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The effect of Vitamin D (1,25-$(OH)_2$-D3) on human theca and granulosa cell function

Short Title:

Vitamin D and human theca and granulosa cells

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Supplementary Figures

Supplementary Figure 1: Progesterone production from theca

Supplementary Figure 2: 17-hydroxy-progesterone production from theca

Supplementary Figure 3: Progesterone production from granulosa-luteal cells

Supplementary Figure 4: Representative western blot of immunoprecipitated proteins.

Keywords:

Vitamin D; Granulosa; Theca; Vitamin D receptor; human ovary; oestradiol; insulin receptor; AMH; CYP27B1

Abstract

Numerous studies have investigated the link between Vitamin D (VD) deficiency and reproductive outcomes, with contradictory results. VD regulates steroidogenic enzymes crucial for human granulosa and cumulus cell function. This study investigated whether deficient levels of 1,25-$(OH)_2$-D3 altered ovarian cell function; and if the ovary could obtain bioactive 1,25-$(OH)_2$-D3 via local enzymatic expression of *CYP27B1*, to counteract systemic deficiency. A variety of cells and tissues were used for the *in vitro* experiments.

We have shown for the first time an increase in VDR expression in theca of large compared to small follicles, which along with the ability of 1,25-$(OH)_2$-D3 to decrease Anti-Mullerian hormone expression, supports a role for 1,25-$(OH)_2$-D3 in theca and granulosa cell function. Conversely, very low levels of 1,25-$(OH)_2$-D3 equivalent to hypovitaminosis, inhibited thecal production of androstenedione and cAMP-driven oestradiol production. Human thecal and un-luteinised GC are incredibly hard to obtain for research purposes, highlighting the uniqueness of our data set. We also demonstrated that deficient levels of 1,25-$(OH)_2$-D3 down-regulated insulin receptor expression, potentially reducing insulin sensitivity. We have shown that the ovary expresses *CYP27B1* potentially allowing it to make local bioactive 1,25-$(OH)_2$-D3 which along with the upregulation in VDR expression in ovarian cellular compartments, could be protective locally in counteracting systemic VD deficiency. To conclude a severely deficient VD environment (<2nM or <1ng/ml) could contribute to impaired ovarian cell function and hence potentially affect folliculogenesis/ovulation, but levels associated with mild deficiency may have less impact, apart from in the presence of hyperinsulinemia and insulin resistance.

Introduction

Vitamin D (VD), is a fat soluble prohormone which has both well-established actions such as its classical role in calcium homeostasis and skeletal integrity (Dusso *et al* 2005). The discovery of vitamin D receptors (VDR) on male and female gonadal cells prompted investigations into the role of vitamin D in reproduction and fertility (reviewed in Lerchbaum & Obermayer-Pietsch, 2012; Lorenzen *et al*, 2017).

VD is derived minimally from the diet in the form of vitamin D2 and D3, with the major source being the photolytic conversion of 7-dehydrocholesterol in the skin to cholecalciferol (vitamin D3) catalysed by UVB radiation (reviewed in Bouillon *et al*, 2008). Both forms of VD are biologically inert and require activation through sequential hydroxylation in the liver and kidneys. The initial hydroxylation in the liver by various 25-hydroxylases results in 25-hydroxyvitamin D3 *aka calcidiol* (25-(OH)-D3), an inert stable metabolite that circulates bound to VD-binding protein (VDBP) and is used as an indicator of VD status in an individual (Holick, 2007). 25-(OH)-D3 is then further metabolised to the biologically active form 1,25-$(OH)_2$-D3 (*aka calcitriol*), primarily in the kidneys, a reaction catalysed by the mitochondrial enzyme 1α-hydroxylase (encoded for by *CYP27B1*) (Schuster, 2011; Luk *et al*, 2012). 1-α-hydroxylase has also been found in extra-renal tissue indicating local conversion of 25-(OH)-D3 to the active 1α,25-$(OH)_2$-D3.

The ability of active 1α,25-$(OH)_2$-D3 to exert its profound actions is mediated predominantly by VDR, a member of the nuclear hormone receptor super-family which acts as a ligand-inducible transcription factor (Haussler *et al*, 2013). Normally 1α,25-$(OH)_2$-D3 enters the cell by diffusion where it binds to and activates VDR to form a heterodimer complex with retinoid X receptor (RXR): this complex interacts with VD response elements (VDREs) found in the

promoter regions of both positively and negatively controlled genes (Cheskis & Freedman, 1994). 1α,25-$(OH)_2$-D3 and VDR are estimated to regulate 3% of the human genome consequently, VD deficiency is recognised to be associated with numerous pathological conditions which is usually reversed upon supplementation (Holick *et al*, 2007; 2011;2012).

The first indication that VD affected female fertility was shown by VD-deficient rats with reduced fertility rates and litter sizes (reviewed in Lorenzen *et al*, 2017). VD has been shown to interact and regulate steroidogenic enzymes that are crucial for human granulosa and cumulus cell function (Merhi *et al*, 2014). Interestingly, genes for insulin receptor *(InsR)*, anti-Müllerian hormone *(AMH)* and *CYP19A1* (encoding aromatase) that play a critical role in folliculogenesis have VDRE on their promoters (Tiejun *et al*, 1998; Maestro *et al*, 2003; Krishnan *et al*, 2007 & 2010), suggesting a role for VD in female fertility.

Yet studies investigating the effects of VD deficiency/VD levels on reproductive outcomes produce extremely variable results (reviewed in Lorenzen . *et al*, 2017). Whilst this could be due to a multitutde of factors, there is also a lack of understanding of the mechanistic actions of VD in ovarian physiology, and the effect of varying levels of VD ligand interaction with its receptor, which may explain the observed outcomes. Current guidelines define VD deficiency as 25-(OH)-D3 levels <20ng/ml (or <50nmol/L); with levels of 21-29ng/ml (52.5-72.5nmol/L) characterized as VD insufficiency and >30ng/ml (>75nmol/L) considered as sufficient (Holick *et al*, 2011; 2012; Pilz et al, 2019).

The aim of our study was to investigate the function of human ovarian cells in a low vitamin D environment (i.e., ≤20nM), thereby providing mechanistic insight to account for the variable reproductive outcomes observed clinically in women with deficient serum levels of VD. We

hypothesized that exposure of ovarian cells to deficient levels of VD would alter steroidogenic output and gene expression.

Materials and Methods

All reagents were obtained from Sigma-Aldrich, Merck Life Science UK Limited, Gillingham, Dorset, UK, unless stated otherwise, and all plasticware was purchased from Fisher Scientific, Loughborough, UK.

Tissue samples for *in vitro* experiments

A variety of cells and tissues were used for the *in vitro* experiments of this study as detailed below.

Human Ovarian Tissues: Informed written consent was obtained from women undergoing trans-abdominal hysterectomy with bilateral oophorectomy for benign gynaecological conditions at St Luke's Hospital, Malta. Ethical approval was granted by the Ethics Committee of The Faculty of Medicine and Surgery, Medical School, Malta. Clinical details were obtained including age, gynaecological history, menstrual frequency, and day of cycle. The ovaries removed from each patient were seen by a pathologist before a portion of each was taken to the laboratory for dissection. Morphology and ovulatory status were assigned as previously published and were based on ovarian size, follicle sizes and numbers, the presence of a dominant follicle or corpus luteum and the amount and density of stroma as determined by dissection, in conjunction with patient history (Mason *et al*, 1994; Gilling Smith *et al*, 1994). The timing of surgery was random. Patient details, number of follicles obtained, and tissue used in each experiment are shown in Table 1. Follicles were isolated from the surrounding stroma, the diameter measured, and granulosa cells (GC) collected as previously described (Mason *et al*, 1990; 1996). When follicles from normal ovaries have reached 9-10mm in

diameter (Willis *et al*, 1998) i.e. the window of dominant follicle selection, their GCs secrete E2 in response to LH. Therefore, follicles <10mm are described as small antral follicles (SAF) and those equal to or greater than this diameter as large antral follicles (LAF).

Finally, the theca cell layer was carefully peeled off and digested in an enzyme cocktail for 30mins at 37^{o}C with gentle agitation (Mason *et al*, 1990; 1996). GC and theca cells were cultured as outlined in subsequent sections, other ovarian tissue samples were immersed in RNA-later or flash-frozen and archived at -80C for further analysis. Use of archived tissues was compliant with HTA regulations (*see statement at end of manuscript*).

Luteinised granulosa cells (GLC) were obtained from follicular aspirates obtained during oocyte retrieval from women undergoing *in-vitro* fertilisation (IVF) treatment at various assisted conception units including those at King's College Hospital and The Lister Hospital, London, UK. GLCs are considered a waste product by the HFEA and do not need ethical approval as such, however local ethics committee approval for each unit was sought and informed consent was obtained from all women.

KGN granulosa cell (KGN-GC) line, that are established to correspond to immature granulosa cells from smaller antral follicles, were used to provide mechanistic insight (Nishi *et al*., 2001). The distribution of VDR and AMH protein in various ovarian tissue compartments was assessed using *ovaries from mice (strain 129Sv)* aged between 4 and 5 months. These were euthanised as part of separate ethically approved projects at SGUL and ovaries donated to this study.

Immunohistochemistry of AMH and VDR in mouse ovarian sections

Ovaries were fixed in paraformaldehyde (4%) at 4^{o}C, dehydrated in a gradient ethanol series, cleared in methyl salicylate, embedded in wax and 5µm serial sections cut. Every 3rd section

was stained with haematoxylin and eosin (H&E). Adjacent sections were used for immunohistochemistry with anti-VDR (1:100, rat) and anti-AMH (1:50, mouse) monoclonal antibodies (Abcam Ltd, Cambridge, UK. Details listed in Table 2) as per manufacturers' protocol with modifications and appropriate controls.

Briefly, slides were dewaxed and rehydrated, and the antigen epitopes exposed by heating the slides in a 0.01M citrate buffer (pH 6) (National Diagnostics, Atlanta, GA, USA) bath until boiling point for 20 minutes. Peroxidases were blocked by 3% hydrogen peroxide (VWR Ltd., Lutterworth, Leic., UK) in methanol (National Diagnostics) for 10 minutes. Sections were then blocked with relevant anti-serum to reduce non-specific binding for 1h at room temperature. Slides were then incubated with the relevant primary antibody overnight at 4^{o}C, followed by 1-hour incubation at room temperature with 0.1% monoclonal biotin-labelled secondary antibody (Vector) (Table 2). Visualisation was carried out with Avidin Biotin Complex (ABC) peroxidase solution followed by 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Newark, CA, USA). A haematoxylin counterstain was also used for 10 seconds to label nuclei blue. After a brief dehydration through an ethanol series, slides were mounted using Histomount (National Diagnostics). Negative controls omitted primary antibody. Images were captured using the Hamamatsu NanoZoomer 2.0-Rs Slide Scanner for analysis with the NDP.view 2 software (Hamamatsu City, Shizuoka Pref.,Japan) (courtesy of the Image Resource Facility, SGUL).

mRNA and protein expression of VDR in ovarian tissue/cellular compartments

The mRNA expression of VDR in human ovarian cortex, stroma and theca tissue compartments was assessed using archived frozen tissue collected as described above. All tissue was stored and used under HTA regulations. Tissues were defrosted on ice and

164 homogenised in lysis buffer (RLT lysis buffer, Qiagen Ltd, Manchester, UK) using the FastPrep®
165 TissueLyser 24 and the FastPrep® Lysing Matrix A 2mL tubes containing a garnet matrix and a
166 ceramic sphere (MP Biomedicals, Santa Ana, CA.). After homogenisation RNA was extracted,
167 reverse transcribed and real-time quantitative PCR (qPCR) performed for VDR relative to L19
168 (the reference gene), as previously described (Rice *et al*, 2006). In addition, the expression of
169 VDR in the KGN-GC was established using standard PCR as well as qPCR (see Table 3 for details
170 of primers and cycling conditions). For protein extraction the tissue was homogenised in RIPA
171 buffer with a cocktail of phosphatase inhibitors (Sigma-Aldrich), using a sonicator three times
172 for 30s each time. Samples were maintained on ice throughout to prevent overheating and
173 denaturation of proteins. The lysed samples were micro-centrifuged at 4^{o}C for 10min at
174 maximum speed. Pelleted debris was discarded, and the protein lysate stored at -80^{o}C prior
175 to Bradford assays (for measurement of protein concentration) and western blotting.

176 **GC, theca and GLC culture experiments**

177 The granulosa or theca cells were pooled from several follicles based on the experimental
178 protocol and cultured in McCoys 5A medium supplemented with penicillin/streptomycin, L-
179 glutamine and amphotericin B (all purchased from Invitrogen, ThermoFisher Scientific,
180 Altrincham, Cheshire, UK). Granulosa cells were plated in 96-well plates at 5x10^{4} and theca
181 cells at 5x10^{5} cells per well as previously described (Mason *et al*, 1990; Willis *et al*, 1996). To
182 increase the adhesion of theca cells to the plate, plates were first coated in ECM gel (McCaig
183 et al, 2002).

184 Cells were incubated in culture medium (plus experimental treatments) for 48 hours with a
185 range of 1,25-$(OH)_2$-D3 concentrations (0.2, 2 and 20nM serially diluted down from a stock
186 concentration of 2µM). Testosterone (5x 10^{-7} M) was used as a substrate for conversion to
187 oestradiol (E2) in the granulosa cells and steroid levels measured in the medium by

radioimmunoassay (Hillier *et al*, 1980; Gilling-Smith *et al*, 1994; Willis *et al*, 1996). To confirm that cells were healthy, 10ng/ml LH was used as a positive control in luteinised GCs and theca, and 5ng/ml FSH in cells dissected from SAF <10mm (LH and FSH supplied by Endocrine Services, Biddeford-upon-Avon, UK). Isolation of GLC was performed as previously described (Wright *et al*, 2002). Cells were plated in 96-wells at $5x10^4$ cells per well in 200µl of serum (5%) supplemented medium for 48 hours, after which the medium was removed and replaced with experimental treatment as detailed above. GLCs were also exposed to the vitamin D analogue ED1089 (kindly donated by Dr. Kay Colston, SGUL), to examine comparative efficacy and demonstrate specificity.

