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Vitamin D receptor and vitamin D metabolizing enzymes are expressed in the human male reproductive tract

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BACKGROUND: The vitamin D receptor (VDR) is expressed in human testis, and vitamin D (VD) has been suggested to affect survival and function of mature spermatozoa. Indeed, VDR knockout mice and VD deficient rats show decreased sperm counts and low fertility. However, the cellular response to VD is complex, since it is not solely dependent on VDR expression, but also on cellular uptake of circulating VD and presence and activity of VD metabolizing enzymes. Expression of VD metabolizing enzymes has not previously been investigated in human testis and male reproductive tract. Therefore, we performed a comprehensive analysis of the expression of VDR, VD activating (CYP2R1, CYP27A1, CYP27B1) and inactivating (CYP24A1) enzymes in the testis, epididymis, seminal vesicle (SV), prostate and spermatozoa.

METHODS: Tissue samples were obtained after orchiectomy (testis $n = 13$; epididymis $n = 7$), prostatectomy (prostate $n = 5$ and SVs $n = 3$) and semen samples obtained after ejaculation ($n = 13$). mRNA was detected with RT–PCR and expression of proteins was determined by immunohistochemistry.

RESULTS: VDR and VD metabolizing enzymes were concomitantly expressed in round and elongated spermatids, vesicles within the caput epididymis, and glandular epithelium of cauda epididymis, SV and prostate. The expression pattern in ejaculated spermatozoa varied, although, concomitant expression of VDR, CYP2R1, CYP27B1 and CYP24A1 was observed in neck and midpiece in a subpopulation of mature spermatozoa.

CONCLUSION: On the basis of the marked expression of VDR and the VD metabolizing enzymes in human testis, ejaculatory tract and mature spermatozoa, we suggest that VD is important for spermatogenesis and maturation of human spermatozoa.

Key words: vitamin D receptor / hydroxylase / reproduction / testis / CYP

Introduction

Development of spermatozoa, and hence male fertility, depends on a complex series of events that occur in the reproductive organs. Following spermiogenesis, the spermatozoa become motile in the epididymis, where they are stored until ejaculation. During ejaculation epididymal content is mixed with secretion from the prostate gland and seminal vesicle (SV), before the spermatozoa undergo capacitation and fertilization competency in the female reproductive tract (Cornwall, 2009). Several endocrine factors have been implicated in sperm production and maturation, but little is known about the potential role of vitamin D (VD) in male reproduction.

VD is a key regulator of calcium homeostasis and bone mineralization, although expression of the vitamin D receptor (VDR) in various tissues has been related to several functions, comprising an important role in cell cycle control (Fleet, 2008). VD affects calcium homeostasis by regulating intestinal absorption, urinary excretion, secretion of PTH, and by altering expression of intracellular calcium binding proteins and calcium-channels, -pumps and -transporters. Calcium is important in the male reproductive tract, where it is essential for spermatogenesis, sperm motility, hyperactivation and acrosome reaction (Yoshida *et al.*, 2008).

VD needs to be activated by two enzymatic steps, before it binds with high affinity to VDR and the systemic activation of VD is a tightly regulated process. It normally starts in the skin, where UVB radiation converts 7-dehydrocholesterol to cholecalciferol (vitamin D_3). Vitamin D_3 is subsequently metabolized by the hepatic 25-hydroxylases (CYP2R1 or CYP27A1), before 25-hydroxycholecalciferol ($25(OH)D_3$)

relocates to the circulation, where the renal 1α-hydroxylase (CYP27B1) converts 25(OH)D_3 to the active 1,25(OH)$_2D_3$ (calcitriol). Calcitriol binds and activates VDR in target cells, until the 24-hydroxylase (CYP24A1) inactivates it (Prosser and Jones, 2004). VD deficiency in male rats results in reduced sperm counts, and female rats inseminated with semen from VD deficient male rats have lower fertility rates (Kwiecinski *et al.*, 1989; Schleicher *et al.*, 1989; Uhland *et al.*, 1992; Audet *et al.*, 2004; Hirai *et al.*, 2009). The impaired reproductive performance induced by VD deficiency is reversible and seems to be mediated predominantly through calcium imbalance, because it can be corrected either by supplying VD or by normalizing calcium levels (Uhland *et al.*, 1992). VD acts through the VDR, and VDR expression has been shown in testis from rodents, chickens, roosters, humans and recently also in mature human spermatozoa (Merke *et al.*, 1985; Johnson *et al.*, 1996; Corbett *et al.*, 2006; Aquila *et al.*, 2008; Oliveira *et al.*, 2008; Hirai *et al.*, 2009). The importance of this expression is highlighted in VDR knockout mice, which show decreased sperm counts, reduced sperm motility and histological abnormalities of the testis, which unlike the VD deficient male rats only partly can be restored by calcium supplements (Kinuta *et al.*, 2000). The liver and kidney were previously thought to be the only organs with the ability to activate VD, but extra-renal expression of CYP27B1, CYP24A1 and CYP27A1 have been shown in tissues thought only to be VD responsive (Nagakura *et al.*, 1986). This complicates the supposed cellular response to VD, because in addition to VDR expression, it also depends on the ability to allow uptake of substrate and metabolize the circulating forms of VD (Fleet, 2008).

To improve our knowledge of VD in male reproduction, we conducted a comprehensive analysis of the expression of VDR and VD metabolizing enzymes (CYP2R1, CYP27A1, CYP27B1 and CYP24A1) in the human male reproductive tissues, including testis, epididymis, prostate, SV and ejaculated sperm. The pattern of expression reported here suggests a role of VD in male reproduction.

