nature cell biology

An epididymis-specific β-defensin is important for the initiation of sperm maturation

Chen Xi Zhou[1], Yong-Lian Zhang[2], Liqing Xiao[2], Min Zheng[2], Ka Man Leung[1], Man Yee Chan[1], Pui Shan Lo[1], Lai Ling Tsang[1], Hau Yan Wong[1], Lok Sze Ho[1], Yiu Wa Chung[1] & Hsiao Chang Chan[1,3]

Although the role of the epididymis, a male accessory sex organ, in sperm maturation has been established for nearly four decades[1], the maturation process itself has not been linked to a specific molecule of epididymal origin. Here we show that Bin1b, a rat epididymis-specific β-defensin with antimicrobial activity[2], can bind to the sperm head in different regions of the epididymis with varied binding patterns. In addition, Bin1b-expressing cells, either of epididymal origin or from a Bin1b-transfected cell line, can induce progressive sperm motility in immotile immature sperm. This induction of motility is mediated by the Bin1b-induced uptake of Ca^{2+}, a mechanism that has a less prominent role in maintaining motility in mature sperm. *In vivo* antisense experiments show that suppressed expression of Bin1b results in reduced binding of Bin1b to caput sperm and in considerable attenuation of sperm motility and progressive movement. Thus, β-defensin is important for the acquisition of sperm motility and the initiation of sperm maturation.

Mammalian sperm are produced in the testis, but they can neither swim nor fertilize an egg when they leave the testis. They become mature and acquire their forward motility and fertilizing capacity during transit through the epididymis, situated next to the testis[1,3]. The epididymal fluid milieu, which is created by specialized regions of the epididymis — namely, the caput (head), corpus (body) and cauda (tail) — is thought to be essential for sperm maturation; however, the molecular basis for the maturation process remains largely unknown. Of about 200 epididymal proteins present in different species, few have been identified that seem to be directly involved in sperm maturation in the epididymis[4,5].

Bin1b is a rat epididymis-specific peptide[2] with a homologue in humans[6]. We have previously shown that Bin1b has molecular structure and antimicrobial activity similar to those of β-defensins[2]. The region-specific localization of Bin1b, which is expressed exclusively in the middle of the caput region and not in other regions of the epididymis, led us to the hypothesis that Bin1b might have a role in sperm maturation other than merely being a β-defensin, because the microenvironment of the caput region has been shown to be essential for sperm to acquire their forward motility[7,8].

As its signal sequence suggests that it is a secretory peptide, we considered that Bin1b might affect sperm maturation by directly binding to sperm. We tested this possibility by immunohistochemical assessment with a Bin1b antibody, the specificity of which was verified by western blot (Fig. 1a). Bin1b immunoreactivity was found in sperm from all epididymal regions except the initial segment (Fig. 1b), and the percentage of sperm binding to Bin1b antibody, determined by flow cytometry, varied across the different regions (Fig. 1c). The patterns of sperm binding also varied within the different regions, with immunoreactivity distributed throughout the caput sperm head and prominent signals concentrated on the post-acrosome region of cauda sperm (Fig. 1b). These distinct sperm-binding patterns of Bin1b in different regions suggest that Bin1b may modify specific sperm function during their transit through the epididymis.

We investigated the possible effects of Bin1b on sperm maturation by using the epididymal epithelial cell culture previously used for elucidating the antimicrobial activity of Bin1b[2]. Caput epididymal cells expressing Bin1b and control cauda epididymal cells were cultured and grown to confluence (4 d) on a floating filter device to ensure formation of epithelial polarity and proper epididymal secretion. Immature sperm, collected from the initial segment of the epididymis, were then added to the apical compartment of the epididymal culture and incubated for various durations. Sperm motility dynamics, which is the most obvious maturational change in sperm[9], was examined by computer-assisted sperm analysis.

The immature sperm showed non-progressive movement when first released into sperm medium and stopped beating within a few minutes, consistent with previous observations[10]. The immotile sperm began to move, however, 3 h after coculture with caput (but not cauda) epididymal epithelial cells and reached a plateau at 5 h, which could be maintained for up to 9 h (Fig. 2a). Analysis of the sperm movement parameters showed that both averaged-path velocity (VAP) and straight-line velocity (VSL) were significantly greater in sperm cocultured with caput cells than in those cocultured with cauda cells (Table 1). By contrast, there was basically no sperm movement

[1]Epithelial Cell Biology Research Center, Department of Physiology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong.
[2]State Key Laboratory of Molecular Biology, Institute of Biochemistry & Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, 320, Yue-Yang Road, Shanghai 200031, China. [3]Correspondence should be addressed to H.C.C. (e-mail: hsiaocchan@cuhk.edu.hk).

