Animal Reproduction Science 200 (2019) 1–6

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Sex control by *Zfy* siRNA in the dairy cattle

Ji-feng Xi[a,b,1], Xiang-zu Wang[a,b,1], Yong-sheng Zhang[a], Bin Jia[a,*], Chao-cheng Li[a], Xu-hai Wang[a], Rui-wen Ying[a]

[a] *College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang, 832003, PR China*
[b] *Xinjiang Agricultural Vocational Technical College, Changji, Xinjiang, 831100, PR China*

ARTICLE INFO

Keywords:
Zfy
siRNA
Bos
Sex control
Spermatogenesis

ABSTRACT

Zinc-finger Y is located in the short arm of the Y-chromosome and is a highly conserved gene that plays an important role in spermatogenesis. The objective of this study was to investigate the influence of silencing the *Zfy* gene during spermatogenesis on Y-sperm formation and offspring sex determination in *Bos taurus* cattle. Three recombinant expression vectors pLL3.7/a, pLL3.7/b and pLL3.7/c were evaluated and only pLL3.7/a effectively silenced the *Zfy* gene. The pLL3.7/a recombinant expression vector was injected into bull testes, using three injections. Semen was collected and preserved by extending and freezing. The frozen semen was subsequently used in artificial insemination of cows during a breeding season in accordance with the production plan on the farm where the experiment was conducted. Results showed that, after exposure to pLL3.7/a, sperm motility decreased ($P < 0.01$), but the sperm density was similar ($p > 0.05$) to the non-treated control semen. Injection of pLL3.7/a resulted in 72.0% female offspring, and was greater than the 49.4% female calves in the control ($P < 0.01$), Results from this research suggests that the *Zfy* gene plays a role in the process of Y-sperm formation, and *Zfy* siRNA is a potential useful approach to control sex of offspring in cattle.

1. Introduction

Milk production is a sex-limited trait and the ratio of bull to heifer calves is potentially economically important in dairy production. Most of the current efforts in sex determination have focused on separation of X and Y-sperm based on physical parameters, such as swimming velocity, density, surface charge, or sex-specific antigens. Of the potentially available methods, only flow cytometry can sort sperm at greater than 90% accuracy, and it is currently commercially available. Although clearly effective, the use of flow cytometry as a routine tool for sex selection in animal breeding is relatively inefficient, requiring specialized, expensive, and immobile equipment and highly skilled technicians. It seems reasonable that interference with Y-chromosome production during spermatogenesis could be of benefit in provision of sex determination technology in cattle.

The *Zfy* gene is located on the short arm of the Y-chromosome Yp1113, a 13″zinc-finger" structure (Palmer et al. 1990). The *Zfy* gene has 11 exons and 1 random repeat region, with about a 30 residue motif and codes 801 amino acids, composed of two cysteines and two histidines coordinating with a zinc ion (Brown et al. 1985). The base sequence belongs to the X-degradation (Skaletsky et al. 2003), and is the initial sex determination factor (TDF). The zinc finger protein motif is associated with the nucleic acid-binding protein and plays an important role in early embryo sex determination (Nagamine et al. 1989; Mardon and Page 1989).

---

[*] Corresponding author.
E-mail address: jiabin@shzu.edu.cn (B. Jia).
[1] Both authors equally contributed to the work.

https://doi.org/10.1016/j.anireprosci.2018.05.015
Received 7 December 2017; Received in revised form 2 May 2018; Accepted 14 May 2018

Table 1
Zfy cDNA templates for siRNA *in vitro* transcription.

Zfy	(NM_177491.1)
A	GTGAGGACTACCTTATGAT
B	GTACCGCTGTACTGATTGT
C	GAAGTCATCAAGGTGTATA

RNA interference (RNAi) is a powerful research tool for gene silencing. It has been widely applied in mammalian cells and organisms (Shi et al., 2003; Dorsett and Tuschl 2004).

In our laboratory *Zfy* was silenced by RNAi to restrict or deteriorate the function of the Y-sperm during spermatogenesis and the results showed more female offspring of mice were produced compared with control (Pen et al.2011). Silencing the expression of *Zfy* in testis during spermiogenesis by using RNAi technology might affect offspring sex ratio in cattle. Thus, the objective of this research was to determine if the bovine *Zfy* gene could be silenced using siRNA vectors and to determine effects of gene silencing on sperm quality and subsequent sex ratios when gene-silence semen is used in artificial insemination in dairy cattle.

