Vitamin D and male reproduction

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Abstract | Vitamin D is a versatile signalling molecule with a well-established role in the regulation of calcium homeostasis and bone health. The spectrum of vitamin D target organs has expanded and the reproductive role of vitamin D is highlighted by expression of the vitamin D receptor (VDR) and enzymes that metabolize vitamin D in testis, male reproductive tract and human spermatozoa. The expression levels of VDR and CYP24A1 in human spermatozoa serve as positive predictive markers of semen quality, and VDR mediates a nongenomic increase in intracellular calcium concentration that induces sperm motility. Interestingly, functional animal models show that vitamin D is important for estrogen signalling and sperm motility, while cross-sectional studies support the positive association between serum 25-hydroxyvitamin D level and sperm motility in both fertile and infertile men. Expression of VDR and enzymes that metabolize vitamin D in fetal testis indicates a yet unknown role during development, which may be extrapolated from invasive testicular germ cell tumours where 1α,25-dihydroxyvitamin D induces a mesodermal differentiation of the pluripotent testicular cancer cells. Taken together, vitamin D signalling has a positive effect on semen quality, increases estrogen responsiveness and differentiates germ cell tumours. Future studies are needed to determine when 1α,25-dihydroxyvitamin D acts in a paracrine manner and whether systemic changes, which are subject to pharmacological modulation, could influence male reproductive function.

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Introduction

The main functions of the testis are synthesis of sex hormones and production of spermatozoa. Steroidogenesis is performed by Leydig cells of the interstitium, whereas spermatogenesis occurs inside the seminiferous tubules (Figure 1).[1] Testicular functions require a multitude of tightly regulated morphogenic and molecular events, and the search for regulatory factors has been intense. Most attention has been paid to effects occurring during fetal life, as testicular development to a large degree predetermines adult testicular function. However, the adult testis is also sensitive to various exposures and numerous investigations have addressed the effect of nutrition, hormones, pharmacological agents, endocrine disrupters and vitamins on reproductive function.[2]

Vitamin D is a versatile signalling molecule, and the male reproductive organs are part of the expanding palette of vitamin D targets, in addition to the classic effects on bone, calcium and phosphate homeostasis.[3,4] In the past decade, an ongoing debate concerning global vitamin D deficiency in the general population[5,6] has prompted intensive research of the nonclassic effects of vitamin D. In this Review, the spatial and temporal localization of the vitamin D receptor (VDR) and enzymes that metabolize vitamin D in the male reproductive organs are discussed with the aim of conceptualizing the role of systemic and local metabolism of vitamin D in male fertility, sperm function, sex and reproductive hormone synthesis and testicular germ cell cancer. Improved knowledge of the effects of vitamin D on male reproduction will provide insights concerning the influence of classic bone factors on gonadal function and aid understanding of human reproduction in general.

Vitamin D metabolism

The inactive form of vitamin D (cholecalciferol) is synthesized in the skin following conversion of 7-dehydrocholesterol by ultraviolet B radiation (Figure 2).[7] Cholecalciferol must undergo two hydroxylation events to form the active form of vitamin D, 1α,25-dihydroxyvitamin D_3. The first step is 25-hydroxylation by the hepatic enzyme CYP2R1 followed by renal 1α-hydroxylation by CYP27B1, whereas CYP24A1 inactivates all circulating forms of vitamin D.[7] Subsequently, 1α,25-dihydroxyvitamin D_3 binds and activates VDR that forms a heterodimer with retinoid X receptor (RXR). This complex recognizes a vitamin D response element (VDRE) in the promoter region of target genes and regulates transcription.[8,9] The genomic ligand-binding pocket of VDR mediates the transcriptional effects, but VDR also has an alternative ligand-binding pocket that mediates rapid nongenomic effects.[8,10]

Vitamin D status is assessed by measuring the serum concentration of 25-hydroxyvitamin D rather than serum 1α,25-dihydroxyvitamin D_3 because levels of serum 25-hydroxyvitamin D are associated with rickets,

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Competing interests
The author declares that he holds two patent applications related to vitamin D and reproduction.

Key points

- Vitamin D is metabolized in the male reproductive organs and the expression levels of vitamin D receptor (VDR) and CYP24A1 in human spermatozoa are positive markers for semen quality
- 1α,25-dihydroxyvitamin D_3 induces a VDR-mediated increase in intracellular calcium concentration in human spermatozoa *in vitro*, leading to increased motility and induction of the acrosome reaction in capacitated spermatozoa
- *Vdr*-null mice and rodents with vitamin D deficiency develop impaired fertility due to decreased sperm production and low sperm motility, which can only partly be restored by calcium supplementation
- Results from human association studies are in line with those in animal models, as men with vitamin D sufficiency have a higher percentage of motile spermatozoa than men with vitamin D deficiency
- 1α,25-dihydroxyvitamin D_3 induces differentiation of embryonal carcinoma cells *in vitro* and *in vivo* and increases cellular susceptibility to cisplatin *in vitro*
- The most important regulators of testicular vitamin D metabolism seem to be fibroblast growth factor 23 and Klotho, other regulators are testosterone, estradiol, calcium, phosphate and 1α,25-dihydroxyvitamin D_3

hypocalcaemia, hypophosphataemia and serum levels of parathyroid hormone (PTH).[3,5,6] Hypocalcaemia often accompanies vitamin D deficiency and is a major obstacle to proper interpretation of proposed vitamin-D-mediated effects. The effect of vitamin D on calcium and phosphate homeostasis necessitates a tight regulation of the activity of the enzymes that metabolize vitamin D; however, the spatial expression of these enzymes is not restricted to the liver and kidney.[9] Extra-renal vitamin D metabolism is not involved in calcium homeostasis, but rather in paracrine and autocrine functions (for instance, the regulation of cell cycle control) and, therefore, has a different system of regulation than systemic vitamin D metabolism.[9]

Vitamin D and male reproductive organs

A prerequisite for being a vitamin D target organ is the expression of VDR. However, the cellular responsiveness in target cells depends on not only circulating levels of 1α,25-dihydroxyvitamin D_3, but also cellular expression of CYP2R1, CYP27B1 and CYP24A1. The encoded proteins activate or inactivate circulating cholecalciferol or 25-hydroxyvitamin D, which modulates the intracellular concentration of 1α,25-dihydroxyvitamin D_3. VDR and enzymes that metabolize vitamin D are concomitantly expressed in Sertoli cells, germ cells, Leydig cells, spermatozoa and in the epithelial cells lining the male reproductive tract (Figure 1).[4,11–17] The presence of vitamin D metabolizing enzymes indicates that the reproductive organs can modulate the local vitamin D response, which is supported by the high concentration of 25-hydroxyvitamin D, 1α,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 in rat testis and epididymis compared with other organs following injection of tritiated vitamin D progenitors.[18] VDR has no testis-specific splice variants[19] and the high binding affinity (VDR_{kd} 50–100 pM)[20–22] for 1α,25-dihydroxyvitamin D_3 in the testis indicates that the concentration of 1α,25-dihydroxyvitamin D_3 in serum is adequate for gonadal VDR activation.

