The RNAi vecter construction and verification of the functions in sexual control of *Cervus elaphus Zfx* gene

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Abstract

Background: Zinc finger protein X-linked (*Zfx*) was regarded to be a sex determination factor, and plays a critical role in spermatogenesis. RNAi is an effective method of silencing *Zfx*/*Zfy* mRNA expression. However, there has been little research on the use of RNAi technology to control the sex of the offspring of *Cervus elaphus*. The objective of this study was first to explore an efficient method to alter the *Cervus elaphus* offspring sex-ratio by silencing the genes *Zfx* during spermatogenesis.

Results: Three recombinant expression vectors pLL3.7/A, pLL3.7/B and pLL3.7/C were constructed to interrupt the *Zfx* gene. The results showed that the expression of *Zfx* mRNA was significantly silenced by pLL3.7/A ($P < 0.01$), compared with the control group. The group injected with pLL3.7/A produced 94 *Cervus elaphus*, including 68 males and 26 females. The male rates (72.34%) were significantly higher than the control groups ($P < 0.01$).

Conclusions: Our experiment suggest that the *Zfx* gene plays a significant role in the process of X-sperm formation. *Zfx* siRNA may be a useful approach to control offspring sex in *Cervus elaphus*.

Background

The Cervus elaphus is the second-class protected animal in China, and an important economic animal. It has high output of antler and great economic value. Antler is a kind of precious traditional Chinese medicine and a valuable nourishing health product [1, 2]. Velvet antler is considered to have various pharmacological activity, such as immunomodulatory, wound-healing effects, anti-inflammatory and enhancement of sexual function [2–4]. The main purpose of raising deer is to obtain antler and only male produces antler, so the producers have great interest in obtaining more male fawns for the economic benefits of Cervus elaphus breeding. Therefore, in this study, RNAi technology was used to control the sex of the Cervus elaphus and improve the male ratio, which is an important means to increase the production of antler and increase economic benefits.

Up to now, flow cytometry (FCM) analysis has been the most accurate method for sperm separation, which accuracy can be reached up to 90% [5]. But it is relatively inefficient, requiring specialized, expensive and immobile equipment as well as skilled technicians. Therefore, an alternative way is to find a new sex control method which could interfere with X chromosome generation during spermatogenesis. Zfx/Zfy is thought to play a major role in sex determination during spermatogenesis, which is located on the X/Y chromosome [6–9]. The binding of zinc finger protein motifs to nucleic acid binding proteins plays an important role in sex determination of early embryos [10, 11]. Recent reports on mice and humans have shown that Zfy is essential for sperm formation, fertilization and reproduction [12]. Zfx is concerned with X spermatogenesis, can serve as a strong transcription activation factor, and guides the target genes positioning in X sperm nuclei through the nuclear membrane, it may also have a transcription activation function for some genes during X spermatogenesis [13]. Silencing the expression of Zfx may interfere with the normal function of these sex-specific genes after meiosis I [14]. Therefore,

simple methods of identifying Zfx at the level of RNA alone may allow for directional manipulation of X or Y sperm, respectively.

The RNAi technology has the advantages of simple operation, low cost, quick effect, high accuracy and good repeatability. RNAi is an effective method of silencing Zfx/Zfy mRNA expression [14–17]. However, there has been little research on the use of RNAi technology to control the sex of the offspring of Cervus elaphus. Therefore, silencing Zfx expression in testis by RNAi during spermatogenesis may change the sex ratio of Cervus elaphus offspring. In this study, specific RNA interference fragments were first designed to interfere with development of X sperm, in order to change the male proportion of Cervus elaphus offspring and improve the market economic benefits of Cervus elaphus industry in world.

