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NOVEL GENES AND REGULATORY SYSTEMS IN EPIDIDYMAL DIFFERENTIATION AND SPERM MATURATION OF THE MOUSE

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ABSTRACT

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Novel genes and regulatory systems in epididymal differentiation and sperm maturation of the mouse.

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Mammalian spermatozoa gain their fertilizing ability during maturation in the epididymis. Proteins and lipids secreted into the epididymal lumen remodel the sperm membrane, thereby providing the structure necessary for progressive motility and oocyte interaction. In the current study, genetically modified mouse models were utilized to determine the role of novel genes and regulatory systems in the postnatal development and function of the epididymis.

Ablation of the mouse β-defensin, *Defb41*, altered the flagellar movements of sperm and reduced the ability of sperm to bind to the oocyte *in vitro*. The *Defb41*-deficient *iCre* knock-in mouse model was furthermore utilized to generate *Dicer1* conditional knock-out (cKO) mice. DICER1 is required for production of mature microRNAs in the regulation of gene expression by RNA interference. *Dicer1* cKO gave rise to dedifferentiation of the epididymal epithelium and an altered expression of genes involved in lipid synthesis. As a consequence, the cholesterol:polyunsaturated fatty acid ratio of the *Dicer1* cKO sperm membrane was increased, which resulted in membrane instability and infertility.

In conclusion, the results of the *Defb41* study further support the important role of β-defensin family members in sperm maturation. The regulatory role of *Dicer1* was also shown to be required for epididymal development. In addition, the study is the first to show a clear connection between lipid homeostasis in the epididymis and sperm membrane integrity. Taken together, the results give important new evidence on the regulatory system guiding epididymal development and function.

Keywords: epididymis, sperm maturation, β-defensins, *Dicer1*, miRNA, lipid homeostasis, sperm motility, sperm-oocyte interaction, fertility

TIIVISTELMÄ

Ida Björkgren

Uudet geenit ja säätelyjärjestelmät hiiren lisäkiveksen kehityksessä ja siittiöiden kypsymisessä.

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Nisäkkäiden siittiöt kypsyvät lisäkiveksessä, jossa epiteelisolujen erittämät proteiinit ja lipidit muokkaavat siittiön membraanin rakennetta mahdollistaen siittiöiden liikkuvuuden ja hedelmöityskyvyn. Tässä väitöskirjatyössä tutkittiin lisäkiveksen kehityksen ja siittiöiden kypsymisen säätelyä käyttämällä geenimuunneltuja hiirimalleja. Tutkimuksemme osoitti, että β-defensiinin, *Defb41*, poisto muuttaa siittiön hännän liikettä ja vähentää siittiöiden kykyä sitoutua munasoluun *in vitro*. Osoitimme myös, että *Defb41* -hiirimallia voidaan käyttää lisäkivesspesifisten poistogeenimallien luomisessa.

DICER1 on välttämätön RNAi-signalointia välittävien mikroRNA:den valmistamiselle soluissa. *Dicer1* poisto lisäkiveksessä aiheutti lisäkivesepiteelin regression erilaistumattomaan tilaan. Lisäksi lisäkiveksen epiteelin muuttuneen rasvasynteesin johdosta siittiöiden membraanin kolesteroli:PUFA suhde oli kohonnut. Membraanirakenteen muutos aiheutti siittiöiden hajoamisen normaalia herkemmin ja siten uroshiirten hedelmättömyyttä.

Avainsanoja: lisäkives, siittiöiden kypsyminen, β-defensiini, *Dicer1*, rasva-aineenvaihdunta, siittiöiden liikkuvuus, siittiö-munasolu -vuorovaikutus, hedelmällisyys

SAMMANFATTNING

Ida Björkgren

Nya gener och regleringssystem i utvecklingen av bitestikeln och i spermiens mognad hos möss.

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Däggdjurens spermier får sin förmåga att befrukta äggcellen genom mognad i bitestikeln. Proteiner och fetter som utsöndras i bitestikelns lumen, omformar spermiens membran och ger den därmed den struktur som krävs för progressiv rörelseförmåga och för att komma i kontakt med äggcellen. I denna studie använde vi oss av genmodifierade musmodeller för att undersöka betydelsen av nya proteiner och regleringssystem för bitestikelns postnatala utveckling och funktion. Genom att förhindra uttryck av musens β-defensin gen, *Defb41*, påverkades rörelsemönstret hos spermiens flagell och förmågan att binda till äggcellen *in vitro* försämrades. Vi påvisade även att musmodellen kunde användas för att skapa konditionala knockoutmöss (cKO) för *Dicer1*.

DICER1 behövs för produktionen av mogna mikroRNA vid regleringen av genuttryck. Förhindrande av *Dicer1*'s genuttryck i bitestikeln ledde till en återgång av epitelets differentiering till ett outvecklat stadie. På grund av ett förändrat uttryck av gener viktiga för fettproduktionen i bitestikelns epitel, påvisade även *Dicer1* cKO spermiernas membran ökade kolesterolvärden medan de omättade fettsyrornas andel minskade. Det ändrade fettförhållandet hos spermierna ledde till ett instabilt membran och infertilitet.

Nyckelord: bitestikel, spermiens mognad, β-defensin, *Dicer1*, miRNA, fettomsättning, spermiens motilitet, spermie-äggcell interaktion, fertilitet

TABLE OF CONTENTS

ABBREVIATIONS

ABCA1	ATP-binding cassette, sub-family A, member 1
ABCG1	ATP-binding cassette, sub-family G, member 1
AC	acylcarnitine
AR	androgen receptor
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAP	caput
CATSPER	sperm associated cation channel
CAU	cauda
Ca_v	voltage gated Ca^{2+} channel
cKO	conditional knock-out
CLU	clusterin
COR	corpus
CRISP1	cysteine-rich secretory protein 1
DEFB41	β-defensin 41
DHA	docosahexaenoic acid
DHCR	dehydrocholesterol reductase
DHT	dihydrotestosterone
Elovl	elongation of very long chain fatty acids gene
ES cell	embryonic stem cell
ESR	estrogen receptor
F-actin	filamentous actin
FADS2	fatty acid desaturase 2
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
gACE	germinal angiotensin I-converting enzyme
GPI	glycosylphosphatidylinositol
GPX5	glutathione peroxidase 5
HEZ	heterozygous
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
HOZ	homozygous
IS	initial segment
IVF	*in vitro* fertility
IZUMO1	izumo sperm-egg fusion 1
KI	knock-in
LIN	linearity
LCN	lipocalin
LGR4	leucine-rich repeat domain containing G protein-coupled receptor 4

LH	luteinizing hormone
miRNA	microRNA
NR1H	nuclear receptor subfamily 1, group H
OCTN	Carnitine/organic cation transporter
PC	phosphatidylcholine
PGK1	phosphoglycerate kinase 1
PKA	protein kinase A
pri-miRNA	primary-microRNA
pre-miRNA	precursor-microRNA
pp	postpartum
PPIA	peptidylprolyl isomerase A
PUFA	polyunsaturated fatty acids
RNAi	RNA interference
ROS	reactive oxygen species
ROS1	ros1 proto-oncogene
RPL19	ribosomal protein L19
SACY	soluble adenylyl cyclase
si-RNA	small interfering RNA
SM	sphingomyelin
SNARE	soluble n-ethylmaleimide-sensitive attachment protein receptors
SPAG11	sperm associated antigen 11
SPINK13	serine peptidase inhibitor, Kazal type 13
STR	straightness
TCF8	transcription factor 8
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight line velocity
WD	wolffian duct
WT	wild type
ZP	zona pellucida glycoprotein

LIST OF ORIGINAL PUBLICATIONS

The study is based on the following publications and manuscripts, which are referred to in the text by Roman numerals (I-III). Unpublished data is also included.

I. **Ida Björkgren**, Lauri Saastamoinen, Anton Krutskikh, Ilpo Huhtaniemi, Matti Poutanen, Petra Sipilä (2012). Dicer1 ablation in the mouse epididymis causes dedifferentiation of the epithelium and imbalance in sex steroid signaling. *PLoS One* 7(6):e38457.

II. **Ida Björkgren**, Helena Gylling, Heikki Turunen, Ilpo Huhtaniemi, Leena Strauss, Matti Poutanen, Petra Sipilä (2014). Imbalanced lipid homeostasis in the conditional Dicer1 knockout mouse epididymis causes instability of the sperm membrane. *FASEB J*. Nov 3., accepted for publication.

III. **Ida Björkgren**, Luis Alvarez, Nelli Blank, Heikki Turunen, Teemu Daniel Laajala, Jussi Toivanen, Anton Krutskikh, Ilpo Huhtaniemi, Matti Poutanen, Dagmar Wachten, Petra Sipilä. Targeted inactivation of the mouse epididymal beta-defensin 41 alters sperm flagellar beat pattern and zona pellucida binding. *Biology of Reproduction*. Submitted.

1. INTRODUCTION

Development in testis gives rise to the familiar physiology of the mammalian sperm cell, with a head, where the genomic material is stored, and a tail that drives the cell movements. At this stage, active transcription of the sperm genome is prevented through packaging of the DNA in dense structures. However, the spermatozoon is not yet able to swim progressively or to penetrate the oocyte. Thus, to become fully mature, spermatozoa are dependent on factors outside the cells. The epididymis provides such a specialized milieu where the sperm membrane is modified through interaction with luminal proteins and lipids (Dacheux and Dacheux. 2013). Proteins secreted by the epididymal epithelium either bind to the sperm or process factors already present on the sperm surface. Lipids in the epididymal lumen are also taken up by sperm and give the membrane a more fluent structure (Jones. 2002). After maturation, the spermatozoon is stored in the distal region of the epididymis or, as in humans, in the proximal vas deferens. During ejaculation, sperm are transferred from vas deferens into the urethra and are immersed in secretions from the seminal vesicles, prostate gland and the bulbourethral glands, forming the seminal fluid. Due to maturation in the epididymis, and the nutrients and protective elements provided by the seminal fluid the spermatozoa are able to swim up the female reproductive tract and to react to signals from the oocyte.

Postnatal differentiation of the epididymis divides the duct into several segments, each with its unique pattern of gene expression (Robaire, et al. 2000). When the sperm travels from one segment to the next, the different luminal environments cause a progressive change in the sperm physiology. Due to the complex nature of the duct, *in vitro* studies are not able to fully model sperm maturation. Only *in vivo* analyses can give a more comprehensive overview of epididymal functions and the regulatory processes involved in its development. In this study, we have therefore generated a mouse model with epididymis specific expression of codon-improved Cre (*iCre*) with which it is possible to develop conditional knock-outs of genes in the mouse epididymis. In this way, we are also able to look at the role of a certain gene in isolated segments and at a specific time point of epididymal development.

Male infertility is on the rise in western countries (World Bank, 2010) and many of the idiopathic cases show defective sperm motility or sperm-oocyte interaction. This indicates problems in sperm maturation, however, little is still known about the role of epididymal proteins in this process. To study this further we have utilized genetically modified mouse models together with extensive *in vitro* studies of sperm to determine the role of a novel mouse β-defensin, *Defb41*, in sperm motility and the role of *Dicer1* in regulation of epididymal differentiation and fertility.

2. REVIEW OF THE LITERATURE

2.1. Development of the epididymis

The development of the epididymis begins in the embryo through the formation of the Wolffian ducts. After connecting to the testis, elongation and coiling of the duct takes place. The epididymis increases in size until puberty, when final differentiation of the epididymal epithelium takes place and sperm enter the duct. How the short ductal structure of the embryo can develop into the long, in humans six meters, epididymal duct will be discussed in the following chapters.

2.1.1. Embryonic development

During early embryonic development, the male and female reproductive organs are indistinguishable. It is only through the expression of the male specific sex determining region Y (*Sry*) that differentiation of the urogenital ridge into testis can begin (Koopman, et al. 1991; Hacker, et al. 1995). The epididymal duct stems from a condensation of mesenchymal cells along the urogenital ridge. During early embryonic development, the cells are rearranged to stretch out toward the cloaca and form the Wolffian or mesonephric duct (WD), that later develop into the epididymis, vas deferens, and the seminal vesicles (Fig. 1A). Two paired box gene-transcription factors, *Pax2* and *Pax8*, are required for initiation of mesenchymal-epithelial transition and tubule formation (Torres, et al. 1995; Bouchard, et al. 2002). In mice, the extended WD is present at embryonic day (E) 10.5.

The efferent ducts, connecting the testicle with the epididymal duct develop from mesonephric tubules, which form along the cranical part of the WD (Fig. 1B). A *Sry* dependent activation of a chemotactic signal, fibroblast growth factor 9 (*Fgf9*), causes migration of WD cells into the genital ridge (Capel, et al. 1999; Colvin, et al. 2001). The migrating cells are suggested to form rete testis and to further induce the connection between the testis, the mesonephric tubules and the future epididymis (Upadhyay and Zamboni. 1981). Although the WD is a singular tubule, the efferent ducts differentiate from multiple mesonephric tubules and also form several points of connection with the epididymis of larger mammals. In rodents, however, the efferent ducts converge to form a single tubule before epididymal contact, while the connection to rete testis still goes through multiple points of entry (Guttroff, et al. 1992). The difference in efferent duct development can also been seen in the most proximal part of the rodent epididymis, where cells from the mesonephric tubules are suggested to form part of the first segment of the epididymis, the initial segment (Hinton, et al. 2000). Larger mammals lack this segment, which is known to be of specific importance for the maturation of rodent sperm.

Segment formation. After connecting to the testis, rapid elongation and coiling of the epididymis takes place. An anterior-posterior gradient of inhibin-beta A initiates coiling in the most proximal part of the mouse WD at E16.5 (Fig. 1D, Tomaszewski, et al. 2007). The actual structures of the ductal coils are regulated by testicular fluid, secreted into the duct, and cell proliferation and possible "hotspots" of cell division and/or apoptosis in the duct (reviewed in Joseph, et al. 2009). In rodents, coiling of the duct leads to the formation of a "head" segment (consisting of the initial segment, IS, and caput, CAP), a long more outstretched "body" (corpus, COR) and the highly coiled "tail" (cauda, CAU). In humans, CAU does not form the bulbous compartment typical of rodents, and instead, vas deferens functions as the storage area for sperm before ejaculation (Turner. 1991).

The expression of androgen receptor (*Ar*) in the WD epithelium commence already at E14.5 in mice (Cooke, et al. 1991; Crocoll, et al. 1998; Tomaszewski, et al. 2007; Murashima, et al. 2011), however, only mesenchymal expression of AR is required for WD maintenance. Although the full knock-out of *Ar* displayed regression of the WD due to increased cell apoptosis, an epithelium specific *Ar* knock-out still showed WD elongation and coiling (Murashima, et al. 2011). This is explained by the intricate mesenchymal-epithelial regulatory system of the future epididymis. For example, the expression of epithelial growth factors is regulated by mesenchymal androgen signaling (reviewed in Hannema and Hughes. 2007).

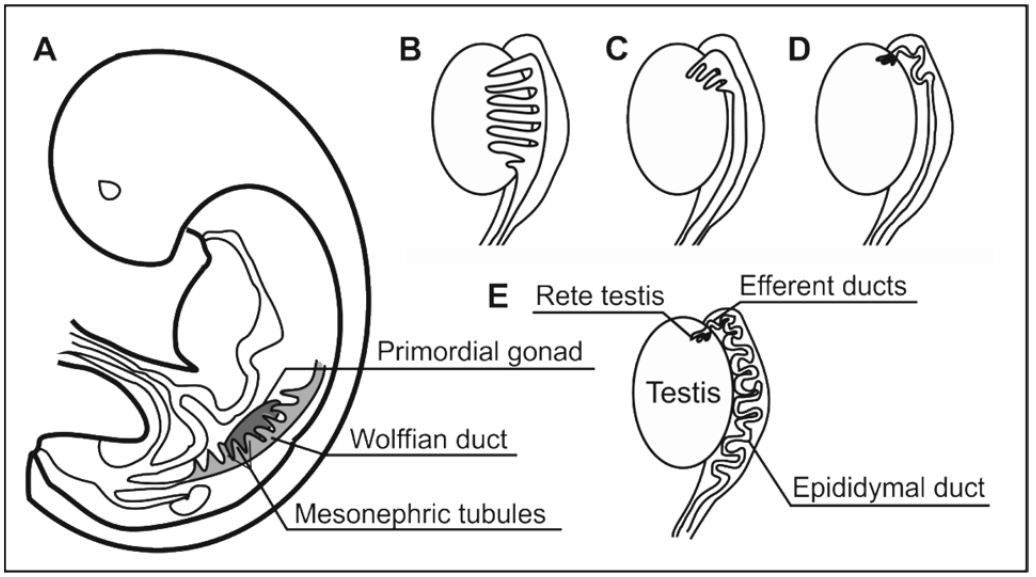


Fig. 1. Embryonic development of the epididymis. A) A sagittal section of a mouse embryo (E11) showing the relative locations of the developing primordial gonad and the Wolffian duct (WD) that will form the testicle and epididymis, respectively. **B)** Detail of the differentiating male reproductive organs, displaying mesonephric tubules extending from the WD to the future testis. **C)** The cords arising from the cranial mesonephric tubules form the efferent ducts that will fuse with rete testis. **D)** Once connected to the testis, proximal-distal coiling of the WD begins (E16.5). **E)** The three-dimensional coiling is completed in the early postnatal period (Modified from Arrotéia, et al. 2012).

2.1.2. Postnatal development

At birth, the proximal part of the epididymis is highly coiled but the epithelium is still undifferentiated and thus, unable to produce the milieu necessary for sperm maturation (Joseph, et al. 2009). To become fully differentiated, the postnatal epididymis goes through three developmental stages: the undifferentiated period, the period of differentiation and the period of expansion (Sun and Flickinger. 1979). As these periods have been extensively studied in rats, the following description of postnatal development uses the rat to show the exact time point of differentiation for the epithelial cell types (Sun and Flickinger. 1979; Hermo, et al. 1992a).

Epithelial cell differentiation. One to two days after birth the epididymal tubule has already begun to form septa between the different segments. The previously mentioned, major segments (IS, CAP, COR and CAU) are further divided into several parts by septa (Robaire, et al. 2006). At this stage, the epididymis goes through extensive cell proliferation which leads to considerable growth of the tubule in the first

two weeks after birth (Hermo, et al. 1992a; Jiang, et al. 1994). On postnatal day 15 the epididymal epithelium consists of immune cells, as well as narrow, and columnar cells (Sun and Flickinger. 1979). At this stage, the columnar cells of distal CAU have differentiated into basal cells and the differentiation continues in a distal to proximal direction during the following month (Shum, et al. 2013). The columnar cells further differentiate into the major cell type of the epithelium, the principal cells, while the narrow cells differentiate into narrow and clear cells (Sun and Flickinger. 1979; Hermo, et al. 1992a). Approximately seven weeks after birth, the epididymal epithelium of rats is fully differentiated (Hermo, et al. 1992a; Shum, et al. 2013). At this time spermatozoa enter the tubule, which gives rise to further expansion (Jiang, et al. 1994).

Androgen signaling. Early development of the postnatal epididymis is mainly dependent on mesenchymal androgen signaling. However, at postnatal day 11, an AR autocrine regulation of epithelial cell differentiation can already be observed in mice (O'Hara, et al. 2011). The secretion of testicular testosterone into the epididymal lumen generates a progressive differentiation of the epididymal epithelium, with cells of IS and CAP differentiating prior to those of CAU. This could further be a result of increased conversion of testosterone to dihydrotestosterone (DHT) in the proximal epididymis (Robaire and Viger. 1995). However, in mice, DHT is thought to mainly amplify the androgen signal and to be redundant when testosterone levels are significantly high (Mahendroo, et al. 2001). Activation of AR signaling is required for IS development and the differentiation of principal and basal cells of CAP (O'Hara, et al. 2011; Murashima, et al. 2011; Krutskikh, et al. 2011). When AR signaling is depleted from adult mice, a regression of IS and dedifferentiation of CAP is also observed (Avram, et al. 2004). CAU is not as dependent on androgen signaling, although CAU principal cell differentiation requires *Ar* expression (Murashima, et al. 2011).

Testicular factors. The proximal epididymis is also dependent on testicular factors other than androgens to fully differentiate. This is observed after castration when testosterone replacement is able to restore most of the epididymal morphology except for that of IS (Fawcett and Hoffer. 1979; Hamzeh and Robaire. 2009). Among others, fibroblast growth factors (FGFs), expressed in testis and transported into the epididymis, are known to regulate epididymal gene expression by binding to receptors in the epithelium (reviewed in Cotton, et al. 2008). Factors like these activate the IS specific expression of ros1 proto-oncogene (*Ros1*), a gene important for the induction of IS differentiation in mice (Sonnenberg-Riethmacher, et al. 1996; Krutskikh, et al. 2011). The expression of *Ros1* begins around the time of epithelial differentiation, when a decline in cell proliferation is observed, and continues until adulthood (Jun, et al. 2014). Mice with complete knock-out of *Ros1* display an undeveloped IS, unable to

regulate luminal pH and osmolality (Sonnenberg-Riethmacher, et al. 1996; Yeung, et al. 2004b). This leads to failure of sperm maturation and animal infertility (Yeung, et al. 1999). Ablation of *Ros1* in the adult mouse epididymis did however, not lead to dedifferentiation of the tissue, which further emphasizes the role of ROS1 as an inducer of differentiation (Jun, et al. 2014). It is also thought that ROS1 would serve in cell-cell and/or epithelial-mesenchymal signaling due to its extracellular domain, however, studies regarding this are yet to be performed (reviewed in Yeung, et al. 1998).

Epithelial-mesenchymal signaling. The importance of a continued epithelial-mesenchymal communication has been observed during IS development in mice. Both the efferent ducts and the proximal mouse epididymis express the leucine-rich repeat domain containing G protein-coupled receptor 4 (*Lgr4*) in the mesenchyme surrounding the duct (Mendive, et al. 2006). The expression is initiated already before birth and affects the expression of estrogen receptor 1 (*Esr1*, also known as *Erα*) and the development of IS (Mendive, et al. 2006; Hoshii, et al. 2007). In the hypomorphic *Lgr4* mutant mouse, the postnatal elongation of the epididymis fails, as cell proliferation and remodeling of the extra cellular matrix do not take place (Hoshii, et al. 2007). This also causes the epithelium of the mouse IS to remain undeveloped, which further emphasizes the role of mesenchymal-epithelial signaling (Hoshii, et al. 2007).

