REPRODUCTION
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Progress of sperm IZUMO1 relocation during spontaneous acrosome reaction

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Abstract

It has been recently shown in mice that sperm undergo acrosome reaction (AR) by passing through cumulus cells; furthermore, the acrosome-reacted sperm can bind to zona pellucida and consequently fertilise the egg. During AR, the relocation of the primary fusion protein IZUMO1 into the equatorial segment is crucial for sperm–egg fusion. There is a high rate of spontaneous AR in rodents, with up to 60% in promiscuous species. The aim of this study was to clarify whether the IZUMO1 relocation in sperm after spontaneous and induced AR is the same, and whether there is a correlation between the speed of IZUMO1 relocation and species-specific mating behaviour in field mice. Immunofluorescent detection of IZUMO1 dynamics during the *in vitro* capacitation, spontaneous, calcium ionophore and progesterone-induced AR was monitored. Our results show that during spontaneous AR, there is a clear IZUMO1 relocation from the acrosomal cap to the equatorial segment, and further over the whole sperm head. In addition, there is positive tail tyrosine phosphorylation (TyrP) associated with hyperactive motility. Moreover, the beginning and the progress of IZUMO1 relocation and tail TyrP positively correlate with the level of promiscuity and the acrosome instability in promiscuous species. The findings that crucial molecular changes essential for sperm–egg fusion represented by dynamic movements of IZUMO1 also happen during spontaneous AR are vital for understanding fertilisation in mice.

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Introduction

Only capacitated sperm are able to undergo acrosome reaction (AR) and fertilise the egg (Yanagimachi 1994) and the progress of capacitation is associated with sperm tail protein tyrosine phosphorylation (TyrP), especially in the mid-piece region (Urner *et al.* 2001). Capacitated sperm penetrate the layer of cumulus cells and are able to begin and complete the AR before reaching the zona pellucida (ZP; Jin *et al.* 2011). It has been documented that after a successfully completed AR, the protein composition of the equatorial segment and post-acrosomal region is modified (Sosnik *et al.* 2009). The key event for a sperm–egg fusion involves the preceding dynamic relocation of the primary fusion protein IZUMO1 in sperm (Miranda *et al.* 2009, Sosnik *et al.* 2009), which starts at the end of capacitation and is completed during the induced AR (Sosnik *et al.* 2010). It was demonstrated that during the induced AR, IZUMO1 moves from the acrosomal membrane into the equatorial segment and the post-acrosomal region of the sperm head in the epididymal and freshly ejaculated sperm (Inoue *et al.* 2011*a*). This IZUMO1 relocation process was later confirmed by microscopic studies of sperm–egg fusion in living spermatozoa (Satouh *et al.* 2012). Further gene disruption experiments showed the necessity of IZUMO1 for the fusion of sperm with egg oolema. In that work, IZUMO1 protein was labelled in live spermatozoa by using a double transgenic mouse line, which expresses IZUMO1 – mCherry fusion protein – and green fluorescent protein (GFP) in the sperm acrosome. It was also shown that IZUMO1 protein gradually moves towards the equatorial segment of the sperm head (Satouh *et al.* 2012).

Relocation of IZUMO1 is dependent on the actin cytoskeleton and on testis-specific serine kinase (Tssk). Tssk6-null sperm (Sosnik *et al.* 2009) as well as *IZUMO1* knockout sperm (Inoue *et al.* 2005) are able to penetrate through the ZP but are not capable of fusing with the egg plasma membrane.

The reason for sperm spontaneous AR is not yet known and has so far been considered disadvantageous or even a self-destructing mechanism leaving these sperm with a further inability to fertilise (Moore *et al.* 2002). On the other hand, the *in vitro* study of Naito *et al.* (1992) using ZP-free mouse eggs showed that spermatozoa in which AR was not mediated by native ZP were functional as they produced healthy offspring.

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In highly promiscuous field mice (*Apodemus*) with documented multiple paternities (Bryja & Stopka 2005, Bryja *et al*. 2008, Johnson *et al*. 2007), where up to 60% of their sperm undergo synchronised spontaneous AR (Moore *et al*. 2002, Johnson *et al*. 2007, Clift *et al*. 2009), every minute may count in the sperm race to fertilise the egg. Several molecular mechanisms may be involved in speeding up the fertilising process. One such mechanism is the absent translation of the specific acrosomal transmembrane protein CD46 (Johnson *et al*. 2007), which is thought to be responsible for acrosome integrity in sperm. This is supported by the phenotype of CD46 knockout sperm displaying an increased spontaneous AR rate (Inoue *et al*. 2003). CD46 disruption probably changes the activity of ion channels and spontaneous AR is triggered (Inoue *et al*. 2003).

It has recently been shown that in mice the AR is triggered not only in the ZP but also through the cumulus oophorus cell (Jin *et al*. 2011) and that sperm after the AR are still able to pass through the ZP and fertilise the egg (Inoue *et al*. 2011*a*, 2011*b*). However, to date the fate of the massive population of sperm well described in rodents where the AR is occurring spontaneously without any exogenous stimuli has not been determined.

Therefore, we aimed to answer three main questions. Firstly, whether the IZUMO1 relocation happens during the spontaneous AR and the timing of the process. In parallel, the status of tail protein TyrP, which is related to the acquisition of the hyperactive motility, was monitored. Secondly, whether there is a correlation between the dynamics of IZUMO1 movements, promiscuous mating behaviour and the rate of spontaneous AR in the relevant field mouse (*Apodemus*) species. Thirdly, whether there are any differences in the time of IZUMO1 relocation between the group of sperm undergoing only spontaneous AR and the sperm group when outer inductors of AR such as calcium ionophore (CaI) or progesterone are added. In our experimental setup, we used promiscuous species of field mice *Apodemus sylvaticus* and *Apodemus microps*, which exhibit a more rapid spontaneous AR in comparison with the laboratory strain of BALB/c mice used as a control.

