

Original Article

The role of *Dby* mRNA in early development of male mouse zygotes

Chen-Jiang Yao¹, Wang-Jie Xu¹, Xiu-Li Gong², Ying Zhou¹, Zhi-Qiang Yan¹, Zi-Jue Zhu¹, Zhao-Xia Wang¹, Qiao-Li Li³, Xin-Bin Guo², Lian-Yun Wang¹, Duan Ma³, Zhong-Dong Qiao^{1,2}

¹Department of Biological Science and Technology, Key Laboratory of Developmental Genetics & Neuropsychiatric Diseases, Ministry of Education, School of Life Science & Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China

²Shanghai Institute of Medical Genetics, Shanghai Jiao Tong University, Shanghai 200240, China

³School of Medicine, Fudan University, Shanghai 200030, China

Abstract

Ejaculated mammalian spermatozoa contain a complex yet specific population of mRNA. However, the possible roles that mRNA has in early zygotic and embryonic development remain unclear. We found that *Dby* mRNA is selectively retained in capacitated mouse spermatozoa, and is transferred into the oocyte during fertilization by reverse transcription-polymerase chain reaction even though no DBY protein expression is detected. The cellular location of *Dby* mRNA is seen in the post-acrosome region, and it comprises nearly half of the mouse spermatozoa in *in situ* hybridization. In contrast, transcripts of the control gene, *Smcy*, are not detected in capacitated mouse spermatozoa, although the H-Y antigen encoded by *Smcy* is expressed on the surface of the spermatozoa. In our micro-injection experiment, the zygotic development rate of the as-*Dby* male pronucleus injection group was significantly lower than that of the as-*Smcy* male pronucleus injection group (35.9% vs. 95%, $P = 0.001$) and the as-*Dby* female pronucleus injection group (35.9% vs. 93.8%, $P = 0.001$). The rate of male-developed zygotes was also lower than that of the as-*Smcy* male pronucleus injection group (17.4% vs. 57.9%, $P = 0.002$) and the as-*Dby* female pronucleus injection group (17.4% vs. 54.1%, $P = 0.002$). Thus, we conclude that *Dby* mRNA is selectively retained in capacitated mouse spermatozoa, and it has an important role in the early zygotic development of male mouse zygotes. This might imply that spermatozoa mRNA is involved in early zygotic and embryonic stages of reproduction.

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1 Introduction

Fertilization begins at the physical union of the sperm and oocyte, and ends at the activation of the

embryonic genome. The molecular contribution of the oocyte in this process is well understood. The oocyte contributes most of the molecular components, including cytosol, organelles and large stores of maternal mRNA required for embryogenesis. In contrast, the paternal contribution of sperm is rarely considered because the mammalian ejaculated spermatozoa are highly differentiated terminal cells that have an extremely compacted nucleus with a haploid genome. Transcription and translation seem unlikely in ejaculated spermatozoa due to the nuclear DNA binding with protamine

Correspondence to: Prof. Zhong-Dong Qiao, School of Life Sciences Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China.

Fax: +86-21-5474-7330

E-mail: zdqiao@sjtu.edu.cn

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and shedding cytoplasm during the later stage of spermatogenesis. However, recent evidence suggests that a complex specific mRNA population is retained in freshly ejaculated spermatozoa of several species [1–4]. In addition, some spermatozoa mRNAs are transferred into the oocyte during fertilization, and remain stable until the activation of the embryonic genome [5]. These findings suggest that the paternal contribution might not be limited to the male haploid genome and the spermatozoa RNA, but may also be involved in early zygotic and embryonic development.

Recently, we reported that transcripts of three Y-chromosome genes, *DBY*, *SRY* and *RPS4Y*, are selectively retained in human ejaculated spermatozoa [6]. Functional analysis indicates that all three Y-chromosome genes might be involved in early zygotic and embryonic development. However, because of ethical concerns, it is difficult to further investigate their biological functions in zygotic and embryonic development. As a result, the Y-chromosome gene transcripts were analysed in mouse capacitated spermatozoa. The expression of a panel of non-recombining region of the Y chromosome (NRY) genes in mouse testis and capacitated spermatozoa was screened first using reverse transcription-polymerase chain reaction (RT-PCR). *Dby* is the only gene that was detected in capacitated mouse spermatozoa. *DBY* encodes a putative ATP-dependent RNA helicase, which belongs to DEAD-box proteins. In humans, 36 members of the DEAD families of putative RNA helicases have been identified, and are hypothesized to have roles in the differentiation and carcinogenesis [7]. *DBY* has an X-homologue, *DBX*, located on Xp11, which escapes X-inactivation and has 91.7% sequence identity at the protein level in humans. Slightly different from the human *DBY* gene, mouse *Dby* has a structural homolog called *Ddx3x* on the X chromosome and an autosomal homolog, *DIPas1*, on mouse chromosome 1 [8, 9]. Furthermore, the spermatogenesis-specific function of *Dby* is not obvious in mice when compared with humans [10]. Naturally, these results lead to the question of why only *Dby* is retained in capacitated mouse spermatozoa and what possible role it could have.

To investigate the possible mechanisms of selective mRNA retention in capacitated spermatozoa, two genes, *Dby* and *Smcy*, that manifest contrasting functions were selected and analyzed. As the transcripts of *Smcy*, which encode the H-Y antigen, have not been detected in capacitated spermatozoa, and the H-Y antigen has been defined as a membrane protein on almost

all male cells, *Smcy* was selected as a control. Using *in situ* hybridization, the cellular locations of the mRNA encoding the two genes were compared, and their expression patterns were analyzed using Western blot and immunohistochemistry. Their possible functions during fertilization and early zygotic development were evaluated using antisense RNA microinjection into the male and female pronuclei of zygotes.