Results

Spermatogenic cells separation and transfection

Collagenase IV, hyaluronidase and trypsin were used to digest the testicular tissue of Cervus elaphus into spermatogenic cells and Sertoli cells, the segregated spermatogenic cells were round and full in shape with a clear outline and uniform size. However, the isolated Sertoli cells were irregular, mostly of them were polygonal and triangular (Fig. 1). After culturing 24 h, reconstructed vectors were transfected into cultured cells. There was no fluorescence expression at 24 h after transfection, however, there was some fluorescence expression at 48 h, and the highest fluorescence expression was at 58 h, moreover the transfection efficiency could reach about 92%. However, at 72 h, the viability of the cells decreased, and some cells showed apoptosis. So, after transfection for 58 h, the cells mRNA was extracted and then reverse-transcribed into cDNA. The results of transfection at 58 h showed that the Zfx gene RNAi vector were successfully transferred into spermatagonia of the Cervus elaphus (Fig. 2).

The expression of Zfx mRNA in injection groups and control groups by qRTPCR

The Zfx mRNA levels results showed that all recombination vectors were lower than the control groups which were the blank and vector-free group (Fig. 3). Specifically, the Zfx mRNA level of test groups injected with pLL3.7/A ($P < 0.01$) were significantly lower than control groups, and transfected pLL3.7/B were lesser than the control group ($P < 0.05$). Therefore, pLL3.7/A vector was selected for use in the in vivo experiment because it had the least expression of Zfx mRNA ($P < 0.01$) (Table 2).

Table 2
Zfx RNAi oligonucleotide sequence with HpaI and XhoI enzyme sites.

Vector shRNA	Oligonucleotide sequence
pLL3.7 A	F: 5'- TGTGAGGACTACCTTATGATTTCAAGAGAATCATAAGGTAGTCCTCACTTTTTTC-3'
	R: 5'- TCGAGAAAAAAGTGAGGACTACCTTATGATTCTCTTGAAATCATAAGGTAGTCCTCACA-3'
pLL3.7 B	F: 5'- TGACGTCTTAGCTTCTGATATTCAAGAGATATCAGAAGCTAAGACGTCTTTTTTC-3'
	R: 5'- TCGAGAAAAAAGACGTCTTAGCTTCTGATATCTCTTGAATATCAGAAGCTAAGACGTCA-3
pLL3.7 C	F: 5'- TGAAGCAGATGTATCTGAAATTCAAGAGATTTCAGATACATCTGCTTCTTTTTTC-3'
	R:5'- TCGAGAAAAAAGAAGCAGATGTATCTGAAATCTCTTGAATTTCAGATACATCTGCTTCA-3'
Notes: Using pLL3.7 as the vector construction of shRNA interference fragment.	

Statistical analysis sex proportion of the offspring

The non-injection group, as control group, produced 98 Cervus elaphus, including 50 males and 48 females. While, the group injected with pLL3.7/A produced 94 Cervus elaphus, including 68 males and 26 females. The male rates (72.34%) were significantly higher than the control groups ($P < 0.01$) (Table 4). In addition, the fertility rate, dystocia rate, abortion rate, reproduction rate, empty embryo rate, offspring survival rate and offspring deformity rate of the female deer experimental group were not abnormal compared with the control group.

Table 4
The number of female and male offspring in each group of Cervus elaphus.

Subgroup	Pregnancy rate	Stillbirth rate	Female	Male	Male rate
Non-injection	92.3%	2%	48	50	51.02%
Injection	89.8%	3%	26	68	72.34%**
** means extremely significant difference between non-injection group and treatment groups ($P < 0.01$).					

This result suggest that the Zfx gene plays an important role in the process of X-sperm formation. Zfx siRNA is an advanced method to control Cervus elaphus X spermatogenesis and sex ratio of offspring.

Discussion

Although the reproduction of sex determination mechanism requires multiple gene interactions, the sex ratio in nature is stable at 1:1 [18]. The formation of X or Y sperm during spermatogenesis plays an important role in sex determination of offspring [19]. Previous studies in our laboratory found that Zfx or Zfy siRNA can change the sex ratio of animal offspring, such as cows, mouse, sheep, swine [14, 17, 20]. Cervus elaphus is a special economic animal and its antler has great market economic value. However, there has been little research on the use of RNAi technology to control the sex of the offspring of Cervus elaphus. In our study, the Cervus elaphus Zfx siRNA was first designed to change the sex ratio of the offspring, once again demonstrating the important role of Zfx in gender. More importantly, it is of great significance to increase the male ratio of Cervus elaphus offspring to increase antler yield and economic benefits.

