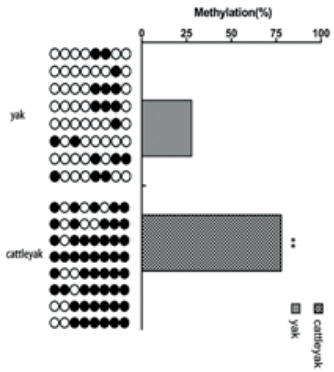

Fig. 5. Methylation analysis of SYCE1 promoter in testes of cattleyaks and yaks testes. Bisulfite sequencing for yaks and cattleyaks. Each line represents an individual bacterial clone that was sequenced. Open and filled circles represent unmethylated and methylated CpG sites, respectively.

correctness of PCR amplification products via sequencing. The DNA methylation pattern of the *SYCE1* promoter in the testes of yaks and cattleyaks analysed by BSP is shown in Figure 5. A total of 64 sites were identified in all of the clones. Methylation data from BSP sequencing were analysed by calculating the percentage of methylated CpGs in the total number of CpGs using the QUMA software. For the CpG islands the methylation status varied remarkably in different species. More specifically, methylation analysis indicated that the level of *SYCE1* promoter methylated CpG sites was 28.125% and 78.125% in yaks and cattleyaks, respectively. The *SYCE1* CpG methylation level in cattleyaks was significantly higher than in yaks (P<0.01) (Fig. 5).

The SC in mammals was discovered over 47 years ago through elegant embryological and genetic experiments on mouse spermatocytes. Successful progression of mammalian meiosis depends on the assembly of the SC [Bolcun-Filas *et al.* 2009, Schramm *et al.* 2011, Fraune *et al.* 2012]. During the preleptotene to the zygotene stage of meiosisI, a ladder-like structure is assembled to the autosomes and the sex chromosomes. The tripartite SC structure consists of a CE flanked by two parallel lateral elements (LE), which has been highly conserved throughout evolution. Undoubtedly, technical progress has greatly promoted advances in the field of molecular structure, resulting in a ful understanding of the mammalian SC protein structure. Four CE proteins (SYCE1, SYCE2, SYCE2 and TEX12) contributing to the SC accomplishment and synapsis have been identified employing the knockout models and immune-EM localisation tools. The *SYCE1* is stage- and testis-specific expressed during the spermatogenesis and it is essential for stable homologue interactions mediated by the SC and crossover formation [Maor-Sagie *et al.* 2015]. Unlike the normal spermatogenesis of other mammals, this process in the cattleyak is blocked seriously from the pachynema of the primary spermatocyte to the stage of subsequent formation of spermatozoa. A number of researches provided evidence that the spermatogenetic arrest is mainly caused by the failure of SC assembly. In

this study we observed that the mRNA expression level of the *SYCE1* in the testes of yaks with normal meiosis and spermatogenesis was significantly higher than that of cattleyaks with spermatogenesis arrest and male infertility, while the level of the *SYCE1* promoter in the cattleyaks was characterised by a remarkably higher rate of methylation compared with their maternal species, providing novel evidence that the expression of the *SYCE1* gene was epigenetically altered.

Central element proteins may be divided into two apparently distinct groups. Previous studies show that *SYCE1* and SYCE3 are essential for the SC three-dimensional structure formation, thus they are considered as synapsis initiation factors. In detail, *SYCE1* and *SYCE3* are co-localised in a continuous pattern identical to that of *SYCP1*, while their disruption leads to a complete failure of tripartite structure formation [Bolcun-Filas *et al.* 2009]. By contrast, *SYCE2* and *TEX12* co-localise in a constitutive complex that self-assembles into long filaments in vitro. They are required for long-range synapsis, but not for the formation of short stretches of the SC tripartite structure in vivo, so they are considered as synapsis elongation factors [Bolcun-Filas *et al.* 2007]. In general, *SYCP1* forms a complex with *SYCE1*, while *SYCE3* and *SYCE2* form a separate complex with TEX12 [Bolcun-Filas *et al.* 2007, Davies *et al.* 2012]. These findings are also consistent with data on the gene knockout mouse phenotype [Costa *et al.* 2005, Bolcun-Filas *et al.* 2009, Maor-Sagie *et al.* 2015]. A complete loss of the *SYCE1* function blocks the progression of spermatocytes into the first meiotic division, resulting in complete male sterility.