2 Materials and methods

2.1 Mice and spermatozoa samples

Inbred C57BL/6j mice were housed in a temperature- and light-controlled room, with a standard diet and water *ad libitum*. The protocol was fully accredited by the Chinese Ministry of Science and Technique for the Accreditation of Laboratory Animal Care. Mice were anesthetized with ethyl ether and killed by cervical dislocation. Mouse spermatozoa samples used in the experiment were obtained from the caudae epididymides and were subject to capacitation, which was carried out in an NTF medium at 37°C for 1.5 h before use.

2.2 RT-PCR

Capacitated mouse sperm were first purified using a swim-up technique to avoid somatic contaminations [11]. The total RNA from the purified spermatozoa and the tissue samples were extracted using a Trizol RNA isolation reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol (<http://www.invitrogen.com/content/sfs/manuals/10296010.pdf>). Reverse transcription of all RNA samples was carried out with the M-MLV reverse transcriptase kit (Promega, Madison, WI, USA) in a 20- μ L reaction volume containing 0.5 μ g oligo (dT) and 200 U M-MLV reverse transcriptase for 1 h at 42°C. The reaction was terminated by incubation at 70°C for 10 min and the cDNA was stored at –20°C for further use. A 1- μ L aliquot was then added to a 25- μ L PCR reaction and contained 1 μ mol L^{–1} of each primer (sequences are shown in Table 1). PCR was carried out as described previously, but with slight modifications. In our experiments, 30–35 cycles of amplification were used. The products were separated on a 1.2% agarose gel stained with ethidium bromide, and all PCR reactions were replicated at least once.

2.3 Nested PCR

The mouse Y-chromosome-specific primers MY1-f

Table 1. Sequences of primers used for the detection of Y chromosome gene transcripts.

Name	Gene	Direction	Sequence (5' to 3')
β -actin-F	Mouse <i>β-actin</i>	Forward	TCACCCACACTGTGCCCATCTACGA
β -actin-R	Mouse <i>β-actin</i>	Reverse	CCACGTCACACTTCATGATGGA
Smcy-F	Mouse <i>Smcy</i>	Forward	TTGACAGTGAAGAAAGAGACAAGG
Smcy-R	Mouse <i>Smcy</i>	Reverse	TAAACTGGGTACTGCTGTGATTGT
Dby-F	Mouse <i>Dby</i>	Forward	CAGATGAAGATGACTGGTCAAAAC
Dby-R	Mouse <i>Dby</i>	Reverse	CCAAGGAGATTGGGTATTGTTTAC
Eif2s3y-F	Mouse <i>Eif2s3y</i>	Forward	GGTGCTGTTGGAGCATTACC
Eif2s3y -R	Mouse <i>Eif2s3y</i>	Reverse	TCAACTCGTCGGCTTAGAGC
Uty-F	Mouse <i>Uty</i>	Forward	GAGGAAGAYTCTAATTCTGGTCAA
Uty-R	Mouse <i>Uty</i>	Reverse	GTATCAGAAACACTCTGCTGTGCT
Zfy1-F	Mouse <i>Zfy1</i>	Forward	CACTGGTCTGGAGCTGACTTAGTA
Zfy1-R	Mouse <i>Zfy1</i>	Reverse	AGACTGCTTCAGAAACACAATCTG
Ube1y1-F	Mouse <i>Ube1y1</i>	Forward	CTCTGAGTACATCCGTGG
Ube1y1-R	Mouse <i>Ube1y1</i>	Reverse	GCAATCCTGCTGAACTGC
Sry-F	Mouse <i>Sry</i>	Forward	ATCACATACAGGCAAGACTGGAGTAGAG
Sry-R	Mouse <i>Sry</i>	Reverse	TTAGTAAGTAGGTAAGCTGCTGGTCGTG
Usp9y-F	Mouse <i>Usp9y</i>	Forward	ATGTGAATCAGTGTCTCAAGTGT
Usp9y-R	Mouse <i>Usp9y</i>	Reverse	GCCACTCTTCTTCAGAATTACCAT
Rbmy1a1-F	Mouse <i>Rbmy1a1</i>	Forward	CACTTGAAAGTGGTAGCAAGAAGA
Rbmy1a1-R	Mouse <i>Rbmy1a1</i>	Reverse	GAGTGGTAATTGCCATAGTCACAG
Sly-F	Mouse <i>Sly</i>	Forward	GAAGGTGATACCAAAGGAAGGTTA
Sly-R	Mouse <i>Sly</i>	Reverse	TGTTGTTGATGTTCTTCTCTCTC

(5'-AGCAATGGTTTGTGTATGGGTTGGTT-3') and MY1-r (5'-AGGCAAGGTAGGGGCTTCTTATGT-3') were used as outer primers to amplify the Y-chromosome gene *Smcy*. Specific inner primers MY2-f (5'-AAGGTATCTTGCATTACGTCACAT-3') and MY2-r (5'-TAGCAGTTGAGATGAGGTATGACTG-3') were designed based on the first-PCR product. PCR amplification was carried out at a final volume of 25 μ L, and the first-PCR conditions were used as follows: 95°C for 5 min, followed by 25 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and a final cycle of PCR extension at 72°C for 5 min. For the nested PCR, amplification was carried out as described previously, except that the cycles were increased to 35. One μ L of the first-PCR product was used as the template. Then the nested PCR products were subjected to electrophoresis on a 1.2% agarose gel containing ethidium bromide.

