# Attenuation of VDR hinders pre-implantation development of porcine embryos *in vitro*

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# Abstract

The active form of Vitamin D ($1\alpha,25(OH)_2D_3$; VD3) and the Vitamin D Receptor (VDR) are well known to regulate growth, development and survival in various cell types. However, to our knowledge, the role of VD3 and its receptor, VDR, has not been characterized during oocyte maturation and early embryo development. Therefore, this study aimed at evaluating the effect of VD3 supplementation and VDR attenuation during in vitro development of porcine embryos. Immunostained VDR protein was observed around the nuclear envelope of immature oocytes and in their surrounding cumulus cells (CCs). VDR mRNA expression in cumulus-oocyte complexes (COCs) was evaluated by qRT-PCR, and was not significantly altered before or after (46 hours) *in vitro* maturation (IVM), nor after VD3 supplementation (100 nM). Under our conditions, VD3 supplementation did not improve oocyte developmental potential *in vitro*. Meanwhile, on day 3-5 of embryonic development, VDR mRNA expression decreased by 42% ($p<0.01$) and the VDR protein was detected in both the cytoplasm and the nucleus. Whereas on day 7, the VDR was predominantly nuclear localized. VD3 supplementation (100 nM) during *in vitro* culture (IVC) upregulated VDR mRNA expression by 1.6 fold ($p<0.01$), but reduced blastocyst development by 15% ($p<0.05$) compared to controls. Targeted knockdown of the VDR by DsiRNA hindered blastocyst development (by 46%; $p<0.01$), and reduced the average number of cells per embryo (by 33%; $p<0.05$) and reduced expansion compared to controls. Taken together, these findings indicate that VD3 supplementation does not benefit oocyte competence or embryo development, but the VDR is important for regulation of pre-implantation embryo development in swine.

# Résumé

La forme active de la Vitamine D (1α,25$(OH)_2D_3$; VD3) et le récepteur de la vitamine D (VDR) sont bien connus pour réglementer la croissance, le développement et la survie dans diverses lignées cellulaires. Pourtant, à notre connaissance, les roles de la VD3 et son récepteur, VDR, n'ont pas encore été caractérisés durant la maturation des ovocytes et les stades initiales du développement embryonnaire. Ainsi, cette étude visait à évaluer l'effet de la supplémentation de VD3 et l'attenuation du VDR durant le développement *in vitro* des embryons porcins. Les protéines de VDR ont été observées, par immunofluorescence, autour de l'envelope nucléaire des ovocytes immatures et dans leurs cellules de cumulus (CCs). L'expression de l'ARNm VDR dans les complexes ovocytes-cumulus (COC) a été évaluée par qRT-PCR, mais n'as pas changé de manière significative avant ou après (46 heures) la maturation *in vitro* (MIV), ni après la supplémentation de VD3 durant cette période. Avec nos conditions, la supplémentation de VD3 n'améliore pas la compétence des ovocytes *in vitro*. Par contre, du troisième au cinquième jour de développement embryonnaire, l'expression de l'ARNm VDR a diminué de 42% ($p<0.01$) et les protéines de VDR étaient localisées dans le cytoplasme et le noyau. Tandis que le septième jour, le VDR était principalement localisé dans le noyau. La supplémentation de VD3 (100 nM) durant la culture *in vitro* (CIV) a surrégulée l'expression de l'ARNm VDR par 1.6 fois ($p<0.01$), mais a diminuée le développement au stade blastocyste par 15% ($p<0.05$), comparativement aux contrôles. Le knockdown ciblé du VDR par DsiARN a empêché le développement au stade blastocyste (par 46%; $p<0.01$), a réduit la moyenne de nombres de cellules par embryon (par 33%; $p<0.05$), et a reduit l'expansion des blastocystes, comparativement aux contrôles. Ensembles, ces résultats indiquent que la supplémentation de VD3 ne contribue pas de manière positif à la compétence des ovocytes ni le développement embryonnaire, mais le VDR est important pour le bon déroulement du développement pré-implantoire porcin.

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# Contribution of Authors

**Authors of the presented manuscript:**

Rosalba Lopez, Werner G. Glazner, Karina Gutierrez, Luke Currin, Mariana Priotto de Macedo, and Vilceu Bordignon.

