Male congenital hypothyroid $Pax8^{-/-}$ mice are infertile despite adequate treatment with thyroid hormone

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Abstract

Severe forms of congenital hypothyroidism lead to serious clinical symptoms if thyroid hormone replacement therapy is not instituted immediately after birth. In this study, $Pax8^{-/-}$ mice that are born without a thyroid gland were used as an animal model to study the consequences of congenital hypothyroidism. As expected, adequate treatment of these animals with thyroxine restored the general deficits of congenital hypothyroidism; however, $Pax8$ -deficient male mice were infertile. We report here that in these mice, the efferent ducts and epididymides are either absent or the efferent ducts exhibit a reduced lumen and extensive connective tissue, which appears to impair testicular drainage and subsequently leads to complete absence of spermatozoa from the epididymis. The results suggest that, starting with the onset of pubertal testicular fluid secretion, a backpressure is created

in the testis by the absence of efferent ducts or constriction of their tubule lumen when present. This subsequently leads to secondary disorganization of the seminiferous epithelium that increases with age, resulting in mixed atrophy of the testis in the adult. Serum testosterone levels as well as mRNA expression of anterior pituitary hormones are in the normal range, indicating that the observed infertility is not due to hormonal imbalance, but rather to a developmental defect of the efferent ducts. The demonstration of $Pax8$ expression in the epithelia of the epididymis and the efferent ducts suggests a direct morphogenic role of $Pax8$ in the development of these organs. It remains to be elucidated whether congenital hypothyroid male patients with mutations in the $Pax8$ gene are similarly affected.

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Introduction

Thyroid hormones (TH) are essential for development, growth, metabolism, and reproduction (Porterfield & Hendrich 1993, Oppenheimer & Schwartz 1997, Anderson *et al.* 2003, Choksi *et al.* 2003, Bernal 2005). The physiological importance of TH becomes most evident under the conditions of congenital hypothyroidism (CH), a common disorder mostly caused by thyroid dysgenesis or agenesis, affecting 1 in 3500 newborns (Kopp 2002, De Felice & Di Lauro 2004). If not treated immediately, CH leads to cretinism, a syndrome characterized by metabolic disturbances, growth retardation, severe neurological defects, and mental retardation.

Among other factors, mutations in the paired box gene 8 ($Pax8$) have been associated with CH and severe thyroid hypoplasia in humans (Macchia *et al.* 1998). Correspondingly, in mice with deletions of the $Pax8$ gene, the thyroid gland is completely devoid of TH-producing follicular cells (Mansouri *et al.* 1998). Therefore, these athyroid mice provide an ideal animal model for CH, especially since no

further defects have been observed in other tissues expressing $Pax8$ such as the kidney or some hindbrain regions, which is probably due to a partial redundancy with the highly homologous $Pax2$ and $Pax5$ genes (Mansouri *et al.* 1994, 1998).

As a consequence of their athyroidism, $Pax8^{-/-}$ mice are deaf, growth retarded, ataxic, and do not survive weaning. As in most cases of congenital hypothyroid patients (Larsen *et al.* 2003, Roberts & Ladenson 2004), the symptoms can be reversed in $Pax8$ -deficient mice by TH replacement therapy if instituted within the first days of postnatal life (Christ *et al.* 2004, Friedrichsen *et al.* 2004). However, male $Pax8^{-/-}$ mice that were properly substituted with TH and developed otherwise normally without any overt deficits, did not become fertile. Therefore, we decided to study the reproductive system of these animals. Our analysis revealed that the infertility of these mice is not caused by any hormonal disturbance, but rather by an abnormal development of the posttesticular ducts, especially the efferent ducts, leading to mixed atrophy of the testis and complete absence of spermatozoa from the epididymis.

Materials and Methods

Experimental animals

Animal procedures were approved by the animal welfare committee of the Medizinische Hochschule Hannover. Animal treatment was performed in accordance with the German Federal Law on the Care and Use of Laboratory Animals. Mice were kept at a constant temperature (22 °C) and light cycle (12 h light, 12 h darkness) and were provided with standard laboratory chow and tap water *ad libitum*. *Pax8*^{-/-} males obtained by breeding *Pax8*^{+/-} animals (Mansouri *et al.* 1998) were injected daily with thyroxine (T4, 18 ng/g body weight sc; Sigma Aldrich, Germany) from postnatal day 2 (P2) onwards, which restores the euthyroid status in these animals (Friedrichsen *et al.* 2004). Wild-type controls were littermates of the *Pax8*^{-/-} mice. Genotyping of *Pax8* mice was performed as described previously (Flamant *et al.* 2002). *TRα1*^{-/-} *TRβ*^{-/-} double-mutant mice which are devoid of all functional TH receptor (TR) isoforms were generated by intercrossing *TRα1*^{-/-} and *TRβ*^{-/-} animals that were obtained from The Jackson Laboratory (Bar Harbor, ME, USA).

