DICER1 regulates antibacterial function of epididymis by modulating transcription of β-defensins

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DICER1 is a key enzyme responsible for the maturation of microRNAs. Recent evidences suggested that DICER1 and microRNAs expressed in epididymis were involved in the control of male fertility. However, the exact mechanism remains to be elucidated. Here, we created a mouse line by targeted disruption of Dicer1 gene in the principal cells of distal caput epididymis. Our data indicated that a set of β-defensin genes were downregulated by DICER1 rather than by microRNAs. Moreover, DICER1 was significantly enriched in the promoter of β-defensin gene and controlled transcription. Besides, the antibacterial ability of the adult epididymis significantly declined upon Dicer1 deletion both in vitro and in vivo. And a higher incidence of reproductive defect was observed in middle-aged Dicer1−/− males. These results suggest that DICER1 plays an important role in transcription of β-defensin genes, which are associated with the natural antibacterial properties in a microRNA-independent manner, and further impacts the male fertility.

Keywords: epididymis, antibacterial property, DICER1, β-defensin

Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs (21–24 nucleotides) as the regulator of translation initiation and mRNA stability (Bartel, 2018). In mammals, two key enzymes participate in the biogenesis of canonical miRNAs: the RNase III DROSHA executes the nuclear processing of primary miRNAs (pri-miRNAs) with DiGeorge syndrome chromosomal region 8 (DGCR8) to produce pre-miRNAs, the RNase III DICER cleaves the pre-miRNA hairpin into mature miRNAs in the cytoplasm (Bartel, 2018). Then one single-strand of mature miRNA is incorporated into the miRNA-induced silencing complexes (miRISCs), interacts with mRNA targets that have similar sequences and silences the gene expression (Jonas and Izaurrealde, 2015). Quantitative studies have suggested that the miRNAs establish an expression threshold for their target genes to provide a safety mechanism against random fluctua-
sions and transient signals (Legewie et al., 2008; Levine and Hwa, 2008). Therefore, dysregulation of miRNAs induces the disorder of functional gene expression, which leads to dysontogenesis and pathogenesis. A great number of indications reveal that miRNAs are involved not only in the development of different organs including hearts, skeletal muscles, brains, kidneys, lungs, liver and pancreas but also in various diseases such as cardiac hypertrophy, rhabdomyosarcoma (Sayed and Abdellatif, 2011). Recently, the miRNA is also involved in male reproductive system. miRNAs in mouse testis were reported to differentially express from pre-puberty to adulthood (Yu et al., 2005). Deletion of testicular miRNAs, such as the mir-34b/e cluster and the mir-449 cluster, was noted to impair spermatogenesis and male fertility (Comazzetto et al., 2014; Wu et al., 2014). However, the source, function and molecular mechanism of miRNAs in the reproductive system are largely unknown.

The epididymis is a male reproductive organ. In mammals, spermatozoa are produced in the testis. However, it is often overlooked that testicular sperm cannot swim to recognize and fertilize ova until they undergo a programmed maturation process during their migration through the epididymis. The epididymis can be roughly divided into the caput, corpus and cauda...
regions (Cosentino and Cockett, 1986). More precisely, for example, the epididymis of mouse can be divided into 10 intraregional segments (Jelinsky et al., 2007). The principal cell is the main cell type found in the epididymis of all mammals. Within these columnar epithelial cells, protein expression and secretion is segment-dependent and tightly regulated, therefore characterizing a special luminal fluid to protect and modify sperms (Johnston et al., 2005; Jelinsky et al., 2007). Actually, 70%–80% of proteins secreted into the epididymal lumen are from the caput region (Cornwall, 2009). The gene expression in principal cells of caput epididymis is essential for sperm maturation. However, the molecular mechanisms involved in the control of regionalized gene expression are not fully understood.

