Characterization of X-linked RNA Transcripts in Matured Bovine Spermatozoa

Byeong-Gyun Jeon[1], B. Mohana Kumar[1,2] and Gyu-Jin Rho[1,2,†]

[1]*OBS/Theriogenology and Biotechnology, College of Veterinary Medicine, Gyeongsang National University, Jinju 660-701, Republic of Korea*

[2]*Research Institute of Life Science, Gyeongsang National University, Jinju 660-701, Republic of Korea*

ABSTRACT

Although the function and utility of RNA transcripts derived from matured spermatozoa remains unclear, they might play important roles in the establishment of a paternal genome and subsequently embryo development. Herein, we investigated the expression of X-chromosome linked RNA transcripts in matured bovine spermatozoa. The total RNA was extracted from the matured spermatozoa, and then converted to cDNA. Autosomal genes (ACT-β and H-2A) and X-chromosome linked genes (ANT3, HPRT, MeCP2, RPS4X, XIAP, XIST and ZFX) were analyzed for the characterization of X-chromosome linked RNA transcripts and compared to female fibroblasts by RT-PCR. The transcripts of autosomal genes (ACT-β and H2A) and X-chromosome linked genes (ANT3, HPRT, MeCP2, RPS4X and ZFX) were not detected in spermatozoa. However, XIAP (X-linked inhibitor of apoptosis protein) and XIST (X-chromosome inactive-specific transcript, a kind of paternal imprinted gene) transcripts were detected in spermatozoa, and relative levels of XIAP and XIST transcripts were similar and 0.5-fold lower when compared to female fibroblasts, respectively. Based on the findings, it is summarized that the presence of RNA transcripts of XIAP and XIST in the isolated spermatozoa may imply their role in inhibition of apoptosis and induction of X-chromosome inactivation in embryo development.

(Key words : Bovine, Spermatozoa, X-lined genes, XIAP, XIST)

INTRODUCTION

Spermatozoa carry the paternal haploid nucleus that contributes approximately half of the nuclear genetic materials to the diploid offspring thorough fertilization. The matured and ejaculated spermatozoa generally contain an extremely condensed nuclear DNA packaged with protamine proteins, and their cytoplasm and cellular organelles are nearly removed during spermatogenesis for mobile characterization. Therefore, the matured and ejaculated spermatozoa are dormant status with minimal size.

Meanwhile, several components derived from spermatozoa, including paternal centriole and the soluble oocyte activating factor(s) are also delivered to the oocytes on fertilization. The mitotic spindles for first cleavage at the zygote stage are originated from the paternal centriole, and the signals to initiate metabolic activation of the fertilized oocyte are induced by the oocyte activating factor(s) from spermatozoa. It is also well proven that the paternal centriole and the oocyte activating factor(s) are critically important roles for subsequent development of post-fertilization embryos (Barroso *et al.*, 2009).

Furthermore, with the paternal centriole and the soluble oocyte activating factor(s) which enter to the oocyte on fertilization, it has been reported that specific populations of RNA transcripts are detected in the matured and/or ejaculated human, bovine and porcine spermatozoa with dormant status by RT-PCR and microarray, and these RNA transcripts are also delivered to the oocyte in mouse, human and porcine (Hayashi *et al.*, 2003; Kempisty *et al.*, 2008; Ostermeier *et al.*, 2004). The biological functions and translation of RNA transcripts to protein still remains unclear, but growing evidences have been speculated that spermatozoa RNA transcripts might be important roles in the establishment of subsequently embryo development in human (Dadoune, 2009; Barroso *et al.*, 2009; Ostermeier *et al.*, 2004).

* This study was supported by grants from BioGreen 21, 20070301034041 and 200908FHT010204005, Rural Development Administration, Republic of Korea. The authors declare no conflict of financial interests.

† Corresponding author : Phone: +82-55-772-2347, E-mail: jinrho@gnu.ac.kr

Other reports have been suggested that spermatozoa RNA transcripts are obviously associated with establishment of genetic imprinting during the maternal-zygotic transition in the post-fertilization embryos (Ostermeier *et al.*, 2004; Ostermeier *et al.*, 2005).

In female, one of the two X-chromosome is generally inactivated for dosage compensation, which overcome imbalance of X-linked genes between the male and the female (Chow and Brown, 2003). Meanwhile, a large number of X-linked genes which are important for early embryo development as well as cellular function and metabolism are expressed from the activated X-chromosome. It is well known that hypoxanthine phosphoribosyltransferase (HPRT) and ADP/ATP translocase 3 (ANT3, known as SLC25A6) for metabolism, X-inactive specific transcript (XIST) for X-chromosome inactivation, ribosomal protein S4 (RPS4X), methyl CpG binding protein 2 (MeCP2), X-linked inhibitor of apoptosis protein (XIAP), zinc finger X-chromosomal protein (ZFX) are important genes expressed from the activated X-chromosome (Nino-Soto *et al.*, 2007).

