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CHARACTERIZATION OF NOVEL GENES PREDOMINANTLY EXPRESSED IN THE EPIDIDYMIS – FROM GENOME ANALYSES TO GENETICALLY MODIFIED MICE

by

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ABSTRACT

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Characterization of novel genes predominantly expressed in the epididymis – from genome analyses to genetically modified mice

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In mammals, post-testicular sperm maturation taking place in the epididymis is required for the spermatozoa to acquire the abilities required to fertilize the egg *in vivo*. The epididymal epithelial cells secrete proteins and other small molecules into the lumen, where they interact with the spermatozoa and enable necessary maturational changes. In this study different *in silico*, *in vitro* and *in vivo* approaches were utilized in order to find novel genes responsible for the function of the epididymis and post-testicular sperm maturation in the mouse. Available online genomic databases were analyzed to identify genes potentially expressed in the epididymis, gene expression profiling was performed by studying their expression in different mouse tissues, and significance of certain genes to fertility was assessed by generating genetically modified mouse models. A recently discovered *Pate* (prostate and testis expression) gene family was found to be predominantly expressed in the epididymis. It represents one of the largest known gene families expressed in the epididymis, and the members code for proteins potentially involved in defense against microorganisms. Through genetically modified mouse models CRISP4 (cysteine-rich secretory protein 4) was identified to regulate sperm acrosome reaction, and BMYC to inhibit the expression of the *Myc* proto-oncogene in the developing testis. A mouse line expressing iCre recombinase specifically in the epididymis was also generated. This model can be used to generate conditional, epididymis-specific knock-out models, and will be a valuable tool in fertility studies.

Keywords: Epididymis, fertility, gene knockout, *Pate*, *Bmyc*, *Crisp4*

TIIVISTELMÄ

Heikki Turunen

Uusien vallitsevasti lisäkiveksessä ilmenevien geenien karakterisointi – genomianalyyseistä geenimuunneltuihin hiiriin

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Nisäkkäillä siittiöiden kypsyminen ja hedelmöityksessä tarvittavien ominaisuuksien kehittyminen tapahtuu pääosin lisäkiveksessä sen epiteelisolujen erittämien proteiinien ja muiden pienmolekyylien välityksellä. Tässä väitöskirjatyössä tutkittiin uusia lisäkiveksen toimintaan ja siittiöiden kypsymiseen vaikuttavia geenejä hiirellä erilaisia *in silico*, *in vitro* ja *in vivo* menetelmiä käyttäen. Lisäkiveksessä mahdollisesti ilmeneviä geenejä etsittiin julkisista hiiren genomitietopankeista, geenien ilmenemistä kartoitettiin useissa hiiren kudoksissa, ja tiettyjen geenien vaikutusta hedelmällisyyteen arvioitiin tutkimusta varten kehitettyjä geenimuunneltuja hiirimalleja tutkimalla. Tutkimus osoitti, että hiljattain löydetyn *Pate* (prostate and testis expression; eturauhasessa ja kiveksessä ilmenevä) geeniperheen jäsenet ilmenevät voimakkaasti lisäkiveksessä. Kyseessä on yksi suurimmista tunnetuista pääasiassa lisäkiveksessä ilmenevistä geeniperheistä, ja sen jäsenet ohjaavat mahdollisesti mikro-organismien vastaiseen puolustukseen osallistuvien proteiinien tuottamista. Geenimuunneltuja hiirimalleja tutkimalla havaittiin, että CRISP4 (cysteine-rich secretory protein 4; kysteiinirikas eritettävä proteiini 4) osallistuu siittiöiden akrosomireaktion säätelyyn, ja että BMYC heikentää *Myc* esisyöpägeenin ilmenemistä kiveksen kehittymisen aikana. Väitöskirjatyön osana kehitettiin myös iCre rekombinaasia lisäkiveksessä ilmentävä hiirimalli, jota voidaan käyttää geenien sammuttamiseen yksinomaan lisäkiveksen soluissa. Kehitetty malli on hyödyllinen tutkittaessa lisäkiveksessä ilmenevien geenien vaikutusta hedelmällisyyteen hiirillä.

Avainsanat: Lisäkives, hedelmällisyys, poistogeeniset hiirimallit, *Pate*, *Bmyc*, *Crisp4*

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ABBREVIATIONS

ABP	androgen binding protein
ADAM	a disintegrin and a metalloprotease
AR	androgen receptor
BAC	bacterial artificial chromosome
CAP	cysteine-rich proteins, antigen-5, and pathogenesis-related 1
CDK4	cyclin dependent kinase 4
CRISP	cysteine-rich secretory protein
DEFB	β(beta)-defensin
DHT	5α-dihydrotestosterone
E#	embryonic day #
ECM	extracellular matrix
ED	efferent duct
ES	embryonic stem
EST	expressed sequence tag
FSH	follicle-stimulating hormone
GOI	gene of interest
GPX	glutathione peroxidase
HA	homology arm
HE	heterozygous
ICM	inner cell mass
iCre	improved cre-recombinase
IS	initial segment
IVF	*in vitro* fertilization
KI	knock-in
KO	knock-out
LH	luteinizing hormone
NHEJ	non-homologous end joining
PATE	prostate and testis expression
PCR	polymerase chain reaction
qRT-PCR	quantitative reverse-transriptase polymerase chain reaction
RAR	retinoic acid receptor
RNAi	RNA interference
ROS	reactive oxygen species
RT-PCR	reverse-transcriptase polymerase chain reaction
SHBG	sex hormone-binding globulin
T	testosterone
TF	transcription factor
TG	transgenic

TRE	tetracycline response elements
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
WT	wild type
ZP	zona pellucida

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, to which the text refers by their respective Roman numerals (I–III).

I **Heikki T. Turunen**, Petra Sipilä, Anton Krutskikh, Jussi Toivanen, Harri Mankonen, Veera Hämäläinen, Ida Björkgren, Ilpo Huhtaniemi, Matti Poutanen (2011). Loss of Cysteine-Rich Secretory Protein 4 (*Crisp4*) Leads to Deficiency in Sperm–Zona Pellucida Interaction in Mice. *Biol Reprod, in press*. DOI: 10.1095/biolreprod.111.092403.

II **Heikki T Turunen**, Petra Sipilä, Dwi Ari Pujianto, Anastasios E Damdimopoulos, Ida Björkgren, Ilpo Huhtaniemi, Matti Poutanen (2011). Members of the murine *Pate* family are predominantly expressed in the epididymis in a segment-specific fashion and regulated by androgens and other testicular factors. *Reprod Biol Endocrinol* 2011, **9**:128

III **Heikki T. Turunen**, Petra Sipilä, Leena Strauss, Ilpo Huhtaniemi, Matti Poutanen. Loss of *Bmyc* Results in Smaller Male Reproductive Organs and Increase in *Myc* Expression in Juvenile Murine Testis. Submitted.

1. INTRODUCTION

The epididymis is a highly coiled tubular organ situated adjacent to the mammalian testis, which provides a location for both maturation and storage of developing spermatozoa to leave the testis. Proteins and other small molecules secreted by the epididymal epithelial cells participate in sperm maturation both directly by interaction with the spermatozoa, and indirectly by modification of the conditions of the luminal fluid. Epididymal sperm maturation enables the spermatozoa to undergo capacitation and acrosome reaction in the female reproductive tract, to enable them to reach the egg, penetration of the layer of cumulus cells and zona pellucida which surround it, and to also provide proteins that are required to bind to and fuse with the egg membrane, thereby to ultimately lead to fertilization. In addition to its role in sperm maturation, the epididymis also functions as a storage for the spermatozoa prior to ejaculation, protecting them from damage caused by reactive oxygen species and/or harmful micro-organisms. Although the epididymis is comprised of a single, highly convoluted duct, specific segments can be distinguished by morphology, histology, function, and thereby by gene expression, too. The proximal part, the caput, is active in both secretion and absorption of components of the luminal fluid derived from the testis. The caput can also be divided into several sub-segments, of which the initial segment in rodents is the most prominent. The distal epididymis is named the cauda, with the corpus a thin connecting segment between the caput and cauda. No specific functions for the corpus have been identified, although the cauda acts mainly as a storage for mature sperm.

As testicular spermatozoa are incapable of fertilization of an egg *in vivo*, maturational changes which take place in the epididymis are required, to allow abilities required for fertilization to develop. With the DNA in their nuclei highly condensed, the spermatozoa are transcriptionally mostly inactive; thus, factors to facilitate sperm maturation must originate from outside, namely from epididymal epithelial cells. Study of the proteins produced in the epididymis together with their interactions with the spermatozoa, the details in respect of sperm maturation can be unraveled.

Although unknown proteins are challenging objects to study, the genes which encode them are not. In addition to the fully sequenced genomes of several species, corresponding databases on gene expression profiles are readily available. Thus, genes predominantly expressed in the epididymis can be identified by database analyses. Furthermore, the characteristics of the proteins encoded by these genes can be predicted by comparison of their amino acid sequences to those of proteins with known functions. Following initial identification and prediction of function, results are confirmed by extensive *in vitro* studies. However, the staggeringly

complex interactions between millions of different molecules in a living organism are impossible to simulate in detail. Thus, an efficient, if laborious, approach to study the significance of a single gene *in vivo* is with a loss of function, or gene knock-out, animal model.

The technique to generate knock-out mice is based on the abilities of 1) isolation and culture of mouse embryonic stem cells, 2) accurate manipulation of their genomes, and 3) incorporation of the manipulated stem cells into a mouse embryo, in order to produce mice able to pass the mutation onto their offspring. The genetic manipulation which leads to gene knock-out is made possible by sequence homologies between two DNA molecules, and cellular DNA repair mechanisms. The embryonic stem cells are transfected with a gene knock-out construct designed to replace the endogenous gene with a non-functional version of the gene. Mice which carry the mutated gene are bred with each other to obtain homozygous knock-out mice, and the effect of the gene's loss is evaluated by analysis of phenotypic changes in the mice. However, if the gene's loss requires study in a specific organ, a conditional knock-out model is required. The conditional knock-out approach relies on two separate mutations: first, a transgenic model which provides expression of a recombinase enzyme under a promoter to drive its expression into the desired organ(s), and secondly, the gene of interest flanked by the recombinase target sequences. Thereby, the gene of interest is rendered non-functional only in cells which express the recombinase.

The aim of this research was to study novel genes expressed in the epididymis through different *in silico*, *in vitro* and *in vivo* approaches. Specifically, the expression profiles and regulation of the *Pate* family genes were characterized in male reproductive organs, and the effects of *Bmyc* and *Crisp4* on male fertility were studied through knock-out mouse models. We also generated a mouse model expressing the *iCre* recombinase under an epididymis-specific promoter, to allow generation of conditional, epididymis-specific knock-outs.

2. REVIEW OF THE LITERATURE

2.1 The epididymis

The epididymis is an organ responsible for post-testicular sperm maturation in amniotic vertebrates. Essentially, it is a single, highly coiled duct which connects the efferent ducts (ED) to the vas deferens (Figure 1A). Although most anamniotic vertebrates also have a similar system of ducts, among vertebrates the terminology for the ducts is not consistent; it is unresolved as to which species this structure can be labelled as the epididymis (Jones, 1998). Testicular spermatozoa, still immotile and unable to bind to and pass through the zona pellucida (ZP) of the egg, enter the epididymal lumen via the ED and undergo their final maturation during their transit through the epididymis (Jones, 1999). The minimal requirement for epididymal sperm maturation is the passage of sperm through the proximal caput epididymidis, which is the most active segment in secretion of proteins and other small molecules to the epididymal lumen, wherein they interact with the developing sperm and modify the protein and lipid components of their cell membranes (Jones, 1999). In addition, the epithelial cells regulate the composition of the luminal fluid by secretion and absorption to enable the conditions required for the sperm maturation to take place (Turner, 2002). The luminal microenvironment also protects the developing sperm from pathogens, through the presence of antimicrobial proteins (Hall *et al.*, 2002; Li *et al.*, 2001), and acts as storage for the mature sperm until ejaculation (Jones, 1999; Glover *et al.*, 1971; Hinton *et al.*, 1996).

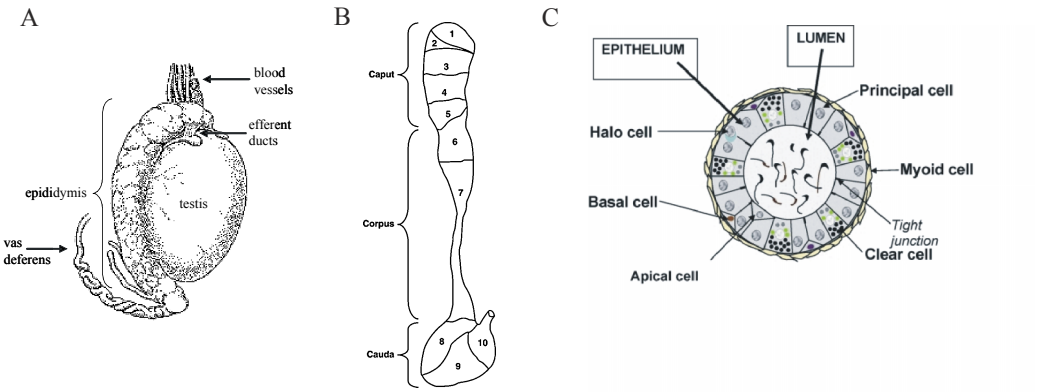


Figure 1. (A) The epididymis is situated adjacent to the testis and connects the ED to the vas deferens. **(B)** The epididymis is divided into caput, corpus and cauda. Further divisions can be made based on connective tissue septae separating the segments (1–10). (Modified from (Johnston *et al.*, 2005).) **(C)** Cross-section of the caput epididymal duct, to illustrate different epididymal cell types. (Modified from (Robaire *et al.*, 2006).)

2.1.1 Development of the epididymis

The epididymis develops from the cells of intermediate mesoderm along with the kidney, the primordial gonad, and other accessory reproductive organs. Development of the whole male urogenital system starts with the expression of the Y-chromosomal *Sry* gene in the somatic cells of the bipotential primordial gonad (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990; Koopman *et al.*, 1991; Piprek, 2009). This initiates a cascade which leads to male-specific developmental pathway. Initially, SRY causes differentiation of the Sertoli cells in the gonad (Burgoyne *et al.*, 1988; Palmer *et al.*, 1991; Patek *et al.*, 1991) which commence production of anti-Müllerian duct hormone (AMH) which induces the regression of the Müllerian duct in the male embryo (reviewed in (Rey *et al.*, 2003)). Soon after Sertoli-cell differentiation, Leydig cells also commence differentiation and production of testosterone (T). T prevents cell death in the Wolffian duct and, through the conversion into a more potent androgen, 5α-dihydrotestosterone (DHT), leads to the generation of male genitalia and accessory sex organs, inclusive of the epididymis (reviewed in (Griswold *et al.*, 2009)).

The mammalian kidney develops through three different phases: pronephros, mesonephros, and metanephros, the latter of which becomes the permanent kidney. During the second phase, around embryonic day (E) 11 in mice, the nephric duct, or Wolffian duct as it is known in males, forms after degradation of the pronephros. At E15 the T secretion of the Leydig cells initiates the differentiation of the Wolffian duct into the epididymis, vas deferens and the seminal vesicles. It has also been hypothesised that the initial segment (IS) is partially derived from some of the mesonephric tubules (Hinton *et al.*, 2000). However, T alone is insufficient to generate a functional epididymis directly, since the initial segment fails to form in certain genetically modified mouse models, even in the presence of normal amounts of T (LeBarr *et al.*, 1986; Sonnenberg-Riethmacher *et al.*, 1996). There is also evidence that suggests that DHT may be required for proper coiling and cell proliferation in the epididymis (Tsuji *et al.*, 1991).

Post-natal development of the epididymis is divided into three periods: the undifferentiated period, the period of differentiation, and the period of expansion (Sun *et al.*, 1979; Hermo *et al.*, 1992a). The most extensive studies have been done with rats, and in general, development seems similar in other species, too (reviewed in (Rodriguez *et al.*, 2002)). All the following time points refer to rat postnatal development. At birth the epithelial cells throughout the epididymis remain undifferentiated low columnar cells. However, coiling of the duct has initiated during embryonal development, and considerable coiling can be detected in the caput and corpus epididymidis already at birth (Bomgardner and Hinton, unpublished observations: in (Robaire *et al.*, 2006)). During the undifferentiated

period, days 1–15, the duct continues to grow in length and reaches the length of almost 2 m by day 15 (Hermo *et al.*, 1992a; Jiang *et al.*, 1994). The first differentiated cell types, the halo cells, can be detected by day 14, and narrow cells by day 15 (Rodriguez *et al.*, 2002). The remainder of the cell types appears during the period of differentiation, days 16–44. Further, the columnar cells change in appearance; by day 35 growth to tall columnar cells, the gain of a distinct brush border, together with a prominent Golgi apparatus, and numerous vesicles and coated pits indicative of active endocytosis. The period of expansion starts at day 44 which will last until adulthood, and by day 49 all epithelial cell types are differentiated (reviewed in (Rodriguez *et al.*, 2002)). Although circulating androgens are known to have a critical role in the development of the epididymis, there is also evidence that testicular factors associated with the luminal fluid on the fluid flow commencement affect cellular differentiation as well (Alexander, 1972; Abe *et al.*, 1984). However, no such factors have been identified, to date.

2.1.2 Morphology, histology and cell types of the epididymis

The epididymis is a part of the ductal system through which testicular sperm are able to exit the male body. Seminiferous tubules in the testis converge and form the rete testis, which give rise to the ED. The amount of tubules in the ED is species specific, ranging from 4 to 20 (Hemeida *et al.*, 1978; Nistal *et al.*, 1984). ED join and form a single duct, the epididymis. The mammalian epididymis is commonly divided into four segments, based on morphology and histology. From proximal to distal there is the IS, caput (head), corpus (body) and cauda (tail) (Jones, 1998; Benoit, 1926). Other distinct sub-segments, such as the intermediate zone between the IS and the caput, can also be identified in several mammalian species, although these divisions are not so consistently established (Figure 1A–B and reviewed in (Jones, 1998)). Gross anatomy of the epididymis in most species resembles two globular ends, caput and cauda, connected to one another by the thin corpus. The proximal end is easily distinguished from the distal by the characteristic reddish hue of the IS caused by ample vascularization. The epididymal segments are divided by connective tissue septae, which also forms a capsule to cover the whole organ. After the cauda epididymidis the duct continues as vas deferens, a straight tube connected to the urethra, to finally lead to externalization from the body. Accessory sex glands which contribute to the ejaculate, such as the prostate and seminal vesicles, also connect to the vas deferens.

Histologically, the epididymal segments are readily identifiable by separation borders created by connective tissue. Small capillaries can be seen amidst the connective tissue. The appearance of the epithelium also changes characteristically to each segment. The epithelium is comprised of essentially only a single layer of cells, situated between the basal lamina and the lumen of the duct. The structure of

the epithelium also differs clearly between the segments. The IS has thick epithelium and small luminal volume with no visible spermatozoa in the lumen. The epithelium thins in the caput and numerous spermatozoa can be observed in the lumen. The same development continues in the cauda, and the diameter of the whole duct increases dramatically. With an accompanying increase in luminal volume, the cauda is thus able to better fulfill its role as a storage for mature sperm.

Several different types of cells can be identified in the epididymal epithelium, including multiple layers of peritubular myoid cells surrounding the epithelium. Although the exact regional localization of the cell types differs between species, (in part due to the inconsistency of identification of the different regions) in general, all the cell types appear to be present in all mammalian species, inclusive of the human (Figure 1C and reviewed in (Robaire *et al.*, 2006)).

The most abundant cell type, comprising approximately 65–80% of the total epithelial cell population, depending on the segment, is the principal cell (Robaire *et al.*, 2006; Trasler *et al.*, 1988). These are active in the secretion of proteins to, and also the endocytosis of proteins from, the epididymal lumen (reviewed in (Hermo *et al.*, 2002)). Although found in each epididymal region, each differs in structure and function between the different segments (Hermo *et al.*, 1994). The segment specific changes in structure reflect the alternating activities in secretion and endocytosis. Thus, the most notable changes are detected in the appearance and organization of the secretory organelles, including the endoplasmic reticulum (ER), the Golgi apparatus and secretory granules, and in the endocytic organelles, including coated pits, endosomes, multivesicular bodies (MVB's) and lysosomes (reviewed in (Hermo *et al.*, 2002). Furthermore, the abundance of lipid droplets marks the principal cells of the corpus epididymidis, although their significance is not known (Robaire *et al.*, 2006; Byers *et al.*, 1985).

Apical and narrow cells are primarily present in the IS and intermediate zone of adult epididymis, and share certain similarities (Hermo *et al.*, 2002; Sun *et al.*, 1980; Adamali *et al.*, 1996). Both are spherical and are located near the apical border of the epithelium. However, narrow cells are also connected to the basement membrane by a thin process of cytoplasm, whereas apical cells have no such contacts. Although similar, narrow and apical cells differ in morphological appearance, relative distribution and expression of proteins. The most prominent morphological differences are the abundance of apical vesicles and coated pits in narrow cells, as compared to apical cells. The narrow cells are involved in endocytosis and secretion of H^+ ions into the lumen (Hermo *et al.*, 2000). The apical cells are also active in endocytosis, and additionally contain many proteolytic enzymes (Adamali *et al.*, 1996; Adamali *et al.*, 1999).

Clear cells are characterized by their lack of microvilli, as well as the presence of numerous coated pits, vesicles, endosomes, MVB's and lysosomes, all of which indicate their active role in endocytosis (Robaire *et al.*, 2006; Abou-Haila *et al.*, 1984; Hermo *et al.*, 1988), and they are found in the caput, corpus and cauda epididymidis in several species, including the human (Robaire *et al.*, 2006; Hamilton, 1975; Cooper, 1986). These are thought to be the most actively endocytosing cells in the epididymis, and normally absorb the contents of cytoplasmic droplets released by the spermatozoa (Hermo *et al.*, 1988; Moore *et al.*, 1979). The clear cells endocytose several different proteins in a region-specific manner (Lea *et al.*, 1978; Flickinger *et al.*, 1988; Hermo *et al.*, 1992b; Vierula *et al.*, 1995), and also participate in the regulation of luminal fluid acidification (Jensen *et al.*, 1999; Pastor-Soler *et al.*, 2003; Isnard-Bagnis *et al.*, 2003).