Regulation by non-coding RNAs. An increasing number of studies suggest a role for non-coding RNAs in the regulation of epididymal development. Different length transcripts of RNA can function as regulators of mRNA transcription and translation, either by binding to the DNA strand itself, targeting the strand for epigenetic modifications, or by binding to the complementary mRNA transcripts and thereby inhibiting translation or targeting the mRNA strand for degradation (reviewed in Goto and Nakayama. 2012; Castel and Martienssen. 2013; Dogini, et al. 2014). One of the most studied non-coding RNAs are microRNAs (miRNAs) which are said to regulate about one third of all human genes (Lewis, et al. 2005). MiRNAs are synthesized as longer primary-miRNAs (pri-miRNAs), either from independent transcripts or from the introns of protein-encoding genes. Already in the nucleus the pri-miRNAs are cleaved by DROSHA, a ribonuclease III enzyme, to produce precursor-miRNAs (pre-miRNAs) before transportation to the cytoplasm by EXPORTIN-5 (Fig. 2, Lee, et al. 2003; Yi, et al. 2003; Bohnsack, et al. 2004). In the cytoplasm, the enzyme DICER binds to the stem-loop formation of the pre-miRNA and further cleaves it to produce a ~22 nucleotide long double stranded RNA (Bernstein, et al. 2001). The product is loaded onto the RNA-induced silencing complex (RISC) which exposes the guide strand to the target mRNA (Fig. 2, Martinez, et al. 2002). DICER is also involved in the formation of heterochromatin by prosessing of endogenous small interfering RNAs (endo-

siRNAs) that target the heterochromatin loci (Fukagawa, et al. 2004). After cleavage by DICER, the nuclear siRNAs form RNA-induced initiation of transcriptional gene silencing (RITS) complexes that can reqruit histone deacetylases and DNA methyltransferases to the targeted sites and thereby cause heterochromatin formation (Morris, et al. 2004; Verdel, et al. 2004).

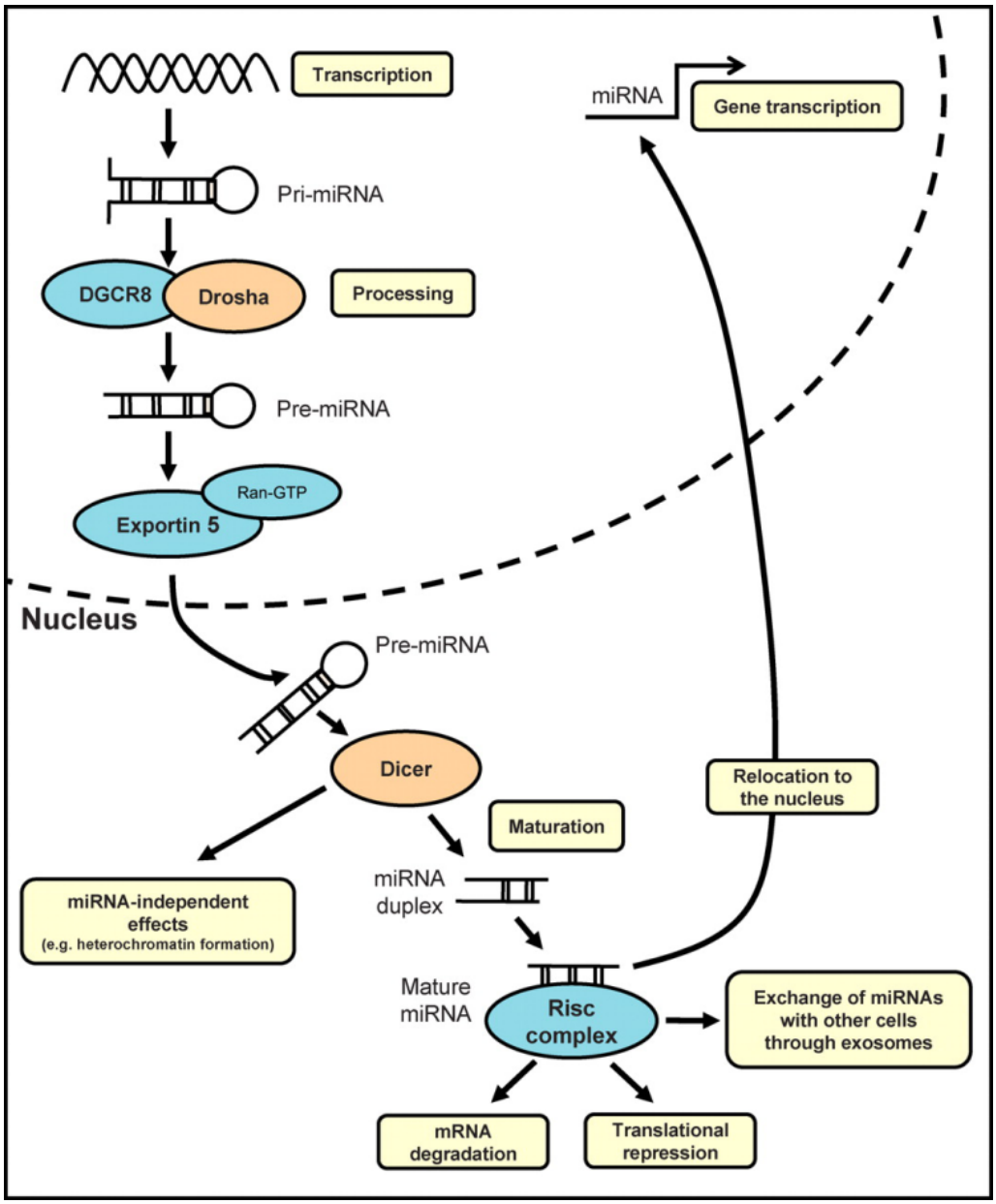


Fig. 2. Production and function of microRNAs (miRNAs). (Urbich, et al. 2008)

The importance of miRNA regulation in the epididymis was suggested by the pattern of miRNA expression that differs during postnatal development of the epididymis (Zhang, et al. 2010; Wang and Ruan. 2010a; Belleannee, et al. 2012a; Belleannee, et al. 2013). Some miRNAs have also been shown to be specific for the rat or primate epididymis (Li, et al. 2010; Ni, et al. 2011). The epididymides of newborn humans show the highest expression of miRNAs, while the epididymides of adult and aged men show less age-specific miRNA expression (Zhang, et al. 2010). This led Zhang et al. to suggest that the epididymides of young and adult men have different regulatory systems for gene expression. While the young epididymis would be regulated by miRNAs, the gene expression in the adult men epididymis would primarily be governed by transcription factors (Zhang, et al. 2010). By comparing microarray miRNA and mRNA expression data from the rat epididymis, several studies have suggested specific roles for miRNA in epididymal development. Cell proliferation and the development of the epididymis-blood barrier especially seem to be under miRNA regulation. For example rat miR-200c targets the expression of transcription factor 8 (*Tcf8*) and thereby indirectly causes an increase in E-cadherin expression (Wang and Ruan. 2010a). During rat epididymal development, miR-200c expression increases to promote the high levels of E-cadherin needed for cell adhesion during the late expansion period of epididymal development (Cyr, et al. 1993; Wang and Ruan. 2010a). Rat miR-200a serves a similar function by targeting β-catenin mRNA and thereby regulating cadherin-catenin interaction in the epididymal epithelium (Wu, et al. 2012). Excess β-catenin expression could potentially cause tumour formation and the regulation by miRNAs was also suggested to play a role in the low tumour incidence observed in the epididymis (Wang, et al. 2013). Another study shows a role for rat miR-29a in cell proliferation (Ma, et al. 2012). The reduction in cell proliferation after the first undifferentiated period is suggested to be partly dependent on the expression of miR-29a through down-regulation of its target gene autoantigenic sperm protein (*Nasp*) in the rodent epididymis (Ma, et al. 2012). However, cell proliferation is also suggested to depend on a reduction in the expression of rat miR-335 during postnatal development of the epididymis. Decreased levels of miR-335 would promote RAS p21 protein activator 1 (*Rasa1*) expression and an increased cell proliferation (Wang and Ruan. 2010b). Thus, the role of non-coding RNAs is multifaceted and would give rise to a complex regulatory network promoting the correct development of the epididymis.

2.2. Structure of the epididymal epithelium

At the time of sperm entry into the epididymis, the tubule consists of a highly differentiated epithelium (Hermo, et al. 1992a). This can especially be appreciated when studying the physiology of the different segments. Although they consist of similar epithelial cells, the morphology of the segments differs. The more caudal the segment lies, the thinner the epithelium (Fig. 3). At the same time, the diameter of the tubule broadens until CAU, where the storage of millions of sperm requires the largest area (Markey and Meyer. 1992). Although most differences can only be observed in histological sections, the high vascularization of the initial segment allows it to be distinguished from the other segments macroscopically (Markey and Meyer. 1992). The following sections will give a brief introduction to each of the epithelial cell types and their functions in the epididymis.

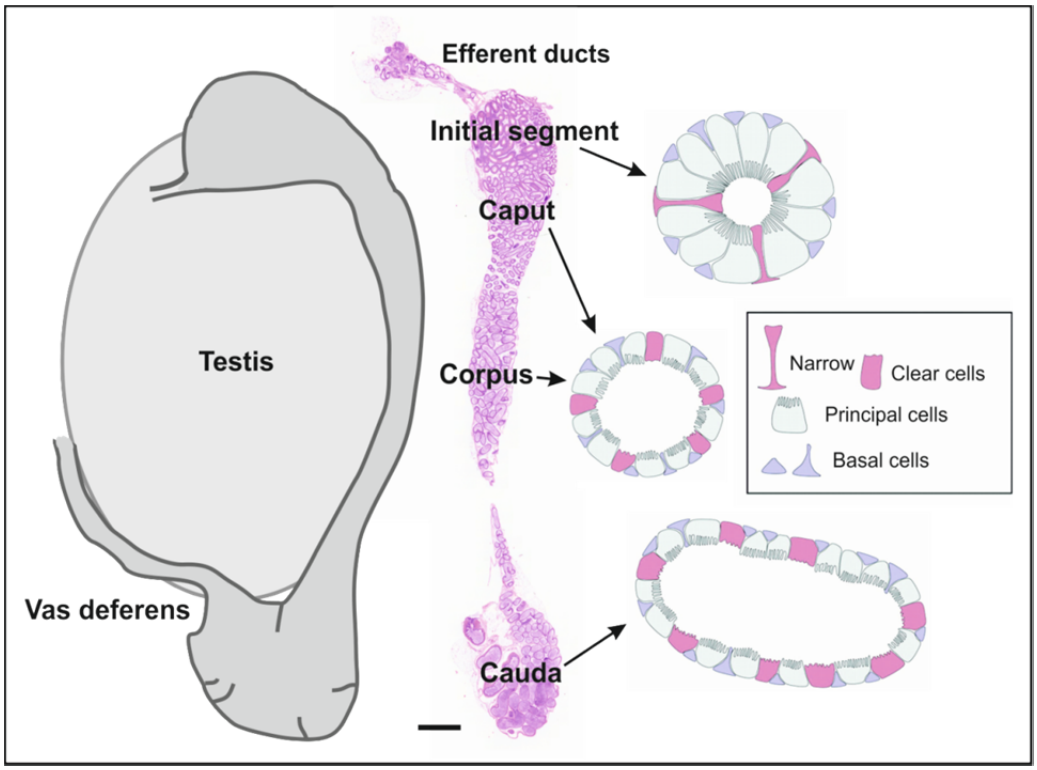


Fig. 3. Morphology of the epididymis. Schematic drawing of the epididymis and testis flanked by a hematoxylin-eosin stained section of the epididymis of an adult mouse. Drawings of epithelial cross-sections depict several of the epithelial cell types and their physiology in the different segments of the epididymis. Scale bar 1 mm (Modified from Shum, et al. 2009).

2.2.1. Principal cells

The principal cells constitute up to 80% of all the epithelial cells of the epididymis (Robaire and Hermo. 1988). The cells are highly specialized and differ in morphology and function between the epididymal segments (Fig. 3, reviewed in Hermo and Robaire. 2002). In the most proximal part of the epididymis, the principal cells have a columnar shape and express and secrete high amounts of proteins. In rodents the principal cells in the initial segment contain a specialized endoplasmic reticulum and golgi apparatus that are thought to be involved in this process (Hermo, et al. 1991). More distal segments form a thinner epithelial layer with square-shaped principal cells. Throughout the epididymis, the principal cells extend long microvilli with which they detect changes in the luminal fluid (Primiani, et al. 2007). The physiology of the epididymis is highly dependent on the secretion of principal cells. Not only do they contribute with ions, water, and ATP but essentially all proteins secreted into the luminal fluid stem from the principal cells (Hermo and Jacks. 2002). The gene expression of principal cells is segment dependent. However, adjacent cells are thought to communicate and thereby regulate each other's gene expression, which gives rise to a checkered expression pattern along the duct (Robaire, et al. 2000). The proteins and glycoproteins produced by the principal cells are involved in the acquisition of sperm progressive motility, sperm-oocyte interaction, and in the protection of sperm from reactive oxygen species (ROS) or inflammatory factors. These will be discussed in more detail in a later chapter. The principal cells also serve in the removal of defective spermatozoa by phagocytosis (Sutovsky, et al. 2001).

Apart from the merocrine and channel based secretion, the principal cells also secrete large lipid vesicles, so called epididymosomes, that are suggested to transport lipids, proteins, glycoproteins and miRNA between the different epididymal segments (Hermo and Jacks. 2002; Rejraji, et al. 2006; Belleannee, et al. 2013). *In vitro* studies of the epididymosomes also showed exchange of material between the vesicles and sperm (Caballero, et al. 2013). It is interesting to note that apocrine secretion mainly takes place in IS and CAP even though the principal cells of COR contain a high number of lipid droplets (Hermo and Jacks. 2002).

The blood-epididymis barrier. The principal cells also form the blood-epididymis barrier, which separates the luminal fluid of the epididymis from the blood stream. The barrier consists of tight junction complexes, composed of cadherins, catenins, occludin, and claudins, on the apical side of principal cells (reviewed in Cyr, et al. 2007). In rats, these junctions start to form already during WD development but are not completed until postnatal day 21 (Agarwal and Hoffer. 1989; Cyr, et al. 1999). The proximal epididymis has the highest number of tight junctions which span a considerable length of the apical plasma membrane (Suzuki and Nagano. 1978). In the distal epididymis

the junctions become more scarce and instead, numerous desmosomes are found in the apical area (Cyr, et al. 1995). The blood-epididymis barrier is thought to be required both for protection of spermatozoa and in the formation of the luminal environment needed for sperm maturation. The barrier efficiently restricts movement of ions, solutes, and macromolecules across the epididymal epithelium and thereby, enables the luminal fluid to have a considerably higher concentration of, for example, inositol and carnitine than blood plasma (Hinton and Howards. 1981). The barrier also functions as an extension of the blood-testis barrier and may serve a protective role by preventing sperm from interacting with immune cells or prohibiting certain toxins from reaching the epididymal lumen (Wong, et al. 1983; Hoffer and Hinton. 1984).

2.2.2. Narrow and clear cells

The narrow cells of IS and the clear cells of more distal segments are both involved in the regulation of epididymal luminal pH. The cells keep the luminal fluid acidic by secretion of protons and thereby protect sperm from early activation of motility (Wong, et al. 1981; Vijayaraghavan, et al. 1985; Caflisch and DuBose. 1990). Although rodents are the only mammals with an IS, other species, including humans, are also known to have cells similar to narrow cells in the most proximal part of the epididymis (Palacios, et al. 1991). The narrow cells are, as the name implies, thin in shape, lie in-between the principal cells and contain cup-shaped vesicles in the apical membrane that are involved in endocytosis and secretion of protons into the epididymal lumen (Fig. 3, Hermo, et al. 2000). Clear cells are much larger in size (Fig. 3), but like narrow cell they contain numerous mitochondria and regulate luminal pH by expression of vacuolated [V]-ATPase, carbonic anhydrase II, and soluble adenylate cyclase (Hermo, et al. 2000; Pastor-Soler, et al. 2003). When the clear cells detect a change in pH, the cell plasma membrane forms elongating microvilli out into the epididymal lumen to which the V-ATPase protein is transported (Pastor-Soler, et al. 2003; Beaulieu, et al. 2005; Shum, et al. 2008; Belleannee, et al. 2010). Clear cells also take up proteins from the luminal fluid in a region-specific manner and are said to rid the epididymal lumen of degrading sperm material (Hermo, et al. 1992b; Vierula, et al. 1995).

2.2.3. Apical cells

The apical cells are primarily found in the initial segment of the epididymis. The cells have an apically located nucleus and do not form any contact with the basement membrane (Sun and Flickinger. 1980; Adamali and Hermo. 1996). Not much is known about the function of apical cells but they display a very different protein expression profile compared to other epithelial cell types (Adamali and Hermo. 1996). They contain many proteolytic enzymes with which they are thought to break down substances that are endocytosed from the epididymal lumen (Adamali and Hermo. 1996; Adamali, et al. 1999). Anion transporters, similar to those found in narrow and

clear cells were also present in human apical cells (Kujala, et al. 2007). This indicates an additional, species specific, role in regulating luminal fluid pH.

2.2.4. Basal cells

During postnatal development, the basal cells appear as dome-shaped cells close to the basement membrane. However, after the entry of fluids and sperm into the epididymis the cells change shape, become flat and send out protrusions that encompass a large portion of the epididymal tubule (Fig. 3, Veri, et al. 1993; Shum, et al. 2013). Although the basal cells are not thought to divide in adults, the differentiated cells are very adaptable and can send out narrow cytoplasmic extensions that cross the tight junctions at the apical pole of the epithelium (Shum, et al. 2008). These extensions contain angiotensin II type 2 receptor (AGTR2) and are able to detect angiotensin II directly from the luminal fluid. This "message" is passed on to adjacent clear cells that start to secrete protons through the activity of V-ATPase, thereby regulating the pH of the luminal fluid (Shum, et al. 2008). The basal cells are also said to regulate the transportation of electrolytes and water from principal cells through prostaglandin signaling (Leung, et al. 2004).

The number of basal cells increases in the distal epididymis to 21% of the total epithelial cells in COR and CAU compared to 12% in the initial segment (Trasler, et al. 1988). An explanation for this is the increased need for immune response and the protection against ROS in CAU, where sperm are stored for long periods of time. The expression of superoxide dismutase 1 (SOD1) and glutathione-S-transferase (GST) is highest in caudal basal cells and is thought to protect stored sperm from damage by ROS (Nonogaki, et al. 1992; Veri, et al. 1993; Jervis and Robaire. 2001). Basal cells are also said to have macrophage like functions that could eliminate the immune recognition of degenerating spermatozoa (Seiler, et al. 2000). This is especially important in extreme conditions, for example during increased epididymal temperatures, when the amount of luminal sperm antigens increase and could cause harm if they come into contact with cells of the immune system (Seiler, et al. 2000).

2.2.5. Halo cells

Immune cells, the so called halo cells, are found in all segments and at all levels of the epididymal epithelium. They can be distinguished by their dark-stained nucleus surrounded by a pale cytoplasm (Sun and Flickinger. 1979). Although they consist of a mixture of T lymphocytes, cytotoxic T lymphocytes and macrophages, their exact immunological function is not known. The proximal epididymis contains the highest number of halo cells, and their number increase throughout the epididymis during ageing (Flickinger, et al. 1997; Serre and Robaire. 1999). In older animals also

eosinophils and B lymphocytes are occasionally found among the halo cells (Serre and Robaire. 1998; Serre and Robaire. 1999).

2.2.6. Smooth muscle cells

Surrounding the epididymal duct is a layer of smooth muscle cells that drive sperm forward in the tubule through rhythmic contractions (Baumgarten, et al. 1971; Jaakkola and Talo. 1983). Although it serves the same function, the morphology of the muscle layer differs between species. Larger mammals have a much thicker muscle layer than, for example, rodents. Birds on the other hand have additional muscle cells in the interductal space (Abd-Elmaksoud. 2009; Egger and Witter. 2009; Alkafafy, et al. 2011). Androgen and estrogen signaling is suggested to work together in regulating muscle contractions and the rate with which sperm travels through the epididymis. The signals have an opposite effect with androgen depletion and estrogen treatments giving rise to increased muscle contractility and faster transport of spermatozoa (Meistrich, et al. 1975; Din-Udom, et al. 1985; Sujarit and Pholpramool. 1985). In addition to steroid signaling, neurohypophysial hormones and neuronal regulation affects contractility and sperm transit (Hib. 1977; Hib, et al. 1979; Ricker and Chang. 1996).

2.3. Epididymal luminal fluid and sperm maturation

Sperm obtained from testis are fully developed and consist of a head, containing the genetic material, and a flagellum, divided into the mid piece, containing mitochondria, and the principal and end piece. Over the anterior part of the sperm head lies the acrosome, a membrane-bound structure that stems from the Golgi apparatus, and is filled with enzymes needed to penetrate the oocyte (Fig. 4, Yanagimachi. 1994). However, when spermatozoa leave the testis they still lack progressive motility and the ability to react to signals in the female reproductive tract. To be able to fertilize the oocyte, sperm first have to travel through the epididymal duct where proteins secreted by the epithelium gradually remodel the sperm membrane (reviewed in Dacheux and Dacheux. 2013). This maturation process includes both processing of proteins already present on the sperm surface and binding of new proteins to the sperm. Furthermore, the lipid layer of the sperm membrane becomes reorganized and more fluent during epididymal transit (reviewed in Jones. 2002). Before ejaculation, sperm are stored in a quiescent state in CAU. To achieve this, the environment of the epididymal lumen differs significantly from that of the female reproductive tract, with lower pH and higher osmolality (Cooper and Barfield. 2006; Cooper, et al. 2008; Liu, et al. 2012; Dacheux and Dacheux. 2013). Although epididymal transit takes between 3 to 10 days, spermatozoa can be stored in CAU for more than 30 days (Robaire, et al. 2006). Thus, the epididymis also needs to secrete factors that serve to protect sperm during this time. Problems in any of the epididymal functions concerning sperm maturation, protection or storage leads to reduced fertility of the sperm. The following sections will explain in

more detail how the luminal environment of the epididymis contributes to sperm maturation.