Materials and methods

Ethics statement

All animal procedures were carried out in strict accordance with the law of the Czech Republic, paragraph 17, no. 246/1992, and Animal Scientific Procedures, paragraph 11, no. 207/2004. The local ethics committee of the Faculty of Science of Charles University in Prague specifically approved this study in accordance with accreditation no. 24773/2008-10001.

Animals

Inbred BALB/c and wild-caught field mice *A. sylvaticus* and *A. microps* were obtained from a breeding colony of the Laboratory of Reproduction, Faculty of Science, Charles University in Prague. Mice were housed in the animal facilities of the Faculty of Science, Charles University, and food and water were supplied *ad libitum*. The male mice used for all experiments were a reproductive age of 10–12 weeks.

Experiment 1: capacitation and spontaneous AR

Sperm from the distal regions of the cauda epididymis were released into a 200 μl droplet of M2-fertilising medium (Sigma–Aldrich) under paraffin oil in a Petri dish and pre-tempered at 37 °C in the presence of 5% CO_2. Released sperm were assessed for motility and viability under a light inverted microscope with a thermostatically controlled stage at 37 °C. Sperm stock was diluted to the required concentration (5×10^6/ml) in 100 μl of M2 medium under paraffin oil. Sperm were left freely to capacitate and undergo the spontaneous AR without the addition of other stimuli. Sperm samples were collected at 0, 5, 10, 20, 40, 60, 80 and 90 min. The time marked as 0 was the minimum time required for the sperm to be added to the M2 medium, removed and added to 500 μl of PBS for washing. This manipulation did not exceed 1 min. The spermatozoa were washed twice with PBS for 5 min at 600 ***g***.

Experiment 2: capacitation and induced AR

Sperm from the distal regions of the cauda epididymis were released into M2 fertilising medium (as described above) with either 5 μM CaI A-23187 or 10 μM progesterone P8783 (both Sigma–Aldrich, CR). Sperm samples were collected at each experimental time of 0, 5, 10, 20, 40, 60, 80 and 90 min, and CaI or progesterone were left in the M2 medium for up to 90 min.

Monitoring of sperm quality and acrosome status

All the sperm samples were incubated at 37 °C under 5% CO_2. Sperm motility and viability were assessed at every experimental time point, when a drop of spermatozoa was also placed onto a glass slide and 2.5 μM PNA lectin (Molecular Probes, Prague, Czech Republic) was added. The status of the acrosome was examined immediately under a fluorescent microscope. The obtained results were consistent with data published in Johnson *et al*. (2007).

Immunofluorescence detection of IZUMO1 and protein TyrP with co-staining for acrosome status

Sperm smears were prepared for every *in vitro* incubation time stated above. Sperm were washed twice in PBS, smeared onto glass slides and air dried.

Sperm smears were fixed with acetone for 10 min at −20 °C, washed with PBS, blocked with 10% goat serum, incubated with primary antibody IZUMO1 (sc-79539, Santa Cruz Biotechnology, Inc.), 1:100 in 1.5% goat serum overnight,

followed by a secondary antibody donkey anti-goat IgG-FITC (2024, Santa Cruz Biotechnology) 1:1000 in PBS for 1 h at room temperature.

Tyrosine phosphorylation

Sperm smears were air dried and fixed with 3.7% formaldehyde in PBS (pH 7.34) at room temperature for 10 min, followed by washing in PBS, incubation with 15 mM ammonium chloride (NH_4Cl) for 5 min and with 0.1% detergent TritonX100 for 3 min. Slides were washed with PBS, blocked with 10% BSA in PBS for 1 h and incubated with primary MAB anti-phosphotyrosine (PTyr 01 112630025, Exbio Antibodies, Prague, Czech Republic) diluted 1:500 in 1% BSA in PBS for 2 h, followed with Alexa Fluor 488, donkey anti-mouse IgG (A21202, Molecular Probes) secondary antibody 1:1000 in PBS for 1 h. Irrelevant isotype-matched MABs and secondary antibodies (used without primary antibodies) served as negative controls.

Furthermore, PNA lectin (Molecular Probes) was added at a concentration of 2.5 μM in PBS. After washing, the slides were mounted into Vectashield mounting medium with DAPI (Vector Lab., Burlingame, CA, USA). The samples were examined with an Olympus IX81 fluorescent microscope equipped with 60× UPLSAP and photographed with Hammatsu ORCA C4742-80-12AG, using Olympus Soft Imaging Solutions software (Laboratory Imaging Ltd., Prague, Czech Republic). Representative results are shown.

Statistical analysis

The depicted immunofluorescent staining pattern of IZUMO1 relocation and tail protein TyrP during *in vitro* incubation in spontaneous AR group was monitored in 200 sperm in six individual experiments for each species and at each time point. The presented values for IZUMO1 (Fig. 4A) and protein TyrP (Fig. 5A) were only counted from the sperm undergoing spontaneous AR, and this population represented 100% of sperm. The data provided in Fig. 4B represent modified data published by Johnson *et al.* (2007) and show the percentage of spontaneously acrosome-reacted sperm in the sperm population.

Experimental data were analysed using STATISTICA 6.0. (Statsoft, Prague, Czech Republic) and GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, CA, USA). The differences in the IZUMO1 relocation among individual species and times of incubation were analysed by two-way ANOVA and the Kruskal–Wallis test. For the data on the differences in the IZUMO1 pattern proportions in BALB/c mouse spermatozoa during spontaneous AR and the timing of IZUMO1 relocation during spontaneous AR among individual species (Fig. 2B and 3B), no *post-hoc* analysis was performed. For the data presented on differences in the IZUMO1 pattern proportions among species at selected times of production (Fig. 4A), the Bonferroni multiple comparison test was applied as the *post-hoc* analysis and was used to indicate the specific differences among individual species in the appropriate time of incubation. The differences in tail protein TyrP in the spontaneously acrosome-reacted sperm (Fig. 5A), the percentage of spontaneously acrosome-reacted sperm in the whole sperm population (Fig. 4B) among individual species and times of incubation were analysed by the Kruskal–Wallis ANOVA and Dunn's *post-hoc* test. A P value ≤ 0.05 was considered to be statistically significant (*P value ≤ 0.05).

