Targeted inactivation of the mouse epididymal beta-defensin 41 alters sperm flagellar beat pattern and zona pellucida binding

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ABSTRACT

During epididymal maturation, sperm acquire the ability to swim progressively by interacting with proteins secreted by the epididymal epithelium. Beta-defensin proteins, expressed in the epididymis, continue to regulate sperm motility during capacitation and hyperactivation in the female reproductive tract. We characterized the mouse beta-defensin 41 (DEFB41), by generating a mouse model with iCre recombinase inserted into the first exon of the gene. The homozygous Defb41iCre/iCre knock-in mice lacked Defb41 expression and displayed iCre recombinase activity in the principal cells of the proximal epididymis. Heterozygous Defb41iCre/+ mice can be used to generate epididymis specific conditional knock-out mouse models. Homozygous Defb41iCre/iCre sperm displayed a defect in sperm motility with the flagella primarily bending in the pro-hook conformation while capacitated wild-type sperm more often displayed the anti-hook conformation. This led to a reduced straight line motility of Defb41iCre/iCre sperm and weaker binding to the oocyte. Thus, DEFB41 is required for proper sperm maturation.

1. Introduction

After leaving the testis, immature sperm travel through the different epididymal segments, the initial segment, caput, corpus and cauda, where proteins secreted by the epithelial cells interact with and modify the sperm plasma-membrane. During this transit, the sperm mature and acquire the ability to swim progressively, although they are kept in an immotile state until ejaculation (Robaire et al., 2000; Cornwall, 2009; Yanagimachi, 1994). In passage through the most proximal segments, the sperm tail is stabilized, allowing the straight-line movement that is necessary to localize the oocyte (Yeung et al., 1992, 1993; Jeulin et al., 1996). In addition, the sperm membrane is modified to promote capacitation in the female reproductive tract (Visconti et al., 1995; Lewis and Aitken, 1996). Throughout the epididymal transit, sperm are protected from autoimmunity and potentially harmful bacteria by antimicrobial proteins secreted by the epididymal epithelium (Cobellis et al., 2010; Yenugu et al., 2004; Lin et al., 2008; Yu et al., 2013).

Beta-defensins form a protein family involved in both sperm maturation and antimicrobial defense (Klüver et al., 2006; Selsted and Ouellette, 2005; Klotman and Chang, 2006; Yenugu et al., 2004; Zhou et al., 2004; Tolnai et al., 2004; Lin et al., 2008; Zhao...
et al., 2011; Yu et al., 2013). A majority of the currently known beta-defensin genes (39 genes in humans, 43 in rats and 52 in mice) are evolutionarily conserved in mammals, where they form four to five syntenic gene clusters (Patil et al., 2005; Schutte et al., 2002). Beta-defensins are mainly expressed in the epithelial cells of the male reproductive tract, especially in testis and in the different segments of the epididymis (Patil et al., 2005). Two beta-defensins have been shown to have antimicrobial functions in vivo: deletion of mouse beta-defensin1 lead to delayed bacterial clearance from the lung (Moser et al., 2002) or higher number of bacteria in the bladder (Morrison et al., 2002), whereas overexpression of the mouse beta-defensin Spag11a (also known as B1N1b), showed an effect on epididymal infection resistance (Fei et al., 2012). However, despite the antimicrobial function of beta-defensins, concurrent deletion of nine beta-defensins, expressed in the reproductive tract, did not cause inflammation under normal animal housing conditions (Zhou et al., 2013). In contrast, several studies have shown a role for beta-defensins in sperm maturation, especially regulating sperm motility. In rats, the beta-defensins Defb15 and Spag11b bind to the sperm head and contribute to the maintenance and acquisition of sperm motility, respectively (Zhou et al., 2004; Zhao et al., 2011). Knock-down of either of the two genes led to reduced motility of capacitated sperm (Zhou et al., 2004). In the case of Spag11b, this was attributed to a reduced uptake of Ca2+ during epididymal transit (Zhou et al., 2004). Similarly the knock-out of a cluster of nine mouse beta-defensins caused altered Ca2+ signaling in epididymal sperm, which led to premature capacitation and acrosome reaction (Zhou et al., 2013). Defb22/DEFB126 is secreted from corpus epididymis and binds to the sperm membrane, forming the most external part of the glyccalyx (Yudin et al. 2003, 2008). The macaque DEFB126 is required for sperm to penetrate the cervical mucus (Tollner et al. 2008) and, interestingly, a common deletion in human DEFB126 hinders sperm from penetrating the mucus, thereby reducing pregnancy rates (Tollner et al., 2011). In addition, release of DEFB126 from the sperm membrane during capacitation is required for oocyte fertilization (Tollner et al., 2004).

To further clarify the role of beta-defensins in sperm maturation, we have studied the role of mouse Defb41, which is expressed in the most proximal part of the epididymis (Jalkanen et al., 2005). Defb41 is located on chromosome 1qA4 and, like most beta-defensins consists of two short exons, which are 113 bp and 353 bp in size. The 62 amino acid long gene product contains a putative signal sequence, which is cleaved between amino acids 21 and 22 to produce the mature 41 amino acid DEFB41 protein. Further, Defb41 contains six highly conserved cysteines typical for beta-defensins (Jalkanen et al., 2005). Similar to several other epididymal beta-defensins, Defb41 is regulated by androgens. The expression of Defb41 begins between day 8 and 13 after birth and increases until adulthood, due to the rise in testosterone levels during puberty (Jalkanen et al., 2005; Hamil et al., 2000; Liu et al., 2001; Ibrahim et al., 2001; Palladino et al., 2003). The mouse Defb41 is orthologous to the human and macaque Defb110, which both have 69% identity to the mouse protein (human Gene ID: 245913/Ensembl: ENSG00000203970; macaque Gene ID: 707363/Ensembl: ENSMUG0000029616), however, no studies on gene function have been performed for these species.