2. Material and methods

2.1. *Experimental animals*

All procedures used in this experiment were reviewed and approved by the Animal Care and Use Committee of Shihezi University.

Three 18-mo healthy Holstein bulls were selected from the Xinjiang Tianshan Animal Husbandry and Biological Engineering, Ltd. for this experiment. Breeding soundness was assessed and semen quantity and quality were normal for these bulls. Semen from these bulls was collected prior to treatment and used as the control.

2.2. *Construction of Zfy gene RNAi vector*

Consistent with the *Bos taurus Zfy* sequence (NM_177491.1) and siRNA design principles (Elbashir et al., 2002; Reynolds et al., 2004; Ui-Tei et al., 2004; Jagla et al., 2005; Fang et al., 2007) 3 *Zfy* siRNA sequences were evaluated and selected (Table 1) and synthesized (Sangon, China). Restriction endonucleases (HpaI and XhoI) were added at the 5′ and 3′ end of siRNA sequences. The sense and antisense strands of each oligonucleotide sequence (Table 2) were annealed. The annealed product was linked with pLentiLox3.7 (pLL3.7) which was incised by HpaI and XhoI (TaKara, Biotechnology Co.,Dalian,China). The recombinant siRNA expression vectors were constructed, designated pLL3.7/a, pLL3.7/b and pLL3.7/c, then transformed into E. coli DH5α competent cells (Tiangen Biotech Co., Beijing, China). The plasmids were extracted from the bacterial solution using TIANprep Mini Plasmid Kit (Tiangen Biotech Co., Beijing, China) and stored at -20°C.

2.3. *Zfy gene RNAi experiment in vitro*

2.3.1. *Isolation and culture of spermatogenic cells*

Spermatogenic cells of the bovine testis were isolated using the improved two-step enzymatic digestion method. (Izadyar et al.2002; Chang et al., 2011). The spermatogenic cells were co-cultured with Sertoli cells (Gu et al.2009; Wang et al.2015). The cells were cultured in 6 well cell culture plates with DMEM/F12 (supplemented with 10% fetal bovine serum; 10 μg/mL insulin; 10 μg/mL transferrin; 25U/L follicle-stimulating hormone ; 10^{-7} mol/L testosterone; 10^{-3} mol/L sodium pyruvate; 10^{-4} mol/L Vitamin C; 10^{-3} mol/L pyruvic acid, 100 U/mL penicillin, and 100 μg/mL streptomycin) at density of $1 \times 10^6/\text{cm}^2$ in an incubator at 37°C temperature, 5% CO_2, and 95% relative humidity.

2.3.2. *Transfection of zfy gene RNAi vector and qRT-PCR analysis*

After culturing 24 h, spermatogenic cells were transfected using the recombined plasmid. After 96 h, the cells were observed under inversion fluorescence microscope (LeicaDMi8, Leica Microsystems, Wetzler, Germany), and then mRNA was extracted from

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

Vector shRNA	Oligonucleotide sequence
pLL3.7 a	F: 5'- T GTGAGGACTACCTTATGATTTCAAGAGATCACTAGAGTTTGAACTCCTTTTTTC-3'
	R: 5'- TCGAGAAAAAAAGTGAGGACTACCTTATGATCTCTTGAATCACTAGAGTTTGAACTCCA-3'
b	F: 5'- TGTACCGCTGTACTGATTGTTTCAAGAGAACAATCAGTACAGCGGTACTTTTTTC-3'
	R: 5'- TCGAGAAAAAAGTACCGCTGTACTGATTGTTCTCTTGAAACAATCAGTACAGCGGTACA-3'
c	F: 5'- TGAAGTCATCAAGGTGTATATTCAAGAGATATACACCTTGATGACTTCTTTTTTC-3'
	R: 5'- TCGAGAAAAAAGAAGTCATCAAGGTGTATATCTCTTGAATATACACCTTGATGACTTCA-3'

Notes : Using pLL3.7 as the vector construction of shRNA interference fragment.