Results

PNA and IZUMO1 staining pattern distribution in BALB/c spermatozoa

The PNA and IZUMO1 display six different staining and co-staining patterns in the BALB/c mouse sperm head (Fig. 1A) during *in vitro* incubation. The abundance of these patterns among experimental time points of *in vitro* incubation in BALB/c spermatozoa is presented in Fig. 1B. Presented IZUMO1 and PNA staining patterns represent the whole heterogeneity of the sperm population in each sample (no staining patterns or cells were excluded from the analysis). The majority (82–90%)

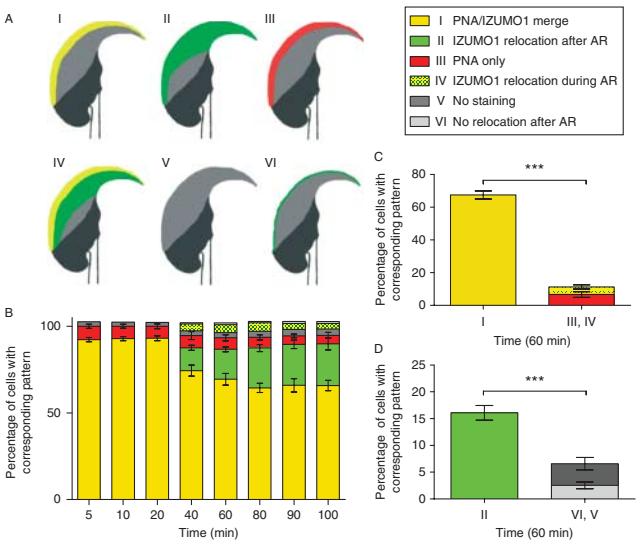

Figure 1 The whole heterogeneity of the sperm population with regard to IZUMO1 and PNA detection. (A) Scheme of IZUMO1 and PNA staining pattern distribution in BALB/c spermatozoa during *in vitro* incubation (detected at 60 min). (I) PNA/IZUMO1 merge. (II) IZUMO1 relocation after AR. (III) Positive PNA staining only. (IV) IZUMO1 relocation during AR when PNA labelling is still visible. (V) No sperm head staining. (VI) No IZUMO1 relocation. (B) Changes in the PNA and IZUMO1 staining pattern distribution in BALB/c spermatozoa during *in vitro* incubation. Individual bars denote the distribution of IZUMO1 and PNA staining patterns among individual times during sperm incubation. A total of 200 cells were counted in six individual samples at one time. Error bars denote S.D.s (S.D.s are presented only for patterns 1,2,3). Statistical analysis of PNA and IZUMO1 staining patterns prior and after AR in BALB/c spermatozoa. (C) Distribution of PNA and IZUMO1 staining patterns prior to AR in BALB/c spermatozoa (counted at 60 min). A total of 200 cells were counted in six individual samples. Error bars denote S.D. The statistical significance of the differences among individual IZUMO1 pattern abundances is indicated by asterisks (***$P \leq 0.001$). (D) Distribution of PNA and IZUMO1 staining patterns after AR in BALB/c spermatozoa (counted at 60 min). A total of 200 cells were counted in six individual samples. Error bars indicate the S.D. The statistical significance of the differences among individual IZUMO1 patterns abundances is indicated by asterisks (***$P \leq 0.001$).

of sperm populations during all incubation times represent an intact acrosome with PNA and IZUMO1 co-localisation (Fig. 1AI and B) occurring in the acrosome cap. On the other hand, patterns without IZUMO1 or PNA staining belong to a minority (8–9%) out of all pattern groups (Fig. 1AIII, V, VI and B). Minority representation was also found in the case of the parallel PNA and IZUMO1 labelling after the AR when IZUMO1 starts to relocate and PNA was still detected on the acrosome vesicle (Fig. 1AIV and B). From 40 min of incubation and onwards there is an increasing number of sperm with IZUMO1 relocation (Fig. 1AII and B). The statistical data of the whole heterogeneity of the sperm population is shown in Supplementary Table 2, see section on supplementary data given at the end of this article. Statistical analysis of the distribution of PNA and IZUMO1 staining patterns pre and post-spontaneous AR during 60 min of *in vitro* sperm incubation was made (Fig. 1C and D). Before the spontaneous AR, IZUMO1 and PNA co-localisation in the acrosomal area belongs to a large majority (85%) of the sperm cell population. The proportion of IZUMO1-negative sperm is ∼6%. Around 5% of sperm cells express the staining pattern, which is specific for the ongoing AR (Fig. 1C). In the majority (71%) of the sperm cell population, IZUMO1 relocalised after spontaneous AR.

The number of sperm with no IZUMO1 relocation pattern (without the IZUMO1/PNA-negative sperm population) was ∼17 times lower compared with the number of sperm cells with IZUMO1 relocation after spontaneous AR. The number of sperm cells with IZUMO1 relocation is significantly higher even in the case where sperm with no relocation as well as unstained sperm were counted and compared as a sum (Fig. 1D).

The IZUMO1 relocation progresses after spontaneous AR in BALB/c spermatozoa

IZUMO1 further relocalises across the sperm head to other compartments after spontaneous AR during subsequent incubation times. During spontaneous AR, there was IZUMO1 relocation starting at the acrosomal cap (AC), moving via the apical equatorial segment (AES) across the whole equatorial segment (WES) towards the post-acrosomal segment, and finally IZUMO1 covered the whole sperm head (WSH; Fig. 2A and B). The beginning of IZUMO1 relocation from the AC into the AES was not dependent on the PNA-detectable onset of the AR (cells here expressing staining pattern IV, Fig. 1A); however, the further relocation stages corresponded positively to the detection of the on-going spontaneous AR. The IZUMO1 relocation over the WSH, which is crucial for gamete fusion, happened after the spontaneous AR was completed (Fig. 3AI). No sperm in the whole sperm population showed PNA staining when IZUMO1 was relocated to the WES or even to the WSH segment. The analysis of the progression of IZUMO1 relocation shows the gradual increase in the abundance of WES and WSH IZUMO1 staining patterns in the incubation times starting at 60 min (Fig. 2B).