In light of the previous studies on beta-defensins, and because of the segment-specific expression of Defb41 in the initial segment (IS) and caput (CAP) of the mouse epididymis, we hypothesized that the protein has a function in sperm maturation. Thus, we generated a Defb41 knock-in (KI) mouse model in which the cDNA coding for codon improved Cre recombinase (iCre, codon usage optimized for mammals (Shimshek et al., 2002)) is inserted into the translation start site of the Defb41 locus. Homozygous Defb41iCre/iCre mice show deletion of both Defb41 alleles, while the heterozygous Defb41iCre/+ mice can be used to generate conditional knock-out models through the specific expression of iCre in the principal cells of the epididymal epithelium and the consequent deletion of floxed alleles.

2. Materials and methods

2.1. Ethic statement

Mice were housed in individually ventilated cages under controlled conditions of light (12 h light/12 h dark), temperature (21 ± 3 °C), and humidity (55% ± 15%). The mice were given soy-free natural-ingradient feed (RM3 (E), Special Diets Services), tap water ad libitum, and were housed in specific pathogen-free conditions at the Central Animal Laboratory, University of Turku, complying with international guidelines on the care and use of laboratory animals. All animal handling was conducted in accordance with the Finnish Animal Ethics Committee license, and the institutional animal care policies, which fully meet the requirements of European Union Directive 2010/63/EU and European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS No. 123, appendix A).

2.2. Generation of the Defb41 iCre KI mouse line

For cloning the Defb41 iCre KI targeting construct, the BAC clone containing the Defb41 locus was purchased from RZPD German Resource Center for Genome Research (ImaGenes GmbH). The first exon of Defb41 together with the two homology arms (1700 bp and 7591 bp long) were subcloned into the pACYC177 minimal backbone vector using Red/ET recombination technology (Angrand et al., 1999). An iCre-neomycin phosphotransferase (neo*) expression cassette was inserted into the translation initiation site of the Defb41 gene. Mutated FRT sites were located around the iCre-neo* cassette to allow recombinein mediated cassette exchange if needed (Fig. 1A). All primer sequences used in cloning are available on request.

The targeting construct was electroporated into AB12.2 embryonic stem (ES) cells, (129/Sv/Ev background, Lexicon Genetics). The clones were screened for homologous recombination by PCR over the 1700 bp 5’-homology arm using the Expand Long Template PCR System (Roche). The primers used for screening were DefbScrf1: CGGATAGGTTACTGTCTGACCT and DefbScrR1: ATTCTCCTTTCTGATTCTCCTCATC (Fig. 1A). The correct targeting was further confirmed by PCR over the 7591 bp 3’-homology arm using the following primers, Defb41 3’ Fw: TCAAAAGACCCCCGCACAA and Defb41 e2 Re: TGGTGCTAGGATGGAGATT. The PCR was carried out using LongAmp Taq DNA Polymerase according to the manufacturer’s instruction (New England BioLabs). Finally, the PCR products were sequenced to ascertain correct recombination. Chimeric mice were generated by injecting a correctly targeted ES cell clone into C57BL/6N blastocysts. Chimeric males were bred with wild type (wt) C57BL/6N females to obtain heterozygous Defb41iCre/+ mice. Heterozygous hybrid males were backcrossed to C57BL/6N for three generations and then bred with each other to obtain homozygous, Defb41iCre-deficient, iCre KI mice, referred to as Defb41iCre/Cre iCre mice in the text. Defb41iCre/Cre mice were genotyped as previously described (Björkgren et al., 2012). For the experiments the control wild type Defb41+/+ mice and the Defb41iCre/+ and Defb41iCre/Cre mice were all obtained from the same litters.

The histology of the Defb41iCre/Cre male epididymis was determined by dissecting the epididymides of Defb41+/+ and Defb41iCre/+ male mice at different ages, from two-months to seven-months of age. The tissue was fixed overnight in 4% paraformaldehyde (PFA) and embedded in paraffin. Hematoxylin and
2.3. RNA isolation and RT-PCR