It is well established that the gene expression in the epididymis is regulated by several factors, including hormones, transcription factors and paracrine factors in a region-specific manner (Belleannée et al., 2012b). Recent evidence has revealed a novel and extremely important role of non-coding RNAs and DICER1 in the regulation of epididymal gene expression. Our studies have documented the presence of miRNAs in human epididymis and several are differentially expressed in newborn and adult (Zhang et al., 2010). The comparative profiling of epididymal miRNAs has revealed the distinct temporospatial patterns in human and mouse epididymis (Zhang et al., 2010; Belleannée et al., 2012a). Therefore, by regulating target genes, miRNAs may play roles in the proliferation of epitheliums, the development and maintenance of epididymis (Ma et al., 2012). Moreover, the deficiency of DROSHA or DICER1 usually leads to the depletion of bulk of miRNAs (Kim et al., 2016). Some studies have shown that the conditional knockout of Dicer1 in the proximal caput epididymis results in development failure associated with dedifferentiated epithelium and instability of the sperm membrane and reduced ability of fertilize the oocyte (Bjorkgren et al., 2012, 2015). Related gene expression changes are triggered by miRNAs in this distinct region (Jerczynski et al., 2016). Besides, Dicer1 is not restricted to the miRNA processing. DICER1 is involved in removing the accumulated endogenous dsRNA and contributes to the integrity of rDNA (Sinkkonen et al., 2010; White et al., 2014). The deficiency of Dicer1 halts the glowing of embryonic stem cells (ES cells) (Kanellopoulou et al., 2005); however, Dgcr8-null ES cells do not stop glowing and differentiation although Dgcr8 is essential for miRNA biogenesis (Wang et al., 2007). The phenotypic difference suggests that DICER1 regulates the differentiation of ES cells without miRNAs. Similar incidents occur in other tissues. DICER1 is required by murine follicular development, but DROSHA is dispensable (Yuan et al., 2014). By comparing the non-overlapping functions between the main components in miRNA processing approaches, the role of DICER1 in retrotransposon transcript degradation is revealed that losing of DICER1 induces Alu RNA toxicity which is related with macular degeneration (Kaneko et al., 2011). The particularity intriguing point is the genome-wide interaction of DICER1 with RNA polymerase II (POL II) (White et al., 2014). It may suggest the function of DICER1 in regulating gene transcription. A research work has shown that double hydrogen testosterone (DHT) can stimulate DICER1 to occupy the promoter of prostate-specific antigen gene (Redfern et al., 2013). But the detailed mechanism is unknown. The miRNA-independent fuctions of DICER1 and DROSHA in epididymis are still to be explored.

In this study, we have created a conditional gene knockout mouse line, in which Dicer1 was deleted in the principal cells of distal caput epididymis (epididymal segment IV & V, CAP4/5) by using Lcn5-Cre-loxP system. We assessed the role of Dicer1 in male fertility and antibacterial ability of epididymis. The Dicer1-null mice showed a phenotype resembling the human disease epididymitis and concomitantly infertility. Furthermore, we used multiple genome-wide sequencing data to obtain the maps of miRNAs and mRNAs in the epididymis of adult Dicer1−/− mice. By analyzing these expression profiles and functional studies, we revealed a set of β-defensin genes transcriptional controlled by DICER1 with the miRNA-independent manner in epididymis. This is the first demonstration that DICER1 regulates the antibacterial ability of epididymis through controlling the transcription of β-defensin genes in epididymial cells directly and influences the latter male fertility. This study likely contributes to understand the significance of DICER1 expressed in principal cells in maintaining the epididymis homeostasis and causes of epididymitis.

Results

Dicer1 is highly expressed in the caput region of adult mouse epididymis

Dicer1 is known to express persistently in the epididymis of mouse from birth into postnatal day 42 (Bjorkgren et al., 2012), but its tissue- or region-specific expression pattern in adult mouse is ambiguous. We profiled the Dicer1 mRNA expression of multiple organs by real-time quantitative PCR (qPCR) and found the highest level of Dicer1 mRNA in the caput region of epididymis (caput EP) in 2-month-old (2 M) mouse (Figure 1A). Immunohistochemical staining also showed a protein expression trend in accordance with mRNA (Figure 1B). Moreover, DICER1 protein was wildly located in epididymal epithelium (Figure 1C).

Dicer1 is conditionally disrupted in the CAP4/5 of mouse epididymis

Since the systematic knockout of Dicer1 is embryonic lethal, we generated a conditional knockout mouse line in which Dicer1 was ablated in the CAP4/5 of adult epididymis by crossing the Dicer1mT/mG mice with the Lcn5-Cre mice (Xie et al., 2013). To verify the spatial and temporal disruption of Dicer1 gene, genomic DNA from the epididymis and several other organs were analyzed by PCR using the specific primer pair DicerF and DicerDel1 which bind to the sequence near flox sites (Figure 2A). The recombination of Dicer1 was observed in 30-day-old Dicer1l−/− mice and reached maximum in 60-day-old Dicer1−/− mice (Figure 2B). The deletion of Dicer1 occurred specifically in CAP4/5 (Figure 2C). The mT/mG mouse line can be used as a tool for visualization of CRE activation. We crossed mT/mG mice with Dicer1l−/− mice. The triple transgenic progeny demonstrated a powerful CRE activation in CAP4/5 (Figure 2D) and a dramatically upregulated Cre mRNA level (Figure 2E). And
the Dicer mRNA was significantly downregulated in the same region (Figure 2f).

