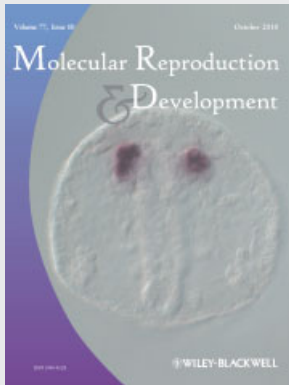
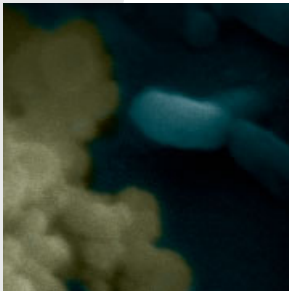


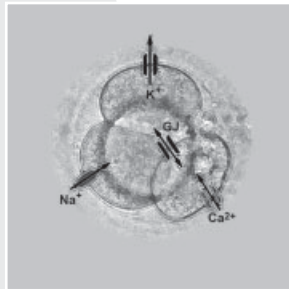
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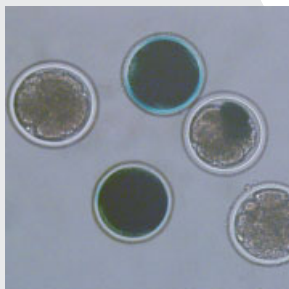
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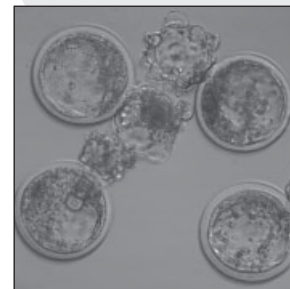
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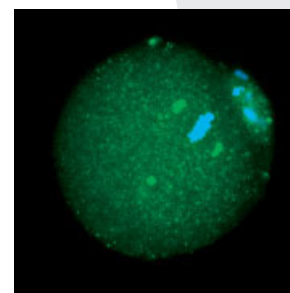
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*AMP-activated protein kinase is a ubiquitous signaling molecule whose phosphorylating activity is triggered by a variety of stimuli. In oocytes, this enzyme is intimately involved with resumption of meiosis, and its activity persists beyond germinal vesicle breakdown. Downs et al. report that AMPK activity in the oocyte regulates the timing of polar body extrusion and subsequent oocyte activation.*



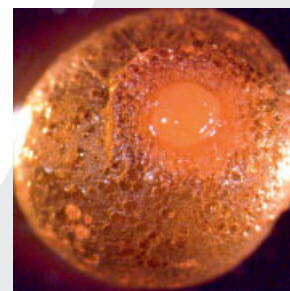
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*Stress responses in vertebrates generally involve increases in circulating cortisol levels, allowing the individual to adapt to the new situation by adjusting metabolic acuity. Yet cortisol also accumulates in oocytes and eggs, presumably from circulating maternal concentrations. Li et al. document that elevated levels of cortisol in rainbow trout eggs are linked to altered embryonic expression of metabolic genes, lower rates of hatching, and the health and life-span of the resultant individuals.*

# Relationship of Protein Tyrosine Phosphorylation State With Tolerance to Frozen Storage and the Potential to Undergo Cyclic AMP-Dependent Hyperactivation in the Spermatozoa of Japanese Black Bulls

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## SUMMARY

The aim of this study was to elucidate the relationship between protein tyrosine phosphorylation state and sperm characteristics in frozen-stored spermatozoa of Japanese Black bulls. The spermatozoa were washed with PBS containing polyvinyl alcohol and then incubated with cell-permeable cAMP analog cBiMPS to induce flagellar hyperactivation. Before and after incubation, the spermatozoa were used for immunodetection of tyrosine-phosphorylated proteins, assessment of morphological acrosome condition and evaluation of motility. In bulls whose frozen-stored spermatozoa were classified as having a high-grade acrosome condition before incubation, sperm tyrosine-phosphorylated proteins, including the 33-kDa tyrosine-phosphorylated SPACA1 protein, were localized in the anterior region of the acrosome and equatorial subsegment. The immunodetection level of the 41- and 33-kDa sperm tyrosine-phosphorylated proteins in the Western blots and the immunofluorescence of tyrosine-phosphorylated proteins and SPACA1 proteins in the anterior region of the sperm acrosome were lower in bulls whose frozen-stored sperm were classified as having a low-grade acrosome condition. On the other hand, after incubation with cBiMPS, immunodetection levels of at least 10 tyrosine-phosphorylated proteins increased in the connecting and principal pieces of spermatozoa, coincident with the induction of flagellar hyperactivation. Many of the spermatozoa also exhibited detection patterns similar to those of boar hyperactivated spermatozoa. These results are consistent with the suggestion that immunodetection levels of tyrosine-phosphorylated proteins are valid markers that can predict the level of tolerance to frozen storage and the potential to undergo cAMP-dependent hyperactivation for the spermatozoa of individual Japanese Black bulls.



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Additional Supporting Information may be found in the online version of this article.

INTRODUCTION

Mammalian spermatozoa undergo capacitation-related changes in the female reproductive tract. These changes are terminated after several hours, and consequently fully capacitated spermatozoa are capable of undergoing the acrosome reaction in the head and hyperactivation in the flagellum (Yanagimachi, 1994; Rodriguez-Martinez, 2007). We previously showed that hyperactivation was induced in boar ejaculated spermatozoa by a long (90–180 min) incubation with cell-permeable cyclic AMP (cAMP) analog “Sp-5,6-DCI-cBiMPS” (cBiMPS) in vitro (Harayama and Miyake, 2006; Harayama and Nakamura, 2008). Similar effects of cBiMPS on the transition of motility to hyperactivation were recently observed in frozen-stored spermatozoa of cattle (Japanese Black bulls) (Murase et al., 2010). Moreover, our previous findings (Harayama et al., 2004a, 2005) indicate that cBiMPS-induced protein tyrosine phosphorylation is involved in the activation of spleen tyrosine kinase (SYK)-phospholipase Cgamma1 (PLCgamma1) signaling cascades in the connecting piece of boar spermatozoa. In addition, the importance of flagellar protein phosphorylation in the transition to hyperactivation has also been indicated in the spermatozoa from other animal species (Si, 1999; Si and Okuno, 1999; Baker et al., 2006; Kaneto et al., 2008; Goto and Harayama, 2009).

Boar ejaculated spermatozoa contain tyrosine-phosphorylated proteins in the acrosome and equatorial segment of the heads before incubation with cBiMPS to induce hyperactivation (Harayama et al., 2004b). The tyrosine-phosphorylated proteins of the equatorial segment are localized in the triangular compartment (equatorial subsegment), and one of them is identified as an ortholog of human SPACA1 proteins (formerly sperm acrosomal membrane-associated protein, SAMP32, Jones et al., 2008). The human SPACA1 proteins are localized in the acrosome and equatorial segment, and they contain four isoforms (pls: 4.5–5.5, molecular masses: 32–34 kDa) that are products of a single gene (*SAMP32*) encoding a 32-kDa protein with a pI of 4.57 (Hao et al., 2002).

Cases of male subfertility in the artificial insemination (AI) program using frozen-stored spermatozoa have been found in Japanese Black bulls. Many of them are hardly detectable by conventional examination of sperm characteristics because there is almost no difference in sperm motility after thawing between AI-fertile and AI-subfertile bulls (Murase

et al., 2001; Kuroda et al., 2007). Thus, farmers have an urgent need for markers of AI-subfertile bulls. However, the causal factors of AI subfertility remain to be determined at the molecular level, although we have suggested that it is associated with cryo-capacitation and precocious acrosome reaction (Murase et al., 2001; Kuroda et al., 2007).

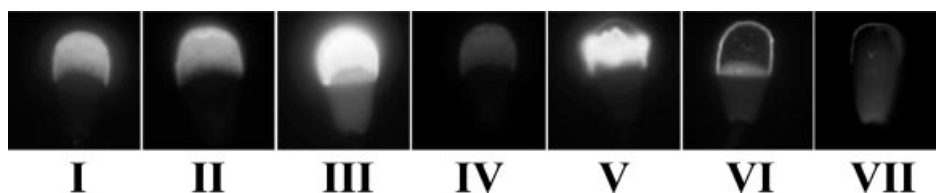
The purposes of this study were to characterize protein tyrosine phosphorylation patterns in the heads and flagella of frozen-stored spermatozoa of Japanese Black bulls before and after incubation with cBiMPS to induce hyperactivation and to examine their relationship with sperm characteristics. Additionally, we compared the patterns of cBiMPS-induced protein tyrosine phosphorylation among frozen-stored spermatozoa of Japanese Black bulls, boar ejaculated spermatozoa, and mouse epididymal spermatozoa.

