Foxi1, an important gene for hearing, kidney function and male fertility

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Is life just a game where we make up the rules
while we're searching for something to say,
or are we just simply spiralling coils
of self-replicating DN-- nay, nay, nay, nay, nay, nay, nay

Monty python - The Meaning of Life, 1983

ABSTRACT

Foxi1, an important gene for hearing, kidney function and male fertility

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When mice lacking the winged helix transcription factor Foxi1 were compared to their wt littermates in their response to acidic load, we discovered that Foxi1 deficient mice develop distal renal tubular acidosis (dRTA). Moreover, during breeding we observed the inability of Foxi1-/- males to generate offspring, indicating male infertility. In the light of these observations, together with our previous results from the inner ear of Foxi1-/- mice, we set out to examine the biological importance of Foxi1 in the inner ear, kidney and the epididymis. In this thesis we assess the expression pattern of Foxi1 and its potential downstream target genes in these three different tissues by using Northern blot analyses, *in situ* hybridization and immunohistochemistry. We demonstrated that Foxi1 is exclusively expressed in cells positive for the “kidney specific” vATPase subunits B1and a4, as well as the anion exchangers AE1 and pendrin, all characteristics for acid/base regulating cells of the inner ear, kidney and epididymis, called “mitochondria rich” cells. We present evidence that Foxi1 is required for the expression of these genes. Furthermore, in transfection experiments and in electrophoretic mobility shift assays (EMSA) we established direct transcriptional activation of promoter constructs by Foxi1. This activation was abolished when specific Foxi1 *cis*-elements on the promoters were mutated.

Previously we have shown that mice with target disruption of the Foxi1 locus exhibit severe cochlear and vestibular malformation and that Foxi1 is expressed in endolymphatic epithelial cells important for regulation of volume and composition of endolymph fluid. We have also shown that pendrin is missing from endolymphatic epithelium in Foxi1 deficient mice, making human FOXI1 a potential transcriptional activator of the gene coding for pendrin, the *SLC26A4* gene. While recessive mutations in the *SLC26A4* gene are known to be responsible for Pendred syndrome (PS) and non-syndromic hearing loss associated with enlarged vestibular aqueduct (EVA), a large percentage of patients with this phenotype lack mutations in the *SLC26A4* coding region in one or both alleles. We have identified and characterized a key transcriptional regulatory element in the *SLC26A4* promoter that binds FOXI1. Moreover, we have identified six PS or non-syndromic EVA patients with mutations in *FOXI1* that inhibits its activation of *SLC26A4* transcription

In summary, we present molecular data showing that Foxi1 is required for the expression of some of the most important transporter molecules of acid-base regulation in the inner ear, kidney and epididymis, establishing Foxi1 as an essential regulator of fluid homeostasis in these organs. Hence, mice deficient for Foxi1, beside their early onset deafness, develop dRTA and male infertility. We also demonstrate that mutations in the human *FOXI1* gene are involved in pathogenetic mechanisms underlying human deafness. Finally, these results together with our earlier findings in mice support the hypothesis that mutations in the human *FOXI1* gene might prove to cause a sensorineural deafness syndrome with distal renal tubular acidosis and male infertility.

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LIST OF PUPLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals, I - IV:

I. Tao Yang, **Hilmar Vidarsson**, Sandra Rodrigo-Blomqvist, Sally S. Rosengren, Sven Enerbäck, and Richard J.H. Smith (2007) Transcriptional Control of SLC26A4 Is Involved in Pendred Syndrome and Non-syndromic Enlargement of Vestibular Aqueduct (DFNB4). *Am. J. Hum. Genet.,* 80:1055-1063

II. Blomqvist SR, **Vidarsson H**, Fitzgerald S, Johansson BR, Ollerstam A, Brown R, Persson AE, Bergstrom G G, Enerback S (2004) Distal renal tubular acidosis in mice that lack the forkhead transcription factor Foxi1. *J Clin Invest.* 113:1560-70.

III. Blomqvist SR*, **Vidarsson H*,** Soder O, Enerback S (2006) Epididymal expression of the forkhead transcription factor Foxi1 is required for male fertility. *EMBO J.* 25:4131-41.* authors contributed equally

IV. **Vidarsson H,** Blomqvist SR and Enerback S. The transcription factor Foxi1 is a master regulator of vacuolar H+-ATPase proton pump subunits in the inner ear, kidney and epididymis. *Manuscript 2007*

LIST OF ABBREVIATIONS

To distinguish between genes and proteins, italicized (*genes*) versus roman (proteins) letters will be used in this thesis. All abbreviations for the human FOXI1 protein will contain capital letters, while for the mouse Foxi1 protein only the first letter is capitalized.

AQP	Aquaporin
ATP6V0A4	Gene encoding a4 subunit of the vATPase
ATP6V1A1	Gene encoding A1 subunit of the vATPase
ATP6V1B1	Gene encoding B1 subunit of the vATPase
ATP6V1E2	Gene encoding E2 subunit of the vATPase
CAII/CAIV	Carbonic anhydrase type II / type IV
dRTA	Distal renal tubular acidosis
E16	Embryonic stage (day) 16
EVA	Enlarged vestibular aqueduct
Fkh	Forkhead homolog
FORE cells	Forkhead related cells
FOX	Forkhead box
FREAC	Forkhead-related activator
HFH	HNF-3/forkhead homolog
IC	Intercalated cells
kAE	Kidney specific anion exchanger
NBC	Natrium/bicarbonate co-transporter
NHE	Natrium/proton exchanger
Pds	Pendrin
PS	Pendred syndrome
SLC	Solute Carrier Family of anion exchanger proteins
vATPase	Vacuolar H^+-ATPase proton pump
wt	Wild type

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INTRODUCTION

Inner Ear

The inner ear is one of the most complex sensory organs of vertebrates. In addition to sensation of sound by the cochlea, the vestibular system serves to detect gravity and linear as well as angular acceleration. Much progress has been made in the understanding of the molecular and genetic factors regulating the inner ear development, from the undifferentiated embryonic ectoderm to a fully formed spherical vesicle, named the *otocyst*. Later in the developmental process, during inner ear morphogenesis, the otocyst undergoes many structural changes to form the different parts of the inner ear, the *cochlea*, the *saccule*, the *utricle* and the *semicircular canals* (Figure 1). The saccule, utricle and the semicircular canals are known as the vestibular system of the inner ear and together with the cochlea form the

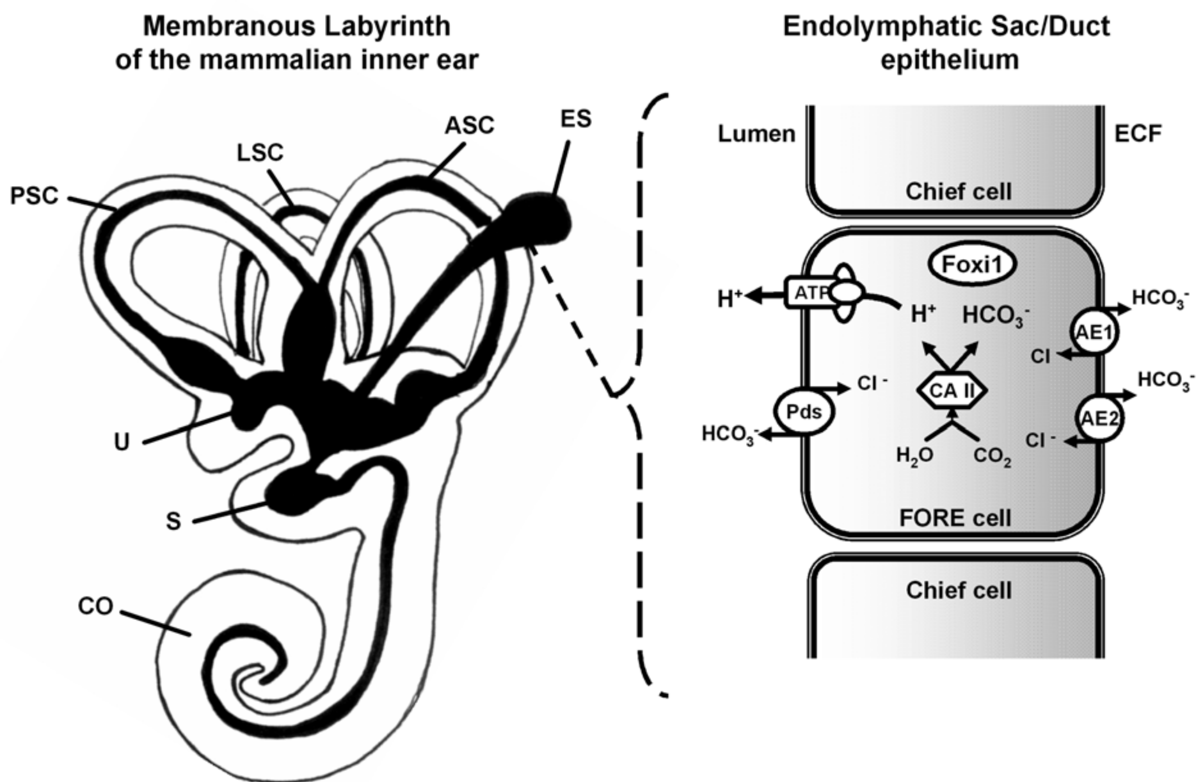

Figure 1: Schematic figure illustrating the different compartments of the membranous labyrinth of the inner ear and a hypothetical model of acid-base regulation of the "mitochondria-rich" FORE cells of the endolymphatic sac/duct epithelia. Acid-base regulation is controlled by proton/bicarbonate secretion by apically localized vATPase proton pump and anion exchanger pendrin (Pds), coupled with HCO_3^- reabsorption by basolaterally localized anion exchangers AE1 and AE2. Carbonic anhydrase II (CAII) catalyzes the formation of H^+ and HCO_3^- in the cytobplasm of the FORE cells. Cochlear duct (CO), endolymphatic sac (ES), Saccule (S), Utricle (U), anterior (ASC), posterior (PSC) and lateral (LSC) semicircular canals, (ECF) extracellular fluid.

membranous labyrinth, a series of communicating ducts and tubules. The membranous labyrinth lies within the *bony labyrinth* and is connected to the endolymphatic sac via the endolymphatic duct (Figure 1).

Endolymph and the membranous labyrinth

Endolymph is the fluid inside the membranous labyrinth. It fills the spaces where the sensory cells of the inner ear are found, i.e. in the sensory organs of the semicircular canals (the ampulla), the utricle, the saccule and finally in the cochlear duct, or the *Scala media*, where the hair cells of the *Organ of Corti* are located (Figure 2) (Kimura, 1975). The endolymph fluid is very similar in ionic composition to intracellular fluid, with low Na^+ concentration but relatively high levels of K^+ ions. The cochlear duct is surrounded by two additional fluid filled spaces, *Scala tympani* and *Scala vestibuli*, separating the membranous labyrinth from the bony labyrinth. *Scala tympani* and *Scala vestibuli* are filled with perilymph, an extracellular-like fluid with high Na^+ but low K^+ concentration (Figure 2) (Salt and DeMott, 1998; Thorne et al., 1999). The high level of K^+ concentration in the endolymph is necessary for establishing the membrane potential difference across the borders of the sensory hair cells, which then supports the vectorial influx of K^+ ions into the hair cells under mechanical stimulation (Raphael and Altschuler, 2003). This ionic difference, which generates the *endocochlear potential* of the inner ear (approximately 100-120 mV), is dependent on the activity of various ionic pumps and transporters located in the plasma membrane of cells lining the endolymphatic compartment (Neyroud et al., 1997; Schulze-Bahr et al., 1997; Spicer and Schulte, 1991). The marginal cells of *stria vascularis*, the major site of endolymph production (Figure 2), regulate the output of K^+ ions into Scala media (Takeuchi et al., 2000; Wangemann et al., 2000).

