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REPRODUCTION
REVIEW

Molecular actions of vitamin D in reproductive cell biology

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Abstract

Vitamin D (VitD) is an important secosteroid and has attracted attention in several areas of research due to common VitD deficiency in the population, and its potential to regulate molecular pathways related to chronic and inflammatory diseases. VitD metabolites and the VitD receptor (VDR) influence many tissues including those of the reproductive system. VDR expression has been demonstrated in various cell types of the male reproductive tract, including spermatozoa and germ cells, and in female reproductive tissues including the ovaries, placenta and endometrium. However, the molecular role of VitD signalling and metabolism in reproductive function have not been fully established. Consequently, the aim of this work is to review current metabolic and molecular aspects of the VitD–VDR axis in reproductive medicine and to propose the direction of future research. Specifically, the influence of VitD on sperm motility, calcium handling, capacitation, acrosin reaction and lipid metabolism is examined. In addition, we will also discuss the effect of VitD on sex hormone secretion and receptor expression in primary granulosa cells, along with the impact on cytokine production in trophoblast cells. The review concludes with a discussion of the recent developments in VitD–VDR signalling specifically related to altered cellular bioenergetics, which is an emerging concept in the field of reproductive medicine.
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Introduction

Insufficient vitamin D (VitD) status is a worldwide health issue, and deficiency has been inversely associated with the incidence of various chronic ailments including cardiovascular disease, obesity, diabetes, cancer and infertility (Kayaniyil *et al.* 2010, Garbedian *et al.* 2013, Rudick *et al.* 2014, Brożyna *et al.* 2015, Calton *et al.* 2015*b*, Gibson *et al.* 2015). Although progress has been made in understanding the functional and biological action of VitD, the causal links with many diseases have proven difficult to verify. There are two plausible direct mechanisms by which VitD may provide protection from chronic disease. First, VitD is a well-known modulator of immune cell activation *in vitro* (Matilainen *et al.* 2010), and as such may dampen inflammation, which is primarily responsible in chronic auto-immune diseases like type 1 diabetes. Second, VitD and its direct metabolites have recently been suggested to stabilise the endothelium and limit vascular leak (Gibson *et al.* 2015). Impairment of endothelium integrity can be induced by pro-inflammatory cytokines, and subsequently leads to dysfunctional nutrient exchange and clotting, along with immune cell infiltration – key aspects that are dysregulated in chronic disorders such as cardiovascular disease, diabetes and pre-eclampsia (Noyola-Martinez *et al.* 2013, Gibson *et al.* 2015, Keane *et al.* 2015).

However, the difficulty in establishing clear functional effects in disease is partly due to the various limitations associated with techniques to measure VitD status, the importance of specific hydroxylated metabolites (Arneson & Arneson 2013) and importantly the lack of well-designed clinical trials (Lappe & Heaney 2012). Together, they stimulate debate for defining the specific ranges for VitD deficiency, insufficiency and sufficiency (Holick 2009). The uncertainty is further compounded by the extremely complex nature of VitD signalling, which is slowly beginning to be understood with advances in '-omic' technologies, including chromatin immunoprecipitation (ChIP) assay coupled to genomic array (ChIP-chip) or next-generation sequencing (ChIP-seq) (Pike & Meyer 2014).

In reproductive medicine, the precise clinical function of VitD in relation to male/female fertility remains highly elusive. Recent reports have suggested that 25 hydroxyvitamin D ([25(OH)D]) was positively correlated with IVF pregnancy rate (Garbedian *et al.* 2013, Paffoni *et al.* 2014, Rudick *et al.* 2014), whereas others found no significant association

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(Fabris *et al.* 2014, Franasiak *et al.* 2015, Abadia *et al.* 2016). However, these studies were relatively small with between 100 and 150 participants in each deficient/sufficient arms, and they defined different cut-off values for VitD insufficiency (<75 or <50 nmol/L), thus making comparisons difficult. Nonetheless, in a well-designed study, with one of the largest cohorts ($n=239$ and $n=129$, <50 and ≥50 nmol/L respectively) and incorporating 16 confounding variables, (Polyzos *et al.* 2014) showed that low 25(OH)D level was independently associated with reduced pregnancy rates (OR 0.56). Conversely, in the most recent prospective study, albeit in a small cohort ($n=100$), it was demonstrated that increasing serum 25(OH)D quartiles were associated with enhanced fertilisation rate, but this did not translate to improved clinical pregnancy or live birth rate (Abadia *et al.* 2016). However, most women had serum concentrations between 50 and 125 nmol/L, and the authors advised that a much larger cohort was required in future studies. It must also be remembered that the use of specific ranges for defining VitD sufficiency and insufficiency is critical, and the Institute of Medicine has indicated that applying 75 nmol/L may overestimate VitD inadequacy in some studies (Ross *et al.* 2011).

In males, 1α,25 dihydroxyvitamin D (1α,25(OH)$_2$D) has been linked to increased sperm motility suggesting an impact on mitochondrial function (Blomberg Jensen *et al.* 2011). Others have reported that excessively low and excessively high levels of 25(OH)D may be detrimental to sperm motility and survival (Hammoud *et al.* 2012). However, this latter study had a very small sample size in the low (<50 nmol/L) and high (≥125 nmol/L) VitD categories ($n=19$ and 20 respectively), compared with the middle VitD group (50–124 nmol/L, $n=108$). Conversely, in a much larger sample size ($n=1248$), it was shown that increasing levels of 25(OH)D correlated positively with semen parameters (Blomberg Jensen *et al.* 2016), adding further complexity to the area.

Taken together, most clinical studies investigating the impact of VitD-related metabolites on fertility/IVF outcomes, measure 25(OH)D and have demonstrated inconclusive results. Therefore, an understanding of the molecular regulation of VitD signalling and action in reproductive tissues may clarify the physiological role in fertility. In this review, the latest advancements in understanding molecular VitD signalling will be first discussed, followed by the current functional roles of this signalling network in cells derived from the reproductive system. The aim is to identify limitations in current knowledge regarding molecular VitD actions in reproductive biology and to propose the direction of future research.