RESULTS

Characterization of Immunodetection Patterns of Tyrosine-Phosphorylated Proteins and SPACA1 Proteins in Frozen-Stored Spermatozoa From the AI-Fertile Bull

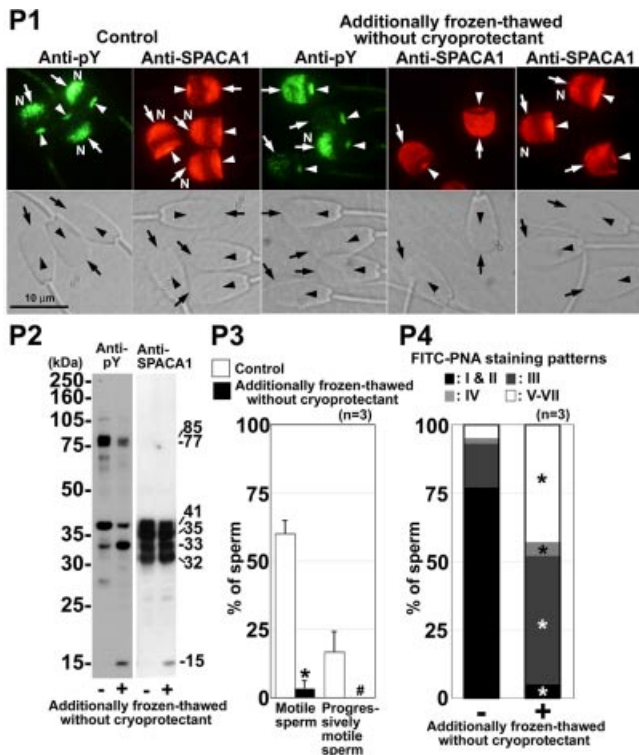
First, localization and Western blotting patterns of tyrosine-phosphorylated proteins and SPACA1 proteins were observed in frozen-stored spermatozoa from AI-fertile bull A. The AI fertility of bull A was confirmed by routine AI tests (No. of AI tests: 68 tests and rate of pregnancy: 66%). In the spermatozoa (Figs. 1 and 2, control samples) characterized by high-grade motility and morphological acrosome conditions, both tyrosine-phosphorylated proteins (major bands: 85, 77, 41, and 33 kDa) and SPACA1 proteins (major bands: 41, 35, 33, and 32 kDa) were strongly detected in the equatorial subsegment and the anterior region of the acrosome of sperm heads. SPACA1 proteins were also distributed in the equatorial segment surrounding the equatorial subsegment. These localization patterns of tyrosine-phosphorylated proteins and SPACA1 proteins (normal localization patterns, indicated by “N” in Panel (P)1 of Fig. 2) were observed in 94% and 91% of spermatozoa, respectively (as determined by the observation for approximately 100 spermatozoa).

In order to examine the influences of acrosomal damages on the immunodetection levels of tyrosine-phosphorylated



**Figure 1.** Classification of fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) staining patterns of bull sperm acrosomes. The stained samples were frozen-stored spermatozoa of Japanese Black bulls. I pattern: normal acrosome, II pattern: slightly disordered acrosome, III pattern: severely disordered acrosome with highly bright fluorescence, IV pattern: acrosome with less fluorescence in the whole part, V pattern: severely disordered acrosome with less fluorescence in the anterior region, VI pattern: acrosome with fluorescence only along its outline, and VII pattern: acrosome with almost no fluorescence.





**Figure 2.** Immunodetection of tyrosine-phosphorylated proteins and SPACA1 proteins in frozen-stored spermatozoa of the artificial insemination (AI)-fertile Japanese Black bull. The frozen-stored spermatozoa from bull A were washed with PBS containing polyvinyl alcohol (PVA-PBS) three times [control or (–) of additionally frozen-thawed without cryoprotectant]. Otherwise, they were washed once with PVA-PBS, additionally frozen-thawed without cryoprotectant and then washed twice [additionally frozen-thawed without cryoprotectant or (+) of additionally frozen-thawed without cryoprotectant]. In Panel 1 (P1) (each photograph of indirect immunofluorescence: a representative of three replicates), aliquots of each sperm sample were treated with mouse anti-phosphotyrosine monoclonal antibody or guinea pig anti-SPACA1 polyclonal antibody, and subsequently detected with fluorescein isothiocyanate (FITC)-conjugated (green) or TRITC-conjugated goat secondary antibody. In P2 (each of Western blots: a representative of three replicates), aliquots of each sperm sample ( $5 \times 10^5$  cells/lane) were used for SDS–PAGE (the final concentration of acrylamide: 10%) and Western blotting, and then treated with anti-phosphotyrosine antibody or anti-SPACA1 antibody and subsequently with horseradish peroxidase (HRP)-conjugated goat secondary antibody. In P3 and P4, the balance of each sperm sample was used for the observation of sperm motility and assessment of the morphological acrosome condition by the FITC-PNA staining. In P3, values are means  $\pm$  SD. In P3 and P4, values with asterisks are significantly different from those of the control samples (paired *t*-test,  $P < 0.05$ ). The value with # is zero  $\pm$  zero, which was not subjected to the statistical analyses. [Color figure can be viewed in the online issue which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

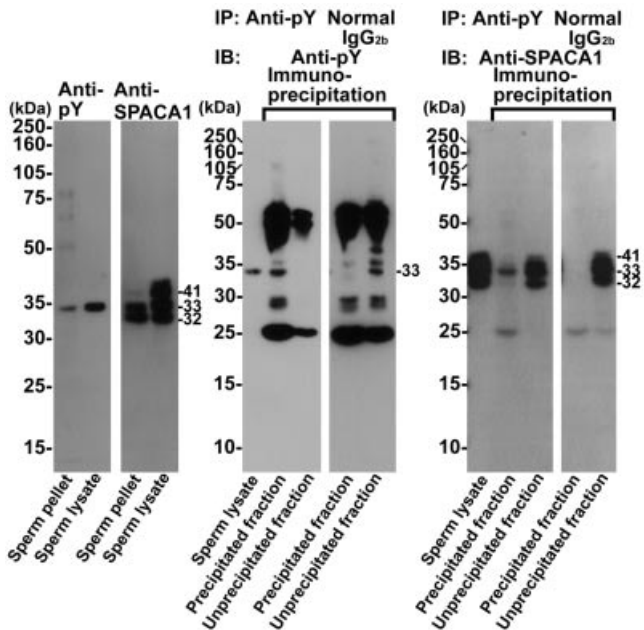
proteins and SPACA1 proteins, frozen-stored spermatozoa of AI-fertile bull A were washed once with PVA-PBS, additionally frozen ( $-80^\circ\text{C}$  for 20 min)-thawed without cryoprotectant, and then washed twice. This additional freezing-thawing treatment severely damaged the acrosome and significantly reduced motility (P3 and P4 of Fig. 2). After many sperm underwent the additional freezing-thawing treatment, the acrosomal tyrosine-phosphorylated proteins

were only faintly detected or irregularly spread while the acrosomal SPACA1 proteins were spread uniformly or irregularly (P1 of Fig. 2). In the Western blots, the immunodetection level of the 33-kDa tyrosine-phosphorylated protein increased, whereas that of the three other major tyrosine-phosphorylated proteins (85, 77, and 41 kDa) decreased. On the other hand, four major bands of SPACA1 proteins (41, 35, 33, and 32 kDa) were strongly detected even after the additional freezing-thawing treatment. In addition, the 15-kDa protein appeared in both blots immunodetected with anti-phosphotyrosine antibody and anti-SPACA1 antibody (P2 of Fig. 2).

To clarify the relationship between tyrosine-phosphorylated proteins and SPACA1 proteins, lysates were prepared from AI-fertile bull A frozen-stored spermatozoa and subjected to immunoprecipitation with the anti-phosphotyrosine antibody followed by immunodetection for phosphotyrosine and SPACA1 (Fig. 3). During the preparation of sperm lysates, three major tyrosine-phosphorylated proteins (85, 77, and 41 kDa) were lost, and finally only the 33-kDa tyrosine-phosphorylated protein was recovered. The decrease in the three proteins might be due to acrosome damage occurring during the preparation of sperm lysates, as shown in Figure 2. At least three isoforms of SPACA1 proteins (41, 33, and 32 kDa) were also extracted into the sperm lysates (left panel of Fig. 3). As shown in the central and right panels of Figure 3, almost all of the 33-kDa tyrosine-phosphorylated protein was immunoprecipitated with the protein A agarose beads bound to the anti-phosphotyrosine antibody and recognized by the SPACA1 antibody. These results reveal that the 33-kDa tyrosine-phosphorylated protein is one form of SPACA1 proteins. However, the fraction unprecipitated with the anti-phosphotyrosine antibody abundantly contained 33-kDa SPACA1 proteins (right panel of Fig. 3), though almost all of the 33-kDa tyrosine-phosphorylated protein was detected in the precipitated fraction but not in the unprecipitated fraction (central panel of Fig. 3). These results indicate that the 33-kDa SPACA1 proteins contain not only the tyrosine-phosphorylated form but also the nontyrosine-phosphorylated form.