We compared postpubertal (age > 45 days pp) controls (*n*=8), T4-treated *Pax8*^{-/-} mice (*n*=9), and *TRα1*^{-/-} *TRβ*^{-/-} double-mutant mice (*n*=7). Before puberty (23–29 days pp), controls (*n*=4) and T4-treated *Pax8*^{-/-} males (*n*=3) were analyzed.

Organ and tissue collection

Animals were weighed and subsequently killed by decapitation. Trunk blood was collected and serum was stored at -20 °C for later evaluation. Testes, epididymides, and pituitaries were dissected out and weighed. Testes were either fixed in Bouin's fixative or snap-frozen in liquid nitrogen and subsequently stored at -80 °C; epididymides were fixed in Bouin's or 4% (w/v) paraformaldehyde in PBS. Pituitaries were frozen on dry ice.

Histology

Pieces of testicular tissue, epididymides, and efferent ducts were fixed for several hours and afterwards routinely embedded in paraffin and cut into 5 µm serial sections. Periodic acid-Schiff/hematoxylin staining was used for histological analysis. Histological slides were analyzed with an Axiovert 200 microscope (Zeiss, Oberkochen, Germany) and Axiovision 3.1 (Zeiss) software. Representative images were taken at magnifications of 10×, 25×, and 40× (AxioCam, Zeiss).

Immunohistochemistry

As a specific marker for the intact contractile apparatus of peritubular cells, α-smooth muscle actin was used (Schlatt *et al.* 1996) and detected with a specific monoclonal mouse antibody (1:500; A2547, Sigma) in the testicular tissues of

Pax8-deficient mice and matched controls. Antibodies were applied for 60 min at room temperature (RT) in a blocking buffer. For staining, Dako-LSAB 2 System (Dako Diagnostika, Glostrup, Denmark) was added for 30 min after washing, followed by an additional washing step. Antibodies were visualized by a secondary horseradish peroxidase-labeled goat anti-mouse IgG (1:100 dilution, Dako) with diaminobenzidine (DAB) used as a substrate, producing a dark brown signal. Briefly, the staining process was as follows: after washing, the slides were incubated in 3% (v/v) hydrogen peroxide to suppress endogenous peroxidase activity. After washing in Tris-buffered saline (10 mM Tris, 150 mM NaCl (pH 7.6)), nonspecific background was blocked by incubation in 5% (v/v) normal goat serum diluted in incubation buffer (0.1% (w/v) BSA in washing buffer). The sections were incubated with the primary antibody for at least 90 min at RT. After extensive washing, the secondary antibodies were added and incubated for more than 90 min at RT. DAB was finally added for 10–20 min followed by several washing steps. In a final step, the slides were counterstained with hematoxylin for 10 s and covered with Faramount (Dako) before microscopic examination (Axioskop, Zeiss).

In situ hybridization

Tissues were removed rapidly, embedded in Tissue-Tek medium (Sakura Finetek, Torrance, CA, USA) and frozen on dry ice. Sections (16 µm) were cut on a cryostat (Leica, Bentheim, Germany), thaw-mounted on silane-treated slides, and stored at -80 °C until further processing. *In situ* hybridization histochemistry was carried out as described previously (Schäfer & Day 1995). Briefly, frozen sections were fixed in 4% (w/v) phosphate-buffered paraformaldehyde (pH 7.4) for 1 h at RT, rinsed with PBS and treated with 0.4% (v/v) Triton X-100 in PBS for 10 min. After washing with PBS and water, tissue sections were incubated in 0.1 M triethanolamine (pH 8) containing 0.25% (v/v) acetic anhydride for 10 min. Following acetylation, sections were rinsed several times with PBS, dehydrated by successive washing with increasing ethanol concentrations and air dried.

Radioactive-labeled probes were generated from cDNA subclones in Bluescript SKII+ plasmids. *In vitro* transcription was carried out according to standard protocols with [³⁵S]UTP and [³⁵S]CTP as labeled nucleotides (Melton *et al.* 1984). Probes were prepared from a cDNA fragment corresponding to nt 887–1177 (Accession no. NM011040) of *Pax8*.

