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

Ida Björkgren [a, b, 1], Luis Alvarez [c], Nelli Blank [d], Melanie Balbach [d], Heikki Turunen [a, b], Teemu Daniel Laajala [e, f], Jussi Toivanen [a], Anton Krutskikh [g], Niklas Wahlberg [h], Ilpo Huhtaniemi [g], Matti Poutanen [a, i], Dagmar Wachten [d], Petra Sipilä [a, *]

[a] Department of Physiology and Turku Center for Disease Modeling, Institute of Biomedicine, University of Turku, Turku, Finland
[b] Turku Doctoral Programme of Biomedical Sciences, Turku, Finland
[c] Center of Advanced European Studies and Research (Caesar), Department of Molecular Sensory Systems, Bonn, Germany
[d] Center of Advanced European Studies and Research (Caesar), Minerva Research Group Molecular Physiology, Bonn, Germany
[e] Department of Mathematics and Statistics, University of Turku, Turku, Finland
[f] Institute for Molecular Medicine Finland (FIMM), University of Helsinki, Helsinki, Finland
[g] Institute of Reproductive and Developmental Biology, Imperial College London, Hammersmith Campus, London, United Kingdom
[h] Department of Biology, Lund University, Lund, Sweden
[i] Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

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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 *Defb41*$^{iCre/iCre}$ knock-in mice lacked *Defb41* expression and displayed iCre recombinase activity in the principal cells of the proximal epididymis. Heterozygous *Defb41*$^{iCre/+}$ mice can be used to generate epididymis specific conditional knock-out mouse models. Homozygous *Defb41*$^{iCre/iCre}$ 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 *Defb41*$^{iCre/iCre}$ 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, 2001). 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; Tollner et al., 2004; Lin et al., 2008; Zhao

Abbreviations: Defb, Beta-defensing; KI, Knock-in; AR, Androgen receptor; IVF, *In vitro* fertilization; PKA, Protein kinase A; SACY, Soluble adenylyl cyclase; ZP, Zona pellucida.

* Corresponding author. Department of Physiology, Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10, FI-20520, Turku, Finland.
E-mail address: petra.sipila@utu.fi (P. Sipilä).
[1] Present address: Department of Molecular and Cell Biology, UC Berkeley, Berkeley, CA, USA.

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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 BIN1b), 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 caput sperm (Zhou et al., 2004; Zhao et al., 2011). In the case of SPAG11b, this was attributed to a reduced uptake of Ca^{2+} during epididymal transit (Zhou et al., 2004). Similarly the knock-out of a cluster of nine mouse beta-defensins caused altered Ca^{2+} 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 exterior part of the glycocalyx (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: ENSMMUG00000029616), 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 $Defb41^{iCre/iCre}$ mice show deletion of both *Defb41* alleles, while the heterozygous $Defb41^{iCre/+}$ 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-ingredient 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 7951 bp long) were subcloned into the pACYC177 minimal backbone vector using Red/ET recombination technology (Angrand et al., 1999). An *iCre*-neomycin phosphotransferase (neo^r) expression cassette was inserted into the translation initiation site of the *Defb41* gene. Mutated FRT sites were located around the $iCre\text{-}neo^r$ cassette to allow recombinase mediated cassette exchange if needed (Fig. 1A). All primer sequences used in cloning are available on request.

The targeting construct was electroporated into AB2.2 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: CGGTATGAAATAGTGCTCTGAACTT and DefbScrR1: ATTCTCCTTTCTGATTCTCCTCATC (Fig. 1A). The correct targeting was further confirmed by PCR over the 7951 bp 3'-homology arm using the following primers, Defb41 3′ Fw: TCAAACAAGACCCCGTACAA and Defb41 e2 Re: TGTGTGCATGGATGGAGATT. 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 $Defb41^{iCre/+}$ mice. Heterozygous hybrid males were backcrossed to C57Bl/6N for three generations and then bred with each other to obtain homozygous, *Defb41*-deficient, *iCre* KI mice, referred to as $Defb41^{iCre/iCre}$ mice in the text. $Defb41^{iCre/iCre}$ mice were genotyped as previously described (Björkgren et al., 2012). For the experiments the control wild type $Defb41^{+/+}$ mice and the $Defb41^{iCre/+}$ and $Defb41^{iCre/iCre}$ mice were all obtained from the same litters.

The histology of the $Defb41^{iCre/iCre}$ mouse epididymis was determined by dissecting the epididymides of $Defb41^{+/+}$ and $Defb41^{iCre/iCre}$ 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