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Original Article

Identification of Sperm mRNA Biomarkers Associated with Sex-Determination in Korean Native Cows

Kwan-Sik Min[1,2,*], Munkhzaya Byambaragchaa[1], Hyun Kim[3] and Myung-Hum Park[3]

[1]Animal Biotechnology, Graduate School of Future Convergence Technology, Anseong 17579, Korea
[2]Department of Animal Life Science, Institute of Genetic Engineering, Hankyong National University, Anseong 17579, Korea
[3]TNT Research, Anyang 14059, Korea

*Correspondence
Kwan-Sik Min
Animal Biotechnology, Graduate School of Future Convergence Technology, Hankyong National University, 327 Joongang-ro, Anseong 17579, Korea
Tel: +82-31-670-5421
Fax: +82-31-670-5616
E-mail: ksmin@hknu.ac.kr
ORCID
https://orcid.org/0000-0002-5451-3085

ABSTRACT This study was conducted to analyze the specific genes associated with sex-determination in Korean native cow. The highly organized spermatogenesis requires accurate spatial and temporal regulation of gene expression, which is governed by transcriptional, post-transcriptional, and epigenetic processes. Recently, farmers have been interested in determining the sexual identity of the calves in their farm. We analyzed the sperm of Korean native and Holstein cows, which were supplied from Hanwoo Improvement Center. We evaluated sperm motility and expression of sperm-specific genes after treating semen with both male- and female reagents. Sperm motility in Korean native cows decreased by approximately 10% in the first 30 minutes after treatment with sex-determination reagent. However, sperm motility of Holstein cows decreased to 60-70% after 15 minutes and to 20-30% after 30 minutes. We selected six specific genes expressing in the spermatozoa to analysis the gene expression level. The Real-time PCR results suggest that the selected genes (*Gimap4*, *Tmeff1*, *Rac2*, *Abi2*, *Rac1*, and *Clu*) were highly expressed in the group treated with the male reagent compared to the group treated the female reagent and to the untreated-group (control). In the present study, we suggest that the selected genes play a pivotal role in sex-determination.

Keywords: Korean native cows, sex-determinant reagent, sperm mRNA

INTRODUCTION

Sex determination (male fertility) depends on the successful and constant process of of spermatogenesis, which is a highly organized process where spermatozoa develop from germ cells in the seminiferous tubules of the testis (Chen et al., 2017). The testis is one of many organs routinely and systematically assessed in comparative preclinical drug studies (Dere et al., 2017). DNA damage

to sperm cells has been identified as a major contributor to male infertility and to negative outcomes following assisted reproduction treatment, including impaired embryo development and birth defects in the offspring (Ebner et al., 2011; Zribi et al., 2011). However, conventional semen analysis does not measured all parameters related to sperm quality (Cissen et al., 2016). Several papers have investigated the link between pregnancy failure and DNA damage to sperm cells (Ozmen et al., 2007; Lin et al.,

2008;). Thus, DNA integrity of sperm cells is one of the key determinants of successful fertilization and embryo development (Robinson et al., 2012). Sperm with DNA damage caused by specific reagents are capable of fertilizing eggs (Aitken et al., 1998; Gandini et al., 2004), but may be associated to low pregnancy rates (Bungum et al., 2007; Collins et al., 2008; Frydman et al., 2008; Lin et al., 2008).

These technique for sex-determination relies on flow cytometric assays that separates spermatozoa bearing X and Y chromosome with an accuracy greater than 90% (Garner et al., 1983; Morrell et al., 1988). However, it is expensive, time-consuming (Seidel, 2003), and accelerates changes in mRNA expression levels of certain genes in the embryo (Morton et al., 2007), which yields lower fertility rates *in vitro* (Lu et al., 1999; Xu et al., 2009) and *in vivo* (Maxwell et al., 2003; Bodmer et al., 2005). Recently, many methods to isolate sperm have been developed using PCR (Tan et al., 2008), such as DNA quantification of sex sorted sperm (Welch and Johnson, 1999), fluorescence in site hybridization (Rens et al., 2001), real-time quantitative PCR (qPCR) (Joerg et al., 2004), and SYBR green qRT-PCR (Husna et al., 2016).

In order to determine the sexual identity of Korean native cows, we investigated sperm motility in semen treated with specific sex-determination reagents. We also analyzed the expression level of sperm mRNA in semen treated with male and female reagents.