Radioactive-cRNA probes were diluted in hybridization buffer (50% (v/v) formamide, 10% (w/v) dextran sulfate, 0.6 M NaCl, 10 mM Tris-HCl (pH 7.4), 1× Denhardt's solution, 100 µg/ml sonicated salmon sperm DNA, 1 mM EDTA, and 10 mM dithiothreitol) to a final concentration of 5×10⁴ c.p.m./ml. After application of the hybridization mix, sections were mounted under coverslips and incubated in a humid chamber at 58 °C for 16 h. Following hybridization, coverslips were removed in 2× SSC (0.3 M NaCl, 0.03 M

sodium citrate (pH 7.0)). The sections were then treated with RNase A (20 µg/ml) and RNase T₁ (1 U/ml) at 37 °C for 30 min. Successive washes followed at RT in 1×, 0.5×, and 0.2× SSC for 20 min each and in 0.2× SSC at 65 °C for 1 h. The tissue was dehydrated and exposed to Biomax MR Film (Kodak) for 48 h. For microscopic analysis, sections were dipped in Kodak NTB2 nuclear emulsion and stored at 4 °C. After exposure for 14 days, autoradiograms were developed in Kodak D19 for 4 min and fixed in Rapid Fix (Kodak) for 4 min. If required, sections were counterstained with cresyl violet and then photographed under dark field or bright field illumination.

Digoxigenin-labeled probes were generated from cDNA subclones in pGEM-plasmids (Promega) with a DIG RNA Labeling Kit (Boehringer, Mannheim, Germany). *In vitro* transcription was carried out according to standard protocols. Probes were generated from cDNA fragments corresponding to nt 190–445 (Accession no. M10902) of thyroid stimulating hormone (β-TSH), nt 248–445 (Accession no. U62779) of growth hormone (GH), nt 1566–1749 (Accession no. J00769) of prolactin (PRL), nt 56–526 (Accession no. J00759) of prolactin (PRL), nt 56–526 (Accession no. J00759) of prolactin (PRL), nt 1–880 (Accession no. M36804) of β-FSH, and nt 31–488 (Accession no. NM012858) of luteinizing hormone (β-LH).

The digoxigenin-labeled probes were diluted in hybridization buffer to a final concentration of 5 ng/µl. Hybridization and posthybridization procedures were performed as described for radioactive *in situ* hybridization. Sections were then rinsed with wash buffer (100 mM Tris, 150 mM NaCl (pH 7.5)) and incubated for 2 h in blocking solution provided by the manufacturer of the kit. After overnight incubation with anti-digoxigenin antibody conjugated with alkaline phosphatase (1:1000 dilution; Boehringer), the tissue sections were washed with wash buffer. Staining proceeded for 2–6 h in substrate solution containing NBT (nitroblue tetrazolium chloride, 340 µg/ml; Biomol, Hamburg, Germany), X-phosphate (5-bromo-4-chloro-3-indolyl phosphate, 175 µg/ml; Biomol), 100 mM Tris, 100 mM NaCl, and 50 mM MgCl₂ (pH 9.0).

Hormone analysis

Serum testosterone was measured by RIA (Chandolia *et al.* 1991). Each sample was processed in duplicate after extraction with diethyl ether. The intra- and interassay coefficients of variation were 5.8 and 4.8% respectively. The detection limit of the assay was 0.68 nmol/l.

Analysis of sperm motility

Spermatozoa from a segment of the cauda epididymal tubule were immediately released into modified Biggers Whitten Whittingham medium. Motile cells were counted and >200 in each sample were recorded on videotape for computerized analysis of kinematic parameters as described in Yeung *et al.* (2002).

Statistical analysis

Data were analyzed by one-way ANOVA. Values of tubular and lumen diameter were compared by ANOVA on ranks followed by Tukey's post hoc test. Computations were performed using the statistical software package SIGMA-STAT 2.03 (SPSS, Inc., Chicago, IL, USA). All data are expressed as means ± s.d., unless stated otherwise. Values were considered significantly different if $P < 0.01$.

