CRISPR/Cas9-mediated mutation revealed cytoplasmic tail is dispensable for IZUMO1 function and male fertility

Samantha A M Young^{1,4}, Haruhiko Miyata¹, Yuhkoh Satouh¹, Masanaga Muto^{1,2}, Martin R Larsen³, R John Aitken⁴, Mark A Baker⁴ and Masahito Ikawa^{1,2}

¹Research Institute for Microbial Diseases, ²Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan, ³Institute for Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark and ⁴Priority Research Centre in Reproductive Science, Discipline of Biological Sciences, Faculty of Science and IT, University of Newcastle, Callaghan, New South Wales, Australia

Correspondence should be addressed to M Ikawa; Email: ikawa@biken.osaka-u.ac.jp

Abstract

IZUMO1 is a protein found in the head of spermatozoa that has been identified as essential for sperm–egg fusion. Its binding partner in the egg has been discovered (JUNO); however, the roles of several domains within IZUMO1 remain unexplored. One such domain is the C-terminus, which undergoes major phosphorylation changes in the cytoplasmic portion of the protein during rat epididymal transit. However, the cytoplasmic tail of IZUMO1 in many species is highly variable, ranging from 55 to one amino acid. Therefore, to understand the role of the cytoplasmic tail of IZUMO1 in mouse, we utilised the gene manipulation system of CRISPR/Cas9 to generate a point mutation resulting in a premature stop codon, producing mice with truncated IZUMO1. Mice without the cytoplasmic tail of IZUMO1 showed normal fertility but decreased the amount of protein, indicating that whilst this region is important for the expression level of IZUMO1, it is dispensable for fertilisation in the mouse.

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Introduction

The process of fertilisation involves a series of orchestrated, complex biological steps that culminate when the spermatozoa adhere to, and then fuse with, the oocyte plasma membrane in order to gain entry into the egg cytoplasm (Yanagimachi 1994, Gupta 2014). The processes and proteins involved in sperm–egg fusion are slowly being unraveled. Previously, our group generated an antibody against an unknown antigen, which was shown to inhibit sperm–egg fusion (Okabe *et al.* 1987). The antigen was identified by initial 2D-PAGE separation, and the parent protein is called ‘Izumo’ after the Japanese shrine dedicated to marriage (Inoue *et al.* 2005). Knockout studies have clearly shown that IZUMO1 is essential for male fertility, as male *Izumo1*^{−/−} mice are infertile due to a failure of their gametes to fuse with the egg (Inoue *et al.* 2005).

As a testis-specific protein, IZUMO1 belongs to the immunoglobulin superfamily (IgSf) and is initially located on the outer and inner acrosomal membranes of the sperm head (Inoue *et al.* 2011, Satouh *et al.* 2012). However, during the acrosome reaction (both spontaneous and induced), IZUMO1 translocates, spreading to the postacrosomal region as well as the equatorial segment in order for fusion to take place

(Okabe *et al.* 1986, Okabe *et al.* 1987, Kawai *et al.* 1989, Ellerman *et al.* 2009, Miranda *et al.* 2009, Satouh *et al.* 2012, Sebkova *et al.* 2013). Although the mechanism by which this occurs is unclear, it is important to understand, as the equatorial segment is the site where sperm–egg fusion begins.

IZUMO1 contains a single transmembrane domain, and both extracellular and intracellular domains. Studies investigating the extracellular portion of IZUMO1 have clearly shown that this region binds to JUNO (formerly folate receptor 4) which is found on the surface of the plasma membrane of the egg (Bianchi *et al.* 2014). Female *Juno*^{−/−} mice are infertile, due to a lack of adhesion with the extracellular region of IZUMO1 on the spermatozoa. Furthermore, we have shown that the extracellular part of IZUMO1 is N-terminally glycosylated, which, whilst not essential for fertilisation, does appear to have a protective role against protein degradation during epididymal transit (Inoue *et al.* 2008).

Interestingly, the cytoplasmic (intracellular) side of IZUMO1 has also been suggested to play an important role in mice (Ellerman *et al.* 2009). The cytoplasmic region of IZUMO1 contains several potential phosphorylation sites, one of which was confirmed by Ellerman *et al.* (2009) with the amino acid sequence SDYSGDK. Furthermore, Baker *et al.* (2012) performed

an in-depth proteomic analysis of the IZUMO1 peptides in rat spermatozoa and found significant post-translational changes, associated with a dynamic modification of IZUMO1 phosphorylation status during epididymal transit. All of these phosphorylation changes occurred in the cytoplasmic region of the IZUMO1 protein (Baker *et al.* 2012).