Materials and Methods

Patients and ethics

Patients were recruited from the andrology clinic at University Department of Growth and Reproduction at Rigshospitalet, Denmark in accordance with the Helsinki Declaration and after approval from the local ethics committee (permit no. KF 01 2006-3472). Freshly delivered semen samples from men from the general population with no andrological problems ($n = 13$) went through a routine semen analysis, before immunocytochemistry was performed. Samples of 100 μl semen were diluted in PBS (25×10^6 spz/ml PBS), and were centrifuged using Shandon Cytospin 2 centrifuge (Anatomical Pathology International, Runcorn, UK) at 200*g* for 5 min onto SuperFrost^{R}Plus microscope slides (Menzel-Glaser, Braunschweig, Germany). The cytospins were not fixed.

Epididymis and adult testis samples ($n = 7–13$) were obtained from orchidectomy specimens, performed due to testicular cancer. Each sample was divided into fragments, which were either snap-frozen at 80°C for RNA extraction, or fixed overnight at 4°C in Stieve's fluid, Bouin's fixative, formalin or paraformaldehyde and subsequently embedded in paraffin. All testicular tumours were germ cell tumours, and the surrounding tissue contained tubules with normal spermatogenesis. One specimen for RNA extraction contained tubules with Sertoli-cell-only (SCO). Tissue from the prostate and SV were obtained after prostatectomy. All tissues used for immunohistochemistry (IHC) were regarded as normal after histological evaluation, although the normal tissue was adherent to pathological parts. For additional normal control, we purchased normal testis RNA from three different companies (Clontech-Takara Bio Europe, Abcam Plc, Cambridge, UK, Biopharm US), although normal ovary (used for comparison) was purchased from Abcam Plc, Cambridge, UK. The kidney samples used for positive controls have been described earlier (Blomberg Jensen *et al.*, 2009).

RT–PCR

We used the same primers and conditions as described previously (Blomberg Jensen *et al.*, 2009), except for the investigation of the CYP24A1-splice variant lacking the mitochondrial-targeting signal (CYP24SV: TCCTGAAGTTGCAGCTGGAGT & GAGCTCATCTATTC TGCCCATA 213 bp). Representative bands from each primer combination were excised and sequenced for verification.

Immunohistochemistry

Paraffin sections were deparaffinized and rehydrated. Antigen retrieval was accomplished by microwaving the sections for 15 min in TEG buffer (Tris 6.06 g, EGTA 0.95 g in 5 l, pH 9.0). Afterwards all sections were incubated with 2% non-immune goat serum (Zymed Histostain kit, San Francisco, CA, USA) or 0.5% milk powder diluted in Tris buffered saline (TBS) to minimize cross-reactivity. Primary antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA [CYP2R1 (C-15) sc-48985, CYP27A1 (P-17) sc-14835, CYP27B1 (H-90) sc-67261, VDR (H-81): sc-9164, CYP24 (H-87): sc-66851]. CYP2R1 and CYP27A1 were goat polyclonal antibodies and CYP27B1, VDR and CYP24 were rabbit polyclonal antibodies. All antibodies were tested by western blot in our laboratory (data not shown) and have been validated in the normal human kidney (Blomberg Jensen *et al.*, 2009). In the human kidney we showed that VDR, CYP24A1 and CYP27B1 were strongly expressed in the proximal tubules in addition to a minor expression in the distal tubules, while CYP2R1 and CYP27A1 had a marked expression in distal tubules. Presence of the proteins in the testis and kidney was determined by Western blot, and subsequently initial experiments were carried out in the male reproductive tract with different antibody dilutions (1:50 to1:500), different buffers (TEG, citrate, Urea) and with different fixatives (Stieve's, Bouin's, formalin or paraformaldehyd). The optimal dilutions of the primary antibodies in formalin fixed tissues were: CYP2R1 1:100, CYP27A1 1:200, CYP27B1 1:200, VDR 1:100 and CYP24 1:200. After 16 h of incubation at 5°C, the sections were incubated with biotinylated goat anti-rabbit IgG (Zymed Histostain kit) or biotinylated donkey anti-goat IgG (1:400), before a peroxidase-conjugated streptavidin complex (Zymed Histostain kit) was used as a tertiary layer. Visualization was performed with amino ethyl carbasole (Zymed Histostain kit). Between incubation steps, the slides were washed with TBS. When needed, counterstaining was performed with Meyer's haematoxylin. The procedure for staining of the Leydig cells was performed with a slightly different procedure, as described previously in the kidney (Blomberg Jensen *et al.*, 2009), because a biotin and peroxidase based development is inappropriate in Leydig cells due to an unspecific background staining. We used alkaline phosphatase (AP)-conjugated secondary antibodies (Santa Cruz) and evaluated expression after visualization with NBT-BCIP [Nitro-Blue Tetrazolium Chloride-5-Bromo-4-Chloro-3′-Indolyphosphate p-Toluidine Salt] after blocking endogenous AP with Levamisol. All experiments were performed with control staining without the primary antibody, which were carefully examined to ensure that negative controls remained unstained.

The immunocytochemical staining of the ejaculated spermatozoa was performed as described above, except that the slides were not fixed, and we used 2% mouse serum instead of goat serum to avoid cross-reactivity. Two independent investigators evaluated all slides (M.B.J. and

J.E.N.). Staining was classified according to an arbitrary semi-quantitative reference scale depending on the intensity of staining and the proportion of cells stained: +++, strong staining in nearly all cells; ++, moderate staining or only a minority of cells positive; +, weak staining or a low percentage of cells stained; +/−, very weak staining in single cells; neg., no positive cells detected. Finally, the subcellular location of the expression was noted.

