Importance of β-defensins in sperm function

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Submitted on May 14, 2014; resubmitted on June 17, 2014; accepted on June 27, 2014

Abstract: Recent work in humans and mouse has confirmed the involvement of the host defence β-defensin peptides in male fertility. We discuss here the work that has implicated β-defensins in sperm function including the identification of the epididymis as the predominant site of expression of the peptides and the in vivo consequences of mutation and deletion. The potential dual role of these peptides in the regulation of infection and control of sperm maturation is compelling and may combine their antimicrobial activity with the ability of these molecules to interact with cell membrane receptors and modulate ion transport.

Key words: β-defensins / sperm / defensin / acrosome reaction / infertility

Introduction

Defensins are host defence peptides that are cationic and have a canonical series of cysteines held in a classic antiparallel β-sheet structure stabilized by three intramolecular disulphide bonds. In humans there are two classes of defensins, α-defensins and β-defensins, distinguished by their pattern of cysteine spacing and disulphide bond arrangement. α-Defensins are present at high levels in the granules of either neutrophils or paneth cells in the small intestine, although expression has also been detected in male reproductive tract (Com et al., 2003). β-Defensins are inducible in response to various inflammatory signals at mucosal surface epithelia.

In mammals, it is generally believed that mature β-defensin molecules are secreted host defence peptides with antimicrobial activity and their potent ex vivo ability to kill bacteria, fungi and enveloped and non-enveloped viruses supports this assumption. Consistent with this, knockout mice for Defb1 [the clear orthologue of DEFB1 (coding for the human β-defensin 1, hBD1, peptide in humans)] have increased Staphylococcus species in the bladder and a reduced ability to clear Haemophilus influenzae when introduced into the airway (Morrison et al., 2002; Moser et al., 2002). Increased expression of SPAG11/Bin1b in mice results in enhanced resistance to bacterial epididymal infection (Fei et al., 2012). Additionally, β-defensins have been found to have antiviral activities including an activity against HIV (Wilson et al., 2013). In addition to their antimicrobial properties, β-defensins have other diverse functions including actions as immune modulators, with the capacity for immune cell attraction and with anti-inflammatory and proinflammatory effects. In addition, they appear to be promiscuous ligands for a variety of receptors; for example, human β-defensin 3 (hBD3) has been shown to interact with chemokine receptors CCR6 and CCR2 and melanocortin receptors (Semple and Dorin, 2012).

β-Defensins are present throughout evolution and homologues have been described, for example, in platypus, birds and fish (Zou et al., 2007; Whittington et al., 2008a; Hellgren and Ekblom, 2010). In platypus, some β-defensin genes have adapted to become venom peptides but these are evolutionarily distinct from reptilian venom genes, although the peptides have a similar structure, implying convergent evolution (Whittington et al., 2008a). The β-defensin gene clusters found in many species arise by gene duplication and are subject to complex evolutionary pressures. In the rodent lineage, there is strong positive selection pressure following the birth of novel, rodent-specific beta-defensin genes. In humans, there are over 40 β-defensin genes at five different loci with the main cluster on chromosome 8p23.1 (Patil et al., 2005) and in the primate lineage, there are episodes of both negative and, more rarely, positive selection (Semple et al., 2005). Of interest is the fact that the 8p23.1 locus in humans contains six β-defensin genes that are highly copy number variable and it is intriguing to think that variation in copy number may have effect on phenotype. The average copy number of this cluster in the population is 4, but some individuals have up to 12 copies, while others have only 1 copy (Hollox et al., 2003). It has been shown that individuals with an increased copy number have an increased risk of the autoimmune immune disease, psoriasis (Hollox et al., 2008).