KGN-GC culture experiments

To model the effect that VD deficiency may have on ovarian function, KGN-GC were grown and passaged in 10% DMEM-F12 supplemented with L-glutamine and penicillin/streptomycin (Invitrogen), at 37°C in 95% air/CO_2. Cells were plated in 12-well plates ($3×10^3$ cells/well) and cultured in 1% DMEM-F12 (charcoal-stripped) overnight. Cells were serum-starved for 2h and then treated with forskolin (25µM), to reproduce the effect of LH on cAMP stimulation, ± 1,25-$(OH)_2$-D3 at 0.02, 0.2, 2 and 20nM and cultured for a further 48h. Testosterone ($5x10^{-7}$M) was added to all cultures as the aromatase substrate for conversion to oestrogen. Similarly, the effect of VD on AMH mRNA expression was investigated in cells treated with forskolin and VD as described above, using only the upper and lower doses of 1,25-$(OH)_2$-D3 from the range above at 0.02 and 20nM. At the end of the relevant culture time, RNA was extracted, reverse transcribed and real-time quantitative PCR (qPCR) performed for *CYP19A1* (encoding aromatase) as described previously (Rice *et al*, 2013). We have previously established that *CYP19A1/aromatase* mRNA, protein expression and enzyme activity are all

tightly correlated in this model and hence any of these techniques can be used interchangeably to assess changes (Rice *et al*, 2006; Rice *et al*, 2013).

To investigate the effect of VD on aromatase promoter II (PII) activity, cells were plated at a density of $2x10^4$ cells/well in 96-well plates. After overnight incubation, they were transfected with a *CYP19A1 PII-516* reporter construct expressing firefly Luciferase, along with a control plasmid expressing Renilla luciferase from a constitutive promoter, and a transcription enhancing element, PVAi, as previously described (Rice *et al*, 2013). After 2h serum-starvation, cells were treated as described above for 24 hours with quadruplicate replicates/treatment, and luciferase reporter assays were carried out using the Dual-Glo Luciferase Assay System (Promega, Chilworth, Southampton, UK).

To mimic a situation of chronic insulin exposure, cells were treated with insulin for 48h at post-prandial (10ng/ml) and hyperinsulinemic (100ng/ml) levels in the presence and absence of the lowest [0.02nM] and highest [20nM] 1,25-$(OH)_2$-D3 levels tested in the experiments. The effect of 1,25-$(OH)_2$-D3 on aromatase and insulin receptor (InsR) mRNA expression was measured by qPCR.

Radioimmunoassay (RIA) for Progesterone (P), Oestradiol (E2) and Androstenedione (A4)

Conditioned media was collected from the cells at 48 hours, frozen at -20C and E2, P and A4 measured using a modified 'in-house' RIA, with tritiated steroids and charcoal separation (Hillier *et al*, 1980; Gilling-Smith *et al*, 1994; Willis *et al*, 1996). The components of the assay were: tritiated steroid label (Amersham Pharmaceutical Diagnostics, Chalfont St. Giles, Buckinghamshire, , UK), steroid antiserum (sheep anti-human from Guildhay Antisera Lts, Guildford, Surrey, UK) and standards and quality controls (QCs) (approximately 1, 6 and 35% of the top standard), prepared from powdered steroid (Sigma-Aldrich). Conditioned medium

was diluted to be within the midrange values for each assay and therefore on the linear part of the standard curve. Each sample was tested in duplicate.

Immunoprecipitation (IP)

To investigate the effect of 1,25-$(OH)_2$-D3 on VDR:RXR dimer formation, KGN cells were plated at a density of $5x10^5$ cells/well in a 6-well plate and treated as described above with and without forskolin, testosterone and 1,25-$(OH)_2$-D3 at 0.02 and 20nM for 48h. At the end of culture time, media was removed, cells washed with ice-cold PBS and lysed with ice-cold RIPA buffer as previously described (Dilaver *et al*, 2019). The protein concentration of the lysate was measured by Bradford assay and equalised to the lowest amount obtained in each experimental group (range 40-300µg/sample). Prior to immunoprecipitation, equal amounts of protein lysate from each treatment group were pre-cleared on A/G-coupled Sepharose beaded (Pierce, Thermo Fisher Scientific) support (binding capacity 27mg mouse IgG or ≥ human IgG/ml settled resin) for one hour at 4°C to reduce non-specific ligand binding. Immunoprecipitation of the cleared lysates was performed overnight at 4°C with 5µg of anti-VDR antibody or anti-RXRα monoclonal antibodies (Abcam – Table 2). The beaded complexes were washed several times with NP-40 lysis buffer, after which protein complexes were dissociated by boiling for 10 mins with 1X SDS-DTT reducing buffer (protocol adapted from Luderer *et al*, 2011). The collected supernatant was stored at -80°C for subsequent Western blotting analysis. Negative controls included retention of pre-cleared beads which were eluted and run on western blots, alongside the total lysates (data not shown). IP samples were checked for specificity using the corresponding specific antibody (Supplementary fig S4).

Western Blotting of VDR and RXR

Ovarian tissue protein concentrations were measured using the Bradford assay (Bio-Rad Labs Ltd, Watford, Hertfordshire, UK). 10µg/ml of cortical/stromal protein or equalised amounts

(as described in previous section) from immunoprecipitation experiments, were resolved with Western blotting as previously described (Dilaver N *et al*, 2019). PVDF(fl) (Immobilon, Merck Life Sciences, Gillingham, Dorset, UK) membranes with transferred proteins were incubated with rat anti-human VDR or rabbit anti-human RXR (both used at 1:1000) and/or mouse anti-human β-actin (1:2000). Fluorescently conjugated relevant secondary antibodies (1:5000) (see Table 2 for details of antibodies) were used for visualisation using the Odyssey Imaging System (Li-Cor Biosciences Ltd, Milton, Cambridge, UK) (Dilaver *et al*, 2019).

Experimental Quantification and Statistical Analysis

All data are represented as the mean ± SEM of triplicate or more observations (detail in legends) from a minimum of 3 or more independent experiments, unless otherwise stated. qPCR data were analysed using the ΔΔCt method as described in detail previously (Rice *et al*, 2006), with normalisation to L19 and subsequent normalisation to the Ct value of the control (untreated). To use the ΔΔCt method, the amplification efficiency for each GOI and reference gene must be in the recommended range of 90-100%. This was rigorously applied to our study by the inclusion of a standard curve for every qPCR assay conducted. Data from Western blots represent the mean densitometry measurements taken from all individual experiments using Image Studio software (Licor™) and normalised to ß-actin (loading control) and where relevant, to the untreated (control) samples. For the IP experiments, the densitometry values were also adjusted for the quantity of protein in the sample. Results for steroid RIA were calculated using Assay Zap V2.69 software (Biosoft, Great Shelford, Cambridge, UK). Intra- and inter-assay coefficients of variation were below 5% and 6% respectively.

Statistical significance was determined by ANOVA followed by post hoc multiple comparison tests; unpaired Student's or paired t test when 2 groups were compared (depending on the

design of the experiment) or a one-sample t test when comparing with normalised control values, using GraphPad Prism™. Significance was set at $P \leq 0.05$.

Results

We analysed expression (via qPCR, IHC and WB) of key elements (VDR and *CYP27B1)* involved in the synthesis and signalling pathway of VD in various ovarian cellular compartments. We then proceeded to investigate the effect of a range of doses of VD on steroid output (RIA) from primary theca and un-luteinised granulosa cells dissected from antral follicles of varying sizes cultured *in vitro*. The effect of VD on oestradiol (E2) production (RIA and qPCR) was also analysed in luteinised GLCs and the KGN cell-line. Since VD is known to interact with other factors involved in granulosa cell proliferation, we chose two of particular interest, namely AMH and insulin to investigate via qPCR. Finally, we examined the relationship via IP between varying doses of VD and the degree of binding to VDR and RXR receptors, which then complex to VDREs to modulate its actions.

Expression of VDR and *CYP27B1* in ovarian tissue and cellular compartments

IHC was used to detect the extent of VDR protein expression in sequential mouse ovary sections, and the anti-VDR antibody revealed extensive staining throughout all mouse ovarian tissue compartments including the cellular components of the follicles (figure 1a (VDR negative control) and 1b). Closer examination revealed staining in GC and thecal cells of both LAF and SAF (inset figures 1b). The general uniformity and ubiquity of VDR expression was in marked contrast to staining with anti-AMH antibody in the sequential section, which showed

a distinctive pattern of expression i.e., greater staining in GC of SAF compared to LAF, with no staining in theca or ovarian compartments (figure 1d; figure 1b AMH negative control).

We then measured VDR mRNA levels in human theca cells dissected from human follicles of varying sizes to ascertain whether expression levels changed with follicle size. VDR mRNA levels were significantly higher in theca from antral follicles >10mm compared to those of 5-6mm (Mann-Witney, *p=0.01, n=4 in each group; figure 2a). Whilst there was greater variability in antral follicles 7-8mm (n=3), the increased trend in thecal VDR mRNA expression is clearly apparent as follicles progress in size (fig 2a).

In addition, VDR mRNA (fig 2b) and protein (fig 2c) expression were also detected in human cortex and stroma. There was no statistically significant difference between the two compartments *(fig 2b, unpaired t-test p=0.61; fig 2c, unpaired t-test p=0.31)*. *CYP27B1* mRNA was expressed in human ovarian stroma as assessed by qPCR, though there was considerable variation in expression levels between different biological samples (fig 2d).

Effect of 1,25 (OH)2-D3 on steroid production in ovarian cells

VD and theca steroidogenesis: Overall, in theca from LAF (15-22mm), exposure to all doses of 1,25-$(OH)_2$-D3 consistently suppressed A4 production (mean 22% suppression) but had no effect on A4 from SAF (*n=4-8, Two-way ANOVA, p=0.048 for treatment and p=0.002 for follicle size*) (fig 3a). Treatment with 1,25-$(OH)_2$-D3 had no effect on either P (*ANOVA p=0.69*) or 17-OH-P (*ANOVA p=0.97*) production (supplementary figures S1 and S2).

VD and unluteinised GC steroidogenesis: There was no effect of 1,25-$(OH)_2$-D3 at any dose on E2 production in GCs taken from follicles <10mm diameter; however, in follicles >10mm

there appeared to be a trend to increasing E2 with 1,25-$(OH)_2$-D3 though this was not significant (n=4; ANOVA p=0.55) (figure 3b).

VD and E2 production in GLC: The effect of VD on E2 production from luteinised granulosa cell culture was then investigated. Luteinisation of GC occurs once follicles have been exposed to the mid-cycle ovulatory LH surge. As expected, exposure of the cells to LH produced a substantial stimulation in the production of E2, surprisingly this was considerably attenuated by 1,25-$(OH)_2$-D3 at both 2nM and 20nM doses (figure 3d) (*ANOVA p<0.0025; post-hoc t-test b**p<0.005*). This contrasts with the results from non-luteinised GC from small follicles in which 1,25-$(OH)_2$-D3 had no effect on FSH-stimulated E2 production (figure 3c) (*n=4; ANOVA p<0.0001; post-hoc t-test b*p<0.05*). Interestingly culturing GLC with the VD analogue EB1089 showed a non-significant dose-related trend in suppressing E2 production (figure 3e) (*ANOVA p=0.27*). There was, however, no effect on progesterone production in the cells by any dose of VD (*ANOVA p=0.22*) (supplementary figure S3).

Effect of VD on aromatase expression in KGN-GC: To determine the mechanism by which 1,25-$(OH)_2$-D3 was altering E2 production in primary cells, KGN cells were cultured with forskolin to increase cAMP and hence aromatase activity (equivalent to stimulation by either gonadotrophin, which both act via cAMP). Very low 1,25-$(OH)_2$-D3 levels [0.02 & 0.2nM] significantly down-regulated Fsk-stimulated aromatase mRNA expression (*n=6, One-way ANOVA**p=0.0018; post-hoc t-tests *p<0.05, **p<0.005*), but when doses approached sufficiency levels [2 and 20nM] this attenuation was reversed. An identical pattern was seen in PII transfected KGN cells demonstrating that 1,25-$(OH)_2$-D3 affected aromatase activity as

well as expression (figures 4a & 4b) (*n=3; ANOVA****p<0.0001; post-hoc t-tests *p=0.02, **p=0.0095*).

Effect of VD on other factors implicated in regulation of follicle growth

Insulin: There is compelling evidence that systemic VD levels are correlated with insulin sensitivity (Łagowska *et al*, 2018); however, there is little supportive evidence at a cellular level. Chronic exposure of granulosa cells to high (100ng/ml) insulin significantly downregulated expression of total InsR mRNA (*p=0.001*), and this reduction was surprisingly potentiated by both doses of 1,25-$(OH)_2$-D3: 20nM (<50% of basal) and 0.02nM (<25% of basal) (*n=5-8; One-way ANOVA *p=0.048, one-sample t-tests to the control ***p<0.0005, **p<0.005, *p<0.05*) (figure 5a). This profound attenuation of insulin receptor expression by 1,25-$(OH)_2$-D3 in the presence of chronic insulin exposure did not have a concomitant reduction on aromatase expression in the same cells. In fact, the presence of insulin reversed the attenuation of basal aromatase levels brought about by low dose VD (figure 5b).

AMH: Forskolin down-regulated AMH expression compared to basal (<50%), which was further potentiated in the presence of 1,25-$(OH)_2$-D3, with a more potent attenuation by 20nM 1,25-$(OH)_2$-D3 compared to 0.02nM (figure 5c) (*n=3, ANOVA *p=0.02; multiple comparison t-test *p<0.05*).

Investigating the differential effects of varying doses of VD ligand

Data presented so far indicate that those doses of 1,25-$(OH)_2$-D3 equivalent to extremely deficient levels have differential effects compared to those approaching sufficiency. To determine a possible mechanism, the relationship between levels of VD ligand and the proportion of either VDR or RXR was investigated. KGN-GC treated with extremely low or

sufficient doses of 1,25-$(OH)_2$-D3 in the presence of Fsk, were immunoprecipitated (IP) with either anti-VDR or anti-RXR antibodies. The VDR-IP pulls down all forms of VDR:homodimers, heterodimers and solo receptor as does the RXR-IP. The immuno-precipitated samples were then immunoblotted (IB) with corresponding anti-VDR or anti-RXR antibodies to assess just the heterodimer association between RXR and VDR in the relevant IP samples (figure 6a,b,c). The subsequent western blot membranes were analysed in a variety of ways i.e. corresponding IB adjusted for protein concentration or ratio of corresponding IB:IP adjusted for protein concentration (table).There was a significant reduction in levels of VDR associated with RXR-IP in the presence of VD [20nM] combined with Fsk compared to basal, FSk alone or Fsk+ 0.02nM 1,25-$(OH)_2$-D3 (table 4) However, there was no change in RXR levels associated with VDR-IP (table 4).

Discussion

We have discovered widespread distribution of VDR in the human ovary, pointing to a likely critical role for VD in female reproduction. We have demonstrated for the first time, a clear relationship between increasing VDR expression in theca and follicle size, highlighting a possible important functional role for Vitamin D in antral follicle progression.