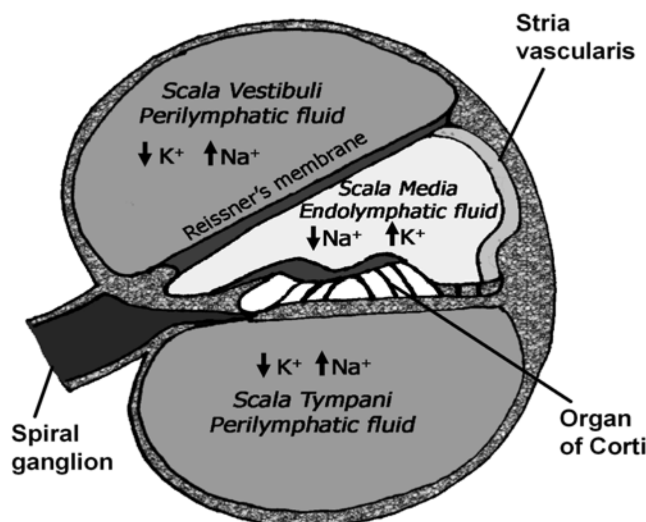

Figure 2: Schematic drawing of the cochlea in cross section. The cochlear duct (*Scala media*) contains K^+ rich endolymph, while *Scala vestibuli* and *Scala tympani* are filled with Na^+ rich perilymph. The high K^+ concentration is important for the endocohlear potential of the inner ear, establishing the importance of appropriate regulation of the ionic composition of the endolymphatic fluid.

Endolymphatic sac/duct epithelium

Endolymph homeostasis is essential for adequate auditory function (Dou et al., 2003; Peters et al., 2001). Under normal circumstances the endolymph fluid is separated from other compartments of the inner ear and this separation is strictly controlled by the tight junctions of the epithelium lining the membranous labyrinth. The only space endolymph can drain into is the endolymphatic sac, via the endolymphatic duct (Figure 1) (Salt and DeMott, 1999). The endolymphatic duct arises from the junctions of the saccule and utricle and ends in the wider endolymphatic sac. The most important function of the endolymphatic sac/duct is to maintain fluid composition in the membranous labyrinth, through its ability to reabsorb waste products of the endolymphatic circulation and through its secretion of ions and protons into the lumen (Rask-Andersen et al., 1999). At least two types of cells can be identified in the endolymphatic sac/duct epithelium, i.e. the "mitochondria-rich" light cells, also called FORE cells (Forkhead Related cells) and the "ribosome-rich" Chief cells, the most abundant cells of the endolymphatic epithelium (Hulander et al., 2003; Hultcrantz et al., 1987; Manni and Kuijpers, 1987; Peters et al., 2002). These are morphologically different cell types with distinct transport properties as well (Dahlmann and von During, 1995; Rask-Andersen et al., 1999). FORE cells are characterized by their expression of Foxi1 and high concentration of mitochondria, but they are also known for their expression of the vacuolar H^+-ATPase (vATPase) proton pump and the carbonic anhydrase type II (CAII), as well as the anion exchangers pendrin, on their apical membrane, and AE1 and AE2 on the basolateral side (Figure 1) (Dou et al., 2004; Stankovic et al., 1997). FORE cells specific expression of these transporter proteins is consistent with their importance in regulation of fluid and ionic composition of the endolymph fluid. Mutations in genes coding for ionic exchangers or proton transporters, such as pendrin and the vATPase, have been shown to cause human deafness due to the disrupted endolymphatic homeostasis in the inner ear (Karet et al., 1999b; Li et al., 1998).

Pendred syndrome

Pendred syndrome (PS) is an autosomal recessive disorder caused by mutations in the *SLC26A4* gene that encodes the anion exchanger pendrin (Everett et al., 1997). PS is characterized by congenital sensorineural hearing loss, in some cases goiter and an enlarged endolymphatic aqueduct (Fugazzola et al., 2000; Reardon et al., 2000; Reardon and Trembath, 1996). This is the most common form of syndromic deafness, accounting for about 10% of hereditary hearing loss (Phelps et al., 1998). In more than 80% of PS patients the inner ear is malformed with an enlarged vestibular aqueduct (Gill et al., 1999; Phelps et al., 1998). Goiter usually develops in late childhood to early adulthood but is absent in about one fifth of patients (Reardon et al., 1999). In 1997, Everett and colleagues first reported the molecular structure of the gene mutated in the PS, the *SLC26A4* gene (also known as the *PDS* gene) (Everett et al., 1997). Numerous mutations in the *SLC26A4* gene have now been identified in PS patients, including small deletions and insertions as well as altered splice sites and

missense mutations (Li et al., 1998; Taylor et al., 2002). Several reports have documented certain *SLC26A4* mutations that cause deafness in the absence of other syndromic features. This has established direct connections between *SLC26A4* mutations and nonsyndromic hearing loss associated with enlarged vestibular aqueduct (EVA) (Campbell et al., 2001; Usami et al., 1999).

The Kidney

Kidneys provide several important physiological functions, including regulation of water and electrolyte balance, regulation of body fluid osmolarity and the excretion of waste materials that are either ingested or produced by metabolism. One of the most important functions of the kidney is regulation of acid-base homeostasis. Every cell in the body is dependent on a strict regulation of acid-base homeostasis for growth, development and normal cellular function (Alper, 2002; Batlle et al., 2001). The human kidney is made up of about one million nephrons, the functional units of the kidney (Figure 3). Each nephron can be divided into at least four main functional regions, the *glomerulus*, the *proximal tubule*, the *loop of Henle* and finally the *distal tubule*, including the *collecting duct*. The glomerulus, encased in the Bowman's capsule, is the most proximal part of the nephrons and there filtration of fluid from the glomerular capillaries occurs. The filtered fluid flows from the Bowman's capsule into the proximal tubules, where approximately 80 % of the filtered bicarbonate (HCO_3^-) is reabsorbed (Baum et al., 2003). The function of the Loop of Henle is mainly to allow simple diffusion of substances through its wall before emptying the load into the distal tubule, where the final tuning of urinary acidification occurs (Figure 3) (Wagner et al., 2002).

Acid-base regulation in the kidney

Overall body acid-base homeostasis is controlled mainly by exhalation of CO_2 and by reabsorption of HCO_3^- and proton (H^+) secretion in the kidneys. The average net result from a normal food intake of an adult person in the Western world today is the production of an acid load of ~70 mmol H^+ per day (Karet et al., 1999a). To maintain acid-base homeostasis under these conditions and to preserve the arterial pH within a narrow range of approximately pH 7.4, this acidic load must be excreted by the kidney. These homeostatic functions are disrupted in acute or chronic renal failure, leading in most cases to severe abnormalities in body fluid volume and composition (Alper, 2002). Filtered HCO_3^- is almost completely reabsorbed by the cells of the proximal tubule. This HCO_3^- reabsorption is mediated by the apically expressed Na^+/H^+ exchangers NHE2 or NHE3 and the basolaterally located Na^+/HCO_3^- cotransporter NBCe1. But the reabsorption process also requires the catalytic activity of the cytosolic CAII as well as the membrane bound CA type IV (CAIV), which is found within the tubular lumen (Figure 3) (Goyal et al., 2003; Wagner et al., 2006). The distal tubules are divided into the connecting tubule and the two distinctive parts of the collecting duct, the cortical and the medullary collecting ducts. Even if the primary role of the distal

nephron is sodium (Na^+) reabsorption and potassium (K^+) secretion, the collecting duct plays a very important role in the regulation of acid-base homeostasis (Wagner et al., 2002). Under normal conditions net acid excretion occurs in the distal tubule, mainly in the collecting duct, but only minimal (~5%) reabsorption of HCO_3^- (Wagner et al., 2002).

The collecting duct epithelia is highly heterogeneous and consists of at least three cell types, the *principal cells* and the two functionally distinct subtypes of *intercalated cells* (IC), the H^+-secreting α-intercalated cells (α-IC) and the HCO_3^--secreting β-intercalated cells (β-IC) (Wagner et al., 2006). The principal cells of the collecting duct comprise about two thirds of the epithelia and these cells, characterized by their expression of the water channel AQP2, are mainly involved in Na^+ reabsorption and K^+ secretion. The intercalated cells are the main