Vitamin D metabolism, transport and storage

VitD is sourced as part of the diet or generated *in vivo* through exposure to UVB radiation and subsequent enzymatic hydroxylation (Haussler *et al.* 2013), summarised in Fig. 1. To discuss the entire clinical and molecular details of the various aspects regulating VitD homeostasis *in vivo* and in non-reproductive tissues is beyond the scope of the current review, but readers are directed to appropriate publications (Bikle 2014, Heaney & Armas 2015, Heaney 2016). However, in the classical example, 7-dehydrocholesterol in keratinocytes is converted to cholecalciferol (D3) via an electrocyclic reaction, which is then subsequently metabolised by liver microsomal cytochrome oxidase, CYP2R1, to 25(OH)D$_3$ (calcidiol). Alternatively, ergocalciferol (D2) from the diet is converted by CYP2R1 to 25(OH)D$_2$. These 25(OH)D configurations are the main circulatory

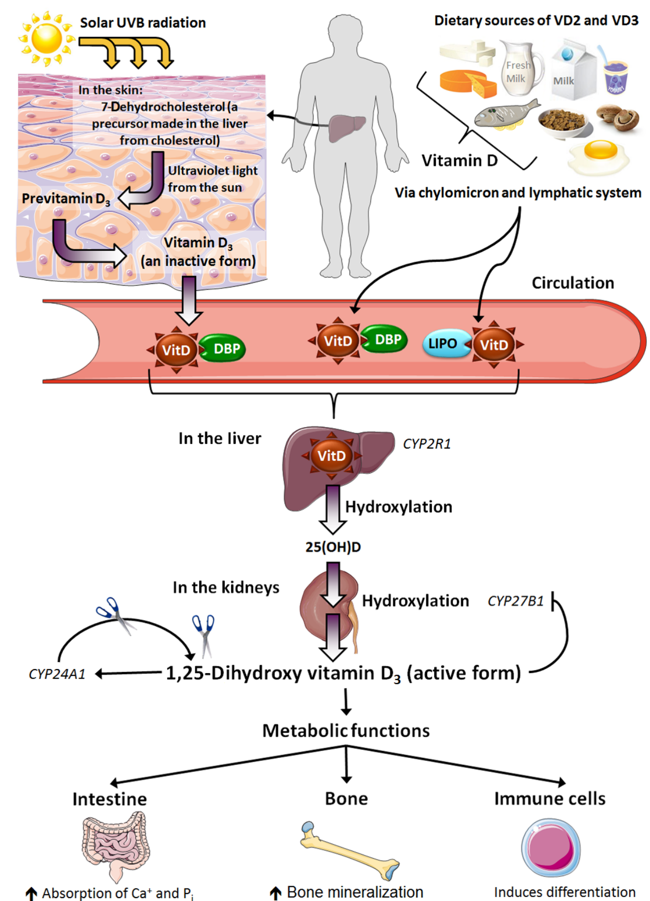

Figure 1 Basic metabolism of vitamin D in humans. Cholesterol from the diet undergoes conversion to 7-dehydrocholesterol and circulates through the bloodstream attached to serum proteins. It can be taken up by or synthesised by cells such as keratinocytes, where it is converted to cholecalciferol (VitD) via exposure to UVB radiation. Cholecalciferol (VitD) or ergocalciferol (vitamin D2, VitD) from the diet enters the bloodstream and is transformed to 25-hydroxyvitamin D (25(OH)D) by the liver. It then goes to the kidneys where conversion to the active form 1α,25 dihydroxyvitamin D (1α,25(OH)$_2$D) occurs. Local production of 1α,25(OH)$_2$D also takes place as many other organs and cells contain the enzyme CYP27B1. DBP, VitD-binding protein; LIPO, lipoprotein; SA, serum albumin; VitD, cholecalciferol or ergocalciferol.

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VitD metabolites, with a half-life of approximately 25 h and are routinely used to assess whole-body VitD status. 25(OH)D is further hydroxylated by mitochondrial CYP27B1 in the proximal tubule of the kidney to the active ligand 1α,25$(OH)_2$D. This ligand can bind the vitamin D receptor (VDR), a potent transcription factor in target tissues, notably bone and enterocytes (Haussler *et al.* 2013). CYP27B1 activity is controlled directly by various growth factors, cytokines, sex steroids and inorganic minerals (Hendrix *et al.* 2004, Adams *et al.* 2014). Furthermore, parathyroid hormone (PTH) is a classical regulator of whole-body VitD levels and enhances the expression of CYP27B1 under low calcium conditions to promote 1α,25$(OH)_2$D-mediated calcium absorption from the intestines (Fig. 1). Positive regulation is mediated by changes in gene transcription, but lately, elements of complex epigenetic modifications have been proposed (Wjst *et al.* 2010).

Production of 1α,25$(OH)_2$D also functions in a negative feedback fashion to inhibit CYP27B1 expression and in a feed-forward manner to transcriptionally promote CYP24A1 expression, which subsequently degrades 1α,25$(OH)_2$D to inactive calcitroic acid (St-Arnaud 2010). Consequently, active 1α,25$(OH)_2$D has a short serum half-life of approximately 6 h because both regulatory mechanisms prevent hypervitaminosis D, hypercalcaemia and hypercalciuria.

Importantly, generation of 25(OH)D and 1α,25$(OH)_2$D from VitD (D3 and D2) can occur locally not only in tissues including the colon and pancreatic islets (Zehnder *et al.* 2001) but also in many cell types that express functional CYP27B1, such as immune cells (Viaene *et al.* 2012, Moran-Auth *et al.* 2013), syncytiotrophoblasts (Halhali *et al.* 1999) and cells of the male reproductive tract (Blomberg Jensen *et al.* 2010). Collectively, this suggests that VitD and metabolites have extra-skeletal properties, but autocrine production of these intermediates in non-classical tissues is dependent on the delivery of substrates to target tissues via blood. VitD-binding protein (DBP) is the primary serum carrier with three isoforms described, and all have different affinities for VitD-related metabolites (Bikle 2014, Heaney & Armas 2015). The serum isoform composition and concentration are dependent on ethnicity (Heaney & Armas 2015), and various VitD analogues such as D2, D3 and 25(OH)D also have distinct affinities for specific DBPs. Dietary D2 has a lower affinity for DBP and can be rapidly cleared from the body (Bikle 2014). Therefore, these elements regulating ligand–protein interactions control *in vivo* transportation of VitD metabolites. However, these metabolites bind to other carrier proteins including serum albumin and lipoproteins such as chylomicrons that allow transportation in the blood and also facilitate absorption from the gut and subsequent hydroxylation in the liver (Haddad *et al.* 1993). Hydroxylated VitD (25(OH)D) is mainly transported by DBP, and the bioavailability of all metabolites is dependent primarily on the route of VitD entry (cutaneous or dietary) and the mode of plasma transport via association with serum carriers (Haddad *et al.* 1993). The level of these carriers can also vastly alter the free-serum concentration of metabolites, and consequently should be factored into clinical determinations of VitD status.