Minor differences in the expression of VDR and enzymes that metabolize vitamin D between species exist: in humans, VDR is expressed mainly in germ cells as well as fetal or immature Sertoli cells,[4,23] whereas in mice VDR is expressed in germ cells in addition to both immature and mature adult Sertoli cells,[24,25] which impedes translation of results from mice models to humans. Interestingly, specific expression profiles of VDR and enzymes that metabolize vitamin D in human spermatozoa[4,16,17,21] from healthy normospermic and subfertile men distinguish the two populations of sperm with high specificity and might thus prove clinically relevant.[21,26] In particular, the distinct localization of CYP24A1 at the sperm annulus serves as a predictive marker of good quality sperm,[4,26] and the fraction of CYP24A1-positive spermatozoa has been positively associated with sperm count, concentration, motility and morphology (Figure 1).[26]

Importantly, >80% of CYP24A1-positive spermatozoa concomitantly express VDR in the neck and head region.[26] Co-expression of both proteins might be linked to transcriptional induction during spermatogenesis, wherein the VDR complex binds to one of the two positive VDREs in the promoter region of *CYP24A1*.[8] During the later stages of spermatogenesis, cellular organelles are lost and most histones are replaced by protamines that prevent transcription and spermatozoa are thus almost transcriptionally silent.[27] Human spermatozoa are therefore a unique model to study nongenomic effects exerted by VDR (discussed later).[10] The low expression levels of VDR and CYP24A1 in spermatozoa from men with infertility serves not only as a marker but implies unresponsiveness to treatment or exposure to 1α,25-dihydroxyvitamin D_3 in the male or female reproductive tract.[26] Indeed, the proposed functional consequences of low expression of CYP24A1 in spermatozoa were supported by showing an increase in sperm motility after treatment with 1α,25-dihydroxyvitamin D_3 *in vitro* exclusively in healthy normospermic men and not in spermatozoa from men with infertility[3] who had a very low average proportion (<3%) of CYP24A1-expressing sperm.[26]

The enzymes 1α-hydroxylase (CYP27B1) and 25-hydroxylase (CYP2R1) in particular, are highly expressed in the testis compared with other tissues.[28,29] CYP2R1 is expressed in both Leydig and germ cells[4,23,25] and reduced CYP2R1 expression has been reported in testis from men with impaired spermatogenesis, probably due to a decreased number of germ cells.[30] These patients also display a lower serum level of 25-hydroxyvitamin D than normospermic men, and it has been proposed that testicular CYP2R1 is important for systemic 25-hydroxylation.[30] This suggestion is attractive because the relative expression of CYP2R1 is higher in human testis than in the liver.[28] Nevertheless, several studies have shown that systemic 25-hydroxylation mainly takes place in the liver. This theory is strongly supported by the low circulating serum level of 25-hydroxyvitamin D in mice following hepatectomy.[31] However, an increase in hepatic 25-hydroxylase has been observed following castration in rats, which indicates that the testis might contribute to, or at least produce, a regulator of hepatic 25-hydroxylase and therefore also influence serum levels

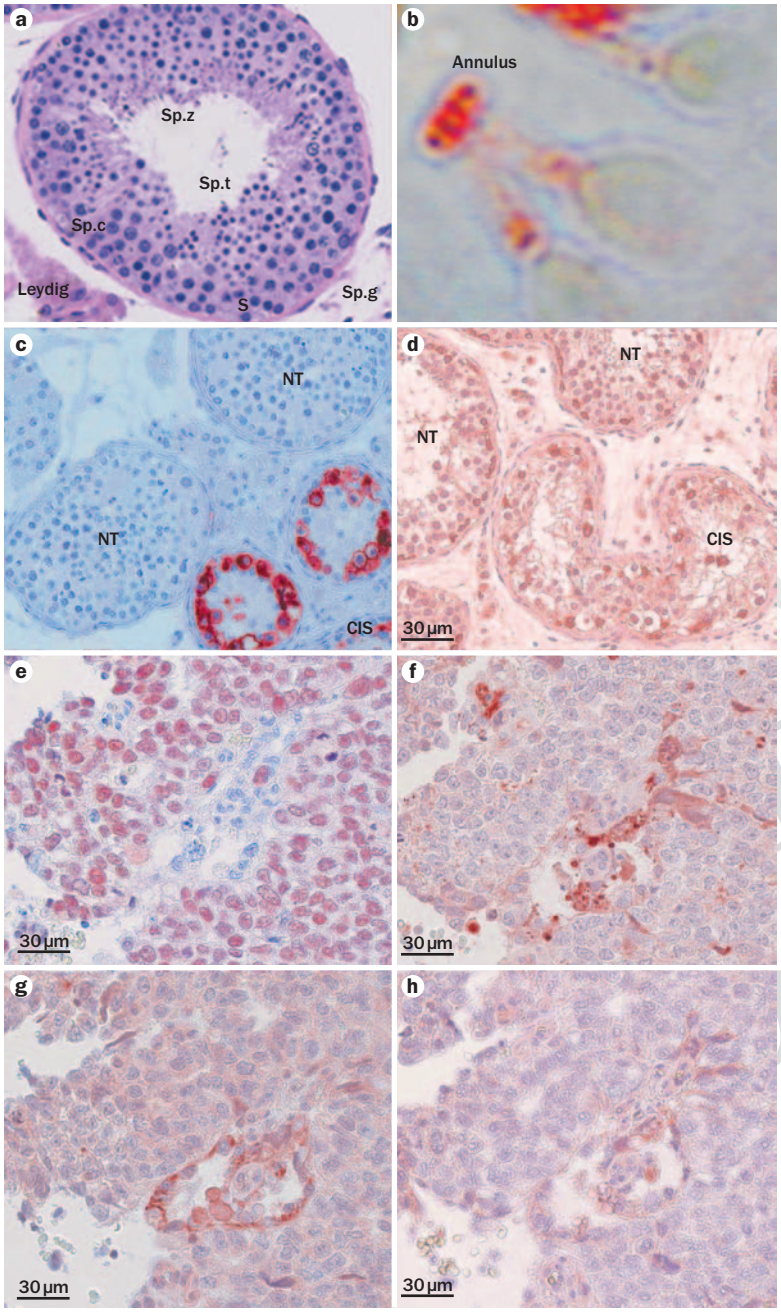

Figure 1 | Immunohistochemistry from the male reproductive organs. **a** | Haematoxylin and eosin staining of intratubular normal spermatogenesis with spermatogonia (Sp.g.), Sertoli cells (S), spermatocytes (Sp.c.), spermatids (Sp.t), spermatozoa (Sp.z) and interstitial Leydig cells marked. **b** | Immunohistochemical staining of CYP24A1 in human spermatozoa with a distinct expression at the annulus separating the midpiece from the tail. **c** | Alkaline phosphatase placental-like (PLAP) is a marker of carcinoma *in situ* (CIS) cells, whereas there is no detectable expression of PLAP in normal testis (NT). **d** | Vitamin D receptor (VDR) expression in CIS and NT. **e**–**h** | Serial sections of OCT4, CYP27B1, VDR and osteocalcin, respectively, in a nonseminona show that VDR and enzymes that metabolize vitamin D in addition to a VDR-regulated gene *BGLAP* (osteocalcin) are expressed in cancer cells with none or low OCT4 expression.