Theca contains the blood supply of the follicle, so increased VDR expression here will allow the follicle to access circulating VD3. That activation of these receptors is playing a role in follicle growth was demonstrated by the fact that VD3 supplementation promoted the survival and growth of *in vitro* cultured preantral follicles to the antral stage in the primate ovary (Xu et al, 2018). Our finding of protein expression of VDR in human cortex and stroma was also matched by extensive and uniform expression of VDR protein in granulosa cells,

cortex, and stroma of mouse ovaries, further indicating an important role for VD throughout the ovary. This is also supported by the fact that female VDR knockout mice have impaired folliculogenesis with no progression beyond primary and secondary stages and low oestradiol levels (Yoshizawa et al, 1997). Ideally, we would have preferred to use either human whole ovary sections or other mono-ovulatory species such as sheep which would be a better model, but these were not available to us. Another limitation of our study was that we had insufficient archived human theca material to allow for detection of protein expression of VDR. However, mRNA copy number has shown to be a good determinant of VDR protein concentration in many cell types (Ogunkolade BW *et al*, 2002; Seoane S *et al*, 2007).

The detection of *CYP27B1* mRNA expression in human ovarian stroma was intriguing as it indicated that the human ovary is capable of being an extra-renal site of active 1,25-$(OH)_2$-D3 synthesis, as supported by studies in other species (Xu *et al*, 2018). The contribution of any locally produced bioactive 1,25-$(OH)_2$-D3 is difficult to determine but could play an important role in counteracting systemic VD deficiency. Active 1,25-$(OH)_2$-D3 negatively regulates expression of CYP27B1, hence regulating its own circulating concentrations (Bouillon *et al*, 2008). This may account for the considerable variation seen in our *CYP27B1* mRNA expression data as our stromal samples were taken from women whose VD status was unknown. Moreover, five of the stromal samples came from women with normal ovaries and three from women with polycystic ovaries. Women with PCOS have lower serum 25-(OH)-D3 levels compared to fertile controls (Krul-Poel *et al*, 2018; Eftekhar *et al*, 2020). Again, we were limited in the amount of protein available from the archived tissue so could not assess CYP27B1 protein expression, but there is good correlation between CYP27B1 mRNA and protein expression and activity (Lechner *et al*, 2007). Moreoever, 1α-OHase knock-out mice were acyclical, did not ovulate and infertile with small ovaries and immature follicles

compared to wild-type, indicating the critical role that this enzyme plays in normal ovarian folliculogenesis. Interestingly this only affected female and not male mice (Panda *et al*, 2001).

This increasing expression of VDR in the growing follicle at the LAF stage (>10mm) corresponds to increasing steroidogenesis and the acquisition of LH receptors. It had been documented that incubation of human cumulus GC with VD at normal levels affects steroid output (Merhi *et al*, 2014), but we wished to investigate the extent of and mechanisms by which exposing cells to a 1,25-$(OH)_2$-D3-deficient environment affected steroidogenesis. To that end we incubated cells with 20, 2, 0.2 or 0.02nmol/L (since serum levels <50nmol/L is defined as severe deficiency) (Holick *et al*, 2011). A recent study of women of childbearing age in rural northern China found the prevalence of severe VD deficiency was 16% in the 1151 women studied, with a median serum level of only of 5.63ng/ml (equivalent to 14nmol/L) (Lin *et al*, 2021); indicating that severe hypovitaminosis of this magnitude occurs in the population. Interestingly, 1,25-$(OH)_2$-D3 is established to act at nanomolar, or even picomolar, concentrations, as a direct regulator of specific target genes in VDR-expressing cell and tissues. It also binds to VDR with an affinity of 0.1nM (reviewed in Carlsberg, 2022).

In GC, gonadotrophins stimulate oestrogen synthesis via cAMP-mediated signalling, and it is well-established that a cAMP-response element (CRE) sequence has been identified within promoter II (PII); the predominant aromatase promoter used in the ovary (Michael et al, 1997). Changes in the 1,25-$(OH)_2$-D3 environment had no effect on E2 production from GC of either SAFs/LAFs, in the presence or absence of FSH and FSH responsiveness was retained. Conversely in luteinised granulosa cells (GLCs), 1,25-$(OH)_2$-D3 did prevent the LH-mediated stimulation of E2 production. It is without doubt that 1,25-$(OH)_2$-D3 can decrease E2 production as the same effect was observed with the VD analogue in GLCs.

This would imply that attenuation by VD only occurred when GC acquired LHR or else is dependent on levels of cAMP generated, since LH stimulates more cAMP activity than FSH (Aharoni *et al*, 1995). This is supported by the observation that extremely low doses of 1,25-$(OH)_2$-D3 down-regulated forskolin-stimulated aromatase expression and activity in KGN cells (which do not have LHR), an effect that was lost once dosing levels were increased.

This contrasted with theca, where a low 1,25-$(OH)_2$-D3 significantly attenuated androstenedione production from theca of LAF (15-22mm) but not of SAF (<10mm). Surprisingly, 1,25-$(OH)_2$-D3 had no effect on progesterone or 17-OH-P production from theca of all sized follicles indicating that this inhibitory action presumably occurs in the *CYP17* pathway, but only in LAFs when they have upregulated VDR expression. Like observations in theca, there was no effect of 1,25-$(OH)_2$-D3 on progesterone production from GLCs.

The bi-phasic dose-response effects of 1,25-$(OH)_2$-D3 on aromatase expression, was a direct influence on the transcriptional activity of PII, as seen in the PII transfection assay results. Analysis of PII has revealed the presence of two VDRE - proximal and distal, with an overlap between the proximal VDRE and CRE (Krishnan *et al*, 2010). A key factor could be the mechanism of ligand-binding of VD to its receptor. VDR functions as both a monomer, homodimer, and a heterodimer with RXR, and the alteration of the ratio of these complexes within a cell is dependent on the amount of VD ligand present (Cheskis & Freedman, 1994). Depending on the proportion of each, these complexes will bind to VDRE on genes and attract either co-repressors/activators to enhance or suppress gene expression and activity. Increased levels of VD decreased the amount of DNA-bound VDR homodimer complexes and promote the formation of VDR-RXR heterodimers (fig 7a) (Carlsberg , 2022; Cheskis *et al*,

1994; Haussler *et al*, 2013). The proportion and effect of VDR:RXR heterodimers binding to the VDRE of the aromatase gene remains to be determined in future studies.

Unliganded VDR-RXR heterodimers are initially bound to a VDRE and recruit co-repressor complexes, which prevents basal transcription through the activity of histone deacetylase. Once sufficient ligand has bound, the repressors are substituted by co-activator complexes, allowing gene transcription to commence (Carlsberg , 2022; Dwivedi *et al*, 1998; Perissi *et al*, 2010). The results of our IP experiments showed a difference in the levels of VDR associated with immunoprecipitated RXR, at different concentrations of VD ligand in the presence of forskolin. In the presence of higher concentrations of VDR ligand there appears to be less VDR associated with immunoprecipitated RXR. However, it must be pointed out that it was not possible from our experiments to determine the proportion of heterodimers in the cytoplasmic or nuclear compartments (and also non-genomic/genomic forms) as we used whole cell lysates. To answer this, we would need to carry out further experiments (beyond scope of this study) to explore VDR sub-cellular trafficking between nuclear and cytoplasmic compartments and components of the repressor/activator complexes bound to chromatin. Interestingly, the ability of forskolin to alter the association of the intracellular dimers would indicate the presence of a ligand-independent cAMP activated pathway outside the nucleus (Luk *et al*, 2012; Haussler *et al*, 2013) which could account for the different outcomes observed when using either FSH or LH.

Women with PCOS commonly present with increased serum levels of AMH (reflecting the pool of stalled SAF) and hyperinsulinemia, both of which are also linked to VD status (Luk , 2012; Lorenzen et al, 2017). To investigate this link, we replicated hyperinsulinemia and insulin resistance *in vitro* by chronically exposing KGN cells to insulin at either post-prandial

(10ng/ml) or hyper-insulinemic (100ng/ml) doses. The HI dose downregulated expression of insulin receptor mRNA by more than 50% reproducing insulin resistance, whereas there was no significant effect noted at post-prandial treatment levels. To our surprise, culture of cells in an extremely low and deficient VD environment caused an even further attenuation of insulin receptor expression in the presence of both doses of insulin. This effect only occurred in the presence of insulin, as 1,25-$(OH)_2$-D3 alone had no effect on insulin receptor expression. Systemic VD deficiency is clearly linked to a reduction in insulin sensitivity (independent of BMI) as others have shown (Muscogiuri *et al*, 2017), and this insulin receptor reduction may be a contributory mechanism. Interestingly, in the same cells a combination of insulin and 1,25-$(OH)_2$-D3 had no effect on aromatase, unlike in the forskolin experiments, again indicating that this was a cAMP-driven process, possibly linked to overlap between VDRE and CRE on PII as described previously.

It is well established that follicle progression in the normal human ovary requires down-regulation of AMH expression to permit FSH-driven activity (Pellatt *et al*, 2010). The IHC analysis of mouse ovaries also revealed high AMH expression in SAF which was substantially reduced in pre-ovulatory follicles. This is thought to normally occur via LH down-regulating AMH expression (Pierre *et al*, 2013). Using forskolin instead of LH to achieve down-regulation, we demonstrated that this reduction in AMH was potentiated by 1,25-$(OH)_2$-D3 [0.02nM] with a further reduction occurring with higher doses of 1,25-$(OH)_2$-D3 [20nM]. Interestingly treatment with 1,25-$(OH)_2$-D3 alone had no significant effect on AMH mRNA levels, indicating again that this only occurs when the cAMP pathway is activated.

In hen GC from 3-5mm and 6-8mm follicles, vitamin D3 was shown to substantially decrease AMH mRNA expression, with a more robust effect seen in the LAF (Wojtusik & Johnson, 2012).

In addition, Mehri *et al* showed that in AF (<14mm) from women with insufficient/deficient follicular fluid levels of 25-OH-D3, there was significantly higher AMHR-II mRNA level compared to those with normal VD levels (Merhi *et al*, 2014). In the same study, 25-OH-D3 was shown to decrease AMH-mediated pSMAD 1/5/8 nuclear localisation in cumulus GC, indicative of reduced AMH signalling. Hence at a local ovarian level, our data and other studies, clearly showed that VD is involved in AMH regulation and expression, which could impact on follicle progression particularly in conditions such as PCOS. At a systemic level however, evidence for correlations between serum vitamin D and serum AMH levels are contradictory and dependent on a woman's ovulatory status (Moridi *et al*, 2020).

To summarise, we have shown that Vitamin D clearly has a role to play in the theca and granulosa cell function and hence growth of ovarian follicles, as shown by the increased expression of VDR in follicles of increasing size. In addition, we have shown that Vitamin D may promote follicle progression by downregulating the expression of AMH, thereby reducing AMH's well-documented inhibitory effect on follicle growth (Pellatt *et al*, 2010). Vitamin D is known to affect steroidogenesis, and we have demonstrated that levels of 1,25-$(OH)_2$-D3 equivalent to hypovitaminosis, inhibited thecal production of androstenedione. In addition, extremely low levels of vitamin D had an attenuating effect on cAMP-driven aromatase expression, which translated to decreased E2 production. Encouragingly this reduction in E2 is reversed as levels of 1,25-$(OH)_2$-D3 increased; apart from in the presence of LH in luteinised GC, which could have consequences for regular ovulation in women with severe Vitamin D deficiency. For the first time we have demonstrated that deficient levels of 1,25-$(OH)_2$-D3 also down-regulated insulin receptor expression, potentially reducing insulin sensitivity (fig 7b). This could have serious implications for women with hyperinsulinemia and insulin resistance, typically seen in PCOS; indicating that insulin resistant women should try and maintain

sufficient levels of systemic vitamin D for regular ovarian function. The detailed mechanism by which vitamin D regulates the insulin signalling pathway in ovarian cells remains to be determined. The ability of the ovary to make local bioactive 1,25-$(OH)_2$-D3, was demonstrated by the expression of *CYP27B1.* This together with the upregulation of VDR expression in all ovarian cellular compartments, could potentially counteract the effect of systemic VD deficiency and protect the local ovarian environment (figs 7a & 7b). To conclude a severely deficient VD environment (<2nM or <1ng/ml) could contribute to impaired ovarian cell function and hence potentially affect folliculogenesis/ovulation, but levels associated with mild deficiency may have less impact, apart from in the presence of hyperinsulinemia and insulin resistance.

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681

682 **Data Availability:** *The data underlying this article are available in the article and in its online*
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694 ***Statement regarding use of archived tissue***: Research and the Human Tissue Act 2004 -
695 Consent IS REQUIRED to use and store tissues for research; UNLESS: The relevant material is
696 classed as an existing holding i.e., held prior to 1st September 2006 and/or the relevant
697 material is imported.

698

699 **Authors Role:**

700 Henrietta Brain: Data curation, Analysis, Methodology - all experiments from human ovaries
701 i.e. GC and theca cells cultures, RIA etc

702 Christiana Georgiou: Data curation, Analysis, Methodology - transfection experiments

703 Helen D. Mason: Conceptualization, Analysis, Writing – review & editing.

Suman Rice: Conceptualization, Data curation, Methodology, Analysis, Writing manuscript, review & editing.

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We have no conflict of interest to declare

Figure Legends:

Fig 1: Immunohistochemistry images of a serially sectioned mouse ovary.

1a: Negative control (no VDR primary antibody) of the sectioned mouse ovary with a variety of small antral follicles (SAF) and large antral follicles LAF). **1b:** Negative control (no AMH primary antibody) of the sectioned mouse ovary **1c:** VDR expression in the corresponding adjacent section of the same mouse ovary showing extensive expression of VDR protein throughout cortex, stroma and cellular compartments of the follicle i.e. both GC (arrowhead) and theca (arrows) of SAF and LAF. Intensity of staining in theca appears to be greater in the LAF compared to SAF *(inset pictures enlarged).* **1d:** AMH expression in the corresponding adjacent section of mouse ovary, showing strong expression in GC of SAF (white arrowhead) with minimal expression in GC of LAF (black arrowhead). There is no expression in theca cells (black arrows) or in cortex/stroma.

Fig 2: Expression of VDR and *CYP27B1* in human theca, cortex and stromal cells.

2a: mRNA expression of VDR quantified by qPCR, in human theca taken from human ovarian follicles of different sizes. VDR mRNA is significantly upregulated in theca as follicles increase in size, with a significant difference in follicles >10mm compared to 5-6mm. Intermediate follicles (7-8mm) showed a wider range of VDR expression with an upward trend. *(Unpaired t-test, two-tailed*p=0.0128; 5-6mm (n=4); 7-8mm (n=3); >10mm (n=4)).* **2b:** VDR mRNA expression was quantified by qPCR in human cortex (grey bar ●, *n=6*) and stroma (white bar ■, *n=5*). Whilst VDR was present the variability between samples, meant there was no significant difference in expression *(Unpaired t-test, two-tailed p=0.61)*. **2c:** VDR protein expression levels were quantified by western blotting, in human ovarian cortex (*n=5; grey bar ■)* and stroma (*n=4; white bar* ●), showed the same pattern of expression as mRNA with no

significant difference in expression between the two compartments. *(Unpaired t-test p=0.31. Densitometry values on y-axis multiplied by 1000 for scaling purposes)*. **2d:** Expression of *CYP27B1* mRNA was quantified by qPCR, in human ovarian stroma samples *(n=8).* Results showed that *CYP27B1,* which encodes for 1-α-hydroxylase (the enzyme that makes active $1,25(OH)_2D_3$) is present in the ovarian stroma.