To investigate the role of the C-terminus of IZUMO1 in mice, we generated a mutant mouse line where the cytoplasmic portion of IZUMO1 has been removed via CRISPR/Cas9 genome editing. Our data suggest that the C-terminal, cytoplasmic region of IZUMO1 affects the expression levels of this protein yet is not essential for male fertility.

Materials and methods

Animals

All animal experiments were approved by the Animal Care and Use committee of the Research Institute for Microbial Diseases, Osaka University.

Purification of phosphopeptides

The phosphopeptides were purified using titanium dioxide (TiO₂) (Larsen *et al.* 2005, Jensen & Larsen 2007) performed essentially as described in the TiSH protocol (Engholm-Keller *et al.* 2012) without the SIMAC step but with inclusion of a de-glycosylation step between the first and second TiO₂ enrichment. The acidified phosphopeptides were purified by a reversed-phase Oligo R3 micro-column as described elsewhere (Palmisano *et al.* 2010) and lyophilised by vacuum centrifugation.

Labelling of peptides with TMT10 plex

Sperm peptides derived from the caput, corpus and cauda regions of the epididymis in biological duplicates were labelled with the isobaric tag TMT (Tandem Mass Tag) 10 plex reagents for simultaneous quantitation. Aliquots (60 µg) of each of the peptide samples were labelled with TMT10 plex according to the manufacturer's protocol. The labelling was performed as follows: caput (TMT 127C/128N), corpus (TMT 128C/129N) and cauda (TMT 129C/130N).

Hydrophilic interaction liquid chromatography (HILIC) fractionation of peptides

The phosphopeptide sample and a reversed-phase Oligo R3 micro-column-purified non-modified peptide sample (approximately 50 µg) were re-dissolved in 40 µL 90% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) by first dissolving the peptides in 0.4 µL 10% TFA, then adding 3.6 µL H₂O and finally adding 36 µL ACN. The samples were injected onto an in-house-packed TSKgel Amide-80 HILIC 320 µm × 170 mm capillary HPLC column using an Agilent 1200 capillary HPLC system. The peptides were eluted using a gradient from 90% ACN, 0.1% TFA to 60% ACN, 0.1% TFA

for over 48 min at a flow rate of 6 µL/min. The fractions were automatically collected in a microtitre plate at 1-min intervals after UV detection at 210 nm, and the fractions were pooled according to the UV detection to a total of 18 and 8 fractions for the phosphopeptides and non-modified peptides respectively. The fractions were dried by vacuum centrifugation. Prior to LC-MS/MS the samples were re-dissolved in 5.5 µL 0.1% TFA. A total of 5 µL of each fraction were analysed by reversed-phase nanoLC-MS/MS.

NanoLC-MS/MS and data analysis

The peptides were loaded onto an Easy-nanoLC (Thermo Fisher Scientific) coupled to a Q-exactive HF mass spectrometer (Thermo Fisher Scientific). Peptides were loaded onto a pre-column (4 cm Reprosil – Pur C18 AQ 5 µm RP material (Dr. Maisch, Ammerbuch-Entringen, Germany)) using the EASY-LC system and eluted directly onto a 20 cm long fused silica capillary column (75 µm ID) packed with Reprosil – Pur C18 AQ 3 µm RP material. The peptides were separated using a gradient from 1–28% B (A buffer: 0.1% formic acid (FA); B buffer: 90% ACN/0.1% FA) at a flow rate of 250 nL/min over 50–110 min and then 28–45% B for 10 min. Peptides (*m/z* 450–1600) were analysed in full MS mode using a resolution of 120,000 FWHM at 200 *m/z*. A maximum of 15 peptides were selected and fragmented using HCD per MS spectrum, and the fragment ions were recorded in the orbitrap using a resolution of 60,000 FWHM. Ion filling time was set to 100–200 ms depending on the sample and an AGC target value of 1E5 ions.