**Dicer deletion induces age-associated fertility defects**

Macroscopic evaluation of epididymides in the 2 M Dicer−/− mice revealed no obvious difference from those of wild-type mice (Figure 3A). The hematoxylin-eosin (HE) staining assay showed no obvious difference of cell layer and sperm (Figure 3B). Bromodeoxyuridine (5-bromo-2′-deoxyuridine, BrdU) can be incorporated into the newly synthesized DNA of replicating cells as an analog of thymidine and indicates the proliferative capacity of sperm from mice (Figure 3C). The expression of Dicer mRNA in different organs of the mouse. The expression of Dicer in heart is set as 1. Data in the bar graph are expressed as mean ± SEM (n = 3). (B) Immunohistochemical staining analysis of Dicer protein in mouse epididymis. (C) The distribution of Dicer protein in mouse epididymal cells. IS, initial segment; CAP, caput epididymis; COR, corpus epididymis; CAU, cauda epididymis.

![Figure 1](image1.png)

**Figure 1** Dicer1 expressed highly in the caput of epididymis. (A) RT-qPCR analysis of Dicer1 mRNA in different organs of the mouse. The expression of Dicer1 in heart is set as 1. Data in the bar graph are expressed as mean ± SEM (n = 3). (B) Immunohistochemical staining analysis of Dicer1 protein in mouse epididymis. (C) The distribution of Dicer1 protein in mouse epididymal cells. IS, initial segment; CAP, caput epididymis; COR, corpus epididymis; CAU, cauda epididymis.

To panoramically understand the change of mRNAs by Dicer1 knockout in CAP4/5, we performed the transcriptome sequencing (Figure 4A). We identified 15847 unique mRNAs that were mapped to the mouse transcriptome. Among them, about 77% mRNAs were unchanged, only 9% genes were downregulated and 14% genes were upregulated (Figure 4B). Interestingly, the highly expressed genes seemed more inclined to be downregulated upon Dicer1 knockout (Supplementary Figure S1). Moreover, the highly expressed genes in the top, such as β-defensin 30 (Defb30), Bin1b, and β-defensin 17 (Defb17), were all the members of β-defensin gene family. The gene ontology (GO) pathway enrichment analysis showed that these downregulated genes were involved in the defense response to bacterium which was mainly responsible by β-defensin genes (Figure 4C). And the vast majority of β-defensin genes were downregulated upon Dicer1 ablation (Figure 4D).

In order to confirm the changes of β-defensin genes, we picked up several β-defensin genes and performed quantitative analysis by qPCR. The results showed that the mRNA level of these β-defensin genes increased dramatically in 13 M mice. Interestingly, the expression of Dicer1 also increased with age in epididymis. The expression of β-defensin genes decreased in the epididymis of 2 M Dicer1−/− mice, and the reduction of β-defensin gene expression was also identified in the CAP4/5 of 13 M Dicer1−/− mice (Figure 5A).
Furthermore, we singled out the Bin1b which expressed in CAP3/4/5 as a representative gene to investigate the change of β-defensin protein. The immunohistochemical staining assay showed a decreased level of BIN1B protein expression in the Dicer1−/− group (Figure 5C). β-defensins genes are androgen-dependent. Here, we did not find changes of the androgen receptor (AR) expression in the CAP4/5 (Figure 5B and D).

Escherichia coli and Candida albicans are the frequent pathogens of acute human epididymitis while Staphylococcus aureus is the seldom one (Michel et al., 2015). To verify the effect of β-defensins reduction on epididymal functions, we tested the antibacterial ability of CAP4/5 tissue homogenate using three different strains. The results indicated that the suppressive effect of tissue homogenate on E.coli dropped significantly in Dicer1−/− mouse (Figure 6A). Therewith, the result of E.coli challenge in vivo also showed the impaired antibacterial ability of Dicer1−/− epididymis. And the E.coli infection contributed to the inflammation of epididymis in Dicer1−/− group (Figure 6B and C). Actually, Dicer1−/− epididymis of 13 M mice showed swelling (Figure 6D) and retrograde infection (Supplementary Figure S2). And a large number of leukocyte cells were observed in the lumen gap of Dicer1 KO epididymis (Figure 6E). Besides, epithelial damage and upregulated inflammatory cytokines including interleukin-1beta (Il1b), interleukin-6 (Il6) and tumor necrosis factor alpha (Tnfa) were present in Dicer1 KO mice (Figure 6F).
The transcription of β-defensin genes are regulated by DICER1 in a miRNA-independent manner