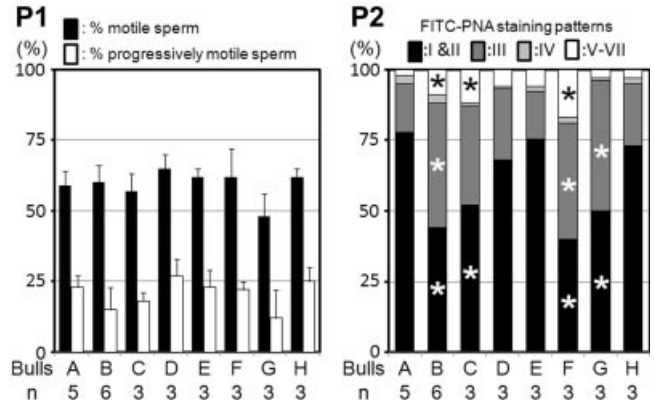
#### Individuality of the Immunodetection Patterns of Tyrosine-Phosphorylated Proteins and SPACA1 Proteins in Bull Frozen-Stored and Freshly Ejaculated Spermatozoa

Bulls A and D (No. of AI tests: 57 tests, rates of pregnancy: 65%) were fertile by the routine AI test, whereas bull B (No. of AI tests: 74 tests and rate of pregnancy: 1%) was apparently AI-subfertile. The AI fertility of the other bulls was still under investigation. Bull A-H frozen-stored spermatozoa before incubation were used to examine the motility and morphological acrosome condition and to detect tyrosine-phosphorylated proteins and SPACA1 proteins. In bulls A, D, E, and H, whose spermatozoa were classified as having a high-grade acrosome condition (P2 of Fig. 4), both tyrosine-phosphorylated proteins (41 and 33 kDa) and SPACA1 proteins (41, 35, 33, and 32 kDa) were strongly detected in the equatorial segment and the anterior part of the acrosome of sperm heads [% of the spermatozoa show-



**Figure 3.** Immunoprecipitation of a tyrosine-phosphorylated form of SPACA1 proteins in the lysates from frozen-stored spermatozoa of A-fertile Japanese Black bull. Frozen-stored spermatozoa of bull A were washed, sonicated in the ice-cold lysis buffer and then gently rocked for 60 min. The obtained lysates (Sperm lysate:  $5 \times 10^5$  cells/lane) were separated from sperm debris (Sperm pellet:  $5 \times 10^5$  cells/lane) by centrifugation and gently rocked with the protein A agarose beads bound with the mouse anti-phosphotyrosine antibody (Anti-pY) or normal mouse immunoglobulin G<sub>2b</sub> (Normal IgG<sub>2b</sub>) in wet ice. The protein A agarose beads (Precipitated fraction:  $5 \times 10^5$  cells/lane) were separated from the supernatants (Unprecipitated fraction:  $5 \times 10^5$  cells/lane). All samples were subjected to SDS-PAGE (the final concentration of acrylamide: 10% or 12.5%) and Western blotting using the anti-SPACA1 antibody (Anti-SPACA1) and anti-phosphotyrosine antibody (Anti-pY), as described in Materials and Methods.

ing normal localization patterns of tyrosine-phosphorylated proteins and SPACA1 proteins (see P1 of Fig. 2), respectively: A (94% and 91%), D (87% and 77%), E (89% and 83%), and H (94% and 94%)]. However, in bulls B, C, F, and G, whose samples were in the low-grade acrosome condition (patterns I and II: significantly lower than bull A samples, P2 of Fig. 4), immunofluorescence levels of tyrosine-phosphorylated proteins and SPACA1 proteins were much lower in the anterior region of the acrosome, even though these proteins were detected in the equatorial subsegment (P1 of Fig. 5) [% of frozen-stored spermatozoa showing normal localization patterns of tyrosine-phosphorylated proteins and SPACA1 proteins, respectively: B (0% and 3%), C (23% and 31%), F (19% and 23%), and G (14% and 20%)]. In Western blots of these samples, 41- and 33-kDa tyrosine-phosphorylated proteins were only faintly or weakly detected, though four major bands of SPACA1 proteins (41, 35, 33, and 32 kDa) were clearly observed at levels almost equivalent to those of the samples from bulls A, D, E, and H (P2 of Fig. 5). Considering that the 33-kDa SPACA1 proteins contains both tyrosine-phosphorylated and nontyrosine-phosphorylated forms (central and right



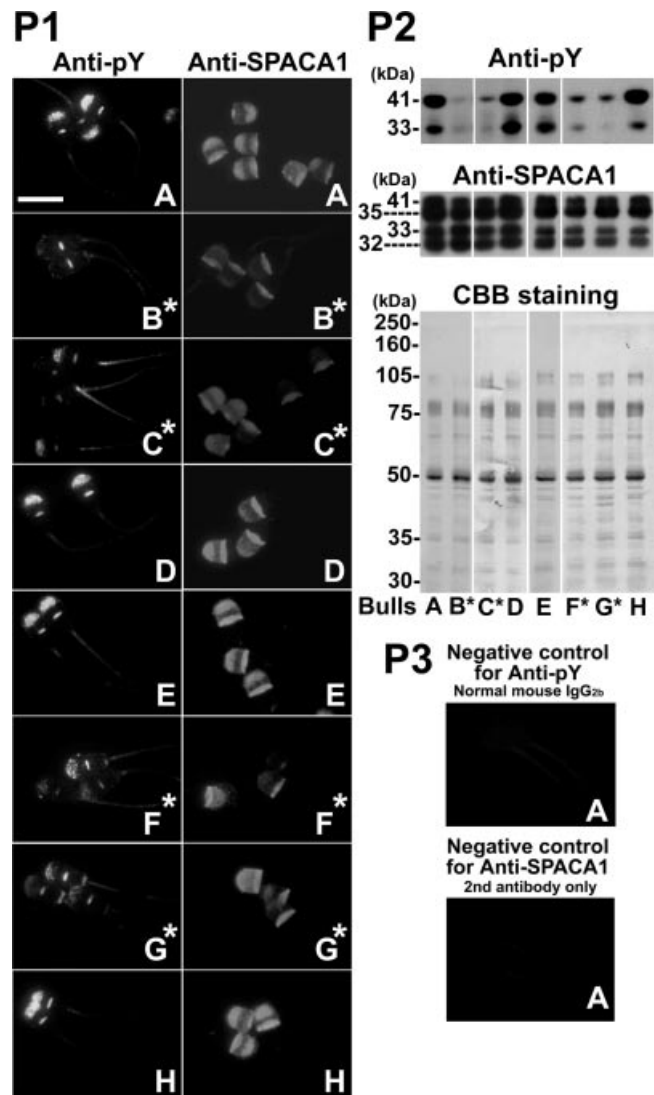
**Figure 4.** Motility and morphological acrosome condition of frozen-stored spermatozoa of individual Japanese Black bulls. Frozen-stored spermatozoa of bulls (A–H) were washed with PVA-PBS three times. Aliquots of each sperm sample were subjected to the assessments of sperm motility (P1) and morphological acrosome condition by the FITC-PNA staining (P2), as described in the Materials and Methods Section. In P1, values are means  $\pm$  SD. In P2, values with asterisks within the same classifications (I and II, III, IV, and V–VII) are significantly different from those of bull A spermatozoa (one-way ANOVA-Tukey's multiple range test,  $P < 0.05$ ).

panels of Fig. 3), the Western blot results indicate that the tyrosine-phosphorylated form reduced compared to the nontyrosine-phosphorylated form in the 33-kDa SPACA1 proteins. In addition, the percentages of motile spermatozoa and progressively motile spermatozoa were relatively constant among all bulls (P1 of Fig. 4).

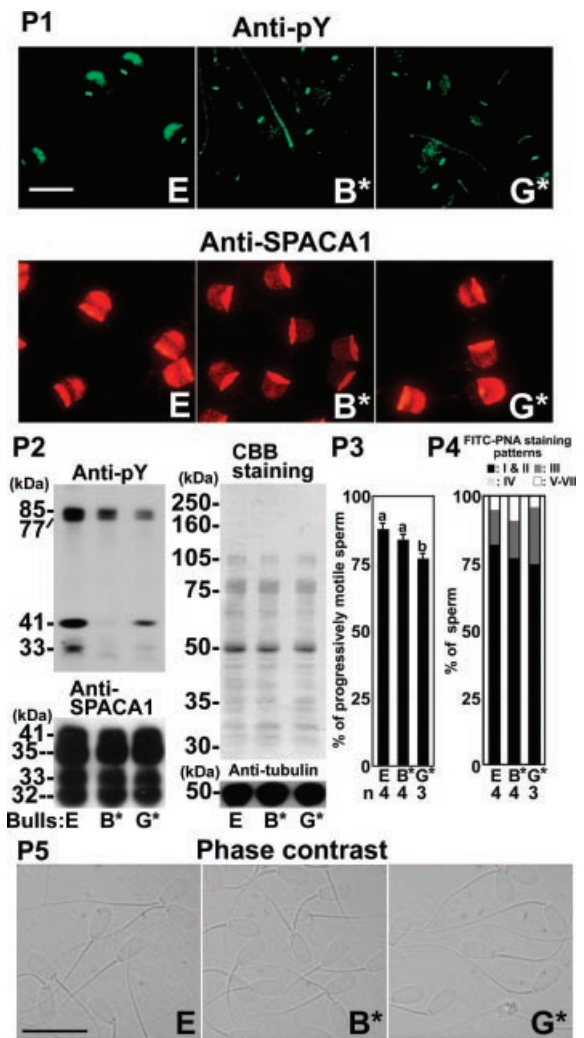
Freshly ejaculated spermatozoa of bulls E, B, and G were used to examine the motility and morphological acrosome condition and to detect tyrosine-phosphorylated proteins and SPACA1 proteins (Fig. 6). The percentages of progressively motile spermatozoa and spermatozoa classified into FITC-PNA patterns I and II were both higher than 70% in these bulls (P3 and P4 of Fig. 6). Most of the spermatozoa from these bulls were morphologically normal (P5 of Fig. 6). However, abnormal patterns of acrosomal tyrosine-phosphorylated proteins and SPACA1 proteins, which were immunodetected in frozen-stored spermatozoa of bulls B, C, F, and G by indirect immunofluorescence and Western blot (the low grade in the acrosome condition, P2 of Figs. 4 and 5), were also observed in freshly ejaculated spermatozoa (with normal acrosomes) of bulls B and G (P1 and P2 of Fig. 6) [% of the freshly ejaculated spermatozoa showing normal localization patterns of tyrosine-phosphorylated proteins and SPACA1 proteins, respectively: E (95% and 87%), B (4% and 10%), and G (27% and 29%)]. Moreover, the immunodetection levels of 85- and 77-kDa tyrosine-phosphorylated proteins were lower in the spermatozoa from bulls B and G than in those from bull E (P2 of Fig. 6).