Published online: 1 May 2004; DOI: 10.1038/ncb1127

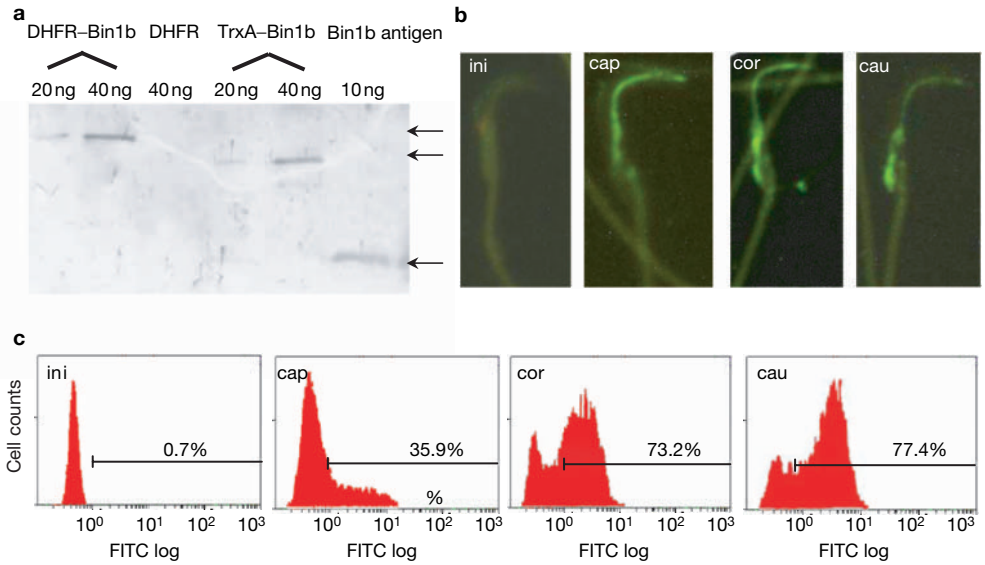

Figure 1 Bin1b binds to sperm from different regions of rat epididymis. (**a**) Rabbit polyclonal antisera were raised against Bin1b recombinant protein and the specificity of the antibody towards Bin1b was verified by western blot. The antibody (1:1,000 dilution) detected Bin1b antigen and the two positive controls of a DHFR–Bin1b (26K) and a TrxA–Bin1b (22K) fusion protein, but not the negative control of DHFR protein alone. (**b**) Immunostaining showing Bin1b binding to sperm from different regions of rat epididymis. (**c**) Percentage of Bin1b binding to sperm of different regions assessed by flow cytometry. ini, initial segment; cap, caput; cor, corpus; cau, cauda.

when sperm were incubated in sperm medium or fresh culture medium for epithelial cells (Fig. 2a).

Because progressive movement of sperm is a characteristic of sperm maturation[9], we determined the effects of caput cells on progressive movement. In the presence of caput cells, sperm acquired slow progressive motility at 3 h, which increased further and could be sustained for up to 9 h, whereas no significant change in progressive movement was observed after other cell treatments (Fig. 2b). Thus, by coculturing with caput epididymal cells *in vitro*, immotile immature sperm could be induced to acquire and sustain progressive motility.

To determine whether the increased sperm motility and gain of progressive movement were due to the effects of Bin1b, we incubated the cocultures with an antibody against Bin1b. Treatment with Bin1b antibody reversed the increase in sperm motility and the gain of progressive movement induced by caput cells, whereas no significant changes in these parameters were observed when the cocultures were treated with control preimmune serum (Fig. 2a, b). These results indicate that Bin1b may have a role in inducing sperm motility in immature sperm.

To confirm this role of Bin1b and to exclude the involvement of other factors secreted by caput epididymal cells, we transfected the Bin1b complementary DNA into a colonic epithelial cell line, T84, that does not express Bin1b endogenously. A cell line stably expressing Bin1b (Bin-T84) and a control cell line transfected with empty expression vector (Vet-T84) were generated, and expression of Bin1b was confirmed by polymerase chain reaction with reverse transcription (RT–PCR) (Fig. 2c). Sperm coculture experiments were then conducted with immotile immature sperm, as described above.

Table 1 Movement parameters of immature rat caput sperm cocultured with caput or cauda epididymal epithelial cells

Parameters	Time	Caput coculture	Cauda coculture
VAP	5h	125.4±3.08*	80.6±16.5
(μm s^{-1})	9h	143.3±11.0	80.7±35.5
VSL	5h	72.9±1.5**	58.8±4.0
(μm s^{-1})	9h	68.18±2.8*	37.7±16.1

Data were obtained using computer-assisted sperm analysis and are expressed as mean ± s.e.m. *$P < 0.05$, **$P < 0.01$, compared with corresponding cauda values (unpaired *t*-test). VAP, averaged path velocity; VSL, straight line velocity.

The increases in sperm motility and percentage of progressive movement, as well as the time course of these increases, were similar in sperm cocultured with Bin-T84 cells (Fig. 2d, e) and sperm cocultured with caput epididymal cells (Fig. 2a, b); by contrast, no significant increase was observed in cocultures with Vet-T84 cells or when Bin1b antibody was present (Fig. 2d, e). Positive Bin1b immunoreactivity was observed in more than 80% of immature sperm (which initially stained negative for Bin1b) after 1 h of incubation with Bin-T84 conditioned medium containing secreted Bin1b (Fig. 2f, g). By contrast, no significant increase in Bin1b immunoreactivity was found in mature sperm from the cauda region that initially stained positive for Bin1b (Fig. 2h). Taken together, the coculture experiments with caput epididymal cells and with Bin-T84 cells confirm our hypothesis that Bin1b is involved in the sperm maturation process at the point where immature sperm start to gain progressive motility; a characteristic change in sperm movement that is known to be associated with sperm from the distal caput epididymis[9]. The involvement of Bin1b in the initiation of sperm maturation may explain why this β-defensin is expressed exclusively in the middle caput region.

