RESEARCH ARTICLE

The epididymis-specific antimicrobial peptide *β*-defensin 15 is required for sperm motility and male fertility in the rat (*Rattus norvegicus*)

Yue Zhao · Hua Diao · Zimei Ni · Shuanggang Hu · Heguo Yu · Yonglian Zhang

Abstract Spermatozoa acquire forward motility and fertilizing capacity during their transit through the epididymis. The maturation process involves modifications of the sperm surface by different proteins that are secreted by a series of specialized regions in the epididymal epithelium. Here we show that the rat epididymis-specific *β*-defensin 15 (*Defb15*) exhibits an androgen-dependent expression pattern, and it can bind to the acrosomal region of caput sperm. Coculture of caput spermatozoa with Defb15 antibody in vitro resulted in a significant decline in sperm motility. Moreover, the total and progressive motility of the spermatozoa dramatically decreased in rats when *Defb15* was downregulated by lentivirus-mediated RNAi in vivo. Remarkably, knock down of *Defb15* led to a reduction in fertility and embryonic development failure. In addition, the recombinant Defb15 showed antimicrobial activity in a dose-dependent fashion. These results suggest that Defb15 plays a dual role in both sperm maturation and pathogen defense in rat epididymis.

Keywords *β*-Defensin · Epididymis · Sperm maturation · Sperm motility · Male fertility · Antimicrobial activity

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Introduction

Spermatozoa leaving the testes are incomplete as they can neither swim nor fertilize an egg. During their transit through the epididymis including the caput (head), corpus (body) and cauda (tail) regions, sperm undergo a series of physical, chemical and morphological alterations to acquire maturity [1–3]. The luminal fluid microenvironment is not only crucial for sperm maturation, but also protects the sperm against autoimmunity and other potentially hazardous factors such as bacteria. At present, some known and some putative host defense proteins produced and secreted by the epithelial cells of epididymides have been characterized in different mammalian species [4–12], and *β*-defensins are the best studied effector molecules.

The defensins are a family of cationic antimicrobial peptides that contribute to the host immune response [13]. They are classified as alpha, beta and theta defensins based on the spacing and pairing patterns of six conserved cysteine residues [14]. *β*-Defensins exhibit distinct antibacterial, antifungal and antiviral capacities against a wide variety of microorganisms [15–18]. Recent studies have also revealed that certain epididymal *β*-defensins are actively involved in sperm maturation and fertilization, physiological properties that are not related to host defense. We first reported that Spag11e/Bin1b, a rat epididymis-specific *β*-defensin, is not limited to the killing of bacteria but also initiates sperm

motility by inducing Ca^{2+} uptake [19]. Moreover, another epididymal secretory β-defensin 126 (DEFB126/ESP13.2) coats the entire surface of macaque sperm and may be an important decapacitation factor that needs to be released before sperm–zona interaction and fertilization [20, 21]. On the basis of the multifunctionality of β-defensins, this family has been attracting steadily growing interest.

In follow-up studies, the complete repertoires of mammalian β-defensins have been identified using genome-wide computational searches. To date, a total of 39 human, 52 mouse and 43 rat β-defensin genes have been discovered [10], and they form four or five syntenic clusters in the respective chromosomes [10, 22]. Surprisingly, almost all rat β-defensins have been found to be preferentially expressed in the male reproductive system, particularly in the testes and different regions of the epididymis [10]. Such a unique property implies that these molecules may constitute an essential component in maintaining the normal reproductive process. Therefore, further investigations of potential roles of β-defensins in the epididymis may shed new light on our understanding of sperm maturation and reveal the therapeutic implications of sperm abnormalities.

Recently, the epididymis of Sprague-Dawley rat was microdissected into 19 intraregional segments [23]. According to the expression levels of β-defensins in each individual section, *Defb15* was singled out among all family members for its higher relative abundance across the epididymis. The *Defb15* gene (NM_001037520) is located on chromosome 16q12.5, and it has 75% homology with human β-defensin 6 (DEFB106, NP_001035794) and 81% homology with mouse β-defensin 15 (mDefb15, NP_631968) at the protein level (Fig. S1). Although the caput region-specific expression pattern of *Defb15* mRNA has been examined by RT-PCR [10], little is known about the function of this protein. In our current study, the recombinant Defb15 protein was demonstrated to be a potent antimicrobial agent. Moreover, we prepared the sensitive and specific antibody against Defb15, and the location of Defb15 on spermatozoa and its role in maintaining sperm motility was characterized. Furthermore, by using RNAi in vivo, knock down of *Defb15* gene expression in the rat epididymis provided direct evidence that Defb15 is a novel and critical molecule in the regulation of sperm motility and male fertility.

Materials and methods

Animals

Healthy Sprague-Dawley rats and New Zealand white rabbits were supplied by the Animal Center of the Chinese Academy of Sciences (Shanghai, China). Animals were housed under standard laboratory conditions. Experiments were conducted according to a protocol approved by the Institute Animal Care Committee. The protocol conformed to internationally accepted guidelines for the humane care and use of laboratory animals.

Androgen regulation and developmental change of *Defb15*

Castration and androgen replacement were conducted following a previously described protocol [24]. For the developmental pattern analysis, the epididymides of normal male rats were sampled at the ages of 15, 30, 45, 60, 90, 120, 270, 360 and 720 days. The epididymides of each group were excised and mixed for real-time PCR analysis (Qiagen, Valencia, CA).

Production of antibody and immunoblotting of Defb15

To raise the specific antibody to Defb15, we adopted the double-copy protein expression scheme described by Xiao et al. [25] in detail. Two *Defb15* fragments corresponding to the mature Defb15 peptide of 59 amino acids (from amino acid 33 to the end) were inserted into pET-28(a) vector (Invitrogen). The recombinant protein was expressed and purified using a Novagen pET Expression Systems kit (Invitrogen). The polyclonal antisera against Defb15 were obtained and purified according to previous methods [26], and the preimmune sera were also purified as negative control following the same protocol. Western analysis, immunohistochemical staining of tissues and indirect immunofluorescence detection of proteins associated with spermatozoa were conducted following a previously described protocol [24]. Lectin peanut agglutinin (PNA) conjugated with Alexa Fluor 488 (diluted 1:500; Molecular Probes) was used to monitor the acrosomes of the spermatozoa.