Interestingly, DBP proteins along with bound VitD metabolites are reabsorbed in the proximal tubule of the nephron by the megalin–cubilin endocytotic transport system (Kaseda *et al.* 2011). This prevents loss of DBP and VitD metabolites by urinary excretion, but can be dysfunctional in chronic disease like diabetes (Thrailkill *et al.* 2011). Megalin and cubilin are both glycoproteins expressed in proximal tubule cells (PTC) and in placenta cells including syncytiotrophoblasts and cytotrophoblasts (Burke *et al.* 2013). Both work in cooperation to facilitate the endocytosis of many ligands and proteins not captured by the glomerulus, such as members of the vitamin B_{12} family, albumin and low-molecular-weight proteins. This transport mechanism is key for delivery of 25(OH)D bound to DBP, to kidney CYP27B1 where it can be converted to the active VitD ligand, 1α,25$(OH)_2$D (Kaseda *et al.* 2011). In addition, this nutrient transport process is important for the maintenance of foetal nutrient supply especially before 10-week gestation, where histiotrophic nutrition is supported by the visceral yolk sac and trophoblasts from endometrial secretions (Burke *et al.* 2013). Interestingly, the expression of these receptors increases with gestation. Therefore, dysfunction of this system could lead to complications during pregnancy, some of which may be associated with decreased VitD levels, such as preeclamsia (PE), but this remains to be proven. However, decreased placental expression of megalin and cubilin has been linked to low birth weights in malaria-infected patients (Lybbert *et al.* 2016), demonstrating its potential role in foetal development. Overall, the megalin–cubilin nutrient transport process is important for organogenesis and facilitates appropriate foetal development against the backdrop of a limited blood supply in early gestation.

Finally, lipophilic D3 has been detected in adipose tissue with levels approximately 12 times higher than those in serum (Blum *et al.* 2008, Heaney & Armas 2015), and it is believed that this may be a storage site for excessive VitD. However, very few studies have investigated VitD content in adipose, and it is not known what the molecular transport mechanisms between blood and adipose are, beyond passive diffusion (Heaney & Armas 2015). Furthermore, it was reported that D3 levels were different between subcutaneous and visceral fat (Pramyothin *et al.* 2011), indicating that the mechanisms allowing distribution among tissues are much more complex than those previously thought. Nonetheless, it is the combination of metabolism, transport and storage that affect circulating VitD levels, and until we understand the full molecular aspects of this VitD 'economy',

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it will be difficult to draw distinct conclusions from clinical VitD studies.

Molecular control of vitamin D signalling

Canonical vitamin D signalling and regulation

The VitD hormone elicits many of its pleiotrophic effects through classical ligand–receptor signalling and downstream modulation of gene expression or indeed through non-canonical genomic-independent mechanisms (Haussler *et al.* 2011). The VitD receptor, VDR, was first reported in 1969 as a chromatin-associated protein (Haussler & Norman 1969). The presence of proteins and enzymes involved in VitD–VDR axis has been demonstrated in various cell types of the male reproductive tract, including spermatozoa, Sertoli, Leydig and germ cells (Blomberg Jensen *et al.* 2010, Blomberg Jensen 2014), as well as in female reproductive tissues including the ovaries, endometrium and placenta (Lerchbaum & Obermayer-Pietsch 2012, Ma *et al.* 2012). The VDR is a transcription factor, and the VitD–VDR signalling cascade results in either activation or suppression of gene transcription. The outcome is dependent on several specific points of control that alters the VDR conformation when it complexes with DNA. The classical signalling mechanism consists of interaction of VitD ligand with VDR, along with subsequent translocation through the cytosol to the nucleus, where it binds specific segments of DNA, known as vitamin D response elements (VDRE). The VDR can associate with its binding partner, retinoid X receptor (RXR) in the cytosol before the association with DNA. Alternatively, RXR may be interspersed throughout VDREs on the target DNA strand, already 'marking' it for VDR docking (Pike & Meyer 2010). The resulting heterodimeric complex recruits co-regulatory proteins that may have endogenous biological activity (e.g. enzymatic, ATPase, phosphatase, kinase), that induce epigenetic changes such as histone acetylation, deacetylation or methylation, which ultimately leads to activation or suppression of gene transcription (Fig. 2).

Central to this process of regulation is the specific conformation of the VDR protein and the entire regulatory complex itself. This can be affected at several critical stages of the signalling cascade, most of which have not been fully characterised (Fig. 2). Changes in the structural configuration will determine the type of co-regulatory proteins that are recruited when VDR-RXR is complexed to DNA, as well as the type of epigenetic alterations and consequently the genomic output. These control points that affect VDR configurations, and subsequently, gene expression will be discussed below.

Ligand and receptor interactions

The VDR ligand-binding domain can interact with various ligands (*trans*- or *cis*-VitD and other analogues), which have different affinities for the receptor and consequently impact differently on final VDR conformations, and ultimately, the transcriptional activity. *In silico* analysis has demonstrated that the VDR ligand-binding domain possesses two overlapping binding sites (Fig. 2) (Mizwicki *et al.* 2004), and they have been identified as the VDR-genomic pocket (VDR-GP) and the VDR-alternative pocket (VDR-AP), which mediate genomic and rapid non-genomic responses (within minutes)