We considered how Bin1b might mediate this change in sperm motility. Defensins are well-characterized cationic (polar) molecules with spatially separated hydrophobic and charged regions that allow them to insert into phospholipid membranes and to form holes or channels in biological membranes. This mechanism of action underlies the antimicrobial capability of defensins[11], which have also been shown to modulate membrane ion channels, including the activation of L-type Ca^{2+} channels[12,13]. Thus, Bin1b might either form Ca^{2+}-permeable channels or activate Ca^{2+} channels in sperm, thereby enabling

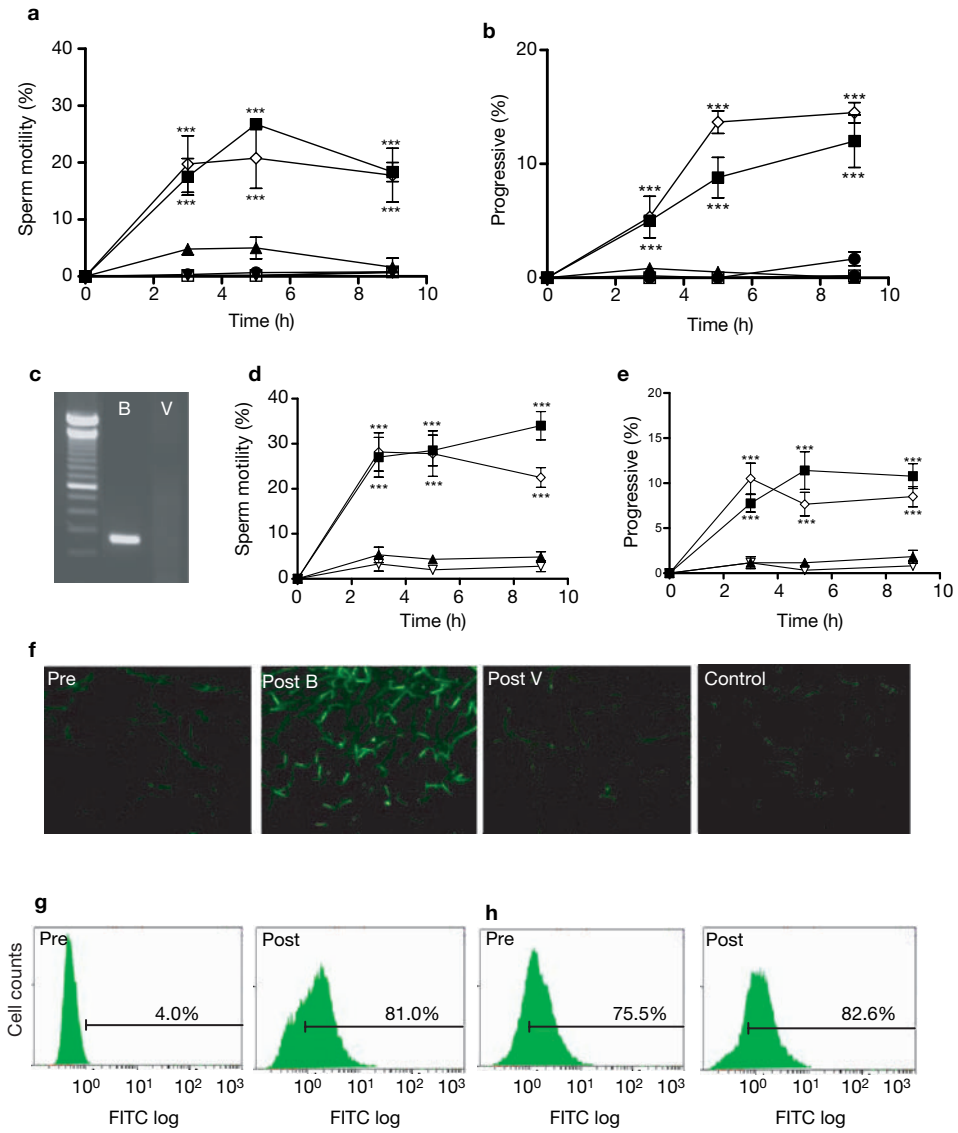

Figure 2 Effect of Bin1b expressing epithelial cells on sperm motility and Bin1b binding after coculture. (**a**, **b**) Sperm motility (**a**) and percentage of progressive movement (**b**) induced by coculture with caput epididymal epithelial cells, but not by other treatments, were greatly attenuated by treatment with Bin1b antibody (1:800 dilution). Filled squares, cocultured with caput cells; filled 'upwards' triangles, cocultured with cauda cells; filled 'downwards' triangles, cocultured with caput cells treated with Bin1b antibody; open diamonds, cocultured with caput cells treated with preimmune serum; open squares, incubated in cell-culture medium (Eagle's minimum essential medium); filled circles, incubated in sperm medium. (**c**) RT–PCR showing Bin1b expression in Bin1b-transfected T84 cells (B) but not in vector-transfected T84 cells (V). (**d**, **e**) Sperm motility (**d**) and percentage of progressive movement (**e**) induced by coculture with Bin-T84 cells, but not by other treatments, were greatly attenuated by incubation with Bin1b antibody (1:800 dilution). Filled squares, cocultured with Bin-T84 cells; filled triangles, sperm cocultured with Vet-T84 cells; open triangles, cocultured with Bin-T84 cells treated with Bin1b antibody; open diamonds, cocultured with Bin-T84 cells treated with preimmune serum. Data are expressed as the mean ± s.e.m. ($n = 4$–6). $^{***}P < 0.0001$, as compared with the corresponding control (one-way analysis of variance). (**f**) Immunostaining of Bin1b in immature sperm before (Pre) and after incubation with Bin-T84 (Post B) and Vet-T84 (Post V) conditioned medium, and after incubation with a negative control of preimmune serum (Control). (**g**) Bin1b binding percentage in immature sperm before (Pre) and after (Post) incubation with Bin-T84 conditioned medium. (**h**) Percentage of Bin1b binding in mature cauda sperm before (Pre) and after (Post) incubation with Bin-T84 conditioned medium.

sperm to accumulate Ca^{2+}, which is well known to have a central role in sperm function, including sperm motility, sperm capacitation and the acrosome reaction[14,15].