Data analysis

The raw data from the Q-exactive HF were processed using the Proteome Discoverer 2.1 using the TMT10 plex quantitation method. The data were searched using both Mascot and Sequest HT search engines. For the Mascot database search the data were searched in the Sprot database with mouse as entry, with phosphorylation on S/T/Y, N-terminal acetylation as variable modifications and TMT (N-terminal amines and lysine) and carbamidomethyl (cysteine) as fixed modifications. For all searches the mass accuracy was set to 10 ppm for MS data and 0.05 kDa for MS/MS analysis.

Plasmid and oligonucleotide preparation

By injecting a sgRNA/Cas9-expressing plasmid and oligonucleotide into the pronucleus of a zygote, we generated mice with the insertion of T near the end of the transmembrane region, creating a stop codon. The CRISPR strategy and preparation was as follows: the plasmids expressing hCas9 and sgRNA were prepared by ligating oligos into the BbsI site of pX330 (<http://www.addgene.org/42230/>; (Cong *et al.* 2013)). The pCAG-EGFP reporter plasmid was prepared as described previously (Mashiko *et al.* 2013). A single stranded oligonucleotide containing the desired mutation with approximately 60 nts homozygous to the target region on either side was designed and ordered. The sequence of oligonucleotide is 5'-GATCTGACTTGG

ATCCTGACGCGGTGGGTTTGAAGTTCGTCACCTTGCATTC
TTCAATTTAGCGCTaGACTTTCTAAAGTGAAGTACCCT
GGGGCAGGACATAGGTGCACAGAACCACATTAGG
TCACAGGT-3' (insertion is lower case and italicised).

Off-target analysis

Potential off-target sites were found using free software, Bowtie (<http://bowtie-bio.sourceforge.net/index.shtml>) with rules outlined previously (Mali *et al.* 2013, Wang *et al.* 2013, Yang *et al.* 2013). Twelve to fourteen bases preceding the PAM sequence with AGG, GGG, CGG and TGG were aligned with the mouse genome (mm9). sgRNA with less number of potential off-target sites were chosen for pX330 plasmid construction.

sgRNA validation assay

Validation of sgRNA/CRISPR cleavage activity was done by transfection of HEK293T cells as reported previously (Mashiko *et al.* 2013).

Pronuclear injection

B6D2F1 female mice were superovulated and mated with B6D2F1 males, and fertilised eggs were collected from the oviduct. The pX330 plasmids and oligonucleotides were injected into one of the pronuclei at 5 and 100 ng/μL respectively. The sgRNA sequence used for injection was 5'-gTTCCTAAAGTGAAGTACCCT-3' (the initial 'g' was added as at the time of generation this was thought to be necessary for sgRNA expression driven by U6 promoter). The injected eggs were cultivated in potassium simplex optimisation medium (kSOM) (Ho *et al.* 1995) overnight and then two-cell stage embryos were transferred into the oviducts of pseudopregnant ICR females. The pups were genotyped by PCR and subsequent sequence analysis.

Genotyping

For genotyping of ΔCyt mutants, PCR was performed using following primers (Forward: aagaattcGGATGGATGGAAAGGAAACG; reverse: aagatcCGAGCCAGAAGTCAGAACCA; initial sequence has an 'a' nucleotide followed by the restriction enzyme sequence for EcoRI (forward) and EcoRV (reverse), as these primers were also used in sgRNA validation). The PCR product was then digested with Bfal restriction enzyme. Mutant mice were assigned labels as follows: B6D2-Izumo1<em1(S346Stop) Osb>. They have been deposited into the Riken BioResource Centre along with the plasmid used to create them; pX330-Izumo1#2.

Quantitative PCR (qPCR)

Mouse cDNA was prepared from testis. Each cDNA was subjected to quantitative RT-PCR analysis with a Thermal Cycler Dice TP800 (Takara Bio) using SYBR Premix Ex Taq II RR820S (Takara Bio). The relative expression level of each target gene mRNA was normalised to the amount of

b-actin control. Each reaction was performed in triplicate and data are presented as the mean plus S.D. Primer sets were as follows: 5'-CTGCTCAGTTGGCATCGTGC-3' and 5'-GGAGTCTCGTGCTCCTCTTG-3' for *Izumo1*, 5'-AAGTG TGACGTTGACATCCG-3' and 5'-GATCCACATCTGCTGGAA GG-3' for *b-actin*.