At present, there are only a little research on Zfx gene silencing to make the sex shift of offspring, so the mechanism of action of Zfx in sperm is not clear. Some studies have shown that reducing the level of Zfx/Zfy mRNA expression will make the physiological function of X/Y sperm worse, which can influence offspring gender [14, 17, 20–22]. Zfy is closely related to sperm head and tail formation and neck development [23], which is highly expressed between meiosis I and II. It can regulate sperm morphology and ROSI efficiency, and promotes second meiosis [24, 25]. Similarly, Zfx gene is female-specific after meiosis I and is always expressed throughout spermatogenesis [13]. The high expression of Zfx in round sperm cells of adult mouse testis is closely related to the self-renewal of spermatocytes. Generally speaking, Zfy gene is to ensure the occurrence of the second meiosis, and the X-related gene Zfx on the chromosome has a promoting effect [26]. Reducing the expression level of Zfx mRNA will affect the development of haploid X sperm, which will lead to the decrease of female offspring [14].

However, there was no health abnormality and decreased reproductive performance in experimental animal group, compared with the control animal group. Then male Cervus elaphus were placed in female breeding enclosures for natural mating within 10–40 days after the fourth injection of the vector. It depends on the sheep's spermatogenic cycle and spermatogenic wave [27] which's Zfx homology is closest to Cervus elaphus. In addition, the fertility rate, dystocia rate, abortion rate, reproduction rate, empty embryo rate, offspring survival rate and offspring deformity rate of the female deer experimental group were not abnormal compared with the control group. In conclusion, under the same feeding conditions, compared with the normal control group, the sex ratio of offspring in the experimental group shifted significantly, and the male ratio reached 72.34%. It indicated that the injection of interfering fragments changed the sex ratio of offspring without effecting on the physiological function and reproductive performance of animals. Therefore, silencing of the Zfx maybe only interferes with the formation of haploid X sperm, but not the normal development of embryo.

Spermatogenesis is a highly regulated and orderly process. It is regulated by many genes, but its mechanism is still unclear [28, 29] and further research is needed. However, this study, the Zfx gene silencing technology was successfully used to significantly increase the number of male Cervus elaphus in the offspring, which not only increased the economic benefits in production, but also provided theoretical reference for the subsequent research on gender control mechanism in scientific research.

Conclusion

The results of this study indicate that the use of RNAi to interrupt Zfx may be a useful approach to control Cervus elaphus sex which lays a foundation for producing sex control semen.

Methods

Experimental animals

Healthy Cervus elaphus were selected from the Xinjiang Production and Construction Corps Second Division 34, including16 stags and 216 does. Among them, we randomly choose 8 stags and 108 does as an experimental group and the rest as a control group. All experimental animals were fed under the same conditions. All procedures and experimental animals used in this experiment were reviewed and approved by the Animal Care and Use Committee of Shihezi University. All orchiectomy surgery was performed under anaesthesia with 4 ml Xylazine Hydrochloride Injection(100 mg/ml) per deer and resuscitation with 8 ml Nixamide Injection (250 mg/mL) per deer according to the instructions, and all efforts were made to minimise suffering. This study of injection plasmid did not cause any health effects to the animals. After the experiment, all experimental animals were kept as normal.

Construction of PLL3.7-shZfx recombination vectors

Zfx siRNA sequences were evaluated and selected by Cervus elaphus Zfx sequence (KP257294.1) and siRNA design principles [30, 31]. According to the design principle on siRNA, preliminary screening of the designed siRNA sequences was processed, and then the siRNA sequences were selected and compared the homology with Cervus elaphus Zfy sequence (MN560153) through BLAST. Nonspecific fragments were eliminated and 3 specific siRNA sequences was finally selected (Table 1). The restriction endonucleases (Hpai and Xhoi) were added to the 5' and 3' ends of siRNA sequence. The sense and antisense strands of each oligonucleotide sequence (Table 2) were annealed. The annealed product was connected to pentilox 3.7 (pLL3.7) cut by Hpai and Xhoi (TaKara, Biotechnology Co, Dalian, China). The recombinant siRNA expression vectors were named as pLL3.7/A, pLL3.7/B and pLL3.7/C, and then transformed into E. coli dh5α active cells (Tiangen Biotech Co., Beijing, China). The plasmids were extracted from the bacterial solution using TIANprep Mini Plasmid Kit (Tiangen Biotech Co, Bei-jing, China) and stored at -20℃.