Table 3
Conditions of PCR and oligonucleotide primer pairs.

Target gene Products	GenBank no.	Sequence of primer (5'-3')	Annealing	temperature
Zfy 100bp	NM_177491.1	F: TTTAGGCAACAGAATGAGG R: ACAACGGTGAGGATAGTCTT		58.8℃
β-actin bp	AY141970.1	F: GATGTGGATCAGCAAGCA R: CCTTCACCGTTCCAGTTT	58.8℃	230

cultured cells by RNA simple Total RNA kit (Tiangen Biotech Co., Beijing, China). RNA was quantified by UV absorption at 260 nm. The RNA was then reverse-transcribed into cDNA by RT reagent kit (Takara Bio Inc.,Kusatsu,Shiga,Japan) according to the manufacturer's instructions.

The mRNA level of *Zfy* and β-actin was determined by RT-qPCR (LightCycler2.0, Roche, Basel, Switzerland), and the primers sequences used to amplify are listed in Table 3. All experiments were performed in triplicate. The RT-qPCR reaction system and programmed as follow: the total volume was 20 μL: 10 μL SYBR@ Premix Ex TAqTM (2 ×), 0.4 μL each oligonucleotide primers, 2 μL template and sterile distilled water to 20 μL. initial denaturation step (30 s at 95 °C), 45 cycles of 95 °C for 10 s, 61℃ for 30 s, and 72 °C for 30 s. Each sample was tested three times to improve accuracy.

The difference in expression of mRNAs was calculated using the equation $2^{\triangle\triangle ct}$, where△Ct = Ct (ZFY) − Ct (GAPDH) and△△Ct = △Ct(ZFY) − △Ct (control). An observed significance level of < 0.05 was considered to be statistically significant. Graphs were generated with MS Excel. Bar graphs show the mean ± SEM.

2.4. zfy gene RNAi experiment in vivo

2.4.1. The injection of plasmid in testes of Holstein bulls

In this experiment, we selected three breeding bulls as experimental animals. Bulls were injected in testicular net with pLL3.7/a (5 mg/bull) which was encased in liposomes,and the needle was pulled slowly while injecting.Bulls were injected once every nine days for a total of three injections.

2.4.2. Analysis of semen quality

Semen was collected every five days following the seventh day after the third injection using an artificial vagina. Fresh semen was evaluated for volume, initial sperm motility and sperm density and then preserved as frozen semen according to procedures of Xinjiang Tianshan Animal Husbandry and Biological Engineering, Ltd. Semen samples were assessed for post-thaw cryopreserved sperm motility and sperm density. A month before injection, semen were also analyzed for the three indicators.

2.4.3. Breeding

Holstein cows (n = 400) under good management were chosen for artificial insemination with the *Zfy*-silenced semen. For the sake of higher fertilization rate two straws of *Zfy*-silenced frozen semen were used for each cow inseminated.

2.4.4. Statistical analysis

Data were analyzed using SPSS 17.0 (Chicago,Illinois,America). With the exception of sex rations, data were analyzed as a completely randomized design with test of hypotheses done with t statistics where $P < 0.05$ was considered statistically significant. A χ^2 test was used to determine the difference of male and female proportion of offspring between the treatment and control groups.

3. Results

3.1. The transfection of Zfy gene RNAi vector and the expression level of Zfy mRNA in spermatogenic cells

The results of transfection in spermatagonia showed that the *Zfy* gene RNAi vector were successfully transferred into spermatagonia of the bulls (Fig. 1). The RT-qPCR of the *Zfy* gene showed that *Zfy* mRNA levels of spermatogonial cells which were transfected using plasmid pLL3.7/a were less than both the blank and vector-free group ($P < 0.01$), pLL3.7/b were numerically lesser than the blank and vector-free group ($P > 0.05$), and transfected plasmid pLL3.7/c were lesser than the blank group ($P < 0.05$) and the vector-free group ($p < 0.01$) (Fig. 2). Of the 3 plasmids evaluated, pLL3.7/a had the least expression ($P < 0.01$) and was selected for use in the *in vivo* experiment (Table 2).