Cell culture and siRNA transfection

Two potential siRNAs of 21 nucleotides (nt) specific to *Defb15* mRNA (15si1 and 15si2) were selected empirically with a design tool incorporating standard parameters [27], and siRNA targeting enhanced green fluorescent protein (EGFP) was used as control for sequence specificity (Csi). The sense strands of siRNAs were: 15si1 (sequence 1), beginning at nt 124, 5′-AATGGGAGATGCACAGA ATCT-3′; 15si2 (sequence 2), beginning at nt 226, 5′-AA GGGCGACGAGTTGGATGAA-3′; and Csi, beginning at nt 106, 5′-GGCGATGCCACCTACGGCAAG-3′. To examine the silencing effect of siRNAs, psiRNA vectors producing shRNAs targeting *Defb15* were constructed according to the kit instructions (Invivogen), then the

psiRNA constructs and a pcDNA 3.0 vector expressing *Defb15* (mass ratio 1:5) were cotransfected into the mouse epididymal epithelial cells using Lipofectamine 2000 (Invitrogen). The knock-down efficiency was determined by real-time PCR and western blotting of extracts from transfected cultures 48 h after transfection.

Lentiviral vector production and injection

Lentiviral particles were produced by transient cotransfection of 293T cells by pRNAi/Lenti vectors (HaiGene, Harbin, China), an encapsidation plasmid (p8.9), and a vesicular stomatitis virus (VSV) expression plasmid as previously described [28, 29]. The surgical procedures were performed according to a previously described method with modification [30]. Briefly, 1×10^6 transducing units of shRNA lentivirus (the viral titer was 4×10^7 transducing units per milliliter) was injected into the interstitial space of the caput epididymis, *Defb15*-shRNAs lentivirus (15si1 and 15si2) were delivered into the left epididymis and the right epididymis was treated with EGFP-shRNA lentivirus (Csi). The total RNA and protein of the caput epididymis and the spermatozoa were collected for assessment 7 days after injection.

Sperm motility analysis

We examined the sperm motility in the epididymis by computer-assisted semen analysis using sperm motility analyzer (HTM-TOXIVOS; Rat Head Toxicology, version 12.3A; Hamilton-Thorn Research, MA) [30].

Evaluation of sperm capacitation and the acrosome reaction

We applied the protocol of protein tyrosine phosphorylation and the chlortetracycline (CTC) staining assay as used in previous studies [30, 31] to assess sperm capacitation and spontaneous acrosome reaction. For the induction of acrosome reaction, spermatozoa were preincubated in the capacitation medium for 5 h, exposed to 15 μM progesterone or its solvent (DMSO; maximal concentration 1%) as control; and further incubated for 30 min. At the end of the incubations, samples were stained with CTC to assess the acrosome reaction rate.

Fertility assay

We carried out in vitro fertilization (IVF) following a standard protocol [32]. After a 48-h incubation, eggs were examined for the presence of two-cell-stage embryos as an indication of successful fertilization. The mating experiments were performed following our modified protocol

[30]. Each male rat was mated with two normal females in succession 7 days after injection of the same lentivirus into the bilateral epididymides (the treated group was injected with *Defb15*-shRNA lentivirus; the control group with EGFP-shRNA lentivirus). The mated male rats were sampled for determining the knock-down efficiency. At 18 days the pregnant females were subjected to hysterectomy to determine the number of embryos or fetuses. After birth, the number, viability and size of the offspring in a litter were recorded.

Soluble recombinant β-defensin production and antibacterial assay

An intein-mediated expression and purification system was used to produce the recombinant β-defensins [33]. The expression plasmids were constructed by insertion of putative sequences of mature β-defensins into the pTWIN1 vector (NEB) between *NcoI/XhoI* sites. Protein expression was induced with 0.5 m*M* IPTG at 22°C for 6 h, and the optimal pH for cleavage was 5.0. Colony-forming unit (CFU) assays were employed to test the antibacterial activity as described previously [33, 34]. The elution buffer of the recombinant peptides with the same volume and pH was used as control in the antimicrobial tests.

Statistical analysis

Statistical analysis was performed using the SigmaPlot 10.0. *P* values were calculated using the standard *t*-test, and differences with a *P* value <0.05 were considered statistically significant.

Results

Characteristics of *Defb15* mRNA expression

Real-time PCR analysis revealed intense expression of *Defb15* mRNA in the caput epididymis (Fig. 1a), which is consistent with a previous report [10]. Monitoring Defb15 expression throughout the life-span of rats indicated that it was developmentally regulated (Fig. 1b). Its expression started at 15 days of age, reached a peak at 1 month, remained at a stable level in mature animals, and increased dramatically in aged rats. Moreover, the expression of *Defb15* mRNA was upregulated by androgen in vivo as are some epididymis-specific genes reported previously [34, 35]. In castrated animals, the abundance of *Defb15* mRNA in the epididymis decreased as serum testosterone levels declined rapidly. Testosterone replacement in the animals 7 days after castration resulted in a rapid increase in *Defb15* mRNA levels (Fig. 1c).
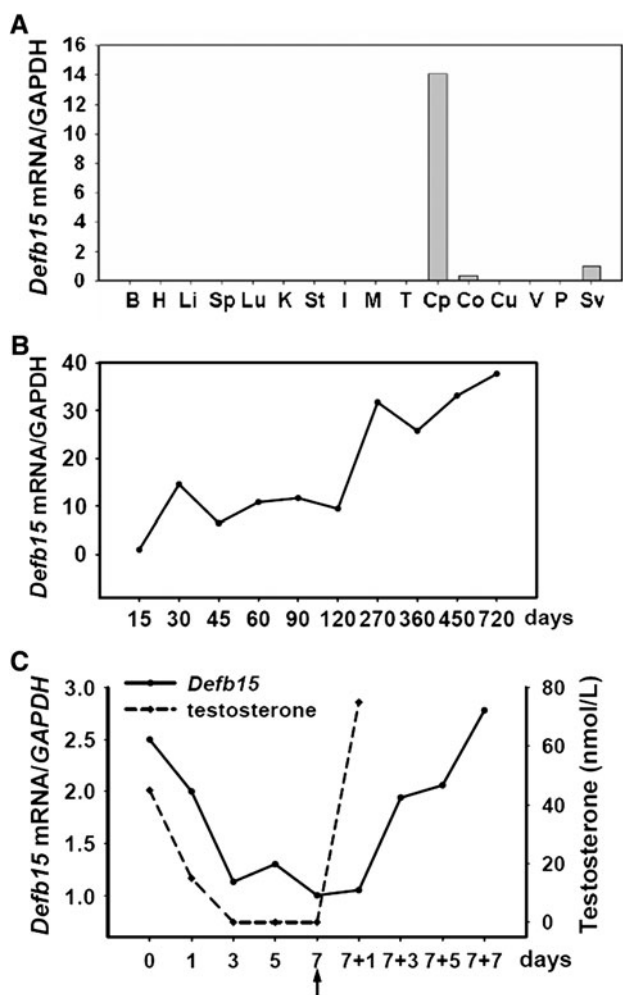
Fig. 1 Expression and regulation of *Defb15* mRNA. **a** Real-time PCR analysis of *Defb15* gene expression in various tissues, including the brain (*B*), heart (*H*), liver (*Li*), spleen (*Sp*), lung (*Lu*), kidney (*K*), stomach (*St*), intestine (*I*), muscle (*M*), testes (*T*), caput (*Cp*), corpus (*Co*), cauda (*Cu*), vas deferens (*V*), prostate (*P*), and seminal vesicle (*Sv*). **b** Relative amounts of *Defb15* mRNA (real-time PCR) in rat epididymis at different developmental stages ($n = 3$). **c** Relative expression levels of *Defb15* mRNA (real-time PCR) in the rat epididymis and the serum testosterone levels (expressed as nanomoles per liter) during androgen manipulation ($n = 4–7$). The *black arrow* indicates the time of androgen supplementation. GAPDH was used as internal control in **a–c**