Despite the identification in the genome of many β-defensin genes, determination of their main in vivo functions has remained elusive. Functional redundancy, the likelihood of very specialized gene-specific function and species-specific functional specialization remain challenges for using single gene knockouts in the mouse and identifying phenotype. Multi-copy gene families are most commonly found genes involved in
immunity and/or reproduction. Recent work has revealed that the dominant site of expression of β-defensins and defensin-like peptides is in reproductive organs particularly in the male (Yamaguchi et al., 2002; Zaballos et al., 2004; Zhou et al., 2004, 2013; Patil et al., 2005; Whittington et al., 2008b; Jin et al., 2010; http://mrgd.org/index.cgi). In mammals, the main site of β-defensin expression is the epididymis and secretion from here is believed to result in their detection on the plasma membrane of sperm (Zhou et al., 2004; Yudin et al., 2005; Zhao et al., 2011). Indeed even more evolutionarily distant animals have β-defensin expression in the reproductive tract, e.g. fish and platypus (Whittington et al., 2008b; Jin et al., 2010). A reproductive function has been demonstrated for defensin-like peptides in the plant Torenia fournieri and these LUREs have been characterized as pollen tube attractants secreted from the synergid cells on the side of the egg cell (Okuda et al., 2009). In mammals, it seems likely that β-defensins are involved in reproductive functioning and here we consider the growing number of studies suggesting that β-defensins have an influence on sperm function.

**Defensins affect sperm function in rodents**

The first study that reported a defensin peptide with epididymal expression was carried out in the rat (Li et al., 2001). The peptide Bin1b (Spag11a), one of the alternatively spliced transcripts from SPAG1 (sperm associated antigen 11), was found to have similar cysteine residue spacing to a β-defensin and to be exclusively expressed in the mid-region of the caput epididymis. Its expression is only observed 30 days after birth and is maximal at sexual maturity (Li et al., 2001). Ligation of the vas deferens to induce inflammation increases its expression further. In a subsequent study, heterologous expression of Bin1b demonstrated that it was involved in the maturation of immotile, immature sperm. Exposure of sperm isolated from the initial region of the caput epididymis to Bin1b caused the sperm to become progressively motile by a calcium uptake-dependent mechanism. The mouse equivalent gene is also exclusively expressed in the caput epididymis and this expression is reduced following castration but restored with testosterone replacement (Pujianto et al., 2013). Since sperm maturation in the epididymis is androgen dependent, this is an important finding. Further functional work in the rat has shown that incomplete knockdown of another β-defensin in the same cluster, Defb15, (which is also expressed in the caput epididymis) affects sperm motility but not the capacitation process or the acrosome reaction (Zhao et al., 2011).

The murine genome has more genes than humans at the main chromosome 8 defensin locus and some have evolved following their divergence and so functional redundancy is a problem when gene knockout technology is used to study function. A recent study (by the Dorin group) has addressed this problem by deleting nine genes (Defb1, 2, 9, 10, 11, 13, 15, 35 and 50) which are located together at the end of the main β-defensin cluster in the mouse (Fig. 1) and expressed in the epididymis (Fig. 2). The resulting male DefbΔ9/DefbΔ9 mice had a profound defect
in the ability to sire offspring. When backcrossed four generations onto the C57Bl/6N mouse strain, they were completely infertile (Zhou et al., 2013). The sperm isolated from the cauda epididymis of the homozygous mice had significantly reduced motility and reduced progressive motility and were more fragile, resulting in increased head and tail separation when dropped onto a slide. In addition, these sperm had three times the number of spontaneously acrosome reacted sperm compared with controls and the frequency of capacitated sperm was significantly increased. Such changes may be expected to occur anyway near the point of fertilization but in an IVF sperm–oocyte binding assay, there was no evidence that the mutant sperm could bind the wild-type donor oocytes, although sperm from littermate controls were competent at binding. The lack of ability of the defensin-deletion sperm to bind to the oocyte, even under IVF conditions, may have several potential explanations: (i) disruption of the membrane composition and loss of non-defensin molecules essential for oocyte binding, (ii) loss of the defen-
sin(s) that would normally interact with the oocyte and/or (iii) lack of sperm motility meaning a reduction of propulsive force.