Fig 3: Steroid assay measurements from theca and granulosa cells dissected from follicles and granulosa-luteal cells (GLCs) cultured *in vitro*.

3a: Effect of $1,25(OH)_2D_3$ on androstenedione production from theca taken from small (<10mm, white bars, *n=4 subjects, total follicles=15*) and large follicles (15-22mm, grey bars, *n=5 subjects, total follicles=5*) from normal ovaries. $1,25(OH)_2D_3$ suppressed androstenedione production significantly but only in theca from large follicles, with no effect in those from SAF. Results were expressed as a percentage change from control, where the control was taken as 100%. *(Two-way ANOVA p=0.05, source of variation follicle size **p=0.002, treatment*p=0.048; Tukeys post-hoc multiple comparison *p<0.05).* **3b**: $1,25(OH)_2D_3$ at all doses had no effect on E_2 production from granulosa cells taken from small antral follicles (<10mm, white bars; *n=3 subjects, total follicles=11*) and large antral follicles (>10mm, grey bars; *n=6 subjects, total follicles=8*) from normal ovaries. Results showed no effect of $1,25(OH)_2D_3$ (vd 0.2-20nM) on E_2 production. The results are expressed as mean percentage change from control where the control is taken as 100%. **3c:** $1,25(OH)_2D_3$ at 2 (spotted bar) & 20nM (hashed bar) had no effect on E_2 production from granulosa cells in the absence (white bars)/presence (dark grey bars) of FSH (5ng/ml), which as expected significantly simulated E2 production. The results are expressed as mean percentage change from control where the control is taken as 100%. *(n=4 subjects; total follicles=49. ANOVA ****p<0.0001;*

Post-hock Sidak's multiple comparison test, Alphabetical annotations are used to denote differences in statistical significance b=p<0.05). **3d:** Effect of 1,25$(OH)_2D_3$ (at 2 & 20nM) on E_2 production from granulosa-lutein cells in the presence of LH (10ng/ml). LH significantly stimulated E2 production, which was significantly attenuated in the presence of VD at both doses *(n=4; ANOVA **p<0.0025; Post-hock Sidak's multiple comparison test. Alphabetical annotations are used to denote differences in statistical significance b=p<0.005).* **3e:** A similar dose-dependent reduction in E2 production was seen in granulosa-lutein cells cultured in the presence of the VD analogue EB1089, though this did not reach statistical significance (*n=3; ANOVA p=0.27*).

Fig4: The effect of Vitamin D doses on aromatase expression and activity in KGNs.

4a: Aromatase mRNA expression levels were measured in KGNs cultured with forskolin (Fsk) to stimulate aromatase and different doses of VD (0.02-20nM). Testosterone ($5x10^{-7}$M) was used as an aromatase substrate. VD at the lowest two doses significantly down-regulated Fsk-stimulated aromatase expression, but as the doses increased this attenuation was lost. *(One-way ANOVA ***p=0.0002; Tukey's multiple comparisons test con vs fsk **p<0.005; con vs 2 **p<0.005; con vs 20 *p<0.05; fsk vs VD0.02 *p<0.05; fsk vs VD0.2*p<0.05 (n=6). Alphabetical annotations are used to denote differences in statistical significance).* **4b**: KGN cells were transfected with PII-specific-Luc reporter construct and treated as for the qPCR experiments. The luciferase assay showed that the effect of VD doses on aromatase expression was direct action on aromatase transcription, with very low doses down-regulating Fsk-stimulated PII activity. As VD concentrations increased this attenuation was lost. *(One-way ANOVA****p<0.0001; Tukey's multiple comparison- C/C+T vs Fsk***; Con vs 2***; Con vs*

*20***; Fsk vs 0.02**; Fsk vs 0.2*; 0.02 vs 2* (n=3). Alphabetical annotations are used to denote differences in statistical significance).*

Fig 5: The effect of Vitamin D (VD) on expression of other ovarian factors in KGNs.

5a: Expression of Insulin receptor (InsR) mRNA expression in KGNs was measured ± insulin and VD at various doses. Chronic exposure (48h) to high insulin (100ng/ml) significantly reduced InsR expression (spotted white bars), which was further attenuated in the presence of very low VD (light grey spotted bars). This combined suppression of InsR was seen even with VD at sufficient levels compared to basal (dark grey spotted bars). *(One column t-test p=0.0014 Ins100; p=0.001 VD20+Ins10; p=0.0003 VD0.02+Ins100; p=0.0522 VD20+Ins10; p=0.0318 VD20+Ins100. One-way ANOVA*p=0.048; (n=5-8). Values expressed as mean±SEM).*

5b: Expression of aromatase mRNA expression in KGNs was measured ± insulin and VD at various doses. Insulin had no effect on aromatase expression but reversed the attenuation of basal aromatase expression brought about by low dose VD exposure (light grey spotted bars). *One way ANOVA *p=0.0307; Unpaired t-test: VD0.02 vs VD0.02+Ins100 *p=0.0118 (2 tail); VD0.02 vs VD20 +p=0.0324 (1-tail) (n=5-8) Values expressed as mean ± SEM).* **5c:** Expression of AMH mRNA expression in KGNs cultured in the presence of VD (at 0.02 and 20nM) and forskolin (Fsk) was measured. Fsk reduced AMH expression below basal (white spotted bars) and this was further attenuated in the presence of 1,25$(OH)_2D_3$ with the strongest reduction seen in the presence of 20nM VD (dark grey spotted bars) *(ANOVA*p=0.02; Tukey's multiple comparison *p<0.05).*

Fig 6: Representative images of western blots from KGNs immunoprecipitated (IP) with either VDR/RXR antibodies.

6a: Representative image of western blot of protein from KGN cells cultured ± Fsk and VD at 0.02 and 20nM; immuno-precipitated with anti-VDR antibody (5µg) and immuno-blotted (IB) with anti-RXR antibody (1:1000) and anti-VDR antibody (1:1000) to detect RXR:VDR heterodimers. Equal quantities of protein were loaded onto each lane to allow for comparisons. **6b:** Representative image of western blot of protein from KGN cells cultured as above but IP with anti-RXR antibody (5µg) and IB with anti-VDR antibody (1:1000) to detect VDR:RXR heterodimers and **6c:** anti-RXR antibody (1:1000) to allow for quantification. Equal quantities of protein were loaded onto each lane to allow for comparisons *Lanes: 1=control, 2=Fsk, 3=Fsk+VD20,4=Fsk+VD0.02 Red band=anti-RXRα, Green band=anti-VDR*

Fig 7: Illustrations of proposed mechanism of VD actions and VDR expression in the ovary.

7a: VDR functions as both a monomer, homodimers and heterodimers with RXR and the amount of VD ligand available will cause an alteration in the ratio of these complexes within a cell. Depending on the proportion of each of these, the complexes will bind to VDRE on genes and attract either co-repressors/activator to enhance or suppress gene expression and activity. The theca and GC of the ovarian follicle can access active VD from the circulation in the ovarian medulla and theca. In addition, we propose that the stroma, via expression of 1-α-OHase (encoded by *CYP27B1*) can form active 1,25-(OH)2-D3 from circulating 25-(OH)-D3.

7b: VD has a role to play in follicles, as shown by increasing expression of VDR in theca of follicles as they increase in size. VD is capable of down-regulating AMH expression, which would aid follicle progression. However, very low levels of VD (equivalent to hypovitaminosis) affect steroidogenesis by inhibiting thecal production of androstenedione, cAMP-mediated

822 E2 production from GC and InsR expression. These attenuating effects are reversed by
823 increasing VD to replete levels.

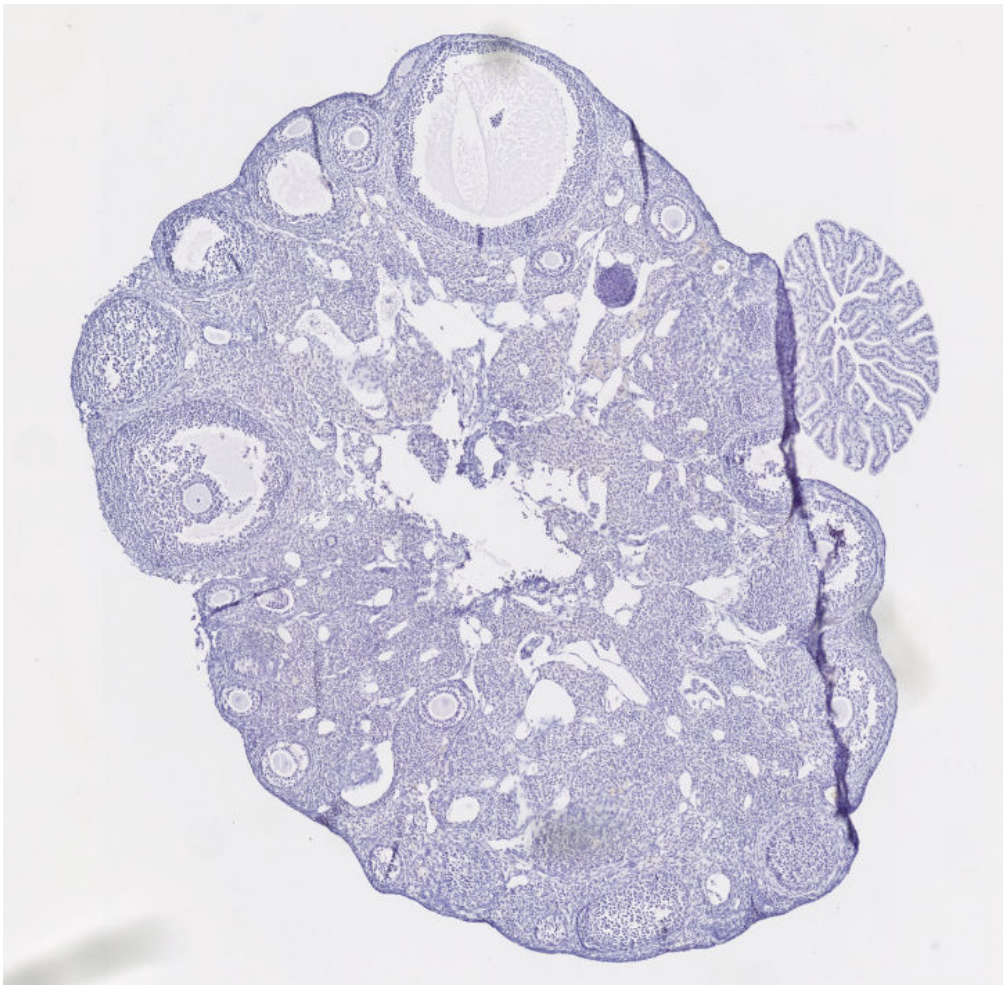

Fig1a

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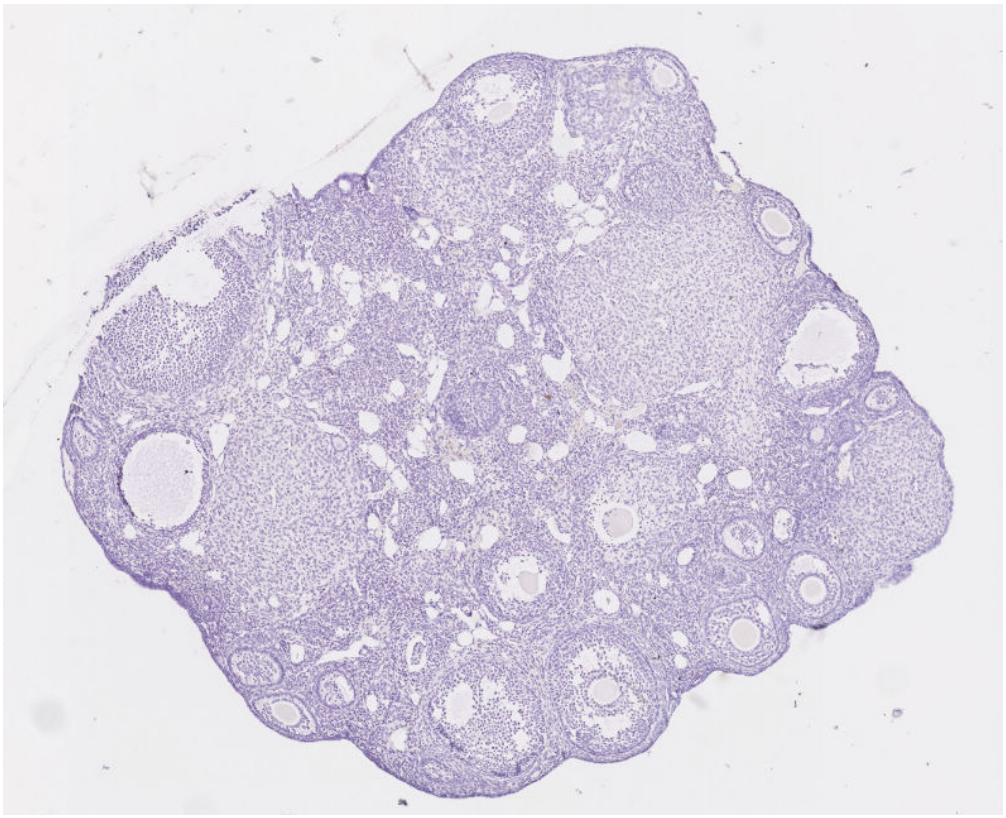