**Hyperactivation and Tyrosine-Phosphorylated Proteins in the Frozen-Stored Spermatozoa After Incubation With Cell-Permeable cAMP Analog**

Tables 1 and 2 show the motility of spermatozoa before and after incubation with cBiMPS. Bull frozen-stored



**Figure 5.** Patterns of tyrosine-phosphorylated proteins and SPACA1 proteins in frozen-stored spermatozoa of individuals Japanese Black bulls. Frozen-stored spermatozoa of bulls A–H were washed with PVA-PBS three times. In **P1** and **P3** (each photograph of indirect immunofluorescence: a representative of three replicates), aliquots of each sperm sample were treated with mouse anti-phosphotyrosine monoclonal antibody [Anti-pY], normal mouse immunoglobulin G<sub>2b</sub> [Normal mouse IgG<sub>2b</sub> (control)], guinea pig anti-SPACA1 polyclonal antibody [Anti-SPACA1] or a blocking buffer without primary antibody [2nd antibody only (control)], and subsequently with FITC-conjugated or TRITC-conjugated goat secondary antibody. A scale bar indicates 20  $\mu$ m. In **P2** (each of Western blots: a representative of three replicates), aliquots of each sperm sample ( $5 \times 10^5$  cells/lane) were used for SDS-PAGE (the final concentration of acrylamide: 10%) and Western blotting, and then treated with anti-phosphotyrosine antibody or anti-SPACA1 antibody, and subsequently with HRP-conjugated secondary antibody. After Western blotting, each membrane was stained with Coomassie Brilliant Blue G-250 (CBB staining). The frozen-stored spermatozoa of bulls B, C, F, and G with asterisks were classified as having a low-grade morphological acrosome condition, as shown in Figure 4.



**Figure 6.** Immunodetection of tyrosine-phosphorylated proteins and SPACA1 proteins in freshly ejaculated spermatozoa of individual Japanese Black bulls. Freshly ejaculated spermatozoa of bulls E, B, and G were subjected to the assessments of sperm motility (**P3**), and were then washed with PVA-PBS three times. Each sperm sample was used for the immunodetection of tyrosine-phosphorylated proteins and SPACA1 proteins by indirect immunofluorescence and Western blotting (**P1** and **P2**, respectively, a representative of three replicates) and the assessment of morphological acrosome condition by the FITC-PNA staining (**P4**). In **P2**, each membrane was stained with Coomassie Brilliant Blue G-250 (CBB staining) after Western blotting. In **P3**, values are means  $\pm$  SD. Values with different letters (a and b) are significantly different (one-way ANOVA-Tukey's multiple range test,  $P < 0.05$ ). In **P5**, phase contrast photographs of the immunostained spermatozoa were taken to determine if morphology was indeed normal. Scale bars indicate 20  $\mu$ m.

spermatozoa (bulls I and J, see supplemental movies 1-1 and 1-2) and boar ejaculated spermatozoa exhibited hyperactivation partially after 90 min, and then highly after 180 min. In mouse epididymal spermatozoa, hyperactivation was highly induced after 30–90 min of incubation with cBiMPS. After 180 min of incubation, however, the number of hyperactivated mouse spermatozoa greatly decreased.

**TABLE 1. Motility of Frozen-Stored Spermatozoa of Japanese Black Bulls, Boar Ejaculated Spermatozoa, and Mouse Epididymal Spermatozoa Incubated With or Without Cell-Permeable Cyclic AMP (cAMP) Analog**

Donor animals	Sperm samples examined	cBIMPS (mM)	Motile sperm (%) <sup>a</sup> , incubation time (min)		Hyperactivated sperm (%) <sup>b</sup> , incubation time (min)	
			0	180	0	180
Bulls I and J	Frozen-stored (I: n = 10, J: n = 12, total: n = 22)	0	72 ± 3 <sup>c</sup>	68 ± 4 <sup>d</sup>	–	–
		0.1	73 ± 4 <sup>c</sup>	64 ± 5 <sup>d</sup>	–	+(n = 7)/++(n = 15)
		0	86 ± 8 <sup>c</sup>	77 ± 10 <sup>d</sup>	–	–
Boars K–M	Ejaculated (K: n = 6, L: n = 7, M: n = 2, total: n = 15)	0.1	86 ± 8 <sup>c</sup>	77 ± 10 <sup>d</sup>	–	+(n = 3)/++(n = 12)
		0	–	–	–	–

Donor animals	Sperm samples examined	cBIMPS (mM)	Motile sperm (%) <sup>a</sup> , incubation time (min)		Hyperactivated sperm (%) <sup>b</sup> , incubation time (min)	
			0	90	0	90
Mice N–T	Epididymal (N–T: n = 1 each, total: n = 7)	0	69 ± 7	64 ± 7	–	–
		0.1	69 ± 7	66 ± 6	–	++

Motility of spermatozoa was recorded with a CCD camera and DVD recorder. The recorded movies were played back and subjectively observed to determine the percentages of motile spermatozoa and hyperactivated spermatozoa. Values with different letters (c, d) within the same line are significantly different (paired t-test,  $P < 0.05$ ).

<sup>a</sup>Values are averages of percentages of motile spermatozoa ± SD.  
<sup>b</sup>Hyperactivated spermatozoa, (–) 0–9%, (+) 10–29%, (++) >29%.

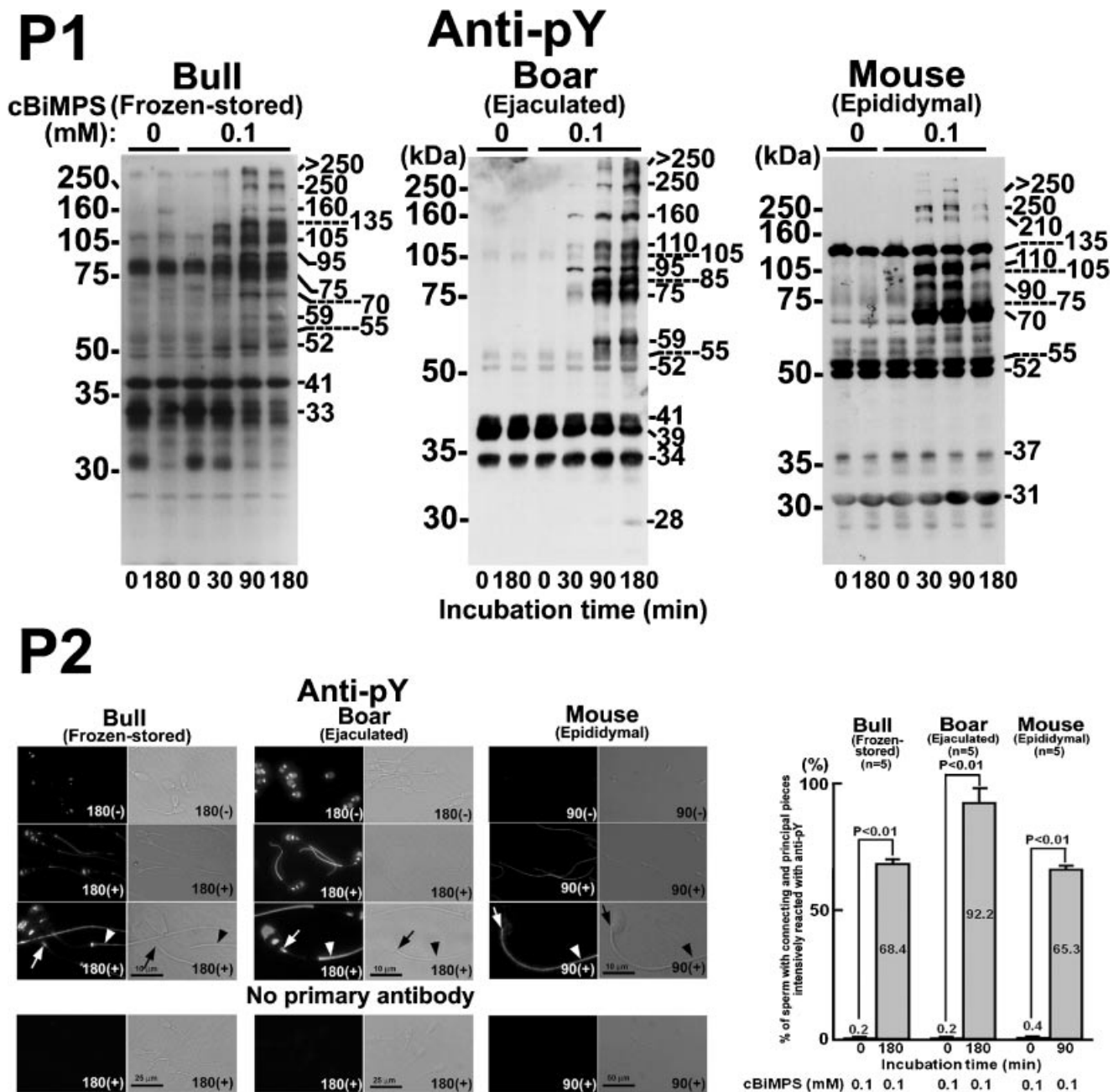
**TABLE 2. Incubation Time-Related Changes in Motility of Frozen-Stored Spermatozoa of Japanese Black Bulls, Boar Ejaculated Spermatozoa, and Mouse Epididymal Spermatozoa Incubated With Cell-Permeable cAMP Analog**