To search the mechanism underlying the change of β-defensin expression, we performed the small RNA sequencing and miRNA binding sites analysis. As expected, miRNAs were significantly decreased in Dicer1−/− tissue (Supplementary Figure S3). Strikingly, 24% detectable genes in mouse CAP4/5 contained no miRNA binding sites while 74% genes contained more than two miRNA binding sites by scanning the whole genome to search conserved miRNA target sites using TargetScanMouse 7.1. (Figure 7A). Another interesting result was that in addition to Bin1b (two binding sites), β-defensin 25 (Defb25, two binding sites), β-defensin 36 (Defb36, two binding sites) and β-defensin 48 (Defb48, one binding site), β-defensin genes practically had no miRNA target site, while average target sites of whole genome were 45.44 (Figure 7B). We isolated and cultured the primary cells from the CAP4/5 of mouse and knocked down the expression of Dicer1 and Drosha which initiated miRNA biogenesis at the upstream of Dicer1. The qPCR results showed that downregulation of β-defensin genes was associated with Dicer1

Figure 3 Morphology of the epididymis and fertility in Dicer1−/− mice. (A) The representative light microphotographs of epididymis in control mice and Dicer1−/− mice. (B) HE staining visualizing the histological structure of CAP4/5 in control mice and Dicer1−/− mice. (C) BrdU and hochest staining detecting the cell proliferation and apoptosis in epididymis. Cells with the ability to proliferate were labeled by green fluorescent (BrdU). Nuclei were labeled by blue fluorescent (Hoechst). (D) Computer-assisted sperm analysis (CASA) measuring the motility of the sperm. Two primary parameters, ‘motile’ and ‘progressive’, were used to characterize the sperm motility. Data in the bar graph are expressed as mean ± SEM (n = 5). ns, not significant, ****P < 0.0001. Each parameter is analyzed, respectively, using two-way ANOVA followed by Sidak’s multiple comparisons test. (E) Mating test detecting the fertility of Dicer1−/− male mice. The horizontal lines indicate mean ± SEM (n = 5). ns, not significant; *P < 0.05 (Mann–Whitney test).
rather than Drosha (Figure 7O). These results suggested that DICER1 could regulate the transcription of β-defensin genes with a miRNA-independent manner. Next, we found an enrichment of DICER1 in the promoter of Bin1b gene by ChIP-qPCR. And POL II failed to bind to the transcription start site (TSS) of Bin1b upon Dicer1 knockout (Figure 7D). Moreover, the transcriptional activity of Bin1b declined in Dicer1−/− cells and wild-type DICER1 could rescue it (Figure 7E). The RNase III domains and PAZ domain of DICER1 are essential for miRNA biogenesis (Yuan et al., 2014). Double mutation of the RNase IIIa and IIIb (D1310A/D1693A) or mutation of the PAZ (Y961A/Y962A) resulted in failing to generate mature miRNAs (Supplementary Figure S4A). The DICER1 with mutations still rescued the gene transcription (Figure 7E). These results revealed that DICER1 could exploit DNA to reach its targets and regulated the transcription of β-defensin genes (Figure 7F).

**Discussion**

The sperm maturational disorders were identified among up to 40% of infertile men (Cornwall, 2009). It is truly frightening that couples who desire a child turn to assisted reproductive techniques such as intracytoplasmic sperm injection using sub-optimal sperm regardless of increased defects in offspring. The better acquainted with encounters of sperm in epididymis may drive an alternative therapy. In this study, we created a conditional knockout mouse line in which Dicer1 was deleted in the principal cells of distal caput epididymis in adult mice to explore the role of epididymis in sperm maturation.
Consistent with previous studies, the biogenesis of miRNAs, especially the biogenesis of high expressed miRNAs, was disrupted obviously upon Dicer1 ablation in the mouse epididymis. As the result of miRNA action, target mRNAs usually are silenced. So, the downregulation of miRNAs which is due to the deletion of Dicer1 certainly increases the expression of target mRNAs. As expected, quite a number of the mRNAs were upregulated in the Dicer1−/− tissue. GO pathway analysis of differentially expressed mRNAs revealed that the deletion of Dicer1 caused disturbance of antibacterial ability, which was associated with the β-defensin gene family. Interestingly, β-defensin genes were downregulated in the Dicer1−/− tissue. It may offer a clue to the new mode of gene regulation.

β-defensins are a set of secreted peptides involved in diverse processes including antibiosis, immune modulation, and sperm motility (Li et al., 2001; Diao et al., 2014; Dorin and Barratt, 2014). In the 2 M and 6 M Dicer1−/− mice, the sperm motility was not affected following the depletion of β-defensins. It could be that the depletion of β-defensins only existed in CAP4/5 while the expression in other regions was normal. But the ability of antibacteria was impaired significantly in the Dicer1−/− tissues both in vitro and in vivo. The phenotype of Dicer1-null epididymis looks like the human disease epididymitis extremely. The epididymitis is a common disease that may attack men at any age. And up to 40% of patients become sterile even after the cure (Rusz et al., 2012). In lipopolysaccharide- and microorganisms-induced epididymitis, the mRNA expression of β-defensins usually changes dramatically, which showed the relation of β-defensins and epididymitis (Michel et al., 2015). It is different from these models that our mouse model simulates