Vilceu Bordignon designed the experiments, analyzed the data, reviewed the manuscript and supervised the primary author. Rosalba Lopez conducted all the experiments, analyzed the data and wrote the manuscript. Werner G. Glazner executed the intra-ooplasmic injections, analyzed the data and trained the primary author. Karina Gutierrez, Luke Currin, and Mariana Priotto de Macedo helped with weekly oocyte collections and media preparation.

# List of Abbreviations

| | |
|---|---|
| 3β-HSD | 3-beta–hydroxysteroid dehydrogenase |
| AF-2 | Transactivation function |
| AKT | Serine/threonine protein kinase; protein kinase B (PKB) |
| AMH | Anti-Müllerian hormone |
| AMOTl2 | Angiomotin-like protein 2 |
| AP | Alkaline phosphatase |
| BMF | Bone morphogenic factor |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| CC | Cumulus cell |
| CDK | Cycline-dependant kinase |
| CDKi | Cycline-dependant kinase inhibitor |
| cDNA | Complementary DNA |
| CDX2 | Claudal type homeobox 2 |
| ChIP | Chromatin Immunoprecipitation |
| cGMP | Cyclic guanosine monophosphate |
| c-Myc | Myc proto-oncogene |
| COC | Cumulus-oocyte complex |
| Cx43 | Connexin 43 |
| CYP11A1 | Cholesterol side chain cleavage enzyme; p450scc |
| DAG | Diacylglycerol |
| DAPI | 4,6-Diamidino-2-phenylindole |
| DKK-1 | Dickkopf WNT signaling pathway inhibitor 1 |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic acid |
| DsiRNA | Dicer-substrate small interference RNA |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |

| | |
|---|---|
| ER | Endoplasmic Reticulum |
| ERα/β | Estrogen receptor α/β |
| ERK | Extracellular-signal-regulated kinase |
| ESC | Embryonic stem cell |
| FBS | Fetal bovine serum |
| FF | Follicular fluid |
| Fgf23 | Fibroblast growth factor 23 |
| FoxO | Forkhead box O |
| FSH | Follicle stimulating hormone |
| FSHR | Follicle stimulating hormone receptor |
| GC | Granulosa cell |
| GDF-9 | Growth differentiation factor-9 |
| GPCR | G protein-coupled receptor |
| GV | Germinal vesicle |
| GVBD | Germinal vesicle breakdown |
| ICM | Inner cell mass |
| IVC | *In vitro culture* |
| IVF | *In vitro fertilization* |
| IVM | *In vitro maturation* |
| $IP_3$ | Inositol triphosphate |
| LH | Luteinizing hormone |
| MI/II | Meiosis I/II |
| MAPK | Mitogen activated protein kinase |
| mGC | Mural granulosa cell |
| miRNA | micro RNA |
| MPF | Maturation promoting factor |
| mRNA | Messenger RNA |
| mTOR | Mammalian target of Rapamycin |
| mTORC1 | Mammalian target of Rapamycin complex 1 |

| | |
|---|---|
| Oct 3/4 | Octomer-binding transcription factor 3 and 4 |
| p21 | Cyclin dependant kinase inhibitor 1A |
| p27 | Cyclin dependant kinase inhibitor 1B |
| p53 | Tumor suppressor protein 53 |
| PA | Parthenogenic activation |
| PAK1 | p21 protein-activated kinase 1 |
| PBS | Phosphate-buffered saline |
| PCOS | Polycystic Ovary Syndrome |
| PCNA | Proliferating cell nuclear antigen |
| PI3K | phosphatidylinositol 3-kinase |
| $PIP_2$ | Phosphatidylinositol (4,5)-biphosphate |
| PKA | Protein Kinase A |
| PKB (AKT) | Protein Kinase B |
| PKC | Protein Kinase C |
| PLC ζ | Phospholipase C zeta |
| PTEN | Phosphatase And Tensin Homolog |
| PUMA | p53 upregulated modulator of apoptosis |
| PVA | Polyvinyl alcohol |
| PZM-3 | $Ca^{2+}$-free porcine zygote medium |
| qPCR | Quantitative Polymerase Chain Reaction |
| RAC1 | Ras-related C3 botulinum toxin substrate 1 |
| rpm | Revolutions per minute |
| RNA | Ribonucleic acid |
| RXR | Retinoid X receptor |
| siRNA | Small interfering RNA |
| SMAD | Contraction of Caenorhabditis elegans Sma and the Drosophila Mothers against decapentaplegic (Mad) gene names |
| SOC | Store-operated calcium |
| StAR | Steroidogenic acute regulatory protein |
| sVD | serum vitamin D |