Table 1
Zfx cDNA templates for siRNA in vitro transcription.

Zfx (KP257294.1)	
A	GTGAGGACTACCTTATGAT
B	GACGTCTTAGCTTCTGATA
C	GAAGCAGATGTATCTGAAA

Zfx gene RNAi experiment in vitro

Isolation and culture of spermatogenic cells

The Cervus elaphus was anesthetized with 4 ml Xylazine Hydrochloride Injection(100 mg/ml) to make it lose consciousness, then the common artery was cut to bleed, and it died immediately. Then take the testicles aseptically, put them in the ice box, and reach the laboratory within 1 h. The improved two-step enzymatic digestion method was used to isolate spermatogenic cells of the Cervus elaphus testis [32]. The spermatogenic cells were co-cultured with Sertoli cells in vitro in a CO_2 incubator (Gu et al.2009; Wang et al.2015). The cells were cultured in 6 well cell culture plates and each well contained 3×10^5-6×10^5 spermatogenic in an incubator at 37℃ temperature, 5% CO_2, and 95% relative humidity.

Transfection of Zfx RNAi vector and qRT-PCR analysis

After culturing 24 h, reconstructed vectors were transfected into cultured cells using Lipofectamine 3000 (Invitrogen, USA). The nutrient solution was changed and the cells were observed under Lipofectamine 3000 every 24 h during culturing. In this experiment, the expression of fluorescent protein was observed by fluorescence inverted microscope after transfection for 24 h, 48 h, 58 h, and 72 h, respectively. The highest transfection efficiency of cells RNA were extracted by Tiangen total RNA extraction reagent and then reverse-transcribed into cDNA by RT reagent kit (Takara).

The RNAi efficiency of each vector in vitro

The mRNA level of Zfx and Gapdh (Table 3) was determined by RT-qPCR (LightCycler2.0, Roche, Basel, Switzerland). The RT-qPCR reaction system contained 10 μL SYBR@ Premix Ex TAqTM (2×), 0.4 μL each oligonucleotide primers, 2 μL template and sterile distilled water up to 20 μL. And the program was as follows: initial denaturation step (30 s at 95 °C), 40 cycles of 95 °C for 10 s, 60℃ for 30 s, and 72 °C for 30 s. Each sample was replicated three times to increase the degree of accuracy.

Table 3
Conditions of PCR and oligonucleotide primer pairs.

Target gene	Gene bank	Sequence of Primer (5'→3')	Annealing temperature	Product /bp
Zfx	KP257294.1	F: TATGGATTCACTCGTCAA	60°C	120
		R: CTCAGATGTAACAGAAGAAG		
GAPDH	AY650282.1	F: AAGGCCATCACCATCTTCCA	60°C	80 bp
		R: CCAGCATCACCCCACTTGA		

Zfx gene RNAi experiment in vivo

The injection of plasmid in testis of Cervus elaphus

In experimental group, the recombinant plasmid expression vector was diluted with PBS containing Penicillin-Streptomycin, then injected into 8 stag's testis at 3 mg per stag (1.5 mg in each testis), for 4 times in 10 days. After injection, the 8 stags were randomly put into 8 female breeding enclosures (9–15 does in each breeding enclusures) for nature mating. The control group was normal stag without injection of RNAi vectors. During the whole experiment, all the conditions were the same.