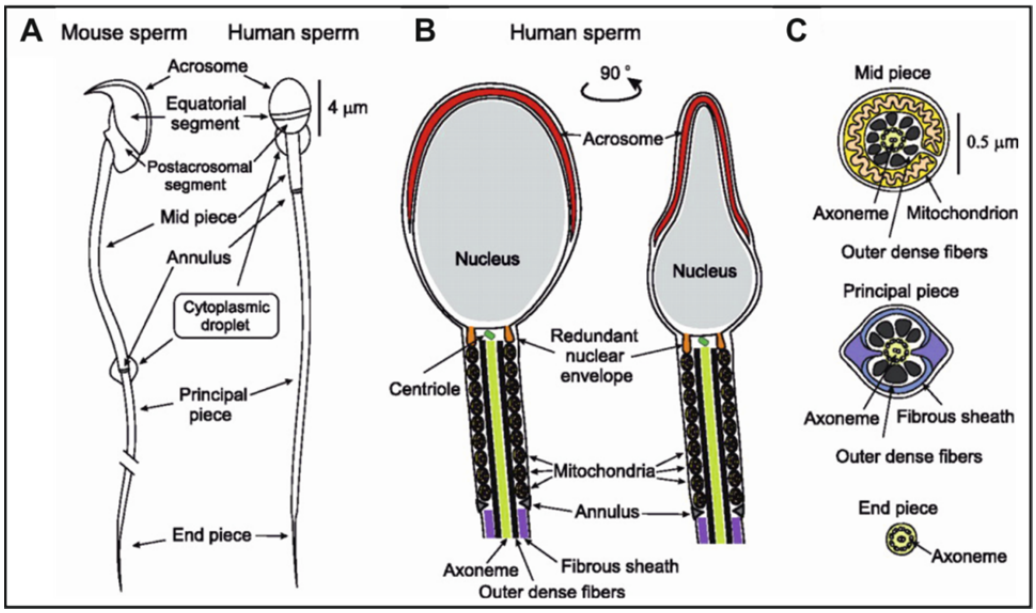


Fig. 4. Morphology of the mouse and human sperm cell. (Modified from Darszon, et al. 2011)

2.3.1. Ions

During transit through the efferent ducts, around 90% of the luminal fluid surrounding spermatozoa is taken up by the non-ciliated cells of the ducts (Crabo. 1965; Levine and Marsh. 1971; Clulow, et al. 1994). The water reabsorption is facilitated by aquaporins, a family of channels driven by the movement of Na^+, Cl^- and HCO_3^- across the epithelial cell membranes (reviewed in Hermo and Smith. 2011). In the proximal part of the epididymis, water reabsorption continues, with a subsequent increase in sperm concentration (Clulow, et al. 1994). The protein concentration of the epididymal luminal fluid also increases, which aids the interaction between spermatozoa and maturation factors secreted by the epididymis. Mice with blocked water reabsorption and consequently more dilute sperm, display a reduction in, or even complete lack of fertility (Zhou, et al. 2001; Hess, et al. 2002).

The cytoplasmic droplet. Due to water reabsorption, the luminal fluid of the epididymis has high osmolality compared to seminal plasma and the female reproductive tract (Cooper and Barfield. 2006; Cooper, et al. 2008). Thus, when sperm leave the epididymis they immediately encounter a hypotonic environment which causes great stress on the cell. To counteract excessive water influx, spermatozoa are

suggested to regulate their volume by shedding the cytoplasmic droplet (reviewed in Cooper. 2011). The droplet is a remnant of the spermatid cytoplasm and is located in the neck region of sperm when they enter the epididymis. During sperm maturation the cytoplasmic droplet migrates caudally to the annulus, at the end of the mid piece (Fig. 4, Hermo, et al. 1988; Cortadellas and Durfort. 1994; Perez-Sanchez, et al. 1997). It is also filled with permeant osmolytes to compensate for the hypertonic surroundings of the epididymal lumen (Jeulin, et al. 1994; Pruneda, et al. 2007; Kobayashi, et al. 2007). After ejaculation, removal of these osmolytes together with the droplet would reduce water uptake by the cell and keep the flagellum of the sperm straight (Cooper. 2011). If the cytoplasmic droplet remains attached to sperm in the female reproductive tract, the droplet swells and the sperm flagellum is bent around it in a hairpin formation (Yeung, et al. 1999; Sipilä, et al. 2002). One cause of droplet retention could be reduced amounts of osmolytes. This has been observed in the previously mentioned *Ros1* knock-out mouse model, whose sperm contain less *myo*-inositol and glutamate than sperm of wild-type mice (Yeung, et al. 2004a). The hairpin formation of *Ros1* knock-out mice sperm leads to infertility (Yeung, et al. 1999). Studies of bull and boar sperm also show lower fertilizing efficiency if sperm retain the cytoplasmic droplet (Amann, et al. 2000; Thundathil, et al. 2001; Lovercamp, et al. 2007). Human sperm is different from other species in that the cytoplasmic droplet remains attached also in the female reproductive tract (Mortimer, et al. 1982; Abraham-Peskir, et al. 2002; Cooper, et al. 2004). It is hypothesized that the droplet continues to regulate fluid intake throughout female duct transit. When sperm reach the oocyte, all osmolytes would be released from the droplet and increased swelling would occur and further activate motility and zona pellucida penetration (Yeung, et al. 2003; Cooper. 2011).

Bicarbonate. A weak motility of the sperm flagellum can be observed already in rete testis and the efferent ducts. However, after entering the epididymis, the acidic luminal fluid keeps sperm in an immotile state until ejaculation (reviewed in Dacheux and Dacheux. 2013 and Liu, et al. 2012). The epididymal epithelium actively reabsorbs HCO_3^- from the lumen to maintain a low pH, but some secretion of HCO_3^- is also observed in the distal parts of the tubule (Levine and Marsh. 1971; Chan, et al. 1996). The slightly higher levels of HCO_3^- in the distal epididymis could contribute to the bulk seminal HCO_3^- needed for sperm motility (Owen and Katz. 2005). During ejaculation, sperm come into contact with the alkaline seminal plasma which causes an influx of HCO_3^- into sperm and activation of flagellar beating (Liu, et al. 2012). Mature sperm are very sensitive to changes in HCO_3^- levels while immature sperm do not react to increased HCO_3^- concentrations in the same way (Baker, et al. 2003). Sperm from caput epididymis also contain the highest levels of HCO_3^-, but during maturation, transporters actively remove HCO_3^- from the cells (Liu, et al. 2012). One such HCO_3^- transporter is even incorporated into the sperm membrane during transit through COR,

and could contribute to the low HCO_3^- concentration observed in mature sperm (Ekstedt, et al. 2004; Wandernoth, et al. 2010).

Calcium. Immature sperm in the proximal part of the epididymis not only contain high amounts of HCO_3^- but also the highest levels of Ca^{2+} (Vijayaraghavan, et al. 1989). As Ca^{2+} -signaling drives sperm motility and fertility in the female reproductive tract, the ion is actively secreted during epididymal transit to avoid early onset of activation in mature sperm (Sanchez-Luengo, et al. 2004). A Ca^{2+} -pump is also transferred from the epididymal epithelium to sperm in the caudal segment to aid in this process (Brandenburger, et al. 2011). Furthermore, several proteins secreted by the epididymal epithelium bind to sperm and regulate Ca^{2+} -channels in the sperm membrane. For example cysteine-rich secretory protein 1 (CRISP1), a protein highly secreted throughout the epididymis, keeps the sperm in an inactive state until they reach the female reproductive tract (Brooks. 1982; Kratzschmar, et al. 1996; Roberts, et al. 2002; Roberts, et al. 2008). Here, the loosely attached protein is rapidly shed from the sperm surface to allow signal activation through ion channels (Kratzschmar, et al. 1996; Nixon, et al. 2006; Roberts, et al. 2008).

The β-defensin family of proteins, predominantly expressed in the male reproductive tract, is also involved in regulating Ca^{2+} -uptake during epididymal transit. The expression of β-defensin genes is often androgen dependent and restricted to certain segments in the epididymis (Fig. 5). The proteins are small, cationic molecules, which are cleaved after translation to produce mature proteins that can be secreted from the epithelial cells into the epididymal lumen. Because of their charged nature they are able to insert into phospholipid membranes, forming holes or channels (Hall, et al. 2007). In this way, they are suggested to regulate sperm ion intake but also possess antimicrobial functions (Selsted and Ouellette. 2005). As the β-defensin genes are found in clusters that have arisen from gene duplication, many family members are believed to be functionally redundant. To eliminate this effect when studying the function of genes from a specific cluster, nine adjacent β-defensin genes (β-defensin 1, 2, 9, 10, 11, 13, 15, 35, and 50) were recently knocked-out from the mouse chromosome 8. The gene deletion resulted in premature activation of sperm motility in the epididymis due to increased Ca^{2+} levels of sperm (Fig. 5, Zhou, et al. 2013). This would indicate a role for one or several of the genes in the regulation of Ca^{2+} -uptake by immature sperm. Especially those genes expressed in CAP and COR were suggested to cause this effect by binding to sperm in the lumen (Fig. 5). However, the Ca^{2+} concentrations of immature sperm need to be carefully regulated as too low sperm Ca^{2+} levels also result in motility defects. For example the rat β-defensin sperm associated antigen 11b (SPAG11b, also known as BIN1b), binds to sperm in CAP where it is proposed to form or activate Ca^{2+} -channels in the sperm membrane and thus induce Ca^{2+} -uptake during epididymal transit (Fig. 5, Zhou, et al. 2004). When

immature sperm were cocultured with cells expressing SPAG11b they showed a significant increase in motility, similar to that of sperm incubated with CAP cells. If *Spag11b* expression was blocked, the number of sperm with progressive motility was significantly reduced (Zhou, et al. 2004).

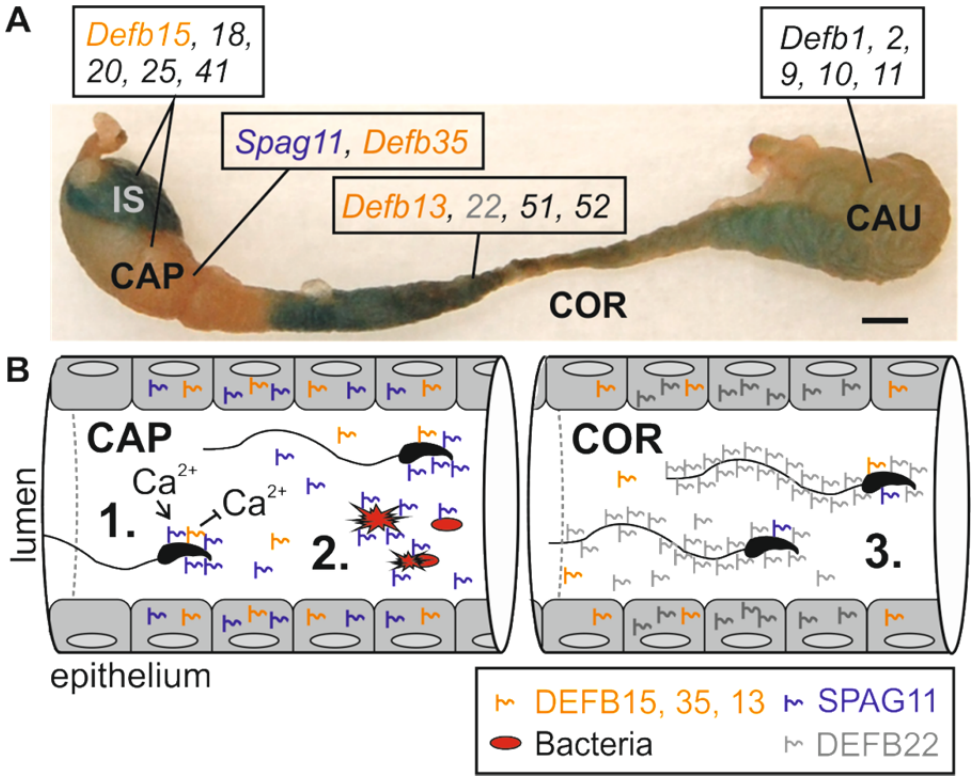


Fig. 5. Expression and function of rodent β-defensins. A) The mouse epididymis, including the expression localization of β-defensins in the epididymal segments (modified from Dorin and Barratt. 2014). The segments are visualized by X-gal staining. Scale bar 1 mm. B) Schematic picture of expression and function of β-defensins in the caput (CAP) and corpus (COR) epididymis. 1. β-defensins expressed in rat and mouse CAP function locally by promoting or inhibiting Ca^{2+} uptake from the epididymal lumen into sperm and thereby regulating sperm motility and activation. 2. Sperm associated antigen 11a (SPAG11a), expressed in mouse CAP, prevents bacterial infection in the epididymis. 3. β-defensin 22 (DEFB22), expressed in COR, forms the major part of the sperm glycocalyx in mice. IS, initial segment; CAU, cauda.

2.3.2. Lipids

The epithelial cells of the epididymis synthesize lipids that are secreted into the lumen. These hydrophobic molecules move into the lumen either by transporters or in larger vesicles, epididymosomes, which are suggested to exchange material with sperm (Rejraji, et al. 2006; Girouard, et al. 2011). Both the epithelial cells of the epididymis and spermatozoa contain many cholesterol and phospholipid transporters. The transporter ATP-binding cassette, sub-family G, member 1 (*Abcg1*) is expressed by principal cells in the epididymis, while the sub-family A, member 1 (*Abca1*) is expressed by apical cells in the proximal segments and in principal cells more distally (Morales, et al. 2008; Ouvrier, et al. 2009). In spermatozoa, ABCA1 is located in the acrosome and middle piece areas while ABCG1 can be found in the neck and principal piece (Morales, et al. 2008). ABCA1 transports cholesterol and phospholipids out from the cell to lipid-poor apoproteins, e.g. APOE and clusterin (CLU), in the lumen. ABCG1, on the other hand, only transports cholesterol to mature HDL particles (reviewed in Oram and Vaughan. 2006). If the epithelial cells detect excess amounts of intracellular cholesterol, *Abca1* expression is increased through the activity of proteins from the nuclear receptor subfamily 1, group H (NR1H) (Ouvrier, et al. 2009). A strong expression of Scavenger receptor class B, type I (*Scarb1*, also known as *SR-B1*) is also detected in the proximal segments of the epididymis, which suggests additional reabsorption of cholesterol (Ouvrier, et al. 2009). The secretion and reabsorption of lipids in the epididymal epithelium, together with the presense of lipid transporters in sperm, would suggest an exchange of lipids between the epididymis and maturating sperm.

Cholesterol. During maturation, sperm membrane cholesterol levels generally decrease while the amount of the cholesterol precursor, desmosterol, increases (reviewed in Keber, et al. 2013). Studies of mouse, rat, hamster, and ram sperm have shown a ~50% reduction in cholesterol concentration between CAP and CAU (Parks and Hammerstedt. 1985; Hall, et al. 1991; Awano, et al. 1993; Rejraji, et al. 2006). The high cholesterol concentration of immature sperm stabilizes the membrane and prevents early exocytosis of the acrosome. In mature sperm, the reduced amount of cholesterol enables sperm progressive motility and the ability of sperm to react to signals from the female reproductive tract (Keber, et al. 2013). For example, *in vitro* studies of human sperm have linked high cholesterol levels with infertility (Sugkraroek, et al. 1991; Meseguer, et al. 2004). However, there are differences between species. Boar sperm did not show a decrease in sterol levels during epididymal transit while goat sperm, in contrast, displayed increased cholesterol levels (Nikolopoulou, et al. 1985; Rana, et al. 1991). Moreover, while desmosterol is mainly added to the sperm membrane during epididymal transit, mature ram sperm only contains trace levels of the sterol (Parks and Hammerstedt. 1985; Nikolopoulou, et al.

1985; Awano, et al. 1993). Due to the additional double bond, desmosterol is suggested to increase membrane fluidity and sperm motility (Connor, et al. 1997). Desmosterol has mainly been found in the sperm flagellum while cholesterol serves a specific purpose in formation of lipid rafts in the sperm head (Lin, et al. 1993; Mourvaki, et al. 2010b). Although desmosterol is structurally able to replace cholesterol in cell membranes, it cannot form the stable lipid rafts which are needed to assemble proteins for sperm-oocyte interaction (Vainio, et al. 2006; Boerke, et al. 2008).

Polyunsaturated fatty acids (PUFAs). A change in cholesterol/desmosterol levels is not the only property of the sperm membrane that contributes to increased fluidity. In addition, most mammals display a shift in the sperm fatty acid content from saturated to polyunsaturated forms during epididymal transit (Parks and Hammerstedt. 1985; Nikolopoulou, et al. 1985; Hall, et al. 1991; Awano, et al. 1993; Haidl and Opper. 1997; Rejraji, et al. 2006; Pyttel, et al. 2014). The only exception found thus far, is goat sperm, which show an increase in saturated fatty acids. In humans, a high level of PUFAs, especially the amount of the omega-3 fatty acid, docosahexaenoic acid (DHA, 22:6), is positively correlated with sperm motility (Aksoy, et al. 2006; Tavilani, et al. 2006). Humans, as well as other mammals with poor sperm motility, have also benefited from increased intake of PUFAs (Rooke, et al. 2001; Safarinejad. 2011). In rabbits this specifically correlates with increased PUFA levels in the sperm flagellum (Mourvaki, et al. 2010a).

Sphingomyelins (SMs). Spermatozoa are unique in that their membrane contains SMs with very long chain PUFAs (Poulos, et al. 1986; Poulos, et al. 1987; Robinson, et al. 1992). At least in mice the proportion of SMs in the sperm head increases during maturation in the epididymis (Rejraji, et al. 2006). When human and rat sperm reach the female reproductive tract, the conversion of SMs to ceramides causes additional loss of cholesterol (Cross. 2000; Zanetti, et al. 2010). This regulatory role of SMs in cholesterol efflux could promote membrane fluidity and acrosome exocytosis (Slotte, et al. 1990; Pörn, et al. 1993).

Carnitine. The acquisition of sperm progressive motility goes hand in hand with an increased uptake of carnitine during epididymal transit (Casillas. 1973; Casillas and Chaipayungpan. 1979; Casillas, et al. 1984). The epididymal lumen contains significantly higher concentrations of carnitine than blood plasma and the concentration increases in more distal segments due to extensive secretion by the epithelial cells (Marquis and Fritz. 1965; Casillas. 1972; Jeulin and Lewin. 1996). Spermatozoa also contain carnitine transporters. Carnitine/organic cation transporter 2 (OCTN2) is found in the principal piece of the murine sperm flagellum while carnitine/organic cation transporter 3 (OCTN3) is located in the mid piece, the place of mitochondrial activity (Kobayashi, et al. 2007). The levels of OCTN3 in the sperm

membrane also increase during epididymal transit and may contribute to the increased levels of carnitine observed in mature spermatozoa (Kobayashi, et al. 2007). In sperm, as in somatic cells, carnitine is involved in transportation of long chain fatty acids into mitochondria. Fatty acids are bound to carnitine in the cytoplasm and the resulting acylcarnitine is transported into the mitochondrion. In mitochondria acylcarnitine is again converted to free carnitine and a long-chain acyl-Coenzyme A that can be used for ATP production through fatty acid oxidation (Fritz. 1963). Although sperm motility is mainly dependent on glycolysis, oxidative phosphorylation also contributes to energy production (Jeulin and Lewin. 1996).

2.3.3. Proteins

The protein content of the luminal fluid changes dramatically between the testis, efferent ducts and the epididymis. This is in part due to reabsorption of testicular proteins, but also because of high protein secretion by the principal cells of the proximal epididymis (del Rio. 1979; Brooks. 1981; Syntin, et al. 1996). The protein concentration of bull and stallion IS luminal fluid is 2-4 mg/ml, after which intense secretion causes peak protein concentrations of 60-80 mg/ml in distal CAP. Thereafter, the protein concentration is again reduced to 20-30 mg/ml in more distal segments due to reabsorption or processing of proteins (Fouchecourt, et al. 2000; Belleannee, et al. 2011). Mass spectrometry studies have shown that around 20 of the secreted proteins represent more than 80% of total luminal protein content. Several of these show high expression in all studied species, for example: CLU, lactoferrin, lipocalin 5 (LCN5), and glutathione peroxidase 5 (GPX5) (reviewed in Dacheux and Dacheux. 2013). However, there are differences in protein concentration between species, for example CLU represents 7.6% of total human epididymal secretions while, when considering data from all species studied, CLU represents 30% of epididymal secretions (Dacheux, et al. 2006; Dacheux and Dacheux. 2013). Due to the concentration differences between the proteins with the highest and lowest secretion, many proteins have also remained unidentified in mass spectrometry analyses. Furthermore, different species have very diverse luminal fluid contents due to post-translational modifications and segment specific secretion and reabsorption of proteins (Syntin, et al. 1996; Fouchecourt, et al. 2000; Belleannee, et al. 2011).

Proteins in sperm protection. The most abundant proteins of the luminal fluid are not the most commonly found in sperm. Instead they are believed to function in preservation of spermatozoa during epididymal transit. The epididymal lumen contains proteins that protect sperm from both bacteria as well as ROS. Inflammation of the epididymis can lead to permanent damage as the tissue lacks regenerative functions (Dohle, et al. 2005). Acute epididymitis is usually caused by urinary tract infections or sexually transmitted bacterial infections, for example Chlamydia trachomatis and

Neisseria gonorrhoeae, but also Escherichia coli (reviewed in Trojian, et al. 2009). The previously mentioned cationic β-defensin proteins have been shown to kill bacteria, fungi and enveloped and non-enveloped viruses *in vitro* (Selsted and Ouellette. 2005). For example, the mouse β-defensin SPAG11a has a role in epididymal infection resistance (Fig. 5, Fei, et al. 2012). Mice overexpressing the gene showed higher resistance to epididymal Escherichia coli infections. In addition, SPAG11a was suggested to regulate inflammatory response in the tissue, by downregulation of IL1α and IL1β expression (Fei, et al. 2012). However, the antimicrobial properties of the β-defensin proteins might be secondary to their function in sperm maturation, as to date this is the only *in vivo* study that has shown a role for β-defensins in microbial defense of the epididymis. Spermatozoa are also sensitive to ROS activity, due to their high numbers of PUFAs. Lipid peroxidation of PUFAs impairs the cell membrane and the oxidative products can further damage DNA (reviewed in Vernet, et al. 2004). An example of an epididymal antioxidant is the GPX5 protein. The secreted protein associates with the sperm membrane in the epididymis and may be incorporated into the membrane through epididymosomes (Taylor, et al. 2013). Caudal sperm of *Gpx5* knock-out mice show high DNA damage, probably because of failure to eliminate the hydrogen peroxide produced in sperm (Chabory, et al. 2009). Antioxidants are important for sperm fertilizing ability as malfunctions in this process may lead to increased incidence of subfertility, spontaneous abortion, developmental defects, and pathologies in offspring (Kodama, et al. 1997; Zini, et al. 2001; Tesarik, et al. 2004; Zini and Libman. 2006).