MATERIALS AND METHODS

Experimental design

Frozen semen of Korean native and Holstein cows was obtained from the Hanwoo Improvement Center-Semen Production Unit. For both breeds, semen was divided into three groups. Cryopreserved treated and untreated (control) semen samples were analyzed for sperm motility after thawing for 0, 15 minutes, and 30 minutes. Sperm was then collected and stored at -80°C.

Primer design

A pair of specific primers were designed for GTPase immunity-associated protein family member4 (*Gimap4*), a transmembrane protein containing two follistatin and an epidermal growth factor-like (EGF) region (*Tmeff1*), Rac1, Rac2, abi-interactor 2 (*Abi2*), and clusterin (*Clu*),

all known as sperm specific genes in cattle. According to the parameters required for the SYBR green real-time PCR, the NCBI dataset was used. An internal housekeeping gene (GAPDH) was needed as a positive control. Gene sequences of the forward and reverse primers are shown in Table 1.

Sperm motility after reaction with sex-determination reagent

Frozen semen' straws of Korean native (KPN 998) and Holstein (334) cows were thawed for 20-25 seconds in a water bath at 37°C. Semen was then put into 6 or 12-well plates, and female and male reagents bought from TNT research (Anyang, Korea) were added to each sample (each well). After which, semen was incubated for 0, 15, and 30 minutes at room temperature. Sperm motility of each sample was objectively analyzed with a microscope by two researchers. After sperm motility was evaluated, RNA was extracted from samples. The sperm motility was analyzed in triplicate independent repeats by two researchers.

RNA isolation of sperm and qRT-PCR

After samples were treated with the specific reagents, sperm was collected and placed in 1.5 mL tubes. Following centrifugation for five minutes at 2,000 × g, the pellet

Table 1. Specific primers used to amplify genes of Korean native cows

Gene	Sequence (5'→3')	Length (bp)
bClus		
Forward	tca gca ggc tgt acg acc agc tg	317
Reverse	tc cgg cgg tat tgc tgc agt gct	
bGIMAP4		
Forward	ctc tca agg aga tta ccc gct gca	412
Reverse	gc tct gta ata ttc ttg taa cac	
bTMEFF1		
Forward	atg atg gac tac agt atc gac cag	314
Reverse	ggt tat gca cat gac aat tgc tac	
bABI2		
Forward	gta cca aat gat tac gta cct agc	254
Reverse	at tca tgc tgt aga act gaa cag	
bRAC1		
Forward	tgt cct acc cgc aga cag atg tat	261
Reverse	agc gcc gag cac tcc agg tat	
bGAPDH		
Forward	acc act gtc cac gcc atc act	452
Reverse	tcc acc acc ctg ttg ctg ta	

of sperm was saved and the supernatant centrifuged again for three minutes at 2,000 × g (Pacheco et al., 2012), the pellets were incubated in a lysis buffer (0.15 M ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA (Thermo Fisher Scientific Inc.,) for 30 seconds prior to centrifugation at 16,000 × g for one minutes to remove contaminants, leaving the sperm intact. RNA was extracted from the samples using the Trizol reagent (Invitrogen) and the *mir*Vana miRNA Isolation kit (Applied Biosystems/Ambion, TX). RNA purity was confirmed by the absence of 18/28S ribosomal RNA peaks with an UV spectrophotometer (Biorad). Sperm mRNA content was assessed using qRT-PCR arrays. The qRT-PCR reaction followed the manufacturer's instructions using an ABI 7900 HT thermocycler (Applied Biosciences, Life Technologies Corporation, CA). Raw C_t values were normalized to the geometric mean of the housekeeping gene (GAPDH). C_t values and C_t expression were analyzed using the $\Delta\Delta C_t$ method following ABI's guidelines. The fold change ratio range was generated using the formula $2^{-\Delta\Delta}$, the standard error of the treatment groups was generated after calculating the average ΔC_t values.

Statistic analysis

Sperm mRNA transcript data was analyzed using one-way ANOVA to identify significant changes relative to vehicle control. The analysis was carried out in Prism 6 software (GraphPad Software, CA).