Native status and localization of Defb15 protein in rat epididymis

With the aid of the sensitive and specific polyclonal antibody against mature Defb15 protein (Fig. 2a), a distinct single band of approximately 8 kDa was detected in the caput but not in other portions of the epididymis or other tissues tested (Fig. 2b). Immunohistochemistry further confirmed that the positive signal was confined to the distal caput epididymis and was also present in the lumen (Fig. 2c, d). We next investigated whether Defb15 protein

could interact with spermatozoa since it was subsequently secreted into the luminal fluid. Defb15 immunoreactivity was only found in the caput sperm (Fig. 2e), and indirect immunofluorescence staining further showed that the prominent signals of Defb15 sperm binding were concentrated in the acrosomal region of the caput sperm surface (Fig. 2f, g).

Defb15 protein is involved in maintaining sperm motility in vitro

The caput region-specific localization and sperm-binding pattern of Defb15 indicate its importance in certain sperm functions during epididymal transit, since the microenvironment of the caput epididymis has been shown to be vital for sperm maturation [36]. We tested the possible role of Defb15 in sperm motility by incubating spermatozoa collected from the distal caput epididymis with the antibody in vitro. To rule out nonspecific interferences, preimmune serum and rabbit polyclonal antibody against Glb1l4 protein specifically expressed in the caput region and having no influence on sperm motility [26] were used as controls. As shown in Fig. 3a, sperm motility of samples was greatly attenuated in the presence of Defb15 antibody, whereas none of the control treatments had any effect on the spermatozoa. Moreover, we found an almost complete loss of Defb15 protein from the sperm incubated with the specific antibody for 3 h (Fig. 3b). The indirect immunofluorescence staining assay further indicated that the acrosomes of antibody-treated spermatozoa were still intact even though the signals of Defb15 had disappeared (Fig. 3c). These results suggest that the lack of Defb15 protein in the caput sperm was associated with a significant decline in sperm motility. We thus consider that Defb15 may be essential in maintaining sperm motility at the caput region of the epididymis where it binds to the sperm.