2.4 Quantitative real-time PCR

Quantitative real-time PCR was carried out with SYBR Green PCR Master Mix (Qiagen, Valencia, CA,

USA) using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The primers for *β -actin* and *Dby* are shown in Table 1. Due to the high homology of *Dby*, *Ddx3x* and *DIPas1*, the *Dby* primers were designed using the most diverse sequences of the three genes. The reaction conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. Fluorescence was detected at 585 nm during each step at 72°C. The *β -actin* mRNAs were used as internal controls. The relative mRNA abundance was determined by the ratio of sample to control.

2.5 In situ hybridization (ISH)

2.5.1 Preparation of the probes

Dby and *Smcy* were transcribed and PCR amplified from mRNA from a male mouse brain. The PCR products were cloned into PMD18-T. The hybridization probes were prepared through PCR amplification of plasmid DNA using the corresponding primers and DIG-dUTP. Digoxigenin-labelled PCR products were separated on agarose gel and eluted.

2.5.2 Sperm fluorescent ISH

All reagents used for ISH were purchased from Roche Molecular Biochemical (Basel, Switzerland) and Sigma (St. Louis, MO, USA). To inactivate the RNase, glasswares were autoclaved and baked, whereas plastic containers and solutions were treated with diethylpyrrocarbonate (DEPC). The capacitated spermatozoa of C57BL/6j mice were first spread on polylysine-coated glass slides at 37°C for 1 h. The slides were then treated with pre-chilled CSK buffer (100 mmol L⁻¹ NaCl, 300 mmol L⁻¹ sucrose, 10 mmol L⁻¹ Pipes (pH 6.8), 3 mmol L⁻¹ MgCl₂, 1 mmol L⁻¹ EGTA, 0.5% Triton X-100, 1.2 mmol L⁻¹ phenylmethylsulphonyl fluoride and 1.2 mmol L⁻¹ vanadyl adenosine) for 10 min on ice after being washed with phosphate-buffered saline (PBS) twice. The slides were fixed with PBS, containing 4% paraformaldehyde and 15 mmol L⁻¹ MgCl₂, at room temperature for 15 min. Then the slides were washed with PBS and stored in 70% ethanol at 4°C. Before hybridization, the cells were re-hydrated in PBS and incubated in 50% formamide in 2 × SSC at room temperature for 15 min. Fluorescent ISH was carried out as described previously [12]. The hybridization mixture (20–40 ng labelled probe, 6 µg salmon sperm DNA, 50% formamide, 10% dextran sulphate and 2 × SSC) was briefly denatured at 75°C for 5 min before being applied to the slide. Hybridization was carried out at 42°C, overnight, in a humidified chamber. Next, the slide was washed twice in 2 × SSC containing 50% formamide at 45°C for 3 min and washed again in 1 × SSC for another 3 min at room temperature. Blocking was carried out in 5% normal goat serum for 1 h, and the slide was washed and incubated in rabbit anti-digoxigenin antibody for 1 h at 37°C. The slide was then washed extensively to remove any unbound antibody and incubated in an FITC-conjugated goat anti-rabbit IgG antibody for 30 min at 37°C. Subsequently, the slide was mounted in antifade medium with propidium iodide (PI) as counterstain. The signals were viewed under an LSM 510 META Olympus Confocal Laser Scanning Microscope (Olympus Corp, Tokyo, Japan), and the images were captured using image-analysis software (Image Pro Plus 3.1; Image Pro, Media Cybernetics, Singapore).

2.6 Western blotting

Mouse testis tissue and spermatozoa were homogenized and lysed in ice-cold Nonidet-P40 buffer (20 mmol L⁻¹ Tris-HCl, pH 8, 137 mmol L⁻¹ NaCl,

10% glycerol, 1% nonidet P-40, 2 mmol L⁻¹ EDTA), supplemented with 1 × Proteinase Inhibitor Cocktail (Pierce, Rockford, IL, USA). The lysate was gently rocked at 4°C for 1 h, and centrifuged at 12 000 × g for 15 min at 4°C before the supernatant was recovered. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was carried out according to the Laemmli method [13] using 50 µg of protein per lane. The separated protein was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, France). The membranes were incubated with 1 µg mL⁻¹ anti-DBY antibodies (Santa Cruz, Biotechnology), diluted in 10 mmol L⁻¹ Tris-HCl (pH 7.5), 0.15 mmol L⁻¹ NaCl and 0.05% Tween (TST) at 25°C for 1 h. The membrane was washed thrice with TST for 10 min, and incubated at 25°C for 1 h with horseradish peroxidase-conjugated IgG (second antibody diluted at 1:5 000 in TST). A GAPDH antibody was used as an internal control. The signal was visualized using an enhanced chemoluminescence kit (Biocolors, Shanghai, China) exposed on Kodak XR-5 X-ray film (Kodak, Rochester, NY, USA).

2.7 Immunohistochemistry

Formalin-fixed testes removed from C57BL/6j mice were dehydrated and embedded in paraffin according to standard protocols. Five µm paraffin sections were cut and mounted onto poly-L-lysine-coated glass slides. The sections were de-paraffinized using xylene (3 × 30 min) at 60°C and a series of decreasing ethanol concentrations according to standard protocols. Endogenous peroxidase activity was blocked by submerging the slides in methanol containing 3% hydroperoxide for 30 min. Samples were then heated in a microwave oven for antigen retrieval (10 mmol L⁻¹ citrate buffer, pH 6, 20 min, 700 W). The sections were allowed to cool in citrate buffer, washed thrice (de-ionized water, PBS, pH 7.4, 5 min each) and incubated in blocking solution for 30 min. Next, they were washed with PBS (3 × 10 min) and incubated overnight with anti-H-Y IgY antibody at 4°C. After being washed with PBS (3 × 10 min), the sections were incubated with horseradish peroxidase-conjugated rabbit anti-chicken IgG (1:5 000 Jackson) for 2 h at room temperature (RT). Finally, peroxidase was visualized using 0.05% diaminobenzidine (DAB; Sigma), in 0.05 mol L⁻¹ Tris buffer, pH 7.6, containing 0.01% hydrogen peroxide.