RESULTS

Semen samples treated with male and female reagents are shown in Fig. 1. The upper lanes refer to the control group, the second lane refer to samples treated with the male reagent, and the third lane to samples treated with female reagent (Fig. 1). Sperm motility of Korean native and Holstein breeds after being treated with the corresponding reagent are shown in Table 2. In samples treated with the specific reagents, sperm motility of Korean native cows was 80, 70-80, and 70-75% at 0, 15, and 30 minutes, respectively. Sperm motility of Korean

Table 2. Sperm motility of Korean native and Holstein cows in each treatment group

Treatment Groups		Treatment time and motility (%)		
		0 min	15 min	30 min
Korean native	Control	80	75–80	75–80
		80	75–80	75–80
	Female	75–80	70–75	70–75
		80	75–80	70–75
	Male	80	75–80	70–75
		80	75–80	70–75
Holstein	Control	75–80	60–70	30
		80	60–70	40
	Female	80	60–70	20–30
		75–80	60–70	20–30
	Male	75–80	50–60	30
		75–80	50–60	20–40

The sperm motility was analyzed in triplicate independent repeats as described in the Material and Methods.
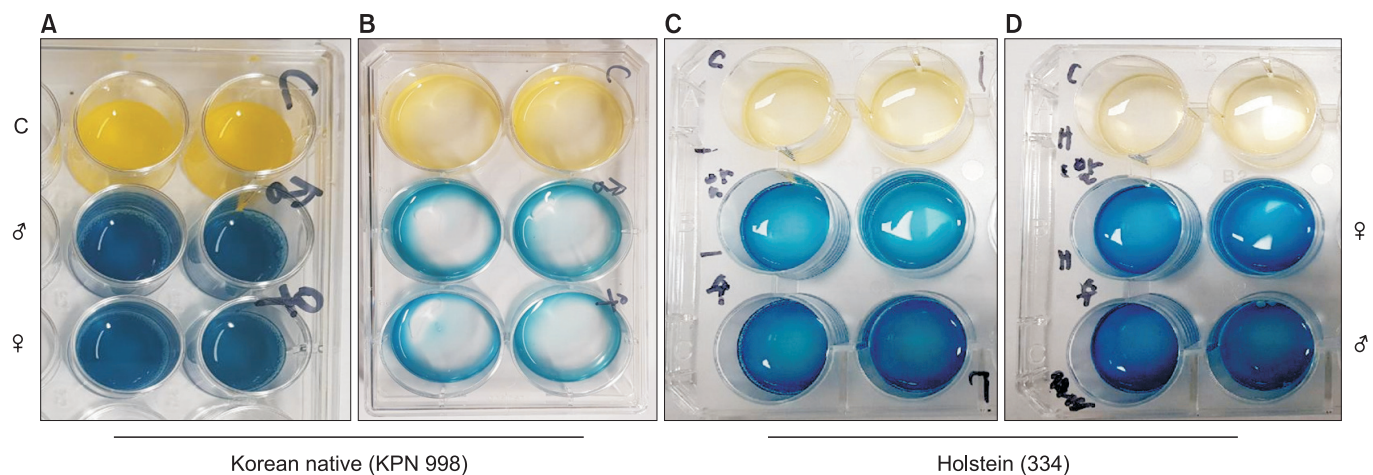

Fig. 1. Reaction of semen with male and female reagents in Korean native Cattle and Holstein. (A) and (B) are shown the Korean Native Cattle. (C) and (B) are shown the Holstein. These images were taken 30 minutes after the corresponding reagents were added. C: control.

native cows decreased after 30 minutes of thawing in all samples, including the control group where sperm motility decreased by 15%. However, sperm motility of Holstein cows was even lower, decreasing to 20-40% after 30 minutes (Table 2).

Sperm mRNA transcript levels on the qRT-PCR array panel was significantly greater in sperm treated with the male reagent (Fig. 2). Interestingly, transcript level of *RAC1*, *Abi2*, *Tmeff1*, *GIMP4*, and *Clu* observed significantly different 30 minutes after reagents were added. When treated with the male reagent, transcript levels of *Rac1*, *abi2*, *Tmeff1*, and *Clu* were significantly greater in comparison to the control group. Transcript levels of *Clu* were 5 times greater when treated with the male reagent in comparison to the female reagent. Transcript levels of the other genes were just a 1.2 to 1.8 times greater when treated with the male reagent in comparison to the female reagent.

