Epididymal expression of the forkhead transcription factor Foxi1 is required for male fertility

Sandra Rodrigo Blomqvist^{1,3},
Hilmar Vidarsson^{1,3}, Olle Söder²
and Sven Enerbäck^{1,*}

¹Center of Medical Genetics, Institute of Biomedicine, Göteborg University, Göteborg, Sweden and ²Department of Woman and Child Health, Pediatric Endocrinology Unit, Karolinska Institute and University Hospital, Stockholm, Sweden

An essential aspect of male reproductive capacity is the immediate availability of fertilization-ready spermatozoa. To ensure this, most mammals rely on post-testicular sperm maturation. In epididymis, germ cells are matured and stored in a quiescent state that readily can be altered to produce active spermatozoa. This depends on active proton secretion into the epididymal lumen. We have identified Foxi1 as an important regulator of gene expression in narrow and clear cells—the major proton secretory cells of epididymal epithelia. Foxi1 appears to be required for the expression of the B1-subunit of the vacuolar H⁺-ATPase proton pump and for carbonic anhydrase II as well as the chloride/bicarbonate transporter pendrin. Using transfection experiments, we have identified a Foxi1 binding *cis*-element in the *ATP6V1B1* (encoding the B1-subunit) promoter that is critical for reporter gene activation. When this site is mutated to eliminate Foxi1 binding, activation is also abolished. As a consequence of defect Foxi1-dependent epididymal sperm maturation, we demonstrate that spermatozoa from Foxi1 null males fail to reach the female genital tract in sufficient number to allow fertilization.

The EMBO Journal (2006) 25, 4131–4141. doi:10.1038/sj.emboj.7601272; Published online 24 August 2006

Subject Categories: molecular biology of disease

Keywords: epididymis; fertility; forkhead; foxi1; transcription

Introduction

Most mammalian spermatozoa are not capable to move progressively or to fertilize an oocyte at the time when they leave testis. To acquire these abilities, they require a post-testicular maturation process that to a large extent takes place in epididymis. Strictly speaking, epididymal maturation is not sufficient, mammalian spermatozoa must undergo capacitation in the female reproductive tract before they can penetrate

and fertilize an ovum (Bedford, 1975). However, in epididymis the spermatozoa are exposed to a highly complex micro-environment characterized by a wealth of secreted proteins, for example, sulfated glycoprotein-1 and 2, the neuroendocrine peptide proopiomelanocortin, human epididymal gene product (HE1) and acidic epididymal glycoprotein (reviewed by Kirchhoff, 1999). While the exact functional role of the various secreted factors in most instances remains obscure, it is clear that acidification of the epididymal fluid is essential for proper germ cell maturation and maintaining sperm in a quiescent state (Acott and Carr, 1984; Carr and Acott, 1984, 1989; Kirchhoff, 1999). This is obviously of importance for maximum functional mobility to occur only after ejaculation when mixing with the alkaline prostatic fluid restores pH and sperm mobility. Acidification of the epididymal lumen rely on the vacuolar H⁺-ATPase proton pump that is localized to the apical pole of narrow and clear cells where it secretes protons over the luminal plasma membrane (Breton *et al*, 1996; Brown *et al*, 1997; Brown and Breton, 2000). Although inhibition of epididymal acidification has been suggested to inhibit proper sperm function and as a potential way to regulate male fertility (Brown and Breton, 2000), there is to our knowledge no *in vivo* data on how altered narrow and clear cell function in epididymis would *per se* affect male fertility.

We show that mice lacking Foxi1 in narrow and clear cells also have lost the expression of the B1 subunit of the vacuolar H⁺-ATPase proton pump. Furthermore, we demonstrate that the chloride/bicarbonate transporter pendrin (pds, encoded by *SLC26A4*) is expressed in these cell types and that pendrin also depends on Foxi1 for its expression. While Foxi1 and pendrin are expressed throughout the epididymis in H⁺-ATPase positive narrow and clear cells no expression can be found in principal cells identified by expression of aquaporin 9 (aqp9) in their brush border membrane (Elkjaer *et al*, 2000; Pastor-Soler *et al*, 2001). In more proximal parts of epididymis such as the initial segment and caput, we find that expression of carbonic anhydrase II (CAII) in narrow cells also require Foxi1 expression. In transfection experiments, we can demonstrate that Foxi1 activates an *ATP6V1B1* (encoding the B1-subunit of the vacuolar H⁺-ATPase proton pump) promoter construct. This activation is abolished when a forkhead *cis*-element in the *ATP6V1B1* promoter, at –102 to –96, is mutated to prevent its ability to interact with Foxi1. Although mice lacking Foxi1 expression in narrow and clear cells are capable of mating, ejaculation and formation of vaginal plug, they fail to generate pregnancies and offspring. Based on sperm counts we conclude that this is due to insufficient number of sperms reaching the female genital tract. Thus, Foxi1 appears to be required for male fertility as a regulator of genes that are expressed in narrow and clear cells of importance for the epididymal dependent maturation and storage of spermatozoa, for example, the B1 subunit of the vacuolar H⁺-ATPase proton pump and CAII.

*Corresponding author. Center of Medical Genetics, Institute of Biomedicine, Göteborg University, Medicinargatan 9A, Box 440, Göteborg 405 30, Sweden. Tel.: +46 31 7733334; Fax: +46 31 416108; E-mail: sven.enerback@medgen.gu.se

³These authors contributed equally to this work

Received: 11 April 2006; accepted: 18 July 2006; published online: 24 August 2006

Results

Foxi1^{-/-} males but not females are sterile

During breeding of mice lacking Foxi1, we discovered an inability of Foxi1^{-/-} males to generate offspring when mated to wild type (wt) females. On the other hand, Foxi1^{-/-} females produced normal litters when mated to wt males, as did breeding with Foxi1^{+/+} males and females. To further study this we set up the following matings: Foxi1^{-/-} males × wt females and wt males × Foxi1^{-/-} females. We identified and followed 10 females with macroscopically normal vaginal plugs from each of the two groups. While normal litters with approximately eight pups each were produced by each Foxi1^{-/-} female impregnated by wt males and no pups were generated from any of the matings of Foxi1^{-/-} males × wt females. Although Foxi1^{-/-} males appear to recognize females, mate, ejaculate and produce macroscopically normal vaginal plugs, they fail to give rise to pregnancies and produce offspring. A failure to produce or maintain viable spermatozoa would be compatible with these findings. This made us interested in the possibility that Foxi1 in some way could play a role in this process.

Foxi1 is expressed in epididymis

Foxi1, also known as Fkh10 or HFH-3, has previously been shown to be expressed in kidney and inner ear (Overdier *et al*, 1997; Hulander *et al*, 1998). To investigate whether Foxi1 is also expressed in the male genito-gonadal tract, we isolated total RNA from adult wt and Foxi1^{-/-} testis and epididymis. As can be deduced from Figure 1B, Foxi1 is transcribed in epididymis of wt mice while testis appears negative for Foxi1. In order to localize the particular cell type(s) expressing Foxi1 in epididymis, we performed cRNA *in situ* hybridization on epididymis sections from initial segment, caput, corpus and cauda (Figure 1A). Foxi1 positive cells were identified in all these regions and representative results shown in Figure 1C–E. Foxi1 positive cells are scattered throughout epididymis constituting a distinct subpopulation of epithelial cells. Comparable sections from Foxi1^{-/-} males were used as negative controls (Figure 1F–H). Using an antiserum against Foxi1 we were able to identify cells expressing Foxi1 protein in their nuclei from all parts of epididymis (Figure 1I–L). As a negative control we used corresponding sections from Foxi1^{-/-} males (Figure 1M–P). In conclusion, Foxi1 transcript and protein are localized to a distinct cell type that is scattered throughout the epididymal epithelium. In the initial segment and caput the nuclei of Foxi1 positive cells are slightly more luminal than the majority of epithelial cells (Figure 1E, I and J).