of 25-hydroxyvitamin D.[32]

The transmembrane proteins LRP-2 (also known as megalin) and cubulin facilitate cellular entry of the protein-bound fraction of steroid hormones, including 25-hydroxyvitamin D, in the kidney, prostate and breast. Both proteins are expressed in the male reproductive tract[33] and might facilitate cellular entry of the protein-bound forms of vitamin D (Figure 3). VDR and enzymes that metabolize vitamin D are also expressed in the different segments of the epididymis (caput, corpus and cauda), prostate and seminal vesicle of mice,[20] rats,[13] roosters[14] and humans.[4] The epididymis regulates the composition of fluid surrounding the spermatozoa during transition through the caput, corpus and cauda. The concentration of calcium decreases, whilst the concentration of phosphate increases in the fluid[34] from the proximal to the distal part of the epididymis, which might be important for sperm maturation and involved in the induction of sperm motility. During ejaculation, the spermatozoa meet the secretions from the prostate and seminal vesicle with a calcium concentration more than twofold higher than serum,[35] which might prepare the spermatozoa for the environmental shift in the female reproductive tract.

The effect of vitamin D in the efferent ducts and epididymis might be comparable with that in the kidney because they share developmental origin.[36] The main function of vitamin D in the kidney is transcellular calcium transportation, which involves concerted action of TRPV5, TRPV6, calbindin, PMCA1 and NCX1.[3] The hypothesized conserved function of vitamin D in both organs is supported by the fact that TRPV6 is expressed in the epididymis, and ablation of TRPV6 impairs epididymal calcium absorption, which results in low sperm motility and male infertility in mice.[37]

Vitamin D and gonadal hormones

Testosterone

Testosterone is produced by Leydig cells and is responsible for primary and secondary male sex characteristics. The concentration of testosterone is 100-fold higher in the testis than in serum, and testosterone biosynthesis is controlled by placental human chorionic gonadotropin (hCG) in early fetal life until the pituitary starts to secrete luteinizing hormone (LH).[38] LH induces steroidogenesis by increasing cyclic AMP production and the intracellular concentration of calcium ions (Ca^{2+}) in Leydig cells,[38,39] and 1α,25-dihydroxyvitamin D_3 might exert an influence by modulating this calcium-dependent LH response.

The testosterone:LH ratio is a good indicator of LH sensitivity and Leydig cell function. In *Vdr*-null mice, a tendency towards a low testosterone:LH ratio and Leydig cell hyperplasia (potentially as a consequence of the low ratio) were noted.[25] However, serum levels of testosterone and LH were not significantly different between wild-type and *Vdr*-null mice generated by two different

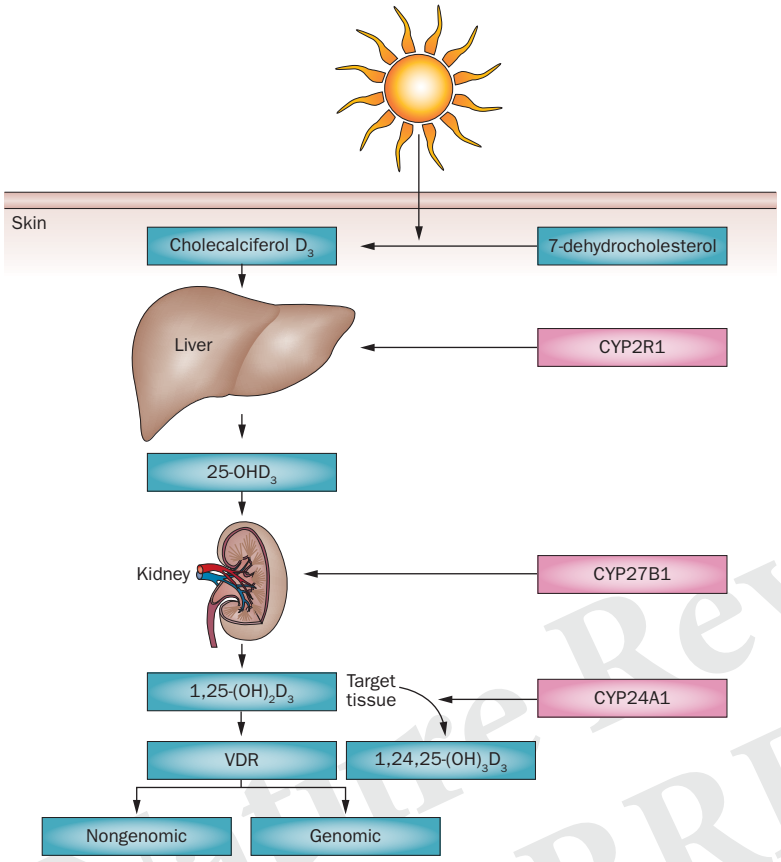

Figure 2 | Systemic vitamin D metabolism. Vitamin D synthesis normally starts in the skin, where ultraviolet B radiation initiates conversion of 7-dehydrocholesterol to cholecalciferol. Cholecalciferol D_3 is biologically inactive and undergoes two hydroxylation steps before the active 1α,25-dihydroxyvitamin D_3 is formed. Normally, 25-hydroxylation is mediated by the hepatic CYP2R1, whilst 1α-hydroxylation is conducted by the renal CYP27B1. The active form of vitamin D, 1α,25-dihydroxyvitamin D_3, binds to the VDR and mediates rapid effects through the alternative ligand binding pocket or genomic effects through the genomic pocket. All the circulating forms of vitamin D are inactivated by 24-hydroxylation. Abbreviations: 25-OHD_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3; VDR, vitamin D receptor.

laboratories.

Correspondingly, hCG tests in three children with hereditary 1α,25-dihydroxyvitamin D_3-resistant rickets (no functional VDR) elicited a normal testosterone response,[40] and infusion of 1α,25-dihydroxyvitamin D_3 for 3 days in healthy men caused no changes in serum levels of testosterone or LH.[41] Therefore, it is reasonable to assume that no causal relationship exists between VDR activation and serum levels of testosterone or LH sensitivity. This suggestion is in accordance with the comparable serum concentrations of testosterone and LH in adolescents and healthy young men with and without vitamin D deficiency.[42–44] However, in older men (>60 years of age), several cross-sectional studies[45–48] have found positive associations between serum levels of 25-hydroxyvitamin D and testosterone and a seasonal variation in serum testosterone levels that matched the seasonal changes in serum levels of 25-hydroxyvitamin D.[46,49]

The striking difference between young and older men indicates an indirect effect of vitamin D, which was supported by a cross-sectional study reporting significant associations between serum levels of 25-hydroxyvitamin D and testosterone, which disappeared when adjusting for health status and comorbidities.[47] Furthermore, serum levels of 25-hydroxyvitamin D and sex hormones decline with age, whereas serum levels of sex hormone-binding globulin (SHBG; also known as testis-specific androgen binding protein) increase.[50] This pattern might explain some of the discrepancy because serum levels of 25-hydroxyvitamin D are positively associated with those of SHBG in young men[42,43] but not in older men.[46–48] Several factors regulate SHBG biosynthesis, and a small randomized clinical trial tested the combined effect of weight loss and cholecalciferol supplementation on levels of SHBG and testosterone in 54 obese men of an average age of 48 years.[45] The researchers found an increased serum level of SHBG, but no significant changes in serum concentrations of testosterone between placebo-treated and cholecalciferol-treated men.[45] The presumed effect of vitamin D on SHBG in the liver might be direct or indirect, but testicular *Shbg* levels are comparable between *Vdr*-null mice and wild-type littermates,[25] which excludes a gonad-specific effect of vitamin D on SHBG in mice.