Results

Expression of the VDR

VDR mRNA was detected in testis, epididymis, prostate, SV, ovary and kidney, although the level of expression varied (Fig. 1A). High expression, similar to the level observed in kidney, was detected in SV and epididymis, although the expression in the remaining tissues was low. *VDR* mRNA was detectable in all normal testis samples, but not in testis with SCO pattern. The mRNA expression was matched by the presence of VDR protein both in nucleus and cytoplasm of spermatogonia, in round and elongated spermatids and in some spermatocytes (Fig. 1B; summarized in Fig. 4A1). VDR was markedly expressed in Leydig cells, where expression was aggregated in small cytoplasmic clusters, with no concomitant nuclear staining (Fig. 4B1). The lack of VDR mRNA expression in the SCO sample, which contains Leydig cells, suggests that the expression observed in Leydig cells should be interpreted with caution. Expression of VDR in ejaculated spermatozoa varied. In some samples the majority of spermatozoa expressed VDR, although others only had detectable expression in a minor proportion of the ejaculated sperm. Morphologically normal spermatozoa often expressed VDR, although expression also was found in morphologically abnormal sperm (Fig. 4G1; Table I). The predominant locations of expression were in the post-acrosomal region (par), neck and in the midpiece of spermatozoa, whereas some had a marked staining in the head.

VDR expression was detectable in luminal cytoplasmic vesicles in caput epididymis, although expression was dispersed in the cytoplasm of epithelial cells of cauda epididymis (Fig. 4C1 and D1). VDR expression was detected in both nucleus and cytoplasm of the basal glandular prostate epithelium and the epithelia lining the prostate ducts (Figs 1C, 4E1 and Table I). All epithelia of SV had a marked expression of VDR in cytoplasm and a minor expression in the nucleus (Figs 1D and 4F1). An overview of expression in the investigated organs can be found in Table I.

Expression of the activating enzymes (CYP2R1, CYP27A1 and CYP27B1)

The mRNAs encoding *CYP2R1* and *CYP27A1* were expressed in all investigated samples, however, with a lower expression in epididymis and prostate. Highest expression of *CYP2R1* was found in testis, although the level of *CYP27A1* mRNA in testis was comparable to the expression found in the kidney. *CYP27B1* was present in all samples, but the levels differed: high expression in control kidney and in SV, although expression was low in normal testis and barely detectable in testis with SCO, epididymis, prostate and ovary (Fig. 2A).

The CYP2R1, CYP27A1 and CYP27B1 proteins were expressed in Leydig cells, and IHC results matched by detectable bands in the SCO sample (Fig. 2A). All the activating enzymes had a marked expression in round and elongated spermatids (Figs 2B–D and 4A2–A4),

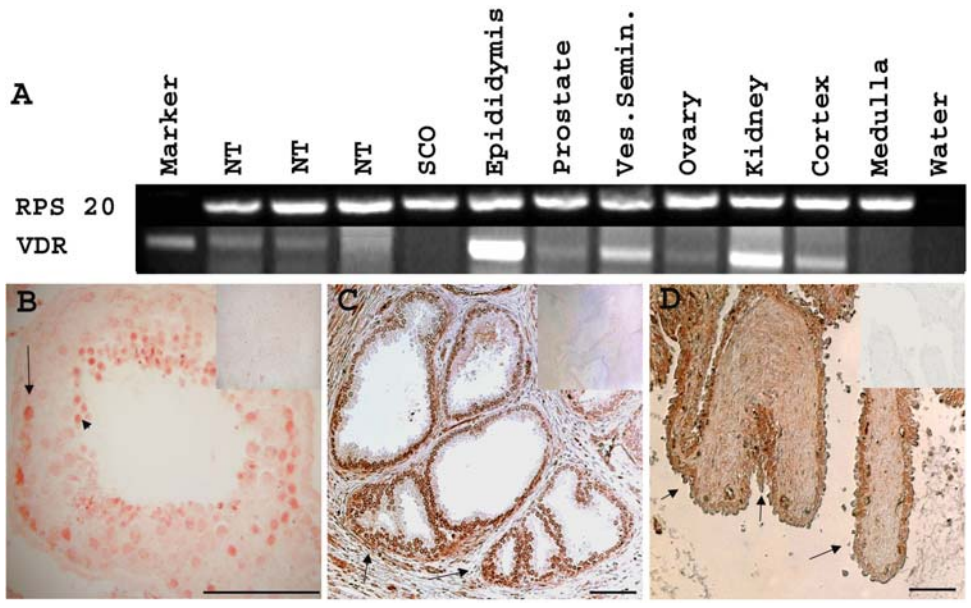

Figure 1 Expression of VDR in the male reproductive tract.

(**A**) RT–PCR analysis of mRNA expression. From left: normal testis (NT), SCO, epididymis, prostate, seminal vesicles (SV), ovary, kidney and kidney tissue divided in cortex and medulla. RPS 20 loading control. (**B**) Normal testis with expression of VDR in spermatogonia (arrow) and in round (arrowhead) and elongated spermatids. (**C**) Nuclear staining in the basal epithelia in addition to a luminal cytoplasmic expression of the prostate gland (arrow). (**D**) A marked cytoplasmic expression in the SV (arrows). Bar corresponds to 50 μm.

Table I Description of specimens and assessment of the expression of VDR, CYP2R1, CYP27A1, CYP27B1 and CYP24A1.