Donor animals	Sperm samples examined	cBIMPS (mM)	Motile sperm (%) <sup>a</sup> , incubation time (min)				Hyperactivated sperm (%) <sup>b</sup> , incubation time (min)				
			0	30	90	180	0	30	90	180	
Bulls I and J	Frozen-stored (I: n = 7, J: n = 8, total: n = 15)	0.1	68 ± 5 <sup>c</sup>	67 ± 3 <sup>c,d</sup>	61 ± 2 <sup>d,e</sup>	59 ± 2 <sup>e</sup>	–	–	–(n = 3)/++(n = 11)/++(n = 12)	–	–
		0.1	87 ± 6	83 ± 12	77 ± 6	73 ± 6	–	–	–	–	–
Boars K and L	Ejaculated (K: n = 1, L: n = 2, total: n = 3)	0.1	71 ± 9	71 ± 9	68 ± 10	65 ± 10	–	–	+(n = 1)/++(n = 3)	–	–
		0.1	71 ± 9	71 ± 9	68 ± 10	65 ± 10	–	–	–	–	–

Motility of spermatozoa was recorded with a CCD camera and DVD recorder. The recorded movies were played back and subjectively observed to determine the percentages of motile spermatozoa and hyperactivated spermatozoa. Values with different letters (c, d, and e) within the same line are significantly different (one-way ANOVA-Tukey's multiple range test,  $P < 0.05$ ).

<sup>a</sup>Values are means of percentages of motile spermatozoa ± SD.  
<sup>b</sup>Hyperactivated spermatozoa, (–) 0–9%, (+) 10–29%, (++) >29%.



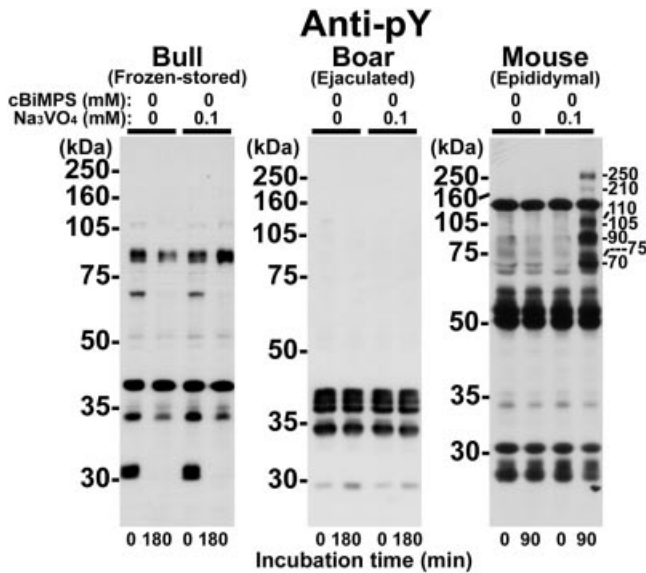


**Figure 7.** Immunodetection of cAMP analog-induced increases of tyrosine-phosphorylated proteins in frozen-stored spermatozoa of Japanese Black bulls, boar ejaculated spermatozoa and mouse epididymal spermatozoa. The spermatozoa from two bulls (bulls I and J), three boars (boars K–M) and five mice (mice N–R) were incubated with or without cell-permeable cAMP analog cBiMPS (0.1 mM) up to 180 min at 38.5°C. Immediately before and after incubation, the spermatozoa were subjected to Western blotting (P1, a representative of five replicates) and indirect immunofluorescence (P2, a representative of five replicates) as described in the legend of Figure 2. Arrows and arrow heads in the photographs indicate the connecting and principal pieces, respectively. In the experiments to assess the flagellar protein tyrosine phosphorylation state (P2), 100 cells on each immunostained sample were examined by fluorescent microscopy to calculate the percentages of spermatozoa with the connecting and principal pieces intensively reacted with the anti-phosphotyrosine antibody. Values are means  $\pm$  SD which were subjected to the statistical analyses (paired *t*-test).

Additionally, hyperactivation was barely induced in all samples incubated without cBiMPS.

Figure 7 shows cBiMPS-dependent increases in tyrosine-phosphorylated proteins in bull frozen-stored spermatozoa, boar ejaculated spermatozoa and mouse epididymal

spermatozoa. In bull and boar spermatozoa incubated with cBiMPS, a few tyrosine-phosphorylated bands (e.g., bulls: 105, 95, 52 kDa, boars: 160, 95 kDa) clearly increased after 30 min, and then several ones (e.g., bulls: >250, 250, 160, 135, 75, 70, 59, 55 kDa, boars: >250, 250, 110, 105, 85, 75,



**Figure 8.** Immunodetection of sodium orthovanadate-induced increases of tyrosine-phosphorylated proteins in frozen-stored spermatozoa of Japanese Black bulls, boar ejaculated spermatozoa and mouse epididymal spermatozoa. The spermatozoa from two bulls (bulls I and J), three boars (boars K–M) and three mice (mice R–T) were incubated with or without the protein tyrosine phosphatase inhibitor sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ , 0.1 mM) for 90–180 min at 38.5°C. Immediately before and after incubation, the spermatozoa were subjected to Western blotting (a representative of three replicates) as described in Materials and Methods.

59 kDa) dramatically increased during the incubation period between 30 and 180 min. These bands likely correspond to proteins in the connecting and principal pieces of many cells (bulls: 68.4%, boars: 92.2% at 180 min. These bands likely correspond to proteins). These cBiMPS-induced increases in tyrosine-phosphorylated proteins were similar to the capacitation-associated increases observed in the bull ejaculated and frozen-stored spermatozoa (Galantino-Homer et al., 1997; Visconti et al., 1999; Pons-Rejraji et al., 2009a,b) and the changes are coincident with the induction of hyperactivation (Table 2). These findings indicate that incubation with cBiMPS has the potential to mimic capacitation-associated changes in tyrosine-phosphorylated proteins in the frozen-stored spermatozoa of Japanese Black bulls. On the other hand, increases of tyrosine-phosphorylated proteins (e.g., >250, 250, 210, 110, 105, 90, and 75 kDa) were observed in the connecting, middle and principal pieces of mouse spermatozoa incubated with cBiMPS for 30–90 min (65.3% at 90 min), after which most of them (>250, 250, 210, 105, 90, and 75 kDa) decreased (Fig. 7). These changes parallel the induction and reduction of hyperactivation (Table 2).

Protein tyrosine phosphorylation patterns are affected by the action of the protein tyrosine phosphatase (PTP). In this study, we examined the effects of a PTP inhibitor, sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ , 0.1 mM), on protein tyrosine phosphorylation patterns of bull frozen-stored spermatozoa, boar ejaculated spermatozoa and mouse epididymal spermatozoa incubated without cBiMPS for 90–180 min. In bull sper-

matozoa, decreases in the 85-kDa and 77-kDa tyrosine-phosphorylated proteins were suppressed by  $\text{Na}_3\text{VO}_4$  (left blot of Fig. 8), whereas this inhibitor showed almost no effect on boar spermatozoa (central blot of Fig. 8). However,  $\text{Na}_3\text{VO}_4$  induced similar increases in tyrosine-phosphorylated proteins in mouse spermatozoa as cBiMPS (P1 right blot of Fig. 7 and right blot of Fig. 8). In addition, the motility of the spermatozoa incubated with  $\text{Na}_3\text{VO}_4$  was severely reduced as this reagent also might inhibit dynein-ATPases (data not shown, see the catalog of Sigma–Aldrich Co., St. Louis, MO). Thus, hyperactivation was not observed in mouse spermatozoa incubated with  $\text{Na}_3\text{VO}_4$ .