Basal cells, as their name suggests, are situated on the basal side of the epithelium, and only rarely extend cytoplasmic processes reaching the luminal surface (Veri *et al.*, 1993). They are present in all epididymal segments, inclusive of the vas deferens (Robaire *et al.*, 2006), and in all species studied (reviewed in (Hermo *et al.*, 2002)). Basal cells present morphological structures typical to endocytotic cells, and they have been suggested to endocytose material derived from the blood or principal cells (Hermo *et al.*, 2002). It has been proposed that basal cells may further have a role in the regulation of electrolyte and water transportation by principal cells (reviewed in (Leung *et al.*, 2004)) or as immune cells (Seiler *et al.*, 2000). Thereby, basal cells may be of extratubular origin (Holschbach *et al.*, 2002).

Halo cells are easily recognized in several histological stainings by their narrow rim of clear cytoplasm, and are present in all epididymal segments (Robaire *et al.*, 2006). In young adult animals, halo cells consist of helper T lymphocytes, cytotoxic T lymphocytes and monocytes, and their quantity increases with age (Flickinger *et al.*, 1997; Serre *et al.*, 1998; Serre *et al.*, 1999). In older animals, eosinophils are also occasionally observed (Serre *et al.*, 1998). Thereby, halo cells are responsible for immune defense in the epididymis.

2.1.2.1 Blood–epididymis barrier

Since certain cell surface proteins on spermatozoa identify them as foreign bodies in the view of the immune system, a barrier to prevent the immune cells entering the epididymal lumen is required. This blood–epididymis barrier can be seen as an extension of the blood–testis barrier, and is also necessary to maintain the correct composition of the luminal fluid and keeping it separated from blood plasma. The structure of the barrier is created by apically located gap, tight and adherens junctions between the epithelial cells. In addition to this isolation role, the barrier also enables communication between the epithelial cells, necessary for coordination

of luminal changes required for sperm maturation and storage (reviewed in (Cyr *et al.*, 2002) and (Robaire *et al.*, 2006)).

2.1.3 Epididymal luminal fluid

The epididymal luminal fluid is a complex mixture of water, inorganic ions, small organic molecules and proteins, which accompanies the spermatozoa as they travel from the testis to the epididymis through the ED, and is mostly composed of secretions of the Sertoli cells (reviewed in (Rato *et al.*, 2010)). Its constitution, however, starts to change immediately upon entering the ED and continues to do so whilst traversing through the epididymis. These changes are caused by secretory and absorptive functions of the epithelial cells which line the lumen, and are very characteristic to different epididymal regions. Thereby, the luminal fluid composition is a balance between secretion and absorption, and reflects the primary function of the region in question related to maturation or storage of the spermatozoa.

Although the epididymis is known as a highly secretory organ, the absorption of molecules from the luminal fluid is also important to its proper function. Approximately 99 % of testicular fluid that accompanies the spermatozoa is absorbed in the ED and proximal epididymis (Mann *et al.*, 1981). Water absorption and secretion are secondary effects resulting from ion transportation mediated by various Na^+/H^+ exchangers on the apical surface of epithelial cells (Wong *et al.*, 1978; Au *et al.*, 1980; Wong, 1988; Chew *et al.*, 2000; Bagnis *et al.*, 2001). However, the absorption of water leads to exceptionally dehydrated luminal conditions. It is required for the concentration of the spermatozoa and certain luminal components (Mann *et al.*, 1981), but it also generates additional demands for luminal proteins to maintain their correct folding and functionality and to prevent unwanted aggregation (Minton, 2005; Rialdi *et al.*, 2007; Cornwall *et al.*, 2007). Notably, several epididymal proteins have a highly conserved distribution of cysteine-residues, between which structure stabilizing disulfide bridges are formed. Being common structures in various toxin proteins of reptiles, they also are thought to help maintain correct protein folding in the hostile environment of the target prey's bloodstream (Bastolla *et al.*, 2005; Kaplan *et al.*, 2007). Similarly, structural stability is needed in the dehydrated epididymal luminal fluid, which possibly explains the abundance of secreted cysteine-rich epididymal proteins (Cornwall *et al.*, 2007).

In addition to fluid transportation, ions, specifically bicarbonate (HCO_3^-) and Na^+, affect the pH of the luminal fluid, which decreases from 7.2 in the IS to 6.8 in the cauda, with the lowest point being 6.6 in the caput, while HCO_3^- concentration decreases in a corresponding pattern. This marginally acidic environment is

necessary to render the spermatozoa in an immotile state during maturation and storage in the epididymis (reviewed in (Turner, 2002; Shum *et al.*, 2009)). Of other luminal ions, zinc (Zn^{2+}) has been shown to function as an antioxidant (reviewed in (Aitken *et al.*, 2008)).

Essential small organic molecules in the epididymal lumen include, for example, steroid hormones, carbohydrates, amino acids, phospholipids and vitamins (reviewed in (Turner, 2002)). Of these, steroid hormones are the most abundant group in the epididymal lumen, and the epididymis is widely accepted to be regulated by androgens. However, although androgens mostly diffuse through vasculature, the concentrations of T and its derivative DHT in luminal fluid can be up to 100-fold higher than in the serum (reviewed in (Turner, 2002)). Irrespective of their high concentrations, the physiological significance of luminal androgens remains obscure. Evidence of androgen receptor (AR) presence in spermatozoa is controversial; while some studies show AR in spermatozoa of several species by immunolocalization, others claim its absence (reviewed in (Wang *et al.*, 2009)). Furthermore, the germ cell specific KO of AR revealed no deficiencies in spermatogenesis, function of the testis or fertility of the mice (Tsai *et al.*, 2006). Although such results have raised controversy (Wang *et al.*, 2009), they imply that luminal androgens do not affect epididymal spermatozoa. As such, the other logical targets for luminal androgens are the epithelial cells. However, these also receive androgens through circulation, although the luminal concentrations are markedly higher. These luminal androgens are likely bound to androgen binding protein (ABP), an abundant component of the epididymal fluid, and intraluminal delivery of SHBG (sex hormone-binding globulin, a human homologue of ABP) has been shown to stimulate protein synthesis and secretion of the epithelial cells (Turner *et al.*, 1995). Interestingly, co-delivery of DHT and SHBG showed less increase in protein production than SHBG alone, which indicates that DHT might inhibit SHBG functions (Turner *et al.*, 1995). Thus, the roles of intraluminal androgens on the function of the epididymis, remain unknown. In addition to androgens, estrogens (surprisingly) are also essential in male fertility. Estradiol is synthesized by the germ cells and also converted from T by cytochrome P450 aromatase in the epididymis. Although the luminal fluid concentration of estrogens is less than 0.01% of androgens, loss of estrogen function leads to defects in the luminal microenvironment and causes infertility, as a result of decrease in both sperm concentration and motility of spermatozoa (reviewed in (Joseph *et al.*, 2011)).

Hundreds of different proteins have been identified in the epididymal lumen, but they will not be comprehensively listed here (Figure 2) (Dacheux *et al.*, 2009; Belleannee *et al.*, 2011b; Li *et al.*, 2011). Also, most epididymal proteins are expressed in a region specific fashion, which adds to the complexity of the epididymal proteome. The proteins secreted into the lumen are mainly derived from

epididymal epithelial cells. Those which originate from the testis are mostly absorbed by the epithelial cells in the ED and the proximal epididymis (Dacheux *et al.*, 2009; Clulow *et al.*, 1994). Epididymal proteins can be bound on spermatozoa, encased in small vesicles secreted by the epithelial cells known as epididymosomes (Sullivan *et al.*, 2007), or unassociated in the luminal fluid. Major classes of secreted proteins are reviewed by Dacheux and Dacheux (Dacheux *et al.*, 2002), and include glycosidases, antioxidants, proteases, protease inhibitors, clusterin, cysteine-rich secretory proteins, and proteins that bind small hydrophobic molecules, lipids and iron. The known roles of secreted epididymal proteins in sperm maturation and storage are discussed in more detail in chapter 2.1.4.

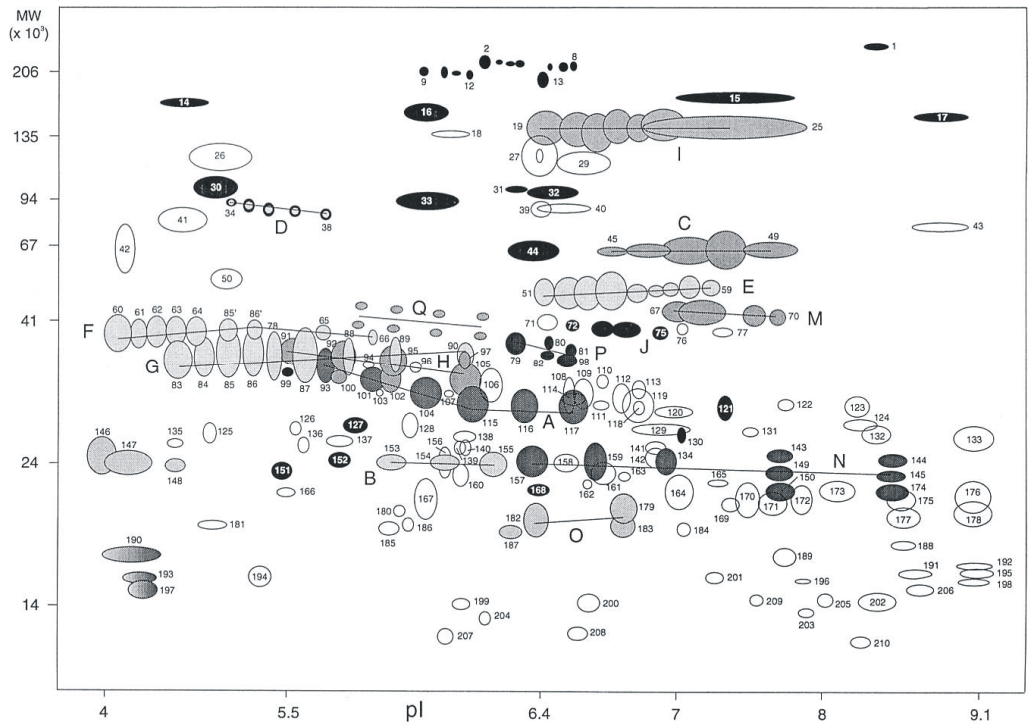


Figure 2. An illustration to demonstrate the multiplicity of secreted proteins detected in the boar epididymis by one- and two-dimensional PAGE analyses. Each spot represents a separate protein (Syntin *et al.*, 1996).

2.1.4 Gene expression and function of the epididymis

Gene expression and, thereby function and anatomy of the epididymis, are mainly regulated by androgens which originate from the testes (Orgebin-Crist, 1996). The two main effectors are T and its more potent metabolite, DHT, which is reduced from T by the enzyme 5α-reductase (Russell *et al.*, 1994). However, there is

evidence of genes that are regulated by other hormones, testicular factors, mesenchyme/epithelium interactions, and even factors which originate from the epididymis itself (reviewed in (Cornwall *et al.*, 2002)). Interestingly, large scale gene and protein expression analyses have revealed that nearly all molecules studied have distinct and highly segment specific expression profiles (reviewed in (Robaire *et al.*, 2006)). Given the different roles of epididymal segments in sperm maturation, transport and storage, this is hardly surprising. However, the strictness of the expression boundaries implies exceptional control of gene expression, making the epididymis an interesting model organ for transcription regulation studies.

Perhaps owing to the different embryonic origins of the IS and the rest of the epididymis, gene expression in the IS and proximal caput epididymidis seems to be mainly controlled by luminal testicular factors rather than circulating androgens (Hinton *et al.*, 2000). This regulation by factors secreted through luminal or ductal system has been termed as lumicrine regulation (Hinton *et al.*, 2000). The evidence for lumicrine regulation in the epididymis originates from experiments with ED ligations and gonadectomies. This results in changes in gene expression which lead to morphological changes and apoptosis of the epididymal epithelial cells. These effects can be countered by the administration of T, except in the IS, indicating that testicular factors other than androgens are required for its proper function (reviewed in (Cornwall *et al.*, 2002) and (Robaire *et al.*, 2006)). Several different lumicrine regulators, including ions, organic solutes, proteins and even the spermatozoa, have been suggested to regulate the IS (reviewed in (Hinton *et al.*, 2000)), although the role of no such factor has been ascertained. The most likely candidates are luminal androgens and estrogens, but also nonsteroidal factors, such as the ABP (Scheer *et al.*, 1980; Robaire *et al.*, 1981), fibroblast growth factors (FGFs) (Lan *et al.*, 1998; Kirby *et al.*, 2003) and neurotrophins (S. Crenshaw and B.T. Hinton, unpublished observation in: (Robaire *et al.*, 2006)), have been suggested to participate in the regulation of epididymal function. It is also possible that the effect of luminal factors extends to more distal parts of the epididymis, too and that lumicrine regulation takes place between different regions of the epididymis (Robaire *et al.*, 2006; Abe *et al.*, 1984)). This has been indicated for example by *c-ros* KO mice, which lack a properly differentiated IS and demonstrate altered gene and protein expression in the caput epididymidis (Cooper *et al.*, 2003a).

Although studying the role of androgens in the function of the epididymis is, in principle, simple by orchidectomy, or removal of the testes, the loss of other testicular factors and spermatozoa from the luminal fluid makes the correct determination of cause and effect more difficult. Hypophysectomy, or the removal of the pituitary, is an alternative approach to achieve the loss of androgens;

however, the Leydig cells are able to produce small amounts of T in the absence of luteinizing hormone (LH), leaving this method lacking, too (Zhang *et al.*, 2001; O'Donnell *et al.*, 2006). Nevertheless, the significance of androgens to the epididymis, starting from its embryonic development, has been well established and documented since their discovery nearly a century ago (Benoit, 1926; Orgebin-Crist, 1996; Butenandt, 1931). The most notable change in the epididymis after orchidectomy is a marked reduction in weight, most of which is attributable to the loss of spermatozoa and luminal fluid (Robaire *et al.*, 1977; Brooks, 1979). Of epithelial cells, the principal cells appear to be the most sensitive to androgen depletion, and changes in their morphology suggest serious compromise in their protein secretion capabilities (Moore *et al.*, 1979; Orgebin-Crist *et al.*, 1974). Epithelial cells begin to undergo apoptotic cell death, which starts from the proximal and advances towards distal epididymidis, over several days (Fan *et al.*, 1998; Turner *et al.*, 1999). Sperm left in the lumen lose their motility, fertilizing capability, and eventually die (Benoit, 1926; Dyson *et al.*, 1973; White, 1932). Total epididymal protein, RNA and DNA contents are reduced (Brooks, 1977), and androgen receptors and 5α-reductase activities are also both decreased, reflecting the loss of androgens (Robaire *et al.*, 1977; de Larminat *et al.*, 1978; Pujol *et al.*, 1979; Zhu *et al.*, 2000). However, most adverse effects can be reversed by the restoration of the levels of circulating testosterone in the caput, corpus and cauda, but not in the IS, which is controlled by other luminal factors (Robaire *et al.*, 1977; Fawcett *et al.*, 1979; Moniem *et al.*, 1978; Ruiz-Bravo, 1988; Holland *et al.*, 1992; Schwaab *et al.*, 1998; Palladino *et al.*, 1994).

2.1.4.1 Sperm maturation

Post-testicular sperm maturation is a chain of events to lead to spermatozoa to attain abilities required for fertilization, which includes gain of motility and the abilities to recognize, bind to and penetrate the ZP, and subsequently to bind to and fuse with the egg cell membrane. Morphologically, the most prominent feature of sperm maturation is the migration of the cytoplasmic droplet from head to flagellum (reviewed in (Cooper *et al.*, 2003b)). Owing to the condensation of the nucleus during spermiogenesis, the spermatozoa are biosynthetically mostly inactive. Thereby, factors to enable the events which lead to sperm maturation must come from outside, *i.e.* the luminal fluid, as secretions by the testes and epididymal epithelial cells. Epididymal secretions affect sperm maturation both indirectly by modification of the luminal microenvironment, and directly by proteins that interact with the spermatozoa. The secreted proteins can either modify existing proteins and other macromolecules on sperm membranes, or be incorporated into the spermatozoa (Figure 3). Hundreds of genes (Johnston *et al.*, 2005; Jelinsky *et al.*, 2007; Guyonnet *et al.*, 2009) and proteins (Dacheux *et al.*, 2009; Belleannee *et al.*, 2011b; Li *et al.*, 2011) have been identified in the epididymis in different

species, but the key factors which affect sperm maturation events have still yet to be identified. Although data on several epididymal transcriptomes and proteomes are readily available in online databanks, functional studies are carried out painstakingly slowly, a gene or a protein at a time. Such task is not made any easier by redundancy in gene families and proteins subject to post-translational and post-secretorial modifications. However, despite the numerous amounts of different proteins present in the epididymal lumen, 60–80% of luminal protein concentration is estimated to be comprised of only 15–20 proteins (Dacheux *et al.*, 2009). Further, recent improvements in methodology have enabled the identification of sperm surface proteins and dynamics in their expression (Belleannee *et al.*, 2011a), which reveal possible candidates for maturation mediating factors. As a result of the lack of *in vitro* methods to study proteins potentially involved in sperm maturation, an effective approach is the use of genetically modified animal models (Lye *et al.*, 2004).

While meiosis and testicular spermatogenesis have been widely studied, the interest towards post-testicular sperm maturation has been considerably lesser in scale. Of late, however, the increased availability and reduced cost of techniques required for the generation of genetically modified animal models has added KO mice to the research methods available to epididymologists. To date, several KO mouse models which unveil details in sperm maturation and the function of the epididymis have been published. The models listed in Table 1 represent confirmed epididymal origin of fertility defects; phenotypic changes caused by loss of genes expressed in the testes or by spermatozoa have not been included. These epididymal KO models include genes coding for secreted proteins that directly interact with spermatozoa, secreted proteins that affect the luminal microenvironment, and transcription factors and receptors that affect the function of epididymal cells in general. Surprisingly, the loss of some of the most abundant epididymal proteins, such as CRISP1 (Da Ros *et al.*, 2008) and GPX5 (Chabory *et al.*, 2009), have led to remarkably minor defects in fertility, which indicates functional redundancy in genes expressed in the epididymis and the plasticity of the sperm maturation process, to ensure stable fertility, which is evolutionarily of paramount importance.

The genetically modified animal models listed in Table 1 mostly represent genes expressed only in the epididymis. However, many important genes potentially involved in sperm maturation are expressed in several male reproductive organs. Thereby, it cannot always be unambiguously stated that a protein is necessary for epididymal sperm maturation, if it is also produced in the testes. In such cases the use of conditional, or tissue specific, KO's may be needed. The first epididymis-specific KO model has recently been published (Krutskikh *et al.*, 2011), and once the necessary tools become more generally available, the details of sperm maturation will be further revealed.

Table 1. Genetically modified animal models concerning genes expressed in the epididymis.

gene	protein category	gene modification	fertility	altered phenotype in	reference
Id3	TF	KO	fertile	luminal fluid composition, sperm motility	Carroll *et al.*, 2011
Cres	enzyme	KO	fertile	acrosome reaction, capacitation	Chau *et al.*, 2011
Defb15	β-defensin	rat RNAi TG	subfertile	sperm motility	Zhao *et al.*, 2011
Pla2g3	phospholipase	KO	subfertile	sperm motility, ZP penetration, sperm morphology, capacitation	Sato *et al.*, 2010
Esr1	receptor	KO	infertile	lumen pH, sperm pH, sperm motility, acrosome reaction, epid. morphology	Joseph *et al.*, 2010a; Joseph *et al.*, 2010b
Sed1	sperm surface	KO	subfertile	ZP binding, lumen pH, epid. morphology	Ensslin *et al.*, 2003; Raymond *et al.*, 2009; Raymond *et al.*, 2010
Cd52	sperm surface	KO	fertile	no change in phenotype	Yamaguchi *et al.*, 2008
Foxi1	TF	KO	infertile	sperm morphology, lumen pH	Blomqvist *et al.*, 2006
Bin1b	β-defensin	rat RNAi TG	fertile	sperm motility	Zhou *et al.*, 2004
Inpp5b	phosphatase	KO	infertile	sperm motility, ZP penetration, fusion with egg membrane; maybe Sertoli cell related	Hellsten *et al.*, 2001
c-ros	receptor	KO	infertile	epid. morphology, sperm morphology	Sonnenberg-Riethmacher *et al.*, 1996; Yeung *et al.*, 1999
Rar	receptor	dominant negative TG	subfertile	epid. morphology; low expression in testes	Costa *et al.*, 1997
Ar	receptor	KO	infertile	azoospermia, epid. morphology	Krutskikh *et al.*, 2011
Dusp6	phosphatase	KO	fertile	epid. morphology	Xu *et al.*, 2010
Pltp	phospholipid transfer protein	KO	subfertile	sperm motility, ZP penetration	Drouineaud *et al.*, 2006
He6	receptor	KO	subfertile	sperm motility, ED morphology	Davies *et al.*, 2004
Apoer2	receptor	KO	infertile	sperm morphology, sperm motility, sperm volume regulation	Andersen *et al.*, 2003
Gpx5	enzyme	KO	subfertile	sperm oxidative stress in older mice	Chabory *et al.*, 2009
Crisp1	sperm surface	KO	fertile	ZP penetration, fusion with egg membrane, sperm capacitation	Da Ros *et al.*, 2008
Crisp4	sperm surface	KO	fertile	acrosome reaction	Gibbs *et al.*, 2011

RNAi, RNA interference; epid., epididymis; TG, transgenic; ED, efferent duct; TF, transcription factor.

2.1.4.2 Sperm storage

Species which practise internal fertilization concentrate and store their maturing spermatozoa in the epididymis prior to ejaculation, which results in the quantity of spermatozoa in the ejaculate more controllable and less dependent on testicular production, which can then be maintained normally while spermatozoa are transported to the epididymis. This also sets demands for storage conditions, *i.e.* the spermatozoa need to be both protected from damage caused by oxidative stress and micro-organisms, and their motility suppressed until required, to conserve energy and maintain structural integrity. Thus, in addition to the importance of the epididymis in sperm maturation, it is also necessary for the safekeeping of spermatozoa. It has even been speculated as to whether the primary role of the human epididymis is either maturation or storage of the spermatozoa (reviewed in (Jones, 1999)).