To analyze the expression pattern of iCre and Defb41, two-month-old Defb41+/+, Defb41iCre+/+, and Defb41iCre/iCre male mice were euthanized and various tissues and different segments of the epididymis were dissected and snap frozen. To analyze the Cre expression of heterozygous Defb41iCre+/+; CAGCre/+ mice, the IS and CAP of mice used in the sperm binding assay were similarly isolated. For time-point expression studies, the whole epididymis of 7-, 14-, 17-, 20-, 25-, 30- and 40-day-old Defb41iCre/iCre mice was dissected (each age n = 3). Total RNA was isolated from the tissues using TRIzol according to the manufacturer’s instructions (Bioline). For generation of cDNA, 1 μg of total RNA was treated with deoxyribonuclease I (DNaseI, Amplification Grade, Invitrogen) and reverse-transcribed using the DyNAzyme cDNA synthesis kit (Thermo Scientific). To detect Defb41 and iCre expression, the RT-reaction was performed using Biotools DNA Polymerase (10.002) according to the manufacturer’s instructions. The cDNA was diluted 1:50–1:100 for qRT-PCR analyses of Cre, iCre, and Defb41 exon 2, expression. Quantitative PCR was performed using the DyNAzyme SYBR Green qPCR Kit (Thermo Scientific). All samples and standards were run in triplicates. Ribosomal protein L19 (Rpl19) and phosphoglycerate kinase 1 (Pgk1) were used as endogenous controls to equalize for the amounts of RNA in the different tissues. Primers for
Defb41 detection were Fw: TCC ATT GCC TTT TCT TGT CC and Re2: TGT GTG CAT GGA TGG AGA TT, Tm 59 °C and primers for Defb41 exon 2 detection were Re2, as previously, and Fw2: AGA TGT GAA AAA GTG CGA GGA, Tm 59 °C. The sequence and qRT-PCR conditions of the Rpl19, Pkg1, Cre and iCre primers were previously described (Turunen et al., 2012; Bjorkgren et al., 2012, 2014; Sakai and Miyazaki, 1997).

2.4. Analyzing epididymal iCre recombinase activity utilizing ArloxP/Miyazaki, 1997).

described (Turunen et al., 2012; Björkgren et al., 2004).

at the age of 35 days and the epididymides were fixed overnight at 4 °C. The epididymides were dissected, washed three times with fresh KSOM medium to remove loosely adhering cell debris, and mounted in Mowiol 488-conjugated rat anti-rabbit antibodies, and for detection of the sample which was put in to two separate fertilization dishes in 200 μl HTF medium. Oocytes in cumulus oophorus from 2 superovulated FVB/N females was added to each fertilization dish as described above. After 6 h incubation, the zygotes were washed through three drops of HTF and then cultured o/n. After reaching 2-cell stage the embryos were moved into KSOM + AA-medium containing BSA, (Millipore) and cultured further until blastocyst stage. Blastocysts were collected and subjected to lysis as described previously (Scavizzi et al., 2015) and genotyped using the same primers and PCR conditions that were used to genotype Defb41iCre/iCre mice (Björkgren et al., 2012) except that in the PCR reaction mix 5 ul of lystate was used and the number of PCR cycles was 50. The mean ± SD were then calculated for both genotypes, Defb41+/− and Defb41iCre/−, from the total number of blastocysts obtained from the competitive IVF.

2.5. In vivo fertility

The breeding performance of six Defb41iCre/iCre male mice was followed in normal mating conditions. Two-month-old males were mated with wt C57Bl/6N female mice and the number of litters and offspring of each male was recorded during a time period of six months. Values were compared to those obtained from Defb41+/− male mice in breeding, at the same time and in the same animal room.

2.6. In vitro fertilization

In vitro fertilization (IVF) was conducted using two-month-old male mice. Sperm from six Defb41+/−, six Defb41iCre/+ and five Defb41iCre/iCre mice were collected during four experimental days, using at least one animal of each genotype per day. Cauda was dissected out and two small incisions were made to allow sperm to swim out into HTF medium containing BSA (K-RVFE-50, William A. Cook Australia Pty. Ltd) for 15 min at 37 °C, 89% N2/6% CO2/5% O2. The oocytes were collected from superovulated FVB/N females and cumulus oophorus was removed by hyaluronidase (0.6 mg/ml, Specialty Media) treatment. 40,000 spermatozoa from each male were incubated with 15–30 zona pellucida intact oocytes for 1 h at 37 °C, 89% N2/6% CO2/5% O2 in 30 μl of KSOM-medium. After co-incubation, the gametes were washed three times with fresh KSOM medium to remove loosely attached spermatozoa, and fixed briefly in 4% PFA. From each male, spermatozoa bound to ten, randomly chosen oocytes were counted under the microscope by assessing the number of sperm in one focal plane.

2.7. Sperm-zona pellucida binding

Next the effect of the loss of Defb41 on sperm-zona pellucida binding efficiency was analyzed. In addition, to ensure that the effects seen are not caused by Cre toxicity, a CAG-Cre mouse line, where Cre is under the cytomegalovirus immediate early enhancer-chicken beta-actin hybrid (CAG) promoter, was utilized (Sakai and Miyazaki, 1997) in the sperm-zona pellucida binding assay. CAG-Cre/− mice were mated with Defb41iCre/iCre mice to produce mice heterozygous for both Cre and iCre (Defb41iCre/−; CAG-Cre/−), and thus having higher total Cre recombinase protein levels than in either mouse line alone. Sperm from two to three month-old wild type control (wt, no genetic modifications), Defb41iCre/+, Defb41iCre/iCre, CAG-Cre/− and double heterozygous Defb41iCre/+, CAG-Cre/− males (n = 3–6), were collected in KSOM + AA-medium for 15 min at 37 °C, 89% N2/6% CO2/5% O2. The oocytes were collected from superovulated FVB/N females and cumulus oophorus was removed by hyaluronidase (0.6 mg/ml, Specialty Media) treatment. 40,000 spermatozoa from each male were incubated with 15–30 zona pellucida intact oocytes for 1 h at 37 °C, 89% N2/6% CO2/5% O2 in 30 μl of KSOM-medium. After co-incubation, the gametes were washed three times with fresh KSOM medium to remove loosely attached spermatozoa, and fixed briefly in 4% PFA. From each male, spermatozoa bound to ten, randomly chosen oocytes were counted under the microscope by assessing the number of sperm in one focal plane.