Results

Male $Pax8^{-/-}$ mice were infertile despite adequate T4 treatment

Since all deficits observed in conditions of CH are generally restored by thyroid hormone replacement in humans (Larsen *et al.* 2003, Roberts & Ladenson 2004) as well as in mice (Christ *et al.* 2004, Friedrichsen *et al.* 2004), male $Pax8^{-/-}$ mice were treated with thyroxine (T4) from postnatal day 2 (P2) onwards in the expectation that they would survive to adulthood and become fertile to set up homozygous breeder pairs. Indeed, when substituted with T4, male $Pax8^{-/-}$ animals developed similarly to wild-type littermates (Friedrichsen *et al.* 2004, Mittag *et al.* 2005). However, these mice were unable to reproduce when caged with fertile female wild-type mice.

The hormonal situation was normal in T4-substituted $Pax8^{-/-}$ mice

To determine the hormonal situation in anterior pituitaries of T4-substituted $Pax8^{-/-}$ mice, the mRNA expression of β-TSH, PRL, GH, β-LH, β-FSH, and POMC was analyzed by *in situ* hybridization (Fig. 1). The pituitaries of T4-treated male $Pax8^{-/-}$ mice did not show any obvious differences in mRNA expression or cellular composition when compared with pituitaries of wild-type littermates. In contrast, untreated $Pax8^{-/-}$ mice exhibited a dramatically distorted cellular composition of the anterior pituitary with hypertrophy and hyperplasia of the thyrotropes, an almost complete absence of lactotropes and a drastically reduced number of somatotropes, as shown before (Friedrichsen *et al.* 2004). $TR\alpha1^{-/-}TR\beta^{-/-}$ double-mutant mice which are unable to transduce any TH signaling owing to the lack of a functional TH receptor (TR) also exhibited increased β-TSH mRNA expression as well as reduced PRL, GH, and gonadotropin transcript levels. As expected from the normal gonadotropin mRNA expression in T4-treated $Pax8^{-/-}$ mice, serum testosterone levels although quite variable were not significantly different between all groups of postpubertal animals (wildtype: (mean ± s.e.m.) 3.59 ± 1.23 nmol/l; T4-treated $Pax8^{-/-}$: 3.12 ± 0.72 nmol/l; $TR\alpha1^{-/-}TR\beta^{-/-}$: 6.64 ± 0.90 nmol/l).

Figure 1 Analysis of the pituitary hormone mRNA expression by *in situ* hybridization histochemistry. mRNA expression of β -TSH, growth hormone (GH), prolactin (PRL), luteinizing hormone (β -LH), follicle-stimulating hormone (β -FSH), and proopiomelanocortin (POMC) in anterior pituitaries of male wildtype (WT), untreated (*Pax8*^{-/-}), and thyroxine-treated *Pax8*^{-/-} mice (*Pax8*^{-/-}T4), and *TRα1*^{-/-}*TRβ*^{-/-} mice (scale bar 500 μ m).

Analysis of the organ weights

As a first approach to identify the cause of the infertility, the organ weights of the reproductive tract were analyzed. No difference was observed between the prepubertal T4-treated *Pax8*^{-/-} males and controls, either in organ or

in body weight (Fig. 2A). In contrast (Fig. 2B), a significantly reduced testis weight compared with controls was observed in the postpubertal T4-treated *Pax8*^{-/-} mice as well as in the postpubertal *TRα1*^{-/-}*TRβ*^{-/-} that were used for comparison. These mice also exhibited a significantly lower

Figure 2 Comparison of body weight and weights of the reproductive organs. (A) Pre-pubertal animals and (B) postpubertal animals. ASGI, accessory sex glands. Values with different superscripts differ significantly ($a > b$, $P < 0.01$). ns, not significantly different.

body weight compared with controls and T4-treated Pax8-deficient animals. While the epididymides of the postpubertal T4-treated Pax8^{-/-} mice were significantly smaller compared with the other animals, the weights of the accessory sex glands (seminal vesicles and coagulating glands) of the postpubertal TRα1^{-/-}TRβ^{-/-} and T4-treated Pax8^{-/-} mice were not different from wildtype.

Body and organ weights of postpubertal animals were analyzed at different ages, but the two groups did not show any differences.

Histology and immunohistochemistry of the testis

The peritubular cells surrounding the seminiferous tubules of the T4-treated Pax8-deficient mice exhibited a strong specific staining for α-smooth-muscle actin, which was also observed in the testicular tissues of the matched controls (data not shown), indicating that in the deficient animals these cells possess mature, functional contractile elements, and most likely also intact peristalsis.