Proteins in sperm maturation. Although many epididymal proteins only serve a protective function, several are bound to the sperm surface during epididymal transit where they affect sperm fertilizing ability. The proteins can either bind loosely to other proteins, or they can be incorporated into the sperm membrane by a glycosyl phosphatidylinositol (GPI) –anchor (reviewed in Dacheux and Dacheux. 2013). Transmembrane and hydrophobic proteins often need transporters to move out from the epithelial cells to the lumen. One way is through biding to CLU that is able to form large protein clusters, or they are moved out from the cells through apocrine secretion, in epididymosomes (Law and Griswold. 1994; Sullivan and Saez. 2013). Interestingly, the epididymal proteins are often incorporated in certain regions of the sperm, for example only in the sperm head or in certain locations of the sperm flagellum as the already mentioned OCTN3 that is specifically located in the mid piece (Kobayashi, et al. 2007). Another example is the GPI-linked serine peptidase inhibitor, Kazal type 13 (SPINK13), which is secreted into the lumen of rat IS, where it binds specifically to the acrosome region of sperm head (Ma, et al. 2013a). SPINK13 is a protease inhibitor and may protect sperm from premature acrosome reaction by an as yet unknown mechanism (Ma, et al. 2013a). Several other epididymal proteins bind to sperm in a similar manner, however, they all appear to work in collaboration with other membrane

proteins as the ablation of any one of these still gives rise to fertile sperm (Dacheux and Dacheux. 2013).

The sperm glycocalyx. During epididymal transit, sperm are further protected and kept in a quiescent state through remodeling of the glycoproteins and –lipids of the sperm glycocalyx (reviewed in Schröter, et al. 1999; Diekman. 2003). Hundreds of glycoconjugates form this, in guinea pig 20-60 nm, thick carbohydrate layer surrounding the cell (Bearer and Friend. 1990). The glycocalyx has a four-dimensional structure in that the N-glycan chains are flexible and can form different conformations as sperm travel through the epididymis and female reproductive tract (Schröter, et al. 1999). In the epididymis, the structure of the glycocalyx changes progressively as sperm encounter the many glycosyltransferases and glycosidases secreted by the different epididymal segments (reviewed in Tulsiani. 2006). Glycoproteins, secreted by the epididymal epithelium, are also added to the glycocalyx sequentially. In this way the added glycoproteins, for example the caudal protein CD52, are thought to prevent the interaction between luminal molecules and proteins already present on the sperm surface (Schröter, et al. 1999). In addition, the glycoconjugates prohibit sperm from binding to each other, i.e. agglutination (Diekman, et al. 1997). The glycocalyx is also essential in sperm transit through the female reproductive tract. For example, the maqacue β-defensin DEFB126 and its mouse orthologue DEFB22 form the major part of the glycocalyx and contribute to the net negative charge of the sperm surface (Fig. 5, Tollner, et al. 2008b). The negative charge is a common feature for all mammalian sperm and is needed for transit through the highly acidic mucins of the female cervix (Yanagimachi, et al. 1973; Holt. 1980; Gould, et al. 1984; Lopez, et al. 1987; Stoffel, et al. 2002). In addition, DEFB126 mediates attachment of the sperm to the oviductal epithelium and the formation of a sperm reservoir (Tollner, et al. 2008a). During sperm capacitation in the fallopian tubes, many of the loosely bound glycoproteins are lost (Rosado, et al. 1973). This is required for further activation of sperm and to uncover proteins important for oocyte binding (Lassalle and Testart. 1994; Tollner, et al. 2008a; Tollner, et al. 2009).

2.4. Regulation of epididymal gene expression

Gene expression in the adult epididymal epithelium is highly regulated, both by testicular factors and factors expressed by the different segments (reviewed in Robaire, et al. 2006; Cornwall. 2009; Belleannee, et al. 2012b). However, how gene expression is restricted to specific segments of the epididymis is still not well known. Especially since the expression of one gene can be regulated at multiple steps by a combination of steroid signals and transcription factors. One example of this is the lipocalins located in the same gene cluster. For example, the expression of lipocalin 5 (*Lcn5*) is restricted to CAP, where AR can bind to its promoter. In more distal segments, the presence of the

transcription factor forkhead box A2 (FOXA2) inhibits induction of *Lcn5* expression by binding to AR. (Yu, et al. 2006; Suzuki, et al. 2007). For some transcription factors this segment bound expression pattern appears to be the results of epididymal development. For example the homeobox (*Hox*) genes, are expressed during epididymal differentiation and set the boundaries between the different segments and vas deferens (Hsieh-Li, et al. 1995; Benson, et al. 1996). In the adult epididymis, the genes continue to be expressed in specific segments where they also regulate gene expression (Bomgardner, et al. 2001; Bomgardner, et al. 2003).

2.4.1. Steroids

The most important regulatory factors of epididymal function are androgens. Microarray studies of rats have shown a reduction or increase in the expression of 6-7% of epididymal genes after orchidectomy. For over two-thirds of these genes the effect was reversed after DHT treatment (Hamzeh and Robaire. 2010). Although AR is expressed throughout the adult epididymal epithelium, the response to androgens differs between segments. CAP expresses the highest levels of androgen-dependent genes, which could be explained by the higher levels of lumicrine androgens found in the proximal epididymis (Robaire and Viger. 1995; Chauvin and Griswold. 2004; Sipilä, et al. 2006; Hamzeh and Robaire. 2010). However, a similar androgen dependency is not observed in the more proximal IS where the expression of segment-enriched genes remains downregulated after treatment of gonadectomized mice with DHT (Sipilä, et al. 2006). Furthermore, a study on the androgen-responsive gene carbonic anhydrase 4 (*Car4*) in rat, shows higher expression in COR than CAP (Kaunisto, et al. 1999). Thus, additional regulation of androgen signaling is required to fully explain the pattern of epididymal gene expression. Such regulatory elements could be the regional expression of androgen receptor coregulators, as seen in the case of *Lcn5*, or a difference in the epigenetic regulation of genes (Yu, et al. 2006; Sipilä, et al. 2011).

In rodents, estrogens are produced in testis and spermatozoa by the activity of cytochrome P450 aromatase (CYP19A1) (Nitta, et al. 1993; Janulis, et al. 1996). Some studies also suggest an expression of CYP19A1 in CAP of rodents and primates (Pereyra-Martinez, et al. 2001; Carpino, et al. 2004). In mice, *Esr1* is mainly expressed in narrow and apical cells of IS, while CAP shows strong expression in all epithelial cell types (Zhou, et al. 2002). In the epididymis, estrogen bound to the receptor is reported to regulate epithelial gene expression in concert with AR. For example, the expression of aquaporin 9 (*Aqp9*) is regulated by both steroids (Pastor-Soler, et al. 2010). Furthermore, treatment of castrated rats with both androgens and estrogens produce a much greater increase in epididymal weight than testosterone alone (Oliveira, et al. 2004). The need for a balanced steroid signaling was also observed

when giving the potent estrogens diethylstilbestrol and ethinyl oestradiol to neonatal rats. The increased levels of estrogen caused a downregulation of AR in the epididymis and a reduction in epithelium height. However, when testosterone was administered to the animals, to compensate for the imbalance in estrogen-testosterone signaling, no phenotypic abnormalities were detected (Williams, et al. 2001). Estrogen receptor 2 (*Esr2*, also known as *Erβ*) is expressed throughout the epididymis, however the role of the receptor is not yet clear, since the knock-out mice of *Esr2* do not show any morphological differencies of the epididymis (Krege, et al. 1998).

2.4.2. Lumicrine factors

As in epididymal development, the regulation of proximal segment function is dependent on factors in the luminal fluid. Growth factors, produced in testis, are continuously secreted into the luminal fluid and transported to the epididymis (Kirby, et al. 2003). Spermatozoa may also function as transporters of testicular proteins, for example germinal angiotensin I-converting enzyme (gACE) (Métayer, et al. 2002). After entering the epididymis the ligands could interact with the epithelial cells of the epididymis either in the released form or still bound to the sperm surface, and thereby affect gene expression. For instance, gACE is known to indirectly regulate luminal acidification and water transport in the epididymis and the expression of proenkephalin in the rat epididymis is also known to be regulated by the presence of sperm (Garrett, et al. 1990; Belleannee, et al. 2012b).

2.4.3. miRNAs

The function of the epididymal epithelium is also suggested to be regulated by transportation of miRNAs from one segment to another. MicroRNAs are transferred into the epididymal lumen by apocrine secretion from the epithelium and, if taken up by more distal cells, are believed to control the gene expression of that cell (Belleannee, et al. 2013). Furthermore, miRNAs specifically expressed in the testis have been found in the epididymal fluid and could serve a similar function as other testicular lumicrine factors (Bao, et al. 2012; Belleannee, et al. 2013).

Although the highest number of miRNAs is found in the differentiating epididymis, the epididymides of adults also show segment specific miRNA expression (Zhang, et al. 2010). MiRNAs belonging to the miR-888 cluster in primates are suggested to play a role in sperm maturation as their expression is restricted to the epididymis (Li, et al. 2010). Furthermore, a study on the human epididymis has shown that the expression of epididymal target genes is negatively correlated with the expression of miRNAs. For example, the expression of miR-145 was highest in CAP while its target gene, claudin 10, displayed high expression in CAU (Belleannee, et al. 2012a). This would indicate a role for miRNAs in regulating segment specific gene expression and, indirectly, the

maturation of sperm in the epididymis. This was further shown in rats where the introduction of an epididymis specific non-coding RNA also led to downregulation of the target gene carboxylesterase 7 (*Ces7*) which is suggested to be of importance during sperm maturation (Ni, et al. 2011). The reduction of *Ces7* expression by RNA interference caused reduced capacitation of rat sperm, which could potentially affect the fertility of animals (Ni, et al. 2011). Many miRNAs are also known to be regulated by androgen signaling. For example the expression of miR-29a in rat was upregulated after castration (Ma, et al. 2013b). As miR-29a is known to induce cell apoptosis, it was suggested that the increase in epithelial cell apoptosis observed after androgen withdrawal could be partially miRNA dependent (Ma, et al. 2013b). Because one miRNA can regulate the expression of multiple target genes and one mRNA product can be regulated by many miRNAs, miRNAs could further fine tune the regulation of epididymal gene expression. This complexity was also observed for miR-29a which was not only downregulated by androgen signaling but also able to inhibit AR expression, thereby generating a regulatory circuitry affecting multiple target genes (Ma, et al. 2013b).

2.5. Sperm activation in the female reproductive tract

When sperm enter the female reproductive tract they encounter an environment that is very different from that of the epididymis. The change in pH and osmolality of the surrounding fluid causes the mature spermatozoon to become motile and to go through the different stages of activation; capacitation, hyperactivation, and the acrosome reaction. The following chapters will elucidate the known signaling pathways required for sperm activation.

2.5.1. Capacitation

Fast events. Sperm capacitation is the beginning of the intricate chain of signaling events required for sperm to reach and penetrate the oocyte (Chang. 1951; Austin. 1951; Yanagimachi. 1994). After entering the female reproductive tract, a number of ion-channels in the principal piece (Fig. 4) are activated by the increased pH of the fallopian tubes, causing elevated intracellular HCO_3^- levels and depolarization of the sperm membrane (Demarco, et al. 2003; Wang, et al. 2003). The change in intracellular pH activates sperm specific Ca^{2+}-channels (sperm associated cation channels, Catspers), which bring further activation of a sperm soluble adenylyl cyclase (SACY, also known as ADCY10) and increases the levels of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) (Fig. 6, Chen, et al. 2000; Qi, et al. 2007). This process is very rapid, for example boar sperm show maximum levels of cAMP within 60 s of induction (Harrison and Miller. 2000).

Slow events. The initial fast induction then changes to a slower signaling pathway where phospholipids of the sperm membrane are rearranged, and sterols are oxidized to promote release of cholesterol to external acceptors (Fig. 6, Harrison, et al. 1996; Gadella and Harrison. 2002; Brouwers, et al. 2011). While *in vitro* studies of sperm capacitation is usually performed using bovine serum albumin (BSA) as a cholesterol acceptor, the high density lipoproteins (HDLs) of human and bovine follicular fluids are suggested to serve a similar function *in vivo* (Ehrenwald, et al. 1990; Hamdi, et al. 2010). At this point, many species form a sperm reservoir in the oviductal isthmus by interaction of the sperm glycocalyx and epithelial cells of the oviduct (Fig. 6, Harper. 1973; Hunter and Nichol. 1983; Hunter. 1984; Wilmut and Hunter. 1984; Smith and Yanagimachi. 1990). One purpose of the reservoir is to synchronize the time of further sperm capacitation with ovulation. Signals released at ovulation cause glycoproteins like CD52 and DEFB126 to dissociate from the glycocalyx and sperm to leave the reservoir (Fig. 6, Della Giovampaola, et al. 2001; Talevi, et al. 2007; Gualtieri, et al. 2009; Tollner, et al. 2009). The release of cholesterol and the negatively charged glycoproteins changes the membrane structure and causes additional influx of both HCO_3^- and Ca^{2+} (Xia and Ren. 2009). This, in turn, gives rise to further phosphorylation of protein tyrosines via upregulation of PKA (Chen, et al. 2000). The phosphorylated proteins include ion channels, metabolic enzymes and proteins involved in the structure of the fibrous sheath of the sperm flagellum (Ficarro, et al. 2003). In mice, *in vitro* studies show maximal levels of protein tyrosine phosphorylation around 90 min after addition of the cholesterol acceptor BSA. At this time spermatozoa are said to be capacitated (Visconti, et al. 1995).

Lipid rafts. During sperm capacitation, changes in sperm membrane fluidity and the surrounding glycocalyx, together with activation of phospholipid scramblase proteins, facilitate the relocation of lipid rafts in the sperm head (Shadan, et al. 2004; Bou Khalil, et al. 2006; Boerke, et al. 2008; Nixon, et al. 2009). Lipid rafts are heterogeneous entities containing high amounts of cholesterol, sphingolipids, and proteins related to sperm-oocyte interaction (Simons and van Meer. 1988; Pike. 2006; Boerke, et al. 2008; Nixon, et al. 2009). The relocation of these to the apical side of the sperm head, right above the acrosome, promotes sperm-oocyte binding (Boerke, et al. 2008; Nixon, et al. 2009). The rafts also contain fusion proteins, soluble n-ethylmaleimide-sensitive attachment protein receptors (SNAREs), which aid in acrosome exocytosis (Boerke, et al. 2008). As previously mentioned, SMs regulate cholesterol efflux from the sperm membrane. However, the SMs with very long chain PUFAs are not able to form lipid rafts together with cholesterol. Thus, these may serve in increasing membrane fluidity, while other SMs generate stable lipid rafts and regulate cholesterol efflux.

2.5.2. Hyperactivation

As previously mentioned, proteins secreted by the epididymal epithelium together with the acidic pH of the luminal fluid keep sperm in an immotile state until ejaculation. When sperm reach the female reproductive tract, the increase in the sperm's inner Ca^{2+} levels initiates the symmetrical flagellar beat pattern characteristic of activated motility (Visconti. 2009). During capacitation the Ca^{2+} influx increases further, and causes sperm to hyperactivate (Suarez, et al. 1993). *In vitro* studies show that hyperactivity changes the motility path of sperm, from a straight line trajectory to circular, figure eight, or zigzag movements, depending on the species (Yanagimachi. 1972; Fraser. 1977; Cooper, et al. 1979). The amplitude of the flagellar beat also increases while the frequency decreases to produce steady state slow oscillations of the flagellum (Ohmuro and Ishijima. 2006; Ishijima. 2011).

Activation of hyperactive motility. The main regulator of sperm hyperactive motility is Ca^{2+} (reviewed in Ho and Suarez. 2001; Costello, et al. 2009). Induction of hyperactivation is caused by increased intracellular levels of Ca^{2+} while maintenance of hyperactive motility requires cAMP signaling (Suarez and Osman. 1987; Lindemann, et al. 1991; Yanagimachi. 1994; Mujica, et al. 1994). A major difference between induction of capacitation and hyperactivation is the requirement of a cholesterol acceptor, for example BSA *in vitro*, to reach capacitation while only CHO_3^- is needed to achieve hyperactivation (Neill and Olds-Clarke. 1987). It has been suggested that the main regulators of hyperactive motility are the inner Ca^{2+} -stores located at the base of the flagellum (Ho and Suarez. 2001). The release of Ca^{2+} from these would lead to direct interaction between Ca^{2+} and the axoneme of the flagellum (Mujica, et al. 1994; Ho and Suarez. 2001), while influx of Ca^{2+} from the extracellular milieu would replenish the intracellular stores and maintain continuously high levels of Ca^{2+} in the cytoplasm (Fig. 6, Yanagimachi. 1994; Ho and Suarez. 2001). The mobilization and release of Ca^{2+} from inner stores gives rise to cytoplasmic Ca^{2+} peaks that drive the asymmetric bending of the sperm flagellum (Harper, et al. 2004; Bedu-Addo, et al. 2005; Machado-Oliveira, et al. 2008). An *in vivo* study has shown that the flagellum naturally bends in a prominent pro-hook conformation (in the same direction as the hook of the rodent sperm head) during hyperactivation (Chang and Suarez. 2011). However, if sperm are placed in a medium without Ca^{2+}, the activation of Ca^{2+} release from the inner stores mainly gives rise to an anti-hook conformation (Chang and Suarez. 2011). Interestingly, when observing sperm swimming in a highly viscous media or in the oviduct, the anti-hook conformation was dominant (Suarez and Dai. 1992; Chang and Suarez. 2012). This demonstrates how complex the system that regulates sperm motility is. Although many ion-channels and receptors of the sperm membrane are known, it has proven very difficult to determine how these function *in vivo*, in correlation with each other and together with signals emitted by the oviduct.

Function of sperm hyperactive motility. In rabbits and mice, hyperactivation takes place in the lower oviduct before ovulation (Overstreet and Cooper. 1979; Suarez and Osman. 1987). At this time, sperm are bound to a storage reservoir, but the increase in the transverse force generated by hyperactivation, together with the release of oviduct-binding proteins, causes sperm to detach from the reservoir and move toward the oocyte (Fig. 6, Demott and Suarez. 1992; Pacey, et al. 1995; Gwathmey, et al. 2003; Gwathmey, et al. 2006; Ignotz, et al. 2007; Ho, et al. 2009; Ishijima. 2011). Only sperm that are able to capacitate and hyperactivate thus continue on to interact with the oocyte (Demott and Suarez. 1992). The observed change in flagellar beat pattern during hyperactivation is also required for sperm to penetrate the oviductal mucus layer and the zona pellucida of the oocyte (Suarez, et al. 1991; Suarez and Dai. 1992; Stauss, et al. 1995). This has been shown especially in Catsper knock-outs where sperm fail to hyperactivate and thus to fertilize the oocyte, due to a reduction in Ca^{2+} -uptake (Ren, et al. 2001; Carlson, et al. 2005; Jin, et al. 2007). Furthermore, sperm motility is highly associated with the efficiency with which sperm bind to the oocyte (Coddington, et al. 1991). Due to this, sperm with lower average path velocity have lower fertilizing ability in *in vitro* fertility (IVF) tests (Check, et al. 1990; Liu, et al. 1991).

Sperm chemotaxis is also highly dependent on hyperactive motility. It is known that sperm react to progesterone and signals emitted from the follicular fluid by increased asymmetrical flagellar bends and sharp turning movements, similar to those of hyperactive motility (Spehr, et al. 2003; Fukuda, et al. 2004; Armon and Eisenbach. 2011). However, the change in motility is only brief and enables sperm to turn towards the chemoattractant after which they continue in a more linear motility path (Armon and Eisenbach. 2011). The model proposes that a high stimulus would lead to an acclimated movement pattern where sperm swim in a straight line. If the sperm swims away from the attractant the reduction in signal causes hyperactive motility and the sperm to turn towards the source of the chemoattractant (Armon and Eisenbach. 2011). The activation of motility is caused by an increased Ca^{2+} -influx into the sperm flagellum, either directly, through the activation of Catsper channels by progesterone (Lishko, et al. 2011; Strunker, et al. 2011), or indirectly, by the activation of G-protein-coupled odorant receptors (Spehr, et al. 2003; Fukuda, et al. 2004). Interestingly, only a small subpopulation of sperm is able to react to the attractant *in vitro*. It is suggested that capacitation is a prerequisite for chemoattraction, however, the amount of receptors already present in the sperm membrane also need to be high enough for the spermatozoon to form a proper response (Cohen-Dayag, et al. 1994; Cohen-Dayag, et al. 1995; Spehr, et al. 2003; Fukuda, et al. 2004).

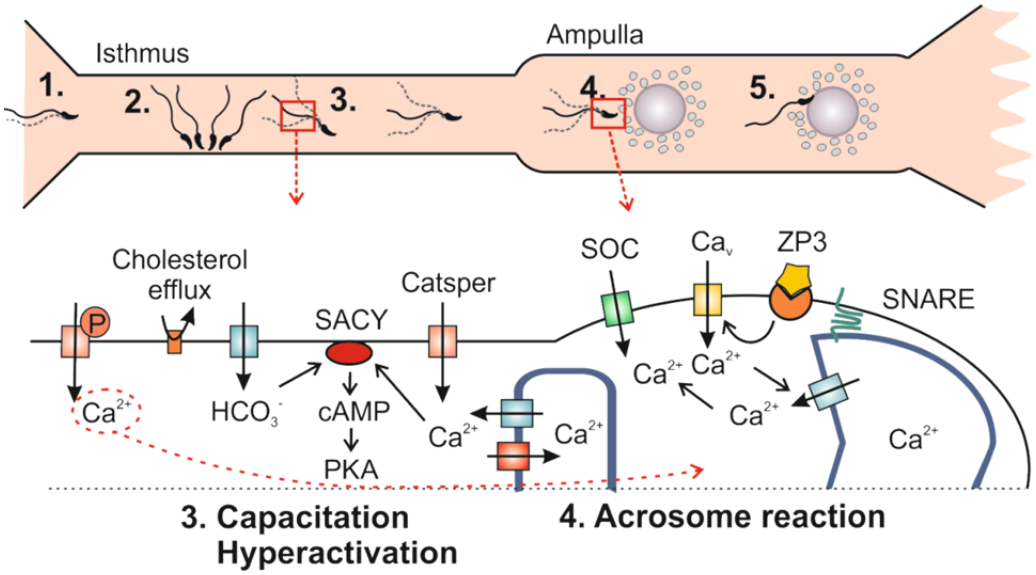


Fig. 6. Sperm activation in the female reproductive tract. Schematic drawing of sperm movements through the female reproductive tract and the major signaling pathways activated in sperm during this time. 1. Sperm motility is activated upon entry into the female. 2. Sperm bind to the oviduct and are kept in a quiescent state until ovulation. 3. Signals from the oocyte cause an efflux of cholesterol from the sperm membrane and an increased influx of HCO_3^- and Ca^{2+}, which finally leads to activation of protein kinase A (PKA) and phosphorylation of protein tyrosines. At the same time, Ca^{2+} is released from the inner stores and the spermatozoon is hyperactivated. This results in the release of sperm from the reservoir. 4. When reaching the oocyte, further Ca^{2+} influx is activated by progesterone (P) binding to Catsper channels and the binding of zona pellucida glycoprotein 3 (ZP3) to receptors in the sperm head. Ca^{2+} is also released from the acrosome stores. High Ca^{2+} levels result in the activation of store operated channels (SOC) in the sperm membrane and the fusion of the outer plasma membrane with the acrosome membrane through soluble n-ethylmaleimide-sensitive attachment protein receptors (SNARE) proteins, i.e. the acrosome reaction. 5. After release of the acrosome content, the sperm can penetrate zona pellucida and fuse with the oocyte (modified from Darszon, et al. 2011; Coy, et al. 2012).