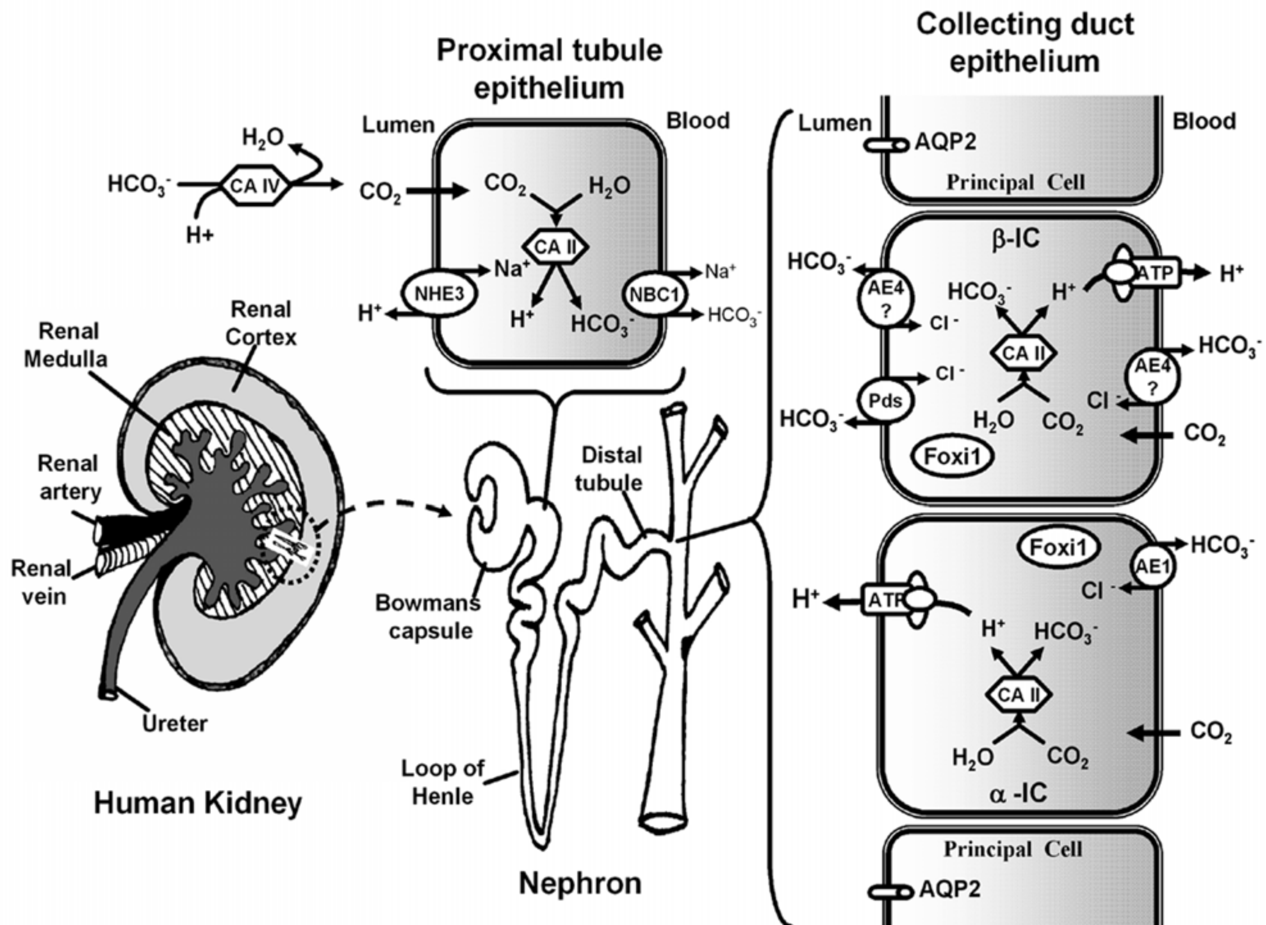

Figure 3: Schematic drawing of a human kidney and the major sites of nephron acid-base handling, i.e. the bicarbonate reabsorbing cells of the proximal tubules and the acid-base secreting intercalated cells (IC) of the collecting duct. In the proximal tubule the HCO_3^- is reabsorbed by the apical Na^+/H^+ exchanger NHE3 and the basolateral Na^+/HCO_3^- cotransporter NBCe1. This is coupled to the formation of H^+ and HCO_3^-, catalyzed by extracellular and cytosolic carbonic anhydrases (CAII and CAIV). The net acid secretion of the kidney occurs in the collecting duct, mainly through the action of intercalated cells alpha (α-IC) and beta (β-IC). The H^+ secreting α-IC express the vATPase proton pump (ATP) on their apical surface and anion exchanger 1 (AE1) on their basolateral side, while the β-IC express the vATPase pump in their basolateral membrane and the anion exchanger pendrin (Pds) on the apical side. Both cell types express high levels of cytosolic CAII.

cells involved in acid-base transport along the collecting duct (Bonnici and Wagner, 2004; Madsen et al., 1988b). Both subtypes, α-IC and β-IC, are found in the cortical collecting duct epithelia, whereas only the α-IC can be found in the medullary collecting duct (Madsen et al., 1988a; Teng-umnuay et al., 1996). The α-IC are responsible for the net acid secretion of the collecting duct (Laing et al., 2005). They can be recognized by their apical expression of the vATPase proton pump and the HCO_3^- reabsorbing anion exchanger AE1 (*SLC4A1*) in their basolateral membrane (Figure 3) (Alper et al., 1989; Kudrycki and Shull, 1989). The main function of the β-IC is to secrete HCO_3^- into the lumen of the collecting duct and they can therefore be identified by their expression of pendrin (*SLC26A4*) on the apical membrane and the vATPase proton pump on the basolateral site (Figure 3). An additional anion exchanger, AE4, has been identified in β-IC. The exact localization and function of AE4 in the β-IC has been disputed, but a growing body of evidence suggests that its subcellular localization is species specific, with a basolateral expression in the mouse (Figure 3) (Ko et al., 2002; Tsuganezawa et al., 2001)(Paper II). A third subtype of intercalated cells, called non-α-non-β-IC, with similar expression pattern as the α-IC has been described having apical staining of vATPase but missing the basolateral expression of the AE1 exchanger (Emmons and Kurtz, 1994; Teng-umnuay et al., 1996).

Distal renal tubular acidosis (dRTA)

The fact that proximal nephrons reabsorb HCO_3^- and distal tubules excrete H^+ has led to the clinical and functional classification of *proximal* versus *distal* forms of renal tubular acidosis (RTA). In distal RTA (dRTA) the ability of α-IC to secrete protons is hampered, leading to an increase in urine pH, which forms the bases for the biochemical diagnose for the disease (Laing et al., 2005). The diagnosis of dRTA in humans must be confirmed by a failure of the kidney to generate sufficiently acidic urine when the systemic pH drops following an imposed acidic load (Laing et al., 2005; Unwin et al., 2002). The ability of α-IC to remove excess load of acid into the urine depends on their expression of various acid-base transporter proteins and enzymes. Mutations in genes encoding some of these proteins have been identified in patients with different forms of inherited dRTA. There are three different types of inherited dRTA, the autosomal dominant dRTA and then the two autosomal recessive forms, with and without sensorineural hearing loss (Laing et al., 2005). Inherited dRTA is a relatively rare disease and the dominant inheritance is much more common then the recessive form (Laing and Unwin, 2006). As the primary cause of dRTA is the failure of α-IC to secrete protons into the lumen of the collecting duct, the vATPase proton pump, expressed in the apical membrane of α-IC (Fig3), was a strong candidate gene for mutation analysis of patients with dRTA (Karet et al., 1998). By now it has been clearly established that mutations in at least two of the subunits of the vATPase pump, the B1 (*ATP6V1B1*) and a4 (*ATP6V0A4*), can cause recessive dRTA (Karet et al., 1999b; Smith et al., 2000). Patients with mutations in the *ATP6V1B1* gene, coding for the B1 subunit, often suffer from sensorineural hearing loss as well, due to its expression in the inner ear (Stankovic et al., 1997; Wagner et al., 2006). Even if it has been

shown that the *ATP6V0A4* gene, encoding the a4 subunit, is also expressed in the inner ear, associated hearing loss is not as common and appears to have a late onset (Dou et al., 2004; Stover et al., 2002; Vargas-Poussou et al., 2006).

Epididymis

When spermatozoa leave the testis most of them are not functionally matured, i.e. their capacity for progressive and sustained motility is very limited and they lack the ability to fertilize an egg (Cosentino and Cockett, 1986; Yeung et al., 1993; Young, 1929). It is in the lumen of the excurrent duct system of the male reproductive tract, the *efferent duct*, *epididymis* and *vas deferens* (Figure 4), that spermatozoa undergo their important maturational changes (Jones and Murdoch, 1996). This is called a "post-testicular maturation process" and it mainly takes place in the lumen of the highly convoluted duct of the epididymis (Bashford et al., 1975). After ejaculation the sperm cells undergo their final maturation when they enter the female genital tract, called *capacitation*, which enables them to penetrate and fertilize an ovum (Bedford, 1975; Zeng et al., 1996).

The epididymis is a single, highly convoluted tubular organ with the important function of sperm maturation, transport and storage. A key event in spermatozoa maturation is the exposure to the luminal environment of the epididymis and the most critical feature is the maintenance of the epididymal fluid at low pH (Levine and Kelly, 1978; Levine and Marsh, 1971). Several changes of the spermatozoa have been presumed to be associated with their maturation during the epididymal passage. These changes include progressive motility, changes in sperm morphology and increased resistance to heat and hostile environment (Kirchhoff, 1999; Lasley, 1951). Regional changes in pH, from pH 7.4 in the proximal region to pH ~ 6.8 in cauda epididymis, and ionic composition are thought to be responsible for these changes (Dott, 1968; Fawcett and Phillips, 1969; Levine and Marsh, 1971). The acidic environment of the distal region is also necessary for keeping the spermatozoa in a quiescent, immotile state during storage, not only for their maturation but also to conserve their energy stores (Moore et al., 1983; Turner and Howards, 1978a; Turner and Howards, 1978b). Failure to provide this highly controlled microenvironment of the epididymal lumen can lead to infertility (Acott and Carr, 1984; Carr et al., 1985; Hinton and Palladino, 1995).

Function of the epididymal epithelium

The long convoluted tubule of epididymis can be divided into four main regions, the *initial segment*, *caput*, *corpus* and *cauda* (Figure 4). These regions are distinguishable by the morphology of the epithelial cells lining the lumen, as well as their specific patterns of gene expression (Kirchhoff, 1999; Robaire and Hermo, 1988). The most proximal part of the epididymis, the initial segment, connects to the testis via the efferent duct and on the distal end the cauda connects to the vas deferens (Figure 4). The epididymal epithelium is composed

of at least three types of epithelial cell, the *principal cells*, which are the most abundant cell type in epididymis, the so called *narrow* and *clear cells*, which are located between the principal cells and finally the *basal cells*, located at the base of the epithelium (Hermo et al., 1994). The narrow and clear cells are functionally similar cell types but with distinct distribution along the epididymal epithelium. The narrow cells can only be found in the initial segment, while the clear cells are located in caput, corpus and cauda epididymis (Robaire and Hermo, 1988; Sun and Flickinger, 1980).

Numerous studies have demonstrated that the post-testicular maturation in the epididymis

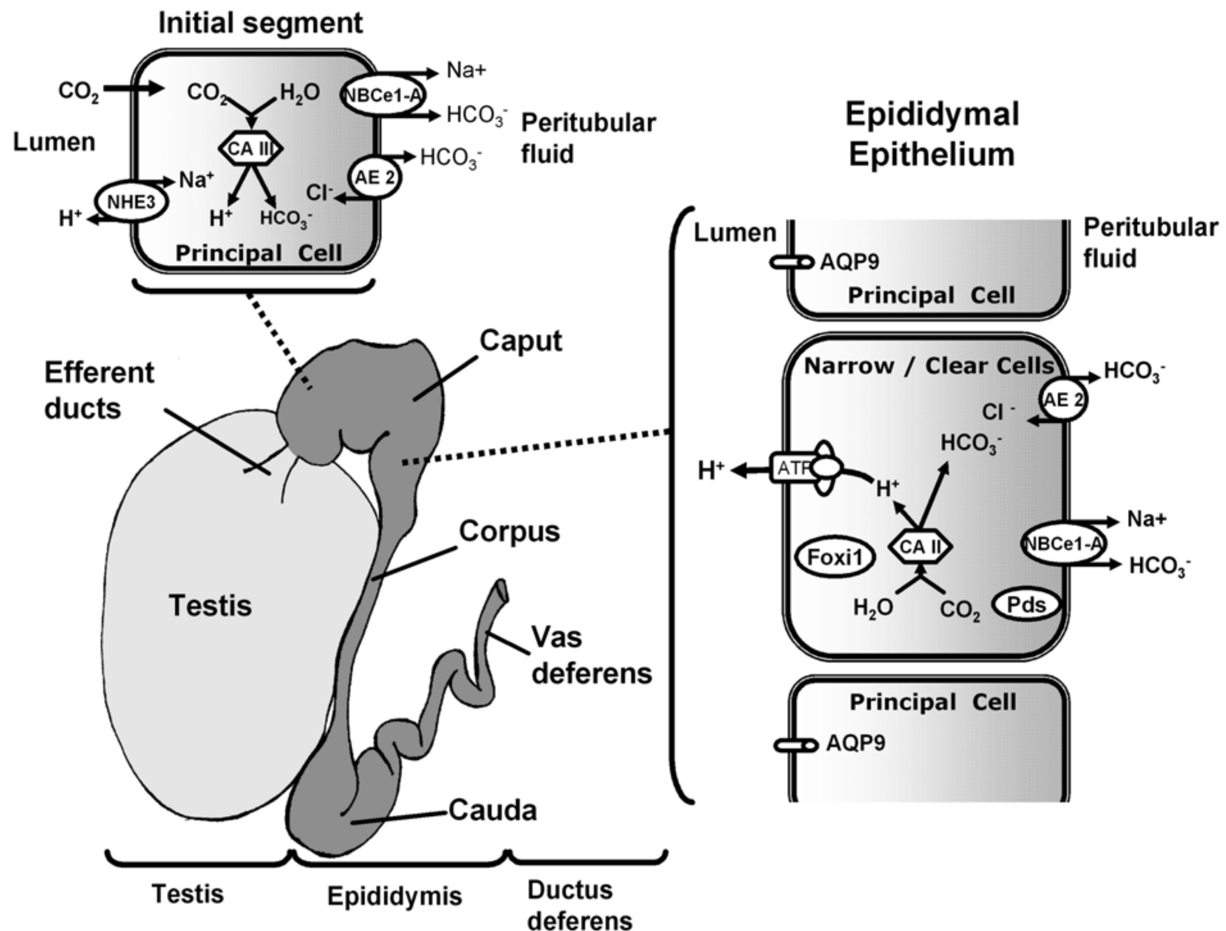

Figure 4: Schematic illustration showing the structure of the testis and the different compartments of the epididymis. Also shown is a hypothetical model of the HCO_3^- absorption of the initial segment and the acid-base regulation of the proton secreting cells of epididymis, the narrow and clear cells. Similarly to the kidneys proximal tubules, the principal cells of the initial segment mainly reabsorb HCO_3^- , which is mainly mediated by the Na^+/H^+ exchanger NHE3 on their apical surface and the basolateral anion exchanger 2 (AE2) and Na^+/HCO_3^- cotransrpoter NBCe1-A. The proton secreting narrow and clear cells express vATPase proton pump (ATP) in their apical membrane, AE2 and NBCe1-A on their basolateral side and pendrin (Pds) in their perinuclear compartment.