Postpubertal wild-type controls as well as TRα1^{-/-}TRβ^{-/-} mice showed qualitatively normal spermatogenesis in all tubule cross-sections (Fig. 3A and C). Spermiogenesis up to the stage of elongated spermatids was observed in almost all tubules. In contrast (Fig. 3B and D), histological analysis

of testicular tissue from T4-treated Pax8^{-/-} mice revealed focally atrophic regions. Such tubules showed arrested spermatogenesis and a variable degree of disorganization. They exhibited a flattened and partially vacuolated seminiferous epithelium, often adjacent to tubules with complete spermatogenesis up to the testicular spermatozoa. In prepubertal animals, spermatogenesis proceeded mainly up to the level of early postmeiotic round spermatids; the proportion of tubules with disorganized epithelia was slightly higher in the T4-treated Pax8-deficient animals but given the limited number of animals analyzed, a statistical significance was not reached compared with the testicular tissue of wild-type controls (Fig. 4A).

Quantification of the spermatogenic progress revealed differences between the groups, reflecting the atrophic changes in the testes of the T4-treated Pax8-deficient animals after puberty (Fig. 4B). Controls and TRα1^{-/-}TRβ^{-/-} mice exhibited a significantly higher percentage of tubules with elongated spermatids as the most advanced germ cell type compared with T4-treated Pax8^{-/-} males. In parallel, the proportion of tubules arrested at the level of spermatocytes (meiosis I) was significantly higher in the T4-treated Pax8-deficient animals compared with controls and TRα1^{-/-}TRβ^{-/-} mice. The number of tubules arrested at the postmeiotic level (round spermatids), at the earlier spermatogonial level or exhibiting Sertoli cells only (SCO), was also higher in the T4-treated Pax8-deficient animals, but the differences were not significant. Between the prepubertal T4-treated Pax8^{-/-} animals and the age-matched controls, significant quantitative differences in spermatogenic progress were not found.

However, regarding the number of analyzed animals, it cannot be excluded, that the subtle differences might already be present at this age.

Analysis of the epididymis and the efferent ducts

In mice, the efferent ducts draining the rete testis run freely as several convoluted tubules among the fat pad for some distance before coiling up to form a small lobule under the epididymal capsule where they join the caput epididymal tubule.

Out of the three, 3–4-week (23–29 days)-old prepubertal T4-treated Pax8^{-/-} mice examined, in one animal there was no detectable efferent ducts on one side and the caput as well as the corpus epididymides were also missing. When efferent ducts were found, tubule cross-sections were smaller, with more interstitium than in the wildtype, but serial sectioning of the caput epididymidis of two animals revealed a patent efferent duct/epididymal junction (data not shown).

In the 6–8-week-old postpubertal T4-treated Pax8-knockout mice around puberty, four out of six animals lacked discernable efferent ducts, two bilaterally and two unilaterally. When efferent ducts were found, they were much smaller in diameter with a small lumen and extensive intertubular connective tissue compared with the control (Fig. 5A).

Figure 3 Testicular histology. Overview and details (inserts). (A) Adult wild-type age-matched controls show complete spermatogenesis in all tubular cross-sections. The scale bar represents 50 μ m. Spermiogenesis up to the stage of elongated spermatids is observed in almost all tubules (insert, arrow, scale bar represents 25 μ m). (B) Adult testes of T4-treated *Pax8*^{-/-} mice frequently exhibiting focal regions of mixed atrophy (*) and disorganized seminiferous epithelium (#) close to tubules with complete spermatogenesis up to elongated spermatids (insert, arrow, scale bar represents 25 μ m). (C) Testicular histology of *TRα1*^{-/-}*TRβ*^{-/-} double knock out mice appeared to be complete and did not show arrested or disorganized tubules. The scale bar represents 50 μ m. (D) Adult testes of T4-treated *Pax8*^{-/-} mice exhibiting extensive disturbance of the epithelial organization caused by backpressure. Lumina are widened and the epithelial height is reduced. Spermatogenesis is arrested in almost every tubular cross-section and in some tubules detached floating cells are seen. The scale bar represents 50 μ m.

A striking feature in the T4-treated *Pax8*-deficient animals at this stage was the occasional and focal dilation of the ducts, some with extremely large lumen and flattened low epithelium (Fig. 5B and C). The abnormality of the small tubules and large interstitium persisted in the postpubertal phase. In the six postpubertal T4-treated *Pax8*^{-/-} mice examined, the epididymis was absent from two animals where only the remnants of the distal tubule with a poorly developed vas deferens were found. In the other four T4-treated *Pax8*^{-/-} mice with a complete epididymis, spermatozoa were not observed in the luminal content expressed from the vas deferens, while in the wild-type spermatozoa were present in the epididymis and the vas deferens.