DISCUSSION

Sexed semen has brought an economic advantages to the dairy industry to Korean native cattle farms. Isolation sperm in order to separate X and Y chromosomes has been widely studied. Sperm with higher motility (Henkel and Schill, 2003) and viability (Lucio et al., 2012) is effective in increasing fertilization success (Rens et al., 2004). However, isolating sperm too expensive. Thus we tested
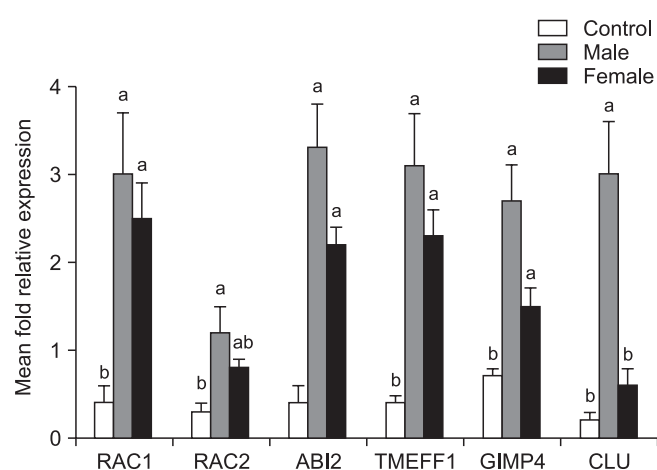

Fig. 2. Expression level of mRNA transcription following treatment with the corresponding sex determination reagent for 30 minutes. Data are expressed as the mean ratio relative to the vehicle control ± SEM.

sex-determination reagents that have been developed without having to isolate sperm. Our results show that sperm motility in Korean native cows was hardly affected by these reagents, suggesting that the sperm is capable to fertilize eggs, both *in vitro* and *in vivo*. However, sperm motility of Holstein semen decreased significantly. The best semen storage method, deep-freezing or stocking in LN2 tank, remains unclear.

The real-time PCR technique is considered a valid, accurate and reliable method to quantity spermatozoa bearing X and Y chromosomes in bovine semen (Maleki et al., 2013; Husna et al., 2017). In this studies, we used qRT-PCR to analyze six specific genes (*Gimap4*, *Tmeff1*, *Rac1*, *Rac2*, *abi2*, and *Clu*) that are involved with important functions of spermatogenesis. Changes in sperm mRNA include increased gene expression in sperm treated with the male reagent.

Clu is a glycoprotein involved in many biological processes that affects germ cell maturation (Anton and Krawetz, 2012). *Clu*, which is also secreted by sertoli cells, maintains the homeostatic balance between cell viability and proliferation (Ammar and Closset, 2008). *Clu* mRNA was detected in rat and pig spermatozoa and mRNA levels were greater in testis exposed to methoxychlor (Ostermeier et al., 2004; Vaithinathan et al., 2009). *Gimap4* is involved in accelerating programmed T-cell death (Schnell et al., 2006) and interacts with the trans-Golgi network to facilitate the proper development of sperm acrosomes (Escalier et al., 1991; Heinonen et al., 2015). Thus, *Gimap4* is very important as it positively affects the quality of sperm (Dere et al., 2017). *Tmeff1* has been reported to be highly expressed in human embryos and nerve tissues (Condomines et al., 2009) and was initially found with differential expression in brain tissues and brain tumors (Eib and Martens, 1996). The expression of *Tmeff1* in an ovarian cancer cells was downregulated after the inhibition of p53 (Nie et al., 2019). Thus, the function of *Tmeff1* in the sperm is not well known.

Recently, Abi1 and Abi2 interact with and dephosphorylate brassinosteroid insentitive2 (BIN2) to regulate its activity toward the phosphorylation of *bri*1-EMS-Suppressor1 (BES1) in the Abi2 (Wang et al., 2018). The genetic and molecular functions for sperm motility in Abi1 and Ab2 were not reported until now. Rac2, a member of the Rho small-GTPase family, is restricted to the hematopoietic lineage, which plays a principal role in regulat-

ing the actin cytoskeleton and neutrophil biology (Hsu et al., 2017). Rac2 is required for adhesion and retention of neutrophils in the hematopoietic tissue, yet not required for their release from this tissue (Deng et al., 2011).

Our study has demonstrated that *Clu*, *Tmeff1* and *Gimap4* play a key role in the male determination of bovine sperm. In the present study, we suggest that these genes play a key role in sperm bearing Y chromosomes. These genes may be used as bio-marker to determine sex in bovine sperm. The reagent used in the present study is adequate and may be used to produce male and female calves in bovine farms, suggesting that these genes could be a new bio-maker for sex-determination in bovine.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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ORCID

Kwan-Sik Min: https://orcid.org/0000-0002-5451-3085
Munkhzaya Byambaragchaa: https://orcid.org/0000-0002-0277-1816
Hyun Kim: https://orcid.org/0000-0001-5540-2857
Myung-Hum Park: https://orcid.org/0000-0002-7920-4756

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