It has been reported that a marked change in the Ca^{2+}-accumulating capacity of sperm occurs during their transit through the epididymis[16] and that the Ca^{2+} concentration of caput sperm is about twofold higher than that of cauda sperm[17]. Caput sperm also accumulate Ca^{2+} from exogenous sources at a rate that is 2–4 times faster than that of cauda sperm[18]. In addition, a Ca^{2+}-dependent mechanism has been implicated in the transformation of an irregular movement of caput sperm into a progressive movement during epididymal sperm maturation[19,20]; however, the mechanism underlying Ca^{2+} transport through the sperm membrane during maturation is not clear.

We therefore considered that Bin1b-formed ion channels or Bin1b-activated Ca^{2+} channels might provide a pathway for a Ca^{2+} influx required for inducing sperm motility. To test this hypothesis, we first

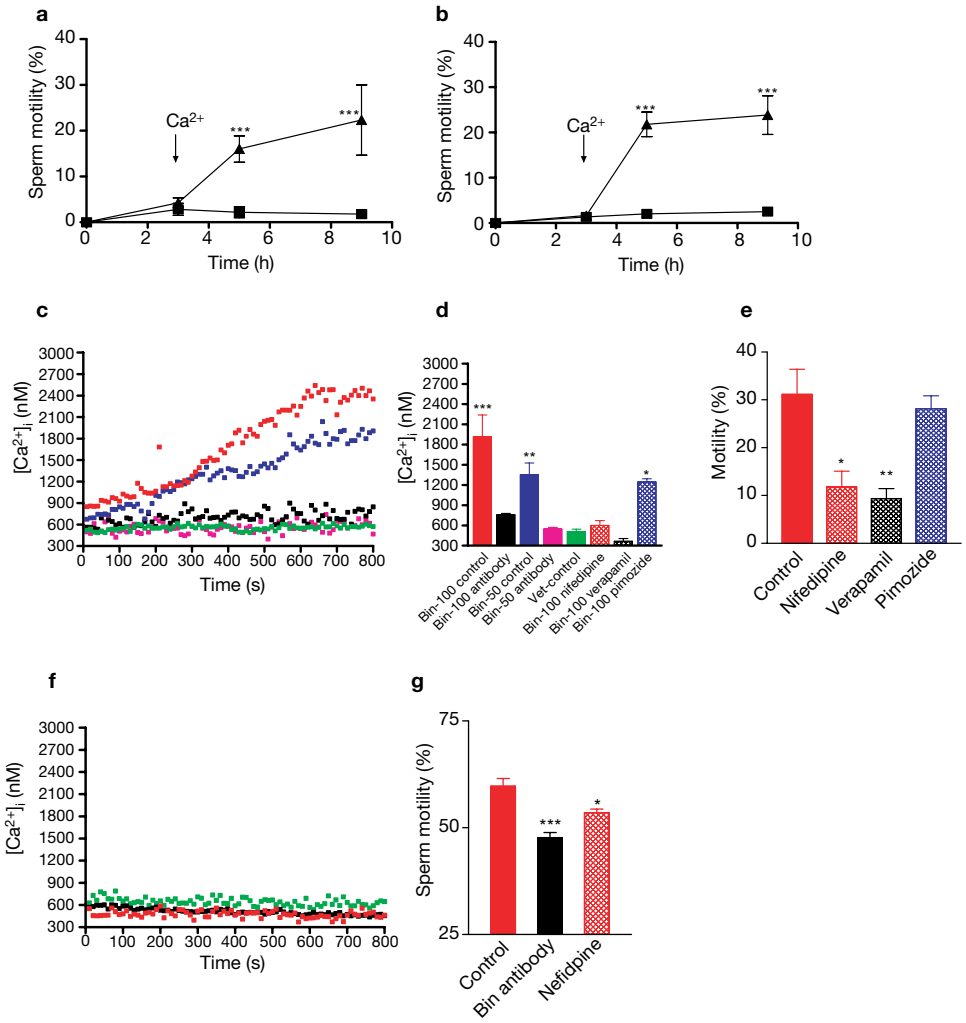

Figure 3 Differential effect of Bin1b on Ca^{2+} uptake in immature and mature sperm. (**a**, **b**) Effect of extracellular Ca^{2+} on caput (**a**) and Bin-T84 (**b**) coculture-induced sperm motility in immature sperm with arrows indicating the addition of Ca^{2+} (2 mM) to the Ca^{2+}-free coculture medium. Squares, Ca^{2+}-free; triangles, Ca^{2+}-free plus Ca^{2+}. (**c**) Representative intracellular Ca^{2+} measurements in immature sperm showing increases induced by different dilutions of Bin-T84 conditioned medium (100%, red; 50%, blue), which could be blocked by Bin1b antibody (black, 100% plus antibody; pink, 50% plus antibody). Increases in Ca^{2+} were not induced by Vet-T84 conditioned medium (green). (**d**) Summary of the intracellular Ca^{2+} measurements in **c**, and effects of the L-type Ca^{2+} channel blockers nifedipine (0.5 μM) and verapamil (1 μM), and the T-type Ca^{2+} channel blocker pimozide (1 μM) on intracellular Ca^{2+}. (**e**) Inhibition of sperm motility induced by Bin-T84 coculture (at 9 h) in immature sperm by nifedipine and verapamil, but not by pimozide. (**f**) Representative intracellular Ca^{2+} measurements in mature cauda sperm showing no response to Bin-T84 or Vet-T84 conditioned medium (colour coding as in **c**). (**g**) Sensitivity of cauda sperm motility to Bin1b antibody and nifedipine after 3 h incubation. Data are expressed as the mean ± s.e.m. ($n = 3$–6). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$, as compared with the corresponding control (unpaired *t*-test).