Foxi1 is expressed in narrow and clear cells and required for expression of the B1 subunit of the vacuolar H⁺-ATPase proton pump

In epididymis low pH is important for post-testicular sperm maturation. Narrow and clear cells are the major proton secreting cell types in the epididymal epithelium (Hermo *et al*, 1994; Breton *et al*, 1996). While narrow cells are restricted to the initial segment and to some degree the caput clear cells are present in caput, corpus and cauda. To

identify the proton secreting narrow and clear cells, we used an antiserum directed against the B1 subunit of the vacuolar H⁺-ATPase proton pump (Brown *et al*, 1992; Breton *et al*, 1996; Herak-Kramberger *et al*, 2001; Paunescu *et al*, 2004). We found a complete co-localization between epithelial cells with apical H⁺-ATPase staining and nuclear Foxi1 staining (Figure 2A–D) indicating that Foxi1 is expressed only in narrow and clear cells. No such staining could be identified in corresponding sections from Foxi1^{-/-} epididymides (Figure 2E). The principal cells are the most abundant cells in the epididymal epithelium and together with narrow and clear cells constituting most of the epithelium. As a marker for principal cells we used the water channel protein aqp9, which is expressed in the apical brush border membrane of principal cells (Elkjaer *et al*, 2000; Pastor-Soler *et al*, 2001). We found that Foxi1 exclusively labeled aqp9 negative cells (Figure 2F–I). Foxi1^{-/-} epididymides were used as a negative control (Figure 2J). According to these experiments, Foxi1 is exclusively expressed in cells positive for the B1 subunit of the vacuolar H⁺-ATPase proton pump, that is, narrow and clear cells while aqp9 positive principal cells do not express Foxi1. It is also evident from these experiments that Foxi1 is required for expression of the B1 subunit of the vacuolar H⁺-ATPase proton pump.

Pendrin expression in narrow and clear cells require Foxi1

In a subpopulation of cells in the endolymphatic epithelium of the inner ear and in intercalated cells of the kidney Foxi1 is necessary for expression of the chloride/bicarbonate transporter pendrin (Pds), encoded by *Slc26a4* (Hulander *et al*, 2003; Blomqvist *et al*, 2004). We used antisera against pendrin and Foxi1 to study if a similar relationship exists in narrow and clear cells of epididymis. In sections from all parts of epididymis epithelia, we found that apical perinuclear pendrin staining co-localizes with the nuclear Foxi1 signal, representative sections are shown in Figure 2K–N. While the epithelial pendrin expression seems to require Foxi1 expression (Figure 2O), there is a residual non-Foxi1 dependent pendrin signal derived from subepithelial septulae of connective tissue located outside of the luminal micro-environment on opposite side of the blood–epididymis barrier (Friend and Gilula, 1972; Figure 2O). Epithelial pendrin expression is located mainly in the apical/perinuclear compartment of narrow and clear cells (Figure 2N). Since this is the first report of pendrin expression in epididymis, we also performed cRNA *in situ* hybridization using an antisense Foxi1 digoxigenin labeled cRNA probe (see Materials and methods). The very same tissue sections containing cells positive for Foxi1 mRNA (Figure 3A) were consecutively used for immunohistochemistry in a double labeling experiment. This enabled us to demonstrate that the B1 subunit of the vacuolar H⁺-ATPase proton pump and epithelial pendrin expression co-localize to the same cells that express Foxi1 mRNA (Figure 3B–D).

Thus, pendrin, the B1 subunit of the vacuolar H⁺-ATPase proton pump and Foxi1 are all expressed by the proton secreting narrow and clear cells while aqp9 positive principal cells do not express these proteins. Furthermore, it appears that expression of the transcriptional regulator Foxi1 is required for expression of these factors.

Figure 1 Foxi1 mRNA and protein expression in epididymis. Line drawing showing epididymal regions (A). Northern blot analysis identifies Foxi1 mRNA in epididymis but not in testis (B). Foxi1 cRNA *in situ* hybridization on wt epididymal tissue sections identified scattered Foxi1 positive cells (dark blue in C–E) while tissue sections from Foxi1^{−/−} epididymis lacked Foxi1 (F–H). Immunohistochemistry using antiserum against Foxi1 (green) showed Foxi1 protein in scattered cells in initial segment (I), caput (J), corpus (K) and cauda (L). No Foxi1 protein could be found in tissue sections derived from Foxi1^{−/−} males (M–P). The nuclei (blue) were visualized using To-Pro 3. Scale bars: 10 μm (L: lumen).

CAII expression in narrow and clear cells of the initial segment and caput of epididymis requires Foxi1

CAII, a cytosolic enzyme catalyzing the reversal formation of protons and bicarbonate from water and carbon dioxide, is found in the epididymal epithelium. The cellular distribution of CAII in the epididymal epithelium is somewhat unclear. There are conflicting data describing CAII in both narrow and clear cells (Miller *et al*, 2005), in both narrow cells of initial segment and principal cells in the rest of epididymis (Kaunisto *et al*, 1995; Hermo *et al*, 2005) or in narrow cells only (Hermo *et al*, 2000). In mouse epididymal tissue sec-

tions from caput, including the initial segment we identified immune-reactive CAII only in cells positive for Foxi1 (Figure 4A–D) while neither CAII nor Foxi1 was expressed in Foxi1 deficient initial segment/caput cells (Figure 4E). In corpus (Figure 4F–I) and cauda (not shown), CAII was found in a large proportion of cells. Among these only a minor fraction was also positive for Foxi1. Although no CAII positive cells could be identified in initial segment or caput from Foxi1^{−/−} animals (Figure 4E), CAII expression in corpus and cauda seems to a large extent Foxi1 independent, since CAII staining is seen in Foxi1^{−/−} epithelia from these regions (Figure 4J).

Figure 2 Confocal analysis of Foxi1, H^+ -ATPase, Aqp9 and Pds expression in wt and Foxi1 $^{-/-}$ epididymides. In confocal images of wt epididymal sections, Foxi1 (green) protein was exclusively found in H^+ -ATPase (red) immunoreactive cells (A–D). Foxi1 is localized to the nuclei, which are stained blue (To-Pro 3), while the proton pump is found on the apical/luminal side of the cell. Neither Foxi1 nor H^+ -ATPase is expressed in Foxi1 $^{-/-}$ epididymis (E). aqp9 (purple) is expressed in the brush border membrane (BBM) of principal cells in epididymides derived from both wt (F–I) and Foxi1 $^{-/-}$ mice (J). These cells are negative for Foxi1. Cells without Aqp9 staining in their BBM have Foxi1 (green) protein in their nuclei (F–I). Pendrin (Pds; orange) is found both in the subepithelial sepulae of connective tissue and in Foxi1 (green) positive epithelial cells (K–N). In Foxi1 $^{-/-}$ epididymis the epithelial expression of Pds (orange) is lost (O). While Aqp9 (purple) is found in the BBM of principal cells in both Foxi1 wt and $^{-/-}$ epididymis epithelial H^+ -ATPase and Pds proteins are exclusively expressed in Foxi1 positive cells in wt epididymis and lost in Foxi1 deficient epididymis. Scale bars: 10 μ m (L: lumen).