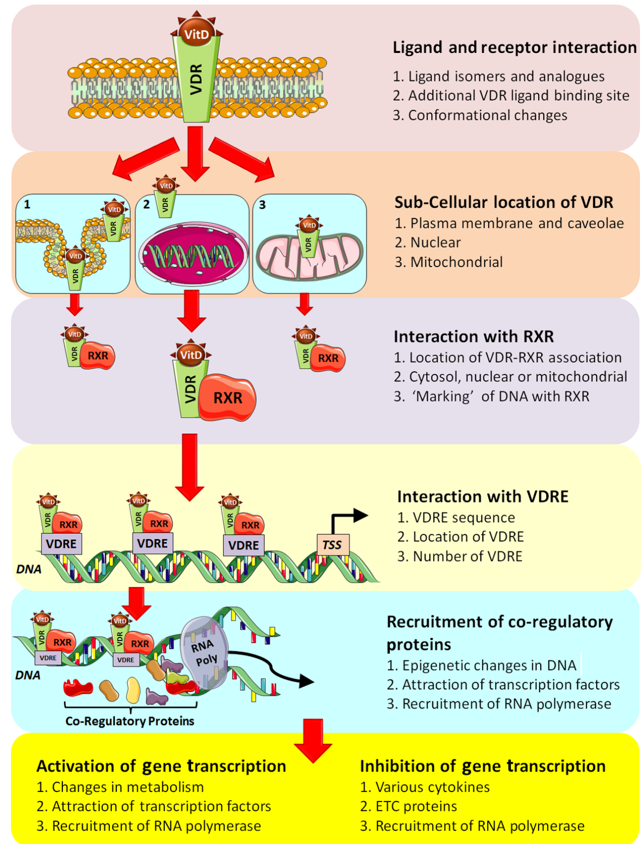

Figure 2 Modes of VitD-VDR signalling with potential aspects that modulate signalling shown on the right. VitD analogues and structurally-related compounds can interact with one of two binding sites on the VDR protein and thereby alter the molecular conformation. The VDR is present in the plasma membrane, nuclear membrane and mitochondria and this localisation will also influence downstream signalling. The liganded VDR forms a heteromeric complex with its binding partner, RXR, and translocates to the nucleus and associates with specific segments of DNA (VDRE). These specific domains are present throughout genomic DNA, and the number and position of the sites along target genes regulate gene transcription. A further element of control is observed as when the VDR-RXR is complexed to VDRE sites on DNA, the whole complex recruits co-regulatory proteins with various endogenous biological activities such as enzymatic, phosphatase, ATPase, which modulate the DNA structure via epigenetic changes. Taken together, these aspects of molecular structural control, ultimately regulate whether or not VitD-VDR signalling leads to activation of gene transcription or repression. DBP, VitD binding protein; RNA Poly, RNA polymerases; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D response elements; VitD, vitamin D.

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respectively (Haussler *et al.* 2011). It is postulated that this 'conformational ensemble' facilitates VDR signalling in both directions, by allowing VDR interaction with different VitD metabolites (Haussler *et al.* 2011). It is suggested that the VDR-GP and VDR-AP associate with the *trans*- and *cis*-analogues respectively. The *trans*-form is compulsory for genomic effects, whereas the *cis* conformation is necessary for rapid effects (expertly reviewed by Haussler *et al.* 2011).

Unfortunately, investigations of VDR-mediated rapid responses have only been performed *in vitro*, showing rapid alterations in insulin secretion in β-cells (Kajikawa *et al.* 1999), vascular smooth muscle cell migration (Rebsamen *et al.* 2002), spermatozoa motility (Blomberg Jensen *et al.* 2011) and calcium flux in Sertoli cells, osteoblasts, spermatozoa and intestinal cells (Norman *et al.* 1997, Zanello & Norman 2004, Menegaz *et al.* 2010, Blomberg Jensen *et al.* 2011). Therefore, whether or not these alternative signalling mechanisms are physiologically relevant remain to be determined. Haussler and coworkers (2011) postulated further that VDR localised in caveolae, may have the potential to activate adjacent signalling proteins such as G-protein-coupled receptors, phosphatases, kinases or ion channels through as yet undefined mechanisms, but probably involving lipid rafts or VDR phosphorylation reactions (Fig. 2) (Haussler *et al.* 2011). Potentially, these interactions may activate specific downstream molecular pathways or cause molecular changes to the VDR, which modulate cell responses. Currently, these proposals are purely speculative and require further research.

Vitamin D response elements (VDREs) and co-regulatory proteins

Possibly, the most important determinant of VDR-mediated gene regulation is the specific sequences of DNA, known as VDREs, that allow docking of the VDR–RXR heterodimer to many sites along target genomic DNA. The first VDRE was identified in the osteocalcin gene in 1989 and consisted of two identical hexanucleotide repeats, separated by either a three (Kerner *et al.* 1989) or six base pair region (Haussler *et al.* 2013). Each of the two adjacent six nucleotide motifs interact with the dual zinc finger DNA-binding domain of VDR and RXR, with each receptor binding at the 3′ and 5′ sequence respectively (Haussler *et al.* 2013). The VDRE sequence can vary, and consequently, this affects the affinity of the receptors for the site. This alters the interaction of receptor C terminals with each other and the whole configuration of the complex. Whether or not VDR is liganded may also influence these interactions and the recruitment of co-regulatory activators or suppressors.

The VDRE location and number of sites will also dictate where exactly on the DNA strand the VDR–RXR heterodimer will bind and therefore what gene is altered (Fig. 2). Gene transcription is influenced by whether this position is on, near, upstream or downstream of promoter regions and transcription start sites (TSS). A target gene can contain one or more VDREs or indeed it is possible that VDREs may be found in adjacent genes or other sites that influence transcription of the target gene through specific epigenetic changes and 'DNA looping'. This second element of multiple control regions for VDRE-mediated gene transcription is dependent on the multiplicity and remoteness of the VDRE (Fig. 2). Single and multiple VDREs have been found in a variety of genomic DNA positions, many kilobase pairs (kbp) from the TSS further complicating their role. VDREs were detected hundreds or thousands of bp upstream from the TSS, in the promoter region of a genes regulating lactogen expression in trophoblasts (Stephanou *et al.* 1994), human FOXO1 gene (Wang *et al.* 2005) and the mouse RANKL gene, a member of the TNF family receptors (Kim *et al.* 2006). Other VDREs are further downstream like those encoding hydroxylase enzymes (Haussler *et al.* 2011).

The most advanced model of how VDRE-regulated gene activation occurs has been proposed in the mouse RANKL gene (Haussler *et al.* 2011) and involves chromatin looping into a 'clover-leaf structure' to allow gene transactivation. Re-structuring of DNA is presumably mediated by various ATPases, phosphatases and histone-modifying enzymes that are recruited to the VDR–RXR complex as co-regulatory proteins. The net result is that the VDRE and VDR–RXR complexes are positioned in close proximity to facilitate the recruitment of RNA polymerase or indeed the inhibition of its activity, thus modifying gene transcription (Haussler *et al.* 2011).

It is very likely that this mechanism of gene control is observed in other genes influenced by VitD–VDR, and it also offers an explanation for the complexity of VDR-mediated effects, i.e. time activation and suppression, particularly for well-studied genes involved in immunomodulation. For example, the interleukin-10 (IL10) gene is negatively regulated by VDR in the short term (up to 8 h), which later becomes active (after 48 h) (Matilainen *et al.* 2010). VDREs were detected upstream of the TSS, and it was suggested that $1\alpha,25(OH)_2D$ induced cyclic epigenetic changes in the DNA strand (Matilainen *et al.* 2010). The immunomodulatory effect of VitD-related metabolites is well established *in vitro* and has been shown to reduce IL1β, IL6, IL8 and TNFα production from trophoblasts isolated from preeclamptic women (Noyola-Martinez *et al.* 2013, Gysler *et al.* 2015). However, the cellular mechanism driving these effects is unknown, but could be similar to RANKL gene regulation.