As a result of the blood–epididymis barrier, the contents of the epididymal lumen are isolated from the rest of the body, which is important as certain proteins on spermatozoa are recognized as foreign objects by the immune system. However, since immune cells are isolated from to the epididymal lumen, alternative methods to protect the spermatozoa against harmful micro-organisms are required. With the high incidence of sexually transmitted infections and effect on fertility in humans, increased interest towards such factors has resulted (Hall *et al.*, 2002). Genes expressed in the epididymis encoding cysteine-rich secreted proteins with antimicrobial properties have been described, inclusive of members of the β-defensin family (Zhao *et al.*, 2011; Hall *et al.*, 2007; Diao *et al.*, 2011). Beta-defensins BIN1b (Li *et al.*, 2001; Zhou *et al.*, 2004) and DEFB15 (Zhao *et al.*, 2011) have been shown to be involved in both sperm maturation and defence against micro-organisms. Further, antimicrobial DEFB22 (Diao *et al.*, 2011; Yudin *et al.*, 2008) and DEFB118 (Yenugu *et al.*, 2004) are also bound to the sperm surface. Similar epididymal protein families, such as CRISPs (cysteine-rich secretory proteins; discussed in chapter 2.5.2) (Gibbs *et al.*, 2007) and ADAMs (a disintegrin and a metalloprotease) (Edwards *et al.*, 2008) have also been identified, but their antimicrobial properties remain to be characterized. However, the detected dual roles of said β-defensins, indicate that antimicrobial epididymal proteins are not necessarily always solely responsible for antimicrobial activities. The epididymal CRISP1 has also been suggested to confer separate functions through different protein domains (Cohen *et al.*, 2011). Thereby, it is reasonable to suppose that β-defensins, CRISPs and ADAMs do not only involve participation in defence against microbes, but have other functions in either addition or instead.

In addition to micro-organisms, spermatozoa also need to be protected from reactive oxygen species (ROS) during their stay in the epididymis, which lasts for

several days. The relationship between spermatozoa and ROS is somewhat paradoxical; ROS cause DNA fragmentation which increases the risk of cancer in offspring, and peroxidation of unsaturated fatty acids on sperm membrane which leads to loss of function, yet the spermatozoa themselves generate ROS and are needed for motility, capacitation and acrosome reaction (reviewed in (Aitken, 2002)). Thus, to maintain a delicate balance in ROS production and recycling is essential to the proper functionality of the spermatozoa. The major antioxidant proteins in the epididymis are indoleamine dioxygenase (IDO) (Hirata *et al.*, 1975; Yoshida *et al.*, 1980), superoxide dismutase (SOD) (Perry *et al.*, 1993) and glutathione peroxidases (GPXs) (Drevet, 2006), and are present in sperm cytoplasm, bound onto sperm membranes, and free in the luminal fluid. It has been estimated that oxidative stress is a contributor in 30–80% of cases of male infertility (Tremellen, 2008), thereby making it an important area of research. However, due to the variability of different ROS and their effects, and changes in their localization patterns within the epididymis, the details of their function in sperm maturation and storage remain to be clarified.

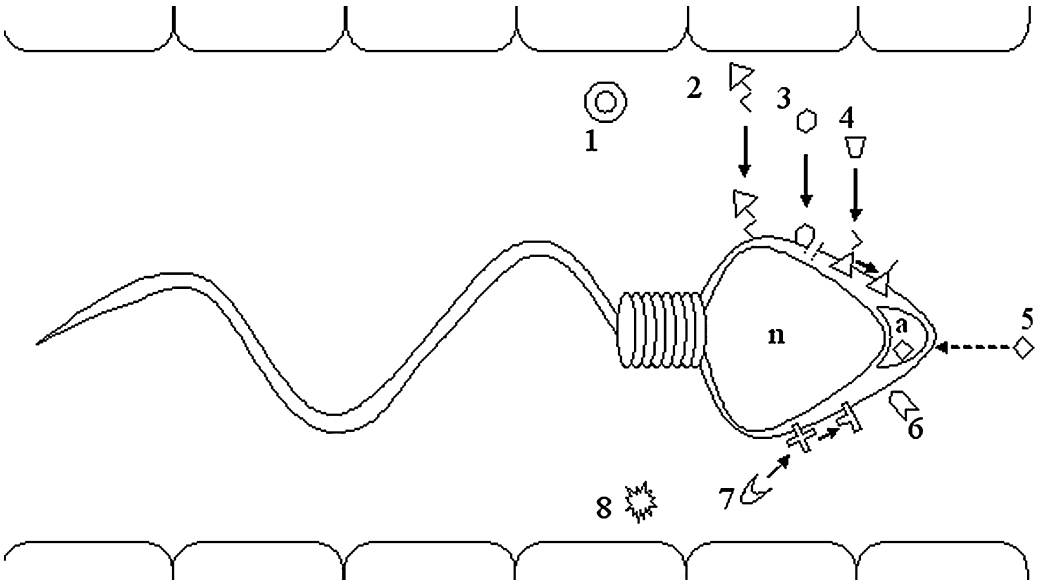


Figure 3. An illustration of epididymal proteins secreted into the lumen with examples of the mechanisms through which sperm maturation and storage are affected. **1)** Antioxidants, **2)** proteins adsorbed to the sperm membrane through glycosyl-phosphatidyl-inositol (GPI) links, **3)** proteins which regulate sperm membrane ion channels, **4)** proteins which modify the sperm glycocalyx, **5)** proteins absorbed into the sperm, **6)** proteins which modify the membrane lipid constitution, **7)** proteolytic proteins which modify existing sperm membrane proteins, **8)** antimicrobial proteins. n = nucleus, a = acrosome.

2.1.5 Diseases of the epididymis

Evolutionarily, the proper maintenance of reproductive capability *i.e.* the passing of genes to the next generation, is of paramount importance. In addition to developmental and structural defects, external factors also pose a threat to fertility. Inflammations of the male reproductive organs are estimated to account for 15% of cases of male infertility, a major target of which appears to be the epididymis (reviewed in (Haidl *et al.*, 2008) and (Trojian *et al.*, 2009)). The most common agents for epididymal inflammation, or epididymitis, are *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, which also cause sexually transmitted diseases gonorrhoea and chlamydia, respectively (reviewed in (Pellati *et al.*, 2008)). Further, several species of enterobacteria, such as *Escherichia coli*, and certain gram-positive cocci have been implicated in epididymitis. Although other bacteria, yeasts, parasites and viruses have been associated with cases of infertility or affecting functional sperm parameters, no definite connections to the function of the epididymis have been made (reviewed by (Pellati *et al.*, 2008)). In addition to micro-organisms, epididymitis can also be caused by certain drugs or other chemicals, trauma or physical stress, alongside with epididymal neoplasms (Chan *et al.*, 2002). Notably, however, epididymal neoplasms and tumours are exceedingly rare, with only a few dozen reported cases of primary adenocarcinomas (Ganem *et al.*, 1998). The epididymal epithelial cells also seem highly resistant to induced tumorigenesis in transgenic mouse models, and high expressions of *Pea3* or *Bmyc*, potential tumour suppressor genes, have been suggested to provide protection against malignant transformations (Sipila *et al.*, 2004). However, the details behind such protection remain unknown.

2.2 Sperm–egg interactions and fertilization

After ejaculation but before fertilization, spermatozoa must 1) reach an egg, 2) penetrate the extracellular matrix of the egg, and 3) bind and fuse with the egg cell membrane (Figure 4A–C). However, testicular spermatozoa lack the abilities required for these tasks (Yanagimachi, 1994; Lacham *et al.*, 1991). Although the final changes in spermatozoa which permit fertilization, occur in the female reproductive tract and upon encounter with the egg (Florman *et al.*, 2006), the processes that enable the enecessary changes, occur in the epididymis during sperm maturation. While in epididymal transit, spermatozoa acquire motility, the potential for capacitation, and modifications to the plasma membrane to facilitate interaction with the egg.

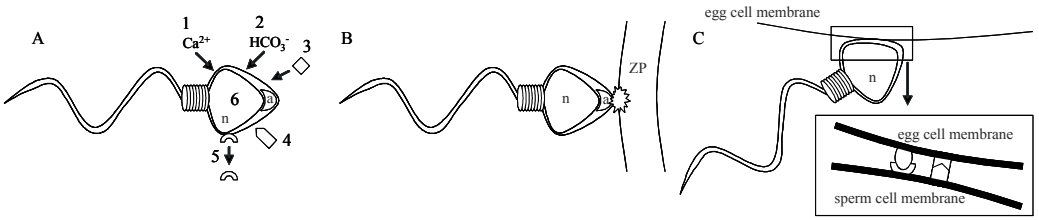


Figure 4. An illustration of events which occur prior to fertilization. **(A)** Sperm capacitation which grants motility and the ability to undergo the acrosome reaction. Capacitation is initiated by the uptake of calcium and bicarbonate ions, along with proteins which act as capacitation factors (1–3), modifications to the plasma membrane (4), loss of decapacitation factors (5), and alterations in intracellular pH and protein tyrosine phosphorylation (6). **(B)** Sperm acrosome reaction (indicated by the explosion symbol) is induced by contact with ZP, which results in an influx of intracellular calcium ions to lead to release of proteolytic enzymes from the acrosome, granting the ability to pass through the ZP. **(C)** Recognition of gametes, which leads to binding and fusion of the membranes. Both recognition and binding are mediated by receptor proteins on egg and sperm cell membranes (box) n = nucleus, a = acrosome, ZP = zona pellucida.

2.2.1 Sperm capacitation

Sperm capacitation refers to the acquisition of fertilization potential, but more exact definitions are controversial (Jaiswal *et al.*, 2002). It has been generally accepted that capacitation confers on spermatozoa the ability to undergo acrosome reaction, although it has been linked to sperm chemotaxis and hyperactivation of motility, too. Since the definition of capacitation is ambiguous, it becomes also difficult to state conclusively whether an individual spermatozoa is capacitated or not. Thus, in the ejaculate, capacitation is not a simple all-or-nothing instance; at any given moment, only a proportion of the sperm will be capacitated, and capacitated spermatozoa can later become decapacitated (Cohen-Dayag *et al.*, 1994). The ratio between capacitated and decapacitated states needs to be controlled both temporally and spatially, and such balance is maintained by different capacitating and decapacitating factors. Due to increased energy consumption of capacitated spermatozoa, premature or over extended state of capacitation would deplete the energy reserves of the spermatozoon before fertilization became possible, to make inhibition of capacitation an important aspect in fertility, too. Although the existence of these inhibitors, or decapacitation factors, has been known for decades, to date their identities still remain elusive, until a few candidates have just recently been suggested. The first decapacitating agents originate from the spermatozoa themselves. Phosphatidylethanolamine binding protein 1 [PBP; also known as Raf kinase inhibitor protein-1 (RKIP-1)] is a sperm membrane protein that has been shown to be

an inhibitor of capacitation (Nixon *et al.*, 2006; Moffit *et al.*, 2007). The epididymis also secretes factors that are adsorbed by the spermatozoa, or that modify existing spermatozoa membrane proteins. HongrES1 (Ni *et al.*, 2009) and SERPINE2 (Lu *et al.*, 2011) are potential epididymal decapacitation factors, of which the latter is also a component of the seminal vesicle fluid. Incidentally, the seminal vesicles are thought to be the main source of decapacitation factors (Jaiswal *et al.*, 2002). In addition to SERPINE2, seminal vesicle protein secretion 2 (SVS2) (Kawano *et al.*, 2007) and secreted serine protease inhibitor Kazal-type-like protein (SPINKL) (Lin *et al.*, 2008) have been suggested as decapacitation factors secreted by the seminal vesicles. However, the abundance of uncharacterized proteins in the seminal fluid and the complexity of the regulation of capacitation, imply that numerous other decapacitation factors have yet to be found.

Although the physiological and functional changes in spermatozoa upon capacitation are well characterized and some key inducers have been identified, the relevant components of the cellular pathways are less well known. Requirements for capacitation vary between species, but commonly include changes in ionic concentrations and pH, a source of energy, and removal of de-capacitation factors (reviewed in (Jaiswal *et al.*, 2002) and (Florman *et al.*, 2006)). The most important ions in capacitation are bicarbonate and calcium, of which the former is considered to be a key regulator of capacitation. Bicarbonate is abundant in the female reproductive tract fluid, and has been linked to changes in sperm membrane, protein phosphorylation, and activation of cyclic adenosine monophosphate signalling pathways (reviewed in (Bailey, 2010)). Although calcium is also considered as a major capacitation factor, its role is more prominent in the acrosome reaction, and will be reviewed in the next section. In addition to ions, progesterone has been shown to stimulate sperm motility and capacitation (reviewed in (Jaiswal *et al.*, 2002)).

During capacitation, spermatozoa undergo a major reorganization of their cell membrane's lipid and protein constitution. The role of cholesterol especially has been widely studied, and as capacitation advances, the quantity of cholesterol decreases, which has resulted in cholesterol being regarded as one of the major decapacitation factors (reviewed in (Cross, 1998)). Its decrease increases fluidity of the membrane, which enables further movements of membrane phospholipids and proteins. Subsequently, this grants the spermatozoa the abilities required in interaction with the egg and its extracellular matrix, and enables the fusion of the plasma and outer acrosomal membranes during acrosome reaction (reviewed in (Cross, 1998) and (Jaiswal *et al.*, 2002)).

Intracellular changes have also been associated with capacitation. In addition to changes in ionic concentrations, extensive protein phosphorylation has been

observed. However, although several phosphorylated proteins have been detected, only a few have been identified, but even these roles in fertility are not known (reviewed in (Urner *et al.*, 2003)).

2.2.2 Extracellular matrix of the egg and the acrosome reaction

Upon reaching the egg, spermatozoa have two major obstacles to overcome before contact with the egg cell membrane: the cumulus oophorus and ZP. The cumulus oophorus is a complex surrounding the egg and consists of cumulus cells and extracellular matrix (ECM) abundant with self secreted hyaluronic acid (reviewed in (Cardullo *et al.*, 2002)). It has many important roles during maturation of the egg and meiosis, in fertilization, and in early embryo development, too: but here, only its functions related to spermatozoa and fertilization are discussed. In respect of its interactions with the spermatozoa, the cumulus oophorus has a dual significance. First, to act as a physical and chemical barrier through which the spermatozoa must pass. Thus, it serves as a screen to prevent abnormal spermatozoa to fertilize the egg, which might result in formation of non-viable embryos (Carrell *et al.*, 1993). Prematurely acrosome reacted and non-capacitated spermatozoa will also be unable to penetrate the oophorus (Cummins *et al.*, 1986). Secondly, however, the oophorus also assists the spermatozoa to reach the egg. The cumulus cells secrete progesterone, which may be the most important chemoattractant that guides the spermatozoa (Oren-Benaroya *et al.*, 2008). Soluble and matrix-associated factors of the oophorus also seem to alter motility patterns of the spermatozoa, to enhance penetration (reviewed in (Cardullo *et al.*, 2002)). To work their way through the oophorus, spermatozoa require hyaluronidases to penetrate the hyaluronic acid-rich ECM (reviewed in (Kim *et al.*, 2008)). To date, three hyaluronidases produced in the testis and/or the epididymis, HYALP1, HYAL5 and PH-20 (SPAM1), have been associated with spermatozoa, which seem to have overlapping functions in degrading the oophorus, as indicated by normal fertility of PH-20 deficient mice (Kim *et al.*, 2008; Baba *et al.*, 2002; Miller *et al.*, 2007).

Although the oophorus has also been indicated in the induction of the acrosome reaction, the traditional model is that the acrosome reaction is initiated as spermatozoa contact ZP (reviewed in (Sun *et al.*, 2011)). ZP is the ECM of the egg, and although relatively simple in structure, yet represents a formidable barrier to spermatozoa. The spermatozoon, however, is well equipped to deal with such an obstacle; within its head is a single large secretory vesicle, known as the acrosome, which contains proteolytic enzymes required for the sperm to penetrate the ZP. Spermatozoa recognize the ZP3 glycoprotein on the egg ZP with β1,4-galactosyltransferase (GalT) (Lu *et al.*, 1997). A flux of Ca^{2+} in the spermatozoa follows and initiates a signalling cascade to result in controlled fusion of the sperm plasma membrane with the outer acrosome membrane, leading to release of

acrosomal contents and the unveiling of a new sperm surface domain (Florman *et al.*, 2006). Since the acrosome reaction is a prerequisite for sperm–egg membrane fusion (Yanagimachi, 1994), it is likely that previously hidden sperm surface proteins responsible for fusion are simultaneously uncovered. The importance of Ca^{2+} in the onset of the acrosome reaction indicates that sperm membrane ion channels and their regulators play a pivotal role. Correspondingly, a host of calcium conducting channels on spermatozoa has been identified (reviewed in (Florman *et al.*, 2006)), and recently CRISP4, a secreted epididymal protein discovered by our group (Jalkanen *et al.*, 2005a), has been shown to regulate ion channel signalling and initiation of acrosome reaction in sperm, although it is not indispensable for fertility (Gibbs *et al.*, 2011). Other potentially important sperm membrane proteins involved in sperm–ZP binding and acrosome reaction, such as acrosin, SED1, fertilin, CD46 and zonadhesin, have also been studied with KO mouse models, but none of the aforementioned, including GalT, are necessary for fertility (reviewed by (Muro *et al.*, 2011)). Interestingly, however, KO models of *Clgn*, *Calr3*, *Adam1a*, *Adam2*, *Adam3* and *Ace* share similar fertility defects, which have been traced to a lack of ADAM3 from the sperm membrane (Muro *et al.*, 2011). Therefore, ADAM3 has been suggested to be one of the key sperm membrane proteins that mediates sperm–ZP interactions, although the specific molecular mechanisms behind its functionality remain to be clarified (Muro *et al.*, 2011). Altogether, numerous studies have revealed the complexities in the regulation of fertility at the level of penetration of the outermost layers of the egg, but such complexity the complexity also generates redundancy, which often guarantees successful reproduction and thereby continuation of the species, even if a cog in the wheel, so to speak, is absent.

2.2.3 Sperm–egg membrane fusion

After breach of the two outer layers of the egg, the spermatozoon finally makes contact with the egg cell membrane. Compatibility of the sperm to the egg is once again confirmed by membrane proteins and their corresponding receptors. The membrane interactions occur in two major phases: first, an initial recognition and binding, and second, fusion of the two membranes (Primakoff *et al.*, 2002). Although a critical protein for fusion on the egg membrane, CD9, has been identified (Kaji *et al.*, 2000), sperm related fusion factors remain more obscure. Candidate receptors and ligands have been suggested, but often subsequently rejected from being indispensable. However, one of the most important sperm-related membrane fusion mediators, is IZUMO1; its loss prevents fusion of the membranes, although the sperm undergo the acrosome reaction and are able to bind to the egg membrane (Inoue *et al.*, 2005). Similarly as with ADAM3 and ZP binding, fusion problems also arise when proteins responsible for correct localization of IZUMO1 on sperm membrane are missing (reviewed in (Inoue *et al.*, 2011)).

In addition to IZUMO1, certain members of the ADAM family, such as fertilin and cyritestin, have received considerable attention due to their ability to bind egg membrane integrins, but have later been found to mainly act in the earlier stages of fertilization (reviewed by (Sutovsky, 2009) and (Muro *et al.*, 2011)). However, there are several unknown or poorly characterized ADAMs produced by the male reproductive organs, and to date studies indicate that many may be either synthesized or modified in the epididymis (Kim *et al.*, 2006; Primakoff *et al.*, 2007). Notably, CRISP1, one of the most abundant epididymal proteins, shares structural characteristics with the ADAM proteins, and has been implicated in participation in several processes during fertilization (reviewed in (Cohen *et al.*, 2011)), and will be discussed in more detail in a later section. However, its role in sperm–egg fusion has recently raised particular interest (Sutovsky, 2009). Although the loss of CRISP1 does not prevent fertilization, its absence may be complemented by CRISP2 (Busso *et al.*, 2007). Thereby, although the role of the epididymis in the acquisition of the spermatozoa of a fertilization function is well established, some light is finally being shed on the molecular mechanisms of epididymal sperm modifications as relevant to fertility.

2.3 Male contraception

From either a global or individual perspective, effective contraceptive methods are needed; the world population continues to rise, causing considerable ecological and socio-economical problems, and a planned parenthood approach is often a preferred strategy for those who desire a family. Compared to the several different female contraceptives available, male contraceptive methods are limited to either condoms, vasectomy, or *coitus interruptus*, but all of which have their respective shortcomings. Thereby, novel male contraceptive methods have been pursued for decades.

As male fertility is heavily reliant on hormones, disruption of the hypothalamic-pituitary-testicular axis to inhibit spermatogenesis has been extensively studied. To date, clinical tests have proved promising; male hormonal contraceptives confer a risk of pregnancy of 1% per year – thus comparable to the efficiency of female hormonal contraceptives –, and fertility is restored after termination of treatment, with adverse side effects relatively minor, yet certain drawbacks still remain. The objective for a male hormonal contraceptive is to reduce sperm concentration to less than 10^6 / ml of ejaculate, which is considered as severe oligozoospermia. However, for such a reduction to occur, will take 2–3 months, and a return to normal sperm concentrations can thereafter take up to a year, and adequate suppression of spermatogenesis is not always achieved. Further, transdermal and oral administration of the treatment are inefficient, which compells reliance on the more impractical injection. Finally, clinical trials groups have been rather small (only up to ~1000 men), and long-term side effects of altered hormone levels are as

yet unknown. In addition to changes in mood and libido, potential risks include, for example, increased susceptibility to thromboembolism and cardiovascular disease. Conversely, however, beneficial effects on muscle, bone and fat mass have also been reported. Altogether, considerable advances have been made in developing male hormonal contraceptives, and further studies may lead to commercially available treatments in the future. (The current status of male hormonal contraceptives is reviewed in (Roth *et al.*, 2011) and in (Ilani *et al.*, 2011).)