Defb15 contributes to sperm motility in vivo

RNAi has been widely applied as a powerful technique for gene silencing in functional studies. Given the advantages over oligonucleotide siRNA, including high transduction efficiency and a stable downregulation of particular genes, we applied lentiviral vector-mediated RNAi to knock down *Defb15* gene expression in rat epididymis to confirm its role in the sperm maturation process. According to the RNAi mechanism-based rules [27], two potential siRNAs (15si1 and 15si2) targeting the different site of *Defb15* gene were designed and a siRNA (Csi) of EGFP was used as control for silencing specificity. Figure 4a shows that both 15si1 and 15si2 were able to inhibit Defb15 expression almost completely at the mRNA and protein levels in the mouse epididymal epithelial cell line. Lentiviral
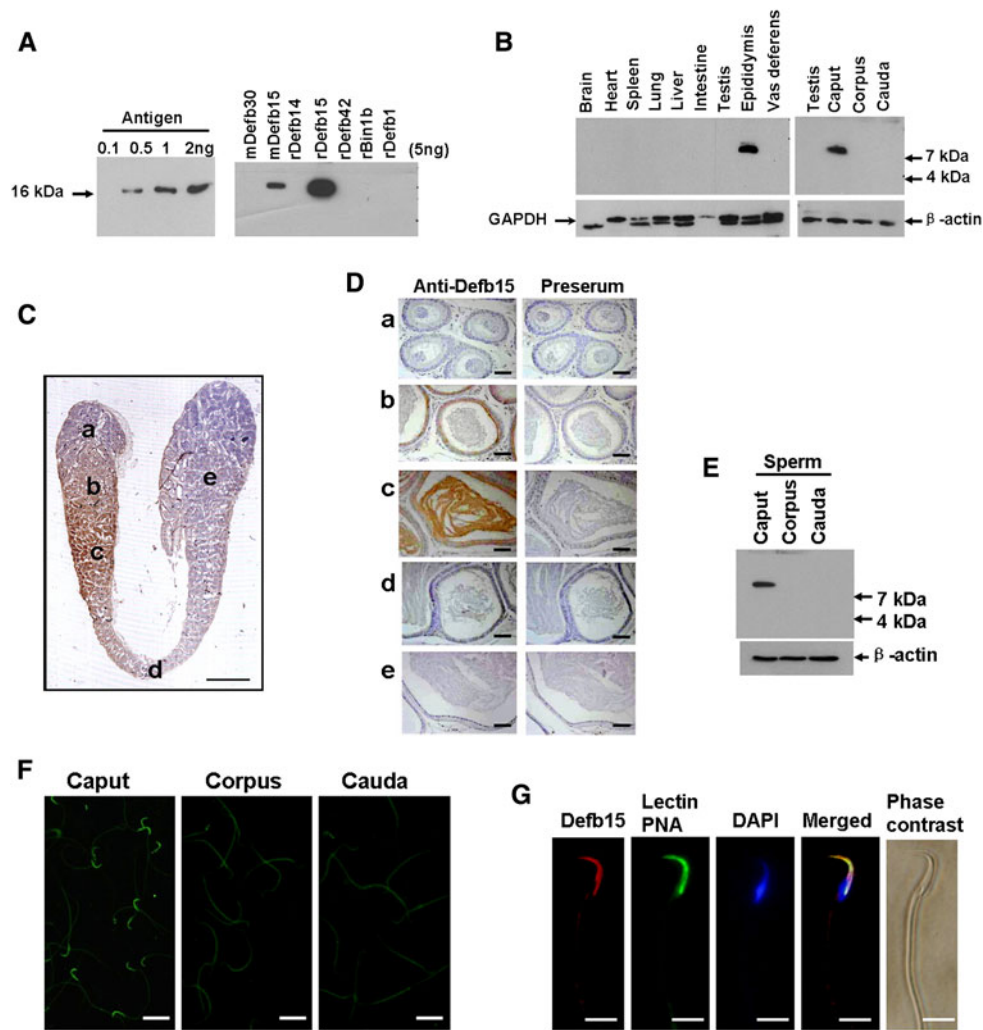
Fig. 2 Localization of Defb15 protein in rat epididymis. a The sensitivity and specificity of rabbit polyclonal antibody towards Defb15 were verified by western blotting. b Protein extracts from various tissues were analyzed by western blotting analysis. GAPDH and β-actin were used to assess protein loading. c Immunohistochemical staining shows the expression pattern of Defb15 protein in the whole epididymis in a 120-day-old rat (bar 4 mm). d Magnified photographs of individual fields of c: a initial segment; b, c caput; d corpus; e cauda. Preimmune serum under the same conditions showed the background level of immunoreactivity (bars 50 μm). e Western blot analysis of Defb15 protein in total protein extracts of sperm from the caput, corpus and cauda regions of the epididymis. β-Actin was used for loading control. f Localization of Defb15 protein (FITC-labeled) on the spermatozoa by indirect immunofluorescence assays. Positive Defb15 immunoreactivity is seen at the head of sperm from the caput region, but no signal is seen on sperm from the corpus or cauda regions of the epididymis (bars 10 μm). g Refined localization of Defb15 (TRITC-labeled) on the caput sperm surface. The acrosome of the sperm was stained by Alexa Fluor 488-conjugated lectin PNA, and sperm DNA was stained with DAPI (bars 5 μm)

particles which generated the effective shRNAs were produced and locally injected into the caput epididymides. As shown in Fig. 4b, expression of the *Defb15* gene in the caput epididymis was suppressed by about 50–60% at the mRNA and protein levels after 7 days by specific siRNAs treatment. Moreover, western blot analysis indicated a nearly 60% decrease in the Defb15 protein bound to the caput sperm in the *Defb15* siRNA-treated male rats (Fig. 4c). Indirect immunofluorescence staining also confirmed the absence of Defb15 signals in partial caput spermatozoa (Fig. 4d). As expected, the reduction in

Defb15 expression and sperm binding led to significant declines in total and progressive sperm motility both in caput and cauda spermatozoa (Fig. 4e, f). These results confirm that Defb15 plays a crucial role in maintaining sperm motility.

Knock down of *Defb15* expression affects the sperm–egg interaction

Capacitation represents the completion of sperm maturation that confers on mammalian sperm fertilization
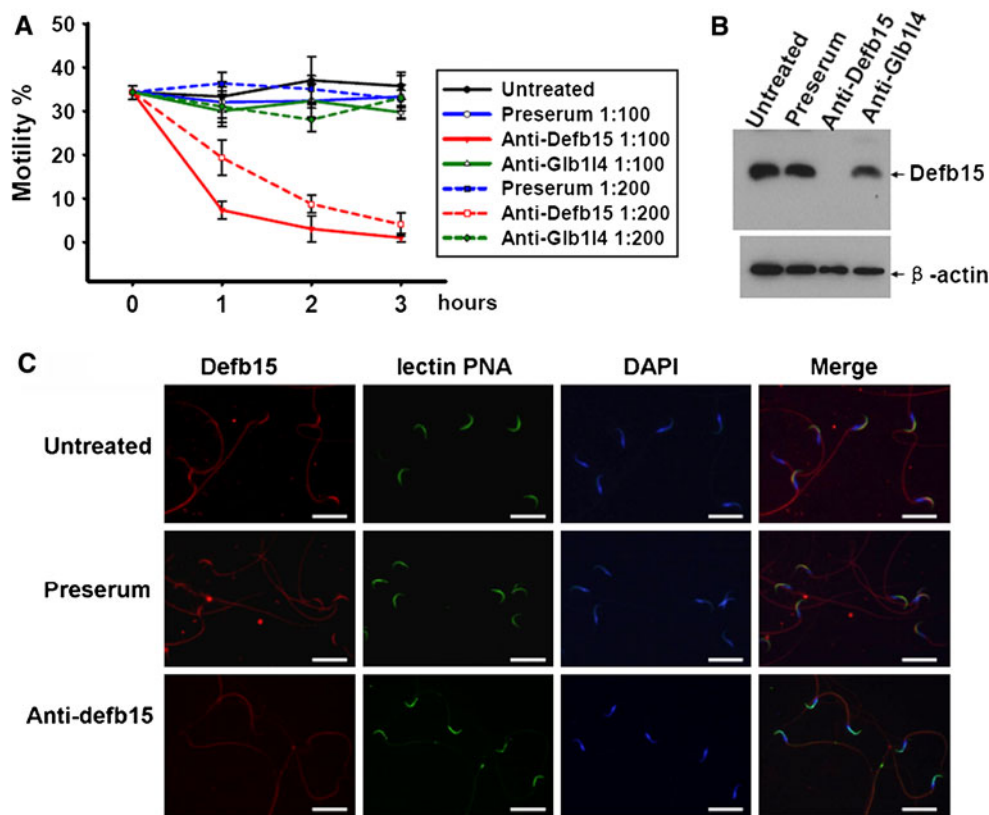
Fig. 3 The effect of Defb15 antibody on sperm motility. **a** Changes in the sperm motility. The treatments with different concentrations of preimmune serum (*Preserum*), Defb15 antibody (*Anti-Defb15*), and Glb1l4 antibody (*Anti-Glb1l4*) were conducted at the time of dilution (0 h), and aliquots of spermatozoa were then taken for recording of motility. The experiment was independently replicated four times. Values are mean $\pm$ SEM. **b** Western blot analysis of Defb15 protein in total protein extracts of caput epididymal sperm with different treatments. β-Actin was used as the internal control. **c** Immunofluorescence staining of sperm from the caput epididymis treated with preimmune serum and Defb15 antibody. The acrosome of the sperm was stained by Alexa Fluor 488-conjugated lectin PNA, and sperm DNA was stained with DAPI (*bars* 10 μm)