2.8. Sperm capacitation

Analyses of sperm capacitation were performed according to the method previously described (Wertheimer et al., 2008). Briefly, caudal sperm from two-month-old Defb41+/−, Defb41iCre/− and Defb41iCre/iCre mice (n = 5) were collected in modified non-capsulating Whitten-HEPES medium (Platt et al., 2009), pH 7.3, for 15 min at 37 °C. Each sperm sample was divided into five aliquots to which 20 mM NaHCO3 and 10 mg/ml BSA were added. Sperm aliquots were collected after 0, 30, 60, 90, and 120 min incubation and the proteins were extracted by boiling in Laemmli sample buffer (Laemmli, 1970). Protein tyrosine phosphorylation was used as a read-out for sperm capacitation. Phosphorylation was assessed by Western blotting using a monoclonal anti-phosphotyrosine antibody (1:5000 dilution; Millipore) as primary antibody followed by a peroxidase-conjugated anti-mouse secondary antibody (1:5000 dilution; GE Healthcare). The detected levels of tyrosine phosphorylation were normalized to the expression level of alpha-tubulin. The membrane was stripped by
incubation with 6 M Guanidine HCl, 10% Triton X-100, 20 mM Tris–HCl, 0.1 M beta-mercaptoethanol, pH 7.5 twice for 5 min at room temperature. Thereafter, the membrane was blocked and rehydrized with tubulin-alpha ab-2 antibody (1:5000 dilution; Lab Vision). The stained proteins were quantified from digital images using the ImageJ software.

2.9. Sperm acrosome reaction

2.9.1. Isolation of mouse zona pellucida

To induce the acrosome reaction with solubilized zonae pellucidae, female mice were superovulated by intraperitoneal injection of 10 IU hCG (human Chorionic Gonadotropin; Intergonan, SymposiumVet) 3 days before the experiment. 14 h before oocyte preparation, mice were injected with 10 IU PMSG (Pregnant Mare’s Serum Gonadotropin; Ovogest, SymposiumVet). Cumulus-enclosed oocytes were prepared from the oviducts in TYH buffer (138 mM NaCl, 4.8 mM KCl, 2 mM CaCl2, 1.2 mM KH2PO4, 1 mM MgSO4, 5.6 mM glucose, 0.5 mM sodium pyruvate, 10 mM L-lactate, pH 7.4) containing 300 μg/ml hyaluronidase (Sigma). After 15 min, cumulus-free oocytes were transferred into fresh buffer and washed twice. Zona pellucidae and oocytes were separated by shear forces generated by expulsion from 50 mm Pasteur pipettes. Zona pellucidae were counted and transferred into fresh buffer.

2.9.2. Analysis of acrosomal exocytosis

For analysis of acrosomal exocytosis, caudal sperm from two-month-old Defb41+/− and Defb41Cre/Cre mice (each genotype n = 3) were collected and 100 μl 1:10 sperm (TYH buffer, 3 mg/ml BSA) were capacitated with 25 mM NaHCO3 for 90 min at 37 °C. As a control, sperm were incubated in BSA buffer only (non-capacitated). Acrosome reaction was induced by incubating sperm with 50 heat-solubilized mouse zona pellucida or 2 μM 4-nitrophenol for 10 min at 37 °C. As vehicle control, sperm were incubated in 1% DMSO. The sperm suspension was spun down, the sperm were resuspended in 100 μl PBS buffer. Samples were air dried on microscope slides and analyzed under non-capacitating and capacitating conditions.

2.10. Intracellular PKA/SACY and cAMP levels

To analyze the expression level of protein kinase A (PKA) and soluble adenyl cyclase (SACY) by Western blot, total lysates were isolated from Defb41+/− and Defb41Cre/Cre sperm and processed as previously described (Krahling et al., 2013). The following primary antibodies were used: PKA [C4], (1:4000 dilution, 610980, BD Transduction Laboratories), SAC R21, (1:1000 dilution, (Zippin et al., 2003)). Primary antibodies were detected with fluorescently-labeled secondary antibodies (anti-mouse IRDye800CW and anti-mouse IRDye680LT) and the LI-COR Odyssey system and analyzed using ImageJ.

For cAMP measurements, sperm were isolated by incision of the cauda epididymis in modified TYH-medium containing 138 mM NaCl, 4.8 mM KCl, 2 mM CaCl2, 1.2 mM KH2PO4, 1 mM MgSO4, 5.6 mM glucose, 0.5 mM sodium pyruvate, 10 mM l-lactate, pH 7.4, supplemented with 3 mg/ml BSA. After 15 min swim out at 37 °C and 5% CO2, sperm were counted. Basal cAMP levels as well as the increase in cAMP levels in the presence of 25 mM NaHCO3 and/or 0.75 mM IBMX was determined using the CatchPoint cAMP Fluorescent Assay Kit (Molecular Devices). The assay was performed according to the protocol provided with the kit.