Alternatives to the systemic inhibition of spermatogenesis by disruption of the endocrine pathway have also been suggested. These novel approaches include the prevention of sperm transportation, contraceptive vaccines, and the pharmacological inhibition of sperm proteins (Cooper, 2002; Cheng *et al.*, 2010). A method termed RISUG (reversible inhibition of sperm under guidance) involves a compound injected into the vas deferens which acts as a spermicide (reviewed in (Sharma *et al.*, 2007)), and is currently undergoing extended phase III clinical trial. However, despite its efficiency and apparent non-toxicity, its invasive method of application is hardly ideal. Thereby, to directly target the spermatozoa, either pharmacologically or by vaccine, would seem a more promising approach. By choice of a suitable target protein (or proteins), an effective contraceptive with no adverse side effects could theoretically be developed. A good candidate protein for inhibition would be specifically associated with spermatozoa, critical for sperm function, and easily druggable. Different approaches to identify such proteins have been utilized, *e.g.* microarrays, proteomics, mass spectrometry, and expressed sequence tag (EST) libraries have proven effective to discover novel putatively specifically sperm-associated proteins (reviewed in (Sipila *et al.*, 2009)). Although immunization against certain sperm-specific proteins has resulted in effective and reversible contraception in animal models, many obstacles still remain. Individual variation in long-term infertility is considerable, possibly due to differences in host immune responses, and suitable antigens for the generation of efficient antibodies have been difficult to identify. As such, no clinical trials with use of immunocontraceptives are currently undergoing, and interest towards vaccination for fertility control appears to have somewhat waned recently (reviewed in (Cheng *et al.*, 2010)). However, pharmacological inhibition of sperm-associated proteins critical for sperm function remains an intriguing option, and is mentioned in practically all publications that describe proteins involved in fertilization. Studies with animal models, including non-human primates, and with human spermatozoa have been promising, and revealed several proteins which render spermatozoa incapable of fertilization when pharmacologically inhibited. Amongst the most notable are, for example, EPPIN (O'Rand *et al.*, 2011), CatSper family proteins (Carlson *et al.*, 2009) and retinoid acid receptor (RAR) (Chung *et al.*, 2011). EPPIN is a sperm-associated protein produced and secreted in the testis, ED and epididymis, and the immunological inhibition of its function results in loss of

motility in spermatozoa. Currently, pharmacological compounds suitable as EPPIN inhibitors are being evaluated for efficiency and safety, and clinical trials will likely follow (reviewed in (O'Rand *et al.*, 2011)). The CatSper members are ion channel proteins which are exclusively expressed in spermatogenic cells, and regulate progesterone mediated Ca^{2+} entry into the cells (Carlson *et al.*, 2009; Lishko *et al.*, 2011; Strunker *et al.*, 2011). A loss of any CatSper protein leads to male infertility in mice due to the inability of the spermatozoa to hyperactivate, and the pharmacological blocking of CatSper channels causes the same effect in mouse spermatozoa (Carlson *et al.*, 2009). However, to date no data from human studies are available. Loss of RAR causes infertility due to vitamin A deficiency (reviewed in (Wolgemuth *et al.*, 2007)). Although pharmacological compounds blocking RAR function cause effective and reversible infertility in mice (Chung *et al.*, 2011), drugs which prevent retinoic acid biosynthesis and thereby cause reversible infertility in humans are unsuitable as contraceptives because of unwanted side effects (Amory *et al.*, 2011). Nevertheless, studies to date have demonstrated that spermatogenesis is very sensitive to changes in retinoic signaling, and thereby its specific disruption may lead to effective male contraceptives.

In conclusion, hormonal male contraception can be achieved, but its poor effectiveness, impracticality of use, and adverse side effects have prevented such from becoming commercially available. Blocking of spermatozoa transportation requires invasive application methods, making it an inconvenient approach, too. Pharmacological inhibition of sperm associated proteins holds the most promise. However, although several interesting target molecules are known, few clinical studies with regard to effectiveness, safety and long term side effects have been made. As such, any new revolution in contraceptive methods seems uncertain for the near future.

2.4 Genetically modified mouse models

To characterize novel proteins, the ultimate test for *in vivo* significance is analysis by alteration of the protein's expression or function in a mammalian animal model through the use of gene manipulation techniques. In transgenic animal models, a protein can be expressed in a non-endogenous temporal or spatial pattern, in excessive quantity, with function altering modifications, in a different species, or in any combination of these. Transgenic animals are produced by introduction of the DNA coding for the desired protein under a promoter of choice, into a zygote or a blastocyst of the animal, with subsequent implantion into the uterus of a surrogate mother. However, the exogenous DNA molecule integrates into a random location in the host's genome, and the transgene's copy number also varies. As such, even careful experimental design may yield unanticipated and irreproducible results. The first transgenic animal model was generated almost four decades ago (Jaenisch *et*

al., 1974), and since, genetically modified organisms have been widely used in biomedical research (reviewed in (Houdebine, 2007)).

Although the introduction of exogenous DNA into an organism has proven a useful method in studying protein function, the significance of a protein is often better revealed by its loss. Despite similarities, generation of loss of function (knock-out) models has required certain key innovations, not needed in traditional gain of function (transgenic) techniques. To lose a protein, an organism's endogenous gene encoding it must be rendered non-functional. Thus, targeted manipulation of the locus with the gene to be knocked out is required, as opposed to the random integration of a transgenic construct into the genome. Due to low efficiency of targeted manipulation of the genome, introduction of the targeting construct into a zygote, as in generation of transgenic models, is not a practical approach. However, targeted mutations can be performed and confirmed in the embryonic stem (ES) cells, which can subsequently be injected into a blastocyst, where they will contribute to the development of the organism. The technique to isolate and culture mouse ES cells became available in 1981 (Evans *et al.*, 1981; Martin, 1981), and the first knock-out mouse model was created in 1989 (Koller *et al.*, 1989). Recently, the novel zinc-finger nuclease based technique has led to the generation of knock-out rats (Geurts *et al.*, 2009), and theoretically allows genomic modifications of any species, including the human (Urnov *et al.*, 2010).

2.4.1 Generation of knock-out models

The process of the generation of knock-out mouse models is summarized in Figure 5. Since gene targeting relies on extensive sequence homology, such targeting constructs mostly consist of a sequence identical to the targeted genomic locus. Usually, several kilobases (kb) of sequence homology is required, and suitable molecules can easily be obtained for example in the form of bacterial artificial chromosomes (BACs). BACs are essentially plasmids of 50–350 kb in size, and often used in the generation of genomic libraries. Several mouse BAC libraries are commercially available, and information on the genomic area encompassed by each individual clone can be accessed from online databases, such as the Ensembl (Hubbard *et al.*, 2009). Thereby, the specific BAC clone which contains the gene of interest can be obtained and subsequently manipulated to generate the desired targeting construct. A typical gene targeting construct consists of two homology arms which surround the disrupted gene of interest. A corresponding segment can be excised from the BAC and subcloned to an appropriate plasmid backbone vector. Finally, the gene of interest is rendered non-functional. Usually this is evoked by insertion of a so-called knock-out cassette in the 5'-end of the gene. The cassette contains a gene conferring resistance to an antibiotic driven by a eukaryotic promoter to facilitate selection after the targeting of the ES cells, and

transcription termination signals to prevent inadvertent transcription of the gene downstream of the cassette. The insertion of the knock-out cassette can be achieved while simultaneously deleting a part of the targeted gene, which is also commonly undertaken to further guarantee the loss of the endogenous gene product.

The complete knock-out construct is linearized by digestion with a restriction endonuclease specific to the plasmid backbone sequence, and electroporated into ES cells. The ES cells are then cultured in selective conditions, to facilitate the survival and proliferation of only the cells which have incorporated the construct into their genomes. However, two separate DNA repair pathways are responsible for such genomic incorporations: non-homologous end joining (NHEJ), which confers random integrations, and homologous recombination repair (HRR) (Huertas, 2010). NHEJ occurs approximately 1000-fold more often than HRR (Vasquez *et al.*, 2001). Thus, subsequent steps in identification of the correct recombinants from the random integrants must be taken. However, certain measures to reduce the number of random integrants can also be considered when designing the construct. One approach is the use of a promoterless antibiotic resistance gene in the knock-out cassette (reviewed in (Friedel *et al.*, 2005)), by which antibiotic resistance is conferred only if the construct is integrated near to a genomic promoter. By placing the knock-out cassette at the 5'-end of the targeted gene, correct recombination will cause the antibiotic resistance gene to be driven by the endogenous promoter of the targeted gene. However, no endogenous promoter elements should be present in the 5'-homology arm of the construct, and the targeted gene should be sufficiently highly expressed in ES cells to provide antibiotic resistance. Another approach is to include a negative selection cassette to the plasmid backbone of the targeting construct. For this, a diphtheria toxin A-chain coding gene is commonly used (McCarrick *et al.*, 1993; Araki *et al.*, 2006). When the construct is correctly targeted, the non-homologous parts (*i.e.* the plasmid backbone) are left out, whereas in random integration the whole construct is incorporated into the genome. Thus, the toxin coding gene should be present only in cells in which random integration of the construct has occurred, and, if correctly expressed, the toxin will kill the cell, to leave only correctly targeted cells behind. A third option to reduce the number of random integrants is to use single-stranded DNA targeting constructs. Double-stranded DNA can be randomly integrated into the genome through NHEJ, whereas single-stranded constructs only participate in homologous recombination. In fact, after electroporation of a double-stranded DNA targeting construct, its digestion into a single-stranded molecule is a prerequisite to homologous recombination (Krogh *et al.*, 2004). However, despite their potential advantages, single-stranded gene targeting constructs are not widely used (Jensen *et al.*, 2011).

Once an ES cell clone in which the correct recombination has occurred has been identified, the targeted cells are used to generate a live animal that carries the

mutation. As ES cells are pluripotent, they can differentiate into any cell type of an adult. This can be accomplished by the incorporation of the targeted ES cells into an early stage embryo. Two approaches are commonly used: microinjection of ES cells into the blastocyst cavity, or aggregation of ES cells with morula stage embryos (Tam *et al.*, 2003). The manipulated embryos are then transferred to the oviducts of a pseudopregnant mouse, and chimeric mice, which consist of cells from the host blastocyst/morula and the ES cells, are born. If the ES cell line is chosen to carry alleles coding for a different fur color than the cells of the host blastocyst/morula, as is usually the case, the chimeric mice can be identified by their coloring. In blastocyst injection chimeras, the injected ES cells incorporate into the inner cell mass of the blastocyst, whereas aggregation chimeras are practically completely derived from the ES cells. Thus, aggregation chimeras are more likely to carry the intended mutation rather than blastocyst injection chimeras. Regardless, the chimeric mice are bred, and once the mutation is passed on to the offspring, a stable mutant mouse line is formed. Since the homologous recombination taking place in the ES cells occurs only in one of two homologous chromosomes, the mutated mice are initially heterozygous for the targeted gene, carrying one wild type (WT) and one targeted allele. By breeding two heterozygotes, mice with two targeted alleles will be born, if viable. Thus, mice which completely lack the targeted gene are finally produced, and the effect of the gene's loss can be characterized.

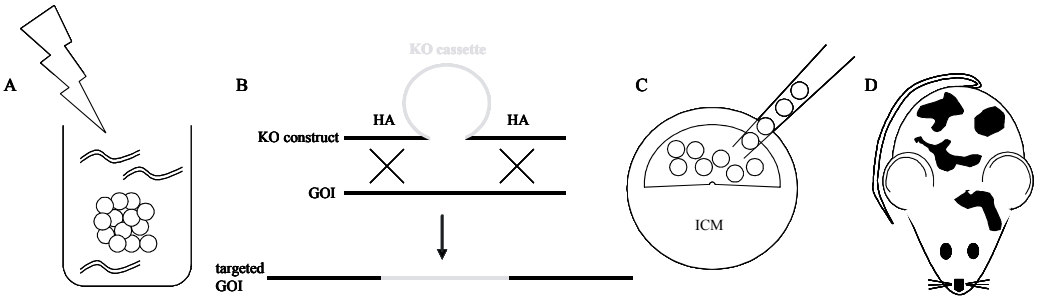


Figure 5. An illustration which summarizes the generation of knock-out mouse models. **(A)** The targeting construct is electroporated into ES cells. **(B)** Within the ES cells the construct locates the gene of interest (GOI) through sequence homology between the homology arms (HA) of the construct and the targeted locus. Crossing over occurs between the two DNA molecules, and repairs made by the DNA repair machinery result in the GOI to be replaced by the KO construct. **(C)** The ES cells which carry the targeted mutation are injected into the blastocyst cavity, whereby incorporated into the inner cell mass (ICM) of the blastocyst. **(D)** The manipulated embryos are transferred to the oviducts of pseudopregnant mice, and chimeric mice which carry the targeted mutation are born.

2.4.1.1 Knock-in models

Homologous recombination enables the targeted insertion of exogenous DNA into the host genome. Although it has mainly been used to disrupt gene function to generate knock-out models, gain of function applications are also possible (Hacking, 2008). As opposed to traditional transgenic models, knock-in models offer certain advantages. First, homologous recombination allows the targeting of the gene construct to a chromosomal area in which the transgene is stably expressed, and does not impede on the function of endogenous genes, whereas random insertion may cause unwanted effects which complicate the interpretation of results. A popular area for targeted transgenics is the ROSA26 locus, which is efficiently targeted and no adverse effects as a result from its disruption have been reported (Casola, 2010). Second, a transgene can be targeted to be expressed under the promoter of a gene of interest. Thereby, the transgene closely mimics the expression pattern of the gene of interest, which can be applied to gene expression studies or in the generation of models with tissue or cell type specific transgene expression. Third, the generation of humanized mouse models is possible by the complete replacement of the endogenous gene with its human orthologue. Further, genes can also be modified only partially to introduce specific mutations. Such models are valuable in biomedical research concerning human biological systems and diseases (Shultz *et al.*, 2007). Fourth, specific recombinase enzyme target sequences can be inserted into the genome to serve as docking sites for recombinase mediated cloning (Sakurai *et al.*, 2010). Thus, after a single round of targeting, a desired cassette can be integrated to (and subsequently removed from) the docking site by using recombinases, a technique which is easier and more effective than repeated homologous recombination.

2.4.1.2 Conditional knock-out models

It is often advantageous, or even necessary, to study the loss of gene function only in a certain tissue or cell type, or at a specific time. Such a case can arise, for example, when a gene is vital during development, and its absence will result in lethality. To study its function in the adult, the embryonic expression must be maintained, while the recombination event which leads to loss of function needs to be induced at a later stage. Dependant on the specifics of the model system, they can be called conditional or inducible knock-outs. Both rely on the same molecular mechanisms.

Conditional knock-outs can be achieved with site-specific DNA recombinase (SSR) technology. In these cases two separate genetic modifications are required. First, the genomic area to be knocked out needs to be flanked with the recombinase recognition sequences, usually 30–50 bp in length, and secondly, the recombinase

coding gene needs to be expressed in a controlled fashion. The recombinase enzyme recognizes two copies of its target sequence and mediates recombination between them. Several SSR systems are available. The most widely used is the Cre-loxP (Kuhn *et al.*, 1995) followed by the FLP-FRT (Dymecki, 1996). More novel systems, not yet used in *in vivo* models, include φC31-attB/attP (Belteki *et al.*, 2003), Dre-rox (Anastassiadis *et al.*, 2009), VCre/VloxP and SCre/SloxP (Suzuki *et al.*, 2011). With the use of mutated recombinase target sites, elaborate constructs for integration, excision and inversion of genomic sequences can be generated (Adams *et al.*, 2008).

A basic strategy to generate conditional knock-outs, involves targeting two loxP sites to flank an exon of the gene of interest in a way that its expression is not disrupted, and then introduction by mating of *Cre* recombinase expression under a tissue-specific promoter. Thereby, recombination between the two loxP sites occurs only in cells that express the recombinase, which is determined by the choice of the promoter. As floxed (flanked by loxP) alleles of many genes are readily available, the limiting factor in the generation off tissue-specific knock-outs, is often the availability of *Cre* expressing lines.

In addition to spatial, temporal control of gene expression can also be achieved (reviewed in (Saunders, 2011)). In a 4-hydroxytamoxifen (4HT) regulated model, a fusion protein of Cre and human estrogen receptor is used. Briefly, treatment of the transgenic mouse with 4HT will cause the Cre-fusion protein to be transported into the cell nucleus, where it will facilitate recombination with its target loxP sequences. This can be used to knock out gene expression with a floxed allele as described above, or to induce expression of a transgene with a floxed stop cassette to prevent its expression. Another method of chemically to either suppress or induce transgene expression, utilizes a fusion protein tTA with a transactivator domain and a tetracycline repression domain, and a second transgene with tetracycline response elements (TRE) in its promoter. In the absence of a ligand (tetracycline or its analog doxycycline) the fusion promoter binds to the TRE and induces gene expression. Treatment with tetracycline causes the protein to dissociate from the promoter, terminating the expression. A mutant version of tTA called rtTA functions the other way around: the fusion protein is able to bind the TRE and initiate transcription only in the presence of tetracycline. Thereby, dependant on the use of either tTA or rtTA, treatment with tetracycline can be used to induce or repress gene expression. However, so far their efficiency *in vivo* has been limited (Saunders, 2011).

Altogether, several ways to generate genetically modified animal models are within grasp, and new opportunities continue to emerge. By the innovative combination and modification of the methods, elaborate experimental setups can be created, and

answers to vexatious biological problems found. Although the expanding array of cloning techniques has often been referred to as a tool box for researchers, for an open-minded scientist, a playground might be the more appropriate metaphor.

2.5 Genes expressed in the epididymis

Recent technological advances have led to an increase in data regarding gene expression profiles. EST databases and microarray studies have suggested epididymal expression of several genes, and these have subsequently been confirmed with *in vitro* methods. However, mere gene expression profile is rarely enough to reveal the *in vivo* significance of a gene. Therefore, experiments to study individual genes and proteins in a living organism are needed. In this thesis, the *in vivo* significance for the function of the epididymis and epididymal sperm maturation of two genes, *Bmyc* and *Crisp4*, were studied.

2.5.1 Bmyc

Bmyc is a relatively poorly studied member of the *Myc* family of transcriptional regulators (Ingvarsson *et al.*, 1988; Asker *et al.*, 1995). The family is best known due to *Myc* (formerly known as c-*myc*), *Nmyc* and *Lmyc*, and their roles as proto-oncogenes; an increase in their expression is linked to many forms of tumorigenesis (Eilers *et al.*, 2008; Nesbit *et al.*, 1999; Albihn *et al.*, 2010). Interestingly, in contradiction to its well established role in proliferation and tumorigenesis, MYC is also a regulator of apoptosis (Nieminen *et al.*, 2007; Hoffman *et al.*, 2008). In fact, MYC has been linked to most key biochemical pathways of the cell in respect of growth, proliferation, differentiation and death (Eilers *et al.*, 2008). It has been estimated that MYC participates in regulation of up to 15% of genes in genomes of different species, although its effect on the expression of an individual gene is usually modest (Dang *et al.*, 2006). All *Myc* family members are expressed during development with varying intensities in different cell types and developmental stages, and studies with knock-out mice indicate that *Myc* and *Nmyc* are indispensable, whereas *Lmyc* is not (Domashenko *et al.*, 1997; Downs *et al.*, 1989; Davis *et al.*, 1993; Hatton *et al.*, 1996; Hurlin, 2005). Expression levels are generally diminished in the adults, although *Myc* is expressed at low levels in most proliferating tissues (Marcu *et al.*, 1992). As such, the *Myc* family members are considered as housekeeping genes in different cells. However, *Bmyc* is an interesting exception. Although its expression pattern mostly mimics that of *Myc*, an exceptionally high expression in the epididymis sets it apart from the rest of the family (Gregory *et al.*, 2000). The BMYC protein has been demonstrated to inhibit MYC and especially its transcriptional activity and ability to induce neoplastic transformations (Resar *et al.*, 1993). As the two proteins share significant sequence similarities but BMYC lacks a DNA binding domain, its effect

in regulating MYC is believed to occur through the competition of shared binding partners (Resar *et al.*, 1993).

BMYC is localized in the epithelial cells predominantly in the proximal epididymis, and its expression is regulated by androgens and other testicular factors (Cornwall *et al.*, 2001). Despite *in vitro* data which indicates it to be a transcriptional regulator and an inhibitor of MYC, no *in vivo* role for BMYC in the epididymis, or in any other tissues, has yet been discovered. However, almost complete loss of *Bmyc* expression in the epididymis was detected in a mouse model for epididymal tumorigenesis, which expressed the oncogenic simian virus 40 T antigen under *Gpx5* promoter (Sipila *et al.*, 2004). As epididymal tumors are practically non-existent (Ganem *et al.*, 1998) and BMYC has been shown to inhibit MYC mediated neoplastic transformation, it has been hypothesized that BMYC may act as a tumor suppressor in the epididymis (Sipila *et al.*, 2004). However, no further evidence in support of this is available. Although MYC is expressed in the testis and is an important cell cycle regulator, its role during meiosis, if any, has not been studied (Koji *et al.*, 1988; Uetani *et al.*, 1994). Overall, very little is known of the possible roles of *Myc* family genes in gamete development or the events that lead to their maturation.

2.5.2 Crisp *gene family*

Members of several gene families that code for cysteine-rich proteins, including CRISPs, β-defensins and ADAMs, are highly expressed in the epididymis. Common features for these proteins include signal peptide sequences, which indicate secretion into the lumen, and conserved distribution of cysteine-residues. Intramolecular disulfide bonds form between the cysteine-residues, to confer structural integrity to the proteins. Notably, orthologous proteins have been identified in venoms of various reptiles and insects. For toxin proteins the hostile environment of a target prey's bloodstream is a challenge to protein stability and thereby its functionality, which provides an explanation for the conservation of distribution of cysteine-residues (Bastolla *et al.*, 2005; Kaplan *et al.*, 2007). Similarly, the severely dehydrated epididymal luminal fluid may pose a threat to epididymal proteins, which possibly explains the abundance of cysteine-rich proteins in the epididymis (Cornwall *et al.*, 2007). However, members of some protein superfamilies, such as the CAP (cysteine-rich proteins, antigen-5, and pathogenesis-related 1) superfamily, to which certain epididymal and toxin proteins belong to, can be found in species that range from archaea and bacteria to eukaryotes (Gibbs *et al.*, 2008). Thereby, these types of cysteine-rich proteins are likely of early evolutionary origin, and have specialized to perform diverse tasks in different, even distantly related, species.