Statistical analysis

Data were analyzed by SPSS Statistics 20.0 (IBM Corp.). The quantitation results were analyzed by using Gapdh expression as internal standard and double standard curve. The difference in expression of mRNAs was calculated using the Eq. $2^{\triangle\triangle Ct}$, where $\triangle Ct = Ct\ (Zfx) - Ct\ (Gapdh)$ and $\triangle\triangle Ct = \triangle Ct\ (Zfx) - \triangle Ct\ (control)$. The significant difference of differences in mRNA levels was quantified by one-way ANOVA (LSD). The significance of differences of male and female proportion of offspring was assessed using $\chi 2$ test. Figures were constructed by Graph Pad Prism 8.0.1.

Declaration

Ethics approval and consent to participate

All procedures used in this experiment were reviewed and approved by the Animal Care and Use Committee of Shihezi University. All *Cervus elaphus* care and use were conducted in strict accordance with the Animal Research Committee guidelines of Shihezi University.

Consent for publication

Not Applicable.

Availability of data and material

Cervus elaphus Zfx sequence and *Cervus elaphus* Zfy sequence are respectively archived at NCBI GenBank under accession numbers KP257294.1 and MN560153.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LMW carried out the experiments, analysed the data and wrote the manuscript under the supervision of BJ. BJ conceived the study and reviewed the data. SJ, JFX and YSZ conducted sample collection, data analyses and assisted with writing. BZ, FL, SYZ and KSW conducted sample collection, Plasmid extraction and assisted in writing. XTZ, YL and HS conducted Contacting deer farm and assisted with writing. All authors have read and approved this manuscript.

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Abbreviations

ZFY: zinc finger protein Y-linked; *ZFX*: zinc finger protein X-linked; *Gapdh*: glyceraldehyde-3-phosphate dehydrogenase; RNAi: RNA interference; pLL3.7: pentilox 3.7