3.2. Sperm quality

The effects of *Zfy* gene RNAi vector on fresh and frozen sperm quality of bull are shown in Table 4. For both fresh and frozen semen, sperm motility of the pre-injection group was greater than the post-injection group ($P < 0.01$), and the sperm abnormality rate in pre-injection group was lesser than the post-injection group ($P < 0.01$). However, there were no significant difference between the two groups in the sperm density for either fresh or frozen semen ($p > 0.05$).

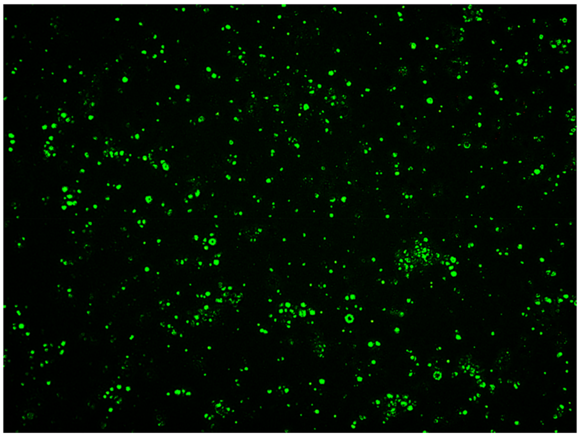

Fig. 1. Zfy gene RNAi vector were transferred into spermatogenic cells of cattle; Fluorescent label is transfected cells.the cells were observed under inversion fluorescence microscope (LeicaDMi8, Leica Microsystems, Wetzler, Germany) and cells were magnified 5×.

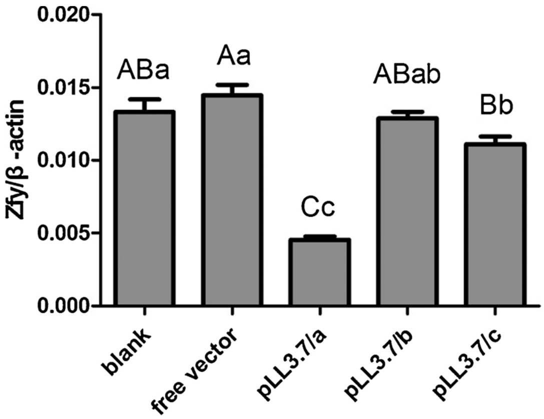

Fig. 2. mRNA relative abundance of Zfy. Columns with same letters indicate no difference between the two groups; Different capital letters indicate a difference ($P < 0.01$) between the two groups and different lowercase letters indicate a difference ($P < 0.05$).

Table 4

Effect of Zfy siRNA pLL3.7 / a plasmid injection of testicular net on motility, sperm density and abnormality rate of bull fresh and frozen sperm.

	Fresh semen		Frozen semen	
	pre-injection	post-injection	pre-injection	post-injection
Motility (%)	63.3 ± 2.9^{Aa}	33.3 ± 2.9^{Bb}	38.7 ± 1.2^{Bc}	18.3 ± 1.5^{Cd}
Sperm density(ten million/ml)	152 ± 6.2^{Aa}	141.3 ± 10.0^{Aa}	1.1 ± 0.8^{Bb}	0.9 ± 0.1^{Bb}
Sperm abnormality rate (%)	0.3 ± 0.6^{Aa}	5.3 ± 0.6^{Bb}	8.7 ± 0.6^{Cc}	20 ± 1.0^{Dd}

[a,b,c] Means in the same row with differing superscripts differ ($P < 0.05$).

[A,B,C] Means in the same row with differing superscripts differ ($P < 0.01$).

3.3. Statistical analysis sex proportion of the offspring

The non-injection group produced 160 calves, including the 49.4% (79) heifer calves. The group injected with pLL3.7/a produced 157 calves, including 72.0% (113 heifer calves which differed from the no-injection group ($P < 0.01$) (Table 5).There was little difference in pregnancy rate and birth weight of the offspring between the two groups ($P > 0.05$).

Table 5
The number of female and male offspring in each group of cows.

Subgroup	Pregnancy rate	Female	Male	Female rate	Offspring birth weight
Non-injection	83%	79	81	49.4%^{A}	44.14 ± 5.74
Injection	80.5%	113	44	72.0%^{B}	41.30 ± 6.62

^{A,B} Means in the same column with differing superscripts differ ($P < 0.01$).