examined the Ca^{2+} dependence of the Bin1b effect on sperm motility. When Ca^{2+}-free medium was used in the coculture experiments with caput epididymal cells or Bin-T84 cells, no increase in sperm motility (Fig. 3a, b) or progressive movement (data not shown) was observed for up to 9 h. When Ca^{2+} was added back to the medium at 3 h, however, an increase in both sperm motility (Fig. 3a, b) and progressive movement (data not shown) was observed 2 h after the addition, indicating that the Bin1b effect on sperm motility depends on extracellular Ca^{2+}. These results indicate that Bin1b may be able to regulate Ca^{2+} uptake in sperm.

As shown in Figure 3c and d, incubating the immature sperm with Bin-T84-conditioned medium, but not control Vet-T84-conditioned medium, resulted in a concentration-dependent increase in Ca^{2+} uptake that could be substantially blocked by Bin1b antibody or the L-type Ca^{2+} channel blockers nifedipine (0.5 μM) and verapamil (1 μM), but not by the T-type Ca^{2+} channel blocker pimozide (1 μM). Similarly, nifedipine and verapamil significantly reduced Bin1b-induced sperm motility, whereas pimozide was less effective (Fig. 3e). The dependence of Bin1b-induced sperm motility on extracellular Ca^{2+}, the ability of Bin-T84 conditioned medium to induce sperm Ca^{2+} uptake, and the inhibition of the Bin1b effect by Ca^{2+} channel blockers indicate that Bin1b may activate sperm Ca^{2+} channels, leading to the Ca^{2+} influx necessary for immature sperm to acquire motility.

The distinct binding patterns of Bin1b to sperm in different epididymal regions — for example, its concentration on the post-acrosome region of cauda sperm versus its uniform distribution throughout the

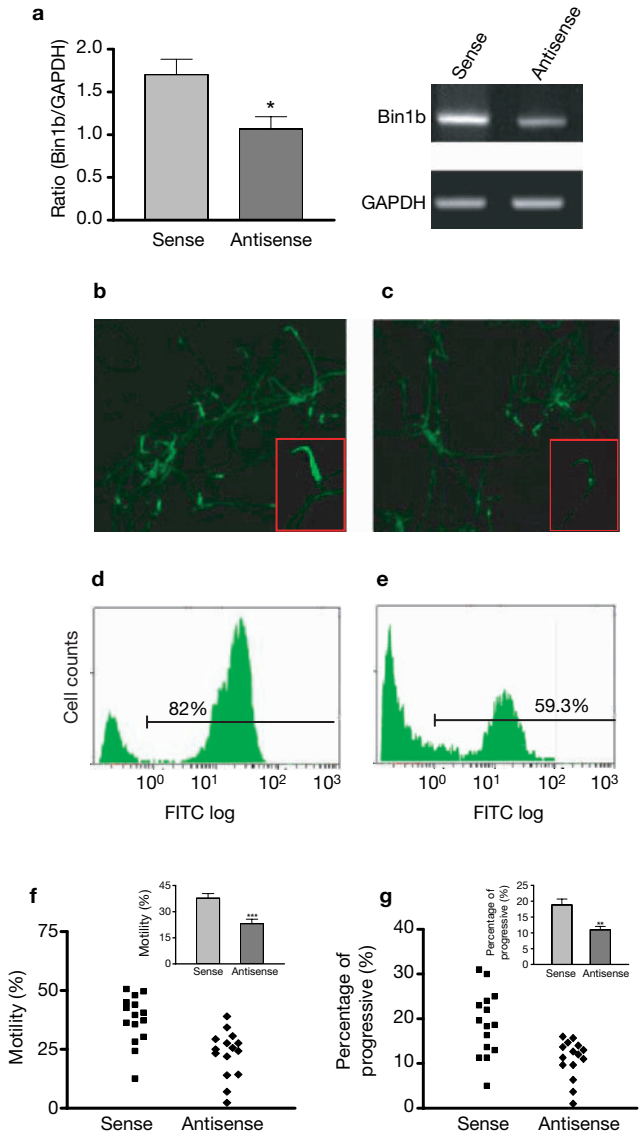

Figure 4 *In vivo* suppression of Bin1b expression and the effect of Bin1b antisense oligonucleotides. (**a**) Results from semi-quantitative RT–PCR show reduced expression of Bin1b mRNA in caput epididymis 48 h after antisense treatment. GAPDH was measured as the internal control. (**b**–**e**) Representative images of Bin1b immunofluorescence staining of sperm from the distal caput epididymis treated with sense control (**b**) and antisense (**c**) oligonucleotides, and corresponding binding percentages obtained by flow cytometry in the sense (**d**) and antisense (**e**) samples. (**f**, **g**) Distribution of results of sperm motility (**f**) and percentage of progressive movement (**g**) obtained from sense control and antisense samples. Insets show a summary of the results. Data are expressed as the mean ± s.e.m. (n = 5–15). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$, as compared with the corresponding control (unpaired t-test).