Fig1b

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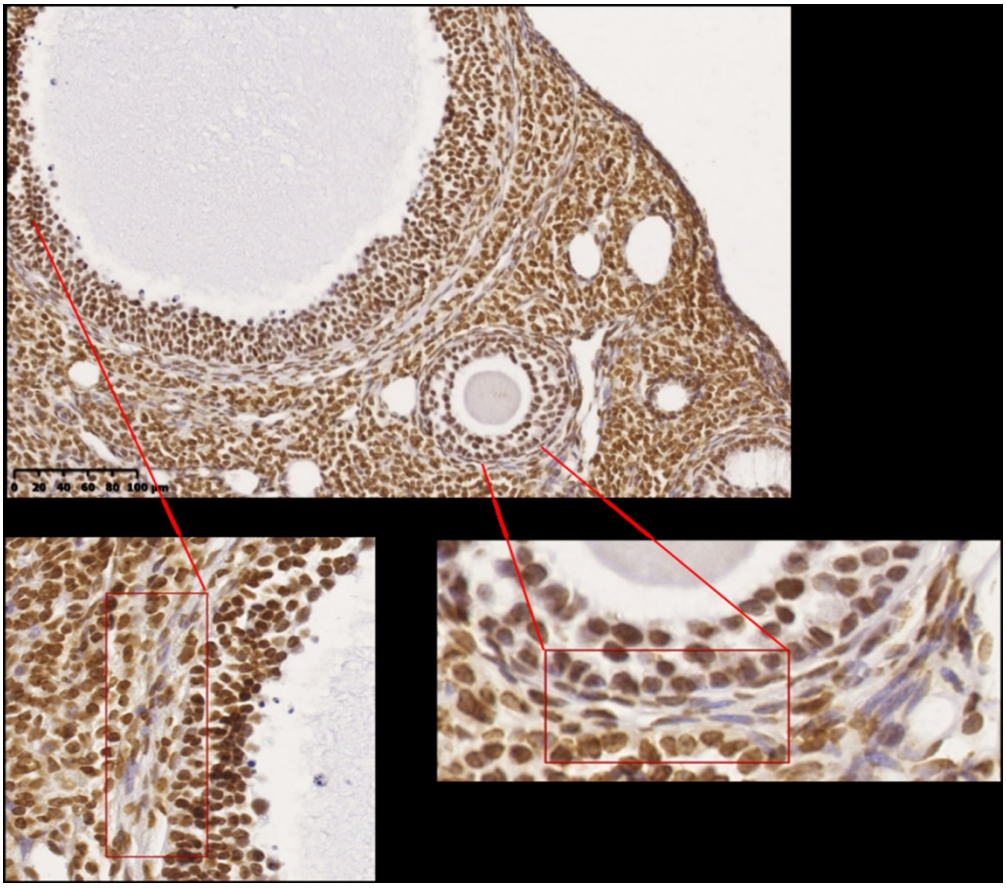

Fig 1c

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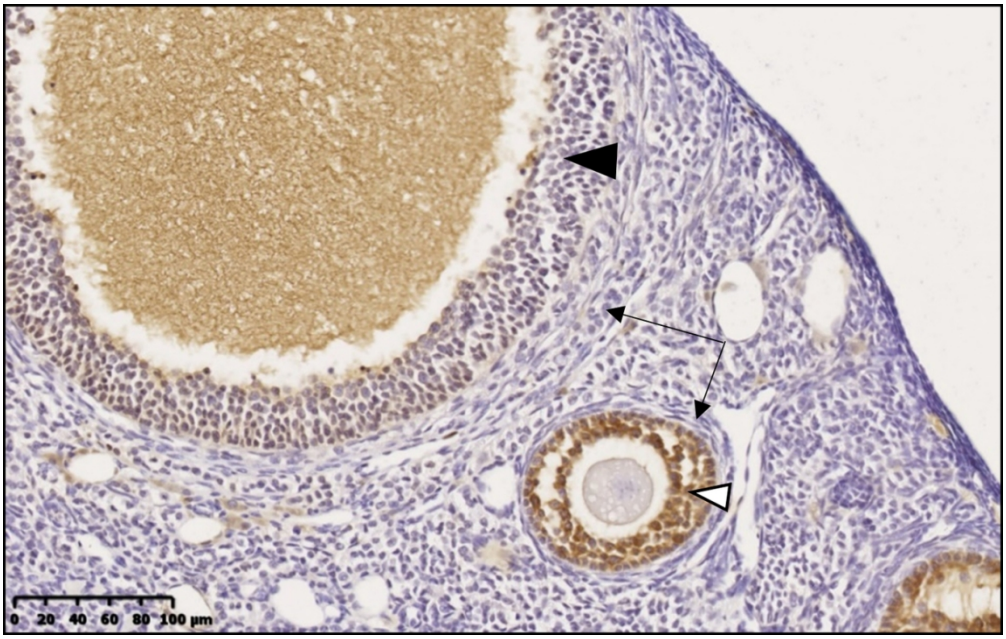

Fig 1d

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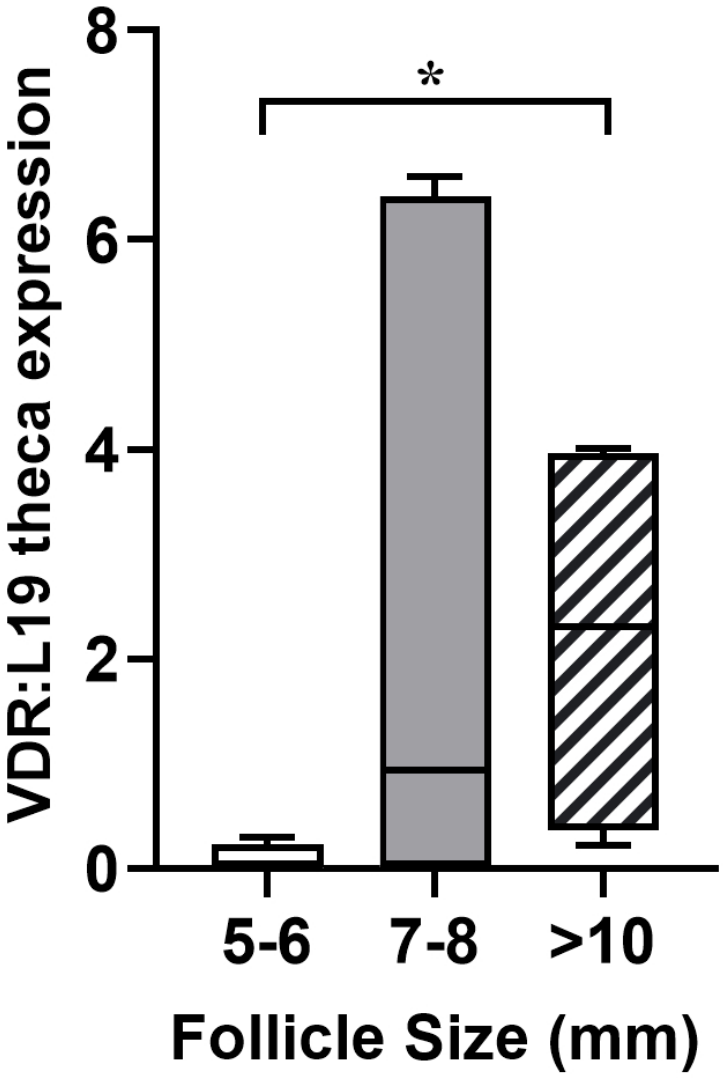

Fig 2a

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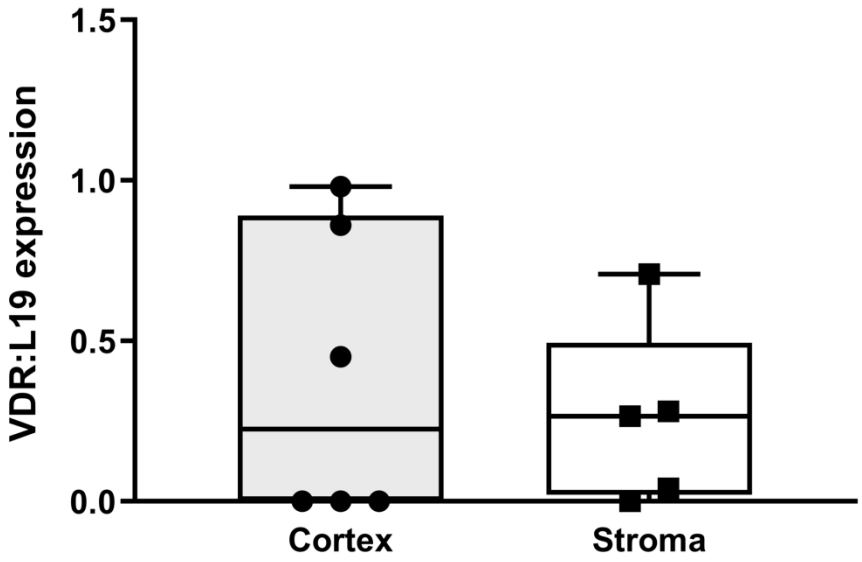

Fig 2b

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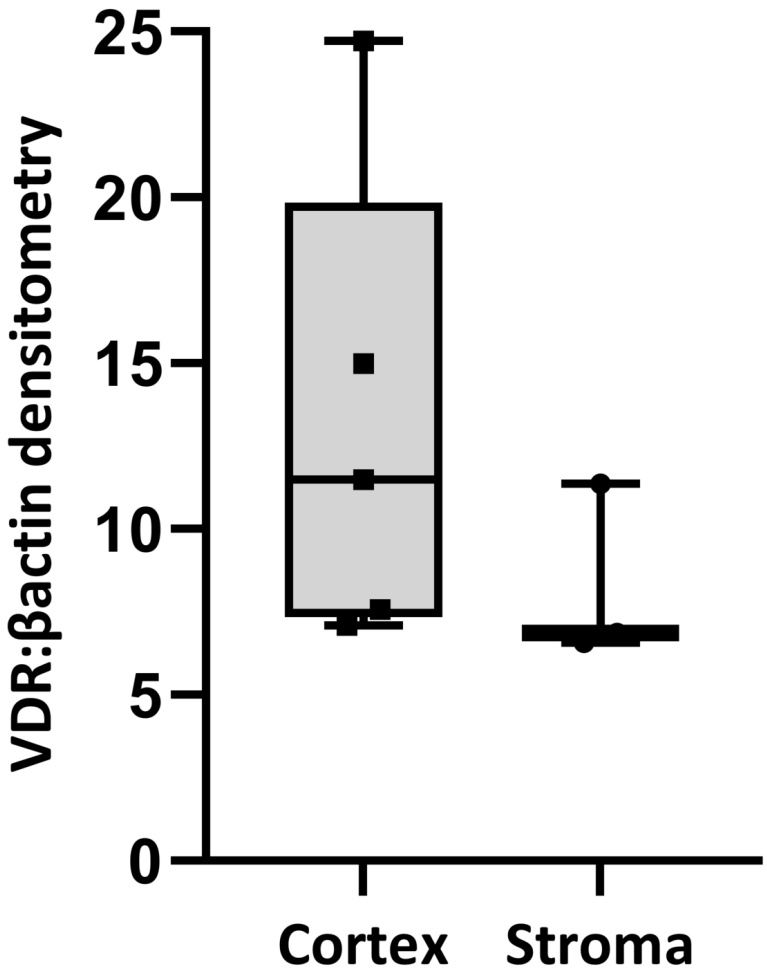

Fig 2c

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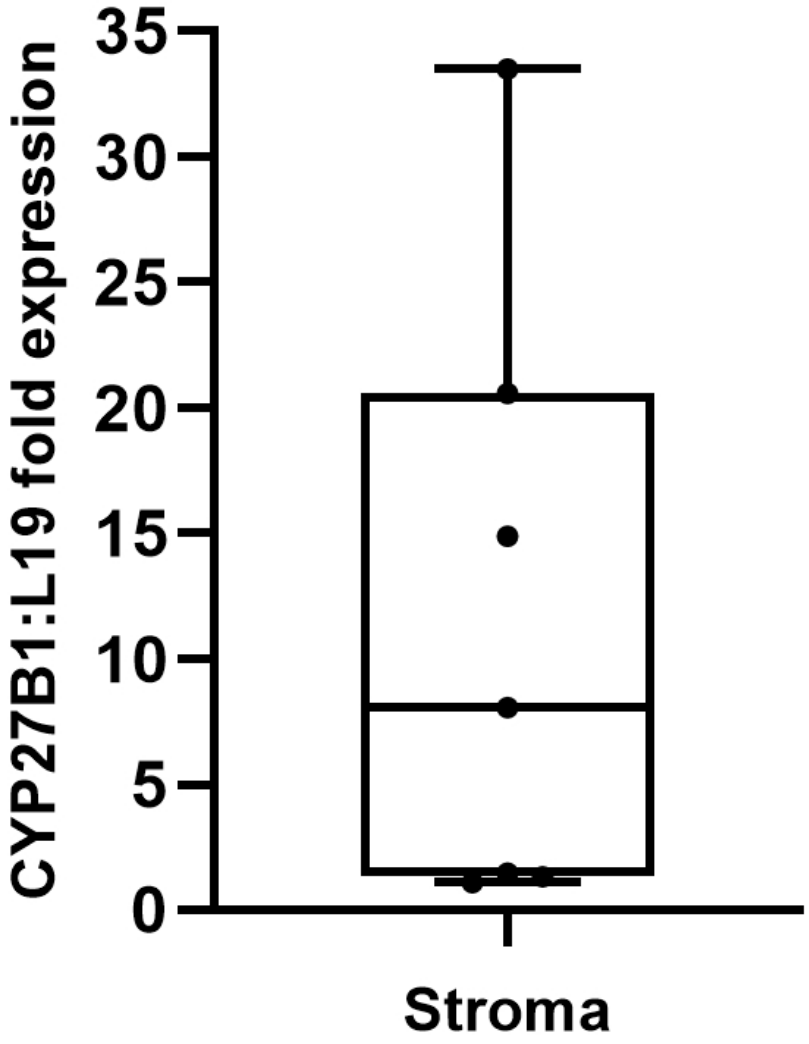

Fig 2d

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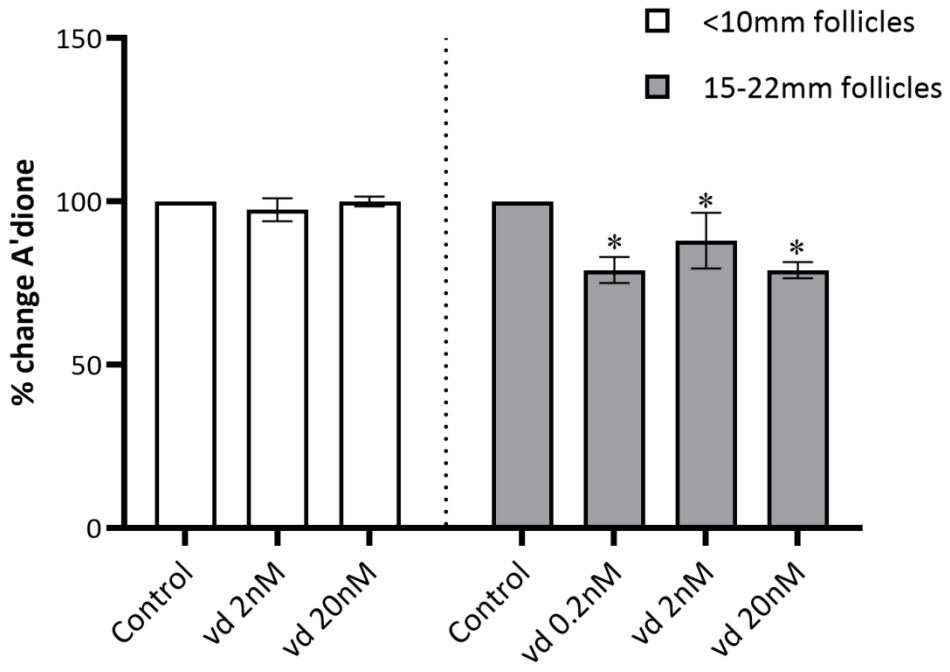