4. Discussion

The *Zfy* gene, isolated from the Y-chromosome, is the first gene identified in the search for a male developmental gene on the Y-chromosome and is thought to be a testes determinant. The *Zfy* DNA regulates the expression of other genes while the protein produced by *Zfy* is encoded by a zinc finger domain and the gene plays an important role in the process of sperm production (Mardon and Page, 1989; Vernet et al., 2014).

In order to verify the hypothesis that the *Zfy* gene is the major determinant in testicular development, most of the early research focused on *Zfy* gene expression in the embryo during gonadal development (Nagamine et al., 1990; Zambrowicz et al., 1994). As the hypothesis was rejected (Palmer et al., 1989) and with the discovery of Sry as the testicular determinant in humans and mice (Koopman et al., 1991), *Zfy* studies progressively declined, with some studies using *Zfy* and *Zfx* genes in evaluation of sex determination (Hasler et al., 2002; Jiang et al., 2004; Vernet et al., 2016). The transcription of the *Zfy* gene during spermatogenesis was evaluated, and results showed that the expression in germ cells increased as they enter meiosis; in the mouse, *Zfy* is involved in triggering the apoptotic elimination of specific types of sex chromosomally aberrant spermatocytes (Decarpentrie et al., 2012). In another study, it was found that another important role of the *Zfy* gene is to ensure the occurrence of the second meiosis, and *Zfx* which is the X-related genes on the chromosome, had a facilitative effect (Vernet et al., 2014). Our study builds on this previous work to test the contribution of *Zfy* in the formation of Y-sperm by RNAi in cattle.

Spermatogenesis is a highly regulated and orderly process and there remain many genes and genetic interactions that are uncharacterized (Russell et al., 2008; Franca et al., 1998). In particular genes responsible for fertility are located on the Y-chromosome; conventional targeted disruption of such genes in embryonic stem (ES) cells will result in the failure of germ-line transmission (Rohozinski et al., 2002). With the extensive application of RNAi to probe gene function, it was used for genes that are expressed and functionally required in spermatogenesis and the results showed RNAi is effective throughout spermatogenesis, including during meiosis and haploidy (Shoji et al., 2005; Bettencourt-Dias et al., 2005; Rao et al., 2006; Williams and Schultz, 2006). In our research, *Zfy* or *Zfx*, which is involved in spermatogenesis in mouse, sheep, swine, and red deer, is silenced by RNAi to restrict or deteriorate the function of Y or X sperm during spermiogenesis (Pen et al., 2011; Zan et al., 2015; Ning et al., 2015).In this study *Zfy* siRNA was injected into bull testes to disturb Y-sperm generation and thus produce female calves.

The siRNA, which was encased by micro-lipoids, was involved each stage of differentiation during spermatogenesis through the vector injection in bull testes. Therefore, *in vitro* spermatogenic cells, which contained different spermatogenic cells, were isolated from the bull testes and cultured for the best RNAi vector selection. After interference, the mRNA level of *Zfy* genes was monitored by Real-time Quantitative Polymerase Chain Reaction(RT-qPCR). Results showed that the difference was significant in three test groups. It indicated that the silencing effect of three siRNA expression vectors was different. Perhaps this depends on the structure of these three interference sequences.

In this study it was demonstrated that, after the injection of *Zfy* gene RNAi vector, sperm motility of fresh and frozen sperm is extremely decreased, and the abnormality rate is significantly increased. However, there was little evidence of changes in sperm density with injection of *Zfy* gene RNAi vector (Table 4). Results of this study suggest that, during spermatogenesis, *Zfy* silencing may not impact the number of sperm but does impact sperm viability. It is possible that the *Zfy* gene is related to sperm structure. Mouse Y-encoded transcription factor *Zfy*2 is essential for sperm head remodeling and sperm tail development (Nadege et al., 2016). Our research showed that the proportion of X-bearing sperm was greater in the injection group compared to the non-injection group, which suggest that, during spermiogenesis, the silence of *Zfy* will impact the formation of Y-sperm. The results also showed that there was little difference on the number and body weight of the offspring between the two groups (Table 5). Thus, it appears that *Zfy* silencing does not impact embryonic development.