2.11. Sperm motility analyses

For motility analyses, the sperm were collected as described above. Flagellar movements and the different motility parameters were analyzed under non-capacitating and capacitating conditions as previously described (Krahling et al., 2013), with the exception of using smaller moving windows (0.5 s side) to quantify the different parameters. To quantify the flagellar movement, a portion of the flagellum (arc length distance of 50 μm from the head) was followed. The angle φ(t) between the long axis of symmetry of the cell and a line crossing the middle flagellar portion and the sperm head described a periodic movement. The flagellar beat asymmetry index corresponded to the average angle (⟨φ⟩) within the 0.5 s side moving window. The flagellar amplitude was calculated by fitting a sinusoidal time trace to the angle around its mean (φ(t) - ⟨φ⟩). The average flagellar beat asymmetry index, amplitude and frequency were calculated from all frames in the recording (between 4000 and 40000 frames). Between 4 and 14 sperm were analyzed in non-capacitating and capacitating conditions for each animal (four Defb41+/− and five Defb41Cre/Cre animals). To assess if the sperm flagellum moved in both pro- and anti-hook conformations and to detect the average angle of each conformation, 120 frames (approximately 5 full beat cycles) depicting pro- and anti-hook motility were quantified separately. Four sperm from each animal were analyzed under non-capacitating and capacitating conditions (three Defb41+/− and three Defb41Cre/Cre animals).

2.12. Sequence alignment and phylogenetic analysis

To reveal which epididymal beta-defensin family members are most similar to DEFB41, amino acid sequences of 23 family members (and different isoforms of SPAG11B) expressed in the mouse caput epididymis were acquired along with rat and human orthologs from the NCBI database (total of 60 sequences, Supplemental Table S1). The amino acid sequences were then aligned using the T-COFFEE algorithm (Notredame et al., 2000) utilizing the BLOSUM matrix during the alignment procedure (Supplemental Fig. S1). The aligned sequences were imported into MEGA6 (Tamura et al., 2013) and analyzed using Maximum likelihood. Uniform rates were assumed across sites and the JTT-model of amino acid evolution was used. To evaluate the robustness of the phylogenetic hypothesis, bootstrapping was used with 500 replications.

2.13. Statistical analyses

For sperm cAMP measurements and motility analyses, statistical comparisons were carried out using Students t-test. For statistical analyses of organ weights, sperm count and animal fertility, One-way ANOVA and for sperm flagellar movement Mann–Whitney test in the GraphPad Prism 5 software (GraphPad Software, Inc.) was used. Statistical analyses for iCre expression at different age points and sperm — oocyte binding SigmaPlot 13.0 software was used. For iCre expression at different age points One-way ANOVA was performed and in the case of statistically significant results a pair-wise multiple comparisons with Holm-Sidak method was used. For sperm — oocyte binding Kruskal–Wallis one-way analysis was performed, and in the case of statistically significant results, Dunn’s test was used for pair-wise multiple comparisons. p ≤ 0.05 was assigned as the limit of statistical significance.

To test each of the seven sperm motility parameters separately (VCL, curvilinearm velocity; VAP, average path velocity; VSL, straight
linear velocity; LHD, lateral head displacement; Wobble, LIN, linearity; and STR, straightness), statistical analyses were performed using a mixed-effects model:

\[ y = b_0 + b_1 \cdot K + b_2 \cdot C + b_3 \cdot K \cdot C + u_{ij} + e_{ij} \]  

(1)

The model Eq. (1) shows the effect of Defb41 ablation and/or capacitation on sperm motility and takes into account animal specific variation and the difference in number of films recorded for each animal. Motility data from 4 to 16 films per animal was recorded, and the average motility of the detected sperm was calculated for each of the films (response y). Non-motile sperm with VAP <20 μm/s were excluded from the calculations. The model terms K and C were used as binary indicators for knock-out or capacitation, respectively. Model term K adopted the value zero for Defb41iCre/+ sperm and one for Defb41iCre/iCre and the term C adopted the value zero for non-capacitated and one for capacitated sperm. The fixed effect terms b0–b3 indicated population effects specific for the baseline level (b0 confirms a motility baseline above zero by comparing non-capacitated Defb41iCre/+ sperm motility to zero), mere Defb41 knockout effect (b1, non-capacitated Defb41iCre/+ versus Defb41iCre/iCre sperm motility), mere capacitation effect (b2, capacitated versus non-capacitated Defb41iCre/+ sperm motility), and the interaction of the 2 × 2 factorial design (b3, if Defb41iCre/iCre sperm respond differently to capacitation than Defb41iCre/+ sperm). Calculated average values and statistical significance for the fixed effect terms b0–b3 are presented in Supplemental Table S2. The random effect μ0 captured animal-specific variation (n = 3 Defb41iCre/+), n = 3 Defb41iCre/iCre) and was assumed to be normally distributed. By introducing μ0 we compensated for the different numbers of films that were recorded for each animal. Finally, the normally distributed model error term ε captured the remaining data noise over the individual animals (index i) and recorded films (index j). The model was fitted using the statistical software R (http://www.R-project.org) and its package lme4 (http://CRAN.R-project.org/package=lme4).