## DISCUSSION

### Tyrosine-Phosphorylated Proteins in the Head

A number of reports have accumulated on the involvement of protein phosphorylation in the promotion of mammalian sperm fertilization (Visconti and Kopf, 1998; Urner and Sakkas, 2003; Bailey et al., 2005; Kota et al., 2009; Platt et al., 2009). However, Zarelli et al. (2009) recently indicated the importance of protein tyrosine dephosphorylation in the promotion of sperm fertilization by showing that the inhibition of PTP with PTP inhibitor 1, a substrate-trapping mutant of PTPB1 or anti-PTPB1 antibody prevented the acrosome reaction in human spermatozoa. We similarly observed the suppressive effects of  $\text{Na}_3\text{VO}_4$  (the PTP inhibitor) and calyculin A (protein phosphatase 1 (PP1)/protein phosphatase 2A (PP2A) inhibitor) on the cAMP analog-induced acrosome reaction (Harayama et al., 2004b; Adachi et al., 2008). Further experiments on human spermatozoa (Zarelli et al., 2009) revealed that *N*-ethylmaleimide-sensitive factor (NSF) was dephosphorylated by PTPB1 and consequently was capable of disassembling the SNARE complexes. This disassembly was necessary for the acrosome reaction. As shown in Figure 8 of this study, 85- and 77-kDa tyrosine-phosphorylated proteins, which had similar molecular masses as human sperm NSF (Zarelli et al., 2009), were substrates of the  $\text{Na}_3\text{VO}_4$ -sensitive PTP in Japanese Black bull spermatozoa. Moreover, the immunodetection level of these tyrosine-phosphorylated proteins was lower in the spermatozoa from bulls B and G than bull E (P2 of Fig. 6). These findings suggest that bulls B and G are more effective than bull E at exerting the PTP-mediated signaling systems to promote the acrosome reaction in spermatozoa. Since the procedures for frozen storage can mimic molecular alterations that occur during capacitation (cryo-capacitation, Bailey et al., 2000; Pons-Rejraji et al., 2009b), acrosome disintegration in frozen-stored spermatozoa from bulls B and G (P2 of Fig. 4) may be due partially to a precocious acrosome a reaction.

A morphologically distinct area (equatorial subsegment) within the equatorial segment exists at the junction with the postacrosomal region of mammalian spermatozoa. This subsegment includes tyrosine-phosphorylated SPACA1 proteins in ram and boar spermatozoa (Ellis et al., 2002; Jones et al., 2008). The SPACA1 proteins were originally identified as human sperm membrane antigens with molecular masses from 32 to 34 kDa. *SPACA1* mRNA expression was restricted to the testes. Immunofluorescence microscopy and immu-

noelectron microscopy showed that the SPACA1 proteins were localized on the inner acrosomal membrane of the acrosome and equatorial segment (Hao et al., 2002). Recently, Okabe and coworkers (Fujihara et al., 2010) reported that many epididymal spermatozoa from *Spaca1*-KO mice had morphological abnormalities in the head, indicating that Spaca1 proteins are necessary for the shaping of the sperm head. In spermatozoa from Japanese Black bulls, we showed the existence of tyrosine-phosphorylated forms of SPACA1 proteins (Fig. 3) and the immunodetection of tyrosine-phosphorylated proteins in the equatorial subsegment (P1 of Fig. 2). Moreover tyrosine-phosphorylated proteins were immunodetected in the anterior region of the acrosome whenever SPACA1 proteins existed in the same region (P1 of Fig. 5 and P1 of Fig. 6). These findings are consistent with the suggestion that localization of SPACA1 proteins to the anterior region of the acrosome and the equatorial subsegment is related to their tyrosine phosphorylation state.

A variety of acrosomal molecules have already been characterized as functional factors for passage through the cumulus oophorus, interaction with and penetration into the zona pellucida, and induction of the acrosome reaction (Yanagimachi, 1994; Naor and Breitbart, 1997). Thus, not only morphological integrity but also normal molecular constitutions of the sperm acrosome are required for successful fertilization with oocytes. In this study, both SPACA1 proteins and tyrosine-phosphorylated proteins were immunodetected in the anterior region of the acrosome as well as the equatorial subsegment of the frozen-stored spermatozoa (bulls A, D, E, and H) that had a high-grade morphological acrosome condition (P1 (control samples) of Fig. 2, P2 of Fig. 4, and P1 of Fig. 5). In the low-grade frozen-stored spermatozoa (bulls B, C, F, and G), however, the immunofluorescence levels of these proteins were much lower in the acrosome (P1 of Fig. 5). Moreover, these low-grade spermatozoa exhibited lower immunodetection levels of the 41-kDa tyrosine-phosphorylated protein and the 33-kDa tyrosine-phosphorylated form of SPACA1 protein (P2 of Fig. 5). Interestingly, the same abnormalities of these acrosomal proteins were also observed in freshly ejaculated spermatozoa of bulls B and G, even though most of their spermatozoa were normal in progressive motility and acrosome morphology (Fig. 6). These results indicate that the above-mentioned protein abnormalities of the acrosomal proteins arise before or immediately after ejaculation (e.g., defective spermatogenesis and sperm maturation), and that it may be a causal factor in the decrease of sperm freezability leading to acrosome disintegration in Japanese Black bulls.

### Tyrosine-Phosphorylated Proteins in the Flagellum

It has been considered that the increase in tyrosine-phosphorylated proteins depends on the activation of protein tyrosine kinases and the inactivation of PTPs that are controlled by the action of protein kinase A (PKA) in mammals (Visconti and Kopf, 1998; Urner and Sakkas, 2003). Thus far, several tyrosine kinases have been found in mammalian spermatozoa, including SYK [boar (connecting and principal pieces, Harayama et al., 2004a, 2005)], Src [mouse (flagellum, Baker et al., 2006)], cAbl [mouse (entire flagellum, Baker

et al., 2009)] and LYN [bull (connecting piece, Lalancette et al., 2006)]. Moreover, isoforms of sperm PTPs have pharmacologically and immunologically been characterized in the rat, human, boar, stallion, and dog (Seligman et al., 2004; González-Fernández et al., 2009; Zarelli et al., 2009). On the other hand, the inhibition of protein serine/threonine phosphatases by calyculin A can induce protein tyrosine phosphorylation in mouse sperm flagella in the absence of cAMP-increasing reagents such as bicarbonate, cAMP analogs and phosphodiesterase inhibitors (Goto and Harayama, 2009) or in the presence of Src kinase inhibitors (Krapf et al., 2010), indicating the importance of PP1/PP2A in the capacitation-associated signaling cascades. These findings indicate that multiple signaling cascades form the complicated network leading to flagellar protein tyrosine phosphorylation. As shown in Figure 7, the segments where tyrosine-phosphorylated proteins increased in a cBiMPS-dependent manner were the connecting and principal pieces in bull frozen-stored and boar ejaculated spermatozoa. In mouse epididymal spermatozoa, however, cBiMPS-dependent increases in tyrosine-phosphorylated proteins were observed in the middle piece as well as in the connecting and principal pieces. This different sensitivity of the middle piece to cBiMPS is probably due to the above-mentioned differences in the localization of the tyrosine kinases (Harayama et al., 2004a, 2005; Lalancette et al., 2006; Baker et al., 2009). Moreover, Na<sub>3</sub>VO<sub>4</sub>-induced increases in tyrosine-phosphorylated proteins in the Hepes-buffered BO medium without cBiMPS were observed in mouse epididymal spermatozoa, but were barely found in bull frozen-stored and boar ejaculated spermatozoa. This protein tyrosine phosphorylation pattern was almost the same as those of spermatozoa incubated with cBiMPS (Figs. 7 and 8), though the hyperactivation was hardly induced, probably owing to the effects of Na<sub>3</sub>VO<sub>4</sub> as an ATPase inhibitor (data not shown). These results suggest that Na<sub>3</sub>VO<sub>4</sub>-insensitive PTPs suppress precocious protein phosphorylation in the flagella of bull frozen-stored and boar ejaculated spermatozoa under noncapacitation conditions, or that the basal activity of protein tyrosine kinases in the absence of cAMP-increasing reagents is higher in mouse epididymal spermatozoa. Taking these findings together, we speculate that there are differences between livestock and mice in the regulation systems for the tyrosine phosphorylation of sperm flagellar proteins.

### CONCLUSION

Based on the data obtained in this study, we conclude that the immunodetection levels of tyrosine-phosphorylated proteins in the head are associated with tolerance to frozen storage in the spermatozoa of Japanese Black bulls. Moreover, we suggest that the immunodetection levels of tyrosine-phosphorylated proteins in the sperm flagellum is related to the potential to undergo cAMP-dependent hyperactivation. As low tolerance to frozen storage and low potential to undergo cAMP-dependent hyperactivation cause AI subfertility (Bailey et al., 2000; Kuroda et al., 2007; Murase et al., 2010), we believe that the assessment of sperm protein tyrosine phosphorylation patterns is a valid tool for screening AI-subfertile Japanese Black bulls.

## MATERIALS AND METHODS

### Animal Use Ethics Statement

The Committee of Laboratory Animal Experiments of Kobe University investigated our research plan and the feeding conditions of the animals to be used therein. We undertook the following experiments with the approval of this committee (document Nos. 16-04-06, 16-04-08, 16-08-04, 19-5-31, and 19-5-37).

### Sperm Washing and Incubation With Cell-Permeable cAMP Analog

We examined frozen-stored spermatozoa of Japanese Black bulls, boar ejaculated spermatozoa and mouse epididymal spermatozoa in this study, as these types of spermatozoa are usually used for animal reproduction using AI techniques by farms or for reproductive research by laboratories.