![Figure 5](https://example.com/figure5.png)

**Figure 5** Dicer1 deficiency leads to downregulation of β-defensin genes. (A) RT-qPCR analysis measuring the mRNA level of Defb30, Bin1b, Defb17, and Dicer1 in Dicer1−/− epididymis. The expression of Defb30 and Dicer1 in 2 M WT mice is set as 1. Data in the bar graph are expressed as mean ± SEM (n = 3). ***P < 0.001, ****P < 0.0001. Each gene is analyzed respectively using two-way ANOVA followed by Tukey’s multiple comparisons test. (B) RT-qPCR analysis measuring the mRNA level of AR in Dicer1−/− epididymis. The expression of AR in WT group is set as 1. Data in the bar graph are expressed as mean ± SEM (n = 3). ns, not significant, unpaired t-test. (C) Immunohistochemical staining analyzing the protein level of BIN1B in Dicer1−/− epididymis. (D) Immunohistochemical staining analyzing the protein level of AR in Dicer1−/− epididymis.
**Figure 6** The expression of Dicer1 is essential for the antibacterial properties of epididymis in vitro and in vivo. (A) Detection of the antimicrobial ability of Dicer1−/− epididymis in vitro. Data in the bar graph are expressed as mean ± SEM (n = 3). ns, not significant, *P < 0.05, **P < 0.01. The data of E. coli and C.albicans are analyzed, respectively, using unpaired t-test. The data of S.aureus are analyzed using unpaired t-test with Welch’s correction. (B) Bacterial challenge assay measuring the antimicrobial ability of Dicer1−/− epididymis in vivo. E. coli k12 suspension was injected in mouse epididymis from deferent duct to construct a simulate model of epididymis infection. The contra-lateral epididymis was injected with equal volume of PBS serving as the performance control. The epididymis of Dicer1−/− mice infected
the infection in vivo after the imbalance of homeostasis. Our data showed epididymis dysfunction lead by Dicer1 defect did not include the ability of anti-C. albicans. But, oddly, the ability to fight S. aureus was greatly strengthened in Dicer1−/− mice. In another project of our lab, we found the ability of anti-S. aureus decreased in the epididymis of Bin1b overexpression mice (unpublished). This may indicate the inner link between Bin1b and S. aureus. Nevertheless, more evidence is needed to come to light the inner mechanism.

Further exploration detected the retrograde infection and inflammatory infiltrations in epididymis as the Dicer1−/− mice grew up and became older, displaying the state of epididymitis which is much closer to real illness events. The under reasons could be ascribed that a subset of target genes being regulated by DICER1, such as β-defensin genes, could help epididymis to response to accumulated environmental stimuli. A comparative study of epididymal genes expression in people of different age showed that 9 of 19 β-defensin genes were upregulated in aged human epididymes, compared with young adult group (change fold ≥2; Reynolds et al., 2017). The present qPCR results of β-defensin genes in mouse epididymis agreed with the data from this comparative study. These secretory defensin peptides expressed highly in aged epididymis may contribute to maintain the homeostasis of epididymis. Thus, dysfunction of β-defensins by Dicer1 defect leads to the higher risk of infection. In contrast, overexpression of β-defensin or intravenous administration of β-defensin peptide can reverse the epididymitis in E.coli-infected animals (Fei et al., 2012; Biswas et al., 2015). It may be a creditable attempt that giving supplements of β-defensin peptides to patients.

By the time Dicer1-null male mice reached 13 M, sperm count in the caudal epididymis decreased dramatically (data not shown) and sperms from these mice showed very low motility. The continuous tracking data of mating test showed a higher incidence of fertilizing defects in middle-aged Dicer1-deficient males compared with WT males of the same age, in spite of the seasonal variation of birth rate (Reynolds et al., 2017). The male fertility disorder in middle-aged Dicer1−/− mice appears to be a secondary effect induced by sperm contamination. These findings are supported by some clinical studies. About 15% of male infertility cases are assigned to the infection of urinary tract including infection of the testis, epididymis and prostate (Henkel et al., 2006; Pellati et al., 2008; Domes et al., 2012). The clinical study based on 1256 infertile men shows that about 33.2% of semen samples are contaminated with bacteria. Out of the 246 semen samples included finally in the study, 50 samples (20.3%) show the presence of E. coli, and 67.4% of individuals with E. coli infection are infertile. The under reason is that bacterial contamination contributes to the apoptosis and necrosis of sperm, which leads to serious infertility (Moretti et al., 2009). Moreover, a study demonstrated that the decrease of some β-defensins by immunologic stimulant and testicular inflammation might aggravate the infertility (Cao et al., 2010). Even with antibiotic treatment, up to 40% have fertility problems as a result of the disease (Rusz et al., 2012). So it is very important to develop new therapeutic approaches that can solve the fertility problems.