Immunoblot analysis

Immunoblot analysis was performed as described previously (Inoue *et al.* 2008). Samples were subjected to sodium dodecyl sulphate PAGE followed by western blotting under non-reduced conditions. For IZUMO1, antibody mAb No. 34 was used (Inoue *et al.* 2013). Anti-acetylated tubulin antibody (T7451, Sigma-Aldrich) was used as a loading control. Immunoreactive proteins were detected by an ECL western blotting detection kit (GE Healthcare). The densitometric analyses were performed using the ImageJ 1.44p software (Wayne Rasband, National Institutes of Health).

Immunolocalisation of IZUMO1 and spontaneous acrosome reaction rates

Visualisation of IZUMO1 using confocal microscopy and deconvolution analysis was done as reported previously (Satouh *et al.* 2012).

To assess spontaneous acrosome reaction rates the spermatozoa prepared from cauda epididymis were incubated in a drop of Toyoda, Yokoyama and Hamada (TYH) media (Toyoda *et al.* 1971) for 2 h at 37°C, 5% CO₂ then spotted onto slides, air dried, fixed with 4% paraformaldehyde for 5 min and washed in PBS for 5 min. Non-specific protein binding sites were blocked with 5% BSA/PBS with 0.1% Tween 20 at room temperature for 1 h. The slides were then incubated at 4°C overnight with anti-IZUMO1 (Inoue *et al.* 2005) antibody or anti-CD46 antibody (KS107-116) that was established in our laboratory as a rat anti-mouse sperm antigen monoclonal antibody. Next, slides were incubated in the dark with Alexa Fluor 488 or 546-conjugated anti-rabbit or anti-rat IgGs respectively (Invitrogen) at room temperature in 5% BSA/PBS with 0.1% Tween 20. After washing, the slides were mounted in PBS. The stained cells were observed under a fluorescence microscope. Acrosome reaction rate was calculated by counting the percentage of cells displaying IZUMO1 localised over the full sperm head – confirmed by CD46 localisation indicating the acrosome reaction had indeed taken place.

In vivo and in vitro male fertility tests

Sexually matured *Izumo1* mutant male mice were caged with 2-month-old B6D2F1 female mice for 3 months, and the number of pups was counted at the day of birth. *In vitro* fertilisation (IVF) analysis was done as reported previously (Inoue *et al.* 2005).

Zona-free fusion assay

Zona-free fusion assay was performed as reported previously (Inoue *et al.* 2005), with some minor changes. In brief, the

Figure 1 Phosphopeptide changes in the cytoplasmic tail of IZUMO1. (A) Amino acid sequence of the end of the transmembrane region (indicated by black line above sequence) and cytoplasmic tail of IZUMO1. The red characters indicate the peptides that were analysed in (B). (B) There were significant increases in the peptide (LKNASDEVKPTASGSK) with 1 phosphorylation event of a serine or a threonine. In contrast, no difference or rather a decrease was observed in the peptide (KASQADFNSDYS GDK) with 1 phosphorylation event of a serine, a threonine, or a tyrosine.

zona pellucida was removed with 1 mg/mL collagenase. Zona-free oocytes were then preloaded with Hoechst 33342 by incubation in TYH media containing the dye (1 µg/mL) for 10 min, then washed thoroughly. Spermatozoa were added to a concentration of 2×10^4 spermatozoa/mL. After 30 min of incubation the eggs were fixed with 0.25% glutaraldehyde and then observed under a fluorescence microscope (excitation with ultraviolet light).

Statistical analysis

Statistical analyses were performed using Student's *t*-test or one-way ANOVA followed by Turkey HSD test. Differences were considered significant at $P < 0.05$.