2.8 Immunofluorescence

The capacitated sperm were washed twice in PBS

and spread on the slides coated with poly-*L*-lysine at 37°C for 1 h. Spermatozoa samples were allowed to air dry, and then fixed in PBS containing 4% paraformaldehyde for 15 min. After being washed, the slides were treated with PBS containing 0.5% Triton X-100 at room temperature for 20 min. They were then treated with blocking solution at room temperature and incubated overnight with anti-H-Y IgY antibody at 4°C. The following day, they were incubated with fluorescein-conjugated rabbit anti-chicken IgG diluted 1:100 at room temperature for 2 h. After being washed extensively to remove the unbound antibody, they were mounted in antifade medium with PI as counterstain and examined by fluorescence microscopy (Olympus BX 51, Olympus Corp, Tokyo, Japan).

2.9 Construction of the antisense RNA expression vectors

The 224-bp antisense fragment A, targeting the initiation codon site of the *Dbp* mRNA, was obtained by RT-PCR amplification of the RNA extracted from a male mouse brain with the primers 5'-GTCCGGTTCCGTGAGAAGTT-3' and 5'-AGCTCCATCCTGAAGTGTCTTAT-3'. The 288-bp antisense fragment B, targeting the initiation codon site of *Smcy* mRNA, was obtained by RT-PCR amplification of the RNA extracted from a male mouse brain with primers 5'-GAACATGAAGCCAGGATCTGA-3' and 5'-TTCCCAGAACTTTGCAATCTG-3'. Fragments A and B were separately inserted in the vector, pTG19-T. The respective clones of pTGA and pTGB were obtained and confirmed through sequencing. Both fragments were later removed from the plasmids through double digestion with *Hind*III and *Eco*RI, before being ligated into the *Hind*III and *Eco*RI sites of the mammalian expression vector pcDNA3.1 (Invitrogen). The two antisense RNA expression vectors were confirmed for correct orientation through DNA sequencing.

2.10 Microinjection of zygotes with antisense RNA expression vector

Female mice, aged 8–12 weeks, were subjected to superovulation protocol involving intraperitoneal (IP) injections of 5 IU of pregnant mare serum gonadotropin on day 1 and an IP injection with 5 IU human chorionic gonadotropin (hCG) after 48 h. On the following day, all female mice mated successfully (males had previously been assessed for reproductive performance) and

were killed by cervical dislocation after being anesthetized. Zygotes were retrieved in an M2 medium and placed in culture in KSOM medium under a humidified 5% CO₂ atmosphere. Any zygotes displaying poor morphology (e.g., absence of two polar bodies, granular cytoplasm, fragmentation and, following subsequent processing, post-injection lysis) were recorded and discarded. In all, 1–2 pL of the *Dbp* and *Smcy* antisense RNA (100 nm) was microinjected into the male and female pronuclei of zygotes, respectively, 20 h after hCG injection. Consequently, the successfully microinjected zygotes were cultured in droplets of KSOM medium. Twenty hours after culture, the rate of the two-cell stage was calculated. The DNA genomes of these developed embryos were extracted by boiling and used a template for nested PCR. Male embryos were identified as those with Y-chromosome fragments.

2.11 Statistical analysis

Data were analyzed by chi-square test and Mann-Whitney test where appropriate. All statistical analysis was carried out using SPSS (version 15.0; SPSS, Chicago, IL, USA). All percentage data were subjected to arc sine transformation before the statistical analysis. A probability of $P < 0.05$ was considered statistically significant.

3 Results

3.1 RT-PCR analysis of the Y-chromosome gene mRNA in mouse testis, spermatozoa and zygote

RT-PCR was used to screen for the expression of 10 genes on the NRY in mouse testis and capacitated spermatozoa. The spermatozoa RNA preparation was devoid of somatic contaminants as detected by the swim-up method and the absence of *E-cadherin*, *c-kit* and *CD45*, three somatic genes that are highly expressed in most tissues, germ cells and haematocytes, respectively. The total RNA from male and female mouse brains was used as positive and negative controls, respectively, for Y-chromosome genes. As shown in Figure 1A, transcripts of all the 10 genes were found in mouse testis samples, whereas only *Dbp* was detected in capacitated mouse sperm. None of the 10 genes was expressed from female brain samples. The specificity of the PCR product was verified by sequencing.

To determine whether it was transferred into the oocyte during fertilization, *Dbp* mRNA from mouse

spermatozoa was checked for in unfertilized mouse oocytes and zygotes before activation of the embryonic genome by RT-PCR. *Smcy* mRNA, which was not found in mouse spermatozoa, was also tested. The result showed that *Dby* mRNA was present in zygotes, but not in unfertilized oocytes, whereas *Smcy* transcripts were undetectable in both zygotes and unfertilized oocytes (Figure 1B). *Dby* transcripts were actually delivered into oocytes during fertilization.