3. Results

3.1. Generation of the Defb41 iCre KI mouse line

To inactivate the Defb41 gene in mice, the iCre recombinase expression cassette was targeted into the first exon of the gene (Fig. 1A). Thereby, iCre was expressed under the control of the regulatory region of Defb41 while the expression of functional Defb41 was abolished. After injection of the targeting vector into ES cells, PCR and sequencing of the 5' and 3' homology arms confirmed correct targeting (the sequencing data is available on request). Mice were generated by injection of ES cells into blastocysts and their genotypes were confirmed by PCR (Fig. 1B). Expression of iCre and lack of Defb41 exon 1 and 2 expression in the epididymis of Defb41iCre/iCre mice was confirmed by RT-PCR and qRT-PCR, respectively (Fig. 1C,D). The single wild-type allele of Defb41iCre/+/ mice gave rise to half the amount of exon 2 mRNA products when compared to Defb41iCre/+ mice (relative expression of Defb41iCre/+ IS: 0.9 ± 0.1, 1.0 ± 0.3; Defb41iCre/iCre IS: 0.5 ± 0.1, CAP: 0.5 ± 0.2). No expression was detected in Defb41iCre/iCre mice IS or CAP. Epididymis and testis weight of two-month-old Defb41iCre/+ and Defb41iCre/iCre mice was similar to Defb41iCre/+ mice (Relative weight of epididymis mg/whole body weight g: Defb41iCre/+ 1.13 ± 0.13; Defb41iCre/iCre 1.06 ± 0.1; Relative testis weight: Defb41iCre/+ 3.25 ± 0.5; Defb41iCre/iCre 3.20 ± 0.3; Defb41iCre/iCre 3.07 ± 0.6). Further, HE staining of tissue sections showed similar histology in Defb41iCre/+ and Defb41iCre/iCre mice in all the age groups studied (Supplemental Fig. S2).

3.2. Localization of the Defb41 locus expression driving iCre recombinase

The expression of Defb41 in the mouse epididymis has been previously defined (Jalkanen et al., 2005). To analyze whether the expression pattern was similar for the iCre recombinase under the Defb41 regulatory region, we performed qRT-PCR studies. The results indicated iCre expression in the most proximal segments of the epididymis, with highest expression in the IS (Supplemental Fig. S3A). The expression level in CAP was a third of the expression level in IS and only one animal out of three showed marginal expression in the spleen and testis (Supplemental Fig. S3A). However, we could not detect any recombinase activity in the testis and spleen. The onset of iCre expression in the epididymis takes place between 7 and 14 days after birth, and the expression reaches peak level at 25 days postpartum. A slight decline in expression can be observed in 30 and 40 days of age (Supplemental Fig. S3B). To define the epithelial cell types expressing iCre under the Defb41 locus, we used mice with a floxed Ar (De Gendt et al., 2004). Immunohistochemical analysis revealed that all epithelial cells in the epididymis of Defb41iCre/+ mice expressed AR (Supplemental Fig. S3C, D). AR+/iCre+, Defb41iCre/iCre mice lacked AR expression in the principal cells of the IS and CAP, while the basal and narrow/clear cells still displayed AR expression (Supplemental Fig. S3C, D). Thus, the iCre recombinase is specifically expressed in the principal cells of the proximal epididymis under the control of the Defb41 promoter.

3.3. Fertility analyses

Defb41iCre/iCre males were fertile and showed no difference in litter number and litter sizes compared to Defb41+/+ males (Defb41iCre/+, 6.6 ± 2.5 pups/litter; Defb41iCre/iCre, 6.7 ± 2.3 pups/litter) within the time period analyzed, from 2 months of age until 8 months of age. In vitro fertilization experiments confirmed this result, as Defb41iCre/+ and Defb41iCre/iCre, iCre sperm fertilized similar numbers of oocytes (Fig. 2A). In addition, in competitive IVF from a total of 129 blastocysts obtained, 63 (mean 15.8 ± 6.3) were fertilized with wild type Defb41+ sperm and 62 (mean 15.5 ± 4.8) by Defb41iCre sperm, thus showing equal fertilizing capacity of the two mouse lines. Furthermore, we analyzed the ability of the Defb41iCre/+ and Defb41iCre/iCre sperm to undergo the acrosome reaction. However, there was no difference in the number of acrosome reacted sperm between Defb41iCre/+ and Defb41iCre/iCre mice when utilizing a calcium ionophore or ZP proteins as inducers (Fig. 2B). In addition, both sperm morphology (Fig. 2C–E) and sperm count were similar between Defb41iCre/+ and Defb41iCre/iCre mice (sperm count in mil: Defb41iCre/+, 7.2 ± 3.3; Defb41iCre/iCre, 8.0 ± 7.0; Defb41iCre/iCre, 9.0 ± 3.8).

In contrast, there was a significant decline in the ability of Defb41iCre/iCre sperm to bind to the oocyte as compared to sperm from wt and Defb41iCre/+ mice (Fig. 3B). To show that the sperm-ZP binding phenotype is not caused by Cre toxicity rather than reduced DefB41 levels, we performed a binding assay with the transgenic CAGCre/Cre mouse line with ubiquitous Cre expression throughout the epididymis (Sakai and Miyazaki, 1997). CAGCre/Cre mice express Cre at similar levels compared to heterozygous Defb41iCre/+ mice whereas double heterozygous Defb41iCre/Cre mice produce similar levels of total Cre expression compared to Defb41iCre/+ mice (Fig. 3A). However, the binding efficiency of CAGCre/Cre sperm was indistinguishable from wt sperm and Defb41iCre/+ mice sperm was comparable to that of Defb41iCre/+ mice sperm and significantly higher than that of Defb41iCre/iCre mice (p < 0.001, Fig. 3B). Thus, the level of Defb41 expression was critical for the binding efficiency. In contrast, the level of Cre expression did
Defb41iCre/iCre omycin. The morphology of sperm collected from (C) spermatozoa after incubation with buffer alone, mouse zona pellucida (ZP) or Ion-mean (iCre sperm. We first analyzed the two flagellar conformations separately. However, the bending angle of the Defb41iCre/iCre sperm tail in the pro- and anti-hook conformation was similar to Defb41+/+ sperm (Fig. 4H). Thus, Defb41iCre/iCre sperm were able to bend in both the pro- and anti-hook conformation; however, they seemed to bend more often in the pro-hook conformation compared to Defb41+/+ sperm. We also analyzed the amplitude and frequency of the flagellar beat, but there was no difference between Defb41iCre/iCre and Defb41+/+ mouse sperm under non-capacitating and capacitating conditions (Fig. 4I, J).