Vitamin D, in conjunction with PTH, regulates calcium absorption in the intestine and excretion in the kidney. Thus, serum levels of calcium can change as a result of systemic changes in serum concentrations of vitamin D and might, therefore, exert an indirect effect in the target tissue. Vitamin D deficiency is often accompanied by hypocalcaemia, which might exert an influence on the target tissue rather than having a direct VDR-mediated effect. One example is the low serum level of testosterone in vitamin-D-deficient rats, which increases 2–5-fold following injections of 1α,25-dihydroxyvitamin D_3.[51] By contrast, another study showed comparable serum levels of testosterone between normocalcaemic vitamin-D-deficient chickens and vitamin-D-replete chickens.[52] The proposed effect of calcium on steroidogenesis is biologically grounded, as a low extracellular calcium level diminishes the hCG-mediated effect on steroidogenesis and, therefore, constitutes a potential confounder.[38] Another testosterone-inducing effect of vitamin D could be mediated by osteocalcin, which is produced and secreted by the skeleton and the gene that encodes osteocalcin is regulated by vitamin D.[8] Osteocalcin has been proposed to stimulate testosterone production through activation of the promiscuous receptor GPRC6A in mouse Leydig cells.[53,54] However, human data are limited. Osteocalcin might be positively associated with serum concentration of testosterone but is not a strong determinant of serum levels of testosterone in men.[42,55,56] Combined, the effects of vitamin D on testosterone production appear to be indirectly mediated through calcium and phosphate homeostasis, SHBG or osteocalcin production. The large randomized clinical trials evaluating vitamin D supplementation to prevent fractures might provide additional information in older men while we await randomized clinical trials with serum testosterone levels as a primary end point.

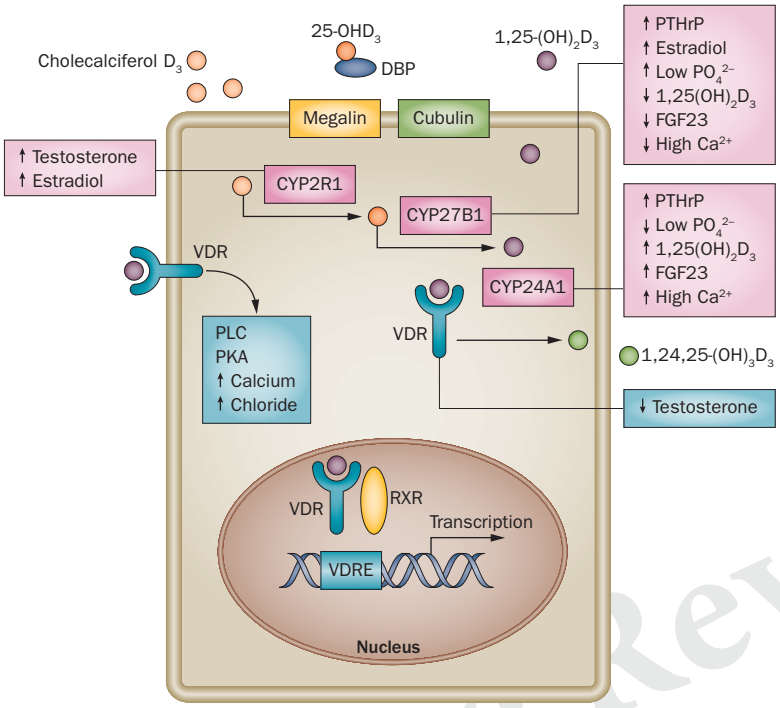

Figure 3 | Factors influencing cellular vitamin D metabolism in the male reproductive organs. The circulating forms of vitamin D (cholecalciferol, 25-hydroxyvitamin D and 1α,25-dihydroxyvitamin D_3) diffuse freely across the plasma membrane, whereas the protein-bound fraction (bound to DBP) are transported actively into the cell by megalin or cubulin. The cell expresses the enzymes that metabolize vitamin D (CYP2R1, CYP27B1 and CYP24A1) and are thus capable of activating or inactivating all circulating forms of vitamin D. 1α,25-dihydroxyvitamin D_3 elicits the effects through the VDR. VDR mediates rapid actions when situated at the membrane or in the cytoplasm through ion channels or modulation of second messengers. VDR heterodimerizes with RXR and binds to a VDRE in the promoter region of the target genes and regulates transcription. The regulators of local vitamin D metabolism in the male reproductive organs and their proposed targets and actions are marked on the figure (stimulators ↑, inhibitors ↓). Abbreviations: 25-OHD_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3; DBP, vitamin D binding protein; FGF23, fibroblast growth factor 23; PKC, protein kinase C; PLC, phospholipase C; PTHrP, parathyroid hormone-related protein; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D response element.

Estradiol

The *CYP19A1* gene encodes aromatase, which converts testosterone to estradiol, has multiple promoters and is regulated differently depending on promoter activity in the target tissue.[57–59] 1α,25-dihydroxyvitamin D_3 binds to VDREs in the promoter of *CYP19A1* and exerts tissue-specific regulation of aromatase by repressing or inducing transcription in breast and bone, respectively.[24,57,60] Several studies have investigated testicular aromatase activity and identified promoter II as the main promoter in the testis,[61,62] although promoter I.4 might contribute in the germ cells.[63] Estrogens are not exclusively produced by Leydig cells as >60% of testicular aromatase originates from germ cells.[58] Estradiol concentration in rete testis is therefore high,[59] whereas the serum level of estradiol is typically low in men.

Aromatase expression was induced by 1α,25-dihydroxyvitamin D_3 in immature rat Sertoli cells *in vitro*.[24] Moreover, two *Vdr*-null mouse models (two different strains of mice expressing a nonfunctional VDR isoform in all tissues due to removal of exon 2 of the *Vdr* gene) convincingly showed a marked influence of 1α,25-dihydroxyvitamin D_3 on estrogen signalling in testis and epididymis (Table 1).[25,60] However, the decreased testicular aromatase level and compensating increases in serum levels of gonadotropins[60] in the initial *Vdr*-null mouse (produced in Tokyo, Japan) were not supported in another *Vdr*-null mouse strain (produced in Leuven, Belgium).[25] Instead, serum levels of gonadotropins were normal but the expression level of estrogen receptor (ER) α and ERβ were altered. ERβ seemed to be a major target, as heterozygous as well as homozygous mutant mice with concomitant hypocalcaemia had low ERβ expression.[25] The aberrant estrogen response was supported by showing an epididymal upregulation of the estrogen regulated gene aquaporin 9.