| | |
|---|---|
| Tcf3 (E2A) | T-cell transcription factor 3; |
| TCM199 | Tissue Culture Media 199 |
| TE | Trophectoderm |
| TGF-β | Transforming growth factor beta |
| VD | Vitamin D |
| VD2 | Vitamin D2; ergocalciferol |
| VD3 | Vitamin D3; 1,25 dihydroxyvitamin d3; calcitriol |
| VDBP | Vitamin D binding protein |
| VDR | Vitamin D receptor |
| VDRE | Vitamin D response element |
| VDRx | Extended Vitamin D receptor |
| Wnt | Wingless-type MMTV integration site family |
| YAP | Yes associated protein |
| ZP | Zona pellucida |
| ZP3 | Zona pellucida sperm-binding protein 3 |

# CHAPTER 1: GENERAL INTRODUCTION

Vitamin D (VD) deficiency has a global impact on all age groups, and plays an important role in reproduction due to its ability to regulate various biological processes [1]. Classically, it was thought that VD signals solely through the vitamin D receptor (VDR), however, there is now evidence that vitamin D3 (the active form of VD; herein abbreviated VD3) signals through several levels of complexity, both genomically [2-5] and non-genomically [6-8]. VD/VDR directly and indirectly regulate the expression of many genes, and furthermore, VD3 and VDR individually are involved in crosstalk between multiple signalling pathways, producing a plethora of cellular responses [9]. It has been demonstrated that VD regulates cell cycle progression (e.g., through FoxOs [10, 11], c-Myc [12-14], p21 [15], and PTEN/AKT/mTOR [16]), hinders cell proliferation (e.g., by inhibiting Wnt signalling [17-21]), and promotes cell differentiation (e.g., by amplifying MAPK/ERK signalling [20, 22, 23]) in various cell types. However, regarding potential roles in the reproductive system, it remains controversial whether VD deficiency is associated with pregnancy outcomes [1, 24-30].

In 2011, 41% of Canadians of reproductive age were VD deficient, and 1 in 3 Canadians were taking VD supplements [31]. With this in mind, the focus of this research was to gain additional insights into the role of VD in the reproductive system, in particular, how VD affects oocyte maturation and early embryo development. Using a well defined *in vitro* porcine model, the expression profile of VDR was characterized, at both the mRNA and protein levels, and confirmed that VDR is expressed in cumulus cells, oocytes and in early developing embryos. Oocytes and embryos were then supplemented with relevant doses of VD3 to investigate whether expression of the VDR was altered and if supplementation during *in vitro* maturation or culture (IVM or IVC respectively) affected development of embryos to the blastocyst stage. Lastly, the expression of the VDR was knocked down to assess its role in the early stages of porcine embryo development.

This thesis presents background information on oocyte maturation, embryo development, VD and VDR signalling (as potentially relevant to reproduction), followed by a manuscript describing experimental design, analyzed results and discussion.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 From follicle to early embryo development