3.2 ISH analysis of *Dby* and *Smcy* transcripts in mouse spermatozoa

To determine the subcellular distribution of *Dby* and *Smcy* in capacitated mouse spermatozoa, fluorescent ISH (FISH) was carried out with *Dby* and *Smcy*

gene-specific cDNA probes. After signal amplification, *Dby*-positive signals were detected in the post-acrosome region of nearly 50% of capacitated mouse spermatozoa. In contrast, no *Smcy*-positive signals were detected. Likewise, no signals were visible in the negative controls in which the sense *Dby* probe was used (Figure 2). These results indicate that only *Dby* transcripts were retained in the post-acrosome region of, presumably, Y-bearing spermatozoa, whereas *Smcy* transcripts were absent in all spermatozoa.

3.3 Expression pattern analysis of *DBY* and *SMCY* in mouse testis and spermatozoa

Western blot analysis was carried out to investigate the pattern of *DBY* expression in mouse testis and spermatozoa. Total proteins were extracted and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. To verify whether the separated proteins were successfully transferred onto the PVDF membrane, a positive control of GAPDH was also examined. *DBY* protein was detected in mouse testis as a single band with a molecular weight of about 73 kDa, whereas no expression was found in the spermatozoa sample (Figure 3A). The result revealed that *Dby* mRNAs in mouse spermatozoa are retained despite the absence of their protein expression.

As it was apparent that *Smcy* encoded two epitopes

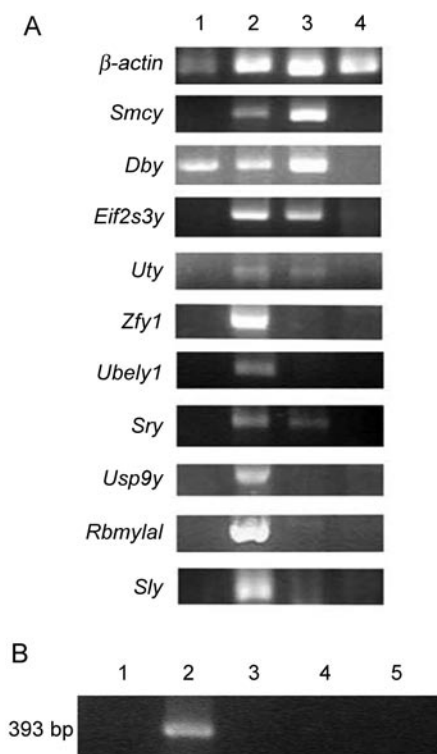


Figure 1. RT-PCR analysis of Y chromosome gene transcripts from mouse testis, spermatozoa and zygote. (A): mRNA screening of 10 mouse Y chromosome gene transcripts in ejaculated spermatozoa. Lane 1, spermatozoa; Lane 2, testis; Lane 3, male brain; Lane 4, female brain. (B): mRNA detection of *Dby* and *Smcy* in mouse zygote and unfertilized oocyte. Lane 1, amplifying *Dby* with mouse oocyte; Lane 2, amplifying *Dby* with mouse zygote; Lane 3, amplifying *Smcy* with mouse oocyte; Lane 4, amplifying *Smcy* with mouse zygote; Lane 5, negative control with water.

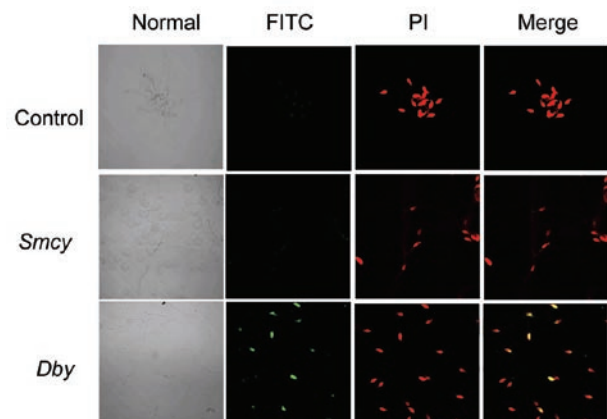


Figure 2. Distribution analysis of *Dby* and *Smcy* mRNA in mouse spermatozoa by *in situ* hybridization. *Dby* mRNA was detectable in the post-acrosome region of the head of mouse ejaculated spermatozoa (green signals). The nucleus is counterstained with propidium iodide (PI) (red signals). *Smcy* mRNA was undetectable in all spermatozoa. Signals were not detected in the negative control. Bar = 20 μ m.

of the H-Y antigen, the expression of *Smcy* in mouse testis and spermatozoa was evaluated based on the existence of H-Y antigen. Thus, the specific IgY antibodies against H-Y antigen obtained in our lab were used to investigate the expression pattern of *Smcy*. Immunohistochemistry analysis of mouse testis treated with anti-H-Y IgY antibodies showed strong positive signals, but no specific H-Y immunoreactivity was observed in the section incubated without the primary antibody. Interestingly, positive signals were also found on part of the mouse spermatozoa (Figure 3B).