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Figures

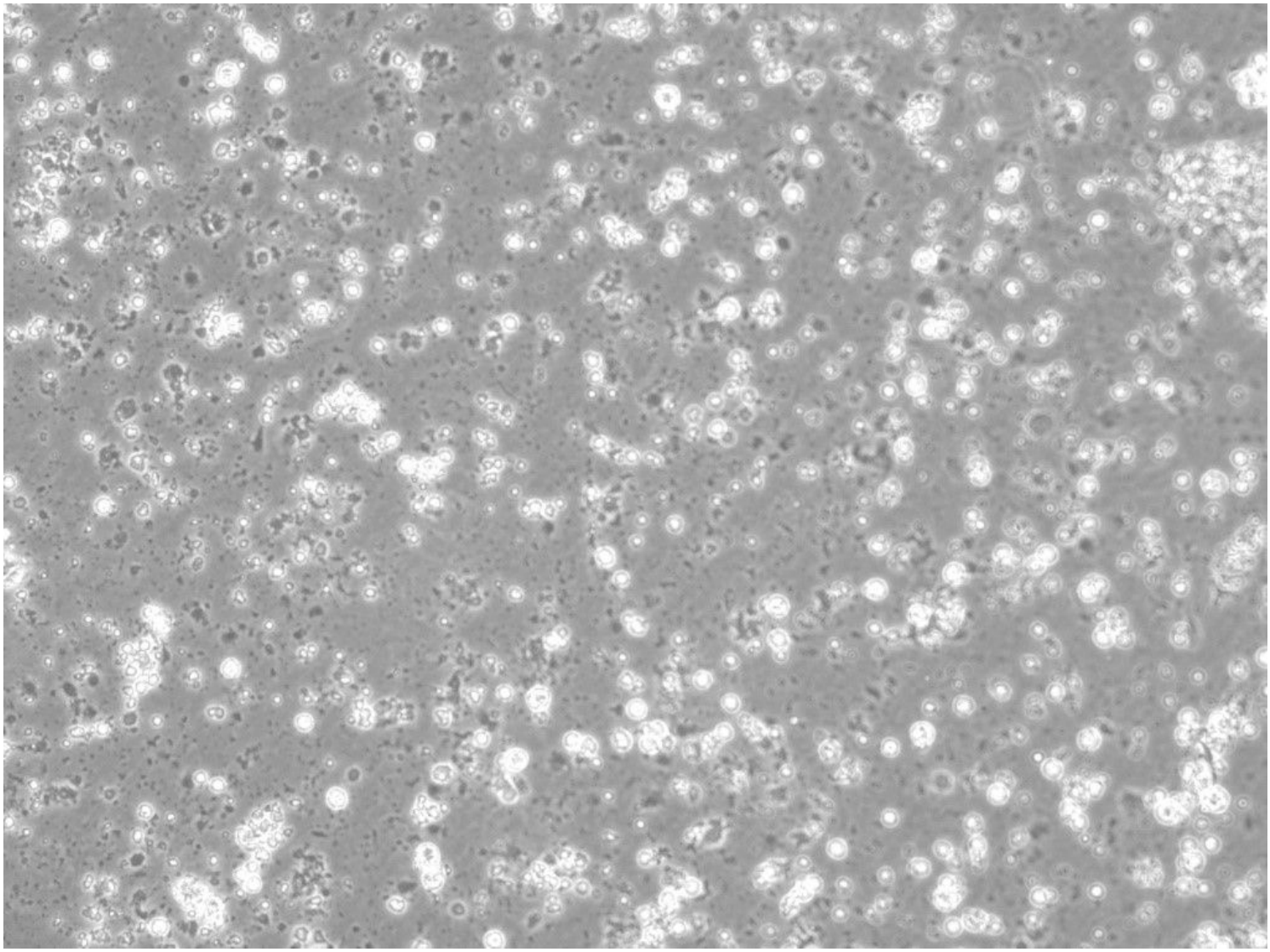
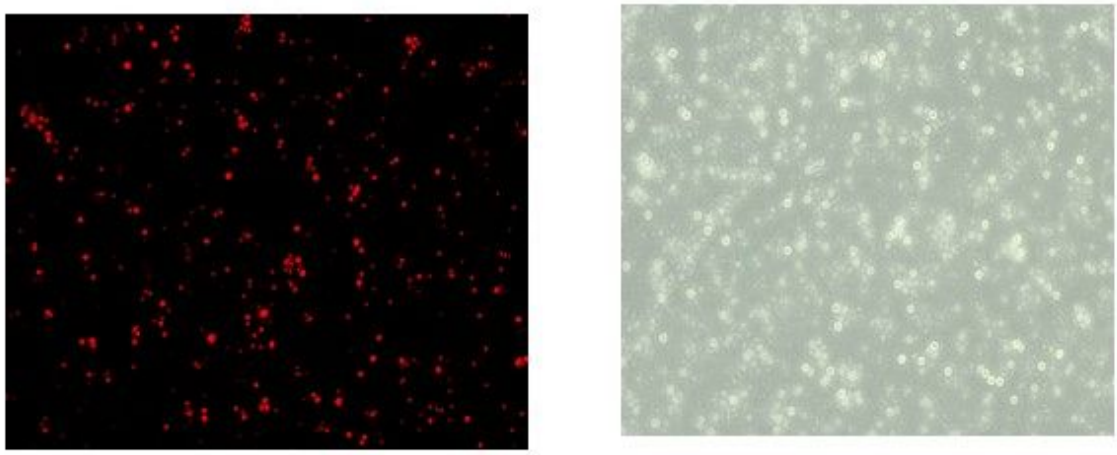


Figure 1

Separation of Cervus elaphus spermatogenic cells. Spermatogenic cells were round and full in shape with a clear outline and uniform size and the isolated Sertoli cells were irregular, mostly of them were polygonal and triangular. The cells were observed under the inversion fluorescence microscope (LeicaDMi8, Leica Microsystems, Wetzler, Germany) and were magnified 5×.



A: Bright field vision B: Dark field vision

Figure 2

Transfection interference vector 58 h of germ cells. Zfx gene RNAi vector were transferred into Cervus elaphus spermatogenic cells, and the fluorescent labels were transfected round spermatids. The cells were observed under the inversion fluorescence microscope (LeicaDMi8, Leica Microsystems, Wetzler, Germany) and were magnified 5× (A：Bright field vision, B：Dark field vision).