### *2.1.1. Folliculogenesis*

During female fetal development, a peak of Follicle Stimulating Hormone (FSH) stimulus at 17 weeks, coincides with the peak of primordial follicle development at 20 weeks [32]. The primordial follicles contain very small oocytes, surrounded by a single layer of flattened granulosa cells (GCs), that keep them arrested at prophase I of meiosis [33-35]. It is only at the onset of puberty, that the GCs finally express sufficient FSH receptors (FSHRs) to receive the adequate gonadotrophin stimuli required to initiate folliculogenesis or the transition from primary, secondary, tertiary and lastly to dominant follicles [35-37]. At the primary follicle stage, the oocyte is protected by a layer of glycoproteins (known as the zona pellucida; ZP) which is surrounded by a single layer of cuboidal GCs, it takes 70 days of low FSH stimulation for the follicle to develop into a 2 mm secondary (or pre-antral) follicle [33, 38-40]. This next stage of development is characterized by the slow proliferation of 3-6 layers of GCs, a “prospective” theca, and a slight increase in the size of the oocyte despite low responsiveness to gonadotrophins [33, 38]. In addition, the oocyte now begins to control its own microenvironment through autocrine and paracrine signalling from granulosa and theca cells via transforming growth factor beta (TGF-β) cytokines [41-43]. As the theca cells receive cues from their associated blood vessels, they differentiate into the theca interna and externa cells which express some receptors for Luteinizing Hormone (LH) and produce androgens [37, 44]. The GCs on the other hand, will increase expression of FSHRs, allowing these tertiary follicles to respond effectively to gonadotrophins [33]. The osmotic gradient created by the theca and GCs allows water to be drawn into the antrum of the tertiary follicle [38, 45]. The dynamic remodelling of these two cell layers facilitates the accumulation of follicular fluid (FF) and differentiation of the GCs (induced by the morphogen gradient of growth differentiation factor-9 (GDF-9) from the oocyte) into cumulus GCs (CCs) directly surrounding the oocyte and mural GCs (mGC) lining the antrum [41, 45, 46]. Both types of GCs remain connected by gap junctions to produce a

functional syncytium that provides important bidirectional communication with the oocyte to maintain it quiescent until ovulation (*Figure 1*) [37, 47].

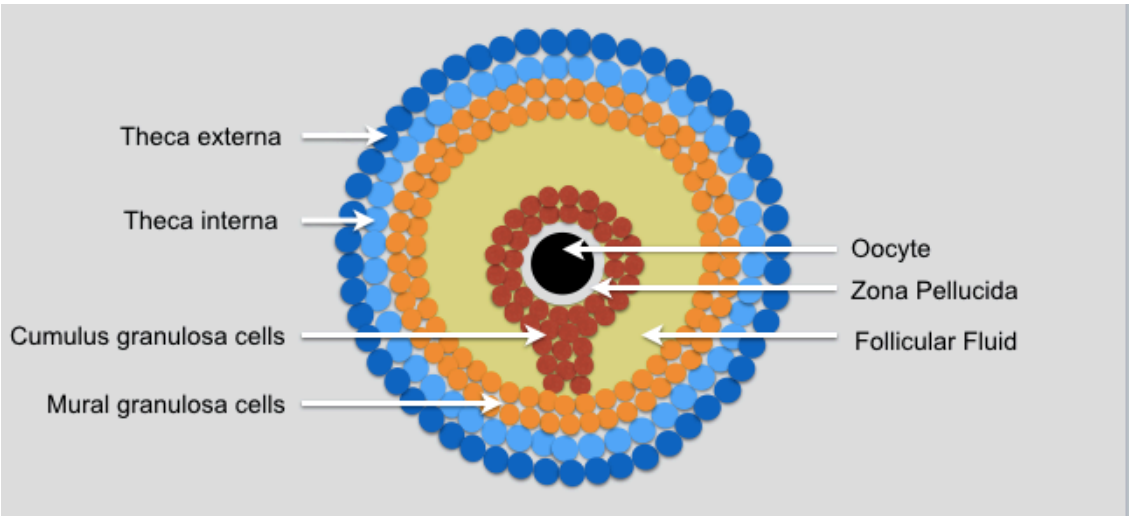


*Figure 1*: **Morphology of an antral follicle**. An oocyte, encapsulated by a glycoprotein layer called the zona pellucida, is directly surrounded by 3-6 layers of cumulus granulosa cells, which are connected to the mural granulosa cells that line the follicular fluid filled antrum. Both theca and granulosa cells are involved in follicular steroidogenesis.

The FF provides a highly metabolic microenvironment for the oocyte to mature in [48]. Since FF has a complex biochemical composition and is composed of many different proteins, in various pathways, it can be used to reveal the conditions of not only the granulosa and theca cells, but also the entire follicle, oocyte competence and quality, fertilization potential and embryo development [48-51]. In fact, a recent and extensive bioinformatic review classified 337 human FF protein factors into five statistically significant groups: (1) regulation of acute inflammatory responses, (2) response to wounding, (3) complement and coagulation cascades, (4) protein-lipid complex/ lipid metabolism and transport and (5) cytoskeleton organization [48].