During pubertal development in the wildtype, the proximal part of the caput epididymidis differentiates into the initial segment characterized by a highly vascularized region

comprising very tall columnar epithelial cells which persist in the adults (Fig. 6B). Such differentiation was not found in the T4-treated *Pax8*^{-/-} mice (Fig. 6A) even when the efferent ducts were present. An abnormality in epithelial development was also noted in the proximal corpus epididymidis, where the epithelium in the knockout was taller than in other regions and the same region of the wildtype. In the more distal regions, especially in the cauda, large vacuoles were often seen in the epithelium of the knockouts.

While the epididymal lumina in the eight postpubertal wild-type mice were full of spermatozoa (Fig. 6C), those from the nine adult T4-treated *Pax8*^{-/-} mice analyzed (older than 12 weeks) were empty (Fig. 6D). One of the three *Pax8*-deficient adults showed unilateral absence of the efferent ducts and the epididymis. When present, epididymides were not significantly lighter than that in the wild-type organs (Fig. 2).

Figure 4 Quantification of spermatogenesis; (A) pre-pubertal animals and (B) postpubertal animals; proportion of most advanced germ cell type determined in cross-sections of testicular tubules are presented together with the proportion of tubules exhibiting an unusual organization of the seminiferous epithelia (disorganized). Elspdt, elongated spermatids; rsptd, round spermatids; sptc, spermatocyte; spg, spermatogonia; SCO, Sertoli cell only (no germ cells). Values with different superscripts differ significantly ($a > b$, $P < 0.005$). ns, not significantly different.

Histological examination of the efferent ducts and epididymis of the postpubertal $TR\alpha1^{-/-}TR\beta^{-/-}$ mice showed no differences compared with the wildtype. Epididymal tubules were full of spermatozoa and those matured and stored in the cauda epididymidis showed the same motility in both phenotypes. Detailed measurement of sperm kinematics using computerized analysis revealed a slight but significant decrease in the swimming velocities of the knockout spermatozoa (data not shown).

Pax8 mRNA expression in the epididymal epithelium

To determine if the reproductive disturbances observed in T4-treated $Pax8^{-/-}$ animals could be directly related to the inactivation of the *Pax8* gene, *in situ* hybridization histochemistry was used to identify *Pax8* transcripts in the male reproductive system of adult wild-type mice. Strong mRNA expression was observed in the epithelium of the epididymis and with weaker intensity in the efferent ducts (Fig. 7B–E), whereas no signal could be found in testis of adult wild-type mice (Fig. 7A).

Discussion

The $Pax8^{-/-}$ mouse is an ideal animal model for congenital hypothyroidism, since apart from the inability to form thyroid follicular structures, no further defects in other *Pax8*-expressing tissues have been reported (Mansouri *et al.* 1998). Thyroid hormone replacement therapy, if instituted immediately after birth, usually prevents all symptoms in patients suffering from CH (Cassio *et al.* 2003, Larsen *et al.* 2003, Roberts & Ladenson 2004). Therefore, it was surprising that despite adequate T4 substitution $Pax8^{-/-}$ mice were unable to reproduce. As a first approach to identify the reasons for this infertility, the hormonal situation in these mice was analyzed. The normal mRNA expression of β -TSH and GH in the pituitary confirmed a proper supply with T4, since these genes are highly sensitive to changes in TH metabolism (Yen 2001, Friedrichsen *et al.* 2004). Normal gonadotropin mRNA expression as well as normal serum testosterone levels also indicated an unimpaired hormonal situation in these animals. We did not find endocrine differences in the T4-treated $Pax8^{-/-}$ -deficient animals, suggesting that the hormonal status was completely rescued in these animals by TH replacement therapy. In addition, this finding is supported by the normal weight of the accessory sex glands that reflect the androgen status in these animals (Mahendroo *et al.* 2001).