Hypocalcaemia complicates the interpretation of the molecular phenotype of *Vdr*-null mice, as it has been demonstrated that low serum levels of calcium induce testicular aromatase transcription and activity in rats.[64] This observation contrasts with the reported decline in aromatase transcription and activity in the first study of *Vdr*-null mice.[60] Moreover, the reproductive phenotype of the *Vdr*-null mouse model[60] does not resemble the inconsistent and late-onset impaired male fertility observed in the three different *Cyp19a1*-null mice.[58] Instead, *Vdr*-null mice have some similarities with *Erα*-null mice, which are characterized by low sperm motility and infertility due to decreased fluid reabsorption in rete testis and caput epididymis.[65,66] However, the complex molecular phenotype with low *Cyp19a1* and *Erβ* expression and high epididymal ERα in *Vdr*-null mice indicates that local estrogen signalling is altered. A relationship between VDR and estrogen in the male reproductive organs is further supported by expression of the estrogen receptors and VDR in the same cells,[36] and by the reversible decrease in spermatogenesis and sperm motility in *Vdr*-null mice[60] following supplementation with calcium and estrogen.

The systemic effects of aberrant estrogen signalling due to loss of VDR are less pronounced than those at the gonadal sites because serum levels of estradiol were not significantly different between *Vdr*-null mice and their wild-type littermates in two different mouse strains.[25,60] Accordingly, no significant associations were found between serum levels of estradiol and 25-hydroxyvitamin D in three cross-sectional studies of healthy young men.[42–44] However, in older men[47] a negative association between serum levels of estradiol and 25-hydroxyvitamin D was reported, which indicates that extra-gonadal conversion of testosterone by aromatase, which mainly occurs in adipose tissue, might contribute to serum levels of estradiol.

Testicular peptide hormones

Inhibin B production reflects the close interaction between Sertoli cells and spermatocytes in the adult testis and is therefore used clinically as an indicator of spermatogenesis.[67] By contrast, anti-Müllerian

Table 1 | VDR-regulated proteins and pathways in male reproduction

Pathway	Proteins and ions	Tissue	Reproductive effect
Calcium and phosphate homeostasis	TRPV5,[118] TRPV6,[37] calbindin 1–3,[52,118] PMCA1–4,[119] NCX1–3,[120] NPT2a–c,[121] CaSR[122]	Testis and/or epididymis	Putative involvement in transcellular calcium and phosphate transportation and calcium storage in germ cells, Leydig cells, efferent ducts and epididymis
Reproductive hormones	CYP19A1,[24,25,60] ERα,[25] ERβ,[25] AMH,[25,68,69] IGF-BP[38,125]	Testis and/or epididymis	Regulation of estrogen signalling in testis, efferent ducts and epididymis Potential regulation of testicular AMH and IGF-BP3 synthesis
Cell cycle control	p21,[93] p27,[93] p53,[93] p73,[93] FOXO1[93]	Testis	Regulation of TGCT proliferation, differentiation and cisplatin resistance
Pluripotency and germ cell layer commitment	OCT4,[23] NANOG,[23] SNAI[123]	Testis	Regulation of pluripotency factors and mesodermal differentiation in TGCT Possible role in fetal development
Regulators of testicular and epididymal vitamin D metabolism	PTHrP,[106] FGF23,[23,123] Klotho,[124] CYP27B1,[25] CYP24A1[25,26]	Testis	Suggested paracrine genetic regulators of testicular vitamin D metabolism
Osteogenic signalling	RUNX2,[23,131] SSP1,[23] BGLAP[23]	Testis	Osteogenic differentiation or transdifferentiation in dysgenetic testis with germ cell tumours
Mixed	ABCA1,[126] GGT[127]	Testis	Cholesterol homeostasis and Sertoli cell function
Nongenomic	Ca^{2+}, Cl^-, K^+-flux,[21,22,128] PKA, PKC, PLC[22,25,26] MAPK[118]	Testis	Rapid nongenomic effects mediated by VDR

The genes of the proteins included in the table are all directly or indirectly regulated by VDR but only some of them have a validated vitamin D response element in the promoter region. Abbreviations: AMH, anti-Müllerian hormone; FGF23, fibroblast growth factor 23; TGCT, testicular germ cell tumour; VDR, vitamin D receptor.

hormone (AMH) is produced predominantly by immature Sertoli cells and is involved in the development of the male reproductive tract, whereas its role in adult life is not resolved.[1] A cohort study published in 2012 reported a positive association between serum levels of 25-hydroxyvitamin D and AMH, and showed that AMH production was modulated by cholecalciferol supplementation in adult men, but not in boys.[68]

Direct stimulation of AMH production by 1α,25-dihydroxyvitamin D_3 is plausible because the *AMH* promoter contains a VDRE, and AMH is solely produced by immature human Sertoli cells that express VDR, unlike mature human Sertoli cells. VDR is also expressed in prostate cancer cells, in which 1α,25-dihydroxyvitamin D_3 has been shown to induce *AMH* transcription *in vitro*.[69] Of note, the proposed influence of vitamin D on testicular AMH production in humans has not been corroborated by functional animal studies. No statistically significant changes in *Amh* expression were observed between *Vdr*-null, heterozygous and homozygous wild-type littermates.[25] AMH is exclusively expressed in immature Sertoli cells, whereas mature Sertoli cells produce and secrete inhibin B and SHBG. Inhibin B exerts negative feedback on FSH secretion, whereas SHBG determines free hormone concentration in the male reproductive tract. No differences were found in the transcriptional levels of testicular *Shbg* or *Inhibin B* levels and, in accordance, comparable serum FSH and LH levels were found between *Vdr*-null and wild-type control mice.[19,25] Moreover, in human cohort studies in healthy men, no associations were found between serum concentrations of 25-hydroxyvitamin D and FSH or inhibin B,[22,42–44] hence VDR seems to be dispensable for testicular *SHBG* or *Inhibin B* expression,[24] whilst the weak association with AMH in humans requires validation. Insulin-like factor 3 (INSL3) is a peptide hormone produced by Leydig cells and is important for inducing testicular descent during development.[70] *Insl3* was not differentially expressed between *Vdr*-null mice and their wild-type littermates and is therefore not dependent on VDR expression.[25] Progesterone synthesis is induced by 1α,25-dihydroxyvitamin D_3 in the placenta and ovaries,[71] but the putative influence of vitamin D on progesterone production in the Leydig cells remains to be investigated in both humans and animals.

Vitamin D and male fertility

An estimated 10–15% of couples are infertile, and the causative contribution seems to be equal between the sexes.[72] Male fertility is evaluated routinely by semen analysis and to date no evidence-based treatment exists for impaired semen quality, which is predominantly idiopathic. A beneficial effect of vitamin D has been shown in rats inseminated with sperm from male rats with vitamin D deficiency, which produced 71% fewer pregnancies compared with sperm from vitamin-D-sufficient rats.[73] A follow-up study suggested that fertility was restored by correcting the concomitant hypocalcaemia in the vitamin-D-deficient rats and therefore not a direct effect of vitamin D.[74] However, re-assessment of the data revealed that pregnancy rates remained 43% lower after insemination with sperm from normocalcaemic vitamin-D-deficient rats than in normocalcaemic vitamin-D-replete rats.[74,75] Thus, a direct effect of vitamin D on male fertility is plausible.