depends on secretion, transport and absorption of ions, solutes and macromolecules by the epithelial cells lining the epididymal lumen (Figure 4) (Carr et al., 1985; Cooper et al., 1986; Hinton and Palladino, 1995). Principal cells of the initial segment are fully equipped for the net HCO_3^- reabsorption that occurs in the proximal epididymis and they utilize a transport mechanism very similar to that found in the proximal tubules of the kidney (Figure 3 and

Figure 4). They use the Na^+/H^+ exchangers NHE2 and NHE3 to secrete protons into the lumen and the Na^+/HCO_3^- co-transporter NBCe1-A and the anion exchanger AE2 to transport the bicarbonate across the basolateral membrane (Figure 4). This results in net bicarbonate reabsorption with no net proton secretion (Bagnis et al., 2001; Jensen et al., 1999a; Jensen et al., 1999b; Kaunisto et al., 2001; Yeung et al., 2004). The very low pH of the distal regions of the epididymis, especially in the distal cauda, indicates that active proton secretion takes place in this part of the epithelium and it is the narrow and clear cells that maintain this low pH by controlling this proton transport, accompanied by HCO_3^- reabsorption (Figure 4) (Levine and Kelly, 1978; Levine and Marsh, 1971). The narrow and clear cells have vATPase proton pump located in their apical membrane, where it translocates protons over the membrane into the lumen (Breton et al., 1996; Brown and Breton, 2000; Brown et al., 1997; Herak-Kramberger et al., 2001). It has also been shown that most of the epithelial cells that contribute to this acid-base regulation in epididymis express CAII or CAIII in their cytoplasm (Hermo et al., 2005; Kaunisto et al., 1995). For bicarbonate reabsorption, narrow and clear cells express the anion exchanger AE2 and the Na^+/HCO_3^- co-transporter NBCe1-A on the basolateral membrane (Figure 4) (Jensen et al., 1999a; Jensen et al., 1999b).

The forkhead family of transcription factors

The forkhead family of transcription factors belongs to the winged helix superfamily of DNA binding proteins (Donaldson et al., 1994; Liang et al., 1994). The name "winged helix" is derived from X-ray crystallography data for the Foxa3 (HNF-3γ) forkhead domain, having "double wing" structure similar in shape to a butterfly (Clark et al., 1993). The first member of the forkhead family, that was described, was the *forkhead* (*fkh*) protein in *Drosophila*, named after "two spiked head" embryos with mutation in this gene (Weigel et al., 1989). The name Fox, for Forkhead box (i.e. the DNA binding domain) was adopted as the unified symbol for all chordate forkhead transcription factors (Kaestner, 2000). Forkhead proteins share a highly conserved DNA-binding motif of 110 amino acids called the forkhead domain (Hacker et al., 1992). To date, over 100 forkhead genes have been identified with approximately 40 known family members in mice and humans (Wijchers et al., 2006). Many of these genes are increasingly being recognized for their importance in a wide range of biological events, such as organogenesis during early development (Ang et al., 1993; Weinstein et al., 1994), cell division (Kops et al., 2002; Laoukili et al., 2005), cell survival (Brunet, A. 2001; Gilley, J. 2003), metabolic regulation (Cederberg et al., 2001; Shih et al., 1999) as well as a regulatory role in the immune system (Coffer and Burgering, 2004; Jonsson and Peng, 2005). All forkhead transcription factors can bind to DNA, but their functional effects can vary since some are activators while others are inhibitors of gene transcription (Coffer and Burgering, 2004).

Foxi1

The winged helix transcription factor Foxi1 (also known as HFH3, Fkh10 or FREAC6) is a member of the forkhead family. Foxi1 was first described as a forkhead gene expressed in the kidney (Clevidence et al., 1993; Pierrou et al., 1994). Overdier and colleagues showed that Foxi1 expression is localized to the distal nephron in mice at embryonic stage E16 (Overdier et al., 1997). At an earlier stage, E9.5, specific expression of Foxi1 is seen in the otic vesicle (otocyst) of the developing inner ear (Hulander et al., 1998). At E11.5, Foxi1 expression gradually becomes restricted to the endolymphatic sac/duct (Hulander et al., 2003). Mice with a targeted disruption of the *Foxi1* locus (Foxi1-/-) display a severe inner ear phenotype. Foxi1-/- mice exhibit circling behaviour, poor swimming ability and abnormal reaching response, all common findings in mice with vestibular dysfunction. They have expanded cochlear and vestibular ducts, where inner ear structures are replaced by a single expanded cavity, resulting in no endocochlear potential and deafness (Hulander et al., 2003). Mice deficient for the anion exchanger pendrin (*SLC26A4*) (Pds-/-) display an inner ear phenotype very similar to that of Foxi1-/- mice (Everett et al., 2001). These observations and the fact that pendrin expression is missing in the endolymphatic epithelium of Foxi1-/- inner ears, establish Foxi1 as an upstream regulator of the *SLC26A4* gene expression in the endolymphatic sac/duct of the inner ear (Everett et al., 2001; Hulander et al., 2003).

Common factors of acid/base regulating cells in the inner ear, kidney and epididymis

Vacuolar H^+-ATPases

The vacuolar H^+-ATPase (vATPase) is a multi-subunit enzyme that utilizes energy from ATP hydrolysis for active transport of protons across various membranes. Since the discovery of the vATPase proton pump in the membrane of chromaffin granules in 1975 (Bashford et al., 1975), accumulating evidence indicates that the proton pump is present in virtually all eukaryotic cells. The pump is primarily expressed in membranes of intracellular vesicles and organelles such as clathrin-coated vesicles, lysosomes and the Golgi apparatus, where it contributes to the regulation process of acidification of these compartments (Johnson et al., 1993; Stevens and Forgac, 1997). Although generally considered to be an intracellular proton pump, the vATPase is also expressed in plasma membranes of certain specialized cells. In the plasma membrane the vATPase serves as an important regulator of extracellular pH of some closed compartments, such as the inner ear, epididymis and the distal tubules of the kidney. Defects in any one of the particular vATPase pumps found at these sites might cause disorders in humans such as deafness, acidosis and infertility (Figure 6) (Al-Awqati, 1996; Breton et al., 1998; Ferrary and Sterkers, 1998; Stankovic et al., 1997).

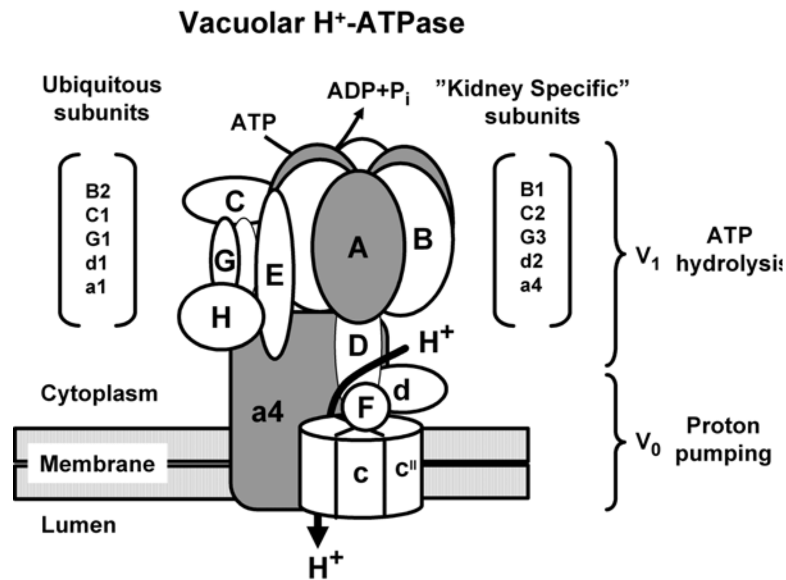

Figure 5: Structural model showing the vATPase complex. The V1 domain is located peripherally and is responsible for ATP hydrolysis. The V0 domain is an integral membrane associated domain and responsible for proton translocation across the membrane. Apart from the five "kidney specific" subunits, which show very specific expression in the kidney, epididymis and inner ear, most of the 14 identified subunits are expressed ubiquitously.

The structure of the vATPase

The vATPase consists of at least 14 different subunits assembled into two main domains, the cytoplasmic V_1 domain (640 kDa), which mediates ATP hydrolysis, and the membrane bound V_0 domain, which translocates H^+ across the membrane (Figure 5). The V_1 domain is composed of eight subunits (A to H; written with capital letters), with the two large subunits A and B arranged three and three together into the so called "head-piece" of the proton pump. The head piece mediates the hydrolysis of ATP, while the other six subunits form the "stalk" that connects the V_1 to the V_0 domain (Arata et al., 2002; Iwata et al., 2004). The proton transporting V_0 domain consists of at least 4 different subunits, a, c, d and e (written in lower-case letters), where six c-subunits form a ring structure and together with the a-subunit, which forms the proton channel, transfer the proton over the membrane (Figure 5) (Leng et al., 1996; Peng et al., 1999). The specific function of the two other V_0 subunits, d and e, is still unknown. When ATP is hydrolyzed by the V_1 complex, a rotation occurs where the stalk forces the ring formed by the c-subunits to rotate causing conformational changes of the a-subunit, which then folds into a proton channel (Figure 5) (Grabe et al., 2000; Gruber et al., 2001; Nelson and Harvey, 1999).