Fig 3a

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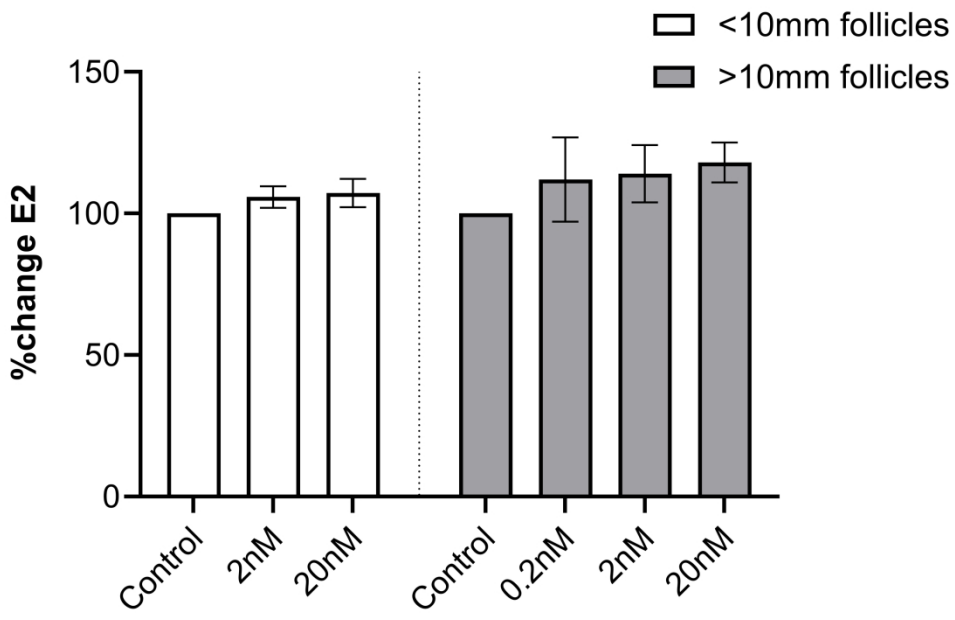

Fig 3b

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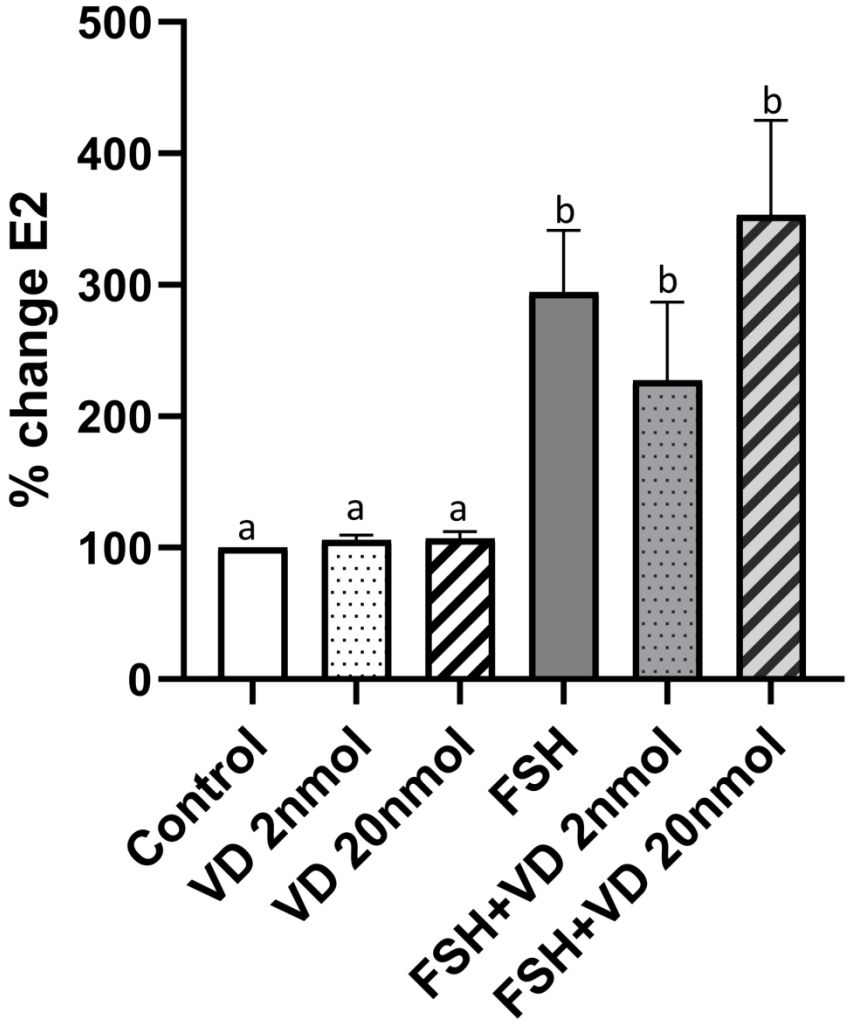

Fig 3c

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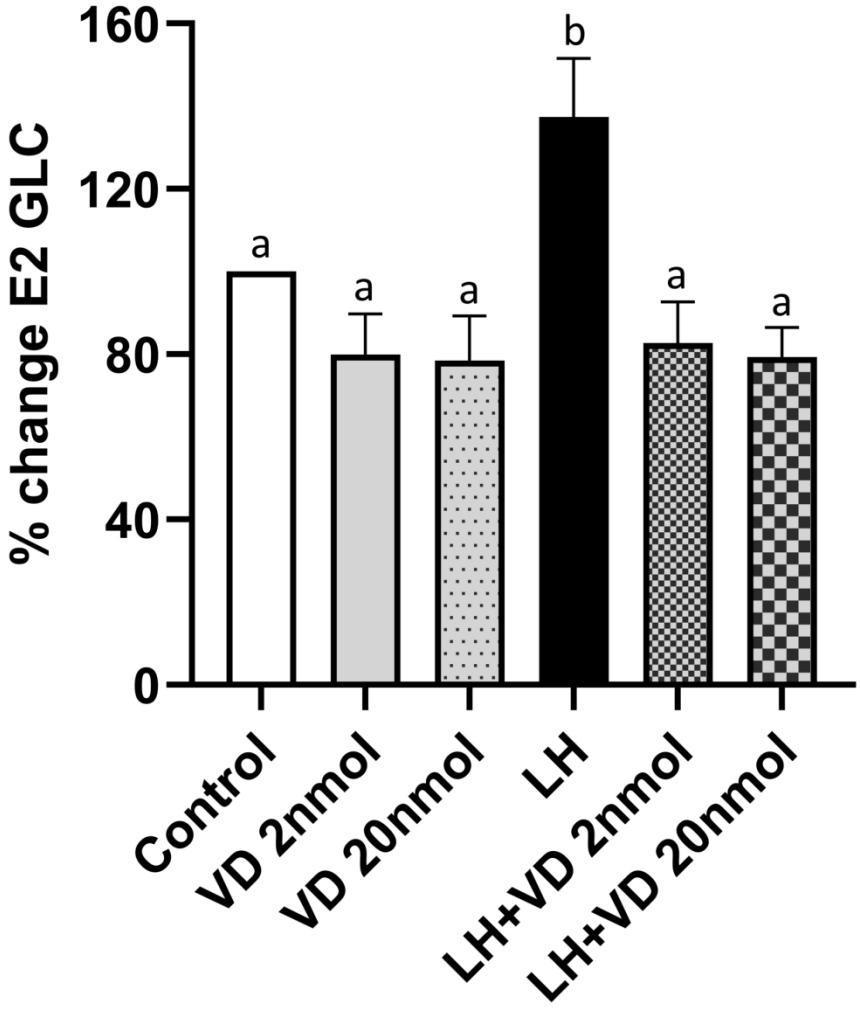

Fig 3d

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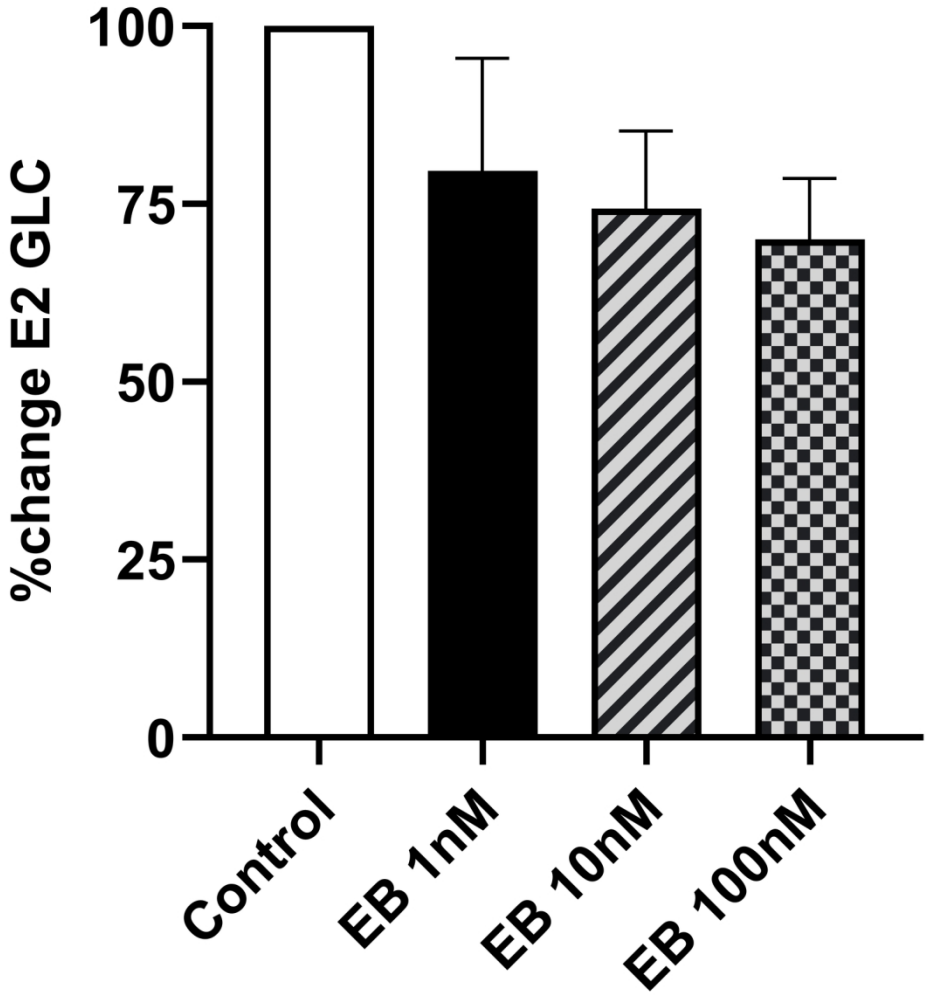

Fig 3e

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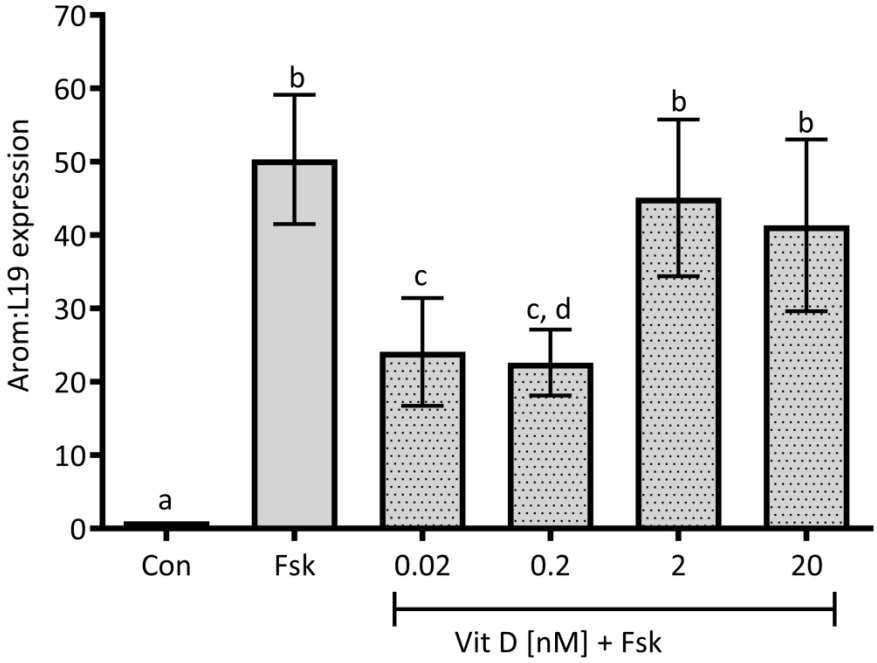

Fig 4a

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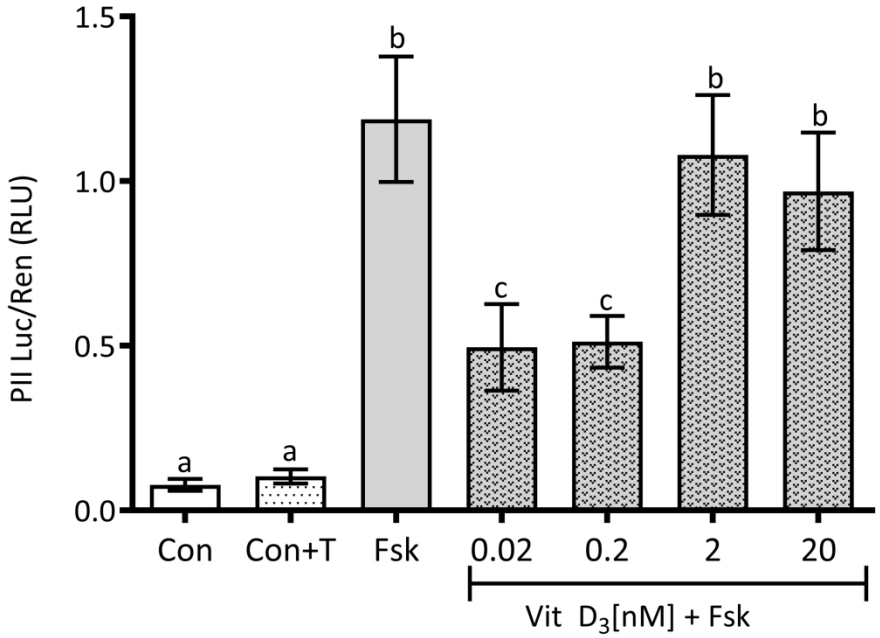