Members of the *Crisp* gene family are amongst the best known epididymal genes. The family's first member was discovered over three decades ago in the rat as one of the most abundant epididymal luminal fluid proteins, and initially named protein D/E (Cameo *et al.*, 1976), and subsequently CRISP1; to date, four members of the family are known (Gibbs *et al.*, 2008). However, the relationship of the family members in different species is ambiguous, and to avoid further confusion, only the mouse and rat CRISPs are discussed here, unless otherwise noted. With the exception of *Crisp3*, the *Crisp* genes are mainly expressed in the male reproductive organs, and although non-reproductive expression has also been reported (Reddy *et al.*, 2008), the main research focus has been in their reproductive functions.

Crisp1 is predominantly expressed in the proximal epididymis, and codes for a secreted protein which interacts with maturing spermatozoa (Kohane *et al.*, 1980a; Kohane *et al.*, 1980b). Two separate forms of CRISP1 have been detected; the majority of the protein is loosely attached to the spermatozoa membrane, whereas a minor fraction is more strongly associated (Cohen *et al.*, 2000). Several studies have indicated that these proteins have separate functions during fertilization. The loosely attached fraction is released from the membrane during capacitation, to indicate that it acts as a decapacitation factor (Kohane *et al.*, 1980b). Furthermore, incubation of the spermatozoa with exogenous CRISP1 has been demonstrated to inhibit capacitation and the acrosome reaction (Roberts *et al.*, 2003). Upon capacitation, the fraction remaining bound to the membrane migrates from the dorsal to the equatorial region of the spermatozoa (Rochwerger *et al.*, 1992b). As the equatorial region is responsible for interaction with the egg during binding and fusion of the gametes, a role for CRISP1 in these events has been suggested. Complementary CRISP1 binding sites on the oocyte were soon detected, and CRISP1 was revealed to mediate the fusion event, as incubation of eggs with exogenous CRISP1 during IVF prevented membrane fusion, yet not the binding of the gametes (Rochwerger *et al.*, 1992a). Despite these clear observations to indicate the importance of CRISP1 during fertilization, the *Crisp1* knock-out mice proved to be fertile with only minor impairment detected in IVF (Da Ros *et al.*, 2008), although it has been suggested that another member of the family may compensate for the loss of CRISP1.

The best candidate as the functional homologue of CRISP1, is CRISP2. Although primarily expressed in the haploid testicular germ cells (Kasahara *et al.*, 1989b; Mizuki *et al.*, 1992) and demonstrated to be partly responsible for the adhesion of the spermatogenic and Sertoli cells (Maeda *et al.*, 1998), the protein has also been shown to localize into the acrosomal region of the spermatozoa and be involved in gamete fusion events (Busso *et al.*, 2007; O'Bryan *et al.*, 2001; Busso *et al.*, 2005). However, since to date the *Crisp2* knock-out mice have not yet been published, the *in vivo* role of CRISP2 remains to be elucidated, and the interplay between both

CRISP1 and CRISP2 will surely be studied with a double knock-out model. Another possible CRISP1 functional homologue is CRISP4.

CRISP4 is predominantly expressed in the epididymis, and is present in the luminal fluid as a soluble form, as associated with the epididymosomes, and bound to the spermatozoa (Jalkanen *et al.*, 2005a; Reddy *et al.*, 2008; Nolan *et al.*, 2006). It has been suggested that *Crisp4* is the true orthologue of human *CRISP1*, and that both mCRISP1 and mCRISP4 may be functional homologues of human CRISP1 (Jalkanen *et al.*, 2005a; Nolan *et al.*, 2006). As human CRISP1 has also been demonstrated to have a role during fertilization (Cohen *et al.*, 2001), the study of CRISP4 function in the mouse should not be overlooked. A *Crisp4* knock-out model was recently published, and although the mice were fertile, a defect in the induction of the acrosome reaction was observed (Gibbs *et al.*, 2011). Thus, CRISP1, CRISP2 and CRISP4 all would seem to be involved in processes both before and during fertilization. As both CRISP1 and CRISP4 are proteins produced by the epididymis, they may also represent the factors responsible for the importance of epididymal sperm maturation.

The final member of the family, *Crisp3*, is expressed in several tissues with highest levels in the salivary gland (Haendler *et al.*, 1993; Haendler *et al.*, 1999). Although the protein has also been detected in the human in seminal plasma, endometrium during pregnancy, and at low levels in the epididymis, its possible role in fertility remains unknown (Kratzschmar *et al.*, 1996; Udby *et al.*, 2005; Horne *et al.*, 2009).

The molecular mechanisms with which CRISPs function are not completely understood. They have two identifiable functional domains: the N-terminal PR-1 (plant pathogenesis-related domain 1) and the C-terminal CRD (cysteine-rich domain) with the CRISP-defining, absolutely conserved distribution of 10 cysteine residues (Jalkanen *et al.*, 2005a; Haendler *et al.*, 1993; Brooks *et al.*, 1986; Kasahara *et al.*, 1989a; Guo *et al.*, 2005). Further, within the PR-1 domain, they have conserved CRISP-specific Signature 1 (S1) and Signature 2 (S2) motifs, of which S2 is responsible for interaction with the egg (Ellerman *et al.*, 2006). Notably, proteins with the CRD domain, which are often also considered as CRISPs, are common in venoms of various reptiles (Gibbs *et al.*, 2007)and have been demonstrated to exert their function by regulation of different types of ion channels (Guo *et al.*, 2005). A similar mechanism has been observed for CRISP2 (Gibbs *et al.*, 2006) and CRISP4 (Gibbs *et al.*, 2011). As changes in intracellular ion concentrations are crucial for the proper function of spermatozoa during fertilization, it is possible that CRISPs affect fertility by regulation of cellular homeostasis through certain ion channels.

3. AIMS OF THE PRESENT STUDY

Epididymal sperm maturation is essential for spermatozoa to gain the abilities required for fertilization of the egg *in vivo*. These abilities develop through complex interactions between the spermatozoa and organic and inorganic factors secreted into the epididymal lumen by the epithelial cells. Although continuous advances in molecular genetics and bioinformatics have led to an explosive increase of available data regarding gene expression and, subsequently, to identification of novel epididymal genes, the data concerning their significance accumulates significantly slower. The work undertaken in this study concentrates on different aspects of the study of the function of the epididymis, from gene identification *in silico*, through expression profiling *in vitro*, to functional characterization *in vivo* with genetically modified mouse models.

The specific aims of the study were:

1. To characterize members of the novel *Pate* gene family *in silico* and to study their expression in the male mouse reproductive organs.

2. To generate a *Bmyc* knock-out mouse model and elucidate its role in male reproduction by analysis of the changes in phenotype caused by the genetic manipulation.

3. To generate a *Crisp4 iCre* knock-in mouse model to study the role of CRISP4 in epididymal sperm maturation and events involved in fertilization.

4. To study the *in vivo* functionality of the Cre-recombinase in the *Crisp4 iCre* knock-in mice, to assess the usability of the model for generation of epididymis-specific conditional knock-out models.

4. MATERIALS AND METHODS

4.1 *In silico* analyses (I–III)

Mouse genome databases in Ensembl (Hubbard *et al.*, 2009) and NCBI (Wheeler *et al.*, 2003) were used to obtain sequences of the genes and proteins studied. Gene expression patterns based on UniGene EST libraries (Pontius *et al.*, 2003) were browsed to find genes expressed in the epididymis. Gene and protein sequence comparisons were made with BLAST (Altschul *et al.*, 1990), and ClustalW (Chenna *et al.*, 2003) and MEGA4 (Kumar *et al.*, 2008) programs were used to build phylogenetic trees. Protein signal peptide cleavage sites were predicted with SignalP 3.0 (Bendtsen *et al.*, 2004), and gene transcript polyadenylation sites with the Poly(A) Signal Miner program (Liu *et al.*, 2003). All statistical analyses were performed with the SigmaPlot 9.0 program (Systat Software Inc., Point Richmond, CA), unless otherwise noted. All pair wise comparisons between different samples or treatment groups were undertaken with Student's t-test, and standard deviations of sample variability are presented, unless otherwise noted. Levels of statistically significant changes are indicated with P values $P > 0.05$ (N.S.), $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

4.2 Generation of the genetically modified mouse models (I, III)

All cloning reactions to generate the targeting constructs were performed with Red/ET recombination technology (Zhang *et al.*, 1998; Muyrers *et al.*, 1999; Zhang *et al.*, 2000) (GeneBridges, Dresden, Germany). Red/ET cloning is based on homologous recombination and *Escherichia coli* bacteria expressing the RecE or Redα 5'→ 3' exonucleases and RecT or Redβ single strand binding proteins. Bacteria that contain three separate DNA molecules are required for each round of cloning: 1) the BAC or a plasmid containing the genomic locus of the gene to be targeted, 2) a plasmid to confer inducible expression of the RecE/Redα and RecT/Redβ proteins, and 3) a PCR product with a selectable marker gene and 5' and 3' homology arms to direct sequence specific targeting. The bacteria are induced to express the RecE/Redα and RecT/Redβ proteins, and bacteria in which recombination has occurred are selected based on antibiotic resistance as conferred by the PCR product used. The correct recombination is confirmed with sequencing. Normally, two rounds of cloning are required: first, the gene of interest is interrupted by the knock-out cassette, and secondly, the BAC is "shaved", or the desired targeting construct is subcloned into a minimal plasmid backbone (pACYC177). The resulting construct is then linearized with a restriction enzyme specific to the plasmid backbone, and electroporated into the ES cells, in which the homologous recombination takes place.

4.2.1 **Bmyc** *knock-out mouse model (III)*

A BAC clone (RP22-380G15) which contains the *Bmyc* genomic locus was purchased from the Children's Hospital Oakland Research Institute (Oakland, CA). The protein coding sequence of *Bmyc* was interrupted by insertion of the *neo*^{r} (neomycin phosphotransferase) gene under eukaryotic PGK and prokaryotic TN5 promoters into exon 1 of the gene. The cloning was designed to simultaneously cause a deletion of 7 nucleotides of *Bmyc* exon 1. Next, the BAC was shaved for the targeting construct to result in 15.3 kb and 10.4 kb long 5' and 3' homology arms, respectively. The construct was linearized and electroporated (230 V, 500 µF: Gene Pulser, Bio-Rad, Hercules, CA) into AB2.2 ES cells as derived from 129/Sv/Ev mice (Lexicon Genetics, Houston, TX). The cells were subsequently cultured in a selective medium containing 350 µg / ml geneticin (G-418, Gibco, Invitrogen, Carlsbad, CA), to result in death of cells which did not contain the targeting construct. After 8 days of culture, 288 cell colonies were manually picked, expanded and stored until screening of correct recombinants.

Screening was undertaken with negative backbone PCR (McDermott *et al.*, 2004) by use of a native DyNAzyme I DNA Polymerase (Finnzymes Diagnostics, Espoo, Finland). When the targeting construct is correctly targeted to the genome by homologous recombination, the plasmid backbone is left out, whereas with random integration, the backbone is also incorporated. Thus, by performance of a PCR reaction with primers specific for the plasmid sequence, random integrants can be identified and excluded. A lack of signal can also result from poor quality of the DNA sample. Thereby, in order to confirm the integrity of the sample, a control PCR was also performed. To summarize the PCR screening process, ES cell clones negative for backbone PCR and positive for control PCR, were potentially correctly targeted. Targeting was further confirmed with Southern hybridization.

Five–15 µg of genomic DNA from the potentially correctly targeted ES cell clones were digested with Spe I restriction enzyme (Promega, Madison, WI), separated on a 0.7% SeaKem Gold Agarose (Cambrex, East Rutherford, NJ) gel, and transferred to Hybond XL (Amersham Biosciences, Buckinghamshire, UK) membranes. The hybridizing DNA probe to recognize both targeted and WT alleles, was generated by PCR and labelled radioactive with [α-^{32}P] dCTP by use of the Prime-a-Gene Labelling System (Promega). The hybridization reaction was undertaken in ULTRAhyb buffer (Ambion, Austin, TX) at 42 °C overnight. The following day the membranes were extensively washed to remove non-specifically bound probe molecules, and the radioactive signal was detected by exposure of the membranes on Fuji Super RX film (Fujifilm, Tokyo, Japan).

The correctly targeted ES cell clone was expanded, and cells injected into blastocoeles of C57BL/6N mouse blastocysts. The mice born were chimeric, partly derived from the C57BL/6N cells, and partly from the AB2.2 cells. Chimerism was identified by fur coat color; the AB2.2 cells provide agouti color, whereas the C57BL/6N mice are otherwise black. Chimeric males were bred with black C57BL/6N females, and agouti pups derived from the targeted cells were genotyped to detect whether they carried the targeted *Bmyc* allele. The heterozygous mice were subsequently bred with one another to produce homozygous, *Bmyc* knock-out mice.

4.2.2 Crisp4 iCre *knock-in mouse model (I)*

A BAC clone (REG_B124E06368Q2) containing the *Crisp4* genomic locus was purchased from RZPD German Resource Center for Genome Research (ImaGenes GmbH, Berlin, Germany). The protein coding sequence of *Crisp4* was interrupted by insertion of an *iCre-neo*^{r} knock-in cassette into the third exon. The targeting resulted in deletion of 7 nucleotides, inclusive of the translation initiating ATG codon, and placement of the *iCre* recombinase coding sequence directly downstream of the *Crisp4* promoter. The BAC was shaved to produce 1.7 kb and 8.3 kb long 5' and 3' homology arms, respectively. The targeting construct was linearized and electroporated (230 V, 500 µF) into AB2.2 ES cells. Subsequently, the cells were cultured in a medium containing 350 µg / ml geneticin, and after 8 days, 384 antibiotic resistant cell clones were manually picked, expanded and stored until screening for correct recombinants.

Screening was undertaken by positive PCR over the 1.7 kb 5' homology arm. The 5' primer was situated outside the targeting construct, and the 3' primer in the *iCre* (improved Cre-recombinase; GeneBank Accession AY056050) coding sequence of the construct. Thereby, a PCR signal is produced only from samples with correctly targeted *Crisp4* locus. The targeting was further confirmed with long range PCR over the 8.3 kb 3' homology arm. The principle is the same as in the 5' PCR, but to produce an almost 10 kb PCR product from genomic DNA template poses certain technological challenges. Thus, the Expand Long Template PCR System (Roche Applied Science, Basel, Switzerland) was used. Those clones producing a positive PCR signal from both 5' and 3' PCR were deemed correctly targeted. The chimeric and *Crisp4 iCre* knock-in mice were produced as described previously (4.2.1).

4.3 Animal handling and sample collection (I–III)

All animal handling was conducted in accordance with the Finnish Animal Ethics Committee and the Institutional animal care policies of the University of Turku (Turku, Finland), which fully meet the requirements as defined in the NIH Guide on animal experimentation. Mice were housed under controlled environmental

conditions (12 h light / 12 h darkness, temperature 21 ± 1 °C) and fed with standard pelleted chow and tap water *ad libitum*. The mice were genotyped by PCR from DNA extracted from tissue samples obtained in earmarking.

The mice were euthanized by anesthetizing with ip injection of 400–600 µl 2.5% Avertin (2-2-2 tribromoethanol) and cardiac puncture to collect blood, or by cervical dislocation. The mice and tissues collected were weighed, and the tissues were fixed in 4% paraformaldehyde, or Bouin's solution (Sigma-Aldrich, St. Louis, MO) for histological studies, or snap-frozen in liquid nitrogen for subsequent DNA, RNA or protein extraction. Blood samples were centrifuged to isolate the serum, which was stored at –20 °C until use. Samples for histological analyses were dehydrated after fixing, embedded in paraffin, and sectioned at 3–5 µm thickness. RNA was extracted from frozen tissue samples by use of TRIzol or TRIsure reagents (Invitrogen). Spermatozoa were collected by incubation of a dissected cauda epididymidis in PBS at 37 °C, 5 % CO_2 to allow the spermatozoa to swim out. Proteins from spermatozoa were extracted by boiling in Laemmli sample buffer (Laemmli, 1970).

4.3.1 Collection of oocytes (I)

Oocytes were collected for IVF and sperm–egg binding assays from 3–4 week-old superovulated C57BL/6N or FVB/N female mice. Superovulation was induced by stimulation of the follicles to mature by administration to the mice of an ip injection of 5 IU of pregnant mare serum gonadotrophin (PMSG) in PBS, and after 47–48 h a further ip injection of 5 IU of human chorionic gonadotrophin (hCG) in PBS, to cause to follicles to rupture. After 12–13 h the mice were euthanized by cervical dislocation and the oviducts dissected out. The ampullae were cut open and the oocytes surrounded by the cumulus oophorus collected. The cumulus cells were removed by a short incubation in FHM (Specialty Media, Phillipsburg, NJ), which contained 0.6 mg/ml hyaluronidase.

4.3.2 Serum hormone measurements (I, III)

Serum hormone levels were compared between adult male WT and *Bmyc* KO or *Crisp4 iCre* KI mice. Testosterone was measured from diethyl ether extracts using RIA (Huhtaniemi *et al.*, 1985), and follicle-stimulating hormone (FSH) and LH were measured by immunofluorimetric assays as previously described (van Casteren *et al.*, 2000; Haavisto *et al.*, 1993).

4.3.3 Gonadectomies (II)

To study gene expression and regulation in the epididymis by androgens and other testicular factors, samples from gonadectomized mice were obtained. The mice were

anesthetized by isoflurane, and both testes removed through an incision to the scrotum or abdomen, after which mice were sutured and left to recover. Simultaneously, a 1 cm long silicon tube (Dow Corning, Inc., Midland, MI; inner diameter 1.98 mm, outer diameter 3.18 mm) filled with testosterone powder (Sigma-Aldrich) was subcutaneously inserted into a group of mice, to provide serum testosterone levels above the normal levels of WT male mice (Pakarainen *et al.*, 2005). The gonadectomized mice were sacrificed after 8 h or 1 d following gonadectomy, and the testosterone treated gonadectomized mice 7 d following gonadectomy. The epididymal segments were dissected and stored for subsequent RNA extraction.

4.4 Gene expression analyses

4.4.1 RT-PCR and quantitative (q) RT-PCR (I–III)

To determine whether a gene is expressed in a biological sample, the presence of its mRNA was studied non-quantitatively (RT-PCR) or quantitatively (qRT-PCR). Total RNA extracted from the sample was reverse transcribed into DNA, of which a PCR reaction was performed with primers specific to the gene of interest: for RT-PCR 1 µg of total RNA was DNase treated with Amplification Grade DNase I (Invitrogen) and reverse transcribed with DyNAmo cDNA Synthesis Kit (FinnZymes), or with AMV reverse transcriptase (Promega). PCR was performed with Biotools DNA polymerase (Biotools, Madrid, Spain) or with DyNAzyme II DNA polymerase (FinnZymes). For qRT-PCR DNase treatment and cDNA synthesis were undertaken as described above, and DyNAmo Flash SYBR Green (FinnZymes) was used for qPCR. All qRT-PCR analyses were carried out in triplicates. The relative standard curve method was used to calculate the gene expression levels, and qRT-PCR results from *L19* were used for normalization.

4.4.2 Microarray analyses (III)

Microarray studies were performed with Illumina Sentrix BeadArrays at the Finnish DNA Microarray Center, Turku Center for Biotechnology (University of Turku, Finland) to study changes in gene expression patterns in the epididymides of WT and *Bmyc* KO mice. Three WT and three KO samples were analyzed. One µg of purified RNA from each sample was hybridized to Sentrix Mouse WG-6 v2.0 Expression BeadChip following the protocols provided by the manufacturer (Illumina Inc., San Diego, CA). The results were analyzed at the Microarray Center, and by use of DAVID Bioinformatic Resources (Dennis *et al.*, 2003; Huang da *et al.*, 2009).

4.4.3 Northern hybridization (II)

Northern hybridization was performed to identify expressed transcripts of the *Pate-C* gene, and to compare their expression levels in different epididymal segments.

Twenty µg of total RNA or 4 µg of poly-A mRNA extracted from IS, caput, corpus and cauda epididymidis were denatured, separated on a 1% denaturing agarose gel, and transferred onto a nylon membrane (Hybond-XL, Amersham Biosciences). The labeled probe was produced by RT-PCR with *Pate-C* specific primers from epididymal RNA, and labeled radioactive with [^{32}P]αCTP by application of the Prime-a-Gene labeling system (Promega). The hybridization signals were detected by autoradiography by use of X-ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan) or a phosphor imager (Fuji Photo Film Co. Ltd.).

4.5 Protein studies

4.5.1 Western hybridization (I)

Western hybridization analyses were performed to assess the protein tyrosine phosphorylation of spermatozoa, to indicate the level of capacitation. Spermatozoa from WT and *Crisp4 iCre* KI mice were incubated in capacitating conditions, and protein samples were collected at different time points. The proteins were size-fractioned on 10% polyacrylamide gel with 0.1% SDS, and transferred to PolyVinyliDine Fluoride (PVDF) membranes (GE Healthcare, Little Chalfont, UK). The unspecific antigens were blocked by incubation of the membrane in tris-buffered saline with 0.1% Tween20 (TBS-T) with 5% bovine serum albumin (BSA) for 1 h at room temperature (RT). Tyrosine phosphorylated proteins were recognized with a monoclonal anti-phosphotyrosine antibody (Millipore, Billerica, MA; 1:5,000) in blocking solution for overnight at +4 °C. Subsequently, the membranes were washed with TBS-T to remove unspecifically bound primary antibody, and incubated with a peroxidase-conjugated anti-mouse secondary antibody (GE Healthcare; 1:5,000) in TBS-T for 1 h at RT. The membranes were again washed with TBS-T, and the horseradish peroxidase was detected with a chemiluminescence substrate (ECL plus Western blot detection system, GE Healthcare) and visualized with a LAS-4000 imaging system (FUJIFILM Holdings Corporation, Tokyo, Japan). To ensure equal loading of the samples, the membranes were stripped in a buffer containing 62.5 mM Tris-Cl, 2% SDS and 100 mM 2-β-mercaptoethanol, pH 6.7 for 30 min at 50 °C, blocked with 5% BSA in TBS-T, re-probed with tubulin-alpha ab-2 antibody (Lab Vision, Fremont, CA; 1:5,000), and detected as above.