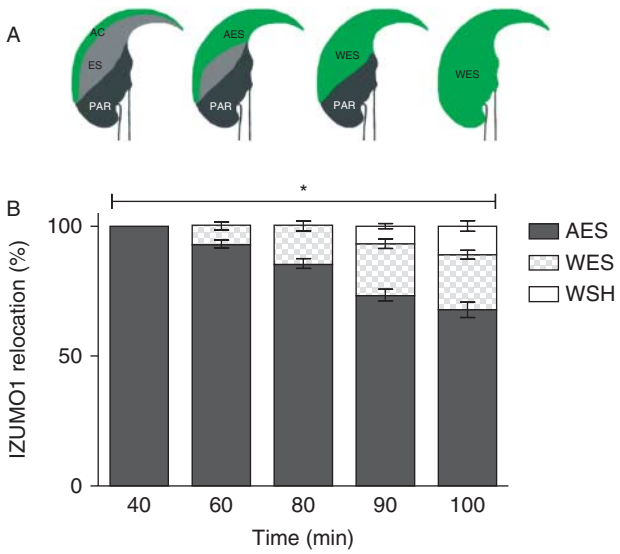

Figure 2 IZUMO1 relocation progression into other sperm head compartments after spontaneous AR. (A) Scheme of IZUMO1 relocation across the mouse sperm head. Acrosomal cap (AC), equatorial segment (ES), post-acrosomal region (PAR), apical equatorial segment (AES), whole equatorial segment (WES), whole sperm head (WSH). (B) Differences in the IZUMO1 pattern proportions in BALB/c mouse spermatozoa during spontaneous AR. A total of 200 described cells were counted and three specific IZUMO1 patterns are presented at one time. Whiskers indicate the S.D. The statistical significance of the differences among individual IZUMO1 patterns abundances is indicated by an asterisk (*$P \leq 0.05$).

Progression of IZUMO1 relocation in individual sperm populations during spontaneous AR correlates with species-specific promiscuity behaviour

Timing of IZUMO1 relocation in the sperm population was compared during *in vitro* incubation (0, 5, 10, 20, 40, 60, 80 and 90 min) in the sperm population undergoing spontaneous AR in two wild-caught field mouse species. These were the highly promiscuous *A. sylvaticus* in which up to 100% of the litter show multiple paternities, i.e. each offspring can be fathered by a different male, and the facultatively promiscuous *A. microps*, where only up to 40% of the litter show multiple paternities (Moore *et al.* 2002, Bryja & Stopka 2005, Bryja *et al.* 2008) and a laboratory mouse strain of BALB/c (Fig. 3A). Before the initial start of the AR, IZUMO1 was anchored in the apical AC membranes (ca. 90% of the whole sperm population, ∼90% WSP). The relocation of IZUMO1 in sperm with spontaneous AR is summarised in Supplementary Table 1, see section on supplementary data given at the end of this article. The comparative data on the time-dependent IZUMO1 relocation during