### *2.1.2. Follicular steroidogenesis*

Both theca and GCs are involved in follicular steroidogenesis, and can convert cholesterol to progesterone, however, the progesterone in the GCs needs to diffuse

across the basement membrane into the theca cells to be converted into androgens (e.g. testosterone) [52]. Then, some of the androgens diffuse back into the GCs to be converted to estradiol (by aromatase), which is involved in the recruitment and selection of dominant follicles [52].

### *2.1.3. Recruitment, Selection and Ovulation of Dominant Follicles*

FSH secretion often recruits bigger follicles with more FSHRs to the surface of the ovary [53]. As they continue to grow, these follicles secrete increasing amounts of estradiol, which positively stimulates the anterior pituitary gland to produce LH. Consequently, larger, dominant follicles (which produce more estradiol) are selected for, whereas the smaller follicles undergo waves of atresia. In the hours leading up to ovulation, the increased intrafollicular pressure weakens the follicular wall [37]. Then, 7-10 minutes prior to ovulation, the thecal capillaries vasodilate, providing an increased blood supply and proteolytic enzymes, that will degrade the thick extracellular matrix (ECM), releasing the cumulus-oocyte complex (COC) [37, 46, 47, 53, 54].

Mammalian ovulation can be considered an acute inflammatory reaction since it is essentially followed by coagulation (formation of a blood clot) at the site of rupture, tissue repair mechanisms a few hours later, and the steroid secreting cells on the follicular surface begin creating a progesterone-secreting corpus luteum [37, 48, 50, 55, 56] . Progesterone plays an important role in maintaining the uterine lining for a potential pregnancy, by sending negative feedback to the hypothalamus and pituitary gland to reduce secretion of the gonadotrophins [56].

### *2.1.4. Meiotic Arrest and Resumption of Oocytes*

During female fetal gametogenesis, oocytes in primordial follicles are arrested at prophase I of meiosis, otherwise known as the germinal vesicle (GV) phase [34]. This occurs because cGCs can transfer cyclic guanosine monophosphate (cGMP) into the oocyte through gap junctions, inhibiting phosphodiesterase 3, which is responsible for cyclic adenosine monophosphate (cAMP) degradation [47, 57]. Consequently, a high concentration of cAMP hinders the activity of the maturation promoting factor (MPF),

directly responsible for meiotic resumption [34], and keeps the oocyte in meiotic arrest [47, 58, 59]. This arrest during early folliculogenesis is important because it allows the oocyte to synthesize ribonucleic acid (RNA) and proteins required to become “meiotically competent” [47, 59]. In particular, mammalian target of rapamycin (mTOR) is a key player in this dynamic meiotic process because it activates the synthesis of proteins involved in cap-dependant translation, chromosomal alignment, spindle formation and cell cycle progression [60].

During ovulation, LH binding to its receptors on the theca interna, granulosa and steroid secreting cells initiates signalling cascades through $G_s$ protein coupled receptors (GPCRs), or more specifically, GPCR3 [61, 62]. Each different type of cell produces a slightly different pathway. For example, LH binding to the GPCR3 on the mGCs, causes a conformational change in the intracellular portion of the $G_s$ protein, which activates adenyl cyclase to produce cAMP [57]. This second messenger activates various kinases such as protein kinase A (PKA), which induces the downstream expression of amphiregulin and epiregulin RNA 1-3 hours later [62]. These two epidermal growth factor (EGF)-like proteins close the gap junctions between the cGCs and the oocyte, preventing both cGMP and cAMP from travelling between the cytoplasm of adjacent cells [47, 57]. As a result of the decrease in cAMP in the oocyte, cycline-dependant kinase (CDK) 1, a catalytic subunit of the MPF, which is suppressed during meiotic arrest, is phosphorylated and activated, allowing the resumption of meiosis until metaphase II [34, 47, 59, 62] [34, 47, 59, 62]. At this point, the oocyte is subjected to a second meiotic arrest and only completes maturation upon fertilization [34].