4.5.2 Immunohistochemistry (III)

The numbers of apoptotic and proliferating cells in the testis and in the epididymis were compared between WT and *Bmyc* KO mice by immunohistochemical stainings. The organs were collected from 3 week-old and 2 month-old mice, fixed in PFA, dehydrated, and sectioned at 3–5 µm thickness. The tissue sections were de-paraffinated and re-hydrated in xylene and in a series of lowering concentrations

of ethanol. Antigen retrieval was performed by boiling in a pressure cooker in 10 mM sodium-citrate buffer. Apoptotic cells were detected with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, which is based on detection of fragmented DNA present in apoptotic cells. Autofluorescence was blocked by incubation of the slides in 100 mM NH_4CL for 5 min. The terminal transferase enzyme (1 U/µl; Roche) was used to attach biotin-labeled dUTP nucleotides (30 nM; Roche) to fragmented DNA ends, which were subsequently detected with TexasRed conjugated avidin (1:50 dilution in TBS with 1% BSA; Invitrogen) and visualized under fluorescence microscope. The proliferating cells were identified with a goat polyclonal anti-Ki-67 antibody (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), detected with a horseradish peroxidase (HRP) linked anti-goat antibody (1:2,000; GE Healthcare) and visualized with a HRP detection kit (Dako, Glostrup, Denmark).

4.6 Functional sperm analyses

4.6.1 Sperm acrosome reaction (I)

To compare the percentages of acrosome reacted and acrosome intact spermatozoa of WT and *Crisp4 iCre* KI mice, the spermatozoa were stained to reveal the structure of the acrosomes. Uninduced WT and KI spermatozoa incubated in capacitating conditions were used as controls, and induction of capacitation was assessed from spermatozoa induced to undergo the acrosome reaction with progesterone or calcium ionophore A23187. Cauda epididymides of adult WT and KI mice were dissected and incubated in a non-capacitating medium at 37 °C, 5% CO_2 for 1 h to allow the spermatozoa to swim out. The spermatozoa were transferred to a capacitating medium, and the incubation was continued for 1 h under the same conditions for capacitation to occur. At this point the control samples were collected. To induce the acrosome reaction, 15 µM progesterone (Fluka Chemicals, Sigma-Aldrich) or 10 µM calcium ionophore A23187 (Sigma-Aldrich) was added to the medium. Incubation was continued for further 15 min, following which the spermatozoa were collected.

Following incubation, the control and induced spermatozoa were fixed in 4% PFA at 4 °C for 1 h. The sperm were then washed by centrifugation for 5 min at 1,000 g in 0.1 M ammonium acetate, pH 9.0, spread on microscope slides, and allowed to air-dry. The slides were subsequently washed in water, methanol and water, for 5 min each, and the spermatozoa stained with 0.22% Coomassie brilliant blue (Sigma-Aldrich) in 50% methanol and 10% acetic acid for 2 min. The slides were then washed in distilled water, mounted with Pertex Mounting Solution (Histolab, Göteborg, Sweden) and observed under microscope. The spermatozoa were classified as acrosome intact when bright blue staining in the dorsal region of the

head was observed, and as acrosome reacted when staining was patchy or absent. One hundred–300 spermatozoa from each mouse were counted, and the ratios of acrosome reacted and acrosome intact spermatozoa compared.

4.6.2 Sperm capacitation (I)

Sperm capacitation was assessed by analysis of the amounts of tyrosine phosphorylated proteins by Western hybridization. Cauda epididymides of adult WT and *Crisp4 iCre* KI mice were dissected, and incubated in non-capacitating conditions at 37 °C, 5% CO_2 for 1 h, to allow the spermatozoa to swim out. Uncapacitated control samples were collected after incubation. The spermatozoa were then transferred into capacitating medium, and samples collected at 30 min intervals until 120 min. The spermatozoa were washed twice by centrifugation for 1 min at 13,000 rpm in PBS with 1 mM sodium orthovanadate and 20 mM sodium fluoride to inhibit de-phosphorylation. Proteins were extracted and Western hybridization performed, as described earlier.

4.6.3 Sperm–egg binding assay (I)

The capabilities of WT and *Crisp4 iCre* KI spermatozoa to bind to ZP intact eggs were compared. Cauda epididymides from adult WT and KI mice were dissected and incubated in HTF medium (William A. Cook Australia Pty. Ltd., Brisbane, Australia) at 37 °C, 5% CO_2 for 15 min, to allow the spermatozoa to swim out. Eggs were collected as described earlier. Approximately 40,000 spermatozoa were incubated with 10–30 eggs in 30 µl of HTF medium for 1 h at 37 °C, 5% CO_2. After the gamete co-incubation, the eggs were washed by transferrence to three drops of fresh HTF to remove loosely attached spermatozoa, and briefly fixed in 4% PFA. The number of spermatozoa bound to the ZP of the egg was counted from a single focal plane under microscope. The number of spermatozoa from 6–12 eggs per sample were counted and compared between WT and KI with Mann-Whitney's rank sum test.

4.6.4 **In vitro** *fertilization (IVF) (I)*

To study the fertilization capability of *Crisp4 iCre* KI spermatozoa, an IVF assay was performed. Fertilization of ZP free and ZP intact eggs incubated with spermatozoa from WT and KI mice were compared. Cauda epididymides from adult WT and KI mice were dissected and incubated in HTF medium (William A. Cook Australia Pty. Ltd.) at 37 °C, 5% CO_2 for 15 min to allow the spermatozoa to swim out. Eggs were collected as previously described. The ZP were dissolved by incubation of the eggs in acidic (pH 3.0) Tyrode's solution (Specialty Media). IVF was performed by incubation of 26–150 ZP free or ZP intact eggs with 50,000–

500,000 spermatozoa in HTF medium (William A. Cook Australia Pty. Ltd.) for overnight at 37 °C, 5% CO_2. Fertilization was deemed to have occurred when the egg had cleaved to 2-cell stage the following day. The percentages of fertilized ZP free and ZP intact eggs incubated with WT or KI spermatozoa were compared.

4.7 Assessment of Cre-mediated recombination

To study whether the Cre-recombinase activity in *Crisp4 iCre* KI mice was sufficient to induce recombination with its target loxP sequences, the KI mice were bred with mice carrying floxed alleles, and the recombination event analyzed both *in vitro* and *in vivo*.

4.7.1 Determination in vitro *by PCR (I)*

The *Crisp4 iCre* KI mice were bred with mice carrying a floxed *Runx1* allele (Putz *et al.*, 2006). Since *iCre* should only be expressed in a pattern identical with the endogenous *Crisp4* expression, tissue samples normally expressing and not expressing *Crisp4* were collected from *Crisp4*$^{+/-}$ / *Runx1*$^{+/fl}$ mice. The epididymides from 17, 20, 30 and 40 day-old mice, and IS, caput, corpus and cauda epididymidis and testis from adult mice, were collected. Genomic DNA from all samples was extracted, and PCR were performed with primers to recognize the WT, floxed, and Cre-recombined *Runx1* alleles.

4.7.2 Determination in vivo *(I)*

The Cre-mediated recombination was also analyzed *in vivo* by crossing the *Crisp4 iCre* KI mice with the Cre-recombinase excision reporter mouse line, named Z/RED (Vintersten *et al.*, 2004). Z/RED mice carry a transgenic construct comprised of a floxed *lacZ* gene followed by a *DsRed-T3* expression cassette coding for a red fluorescent protein. The cells carrying this construct express *lacZ* and not *DsRed-T3*, but when Cre is present, *lacZ* is excised and *DsRed-T3* is expressed. Thus, the recombination event can be assessed by detection of a red fluorescence signal from tissue samples. The epididymis, testis, vas deferens, lung, brain, spleen, skeletal muscle, seminal vesicles and thymus were collected from adult male *Crisp4*$^{+/-}$ / Z/RED^{+} mice, and analyzed under fluorescent stereomicroscope or confocal microscope for red fluorescence emission.

5. RESULTS

5.1 Characterization of the *Pate* gene family (II)

Novel epididymal genes coding for toxin-like, secreted, cysteine-rich proteins in the mouse were discovered by our group (Pujianto, 2007). However, simultaneously and independently two other research groups published these genes as members of a larger gene cluster, named as *Pate* (Levitin *et al.*, 2008) and *Anlp* (α-neurotoxin-like protein) (Kaplan *et al.*, 2007). These genes were reported to be predominantly expressed in the testis and the prostate, while epididymal expression was not studied (Levitin *et al.*, 2008), although the UniGene EST databank and our experimental data suggested high expression in the epididymis. Thus, we set out to characterize the members of the *Pate* family and their expression in the male mouse reproductive organs, with emphasis on the epididymis, in greater detail.

In silico analyses based on sequence comparisons of published family members and available annotations in Ensembl and NCBI genome databases led to minor revisions in the constitution of the family. First, although published, sequences that correspond to *Pate-F* (Levitin *et al.*, 2008) and *Anlp4* (Kaplan *et al.*, 2007) were not found in the genome databases, and *Pate-G* expression was not detected by RT-PCR. Thereby, these genes were deemed not to be expressed members of the *Pate* family. Second, phylogenetic analyses based on protein sequences demonstrated that proteins coded by *Acrv1* (acrosomal vesicle protein 1) (Wright *et al.*, 1990) and *Sslp1* (secreted seminal vesicle Ly-6 protein 1) (Li *et al.*, 2006) share significant similarities with the PATE proteins, and should be considered as members of the family, as has also been suggested previously (Kaplan *et al.*, 2007; Levitin *et al.*, 2008). However, as the names *Acrv1* and *Sslp1* are already established, their possible re-naming should be considered carefully. Third, by analysis of the genomic cluster in which all known *Pate* genes reside, a previously uncharacterized gene *9230113P08Rik* was detected, and recognized as a novel member of the *Pate* family based on gene structure and sequence. To maintain consistency with the previous members, we named it *Pate-X*. A phylogenetic tree based on amino acid sequences of the PATE proteins is presented in Figure 6.

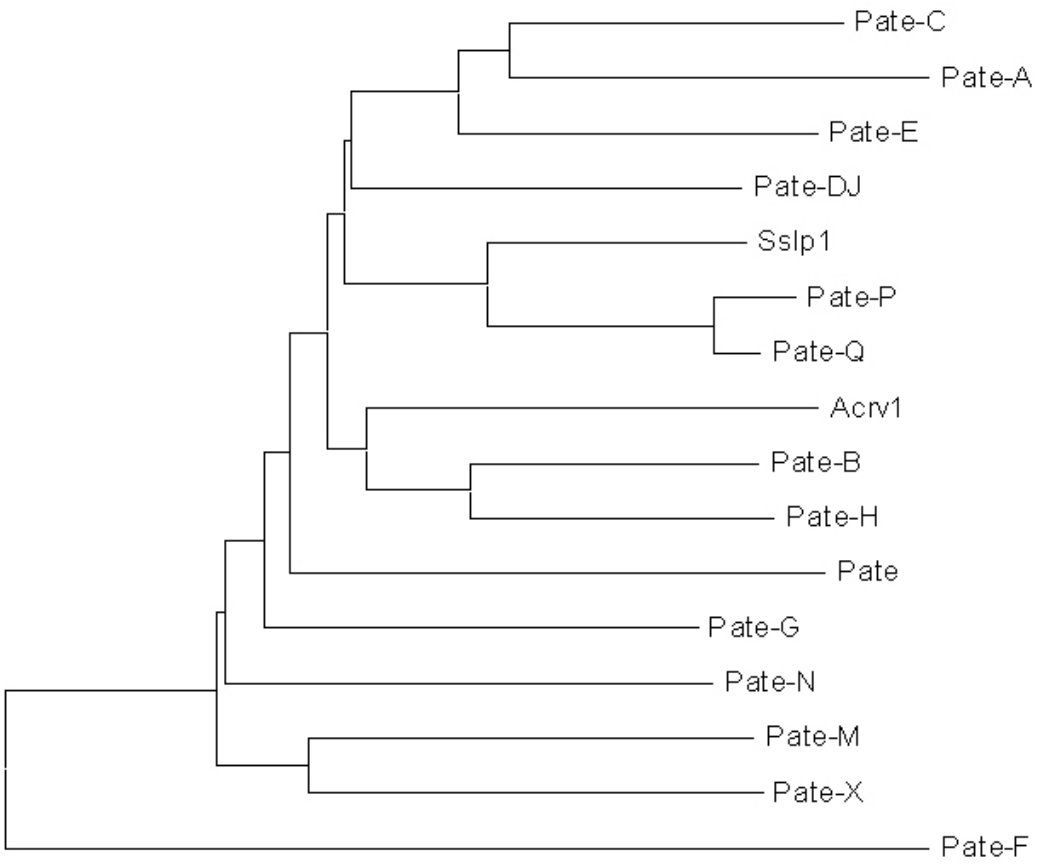


Figure 6. A phylogenetic tree of the murine PATE proteins. PATE-F stands out as a non-member, whereas SSLP1, ACRV1 and PATE-X fit within the family.

5.1.1 Expression of the **Pate** *family genes (II)*

Expression of several *Pate* family genes was studied by RT-PCR. In total, 24 male mouse tissues, as well as segment-specific expression in the epididymis (IS, caput, corpus and cauda) and the prostate (dorsal and ventral lobes), were studied. The results reveal all members to be predominantly expressed in male reproductive organs, with *Pate*, *Pate-A*, *Pate-C*, *Pate-DJ*, *Pate-N*, *Pate-X* and *Sslp1* showing no expression in non-reproductive organs. Expression in the testis was detected only for *Pate-B* (barely detectable), *Pate-E* (barely detectable), *Pate-M* and *Acrv1*, and in the prostate for *Pate-B*, *Pate-H*, *Pate-X* (barely detectable), *Sslp1* and *Acrv1*. However, all members studied were strongly expressed in the epididymis, and epididymal expression levels were highest, except for *Pate-B*, *Pate-H*, *Acrv1* and *Sslp1*. Expression in the seminal vesicles and the vas deferens was also common within the family. In addition, several members showed segment-specific

expression patterns in the epididymis, the most prominent of which was the expression of *Pate-H* strictly limited to cauda epididymidis. The results are summarized in Table 2.

We also detected alternately spliced mRNAs produced from *Pate-C* and *Pate-M*. *Pate-C*, a gene of four exons, expresses two mRNAs, of 1.3 kb and 0.3 kb in length, both containing the first two exons, and with alternate third exons. In addition, a 0.9 kb form of the 1.3 kb mRNA is produced by earlier polyadenylation. Of the three mRNAs the 0.9 kb one was predominant. *Pate-M* has 11 exons, of which at least four mRNAs were detected. The mRNAs consist of 11, 10, 4 and 3 exons, and are 1.7 kb, 1.7 kb, 2.2 kb and 2.2 kb in length, respectively. The predominant form was the 2.2 kb mRNA consisting of 4 exons.

Table 2. Expression patterns of *Pate* genes in mouse tissues.

	pituitary	adrenal	thymus	liver	lymph node	brain	saliv. gland	lung	stomach	s. intest.	kidney	l. intest.	heart	pancreas	eye	sk. muscle	spleen	bladder	bulb. gl.	testis	sem. ves.	epididymis	v.d.	d.prost.	v.prost.
Pate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-	-	-	-
Pate-A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	+	-	-	-
Pate-B	+	-	+	-	-	-	-	-	++	+	-	+	-	-	+	-	-	-	-	+	+++	+++	+++	+++	++
Pate-C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	+++	++	-	-
Pate-DJ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	++	-	-
Pate-E	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	++	+++	++	-	-
Pate-H	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	++	-	+	++	-	+++	+++	-	+++	++
Pate-M	+	+	+	-	-	++	++	++	+	++	++	++	-	-	-	-	+	++	++	+++	+++	+++	++++	++++	-	-
Pate-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+++	-	-	-
Pate-P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pate-Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	++	-	-	-
Pate-X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	+	+
Sslp1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	+++	+++	+++
Acrv1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	++++	+++	+++	+	+++	+

Expression profiles of *Pate* family members based on RT-PCR results. Levels of expression were subjectively graded based on the intensity of the PCR band. Detectable expression is graded from + to ++++, where + indicates barely detectable and ++++ very strong expression. Dash (-) indicates no detectable expression. saliv.gland, salivary gland; s. intest., small intestine; l. intest., large intestine; sk. muscle, skeletal muscle; bulb. gl., bulbourethral gland; sem. ves., seminal vesicle; v.d., vas deferens; d. prost., dorsal prostate; v. prost., ventral prostate. (Modified from *II*: Table 1.)

5.1.2 Regulation of the Pate *family genes by the testis (II)*

Testicular regulation of the expression of *Pate* family genes was studied by comparison of gene expression levels with qRT-PCR in the proximal epididymis between intact mice, gonadectomized mice, and gonadectomized mice under testosterone replacement treatment. Samples from gonadectomized mice were collected 8 h and 1 d after gonadectomy, and 7 d after gonadectomy from those mice that received exogenous testosterone. All gene expression levels were compared to those of the intact mice. The results demonstrate that most genes reacted to the loss of testosterone and other testicular factors, with an increase in expression 8 h after gonadectomy. However, 1 d after gonadectomy, the expression levels returned to the levels of the intact mice, with only a few exceptions. In the IS *Pate-C* expression was significantly up- and *Pate-N* down-regulated 1 d after gonadectomy ($P = 0.009$ and $P = 0.033$, respectively). No statistically significant changes were detected in the caput epididymidis 1 d after gonadectomy. However, clear reductions in gene expression of *Pate-A*, *Pate-N*, *Pate-X* and *Acrv1* in the proximal epididymis of gonadectomized mice under testosterone treatment were detected 7 d after gonadectomy ($P < 0.001$, $P = 0.005$, $P = 0.012$ and $P = 0.045$, respectively). *Pate-A* and *Pate-N* expressions were almost non-detectable, whereas *Pate-X* and *Acrv1* presented with an approximately 50% decrease in expression levels. The results also demonstrate the majority of the members studied to be more strongly expressed in the caput than in the IS, with the exceptions of *Pate-A* and *Pate-N*, predominantly expressed in the IS, and *Acrv1*, which was equally strong in the two segments. *Pate-H* and *Sslp1*, which were not expressed in the proximal epididymis, did not show any gain in expression after gonadectomy. The results are summarized in Table 3.

Table 3. Regulation of the *Pate* family genes by the testis.

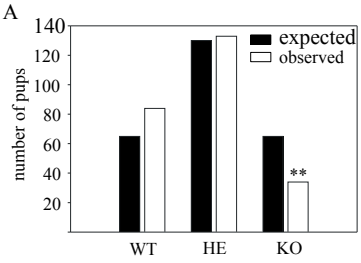
	IS 8h	cap 8h	IS 1d	cap 1d	7d + T
Pate-A	↑ N.S.	↑ N.S.	-	-	↓ ***
Pate-B	↑ N.S.	↑ **	↑ N.S.	-	-
Pate-C	↑ N.S.	↑ *	↑ **	-	-
Pate-DJ	↑ N.S.	-	-	-	-
Pate-E	↑ N.S.	↑ *	↑ N.S.	-	-
Pate-M	↓ N.S.	↑ *	-	↓ N.S.	-
Pate-N	↑ N.S.	↑ *	↓ *	↓ N.S.	↓ **
Pate-X	-	-	-	-	↓ *
Pate	↓ N.S.	-	-	↓ N.S.	-
Acrv1	↑ N.S.	-	-	-	↓ *

The arrows indicate at least 2-fold up- or down-regulation of expression as compared to control levels. Pairwise comparisons were performed with t-test, and statistical significance is marked afterwards ($P > 0.05$, N.S.; $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***).

5.2 Generation of the genetically modified mouse models

5.2.1 Bmyc *KO mouse model (III)*

ES cells were targeted with the *Bmyc* KO targeting construct, and after screening with negative backbone PCR and Southern hybridization, one correctly targeted clone was found. Chimeric mice were produced to establish the *Bmyc* KO mouse line. Mice carrying the targeted *Bmyc* allele were identified by genomic PCR, and the PCR products were sequenced to validate the targeting. Heterozygous (HE) mice were bred with one another, and WT, HE and KO pups were born. However, out of 260 pups genotyped from HE x HE breedings, fewer homozygotes were born than expected by equal Mendelian distribution (Figure 7A, χ^2-test $P < 0.01$). The gender ratio was approximately 50:50 in all genotypes. The KO mice were fertile and produced litters in sizes and frequencies comparable to WT and HE mice (Figure 7B). Phenotypically, the KO mice appeared indistinguishable from their WT and HE littermates, although male homozygotes tended to weigh less than WT males. However, such weight differences were statistically significant only in 3 week-old mice (Figure 7C, 11.2 ± 1.1 g and 9.4 ± 1.3 g, n = 7 and n = 19; P = 0.003); in 4 week-old and older mice, the differences leveled off. The absence of *Bmyc* expression from KO mice was confirmed by RT-PCR from epididymal RNA.



B

Breeding pair	Number of litters	Mean litter size ± SD
WT X WT	14	6.6 ± 2.6
HE X HE	32	7.7 ± 2.9
KO X KO	20	7.7 ± 1.8

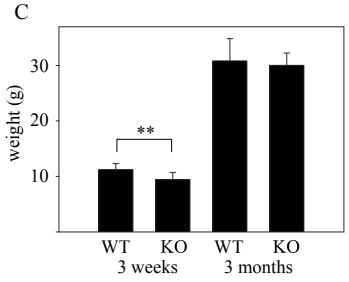


Figure 7. (A) Expected and observed genotype ratios of pups born from HE x HE breedings. χ^2-test $P < 0.01$ (**), n = 260. **(B)** Mean litter sizes born from WT, HE and KO breedings. **(C)** Total body weights of 3 week- and 3 month-old male WT and KO mice. Pairwise comparisons were performed with t-test, and standard deviations are shown as error bars. $P < 0.01$ (**), n = 7 and n = 19 for 3 week-old WT and KO, respectively, and n = 21 and n = 45 for 3 month-old WT and KO, respectively.