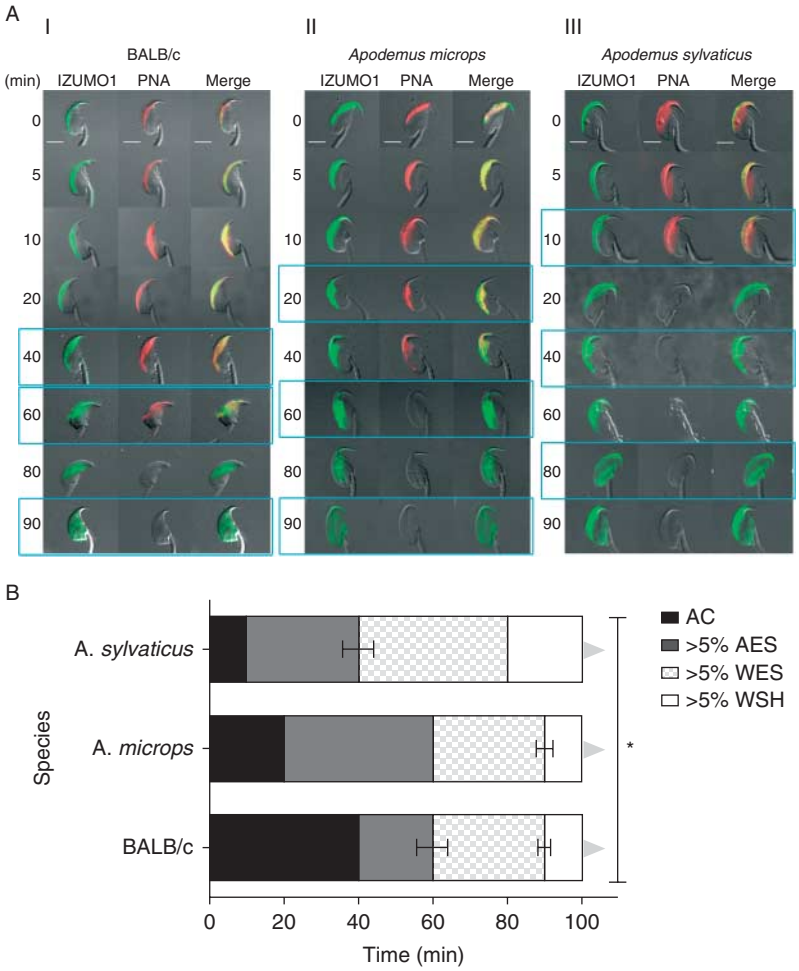

Figure 3 Progression of IZUMO1 relocation during spontaneous AR. (A) The progression of IZUMO1 relocation during spontaneous AR. (I) BALB/c. (II) *A. microps*. (III) *A. sylvaticus*. Immunofluorescent detection of IZUMO1 (green) and acrosome integrity by PNA-lectin (red) during *in vitro* incubation (0, 5, 10, 20, 40, 60, 80 and 90 min) as indicated in the Materials and Methods. Progression of IZUMO1 relocation into the apical equatorial segment (AES), whole equatorial segment (WES) and whole sperm head (WSH) is marked by blue rectangle. IZUMO1 was detected in the capacitated sperm population: in AES after 40 min (I) BALB/c, 20 min (II) *A. microps*, 10 min (III) *A. sylvaticus*; WES after 60 min (I) BALB/c, 60 min (II) *A. microps*, 40 min (III) *A. sylvaticus*; WSH after 90 min. (I) BALB/c, 90 min (II) *A. microps*, 80 min (III) *A. sylvaticus*. Time points and depicted images correspond to the arbitrary threshold of 5% of the population acquiring the depicted staining pattern. Representative results were shown. Scale bar represents 4 μm. (B) Bars indicate the modus time ($n=6$), when more than 5% of sperm show the appropriate IZUMO1 pattern for the first time, from the whole IZUMO1-positive sperm population (AES) or from the population of spontaneously acrosome-reacted sperm (AES, WES, WSH), respectively. Whiskers indicate the S.E.M. The statistical significance of the differences in the individual IZUMO1 patterns abundances among individual strains is indicated by the asterisk ($*P \leq 0.05$). Arrows at the end of the bars indicate the further progression of the IZUMO1 relocation to the WSH in subsequent times of incubation. Acrosomal cap (AC).

spontaneous AR between BALB/c, *A. microps* and *A. sylvaticus* are summarised graphically in Fig. 3B.

In BALB/c sperm (Fig. 3AI), which were left free to capacitate *in vitro*, the IZUMO1 relocation into the AES, which corresponds to the arbitrary threshold of 5% of the population acquiring the staining pattern described in Fig. 1AIV, occurred after 40 min of capacitation. After 60 min of capacitation, IZUMO1 began to be relocated towards the equatorial segment of the sperm heads (more than 5% of the sperm population after AR showed this pattern, >5% ARSP) (pattern Fig. 1AII and heterogeneity graph Fig. 1B). At 40 min, the majority of sperm heads (~80%) still showed positive for PNA labelling, indicating the presence of acrosome, and no PNA-visible onset of the AR was detected. At 60 min of incubation, the acrosome vesicle was clearly modified and the on-going AR could be seen in ~20% of the whole sperm population (20% WSP). The WES was positive for IZUMO1 at 60 min of *in vitro* incubation (>5% ARSP) and at 90 min the IZUMO1 was relocated throughout the WSH and also covered the post-acrosomal region (>5% ARSP). Laboratory mouse BALB/c represents an organism with a low level of spontaneous AR (Johnson *et al.* 2007) and IZUMO1 relocation into the equatorial segment was obvious only in the population of sperm that completed at least a 40-min capacitation. The earlier acrosome-reacted sperm did not display the same pattern.

In the sperm of *A. microps* (Fig. 3AII), IZUMO1 relocation into the AES appears after 20 min of *in vitro* incubation (>5% ARSP), which is about 20 min earlier than in BALB/c. We can track its relocation across the equatorial segment, during the visibly on-going AR. The IZUMO1 location into the WES was completed at 60 min of *in vitro* incubation (>5% ARSP). At that time, the AR was completed and no visible PNA acrosome labelling could be detected. The complete IZUMO1 relocation over the WSH occurred at 90 min of incubation (>5% ARSP). This time was, therefore, the same as in BALB/c.

Compared with the above, IZUMO1 relocation towards the AES in *A. sylvaticus* sperm already began after 10 min of *in vitro* incubation (>5% ARSP), which is about 30 min earlier than in the laboratory species and about 10 min earlier than in *A. microps* (Fig. 3AIII).

At that time, a strong PNA labelling of acrosome vesicle was still detected. Although IZUMO1 relocation started after 10 min, it took until 40 min of *in vitro* incubation for it to move across the WES (>5% ARSP), which was 20 min earlier than in *A. microps* and BALB/c. However, it is important to note that at this time, in *A. sylvaticus*, over half of the sperm population completed the spontaneous AR, which is in contrast to *A. microps* (30%) or BALB/c (20%), and the IZUMO1 relocation over the WES happened after the acrosome vesicle could not be detected by PNA labelling any more. IZUMO1 was relocated into the post-acrosomal region and covered the WSH at 80 min of capacitation (>5% ARSP), therefore, about 10 min earlier than in *A. microps* and BALB/c.

IZUMO1 relocation in the sperm population of promiscuous species is faster and correlates with a higher AR

The presented values of IZUMO1 staining (Fig. 4A) were counted only from sperm undergoing spontaneous AR, and this population represents the starting 100% of sperm. However, the actual percentage of spontaneously acrosome-reacted sperm in the whole sperm populations of BALB/c, *A. microps* and *A. sylvaticus* was correlated with previously published data by Johnson *et al.* (2007) and the modified graph is shown as Fig. 4B.

Changes in IZUMO1 relocation was graphically depicted from 40 min (Fig. 4A), in which sperm in at least one type of mice species began to relocate through the WES (Fig. 2A). The graph (Fig. 4A) shows the time-dependent increase in the progress of IZUMO1 relocation across the mouse sperm heads, occurring in all strains of mice. In correlation with Fig. 1A, in *A. microps* and *A. sylvaticus* the faster relocation occurs at all stages of IZUMO1 relocation during sperm incubation in contrast to BALB/c (Fig. 2A). The progression of IZUMO1 relocation in the sperm population correlates with the rate of spontaneous AR in different mice species (Fig. 5B) when an increasing incubation time leads a rise in the number of sperm undergoing spontaneous AR.