The low fertility rates in vitamin-D-deficient male

animals (such as mice, rats and wild boars) seems to be caused by impaired sperm motility and, occasionally, poor sperm morphology.[60,73,76–78] Accordingly, a comprehensive reproductive investigation in a *Vdr*-null mouse model revealed a marked decrease in sperm number (40%) and sperm motility (ninefold), which resulted in infertility.[60] Semen quality and fertility improved after calcium supplementation but was only normalized after supplementation with both calcium and estrogen.[60] This finding indicates that male fertility, besides being influenced by vitamin D, is susceptible to systemic changes in calcium and estradiol. Interestingly, testicular histology was grossly normal in two out of three *Vdr*-null mouse strains[19,25,60] and in chickens with vitamin D deficiency.[52] However, impaired spermatogenesis was detectable in seminiferous tubules concomitantly with low aromatase expression and activity in another *Vdr*-null mouse strain.[60] The low ERβ and increased ERα expression in the reproductive organs of another *Vdr*-null mouse model did to some extend corroborate the diminished estrogen responsiveness, which ultimately might be mediating the influence of vitamin D on sperm motility (Table 2).[25,37,59]

Knowledge of the reproductive effects of vitamin D in adulthood are largely based on data from cross-sectional studies, in which men with vitamin D deficiency (<25 nmol/l) or insufficiency (<50 nmol/l) had significantly lower sperm motility than men with vitamin D sufficiency.[26,22,79] The positive association with motility has been shown in young men from the general population,[22] fertile men[79] and men with infertility.[26,79] Another cohort study comprising mainly vitamin-D-sufficient young men[43] reported an unadjusted nonsignificant ($P = 0.06$) positive trend between serum levels of 25-hydroxyvitamin D and sperm motility. Furthermore, a small study showed that men with serum levels of 25-hydroxyvitamin D of 50–125 nmol/l had more motile sperm than men with levels <50 nmol/l or >125 nmol/l.[44] Sperm morphology was positively associated with serum levels of 25-hydroxyvitamin D in two studies,[22,79] whereas all larger studies[22,26,43,79] found no association with total sperm count, sperm concentration or serum levels of inhibin B or FSH (Table 2).

The effect of 1α,25-dihydroxyvitamin D_3 on sperm motility might be mediated through the aforementioned epididymal changes, but 1α,25-dihydroxyvitamin D_3 is also a potent inducer of nongenomic effects in human spermatozoa.[8] VDR elicits a rapid increase in intracellular Ca^{2+} concentration through inositol trisphosphate(IP_3)-mediated Ca^{2+}-release from an intracellular IP_3-receptor-gated calcium store in the neck of human spermatozoa (Figure 4).[10,21,26,80] The nongenomic nature of this response was evident due to the rapid kinetics (seconds) initiated in the compartment expressing VDR[17] and validated by using the 6-cis-locked agonist 1α,25-dihydroxylumisterol for rapid effects mediated by VDR. 1α,25-dihydroxylumisterol and 1α,25-dihydroxyvitamin D_3 induced a similar effect, whereas pretreatment with the nongenomic competitive VDR antagonist 1β,25-dihydroxyvitamin D_3 inhibited the effect of 1α,25-dihydroxyvitamin D_3.[22,81] The increase in intracellular Ca^{2+} concentration leads to induction of sperm motility in both capacitated[21] and uncapacitated sperm,[22,26] improves sperm–egg binding *in vitro*[82] and triggers the acrosome reaction,[22] which is a prerequisite to fertilize the oocyte. The *in vitro* effects of 1α,25-dihydroxyvitamin D_3 corroborate the effect on sperm motility shown in both functional animal studies and human association studies. The suggested relationships between vitamin D and fertility are also supported by epidemiological data showing a seasonal variation in the conception rate in the northern countries of the Northern Hemisphere,[83] which peaks in the summer and matches the seasonal variation in serum levels of 25-hydroxyvitamin D.

Vitamin D is not only important in adulthood, as VDR is already expressed in human gonocytes, immature Sertoli and Leydig cells from gestational week 16,[23] which strongly indicates an early effect of vitamin D in the developing gonad. However, the role of vitamin D in fetal life is largely unexplored and can so far only be extrapolated from studies on testicular germ cell cancer cells that resemble embryonic stem cells.

Table 2 | Reproductive effects of vitamin D

Reproductive function	Species		
	Mouse	Rat	Human
Fertility			
Conception	↑↑[60,129]	↑↑[73–75]	↑[83]
Time to pregnancy	↑[60,129]	↑↑[73–75]	ND
Littersize	↑↑[25,60]	↑↑[73–75]	NA
Semen quality			
Concentration	↑↑[60,126]	↑↑[73–75]	→→[22,26,43,44,79]
Motility	↑↑[60]	↑[73–75]	↑↑[22,26,43,44,79]
Morphology	ND	ND	↑[22,26,43,44,79]
Reproductive hormones			
Testosterone	→→[19,25]	↑[51]	→[40–48]
Estradiol	↑[25,60]	↑[24]	→[42–44]
AMH	→[25]	ND	↑↑[68,69]
Inhibin B	→[25]	ND	→[22,43]

↑, increase; →, no effect. One arrow indicates an association proposed by at least one study or conflicting data with the majority of studies indicating this effect. Two arrows indicate that at least two different studies show a reproducible effect. Abbreviations: AMH, anti-Müllerian hormone; NA, not available; ND, not determined; TTP, time to pregnancy.

Vitamin D and testicular cancer

Testicular germ cell tumours (TGCT) are the most frequent type of solid tumour in young men, and their embryonic stem cell-like totipotentiality manifested by the ability to differentiate into all tissue types separates them from somatic cancers.[84] TGCTs originate from a precursor, carcinoma *in situ* (CIS),[85] which has been identified as transformed fetal gonocytes or primordial germ cells (Figure 1).[86] After puberty, CIS cells undergo malignant transformation and form either an invasive seminoma, which retains germ cell characteristics, or a nonseminoma that contains a de-differentiated

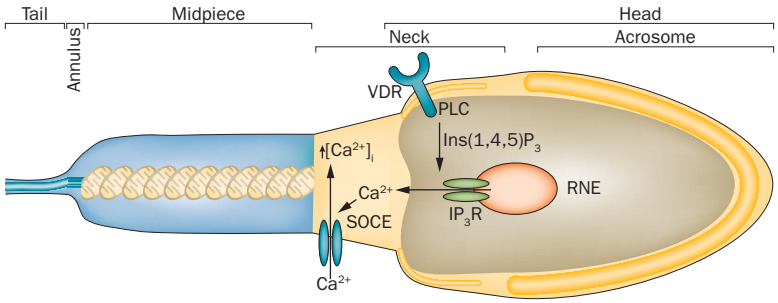

Figure 4 | Proposed mechanism for the nongenomic effect of VDR in human spermatozoa. 1α,25-dihydroxyvitamin D_3 activates VDR in the neck region that elicits PLC activation and generation of IP_3 production that subsequently opens IP_3R gated calcium channels in the RNE and increases intracellular Ca^{2+} concentration. The initial Ca^{2+} release from RNE might be supported by SOCE, but this seems not to be through L-type channels as nifedipine is unable to influence the 1α,25-dihydroxyvitamin D_3-mediated increase in $[Ca^{2+}]_i$. Abbreviations: $[Ca^{2+}]_i$, intracellular concentration of calcium ions; PLC, phospholipase C; RNE, redundant nuclear envelope; SOCE, store-operated calcium entry; VDR, vitamin D receptor.