Mice were sacrificed at different ages, and tissue and blood serum samples collected. The epididymides and testes were smaller in KO and weighed less at all ages studied than in WT mice (Figure 8A–C). Testicular and epididymal weights

relative to whole body weights were also statistically significantly smaller at all time points, except for the epididymis of 3 week-old mice. No macroscopic differences in other organs were detected. Histology of testes and epididymides revealed no consistent gross abnormalities, although the average diameter of the epididymal lumen in segment 3 was significantly smaller in 3 month-old KO mice as compared with WT mice (96.2 ± 7.1 µm and 108.9 ± 8.6 µm, n = 5 and n = 5, respectively; P = 0.035). No changes in the number of proliferating or apoptotic cells were detected in the epididymides or the testes of 3 week-old or adult WT and KO mice.

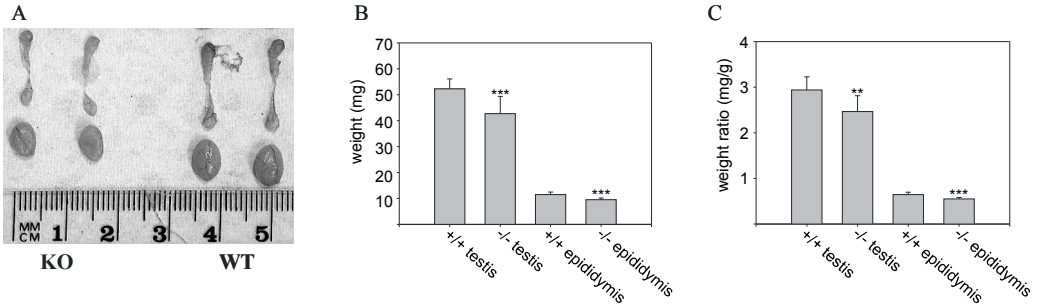


Figure 8. (A) Testes and epididymides of 3 month-old KO and WT mice. **(B)** Testis and epididymis weights of 4 week-old WT and KO mice. Pairwise comparisons were performed with t-test, and standard deviations are shown as error bars. P < 0.001 (***), n = 10 (WT) and 8 (KO). **(C)** Testis and epididymis weights relative to whole body weights of 4 week-old WT and KO mice. Pairwise comparisons were performed with t-test, and standard deviations are shown as error bars. P < 0.01 (**) and P < 0.001 (***), n = 10 (WT) and 8 (KO).

As testicular and epididymal weights are determined by the quantity of spermatogenic cells, the caudal sperm concentrations were compared between adult WT and KO mice. However, no statistically significant differences were detected (7.4 ± 4.5 and 6.1 ± 2.1 x 10^6 / ml, n = 9 and n = 6 for WT and KO, respectively). Further, the motility and morphology of the KO spermatozoa appeared normal, as already indicated by the normal fertility of the mice. Another factor which affects the size of reproductive organs is the quantity of circulating testosterone, yet no changes in serum testosterone levels were detected, although notably, serum LH levels were significantly higher in KO than in WT mice (LH concentrations 63 ± 46 pg/ml and 231 ± 146 pg/ml, n = 10 and n = 5 for WT and KO, respectively; P < 0.05).

To study whether the loss of *Bmyc* alters gene expression in the proximal epididymis, a microarray analysis from three adult WT and KO mice was performed. Based on criteria for significantly changed expression 32 up- and 20 down-regulated genes were identified. Functionally linked gene clusters were identified with the Bioconductor package "globaltest" (Goeman *et al.*, 2008) and

Gene Ontology (GO) (Harris *et al.*, 2004) and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa *et al.*, 2004) databases. Several biological pathways linked to defence responses against micro-organisms were identified, and within these, down-regulation of *Defb37*, *Defb39*, *Defb47* and *Defb50*. Further, known epididymal genes *Bfk* and *Lcn13* were significantly down- and up-regulated, respectively. However, upon validation of these observations with qRT-PCR, no significant changes in gene expression levels were detected.

As the loss of *Bmyc* did not cause any hyperplasic cell proliferation in the epididymis, the *Bmyc* KO mice were cross-bred with Gpx5-Tag2 transgenic mice (Sipila *et al.*, 2004). Although developed as a mouse model for epididymal tumorigenesis, the transgene expression of the Gpx5-Tag2 mice is insufficient to induce hyperplasia in the epididymis, as opposed to the higher expression in the Gpx5-Tag1 mouse line which is. Thus, it was hypothesized that the combined low expression of the *Tag* oncogene and the loss of *Bmyc* could be sufficient to induce epididymal hyperplasia or tumorigenesis. The epididymides of the Gpx5-Tag2$^{+/WT}$ / *Bmyc*$^{-/-}$ mice were analyzed at the ages of 3 m and 12 m, but no changes as compared to Gpx5-Tag2$^{+/WT}$ / *Bmyc*$^{+/+}$ control mice were detected.

5.2.1.1 Loss of Bmyc *in the testis (III)*

The small size of the *Bmyc* KO testes indicates that the gene has a role in the function or development of the organ, although in previous studies *Bmyc* expression in the testes has not been detected (Gregory *et al.*, 2000; Cornwall *et al.*, 2001). Due to the expression of *Myc* family genes in proliferating tissues and during development (Domashenko *et al.*, 1997), *Bmyc* expression in the testes of 2–6 week-old mice was studied by qRT-PCR, to, indeed, find the gene to be expressed at low levels (data not shown). Since BMYC has been demonstrated to inhibit MYC, it was hypothesized that the loss of BMYC would lead to an increase in MYC activity. Thus, MYC activity was assessed by the comparison of expression levels of known MYC regulated genes in the testes of juvenile and adult KO and WT mice. An increase in expression was detected in 4 week-old mice for *Cdk4* (cyclin dependent kinase 4), *Cdc2l1*, *Brca1* and *Mina*, although such increase was statistically significant only for *Cdk4*. The expression of *Myc* itself was also statistically significantly up-regulated in 4 week-old KO mice. Interestingly, however, no changes in gene expression levels between WT and KO in the testes of adult mice were detected. Despite the small size of the KO testes, histology revealed no gross abnormalities in adult or in pre-pubescent mice. However, apoptotic cells in the juvenile KO testes were often observed, as was also confirmed by the TUNEL analysis (Figure 9 A–B), and in two mice (out of 37 studied) severely atrophied seminiferous tubules devoid of spermatogenic cells were also detected (Figure 9 C).

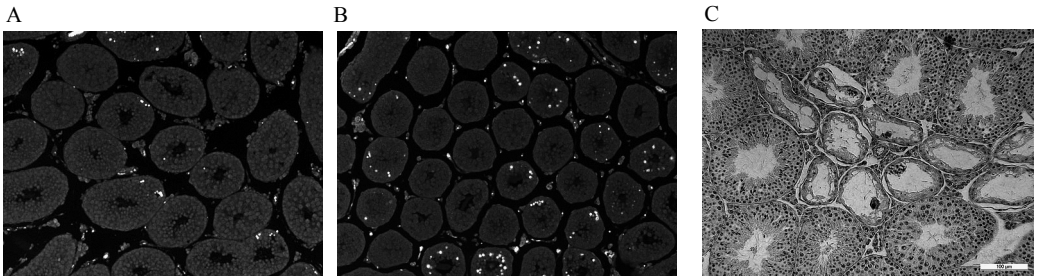


Figure 9. (A) TUNEL staining of 3 week-old WT testis. **(B)** TUNEL staining of 3 week-old *Bmyc* KO testis. **(C)** Hematoxylin and eosin staining of adult *Bmyc* KO testis. (From *III*: Figure 5.)

5.2.2 Crisp4 iCre *knock-in mouse model (I)*

Mice that carried the targeted *Crisp4* allele were identified by genomic PCR, and the PCR products sequenced to validate correct targeting. Heterozygous mice were bred with one another, and produced male and female WT, HE and KI offspring in the expected Mendelian distributions. The KI mice were indistinguishable from their WT and HE littermates. Loss of *Crisp4* expression was confirmed by RT-PCR from epididymides of adult KI mice. Both KI males and females proved to be fertile, and produced litters comparable in size and frequency to WT breeding pairs. The KI males were sacrificed at the age of 3 months, and tissue and blood serum samples collected. The testes of the KI mice weighed significantly less than those of WT mice (76 ± 11 mg and 103 ± 22 mg, n = 22 and n = 7, respectively; P = 0.004), which remained significant even after normalization against total body weights (P = 0.013). The epididymides also weighed less in KI than in WT mice (30 ± 3 mg and 35 ± 6 mg, n = 21 and n = 7), but such difference was not statistically significant. However, no abnormalities were detected in testicular or epididymal histology. No differences in serum hormone levels were either observed. Further, the caudal sperm concentrations were similar between both WT and KI mice, and the KI sperm appeared to have normal motility and morphology.

5.2.2.1 Fertility in vitro *(I)*

Fertility of the *Crisp4 iCre* KI males was further assessed through an IVF assay. Spermatozoa from KI mice were significantly less able to fertilize ZP intact eggs than WT spermatozoa (Figure 11A, 11.2 ± 4.6% and 58.2 ± 26.1%, respectively P < 0.001). However, when the eggs were treated to dissolve the ZP prior to the gamete co-incubation, no differences in fertilization rates between KI and WT spermatozoa were detected (Figure 10A, 80.9 ± 11.9% and 79.2 ± 23.8%, respectively).

5.2.2.2 Functional sperm studies (I)

In addition to the IVF assay, different functional characteristics of the KI spermatozoa were also determined. Based on levels of protein tyrosine phosphorylation, the KI spermatozoa were capacitated normally upon induction. Further, staining of the spermatozoa revealed that the proportion of spontaneously acrosome reacted spermatozoa was similar between WT and KI, and that the spermatozoa underwent the acrosome reaction normally upon induction with calcium ionophore. However, when induced with progesterone, the KI spermatozoa were unable to undergo the acrosome reaction as well as the WT spermatozoa (Figure 10B)**.** Finally, the sperm–egg binding assay indicated that the KI spermatozoa were less able to bind with the egg ZP than the WT spermatozoa (Figure 10C).

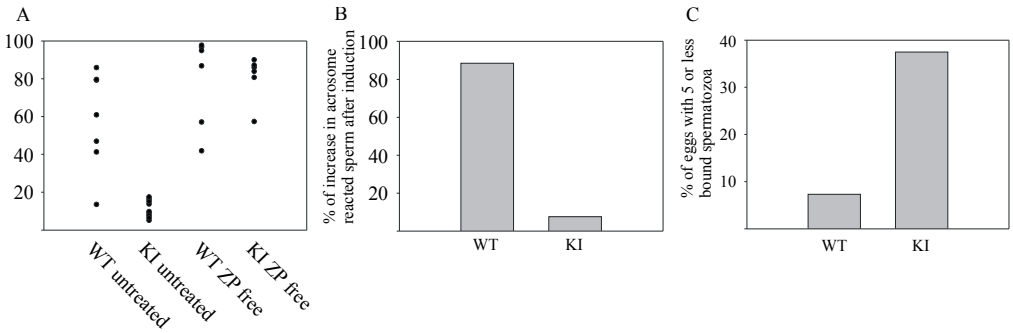


Figure 10. (A) IVF results with WT and KI spermatozoa and intact and ZP free eggs. Each dot represents the percentage of fertilized eggs in a single experiment. n = 7 (WT untreated), n = 9 (KI untreated), n = 6 (WT ZP free) and n = 6 (KI ZP free). **(B)** Percentual increase in the amount of acrosome reacted spermatozoa after induction with progesterone versus uninduced controls. n = 4. **(C)** Percentages of eggs with five or less bound spermatozoa after gamete co-incubation. n = 109 and 88, respectively.

5.2.2.3 Characterization of iCre *expression and function (I)*

To determine whether the *iCre* transgene was expressed in a pattern similar to that of the endogenous *Crisp4*, qRT-PCR analyses from several mouse tissues and from the epididymis at different ages were performed. Results demonstrated that *iCre* expression was highest in the IS and caput epididymidis. Expression in the corpus epididymidis was notably lower, with barely detectable expression levels in the cauda epididymidis, testis, skeletal muscle, lung, brain, and vas deferens (Figure 11A). No expression was detectable in any other organs studied. Epididymal expression was first detected in 20 day-old mice, which increased towards adulthood (data not shown).

In vivo functionality of the Cre-recombinase was assessed by crossing the *Crisp4 iCre* KI mouse line with mice carrying floxed alleles and by study of the occurrence of the recombination event in the offspring. After recombination, mice carrying the Z/RED allele express *DsRed-T3* gene producing red fluorescence in the cells in which the recombinase is expressed. Tissues with known expression of endogenous *Crisp4* and tissues of *Crisp4 iCre* KI mice as presented with detectable *iCre* expression were analyzed under fluorescent stereomicroscope. A fluorescent signal was detected only in the proximal epididymis. Furthermore, fluorescence confocal microscopy revealed the epididymal epithelial cells to emit fluorescence, although such fluorescence was not ubiquitous (Figure 11B). The recombination event was also assessed from *Crisp4*$^{+/-}$ / *Runx1*$^{+/fl}$ mice by genomic PCR with primers that recognize the WT, floxed and recombined *Runx1* allele. The results confirmed that the recombined allele could be detected only in the epididymis, which indicates that the recombinase is unable to induce detectable amounts of recombination in any tissues with low *iCre* or endogenous *Crisp4* expression levels (data not shown).

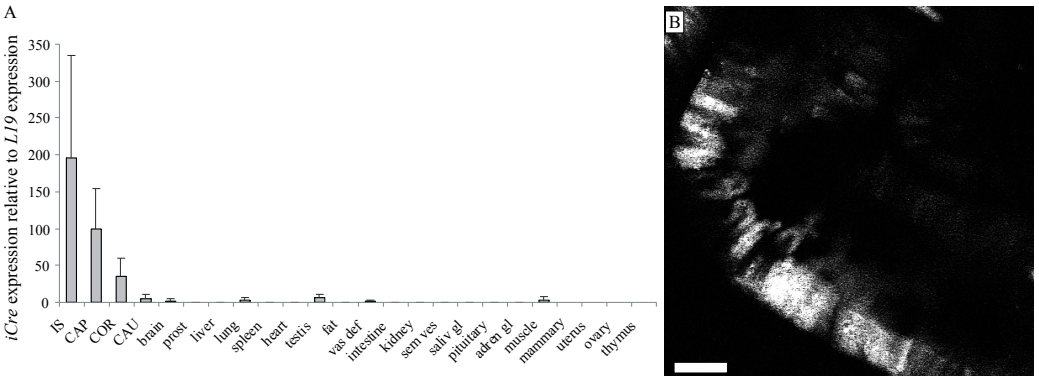


Figure 11. (A) Relative expression of *iCre* in 2 month-old heterozygous *Crisp4 iCre* KI mice. Standard deviations are shown as error bars. (Modified from *I*: Figure 2.) **(B)** Confocal microscope image of a cross section of the caput epididymis of *Crisp4*$^{+/-}$ / Z/RED^{+} mouse. Fluorescent columnar epithelial cells are clearly visible. Scale bar = 50 µm.

6. DISCUSSION

6.1 The *Pate* gene family

Members of the *Pate* family were identified from epididymal EST databases, and the family has also been described by two other research groups independently (Kaplan *et al.*, 2007; Levitin *et al.*, 2008). The first *PATE* gene was discovered in the human by Bera *et al*. and named after high expression in the testis and the prostate (Bera *et al.*, 2002). Later, expression of the other family members was characterized by Levitin *et al*., who also reported predominant expression in the testis and the prostate, to thereby classify the genes into the *Pate* family (Levitin *et al.*, 2008). All known *Pate*-genes reside in a single genomic cluster 1.13 Mb in length in chromosomal region 9A4 of the mouse. Non-expressed pseudogenes have also been observed in the area (Kaplan *et al.*, 2007; Levitin *et al.*, 2008). The availability of the mouse genome sequence, along with gene prediction algorithms and sequence comparison tools, have enabled the characterization of the *Pate* cluster *in silico*. Thus, by comparison of sequences of published *Pate* members to the annotated genes in genomic databases, a revised family tree of the members was constructed. Purely based on *in silico* analyses, the following observations were made: 1) the genes *Pate-F* and *Anlp4* do not exist, 2) *Acrv1* and *Sslp1* should be considered as members of the *Pate*-family, and 3) a previously uncharacterized gene, *9230113P08Rik*, also belongs to the *Pate*-family. These findings were subsequently confirmed by RT-PCR analyses, which also revealed no expression of *Pate-G* in any of the tissues studied, to suggest that it may also be a pseudogene. In addition, and more remarkably, with few exceptions all *Pate* genes studied were observed to be predominantly expressed in the epididymis, to correspond to the predicted expression patterns in EST databases. Although the epididymis had not been included in previous *Pate* expression profiling studies, previously published high expression levels in the testis or the prostate could neither be confirmed (Levitin *et al.*, 2008), which thus makes the *Pate* acronym as somewhat misleading. Altogether, the study validated the use of available genomic databases and *in silico* methods, together with *in vitro* analyses in the search for and characterization of novel genes.

Gene expression studies revealed that most of the *Pate* genes react to loss of androgens or other testicular factors caused by gonadectomy. However, in most cases the expression returned to pre-gonadectomy levels 1 day after gonadectomy, which indicates that the *Pate* genes expressed in the epididymis are not solely regulated by the testis. The regulation of expression of certain *Pate* genes by androgens has also been previously reported in the seminal vesicle and the prostate (Levitin *et al.*, 2008; Li *et al.*, 2006). Furthermore, *Pate-A* and *Pate-N* were detected to be predominantly regulated by luminal testicular factors, since their

expression in the epididymides was not maintained after gonadectomy in gonadectomized mice under testosterone replacement treatment. Interestingly, both *Pate-A* and *Pate-N* are more strongly expressed in the IS than in the caput epididymidis. Thus, their expression patterns support current models of regulation of predominantly IS expressed genes by luminal factors rather than circulating androgens (Hinton *et al.*, 1998; Sipila *et al.*, 2006). Partial lumicrine regulation of *Pate-X* and *Acrv1* was also detected, but both were expressed in the caput more strongly than in the IS, which indicates that they may be differentially regulated in the two segments.

The *Pate* genes code for putatively secreted, cysteine-rich proteins. The genes are fairly uniform in structure, consisting of three exons, with the exceptions of *Pate-C*, *Pate-M* and *Acrv1*, which instead have four, eleven and four exons, respectively. The proteins are also similar in size, ranging from 98 to 136 amino acids, with the exception of *Acrv1* of 261 amino acids. All proteins have a signal peptide sequence which suggests that they are secreted and contain a C-terminal TFP/Ly-6/uPAR (three-fingered protein/Ly-6/urokinase-type plasminogen activator receptor) domain with 8–10 highly conserved cysteine-residues. The domain is identified in several snake toxin proteins, in certain membrane receptors, and in murine Ly-6 glycolipid-anchored membrane proteins (Low *et al.*, 1976; Blasi *et al.*, 1987; Ploug, 2003; Ploug *et al.*, 1994). No enzymatic activities of the TFP/Ly-6/uPAR domain have been identified, but it binds to a variety of cell surface receptors, ion channels and enzymes (Menez, 1998; Galat *et al.*, 2008). Certain recombinant PATE proteins have also been demonstrated to modulate nicotinic acetylcholine receptors (nAChRs), although the involvement of the TFP/Ly-6/uPAR domain was not assessed (Levitin *et al.*, 2008).

The structure of the PATE proteins and their probable localization into the epididymal luminal fluid may give rise to certain clues to their functionality. In addition to PATEs, several members of other protein families with a conserved distribution of cysteine-residues, such as CRISPs, β-defensins and ADAMs, have also been detected in the luminal fluid. It has been hypothesized that the rigid structure of the proteins, conferred by intramolecular disulfide bridges formed between the cysteine-residues, may be required for the prevention of unwanted protein aggregation and misfolding in the dehydrated luminal fluid (Cornwall *et al.*, 2007). Notably, similar cysteine-rich proteins, especially of the CRISP family, are common in venoms of various reptiles, in which toxicity is mediated through the inhibition of the normal functionality of ion channels (Gibbs *et al.*, 2007). However, of mammalian toxin-like proteins, only CRISP2 has been demonstrated to regulate ion-channel activities (Gibbs *et al.*, 2006). By the modulation of nAChRs, however, PATE proteins may participate in the acrosome reaction, as the inhibition of sperm nAChRs prevents acetylcholine mediated acrosome reaction

(Bray *et al.*, 2002). The potential ability of PATEs to be adsorbed into the sperm membrane through the TFP/Ly-6/uPAR domain, as indicated by the Ly-6 proteins (Ploug *et al.*, 1994), also indicates that PATEs may be involved in events during the acrosome reaction.

However, it is also possible that PATEs do not function by direct interaction with the spermatozoa. It has been demonstrated that toxin-like proteins in mammals often have antimicrobial properties (reviewed in (Kaplan *et al.*, 2007)). The organization of the genomic locus which contains the *Pate* genes provides further support for this theory. In venomous animals, genes coding for toxin proteins are under constant selective pressure, so that the venom can maintain its effectiveness against target prey. Thereby, a similar evolutionary arms race could be expected between pathogenic microbes and mammalian genes coding for antimicrobial peptides, particularly in systems that are inaccessible to the immune cells, such as the epididymal lumen. The genomic areas in which the *Pate* genes reside, contain several expressed genes, as well as pseudogenes, and the overall consistency varies between species (Levitin *et al.*, 2008), which indicates an evolutionally active gene family, in which new members arise through duplications and mutations, while diversity guarantees the maintenance of essential functions. Although no experimental evidence with respect to the anti-microbial properties of PATEs is available, the family presents as an interesting candidate as a protector of the spermatozoa against microbes.

6.2 Generation of genetically modified mouse models

To study the function of a gene *in vivo*, loss of function, or knock-out, animal models have proven to be an effective approach. *In vitro* models with cell lines may provide important data on the molecular mechanisms of a protein, but lack most of the sophisticated genetic and systemic interactions inherent in whole organisms. However, the generation of even simple genetically modified mouse models is laborious, time consuming and thereby also expensive. Although advances have been made in large-scale generation of knock-outs, such as the Knock-out Mouse Project (KOMP) and the International Gene Trap Consortium (IGTC) (reviewed in (Guan *et al.*, 2010)), there is still a need for custom-made constructs and their individual targeting. For this thesis, two mouse models with targeted genetic mutations were made, and they provide certain insight into the process as a whole.