Fig 4b

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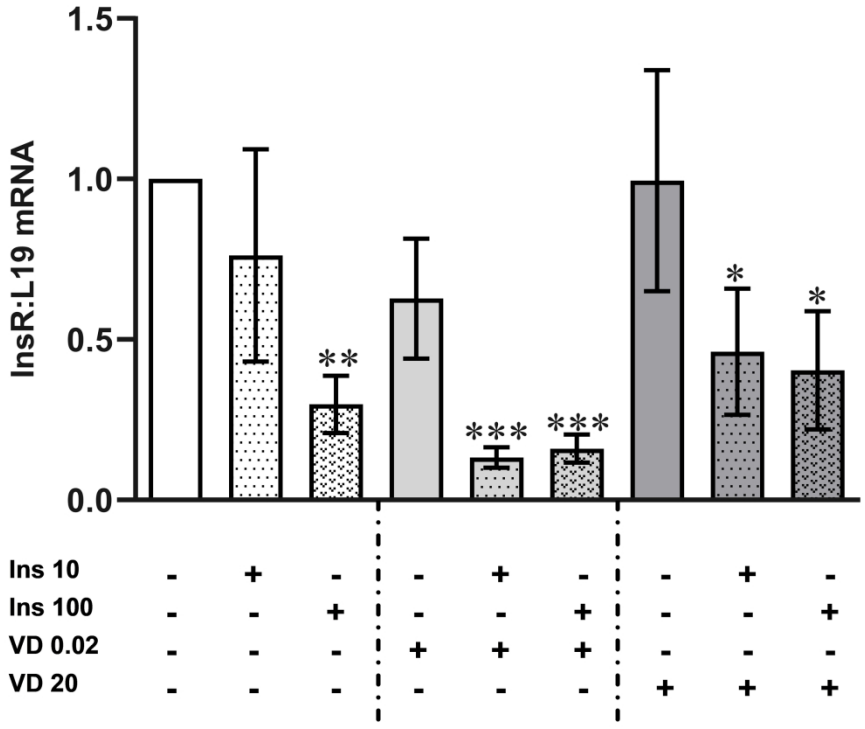

Fig 5a

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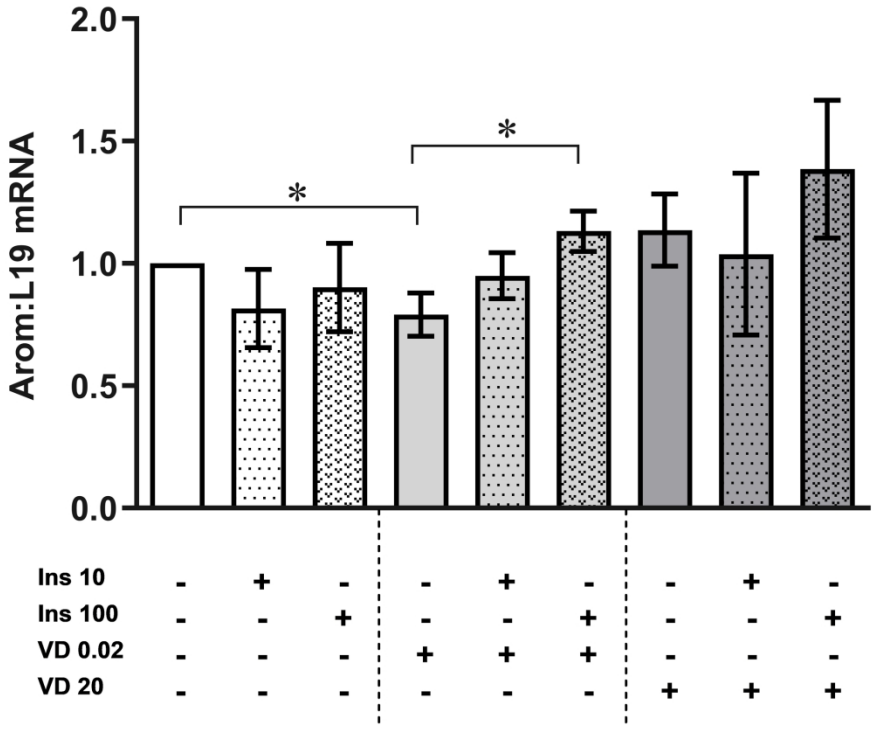

Fig 5b

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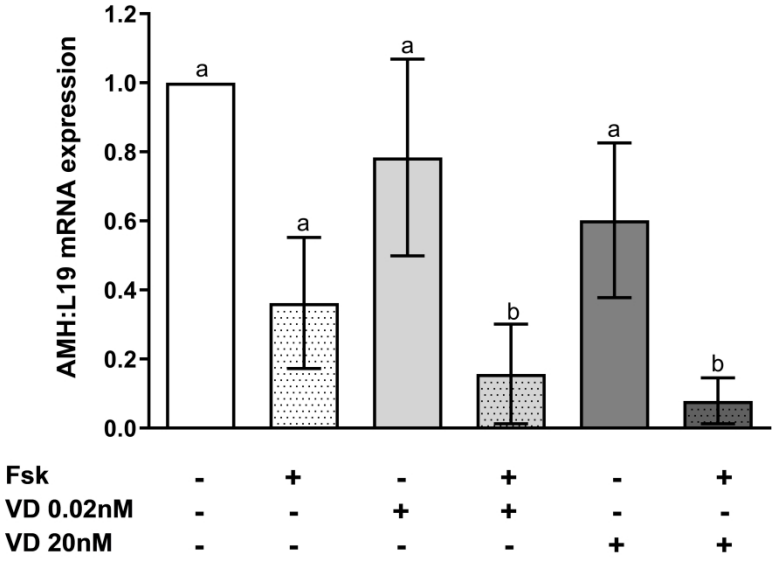

Fig 5c

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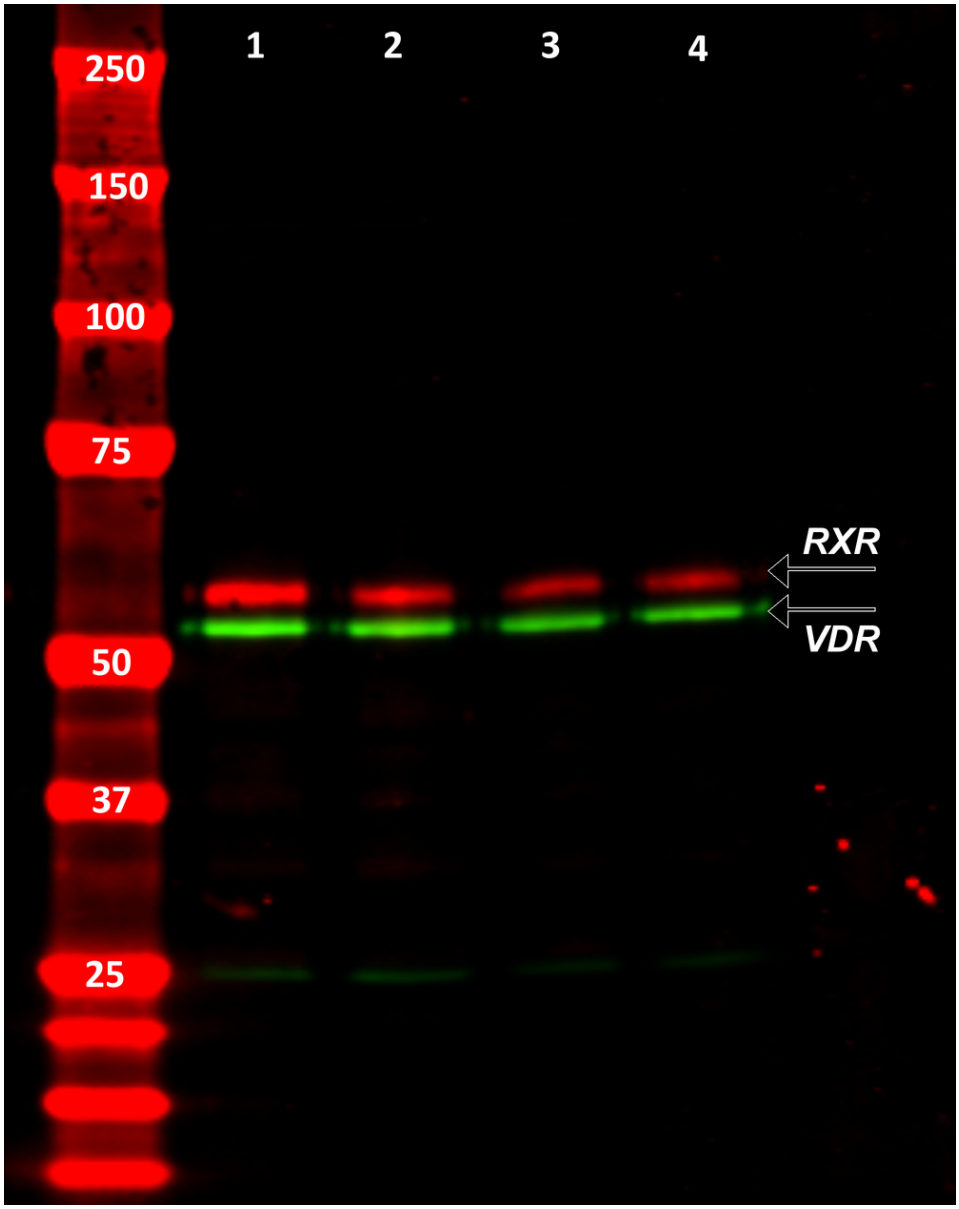

Fig 6a

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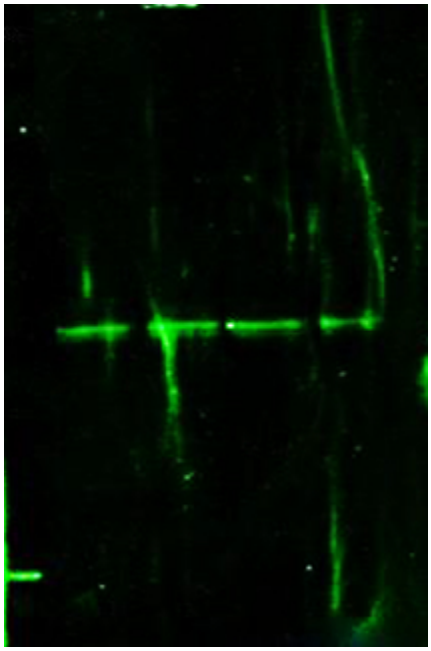

Fig 6b

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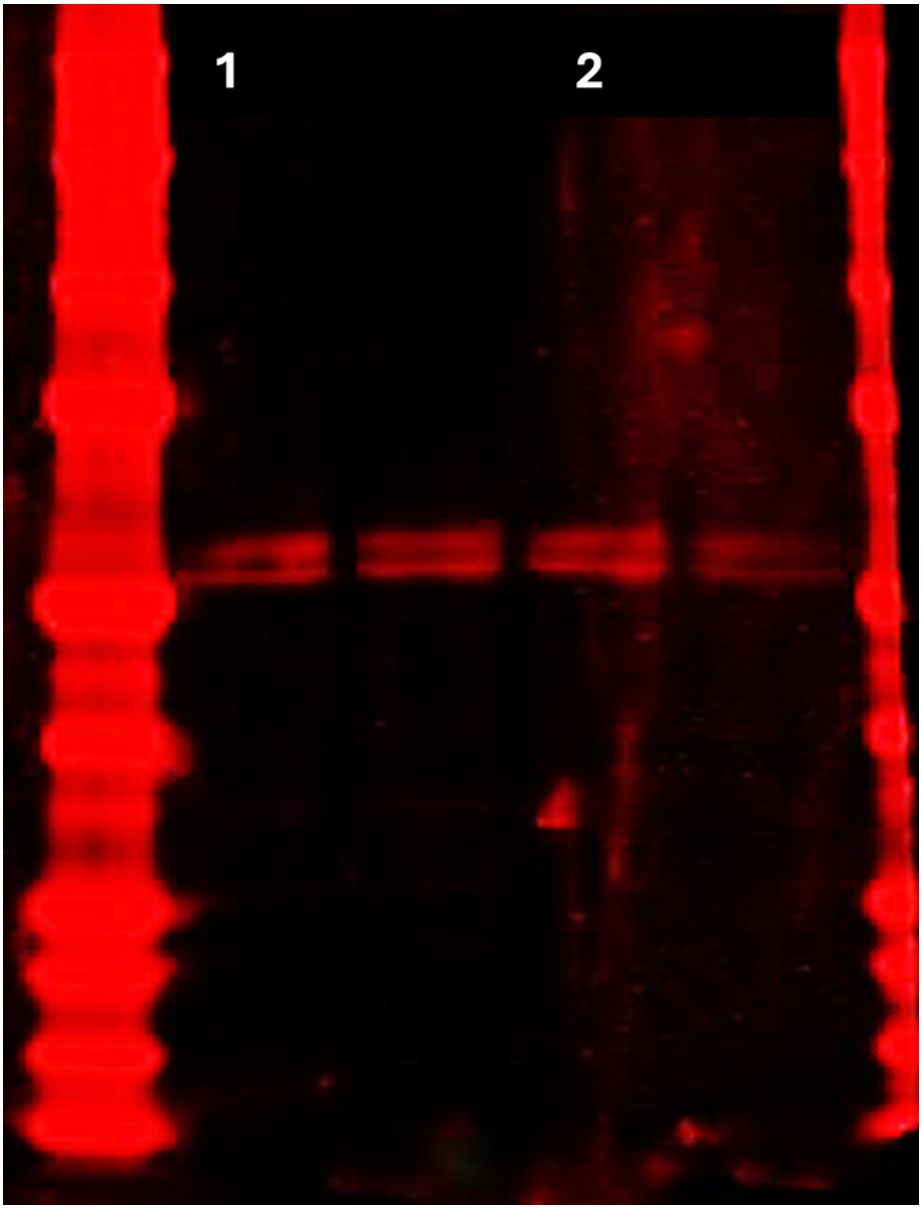