competence. It is widely accepted that tyrosine phosphorylation of sperm proteins is an important indicator of whether the capacitation signaling cascade has been activated [37]. There was no obvious dissimilarity in the protein tyrosine phosphorylation of caudal sperm from *Defb15* siRNA-treated and control rats (Fig. 5a). This is consistent with the observation with CTC staining (Fig. 5c), another indicator for evaluating the acquisition of capacitation state. Additionally, the acrosome reaction rates were further determined by the CTC assay. There was no marked difference in the CTC staining patterns displayed by spermatozoa undergoing spontaneous and progesterone-stimulated acrosome reactions between control and treated groups (Fig. 5d; Fig. S2). Although the knock down of *Defb15* might have no influence on sperm capacitation and the acrosome reactions, IVF assay showed that the fertilization rate of the cauda sperm in *Defb15* siRNA-treated rats was much lower than that of the control groups (Fig. 5e).

Reduced fertility of *Defb15* gene knock-down rats

Finally, we performed mating experiments to evaluate the fertility of *Defb15* downregulated male rats. As shown in Fig. 6b, the numbers of live offspring and normal fetuses were considerably reduced in the litters from receptive female rats that had mated with specific siRNAs-treated male rats, compared with the numbers in the normal and control groups. Moreover, we observed some abnormal fetuses in the treated groups; Fig. 6c shows a representative example. All 17 embryos implanted in the uterus of the female rat mated with the control male rat were normal (Fig. 6c, *a*), whereas five fetuses appeared to be regressing in *Defb15* siRNA-treated group, indicating embryonic development failure (Fig. 6c, *c*). These data confirm that Defb15 is essential in the sperm maturation process, and the sperm lacking Defb15 binding in the caput region seems to be the cause of fertility reduction and embryonic development failure.
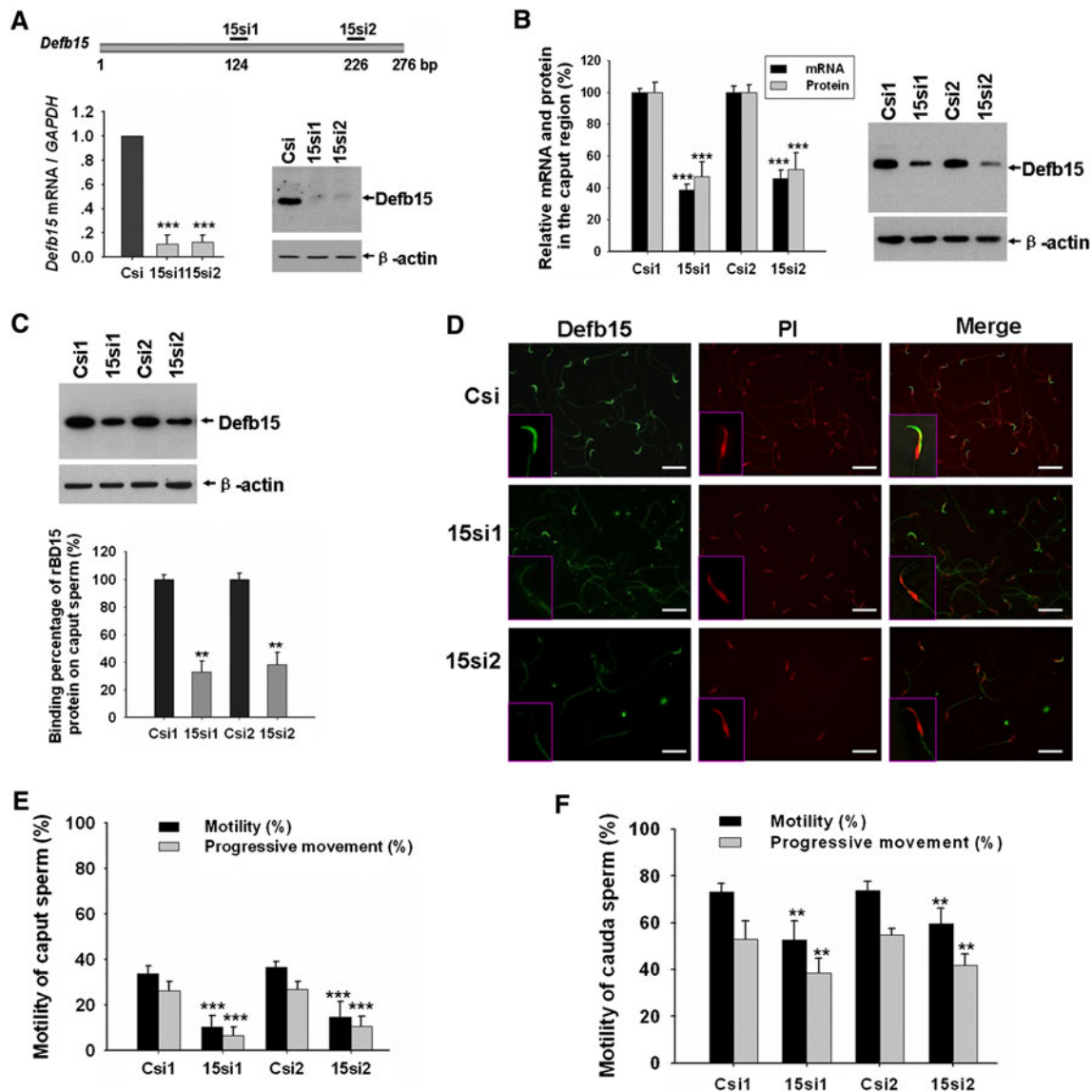
Fig. 4 Knock down of *Defb15* expression by RNAi and its effect on sperm motility. **a** Real-time PCR and western blot analysis show reduced expression of Defb15 mRNA and protein in vitro 48 h after siRNA treatment. Data are expressed as mean ± SEM ($n = 3$; ***$P < 0.001$ vs. control). GAPDH and β-actin were used as the internal control for RNA and protein loading. **b** Expression of Defb15 was significantly suppressed by 15si1 and 15si2 in the caput region of the rat epididymis at the mRNA and protein levels. Data are expressed as mean ± SEM ($n = 16$–18; ***$P < 0.001$ vs. control). GAPDH and β-actin were used as a loading controls. **c** Corresponding binding percentages obtained by western blot analysis. Data are expressed as mean ± SEM ($n = 12$; **$P < 0.01$ vs. control). β-Actin was used as the internal control. **d** Immunofluorescence staining of sperm from the caput epididymis treated with control siRNA and two specific siRNAs (*bars* 10 μm). **e, f** Total and progressive motility of spermatozoa in the caput (**e**) and cauda region (**f**) after *Defb15* expression had been downregulated. Data are expressed as mean ± SEM ($n = 20$–24; ***$P < 0.001$, **$P < 0.01$ vs. control). In **a**–**f**: *Csi* (Csi1 and Csi2), EGFP siRNA control; *15si1* and *15si2*, two siRNAs specifically targeting the different sites of *Defb15* sequence