head of caput sperm — prompted us to examine whether Bin1b contributes to the maintenance of motility in mature cauda sperm. We examined the effect of Bin-T84 conditioned medium on the Ca^{2+} uptake of mature sperm from the cauda region, but observed no significant increase in Ca^{2+} uptake during the incubation period (Fig. 3f). Sperm collected from the cauda epididymal region showed well-documented vigorous sperm motility, of which only about 20% could be inhibited by Bin1b antibody (Fig. 3g), in contrast to the inhibition of more than 90% observed in stimulated immature sperm (Fig. 2a, d). Notably, 0.5 μM nifedipine, which greatly suppressed Bin1b-induced motility in immature sperm, did not have a pronounced effect on cauda sperm motility, at least, not to the same extent as Bin1b antibody (Fig. 3g). These results have two implications: first, unlike its essential role in inducing sperm motility in immature sperm, Bin1b is relatively less important in maintaining sperm motility in mature sperm; second, the action of Bin1b on cauda sperm may be different from its action on caput sperm in that it may involve other Ca^{2+} channels. In short, it seems that mechanisms other than Bin1b may develop during sperm maturation and become important for the maintenance of motility in mature sperm.

To confirm the role of Bin1b in the acquisition of sperm motility in a physiological context, we used an antisense oligonucleotide directed against Bin1b to reduce Bin1b expression *in vivo* and examined its effect on sperm motility and progressive movement. As compared with a sense control, the antisense Bin1b oligonucleotide (20 μg ml^{-1}) significantly suppressed the expression of Bin1b messenger RNA in the caput epididymis, as assessed by semiquantitative RT–PCR (Fig. 4a); this decrease in expression resulted in a considerable reduction in Bin1b binding to sperm collected in the distal caput region, as shown by immunofluorescence staining (Fig. 4b, c) in conjunction with flow cytometry (Fig. 4d, e). Consistent with the reduction in Bin1b expression and sperm binding, the motility and progressive movement of sperm taken from the distal caput epididymis in rats treated with Bin1b antisense oligonucleotide were also found to be significantly lower than those of the sperm from the control treated with sense oligonucleotide (Fig. 4f, g). Taken together, our *in vitro* and *in vivo* studies confirm that Bin1b is essential for the acquisition of sperm motility and thus the initiation of sperm maturation.

Our findings are consistent with the long-recognized central role of Ca^{2+} in sperm motility and provide insight into the possible mechanism that regulates sperm Ca^{2+} uptake, pertinent to the acquisition of sperm motility. Bin1b binding to sperm seems to activate Ca^{2+} channels and to allow Ca^{2+} uptake, leading to the initiation of sperm motility in immature sperm. Sperm Ca^{2+} uptake is considered to be one of the changes that accompanies sperm maturation in the epididymis, and our findings are consistent with the previously observed higher rate of Ca^{2+} accumulation[18] and higher Ca^{2+} concentration[17] in caput sperm as compared with cauda sperm. Our results also suggest that Bin1b does not contribute either to Ca^{2+} uptake or to maintaining motility in mature cauda sperm. The differential effect of Bin1b on immature and mature sperm may be due to the changes in its binding pattern to sperm, which in turn may reflect modification of the sperm membrane; a process that is known to be associated with sperm maturation[21]. Overall, Bin1b seems to be involved in the first uptake of Ca^{2+} that is responsible for the acquisition of motility by immature sperm and, to a much lesser extent, the maintenance of motility in mature sperm.

Our results suggest that Bin1b, an epididymis-specific β-defensin, is not limited to killing bacteria[2] but is also involved in sperm maturation in the epididymis. How is this possible? This question requires further investigation, although we speculate that different modes of action may be exploited by Bin1b under different conditions; in other words, the ever-changing fluid in different regions of the epididymis and the altered environment upon bacterial infection may lead to different modes of Bin1b action. This is because the electrostatic charge interaction within the cell membrane that is required for the initial action of defensins is crucially dependent on the ionic strength of the fluid milieu[11]. Although the detailed mechanisms of Bin1b action remain to be elucidated, the observed dual effects of Bin1b attest to the

importance of this β-defensin in the epididymis. Other studies have also identified a few region-specific β-defensins in human and mouse epididymides, supporting the notion that they may contribute to epididymal regulation as well as innate immunity[22,23]. Notably, the antimicrobial peptide caltrin, which originates from the seminal vesicle, has been also shown to affect sperm capacitation and fertilization[24]. Further investigation of the roles of defensins may shed new light on our understanding of the sperm maturation process and its regulation. Defensins, such as Bin1b, may not only have therapeutic implications for sexually transmitted diseases[11,25], but also offer an interesting lead for work on male infertility and contraception. □

METHODS

Bin1b antibodies and immunolocalization on epididymal sperm. A 135-base-pair cDNA fragment of Bin1b corresponding the mature Bin1b peptide of 45 amino acids was inserted into a modified PET-28(a) vector (Invitrogen). The recombinant protein was expressed and purified by using a Novagen pET Expression Systems kit (Invitrogen). We prepared rabbit polyclonal antisera against this recombinant protein as described[26]. For immunolocalization, the epididymis was divided into four parts: initial segment, caput, corpus and cauda. Sperm were washed out from each part, fixed in 4% paraformaldehyde for 30 min, and immunostained as described[27] with polyclonal antisera against Bin1b (1:600 dilution). Pictures were taken under a BM50 fluorescence microscope (Olympus).