The traditional methods in the cloning of transgenic constructs have relied on taking advantage of endogenous restriction endonuclease recognition sequences in the genomic area to be modified, thus, limiting freedom in experimental design. The cloning method to generate the targeting constructs used in these studies was

based on homologous recombination (Muyrers *et al.*, 1999; Zhang *et al.*, 2000), to allow sequence independent manipulation of the targeted loci. Targeting constructs or targeted ES cell lines suitable for this study's purposes provided by KOMP or IGTC were not available: *Bmyc* only has one exon, and is thereby one of the rare genes excluded from large-scale targeting projects that utilize intronic gene targeting, and although targeted *Crisp4* cell lines do exist, none include the *iCre* coding gene knocked in. Hence, both custom made targeting vector construction, along with independent targeting, were required for these projects.

Although construction of the targeting vectors with recombineering cloning is relatively simple and quick, correct targeting in the ES cells remains a rare phenomenon, and screening for the correct recombinants can be laborious. As correct targeting is based on sequence homology, the length of the targeting construct homology arms is a factor in the targeting efficiency. Although longer homology arms should provide more efficient targeting in theory, in practice a combined homology arm length of 6–10 kb is commonly used (Hasty *et al.*, 2000). Longer homology arms also create additional challenges in screening for correct recombinants, which still relies heavily on PCR over one or both homology arms. For this study two targeting constructs for *Bmyc* were made; one with 2.2 kb and 5.0 kb 5' and 3' long homology arms, respectively, and another with 15.3 kb and 10.4 kb arms. Targeting with the first construct yielded no correct recombinants (0/288), whereas a single correctly targeted clone was obtained with the second construct (1/288). However, no definite conclusions can be made with respect to the effect of homology arm lengths on targeting efficiency from such small numbers of colonies picked and correct recombinants. Further, to overcome the challenges in screening with positive PCR over the homology arms, an alternate method was utilized, the negative backbone screening (McDermott *et al.*, 2004), with which method vector plasmid sequences were amplified from targeted ES cell clones by PCR, to identify clones that carried randomly integrated targeting construct. From 288 clones picked 268 were excluded, to leave only 20 clones to validate for correct targeting. Since identification of correctly targeted clones with Southern hybridization is laborious and requires extensive optimization, the elimination of the vast majority of clones from the analysis is a definite improvement. Further, the Expand Long Template PCR System used for positive screening of the targeted *Crisp4* clones over the 8.3 kb homology arm proved efficient, to obviate the Southern hybridization altogether. As such, targeting constructs with longer homology arms can be used if and when desired, and the negative backbone PCR can be combined with the positive long range PCR to overcome any likely difficulties in screening for correctly targeted clones caused by longer homology arms.

6.3 *Bmyc* in reproductive organs

Although members of the *Myc* family of transcriptional regulators are not known for their functions in reproductive organs, the predominantly epididymal *Bmyc* presents an interesting exception. The protein BMYC has been demonstrated to inhibit MYC and thereby suggested to be a potential tumor suppressor. The practically complete absence of primary epididymal tumors and the loss of *Bmyc* expression from the epididymides of mouse model for epididymal tumorigenesis, the GPX5-Tag1 mice, further support this hypothesis. The possible role of BMYC as an epididymis-specific transcription factor is further of interest. However, no definite evidence for the functions of BMYC *in vivo* has been available.

To address these questions, a *Bmyc* KO mouse model was generated by homologous recombination. Although *Myc* genes are strongly expressed during embryonic development, *Bmyc* deficient mice were born and appeared indistinguishable from their WT and HE littermates. However, the observed lower than expected percentage of KO pups born from HE x HE breedings along with their lower body weight, indicated a developmental defect. Irrespectively, litter sizes and frequencies from KO x KO breedings were comparable with WT x WT breedings, and no defective embryos were detected. In addition to these observations, the high *Bmyc* expression during pre-implantation development (Domashenko *et al.*, 1997) suggests that loss of *Bmyc* may cause a delay in development, to give the WT and HE embryos a competitive advantage, resulting in the detected skewered ratio of genotypes. The normal litter sizes from KO x KO breedings would support this hypothesis, as embryos of the same genotype would have no advantage over one another. However, further experimental evidence is required to clarify the role of *Bmyc* during embryonic development.

Otherwise the *Bmyc* deficient mice proved to be healthy and fertile. However, their epididymides and, interestingly, testes were smaller and weighed less than those of WT mice. As the quantity of spermatozoa affects the total weights of both tissues, caudal sperm concentrations were assessed. However, these proved comparable between WT and KO, and the spermatozoa appeared morphologically normal and also presented normal motility. A common cause for smaller male reproductive organs is lower concentration of serum testosterone, and as *Bmyc* is also expressed in the hypothalamus and the pituitary (Gregory *et al.*, 2000), the correct operation of the hypothalamic-pituitary-testicular axis was assessed by measurement of serum hormone concentrations. Although testosterone levels were normal, in the KO mice the concentration of LH was significantly increased. Increased LH levels often indicate a testicular defect, suggesting that the observed small size of the testes was a primary effect that results from the loss of *Bmyc*. However, expression of *Bmyc* in the adult testis has not been detected, although since *Myc* genes are

often more highly expressed in proliferating tissues, the expression of *Bmyc* in the testes of juvenile mice was studied. As predicted, low levels of expression were detected. To further study the link between loss of *Bmyc* and the small size of the KO testes, gene expression levels in the testes of WT and KO mice were compared. As BMYC has been reported to inhibit MYC function at the protein level, expression levels of known MYC regulated genes were measured in addition to *Myc* itself. Notably, expression levels of *Myc* and its target genes were higher in the KO mice than in the WT mice at the age of 4 weeks, whereas no differences were detected in adult mice. However, these increases were relatively minor, and statistically significant only for *Myc* and *Cdk4*. Nevertheless, these data provide evidence that BMYC inhibits MYC activity and expression *in vivo*.

Irrespectively of the smaller size of the KO testes, morphology and histology did not reveal any gross abnormalities. However, in pre-pubescent KO mice, apoptotic cells were often detected in the testes, and this increased quantity of apoptosis was confirmed by TUNEL stainings. Transgenic mice (Suzuki *et al.*, 1996) and rats (Kodaira *et al.*, 1996) overexpressing *Myc* in the testis have been generated, and, interestingly, display excessive apoptosis of spermatogenic cells and atrophy of the seminiferous tubules, which results in male infertility. Although the *Bmyc* KO mice also have increased *Myc* expression in the testis, they are fertile. However, individual cases of testicular atrophy were detected in *Bmyc* KO mice. Although rare and milder than in *Myc* transgenic animals, the resemblance was clear. The mild phenotype of the *Bmyc* KO mice may also be explained by the moderate (~1.5-fold) and transient increase in *Myc* expression. Unfortunately, *Myc* levels in the testes of the transgenic animals were not quantified (Suzuki *et al.*, 1996; Kodaira *et al.*, 1996). Interestingly, a similar phenotype was also detected in the KO mice of $p19^{Ink4d}$, an inhibitor of CDK4 (Buchold *et al.*, 2007). However, testicular atrophy was detected only in 2 out of 7 mice studied, and only few seminiferous tubules were affected. Thereby, the severity of the phenotype in $p19^{Ink4d}$ KO mice resembles that as detected in *Bmyc* KO mice. The loss of a CDK4 inhibitor should lead to an increase in CDK4 activity, and, interestingly, increased *Cdk4* expression was also detected in the testes of the *Bmyc* KO mice. Unfortunately, the *Cdk4* expression level in the testes of the $p19^{Ink4d}$ KO mice was not measured (Buchold *et al.*, 2007). However, increased CDK4 activity in the spermatogenic cells may be one of the factors which ultimately leads to apoptosis and atrophy of the seminiferous tubules. Since *Cdk4* is a direct downstream target gene of *Myc*, increase of its expression in *Bmyc* KO mice results from increased MYC activity caused by the loss of its inhibitor, BMYC. Thereby, the role of *Bmyc* in the developing testis can be conjectured to guarantee normal progression of the cell cycle of spermatogenic cells, by prevention of excess activity of MYC. However, only minor problems caused by the loss of *Bmyc* indicate a more

comprehensive regulatory network. Further, whether BMYC inhibits transcription of *Myc* or the activity of the protein, or possibly both, remains unknown.

Although BMYC lacks a DNA binding domain, it contains a transcriptional activation domain and has been demonstrated to act as a transcription factor *in vitro* with a partner to allow interactions with DNA (Resar *et al.*, 1993). However, the ability of BMYC to regulate transcription *in vivo* has not been shown, and its binding partners are also unknown, although a sequence similarity with MYC would indicate that the two proteins may interact with the same partners (Resar *et al.*, 1993). To study the effect of BMYC in regulation of transcription *in vivo*, gene expression profiles in the epididymides of adult WT and *Bmyc* KO mice by microarray were compared. Although changes in expression levels of several genes were detected, none of them were able to be confirmed with qRT-PCR. This may further indicate that the role of BMYC is to inhibit excessive MYC activity, and the loss of this inhibition from slowly proliferating epididymal epithelial cells with low levels of *Myc* expression is not sufficient to lead to an abnormal proliferation of cells caused by MYC. An attempt to increase proliferation in the epididymis by crossing *Bmyc* KO mice with GPX5-Tag2 mice was also made. The GPX5-Tag2 mice express the oncogenic simian virus 40 T antigen at low levels in the epididymis. Higher SV40-Tag expression in GPX5-Tag1 transgenic mice results in severe epididymal hyperplasia with almost complete loss of *Bmyc* expression. Thus, it was conjectured that the combined loss of *Bmyc* and a lower expression of SV40-Tag in GPX5-Tag2 mice could cause an increase in cell proliferation in the epididymis. However, no such increase was detected, to indicate that *Bmyc* may not be the limitation factor in SV40-Tag mediated proliferation. Since SV40-Tag commonly affects through the retinoblastoma pathway (reviewed in (Saenz Robles *et al.*, 2009)), the lack of effect caused by the disruption of the MYC pathway was not unexpected. Overall, even though previously published *in vitro* data and results from changes in gene expression levels in the testis of juvenile *Bmyc* KO mice strongly support the role of BMYC as an inhibitor of MYC *in vivo*, the significance of its high expression in the epididymis remains indeterminate.

6.4 *Crisp4* in reproduction

Crisp4 is the least known member of the gene family coding for secreted, cysteine-rich proteins (Jalkanen *et al.*, 2005a; Nolan *et al.*, 2006). The family is renowned for its expression in the male reproductive organs, especially in the epididymis, wherein CRISP1 is one of the most abundant protein components of the luminal fluid (Cameo *et al.*, 1976). CRISPs have been demonstrated to be present in practically all stages of sperm development and maturation, commencing from CRISP2 in testis to CRISP1 and CRISP4 in the epididymis and CRISP3 in seminal plasma, and involved in the regulation of sperm capacitation, the acrosome reaction

and sperm–egg interactions (reviewed in (Koppers *et al.*, 2011)). The molecular mechanisms through which CRISPs function are poorly understood, but regulation of ion channels is likely involved. Further, protein motifs responsible for egg interactions have been identified (Ellerman *et al.*, 2006). However, despite the amounts of data which implicate the importance of CRISPs in sperm development and fertility, their roles *in vivo* have been proven minor as based on the results obtained from knock-out models of *Crisp1* (Da Ros *et al.*, 2008) and *Crisp4* (Gibbs *et al.*, 2011).

Although *Crisp4* has only been found from the mouse and the rat, the naming of the family members is unambiguous, and murine *Crisp4* is likely to be the true orthologue of human *CRISP1*, particularly as based on the S2 egg binding motifs of the two proteins, which differ by only one amino acid. It has also been conjectured that both mouse CRISP1 and CRISP4 may be functional homologues of human CRISP1. Notably, the blocking of CRISP1 function of human spermatozoa with anti-human-CRISP1 antibody prevented sperm–egg fusion in a hamster oocyte penetration assay (Cohen *et al.*, 2001). Thereby, to simulate the loss of human CRISP1 in a mouse, the function of both CRISP1 and CRISP4 may need to be prevented, as also indicated by the mild decreases in the fertilization capabilities of *Crisp1*$^{-/-}$ and *Crisp4*$^{-/-}$ spermatozoa, an approach which was taken by Da Ros *et al*. (2008) by the performance of IVF with *Crisp1*$^{-/-}$ spermatozoa incubated with the anti-human-CRISP1 antibody. However, since the antibody had no negative effect on the fertilization rate, it was concluded that CRISP4 is not the protein which compensates for the loss of CRISP1. However, as the ability of the polyclonal human antibody to inhibit, or even bind to, mouse CRISP4 was not demonstrated (Da Ros *et al.*, 2008; Hayashi *et al.*, 1996), to assume this conclusion is premature. Thereby, the interplay and functional redundancies between CRISP1, CRISP2, CRISP4 and even possibly other proteins during sperm–egg interactions still remains to be evaluated.

To study the role of *Crisp4* in reproduction, a *Crisp4* deficient mouse model was generated. During the course of this study a *Crisp4* KO mouse model was also published by Gibbs *et al*. (Gibbs *et al.*, 2011). Although the *Crisp4* KO mice were fertile and did not display any severe phenotypic abnormalities, *Crisp4*$^{-/-}$ spermatozoa were detected to perform poorly in an IVF assay with ZP intact eggs. However, when the eggs were treated to dissolve the ZP, the *Crisp4*$^{-/-}$ spermatozoa were as efficient at fertilization as WT spermatozoa. The inability of the spermatozoa to penetrate the ZP indicates a defect in the acrosome reaction, and, correspondingly, a significant decrease in the percentage of acrosome reacted spermatozoa after induction with progesterone, the natural inducer of the acrosome reaction, was detected. However, induction of the acrosome reaction with calcium ionophore, an ion transporter, revealed no differences between WT and KO

spermatozoa. Thereby, this would indicate that CRISP4 acts during the endogenous progesterone mediated signaling pathway which leads to the acrosome reaction. Indeed, Gibbs *et al*. (2011) have been able to demonstrate that CRISP4 inhibits the activity of the sperm membrane transient receptor potential ion channel TRPM8 which regulates the Ca^{2+} flow within the spermatozoa. However, the normal fertility of the *Crisp4* KO mice *in vivo* suggests compensatory mechanisms, and although CRISP4 would appear to act primarily in regulation of the acrosome reaction and CRISP1 during sperm–egg membrane fusion, possible redundancies between the two proteins should still not be excluded.

6.5 *iCre* expression and recombinase activity in *Crisp4 iCre* KI mice

Many genes which code for proteins potentially involved in epididymal sperm maturation, or in other epididymal functions, are expressed in a wide variety of tissues. Thus, to study these roles strictly in the epididymis *in vivo* with knock-out models, requires the use of conditional knock-outs. However, to date no epididymis-specific Cre-recombinase expressing mouse models have been available. Several novel epididymis-specific genes have been discovered in our previous studies (Jalkanen *et al.*, 2005a; Penttinen *et al.*, 2003; Jalkanen *et al.*, 2005b; Jalkanen *et al.*, 2006b; Jalkanen *et al.*, 2006a), some of which have proven good candidates for the generation of *iCre* knock-in models. The knock-in approach results in useful dual-purpose mouse models: the homozygotes can be used as loss-of-function models, whereas the heterozygotes function as epididymis-specific Cre-recombinase expressing mice. *Crisp4* was one of the genes selected for generation of an *iCre* knock-in model due to its specific expression in the proximal epididymis and its potential relevance in sperm maturation and during fertilization.

The expression of *iCre* in the *Crisp4 iCre* knock-in mice was assessed with qRT-PCR. The minor differences to *Crisp4* expression detected in tissues with very low levels of gene expression likely result from sensitivity of the detection method, and of different genetic backgrounds used in gene expression studies. However, *iCre* was predominantly expressed in the IS and caput epididymidis, and its expression was first detected in 20 day-old mice to closely resemble, as predicted, the endogenous *Crisp4* expression pattern. The functionality of the recombinase *in vivo* was assessed by crossing the *Crisp4 iCre* KI mice with Cre-excision reporter mice which carry floxed alleles whose recombination is detectable. Detection of fluorescence from $Z/RED^{+/WT}$ / $Crisp4^{+/iCre}$ mice indicated recombinase activity only in the epithelial cells of the proximal epididymis. Genomic PCR of the recombined *Runx1* allele from $Runx1^{+/fl}$ / $Crisp4^{+/iCre}$ mice similarly demonstrated recombination only in the epididymis. Neither method provided evidence of any recombinase activity in tissues with reported low levels of *Crisp4* or *iCre*

expression. Although this does not necessarily disprove *Crisp4* to be expressed outside the epididymis, the results indicate that the *Crisp4 iCre* KI mice can be used to knock out genes specifically in the epididymis. However, the data also suggest that the recombinase is not active in all epididymal epithelial cells; the fluorescence in the epididymal epithelium of Z/RED$^{+/WT}$ / *Crisp4*$^{+/iCre}$ mice is mosaic, and the PCR signal from the unrecombined floxed *Runx1* allele in the *Runx1*$^{+/fl}$ / *Crisp4*$^{+/iCre}$ mice can still be detected. Although these observations could be interpreted as proof of selective *Crisp4* expression in the epididymis, Reddy *et al*. (2008) have demonstrated presence of CRISP4 throughout the epididymal epithelium. It can also be argued that the *DsRed-T3* transgene expression of the Z/RED$^{+/WT}$ / *Crisp4*$^{+/iCre}$ mice is not ubiquitous, and that the PCR signal from the unrecombined floxed *Runx1* allele in the *Runx1*$^{+/fl}$ / *Crisp4*$^{+/iCre}$ mice originates from cells in the epididymis that do not express *Crisp4*, such as the spermatozoa or endothelial cells of the blood vessels. However, altogether the data indicates that Cre-recombinase activity of *Crisp4 iCre* KI mice is insufficient to ensure complete knock-out of floxed alleles in the epididymis, which should be taken into consideration in the interpretation of results obtained when this mouse model is concerned.

6.6 Future prospects

With the technological advances which allow increased efficiency in genome sequencing and gene expression profiling, the quantity of freely available data in online databanks have skyrocketed in recent years, a trend likely to continue in the future, too. However, to transform such raw data into information with biological significance still requires work. In this thesis the effectiveness of combined *in silico*, *in vitro* and *in vivo* methods in identifying novel epididymal genes and characterizing their significance in reproductive physiology have been demonstrated. As a result of the long timescale involved from identification of a novel gene to a mouse model revealing its significance *in vivo*, these approaches are evaluated in projects concerning different genes.

The *Pate* genes represent the largest known gene family with predominantly epididymal expression. Although not restricted to the epididymis, the genes code for putatively secreted proteins which eventually end up in the seminal plasma from different organs to contribute to its constitution. The structure of the proteins indicates ion channel regulatory functions, and their localization within the male reproductive tract further indicates direct and/or indirect effects on maturation and/or storage of the spermatozoa. Since this family has also been identified in the human, it would also indicate the members to be potentially clinically relevant, too. However, the relatively large number of PATE proteins and their structural similarities indicate redundancy within the family. Thereby, as based on the current

knowledge of the family, the members prove to be poor candidates for *in vivo* studies with knock-out mouse models, and further *in vitro* studies to clarify their significance are required.

Despite high epididymal expression of *Bmyc*, its loss seemed to have no effect on the gene expression profile in the epididymis. Surprisingly, however, the ability of BMYC to inhibit the activity of MYC *in vivo* was demonstrated in the juvenile testis. Further, up-regulation of *Myc* led to increased apoptosis in the seminiferous tubules. Thus, although *Bmyc* has not been found in human, factors which facilitate the suppression of the activity of MYC may prove to be of clinical interest, particularly in consideration of increased MYC activity in several cancers.

Crisp4 was one of the novel epididymal genes discovered by our research group. It was selected for *in vivo* studies as a result of its specific expression in the epididymis and also previous studies which have indicated roles for other *Crisp* family members in reproduction. Based on analyses of *Crisp4*$^{-/-}$ spermatozoa, CRISP4 is a regulator of the sperm acrosome reaction, and may present as a good candidate target molecule for a male contraceptive drug together with other CRISP proteins.

Overall, our approach to characterize factors involved in epididymal sperm maturation has proven effective, and thanks to the increasing commercial availability of ES cells carrying targeted alleles, the advancement to *in vivo* studies with knock-out mice has been made even easier. However, the need for custom made targeting constructs and individual targeting remains, and insight gained during this study has rendered the process less laborious. Further, with the generation of an epididymis-specific Cre-recombinase expressing mouse model, it is now possible to knock out any floxed allele only in the epididymis, which will assuredly prove a valuable tool for all reproductive biologists concerned with the epididymis.

7. CONCLUSIONS

The details which concern the molecular mechanisms of mammalian fertilization still remain obscure. It is evident that several factors that provide spermatozoa the abilities required for fertilization to occur *in vivo* are produced by the epididymal epithelial cells. The luminal microenvironment of the epididymis is also responsible for protection of the maturing spermatozoa until ejaculation. Since malfunctions during epididymal sperm maturation and storage often lead to reduced fertility, the identification of the proteins involved in these processes may reveal target candidates for the development of male contraceptives. Through pharmacological prevention of the proper function of an epididymal protein, or proteins, an efficient yet reversible non-hormonal contraceptive for men may become available. *In silico*, *in vitro* and *in vivo* approaches have been utilized to identify and characterize novel epididymal genes and proteins. As such, the main findings of this study are:

1. The *Pate* genes constitute the largest known gene family predominantly expressed in the epididymis, and code for secreted proteins putatively involved in the protection of the male reproductive tract from microbes.

2. BMYC would appear not to be a major regulator of gene expression in the epididymis, despite its high expression in the organ. Nevertheless, its loss does lead to up-regulation of the *Myc* proto-oncogene in the developing testis, to result in increased apoptosis of spermatogenic cells.

3. CRISP4, produced in the epididymis, is involved in the regulation of the sperm acrosome reaction. Together with other CRISP proteins this may prove to be an essential regulator of sperm–egg interactions, and thereby a good target for contraceptive studies.

4. The *Crisp4 iCre* knock-in mouse model can be used to knock out floxed alleles specifically in the epididymis.

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