Transmission electron microscopy demonstrated that 40% of the Defb9/Defb9 sperm from the cauda epididymis (but interestingly not from the testes or caput epididymis) had gross disruption of the microtubule structure. When the intracellular calcium concentration was compared with wild-type sperm, the sperm with the 9 gene deletion showed significantly raised intracellular calcium levels. It is likely that the increase in calcium causes the microtubule disruption as treatment of wild-type sperm with the calcium ionophore A23187 also resulted in a similar microtubule disruption. It is interesting to speculate how the observed defect arises. The lack of the β-defensins in the sperm may result in a more permeable membrane, and/or the channels that normally regulate calcium flow may be impaired. As mentioned above, defensins have diverse receptor-binding activity, and pertinently, the defensin-like molecule MsDef1 from Alfalfa seeds has been shown to have the ability to block mammalian L-type channel calcium channel activity (Spelbrink et al., 2004). Thus, it is possible that the rise in calcium that we observed is due to the lack of β-defensin(s) in the membrane resulting in inappropriate transport through CatSper (and/or) other calcium channels in the sperm in the cauda of the epididymis. Other channels are also important in the ability of sperm to fertilize; potassium conductance, for example, increases calcium concentrations and regulates sperm membrane maturity for fertilization. Interestingly, mice deleted for slo3, one of the potassium channels in mouse sperm, display male infertility and their sperm fail to undergo the acrosome reaction (Santi et al., 2010). The microtubule disruption in Defb9/ Defb9 sperm may be a primary and/or secondary effect of the deletions. With the availability of patch clamping for mouse sperm, it is now feasible to examine whether β-defensins (and if so, which ones) directly affect CatSper and/or other relevant channels.

Clearly, disruption of these nine genes results in a sperm maturation defect and it remains to be seen whether it is a single gene effect that creates the whole phenotype or whether several genes need to be dysfunctional for the phenotype to be obvious. Fortunately, the recent realization of murine mutagenesis using the CRISPR/Cas technique will now enable rapid assessment of the effects of single gene deletions (Wang et al., 2013). The single gene Defb1 knockout mouse has no obvious fertility defect (Dorin, unpublished data) despite the expression of Defb1 in tissues that include the cauda epididymis; therefore, the focus of attention should shift to the other candidates.

Evidence that β-defensins may modulate sperm function in humans

There are very significant differences between sperm physiology and function in mice and humans. Key differences exist in the ion channels and consequent signalling pathways that regulate motility of human and mouse sperm. In mice, for example, there is no H1v1 and the principal sperm calcium channel, CatSper, is not activated by progesterone, as it is in humans (Lishko and Kirichok, 2010; Lishko et al., 2011; Smith et al., 2013). Human β-defensins lie at several separate loci in the genome: 8p23.1, 6p12.3, 20p13 and 20q11.21 and most of the genes have a clear rodent orthologue. Of the nine deleted genes described above, four (Defb1, Defb13, Defb15 and Defb35) have known human orthologues (DEFB1, DEFB107, DEFB106 and DEFB105, respectively) and all of these are expressed in the male reproductive tract (Semple et al., 2003). The remaining genes in the deletion are murine-specific paralogues, also expressed in the epididymis. It is likely that conserved homologues of these β-defensins also have functions in human sperm. There are also β-defensin SPAG11 isoforms, within the high copy number variable region in the β-defensin cluster at 8p23.1, which are also expressed in the epididymis and found on the head and neck regions of sperm (Radhakrishnan et al., 2009).

Yu et al. (2013) studied the effect of recombinant human DEFB114 which is located on chromosome 6p12.3. DEFB114, which is significantly expressed in the caput and corpus of the human epididymis, was shown to have an antimicrobial effect and additionally to protect against a negative effect of lipopolysaccharide (LPS) on sperm motility, although the mechanisms of this protection were not elucidated. In addition to demonstrating a direct role in the epididymis, the authors speculated that DEFB114 (and presumably other cationic antimicrobial β-defensins) may act to reduce the toxicity of excessive cytokine production in semen associated with poor sperm function, for example, in men with genital tract infections (Keck et al., 1998). Another human defensin, hBD3, is known to have potent anti-inflammatory effect on LPS-induced inflammation (Semple et al., 2010); however, the deletion of its mouse orthologue, Defb14, does not appear to have any effect on fertility (Dorin, unpublished data).