Tissue and cell type	Receptor VDR	Activating enzymes			In-activating CYP24A1
		CYP2R1	CYP27A1	CYP27B1	
Normal testis					
Spermatogonia	+ to ++	neg. to +/−	neg. to +	+ to ++	+/− to ++
Spermatocytes	neg. to +	+ to +++	neg. to +/−	neg. to +	neg. to +
Spermatids ($n = 7–13$)	++ to +++	++ to +++	++	++ to +++	+ to +++
Leydig cells	neg. to +++	+ to ++	+ to ++	neg. to +++	++
Spermatozoa					
Head	neg. to ++	neg. to +++	neg. to ++	neg. to ++	neg. to +
Neck	neg to ++	neg.	neg. to ++	neg. to ++	neg. to +++
Midpiece	neg. to ++	neg. to ++	neg.	neg. to ++	neg. to +++
Tail	neg.	neg. to +++	neg.	neg.	neg. to +++
Predominant location ($n = 13$)	P. acr., neck and mid.	P. acr. and tail	Head, p. acr. and neck	P. acr., neck and mid.	Neck and annulus
Epididymis					
Caput/corpus	+ to +++	+ to ++	+/− to ++	+/− to ++	++
Cauda ($n = 7$)	++ to +++	neg to +/−	++	+ to ++	++
Prostate					
Gland	+ to +++	+/− to ++	+ to +++	++ to +++	+++
Ductal ($N = 5$)	+ to ++	+/− to +	+	+ to ++	++
Sem vesicle					
Gland ($N = 3$)	++	++	+ to +++	+ to ++	+++
Subcellular location	Nuclear and cytoplasmic	Cytoplasmic	Cytoplasmic	Cytoplasmic	Cytoplasmic

P.acr., post-acrosomal region; gland, glandular epithelia; ductal, ductal epithelia. Staining was classified according to an arbitrary semi-quantitative reference scale depending on the intensity of cellular staining: +++, strong staining; ++, moderate staining; +, weak staining; +/−, very weak staining; neg., no staining.

although CYP2R1 had a marked staining in spermatocytes, where expression was low for CYP27A1 and CYP27B1, and CYP27B1was unlike CYP2R1 and CYP27A1 also present in spermatogonia (Figs 2B–D, 4A2–A4 and Table I). The expression of the activating enzymes in ejaculated spermatozoa varied. CYP27B1 was predominantly expressed in post-acrosomal region and neck, and the majority expressed CYP2R1 predominantly along the tail, post-acrosomal region and neck (Fig. 4G2–G4). CYP27A1 was only expressed in a low percentage of sperm, and the subcellular location varied. The expression of the activating enzymes seemed independent of head morphology (Fig. 4G2–G4).

All the activating enzymes were expressed in epididymal, prostate and SV epithelium. CYP2R1 were strongly expressed in luminal vesicles in caput epididymis, although expression was absent or barely detectable in cauda epididymis (Figs 2E and 4C2/D2). CYP27A1 and CYP27B1 were stronger expressed in distal parts of the epididymis, where expression was dispersed through out the cytoplasm (Fig. 4D3–D4). All the activating enzymes were expressed in the basal layer of the prostate glandular and ductal epithelia (Fig. 4E2–E4). However, CYP2R1 expression seemed lower than CYP27A1 and CYP27B1 in prostate epithelium, although CYP27A1 in one of the investigated prostate samples had a strong expression in few of the epithelial cells (Fig. 4E3). CYP2R1, CYP27A1 and CYP27B1 were all expressed in the epithelium of the SV (Figs 2G, 4F2–F4 and Table I).

Expression of the inactivating enzyme (CYP24A1)

CYP24A1 mRNA had a marked expression in kidney and a minor expression in testis, prostate, SV and ovary. Moreover, since there are splice-variants of *CYP24A1* lacking the mitochondrial targeting domain, we used different primer combinations (Adams *et al.*, 2007). We did not detect the splice-variant of *CYP24A1* lacking the mitochondrial targeting domain in any of the investigated tissues, but other splice variants (with no known functional importance, splice sites in exon 9–11; Muindi *et al.*, 2007) were detected in ovary, epididymis and SV. *CYP24A1* mRNA was detected in testis and kidney with all primer combinations, but expression in ovary, epididymis and prostate was only detected with primers amplifying a fragment in the C terminal part of *CYP24A1* (Fig. 3A).

The CYP24A1 enzyme was present in spermatogonia, round and elongated spermatids, Leydig cells and glandular epithelia of epididymis, prostate and SV (Figs 3B–D, 4A5–F5 and Table I). A large proportion of the ejaculated sperm expressed CYP24 in the neck, midpiece, together with a marked expression in a distinct structure at the annulus (Jensen's ring; Fig. 4G5). A large proportion of sperm with normal midpiece and tail morphology had this distinct expression at the annulus, although it was also detected in sperm with abnormal head morphology. CYP24A1 was expressed in the epithelia of both caput and cauda epididymis. In the proximal parts of the epididymis,