For comparison with T4-treated $Pax8^{-/-}$ -deficient mice lacking TH as signaling molecule, we also analyzed $TR\alpha1^{-/-}TR\beta^{-/-}$ double-mutant mice that are devoid of all functional TRs (Göthe *et al.* 1999) in order to define defects due to impaired TH-signaling mechanisms. Although there are differences between the absence of the ligand TH and the absence of the receptor, either due to nongenomic actions of TH (Yen 2001) or to a possible aporeceptor activity of unliganded TR (Chassande 2003), the general fertility of $TR\alpha1^{-/-}TR\beta^{-/-}$ mice seems to be a good indication that the reproductive phenotype of $Pax8^{-/-}$ mice is not related to TH metabolism. However, a contribution of a mild perinatal hypothyroidism to the observed phenotype before the onset of the T4 replacement therapy cannot be entirely excluded. The impaired velocity of spermatozoa observed in $TR\alpha1^{-/-}TR\beta^{-/-}$ animals is in agreement with previous reports showing decreased sperm motility in hypothyroid rats (Chandrasekhar *et al.* 1985, Kumar *et al.* 1994). Since the reduction of swimming velocities of spermatozoa from the epididymis of these animals could be rescued by T4 injections (Del Rio *et al.* 1998), it was assumed that TH directly acted on the epididymis since TH binding to the epididymal nuclei has been demonstrated (Del Rio *et al.* 2000). The epididymis, in turn, then affects proper sperm maturation in the lumen.

The situation in *Pax8*-deficient mice which completely lack spermatozoa in the epididymal lumen is obviously more severe than in $TR\alpha1^{-/-}TR\beta^{-/-}$ animals. Either the spermatozoa are not shed from the seminiferous epithelium or there is no patent duct system connecting the rete to the

Figure 5 Cross-sections of the convoluted efferent ducts which join the testis to the epididymis in the early postpubertal wild-type (WT, A) and T4-treated *Pax8*^{-/-} mice showing gradual dilation of some ductal lumina in the T4-treated *Pax8*^{-/-} (B) until the cuboidal epithelium becomes very low in height (C). Note that the nondilated parts of the efferent ducts in (C) are much narrower with more extensive intertubular connective tissues than in the wildtype where the ducts are well developed (A). The scale bar represents 50 μ m.

epididymis. In mice devoid of the epididymal-specific G-protein-coupled receptor HE6, spermatozoa fail to enter the epididymis and are retained within the efferent ducts (Gottwald *et al.* 2006), but this was not the case here, since the efferent ducts, when present, were empty. In the animals studied, a patent efferent duct–epididymis junction was confirmed in two animals and could be inferred in the others from the failure to find a site of occlusion in serial sections. Despite the absence of spermatozoa, epididymal weight was maintained in these mice as the tubule lumen did not collapse but was fluid filled.

The histological observations on the existing efferent ducts of pre- and postpubertal T4-treated *Pax8* null mice, namely the apparent expansion of the interstitium with concomitant narrowing of the lumina and the focal distension of proximal efferent ducts, are consistent with the hypothesis that the physical capacity of the posttesticular excurrent ducts, even when present with a lumen, is inadequate to transmit all the testicular fluid to the epididymis. The increasing testicular fluid secretion that occurs at puberty would lead to a gradual buildup of pressure within the testis damaging the seminiferous epithelium, leading to spermatogenetic arrest as found when distal luminal fluid passage is impeded by ligation of the efferent ducts (Ross 1974) or their failure to absorb water as in the estrogen receptor α -knockout mouse (Hess *et al.* 2000). It remains to be elucidated whether there are subtle differences in the testis of T4-treated *Pax8*-deficient mice already before puberty, which would require a thorough analysis of a greater number of animals.

The *Pax8*-knockout mouse is another male mouse model displaying infertility associated with an undifferentiated

epididymal initial segment. Others include the *c-ros* knockout (Sonnenberg-Riethmacher *et al.* 1996, Avram & Cooper 2004), the SH2 domain protein tyrosine phosphatase (SHP1) Ros1 phosphatase knockout (Keilhack *et al.* 2001), and the apolipoprotein E receptor 2 knockout (Andersen *et al.* 2003). These three models differ from the *Pax8* knockout in having full spermatogenesis and an epididymis containing spermatozoa. Another model lacking the initial segment is the chromosomal anomaly sex-reversed pseudohermaphroditism (XXsr; LeBarr & Blecher 1986), and these, like the *Pax8* knockout, lack spermatozoa in the epididymis, but as a result of a complete absence of germ cells, reflecting the chromosomal condition.

The *Pax8*-knockout mouse also differs from the latter animals by displaying at least some stages of spermatogenesis, suggestive of an initiation of normal testicular function that is inhibited with time starting around puberty when testicular fluid production increases. This would explain the increasing atrophy with age and reduction in testicular size and weight with time, despite foci with apparently normal spermatogenesis in the *Pax8*-deficient animals.