embryonic component (embryonal carcinoma) resembling embryonic stem cells and more differentiated somatic (teratomas) or extra-embryonic (yolk sac or choriocarcinoma) components.[87]

Cellular expression of VDR and enzymes that metabolize vitamin D changes during the malignant transformation of most somatic cancers,[88,89] and the changes have been suggested to be implicated in cancer growth and progression as 1α,25-dihydroxyvitamin D_3 promotes differentiation and decreases proliferation of most cancer cells.[90] The VDR and enzymes that metabolize vitamin D have a markedly high expression in CIS, but the expression level decreases when CIS progress to invasive seminomas, and cellular expression of VDR and enzymes that metabolize vitamin D are lost during the transition to embryonic carcinoma (EC).[23] In other words, the presence of vitamin D metabolism machinery in TGCTs reflects the state of differentiation and decreases during de-differentiation from CIS to EC.

The pluripotent EC is characterized by loss of VDR and enzymes that metabolize vitamin D, but expression of VDR and enzymes that metabolize vitamin D is reintroduced when the EC cells start to differentiate into all types of tissue (teratomas) concomitantly with the downregulation of pluripotency factors (Table 1).[23] Correspondingly, treatment with 1α,25-dihydroxyvitamin D_3 downregulates pluripotency factors in seminoma-derived TCam2 cells and EC-derived NTera2 cells *in vitro* and induces a mesodermal transition towards an osteogenic differentiation of the NTera2 cells *in vitro* and in a NTera2 xenograft mouse model.[23] This finding is intriguing because NTera2 cells are pluripotent cells derived from a pulmonary EC metastasis that normally undergoes terminal differentiation into a neuron-like cell following treatment with retinoic acid. By contrast, TCam2 cells express pluripotency markers such as OCT4 and NANOG but retain germ cell characteristics and are unable to complete differentiation with retinoic acid.[23] The pro-differentiating effect of 1α,25-dihydroxyvitamin D_3 was illustrated by downregulation of OCT4 and NANOG and concomitant upregulation of SNAI1, brachyuri, osteocalcin, osteopontin and fibroblast growth factor 23 (FGF23) (Figure 1).[23] Surprisingly, the changes in gene and protein expression had no effect on tumour growth. Instead, the presence of classic bone markers and alkaline phosphatase in TGCTs from patients, xenograft tumours and cancer cells *in vitro* indicates that some testicular cancer cells possess the capacity to differentiate and initiate the bone-specific mineralization process.[23] This finding might be clinically relevant in patients with testicular microlithiasis, which can be detected with ultrasonography due to depositions of hydroxyapatite,[91] and is a frequent finding in the tissue adjacent to TGCTs,[92] and might be linked with the osteogenic differentiation of some testicular cancer cells.

Most of the known anti-proliferative effects of 1α,25-dihydroxyvitamin D_3 *in vitro* are mediated by cyclin-dependent kinase inhibitors such as p21, p27, p63, p73 or by the repression of pro-survival proteins leading to increased apoptosis.[8,9,90] In NTera2 cells, 1α,25-dihydroxyvitamin D_3 induced a twofold to fivefold increase in *p21*, *p27*, *p53*, *p73* and *FOXO1* transcription, but the changes in gene transcription were not reflected by the fraction of surviving or viable cells.[93] 1α,25-dihydroxyvitamin D_3 or cholecalciferol treatment have a growth inhibitory effect and increases the susceptibility to cisplatin in many somatic cancers.[93] This phenomenon might be relevant in TGCTs as the VDR-regulated gene *p21* largely determines cisplatin resistance in testicular cancer.[94] Indeed, co-treatment with cisplatin and 1α,25-dihydroxyvitamin D_3 reduced the proportion of viable and surviving cells more than cisplatin alone in both seminoma-derived and EC-derived cells lines *in vitro*. Interestingly, 1 μM cisplatin plus 100 nM 1α,25-dihydroxyvitamin D_3 caused a larger reduction in surviving EC-derived cells than 5 μM cisplatin treatment alone. The proposed cisplatin-sparing effect of 1α,25-dihydroxyvitamin D_3 is of clinical interest because men with TGCTs are young at the onset of disease and some experience long-term adverse effects of cisplatin treatment, such as early cardiovascular complications following curative treatment of advanced TGCTs.[95] Unfortunately, the antitumour effect of 1α,25-dihydroxyvitamin D_3 plus cisplatin was not significantly improved compared with cisplatin alone in a xenograft mouse model,[93] although animals treated with 1α,25-dihydroxyvitamin D_3 plus cisplatin tended to have a larger reduction in tumour burden than animals that received the monotherapy. The study was compromised by the high dose of cisplatin used (6 mg/kg per week) because it proved very efficient and eradicated the large tumours rapidly.[93] Therefore, future investigations should reduce cisplatin dosage to elucidate a potential cisplatin sparing effect of 1α,25-dihydroxyvitamin D_3 *in vivo*.

Regulation of vitamin D metabolism

The systemic regulators of vitamin D metabolism (PTH, FGF23, calcitonin, calcium, phosphate, 1α,25-dihydroxyvitamin D_3 and estradiol[96,97]) predominantly target the renal 1α-hydroxylase (CYP27B1), but some

of these factor have an additional effect on 24-hydroxylase (CYP24A1) that amplifies their effect on vitamin D metabolism. Extra-renal vitamin D metabolism is regulated differently than systemic vitamin D metabolism probably due to the lack of effect on systemic calcium and phosphate homeostasis by extra-renal vitamin D metabolism.[90,97] Thus, systemic regulators might be less important for extra-renal vitamin D metabolism, and paracrine and autocrine factors such as IFN-γ, IGF-1, BMPs, TGF-β and 1α,25-dihydroxyvitamin D_3 might be more potent regulators depending on the expression profile of the specific regulators in the target tissue.[96,97] The effects of paracrine vitamin D signalling differs between organs and extrapolating proposed regulators of extra-renal vitamin D metabolism from one organ to another might not be applicable, as illustrated by the various effects of FGF23 on different tissues with vitamin D metabolism.[98]

The testis has a unique feature in that the male germ cells express Klotho,[99,100] which interacts with FGFR1 or FGFR3 to create a specific receptor for FGF23.[101] In the kidney, FGF23 is a key regulator of the sodium–phosphate transporter NPT2A[102] and inhibits 1α,25-dihydroxyvitamin D_3 production by lowering CYP27B1 and inducing CYP24A1 expression. FGFR1 and FGFR3 are also expressed in male germ cells,[103] which could render the testis a FGF23 target (Figure 2). In fact, both *Klotho* and *Fgf23* knockout mice display male infertility,[104] which highlights the importance of FGF23 signalling in the testis. Moreover, *Fgf23*-null mice had the highest (10-fold) increase in 1α-hydroxylase (CYP27B1) expression in the testis compared with other nonrenal tissues such as heart, aorta, spleen, bone and intestine.[98] Thus, the bone-secreted FGF23 acts upstream of CYP27B1 in the testis and it is reasonable to imply that testicular CYP24A1 is also a target of FGF23.[98] Interestingly, FGF23 is highly expressed in EC-derived cell lines, suggesting that TGCTs in the testis results in an ectopic FGF23 production.[23] In turn, if this ectopic FGF23 is secreted it could influence vitamin D metabolism and phosphate homeostasis in the normal Klotho-expressing testicular tissue adjacent to the tumour and might thus be involved in the formation of testicular microlithiasis.