Bin1b transfection. The Bin1b gene was cloned to a PCMV-tab expression vector. T84 cells were seeded onto 35-mm plates at a density of 10^5 per plate. The cells were grown to 60–80% confluency, transfected by using Lipofectin transfection reagent, and selected by using G418 (400 μg ml^{-1}) as described[28].

Coculture of sperm and epithelial cells. Immature caput sperm were collected from adult Sprague-Dawley rats (aged 3 months). The proximal caput epididymis was gently squeezed and punctured and then placed in 0.4 ml of warmed sperm medium (123 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 25 mM $NaHCO_3$, 5 mM glucose, 12.5 mM sodium lactate and 0.5 mM pyruvate, 8 μg of phenol red, 4 mg ml^{-1} bovine serum albumin; pH 7.4, osmolarity 310 mOsm kg^{-1}) in an incubator for 5 min to allow the sperm to disperse throughout the media. The sperm concentration was adjusted to 60 × 10^6 per ml. The methods for isolating and culturing epididymal epithelial cells have been described[29]. On confluence (4 d after the start of culture), the culture medium in the apical compartment of cell culture was removed and replaced by 150 μl of fresh sperm medium 2 h before the coculture experiment. For coculture, we added 3 μl of sperm (60 × 10^6 per ml) to the apical compartment of the cell culture and incubated the mixture for different durations at 37 °C and 5% CO_2. Aliquots of 10 μl were used for sperm motility analysis.

Sperm motility analysis. For the motility analysis, we used an HTM-IVOS system (version 10.8, Hamilton-Thorn Research) with the following settings: objective, ×4; minimum cell size, six pixels; minimum contrast, 65; minimum static contrast, 30; low VAP cut-off, 30.0; low VSL cut-off, 20.0; threshold straightness, 50%; static head size, 0.29–10.0; static head intensity, 0.29–1.11; magnification, 0.82. Sixty frames were acquired at a frame rate of 60 Hz. At least 200 tracks were measured for each specimen at 37 °C. We recorded at least 30 points for each tract. The playback function of the system was used to check its accuracy.

Intracellular Ca^{2+} concentration of caput epididymal sperm. Sperm were loaded with 2 μM Fura-2/AM for 45 min at 37 °C and 5% CO_2, washed twice and resuspended in sperm medium. The Fura-2-loaded sperm were then resuspended in different dilutions of conditioned medium from Bin-T84 cells or in conditioned medium from Vet-T84 cells as a control. The fluorescence signal from alternate excitation at 340 and 380 nm was recorded by a LS-50B luminescent spectrometer (Perkin Elmer), and the Ca^{2+} concentration was calculated as described[30] using the 340/380 ratio of fluorescence.

Flow cytometry analysis. Rat sperm were stained with Bin1b polyclonal antibody at a final concentration of 1:200. In control samples, the antibody was replaced by preimmune serum at the same concentration. After incubation at 4 °C overnight, the sperm were washed twice with PBS containing 4 mg ml^{-1} bovine serum albumin and incubated for 45 min at 22 °C with the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat antibody against rabbit IgG (Zymed Laboratories) at a dilution of 1:50. After three washes, the sperm were analysed on a flow cytometer (Beckman Coulter). We analysed 10,000 individual sperm per sample for FITC fluorescence emissions. A negative control of sperm stained by normal rabbit IgG was used to set the threshold for specific Bin1b staining. The percentage of sperm specifically stained for Bin1b was obtained by subtracting background fluorescence.

***In vivo* antisense oligonucleotide treatment.** The skin covering the testis of a male Sprague-Dawley rat (aged >3 months) was cut open under anaesthesia and 30 μl of Bin1b antisense oligonucleotide (5′-AGAGTAACAAAAC-CTTCATG-3′) or control sense oligonucleotide (5′-CATGAAGGTTTTGT-TACTCT-3′) solution (20 μg ml^{-1}) was injected into the proximal side of the mid-caput epididymis. The sperm were collected for assessment 48 h after injection. The use of animals in this project was authorized by the Animal Research Ethics Committee of The Chinese University of Hong Kong.

ACKNOWLEDGEMENTS

The work was supported by RGC of Hong Kong (CUHK4365/03M), the Strategic Program of The Chinese University of Hong Kong, the Distinguished Young Investigator Fund of the National Natural Science Foundation of China (to H.C.C.), the '973' Basic Research Funding Scheme of China (G 1999055901), the CAS Knowledge Creative Program (KSCX2-SW-201) and the Shanghai Development Foundation of Science and Technology (no. 03JC14080).

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

Received 28 January 2004; accepted 1 April 2004
Published online at http://www.nature.com/naturecellbiology.