Using comprehensive analysis of transcriptome map and genome-wide miRNA binding sites, we identified the β-defensin genes not controlled by miRNAs. Then the Dicer1 and Drosha were separately knocked down in the primary cells of mice epididymal epithelial with RNA interference. Using qPCR assay, we were able to verify and validate that the β-defensin genes were regulated by Dicer1 directly but not by miRNAs, no matter whether the β-defensin gene harbored miRNA binding sites or not. To further uncover the mechanism of gene regulation, we selected Bin1b, a member of β-defensin family. We performed ChIP, Dicer1 mutation (leading to the biogenesis disturbance of miRNAs), and fluorescence assay and were able to prove that the Bin1b gene was transcribed directly by Dicer1. These results confirm that DICER1 could shape the antibacterial ability by transcriptional regulatory activity.

Overall, this study may provide a new tool for investigating the underlying reasons of epididymitis and brings to the forefront a miRNA-independent transcriptional regulation role of DICER1 in modulating the antibacterial property of epididymis and consequent male fertility.

Materials and methods
Experimental animals
To obtain mice with inactive DICER1 in the principal cells of the caput epididymis, Dicer1fl/fl mice were bred to the Lcn5-Cre mice strain which can express specially CRE enzyme in principle cells in the distal caput of epididymis. The Cre allele was genotyped by standard PCR with the primers tgCre F and tgCre R. The floxed allele and the deletion allele were genotyped according to the published article (Harfe et al., 2005). All primer sequences used in this study are available in Supplementary Table S1.

RNA isolation and RT-qPCR
For mRNA detection, total RNA from mouse epididymis was isolated with Trizol reagent (Invitrogen), 0.5 μg RNA was reverse transcribed to cDNA according to the user’s manual of ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (TOYOBO). Then

with E.coli k12 became inflamed. (E) Bacteria in the epididymis of Dicer1−/− mice appendix multiply rapidly (the red arrow), causing the retrograde infection (yellow arrow). CAP1/2/3: G5-CAP2/3. (D) The Dicer1−/− mice have higher risks of epididymis infection with age. The epididymis of 13 M mice showed swelling (the red arrow). (E) Tubule edema and massive infiltration of leukocytes were observed in the epididymis of 13 M mice in Dicer1−/− group (yellow arrows). Besides, microvilli missing of principle cells was also observed in Dicer1−/− group, indicating epididymal epithelial damages (red arrows). (F) RT-qPCR analysis measuring the mRNA level of Il1b, Il6 and Tnfa in the epididymis of 13 M mice. The mRNA expression in WT group is set as 1. Data in the bar graph are expressed as mean ± SEM (n = 8). ns, not significant, *P < 0.05, **P < 0.01, unpaired t-test.
Figure 7 DICER1 regulates the transcription of β-defensin genes in a miRNA-independent way. (A) Conjoint analysis of mRNA expression and predicted miRNA binding sites. Y-axis shows the expression value of mRNAs (log FPKM). X-axis shows the corresponding number of miRNA binding sites. This analysis reveals a set of genes that have 0 miRNA binding site, indicating that these genes are not regulated directly by miRNAs (red dots). (B) Analysis of average miRNA binding sites per mRNA. The short horizontal lines indicate the mean. (C) Contrastive analysis of the expression of defensin genes upon Dicer knockdown and Drosha knockdown using RT-qPCR (mean ± SEM; n = 3). ns, not significant, *P < 0.05, **P < 0.01, ***P < 0.001. Each mRNA expression in WT group is set as 1 and is analyzed, respectively, using unpaired t-test. (D) Quantification of DICER1 and POL II in the promoter of Bin1b determined by ChIP (mean ± SEM; n = 3). **P < 0.01, paired t-test and two-way ANOVA followed by Sidak’s multiple comparisons test. (E) Luciferase assay testing the transcription activity of DICER1 (NC is set as 1, mean ± SEM; n = 3). **P < 0.01, ****P < 0.0001, ANOVA followed by Tukey’s multiple comparisons test. (F) A model for transcription control of gene expression by DICER1.
qPCR was performed for quantification using standard procedures on a Rotor-Gene Q 5plex HRM Platform (QIAGEN). For miRNA detection, RNA was extracted with mirVana miRNA Isolation Kit (Ambion), 0.5 μg RNA was reverse transcribed using the stem-loop RT primer as described (Kramer, 2011). Then the qPCR still was manipulated according to the standard procedures on a Rotor-Gene Q 5plex HRM Platform. All primer sequences used in this study are available in Supplementary Table S1.