To characterize the expression of X-linked genes in the matured bovine spermatozoa, transcript level of autosomal genes (ACT-β and H2A) and X-chromosome linked genes (ANT3, HPRT, MeCP2, RPS4X, XIAP, XIST and ZFX) were investigated by RT-PCR and compared to normal female adult skin fibroblasts.

MATERIALS AND METHODS

Preparation of Spermatozoa

Ejaculated spermatozoa were obtained from Korean cows with normal fertility capacity in breeding center. The live spermatozoa were harvested by a swim-up method with Tyrode's albumin lactate pyruvate (TALP) medium containing 2 mg/ml bovine serum albumin (BSA, essentially fatty acid free, Sigma) for 1 h at 38.5 ℃ in a humidified atmosphere of 5% CO_2. After being washed by centrifugation at 350×g for 10 min in D-PBS, sperm at ~1×10^7 sperm/tube were immediately used for RNA extraction or frozen at −80℃ for further analysis.

RNA Extraction and Purification of Spermatozoa

Total RNA was extracted by TRIZOL method. Briefly, spermatozoa samples were treated with 1 ml of TRIZOL reagent (GIBCO BRL, USA), vortexed for 30 sec and incubated at room temperature for 5 min. After being added 200 μl chloroform, the samples were centrifuged for 20,000×g for 15 min. The aqueous phase was transferred to a clean tube. Total RNA was precipitated with 500 μl isopropyl alcohol for 10 min at room temperature, and centrifuged for 20,000×g for 15 min. The resulting pellet was then suspended in 75% cold ethanol, and centrifuged for 20,000×g for 5 min. The pellet was dried at room temperature, resuspended in 100 μl distilled RNAase free-water, and stored at −80℃. Purification of RNA was performed with the Qiagen RNeasy Micro Kit using carrier RNA (Qiagen, USA) according to the manufacturer's procedure with an extra step of DNase I treatment for removal of DNA contamination. The concentration of extracted total RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer (Mecasys, Korea).

cDNA Synthesis and RT-PCR

A total of 1 μg RNA was synthesized for the first-strand cDNA with Omniscript RT Kit (Qiagen, USA). Each of cDNA samples contained 2 μl of 10 μM Oligi-dT12-18 primer (Invitrogen, USA), 1 μl of 10 U/μl RNase Inhibitor (Invitrogen, USA), 2 μl RT buffer, 2 μl dNTP, and 1 μl Omniscript (Qiagen, USA), was adjusted to a total volume of 20 μl using H_2O. The cDNA samples were then incubated in a thermal cycler (Effendorf, Germany) at 42℃ for 1 h, followed by 5 min at 95℃ to inactivate the enzyme. A total of three reverse transcription reactions was used for each RNA sample. The RT-PCR was carried out in a 20 μl reaction volume containing a final concentration of 1× PCR buffer, 2 μl of first-strand cDNA, 2.5 U Taq DNA polymerase, 0.2 mM dNTP mix, 3 mM $MgCl_2$, and 2 μl of each the forward and reverse primer (0.1 μg/μl). The amplification protocol consists of an initial denaturation step at 95℃ for 10 min followed by 25 cycles of denaturation for 15 sec at 95℃, annealing of 6 sec at 57~65℃ and extension of 16 sec at 72℃. Autosomal genes (β-actin; ACT-β and histone 2A; H2A) and X-chromosome linked genes (ADP/ATP translocase 3; ANT3, growth factor receptor bound protein 2-associated binder 3; HPRT, methyl CpG binding protein 2; MeCP2, 40S ribosomal protein S4; RPS4X, X-linked inhibitor of apoptosis protein; XIAP, X-inactive specific transcript; XIST and zinc finger X-chromosomal protein; ZFX) were analyzed for the characterization of the transcripts by RT-PCR. Primer sequences, the size of amplified products and annealing temperatures are shown in Table 1. A sample of 10 μl of each PCR product were confirmed by 1.0% agarose gel electrophoresis containing 0.5 μg/ml ethidium bromide, and the intensity was analyzed with Gelviewer image-processing software (Innogene, Korea).