2.5.3. The acrosome reaction

Activation of the acrosome reaction. The acrosome is an organelle located above the sperm nucleus that contains enzymes and oocyte interacting proteins required for penetration of the egg cell (Fig. 4, Yanagimachi. 1994). When sperm come into close proximity of the oocyte, progesterone and zona pellucida glycoproteins (ZP1-4) induce the fusion of the outer acrosome membrane with the sperm plasma membrane and a subsequent release of the acrosome content (Baldi, et al. 2009; Litscher, et al. 2009). This, so called, acrosome reaction is largely dependent on Ca^{2+} -signaling. As in the case of capacitation, the acrosome reaction requires an initial fast Ca^{2+} -elevation and a later sustained increase in intracellular Ca^{2+} levels (Darszon, et al. 2011). The initial response is thought to be triggered by the binding of receptors on the sperm head to ZP3 (Litscher, et al. 2009). This causes hyperpolarization of the sperm and activation of voltage gated Ca^{2+} (Ca_v) channels (Arnoult, et al. 1999). However, since patch-clamp experiments of mammalian sperm have not been able to show any Ca^{2+} -influx through the Ca_v channel, another hypothesis was recently proposed. Ren and Xia reported elevated Ca^{2+} levels in the sperm head only seconds after activation of Catsper channels in the principal piece and suggested that this might be the source of the initial Ca^{2+} -increase (Ren and Xia. 2010). Furthermore, sperm have been shown to acrosome react already when reaching the cumulus layer, probably due to an increased secretion of progesterone from the cells (Yin, et al. 2009; Jin, et al. 2011). Thus, the acrosome reaction could be a combined action of progesterone induced CatSper activation and ZP3 binding to receptors in the sperm head (Fig. 6). The initial Ca^{2+} -influx is further aided by activation of the inositol triphosphate receptors (IP_3R) of sperm Ca^{2+} -stores (Walensky and Snyder. 1995; O'Toole, et al. 2000). The acrosome itself stores Ca^{2+}, which is released through the indirect action of Ca_v channel Ca^{2+} -influx (Fig. 6, De Blas, et al. 2002; Herrick, et al. 2005; Darszon, et al. 2011). This, in turn, gives rise to a sustained Ca^{2+} -elevation through activation of store operated channels (transient receptor potential cation channel, subfamily C (TRPC), and/or calcium release-activated calcium modulator ORAI) (Fig. 6, Jungnickel, et al. 2001; Costello, et al. 2009).

Fusion of the sperm acrosome and plasma -membranes requires disassembly of the actin filaments of the sperm head and the assembly of SNAREs into intermembrane complexes (Fig. 6, Darszon, et al. 2011). During capacitation, globular actin (G-actin) is polymerized to form filamentous actin (F-actin) that prohibits the fusion of secretory granules with the plasma membrane (Castellani-Ceresa, et al. 1993; Brener, et al. 2003). After capacitation, intracellular Ca^{2+} continues to rise and activates an actin severing protein, gelsolin, which is able to disperse F-actin and cause exocytosis (Cabello-Agueros, et al. 2003; Finkelstein, et al. 2010). The final stage of membrane fusion is administered by the reassembly of SNAREs from the acrosome and plasma

membranes, into interlinked complexes that promote acrosomal exocytosis (Fig. 6, reviewed in (Mayorga, et al. 2007). This step is also Ca^{2+} dependent and caused by inhibition of the SNARE binding protein, complexin (Mayorga, et al. 2007; Castillo Bennett, et al. 2010).

Sperm-oocyte fusion. The acrosome contains a number of hydrolytic enzymes needed to penetrate the zona pellucida (Ferrer, et al. 2012). Exocytosis of the organelle causes local breakdown of the glycosylated zona pellucida proteins, which enables sperm to reach and fuse with the oocyte. The changes taking place in the actin cytoskeleton during the acrosome reaction, also prepare sperm for fertilization. For example, the protein izumo sperm-egg fusion 1 (IZUMO1) is relocated via alterations of the cytoskeleton from the anterior acrosome to other regions in the head, where it contributes to oocyte fusion (Miranda, et al. 2009). So far, IZUMO1 is the only protein known to be specifically required for sperm-egg fusion, although several other proteins are suggested to participate in the interaction between the gametes (Inoue, et al. 2005 and reviewed in Gadella and Evans. 2011).

3. AIMS OF THE PRESENT STUDY

Although recent technical advances have provided extensive knowledge on the transcriptional profile of the epididymis, the role of specific proteins in epididymal development and function is still largely unknown. For example, the epididymis specific β-defensins were initially thought to function solely in microbial defense, but several family members have since been given additional roles in sperm maturation. The β-defensins that have yet to be studied could thus serve similar functions. Recent studies regarding miRNA expression and function in the epididymis also indicate an important role for RNA interference both in epididymal development and sperm maturation. However, as miRNAs are suggested to mainly function as rheostats it would be important to determine if they have a significant purpose in epididymal functions. To study the role of both epididymal protein functions and miRNAs it would be advantageous to utilize the Cre-loxP technique to generate epididymis specific knock-out models. In this way, a time point specific knock-out could also be made as a further aid to determining the function of a protein at a certain developmental stage.

The specific aims of the study were:

1. To determine the role of the epididymis specific mouse β-defensin 41 (*Defb41*) in sperm maturation.

2. To generate an epididymis specific iCre mouse model by knock-in of *iCre* under the *Defb41* promoter.

3. To study the role of *Dicer1* in the postnatal development of the epididymal epithelium by generating a knock-out of the gene in the mouse epididymis.

4. To study how the ablation of *Dicer1*, and in turn miRNAs in the epididymis, affects sperm maturation.

4. MATERIALS AND METHODS

4.1. Experimental animals

4.1.1. Animal handling (I-III)

All the mice were handled in accordance with the institutional animal care policies of the University of Turku (Turku, Finland). The animal experiments were approved by the Finnish Animal Ethics Committee, and the institutional policies on animal experimentation fully met the requirements as defined in the U.S. National Institutes of Health guidelines on animal experimentation. The mice were specific pathogen-free and housed in individually ventilated cages under controlled conditions of light (12 hours light, 12 hours darkness), temperature (21 ± 3°C), and humidity (55% ± 15%). Pelleted soy-free natural-ingredient feed (RM3 (E), Special Diets Services), or complete pelleted chow, and tap water were provided *ad libitum*.

4.1.2. The *Defb41*-deficient *iCre* knock-in mouse model (III)

To inactivate the *Defb41* gene of the mice by *iCre* knock-in (KI), the BAC clone containing the *Defb41* locus was purchased from RZPD German Resource Center for Genome Research (ImaGenes GmbH). Using Red/ET recombination, the codon-improved *Cre*-neomycin phosphotransferase (*iCre-neo*^{r}) KI cassette (Shimshek, et al. 2002) was inserted into the translation initiation site in the first exon of the *Defb41* gene, and the targeting construct containing the *iCre-neo*^{r} sequence surrounded by 1700 bp (5') and 7951 bp (3') long segments of the *Defb41* sequence, was subcloned to the pACYC177 minimal backbone vector (Angrand, et al. 1999). Subsequently, the targeting vector was linearized and electroporated into AB2.2 embryonic stem (ES) cells (129/Sv/Ev background, Lexicon Genetics). Homologous recombination between the vector and the ES cell genome was confirmed by PCR of the homology arms and the correct recombination was ascertained by sequencing the PCR products. Chimeric mice were generated by injecting a correctly targeted ES cell clone into C57BL/6N blastocysts. Chimeric males were bred with C57BL/6N females to obtain heterozygous *Defb41*$^{iCre/+}$ mice that were further bred with each other to obtain homozygous *Defb41*-deficient *iCre* KI (*Defb41*$^{iCre/iCre}$) mice and the wild type (WT, *Defb41*$^{+/+}$) mice used as a control. The mice were genotyped with DNA extracts from ear samples, using primers described in Table 1.

Table 1.

Detected sequence from		Product	Tm˚C
Defb41	Fw 5'-TCCATTGCCTTTTCTTGTCC-3' Re 5'-TTGTCTTACCAGGTTTCCTCCT-3'	269 bp	56
iCre	Fw 5'-TCTCCAACCTGCTGACTGTG-3' Re 5'-AGGGACACAGCATTGGAGTC-3'	343 bp	59

4.1.3. The *Ar* cKO mouse model (III)

The activity of iCre in the epididymal epithelium was studied by crossing *Defb41*$^{iCre/iCre}$ mice with mice carrying on their X chromosome the AR gene with a floxed exon 2 (De Gendt, et al. 2004). The *Ar*$^{fl/Y}$; *Defb41*$^{iCre/iCre}$ (*Ar* cKO) mice and the control *Defb41*$^{iCre/iCre}$ mice were genotyped as previously described (De Gendt, et al. 2004).

4.1.4. The *Dicer1* cKO mouse model (I-II)

To generate a conditional *Dicer1* knock-out of the epididymis, the *Dicer1*-floxed mouse line (Harfe, et al. 2005) was crossed with *Defb41*$^{iCre/+}$ mice. The *Dicer1*-floxed allele contained two loxP sites flanking exon 24, which includes a major part of the second RNase III domain. The obtained *Dicer1*$^{fl/fl}$; *Defb41*$^{iCre/+}$ (*Dicer1* cKO) and the control *Dicer1*$^{fl/fl}$ mice were of mixed genetic background (C57Bl/6N and SV129) and were genotyped as previously described (Harfe, et al. 2005). To detect the recombination of the floxed allele, 12-day-old mice were sacrificed by decapitation, and IS together with CAP, COR together with CAU, and testes were collected. After DNA extraction, the *Dicer1*-floxed allele was detected by genomic PCR as previously described (Harfe, et al. 2005).

4.2. Animal fertility (I)

The breeding performance of *Defb41*$^{iCre/iCre}$ male mice, compared to control males, was followed after mating with C57Bl/6N females. The number of litters and offspring of each male was determined during a time period of 6 months.

To study *Dicer1* cKO male fertility, 2-3 month-old control and *Dicer1* cKO male mice were mated with FVB/N females. The female mice were superovulated by intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG, NHPP, Dr. Parlow) and 5 IU human chorionic gonadotrophin (hCG, Pregnyl, Schering-Plough) 26 and 2 hours before mating, respectively. The following morning, females were checked for copulatory plugs and the number of litters and offspring produced by each male was determined 3-4 weeks after mating.

4.3. Hormone measurements (II)

Hormone measurements were carried out on serum samples from 2-month-old control and *Dicer1* cKO male mice. For testosterone measurements, the serum samples were extracted with diethyl ether and analyzed using standard RIA (Huhtaniemi, et al. 1985). Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were measured by immunofluorometric assays as previously described (Haavisto, et al. 1993; van Casteren, et al. 2000).

4.4. Histology and immunohistochemistry

4.4.1. Sample collection (I-III)

The control, *Defb41*-deficient *iCre* KI, *Ar* cKO and *Dicer1* cKO mice of different ages were sacrificed by CO_2 asphyxiation and cervical dislocation, after which samples were collected. Testes collected for histology were fixed overnight in Bouin while all epididymides and testes samples used in immunohistochemistry were fixed overnight in 4% paraformaldehyde. After fixation, all samples were embedded in paraffin. For detection of F-actin by phalloidin, the epididymides of 2-month-old control and *Dicer1* cKO mice were fixed for 15 min in 4% paraformaldehyde and rapidly frozen in Tissue-Tek O.C.T. (Sakura).

4.4.2. Histology (I-III)

To study the postnatal differentiation of the epididymis, haematoxylin and eosin (HE) staining of control, *Defb41*-deficient *iCre* KI, and *Dicer1* cKO epididymides was performed. Epithelial cell height of control and *Dicer1* cKO epididymides was measured using the Leica IM500 imaging software. The measurements were performed using five randomly chosen tubular cross-sections of IS and CAP each, and measuring the apical to basal height of one point of the ductal epithelium. The average height of each of five control and *Dicer1* cKO epididymides were used for statistical analyses. To determine if *Dicer1* ablation in the epididymis had an effect on testicular morphology, periodic acid-Schiff's reagent/Gill's hematoxylin (PAS) staining of control and *Dicer1* cKO mice testes was performed according to standard protocols.

The endogenous β-galactosidase activity of IS and COR was detected by X-gal staining of 2-month-old control and *Dicer1* cKO epididymides. The tissues were fixed in 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA in PBS for 30 minutes, washed overnight and stained for 2 hours at 37 ºC in 2 mM $MgCl_2$, 0.01% NaDeoxycholate, 0.02% Tergitol-type NP-40, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 1 mg/ml X-gal in PBS.

4.4.3. Immunohistochemistry (I-III)

Four-µm thick paraffin sections and 8-µm thick frozen sections of control, *Dicer1* cKO and *Ar* cKO epididymides and testes were incubated with the listed primary antibodies (Table 2) over night at 4°C. All antibodies were diluted in PBS supplemented with 1% BSA. The antibody-antigen complexes were visualized by incubation for 30 min at room temperature with the corresponding secondary antibodies, either HRP labeled polymers (EnVision, Dako) combined with DAB+ chromogen system (Dako) or 1:200 dilutions of Alexa Fluor -conjugated antibodies (Invitrogen) in combination with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI, Sigma). As controls to detect unspecific binding of the secondary antibody, sections only incubated with the secondary antibody were used.

For detection of cell apoptosis, terminal Uridine Deoxynucleotidyl Nick End Labeling (TUNEL) was used. Labeling was performed by using 0.8 U/µl TdT (Terminal transferase, recombinant, Roche) and Biotin-16-dUTP (Roche), 1 h at 37 °C. Biotin was further coupled to ExtrAvidin-Peroxidase, (1:200, Sigma-Aldrich). The peroxidase complexes were visualized with the DAB+ chromogen system (Dako).

Table 2.

Antibody	Target	Raised in	Dilution	Source
SCP3	primary spermatocytes	rabbit	1:500	Santa Cruz
CREM	round spermatids	rabbit	1:500	Santa Cruz
Ac H4	elongating spermatids	rabbit	1:1000	Millipore
Lectin *Helix pomatia* agglutinin	acrosome region	(Alexa Flour-488)	1:1000	Invitrogen
Keratin 5	basal cells	rabbit	1:100	Thermo Scientific
Cytokeratin 5/6	basal cells	mouse	1:100	Dako
V-ATPase	clear/narrow cells	rabbit	1:100	kind gift from S. Breton, USA
Foxi1	clear/narrow cells	goat	1:100	AbCam
α-actin	smooth muscle cells	mouse	1:500	Santa Cruz
Phalloidin	stereocilia	(TRITC)	1:400	Sigma-Aldrich
AR	androgen receptor	rabbit (N-20)	1:1000	Santa Cruz
ERα	ESR1	mouse	1:200	Dako
ERβ1	ESR2	mouse	1:100	Dako
Ki67	proliferating cells	rat	1:500	Dako

4.5. Gene expression analyses

4.5.1. Real time-PCR (III)

The tissue specific expression of *iCre* was studied by RT-PCR, using RNA isolated from testis, efferent ducts and the different segments of the epididymis of 2-month-old control and *Defb41*$^{iCre/iCre}$ mice. RNA isolation was performed with Tri Reagent, according to the manufacturer's instructions (Molecular Research Center, Inc.). For RT-PCR, 1 µg of total RNA was treated with deoxyribonuclease I (DNaseI, Amplification Grade, Invitrogen) and reverse-transcribed using the DyNAmo cDNA synthesis kit (Thermo Scientific). Thereafter, RT-PCR was performed using Biotools DNA Polymerase (10.002) according to the manufacturer's instructions. Primers for *iCre* detection and the forward primer for *Defb41* were described earlier. *Defb41* reverse primer: TGT GTG CAT GGA TGG AGA TT, Tm 59˚C, product size 1434 bp.

4.5.2. Quantitative RT-PCR (I-III)

The expression of *iCre* was studied by qRT-PCR in the whole epididymis at different timepoints, and in various tissues of 2-month-old *Defb41*$^{iCre/iCre}$ mice. The expression of *Defb41* exon 2 was studied in IS and CAP of 2-month-old control, *Defb41*$^{iCre/+}$, and *Defb41*$^{iCre/iCre}$ mice. The expression of *Dicer1* was studied in testes, efferent ducts, and the epididymal segments of 2-month-old control and *Dicer1* cKO. Furthermore, the WT expression of *Dicer1* and the effect of *Dicer1* ablation on gene expression in the proximal epididymis, was studied by qRT-PCR at various timepoints during the postnatal development of IS/CAP. After RNA isolation from the tissues, using Tri- (Molecular Research Center, Inc.) or TRIsure-reagent (Bioline), the RNA was reverse transcribed, as previously described. The cDNA was diluted 1:50-1:100 after which qRT-PCR was performed using the DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific) with each sample run in triplicates. The detected gene expression in testes was normalized to peptidylprolyl isomerase A (*Ppia*) while expression in efferent ducts and the epididymis was normalized to ribosomal protein L19 (*Rpl19*) and, in some cases (II and III), phosphoglycerate kinase 1 (*Pgk1*) expression. The primer pairs, annealing temperatures, and products sizes of the genes of interest, can be found in Table 3 and in publications I, II and III.

Table 3.

β-Defensins	Primer sequence	Product	Tm˚C
Defb1	Fw 5'-TTTTCTTTCTCCCAGATGGA-3' Re 5'-TGGGCTTATCTGGTTTACAGG-3'	158 bp	59
Defb2	Fw 5'-CTGCTGATATGCTGCCTCCT-3' Re 5'-AGGGGTTCTTCTCTGGGAAA-3'	175 bp	60
Spag11a	Fw 5'-CAAGGAAACTCAGGGGACAT-3' Re 5'-AGAATAGGGGACGCAGCAT-3'	150 bp	58
Spag11b	Fw 5'-CCTTCCTTTCTTTGCCAGTC-3' Re 5'-GCGTGGAGGTAATCGTTTCA-3'	181 bp	58
Defb18	Fw 5'-TGGCATCATCCTGATGGTTA-3' Re 5'-GTATTCTCGCACCACACAGC-3'	190 bp	60
Defb20	Fw 5'-GCTGCTTCAGGTTCTCCTTG-3' Re 5'-CGACTGAGTGCTTCTTGTGC-3'	197 bp	60
Defb21	Fw 5'-GTTCCTGCTGCTGGTTCTTC-3' Re 5'-TAGAGGTTTCCTCCGTGGTC-3'	224 bp	61
Defb25	Fw 5'-CTCATTGTGGCTCTCCTGGT-3' Re 5'-GCGTGAAGGCTTGAAAGAAT-3'	158 bp	60
Defb41	Fw 5'-GGGGCCACAATATTAACAGC-3' Re 5'-TGCAACAGTGAGTTCCAGGT-3'	157 bp	60
Gm6046 (*Defb51*)	Fw 5'-CTCTCTTTGCCGTCTTCCAG-3' Re 5'-TTGCAGCAGACAGTGAAAGC-3'	154 bp	60
Gm15056 (*Defb52*)	Fw 5'-GCACCTTCATCAATGCCTTC-3' Re 5'-TCCTCAGGTCATGGTTCTCA-3'	156 bp	60

4.6. Sperm analyses

4.6.1. Sperm morphology (I, II)

For analyzing sperm morphology, 2-month-old control and *Dicer1* cKO mice were sacrificed and sperm were collected by making incisions in CAU and incubating the tissue in HTF medium (William A. Cook Australia Pty. Ltd) or KSOM +AA -medium (Millipore) at 37°C and 89% N_2/6% CO_2/5% O_2, for 30 min. The samples were spread on microscopic slides, air dried, fixed in ice cold acetone for 30 min and stained using the Papanicolaou technique (Haematoxylin, OG-6 and EA-50) or with hematoxylin and eosin according to standard procedures. A number of 100-200 sperm from each animal were categorized as being either intact or without flagellum and straight or with hairpin formation.

4.6.2. Sperm motility analyses (II, III)

Sperm from control and *Dicer1* cKO mice CAU were collected in KSOM +AA -medium (Millipore) as described above. After collection the sperm were placed on a glass slide and analyzed under a microscope at ×400 magnification. From each sample the motility of 100 intact (i.e., flagellum attached to head) spermatozoa was classified as progressive, non-progressive or immotile. The average of two analyses from each sample was used for statistical comparison.

Motility analyses of *Defb41*-deficient *iCre* KI mice sperm were performed in the group of Dagmar Wachten (Caesar institute, Bonn, Germany). Sperm samples of control and *Defb41*$^{iCre/iCre}$ CAU epididymides were isolated in modified TYH medium (138 mM NaCl, 4.8 mM KCl, 2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1 mM $MgSO_4$, 5.6 mM glucose, 0.5 mM sodium pyruvate, 10 mM L-lactate, pH 7.4) 15 min swim out at 37 °C and 5 % CO_2. Sperm motility was studied, both in non-capacitating conditions and after capacitation, when the medium contained 3 mg/ml BSA and was supplemented with 25 mM $NaHCO_3$. For motility path analyses, sperm were freely swimming in shallow observation chambers (depth 150 µm). For flagellar beat analyses, sperm were tethered to the glass surface at a BSA concentration of 0.3 mg/ml. Sperm motility was recorded at 37 ˚C under an inverted microscope (IX71; Olympus). To obtain sharp images of moving sperm, stroboscopic illumination was achieved using a white LED (K2 star; Luxeon) and a custom-made housing and pulse generator (freely swimming: 1 ms, tethered: 2 ms). Cells were manually tracked during acquisition using a motorized stage (SCAN IM; Märzhäuser), and the position of the stage was recorded for each frame. Quantification of the motility path and the flagellar beat was performed using custom-made programs written in MATLAB (Mathworks) and LabVIEW.