1. Orgebin-Crist, M. C. Sperm maturation in rabbit epididymis. *Nature* **216,** 816–818 (1967).
2. Li, P. *et al*. An antimicrobial peptide gene found in the male reproductive system of rats. *Science* **291,** 1783–1785 (2001).
3. Bedford, J. M. Fertile life of rabbit spermatozoa in rat uterus. *Nature* **213,** 1097–1099 (1967).
4. Fouchecourt, S., Metayer, S., Locatelli, A., Dacheux, F. & Dacheux, J. L. Stallion epididymal fluid proteome: qualitative and quantitative characterization; secretion and dynamic changes of major proteins. *Biol. Reprod.* **62,** 1790–1803 (2000).
5. Dacheux, J. L., Gatti, J. L. & Dacheux, F. Contribution of epididymal secretory proteins for spermatozoa maturation. *Microsc. Res. Tech.* **61,** 7–17 (2003).
6. von Horsten, H. H., Derr, P. & Kirchhoff, C. Novel antimicrobial peptide of human epididymal duct origin. *Biol. Reprod.* **67,** 804–813 (2002).
7. Jeulin, C., Lewin, L. M., Chevrier, C. & Schoevaert-Brossault, D. Changes in flagellar movement of rat spermatozoa along the length of the epididymis: manual and computer-aided image analysis. *Cell Motil. Cytoskeleton* **35,** 147–161 (1996).
8. Yeung, C. H., Oberlander, G. & Cooper, T. G. Characterization of the motility of maturing rat spermatozoa by computer-aided objective measurement. *J. Reprod. Fertil.* **96,** 427–441 (1992).
9. Yeung, C. H. & Cooper, T. G. in *Molecules to Clinical Practice* (eds Robaire, B. & Hinton, B.) 417–434 (Plenum, New York, 2002).
10. Yeung, C. H., Oberlander, G. & Cooper, T. G. Maturation of hamster epididymal sperm motility and influence of the thiol status of hamster and rat spermatozoa on their motility patterns. *Mol. Reprod. Dev.* **38,** 347–355 (1994).
11. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **415,** 389–395 (2002).
12. MacLeod, R. J. *et al*. Corticostatic peptides cause nifedipine-sensitive volume reduction in jejunal villus enterocytes. *Proc. Natl Acad. Sci. USA* **88,** 552–556 (1991).
13. Bateman, A. *et al*. The isolation and characterization of a novel corticostatin/defensin-like peptide from the kidney. *J. Biol. Chem.* **271,** 10654–10659 (1996).
14. Guraya, S. S. Cellular and molecular biology of capacitation and acrosome reaction in spermatozoa. *Int. Rev. Cytol.* **199,** 1–64 (2000).
15. Breitbart, H. Intracellular calcium regulation in sperm capacitation and acrosomal reaction. *Mol. Cell. Endocrinol.* **187,** 139–144 (2002).
16. Hoskins, D. D., Acott, T. S., Critchlow, L. & Vijayaraghavan, S. Studies on the roles of cyclic AMP and calcium in the development of bovine sperm motility. *J. Submicrosc. Cytol.* **15,** 21–27 (1983).
17. Vijayaraghavan, S. & Hoskins, D. D. Low molecular weight factor in bovine caudal epididymal fluid that stimulates calcium uptake in caput spermatozoa. *Gamete Res.* **20,** 343–352 (1988).
18. Vijayaraghavan, S., Bhattacharyya, A. & Hoskins, D. D. Calcium uptake by bovine epididymal spermatozoa is regulated by the redox state of the mitochondrial pyridine nucleotides. *Biol. Reprod.* **40,** 744–751 (1989).
19. Serres, C. & Kann, M. L. Motility induction in hamster spermatozoa from caput epi-

didymidis: effects of forward motility protein (FMP) and calmodulin inhibitor. *Reprod. Nutr. Dev.* **24,** 81–94 (1984).
20. Peterson, R. N. & Freund, M. Relationship between motility and the transport and binding of divalent cations to the plasma membrane of human spermatozoa. *Fertil. Steril.* **27,** 1301–1307 (1976).
21. Hammerstedt, R. H., Hay, S. R. & Amann, R. P. Modification of ram sperm membranes during epididymal transit. *Biol. Reprod.* **27,** 745–754 (1982).
22. Yamaguchi, Y. *et al.* Identification of multiple novel epididymis-specific β-defensin isoforms in humans and mice. *J. Immunol.* **169,** 2516–2523 (2002).
23. Com, E. *et al.* Expression of antimicrobial defensins in the male reproductive tract of rats, mice, and humans. *Biol. Reprod.* **68,** 95–104 (2003).
24. Coronel, C. E., Winnica, D. E., Novella, M. L. & Lardy, H. A. Purification, structure, and characterization of caltrin proteins from seminal vesicle of the rat and mouse. *J. Biol. Chem.* **267,** 20909–20915 (1992).
25. Zhang, L. *et al.* Contribution of human α-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. *Science* **298,** 995–1000 (2002).
26. Hu, Y. X. *et al.* Get effective polyclonal antisera in one month. *Cell Res.* **12,** 157–160 (2002).
27. Welch, J. E. *et al.* Human glyceraldehyde 3-phosphate dehydrogenase-2 gene is expressed specifically in spermatogenic cells. *J. Androl.* **21,** 328–338 (2000).
28. Jiang, J. L. *et al.* The involvement of HAb18G/CD147 in regulation of store-operated calcium entry and metastasis of human hepatoma cells. *J. Biol. Chem.* **276,** 46870–46877 (2001).
29. Chan, H. C., Ko, W. H., Zhao, W., Fu, W. O. & Wong, P. Y. Evidence for independent Cl^- and HCO_3^- secretion and involvement of an apical Na^+-HCO_3^- cotransporter in cultured rat epididymal epithelia. *Exp. Physiol.* **81,** 515–524 (1996).
30. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260,** 3440–3450 (1985).