Localization and biological importance

Some of the identified subunits of the vATPase proton pump have received special attention during the last ten years, due to the fact that these subunits have been found to have tissue specific expression and also, in a mutated form, been linked to human disease (Finberg et al., 2002; Smith et al., 2002; Sun-Wada et al., 2005). These are the subunits B1, one of two isoforms of the B-subunit, encoded by the gene *ATP6V1B1*, and the a4-subunit, one of four known isoforms of the a-subunits and encoded by the *ATP6V0A4* gene (Karet et al., 1999a; Karet et al., 1999b; Smith et al., 2000). B1 and a4, often referred to as the "kidney-specific" subunits, are expressed in cells with specific requirement for acid/base transport across the

plasma membrane (Figure 5). These include the intercalated cells of the renal collecting duct, the FORE cells of inner ear and the narrow and clear cells of epididymis (Breton et al., 1996; Finberg et al., 2003; Nelson et al., 1992; Smith et al., 2001; Stehberger et al., 2003). Mutations in these two subunits have been identified in patients with inherited forms of dRTA with or without sensorineural hearing loss (Karet et al., 1999b; Smith et al., 2000; Stover et al., 2002). Other subunits of the vATPase proton pump that are found in a limited number of tissues are G3, C2 and d2, which all seem to have very specific expression in the kidney and therefore are included in the "kidney-specific" group of subunits (Figure 5) (Sun-Wada et al., 2005). Furthermore, these kidney specific subunits seem to assemble together selectively into the same multi-subunit vATPase pump. Besides being specifically expressed in the intercalated cells of the kidney, all of these subunits show specific expression in the epididymis and the inner ear (Figure 6) (Dou et al., 2004; Karet, 2005; Stankovic et al., 1997). However, even if some subunits have a specific expression pattern, most of the identified subunits of the vATPase proton pump show ubiquitous expression (Figure 5) and these are mostly associated with vATPases that are required for acidification of intracellular organelles (Boesch et al., 2003; Gluck and Caldwell, 1987; Niederstatter and Pelster, 2000; Sun-Wada et al., 2005).

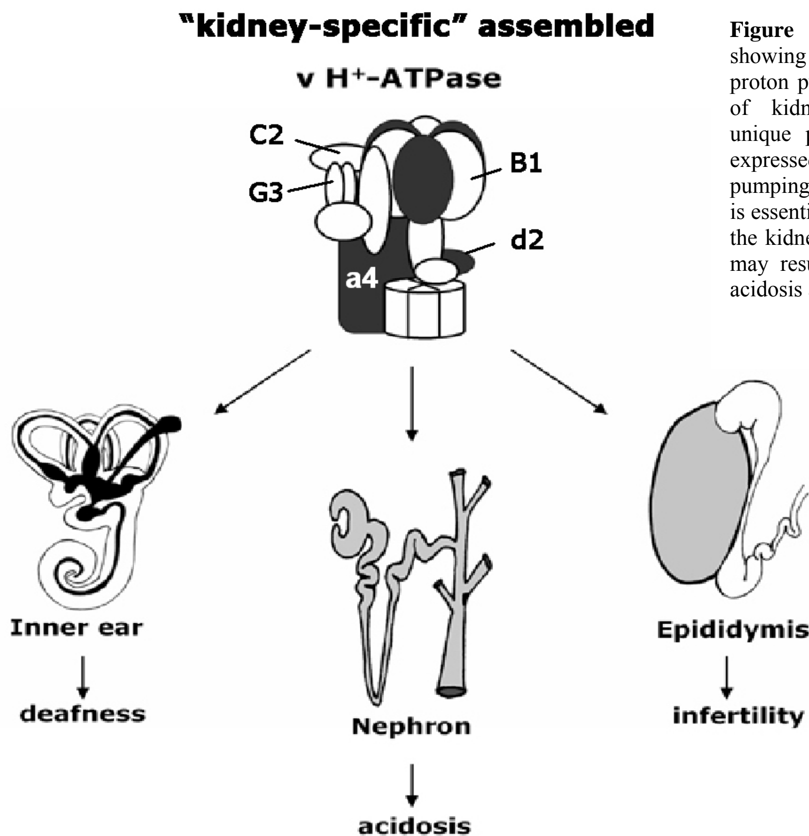

Figure 6: Schematic illustration showing the specific renal apical proton pump with selective assembly of kidney-specific subunits. This unique proton pump is specifically expressed in organs in which proton pumping across the plasma membrane is essential. Malfunction of any one of the kidney-specific vATPase subunits may result in disorders as deafness, acidosis and infertility

Pendrin (*SLC26A4*)

Pendrin belongs to the *Solute Carrier Family 26* (*SLC26*), a gene family of anion exchangers (Everett et al., 1997). Pendrin is a sodium independent Cl^-/HCO_3^- transporter, encoded by the *SLC26A4* gene, also known as the *PDS* gene (Scott et al., 1999). The sensorineural deafness syndrome, Pendred syndrome, is caused by mutations in the *SLC26A4* gene (Coyle et al., 1998). Pendrin is predominantly expressed in the thyroid gland (Bidart et al., 2000), the kidney (Everett et al., 1997; Royaux et al., 2001) and in the inner ear (Everett et al., 1999; Hulander et al., 2003). In the inner ear, pendrin is restricted to the areas composed of specialized epithelial cells thought to play a key role in regulating the composition and reabsorption of endolymph (Royaux et al., 2003). Physiological measurements of inner ear fluid, in the presence and absence of pendrin, have provided insight into the important role of pendrin for the high K^+ concentration in the cochlea and thus for the formation of normal endocochlear potential, both of which are known to be essential for normal auditory function (Royaux et al., 2003). In the kidney pendrin is expressed in the connecting tubule and the collecting duct and within these segments pendrin localizes to the apical plasma membrane of the HCO_3^- secreting β-IC cells (Figure 3) (Kim et al., 2002; Wall et al., 2003).

Anion exchanger AE1 (*SLC4A1*)

AE1 is a Cl^-/HCO_3^- exchanger and a member of the anion exchange family (AE), encoded by the *SLC4A1/AE1* gene. The *SLC4A1/AE1* gene uses different promoters to generate proteins with distinct N-terminal amino acid sequences. The longer transcript eAE1, is abundantly expressed in erythrocytes (red blood cells), while the shorter truncated kidney isoform, kAE1, lacks the first three exons and is abundantly expressed in the α-IC cells of the renal collecting duct (Alper et al., 1989). AE1 has been proposed to contribute to numerous physiological functions, including regulation of intracellular pH and cell volume (Alper et al., 1994; Hebert, 1986) as well as transepithelial Cl^- and acid/base transport in a wide variety of cell types (Al-Awqati, 1996; Kizer et al., 1995). Most mutations found in kAE1 cause autosomal dominant dRTA, but several reports from Thailand, Malaysia and New Guinea show that kAE1 mutations can also be found in patients with recessive dRTA (Bruce et al., 2000; Karet et al., 1998; Tanphaichitr et al., 1998; Vasuvattakul et al., 1999). AE1 has not been detected in the epididymal epithelia, but AE2, another member of the anion exchanger family, has been shown to be expressed in the basolateral membrane of epithelial cells lining the lumen of rat and mouse epididymis (Jensen et al., 1999a; Jensen et al., 1999b). Another member of the SLC4 family of anion exchangers is AE4. The AE4 protein has a very specific expression in the β-IC of the collecting duct epithelia, but the exact subcellular localization in the β-IC is still under debate (Figure 3). All members of the AE family mediate Cl^-/HCO_3^- exchange and their anion specificities appear to be similar (Jensen et al., 1999a; Tsuganezawa et al., 2001).

Carbonic Anhydrase type II (CAII)

Carbonic anhydrases (CA) are zinc metalloenzymes that catalyze the hydration of CO_2 resulting in the formation of HCO_3^- and H^+ (Khalifah, 1971; Maren, 1967). They are involved in the H^+/HCO_3^- secretion process of various epithelia, e.g. in the gastrointestinal tract, kidney, epididymis and inner ear (Tashian, 1989; Tsujikawa et al., 1993). To date, there are 15 known isoforms of CA which differ in their catalytic properties, tissue distribution and subcellular localization. CAI, CAII, CAIII are all cytoplasmic enzymes (Tashian et al., 1983) while CAIV is membrane bound (Wistrand and Knuuttila, 1989). CAII is a 29 kDa cytoplasmic isoenzyme expressed in a wide variety of tissues (Ferrell et al., 1978). CAII is expressed in most parts of the kidney and accounts for more than 95% of the CA activity there (Tashian, 1989; Wistrand and Knuuttila, 1989). CAII can interact with other specific acid/base transporters in the kidney, such as AE1 and pendrin, which markedly enhances H^+/HCO_3^- transportation (Alvarez et al., 2003; Sterling et al., 2001).

AQP2 and AQP9

Aquaporins (AQPs) are water channel proteins of 30 kDa size, known to transport water across various membranes in many different tissues (Agre et al., 1995). To date, ten mammalian AQPs have been identified and their cellular and tissue expression documented (Agre, 1997). Some AQPs are selective water pores (AQP2 and AQP4), while others are also permeable for small solutes (AQP3 and AQP5) (Yang and Verkman, 1997). AQP9, a neutral-solute and water channel, is expressed in the liver, testis, brain and lung in rats (Elkjaer et al., 2000; Tsukaguchi et al., 1998). Moreover, AQP9 is expressed in the apical membrane of principal cells of the epididymis (Pastor-Soler et al., 2001). AQP2 is a selective water pore with restricted expression in the principal cells of the kidney, as well as in specific cells of the endolymphatic sac epithelia (Brown and Breton, 2000; Merves et al., 2000).

PRESENT INVESTIGATION

Aims

In this study we focused on the biological importance of the forkhead transcription factor Foxi1. The specific function of this gene had not yet been determined. Thus, by using our Foxi1 deficient mouse model, as well as molecular information from patients, our overall aim was to elucidate the functional role of Foxi1 in the inner ear, kidney and epididymis.

Specific goals of the different studies reported in this thesis were to

- determine the importance of Foxi1 for hearing, by investigating the involvement of FOXI1 transcriptional regulation of the *SLC26A4* gene in the pathogenesis of Pendred syndrome and nonsyndromic EVA hearing loss (Paper I).
- investigate the importance of Foxi1 for adult kidney function (Paper II).
- elucidate the importance of Foxi1 for male fertility, by studying the expression and function of Foxi1 in epididymis (Paper III).
- address the importance of Foxi1 as a regulator of the vacuolar H^+-ATPase proton pump in the inner ear, kidney and epididymis (Paper IV).

RESULTS AND DISCUSSIONS

1. The importance of Foxi1 for hearing

Transcriptional Control of *SLC26A4* Is Involved in Pendred Syndrome and Nonsyndromic Enlargement of Vestibular Aqueduct (*DFNB4*) (Paper I)

Appropriate control of volume and ionic composition of the endolymph is crucial for normal hearing. Therefore many mutations in genes encoding for ion transporters and pumps have been shown to cause deafness (Delpire et al., 1999; Dixon et al., 1999; Kubisch et al., 1999). One of these genes is the *SLC26A4*, encoding the anion exchanger pendrin. Mutations in the *SLC26A4* gene have been shown to cause the autosomal-recessive disorder Pendred syndrome (PS) and the non-syndromic hearing loss associated with enlarged vestibular aqueduct (EVA; *DFNB4*) (Everett et al., 1997; Li et al., 1998). Even if PS accounts for as much as 10% of hereditary deafness in humans, many patients with clinical diagnosis of PS lack mutations or are heterozygous with only one mutation in the coding region of the *SLC26A4* gene, indicating that there might be other genes contributing to the same symptoms (Reardon et al., 2000). The observations that Foxi1 acts as an upstream regulator of *Slc26a4* in the mouse endolymphatic epithelium and that mice deficient for Foxi1 display very similar phenotype as mice lacking pendrin (Everett et al., 2001; Hulander et al., 2003), raised the question whether the human FOXI1 gene could be a candidate gene for causing PS and EVA. In this study our goal was to elucidate the suggested involvement of FOXI1 transcriptional regulation of the *SLC26A4* gene in both PS and EVA.