Statistical Analysis

One-way analysis of variance was employed to analyze the differences (SPSS 15.0, USA) and the data were expressed as mean±SEM. Comparisons of mean values were analyzed by using a Tukey's multiple com-

Table 1. Sequence-specific primers used for RT-PCR

Gene	Primer sequences (5′-3′)	Band size of PCR product (bp)	Annealing temperature (℃)
ACT-β	F-CGTGACATTAAGGAGAAGCTGTGC R-CTCAGGAGGAGCAATGATCTTGAT	374	63
H2A	F-GTCGTGGCAAGCAAGGAG R-GATCTCGGCCGTTAGGTACTC	181	57
ANT3	F-TTCCCTGTGCTTCGTCTACC R-TGCCCTTGTACATGATGTCG	383	60
HPRT	F-CGAGATGTGATGAAGGAGATGG R-TTTCAAATCCAACAAAGTCTGG	397	61
MeCP2	F-GGGACCCATGTATGATGACC R-ATGTGTCGCCTACCTTTTCG	173	60
RPS4X	F-ATTAAGATCGATGGCAAAGTCC R-AAAAGAACCTGGATGTCTCTCC	408	59
XIAP	F-GGCGACACTTTCCTAATTGC R-AAGCATAAAATCCAGCTCTTGC	213	58
XIST	F-CCTTGTCATGTGGATATCATGG R-AATGTCCTTGGAAAGACTTTGG	224	59
ZFX	F-TCTATCCTTGCATGATTTGTGG R-AGAGTCTGCGGACCTATATTCG	494	57

parisons test. The level of significance was tested at $p< 0.05$.

RESULTS

Transcript Levels of X-linked Genes in Matured Spermatozoa

Transcript levels of X-linked genes were investigated by RT-PCR in the bovine matured spermatozoa and female fibroblasts, as shown in Fig. 1. ACT-β, H2A, ANT3, HPRT, RPS4X and ZFX were not detected in the spermatozoa when compared to female fibroblasts. However, XIAP was detected at a similar level, whereas a transcript level of XIST was about a half level, as compared to female fibroblasts (Table 2).

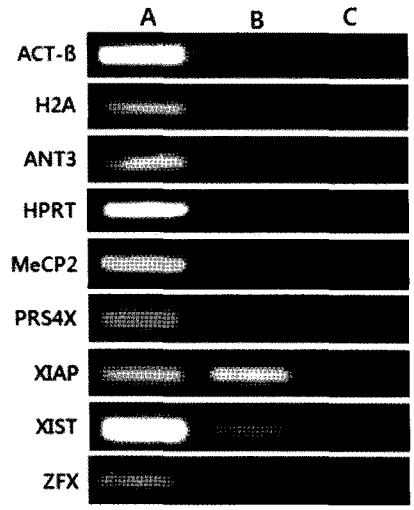

Fig. 1. Transcripts of X-chromosome linked genes detected in bovine female fibroblasts (A), matured spermatozoa (B), and negative control (distilled water, C).

Table 2. Transcript level of X-linked genes expressed in matured spermatozoa

Gene	Ratio* (mean±SEM)
ACT-β	ND
H2A	ND
ANT3	ND
HPRT	ND
MeCP2	ND
RPS4X	ND
XIAP	1.13±0.12
XIST	0.45±0.29
ZFX	ND

* Spermatozoa/female fibroblasts ratio of transcripts. Each transcript band density in female fibroblasts was considered as 1 for comparison. ND, not detected.

DISCUSSION

It has been generally known that the matured oocytes contain large amounts of maternal RNA transcripts,

including mRNA and non-coding RNA transcripts, for subsequent development of post-fertilization embryos. Their RNA transcripts are expressed through development stage and time of embryo and developmental capacity of the embryos is controlled by expressed RNA transcripts (Farley and Ryder, 2008). However, matured spermatozoa are ultimately differentiated and specialized cells with minimal cytoplasm without cell organelles (Jenkins and Carrell, 2011). Despite its compacted nucleus is trancriptionally inactivated, the spermatozoa nucleus contains diverse RNA transcripts at a low level when compared to oocytes (Barroso *et al.*, 2009; Ostermeier *et al.*, 2004; Ostermeier *et al.*, 2005). Herein, we have showed that the matured spermatozoa carry RNA transcripts, especially X-linked genes such as XIAP and XIST.

It has been reported that the spermatozoa RNAs are not transcribed in fully matured spermatozoa, rather than are stored in a stable form prior to spermatogenic differentiation (Steger, 2001). The functional significance of spermatozoa RNA transcripts delivered to the oocyte through fertilization is still a matter of debate, but it has been suggested that mRNAs in spermatozoa RNA transcripts is involved in protein synthesis for subsequent development of embryo after fertilization (Lalancette *et al.*, 2008). Otherwise spermatozoa RNA transcripts might be critically important roles in epigenetic modification of fertilized nucleus by controlling DNA methylation (Dadoune, 2009), and others reports have been also proven that several spermatozoa RNA transcripts is associated with nuclear matrix for structural components of nucleus (Lalancette *et al.*, 2008). Furthermore, antisense RNAs or microRNAs are detected in RNA transcripts derived from spermatozoa, and the increasing evidences have been reported that antisense RNAs or microRNAs in the spermatozoa RNA transcripts are associated with post-transcriptional regulators that bind to complementary sequences on target mRNA transcripts, resulting in translational silencing by mRNA degradation (Amanai *et al.*, 2006; Boerke *et al.*, 2007; Galeraud-Denis *et al.*, 2007; Krawetz, 2005; Lalancette *et al.*, 2008).