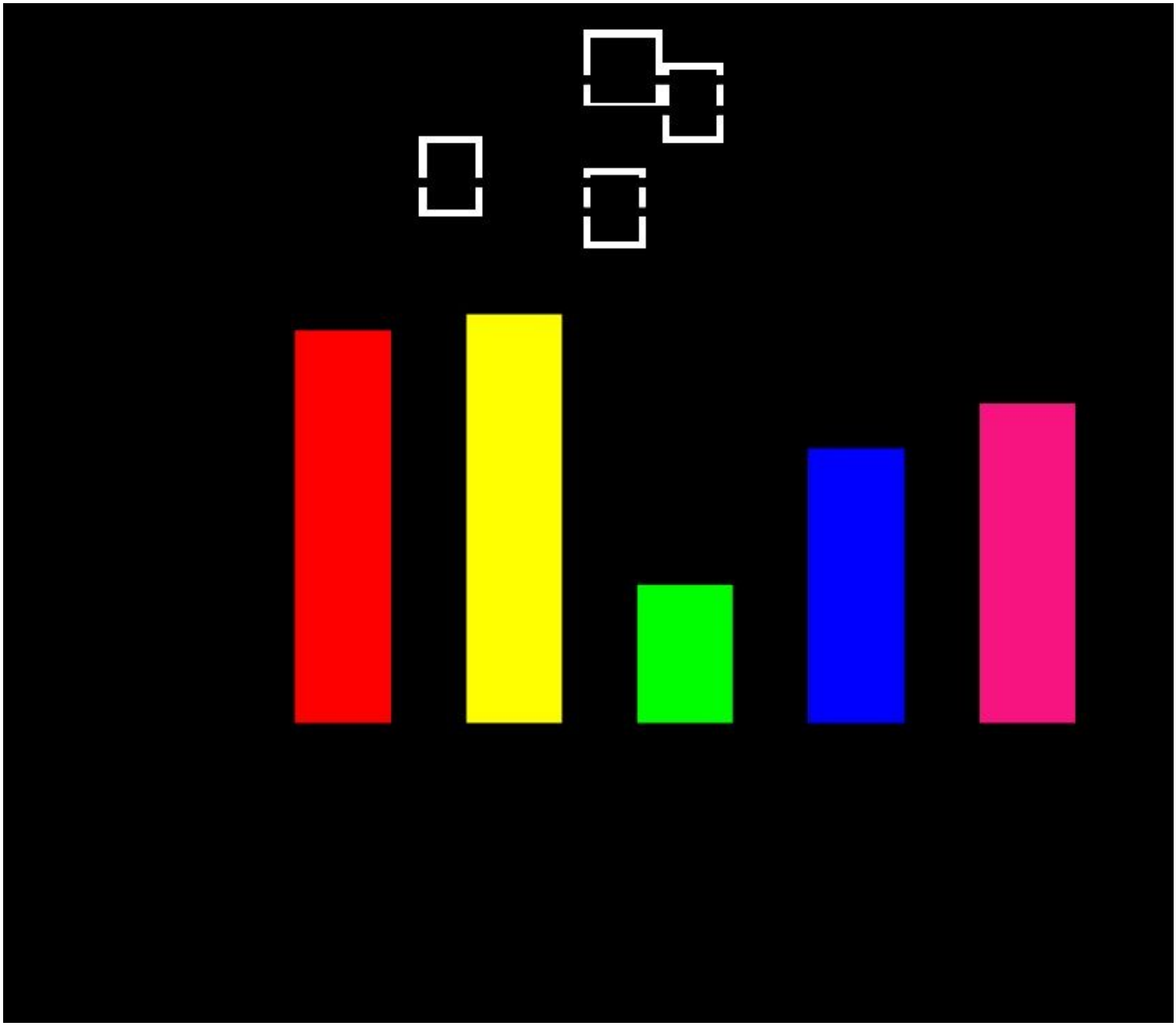


Figure 3

mRNA expression level of Zfx. Zfx mRNA expression was normalized against that of Gapdh, with expression in the control group□Blank group□. Data are the mean±s.d. *P □ 0.05, **P □ 0.01 compared with the control.

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