The spontaneous AR rate in BALB/c and *Apodemus* mice was genus specific. According to KW-ANOVA and Dunn's *post-hoc* test, the BALB/c mice had significantly lower rates of spontaneous AR compared with *A. microps* (from 60 min of incubation and subsequent incubation times) and *A. sylvaticus* (from 20 min of incubation onwards).

The capacitation progress assessed by tail TyrP correlates with speed of spontaneous AR and IZUMO1 relocation

A positive TyrP pattern in the sperm tail was monitored in sperm undergoing spontaneous AR (Fig. 3A), where the status of the acrosome was visualised by PNA lectin as stated in the methods. The progressive protein TyrP in

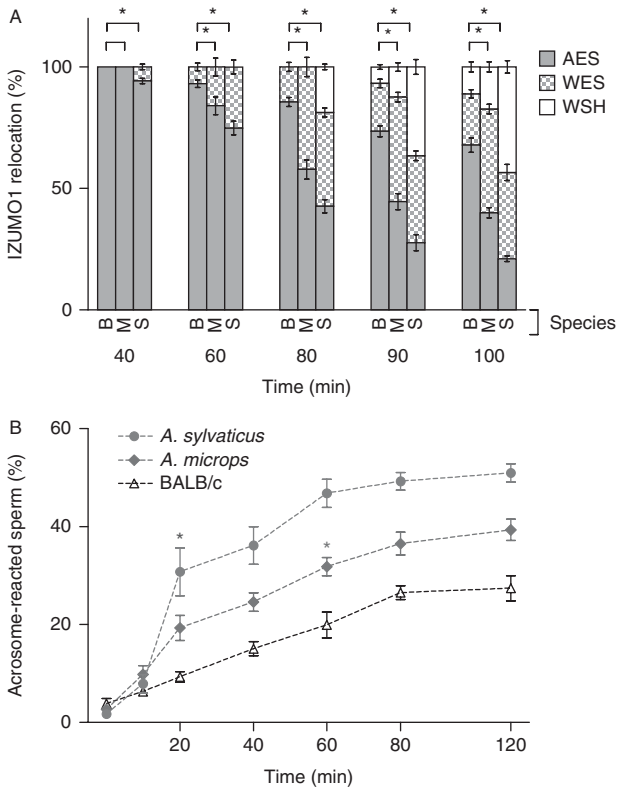

Figure 4 Statistical analysis of IZUMO1 pattern distribution during spontaneous AR among individual species. (A) Differences in the IZUMO1 pattern proportions (AES, WES, WSH) among species in selected times of incubation. Bars denote the arithmetic mean of individual IZUMO1 pattern (AES, WES, WSH) in appropriate species and time of incubation (%). A total of 200 cells were counted in six individual samples at one time. Error bars indicate the S.E.M. The statistical significance of the IZUMO1 patterns abundance differences among individual species in different times of incubation is indicated by asterisks (*$P \leq 0.05$). BALB/c (B), *A. microps* (M), *A. sylvaticus* (S). (B) The species-specific rate of spontaneous AR in the whole sperm population (modified from Johnson *et al.* (2007)). Points indicate the arithmetic mean of the number of acrosome-reacted sperm. Error bars denote S.E.M. Asterisks indicate the first time when the difference between individual species is significant (*$P \leq 0.05$).

both the mid-piece and principal-piece in BALB/c was taken as a marker of capacitating sperm with hyperactive motility, where the positive mid-piece protein TyrP was taken as the crucial pattern for evaluation (Urner *et al.* 2001). It was the same for the *Apodemus* species: a strong mid-piece protein TyrP was detected and it was used as a marker for capacitating sperm with hyperactive motility.

A percentage of positive tail TyrP in sperm that have undergone spontaneous AR (Fig. 3A) positively correlates with the total number of sperm that underwent spontaneous AR (Fig. 5B). This correlation reflects the fact that sperm, which undergo the spontaneous AR, display a pattern typical for hyperactive motility.

The representative immunofluorescence staining of TyrP in mouse spermatozoa is shown in Fig. 5B.

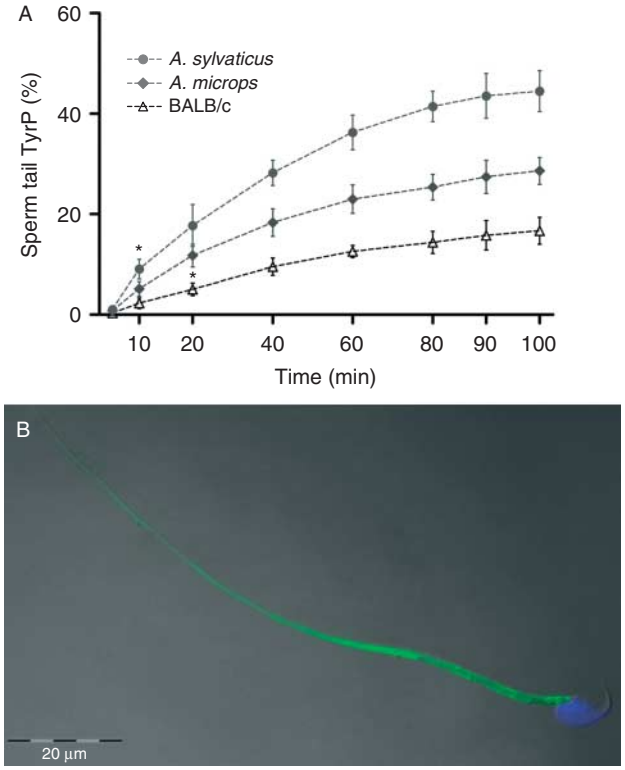

Figure 5 Statistical analysis of protein TyrP distribution during spontaneous AR. (A) The time progression of tail protein TyrP among individual species in the spontaneously acrosome-reacted sperm. A total of 200 cells were counted in six individual samples at one time. Points indicate the arithmetic mean of the tail protein TyrP (%). Error bars denote S.D. Asterisks indicate the first time when the difference between individual species is significant (*$P \leq 0.05$). (B) Immunofluorescent detection of protein TyrP in *A. microps* spermatozoa. TyrP (green) over the mid and principal piece of the sperm tail. Nuclei are counterstained with DAPI (blue). Scale bar represents 20 µm.