5. Conclusion

According to the results of this study, *Zfy* siRNA maybe a useful approach to control dairy cattle sex before fertilization, and lays a foundation for producing sex control semen.

Conflict of interest

There is no conflict of interest.

Acknowledgments

We wish to thank M.A Brown for pertinent suggestion on this article. This study was supported by the Corps Doctor Foundation of Xinjiang, China (grant no. 2012BB016) and a grant from the scientific research foundation of Xinjiang Agricultural Vocational Technical College, China (grant no. XJNZYKJ201704).

References

Bettencourt-Dias, M., Rodrigues-Martins, A., Carpenter, L., Riparbelli, M., Lehmann, L., Gatt, K., et al., 2005. SAK/PLK4 is required for centriole duplication and flagella development. Curr. Biol. 15, 2199–2207.

Brown, R.S., Sander, C., Argos, P., 1985. The primary structure of transcription factor TF IIIA has 12 consecutive repeats. FEBS Let. 186, 271–274.

Chang, Y.F., Lee-Chang, J.S., Panneerdoss, S., Maclean, J.A., Rao, M.K., 2011. Isolation of sertoli, leydig, and spermatogenic cells from the mouse testis. Biotechniques 51, 341–344.

Decarpentrie, F., Vernet, N., Mahadevaiah, S.K., Longepied, G., Streichemberger, E., Aknin-Seifer, I., et al., 2012. Human and mouse ZFY genes produce a conserved testis-specific transcript encoding a zinc finger protein with a short acidic domain and modified transactivation potential. Hum. Mol. Genet. 21, 2631–2645.

Dorsett, Y., Tuschl, T., 2004. siRNAs: applications in functional genomics and potential as therapeutics. Nat. Rev. Drug Discov. 3, 318–329.

Elbashir, S.M., Harborth, J., Weber, K., Tuschl, T., 2002. Analysis of gene function insomatic mammalian cells using small interfering RNAs. Methods 26, 199–213.

Fang, X., Du, Z.P., Cao, Y.C., Guo, X., Liu, P.F., Dong, S.B., 2007. siRNA pro2.0: an online tool for rational design of siRNA. Chin. J. Biochem. Mol. Biol. 23, 751–756.

Franca, L.R., Ogawa, T., Avarbock, M.R., Brinster, R.L., Russell, L.D., 1998. Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biol. Reprod. 59, 1371–1377.

Gu, J., Wang, Y.B., Han, C.H., 2009. Research progress in the in vitro culture of testicular spermatogenic cells. Zhonghua Nan Ke Xue 15, 742–745.

Hasler, J.F., Cardey, E., Stokes, J.E., 2002. Noneleetrophoretic PCR-sexing of bovine embryos in a commercial environment. Theriogenology 8, 1–13.

Izadyar, F., Spierenberg, G.T., Creemers, L.B., denOuden, K., deRooij, D.G., 2002. Isolation and purification of type A spermatogonia from the bovine testis. Reproduction 124, 85–94.

Jagla, B., Aulner, N., Kelly, P.D., Song, D., Volchuk, A., Zatorski, A., et al., 2005. Sequencecharacteristics of functional siRNAs. RNA 11, 864–872.

Jiang, H.Y., Cao, X.R., Zhang, X.R., Hu, J., Xu, C.M., 2004. Cloning of ZFY/ZFX geneof the tufted deer and identification of its sex. Hereditas 26, 465–468.

Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., Lovell-Badge, R., 1991. Male development of chromosomally female mice transgenic for Sry. Nature 351, 117–121.

Mardon, G., Page, D.C., 1989. The sex-determining region of the mouse y-chromosome encodes a protein with a highly acidic domain and 13 zinc fingers. Cell. 56, 765–770.

Nagamine, C.M., Chan, K.M., Kozak, C.A., Lau, Y.F., 1989. Chromosome mapping and expression of a putative testis-determining gene in mouse. Science 243, 80–83.

Nagamine, C.M., Chan, K., Hake, L.E., Lau, Y.F., 1990. The two candidate testis-determining Y genes (Zfy-1 and Zfy-2) are differentially expressed in fetal and adult mouse tissues. Genes Dev. 4, 63–74.