Antimicrobial activity of recombinant Defb15 protein

The antimicrobial properties of β-defensins have been well documented and reviewed [16]. To determine whether Defb15 also possesses microbicidal activity, we prepared some recombinant β-defensin peptides, including Defb15, Defb14 (NP_001032593.1), Bin1b [32] and DEFB106, using an intein-mediated expression and purification system (Fig. S3A). Circular dichroism spectra indicated that they all exhibited certain secondary structures (Fig. S3B). Figure 7a–c shows the antimicrobial activities of these recombinant proteins against three pathogens. By comparison, Defb15 tended to be more active against *Escherichia coli* and *Candida albicans* than against
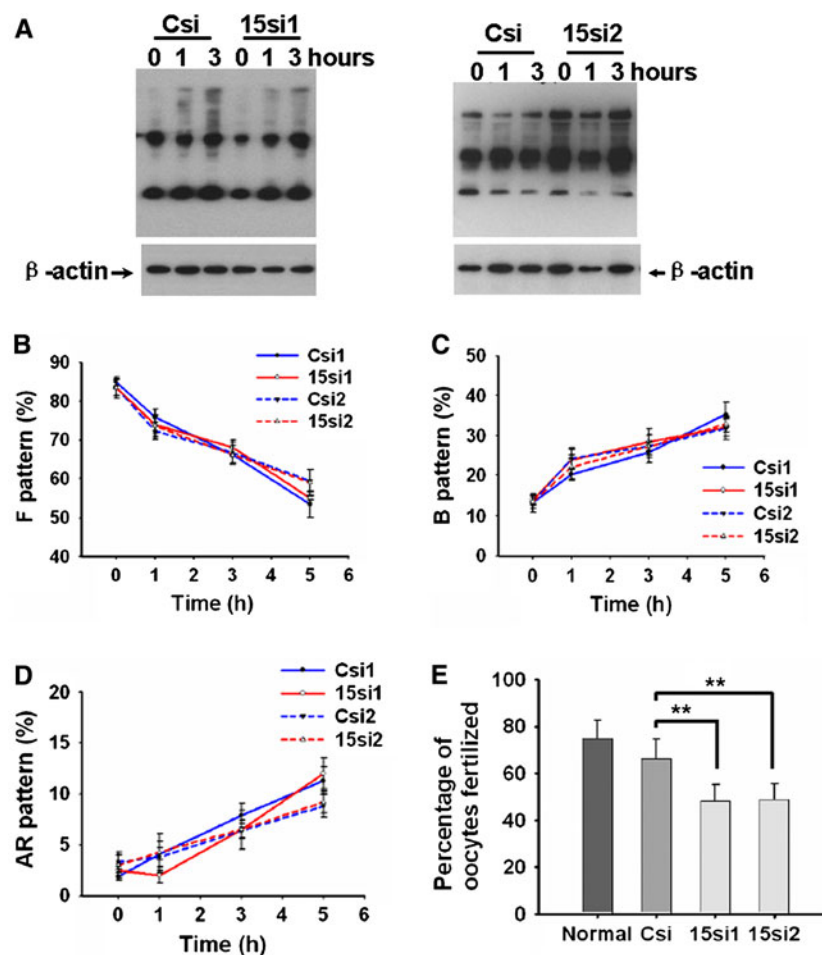
Fig. 5 Assessment of sperm capacitation, acrosome reaction and IVF of *Defb15* knock-down male rats. The spermatozoa of the caudal epididymis from rats treated with siRNAs for 7 days were collected and incubated. Aliquots of spermatozoa were collected for protein tyrosine phosphorylation and CTC assays at different time points during incubation. **a** The pattern of protein tyrosine phosphorylation on caudal sperm after *Defb15* expression is inhibited. *β*-Actin was used as loading control. **b–d** The changes in the sperm state of uncapacitated sperm (*F pattern*, **b**), capacitated sperm (*B pattern*, **c**), and spontaneous acrosome-reacted sperm (*AR pattern*, **d**) in the *Defb15* siRNA-treated and control groups. The experiment was independently replicated three times. Values are mean ± SEM. **e** IVF assay of the sperm in *Defb15* knock-down rats. Data are expressed as mean ± SEM ($n = 6$; **$P < 0.01$ vs. corresponding control). In **a–e**: *Csi* (Csi1 and Csi2), EGFP siRNA control; *15si1* and *15si2*, two siRNAs specifically targeting the different sites of *Defb15* sequence

Staphylococcus aureus; whereas DEFB106, the human orthologue of Defb15, exhibited more powerful resistance to *S. aureus*.