Fig 6c

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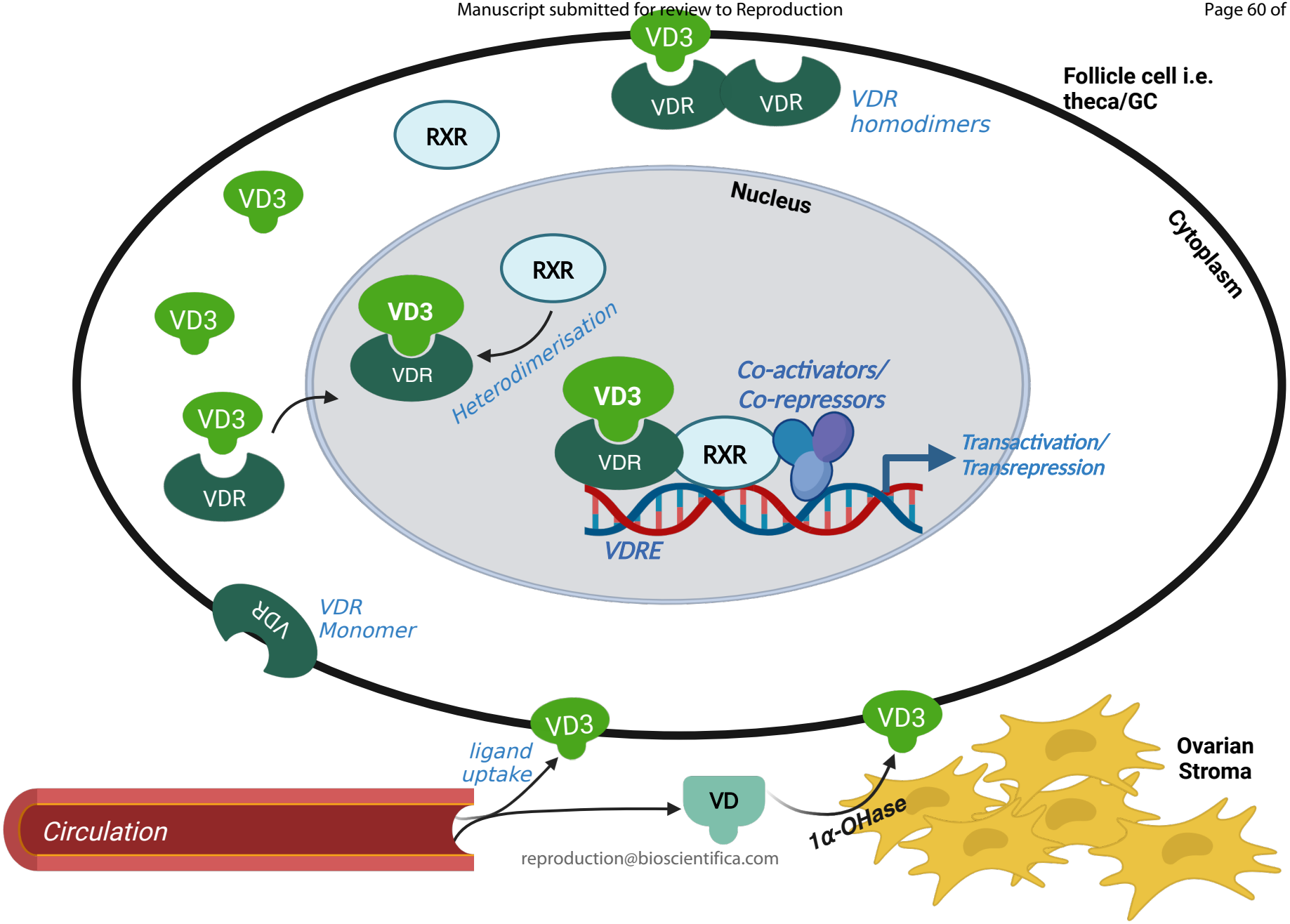
VD3
VDR
VDR
VDR homodimers
Follicle cell i.e. theca/GC
RXR
VD3
Nucleus
Cytoplasm
RXR
VD3
VD3
VDR
Heterodimerisation
VD3
VDR
Co-activators/ Co-repressors
VD3
VDR
RXR
Transactivation/ Transrepression
VDRE
VDR
VDR Monomer
VD3
ligand uptake
VD3
Ovarian Stroma
Circulation
VD
1α-OHase

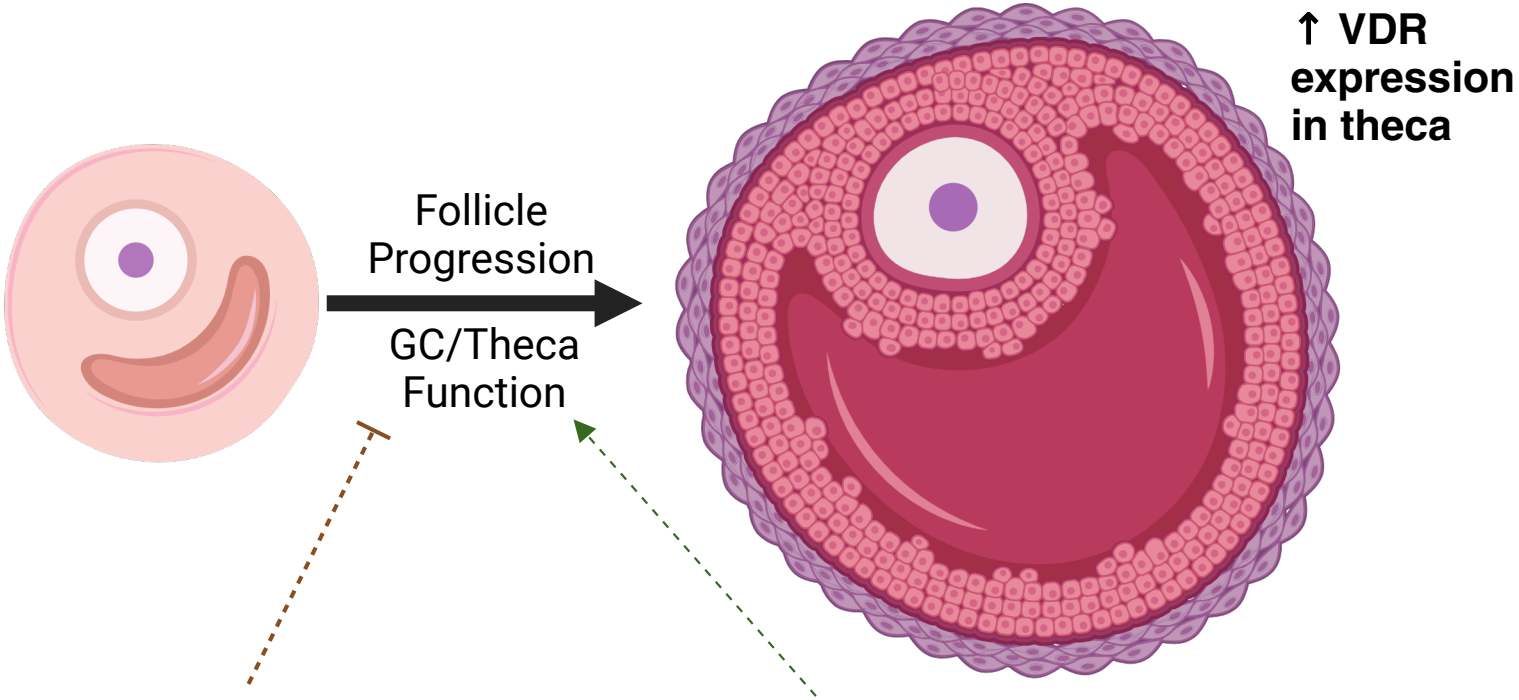
↑ VDR expression in theca
Follicle Progression
GC/Theca Function
↓↓ 1,25-(OH)2-D3
Thecal Androstenedione
cAMP-mediated E2
Insulin R expression
↑ 1,25-(OH)2-D3
via cAMP-mediated pathway
AMH expression

Table 1: Patient details, ovarian morphology and follicles from which GC/theca harvested

No.	Age	Morphology	Day of cycle/cycle length	No.Follicles used (size range mm)	Experiment used for
1	43	Normal	7/28	2 (13+15 pooled)	GC steroids
2	49	Normal	7/28	3 (5+7 pooled + 10)	VD +/- FSH
3	48	Normal	22/30	2 (7+17)	GC/theca steroids
4	38	Normal	14/28	1 (10)	GC steroids
5	48	Normal	2/28-30	2 (8+9)	GC steroids
6	51	Normal	20/28	3 (6+9+19)	GC/theca steroids
7	40	Normal	14/28	7 (5-9 pooled)	GC steroids
8	38	Normal	21/28-30	2 (11+12 pooled)	GC/theca steroids
9	32	ov PCO	10/27-30	17 (3-9 pooled)	VD +/- FSH
10	38	ov PCO	11/28	12 (4-14 pooled)	VD +/- FSH
11	33	anov PCO	0	18 (3-9 pooled)	VD +/- FSH
12	42	Normal	10/28	8 (<10 pooled + 15)	Theca steroids
13	51	Normal	15/28	1 (39)	Theca steroids
14	48	Normal	2/28-30	3 (8,9+12 pooled)	Theca steroids
15	35	Normal	14/28	1 (22)	Theca steroids

Table 2: List of antibodies used and conditions.

Antibody	Conc	Species raised	Source	Secondary used
Anti-VDR 53kDa	1:1000	Rat monoclonal	Abcam ab8756	Biotinylated monoclonal anti-rat (Vector) 0.1% [IHC] Goat anti-rat (Licor) 1:5000 conjugated 780nm IR dye [WB]
Anti-AMH	1:50	Mouse monoclonal	Abcam ab84952	Biotinylated monoclonal anti-mouse (Vector) 0.1%
Anti-RXR 51kDa	1:1000	Rabbit monoclonal	Abcam ab125001	Goat anti-rabbit (Licor) 1:5000 conjugated 680nm IR dye
Anti-βactin 45kDa	1:2000	Mouse monoclonal	Abcam ab125001	Goat anti-mouse (Licor) 1:5000 conjugated 680nm IR dye

Table 3: List of Primer used and conditions

Gene	**Forward sequence 5'-3'** **Reverse sequence 5'-3'**	**Annealing temp °C**	**Primer conc [nM]**	**Accession number**
CYP19A1 (Aromatase)	F – GACTCTAAATTGCCCCCTCTG R – CAGAGATCCAGACTCGCATG	60	100	NM_000103.4
VDR	F-GACTTTGACCGGAA R-CATCATGCCGATGTCCACACA	55	300	NM_001017535.2
AMH	F- GCATGTTGACACATCAGGC R- GAGTGGCCTTCTCAAAGAGC	60	100	NM_000479.5
L19	F – GCGGAAGGGTACAGCCAAT R - GCAGCCGGCGCAAA	60	100	NM_000981.4
CYP27B1	F-CACTGTCCCAAAGCTGGCTA R-GCTTTCTGGCCGAACTTTTC	60	300	NM_000785.4
Insulin Receptor	F-GGTGCAGCCGTGTGACTTAC R-GTCATCAACGGGCAGTTTG	60	300	NM_000208.4

Table 4: Densitometry values of immunoblotted VDR and RXR proteins from cells treated with Forskolin and VD doses and immunoprecipitated with anti-RXR antibody. Values are expressed as the ratio of VDR/µg protein or VDR:RXR/µg protein after adjusting for protein concentration and then normalized to control *(mean ± SEM; n=4; One-Way Anova, Multiple comparisons $^{b}p<0.005$; $^{c}p<0.05$)*.

	RXR-IP	
Treatment	**VDR/µg protein**	**VDR:RXR/µg protein**
Control	1.00 ± 0.0 *a*	1.00 ± 0.0 *a*
Forskolin	0.942 ± 0.14 *a*	1.14 ± 0.25 *a*
Forskolin+VD [20nM]	0.562 ± 0.08 *b*	0.81 ± 0.16 *ab*
Forskolin+VD [0.02nM]	0.838 ± 0.06 *a*	1.62 ± 0.26 *ac*

Table 5: Densitometry values of immunoblotted VDR and RXR proteins from cells treated with Forskolin and VD doses and immunoprecipitated with anti-VDR antibody. Values are expressed as the ratio of RXR/µg protein or RXR:VDR/µg protein after adjusting for protein concentration and then normalized to control *(mean ± SEM; n=6; One-Way Anova, Multiple comparisons)*.

	VDR-IP	
Treatment	**RXR/µg protein**	**VDR:RXR/µg protein**
Control	1.00 ± 0.0	1.00 ± 0.0
Forskolin	0.776 ± 0.19	0.95 ± 0.09
Forskolin+VD [20nM]	0.759 ± 0.18	0.94 ± 0.18
Forskolin+VD [0.02nM]	0.995 ± 0.13	0.78 ± 0.15

Fig S1

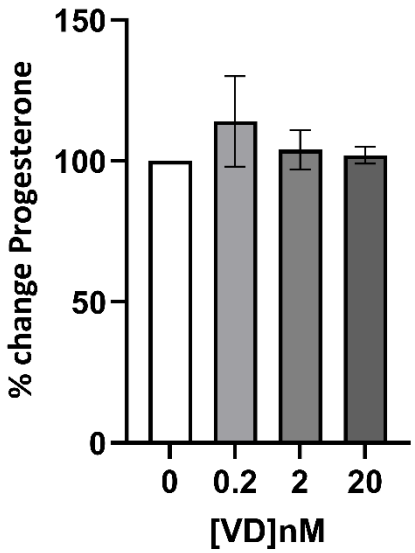

Fig S2

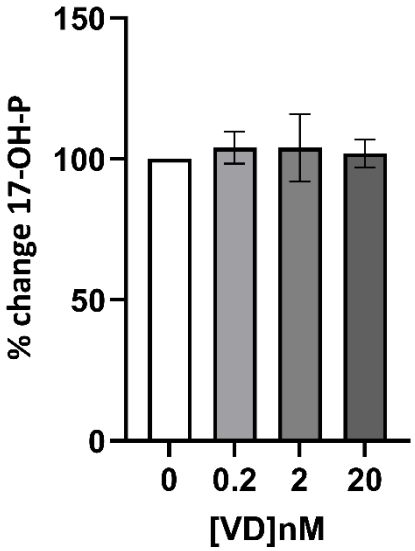

Fig S3

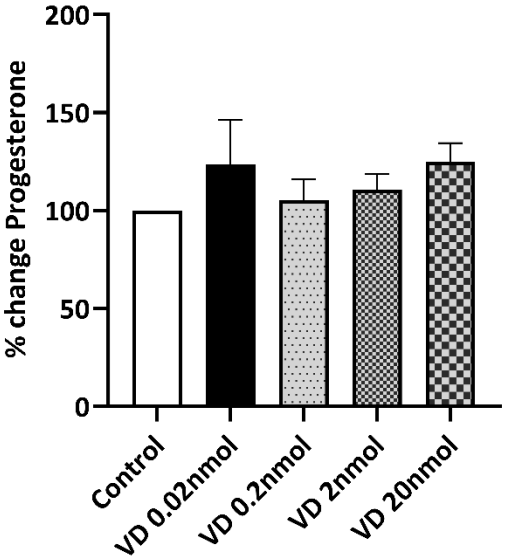

Fig S4