Co-regulatory proteins facilitate the activation or inhibition of gene transcription through epigenetic alterations (Fig. 2). However, very little work has examined VitD–VDR signalling at this level in cells of the reproductive system. Some key chromatin remodelling

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proteins recruited to the VDR–RXR–DNA complex that are possibly conserved in reproductive cells include p160 proteins, the integrator CBP/p300 and the ATP-dependent SWI/SNF complex (Kim *et al.* 2005, Haussler *et al.* 2011). In addition, TAF28, 55, 135, along with basal transcription factors including TFIIb, are induced by the VDR complex and associate with the TATA box, the initiation site for transcription (Blanco *et al.* 1995, Lavigne *et al.* 1999), ultimately leading to RNA polymerase recruitment and gene transcription. However, the ability of these molecules to join the complex is dependent on various factors including the presence or absence of VitD ligand or metabolites, the number and locations of VDREs and ultimately the correct configuration of the VDR–DNA complex (Fig. 2). Given that 100,000 to one million potential VDRE sites are available on the human genome, and 3000 genes are regulated by VDR, the biological effects could be far reaching (Haussler *et al.* 2011, Pike & Meyer 2014). It is clear that VDR-mediated epigenetic changes are central to VitD gene regulation and modulation, and this partially explains the myriad of effects attributed to vitamin D signalling.

Molecular vitamin D studies in reproductive cells

In cells of the reproductive system, the precise function of VitD–VDR signalling is not fully understood beyond the potential influence on calcium and phosphate homeostasis, hormone production and steroidogenesis. The presence of proteins and enzymes involved in VitD–VDR axis has been demonstrated in various cell types of the male and female reproductive tract (Blomberg Jensen *et al.* 2010, Lerchbaum & Obermayer-Pietsch 2012, Ma *et al.* 2012, Blomberg Jensen 2014). These findings suggest that VitD metabolism may play a role in male and female reproductive function. More specific studies in trophoblasts, granulosa cells and spermatozoa have shown the sub-cellular localisation of VDR (Thill *et al.* 2009, Blomberg Jensen *et al.* 2010) and the expression of VitD metabolising enzymes (Smith *et al.* 2009, Blomberg Jensen *et al.* 2010, Ma *et al.* 2012). However, the expression of these elements along with the requirements of human oocytes or embryos for VitD has not been explored.

Molecular mechanistic studies in sperm

Several rodent models have demonstrated that VitD deficiency may directly or indirectly impair testicular and sperm function, along with spermatogenesis (Sun *et al.* 2015), while 1α,25(OH)$_2$D supplementation can restore function (Ding *et al.* 2016). A recent study in humans suggested that lower VitD level correlated with poorer semen quality (Zhu *et al.* 2016). Although it was proposed that the relationship may be more U-shaped rather than linear, as reported in some studies (Hammoud *et al.* 2012, Karras *et al.* 2016), evidence from a large cohort (n = 1248) clearly demonstrated a positive, linear relationship between motility of good grade sperm and VitD level (Blomberg Jensen *et al.* 2016).

In humans, the expression of the VDR in ejaculated spermatozoa was first reported in 2005 using confocal microscopy (Corbett *et al.* 2006). VDR, which is primarily a transcription factor, was mainly detected in the nucleus and postacrosomal region of sperm, but it was also detected in the neck and in the mitochondria-containing midpiece (Corbett *et al.* 2006). A subsequent study using electron microscopy and gold labelling confirmed the main distribution in the nucleus, with a small amount of staining in the neck (Aquila *et al.* 2008). However, in this latter examination, the VDR was entirely absent from the midpiece. The presence of the nuclear VDR (nVDR) in terminally differentiated, transcriptionally inactive and strictly compartmentalised cells such as sperm, presents a conundrum. In particular, the level of nVDR in sperm was variable and changed across different men and was even altered in identical specimens from the same individuals (Blomberg Jensen *et al.* 2010). It has been suggested that the VDR may be a remnant of late-stage spermatogenic processes and could be linked to extratesticular sperm maturation, with higher levels present in final sperm representing more efficient maturation (Aquila *et al.* 2008, Blomberg Jensen *et al.* 2011, Blomberg Jensen 2014). On the other hand, it may be present as a mechanism for genomic stability and protection from DNA strand breaks (Aquila *et al.* 2008), but this requires further study.

Interestingly, VitD processing enzymes were also detected in human sperm including CYP2R1, CYP27B1 and CYP24A1 (Blomberg Jensen *et al.* 2010). The level of CYP24A1 was related to the presence of VDR, but its unique localisation to the sperm annulus has been proposed to be a potential marker for sperm quality (Blomberg Jensen *et al.* 2012). CYP24A1 is responsible for degrading 1α,25(OH)$_2$D to the inactive calcitroic acid, and the expression of this enzyme was found in 25% of sperm from healthy young men, compared with 1% in sub-fertile males. In addition, it correlated positively with typical measures of semen quality including total sperm count, motility, progressive motility and morphology (Blomberg Jensen *et al.* 2012). In functional studies, acute exposure of sperm to 1α,25(OH)$_2$D increased motility in sperm derived from healthy males, but had no effect on sperm from a sub-fertile cohort (Blomberg Jensen *et al.* 2012) (Fig. 3). This may indicate a requirement for active CYP24A1, which is capable of regulating the local 1α,25(OH)$_2$D concentration interacting with the cell. Importantly, the researchers showed that in healthy sperm, the increased motility was due to a transient increase in calcium flux, derived from an intracellular source, which appeared to originate in the neck region (Blomberg Jensen *et al.* 2011, 2012) (Fig. 3). Furthermore, using specific VDR-AP and VDR-GP agonists, the authors concluded that the VDR

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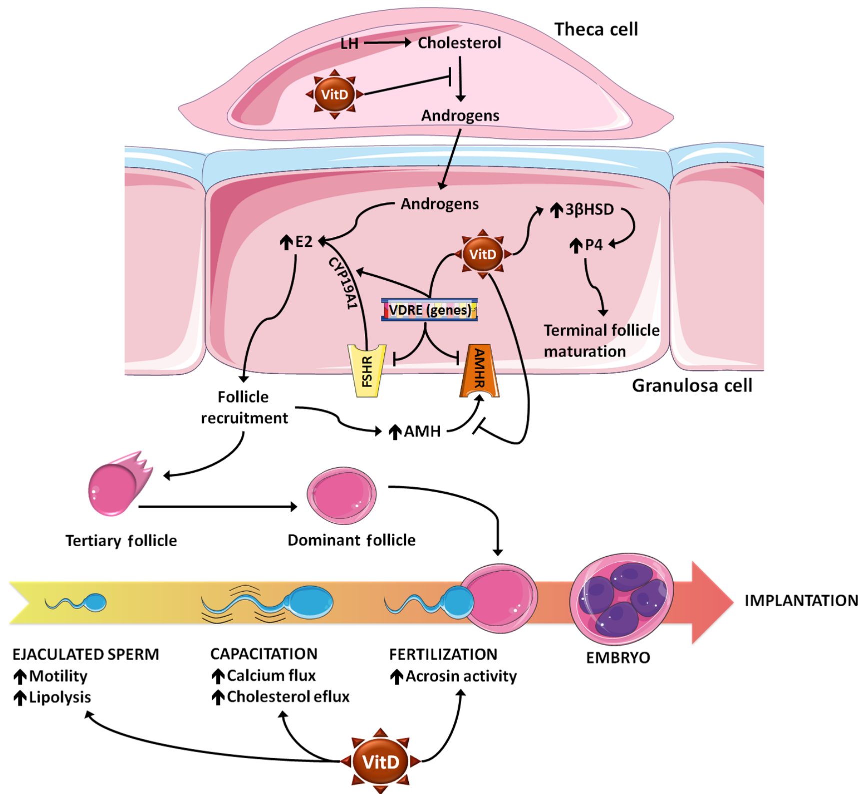