Frozen spermatozoa of 10 Japanese Black bulls (bulls A-J) were produced using an egg yolk-Tris-citrate extender by a straw method in the Northern Center of Agricultural Technology, General Technological Center of Hyogo Prefecture for Agriculture, Forest, and Fishery. The spermatozoa were then stored in liquid nitrogen. They were rapidly thawed in warm water (38.5°C) just before their use in the experiments. Boar ejaculated spermatozoa (sperm-rich fractions) were obtained from three boars (boars K-M, two Meishan pigs and one Large White pig) by a manual method. Bull and boar spermatozoa were washed in PBS containing 0.1% polyvinyl alcohol (PVA, Sigma-Aldrich Co.) (PVA-PBS) by centrifugation at 700 × *g* three times for 5 min per washing. Mouse epididymal spermatozoa were collected from seven ICR mice (mice N-T) as described previously (Goto and Harayama, 2009). All of the sperm samples were resuspended in a Hepes (37 mM, Dojindo Co., Kumamoto, Japan)-buffered Brackett and Oliphant (BO) medium (pH 7.4) (Brackett and Oliphant, 1975; Kuroda et al., 2007) containing 0.1% PVA instead of BSA and lacking NaHCO<sub>3</sub> (BO-H) to adjust the final sperm concentration to 1.0 × 10<sup>8</sup> cells/ml. Aliquots of the spermatozoa were used as samples before incubation for the analyses described below. The balance of the spermatozoa were supplemented with cell-permeable, phosphodiesterase-resistant cAMP analog, cBiMPS (0.1 mM, Biomol International, L.P., Plymouth Meeting, PA) [dissolved in 10%, v/v DMSO (Schaap et al., 1993)] or a PTP inhibitor (Na<sub>3</sub>VO<sub>4</sub>, 0.1 mM, Sigma-Aldrich Co.) (dissolved in the incubation medium), put on the culture dishes, covered with liquid paraffin (Wako Pure Chemical Industry Ltd, Osaka, Japan) and then incubated in 100% air at 38.5°C up to 180 min. The DMSO (10%) was added to the medium for solvent control samples in order to equalize the DMSO concentration with experimental samples. After incubation, the spermatozoa were used for the analyses described below.

### Immunodetection of Tyrosine-Phosphorylated Proteins and SPACA1 Proteins

Western blot (WB) and indirect immunofluorescence (IIF) procedures were described in our previous reports (Harayama et al., 2004b; Harayama and Miyake, 2006; Amano et al., 2007). The antibodies used in this study were mouse anti-phosphotyrosine monoclonal antibody (clone 4G10, Upstate Cell Signaling Solutions, Charlottesville, VA, 1:10,000–1:20,000 for WB and 1:1,000 for IIF), guinea pig anti-SPACA1 (SAMP32) polyclonal antibody (American Research Products, Inc., Belmont, MA, 1:4,000–1:10,000 for WB and 1:400 for IIF), horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin polyclonal antibody (Dako Cytomation Denmark A/S, Glostrup, Denmark, 1:2,000–1:10,000 for WB), HRP-conjugated rabbit anti-guinea pig immunoglobulin polyclonal antibody (Dako Cytomation Denmark A/S, 1:1,000 for WB), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin polyclonal antibody (Dako Cytomation Denmark A/S, 1:100 for IIF) and goat TRITC-conjugated goat anti-guinea pig

immunoglobulin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:100 for IIF). In the experiments to assess the tyrosine phosphorylation state of flagellar proteins, 100 cells on each preparation were examined by fluorescent microscopy to calculate the percentages of spermatozoa with the connecting and principal pieces intensively reacted with the anti-phosphotyrosine antibody.

### Extraction and Immunoprecipitation of Tyrosine-Phosphorylated Proteins and SPACA1 Proteins

Spermatozoa were washed as mentioned above, sonicated with Vibra Cell (Sonics & Materials, Inc., Danburg, CT) at a frequency of 20 kHz ± 50 Hz for 30 sec in ice-cold lysis buffer [50 mM Tris-HCl (pH 7.4), 2% (v/v) Nonidet P-40 (Iwai Chemicals Co., Tokyo, Japan), 1 mM Na<sub>3</sub>VO<sub>4</sub> (Sigma-Aldrich Co.), 2 mM EDTA trisodium salt (Dojindo Co.) and 1 mM (*p*-aminophenyl)methanesulfonyl fluoride hydrochloride (Wako Pure Chemical Industry Ltd)], and then gently rocked for 60 min. The obtained lysates (sperm lysate) were separated from sperm debris (sperm pellet) by centrifugation (12,500 × *g*, 5 min, 4°C).

Mouse anti-phosphotyrosine antibody (clone 4G10, IgG<sub>2b</sub>κappa, 4 μg) was added to 60 μl (30 μl agarose beads) of washed protein A agarose bead slurry (Upstate Cell Signaling Solutions) and gently rocked in wet ice for 90 min. In the control experiments, normal mouse immunoglobulin G<sub>2b</sub> (Dako Cytomation Denmark A/S, 4 μg) was used instead of the anti-phosphotyrosine antibody. After washing three times with ice-cold PBS by centrifugation (12,500 × *g*, 5 sec, 4°C), the protein A agarose beads were reacted with the sperm lysates (50 μl: extracted from 2 × 10<sup>8</sup> spermatozoa) in wet ice for 5 hr. The reactive mixtures were centrifuged (12,500 × *g*, 5 sec, 4°C) to remove the supernatants (unprecipitated fraction), and the resultant pellets of protein A agarose beads (precipitated fraction) were washed twice with ice-cold lysis buffer. The obtained samples (sperm pellet, sperm lysate, precipitated fraction and unprecipitated fraction) were subjected to WB using anti-SPACA1 antibody and anti-phosphotyrosine antibody.

### Assessment of Morphological Acrosome Condition

FITC-conjugated peanut agglutinin (FITC-PNA) staining was performed as described previously (Tabuchi et al., 2008). In brief, the spermatozoa were washed as mentioned above and then fixed with 3% paraformaldehyde (Wako Pure Chemical Industry Ltd) in PBS for 30 min. After washing three times with 1% BSA (Wako Pure Chemical Industry Ltd) and 100 mM glycine (Gly, Wako Pure Chemical Industry Ltd) in PBS [(BSA/Gly-PBS), 1,000 × *g*, 2 min each], the spermatozoa were treated with 1% Triton X-100 (Sigma-Aldrich Co.) in PBS for 5 min and then washed three times with BSA/Gly-PBS. They were stained with FITC-PNA (20 μg/ml, Sigma-Aldrich Co.) in PBS for 30 min and then washed with BSA/Gly-PBS three times. Finally, sperm nuclei were counterstained with 400 μg/ml propidium iodide (Wako Pure Chemical Industry Ltd) in PBS for 10 min. After washing three times, the sperm samples were put on glass slides, covered with 0.22 M 1,4-diazabicyclo [2,2,2] octane (Sigma-Aldrich Co.) dissolved in glycerol-PBS mixture (9:1) and coverslips. The preparations were examined under a differential interference microscope equipped with epifluorescence (mirror unit U-MWB2: excitation filter BP460-490, dichroic mirror DM500, and emission filter BA520IF, Olympus Optical Company Ltd, Tokyo, Japan). Approximately 100 spermatozoa on each preparation were classified into seven categories according to the fluorescent state of FITC-PNA in the acrosome (Fig. 1; I pattern: normal acrosome, II pattern: slightly disordered acrosome, III pattern: severely disordered acrosome with highly bright fluorescence, IV pattern: acrosome with less fluorescence in the whole part, V pattern: severely disordered acrosome with less fluorescence in the anterior region, VI pattern: acrosome with fluorescence only along its outline, and VII pattern: acrosome with almost no fluorescence).

### Evaluation of Sperm Motility

A 2- $\mu$ l drop of sperm suspension was put on the 0.5-mm-deep stage of a glass plate for sperm motility assessment (Fujihira Industry Co., Ltd, Tokyo, Japan), covered with the coverslip and then placed on a heated stage (38.5°C) under a bright-field microscope. Sperm motility was recorded with a CCD camera (CS230B, Olympus Optical Company) and DVD recorder (DVR-7000, Pioneer Corporation, Tokyo, Japan). Sperm motility was assessed using recorded movies that were played back at the slowest mode. Spermatozoa showing any movement were considered motile cells, irrespective of their progressive motility. The percentages of spermatozoa showing hyperactivation were subjectively estimated and the obtained results were classified into the following three categories; (-: 0–9%, +: 10–29%, and ++: >29% of total spermatozoa).

### Statistical Analyses

The obtained data were subjected to *t*-test or one-way ANOVA after arc-sine transformation. When *F*-test results were significant in ANOVA, individual means were further tested by Tukey's multiple range tests (Motulsky, 1995).