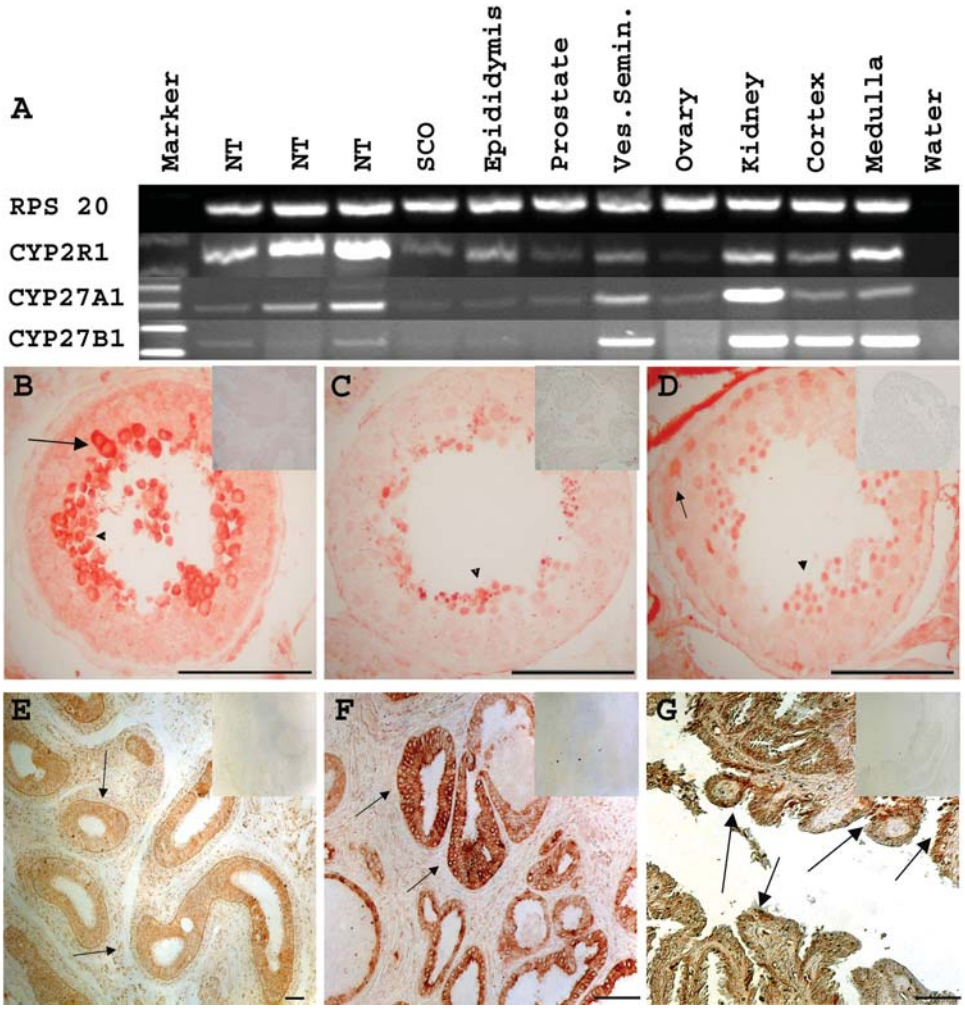

Figure 2 Expression of the VD activating enzymes in the reproductive tract.

(**A**) RT–PCR with the same panel and control as described above (Fig. 1A). (**B**) IHC analysis of CYP2R1 in NT with expression in round spermatids (arrowhead) and arrow indicates expression in spermatocytes. (**C**) IHC analysis of CYP27A1 in NT showing expression in round spermatids (arrowhead). (**D**) IHC analysis of CYP27B1 in NT with expression in spermatogonia (arrow) and in round spermatids (arrowhead). (**E**) CYP2R1 expression in corpus epididymis (arrow). (**F**) Cytoplasmic expression of CYP27A1 in the prostate gland (arrows). (**G**) Cytoplasmic expression of CYP27B1 in epithelium of the SV (arrows). Bar corresponds to 50 μm.

the expression was located only in the luminal vesicles, while it had a dispersed expression in the distal epididymal segments (Fig. 4C5 and D5). In the prostate gland CYP24A1 expression was detected in the basal layer of the glandular epithelia as well as epithelia lining the prostate ducts (Figs 3D, 4E5 and Table I). In SV all epithelial cells expressed CYP24A1 (Fig. 4F5).

Co-localization of all vitamin D metabolizing enzymes and VDR

VDR and all VD metabolizing enzymes co-localized in both Leydig cells and round and elongated spermatids, although only CYP27B1, VDR and CYP24A1 were expressed in spermatogonia (Table I). Some tissue samples contained clusters of Leydig cells and few of these clustered cells did not express any of the investigated enzymes. CYP2R1 stained a larger proportion of ejaculated sperm than CYP27A1, and expression was mainly found along the tail and in the neck and par, where VDR, CYP24A1 and CYP27B1 also were expressed. A large proportion of the spermatozoa from healthy controls seemed to have concomitant expression of the CYP27B1, VDR and CYP24A1 at the post-acrosomal region, neck and midpiece (Fig. 4).

In the proximal part of epididymis the VDR and the metabolizing enzymes were confined to luminal vesicles in the cytoplasm, although cytoplasmic expression of all investigated proteins except CYP2R1were higher in cauda epididymis. The epithelia in prostate and SV co-expressed VDR and the metabolizing enzymes, and they were also expressed in the ducts from both organs. In addition we noticed a minor staining in the endothelium, stroma, peritubular and inflammatory cells.

All the results reported here were reproducible, although expression of VDR in the spermatocytes varied. There were no major discrepancies in the ratings performed by the two observers.