All together, these results indicate that while Foxi1 is necessary for CAII expression in initial segment/caput of epididymis in corpus and cauda, this dependence is only partial since both Foxi1 negative and positive cells appear to express CAII.

To summarize, in wt epididymis Foxi1 is expressed in narrow and clear cells. Inactivation of this forkhead transcription factor results in an epithelium without expression of the B1 subunit of the vacuolar H^+ -ATPase proton pump and the bicarbonate/chloride transporter pendrin in narrow and clear cells. The water channel protein aqp9, a marker of principal cells, is expressed in Foxi1 $^{-/-}$ epididymides. The

Figure 3 Identification of Foxi1 and Pds expressing cells. *In situ* hybridization using a Foxi1 cRNA probe (black) identifies a cell population in caput epithelia that transcribes Foxi1 mRNA (A). (B–D) Confocal images using the same sections as in (A) for immunohistochemistry with H^+ -ATPase and Pds specific ab on slides previously exposed to Foxi1 cRNA *in situ* hybridization. We locate H^+ -ATPase (red, B, D) and Pds (green, C, D) to cells that are the same as the Foxi1 positive cells in (A) (black, A). Thus, epithelial Pds, H^+ -ATPase and Foxi1 are all expressed in epididymal narrow and clear cells. Pds (green) staining is also seen in subepithelial connective tissue (C, D). In upper right corners of (B–D) are higher magnifications of representative clear cells. Scale bars: 10 μ m (L: lumen).

cytosolic CAII expression is lost in initial segment and caput, but still found in corpus and cauda in tissues derived from Foxi1 deficient epididymis (Figure 4K).

Epididymal Foxi1 expression starts at postnatal day 5 (P5) and precedes that of CAII, pendrin and H^+ -ATPase

To determine the onset of Foxi1 expression, we examined genital ridges at E12.5 from wt mice and consecutive time points during epididymal development up to adulthood (3 months). We used real-time RT-PCR (TaqMan) for quantification of Foxi1 mRNA (Figure 5A). No Foxi1 mRNA could be detected at any embryonic time-point. At P5, the first Foxi1 expression was identified comprising only approximately 1% of the level found in epididymis from adult mice. This is followed by a gradual increase in Foxi1 mRNA levels reaching a plateau in epididymides from adult males (Figure 5A). This gradual increase in Foxi1 mRNA steady-state level is paralleled by the first appearance of Foxi1 positive cells in the epididymal epithelium at P5 (Figure 5B–D). At this time point there is no expression of either pendrin, CAII or the B1 subunit of the vacuolar H^+ -ATPase proton pump. At P10 we can demonstrate the first expression of epithelial pendrin and CAII that strictly co-localizes with Foxi1 in proximal epididymis (Figure 5F–H) here Foxi1 mRNA levels are $\sim$ 8.5% of adult expression (Figure 5A). At P15 Foxi1 positive cells do not only express pendrin and CAII they also display an apical staining for the B1 subunit of the vacuolar H^+ -ATPase proton pump (Figure 5J–L). At this stage, Foxi1 mRNA levels are $\sim$ 55.9% of adult expression (Figure 5A). Thus, there is a gradual increase in Foxi1 mRNA that precedes the expression of pendrin, CAII and the B1 subunit of the vacuolar H^+ -ATPase proton pump. This temporal pattern is compatible with the view that Foxi1 directly or indirectly regulates the expression of these proteins.

Foxi1 transactivates an ATP6V1B1 promoter construct *in vitro*

We have previously shown in transfection experiments that a Foxi1 expression plasmid, in a dose-dependent fashion, activates a Pds promoter reporter construct (Blomqvist *et al*,

Figure 4 Distribution of CAII positive cells in wt and Foxi1^{-/-} epididymis. In caput, CAII (orange) is expressed in the cytoplasm of cells that are immunoreactive for Foxi1 (green) (A–D). Some CAII staining is also seen in nonepithelial cells. These cells are identified as erythrocytes, since they lack nucleus (stained blue using To-Pro 3), CAII is known to be highly expressed in the red blood cells. In corpus CAII (orange) is found in both Foxi1 (green) positive and negative cells (F–I). In epididymes derived from Foxi1 deficient males no CAII (orange) protein is found in the caput epithelium (E) while CAII positive cells are present in Foxi1^{-/-} corpus (J) and cauda (not shown). Scale bars: 10 μ m (L: lumen). A schematic view of the proton secreting cell in epididymis (K; narrow and clear cells). Tight junctions constituting the blood–epididymis barrier (black rectangles) and approximate location of the Foxi1 dependent proteins are shown.

2004), a finding that supports the view that Foxi1 can act as an upstream activator/regulator of pendrin. To study if a similar relation exists between Foxi1 and the ATP6V1B1 promoter, encoding the tissue specific B1 subunit of H⁺-ATPase found in kidney, inner ear, and epididymis (Nelson *et al*, 1992; Breton *et al*, 1996; Stankovic *et al*, 1997; Karet *et al*, 1999; Dou *et al*, 2003; Miller *et al*, 2005), we isolated a 1.0 kb region immediately upstream of the ATP6V1B1 initiation codon (Figure 6A). This sequence stretch was cloned into a pGL3 luciferase reporter (see Materials and methods) and used in transfection experiments. As can be seen in Figure 6B, co-transfections using a Foxi1 expression plasmid in COS-7 cells induces a dose-dependent increase in reporter gene activity. In the promoter sequence used, we could identify only one potential Foxi1 *cis*-element conforming to the previously published consensus sequence for Foxi1 binding sites (T(g/a)TTT(g/a)(t/c); Overdier *et al*, 1997). This site is located at –102 to –96 relative to the transcription start and agrees well with the findings of Finberg *et al* (2003). When this site is mutated by substitution of the core T-triplet, of the putative Foxi1 binding site, to a G-triplet both Foxi1 binding and transactivating capacity is abolished (Figure 6B and C). The location and sequence identity of this *cis*-element is conserved between mouse and human (Finberg *et al*, 2003). These findings further support the view that Foxi1 acts as an early activator of a gene repertoire necessary for proper function of narrow and clear cells in epididymis. In executing this task Foxi1 appears to, at least in part, rely on a particular *cis*-element located at –102 to –96 in the promoter that regulates transcription of the B1 subunit of the H⁺-ATPase proton pump.