In present study, two X-linked genes, XIAP and XIST, in the bovine spermatozoa RNA transcripts were detected in RNA transcripts derived from matured bovine spermatozoa. The level of XIAP transcripts was similar when compared to the level of normal female fibroblasts, as shown in our results. XIAP is an X-linked inhibitor of apoptosis protein, known as inhibitor of apoptosis protein 3 (IAP3) or baculoviral IAP repeat-containing protein 4 (BIRC4). The incidence of cellular apoptosis was strongly inhibited by up-regulated XIAP expression in the cells (Harada and Grant, 2003) and was also inhibited in the bovine embryos with up-regulated XIAP expression (Knijn *et al.*, 2005). The translation from XIAP RNA transcript derived from spermatozoa to XIAP protein was still unclear in present study. However, we have considered that the incidence of cellular apoptosis may be strongly inhibited in the normally fertilized embryos with spermatozoa XIAP transcript which can be translated to XIAP protein.

Furthermore, XIST was also detected in matured spermatozoa, as shown in present results. XIST (X-inactive specific transcript) is a non-coding RNA transcript which acts as major controller of the X-inactivation process on the X chromosome in the female cells and embryos (Chang *et al.*, 2006; Heard, 2004). X-inactivation is a process by which one of the two copies of the X chromosome present in female cells and embryos is inactivated, resulting in silencing of genes on the inactivated X-chromosome, and one of the two X-chromosome is generally inactivated in most differentiated female somatic cells for dosage compensation which equally expressed between the male and the female, except for germ cells (Chang *et al.*, 2006). X-inactivation is firstly mediated and continually maintained by coating of XIST non-coding RNA on one of the two X-chromosome which will be inactivate in female embryo cells. Subsequently, modification of histone tail and DNA methylation is synergistically required for maintenance of the inactivated X-chromosome (Chang *et al.*, 2006; Heard, 2004). In mouse, X-inactivation and XIST expression were generally initiated at the 4-cell stage of embryos. Otherwise, it has been reported that XIST expression is detected at the 1-cell stage in mouse (Zuccotti *et al.*, 2002). Further, X-inactivation is induced on the paternally-derived X chromosome in 2-cell or 4-cell stage embryos of mouse. Another report has been also suggested that inactivation of paternally-derived X chromosome results from inheritance of a pre-inactivated X chromosome from the paternal germ line, and X-inactivation is induced by autosomal XIST (Huynh and Lee, 2003). In the early embryo stage, the process of inactivation of the X-chromosome is namely imprinted by paternal factor, and XIST is a kind of imprinted genes (Chang *et al.*, 2006). The inactivated X-chromosome is reactivated at implantation time of embryos, and one copy of the activated two X chromosome is randomly inactivated at implantation time of embryos (Okamoto *et al.*, 2004). As shown in our results, the expression level of XIST was interestingly detected at a half of normal female fibroblasts in the matured spermatozoa. Approximately a half of spermatozoa in the samples for RNA extraction might be X chromosome-bearing spermatozoa and XIST RNA transcripts might be derived from the X chromosome-bearing spermatozoa. In present study, even though the major function of XIST RNA transcript is unclear in spermatozoa and embryos after fertilization, we have suggested that the XIST RNA transcript may be contributed as controller or mediator of inactivation of paternal derived X-chromosome. Many studies have also suggested that se-

veral spermatozoa RNA transcripts play a critically important role for establishment of male-specific genomic imprinting and epigenetic reprogramming (Dadoune, 2009).

Apart from the paternal centriole and the soluble egg-activating factor as well as the haploid genome for a diploid offspring, additional spermatozoal components such as RNA transcripts is delivered to the oocytes through fertilization. Even though the biological functions of spermatozoa RNA transcripts is still debated in the embryo development, paternal contributions by spermatozoa RNA transcripts in the embryos should be considered for more successful reproductive biotechnology, especially in somatic-cell nuclear transfer. The results of this study show that the presence of RNA transcripts of XIAP and XIST in the isolated spermatozoa may have role in inhibition of apoptosis and induction of X-chromosome inactivation in embryo development. Further, the biological functions of these spermatozoa RNA transcripts should be elucidated as they affect the development of embryos.

ACKNOWLEDGEMENTS AND DISCLOSURE

The authors declare no conflict of financial interests.

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(Received: August 18 2011/ Accepted: August 25 2011)