Male sterility in hybrids and the disruption of reproductive isolation by spermatogenesis are well-known, but the underlying mechanisms remain elusive [Bhattacharyya *et al.* 2013, Wang *et al.* 2016]. Meiotic chromosomes align and synapses play an important role in meiosis, ensuring precise separation of homologous chromosomes. Multiple chromosomal asynapsis is a fairly common meiotic aberration that triggers pachynema checkpoints and meiosis arrest, ultimately leading to germ cell arrest and apoptosis [Wang *et al.* 2016]. Recently, the studies searching for genes that are specifically responsible for male infertility, especially meiosis-related events, have identified them based on the single-cell multi-omics sequencing analysis and phenotypes of knockout mice [Bolcun-Filas *et al.* 2007, Bolcun-Filas *et al.* 2009, McDermottNoor 2010, Chen *et al.* 2018b, Wang *et al.* 2018b], providing insight into the meiosis and the mechanisms of hybrid male infertility.

As an important epigenetic modification, DNA methylation at the promoter region usually down-regulates the expression of the gene [Jones 2012]. Meantime, as a crucial factor of transcriptional repression, many typically biological events may be influenced in different manner under this epigenetic regulation, such as chromatin structure, DNA conformation and chromatin stability. Recently, studies have focused on the connection between DNA methylation patterns and interspecific crosses showing that an inappropriate regulation of a specific pattern in the organisms of interspecific hybrids can lead to reproduction arrest [Watson *et al.* 1998, Brown *et al.* 2012, Bhattacharyya *et al.* 2013]. Furthermore, in mammals the variable DNA

methylation in promoter regions observed in interspecific cross hybrids alters transcriptional regulation [Guo *et al.* 2017, Jiang *et al.* 2017]. Male cattleyaks of the F1 hybrids are sterile due to the failure of normal spermatogenesis, resulting in no XY bivalents in primary spermatocytes of cattleyaks [Yu *et al.* 2016], with the SC structure of autosomes observed only in a few primary spermatocytes of cattleyaks. In recent years many researchers have found that the high methylation level of promoter regions for corresponding several meiosis-related genes could suppress gene transcriptional activity and thus result in downregulated gene expression, such as the expression of *SYCP3* and *FKBP6* was significantly lower in testes of the cattleyak hybrids than in their parental species [Wang *et al.* 2012, Li *et al.* 2016]. Some of the primordial germ cell marker genes illustrated developmental-stage-specific expression patterns [Guo *et al.* 2015, Li *et al.* 2018]. For instance, it has been found that the essential part of SC components, *SYCE1*, was upregulated from the leptotene to the zygotene stage, indicating that the accumulation of *SYCE1* mRNAs prepared for the next step double-strand DNA breaking and homologous chromosome aligning and synapsis [Monesi 1964, Wang *et al.* 2018a]. Coincidently, a study revealed that the low methylation levels at the promoter regions of *SYCE1* in the consecutive stages of leptotene and pachytene spermatocytes were maintained, demonstrating clearly that DNA methylation in the gene promoter was usually negatively correlated with gene expression [Guo *et al.* 2015, Chen *et al.* 2018a]. As expected, our study observed the dramatic DNA methylation level of the promoter region in the *SYCE1* gene in the testes of cattleyaks when compared to that of yaks. In normal testes, the SYCE1 gene of primary spermatocytes exhibited a hypomethylated status by the pachytene, but this process was markedly inhibited in cattleyak testes. Therefore, we speculate that the DNA methylation status of the *SYCE1* promoter may play a role in regulating its transcriptional activity and be responsible for silencing of *SYCE1*.

To our knowledge, the high methylation level of the *SYCE1*promoter in cattleyaks coincides with a low expression of *SYCE1*. We propose that *SYCE1* in the testes of cattle yaks is a specific gene that is silenced by methylation and inactivation in interspecific crosses that occurs as a result of species incompatibility of the methylation machinery. Overall, we provide new evidence for the involvement of the *SYCE1* gene in the underlying regulatory mechanism of male infertility in cattleyaks.

Taken together, our work is the first to report changes of the promoter methylation status and mRNA expression of the *SYCE1* gene in the testes of yaks and cattleyaks. The interspecific hybrids exhibited a strikingly lower mRNA level of *SYCE1* and a significantly higher DNA methylation level in the *SYCE1* promoter than in the yaks. This new evidence is consistent with the results of single-cell multi-omics sequencing of spermatogenic cells in mammals. In brief, spermatogenic arrest may be caused by the inhibition of the mRNA expression of the *SYCE1* gene by high methylation of the promoter in cattleyaks.

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