Figure 3 Molecular overview of VitD action in cells from the reproductive system. VitD regulates hormone production and receptor expression in theca and granulosa cells of developing follicles. This in turn affects follicle recruitment and maturation. VitD alters sperm motility and metabolism, and also impacts on the ability of sperm to undergo the acrosome reaction and consequently the ability to fertilise eggs. 3βHSD, 3-β-hydroxysteroid dehydrogenase; AMH, anti-Mullerian hormone; AMHR, anti-Mullerian hormone receptor; E2, oestrogen; FSHR, follicle-stimulating hormone receptor; LH, leutinizing hormone; P4, progesterone; VDRE, vitamin D response elements; VitD, vitamin D. **?** indicates unconfirmed effect; ↑ indicates increased expression, production or activity.

was mediating these effects via non-genomic signalling. Although it is well known that VitD alters calcium mobilisation in many cell types, the precise cellular mechanism regulating this calcium flux in human sperm is not understood, but it is speculated that it may be related to second messenger signalling systems involving molecules such as phospholipase C or protein kinase C (Blomberg Jensen & Dissing 2012). In addition, it would be interesting to examine whether the production of calcitroic acid from CYP24A1 was enhanced from these spermatozoa, which would definitively demonstrate that the biological activity of the enzyme, and the subsequent degradation of 1α,25$(OH)_2$D to calcitroic acid was intimately involved.

Other studies suggest that sensitivity to VitD-related metabolites may control the capacity of sperm to undergo the acrosome reaction and consequently the fertilisation ability (Aquila *et al.* 2008) (Fig. 3). Sperm were acutely exposed to 1α,25$(OH)_2$D, and capacitation was measured indirectly by detecting cholesterol efflux into culture medium, which leads to intracellular signalling and enhanced permeability of the plasma membrane to ions (Aquila *et al.* 2008). Cholesterol efflux was increased in a bi-phasic manner, peaking at 0.1 nM and reducing at 1 nM (Aquila *et al.* 2008). This bi-phasic trend was also reflected in sperm survival assays and acrosin activity (Aquila *et al.* 2008, 2009) and echoes the cyclic gene expression responses observed for cytokines discussed earlier in somatic cells (Fig. 3). An additional experiment by this group showed that 1α,25$(OH)_2$D increased lipase activity, while triglyceride content decreased, and they proposed that lipolysis provided the energy required to drive capacitation (Aquila *et al.* 2009, Lerchbaum & Obermayer-Pietsch 2012).

It is clear that VitD-related metabolites alter sperm motility and that this is coupled to a transient modulation of intracellular calcium (Blomberg Jensen *et al.* 2012). For efficient movement, spermatozoa require enormous amounts of energy. However, no studies have been performed to determine if ATP levels or demand are altered by exposure of sperm to 1α,25$(OH)_2$D. This presents several research questions. Does VitD alter sperm bioenergetics, glycolysis or mitochondrial function, critical for motility? Does VitD enhance nutrient and substrate metabolism, particularly glucose and fructose, along with lipids and energy-producing amino acids like glutamine? Finally, if VitD alters these parameters in sperm, are they mediated by non-genomic processes, including phospholipase C and protein kinase C signalling? Does the VDR play an active, but overlooked role?

Molecular mechanistic studies in female reproductive cells

The relationship between VitD status and reproductive disorders in females is mainly associative, with no direct causative role established (Luk *et al.* 2012). In addition, minimal progress has been made in understanding the participation of VitD–VDR

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signalling in human female reproductive function at the molecular level, but it is largely acknowledged that this axis regulates aspects of hormone production and secretion control, critical for folliculogenesis (Lerchbaum & Obermayer-Pietsch 2012).

The VDR and related enzymes are expressed in a variety of ovarian tissues (Lerchbaum & Obermayer-Pietsch 2012) including granulosa cells (Smith *et al.* 2009, Thill *et al.* 2009) and trophoblasts (Ma *et al.* 2012). As far as we are aware, the expression of this system has not yet been shown in human theca cells, but it has been demonstrated in rat theca cells (Ahonen *et al.* 2000). Nonetheless, human theca cells are purported to respond to 1α,25(OH)$_2$D (Brain *et al.* 2003), where it decreased androstenedione production in the absence or presence of luteinising hormone (LH), but this is yet to be confirmed. Consequently, the regulatory influence of VitD-related metabolites in theca cells still remains largely unknown and should be explored in future investigations. Conversely, a little more is known about granulosa cell VitD metabolism.

Granulosa cells are critical for driving folliculogenesis and promote follicle development by secreting oestrogen in response to biochemical crosstalk from the pituitary (FSH release) and theca cells (androgen release). In early tertiary follicles, LH-activated theca cells convert cholesterol to progesterone, which is further converted to androstenedione via cyclic AMP and protein kinase A signalling, as substrates ultimately for oestrogen production by granulosa cells (Fig. 3) (Fukuda *et al.* 2009). Specifically, incoming androstenedione is converted in the granulosa cell to oestrone by aromatase (CYP19A1), an enzyme activated by FSH derived from the pituitary (Fig. 3). Oestrone is then hydroxylated to active oestradiol by 17β-hydroxysteroid dehydrogenase (17β-HSD), an enzyme which was recently shown to be regulated by 1α,25(OH)$_2$D in mouse granulosa cells (Lee *et al.* 2014). Oestrogen levels steadily increase as the follicle grows and granulosa cells increase in number up until ovulation. After ovum release, the remaining follicle becomes the corpus luteum and primarily secretes progesterone along with a small level of oestrogen.