To investigate whether the difference in flagellar bending affected the motility pattern of freely swimming sperm, we analyzed the swimming path of Defb41+/+ and Defb41iCre/iCre sperm. Whereas there was no difference between Defb41+/+ and Defb41iCre/iCre under non-capacitating conditions, Defb41iCre/iCre sperm showed a significant decrease in straight line velocity (VSL) after capacitation compared to Defb41+/+ sperm (p = 0.037, Fig. 5A, Supplemental Table S2). Consequently, sperm linear motility (LIN, calculated as VSL/VCL) and the straightness of sperm movements (STR, calculated as VSL/VAP) were also significantly decreased (LIN: p = 0.00034, Fig. 5B; STR: p = 0.00055, Fig. 5C, Supplemental Table S2). Taken together, our results indicate that Defb41iCre/iCre sperm swim in more curved paths after capacitation, and consequently, show lower progressive motility compared to the sperm of Defb41+/+ mice.

Flagellar beat frequency and, thereby, sperm motility crucially rely on PKA-dependent phosphorylation of proteins in the axoneme, activated by an increase in the intracellular cAMP concentration through the atypical adenylyl cyclase SACY (Esposito et al., 2004; Wandernoth et al., 2010). Thus, we investigated whether changes in the cAMP signaling-cascade underlie the motility defects observed in Defb41iCre/iCre sperm. Protein expression of SACY and PKA did not differ between Defb41+/+ and Defb41iCre/iCre mice (Fig. 6A). Furthermore, neither basal cAMP levels nor cAMP levels after activation of SACY by bicarbonate were different between genotypes (Fig. 6B). Downstream of PKA, protein tyrosine phosphorylation occurs, which is considered a hallmark of capacitation (Visconti et al., 1995). Since Defb41iCre/iCre sperm displayed a defect in flagellar bending after capacitation, we analyzed tyrosine phosphorylation in Defb41+/+, Defb41iCre/iCre, and Defb41iCre/+ sperm. However, there was no difference between genotypes (Fig. 6C, D).

3.5. Phylogenetic analysis of beta-defensins expressed in the caput epididymidis

The highest amino acid sequence similarity to Defb41 was found from its human and rat orthologs, DEFB110 and DEFB17, respectively (Supplemental Fig. 5A). Similar close clustering of known beta-defensin orthologs was observed in the analyses. DEFB41 formed a clade with DEFB18, which is also physically close to DEFB41 in chromosome 1 (Supplemental Fig. S4).

4. Discussion

Sperm maturation in the epididymis depends on the segment specific expression and secretion of proteins from the epididymal
epithelium (Robaire et al., 2000; Cornwall, 2009). The beta-defensin family is of particular interest, as the proteins have been shown to interact with sperm after release into the epididymal lumen (Zhou et al., 2004; Zhao et al., 2011; Yudin et al., 2003, 2008) and thereby promote fertility by regulating sperm motility (Zhou et al., 2004; Zhao et al., 2011; Yu et al., 2013). To further clarify the role of the beta-defensin family in sperm maturation, we generated a knock-out for Defb41, which is specifically expressed in the most proximal part of the juvenile and adult mouse epididymis (Jalkanen et al., 2005).

Defb41<sup>Cre/iCre</sup> male mice showed normal epididymal morphology and fertility. The rate of spermatogenesis did not differ between Defb41<sup>/+</sup> and Defb41<sup>Cre<i>/i</i>Cre</sup> mice as indicated by comparable numbers and morphology of spermatozoa. However, similar to other published beta-defensin models (Zhou et al., 2004; Zhao et al., 2011), sperm from Defb41<sup>Cre/iCre</sup> mice showed changes in motility. After capacitation, the flagellum of Defb41<sup>Cre/iCre</sup> sperm resided longer in the pro-hook conformation compared to Defb41<sup>/+</sup> sperm. In addition, linearity and straightness of Defb41<sup>Cre/iCre</sup> sperm velocity was reduced after capacitation. Thus, the change in flagellar bending seemed to result in a different motility pattern. Although the different motility pattern was detected after capacitation, there was no discernable change in Defb41<sup>Cre/iCre</sup> sperm capacitation or acrosome reaction. The signaling pathways leading up to these events were not altered, as indicated by similar levels of PKA and protein tyrosine phosphorylation between control and Defb41<sup>Cre/iCre</sup> sperm.