Discussion

Although the role of the epididymis in sperm maturation has been firmly established for nearly four decades [1, 3], the molecular basis for this process remains largely unknown. Our studies identified Defb15 as a new molecule directly linked to sperm maturation. Defb15 is a rat caput region-specific peptide with a homologue in humans (DEFB106), and it can bind in a secreted form to the caput sperm acrosome. In *Defb15* gene knock-down male rats,

the reduced binding of Defb15 protein to caput sperm led to a considerable attenuation of sperm motility. Our previous study has demonstrated that Bin1b, another caput region-specific *β*-defensin, is crucial in inducing sperm motility [19]. Taking into account the potential interaction among *β*-defensin members, we questioned whether the loss of sperm motility was entirely due to the effect of Defb15. When *Defb15* was notably downregulated, there were no significant changes in the mRNA level of 11 *β*-defensins expressed predominantly in the caput epididymis [10] and the sperm-binding pattern of Bin1b protein (Fig. S4). Thus we excluded the involvement of other subfamily members in maintaining the sperm motility.

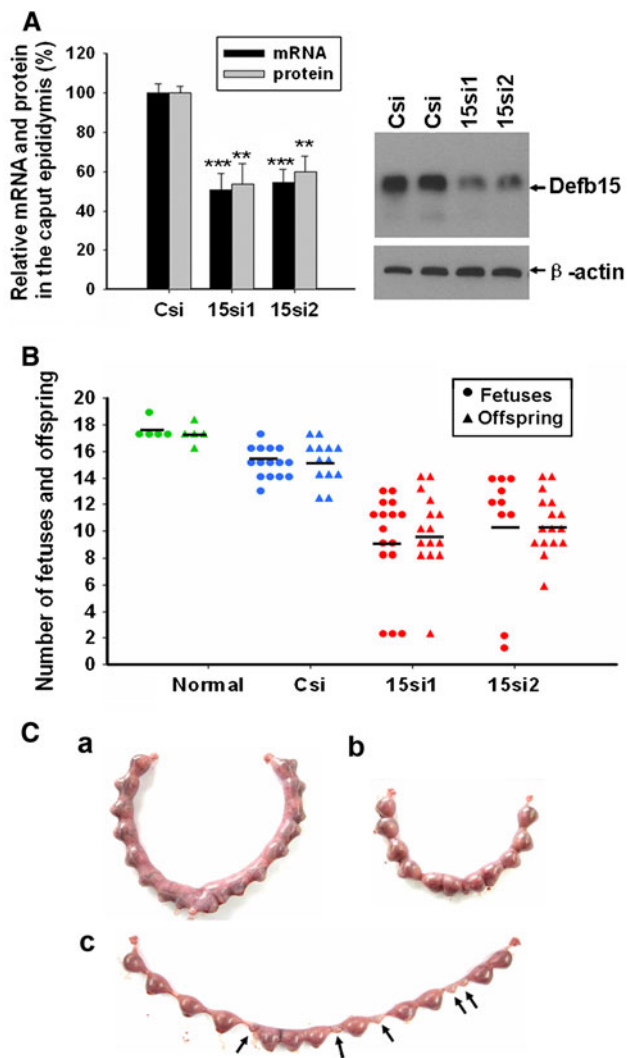Moreover, our results showed that Defb15 binds to the sperm exclusively in the caput region. Despite the absence

Fig. 6 Fertility of *Defb15* knock-down male rats. **a** Relative expression levels of Defb15 including mRNA and protein in the caput region of the epididymis from all successfully mated rats after RNAi treatment. Data are expressed as the mean ± SEM (n = 12–16; ***$P < 0.001$, **$P < 0.01$ vs. corresponding control). *Csi*, EGFP siRNA control; *15si1* and *15si2*, two siRNAs specifically targeting the different sites of *Defb15* sequence. **b** Numbers of normal fetuses (*circles*) and live offspring (*triangles*) from normal female rats mated with normal, control (*Csi*) and knock-down (*15si1*, *15si2*) male rats; *horizontal lines* indicate the averages. **c** Examples of implantation in the control and *Defb15* siRNA-treated groups. There are 17 and 11 normal embryos in the control (*a*) and treated (*b*) groups, respectively. Abnormal embryos (*c*, *arrows*) appeared in the uterus of a female rat mated with a *Defb15* siRNA-treated male rat

of Defb15 protein, the total and progressive motility of caudal sperm in *Defb15* siRNA-treated male rats was also decreased (Fig. 4f), but it seemed less significant than in caput sperm ($P < 0.01$ in caudal sperm, $P < 0.001$ in caput sperm). Based on the previous research, the essential elements of sperm maturation may be "setting a series of triggers" for a sequence of cellular changes, and also "setting a safety" for each trigger to prevent inappropriate

occurrence of the event [36]. Accordingly, we developed the following hypothesis that a series of molecules are involved in the achievement of sperm motility in the epididymis, and they form a long distance relay. It might be conceivable that some other proteins secreted by the epithelia of the corpus and cauda regions modulate the spermatozoa in succession to gain complete motility. As a result, the motility defect in *Defb15* siRNA-treated rats could be partially rescued when poorly motile spermatozoa in the caput travel through the corpus and cauda epididymis. Further studies are needed to elucidate the molecular mechanisms by which Defb15 maintains sperm motility. Examination of target proteins of Defb15 in the sperm and related signaling pathways may contribute to addressing this issue.