Functional and physiological properties of wt and Foxi1^{-/-} gonads and sperms

Microscopic morphology of testis, rete testis, epididymis and interconnecting ducts did not reveal any morphological alteration. However, a more detailed examination of sperm morphology reveals a significant increase of sperms with tail angulation in Foxi1^{-/-} mice ($P < 0.001$; Figure 7A–E). Increased frequency of tail angulation is a sign of disturbed epididymal sperm maturation and has previously been linked to infertility based on elevated epididymal pH (Yeung *et al*, 2002). In terms of quantity, there is no significant difference in number of sperms released from minced epididymides, wt ($34.2 \times 10^6 \pm 3.0 \times 10^6$; $n = 6$) and $-/-$ ($34.6 \times 10^6 \pm 4.0 \times 10^6$; $n = 6$) mice. Vaginal plugs from wt females mated with either wt or Foxi1^{-/-} males were minced with a razor blade in PBS buffer and the sludge was centrifuged to isolate the germ cells. Qualitative examination of the sperms showed normal motility (not shown). To make a quantitative analysis of sperms reaching the female genital tract sperms were flushed from uterus from mated wt females, centrifuged and re-dissolved in a fixed volume of buffer. At this point, the morning of plug appearance, uterus contained most of the ejaculated sperms, while less was found in oviduct (not shown). Wt females mated with Foxi1^{-/-} males had reduced number of sperms in uterus $\sim 1.1\%$ of the sperm count of wt females mated with wt males ($P < 0.05$; Figure 7F). We have also noted an increased organ/body weight ratio and increased luminal area of epididymal ducts for Foxi1^{-/-} mice (Figure 7G and H). These findings are compatible with insufficient post-testicular maturation, due to failure of

Figure 5 Foxi1 expression precedes that of Pds, CAII and H⁺-ATPase. In real-time RT-PCR quantification assays (TaqMan) of RNA from genital ridges and epididymes at time points from E12.5 to adult, measurable levels of Foxi1 transcripts are first detected at P5 and increase during postnatal development (A). This is in accordance with immunohistochemistry results (B–M). At P5, only Foxi1 (green) is found in a few cells of wt epididymis epithelium (B–D). Extra-epithelial Pds (purple) derived from connective tissue is seen at all time-points regardless of genotype (B, E, F, I, J, M). Foxi1 deficient P5 epididymis lacks epithelial Pds (purple; E). At P10, Pds (purple) is found in epithelial cells, colocalized with Foxi1 (green) positive cells (F). CAII (orange) protein is also expressed at P10, in the cytoplasm of Foxi1 (green) expressing cells (G). Also a higher number of wt epididymis cells are Foxi1 (green) positive (H). At P15, Pds (purple, J) and CAII (orange, K) are present in Foxi1 (green) immune-reactive cells. At this time-point H⁺-ATPase (red) is identified, located in the luminal part of the Foxi1 (green) expressing cells (L). No epithelial expression of Foxi1 (E, I, M, left panel), Pds (E, I, M, right panel), CAII (E, I, M, left panel) and H⁺-ATPase (E, I, M, left panel) could be found on Foxi1^{−/−} tissue sections at any of these time points. Nuclei are stained blue. ***P* < 0.01. Scale bar: 10 μm (L: lumen).

proper acidification of epididymal luminal content caused by defective narrow and clear cell function. This is further supported by direct measurement of epididymal luminal fluid pH using a highly sensitive pH strip as has previously been described (Yeung *et al*, 2004). Using this method, we could demonstrate a significant difference (*P* < 0.001) when pH was compared between wt (pH = 6.4 ± 0.03; *n* = 6) and Foxi1^{−/−} (pH = 6.9 ± 0.06; *n* = 6). Thus, it appears that Foxi1 expression in narrow and clear cells of the epididymal epithelium is necessary for proper acidification to occur.

Discussion

It is well established that an acidic luminal pH in epididymis is involved in sperm maturation (Carr *et al*, 1985; Caflisch and DuBose, 1990) to the extent that the acidification mechanism has been suggested as a potential target for male contraceptives (Breton *et al*, 1996). Narrow and clear cells are responsible for the bulk of proton secretion into the epididymal lumen. This is achieved by the vacuolar H⁺-ATPase proton pump located in their apical plasma membrane and

Figure 6 FOXI1 specifically binds to and activates the ATP6V1B1 promoter. Schematic view of the reporter gene constructs used in the luciferase assays (**A**). A 1.0 kb nonmutated luciferase reporter construct (5-ATP6V1B1) and a mutated construct (5ATP6V1B1 TTT>GGG), in which the core T-triplet at position -102 to -96 has been replaced by GGG (**A**). Transient transfection using COS7 cells. When co-transfected with a FOXI1 expression vector (40, 80 and 100 ng) the wt reporter construct (5ATP6V1B1) showed a significant induction of reporter gene activity ($***P<0.001$). In contrast, the mutated variant (5ATP6V1B1 TTT>GGG) displayed no activity above what was generated with 100 ng of expression vector without insert. Reporter gene activity is shown as fold induction relative to activity generated in transfections using 100 ng of expression vector void of FOXI1 (**B**). EMSA of *in vitro* transcribed/translated FOXI1 protein (see Materials and methods) using radioactively labeled oligonucleotides encoding the putative FOXI1 binding site (-102 to -96) of the 5ATP6V1B1 promoter as well as a mutated site (same as in **A**, **B**). As can be deduced wt labeled oligonucleotide together with FOXI1 protein generates a band with altered mobility (**C**; lane 2). Using increasing amounts of wt nonlabeled (cold) oligonucleotide as competitor (lanes 3–6; 2-, 10-, 20- and 50-fold molar excess) demonstrates a gradually decreased intensity of the signal as would be expected for a specific DNA–protein interaction. In a similar experiment using mutated oligonucleotide instead of wt (lanes 7–10), no specific competition could be detected.

in subapical vesicles (Brown *et al*, 1992, 1997; Breton *et al*, 1996). Furthermore, acidification appears to be most pronounced in proximal parts of epididymis such as the initial segment and only to a lesser degree present in more distal regions (Levine and Kelly, 1978). In this context it is interesting to note that expression of CAII (providing protons; Fig

4) and the B1-subunit of the vacuolar H⁺-ATPase proton pump (Figures 2 and 3) is entirely dependent on Foxi1 expression in the initial segment and caput (Figures 2 and 4). Moreover, we demonstrate expression of the transcription factor Foxi1 in narrow and clear cells of epididymis (Figure 1). In these cells, Foxi1 is necessary for expression of

Figure 7 Wt and Foxi1^{-/-} sperm morphology and quantification. Light microscopy studies of sperms from wt (A, B) and Foxi1 deficient (C, D) males revealed a significantly higher number of sperms with tail angulation in ^{-/-} mice (C–E). Direct sperm counts from flushed uteruses (F) revealed a significant difference ($P < 0.05$) in number of sperms derived from wt females mated with Foxi1^{-/-} males as compared with wt males. As a likely sign of altered luminal microenvironment Foxi1^{-/-} males have increased epididymis/body weight ratio (G; $**P < 0.01$) as well as an increased epididymal area (H). Scale bar: 30 μm (A, C), 20 μm (B, D).

the B1-subunit and CAII (Figures 2–5) both playing crucial roles in the proton secretory machinery. It is a well-founded hypothesis that lack of these two factors will abolish or severely inhibit proton secretion from narrow and clear cells and hence produce a luminal microenvironment of the epididymis that is less well suited for proper post-testicular sperm maturation and storage. Hence, very few spermatozoa have the capacity to reach the female reproductive system after an ejaculation and as a consequence hereof Foxi1^{-/-} males are sterile. It is possible that lack of the chloride/bicarbonate transporter pendrin contributes to this phenotype (Figures 2, 3 and 4K). Direct measurements of epididymal pH reveal a significant difference ($P > 0.001$) 6.4 for wt and 6.9 for Foxi1^{-/-}. These values are very similar to what has been published for the *c-ros* knock out mouse that also display male infertility (Yeung *et al*, 2002, 2004). Furthermore, such mice also demonstrate altered sperm morphology with tail angulation (Yeung *et al*, 2002; Figure 7A–E). This phenotype is linked to infertility based on alterations in the regulation of epididymal volume/composition (Yeung *et al*, 2002). pH measurements indicating a significantly higher pH in Foxi1^{-/-} epididymides together

with increased luminal area and higher organ to body weight ratio (Figure 7G and H), which all are findings in agreement with altered epididymal microenvironment. While there is no significant difference in the number of spermatozoa in epididymis (see Functional and physiological properties of wt and Foxi1^{-/-} gonads and sperms) the altered microenvironment leads to an inability of Foxi1^{-/-} sperms to reach the female genital tract in sufficient number to allow fertilization. This is consistent with an important role for Foxi1 in regulating genes necessary for creating an microenvironment needed for proper epididymal post-testicular sperm maturation.