The counterpart to FGF23 is PTH or PTH-related peptide (PTHrP),[102] which normally increases the production of 1α,25-dihydroxyvitamin D_3. PTHrP is produced locally by germ cells and the specific receptors PTHR1 and PTHR2 are also expressed in the testis.[105,106] Nevertheless, the effect of PTH and PTHrP on testicular vitamin D metabolism remains to be elucidated. Calcitonin and calcitonin-related peptide are important for male reproductive function, but are also known stimulators of renal vitamin D metabolism.[107] However, both receptors are only expressed in Leydig cells and spermatozoa, which limits the effect on intratubular vitamin D metabolism.[107]

Sex steroid hormones are known regulators of 1α-hydroxylase, but they also induce sex-specific changes in microsomal 25-hydroxylase activity in rats following castration or hypophysectomy[108,109] and in healthy men following injection of anabolic steroids.[110] The up to 100-fold higher testicular concentrations of testosterone and estradiol might thus induce an even stronger influence on testicular and epididymal vitamin D metabolism compared with the reported effects of serum sex hormone levels on systemic vitamin D metabolism.[1,111] The global expression of ERs in the male reproductive organs[36,112] indicates a regulatory role of estradiol in all compartments of the reproductive tract, although a testis-specific regulation of the metabolizing enzymes by ERs remains to be demonstrated.[58,113] By contrast, the androgen receptor (AR) has a restricted expression pattern. AR is expressed in the epithelial cells lining the reproductive tract and peritubular cells, and the expression profile in Sertoli cells is complex. Immature human Sertoli cells[1] that express VDR do not express AR, whereas adult Sertoli cells that express AR have almost no expression of VDR.[4,23] VDR expression might thus decrease simultaneously with the induction of AR expression during Sertoli cell maturation.[1] It is possible that AR regulates VDR expression directly in the Sertoli cells because testosterone lowers VDR expression in NTera2 cells,[114] which strongly supports a regulatory function of testosterone on testicular vitamin D metabolism (Figure 2).

Functional studies have provided substantial evidence for a direct 1α,25-dihydroxyvitamin D_3-mediated downregulation of CYP27B1 and upregulation of CYP24A1.[115,116] This finding indicates that local vitamin D metabolism is stongly influenced by a testicular feedback system. The high CYP27B1 expression and low CYP24A1 expression in the testis from *Vdr*-null mice confirmed that 1α,25-dihydroxyvitamin D_3 depends on a functional VDR to modulate the expression of the metabolizing enzymes.[25] The intracellular 1α,25-dihydroxyvitamin D_3 concentration seems to be an important factor for testicular function and it is therefore plausible that circulating levels of 25-hydroxyvitamin D or 1α,25-dihydroxyvitamin D_3 in serum could influence the expression level of testicular enzymes that metabolize vitamin D and VDR. However, infusion of 1α,25-dihydroxyvitamin D_3 in rats did not change VDR expression in the testis[117] and serum levels of 25-hydroxyvitamin D were not associated with CYP24A1 expression in spermatozoa in a small cohort of healthy and infertile men.[26] Thus, serum levels of vitamin D metabolites cannot be regarded as strong determinants of testicular vitamin D metabolism.

Conclusions

Vitamin D is metabolized in the testis and male reproductive tract of both rodents and humans. A functional VDR seems to be dispensable for normal testicular development in mice; however, mutant mice with vitamin D deficiency and *Vdr*-null mice develop reduced male fertility later in life. The decreased fertility is mainly caused by impaired sperm motility and gamete quality rather than diminished sperm production, as illustrated by the grossly normal testicular histology in two out of the three VDR-null strains and in CYP27B1-knockout mice. The global expression of VDR implies that the effect on semen quality can be mediated directly in the germ cells, indirectly by modulating Leydig cell

function or through changes in epididymal function, as indicated by the aberrant estrogen signalling in *Vdr*-null mice. Extrapolating results from mice to humans is particularly problematic in the reproductive field as human fertility potential is much lower than that in rodents and even key signalling pathways such as progesterone-mediated activation of CATSPER is not conserved between species.

In humans, vitamin D is metabolized in the fetal testis throughout development. The presence of vitamin D signalling in the fetal gonad is intriguing but the exact role remains to be shown. Some clues can be extrapolated from the pro-differentiation effects found in TGCTs, in which 1α,25-dihydroxyvitamin D_3 is a potent inducer of mesodermal differentiation towards an osteogenic phenotype of cancer cells with embryonic stem cell characteristics. 1α,25-dihydroxyvitamin D_3 might also regulate cell cycle control in normal adult germ cells, but after puberty vitamin D metabolism seems to be more important for postmeiotic germ cell maturation, and the VDR-dependent expression of CYP24A1 in human spermatozoa can be used clinically as a positive predictive marker of semen quality.

The presence of VDR and enzymes that metabolize vitamin D in human spermatozoa also has functional consequences, convincingly shown by the rapid non-genomic 1α,25-dihydroxyvitamin D_3-mediated increase in intracellular calcium concentration that induces sperm motility. This finding corroborates a functional relationship between vitamin D and sperm motility, which already at this stage could be tested during *in vitro* fertilization. Interestingly, serum 25-hydroxyvitamin D concentrations are positively associated with sperm motility in both healthy men and those with infertility, and future studies will show whether sperm motility could be improved by supplementing vitamin D to vitamin-D-deficient fertile or infertile men.

However, most of the vitamin-D-mediated effects in humans are exclusively paracrine effects, wherein VDR acts as a transcription factor. The reproductive changes in gene transcription or protein expression are rarely reflected systemically and are not influenced by serum concentration of 25-hydroxyvitamin D. The putative cisplatin-sparing effect of combining cisplatin with 1α,25-dihydroxyvitamin D_3 in the treatment of advanced TGCTs was not verified in the *in vivo* xenograft model but should be evaluated in well-designed *in vivo* studies with a lower cisplatin dose than that previously used. Finally, emerging evidence corroborates an endocrine interaction between the skeleton and gonads, and suggests that 1α,25-dihydroxyvitamin D_3, besides a direct gonadal effect, is a potent regulator of many bone-specific proteins, including osteocalcin and FGF23 that might modulate testicular function indirectly.

Review criteria

A search for original articles published until May 2013 focusing on vitamin D and male reproductive function was performed in PubMed. The search terms used were "reproduction", "male reproduction", "sperm", "testis", "gonad", "fertility", "epididymis", "seminal plasma", "sex hormones", "testosterone", "estradiol", "AMH", "inhibin B" "INSL3", "testis cancer", "testicular germ cell tumour" in combination with vitamin D. All papers identified were English-language, full-text papers.

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