Comparison of speed of IZUMO1 relocation in individual sperm populations during CaI- and progesterone-induced AR

AR was induced by either CaI or progesterone at the start of *in vitro* capacitation in the sperm of *A. sylvaticus*, *A. microps* and BALB/c. This system exposed sperm to exogenous stimuli and simulated the environment that sperm are facing on the way to reach the egg.

There were no particular differences in the timing of IZUMO1 relocation between the CaI-induced and spontaneous AR. However, the group of sperm exposed to progesterone displayed a different pattern. The whole process of IZUMO1 relocation was initiated and also completed earlier in the progesterone-induced group (Supplementary Table 1).

In BALB/c sperm, the relocation of IZUMO1 into the AES was visible during the CaI-induced AR after 40 min of capacitation in more than 5% of the sperm population (>5% ARSP) and then moving over the equatorial segment during PNA-detectable AR by 60 min and covering the WSH after 90 min of *in vitro* incubation (>5% ARSP) (Supplementary Figure 1AI, see section on supplementary data given at the end of this article). This is not different to the spontaneous AR group (Fig. 4AI). On the other hand, in sperm that were exposed to the presence of progesterone, IZUMO1 started to relocate over the apical into the WES 20 min earlier (>5% ARSP) than in the other two groups and appeared over the WSH by 80 min (>5% ARSP) (Supplementary Figure 1BI).

In sperm of *A. microps* (Supplementary Figure 1AII) and *A. sylvaticus* (Supplementary Figure 1AIII), the general pattern observed in BALB/c (Supplementary Figure 1AI) remained, but the whole process of IZUMO1 relocation began and progressed faster in the sperm population of these species. In both *Apodemus* mice, IZUMO1 relocation during CaI-induced AR (Supplementary Figure 1AII and III) was comparable or identical to the one during the spontaneous AR (Fig. 4AII and III). Using progesterone as an AR stimulus, the IZUMO1 dynamics in the AES for both *A. microps* (Supplementary Figure 1BII) and *A. sylvaticus* (Supplementary Figure 1BIII) was initiated in half the time and its relocation over the fusogenic domain of the WES happened 20 min faster (>5% ARSP) than in the spontaneous or CaI-induced AR. Later IZUMO1 was detectable in the WSH at 80 min for *A. microps* (>5% ARSP) (Supplementary Figure 1BII) or 60 min for *A. sylvaticus* (>5% ARSP) (Supplementary Figure 1BIII) which was 10 resp. 20 min earlier than in the case of the spontaneous AR (Fig. 1AII and III).

Discussion

This study broadly focused on the character of IZUMO1 relocation in the population of sperm that undergo *in vitro* spontaneous AR en masse. The first goal was to describe the time-dependent relocation of IZUMO1 across the sperm head during spontaneous AR. The second aim was to clarify whether there is a correlation between the speed of IZUMO1 relocation and the level of promiscuous behaviour of selected field mouse species compared with BALB/c control. Lastly, the third task was to compare the dynamics of IZUMO1 relocation during spontaneous and induced AR.

IZUMO1 is located in the sperm head and it is essential for sperm–egg fusion (Inoue *et al.* 2011*b*). Its relocation during 30–60 min of induced AR was documented in some studies (Sosnik *et al.* 2010, Inoue *et al.* 2011*b*), but there is a missing gap in the understanding of IZUMO1 dynamics relating to spontaneous AR. It is general knowledge that induced AR would never happen without capacitation and it is related to the molecular changes and dynamic movements of the actin cytoskeleton (Breitbart *et al.* 2005, Dvorakova *et al.* 2005), which is also responsible for IZUMO1 relocation (Sosnik *et al.* 2009). Spontaneous AR is a well-documented phenomenon in the field

mouse (*Apodemus*) species (Moore *et al*. 2002, Johnson *et al*. 2007, Clift *et al*. 2009) and so far it is not understood whether these sperm are capable of fertilisation. Moreover, it is not clear whether sperm that undergo the spontaneous AR previously underwent capacitation. In field, mice more than half of the sperm lose their acrosome during capacitation (Moore *et al*. 2002) and it seems to be related to their general sexual behaviour and the level of promiscuity, which was documented on a behavioural level (Stopka & Macdonald 1998, 1999). It is known that IZUMO1 relocation is crucial for fertilisation and it happens during the AR. So what happens in the case of spontaneously acrosome-reacted sperm? One could speculate whether the ability of these sperm to relocate IZUMO1 is lost due to the lack of time as a consequence of the premature loss of acrosome, or whether the crucial molecular changes including the capacitation, hyperactive motility and IZUMO1 dynamics are fast tracked.

The first question was whether the relocation of IZUMO1 takes place during the spontaneous AR and what was the timing of the process.Our results bring a broader understanding to this topic and show that IZUMO1 relocation is not a sudden process, activated only during the ongoing spontaneous AR; but there is also an earlier gradual IZUMO1 relocation from the AC, towards the AES that happens before the PNA visible onset of the AR and can be located over the WSH after the spontaneous AR is completed (Fig. 5AIV). It has been reported (Jin *et al*. 2011) that acrosin-EGFP sperm lose their signal at the very start and the initialisation of the AR, so-called membrane vesiculation, but at these points the PNA lectin labelling appears and positively stains only the whole compact acrosome vesicle. This can be interpreted that the initial IZUMO1 dynamics start at the undetectable onset of the AR and its further relocation into the WES is indeed happening at the visible beginning of the AR (Satouh *et al*. 2012). At this point, however, the acrosome region is still PNA detectable, so our results are not contradicting the previous ones. It was also reported (Satouh *et al*. 2012) that the dynamics of IZUMO1 relocation to the WES happen within 30 s to 3 min following the AR over the mouse egg. In this study, our aim was to monitor the abundance of the individual IZUMO1 relocation patterns among the different times of the *in vitro* incubation. Our experiment design was set in an egg-free environment, as the capacitation and spontaneous AR happen while the sperm travel through the oviduct. For this reason, the presented results give very valid and important information on how long the mouse sperm remain in the different stages of the IZUMO1 relocation and possibly waiting for ovulation. The differences in the IZUMO1-relocation status among individual sperm in the ejaculation might be an important prerequisite for successful fertilisation, because in different time windows there might always be a subpopulation of sperm with the ideal IZUMO1-relocation status eligible for binding to the ZP.