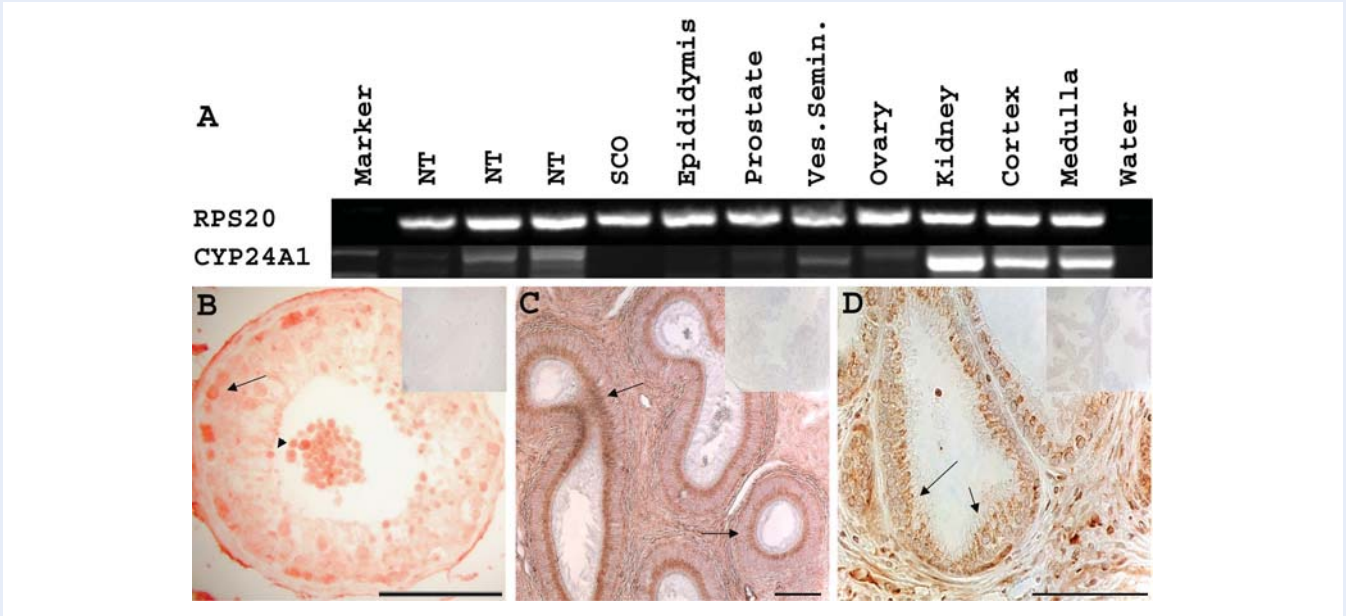

Figure 3 Expression of the inactivating enzyme (CYP24A1) in the reproductive tract.

RT–PCR with the same panel and control as described above (Fig. 1A). (**B**) IHC detection of CYP24A1 in NT with marked expression in spermatogonia (arrow) and round spermatids (arrowhead). (**C**) Marked expression of CYP24A1 in the luminal vesicles in caput epididymis (arrows). (**D**) Cytoplasmic staining in the epithelium of the prostate gland (arrows). Bar corresponds to 50 μm.

Discussion

We show here for the first time that the human testis and epithelia of the ejaculatory tract are capable of metabolizing VD. The expression of VDR and VD metabolizing enzymes is present in germ cells during spermatogenesis and persist during transit through the ejaculatory duct and in ejaculated mature spermatozoa. Extra-renal metabolism of VD has to our knowledge not previously been shown in testis, epididymis, SV or mature spermatozoa, but it has been detected in several other mesenchymal-derived tissues, where local activation of VD is important for cell cycle control (Fleet, 2008). Although this is a descriptive study, we believe the concomitant expression of VDR and VD metabolizing enzymes in male germ cells and reproductive tract indicates that local activation of VD may be important for spermatogenesis and sperm maturation.

VDR expression is required for the cell to respond to activated VD, and the subcellular location of VDR seems to be important for the VD-mediated action. The partial nuclear expression of VDR in spermatogonia indicates a genomic action, where VDR forms a heterodimer with the retinoid receptor (RXR), binds to vitamin D response elements (VDRE) and regulates transcription of several genes involved in mitotic activity, differentiation and apoptosis (Fleet, 2008). VD can also induce a rapid non-genomic action, which is mediated through membrane bound or cytoplasmic VDR and alter cellular activity through second messengers (Fleet, 2008). VDR expression has previously been shown in the reproductive tract in rats, in human testis, prostate and spermatozoa (Johnson *et al.*, 1996; Bieche *et al.*, 2007). The IHC results of our analysis are in agreement with earlier reports (Merke *et al.*, 1985; Johnson *et al.*, 1996; Corbett *et al.*, 2006; Nangia *et al.*, 2007; Aquila *et al.*, 2008; Oliveira *et al.*, 2008; Hirai *et al.*, 2009), although there are a few discrepancies, which could be caused by antibody properties together with use of different fixatives, species and method selection. We evaluated the expression in morphologically normal tissue from pathological specimens, because biopsies or surgery can only be performed if there is a clinical indication for the procedure. The normal tissue, which we used for our investigation, was sometimes present adjacent to pathological tissue such as carcinoma *in situ*, SCO, or a fibroid prostate. This could partly be responsible for the range of expression reported in Table I, because levels of fibrosis, inflammation, metabolism etc. in the tissue may induce variation in the cellular expression between the different tissue samples.

The cellular response to VD is not solely dependent on VDR expression, but depends also upon the presence of VD activating and inactivating enzymes. Spermatogonia express VDR, CYP27B1 and CYP24A1, with no concomitant expression of the 25-hydroxylases. This implies that spermatogonia could have easier access to circulating 25(OH)D_3, while concomitant expression of VDR and all the VD metabolizing enzymes were detected in round spermatids, situated behind the blood-testis barrier. In addition to its role in cell cycle control, VD may be important for the synthesis of VD-dependent calcium transporters (TRPV5, TRPV6), the calcium pump (PMCA), calbindin and calmodulin, which all are important for sperm function (Schuh *et al.*, 2004; Inpanbutr *et al.*, 1996; Schoeber *et al.*, 2007; Fleet, 2008; Li *et al.*, 2009).