3.4 Blocking *Dby* with antisense RNA inhibits zygotic development

To evaluate the potential functions of spermatozoa *Dby* and *Smcy* mRNA in early zygotic development, the antisense RNA of spermatozoa *Dby* and *Smcy* mRNA were microinjected into the male and female pronuclei of mouse zygotes, respectively. After the microinjected

zygotes were cultured for 24 h, the rate of fertilization was calculated by counting the number of eggs that developed into the two-cell stage. Then, the successfully developed embryos were sexed using PCR and a Y-chromosome specific primer. As shown in Table 2, there was a significantly lower development rate in the as-*Dby* male pronucleus-injection group (35.9%) than in the female pronucleus-injection control group (93.8%, $P = 0.001$). In addition, in the as-*Dby* male pronucleus injection group, only 17.4% of the developed embryos were male. In contrast, in the as-*Smcy* male pronucleus injection group, 95.0% of the zygotes successfully developed into the two-cell stage compared with 96.4% in the female pronucleus-injection control group, and 57.9% of the embryos were male. Thus, the development rate of the as-*Dby* group was significantly lower than that of the as-*Smcy* group (35.9% vs. 95.0%, $P = 0.001$). Additionally, the rate of male embryo development was significantly lower

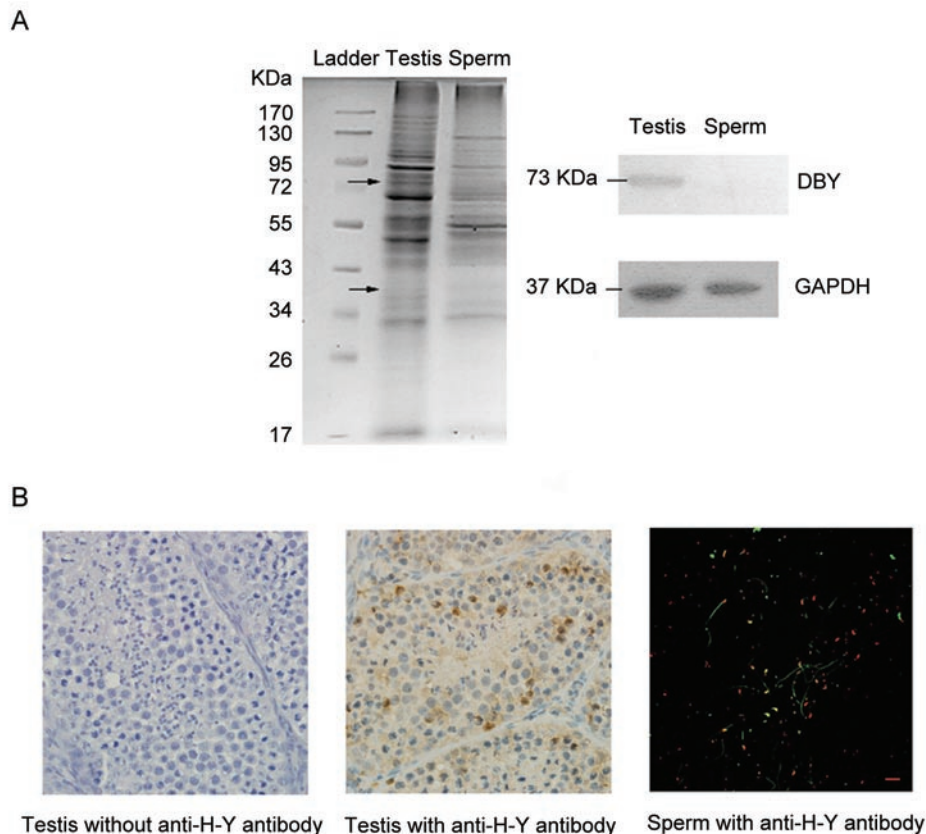


Figure 3. Expression analysis of DBY and SMCY in mouse testis and ejaculated spermatozoa by Western blot and immunohistochemistry. (A): Western blot showing DBY protein existed in mouse testis, while there is an absence of DBY protein in ejaculated spermatozoa. (B): Immunohistochemistry analysis revealing the detection of H-Y antigen on mouse testis and part of the spermatozoa. Bar = 20 μ m.

Table 2. Comparisons of blocking *Dby* and *Smcy* mRNA in mouse zygotes.

Variable	As- <i>Dby</i> pronucleus group		As- <i>Smcy</i> pronucleus group	
	Male	Female	Male	Female
No. of zygotes microinjected	64	65	40	55
No. of developing into two-cell stage	23	61	38	53
No. of male embryos	4	33	22	29
Developed rate (%) ^a	35.9*	93.8	95.0	96.4
Male rate (%) ^b	17.4 [#]	54.1	57.9	54.7

^aRatio of No. of developing into two-cell stage to No. of zygotes microinjected; ^bRatio of No. of male embryos to No. of developing into two-cell stage.

* $P = 0.001$, compared with as-*Smcy* male pronucleus group; [#] $P = 0.002$, compared with as-*Smcy* male pronucleus group.

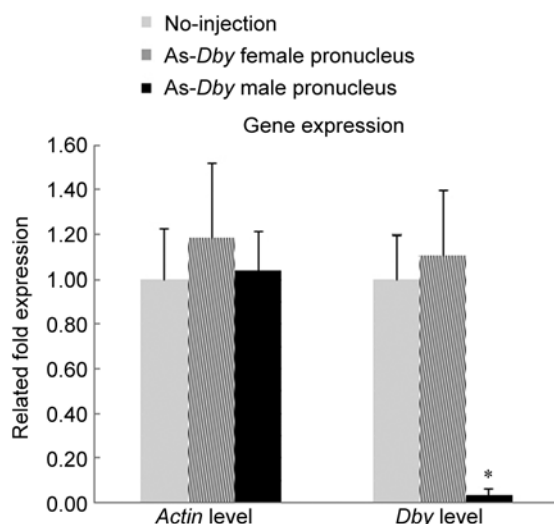


Figure 4. RT-PCR quantitative analysis of *Dby* transcripts in antisense RNA microinjected zygotes. The level of *Dby* transcripts in *Dby* antisense RNA male pronucleus microinjected zygotes was significantly reduced compared with normal zygotes and *SMCY* antisense RNA female pronucleus microinjected zygotes. * $P < 0.01$, compared with normal zygotes and as-*Dby* female pronucleus group.

in the as-*Dby* group than in the as-*Smcy* group (17.4% vs. 57.9%, $P = 0.002$). The microinjection experiment showed that *Dby* antisense RNA strongly affected the zygote's first cleavage, resulting in the observed embryo sex ratio, whereas *Smcy* antisense RNA had no effect on the zygotes. The level of *Dby* transcripts in non-injected zygotes and the as-*Dby* microinjected zygotes was also measured by RT-PCR quantification. The results confirmed that the amount of *Dby* mRNA had been decreased in the as-*Dby* male pronucleus group (Figure 4) ($P < 0.01$, compared with normal zygotes and as-*Dby* female pronucleus group).