### *2.1.5. Fertilization*

Fertilization is a process, in sexual reproduction, whereby a sperm and oocyte (male and female gametes respectively) fuse as a means to restore the original number of chromosomes following meiotic division. In order to acquire the ability to fertilize an oocyte, the sperm must undergo capacitation once inside the female reproductive tract. During this process, the sperm gain directionality and important changes in surface membranes (and ion channels) that ultimately allow them to degrade the hyaluronic acid

in the ECM of the corona radiata (layer of cGCs surrounding the ZP; *Figure 1*) [63, 64]. Then, upon binding to (species-specific) ZP sperm-binding protein 3 (ZP3) the sperm undergo an acrosome reaction to release hydrolytic enzymes that break down the ZP [65, 66]. Immediately after the sperm and ooplasmic membranes fuse together, a cascade of events leading to the "fast" polyspermy block occur to protect the embryo. The introduction of sperm-specific phospholipase C zeta (PLC ζ) into the ooplasm hydrolyzes inner membrane phosphatidylinositol (4,5)-biphosphate ($PIP_2$) into inositol triphosphate ($IP_3$) and diacylglycerol (DAG) [67]. In turn, $IP_3$ binds to and opens calcium channels on the endoplasmic reticulum (ER). As the resulting wave of intracellular calcium spreads to the opposite side of the oocyte, it catalyzes the fusion of the cortical granules stored under the ooplasmic membrane, causing them to release enzymes into the perivitelline space [68]. This "slow" polyspermy block effectively hardens the rest of the ZP, mechanically preventing other sperm from penetrating [40, 63, 69]. After the cytoplasmic contents of the sperm are delivered into the ooplasm, the oocyte completes meiosis II and initiates the necessary physiological changes to extrude the second polar body, start unpacking paternal DNA and become a zygote (1 cell embryo).

### *2.1.6. Early embryo development*

The zygote cleaves and develops into a 2-cell, 4-cell, and subsequently an 8-cell embryo. In pigs, genome activation occurs between the 4-8 cell stage [70], approximately on day 3. During the following two days, porcine embryos will undergo several cycles of compaction and decompaction, and the totipotent embryonic stem cells (ESCs) will begin to differentiate [71]. Blastocysts (32-64 cells) begin to form at day 6 [71], when the outer trophoblast cells begin expressing CDX2 (a transcription factor required to induce trophectoderm genes) and pumping sodium and water into the blastocoel cavity [72, 73]. Meanwhile, in mice, the inner cells exclusively express Oct3/4 transcription factors, and this allows them to aggregate to form the inner cell mass (ICM) [72, 74]. During this crucial preimplantation embryo development, the embryo is sensitive to the autocrine and paracrine growth factors in its microenvironment, as well as pH, light, temperature and humidity of its surroundings [73].

## 2.2. Vitamin D endocrinology

### *2.2.1. Vitamin D*

Vitamin D (VD) is a secosteroid hormone that is well known to play a systemic role in calcium homeostasis, skeletal metabolism, cellular differentiation, apoptosis, antiproliferation, immunosuppression and antiinflammation [75-78]. There are two main types of VD. In animals, cholecalciferol (vitamin D3), is mainly photosynthesized from 7-dehydrocholesterol, a cholesterol precursor taken up by keratinocytes, in the skin, as a result of exposure to solar UVB radiation, or ingested from dietary sources such as fatty fish (ex. salmon, mackerol or tuna) and eggs. Whereas, the other form, ergocalciferol (<10-20%; vitamin D2), is synthesized by yeast and fungi and thus obtained through dietary sources such as mushrooms or fortified dairy products [1, 78, 79]. These inactive vitamin D2 and D3 forms will enter the blood stream and, depending on the individual's ethnicity, be bound by different isoforms of vitamin D binding protein (VDBP) or be absorbed in the small intestine and transported in chylomicrons through lymph veins to the liver where 25-hydroxylase will hydroxylate them [76, 78, 79]. The resulting VD metabolite, 25-hydroxycholecalciferol (25-OHD), will return into circulation and enter the kidneys. Here, under homeostatic control, the VD will either be excreted in urine or undergo the second hydroxylation (by 1 α-hydroxylase) required to convert the molecule to its active form, calcitriol ($1\alpha,25\text{-}(OH)_2D_3$), here on abbreviated VD3, which has a half life of 15 hours [80]. Initially 1 α-hydroxylase was thought to be solely hepatic, but recently has been discovered in other calcium regulating tissues such as the breast, prostate, colon, brain, the female and male reproductive tracts or immune cells - suggesting that circulating VD metabolites can be activated locally and ablation of this enzyme could cause widespread disease from VD deficiency [76, 78, 81-84].