LPS is a microbial product and other microbial products have been shown to bind taste receptors present in both the respiratory and reproductive tract. Indeed some taste receptors have been found directly in sperm membranes (Meyer et al., 2012). It is perhaps relevant that in the airway, stimulation of the bitter taste receptor raises the level of calcium and induces the release of β-defensins, while stimulation of the sweet taste receptor suppresses these effects (Lee et al., 2014). Intriguingly, male knockout mice deficient in the sweet receptor, Tas1R, are infertile, have raised intracellular sperm calcium concentrations and increased spontaneous acrosome reactions (Meyer et al., 2012), consistent with the modulation of sperm calcium dynamics by the loss of defensins.

Recently, the chemokine receptor, CCR6 was recognized in human sperm and it was demonstrated that CCL20, a chemokine ligand of CCR6, modulated sperm motility and had a chemotactic effect on human sperm in vitro (Caballero-Campo et al., 2014). However, hBD2 and hBD3, which have been shown to interact with CCR6 to chemotactract CD4+ T cells in chemotaxis studies (Wu et al., 2003), had no such effect on sperm motility. As CCR6 expression is modulated during
capacitation in humans (but not mice), this may enable a fine tuning system that selects cells that are more responsive to signals from the female reproductive tract.

The primary evidence however for a role of defensins in human fertility comes from the experiments of Tollner et al. (2011) who described a mutation in DEFB126 (chromosome 20p13) which is associated with impaired conception. The β-defensin, DEFB126, was linked to the ability of sperm to penetrate hyaluronic acid gel (a mimic of female cervical mucus) and men homozygous for a frameshift mutation were found not to be infertile but to have a reduced chance of successful conception (Tollner et al., 2011). This β-defensin is quite different from other β-defensins as it has an extensive C-terminal tail containing an extra cysteine and O-linked glycosylation sites which are not commonly seen in other defensins. It is presumed that this glycosylation is important for function. In view of its adverse effect on conception, it is surprising that the mutant allele is reported to be present at an astonishingly high frequency in both European (0.47) and Chinese (0.45) populations. Possibly, the high number of heterozygotes with this mutant allele may imply some advantage in reduced levels of this β-defensin.