Results

Phosphorylation in the cytoplasmic region of mouse IZUMO1

IZUMO1 is the essential sperm-egg fusion protein and as such, it is necessary that we understand how it is regulated. Previous studies have shown that during epididymal transit in the rat, IZUMO1 undergoes major phosphorylation changes (Baker *et al.* 2012). In order to determine whether phospho-changes occurred within the mouse, we enriched phosphopeptides from the spermatozoa obtained from the caput, corpus and cauda region of the epididymides. The peptides were TMT labelled and quantified using liquid chromatography coupled to mass spectrometry. Figure 1A shows a representation of the phosphopeptides observed in the

Figure 2 CRISPR strategy. (A) Structure of *Izumo1* gene and protein structure. Solid bars indicate coding exons, open bars indicate non-coding exons. Primer sequences for genotyping indicated by black arrowheads, forward (Fw) and reverse (Rv). Yellow region depicts transmembrane domain. The final coding exon of the *Izumo1* gene was targeted. (B) Sequence of nucleotides and their corresponding amino acids for both WT and mutated (ΔCyt) *Izumo1*. Red lettering indicates inserted 'T' nucleotide and the corresponding stop codon. Agarose gel showing Bfal digest of PCR amplicons from WT, heterozygote (+/ΔCyt) and homozygote (ΔCyt/ΔCyt) genome. Black line above sequence indicates transmembrane domain. (C) Wave pattern sequence analysis showing mutation (red 'T').

cytoplasmic tail of IZUMO1. Whilst the phosphopeptide (LKNASDEVKPTASGSK) had significantly increased phosphorylations the phosphopeptide (KASQADFNSDYS GDK) remained unchanged or rather had decreased phosphorylation during epididymal transit (Fig. 1B). These data show that like the rat, IZUMO1 undergoes phosphorylation changes during epididymal maturation which may regulate this protein stability and/or function. An overview of *Izumo1* and its protein domains are shown in Fig. 2A.

Generation of *Izumo1* mice with truncation of the cytoplasmic tail

In order to assess the role of the cytoplasmic domain including phosphorylation sites in IZUMO1 we removed this domain via CRISPR/Cas9 gene editing.

This was done by the introduction of a point mutation, a single insertion of T nucleotide to the final exon of *Izumo1* and introducing a premature stop codon, thus producing a truncated protein (Fig. 2B and C). A total of 88 embryos were injected with 7 pups born. Of these, 4 pups were gene modified (GM). The desired mutation was inserted into 3/4 GM pups. There were no homozygous pups for the desired insertion. One mouse was heterozygous with the desired insertion and the other two carried multiple mutations as mosaics. Following selective breeding of the heterozygote mouse, the homozygous mice were obtained (Fig. 2B and C). The insertion of the T nucleotide at this location also introduced a site recognised by the restriction enzyme Bfal (CTAG) (Fig. 2B). This allowed for easier genotyping for distinguishing WT and KO alleles.

Whilst the PCR amplicon size from WT and KO was 410bp and 411bp, respectively, Bfal restriction enzyme digestion gave a single band of 410bp for WT (not digested) and single band comprised of 2 fragments (199 and 212 bp) for the KO allele (Fig. 2B).

Truncation of IZUMO1 was confirmed by immunoblot analysis, where IZUMO1 collected from the testis and from spermatozoa shifted from approximately 56 kDa in the wild-type (WT) to approximately 40 kDa in the homozygous samples, with the heterozygous samples showing both bands (Fig. 3A and B). An anti-acetylated tubulin antibody was used as a loading control (Fig. 3A and B). Because the amount of truncated IZUMO1 was lower than that of WT IZUMO1 in both testis and spermatozoa (Fig. 3A and B), the fold change (the amount of truncated IZUMO1/WT IZUMO1) was quantified ($n = 3$ males for both WT and mutant samples). The fold change in the testis (0.23 ± 0.10) is similar to that in

the spermatozoa collected from the cauda epididymis (0.33 ± 0.11), suggesting that the phosphorylation does not affect IZUMO1 stability during epididymal transit.

To investigate why the protein amount is already lower in the mutated testis, we performed qPCR using testis and found that the amount of *Izumo1* mRNA is lower than that of control (Fig. 3C). However, the fold change in mRNA (the amount of truncated *Izumo1*/WT *Izumo1*, 0.47 ± 0.21) was less than the fold change seen in the amount of protein (truncated IZUMO1/WT IZUMO1, 0.23 ± 0.10), suggesting that the lower protein amount of truncated IZUMO1 is caused by not only mRNA degradation but also protein instability.

Figure 3 Truncated IZUMO1 protein expression. (A) Protein expression of IZUMO1 in WT, +/ΔCyt and ΔCyt/ΔCyt testis. Acetylated tubulin as loading control. (B) Protein expression of IZUMO1 in spermatozoa collected from WT, +/ΔCyt and ΔCyt/ΔCyt cauda epididymis. Acetylated tubulin as loading control. (C) Quantitative PCR analysis of WT vs ΔCyt/ΔCyt *Izumo1* mRNA expression.