As illustrated previously, sufficient production of hormones at specific stages of the cycle is central to folliculogenesis. VDR signalling has a significant effect on hormone production in the female reproductive tract and consequently may potentially affect folliculogenesis. 1α,25(OH)$_2$D was shown to increase production and secretion of progesterone (13%), oestradiol (9%) and oestrone (21%) in human ovarian tissue (Parikh *et al.* 2010), which was mediated by the VDR. In another study, progesterone secretion from porcine granulosa cells was also altered by 1α,25(OH)$_2$D (Smolikova *et al.* 2013). 50–100 nM of 1α,25(OH)$_2$D increased 3β-HSD activity in primary cumulus granulosa cells, which promoted subsequent progesterone secretion (Merhi *et al.* 2014) (Fig. 3). However, it did not alter FSH-induced CYP19A1 expression and consequently no change in oestrogen production. Nonetheless, an atypical VDRE (as outlined in 'Vitamin D response elements (VDREs) and co-regulatory proteins' section) was identified in the CYP19A1 gene (Sun *et al.* 1998), which supports a bona fide mechanism of direct hormonal secretion control by VDR in granulosa cells (Fig. 3).

VitD deficiency has been associated with polycystic ovarian syndrome (PCOS) as expertly reviewed elsewhere (Thomson *et al.* 2012). It is not clear if low VitD is either a cause or consequence of PCOS, but may exacerbate PCOS due to the connection of low VitD status with important PCOS characteristics including obesity and insulin resistance (Thomson *et al.* 2012). At the molecular level, VitD may influence anti-Mullerian hormone (AMH) production and signal transduction, which is important in oocyte maturation and is elevated in PCOS patients (Tal *et al.* 2014), who are often VitD insufficient. VitD increased AMH mRNA in prostate cancer cells were a VDRE site was detected (Malloy *et al.* 2009). Conversely, VitD decreased AMH mRNA in primary granulosa cells from hens (Wojtusik & Johnson 2011). In a study with primary human granulosa cells, 1α,25(OH)$_2$D decreased AMH receptor II and FSH receptor mRNA expression, while promoting 3β-HSD expression and activity leading to increased progesterone secretion (Merhi *et al.* 2014) (Fig. 3). This recent work indicated that VitD altered granulosa cell differentiation and luteinisation potentiation, as reflected by a decreased sensitivity to FSH and AMH (decreased receptor expression), along with a concomitant increase in progesterone secretion. As 1α,25(OH)$_2$D did not alter AMH secretion from these granulosa cells, the authors speculated that VitD metabolites may aid terminal maturation of follicles by decreasing AMH sensitivity, which prevents the inhibitory effect of AMH on transition from the primordial follicle pool to the primary follicle (Fig. 3) (Merhi *et al.* 2014).

VitD status has also been associated with preeclampsia (PE) and pregnancy complications, which are intrinsically linked to inflammation, increased placental cytokine levels and impaired endothelium integrity (Noyola-Martinez *et al.* 2013). Many *in vitro* studies have already shown that VitD is a key modulator of cytokine secretion in immune cells (Matilainen *et al.* 2010, Calton *et al.* 2015*a*) and a similar anti-inflammatory effect has been observed in cytokines derived from trophoblasts *in vitro* (Noyola-Martinez *et al.* 2013, Gysler *et al.* 2015). Specifically, 1α,25(OH)$_2$D reduced TNFα and IL6 mRNA in trophoblasts isolated from PE placentas (Noyola-Martinez *et al.* 2013), and the impact on IL6 was also suggested by the authors to potentially abrogate endothelial dysfunction in PE women induced by excessive Th1 cytokines (Noyola-Martinez *et al.* 2013). However, it is not clear if the low levels of VitD metabolites in PE are a cause or consequence of

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inflammation, as TNFα was shown to enhance CYP24A1 activity leading to increased 1α,25(OH)$_2$D degradation.

Previously it was shown in *in vitro* and *ex vivo* studies that various VitD metabolites, D3, 25(OH)D and 1α,25(OH)2D could all maintain endothelium integrity via non-genomic mechanisms. These metabolites directly prevented cytokine-mediated Rho activation, while also enhancing the expression of cadherin to preserve tight junction integrity in endothelial cells (Gibson *et al*. 2015). These concepts are important for maintenance of placentation and vascularisation against the backdrop of inflammation in PE conditions. In another study, 1α,25(OH)$_2$D reduced IL1β and IL8 secretion in a trophoblast cell line and cells isolated from the placenta of normal first trimester pregnancies. The authors postulated that these anti-inflammatory effects were the result of impaired TRL4 and NFκB signalling, but did not investigate further (Gysler *et al*. 2015). Although studies in trophoblasts demonstrate the potentially anti-inflammatory effect of VitD in pregnancy, and show that cytokine levels may also interfere with VitD signalling, they do not shed any light on the precise cellular mechanisms driving these effects.

Other mechanistic studies have shown that 1α,25(OH)$_2$D reduced trophoblast dysfunction and oxidative stress, at least partially by inhibiting cyclooxygenase 2 (COX2) expression and subsequent thromboxane and 8-isoprostane production (Sun *et al*. 2014). Interestingly, molecular cellular studies in macrophages also found that 1α,25(OH)$_2$D downregulates COX2 expression and subsequent cytokine production by dampening Akt and NFκB signalling via activation of thioesterase superfamily member 4 (THEM4) through a novel VDRE site (Wang *et al*. 2014). It was recently reported that VitD metabolites promoted trophoblast cell invasion by increasing matrix metalloprotease (MMP) secretion, and this may translate to improved embryo implantation (Chan *et al*. 2015). However, although increased expression of MMP-2 and -9 was observed, the cellular signalling mechanism driving this phenotype was also not explored, again demonstrating the limited knowledge in this area. Particularly, considering that other studies in human lung fibroblasts and uterine fibroid cells showed that VitD metabolites actually inhibited MMP-2 and -9 expression by modulating cytokine expression (Halder *et al*. 2013, Kim *et al*. 2014), which are key regulators of immune cell infiltration. Nonetheless, these conflicting effects may be explained by the complex mechanism of VDR and VDRE regulation as outlined earlier.

Taken together, it is clear that VitD and related metabolites play a role in female reproductive function and pregnancy. For instance, VitD metabolism is altered during pregnancy to allow passage of calcium and VitD metabolites to the growing foetus. The levels of maternal VitD and PTH increase and decrease respectively, but the level of 25(OH)D in cord blood is only 65% of circulating maternal serum levels (Roth 2011). Therefore, it is not surprising that neonate VitD status reflects the maternal status (Paxton *et al*. 2013), and that preterm infants are often VitD-deficient due to prematurity (Hollis 2007). Consequently, these infants are at risk of developing bone health problems and possibly other non-skeletal issues. It remains to be seen what precise placental dysfunctions lead to impaired transit of VitD metabolites to the developing foetus. As mentioned previously, the megalin–cubilin transport system could be critical in not only providing key nutrients during early organogenesis, but also for maintaining foetal VitD levels during gestation (Whisner *et al*. 2016). Although these receptors were negatively correlated with maternal 1α,25(OH)$_2$D concentrations, this may be a compensatory mechanism to preserve maternal and foetal VitD status (Whisner *et al*. 2016).