4 Discussion

Since the discovery of RNA in the nucleus of mature ejaculated spermatozoa, its possible biological functions have been controversial. Spermatozoa appear to have no capacity for transcription and translation because they are highly differentiated, specialized cells with minimal cytoplasm and a compact nucleus. Those transcripts found in ejaculated spermatozoa have traditionally been considered remnants [14]. Recently, however, some spermatozoa RNA, along with sperm, have been shown to be introduced into the oocyte during fertilization, which remains stable until the onset of the expression of the embryonic genome. Furthermore, a functional analysis of 389 clustered genes in ejaculated spermatozoa, identified by serial analysis of gene expression (SAGE), revealed highly selective functional categorizations in nucleus proteins related to DNA-dependent transcription (96/389) and ribosomal subunits involving protein biosynthesis (84/389), while the genes highly expressed in mouse testis were mostly concentrated in spermatogenesis, protein metabolism, energy metabolism, growth and differentiation, and signal transduction [15, 16]. Interestingly, a microarray analysis comparing gene expression profiles of fertile and infertile sperm, without basic sperm abnormality, revealed a significant difference in spermatozoa transcripts [17]. A considerable number of ribosomal proteins, normally expressed from a number of common genes, are significantly reduced in infertile men. These results imply that transcripts related to protein synthesis in ejaculated spermatozoa might have functions in early zygotic and embryonic development. Moreover, a chromosomal distribution analysis of the 510 overlapping, sperm-related genes by SAGE revealed an uneven distribution among the two dozen chromosomes. The highest numbers of genes are located on chromosome 1

[18], which harbours high-frequency breakpoints in infertile males. In contrast, the Y chromosome has the least numbers of genes.

Thus, in this study, the possible functions of Y chromosome gene transcripts in spermatozoa during early zygotic development were analysed, as the genes have some advantages. Unlike other chromosomes, the Y chromosome transcripts found in zygotes originated from the paternal gamete. In addition, the population of Y chromosome-coded mRNA in ejaculated spermatozoa is small and a majority are related to sex determination and differentiation. As such, they could serve as a starting point for the investigation of the possible function of spermatozoa mRNA in early zygotic and embryonic development. The mRNA of 10 genes, on the NRY, in mouse testis and spermatozoa was first screened by RT-PCR and only one *Dbp* gene was detected in capacitated spermatozoa. Furthermore, RT-PCR revealed that the *Dbp* mRNA was delivered into the oocyte during fertilization. Then, the *Smcy* gene, which encodes the H-Y antigen, was selected as a control to compare with *Dbp*. Using *in situ* hybridization, the *Dbp* mRNA was localized along the post-acrosome region, comprising nearly 50% of the mouse spermatozoa. However, *Smcy* mRNA was undetectable in all spermatozoa. Surprisingly, although the *Dbp* transcripts were found in capacitated mouse spermatozoa, no DBY protein expression was detected in sperm. In contrast, immunohistochemistry analysis revealed that H-Y antigen, encoded by *Smcy*, was detected in mouse testis and part of the spermatozoa despite the fact that *Smcy* mRNA was not detectable in all spermatozoa.

To confirm whether they function in early zygotic development, *Dbp* and *Smcy* antisense RNA were microinjected into the male and female pronuclei of mouse zygotes, respectively. The results demonstrated that antisense RNA against *Dbp* can greatly affect the first cleavage of zygotes and the sex ratio of the resulting developed embryos, while *Smcy* antisense RNA had no effect on zygotes. Furthermore, microinjections into the male pronucleus with as-*Dbp* blocked *Dbp* expression in male mouse zygotes during the first cleavages, while microinjection into the female pronucleus did not. It is possible that these antisense RNAs are degraded soon before fusion of male and female pronuclei. It is therefore reasonable that the development rate of the as-*Dbp* male pronucleus group is much lower than that of the as-*Dbp* female pronucleus group. This is very

encouraging because it demonstrates that *Dbp* mRNA actually has an important role in early zygotic development. It suggests that those mRNAs reserved in mature sperm are not merely non-functional remnants of spermatogenesis, but may be selected to exert additional functions in fertilization and early embryonic development. To confirm the expression pattern of DBY in zygotes, the DBY protein was examined by immunofluorescence in single-celled embryos. However, due to the high homology of DBY, DDX3X and DIPas1, there is no available antibody that can detect DBY protein specifically (see Appendix, Figures 1S and 2S). As a result, it is regrettable that further investigation cannot be performed. Additionally, even though the zygotic genome activation is supposed to occur only at the two-cell stage in mice, there is evidence that some specific transcripts are transcribed at the pronuclear stage [19]. Hence, the possibility that *Dbp* is being transcribed from the Y chromosome of the zygote cannot be excluded. Further experiments are required to test this hypothesis.