4.6.3. Intracellular PKA/SACY and cAMP levels (III)

Measurements of PKA, SACY and cAMP were performed in the group of Dagmar Wachten. For PKA and SACY analyses the testis and sperm cells of control and *Defb41*$^{iCre/iCre}$ mice were homogenized in lysis buffer (10 mM Tris/HCl, pH 7.6, 140 mM NaCl, 1 mM EDTA, 1% Triton X 100, 1:500 mPIC protease inhibitor cocktail). Protein detection was performed by Western-blot using antibodies against PKA [Cα], (1:4000, 610980, BD Transduction Laboratories) and sAC R21, (1:1000, (Zippin, et al. 2003). The primary antibodies were detected with fluorescently-labeled secondary antibodies (anti-mouse IRDye680LT and anti-mouse IRDye800CW, 1:20,000, LI-COR Biosciences) and analyzed using the LI-COR Odyseey system.

For cAMP measurements sperm were isolated by incision of the CAU epididymis and 15 min incubation of the tissue in modified TYH-medium, pH 7.4, supplemented with 3 mg/ml BSA at 37 °C and 5 % CO_2. The intracellular resting cAMP concentration as

well as the increase in the presence of 25 mM $NaHCO_3$ and/or 0.75 mM IBMX was determined using the CatchPoint cAMP Fluorescent Assay Kit (Molecular Devices) according to the protocol provided by the manufacturer.

4.6.4. Capacitation (II, III)

Sperm capacitation was assessed as protein tyrosine phosphorylation and analyzed according to the method previously described (Wertheimer, et al. 2008). Briefly, caudal sperm from control, *Defb41*-deficient *iCre* KI, and *Dicer1* cKO mice were collected in modified non-capacitating Whitten-HEPES medium, pH 7.3, at 37°C (Platt, et al. 2009). For capacitation, the medium was supplemented with 20 mM $NaHCO_3$ and 10 mg/ml BSA, which was added to five aliquots of each sample. The sperm aliquots were then collected immediately after adding the reagents and after 30, 60, 90 and 120 min incubation and the proteins were extracted by boiling the specimens in Laemmli sample buffer (Laemmli. 1970). For detection of tyrosine phosphorylation Western-blotting was performed, using a monoclonal anti-phosphotyrosine antibody (1:5000; Millipore) as primary antibody, and a peroxidase-conjugated anti-mouse secondary antibody (1:5000, GE Healthcare). To ensure equal sample loading, the membrane was stripped by incubation with 6 M Guanidine HCl, 10% Triton X-100, 20 mM Tris-HCl, 0.1 M β-mercaptoethanol, pH 7.5. Thereafter, the membrane was re-hybridized with tubulin-alpha antibody (1:5000; Ab- 2, Lab Vision). The level of protein tyrosine phosphorylation was quantified from digital images using ImageJ software and normalized to sample levels of alpha-tubulin.

4.6.5. Acrosome reaction (II, III)

The acrosome reaction of control, *Defb41*-deficient *iCre* KI, and *Dicer1* cKO mice sperm was analyzed by Coomassie brilliant blue staining of caudal spermatozoa, as previously described (Turunen, et al. 2012). To account for the spontaneous acrosome reaction, each sample was divided into two aliquots that were collected either without induction of the acrosome reaction or after induction. A number of 100-200 spermatozoa from each sample were classified as either acrosome intact (a bright blue staining on the dorsal region of the acrosome) or acrosome reacted (patchy or absent staining).

4.6.6. Oocyte-binding assay (II, III)

Caudal sperm from control, *Defb41*-deficient *iCre* KI, and *Dicer1* cKO mice were collected in KSOM +AA -medium (Millipore), after which the sperm were counted using a Bürker hemozytometer chamber (Hawksley). A number of 40 000 spermatozoa from each sample were incubated with 15-30 zona pellucida intact oocytes collected from super-ovulated FVB/N females for 1 h at 37°C, and 89% N_2/6% CO_2/5% O_2.

After co-incubation, the gametes were washed three times with fresh KSOM-medium to remove loosely attached spermatozoa and fixed briefly in 4% PFA. From each male, spermatozoa bound to ten, randomly chosen oocytes were counted under microscope by assessing the number of sperm heads in one focal plane.

4.6.7. IVF (II, III)

After sacrificing the mice, caudal sperm of 2-month-old control, *Defb41*-deficient *iCre* KI, and *Dicer1* cKO and mice were collected in HTF-medium (William A. Cook Australia Pty. Ltd) at 37°, 89% N_2/6% CO_2/5% O_2. IVF was performed using oocytes, collected from sacrificed, super-ovulated FVB/N females, and 50 000 spermatozoa from each male. The percentage of fertilized oocytes, cleaved to two-cell-stage embryos, was determined after incubation in HTF-medium overnight.

4.6.8. Sperm membrane lipid analyses (II)

The lipid content of control and *Dicer1* cKO sperm membranes was measured from caudal sperm samples either collected in modified non-capacitating Whitten-HEPES medium, pH 7.3 or acrosome reacted, as described above. After washing the samples twice in PBS at +4˚C, the total sperm number/sample was counted.

Cholesterol measurements were performed in the laboratory of Helena Gylling (Department of Medicine, University of Helsinki, Helsinki, Finland). Briefly, the pellets of intact and acrosome reacted sperm were homogenized in chloroform-methanol with Ultra Turrax (Ika Works, Inc.) after addition of an internal standard (5α-cholestane). The sterol was saponified, extracted, and subjected to gas-liquid chromatography analysis as trimethylsilyl-derivatives, using a 50-m-long Ultra 2 capillary column (Agilent Technologies) as described previously (Miettinen. 1988). The coefficient of variation for cholesterol measurement was 3.2%.

Lipid analyses were performed by BIOCRATES Life Sciences AG (Innsbruck, Austria) who used commercially available KIT plates (AbsoluteIDQTM p150 kit assay) for quantification of acylcarnitines, sphingomyelins, and phosphatidylcholines. The fully automated assay was based on PITC (phenylisothiocyanate)-derivatization in the presence of internal standards followed by FIA-MS/MS using an AB SCIEX 4000 QTrap™ mass spectrometer (AB SCIEX) with electrospray ionization.

4.7. Statistical analyses (I-III)

Statistical comparisons between between control, *Defb41*$^{iCre/+}$ and *Defb41*$^{iCre/iCre}$ samples and control and *Dicer1* cKO were performed using One-way ANOVA and Student's t-test, respectively. Statistical significance was determined using the

GraphPad Prism 5 software (GraphPad Software, Inc.) and assigning $p \leq 0.05$ as the limit of statistical significance.

Daniel Laajala (Department of Mathematics and Statistics, University of Turku, Turku, Finland) performed statistical analyses of *Defb41*-deficient *iCre* KI sperm motility parameters (VCL, curvilinear velocity; VAP, average path velocity; VSL, straight linear velocity; LHD, lateral head displacement; Wobble, LIN, linearity; and STR, straightness), after the non-motile cells were filtered out from the data (criterion VAP < 20 μm/s). Statistical analyses were performed using a mixed-effects model that adjusted for the hierarchical structure of the data. The model was fitted using statistical software R (http://www.R-project.org) and the package lme4 (http://CRAN.R-project.org/package=lme4).

5. RESULTS

5.1. Generation of the *Defb41*-deficient *iCre* KI mouse line (III)

The expression of *Defb41* was shown by Jalkanen et al. to be epididymis specific (Jalkanen, et al. 2005). To generate a full inactivation of the *Defb41* gene and simultaneously induce an epididymis specific expression of *iCre*, a targeting vector was made by inserting the *iCre*-recombinase expression cassette into the first exon of the gene (III: Fig. 1A). The construct was incorporated into the genomic DNA of ES cells through homologous recombination, and correct targeting was confirmed by PCR and sequencing of the 5'- and 3'-homology arms. The chimeric mice produced by this technique were further mated to give rise to control (*Defb41*$^{+/+}$), *Defb41*$^{iCre/+}$, and *Defb41*$^{iCre/iCre}$ mice. Mice homozygous for the *Defb41*-deficient *iCre* KI allele showed no expression of *Defb41* exon 1 or exon 2 while *iCre* was expressed in IS and CAP (III: Fig. 1C,D). This did, however, not result in any discernable phenotypic changes in the epididymis. The weight of both the epididymis and testis of 2-month-old *Defb41*$^{iCre/+}$ and *Defb41*$^{iCre/iCre}$ mice was similar to that of control mice. Hematoxylin and eosin staining also showed similar histology of the epididymis as compared with the control (III: Fig. S1).

5.2. *Defb41*-deficient *iCre* KI male mice fertility (III)

As β-defensins are known to be secreted into the lumen of the epididymis where they can interact with spermatozoa, studies of *Defb41*-deficient *iCre* KI mice fertility were performed. Sperm count and morphology (III: Fig. 3B-D) did not differ between the control, *Defb41*$^{iCre/+}$, and *Defb41*$^{iCre/iCre}$ mice, and all genotypes were fertile in normal breeding tests and produced similar number of litters and offspring. After entering the female reproduction tract, sperm go through capacitation, which is detected as an increase in protein tyrosine phosphorylation. Control and both *Defb41*$^{iCre/+}$ and *Defb41*$^{iCre/iCre}$ mice sperm showed a close to three-fold increase in phosphorylation of protein tyrosine after incubation in capacitating conditions (III: Fig. 6C,D). Sperm capacitation is a prerequisite for the acrosome reaction, which takes place when spermatozoa come in contact with the oocyte. The *Defb41*$^{iCre/iCre}$ sperm showed a significantly reduced ability to bind to the oocyte, however, we did not observe any decrease in the ability to go through the acrosome reaction (Table 4). *In vitro* fertility tests also showed similar percentage of fertilized oocytes in control, *Defb41*$^{iCre/+}$, and *Defb41*$^{iCre/iCre}$ mice (Table 4).

Table 4.

sperm phenotype	control	*Defb41*$^{iCre/+}$	*Defb41*$^{iCre/iCre}$	*p*-value
% acrosome reacted	72.7 ± 3.2	66.4 ± 5.0	61.1 ± 4.9	*0.320*
nr. of bound sperm	37.3 ± 8.8	25.0 ± 7.0	9.5 ± 4.1	*0.046* *
% fertilized oocytes (IVF)	63.2 ± 8.1	73.6 ± 9.6	51.2 ± 11.7	*0.309*

*statistical significance $p < 0.05$

5.3. *Defb41*-deficient *iCre* KI sperm motility (III)

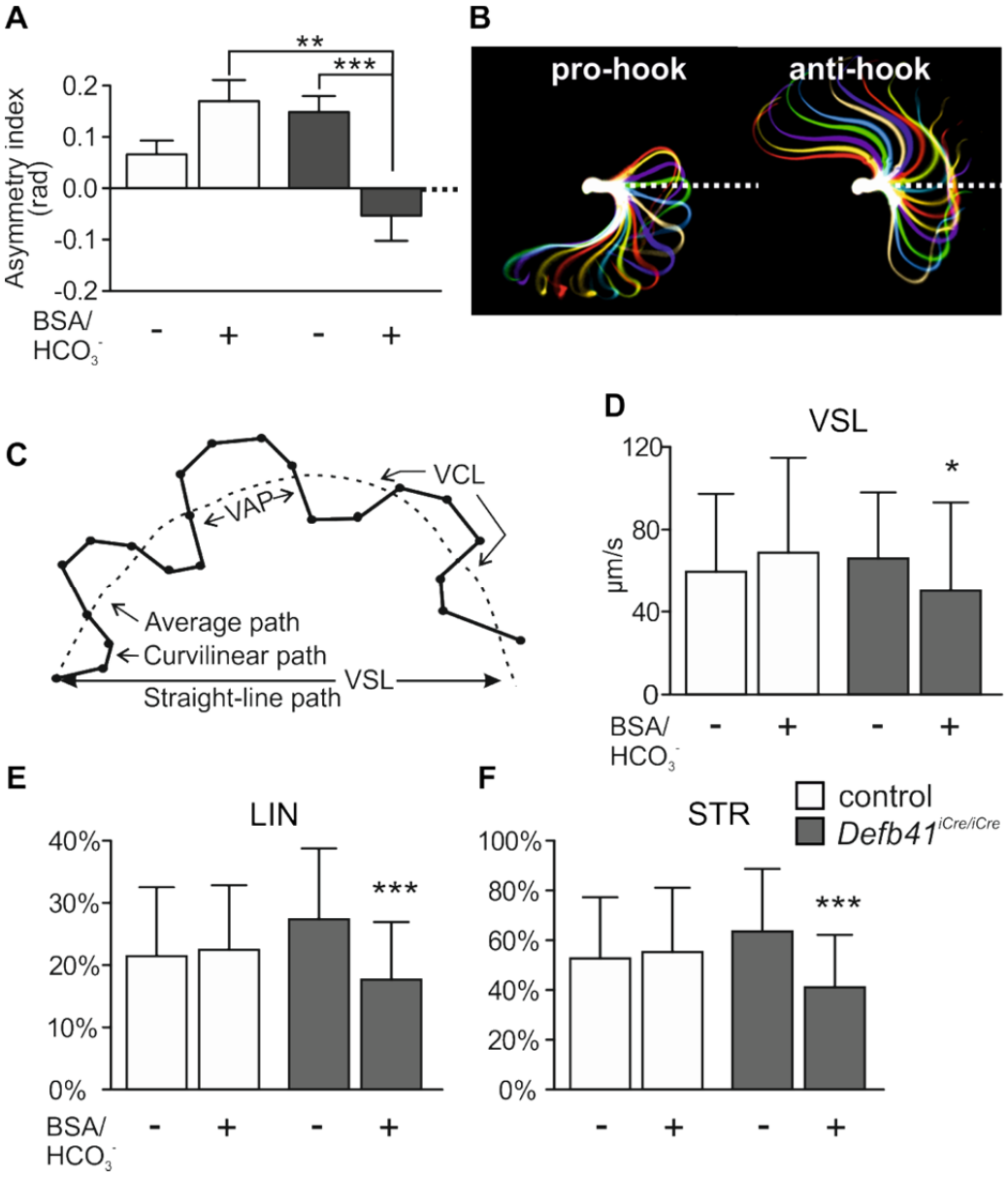
Sperm motility was analyzed prior to and after capacitation, by studying the flagellar movements and the path of motility. When studying the flagellar beat of tethered sperm we detected a significant difference in symmetry of flagellar bending between capacitated control and *Defb41*$^{iCre/iCre}$ sperm (Fig. 7A). Capacitation of sperm results in a change of flagellar movement from a symmetrical beat pattern to bending alternatively in a prominent pro-hook and anti-hook conformation (Fig. 7A,B). While control sperm were more often found in an anti-hook conformation, the capacitated *Defb41*$^{iCre/iCre}$ sperm flagellar bending was biased towards a pro-hook conformation, as indicated by a more negative asymmetry index (Fig. 7A). However, when studying the two flagellar conformations separately, the *Defb41*$^{iCre/iCre}$ sperm were also observed in anti-hook conformation with similar bending amplitude as the control sperm (III: Fig. 4H). This showed that *Defb41*$^{iCre/iCre}$ sperm are able to bend in an anti-hook conformation but spend more time in pro-hook than control sperm after capacitation. We also analyzed the amplitude and frequency of the flagellar beat, but there was no difference between control and *Defb41*$^{iCre/iCre}$ mouse sperm under non-capacitating and capacitating conditions (III: Fig. 4I,J).

The different motility parameters of the swimming path of sperm showed a significant decrease in straight line velocity (VSL) after capacitation of *Defb41*$^{iCre/iCre}$ sperm (Fig. 7D). Consequently, sperm linear motility (LIN) and the straightness of sperm movement (STR) were also reduced as they describe the relationship between VSL and sperm curvilinear motility (VSL:VCL) and average path of motility (VSL:VAP), respectively (Fig. 7E,F). Taken together, our results indicate a reduced progressive motility of *Defb41*$^{iCre/iCre}$ sperm, as their path of motility is more curved after capacitation.

Sperm motility is strongly dependent on PKA signaling which in turn is regulated by intracellular levels of SACY and cAMP. Protein expression of SACY and PKA were similar in control and *Defb41*$^{iCre/iCre}$ sperm (III: Fig. 6A). Furthermore, sperm of both

genotypes showed similar increase in cAMP after activation of SACY by bicarbonate (III: Fig. 6B).

Fig. 7. *Defb41*-deficient *iCre* KI sperm motility. A) Asymmetry index of flagellar bending (radian) in control and *Defb41*$^{iCre/iCre}$ sperm before (-) and after (+) induction of capacitation with BSA/HCO_3^-. Negative values indicate pro-hook and positive values anti-hook conformation. Statistical significance is calculated using the unpaired t-test. B) Representative image of the flagellar waveform of tethered, capacitated sperm in pro-hook and anti-hook conformation. C) Schematic view of the detected motility path of one spermatozoon. VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity. D) VSL, E) linearity (LIN), and F) straightness (STR) before (-) and after (+) induction of capacitation with BSA/HCO_3^- of freely swimming control and *Defb41*$^{iCre/iCre}$ sperm. Statistical significance, indicating difference in response to capacitation between control and *Defb41*$^{iCre/iCre}$ sperm, was calculated using a mixed effects model. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.


A
B
**
***
Asymmetry index (rad)
0.2
0.1
0.0
-0.1
-0.2
BSA/
HCO3-
- + - +
pro-hook
anti-hook
C
VCL
VAP
Average path
Curvilinear path
VSL
Straight-line path
D
VSL
120
80
40
0
µm/s
*
BSA/
HCO3-
- + - +
E
LIN
40%
30%
20%
10%
0%
***
BSA/
HCO3-
- + - +
F
STR
control
Defb41iCre/iCre
100%
80%
60%
40%
20%
0%
***
- + - +

5.4. Generation of epididymis specific conditional knock-out mouse lines (I, III)

The expression pattern of *iCre* in *Defb41*$^{iCre/iCre}$ mice was similar to that of previously published data on WT mice *Defb41* expression (Jalkanen, et al. 2005), as defined by qRT-PCR. *Defb41*$^{iCre/iCre}$ mice began to express *iCre* in IS and CAP between 7 and 14 days postpartum (pp). The expression levels increased until 25 days pp, after which a slight reduction in expression was observed (III: Fig. 2B). To define cell type specificity of *iCre* expression, *Ar*-floxed mice were crossed with *Defb41*$^{iCre/iCre}$ mice. The resulting *Ar* cKO mice displayed deletion of the *Ar*-floxed allele and subsequent depletion of *Ar* expression at the site of *iCre* expression, as visualized by immunohistochemistry. AR expression could be observed in all epithelial cells of the *Defb41*$^{iCre/iCre}$ epididymis while *Ar* cKO mice only showed robust immunohistochemical staining in basal and narrow/clear cells of the epididymal epithelium (III: Fig. 2C,D). This indicates a specific expression of *iCre* in the principal cells which enable the use of *Defb41*$^{iCre/+}$ mice in generation of conditional knock-out mice of the proximal epididymis. Furthermore, the ablation of *Ar* expression led to reduction in epithelial cell height of IS as observed in previous epididymis specific *Ar* knock-out models (III: Fig. 2C,D, Krutskikh, et al. 2011; O'Hara, et al. 2011).

To study the role of RNA interference in postnatal development of the epididymis, *Defb41*$^{iCre/+}$ mice were crossed with a *Dicer1*-floxed mouse line. As DICER1 is the enzyme required for production of mature miRNAs, the ablation of *Dicer1* inhibits the regulation of miRNA target genes. Recombination of *Dicer1* was observed in IS and CAP of 12-day-old *Dicer1* cKO mice by genomic PCR, and additional qRT-PCR studies revealed a significant reduction in *Dicer1* expression levels at the age of 2 months (I: Fig. 1B,C).

5.5. Morphology of the *Dicer1* cKO epididymis (I)

Two-month-old *Dicer1* cKO male mice presented with underdeveloped IS, as seen both in macroscopic evaluation and with X-gal staining. IS and COR of the control epididymis have an endogenous β-galactosidase activity which causes blue coloration of the segments after X-gal staining. However, the *Dicer1* cKO epididymis displayed weak or no staining in the proximal part of the epididymis (I: Fig 2A). The size difference was also observed as a reduction in epididymal weight as compared with control mice (30.4 ± 1.5 mg, control: 35.4 ± 0.7 mg, $p \leq 0.01$). Furthermore, the intense vasculature of control mice IS was not visible in *Dicer1* cKO IS (data not shown). To study the postnatal differentiation of the *Dicer1* cKO epididymal epithelium, histological staining of the epididymis from different time points was performed. The studies showed an initial differentiation of the IS epithelium in one-

month-old *Dicer1* cKO epididymides as an increase in the height of the epithelium (I: Fig. 3D). However, at 45 days pp, histological staining showed a regression of the epithelial cells to an undifferentiated state (I: Fig. 3F). The phenotype of the adult mice epithelium varied, on one end; showing a highly disorganized cell layer and tubules with small diameter and on the other end an IS and CAP which resembled the thin epithelial layer of COR with an increased tubular diameter (I: Fig. S2). A common feature of all *Dicer1* cKO IS was a significant reduction in the height of the epithelial cells (average cell height in control IS: 45 µm, in *Dicer1* cKO IS: 28 µm, $p \leq 0.0001$).

Differentiation of the cell types of the epididymal epithelium takes place after birth. Immunohistochemical staining for cell type specific proteins showed the presence of principal, narrow/clear and basal cells, in similar numbers and locations in the 2-month-old *Dicer1* cKO epididymis as in the epididymis of control mice (I: Fig. 4A-F). However, the smooth muscle cells surrounding the duct consisted of three cell layers, compared to the very thin, one muscle cell layer of control mice (I: Fig. 4G,H). This resembles the ducts of a 14-day-old mice epididymis that have small diameters and are surrounded by thick muscle layers.