Ning, M.Y., Sun, L.R., Zhang, Y.S., Jia, B., Qin, B.Y., Wei, L.M., et al., 2015. Construction of RNAi vectors of swine Zfx gene and their effects on sexual control. J. SHZU 33, 192–195.

Palmer, M.S., Sinclair, A.H., Berta, P., Ellis, N.A., Goodfellow, P.N., Abbas, N.E., et al., 1989. Genetic evidence that ZFY is not the testis-determining factor. Nature 342, 937–939.

Palmer, M.S., Berta, P., Sinclair, A.H., Pym, B., Goodfellow, P.N., 1990. Comparison of human ZFY and ZFX transcripts. Proc. Natl. Acad. Sci. U. S. A. 87, 1681–1685.

Pen, Q., Li, R.Y., Jia, B., Li, H.T., 2011. Sex control by Zfy siRNA in the mouse. Theriogenology 76, 507–511.

Rao, M.K., Pham, J., Imam, J.S., MacLean, J.A., Murali, D., Furuta, Y., Sinha-Hiki, A.P., Wilkinson, M.F., 2006. Tissue specific RNAi reveals that WT1 expression in nurse cells controls germ cell survival and spermatogenesis. Genes Dev. 20, 147–152.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W.S., Khvorova, A., 2004. Rational siRNA design for RNA interference. Nat. Biotechnol. 22, 326–330.

Rohozinski, J., Agoulnik, A.I., Boettger-Tong, H.L., Bishop, C.E., 2002. Successful targeting of mouse Y-chromosome genes using a site-directed insertion vector. Genesis 32, 1–7.

Russell, L.D., Ettlin, R.A., Hikim, A.P., Clegg, E.D., 2008. Histological and histopathological evaluation of the testis. Int. J. Androl. 16, 83.

Shi, Y., 2003. Mammalian RNAi for the masses. Trends Genet. 19, 9–12.

Skaletsky, H., Kuroda-Kawaguchi, T., Minx, P.J., Cordum, H.S., Hillier, L., Brown, L.G., et al., 2003. The male-specific region of the human Y-chromosome is a mosaic of discrete sequence classes. Nature 423, 825–837.

Shoji, M., Chuma, S., Yoshida, K., Morita, T., Nakatsuji, N., 2005. RNA interference during spermatogenesis in mice. Dev. Biol. 282, 524–534.

Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., Ueda, R., Saigo, K., 2004. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res. 32, 936–948.

Vernet, N., Mahadevaiah, S.K., Yamauchi, Y., Decarpentrie, F., Mitchell, M.J., Ward, M.A., et al., 2014. Mouse Y-linked Zfy1 and Zfy2 are expressed during the male-specific interphase between meiosis I and meiosis II and promote the 2nd meiotic division. PLoS Genet. 10, e1004444.

Vernet, N., Mahadevaiah, S.K., Decarpentrie, F., Longepied, G., de Rooij, D.G., Burgoyne, P.S., et al., 2016. Mouse Y-encoded transcription factor Zfy2 is essential for sperm head remodeling and sperm tail development. PLoS One 11, e0145398.

Wang, J.L., Yang, S., Tian, R.H., Zhu, Z.J., Guo, Y., Yuan, Q.Q., et al., 2015. Isolation, culture, and identification of human spermatogonial stem cells. Zhonghua Nan Ke Xue 21, 208–213.

Williams, C.J., Schultz, R.M., 2006. Transgenic RNAi : a tool to study testis specific genes. Mol. Cell Endocrinol. 247, 1–3.

Zambrowicz, B.P., Zimmermann, J.W., Harendza, C.J., Simpson, E.M., Page, D.C., Brinster, R.L., et al., 1994. Expression of a mouse Zfy-1/lacZ transgene in the somatic cells of the embryonic gonad and germ cells of the adult testis. Development 120, 1549–1559.

Zan, H.B., Sun, L.R., Zhang, Y.S., Jia, B., Shen, H., Wang, X.H., et al., 2015. Construction of sheep Zfy gene interference vector and observation of sex control affection. J. SHZU 33, 721–726.