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### REFERENCES

- Adachi J, Tate S, Miyake M, Harayama H. 2008. Effects of protein phosphatase inhibitor calyculin A on the postacrosomal protein serine/threonine phosphorylation state and acrosome reaction in boar spermatozoa incubated with a cAMP analog. *J Reprod Dev* 54:171–176.
- Amano R, Lee J, Goto N, Harayama H. 2007. Evidence for existence of cAMP-Epac signaling in the heads of mouse epididymal spermatozoa. *J Reprod Dev* 53:127–133.
- Bailey JL, Bilodeau JF, Cormier N. 2000. Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon. *J Androl* 21:1–7.
- Bailey JL, Tardif S, Dubé C, Beaulieu M, Reyes-Moreno C, Lefèvre L, Leclerc P. 2005. Use of phosphoproteomics to study tyrosine kinase activity in capacitating boar sperm. Kinase activity and capacitation. *Theriogenology* 63:599–614.
- Baker MA, Hetherington L, Aitken RJ. 2006. Identification of SRC as a key PKA-stimulated tyrosine kinase involved in the capacitation-associated hyperactivation of murine spermatozoa. *J Cell Sci* 119:3182–3192.
- Baker MA, Hetherington L, Curry B, Aitken RJ. 2009. Phosphorylation and consequent stimulation of the tyrosine kinase c-Abl by PKA in mouse spermatozoa; its implications during capacitation. *Dev Biol* 333:57–66.
- Brackett B, Oliphant G. 1975. Capacitation of rabbit spermatozoa *in vitro*. *Biol Reprod* 12:260–274.
- Ellis DJ, Shadan S, James PS, Henderson RM, Edwardson JM, Hutchings A, Jones R. 2002. Post-testicular development of a novel membrane substructure within the equatorial segment of ram, bull, boar, and goat spermatozoa as viewed by atomic force microscopy. *J Struct Biol* 138:187–198.
- Fujihara Y, Satou Y, Inoue N, Isotani A, Ikawa H, Okabe M. 2010. Spaca1, Sperm acrosome associated 1, is required for sperm-head shaping. Proceeding of the 11th International Symposium on Spermatology, P01-37.
- Galantino-Homer HL, Visconti PE, Kopf GS. 1997. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3' 5'-monophosphate-dependent pathway. *Biol Reprod* 56:707–719.
- González-Fernández L, Ortega-Ferrusola C, Macias-Garcia B, Salido GM, Peña FJ, Tapia JA. 2009. Identification of protein tyrosine phosphatases and dual-specificity phosphatases in mammalian spermatozoa and their role in sperm motility and protein tyrosine phosphorylation. *Biol Reprod* 80:1239–1252.
- Goto N, Harayama H. 2009. Calyculin A-sensitive protein phosphatases are involved in maintenance of progressive movement in mouse spermatozoa *in vitro* by suppression of autophosphorylation of protein kinase A. *J Reprod Dev* 55:327–334.
- Hao Z, Wolkowicz MJ, Shetty J, Klotz K, Bolling L, Sen B, Westbrook VA, Coonrod S, Flickinger CJ, Herr JC. 2002. SAMP32, a testis-specific, isoantigenic sperm acrosomal membrane-associated protein. *Biol Reprod* 66:735–744.
- Harayama H, Miyake M. 2006. A cyclic adenosine 3',5'-monophosphate-dependent protein kinase C activation is involved in the hyperactivation of boar spermatozoa. *Mol Reprod Dev* 73:1169–1178.
- Harayama H, Nakamura K. 2008. Changes of PKA and PDK1 in the principal piece of boar spermatozoa treated with a cell-permeable cAMP analog to induce flagellar hyperactivation. *Mol Reprod Dev* 75:1396–1407.
- Harayama H, Muroga M, Miyake M. 2004a. A cyclic adenosine 3',5'-monophosphate-induced tyrosine phosphorylation of Syk protein tyrosine kinase in the flagella of boar spermatozoa. *Mol Reprod Dev* 69:436–447.
- Harayama H, Sasaki K, Miyake M. 2004b. A unique mechanism for cyclic adenosine 3',5'-monophosphate-induced increase of 32-kDa tyrosine-phosphorylated protein in boar spermatozoa. *Mol Reprod Dev* 69:194–204.
- Harayama H, Murase T, Miyake M. 2005. A cyclic adenosine 3',5'-monophosphate stimulates a phospholipase Cgamma1-calcium signaling via the activation of tyrosine kinase in boar spermatozoa. *J Androl* 26:732–740.
- Jones R, James PS, Oxley D, Coadwell J, Suzuki-Toyota F, Howes EA. 2008. The equatorial subsegment in mammalian spermatozoa is enriched in tyrosine phosphorylated proteins. *Biol Reprod* 79:421–431.
- Kaneto M, Krisfalusi M, Eddy EM, O'Brien DA, Miki K. 2008. Bicarbonate-induced phosphorylation of p270 protein in mouse sperm by cAMP-dependent protein kinase. *Mol Reprod Dev* 75:1045–1053.
- Kota V, Dhople VM, Shivaji S. 2009. Tyrosine phosphoproteome of hamster spermatozoa: Role of glycerol-3-phosphate dehydrogenase 2 in sperm capacitation. *Proteomics* 9:1809–1826.
- Krapf D, Arcelay E, Wertheimer EV, Sanjay A, Pilder SH, Salicioni AM, Visconti PE. 2010. Inhibition of Ser/Thr phosphatases induces capacitation-associated signaling in the presence of Src kinase inhibitors. *J Biol Chem* 285:7977–7985.
- Kuroda K, Fukushima M, Harayama H. 2007. Premature capacitation of frozen-thawed spermatozoa from subfertile Japanese Black cattle. *J Reprod Dev* 53:1079–1086.



- Lalancette C, Faure RL, Leclerc P. 2006. Identification of the proteins present in the bull sperm cytosolic fraction enriched in tyrosine kinase activity: A proteomic approach. *Proteomics* 6:4523–4540.
- Motulsky H. 1995. *Intuitive biostatistics*. New York: Oxford University Press.
- Murase T, Mukohjima K, Sakaguchi S, Ohtani T, Tsubota T. 2001. Generation of diacylglycerol during the acrosome reaction induced by  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$  ionophore A23187 in frozen-thawed spermatozoa from fertile and subfertile Japanese Black bulls. *J Reprod Dev* 47:311–316.
- Murase T, El-Kon I, Harayama H, Mukoujima K, Takasu M, Sakai K. 2010. Hyperactivated motility of frozen-thawed spermatozoa from fertile and subfertile Japanese Black bulls induced by cyclic adenosine 3',5'-monophosphate analogue, cBiMPS. *J Reprod Dev* 56:36–40.
- Naor Z, Breitbart H. 1997. Protein kinase C and Mammalian spermatozoa acrosome reaction. *Trends Endocrinol Metab* 8:337–342.
- Platt MD, Salicioni AM, Hunt DF, Visconti PE. 2009. Use of differential isotopic labeling and mass spectrometry to analyze capacitation-associated changes in the phosphorylation status of mouse sperm proteins. *J Proteome Res* 8:1431–1440.
- Pons-Rejraji H, Bailey JL, Leclerc P. 2009a. Modulation of bovine sperm signalling pathways: Correlation between intracellular parameters and sperm capacitation and acrosome exocytosis. *Reprod Fertil Dev* 21:511–524.
- Pons-Rejraji H, Bailey JL, Leclerc P. 2009b. Cryopreservation affects bovine sperm intracellular parameters associated with capacitation and acrosome exocytosis. *Reprod Fertil Dev* 21:525–537.
- Rodriguez-Martinez H. 2007. Role of the oviduct in sperm capacitation. *Theriogenology* 68:S138–S146.
- Schaap P, van Ments-Cohen M, Soede RD, Brandt R, Firtel RA, Dostmann W, Genieser HG, Jastorff B, van Haastert PJ. 1993. Cell-permeable non-hydrolyzable cAMP derivatives as tools for analysis of signaling pathways controlling gene regulation in *Dictyostelium*. *J Biol Chem* 268:6323–6331.
- Seligman J, Zipser Y, Kosower NS. 2004. Tyrosine phosphorylation, thiol status, and protein tyrosine phosphatase in rat epididymal spermatozoa. *Biol Reprod* 71:1009–1015.
- Si Y. 1999. Hyperactivation of hamster sperm motility by temperature-dependent tyrosine phosphorylation of an 80-kDa protein. *Biol Reprod* 61:247–252.
- Si Y, Okuno M. 1999. Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. *Biol Reprod* 61:240–246.
- Tabuchi T, Shidara O, Harayama H. 2008. A 32-kDa tyrosine-phosphorylated protein shows a protease-dependent increase in dead boar spermatozoa. *J Reprod Dev* 54:502–507.
- Urner F, Sakkas D. 2003. Protein phosphorylation in mammalian spermatozoa. *Reproduction* 125:17–26.
- Visconti PE, Kopf GS. 1998. Regulation of protein phosphorylation during sperm capacitation. *Biol Reprod* 59:1–6.
- Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP, Jorgez CJ, Alvarez JG, Kopf GS. 1999. Cholesterol efflux-mediated signal transduction in mammalian sperm. Beta-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. *J Biol Chem* 274:3235–3242.
- Yanagimachi R. 1994. Mammalian fertilization. In: Knobil E, Neill JD, editors. *The physiology of reproduction*. 2nd edition. New York: Raven Press, pp 189–317.
- Zarelli VE, Ruete MC, Roggero CM, Mayorga LS, Tomes CN. 2009. PTP1B dephosphorylates N-ethylmaleimide-sensitive factor and elicits SNARE complex disassembly during human sperm exocytosis. *J Biol Chem* 284:10491–10503.