Figure 4 Immunolocalisation of IZUMO1 and spontaneous acrosome reaction. (A) Deconvolution analysis of confocal image of WT and ΔCyt/ΔCyt IZUMO1 localisation in acrosome intact spermatozoa. CD46 as control. IAM, inner acrosomal membrane; OAM, outer acrosomal membrane; PM, plasma membrane. (B) Location of IZUMO1 over the sperm head before and after acrosome reaction in WT versus ΔCyt/ΔCyt spermatozoa. CD46 as a control for acrosome reaction. Asterisks indicate acrosome-reacted spermatozoa. (C) Acrosome reaction rates of control (Ctr) versus ΔCyt/ΔCyt spermatozoa ($n = 3$ males each for Ctr and ΔCyt/ΔCyt). Percentage acrosome reacted; $15.0 \pm 8.5\%$ vs $11.8 \pm 2.8\%$ (Ctr vs ΔCyt/ΔCyt).

Immunolocalisation of IZUMO1 in relation to spontaneous acrosome reaction

To analyse if the truncated IZUMO1 can translocate correctly following the spontaneous acrosome reaction (Okabe *et al.* 1986, 1987, Kawai *et al.* 1989, Sebkova *et al.* 2013), spermatozoa were collected and incubated for 2 h in TYH medium. Anti-CD46 was used as a marker of the acrosome reaction as this protein also translocates in a similar manner to IZUMO1 in human (Okabe *et al.* 1990, 1992) and in mouse (Fig. 4A and B). In the acrosome-intact spermatozoa, IZUMO1 is located in both the outer and inner acrosomal membranes, and translocates to the plasma membrane then spreads to cover the whole sperm head following the acrosome reaction (Satouh *et al.* 2012) (Fig. 4A and B). Deconvolution analysis of confocal images for IZUMO1/CD46 staining of an acrosome-intact spermatozoon showed that truncated IZUMO1 is localised in both the outer and inner acrosomal membrane (Fig. 4A). It should be noted that immunostaining of truncated IZUMO1 resulted in decreased intensity to that of WT IZUMO1, consistent with the decrease in protein amount observed with western blotting (Figs 3B, 4B and Supplementary Fig. 1 for staining control, see section on supplementary data given at the end of this article).

Figure 5 Analysis of fertility phenotype. (A) Litter size; $n=3$ males each for Ctr and $\Delta\text{Cyt}/\Delta\text{Cyt}$, with 2 WT females per male. Average litter size; Ctr = 8.6 ± 1.5 , $\Delta\text{Cyt}/\Delta\text{Cyt}$ = 9.4 ± 1.9 pups/litter. (B) IVF success rate; $n=3$ males each for Ctr and $\Delta\text{Cyt}/\Delta\text{Cyt}$, Oocytes; 150 vs 195 (Ctr vs $\Delta\text{Cyt}/\Delta\text{Cyt}$), $86.5 \pm 24.2\%$ vs $67.4 \pm 11.2\%$ fertilised (Ctr vs $\Delta\text{Cyt}/\Delta\text{Cyt}$). (C) Zona-free fusion success rate. Fusion index; 2.5 ± 0.8 vs 2.1 ± 0.4 (Ctr vs $\Delta\text{Cyt}/\Delta\text{Cyt}$); $n=3$ males each for Ctr and $\Delta\text{Cyt}/\Delta\text{Cyt}$; Oocytes = 47 vs 42 (Ctr vs $\Delta\text{Cyt}/\Delta\text{Cyt}$); Fusion index; fused spermatozoa/egg.

However, truncated IZUMO1 showed identical localisation with WT IZUMO1 even after acrosome reaction, and co-translocation of truncated IZUMO1 and CD46 was examined further by the comparison of enhanced images of truncated IZUMO1 and CD46 (Fig. 4B). Moreover, the spontaneous acrosome reaction rate of the truncated IZUMO1 spermatozoa was comparable to that of the control mice; 11.8 and 15.0% respectively (Fig. 4C). These results indicate that the cytoplasmic region of IZUMO1 is not necessary for spontaneous acrosome reaction and subsequent IZUMO1 translocation *in vitro*.