DBY is located in the non-recombining region of the Y chromosome (Yq11), belongs to the DEAD box proteins and encodes a putative ATP-dependent RNA helicase [20]. The DEAD-box proteins are implicated in a number of cellular processes involving alterations of the secondary structure of RNA, such as translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly. Based on their distribution patterns, some members of this family are believed to be involved in embryogenesis, spermatogenesis, cellular growth and division [7]. DBY is frequently deleted in infertile patients, and its absence produces severe spermatogenic damage. This damage leads to significant reductions in germ cells (oligozoospermia) or even to their complete absence (azoospermia) [21]. In addition, *Caenorhabditis elegans*, a homolog of DEAD-box protein VBH-1, was proven to perform conserved roles in fertility and development. VBH-1 (RNAi) animals produced fewer offspring than wild type because of defects in oocyte and sperm production and embryonic lethality. VBH-1 was also shown to participate in the sperm/oocyte switch of the hermaphrodite gonad [22]. Moreover, despite slight differences with its human counterpart, mouse *Dbp* has a structural homolog, called *Ddx3x*, on the X chromosome and an autosomal homolog, *DIPas1*, on mouse chromosome 1. Sequence alignment revealed significant homology among *Dbp*, *Ddx3x* and *DIPas1*. When the coding sequences were compared, *Dbp* showed 90% and 84%

overlap with *Ddx3x* at the amino acid and nucleic acid levels, respectively, while *Dby* and *DIPas1* shared 87% and 80% homology at the amino acid and nucleic acid levels, respectively [23]. Studies have shown that the expression patterns and levels of the three homologous genes are different. Although *Dby*'s expression pattern is similar to its homolog *Ddx3x*, the expression level is significantly lower, and further evidence demonstrates that *Dby* is not required at any stage of the male mouse germ line [10]. As such, *Dby*'s spermatogenesis-specific function is not obvious compared to *DIPas1* and *Ddx3x*. However, apart from having an inappreciable function in spermatogenesis, it is not abandoned during the spermatogenesis process. Because its function is involved in DNA replication and transcript initiation during early embryonic development stages, *Dby* may be considered essential during that particular phase. Although no DBY protein was detected, *Dby* transcripts were still retained in capacitated mouse spermatozoa.

In contrast, *SMCY* is widely transcribed in almost all male tissues. It has been established that human and mouse *SMCY* encode two epitopes of the H-Y antigen [24, 25]. The H-Y antigen is a male specific antigenic epitope that was first described in 1955 by Eichwald and Silmsen. It appears to be an integral part of the membrane for most male cells and may have functions in the organ transplants between males and females. In addition, the H-Y antigen was first detected in eight-cell mouse embryos [26]. As such, it is apparent that *Smcy* has no function in the zygote during the early stages of embryonic development. It is thus realistic to assume that *Smcy* transcripts become undetectable in capacitated spermatozoa.

Although it is known that sex chromosomes undergo transcriptional inactivation [27] (meiotic sex chromosome inactivation), during male meiosis, our results revealed that *Dby* mRNA was detectable in about half of capacitated mouse spermatozoa. Thus, it is cautiously speculated that the transcripts in Y bearing sperm are slightly different from those of X bearing sperm, at least in Y chromosome genes. This means that zygotes derived from X and Y bearing sperm would potentially differ in terms of proteins contributed and utilized, in early development. We suggest that this is the reason why as-*Dby* only affects the development of male mouse zygotes. Furthermore, the effects of *Dby* mRNA were possibly limited to the activation of the Y chromosome.

In summary, the results of this study indicate that

Dby mRNAs have an important role in early zygotic development and are selectively retained in mouse spermatozoa during spermatogenesis. Those RNAs that had finished their mission are abandoned before being delivered into oocyte. Therefore, it is reasonable to conclude that preparation for zygote formation and embryo development could have begun during the initial stages of spermatogenesis.

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Appendix

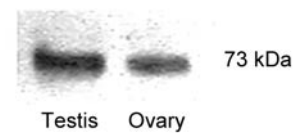


Figure 1S. Western blot analysis on mouse testis and ovary with antibodies against DBY. Obvious bands are detected in mouse testis and ovary with antibody of DDX3Y(B-4):sc-130421.

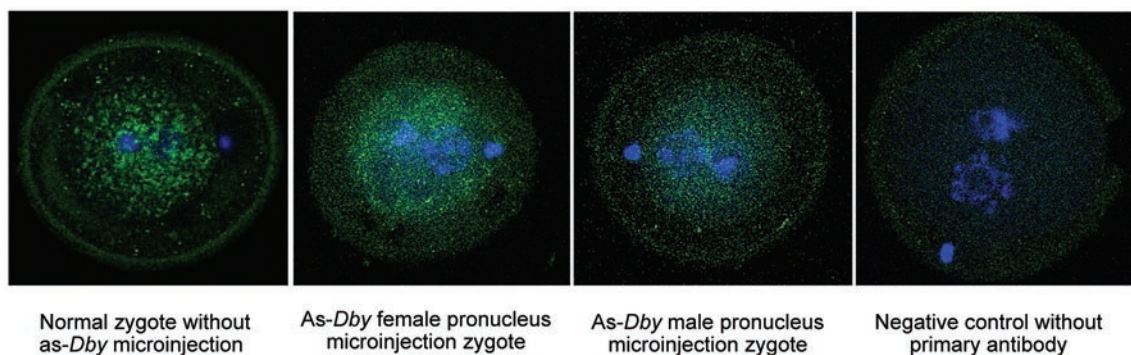


Figure 2S. Expression analysis of *Dby* in as-*Dby* male and female pronucleus microinjected one cell zygotes by immunofluorescence.