The observations on the vacuolization of the cauda epididymal epithelium are reminiscent of what occurs after neonatal and juvenile ligation of the corpus epididymidis of mice. This was considered to indicate failed development of the epithelium as a result of the withdrawal of more proximal epididymal secretions (Abe *et al.* 1982, 1984), which is again consistent with the view that normal luminal irrigation of the epididymis was impeded in the animals of the present study.

In mammals, the male posttesticular reproductive system is derived from the mesonephric duct or Wolffian duct, which

Figure 6 Comparison of the epididymis from adult T4-treated *Pax8*^{-/-} mice (A, D) and the wildtype (B, C). In the wildtype, the initial segment (IS) is situated adjacent to the efferent ducts (ED) under the epididymal capsule and shows a well-differentiated epithelium (B). In the T4-treated *Pax8*^{-/-} mice, there is no differentiation of the epithelium of the caput tubule, with or without the efferent duct in the apex of the epididymis (A). Sperm-filled tubules in the more distal epididymis in the wildtype (C) are in stark contrast to the empty lumen in the knockout (D) as shown here in the corpus epididymidis. The scale bar represents 50 μ m.

degenerates in the female embryo. In mice, around embryonic day (E) 13.5, the derivatives of this mesonephric tubule start forming the efferent ductules that connect the rete testis with the upper part of the epididymis, which itself is

derived from the most rostral mesonephric duct (Brune *et al.* 1999). *Pax8*, being expressed in the mouse mesonephros during development (Mansouri *et al.* 1998, Bouchard *et al.* 2004) and as shown here also expressed in the epididymis

Figure 7 *In situ* hybridization showing mRNA expression of *Pax8* in the epithelium of the epididymis (EP) and the efferent ducts (ED) (B and D) but not in the testis (T) of adult wild-type mice under dark field illumination (A). The corresponding cresyl violet counterstaining of neighboring sections under bright field illumination are shown in (C) and (E) (scale bars A, D, E: 1 mm; scale bars B, C: 500 μ m). Sections of the reproductive tract of *Pax8*^{-/-} mice and sense probes that were used to confirm the specificity of the hybridization did not show any signal (not shown).

during adulthood, thus seems to be a factor that regulates the differentiation of the mesonephric tubules that are the anlage of the testicular efferent ducts, possibly via stromal/mesenchymal interactions. To date, nine *Pax* genes have been identified that regulate fundamental events in body patterning during development (Wehr & Gruss 1996). The highly homologous genes *Pax2* and *Pax8* have been shown to be involved in the early development of the urogenital system. *Pax8* expression is already detectable at E9.5 in the developing Wolffian duct and the kidney and later at E15.5 also in the epididymis of male mice (Kobayashi & Behringer 2003, Bouchard *et al.* 2004). In contrast to *Pax2*-deficient mice which entirely lack kidneys and genital tracts (Torres *et al.* 1995), the anterior–posterior patterning is well established in *Pax8*^{-/-} mice which can display an affected differentiation of the Wolffian duct into efferent duct, epididymis and vas deferens. Kidney development is also normal in these mice (Mansouri *et al.* 1998). Therefore, the loss of *Pax8* can be compensated for by other factors, such as *Pax2* in the early developmental events of the urogenital system, but *Pax8* seems to play a pivotal role in the proper morphogenesis of the efferent duct and the epididymis in later developmental stages.

Taken together, the unimpaired hormonal situation and the more severe phenotype compared with the *TR α 1*^{-/-} *TR β* ^{-/-} double-mutant mice suggest that the infertility in thyroxine-substituted *Pax8*-deficient animals is not related to any hormonal imbalance, but rather is a direct consequence of the lack of the *Pax8* gene, which seems to be a novel key player in the genetic pathway leading to the formation of efferent ducts and epididymis from the Wolffian ducts. It remains to be elucidated whether male congenital hypothyroid patients with mutations in the *Pax8* gene are similarly affected.

Although each *Pax8*^{-/-} male exhibited an abnormal phenotype, the highly variable nature of the epididymal form expressed in the few animals available for study at each age demands that any interpretation of the phenotype be cautious. Nevertheless, it is clear that some aspects of the development of the posttesticular duct system are affected by the absence of the early expressed *Pax8* gene; the eventual phenotype exhibited must reflect the effects of other genes that take over the part of its role during development.

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