From a developmental perspective Foxi1 starts to be expressed rather late during epididymal development. The induction of epithelial pendrin and CAII follows Foxi1 expression and is found in Foxi1 positive cells at P10 (Figure 5F–G). The H⁺-ATPase proton pump is not detected until P15 and it is located apically in Foxi1 expressing cells (Figure 5L). From this we conclude that Foxi1 expression precedes and is a prerequisite for the expression of Pendrin, CAII and H⁺-ATPase in narrow and clear cells of epididymis, since epithelial expression of these proteins could not be found in Foxi1 deficient epididymides (Figure 5E, I and M). The level of Foxi1 mRNA increases progressively during postnatal development, and so does the number of Foxi1 positive cells (Figure 5A, D and H). The rather late appearance of Foxi1 in the developing epididymis argue in favor of a direct effect on target gene promoters as part of juvenile and peripubertal maturation of the epididymis epithelium rather than affecting specification of epididymal epithelia. The fact that no microscopic alteration of the epithelia has been found would be consistent with this view. It should be pointed out that previous cDNA array experiments support our findings regarding Foxi1 mRNA distribution (Figure 1B,) while the entire epididymis was positive for Foxi1 mRNA (Johnston *et al*, 2005) no significant expression was found in testis (Shima *et al*, 2004).

Even though several bicarbonate transporters have been suggested/identified in narrow and clear cells, for example, NBC3 and AE2 (Jensen *et al*, 1999a,b; Pushkin *et al*, 2000; Medina *et al*, 2003), we would like to speculate that pendrin could play a role in basolateral bicarbonate secretion in narrow and clear cells. Both these cell types have an elongated cell shape extending from the lumen towards the basal membrane. In this way, the vacuolar H⁺-ATPase proton pump with its apical location will be on the luminal side of the adluminal blood–epididymis barrier (Farquhar and Palade, 1963; Friend and Gilula, 1972), while the more perinuclearly located chloride/bicarbonate transporter pendrin will be on the ‘blood’ side of the adluminal barrier (Figures 2K–N and 4K).

The kidney and epididymis both originate from the Wolffian duct and surrounding mesenchyme, thus sharing a common embryological origin. As a sign of this, similar epithelial cells that share several transport features populate both epithelia. For example, intercalated alpha cells of the distal kidney tubuli and narrow and clear cells both secrete protons and express the B1 subunit of the vacuolar H⁺-ATPase proton pump at the apical cell pole (Brown *et al*, 1992; Nelson *et al*, 1992; Al-Awqati, 1996). Pendrin and Foxi1 are also expressed by intercalated beta cells (Blomqvist *et al*, 2004), data presented here extend this kidney epididymis comparison to include Foxi1 and pendrin (Figures 1–5).

Moreover, Foxi1, pendrin and the B1 subunit of the vacuolar H^+ -ATPase proton pump are expressed in the endolymphatic duct epithelium of the inner ear (Everett *et al*, 1999; Dou *et al*, 2003; Hulander *et al*, 2003). Mutations in the B1 subunit encoding gene (*ATP6V1B1*) are in humans associated with deafness and distal renal tubular acidosis as an indication of its importance for inner ear and kidney function (Karet *et al*, 1999). To our knowledge, there are no reports on decreased male fertility in such patients. This is supported by the reported finding that mice lacking the B1-subunit are fertile (Finberg *et al*, 2002). Mutations in the pendrin encoding gene (*SLC26A4*) cause Pendred's disease with deafness as an obligatory finding (Phelps *et al*, 1998; Reardon *et al*, 2000; Campbell *et al*, 2001). It appears that such patients have normal kidney function. While mice lacking pendrin develop early onset deafness they have essentially normal *in vivo* kidney function (Everett *et al*, 2001). This is in contrast to mice lacking Foxi1 that develop both early onset deafness (Hulander *et al*, 1998; Hulander *et al*, 2003) as well as distal renal tubular acidosis (Blomqvist *et al*, 2004) and as reported here male infertility. It appears that the Foxi1 expressing cells of the inner ear (FORE cells; Hulander *et al*, 2003), kidney (intercalated cells; Blomqvist *et al*, 2004) and narrow and clear cells of the epididymis require Foxi1 for normal cellular function. The more pronounced dependence on Foxi1 as compared with the B1-subunit or pendrin might be explained by the fact that expression of the transcription factor Foxi1 is required for a cascade of genes that all seem to be involved to some extent in proton/bicarbonate secretion. While it is likely that the B1-subunit to some degree is redundant in epididymis (Finberg *et al*, 2002), it is required for normal function in the inner ear and kidney. Consistent with this notion it is possible that other mechanisms, independent of the B1-subunit, could contribute to epididymal acidification such as the Na^+/H^+ exchanger NHE3 (Pastor-Soler *et al*, 2005). However, if the activities of other proteins such as CAII and pendrin are reduced by the lack of Foxi1 there might be a more pronounced dependence of Foxi1 expression in the epididymal epithelium as compared with the B1-subunit. Thus, Foxi1 appears to be essential for normal function at all of these sites indicating a nonredundant role as a regulator of the B1-subunit, pendrin and CAII as reported here.

The fact that Wolffian duct mesoderm derived kidney intercalated cells and epididymal narrow and clear cells rely on similar mechanisms for proton/bicarbonate transport as does ectodermal FORE cells of the endolymphatic duct epithelium in the inner ear argues in favor of evolutionary convergence of a functional secretory mechanism. Finally, the experiments presented here, together with the findings in inner ears and kidneys that lack Foxi1 expression (Hulander *et al*, 2003; Blomqvist *et al*, 2004), predict that mutations in the human FOXI1 gene, located at 5q34, might prove to cause a sensorineural deafness syndrome with distal renal tubular acidosis and male infertility.

Materials and methods

Northern blot analysis

Total RNA (20 µg), prepared as previously described (Chomczynski and Sacchi, 1987), from wt testis and wt or Foxi1^{-/-} epididymis were electrophoresed on a 2% formaldehyde/1% agarose gel and transferred onto Gene Screen Plus membrane (PerkinElmer Life Science Inc.). The membranes were hybridized as in Blomqvist *et al*

(2004) using ³²P-labeled Foxi1 cDNA probe (corresponding to nucleotide (nt) 1185–1667, GenBank Acc No. NM_023907).