To identify additional disease linked mutations of PS and EVA, we started by searching in non-coding promoter sequences upstream of the *SLC26A4* gene in all PS patients with effected sibling or with classic PS phenotype, that either lacked mutations or only had one mutation (heterozygous) in the *SLC26A4* gene. Three upstream regions were chosen for mutations screening due to their high homology with the mouse sequence. One mutation was identified that affected *SLC26A4* expression. This mutation, a single nucleotide change from C to T (T103C), was localized within a consensus binding sequence important for FOXI1 activation (Pierrou et al., 1994) of the *SLC26A4* gene and moreover, this mutation, which was found in 9 of 429 patients, completely inhibited FOXI1 activation (Figure 1 and Figure 2 in Paper I). During this analysis of FOXI1 *cis*-elements on the *SLC26A4* promoter, we discovered two adjacent FOXI1 binding sites in a unique head-to-head structure, i.e. in opposite direction, and moreover, this specific orientation was required for FOXI1 transcriptional activation (Figure 2 in Paper I). Remarkably, when compared to other two FOXI1 specific binding sites upstream of the two recently identified FOXI1 target genes, *SLC4A9* (encoding AE4) (Kurth et al., 2006) and *ATP6V1B1* (encoding the B1 subunit of the

vATPase proton pump) (Paper III), all motifs had a similar second adjacent binding sites in the opposite direction (Figure 7 in Paper I). This unique structure suggests that the FOXI1 protein needs this conserved head-to-head arrangement to interact in protein-DNA specific manner.

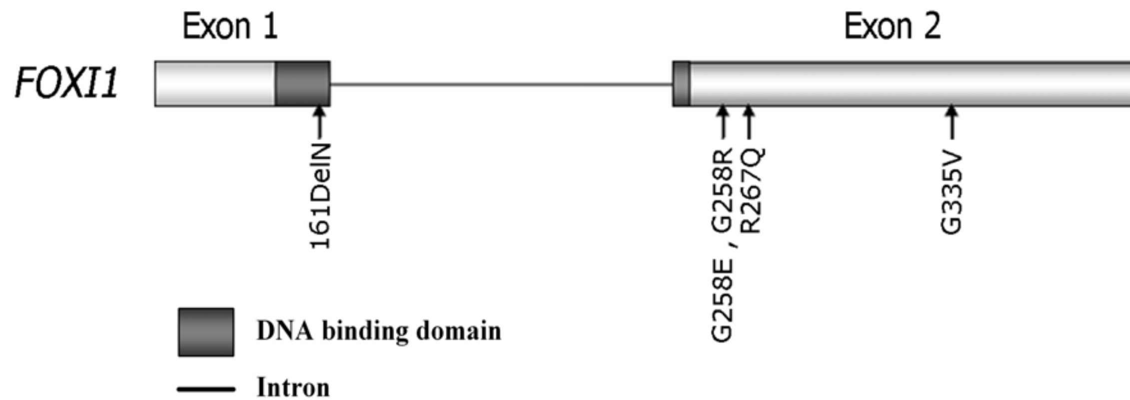

Figure 7: A schematic drawing of the *FOXI1* gene structure, the two exons and the intron in between. Arrows indicate the position of the five mutations found in the *FOXI1* gene in patients with Pendred syndrome and nonsyndromic EVA.

Having obtained the unique and interesting results of elucidating the role of a *cis*-element in the pathogenesis of non-syndromic hearing loss, we continued pursuing the goal of identifying mutations in the *FOXI1* gene that could contribute to the genetic cause of the PS and EVA. We found five mutations in the *FOXI1* gene (Figure 7) that, when introduced into FOXI1 expression vector, all reduced its transcriptional activity (table 2, Figure 3 and Figure 4 in Paper I). Three of these mutations, the 161DelN, R267Q and G335V, were conserved between human and mouse, indicating a specific relevance of these locations for FOXI1 activity and moreover, one of them, the 161DelN mutation, was found in the conserved forkhead domain (Figure 7). This is the first reported mutation analysis connecting the human *FOXI1* gene to sensorineural human deafness. Notably, all five mutations found were non-synonymous, meaning that all patients were heterozygous for the mutations. As PS displays a recessive mode of inheritance, this indicates that *FOXI1* mutations probably work in a combination with other mutations, such as the conventional *SLC26A4* mutations or other as yet unidentified mutations, to cause the disease phenotype. These speculations are based on our dosage-dependent model for the pathogenesis of both PS and EVA that involves both the *SLC26A4* gene and its transcriptional machinery, where mutations lead to reduced expression levels below requirements for normal inner ear function. Moreover, during the mutation screen we identified a patient displaying inheritance in a double-heterozygous manner, i.e. with a single mutation in both genes, *FOXI1* and *SLC26A4*. This inheritance pattern was later supported by the phenotype displayed by our double-heterozygous mouse mutant (Slc26a4+/-;

Foxi1+/-) (Figure 6 in Paper I), where changes in the temporal bone as well as enlargement of the vestibular aqueduct (EVA) could be observed.

Taken together, in this study we show that mutations either in the coding sequence of the human *FOXI1* gene or in the FOXI1-binding *cis*-element upstream of the *SLC26A4* gene, can in a combination with *SLC26A4* mutations or with yet-to-be-identified mutations cause sensorineural hearing loss with either EVA or PS-EVA phenotype. To our knowledge, this is the first time a digenetic inheritance has been shown to cause human deafness. These findings further support our earlier suggestions of the importance of FOXI1 transcriptional control of the *SLC26A4* gene as well as the dosage-dependent model of the molecular pathogenesis of both PS and EVA that involves both *SLC26A4* and its transcriptional regulation.

2. The importance of Foxi1 for kidney function

Distal renal tubular acidosis in mice that lack the forkhead transcription factor Foxi1 (Paper II)

In this study our goal was to investigate a possible role for Foxi1 as a regulator of adult kidney function. As mentioned earlier, in 1997 Overdier and colleagues detected Foxi1 expression in the distal tubules of the embryonic and adult mouse kidney (Overdier et al., 1997). However, Foxi1-/- mice show no obvious signs of any kidney failure. In Paper I we found that Foxi1 regulated pendrin expression in the inner ear and pendrin is missing in the "mitochondria rich" cells, or FORE cells of Foxi1-/- inner ears (Hulander et al., 2003). Pendrin is normally expressed in the kidney and we therefore became interested in the possibility that Foxi1 might also regulate pendrin expression in the kidney, which might then lead to mild alkalosis in Foxi1-/- mice. Unexpectedly, our Foxi1-/- mice developed dRTA with elevated pH in their urine when they were challenged with both chronic and acute acidic load (Figure 7 in Paper II). This observation could not be explained by the lack of pendrin expression, so we set out to find other Foxi1 target genes in the distal nephrons.

Basic defects of dRTA are linked to acid-base homeostasis regulated by the proton/bicarbonate secreting intercalated cells, α-IC and β-IC, of the renal collecting duct (Wagner et al., 2001; Vargas-Poussou et al., 2006). Our primary target genes became therefore those genes known to be specifically expressed in the intercalated cells of the collecting duct as well as causing dRTA when they are mutated. By using tissue sections from Foxi1-/- kidneys we examined the expression level of two of the most important genes for acid-base regulation of the intercalated cells, the genes encoding the kidney specific anion exchanger kAE1 (*SLC4A1*) and the B1 subunit of the vATPase proton pump (*ATP6V1B1*). Apart from these two genes we also studied the potential role of Foxi1 to regulate the pendrin gene, *SLC26A4*, in the kidney, as well as the gene encoding the K^+/Cl^- co-transporter Kcc4 (*SLC12A7*), which has also been linked to dRTA (Boettger et al., 2002).

Our results demonstrate a specific expression of Foxi1 in both α-IC and β-IC. This was established by showing that Foxi1 co-localizes with the α-IC specific marker genes kAE1 and B1, as well as with the β-IC marker pendrin. No expression of kAE1, B1 or pendrin could be detected in tissue sections from Foxi1-/- kidneys (Figure 3 and Figure 4 in Paper II). This indicates a potential role for Foxi1 as a transcriptional regulator of these genes in the renal collecting duct. To confirm this potential Foxi1 regulation of kAE1, pendrin and B1, we set up co-transfection luciferase assays in COS7 cells. As can be seen in figure 5B (Paper II) and figure 6B (Paper III), the luciferase reporters containing the promoter sequence for these genes showed significant activation when co-transfected with Foxi1. However, the Kcc4 co-transporter remained unaffected in kidneys from Foxi1 deficient mice (Figure 5 in Paper II). The anion exchanger AE4, another member of the SLC4 family of anion exchanger factors, has been shown to be specifically expressed in the β-IC, even if its subcellular localization in the β-IC is still under debate (Royaux et al., 2001; Tsuganezawa et al., 2001). In this study we showed a clear evidence of AE4 expression in the basolateral membrane of β-IC (Figure 4 in Paper II) and moreover that the expression is absent in Foxi1-/- kidneys. Later, these results led to the identification of Foxi1 binding sites on the upstream region of the AE4 gene and the establishment of direct transcriptional activation of the AE4 promoter by Foxi1 (Kurth et al., 2006).

Another interesting discovery was that Foxi1 not only appears to regulate gene expression of genes important for acid-base activity of the intercalated cells, but also to regulate their morphological development (Figure 8) (Figure 1 in Paper II). It first became apparent that cells of the renal collecting duct had altered ultrastructure when Foxi1-/- kidneys were analyzed using electron microscopy. Epithelial cells from Foxi1-/- cortical collecting ducts were missing their normal protruding microvilli from their apical borders (Figure 8). This alteration became more evident later when their gene expression profile was revealed, showing a single cell type positive for both AQP2 and CAII (AQP2+,CAII+) instead of two distinctive cells types, the intercalated cells (AQP2-, CAII+) and the principal cells (AQP2+, CAII-), which under normal circumstances form the epithelium of the collecting duct (Brown et al., 1983; Deen et al., 1994; Kurth et al., 2006). It can thus be speculated that this single cell type found in the collecting duct of Foxi1-/- kidneys can be regarded as a possible common progenitor cells for intercalated and principal cells, and that Foxi1 might be the key regulator for the molecular switch to drive this cell into becoming either principal or intercalated cell.

On a normal laboratory diet Foxi1-/- mice have only minor problems in handling their fluid and acid-base regulation. This can probably be explained by the very low acidity of normal animal chow. We have shown that the progenitor cells express AQP2 and CAII. It can not be ruled out that they also express some proton transporters, such as an apically expressed H^+-K^+-ATPase (Fejes-Toth and Naray-Fejes-Toth, 2001), which then might help them to regulate their acid-base balance under moderate challenge. It is however clear, that Foxi1 expression is

needed for proper cellular differentiation into intercalated cells (with Foxi1) and principal cells (without Foxi1) and to maintain the normal acid-base capacity of the collecting duct. This is in good agreement with the rather late appearance of Foxi1 in the developmental process of the kidney (~ E15.5 in mice) (Jouret, 2005), which rules out a major effect on earlier events in that process. Interestingly, it has also been shown that the "kidney- specific" subunits of the vATPase proton pump (B1, G3, C2 and a4), as well as pendrin, are all expressed later then the ubiquitous isoforms in the distal tubules, with a progressive induction after Foxi1 appearance (Jouret, 2005). Altogether, these results strongly support the role of Foxi1 in the differentiation process of the intercalated cells.