**Immunohistochemistry staining assay**

Mouse epididymis was fixed in 4% paraformaldehyde and embedded in paraffin. After deparaffinage and rehydration, sections were dipped into 3% H2O2 for 15 min to block endogenous peroxidase. Then, these samples were boiled in citrate antigen retrieval solution twice, 5 min every time and naturally cooled to room temperature. For IHC, epididymis sections were incubated with second antibody and colored with GTVision Ⅲ detection system/Mo&Rb (GeneTech) followed by hematoxylin counterstaining.

**BrdU incorporation assay**

BrdU (B5002, Sigma-Aldrich) is diluted in PBS to make a sterile solution of 20 mg/ml. The BrdU solution was injected into mice abdominal cavity as concentration of 200 mg/kg. Then these animals were mercy killed using carbon dioxide after 24 h. The epididymis tissues were fixed with 4% paraformaldehyde for 24 h at 4°C, tissues were then dehydrated with the different concentration ethanol, vitrificated by dimethylbenzene, infiltrated with paraffin, and made of paraffin section. These samples were boiled in citrate antigen retrieval solution for 30 min after deparaffinage and cooled in ice water mixture immediately. For BrdU detection, these sections were incubated in 10% (v/v) goat serum/PBS at room temperature for 30 min and with the BrdU monoclonal antibody at 1:50 (05–633, Millipore) at 4°C overnight. After PBS washes three times for ~5 min per wash, the FITC-conjugated secondary antibody (Jackson Immuno Research) was added at 1:1000 at room temperature for 1 h. At last, sections were co-stained with Hoechst 33258 (B2883, Sigma-Aldrich).

**Fertility test**

_Dicer1<sup>−/−</sup> _male mice and control mice were mated with C57BL/6 female mice last for a year. The physiological state and little size of mated female mice were closely observed during the experiment. And every mated female mouse was replaced to the new one after delivering two litters to avoid the effects from physiological changes of female mice.

**Computer-assisted sperm analysis (CASA)**

As previously described (Zhou et al., 2013), the epididymis was stripped away from the surrounding tissues such as fat-pad and blood vessels and so on. The cauda epididymis was cut and transferred into 1 ml EKRB medium which was warmed up to 37°C. To release the sperms sufficiently, cut the tissue in several places and incubate in medium for 5 min. Then the sperm was placed in a Sperm Analysis Chamber (Hamilton Thorne Research) and analyzed using a CASA machine (HTM-TOX IVOS sperm motility analyzer, Animal Motility, version 12.3 A; Hamilton Thorne Research) followed by concentration adjustment.

**RNA-seq and analysis**

For transcriptome sequencing, the total RNA was extracted from the segment IV & V of epididymis (CAPA4/5), the poly-A containing mRNAs were enriched using oligo-dT attached magnetic beads from the total RNA. Then the mRNA library was constructed using TruSeq RNA Sample Preparation Kit V2 (Illumina) as follows: (i) Isolated mRNAs were fragmented into small pieces ~200 nt using divalent cations. (ii) Complementary DNAs (cDNAs) were synthesized. (iii) One microgram cDNA for each sample was used for end repair, A-tailing, and adapter ligation. (iv) The cDNAs with adapters were amplified using PCR. The library was amplified to produce clonal clusters on cBot (Illumina) using HiSeq PE Cluster Kit v4-cBot-HS (Illumina) and then was sequenced with HiSeq SBS Kit v4-HS (Illumina) by Illumina Hiseq 2000 (Illumina). Then, 100 bp paired-end reads were cleaned and mapped to mouse genome by TopHat2 short read mapper version 2.1.0. The differential expression genes were analyzed with fold change. GO analysis of differential expression genes (change fold ≥2) was performed by the Gene Ontology enRichment anaLYsis and visuaLizAtion tool (GORILLA, http://cbl-gorilla.cs.technion.ac.il/).

For small RNA-seq, 1 μg purified small RNA fragments were modified with 3’ adapter and 5’ adapter. Sequencing was performed using Illumina Hiseq 2000 followed reverse transcription and PCR amplification. The clean reads were mapped to the library of mouse miRNAs by Bowtie. The miRNA targets and corresponding annotations mRNAs were downloaded from TargetScanMouse (http://www.targetscan.org/mmu_71/mmu_71_data_download/Conserved_Family_Conserved_Targets_Info.txt.zip).

**Antimicrobial activity in vitro assay and bacterial challenge assay**

_Escherichia coli_ k12 strain ( _E. coli_ k12), _candida albicans_ strain ( _C. albicans_ ) and _staphylococcus aureus_ strain ( _S. aureus_ ) were preserved by our lab which was brought to 50% glycerol. The _E. coli_ k12 was grown in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) with 50 mg/L streptomycin at 37°C for 4 h. The _C. albicans_ was grown in Yeast extract-Trypton-Dextrose medium (2% trypton, 1% yeast extract, and 2% glucose) at 30°C for 4 h. The _S. aureus_ was grown in Mueller–Hinton Broth (0.2% beef extract, 1.75% casein hydrolysate, and 0.15% starch) at 37°C for 4 h.