In parallel the status of tail protein TyrP, which is related to the acquisition of the hyperactive motility (Urner *et al*. 2001), was monitored. According to obtained results, we can report that a percentage of the positive sperm tail TyrP in sperm with a PNA-detectable onset of the spontaneous AR positively correlated with the total number of sperm that underwent the spontaneous AR. This correlation reflects the fact that sperm that undergo the spontaneous AR display a pattern typical for capacitated sperm, with hyperactive motility, and it may be speculated that these processes happen faster in this group of sperm.

The second question was whether there is correlation between the dynamics of IZUMO1 movements, promiscuous mating behaviour and the rate of spontaneous AR in the relevant species. The presented results imply that the actual IZUMO1 relocation may not be fully related to the acrosome stability. The sperm of wild *Apodemus* species do not translate the CD46 protein (Johnson *et al*. 2007, Clift *et al*. 2009) and have the same phenotype as CD46 knockout mice, displaying acrosome instability but with a higher fertility rate (Inoue *et al*. 2003). In *Apodemus* sperm compared with BALB/c, the initial relocation of IZUMO1 takes place 20–30 min earlier, which is significant in terms of the timing of fertilisation. It has been shown that the time required for the processes involving sperm AR, sperm passing through the cumulus cell layer and penetration of ZP was ∼15 min (Hirohashi *et al*. 2011). This could be another selective advantage for sperm facing severe sperm competition. The whole process of molecular changes happening in sperm before fertilisation is then started earlier and correlates with the level of promiscuity in the species. However, the actual time needed for completing the relocation process into the WES and later the WSH remains notably faster only in the highly promiscuous species of *A. sylvaticus* compared with the facultatively promiscuous *A. microps* and BALB/c. On the other hand, the actual final difference between the species in IZUMO1 relocation into the WSH (Fig. 4B) is not so distinct and might depend on other factors rather than the completion of the spontaneous AR. However, sperm fuse with the oolema by the equatorial segment (Inoue *et al*. 2005), so it might be suspected, based on obtained results, that the speed of the IZUMO1 movement into the fusogenic region could be a key factor.

The third question was whether there are any differences in the time of the IZUMO1 relocation between the population of sperm undergoing only spontaneous AR and the group of sperm exposed to the outer inductors of AR such as CaI or progesterone. When performing experiments it may happen to artificially

separate processes, such as spontaneous and induced AR, that are not present in the natural physiological environment. Both these kinds of ARs can likely occur at the same time in the same sperm population with a possible coexistence of sperm undergoing spontaneous or induced AR if the inductor (or the egg) is present. For this reason, the exogenous inductor of AR such as CaI or progesterone was added into capacitating sperm at the beginning of sperm incubation.

Based on the presented results, there were no particular differences between the IZUMO1 relocation in the spontaneous and CaI-induced AR group, including the timing of the process prior, during and after the AR described earlier. However, the whole process of the IZUMO1 dynamics was sped up when sperm were exposed to the parallel progesterone stimuli compared with CaI or spontaneous AR only. On the basis of the presented results from the spontaneous and CaI-induced AR, it can be implied that IZUMO1 relocation is happening independently of the outer or inner calcium ion stimuli triggering the AR. Nevertheless, progesterone secreted by the cells of the cumulus oophorus (Meizel *et al*. 1997, Thérien & Manjunath 2003), which is known to induce the AR (Pietrobon *et al*. 2005), is able to initiate the IZUMO1 relocation. This may be, therefore, a logical mechanism for how crucial molecular changes are triggered in sperm by passing through cumulus cells.

The heterogeneity of the sperm population involves up to six different IZUMO1 and PNA staining patterns occurring before and after the spontaneous AR. When comparing all patterns that have undergone spontaneous AR, it is clear that sperm with IZUMO1 relocation represents the majority (71%).

The mechanism of spontaneous AR is not yet known. These sperm were always considered to be unable to pass through ZP and therefore unable to fertilise the egg, so further investigation was not of any interest. Recently, it has been shown (Inoue *et al*. 2011*a*) that mouse acrosome-reacted sperm pass through ZP and fertilise. In close correlation, it was then suggested that AR is triggered by cumulus oophorus cells (Clift *et al*. 2009). However, again what happens with the amount of mouse sperm undergoing spontaneous AR remains unclear. Mouse sperm after spontaneous AR are capable of IZUMO1 relocation towards the equatorial segment of the sperm head, and the signalling pathways leading to TyrP, the indicator of successful capacitation, are activated. However, only *in vivo* experiments would have shown whether spontaneously acrosome-reacted sperm may have the same fertilising potential as those after induced AR.

In wild species, the time of IZUMO1 relocation positively correlates with the level of species promiscuity and negatively with the sperm acrosome stability. This finding may reflect the fact that IZUMO1 relocation is not only fully dependent on the process of induced AR in mice but can also occur during spontaneous AR.

AR might be set off spontaneously, on ZP or passing through cumulus cells, and each of the mechanisms may serve its purpose, although mouse sperm with an intact acrosome binding to the ZP can be considered an exception (Inoue *et al*. 2011*a*). Based on presented results, it can be concluded that the IZUMO1 relocation happens during the spontaneous AR in mice and its speed correlates positively with the level of sperm competition.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0193.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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