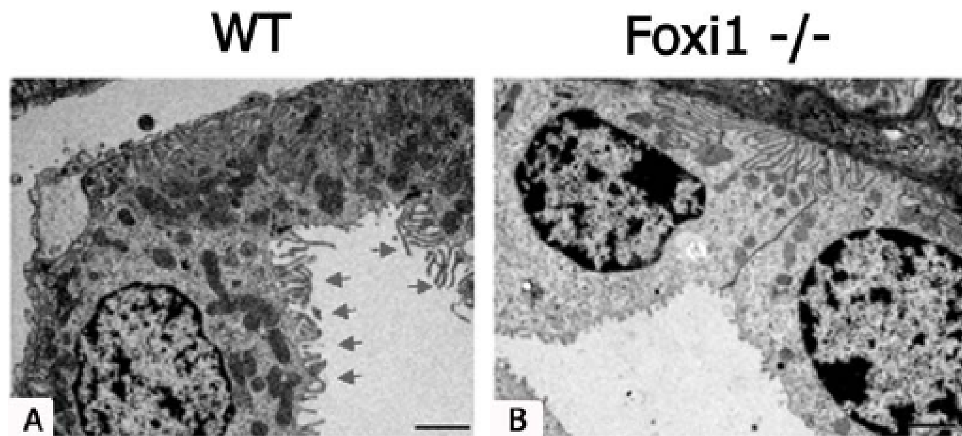

Figure 8: Electron microscopy of the cortical collecting duct in mouse kidney. (A) Cells from a WT kidney with protruding "tussock-like" microvillous apex of the cells (arrows). (B) Cells from Foxi1 -/- kidney which appears to lack these microvillous structures.

Thus, in this study we have demonstrated that Foxi1-/-mice develop dRTA when challenged with an acidic load, due to their failure of inducing proper differentiation of the distal tubule epithelia, as well as their defective transcriptional activation of kAE1 and the B1 subunit of the vATPase proton pump in the α-IC (evidence combined with the results in Paper III). This suggests a crucial role for Foxi1 in the normal function of intercalated cells of the collecting duct, where it regulates the proper gene expression profile needed for these cells to differentiate and to maintain normal proton and bicarbonate secretion.

3. The importance of Foxi1 for male fertility

Epididymal expression of the forkhead transcription factor Foxi1 is required for male fertility (Paper III)

When we discovered that male mice deficient for Foxi1 (Foxi1-/-) never gave rise to any offspring, while Foxi1-/- females became pregnant and produced normal litters, we decided to investigate the possible role of Foxi1 in this phenotype. We set out to examine if Foxi1 was expressed in the male reproductive tissue, i.e. in the testis and/or in the excurrent duct system (the efferent duct, epididymis and vas deferens). Foxi1 was not found to be expressed in the testis but showed a specific expression in a distinct subgroup of cells found scattered throughout the epithelium lining all parts of the epididymis, i.e. in the initial segment, caput, corpus and cauda (Figure 1 in Paper III). These observations, and the fact that in our previous work we had established Foxi1 as an important gene for acid-base regulation of intercalated cells of the renal collecting duct (Paper II), lead us to investigate more closely the proton secreting cells of the epididymal epithelium, the narrow and clear cells.

As mentioned earlier, the epididymis is an essential part of the male reproductive system. In the tubular lumen of epididymis the spermatozoa undergo the final maturation, or “post testicular maturation”, a process dependent on highly regulated microenvironment of the epididymal fluid (Bedford, 1975; Jones and Murdoch, 1996). Altered pH of the epididymal microenvironment results in abnormal sperm maturation which can affect their mobility, morphology and viability (Bedford, 1975). The epididymal fluid in the distal epididymis is relatively acidic (pH 6.8), which is controlled by active proton secretion from vATPase proton pump located apically on the narrow and clear cells. By using antibody against Foxi1 on tissue sections from wt epididymis we found a complete co-localization of Foxi1 and the B1 subunit of the vATPase proton pump in the same subset of cells (Figure 2 in Paper III), meaning that Foxi1 expression is restricted to narrow and clear cells (Figure 4). Remarkably, no expression of B1 could be detected in Foxi1-/- tissue sections, indicating that Foxi1 is required for the expression of the B1 subunit in narrow and clear cells (Figure 2 in Paper III). Furthermore, to study the possible role of Foxi1 to act as an upstream regulator of the *ATP6V1B1* gene, which codes for the B1 subunit, we performed transfection experiments and shift assays, whereupon we could assess if Foxi1 could specifically bind to and activate the *ATP6V1B1* promoter. As can be deduced from figure 6 (Paper III), Foxi1 binds directly to the promoter and induces its activity in a dose-dependent manner. This activation is completely abolished when the potential Foxi1-binding motif on the upstream sequence is mutated. These results suggest that Foxi1 plays an important role in regulating proton secretion in narrow and clear cells of the epididymis. It is also well recognized that CAII expression is crucial for narrow and clear cells proton secretion, so by showing that CAII expression is lost in initial

segment and caput in Foxi1-/- epididymal tissue sections we further supported our suggested role of Foxi1 as a key regulator of acid-base regulation in epididymis (Figure 4 in Paper III).

Since in our previous studies we had shown that Foxi1 regulates the anion transporter pendrin in inner ear and kidney and to our knowledge no one had looked at pendrin expression in the epididymis, we set out to see if similar relationship exists between Foxi1 and pendrin in the epididymal epithelium. By using both *in situ* hybridization and antibody staining, we established a clear expression of pendrin in cells positive for both Foxi1 and B1 expression, i.e. in the narrow and clear cells (Figure 2 and Figure 3 in Paper III). This epithelial expression of pendrin, which was mainly located in the apical/perinuclear compartment of the narrow and clear cells, could not be detected in Foxi1-/- tissue, demonstrating the requirement of Foxi1 for pendrin expression in the epididymis.

One of the most notable differences in the spermatozoa morphology during maturation are the structural changes of the sperm tail, which happens at the same time as the middle-piece cytoplasmic droplet, the remnant of the spermatid cytoplasm, migrates from the sperm-neck to more distal regions until it becomes lost right before ejaculation. As can be seen in figure 7 (Paper III), a much higher proportion of sperm cells from Foxi1-/- males show a clear sign of altered sperm morphology with tail angulation and cytoplasmic droplet when compared to sperm cells from wt males. This phenotype has been linked to infertility (Yeung et al., 2002). It is well known that under normal circumstances the luminal fluid undergoes some very important changes on its way through the epididymal tube, both in ionic composition as well as in pH, from a pH of 7.4 in the proximal segments to the more acidic level of pH 6.8 in the distal cauda epididymis (Cosentino and Cockett, 1986; Levine and Kelly, 1978) and that these regional changes are responsible for these morphological changes of the sperm cells (Dott, 1968; Fawcett and Phillips, 1969). This, together with the fact that the epididymal fluid was measured to be 0.5 pH units higher in the Foxi1-/- males compared with wt littermates, supports our view that the lack of both the B1 subunit of the vATPase and CAII severely inhibits proton secretion from narrow and clear cells in Foxi1-/- epididymis and hence produces a less appropriate luminal microenvironment for proper sperm maturation.

Taken together, the results presented in this paper show that Foxi1-/- males are infertile due to their inability to give rise to fully mature sperm. This is associated with a failure in normal epididymal fluid acidification, together with increased luminal area and organ weight (Figure 7G and Figure 7H in Paper III), all findings indicating abnormal acid-base homeostasis of the epididymal fluid in the Foxi1-/- males. This is consistent with the important role of Foxi1 in regulating genes necessary for creating the microenvironment needed for proper epididymal prost-testicular sperm maturation. It has been estimated that human infertility is associated with defects in the male reproductive tract in as much as 30% of all affected couples (Elzanaty et al., 2002; Pastor-Soler et al., 2005). With our results at hand, and the fact that luminal acidification is achieved by the vATPase proton pump, it could be predicted that male

fertility might be altered in patients harboring mutations in the human *FOXI1* gene, located at 5q34, and that *FOXI1* could prove to be a new candidate gene in therapeutical approaches towards male fertility.

4. The importance of Foxi1 for proton secretion in inner ear, kidney and epididymis

The forkhead transcription factor Foxi1 is a master regulator of vacuolar H^+-ATPase proton pump subunits in the inner ear, kidney and epididymis (Paper IV)

Active translocation of protons across plasma membranes by the vATPase proton pump and thus active acidification of extracellular compartments such as the endolymphatic duct of the inner ear, collecting duct of the kidney and the epididymal lumen, is essential for adequate function of these organs (Al-Awqati, 1996; Breton et al., 1996; Stankovic et al., 1997). Foxi1 has previously been shown to be expressed in the major proton secreting cells of endolymphatic, epididymal and renal collecting duct epithelia and moreover, mice lacking Foxi1 develop early onset deafness (Hulander et al., 2003; Hulander et al., 1998), distal renal tubular acidosis (Paper II) and male infertility (Paper III). With this knowledge at hand, together with the fact that Foxi1 expression in the epithelia of renal collecting duct and epididymis is required for expression of the kidney-specific gene *ATP6V1B1*, encoding the B1 subunit of the vATPase proton pump (Paper II and Paper III), our aim was to investigate the role of Foxi1 as a regulator of vATPase proton pump subunits in inner ear, kidney and epididymis.

We started by extending our previous observations and study the expression of the B1 subunit in the endolymphatic epithelia of mouse inner ear. Since most patients with recessive dRTA associated with sensorineural deafness, have mutations in the *ATP6V1B1* gene (Karet et al., 1999b; Smith et al., 2000; Stover et al., 2002), not surprisingly, the B1 subunit has been located to the apical membrane of the proton secreting cells of the endolymphatic sac (Dou et al., 2004; Karet et al., 1999a). By using immunostaining with antibodies specific for the B1 subunit and Foxi1 we showed a clear co-localization between the two proteins in the same population of epithelial cells in the endolymphatic sac/duct (Figure 1 in Paper IV). No staining could be detected in endolymphatic epithelium from Foxi1-/- inner ears (Figure 1 in Paper IV). These results establish the requirement of active Foxi1 for B1 expression in the proton secreting FORE cells of the endolymphatic epithelia. This together with our earlier findings in kidney and epididymis confirms that Foxi1 is an essential factor for expression of B1 in these three epithelia.

Another interesting kidney-specific gene is *ATP6V0A4*, encoding the a4 subunit of the vATPase proton pump. It is well established that mutations in the *ATP6V0A4* gene cause

recessive dRTA and in most cases affect the hearing of the patients as well (Karet et al., 1999b; Smith et al., 2000; Vargas-Poussou et al., 2006). The a4 subunit is specifically expressed in the IC of the renal collecting duct (Oka et al., 2001; Smith et al., 2000) and narrow and clear cells in epididymis (Pietrement et al., 2006) and recent data have also confirmed a co-localization of the a4 subunit, pendrin and CAII within the endolymphatic epithelia (Dou et al., 2004). With these observations at hand we then asked the question of whether a similar relationship exists between a4 and Foxi1 in the inner ear, kidney and epididymis as was established between B1 and Foxi1. Double immunostaining of tissue sections from these three organs revealed a clear co-localization of apical expressed a4 subunit and the nuclear expressed Foxi1 in FORE cells of the endolymphatic epithelia (Figure 1 in Paper IV), intercalated cells of the collecting duct (Figure 2 in Paper IV) and narrow and clear cells in the epididymis (Figure 3 in Paper IV). This immunostaining was absent in tissue sections from Foxi1-/- mice (Figure 1, 2 and 3 in Paper IV).