Although the weight and size of the epididymis was reduced in 2-month-old *Dicer1* cKO mice, there was a significant increase in the number of proliferative cells. This was especially evident in CAP, which showed 6 times more cells that were proliferative than the control epididymis (control IS: 6.4 ± 0.6 cells/mm, CAP: 1.9 ± 0.5 cells/mm; *Dicer1* cKO IS: 15.2 ± 3.0 cells/mm, CAP: 11.1 ± 1.7 cells/mm). The number of apoptotic cells was also slightly increased in the *Dicer1* cKO epididymis (control IS: 0.07 ± 0.07 cells/mm, CAP: 0.07 ± 0.07 cells/mm; *Dicer1* cKO IS: 0.89 ± 0.39 cells/mm, CAP: 1.12 ± 0.34 cells/mm). At six months of age, the *Dicer1* cKO epididymides were of similar weight to those of the control mice. However, the epithelial cell layer was further disturbed, with neoplastic changes in the efferent ducts, which resulted in their progressive obstruction.

5.6. Testicular phenotype of *Dicer1* cKO mice (II)

Due to the observed increase in efferent duct obstruction, the *Dicer1* cKO mouse testis also displayed a varied phenotype. The fluid back pressure from the epididymis caused areas of dilated seminiferous tubules in some testes of 2-month-old mice (II: Fig. S1A). While the 6-month-old animals had highly enlarged seminiferous tubules with almost no generation of spermatozoa, the younger animals displayed normal spermatogenesis, with primary spermatocytes, and both round and elongating spermatids observed in the testes (II: Fig. S1B-I). The production of testosterone, and the LH, and FSH levels of 2-month-old *Dicer1* cKO male mice was also comparable to those of the control mice (II: Fig. S2).

5.7. Segment specific gene expression of *Dicer1* cKO mice (I, unpublished)

The postnatal differentiation of the epididymis divides the duct into highly specified segments, each with its own unique pattern of gene expression. For example several β-defensins are not only epididymis specific but also segment specific in their expression. Quantitative RT-PCR analyses were performed to determine how *Dicer1* ablation affects segment specific gene expression. *Lcn8*, brain expressed myelocytomatosis oncogene (*Bmyc*) and cystatin 8 (*Cst8*), showed a marked downregulation in their expression in *Dicer1* cKO IS and CAP (I: Fig. 4I). The expression was significantly reduced already 21 days pp, at the time when there were no discernable differences in epididymal morphology (I: Fig. S3A,B). The expression of several β-defensins, which show highest expression in the proximal part of rodent epididymides (Fig. 5), was also downregulated in the 2-month-old *Dicer1* cKO epididymis (Fig. 8). Those family members that have a broader expression pattern (Fig. 5) did not display any reduction in expression (Fig. 8). Neither was the expression of IS specific G protein-coupled receptor 64 (*Gpr64*), *Ros1* (I: Fig. 4I), or the expression of *Fgfrs* reduced when compared with the expression of the control mouse epididymis.

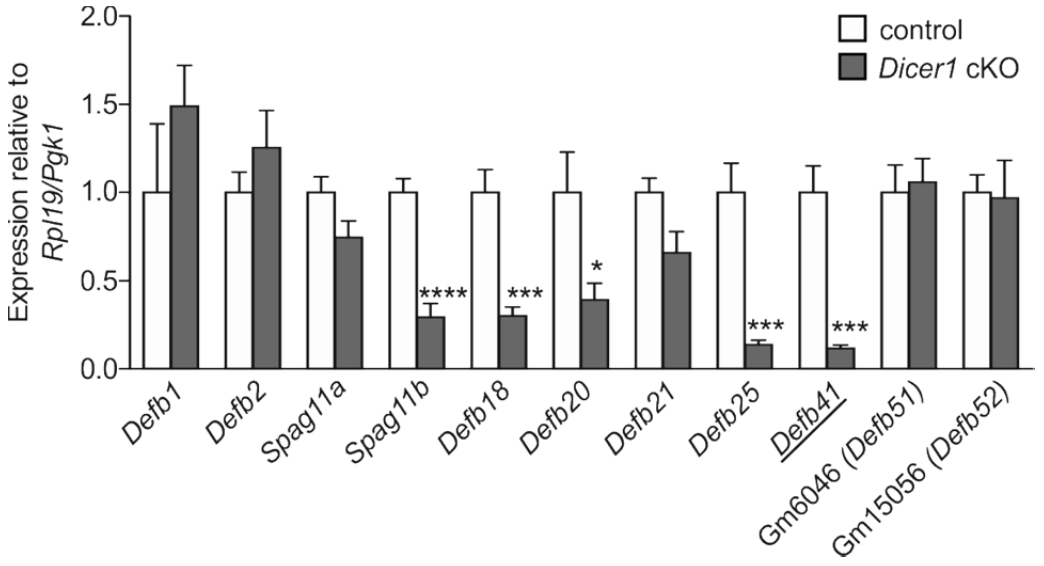


Fig. 8. Expression of β-defensins. Quantitative RT-PCR studies of the expression of β-defensin genes in 2-month-old control and *Dicer1* cKO initial segment and caput. Statistical significance is calculated using the unpaired t-test, *, $p \leq 0.05$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

5.8. Sex steroid receptor expression (I)

The development and function of the epididymis is highly androgen dependent. To study the effect of *Dicer1* cKO on sex steroid signaling, qRT-PCR and immunohistochemical analyses were performed for *Ar, Esr1* and *Esr2*. Quantitative RT-PCR results showed a significant downregulation of *Ar* and *Esr2* in 2-month-old *Dicer1* cKO IS and CAP while no clear difference in *Esr1* expression was detected (I: Fig. 6E). The expression of AR target genes was also affected with *Crisp1*, *Gpx5* and *Lcn5* showing significant downregulation in the *Dicer1* cKO epididymis (I: Fig. 6F). The immunohistochemical staining for AR and ESR1 gave a more varied picture of the observed changes. Although there were *Dicer1* cKO epididymides with weaker AR staining (I: Fig. 6D), some showed similar expression patterns as the control epididymides with AR expressed in all epithelial cells. ESR1 also showed a different expression pattern in *Dicer1* cKO animals. Where control epididymidi only display ESR1 staining in narrow cells, the *Dicer1* cKO epididymis show staining in all cells of the IS epithelium (I: Fig. 6A,B). No significant reduction in ESR2 expression was observed by immunohistochemistry (data not shown).

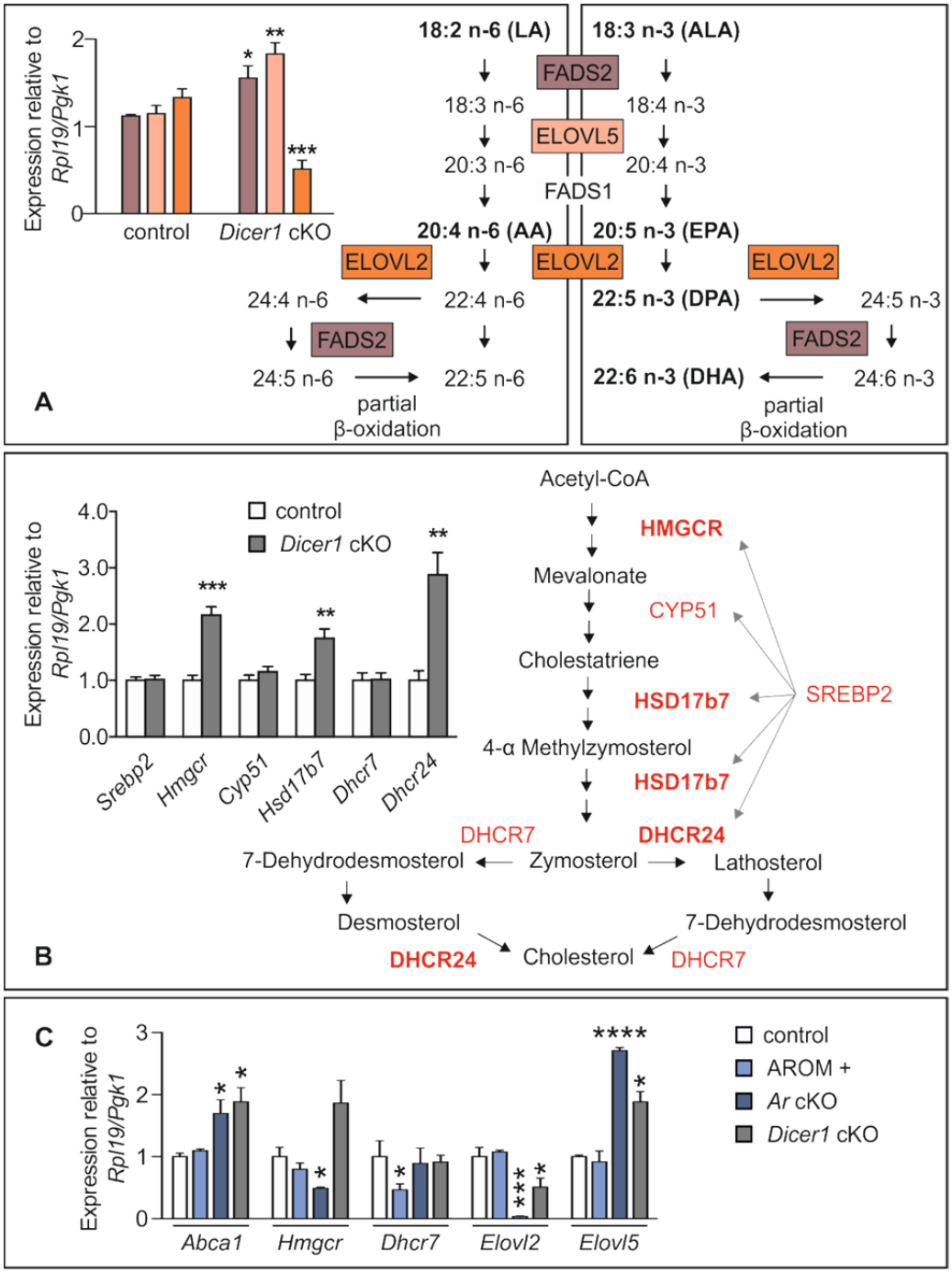
5.9. *Dicer1* cKO epididymis expression of genes in lipid synthesis (II, unpublished)

Androgen signaling also governs the expression of elongation of very long chain fatty acids –like 2 (*Elovl2*), an enzyme involved in the production of 22-carbon chain PUFAs (Kerkhofs, et al. 2012). Quantitative RT-PCR results of *Dicer1* cKO IS and CAP showed downregulation of *Elovl2* expression while the expression of fatty acid desaturase 2 (*Fads2*) and elongation of very long chain fatty acids –like 5 (*Elovl5*), the enzymes that generate 20-carbon long PUFA chains, were upregulated (Fig. 9A). This expression pattern resembles that of younger animals when the testosterone levels are still too low to induce *Elovl2* expression (II: Fig. 3D). Additional changes in the expression of enzymes involved in cholesterol synthesis were observed in the *Dicer1* cKO epididymis. 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (*Hmgcr*), hydroxysteroid (17-beta) dehydrogenase 7 (*Hsd17b7)*, and 24-dehydrocholesterol reductase *(Dhcr24)* showed significant upregulated mRNA levels while sterol regulatory element binding factor 2 (*Srebp2*), cytochrome P450, family 51 (*Cyp51*), and 7-dehydrocholesterol reductase *(Dhcr7)* displayed similar expression levels as in control mice (Fig. 9B). Genes involved in the transportation of cholesterol and phospholipids from the epithelium to the epididymal lumen were also upregulated (*Abca1*, *Abcg1*) while those regulating their expression did not show any significant increase in the *Dicer1* cKO epididymis (*Nr1h3* and *Nr1h2*, II: Fig. 4C). No difference between *Dicer1* cKO and control was observed in the expression of enzymes

regulating sphingomyelin synthesis (long chain base subunit 1 (*Sptlc1*), sphingomyelin synthase 1 (*Sgms1*), sphingomyelin synthase 2 (*Sgms2*), II: Fig. 5B).

To analyze whether or not the observed change in gene expression of *Dicer1* cKO IS and CAP was solely dependent on the imbalance in sex steroid receptor expression, we further compared the expression of control and *Dicer1* cKO mice to that of the *Ar* cKO mice and to mice overexpressing the aromatase enzyme (AROM+). Although we observed some similarities between the *Ar* cKO and *Dicer1* cKO mice (e.g. the expression of *Abca1*, *Dhcr7* and *Elovl*) there were also differences (e.g. *Hmgcr*) and the AROM+ epididymis, which shows high *Esr1* expression and low *Ar* expression, did not have a gene expression comparable to that of the *Dicer1* cKO epididymis (Fig.9C).

Fig. 9. Expression of genes in lipid synthesis. A) The expression of genes involved in the synthesis of long-chain polyunsaturated fatty acids. The role of Fatty acid desaturase 2 (FADS2) and Elongation of very long chain fatty acid –like 5 and 2 (ELOVL5 and ELOVL2) is seen in the outline of the omega-6 and omega-3 fatty acid synthesis pathway. LA, linoleic acid; ALA, α-linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. B) Expression of genes involved in the synthesis of cholesterol together with an outline of the cholesterol synthesis pathway. Sterol regulatory element binding factor 2 (*Srebp2*), 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (*Hmgcr*), cytochrome P450, family 51 (*Cyp51*), hydroxysteroid (17-beta) dehydrogenase 7 (*Hsd17b7)*, 7-dehydrocholesterol reductase *(Dhcr7)*, 24-dehydrocholesterol reductase (*Dhcr24*). C) A comparison of gene expression in control and *Dicer1* cKO mice with the expression of *Ar* cKO mice and mice overexpressing aromatase (AROM+). ATP-binding cassette, sub-family A, member 1 (*Abca1*). All analyses were made using the initial segment and caput of 2-month-old mice. Gene expression was normalized to the expression of ribosomal protein L19 (*Rpl19*) and phosphoglycerate kinase 1 (*Pgk1*). Statistical significance was calculated using the unpaired t-test, *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.


A
Expression relative to Rpl19/Pgk1
control
Dicer1 cKO
18:2 n-6 (LA)
18:3 n-3 (ALA)
FADS2
18:3 n-6
18:4 n-3
ELOVL5
20:3 n-6
20:4 n-3
FADS1
20:4 n-6 (AA)
20:5 n-3 (EPA)
ELOVL2
24:4 n-6
22:4 n-6
22:5 n-3 (DPA)
24:5 n-3
24:5 n-6
22:5 n-6
22:6 n-3 (DHA)
24:6 n-3
partial β-oxidation
B
Srebp2
Hmgcr
Cyp51
Hsd17b7
Dhcr7
Dhcr24
Acetyl-CoA
HMGCR
Mevalonate
CYP51
Cholestatriene
HSD17b7
SREBP2
4-α Methylzymosterol
DHCR7
DHCR24
7-Dehydrodesmosterol
Zymosterol
Lathosterol
Desmosterol
Cholesterol
C
control
AROM +
Ar cKO
Dicer1 cKO
Abca1
Hmgcr
Dhcr7
Elovl2
Elovl5

5.10.*Dicer1* cKO male mice fertility and sperm morphology (I, II)

Dicer1 cKO male mice were infertile in normal breeding and showed a significant reduction in binding to and fertilizing the oocyte *in vitro* (Table 5). Light mechanical force during handling of caudal sperm gave rise to an increased number of *Dicer1* cKO sperm with the head severed from the flagellum, as compared with the control sperm (Table 5). The number of *Dicer1* cKO sperm with a broken acrosome region was also significantly higher than that of control animals (Table 5). In accordance with the number of sperm with loss of acrosome region, the *Dicer1* cKO sperm showed a 50% lower induction of protein tyrosine phosphorylation during capacitation (II: Fig. 2C,D). Sperm motility was also affected by the epididymal ablation of *Dicer1*, with only 3.2% of *Dicer1* cKO sperm displaying progressive motility (Table 5). However, no other morphological defect, for example tail angulation, was observed (Table 5).

Table 5.

sperm phenotype	control	*Dicer1* cKO	*p*-value
% with hairpin flagella	19.1 ± 2.9	22.9 ± 3.4	*0.4123*
% without flagellum	5.6 ± 1.5	31.5 ± 7.3	*0.0036**
% without acrosome	6.4 ± 1.5	51.3 ± 15.1	*0.0253**
% progressively motile	54.6 ± 5.3	3.2 ± 2.3	*< 0.0001**
nr. of bound sperm	23.2 ± 4.7	6.2 ± 2.1	*0.0051**
% of fertilized oocytes	78.8 ± 8.9	10.3 ± 4.8	*0.0005**

*statistical significance $p < 0.05$

5.11. Lipids of the *Dicer1* cKO sperm membrane (II)

The lipid composition of the sperm membrane changes during maturation in the epididymis to promote motility and ability to fertilize the oocyte. To study how the altered expression of factors involved in lipid synthesis of the *Dicer1* cKO epididymis affected the sperm membrane lipid content, mass spectrometry analyses were performed. When measuring total numbers of carbons and double bonds of the two fatty acids bound to phosphatidylcholines (PCs), we observed a reduction in the long-chain PUFAs of *Dicer1* cKO sperm (Fig. 10A). Although the cholesterol levels were similar in control and *Dicer1* cKO sperm before induction of the acrosome reaction (Fig. 10B), the reduced levels of PC PUFAs with 40-42 carbons and 5-6 double bonds caused a 3.6 ± 0.4 fold increase in the cholesterol:long-chain PUFA ratio of *Dicer1* cKO sperm. The reduction in PCs also led to a decrease in total amount of phospholipids (control: 2077 ± 304 pmol/mil. sperm, *Dicer1* cKO: 1124 ± 123 pmol/mil. sperm, $p = 0.0241$), even though *Dicer1* cKO sperm displayed increased

levels of several sphingomyelins (Fig. 10C). In addition, measurements of sperm acylcarnitines (ACs) showed a 4.4 ± 1.1 fold increase in AC C18:0 when comparing *Dicer1* cKO sperm to control sperm (Fig. 8C). After induction of capacitation and the acrosome reaction, a large percentage of the membrane cholesterol is lost from sperm. This was also observed in control sperm, however, *Dicer1* cKO sperm did not display a reduction in cholesterol after induction (Fig. 10B).

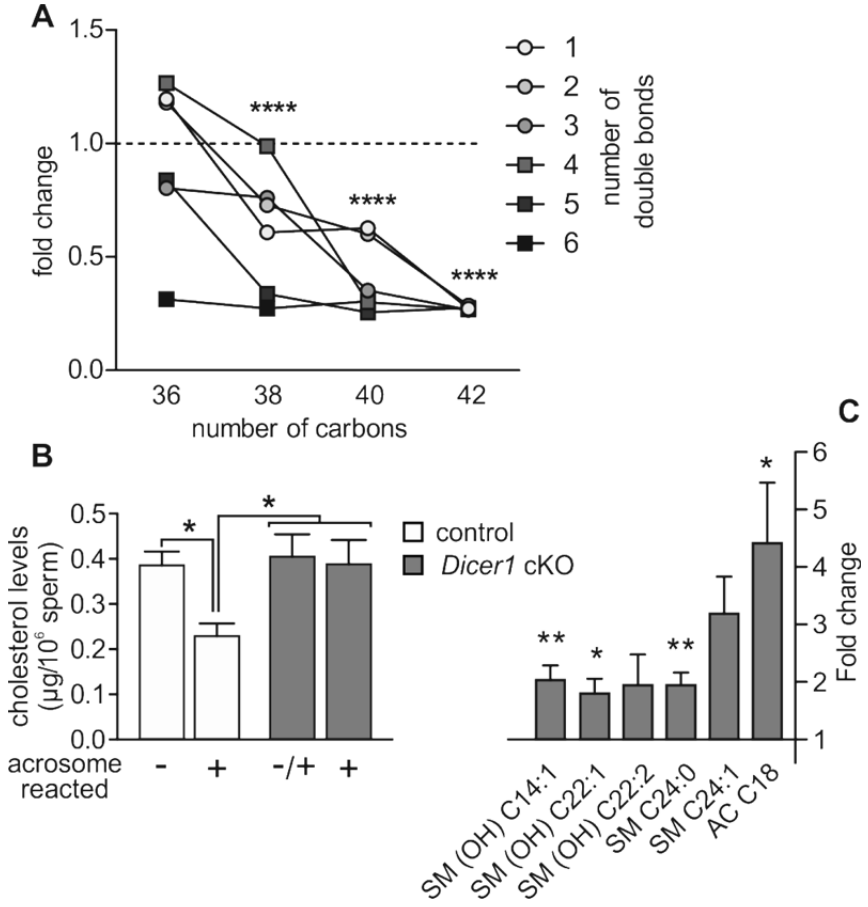


Fig. 10. Lipid composition of the sperm membrane. A) The average fold-decrease in the total number of double bonds and number of carbons of the two fatty acids bound to each phosphatidylcholine in the membrane of *Dicer1* cKO mice sperm, as compared with control sperm. Statistical significance is calculated for the length of the carbon chain. C) The amount of cholesterol (µg/10^6 sperm) before (-) and after (+) induction of the acrosome reaction. (-/+) marks the 51% of *Dicer1* cKO sperm that have lost the acrosome region prior to induction. D) Fold change in the amount of sphingomyelins (SMs) and acetylcholine (AC) C18 in *Dicer1* cKO sperm, as compared with the control. Statistical significance was calculated using the unpaired t-test, *, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$.

6. DISCUSSION

6.1. *Defb41* in sperm motility

The mouse *Defb41* gene shares many features with other members of the β-defensin family. Its expression is androgen dependent and specific to the male reproductive tract (Jalkanen, et al. 2005). The gene is transcribed as two short exons, 113 and 353 bp in length, which give rise to a 62 amino acid long protein product. The putative signal sequence of DEFB41 is further cleaved between amino acids 21 and 22 to produce the mature 41 amino acid protein (Jalkanen, et al. 2005). Our results show that the expression of *iCre* under the *Defb41* promoter is located in the principal cells of IS and CAP. Thus, it is likely that the *Defb41* gene is specifically expressed in the principal cells. The cell specific expression and the observed secretion of other β-defensin family members would further suggest secretion of the DEFB41 protein into the epididymal lumen where it could interact with spermatozoa (Yudin, et al. 2003; Zhou, et al. 2004; Yudin, et al. 2008; Zhao, et al. 2011). Ablation of *Defb41* through *iCre* knock-in, also changed the motility pattern of the sperm. After capacitation, the flagellum of *Defb41*$^{iCre/iCre}$ sperm was more often observed in the pro-hook conformation compared to the control sperm. In addition, the linearity and straightness of *Defb41*$^{iCre/iCre}$ sperm velocity was reduced after capacitation, probably due to the changed motility pattern of the sperm flagellum. Thus, the expression of *Defb41* is required for sperm maturation, specifically in regulating the flagellar bending of spermatozoa. However, the change in motility pattern did not lead to reduced fertility although *in vitro* studies showed less spermatozoa of *Defb41*$^{iCre/iCre}$ mice bound to the oocyte compared to the sperm of control mice.