Capacitation is a unique feature of mammalian sperm that occurs in the female tract and is essential for fertilization. During capacitation, spermatozoa acquire hyperactive motility and the ability to undergo a regulated acrosome reaction. Despite the fact that Defb15 has no effect on sperm capacitation and acrosome reaction (Fig. 5), results of IVF and mating experiments indicated a reduced fertilization rate and fertility in *Defb15* gene knock-down rats. This supports the importance of Defb15 in maintaining normal fertilization. Since sperm motility facilitates the passage of sperm through the zona pellucida, it can be thought of as an indispensable attribute of the sperm in achieving a successful pregnancy. The decrease in progressive motility in caudal sperm may interfere with their ability to fertilize oocytes and appears to be the cause of the fertility reduction. Remarkably, embryonic development failure was observed in *Defb15* siRNA-treated groups (Fig. 6c). We speculated that knock down of *Defb15* might cause some changes in the essential attributes of spermatozoa besides the forward motility, and these changes to some extent influence fertility and developmental potential of embryos. It has been reported that the degree of DNA damage is clearly correlated with the impairment of embryo development and severe DNA damage causes male infertility [38, 39]. We tested whether knock down of *Defb15* induced an inflammatory reaction in the epididymis and sperm DNA damage. As revealed in Fig. S5, downregulation of *Defb15* expression in vivo did not cause changes in the expression of several inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6 and IFN-γ) or the extent of spermatozoal DNA damage. We are trying to investigate other changes in sperm quality in *Defb15* knock-down rats which might account for reduced fertility and abnormal fetuses.

Other questions arise from our data related to the effect of Defb15 on sperm motility. In spite of the reduced proportion of motile sperm, no statistically significant changes in other sperm motion variables were observed in *Defb15*
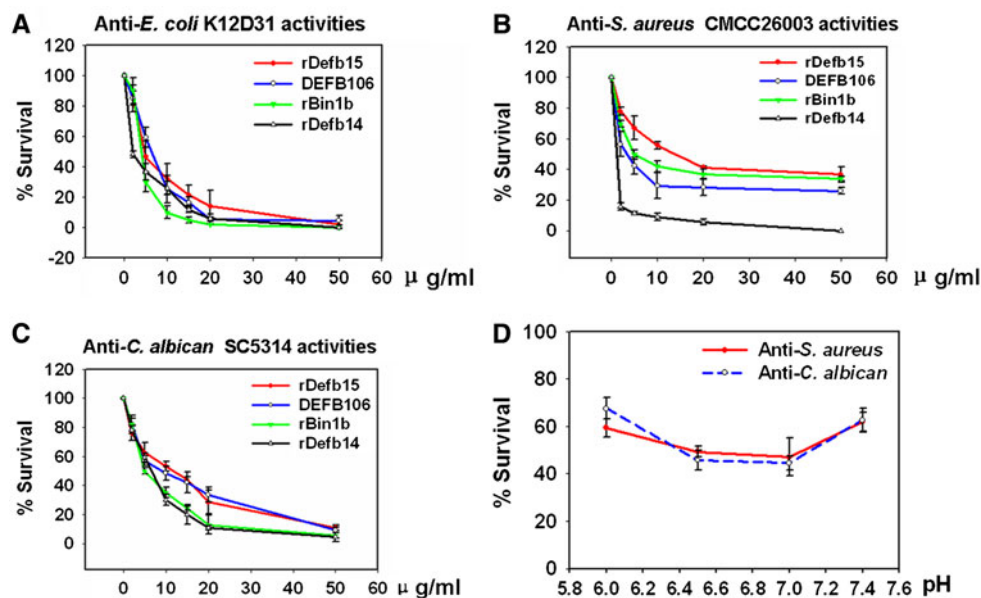
Fig. 7 Antimicrobial activities of recombinant β-defensins. **a–c** Dose effects of recombinant β-defensins on antimicrobial activities. *E. coli* K12D31 (**a**), *S. aureus* CMCC26003 (**b**) and *C. albicans* sc5314 (**c**) were incubated in 10 m*M* phosphate buffer (pH 7.4) in the presence of recombinant peptides for 2 h (*E. coli* and *S. aureus* at 37°C; *C. albicans* at 30°C). Incubation with the same volume of elution buffer was used as control in the antimicrobial tests.

d Antimicrobial activities of Defb15 at various pH values (pH 6.0, 6.5, 7.0 and 7.5). Incubation with the elution buffer of recombinant Defb15 with the same volume and pH value was used as control. The number of CFU was determined by colony counts after serial dilutions. The experiments were independently replicated four times. Values are mean ± SEM

siRNA-treated rats (data not shown). This suggests that the velocity and movement pattern of motile sperm are not affected when *Defb15* is downregulated. Moreover, successful knock-down of about 50% in *Defb15* gene expression in vivo led to distinct sperm-binding patterns of Defb15 protein (Fig. 4d). Some spermatozoa were not attached to Defb15 protein, while others were still bound to Defb15. These differences may be associated with various phenotypes in the subsequent studies. However, it is difficult to separate these varied types of sperm to investigate their individual movement pattern and their roles in fertilization. Hence, our future work will concentrate on the generation of *Defb15* conditional knock-out mice to reveal the phenotype associated with those spermatozoa with no Defb15 binding.

At present, overwhelming evidence is accumulating that a number of epididymal β-defensins function as important effectors by providing a rapid and broad response to invading microorganisms [5–7, 11, 16, 33, 34]. Based on our results, an additional function of Defb15 can be envisioned in sperm protection since the recombinant Defb15 peptide possessed distinct and dose-dependent activity against *E. coli*, *S. aureus* and *C. albicans*. It is well known that the pH value and salt concentration of epididymal luminal fluid are lower than in the physiological environment [40, 41], and many studies

have shown that most β-defensins lose their activity at higher sodium concentrations [6, 15, 33]. To explore whether β-defensin activity is also correlated with the degree of acidity, we examined the capacity of Defb15 to inhibit *S. aureus* and *C. albicans* at various pH values. In accordance with the localization in distal caput region, Defb15 exerted a more powerful effect at pH 6.5 and 7.0 (Fig. 7f). We are now trying to express and characterize more β-defensins of rat epididymal origin. Antimicrobial activity analyses of these peptides under simulated in vivo conditions will reveal their roles in innate immunity. There may also exist synergistic or additive effects among β-defensins clustered in the epididymis [42]. Different combinations of these agents may provide further evidence of their collaboration in microbial killing. A strategy to mimic or enhance their activities may be developed to address the increasing need for new therapeutic agents for sexually transmitted diseases.

Collectively, within the epididymis, Defb15 not only acts as a component of the immune system, but is clearly involved in the sperm maturation process by maintaining the motility of caput sperm. Determination of the molecular events by which relatively immotile quiescent sperm become incapable of fertilization may aid in the development of treatments for motility-related clinical infertility and the development of improved contraceptives.

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