Conclusions

The effects of VitD are largely mediated through the VDR. Although much research has focused on unravelling the VitD-VDR system, the regulation is complex, and it has not been fully elucidated, particularly in relation to non-genomic signalling mechanisms and the type of transactivator co-regulatory proteins recruited. However, VitD and VDR signalling appears to be important for reproduction parameters in both the male and female. At the cellular level, it is clear that VitD acutely increases sperm motility and is potentially important for healthy semen. Some mechanistic insights have demonstrated that 1α,25(OH)2D alters intracellular calcium mobilisation, which significantly affects sperm motility. VitD also has the potential to modulate inflammation and hormones levels, which are both key aspects for female reproductive function (Shahrokhi *et al*. 2016). It appears that acute exposure can alter oestrogen and progesterone production from primary granulosa cells, and weak evidence indicates an impact on theca cell androgen biosynthesis. However, very little is known about the precise molecular mechanisms by which VitD drives these changes, but it is possibly through genomic regulation of enzyme expression. VitD has a classical anti-inflammatory effect, but its impact on cytokine production from trophoblasts is still in its infancy. However, the molecular mechanism is possibly related to advances already made in immune cells and point to a direct regulation through epigenetic changes in target cytokine gene expression. At the clinical level, many studies have attempted to correlate VitD status with various chronic ailments, but apart from Ricketts, there appears to be no causative role, only association. Consequently, new studies should also consider not only the type of VitD metabolite to study but also the key factors that regulate the bioavailability including DBP and cellular transport systems critical for conversion of one form to the other. Finally, whole-body VitD

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storage has not been adequately explored, and this is another important aspect that should be considered in clinical studies.

Perspectives for future studies

Some of the most interesting advances in VitD signalling are the recent studies demonstrating VDR translocation to the mitochondria (mito-VDR) in various cell types and the subsequent direct impact on cell metabolism (Silvagno *et al.* 2010, 2013, Consiglio *et al.* 2014, 2015). These interactions may have important implications for the rapid non-genomic responses observed previously, like the insulinotropic actions in pancreatic β-cells (Kajikawa *et al.* 1999) and are unlike those observed for nuclear or membrane VDR. In addition, mito-VDR is dependent on mitochondrial permeability proteins, where inhibition of these significantly reduced VDR accumulation and resulted in altered cell proliferation, enhanced mitochondrial membrane potential along with increased mitochondrial respiration (Silvagno *et al.* 2013, Consiglio *et al.* 2014, 2015). The biological function of mito-VDR is believed to involve modulation of electron transport chain (ETC) activity and lipid metabolism (Consiglio *et al.* 2014), and the VDR receptor may directly regulate respiratory protein expression coded by mitochondrial DNA. Inhibition of mito-VDR reduced glutathione levels, decreased the *de novo* synthesis of cholesterol, while reducing the lipidation of GTPases Rho and Ras – major players regulating cell proliferation (Consiglio *et al.* 2014). Furthermore, attenuation of mito-VDR boosted ETC activity and when coupled with reduced glutathione levels, could potentially expose cells to excessive ETC-derived oxidative insult, leading to disease states. This is possibly one biological mechanism linking VitD deficiency with mitochondrial dysfunction and chronic diseases (Calton *et al.* 2015*b*), which may also relate to aspects of infertility.

The influence of mito-VDR on cholesterol metabolism has not been explored in trophoblasts, granulosa or theca cells, but it is reasonable to assume that it may be important for supply of cholesterol as a precursor for sex steroid biosynthesis or for cell expansion during folliculogenesis as a constituent of the plasma membrane. Current knowledge indicates that mito-VDR fed lipid in the form of acetyl-CoA, into the biosynthetic pathway, which resulted in intracellular lipid deposition and may be required for continued cell replication (Consiglio *et al.* 2015). Conversely, the direct influence of $1\alpha,25(OH)_2D$ on the other main energy-generating pathways, glycolysis and the Kreb's Cycle (tricarboxylic acid; TCA cycle), is not known. One study demonstrated a potential effect of $1\alpha,25(OH)_2D$ on glycolytic and TCA metabolic flux in a breast cancer cell model in 2013 (Zheng *et al.* 2013), where 10 nM decreased glucose conversion to lactate, 3-phosphateglycerate and acetyl-CoA by 15%, 10% and 24% respectively, while also reducing oxaloacetate generation from acetyl-CoA by 34% (Zheng *et al.* 2013). This restraining of metabolic flux by $1\alpha,25(OH)_2D$ impeded cancer cell progression. However, no comparable studies have been performed in cells derived from the reproductive system including primary granulosa cells or spermatozoa. Overall, these experiments showed that mito-VDR can dampen mitochondrial respiration and glycolytic metabolic flux, while also enhancing cholesterol biosynthesis and anti-oxidant defence. These findings may indicate the first potential metabolic benefits of VDR in cell-based studies, which may ultimately be translated to dysfunction in diseased states.

With specific respect to reproductive medicine, the requirement of oocytes, granulosa, theca, trophoblasts and sperm for VitD should be studied with a particular focus on how it may alter metabolic activity. We speculate that there may also be a role for VitD or related activators present in the female reproductive tract or seminal fluid that regulate fertilisation or implantation. Although there is greater knowledge regarding the influence of VitD on granulosa cell metabolism and hormone production, we still do not know how these phenotypic changes are controlled at the molecular level, and the function of the VitD-VDR system in human theca cells has been completely neglected. Better progress has been made concerning the effect of VitD on trophoblast and sperm function, but the identity of second messengers that mediate placental cytokine secretion, nutrient transport, such as the megalin–cubilin transport systems, endothelium integrity and the regulation of intracellular calcium flux in sperm are by far the most pressing issues.

With the development of highly specialised and sensitive biological techniques, including ChIP-chip and ChIP-seq, we speculate that the next ten years of research will answer a significant proportion of these important questions and their implication for reproductive medicine. We hypothesise that VitD, the VDR axis and its downstream molecular pathways may have a critical role in reproductive processes, particularly in relation to inflammation, cellular metabolism, metabolic substrate utilisation and mitochondrial function.

Declaration of interest

K N K, P N and J L Y have no affiliation, official or otherwise with Merck Serono, but have received seed funding in 2016 from Merck Serono that will allow investigation into some of the areas discussed in this article.

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Author contribution statement

K N K, P N and J L Y conceived the idea, planned and designed the manuscript. K N K wrote the first draft, which was revised initially by V F C and E K C. Figures were designed by K N K and V F C, and all artwork created by V F C. P H H and M J S provided critical insight into the subject matter. All authors participated in manuscript revision and approved the final submitted version.

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