Sperm motility and sperm-oocyte interaction are controlled by Ca<sup>2+</sup> (Darzson et al., 2011). It has been suggested that hyperactive motility of sperm in the female reproductive tract is caused by an increased pH, which triggers influx of Ca<sup>2+</sup> through CatSper channels (Kirichok et al., 2006). This increase in Ca<sup>2+</sup> causes sperm to bend predominantly in a pro-hook formation. At the same time, there is a release of Ca<sup>2+</sup> from inner stores that is thought to promote anti-hook bending (Marguez et al., 2007; Alasmari et al., 2013). Sperm from CatSper knock-out mice lack hyperactivated motility and are infertile (Ren et al., 2001; Quill et al., 2003; Jin et al., 2007; Qj et al., 2007), as they cannot penetrate the zona pellucida (Suarez et al., 1991, 1992; Ren et al., 2001; Quill et al., 2003). However, if the zona pellucida is removed from the oocyte, CatSper knock-out sperm are still able to fertilize the oocyte (Ren et al., 2001). Previous beta-defensin mouse models have shown a motility phenotype due to changes in Ca<sup>2+</sup> signaling (Zhou et al., 2004, 2013). Beta-defensins are cationic molecules, which insert into phospholipid membranes forming holes or channels (Hall et al., 2007). Thereby, they possess anti-microbial properties but also, as in the case of SPAG11b, the ability to form or activate Ca<sup>2+</sup> channels in the sperm membrane (Zhou et al., 2004). Lack of Spag11b in the rat epididymis led to reduced sperm motility, while the introduction of the protein to immature sperm gave rise to increased Ca<sup>2+</sup> uptake and progressive motility (Zhou et al., 2004).

On the contrary, the partial deletion of the mouse chromosome 8 beta-defensin cluster resulted in increased Ca<sup>2+</sup>-uptake with premature hyperactivation and spontaneous acrosome reaction of the spermatozoa (Zhou et al., 2013). It was suggested that one or several of the beta-defensins in this study had a role in regulating CatSper or other calcium channels (Zhou et al., 2013). Thus, binding of beta-defensins to sperm in the epididymis could protect sperm from premature activation by inhibiting the activation of Ca<sup>2+</sup> channels. In the case of Defb41, the predominant pro-hook formation of the sperm flagellum would indicate a role in regulating Ca<sup>2+</sup>-signaling, possibly by direct or indirect inhibition of CatSper channels. Interestingly, sperm motility and VSL has been positively correlated with sperm-oocyte binding efficiency (Mahony et al., 2000; Yogev et al., 2000). Although the Defb41<sup>Cre/iCre</sup> sperm are produced in normal amounts and would capacitate and acrosome react, the more prominent pro-hook movements could lead to a reduced number of spermatozoa with the appropriate pattern of flagellar movement to bind to the oocyte. Few studies have analyzed the role of beta-defensins in sperm-oocyte interaction. It was shown that removal of DEFB126 from sperm promotes binding to the oocyte, while the deletion of chromosome 8 beta-defensins caused reduced amount of bound sperm (Tollner et al., 2004; Zhou et al., 2013). The role of sperm binding affinity in fertilization is debatable as it has been shown that penetration of the oocyte can take place directly after the acrosome reacted sperm encounter zona pellucida (Jin et al., 2011). This could also explain why we did not observe a change in Defb41<sup>Cre/iCre</sup> fertility. If the acrosome reaction took place prior to oocyte contact, the reduced binding efficiency would not have had a significant effect on animal fertility in vitro or in vivo.

The beta-defensin family is a large family of proteins and the function of one particular beta-defensin is most likely compensated by other family members. This has also been observed in previous studies of beta-defensin knockout-mice (Morrison et al., 2002; Moser et al., 2002). In addition to Defb41, there are 22 beta-defensin expressed in the mouse caput epididymis (Hu et al., 2014) and redundancy could explain the mild phenotype of
Defb41Cre/Cre mice. Beta-defensin family members have low sequence similarity among the members suggesting that the molecular diversity of beta-defensins have evolved as a response to a range of diverse pathogens (Semple and Dorin, 2012). However, DEFB41 along with other beta-defensins share a 6-cysteine motif necessary for the disulfide bonding pattern; Cys1-Cys5, Cys2-Cys4, Cys3-Cys6, necessary for secondary structure typical for beta-defensins (Klüver et al., 2006). Phylogenetic analyses performed in our study are consistent with the previous observation that closest sequence similarity is found between family members that are physically close to each other in one genomic cluster (Morrison et al., 2003). The closest family member to mouse Defb41 seems to be Defb18 located next to Defb41 in the chromosome 1. Interestingly, those beta-defensins whose lack has been shown to have
clear effects on fertility have an extended tail region with potential O-linked glycosylation sites after the last two core cysteines (Semple and Dorin, 2012). However, it is not clear how this difference in structure affects the functions of beta-defensins and further studies are required to resolve this question.

5. Conclusion

Our study shows a role for the novel mouse beta-defensin, Defb41, in sperm motility. By utilizing advanced measurement techniques detecting sperm flagellar motility, Defb41iCre/iCre sperm was shown to have an inverse flagellar beat pattern after capacitation. While ablation of Defb41 expression does not give rise to morphological changes in the epididymis or spermatozoa, the change in flagellar movement results in an altered velocity of sperm motility as well as a reduction in oocyte binding. The function of DEFB41, thus, correlates with the role of beta-defensins as regulators of sperm maturation and the results from current study are consistent with view that beta-defensins might regulate Ca2+ uptake in the sperm membrane.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.03.013.

References