The expression of VDR and VD metabolizing enzymes in Leydig cells were evaluated by using AP-conjugated secondary antibodies, because the peroxidase based visualization resulted in a minor staining of Leydig cells in negative controls. The IHC expression is partly reflected by the mRNA level in the SCO sample, which contains

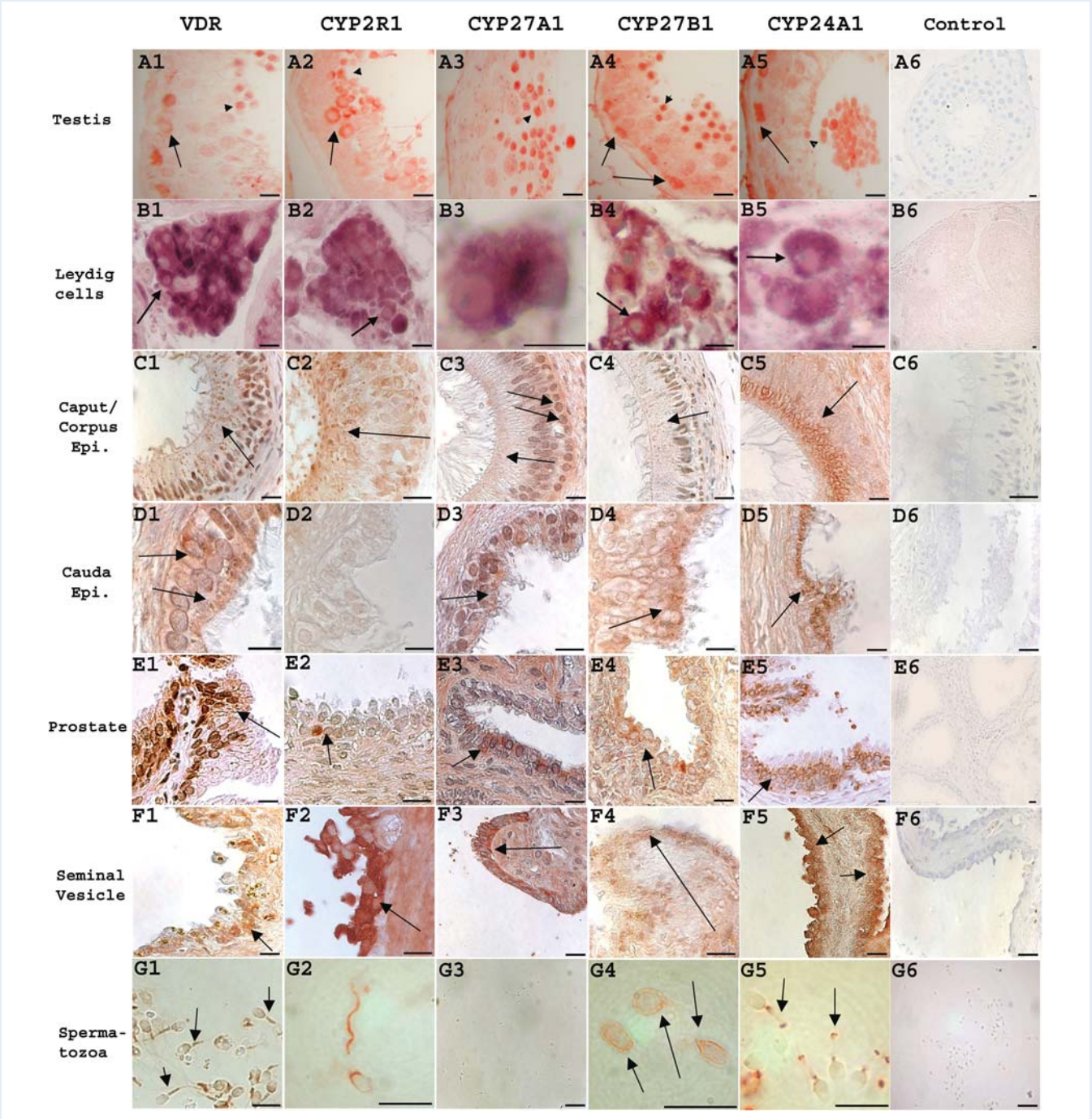

Figure 4 Cellular expression pattern of VDR and the VD metabolizing enzymes in the male reproductive tract.

(**A1**) VDR expression in the nucleus and cytoplasm (black arrow) of spermatogonia and in round spermatids (arrowhead). (**A2**) CYP2R1 expression in spermatocytes (arrow) and round spermatids (arrowhead). (**A3**) CYP27A1 expression in round spermatids (arrowhead). (**A4–5**) Expression of CYP27B1 and CYP24A1 in spermatogonia (arrow) and round spermatids (arrowhead). (**B1–5**) Cytoplasmic expression of VDR and all the metabolizing enzymes in Leydig cells. (**C1–5**) Black arrows indicate expression of VDR and all the enzymes in the luminal vesicles, whereas double arrow points on expression of CYP27A1 in basal cells of caput/corpus epididymis. (**D1–5**) Abundant cytoplasmic expression of all investigated proteins except for CYP2R1 (D2) in cauda epididymis. (**E1**) Nuclear and cytoplasmic staining (arrow) in the epithelium of the prostate. (**E2–3**) A few cells (arrow) with a marked cytoplasmatic expression of CYP2R1 and CYP27A1 in the prostate gland. (**E4–5**) Abundant expression of CYP27B1 and CYP24A1 in the prostate. (**F1–5**) A strong cytoplasmic expression of VDR and all the enzymes in the epithelium of the SV. (**G1**) expression of VDR in the midpiece (arrows). (**G2**) CYP2R1 expression in par, neck and tail of ejaculated spematozoa. (**G3**) no detectable expression of CYP27A1 in sperm. (**G4**) expression of CYP27B1 in par and neck of spermatozoa. (**G5**) detection of CYP24A1 in the neck and at the annulus (arrow). (**A6–G6**) Negative controls, all except B6 and G6 counterstained with Mayer's. A6, B6 and G6 with monkey secondary antibody, although C6, D6, E6 and F6 with goat secondary antibody. Bar corresponds to 10 μm.