In situ hybridization and immunohistochemistry

Nonradioactive *in situ* hybridization of adult wt and Foxi1^{-/-} mouse epididymis cryosections (10 µm) were performed as previously described (Vosshall *et al*, 1999), using a digoxigenin labeled Foxi1 riboprobe (corresponding to nt 966–1668, GenBank Acc No. NM_023907). Cryosections (10 µm) were treated for antigen-retrieval as described in Brown *et al* (1996) and blocked in 1% BSA/0.5% Triton X-100 in 1 × PBS buffer. The primary antibodies (ab) were diluted in 0.2% BSA/0.1% Triton X-100 and incubated over night at 4°C. Three 10-min washes in 1 × PBS were followed by 1-h incubation of diluted secondary ab at room temperature. Nuclei were visualized using ToPro3 1:1000 (T3605; Molecular Probes Inc.). After washes in 1 × PBS, the slides were mounted in ProLong antifade (P7481; Molecular Probes Inc.) and imaged with a Zeiss LSM 510 META confocal microscope. Tissues from at least three different animals were analyzed and the results were consistent. ab were diluted as follows: goat anti-Foxi1 1:1000 (ab20454, AbCam); rabbit anti-CAII 1:1000 (a kind gift from Dr W Sly); sheep anti-CAII 1:700 (ab 8953 AbCam); rabbit anti-B1 subunit of H^+ -ATPase 1:100 (kind gift from Dr D Brown); mouse anti-Pendrin 1:100 (K0143-3, BioSite Inc.); chick anti-aqp9 1:75 (ab15129 AbCam); donkey anti-goat Alexa488 1:100 (A11055, Molecular Probes Inc.); donkey anti-goat Cy3 1:100 (705-165-147 Jackson ImmunoResearch Laboratories); donkey anti-mouse Cy5 1:100 (715-175-150 Jackson ImmunoResearch Laboratories); donkey anti-rabbit Rhodamine Red 1:100 (711-296-152 Jackson ImmunoResearch Laboratories); donkey anti-chick FITC 1:100 (703-095-155 Jackson ImmunoResearch Laboratories) and donkey anti-sheep Alexa488 1:100 (A11016, Molecular Probes).

Real-time quantitative RT-PCR

Total RNA was isolated from mouse genital ridges and epididymes and DNase treated using Micro-to-Midi total RNA purification System (12183-018 Invitrogen) and DNA-freeTM (Cat # 1906, Ambion), according to the manufacturer's protocols. RNA (500 ng) was used for first strand cDNA synthesis according to the manufacturer's recommendation (SuperScriptTM First Strand Synthesis for RT-PCR, 12371-019, Invitrogen). TaqMan Real-time PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) and the ABI Prism 7900HT sequence detection system. At least three samples, from different animals, for each time-point were run in triplicates twice. Samples either derived from Foxi1^{-/-} tissue or without reverse transcriptase were used as negative controls and showed no amplification. The change in cycle threshold (ΔC_T) method was used to calculate Foxi1 mRNA levels as percent of adult epididymal Foxi1 expression. The cycling protocol used was step 1: 50°C for 2:00 min; step 2: 95°C for 10:00 min; step 3: 95°C 0:15 min; step 4: 60°C for 1:00 min, steps 1–4 repeated 40 times. The primers and 5'-FAM and 3'-TAMRA labeled probes used were (1) Foxi1 Forward: ACT AAC GCC AGC CCC TTT CT; (2) Foxi1 Reverse: AGG TCG CTG GGC AGT AGC T; (3) Foxi1 Probe: CCC CAG GCC TAT GGC ATG CAG; (4) 18s rRNA Forward: GTT CGG AAC ATC AAG ACA CAT CT; (5) 18s rRNA Reverse: CAG AAC AAC AGA TCC CAC CAT TA; (6) 18s rRNA probe: TTG GCC CTT TCC CCG TGG TCA.

Cell culture, transient transfections and luciferase assays

COS-7 cells (American Type Culture Collection, USA) were cultured in DMEM, containing 4.5 g/l glucose, 10% heat-inactivated calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen) and maintained in a humidified incubator with 5% CO₂ at 37°C. Cells were grown on 24-well plates to approximately 50–80% confluence before transfection. Transient transfections were performed with FUGENE 6 transfection Reagent (Roche), using 140 ng of luciferase reporter plasmid co-transfected with 40–100 ng expression vector, in accordance to the manufacturer's instructions. Differences in transfection efficiencies were assayed by co-transfecting each well with 1.0 ng pRL-SV40 Renilla luciferase plasmid (Promega). After 48 h of transfection, cells were washed with cold PBS buffer, harvested with passive lysis buffer (Promega) and analyzed using Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol. Luciferase activity was determined as fold induction relative to cells

transfected with an expression vector void of insert, normalized to *Renilla* activity. Experiments were performed in triplicates.

Plasmid constructs and mutants

The full-length FOXI1 was cloned into pcDNA3.1/GS expression vector (Genestorm Clone ID: RG001823) was obtained from ResGen, Invitrogen. A 1.0 kb (corresponding to nt 49 977 948–49 978 999, GenBank Acc No. NT_022184) region of genomic sequence immediately 5' of the *ATP6V1B1* gene ATG (Locus ID:525) was PCR amplified from human genomic DNA. Unique *NheI* or *XhoI* restriction sites were incorporated at the 5' and 3' ends of the primer sequence, sequenced verified PCR product was cloned into *NheI* and *XhoI* sites in the reporter vector pGL3-basic (Promega). Primers: (1) FW: 5'-CTA GCT AGC CCA CAC TCC ACT CAC CAG GAG AA-3', (2) REV: 5'-CTA CTC GAC CCA GTG TCT GAG CCT GCT GCT-3'. Based on information from GenBank this promoter region contains at least one putative FOXI1 binding site (–102/–96, relative to transcription start). Introduction of the triple mutation (–98T>G, –99T>G and –100T>G) into the putative FOXI1 binding site of the 5'ATP6V1B1 promoter (5'ATP6V1B1 TTT>GGG) was generated by PCR, using the Quick Change technique (Stratagene), according to the manufacturer's protocol. The following primers were used: Mut-FW: 5'-CCA GGC TTC CTT GGG GAC TGT CAA CCT AGA GG. Mut-REV: 5'-CCT CTA GGT TGA CAG TCC CCA AGG AAG CCT GG. All constructs were sequence verified by using ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Electrophoretic mobility shift assay

In vitro transcription/translations were performed according to the manufacturer's protocol using TnT Quick Coupled Transcription/translation Systems (Promega) together with [³⁵S]methionine and 2 µg expression plasmid. Oligonucleotides were labeled using Klenow polymerase and [α -³²P]dCTP. The following wt pair of oligonucleotides were used (the putative FOXI1 binding site is underlined): wt-fw: 5'-GCA GGC TTC CT T GTT TAC TGT CAA C; wt-rev: 5'-GTA GGT TGA CAG TAA ACA AGG AAG C. The following mutated (mut) pair of oligonucleotides were used (the triple mutation TTT>GGG is underlined): mut-fw: 5'-GCA GGC TTC CTT GGG GAC TGT CAA C; mut-rev: 5'OGTA GGT TGA CAG TCC CCA AGG AAG C. For each binding reaction, 50 000 c.p.m. of labeled probe was incubated with 3 µl of *in vitro* translated FOXI1 protein and 1.0 µg of poly dI:dC in a binding-buffer containing 5 mM HEPES, pH 7.9, 26% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM PMSF with 100 mM KCl at room temperature for 15 min. Competition experiments were performed in the presence of 10- to 50-fold molar excess of unlabeled wt or mutated oligonucleotides, respectively. Samples were run in 6% nondenaturing polyacrylamide gels at 200 V in Tris-glycine buffer

(50 mM Tris, pH 8.5; 380 mM glycine; 2 mM EDTA) at 4°C. Subsequently, gels were dried and subjected to autoradiography.