What was particularly interesting in this study was that despite having normal semen parameters and sperm motility, men with the DEFB126 mutation, had sperm with a reduced ability to penetrate the cervical mucus substitute. Penetration of viscous material is a robust marker of sperm function, and demonstrates a subtle yet important effect on the fitness of the cell (Egger-Kruse et al., 1989; Mortimer et al., 2013). Although the mechanism of this dysfunction has yet to be determined, the key importance of the sperm glycoalyx to fertilizing capacity is implied. In complementary experiments using macaques, DEFB126 was found to present along the length of the sperm. It is added to the sperm in the epididymis, and remains present throughout the journey in the female tract and during the initial stages of capacitation (Tollner et al., 2008a, b). Detailed studies showed that DEFB126 is critical to binding of the sperm cells to the oviductal epithelium and that release from the oviduct surface (presumably during capacitation) is associated with removal of DEFB126. The defensin was shown to be lost following caffeine treatment or induction of capacitation and this loss seems to be important to facilitate binding to the zona pellucida. Comparable experiments in humans have not been performed but as DEFB126 is important for penetration of cervical mucus substitutes, it is reasonable to presume that this particular defensin (probably via its glycosylation sites) is important for the transport to, and establishment of a functional reservoir in, the human oviduct. With the availability of patch clamping, it would be interesting to examine the effect of DEFB126 (and indeed DEFB105, DEFB106 and DEFB107) directly on CatSper. Activation of human CatSper, for example by progesterone, is important in accelerating penetration of cervical mucus substitutes (Alasmari et al., 2013); thus a logical working hypothesis would be that the defensins modulate CatSper (directly or indirectly) and abnormalities in this system will impair fertility. Consistent with this is the concept that CatSper is a polymodal channel (Brenker et al., 2012). Removal or modulation of the defensin(s) during the latter stages of capacitation in the upper reaches of the female tract may unmask CatSper for activation by the oocyte. Defensins may also have an indirect effect on CatSper. Fundamentally, important channels that regulate membrane potential in human spermatozoa (and hence CatSper) are KCNMA1 and KCNU1 (SlO1/3) (potassium channel, subfamily U, member 1 and potassium large conductance calcium-activated channel, subfamily M, alpha member 1) (Mannowetz et al., 2013; Brenker et al., 2014; Lópe-González et al., 2014; Mansell et al., 2014). Changes in the oviductal milieu, for example a decrease in potassium concentration and increase in bicarbonate may modify the potassium conductance, affecting membrane potential and thus activation of CatSper. At present there is no evidence of a direct effect of defensins on potassium conductance, but modulation could indirectly affect CatSper and hence calcium signalling. These are interesting ideas that are not mutually exclusive to the concept of a direct effect on the surface charge or glycoalyx (Tollner et al., 2011).

**Identification of men with abnormalities in defensins**

As the function(s) of defensins in human sperm are not yet clearly delineated, we do not know how best to identify men who are potentially abnormal in this regard. However, extrapolating from the observations of Tollner et al., biological screening via the Kremer sperm penetration test would presumably identify men with poor sperm penetration of a viscous substance yet with relatively normal sperm motility and normal results for other semen parameters. Perhaps these men represent those previously identified with unexplained infertility where there has been a reported association with poor penetration of cervical mucus and direct fertilizing capacity (Barratt et al., 1989). Such men are not uncommon in an infertility clinic (∼13%; Barratt et al., 1989). It would also be interesting to see if mice deleted for Defb22, the equivalent gene to DEFB126, also display a similar phenotype and what ability they have in IVF.

**Conclusion**

Despite their clear importance in innate immunity, the predominance of expression of defensins in the male and female reproductive tracts and along the surface of sperm does imply that β-defensins have a role in sperm function and fertility. Despite the differences in the regulation of key channels in humans and mice, important clues can be learned from mice and it is interesting to see the phenotype of male infertility in the defensin-deletion mice. In addition, men with the DEFB126 mutation clearly have a reduced ability to successfully reproduce. In macaques, sperm DEFB126 is important in binding to the epithelia of the oviduct through its glycosylation. The loss of this defensin during capacitation seems important in facilitating binding to the zona pellucida. These features may be true for all β-defensins or only highly O-glycosylated peptides like DEFB126.

It is possible that fertility may also be linked to increased chromosome 8 β-defensin copy number and expression and, coincidentally, the added benefit of a raised resistance to viral and bacterial infection. It is most likely that the ability of β-defensins to localize on the sperm membrane and associate to make raft-like structures may result in non-specific channel blocking. During transit through the female reproductive tract, the β-defensins may both protect from infection and stop premature hyperactivation. Subsequently, in the appropriate environment for fertilization, the β-defensins will be lost from the sperm; membrane hyperpolarization will result following potassium efflux and an increase in intracellular calcium will result from CatSper activation by progesterone. Further work, using human and mice sperm, state-of-art genome editing
techniques and direct channel characterization using electrophysiology, will clarify whether this model is correct.

**Authors’ roles**

Both authors contributed equally to writing this review.

**Funding**

J.R.D. is funded by MRC and University of Edinburgh at the MRCIGMM at University of Edinburgh. C.L.R.B.’s laboratory is funded by MRC and NHS Scotland. His salary is funded by University of Dundee.

**Conflict of interest**

None declared.

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