Male fertility of truncated *Izumo1* mice

Litter size analysis was used to determine the fertilizing ability of *Izumo1* mutated mice. Mutated mice and controls were given mature WT females and allowed to copulate and breed for 3 pregnancy cycles (approx. 3 months). Pups were counted on the day of their birth. Homozygous mutated males showed comparable litter sizes to the controls (control: 8.6 ± 1.5 ; mutant: 9.4 ± 1.9) (Fig. 5A). There was no significant difference between control and mutant male mice; therefore, it was concluded that the truncated *Izumo1* mice are fully fertile. Further analysis using IVF also showed no significant difference in sperm fertilizing ability (Fig. 5B). In addition, there was no significant difference in the capacity of the spermatozoa to fuse with the plasma membrane of the oocyte in the zona-free fusion assay (Fig. 5C).

Discussion

IZUMO1 undergoes specific phosphorylation events in the cytoplasmic tail region suggesting that this region of IZUMO1 may be important for the function and/or translocation of IZUMO1 seen following the acrosome reaction. Across several mammalian species IZUMO1 exists with a highly conserved N-terminal region, however the C-terminus differs in three ways. The cytoplasmic region can be long, as seen in rodent species such as the mouse (approx. 55 aa), shorter in humans (approx. 40 aa) and very short in porcine species – only one amino acid in length (an alanine) (Kim *et al.* 2013). In order to investigate the importance of this region, this study produced a mutated mouse line with a truncated version of IZUMO1. The cytoplasmic tail of the mutated mice in this study is 3 amino acids long – very similar to the length seen in the pig, yet their fertility was unaffected. These data indicate that the cytoplasmic region including phosphorylation sites does not play any essential role in fertilisation in mice.

Ellerman *et al.* (2009) demonstrated IZUMO1's involvement in the formation of larger protein complexes. These complexes appear to be homo-tetramers, trimers

and dimers and are formed in conjunction with different regions of the IZUMO1 protein. The extracellular domain of the protein was found to be involved in forming complexes the size of dimers, whereas the cytoplasmic tail region and/or the transmembrane region were found to form larger complexes – the trimers and tetramers (Ellerman *et al.* 2009). Recently, it was found that IZUMO1 is largely monomeric in spermatozoa, a structure that is altered by its binding partner, JUNO, resulting in dimerisation and tight binding between the gametes (Inoue *et al.* 2015). Although the cytoplasmic region may play a role in the formation of larger complexes, this region is apparently dispensable for IZUMO1 relocalisation during acrosome reaction and subsequent fusion ability.

In the pig IZUMO1 still forms complexes; however, these are mainly homodimers of IZUMO1 (Kim *et al.* 2013). As seen in the work by Ellerman *et al.* (2009) complexes of this size are most likely formed by interactions between the extracellular portion of the protein, therefore it is unsurprising that pig IZUMO1 forms complexes of this size, even without a long cytoplasmic region. While the latter is clearly not necessary for the assembly of IZUMO1 complexes essential for fertility, this study has demonstrated that the C-terminus of this molecule is important in maintaining the amount of protein, however, it is not regulated by the phosphorylation changes that occur during epididymal transit.

There are many possible explanations for this impact on protein amount. One reason may be that the mRNA was unstable, leading to a decrease in levels of protein translation. Instability of the mRNA can be caused by nonsense mediated decay (NMD). However, NMD in mammals typically occurs when a premature stop codon appears more than approximately 50 nt upstream of the junction between the final intron and exon (Hentze & Kulozik 1999). In this case, the mutation of the *Izumo1* mRNA was in the final exon, suggesting that NMD did not play a causative role in driving down the amount of the corresponding protein in the mature gamete. That protein instability may also be involved in mediating the decreased manifestation of IZUMO1 in mutant mice is supported by comparing the relative levels of IZUMO1 expression as mRNA and protein, as set out in Fig. 3.

The discovery of the IZUMO1 binding partner JUNO, as well as the dimerisation of IZUMO1 following its recognition by JUNO, explains how the gametes are brought into close proximity during the early stages of fertilisation. Our data demonstrate that IZUMO1's cytoplasmic domain plays some roles in the regulation of IZUMO1 amount but is not involved in IZUMO1 translocation and the subsequent sperm–egg interaction.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0150>.

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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