Sperm count, morphology, morphometric analysis and pH

At the day of vaginal plug appearance, the wt females were killed by cervical dislocation and the oviduct and uterus were flushed with PBS. The flushed buffer was treated with hyaluronidase until all cumulus cells were single cells to make sure no sperms were concealed within the cumulus cloud. The solution was centrifuged and the pellet redissolved in a fixed volume of PBS buffer. Counting the number of sperms was repeated three times for each copulation event (wt males *n* = 3, Foxi1–/– males *n* = 5). Epididymes were minced, centrifuged and dissolved in M16, a medium supporting sperm motility (wt *n* = 6, Foxi1–/– *n* = 6) and counted under a microscope. Sperms were fixated in PBS containing 4% PFA mounted and viewed in a Nikon eclipse E800 microscope using a Nikon DXM1200 digital camera to record micrographs. To score epididymal lumen area, sections from at least five slides each of wt (*n* = 6) and Foxi1–/– (*n* = 6) epididymides were viewed and evaluated in a Zeiss LSM510 confocal microscope. Tubules sectioned perpendicular to the focal plane were used for area calculations using Zeiss LSM Image Browser software. pH was measured as previously described (Yeung *et al*, 2004). In brief, fluid was collected from epididymal lobules after careful dissection to avoid blood vessels and other tissue. Tubules were cut and gently pressed so that the exuded luminal contents immediately could be applied to an ultra sensitive pH strip (Hydronion MicroFine pH strips cat# MF-1606, Micro Essential Laboratory, New York, NY) and compared with standards by two independent observers. Sperm tail morphology was determined for 100 epididymal spermatozoa from wt (*n* = 3) and Foxi1–/– (*n* = 3).

Statistics

All values are given as mean ± s.e.m. Student's *t*-test was used for statistical analysis, a *P*-value of less than 0.05 was considered to be significant.

Acknowledgements

We thank Drs D Brown (B1-subunit) and W Sly (CAII) for providing antisera. ES cell culture work and blastocyst injections were performed by the Transgenic Core Facility at Göteborg University. This work was supported by the Swedish Research Council (Grants K2002-04X-03522-31D to SE, K2002-31X-12186-06A to SE and 8282 to OS), EU grants (QLK3-CT-2002-02149 and LSHM-CT-2003-503041 to SE) The Arne and IngaBritt Foundation (SE) and The Söderberg Foundation (SE), and The Children's Cancer Fund (OS).

References

- Acott TS, Carr DW (1984) Inhibition of bovine spermatozoa by caudal epididymal fluid: II. Interaction of pH and a quiescence factor. *Biol Reprod* **30**: 926–935
- Al-Awqati Q (1996) Plasticity in epithelial polarity of renal intercalated cells: targeting of the H(+)–ATPase and band 3. *Am J Physiol* **270**: C1571–C1580
- Bedford JM (1975) Maturation, transport, and fate of spermatozoa in the epididymis. In *Handbook of Physiology Section 7: Endocrinology, Vol. V. Male Reproductive System*, Greep RO, Astwood EB (eds). pp 303–317. Washington DC: American Physiology Society
- Blomqvist SR, Vidarsson H, Fitzgerald S, Johansson BR, Ollerstam A, Brown R, Persson AE, Bergstrom GG, Enerback S (2004) Distal renal tubular acidosis in mice that lack the forkhead transcription factor Foxl1. *J Clin Invest* **113**: 1560–1570
- Breton S, Smith PJ, Lui B, Brown D (1996) Acidification of the male reproductive tract by a proton pumping (H+)–ATPase. *Nat Med* **2**: 470–472
- Brown D, Breton S (2000) H(+)V-ATPase-dependent luminal acidification in the kidney collecting duct and the epididymis/vas deferens: vesicle recycling and transcytotic pathways. *J Exp Biol* **203**: 137–145
- Brown D, Lui B, Gluck S, Sabolic I (1992) A plasma membrane proton ATPase in specialized cells of rat epididymis. *Am J Physiol* **263**: C913–C916
- Brown D, Lydon J, McLaughlin M, Stuart-Tilley A, Tyszkowski R, Alper S (1996) Antigen retrieval in cryostat tissue sections and cultured cells by treatment with sodium dodecyl sulfate (SDS). *Histochem Cell Biol* **105**: 261–267
- Brown D, Smith PJ, Breton S (1997) Role of V-ATPase-rich cells in acidification of the male reproductive tract. *J Exp Biol* **200**: 257–262
- Caflisch CR, DuBose Jr TD (1990) Direct evaluation of acidification by rat testis and epididymis: role of carbonic anhydrase. *Am J Physiol* **258**: E143–E150
- Campbell C, Cucci RA, Prasad S, Green GE, Edeal JB, Galer CE, Karniski LP, Sheffield VC, Smith RJ (2001) Pendred syndrome, DFNB4, and PDS/SLC26A4 identification of eight novel mutations and possible genotype-phenotype correlations. *Hum Mutat* **17**: 403–411
- Carr DW, Acott TS (1984) Inhibition of bovine spermatozoa by caudal epididymal fluid: I. Studies of a sperm motility quiescence factor. *Biol Reprod* **30**: 913–925
- Carr DW, Acott TS (1989) Intracellular pH regulates bovine sperm motility and protein phosphorylation. *Biol Reprod* **41**: 907–920