To verify the potential role of Foxi1 in regulation of the a4 subunit we identified four potential Foxi1 binding sites (Fk1-Fk4), within the 1.0 kb promoter region immediately upstream of the *ATP6V0A4* gene (Figure 4 in Paper IV). With this promoter region cloned into luciferase reporter vector we performed transient co-transfection experiments with Foxi1 expression vector in COS7 cells. Transfection results showed a dose dependent activation of the reporter constructs by Foxi1 (Figure 4 in Paper IV). Furthermore, by using site-directed mutagenesis a cluster of three Foxi1 binding sites (Fk1- Fk2- Fk3) was shown to be a major contributor to this promoter activation. In electrophoretic mobility shift assays we could also demonstrate a significant Foxi1 binding to the Fk1-3 cluster and much weaker interaction with the Fk4 site (Figure 4 and 5 in Paper IV). Taken together, these results suggest that Foxi1 is able to interact with the *ATP6V0A4* promoter by binding directly to this Fk1-3 *cis*-elements cluster in a sequence specific manner. Apart from our earlier reported discovery of Foxi1 transcriptional regulation of the B1 subunit (Paper II and III), to date relatively little is known about the transcriptional regulation of genes encoding the subunits of the vATPase proton pump. Here we present evidence showing that Foxi1 directly binds to and activates the *ATP6V0A4* promoter in vitro and thus establish the *ATP6V0A4* gene as a direct target of Fox1 trans-activation in FORE cells of the inner ear, intercalated cells of the kidney and narrow and clear cells in epididymis.

Data presented in this paper together with our previously reported results (Paper II and Paper III) establishes Foxi1 as a key regulator of the two subunits a4 and B1 in kidney, epididymis and inner ear, suggesting a specific role for Foxi1 in transcriptional regulation of kidney-specific subunits of the vATPase proton pump at these locations. However, to our surprise we also discovered that the ubiquitously expressed subunits A1 and E2 co-localize with Foxi1 in the endolymphatic (Figure 1 in Paper IV), collecting duct (Figure 2 in Paper VI) and epididymal epithelium (Figure 3 in Paper IV). Furthermore, we also showed that their

expression was absent in tissue sections from Foxi1-/- mice. This indicates that not only the expression of the kidney-specific subunits B1 and a4, but also the ubiquitous A1 and E2 is dependent on Foxi1 expression in these epithelia. Since it has been shown that a proper assembly of subunits into the vATPase protein complex is important for correct cellular localization as well as subunit stability (Forgac, 1999; Liu et al., 1997), we would like to speculate that a possible explanation to the absence of A1 and E2 could be that the lack of the a4 and B1 subunits affects the process of subunit assembly and consequently leads to their destabilization and degradation. Thus, our observations lead to the hypothesis that Foxi1 is of major importance for proper assembly of a functional vATPase complex in proton secreting cells of the inner ear, kidney and epididymis.

CONCLUSIONS AND CLOSING REMARKS

Foxi1 is an essential transcription factor in "mitochondria-rich" cells of the epididymis, the renal collecting duct and the endolymphatic epithelia.

The male reproductive system, including the epididymis, arise from the mesonephric (Wolffian) duct and the surrounding mesenchyme that develops as a part of the mesonephric kidney, that later becomes the definitive metanephric kidney (Kirchhoff, 1999; Saxen and Sariola, 1987). This indicates that all cells of the renal collecting duct and the epididymis share a common embryological origin in the Wolffian duct (Al-Awqati and Schwartz, 2004). Since both organs are actively engaged in fluid and electrolyte regulation it is not surprising that both epithelia contain a subset of very similar epithelial cells that are specialized for proton and/or bicarbonate secretion. These are the intercalated cells of the collecting duct and the narrow and clear cells of the epididymis. Both cell types share several typical features and because of their high concentration of mitochondria compared to adjacent epithelial cells they have been grouped into a family of cells known as "mitochondria-rich" cells (Brown and Breton, 1996).

The ectoderm derived FORE cells of the endolymphatic sac/duct epithelia are also "mitochondria-rich" cells. They show a striking similarity to the mesodermally derived intercalated cells and narrow and clear cells in both cellular morphology and their expression of genes important for ionic and proton transportation (Figure 9) (Hulander et al., 2003; Peters et al., 2001; Qvortrup and Bretlau, 2002). Even if the FORE cells do not share the same Wolffian duct origin as the intercalated cells and the narrow and clear cells, they appear to express key pH regulating proteins in common and rely on similar mechanisms for proton/bicarbonate transport (Figure 9). This argues in favor of evolutionary convergence of the secretory mechanism in these cells. All three cell types express high levels of vATPases on their surface and CAII in their cytosol (Brown et al., 1988; Dou et al., 2004; Hermo et al., 2000) (Papers II, III and IV). Furthermore, all cells show a very specific expression patterns of certain types of HCO_3^- exchangers, such as the AE1, AE2, AE4 and pendrin, either on their basolateral membrane (for reabsorption) or the apical membrane (for secretion) (Figure 9) (Everett et al., 1997; Hulander et al., 2003; Jensen et al., 1999b; Royaux et al., 2001; Tsuganezawa et al., 2001)(Papers II and III). All these common features, as well as the human diseases caused by the disruption of any one of them, confirm their indispensable role in acid-base homeostasis of each organ (Figure 9).

In the light of the results presented in this thesis, we would like to add Foxi1 to the list of common features for these three types of "mitochondria-rich" cells as a new characteristic factor (Figure 9). Our work provides evidence showing that Foxi1 specifically co-localizes in

the same cells as the two "kidney-specific" subunits of the vATPase proton pump, B1 and a4, as well as the anion exchangers AE1, AE4 and pendrin, all being characteristic proteins of the "mitochondria-rich" cells. Moreover, our data show that all of these ionic channel proteins are completely absent in Foxi1-/- tissue, establishing Foxi1 as an essential transcription factor for the expression of their genes and an important regulator for proper assembly of the vATPase complex in "mitochondria-rich" cells (Papers II, III and IV). Even if the importance of many of these acid/base transporters has already been revealed, the regulation of their expression and function has, to date, not been fully understood. Our observations, and the fact that our Foxi1-/- mice display a phenotype very similar to the symptoms seen in patients with inherited mutations in one or more of these genes, lead us to propose that Foxi1 has a fundamental role in the gene regulation in "mitochondria-rich" cells and thus, in the regulation of fluid composition of the inner ear, epididymis and renal collecting duct. These speculations are to some extent confirmed by the discovery that mutations in the human *FOXI1* gene give rise to sensorineural human deafness, Pendred syndrome and nonsyndromic enlargement of vestibular aqueduct (EVA) (Paper I). Finally, based on the results presented here and our earlier findings (Hulander et al., 2003), we would like to predict that homozygous mutations in the human *FOXI1*, located at 5q34, might cause a sensorineural deafness syndrome with distal renal tubular acidosis and male infertility.

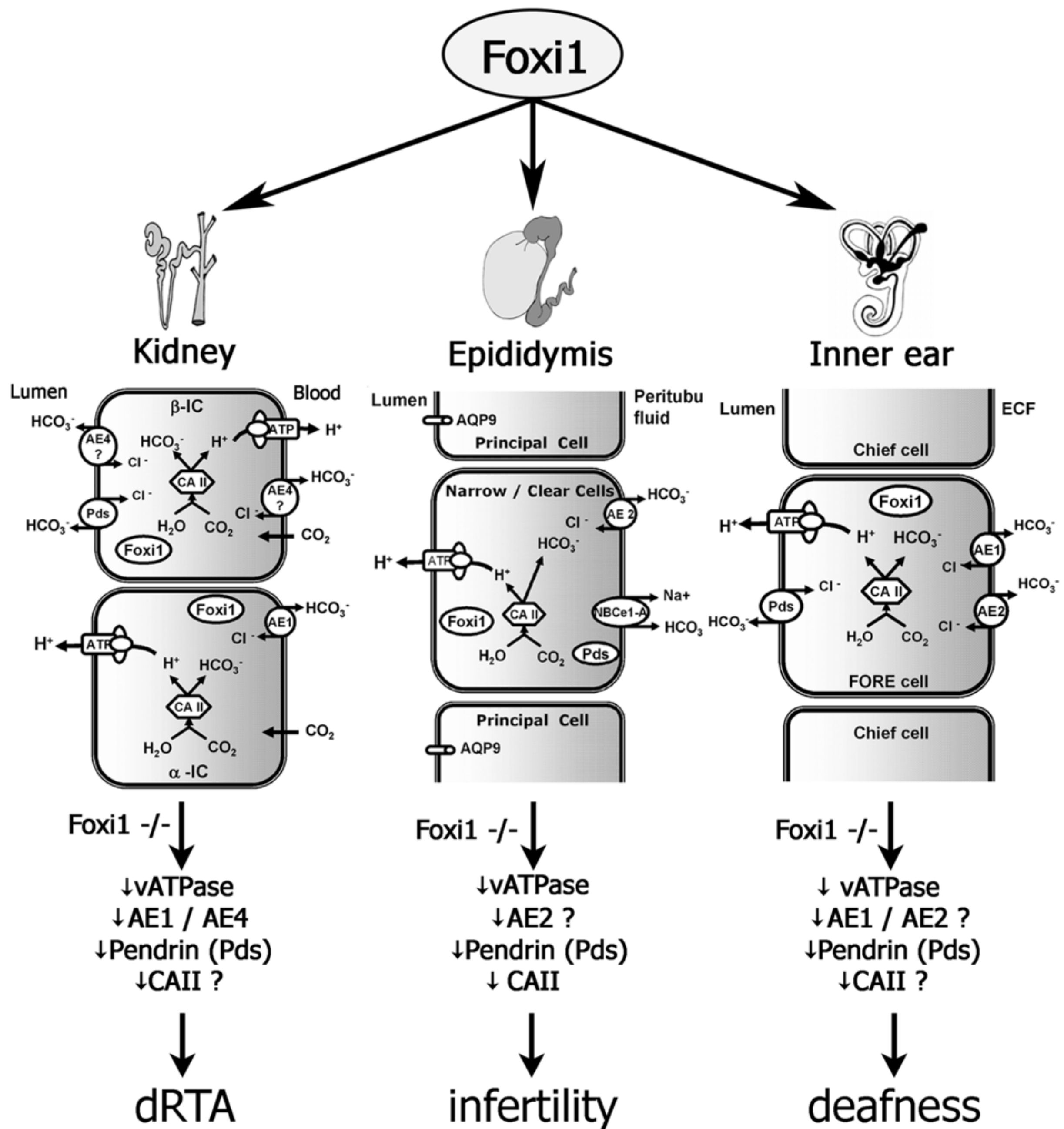

Figure 9: Comparison of the intercalated cells (IC) of renal collecting duct, the Narrow and Clear cells of epididymis and the FORE cells of inner ear. These are all acid-base regulating "**mitochondria rich**" cells and they share many molecular properties, including the presence of carbonic anhydrase II (CAII) and a specific cellular distribution of vATPase proton pumps and anion exchangers (AE and pendrin). Foxi1 is specifically expressed in these "mitochondria rich" cells. In mice deficient for Foxi1 (Foxi1-/-) many of the acid-base regulating molecules are missing resulting in deafness, acidosis (dRTA) and male infertility. (? = not been shown to be missing in Foxi1 -/- mice).

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"And now to something completely different"
Monty Python

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