For antimicrobial activity _in vitro_ assay, six pieces of epididymis segment IV & V from three mice were placed in a clean round-bottom microcentrifuge tube, and ice-cold PBS was added to the tube up to 300 μl. Then tissues were homogenized.
rapidly on ice. *E. coli k12, Calbicans*, and *S.aureus* suspensions were washed with ice-cool PBS and the concentration of suspension was measured and adjusted to $1 \times 10^2 /50 \mu l$, respectively. Tissue homogenate was divided into three parts, and then every part (100 µl/part) was mixed with 50 µl microbial solution and incubated on a plate medium with 1.5% agar at 37°C. The same volume of PBS (100 µl) was incubated with 50 µl microbial solution as performance control. Colonies were counted after 16 h. The antimicrobial ratio was calculated according the formula as follow: antimicrobial ratio = 100% × (colonies\text{test} - colonies\text{performance control}) / colonies\text{performance control}.

For bacterial challenge assay *in vivo*, the frozen *E. coli k12* was grown in LB with 50 mg/L streptomycin at 37°C for 4 h. The concentration of suspension was measured and adjusted. Then, $1 \times 10^3$ *E. coli k12* suspended in 5 µl sterile PBS was injected in mouse epididymis from deferent duct with a microinjection apparatus (NARISHIGE Group). The contralateral epididymis was injected, equal volume PBS serving as the performance control. The mice after surgery were surtured and housed for 3 days. Then the epididymis was dissected carefully and placed into a clean round-bottom microcentrifuge tube. Three pieces of tissues from three mice were homogenized rapidly on ice with 300 µl ice-cold PBS. Tissue homogenate was diluted gradually and printed on the plate medium using a drop-plate method as described (Chen et al., 2003). Colonies were photographed and counted after 16 h.

**Epididymal cell culture**

The primary cells from mouse epididymis were cultured as described (Lin et al., 2002), with modifications. In brief, the epididymal tissues of region 4 and 5 were minced in PBS and then incubated in 1 mg/ml collagenase type IV (C5138, Sigma-Aldrich) for 2 h at 34°C in 5% CO₂. After incubation, fragments of epididymis were centrifuged to remove collagenase, transferred in 1 ml fresh IMDM medium (with 20% FBS, 1 nM DHT, and 10 µg/ml insulin) and separated to small cell clusters using pipette.

**ChIP-qPCR**

ChIP was performed as previously described (Hu et al., 2010), with modifications. In brief, epididymis region 4 and 5 tissues from three mice were pooled together and minced in PBS. Then, 4 µg ChIP Grade antibody to DICER1 (ab14601, Abcam) was used per ChIP reaction followed crosslinking and sonicate. The protein A/G plus agarose beads (sc2003, Santa Cruz Biotechnology) were washed and DNA was eluted and purified. Then DNA was quantified by real-time quantitative PCR using standard three-step approach on a Rotor-Gene Q Splex HRM Platform (QIGEN). The sequences of primer pairs DCR ChIP F, DCR ChIP R, POL II ChIP F and POL II ChIP R used in this study are available in Supplementary Table S1.

**Construction of Dicer1 knockout 293T cell line**

The gRNAs were designed using the sgRNAsCas9 (Xie et al., 2014). Then the gRNA oligos were inserted into the PGL3-U6-sgRNA-PGK-puromycin vector and co-transfected 293T cells with pST1374-N-NLS-flag-linker-Cas9 plasmid. After 24 h, cells were incubated in medium with 1 µg/ml puromycin (Merck) and 10 µg/ml blasticidin (Sigma) for 72 h. Then cells were dissociated and planted in 96-well plate (one cell per well). The final surviving clones were analyzed by sequencing. The sequences of gRNAs hDCR_gRNAa F, hDCR_gRNAa5 R, hDCR_gRNAs10 F and hDCR_gRNAs10 R are available in Supplementary Table S1.

**Statistical analyses**

Statistical analyses were performed by using GraphPad Prism (version 7.00; GraphPad Software). *P*-values are indicated in the figures, and differences were considered significant when *P* < 0.05.

**Supplementary material**

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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**Author contributions:** C.T. performed experiments, made data analysis, and wrote manuscript. M.N. performed experiments. S.X. and Yao Zhang performed sequencing data analysis. C.C. and L.W. performed animal surgeries. C.Z. and Z.N. performed animal surgeries. C.C. and L.W. performed experiments. Yuchuan Zhou performed part of CASA experiments and edited the manuscript. Yonglian Zhang conceived of the study and edited the manuscript.

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