- Carr DW, Usselman MC, Acott TS (1985) Effects of pH, lactate, and viscoelastic drag on sperm motility: a species comparison. *Biol Reprod* **33**: 588–595
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* **162**: 156–159
- Dou H, Finberg K, Cardell EL, Lifton R, Choo D (2003) Mice lacking the B1 subunit of H⁺-ATPase have normal hearing. *Hearing Res* **180**: 76–84
- Elkjaer M, Vajda Z, Nejsum LN, Kwon T, Jensen UB, Amiry-Moghaddam M, Frokiaer J, Nielsen S (2000) Immunolocalization of AQP9 in liver, epididymis, testis, spleen, and brain. *Biochem Biophys Res Commun* **276**: 1118–1128
- Everett LA, Belyantseva IA, Noben-Trauth K, Cantos R, Chen A, Thakkar SI, Hoogstraten-Miller SL, Kachar B, Wu DK, Green ED (2001) Targeted disruption of mouse Pds provides insight about the inner-ear defects encountered in Pendred syndrome. *Hum Mol Genet* **10**: 153–161
- Everett LA, Morsli H, Wu DK, Green ED (1999) Expression pattern of the mouse ortholog of the Pendred's syndrome gene (Pds) suggests a key role for pendrin in the inner ear. *Proc Natl Acad Sci USA* **96**: 9727–9732
- Farquhar MG, Palade GE (1963) Junctional complexes in various epithelia. *J Cell Biol* **17**: 375–412
- Finberg KE, Wagner CA, Bailey MA, Wang T, Mentone SA, Kashgarian M, Giebisch GH, Geibel JP, Lifton RP (2002) Loss of plasma membrane v-H⁺ATPase activity from cortical collecting duct intercalated cells of H⁺-ATPase B1-subunit deficient mice: a mouse model of distal renal tubular acidosis (abstract). *J Am Soc Nephrol* **13**: 267A
- Finberg KE, Wagner CA, Stehberger PA, Geibel JP, Lifton RP (2003) Molecular cloning and characterization of Atp6v1b1, the murine vacuolar H⁺-ATPase B1-subunit. *Gene* **318**: 25–34
- Friend DS, Gilula NB (1972) Variations in tight and gap junctions in mammalian tissues. *J Cell Biol* **53**: 758–776
- Herak-Kramberger CM, Breton S, Brown D, Kraus O, Sabolic I (2001) Distribution of the vacuolar H⁺ atpase along the rat and human male reproductive tract. *Biol Reprod* **64**: 1699–1707
- Hermo L, Adamali HI, Andonian S (2000) Immunolocalization of CA II and H⁺ V-ATPase in epithelial cells of the mouse and rat epididymis. *J Androl* **21**: 376–391
- Hermo L, Chong DL, Moffatt P, Sly WS, Waheed A, Smith CE (2005) Region- and cell-specific differences in the distribution of carbonic anhydrases II, III, XII, and XIV in the adult rat epididymis. *J Histochem Cytochem* **53**: 699–713
- Hermo L, Oko R, Morales CR (1994) Secretion and endocytosis in the male reproductive tract: a role in sperm maturation. *Int Rev Cytol* **154**: 106–189
- Hulander M, Kiernan AE, Blomqvist SR, Carlsson P, Samuelsson EJ, Johansson BR, Steel KP, Enerback S (2003) Lack of pendrin expression leads to deafness and expansion of the endolymphatic compartment in inner ears of Foxi1 null mutant mice. *Development* **130**: 2013–2025
- Hulander M, Wurst W, Carlsson P, Enerback S (1998) The winged helix transcription factor Fkh10 is required for normal development of the inner ear. *Nat Genet* **20**: 374–376
- Jensen LJ, Schmitt BM, Berger UV, Nsumu NN, Boron WF, Hediger MA, Brown D, Breton S (1999a) Localization of sodium bicarbonate cotransporter (NBC) protein and messenger ribonucleic acid in rat epididymis. *Biol Reprod* **60**: 573–579
- Jensen LJ, Stuart-Tilley AK, Peters LL, Lux SE, Alper SL, Breton S (1999b) Immunolocalization of AE2 anion exchanger in rat and mouse epididymis. *Biol Reprod* **61**: 973–980
- Johnston DS, Jelinsky SA, Bang HJ, DiCandeloro P, Wilson E, Kopf GS, Turner TT (2005) The mouse epididymal transcriptome: transcriptional profiling of segmental gene expression in the epididymis. *Biol Reprod* **73**: 404–413
- Karet FE, Finberg KE, Nelson RD, Nayir A, Mocan H, Sanjad SA, Rodriguez-Soriano J, Santos F, Cremers CW, Di Pietro A, Hoffbrand BI, Winiarski J, Bakkaloglu A, Ozen S, Dusunsal R, Goodyer P, Hulton SA, Wu DK, Skvorak AB, Morton CC, Cunningham MJ, Jha V, Lifton RP (1999) Mutations in the gene encoding B1 subunit of H⁺-ATPase cause renal tubular acidosis with sensorineural deafness. *Nat Genet* **21**: 84–90
- Kaunisto K, Parkkila S, Parkkila AK, Waheed A, Sly WS, Rajaniemi H (1995) Expression of carbonic anhydrase isoenzymes IV and II in rat epididymal duct. *Biol Reprod* **52**: 1350–1357
- Kirchhoff C (1999) Gene expression in the epididymis. *Int Rev Cytol* **188**: 133–202
- Levine N, Kelly H (1978) Measurement of pH in the rat epididymis *in vivo*. *J Reprod Fertil* **52**: 333–335
- Medina JF, Recalde S, Prieto J, Lecanda J, Saez E, Funk CD, Vecino P, van Roon MA, Ottenhoff R, Bosma PJ, Bakker CT, Elferink RP (2003) Anion exchanger 2 is essential for spermiogenesis in mice. *Proc Natl Acad Sci USA* **100**: 15847–15852
- Miller RL, Zhang P, Smith M, Beaulieu V, Paunescu TG, Brown D, Breton S, Nelson RD (2005) V-ATPase B1-subunit promoter drives expression of EGFP in intercalated cells of kidney, clear cells of epididymis and airway cells of lung in transgenic mice. *Am J Physiol Cell Physiol* **288**: C1134–C1144
- Nelson RD, Guo XL, Masood K, Brown D, Kalkbrenner M, Gluck S (1992) Selectively amplified expression of an isoform of the vacuolar H⁺(+)-ATPase 56-kilodalton subunit in renal intercalated cells. *Proc Natl Acad Sci USA* **89**: 3541–3545
- Overdier DG, Ye H, Peterson RS, Clevidence DE, Costa RH (1997) The winged helix transcriptional activator HFH-3 is expressed in the distal tubules of embryonic and adult mouse kidney. *J Biol Chem* **272**: 13725–13730
- Pastor-Soler N, Bagnis C, Sabolic I, Tyszkowski R, McKee M, Van Hoek A, Breton S, Brown D (2001) Aquaporin 9 expression along the male reproductive tract. *Biol Reprod* **65**: 384–393
- Pastor-Soler N, Piétrement C, Breton S (2005) Role of acid/base transporters in the male reproductive tract and potential consequences of their malfunction. *Physiology* **20**: 417–428
- Paunescu TG, Da Silva N, Marshansky V, McKee M, Breton S, Brown D (2004) Expression of the 56-kDa B2 subunit isoform of the vacuolar H⁺(+)-ATPase in proton-secreting cells of the kidney and epididymis. *Am J Physiol Cell Physiol* **287**: C149–C162
- Phelps PD, Coffey RA, Trembath RC, Luxon LM, Grossman AB, Britton KE, Kendall-Taylor P, Graham JM, Cadge BC, Stephens SG, Pembrey ME, Reardon W (1998) Radiological malformations of the ear in Pendred syndrome. *Clin Radiol* **53**: 268–273
- Pushkin A, Clark I, Kwon TH, Nielsen S, Kurtz I (2000) Immunolocalization of NBC3 and NHE3 in the rat epididymis: colocalization of NBC3 and the vacuolar H⁺-ATPase. *J Androl* **21**: 708–720
- Reardon W, CF OM, Trembath R, Jan H, Phelps PD (2000) Enlarged vestibular aqueduct: a radiological marker of pendred syndrome, and mutation of the PDS gene. *QJM* **93**: 99–104
- Shima JH, McLean DJ, McCarrey JR, Griswold MD (2004) The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. *Biol Reprod* **71**: 319–330
- Stankovic KM, Brown D, Alper SL, Adams JC (1997) Localization of pH regulating proteins H⁺ATPase and Cl⁻/HCO₃⁻-exchanger in the guinea pig inner ear. *Hear Res* **114**: 21–34
- Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, Axel R (1999) A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell* **96**: 725–736
- Yeung C-H, Anapolski M, Sipilä P, Wagenfeld A, Poutanen M, Huhtaniemi I, Nieschlag E, Cooper TG (2002) Sperm volume regulation: maturational changes in fertile and infertile transgenic mice and association with kinematics and tail angulation. *Biol Reprod* **67**: 269–275
- Yeung C-H, Breton S, Setiawan I, Xu Y, Lang F, Cooper TG (2004) Increased luminal pH in the epididymis of infertile c-ros knockout mice and the expression of sodium–hydrogen exchangers and vacuolar proton pump H⁺-ATPase. *Mol Reprod Dev* **68**: 159–168