Sperm motility is mainly controlled by Ca^{2+} -signaling (Darszon, et al. 2011). The release of Ca^{2+} from intracellular stores is suggested to regulate the beat of the sperm flagellum while Ca^{2+}-channels in the sperm membrane replenish the stores and maintain high levels of Ca^{2+} in the cytoplasm (Yanagimachi. 1994; Ho and Suarez. 2001). If the only source of Ca^{2+} is the inner stores, the sperm flagellum is mainly found in an anti-hook conformation. However, when both Ca^{2+} pathways are utilized the flagellum primarily beats in a pro-hook conformation (Chang and Suarez. 2011). Because of their small cell size, Ca^{2+} fluctuations in sperm are difficult to measure and we do not currently have access to techniques sensitive enough to perform such measurements in living sperm. However, the difference in flagellar bending of *Defb41*$^{iCre/iCre}$ mice sperm would suggest an altered Ca^{2+} -signaling and we hypothesise that the DEFB41 protein functions in Ca^{2+} -uptake either when the spermatozoa travels through the epididymis or during sperm motility in the female reproductive tract.

Studies of other β-defensin have also suggested a regulatory role for the proteins in Ca^{2+} -uptake by spermatozoa. The separated hydrophobic and highly charged regions of β-defensins allow them to be inserted into phospholipid membranes (Hall, et al. 2007). In this way, they both function in bacterial defense and are able to form and/or regulate ion-channels in the sperm membrane. For example the rat β-defensin SPAG11b is expressed in CAP where it binds to sperm and causes an increased Ca^{2+} -uptake, which leads to induction of motility (Zhou, et al. 2004). The rat DEFB15 is thought to function in a similar manner, displaying a CAP specific expression pattern that is needed to maintain sperm motility (Zhao, et al. 2011). Interestingly, both proteins are suggested to work locally in the proximal epididymis since the reduction in sperm motility of *Defb15* knock-down rats is stronger in CAP than in CAU, and the introduction of SPAG11b antibodies to mature caudal sperm does not lead to a reduction in motility (Zhou, et al. 2004; Zhao, et al. 2011). As *Defb41* is primarily expressed in the most proximal part of the epididymis, the DEFB41 protein may also only be needed for the initial uptake of Ca^{2+} in IS and CAP. Sperm of *Defb41*$^{iCre/iCre}$ mice may have stored less Ca^{2+} during epididymal transit, which would lead to decreased intracellular Ca^{2+} levels during hyperactivation, and sperm more prone to move in a pro-hook conformation. However, this would only be true if the Ca^{2+} levels in the sperm stores did not increase during Ca^{2+} -uptake in the female tract.

Another function of β-defensins is identified through the partial deletion of the mouse chromosome 8 β-defensin cluster. Sperm from these mice show increased Ca^{2+} -uptake during epididymal transit with premature hyperactivation and spontaneous acrosome reaction of spermatozoa (Zhou, et al. 2013). If DEFB41 was still bound to sperm during hyperactivation in the female reproductive tract, the protein could have an inhibitory role in Ca^{2+} -uptake and thereby regulate the motility pattern of the sperm. Ablation of *Defb41* would then give rise to increased uptake of Ca^{2+} through membrane channels, which would, again, lead to stronger pro-hook motility patterns. A third possibility for DEFB41 action is found in the inhibition of proteins by β-defensins. For example, the human β-defensin SPAG11B isoform D is reported to inhibit tryptase, a protein that is known to reduce sperm motility (Radhakrishnan, et al. 2009). Thus, the function of DEFB41 could be to regulate the activity of other proteins, either on the sperm surface or those interacting with spermatozoa during transit through the epididymis or the female tract, and thereby regulate the flagellar beat pattern.

Although the *Defb41*$^{iCre/iCre}$ sperm are produced in normal amounts and would capacitate and acrosome react, the more prominent pro-hook movements could lead to a reduced number of spermatozoa with the appropriate pattern of flagellar movement to bind to the oocyte. It is known that hyperactive motility is needed for sperm-oocyte interaction. In *Catsper* knock-out mice, the loss of Ca^{2+} -signaling and the subsequent

reduction in motility led to failure of sperm to penetrate to the zona pellucida (Suarez, et al. 1991; Suarez, et al. 1992; Ren, et al. 2001; Quill, et al. 2003). Interestingly, sperm motility and VSL, which are both affected in *Defb41*$^{iCre/iCre}$ sperm, have also been positively correlated with sperm-oocyte binding efficiency (Mahony, et al. 2000; Yogev, et al. 2000).

The *Defb41*$^{iCre/iCre}$ mice present with a very mild phenotype which could be due to a compensatory role of other β-defensins with similar expression and function. We also expect the change in motility of *Defb41*$^{iCre/iCre}$ sperm to be due to very minute changes in Ca^{2+} -uptake. To confirm our hypothesis regarding the role of DEFB41 in Ca^{2+} -signaling would therefore require highly sensitive measurement techniques, which may not be available at this time. To increase our knowledge on DEFB41 and its role in sperm maturation, it would also be necessary to generate an antibody against the protein. The antibody would not only give us the location of the protein on the sperm surface but also tell us if it is still bound to the sperm during activation of sperm hyperactivity in the female tract. In addition, co-precipitation studies could be performed to determine if DEFB41 binds to other proteins, either in the epididymal lumen or on the sperm surface.

6.2. *Dicer1* cKO epididymal phenotype

As no phenotypic alterations were detected in *Defb41*$^{iCre/+}$ mice, we were able to utilize the mice for generation of epididymis specific knock-out models. The recombination of the floxed genes by iCre begins around 12 days after birth, and could be detected by genomic PCR and qRT-PCR of IS and CAP tissue lysates. Although the generation of *Ar* cKO mice showed a very potent effect in the principal cells of the epididymis, *Dicer1* cKO mice did not display a clear change in epithelial phenotype until 45-days pp. In the mouse epididymis, differentiation of the epithelial cell types takes place between day 14 and day 21 pp (Avram and Cooper. 2004) after which segment-specific gene expression can be observed (Kirchhoff. 1999). At 33-days pp the principal cells of the *Dicer1* cKO epididymis had begun to display the typical high columnar shape of IS. This occurred even though there was a significant reduction in the expression of IS specific genes analyzed. Two weeks later however, a dramatic regression of IS had taken place and the epithelium resembled that of an undifferentiated pre-pubertal epididymis. Despite the morphological changes, *Dicer1* ablation did not affect cell type differentiation as all major epithelial cell types were detected in the epididymides of 2-month-old *Dicer1* cKO mice. However, the reduction in segment specific gene expression indicates a significant loss of function of the principal cells.

As differentiation of the epididymal epithelium continues after *Dicer1* ablation, regulation of gene expression by miRNAs may not be required for initiation of epithelial development. This is also reflected in the continued expression of *Ros1* in the

Dicer1 cKO epididymis. Previous studies have showed that ROS1 is one of the master regulators of IS development, as the lack of both ROS1 and its negative regulator, protein tyrosine phosphatase SHP-1, causes defects in IS differentiation (Sonnenberg-Riethmacher, et al. 1996; Keilhack, et al. 2001). However, the epididymal phenotype of *Dicer1* cKO is likely to be independent of ROS1 signaling as males with one intact *Ros1* allele display normal epididymal development (Sonnenberg-Riethmacher, et al. 1996) and *Dicer1* cKO IS still has ~70% of the *Ros1* expression of control IS. Furthermore, the expression of *Fgfr* genes, which function as inducers of *Ros1*, was not reduced in the proximal *Dicer1* cKO epididymis. Both ROS1 and the FGFs are also suggested to contribute to stromal-epithelial cell interaction (Yeung, et al. 1998; Cotton, et al. 2008). Although *Dicer1* ablation only took place in the principal cells of the epididymis, *Dicer1* cKO mice had a much thicker layer of smooth muscle cells than the control mice. This would indicate a lack in mesenchymal-epithelial communication and a role for principal cell miRNAs in the regulation of other cell types of the epididymis, again, independent of ROS1 or FGFs.

In light of the delayed dedifferentiation of the *Dicer1* cKO epididymis, it is interesting to consider the cumulative effect of miRNA expression in the tissue. For example, rat miR-200c targets the expression of *Tcf8* and thereby promotes accumulation of E-cadherin, throughout the postnatal development of the epididymis (Wang and Ruan. 2010a). E-cadherin is important for intercellular adhesion and the formation of the blood-epididymal barrier (Cyr, et al. 1993). In rats, miR-200c and E-cadherin reach peak levels immediately before spermatozoa enter the epididymal duct and it is hypothesized that this allows expansion of the tubule while maintaining epithelial cell connections (Cyr, et al. 1993; Wang and Ruan. 2010a). The *Dicer1* cKO mice, which would lack mature miR-200c, would then be unable to provide sufficient contact between the principal cells for proper intercellular communication. This could explain why the *Dicer1* cKO epididymis displays an altered morphology at the time of sperm entry into the epididymis. Prior to ductal expansion, a reduction in segment specific gene expression of the *Dicer1* cKO epididymis was observed, however, it is not until sperm enter the duct that the epithelial cell communication would fail and the principal cells go back to an undifferentiated state.

The observed change in expression of *Dicer1* cKO steroid receptors could also be contributing to epithelial dedifferentiation. Although the qRT-PCR and immunohistochemical studies gave somewhat different results, there is strong evidence of a downregulation of AR in most *Dicer1* cKO mice studied. An explanation to the different detection of *Esr1* mRNA and protein expression could be the small surface area of *Dicer1* cKO IS, which might not give rise to high enough mRNA levels of *Esr1* to show significant difference in qRT-PCR studies. Although we observe broader expression of ESR1 in the epithelial cells of IS, the high expression in CAP of both

control and *Dicer1* cKO mice could level out the increased mRNA levels of IS. Interestingly, miRNAs have been suggested to regulate the expression of *Esr1* through AR activity. The 3'-UTR of mammalian *Esr1* is directly targeted by around 14 evolutionarily conserved miRNAs (Pandey and Picard. 2010), while Androgen Responsive Elements (AREs) have been found in the promoter region of several miRNAs (Shi, et al. 2007; Ribas, et al. 2009). AR signaling has, in addition, been shown to induce miRNA expression *in vivo* (Narayanan, et al. 2010) and there is also evidence of a miRNA-induced downregulation of *Esr1* after testosterone treatment in the female mouse liver (Delic, et al. 2010). Taken together, the observed change in steroid receptor expression in the *Dicer1* cKO epididymis could be caused by the reduction in mature miRNAs, which would lead to an increased *Esr1* expression and a subsequent reduction in *Ar* expression. However, further studies are needed to clarify when the change in expression of *Esr1* and *Ar* takes place and whether or not the mouse epididymis expresses miRNAs that would target *Esr1*. As studies of *Esr2* knock-out models have not been able to detect a difference in morphology of the mouse epididymis, we do not expect the increased expression of *Esr2* to have a significant affect on the *Dicer1* cKO phenotype.

In the epididymis, epithelial expression of *Ar* is required for IS and CAP development (O'Hara, et al. 2011; Murashima, et al. 2011; Krutskikh, et al. 2011). Androgen signaling also regulates epithelial gene expression and cell survival, as orchidectomy causes extensive apoptosis throughout the epididymal epithelium of both prepubertal and postpubertal animals (Takagi-Morishita, et al. 2002). *Dicer1* cKO IS and CAP displayed a significant reduction in the expression of known AR target genes, for example *Elovl2* (Kerkhofs, et al. 2012). However, when comparing the gene expression of the *Dicer1* cKO epididymis with that of *Ar* cKO mice there were clear differences. For example, the expression of *Hmgcr*, the limiting factor in cholesterol synthesis, was downregulated in *Ar* cKO mice and upregulated in the *Dicer1* cKO epididymis. Furthermore, the β-defensin family member *Spag11a* has been shown to contain an AR binding site (Hu, et al. 2010) and both *Defb51* and *Defb52* are known to be partially regulated by androgen signaling (Hu, et al. 2014) however, none of these genes were significantly downregulated in *Dicer1* cKO IS and CAP. Moreover, while the expression of *Defb25* and *Spag11b* is androgen independent (Hu, et al. 2014), both genes displayed reduced expression in the *Dicer1* cKO epididymis. Thus, the *Dicer1* cKO phenotype cannot be fully explained by the reduction in *Ar* expression alone. It is also known that miRNAs directly affect lipid homeostasis. For example the expression of lipid and sterol transporters *Abca1* and *Abcg1* in mice, and the expression of *Hmgcr* are regulated by miR-33 and miR-122, respectively (Esau, et al. 2006; Marquart, et al. 2010; Gerin, et al. 2010). We therefore propose that the observed changes in gene expression of the *Dicer1* cKO epididymis are caused by the loss of mature miRNAs in combination with the altered hormone signaling.

6.3. *Dicer1* cKO sperm phenotype

The *Dicer1* cKO mice lack a fully developed IS and are infertile in normal breeding tests and thus resemble *Ros1* knock-out mice and a mouse model where the Simian virus 40 small T-antigen was placed under the *Gpx5* promoter (GPX5-Tag2) (Yeung, et al. 1999; Sipilä, et al. 2002). However, the sperm phenotype of these previous mouse models is mainly caused by a failure to regulate the cell volume, which leads to hairpin formation of the sperm flagellum with no reduction in motility or sperm membrane fluidity (Yeung, et al. 1999; Christova, et al. 2002; Sipilä, et al. 2002). The *Dicer1* cKO mice on the other hand, present predominantly immotile sperm, which display increased breakage of the neck and acrosome region. These are indicators of sperm membrane instability, which could be caused by an altered lipid constitution of the membrane.

When studying the lipid content of the *Dicer1* cKO sperm membrane we noticed a significant reduction in long-chain PUFAs while there was an increase in the amount of cholesterol, saturated sphingomyelins and acylcarnitine C18. This was correlated with a decreased expression of the gene *Elovl2*, which promotes long-chain PUFA synthesis, and upregulation of several genes affecting cholesterol synthesis in the *Dicer1* cKO epididymal epithelium. The addition of PUFAs during sperm maturation is correlated with increased sperm motility and animal fertility (Aksoy, et al. 2006; Tavilani, et al. 2006). There have been studies on knock-out mouse models of *Fads2* and *Elovl2*, which cause inhibition of long-chain PUFA production. The knock-out mice display a disrupted spermatogenesis where the primary spermatocytes fail to develop into elongated spermatids (Stoffel, et al. 2008; Stroud, et al. 2009; Zadravec, et al. 2011). Even though deletion of *Fads2* and *Elovl2* would also take place in the epididymal epithelium, the testis phenotype of the knock-out models is too severe to allow studies of sperm maturation. To our knowledge, the *Dicer1* cKO mouse model is thus the first to show a specific requirement for synthesis of long chain PUFAs in the proximal epididymis in sperm membrane integrity. Since *Elovl2* is the limiting factor in long-chain PUFA synthesis (Zadravec, et al. 2011), the increased expression levels of *Elovl5* and *Fads2* in the *Dicer1* cKO epididymal epithelium may not have any significant effect on PUFA production.

The *Dicer1* cKO sperm showed signs of increased membrane rigidity, with 51% of the sperm displaying a broken acrosome region. When compared to acrosome intact control sperm, sperm of *Dicer1* cKO mice contained similar levels of cholesterol. However, as the acrosome region contains high levels of cholesterol (Suzuki. 1988; Lin and Kan. 1996), the acrosome loss detected in *Dicer1* cKO sperm could cause the sperm to have cholesterol levels comparable to those of the intact control sperm. This was also evident after induction of the acrosome reaction with a Ca^{2+}-ionophore, when

almost all the sperm of both *Dicer1* cKO and control mice had lost the acrosome, but only the control sperm showed a reduction in cholesterol levels. Thus, we hypothesize that the *Dicer1* cKO sperm membrane contains significantly higher amounts of cholesterol. This, in combination with the decrease in PUFAs, would cause membrane instability and spontaneous breakage of the acrosome. Interestingly, a similar sperm phenotype is found in an *Nr1h2* and *Nr1h3* double knock-out mouse model with altered lipid homeostasis of the epididymis. When challenging the mice by excess dietary cholesterol, Ouvrier et al. detected increased breakage of the sperm neck and acrosome region, together with sperm immotility (Ouvrier, et al. 2011). Lack of *Nr1h* regulators caused downregulation of *Abca1* and accumulation of cholesteryl esters in the proximal epididymis (Ouvrier, et al. 2011). However, the lipid constitution of the sperm membrane was never described and we cannot, at this stage, say whether or not the sperm have a similar cholesterol:PUFA ratio as those of the *Dicer1* cKO mice. Nevertheless, it is clear that high cholesterol levels have a detrimental effect on sperm physiology, among others; a study on human sperm correlates high membrane cholesterol with decreased sperm motility (Buffone, et al. 2009).

Dicer1 cKO sperm also displayed increased SM levels. Due to limitations in detection, we were not able to measure SMs coupled to long-chain PUFAs, which is a specific feature of the sperm membrane (Poulos, et al. 1986; Poulos, et al. 1987; Robinson, et al. 1992). Therefore, it is not possible to determine if all SMs were increased or if the detected amount of saturated SMs compensated for a lack in SMs with PUFAs. If the latter is true, the SMs would contribute to a more rigid structure of the sperm membrane. Furthermore, SMs are known to bind cholesterol and to regulate sterol efflux during capacitation and the acrosome reaction (Slotte, et al. 1990; Pörn, et al. 1993). Thus, increased amounts of SMs could keep cholesterol bound to the membrane which would further promote membrane rigidity and instability.

The increased levels of acylcarnitine C18 in *Dicer1* cKO mouse sperm would suggest deficient β-oxidation in mitochondria. Carnitines taken up from the epididymal lumen are readily transported into sperm mitochondria as acylcarnitines. After β-oxidation in the mitochondrion, acetylcarnitines are formed and transferred back to the cytoplasm (Fritz. 1963; Jeulin and Lewin. 1996). Thus, *Dicer1* cKO mice either lack transportation of acylcarnitines into mitochondria or have reduced fatty acid oxidation. Although sperm are said to mainly rely on glycolysis for energy production, a recent study on human sperm showed reduced motility after inhibition of β-oxidation (Amaral, et al. 2013). The change in sperm membrane lipid constitution, might thus affect energy production and the motility of *Dicer1* cKO sperm.

Although the current study mainly focused on lipid homeostasis of spermatozoa, the reduction in fertility of *Dicer1* cKO mice could partly be caused by an altered

expression of other factors promoting sperm maturation. For example, loss of expression of multiple β-defensins has been shown to cause an altered Ca^{2+} -uptake and thereby affect sperm activation and motility (Zhou, et al. 2013). As β-defensins may be able to compensate for the function of other family members, the ablation of several β-defensins would have a more severe effect on sperm maturation than the knock-out of a single gene. *Dicer1* cKO mice are heterozygous for the *Defb41*-deficient *iCre* KI allele and only contain one wild-type *Defb41* allele. However, the expression of the gene is still much lower than what would be expected in *Dicer1* cKO IS and CAP. When studying the expression of other β-defensins we observed an additional downregulation of four other β-defensin family members. As we hypothesize that DEFB41 plays a role in sperm Ca^{2+} -signaling, it may be that the other β-defensins further affect Ca^{2+} -uptake and sperm motility. Although DEFB41 has a minor role in sperm flagellar movements, the downregulation of several β-defensins in the *Dicer1* cKO epididymis could thus have an additive effect and in part be responsible for the observed reduction in sperm motility.

It is suggested that miRNAs, transported out from the epididymal epithelium by epididymosomes, can travel to more distal segments of the epididymis and be incorporated into the epithelial cells (Belleannee, et al. 2013). The lack in mature miRNAs of *Dicer1* cKO IS and CAP could therefore lead to altered gene expression also in COR and CAU. However, miRNAs do not seem to function as on/off switches in the regulation of protein output, and the uptake of miRNAs might serve more as a way of the cell to fine tune its gene expression (Baek, et al. 2008). Thus, a lack of regulation by IS and CAP miRNAs on other segments of the *Dicer1* cKO epididymis, might not be enough to cause the severe phenotype of the *Dicer1* cKO mouse sperm. Furthermore, the phenotype of *Dicer1* cKO COR and CAU would not suggest an altered function of the epithelium and we expect that the main reason for infertility is the direct action of *Dicer1* ablation on the proximal part of the epididymis. However, further studies of the miRNAs of epididymosomes and the mRNA expression of both control and *Dicer1* cKO mice epididymides are required to verify this hypothesis.

The expression data for mouse epididymal miRNAs is very limited and thus we cannot say which of the miRNAs influenced the observed phenotype. However, since the lipid homeostasis of the epididymis appears to be vital for fertility, its regulation by miRNAs could function as an additional buffering system to balance any external changes in lipid availability. This would be especially important considering the high cholesterol diet of western men, which could have a negative effect on sperm quality. Based on this knowledge, miRNA studies could also be beneficial in cases of human idiopathic infertility and potentially provide options for future treatment.

7. SUMMARY AND CONCLUSIONS

The luminal environment of the epididymis is a consequence of a highly differentiated epithelium. Proteins and lipids secreted by the epididymal epithelium modify the sperm membrane and enable spermatozoa to fertilize the oocyte. However, the regulation of epididymal differentiation and function, as well as the role of the secreted factors, is not well known. In the present study we have utilized *in vivo* and *in vitro* methods to analyze the role of *Defb41* in sperm maturation and the regulation of epididymal development and function by *Dicer1*. The main conclusions of the study are:

1. The mouse *Defb41* is expressed by principal cells in the proximal epididymis. The protein is required for sperm flagellar movements after capacitation and for sperm-oocyte interaction.

2. The *Defb41*-defecient *iCre* KI mouse model can be utilized to delete floxed alleles in IS and CAP of the juvenile epididymis.

3. *Dicer1* is needed for final differentiation of the initial segment. Lack of *Dicer1* gave rise to a reduction in segment specific gene expression, imbalanced sex steroid signaling, and an altered lipid homeostasis of the epithelium.

4. *Dicer1* depletion in the epididymal epithelium causes an imbalance in the lipid constitution of the sperm membrane. Among other things, the sperm display an increased cholesterol:PUFA ratio, which is likely to give rise to the observed membrane instability and animal infertility.

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