Leydig cells, although, *VDR* mRNA was not detected. VDR has previously been shown in human Leydig cells, and we show here that VDR is expressed concomitantly with all the metabolizing enzymes in Leydig cells, although the intensity of the expression should be interpreted with caution (Nangia *et al.*, 2007). The cytoplasmic co-expression of VDR and the metabolizing enzymes in Leydig cells suggest that VD might affect male reproductive hormone production. However, the impact of VD on sex hormone production is largely unexplored, although VDR knockout mice develop hypergonadotropic hypogonadism with low estrogen levels, because of low aromatase activity (Kinuta *et al.*, 2000). Estrogen is important for reabsorption of water during transit of sperm from testis to epididymis, and low estrogen results in altered osmolarity, which may lead to dysfunction of spermatozoa (Hess *et al.*, 1997).

The ejaculatory tract originate like the kidney from intermediate mesoderm and estrogen dependent iso-osmotic reabsorption of water and electrolytes in rete testis, resembles the iso-osmotic reabsorption (not estrogen-dependent) that takes place in the proximal tubules of the kidney (Clulow *et al.*, 1994). We show here that the distal part of epididymis, SV and prostate all express VDR and VD metabolizing enzymes. These organs also express VD-dependent ion channels and proteins, which are expressed in the distal tubules of human kidney, where VD is important for calcium reabsorption and intracellular transport (Kumar *et al.*, 1994). We propose that VD in the ejaculatory tract is important for transcellular calcium transport, like it is in kidney and intestine (Blomberg Jensen *et al.*, 2009). This hypothesis is supported by high calcium content in fluid from epididymis, SV and prostate, where concentration of calcium is 2–3-fold higher than in serum (Kavanagh, 1985; Wong *et al.*, 2001). The marked difference suggests active transport from the circulation to the lumen of the ejaculatory tract, and this transcellular transport may be VD-dependent, in analogy to the intestine and kidney (Kavanagh, 1985; Wong *et al.*, 2001). VDR and the VD metabolizing enzymes are also expressed in the luminal vesicles of caput/corpus epididymis, which are excreted into the lumen of the ejaculatory tract. Thus, function of VD may be different in the proximal part of epididymis. Here it could affect the activity of the aromatase, acts as a paracrine signalling molecule, or in case the vesicles fuse with the membrane of spermatozoa, it may initiate rapid non-genomic actions in the sperm.

The ejaculated spermatozoa are known to be genetically silent; there is, if any, only limited transcriptional activity after the DNA is packed with protamines (Galeraud-Denis *et al.*, 2007). Since mature spermatozoa co-express VDR and the metabolizing enzymes in the post-acrosomal region/neck and midpiece, we speculate that activated VD may have a non-genomic action in spermatozoa. Activated VD in small concentrations has been shown to prolong survival of ejaculated spermatozoa, modulate cholesterol composition and induce phosphorylation of selected proteins (Aquila *et al.*, 2008). Immature mice Sertoli cells, which unlike mature human Sertoli cells express VDR, can respond to VD by an increase in intracellular calcium content, which is caused by an increased calcium-influx either by activating membrane-channels, -transporters, intracellular store-operated calcium channels or through second messengers like cAMP (Akerstrom and Walters, 1992; Fleet, 2004; Zanello and Norman, 2006). VD may be a regulator of the intracellular calcium content in mature sperm, which is supported both by a study showing that the effect of VD on spermatozoa can be diminished by using a calcium channel blocker, and by the positive effect of calcium in the VD deficient male rats (Uhland *et al.*, 1992; Menegaz *et al.*, 2009).

Kidroni *et al.* (1983) showed that tissue concentration of 25-hydroxycholecalciferol in male rats after eight subcutaneous injections of tritiated 25-hydroxycholecalciferol was highest in the kidney, followed by epididymis, prostate, testis and SV (Kidroni *et al.*, 1983). They also measured $24,25(OH)_2D_3$, and found that the concentration of this metabolite was higher in both epididymis and prostate compared with the kidney, which could be explained by the marked expression of CYP24A1 we show in cauda epididymis and prostate gland. Following intravenous injections of [^{3}H] 25-hydroxycholecalciferol they found a high concentration of $1,25(OH)_2D_3$ in the kidney, epididymis and testis, which is in accordance with the presence of the metabolizing enzymes documented in this study.

In conclusion, we analysed the expression pattern of the vitamin D metabolizing machinery, and showed that VDR, CYP2R1, CYP27A1, CYP27B1 and CYP24A1 were concomitantly expressed in the human testis, epididymis, prostate, SV and mature spermatozoa. This finding is novel and provides a necessary reference for further mechanistic studies, which are needed to elucidate the precise role of VD in human spermatogenesis and reproduction.

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