### *2.2.2. Defining vitamin D status*

The literature reports discrepancies in classifications of serum VD (sVD) status *(Figure 2).* Some considered individuals with >10 ng/mL sVD as deficient, 10-29 ng/mL as insufficient, and >30 ng/mL as sufficient (*Figure 2A*) [85-87]. While other groups,

including the Endocrine Society (2011) defined deficiency as >20 ng/mL, insufficiency as 20<30 ng/mL, and sufficiency as 30-100 ng/mL (*Figure 2B*) [1, 26, 88, 89]. In contrast, in 2011, the US Institute of Medicine recommendations were to have <12 ng/mL to prevent deficiency and at <20 ng/mL to ensure sufficiency (Figure 2C) [90]. Nevertheless, gestational sVD levels should be between 40-60 ng/mL (Figure 2D) [86].

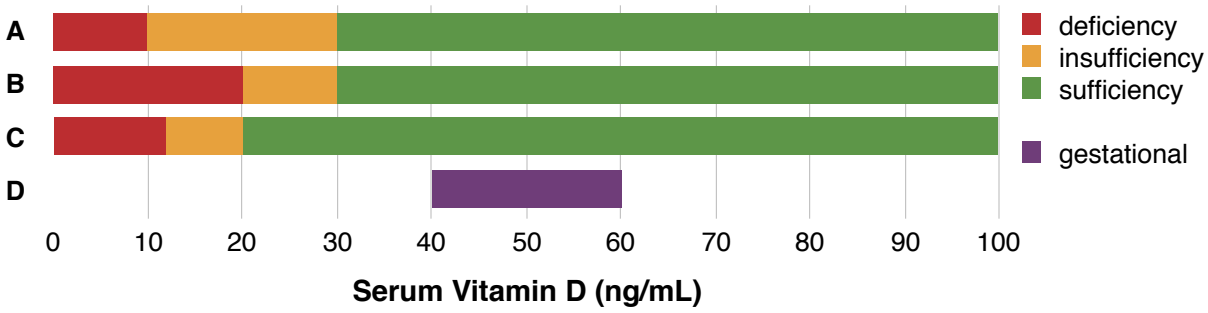


*Figure 2*: **Comparison of serum Vitamin D status classifications by different groups.** See text for discussion.

Although there are studies that claim too much or too little VD can increase risk of some cancers, cardiovascular disease and mortality, Grant (2009) disagrees with these findings, and states they were “taken out of context” because his results show increasing sVD to 45 ng/mL could significantly decrease mortality rates in the US [91].

Nonetheless, it is important to note that since less than 1% of VD is free (99% is bound to VDBP or to albumin), measuring circulating VD levels may not always reflect bioavailable VD levels in a given tissue [30]. On the other hand, the VDBP was found in human FF [51] and sVD has been positively correlated to VD levels in FF [85, 87, 92].

### *2.2.3. Vitamin D deficiency and supplementation during early pregnancy*

It remains controversial whether VD deficiency can be definitively associated with pregnancy outcomes [1, 24-29]. A 2017 cross-sectional study looking at 283 infertile women concluded that VD deficiency was unlikely to have a negative effect on ovarian reserve [93]. However, serum levels of VD increase during pregnancy, as VD is

secreted by the endometrium to act locally to mediate mineral homeostasis, endometrial to decidual differentiation and regulate expression of genes essential for implantation and immunological protection of the embryo [1, 24-29, 94]. In fact, some report that women with less VDR are more likely to lose the pregnancy during the first trimester [95] and VD deficiency further increases the risk of pregnancy loss [96], complications such as preeclampsia, and premature birth [97, 98]. Risk factors that predispose VD deficiency include: lack of sunlight exposure, skin hyperpigmentation, low dietary intake, smoking, pollution, ageing, intestinal malabsorption, kidney disease, liver disease, obesity [99-101].

During pregnancy, high levels of estrogen increase intestinal calcium absorption so that 30-300 mg/day of maternal calcium can be transferred to the fetus through the placenta [102]. Since maternal VD can also be transferred, and is required for optimal calcium absorption and bone development of the neonate, the recommended VD dosage for pregnant women varies between 1500-400 IU per day, and can be increased to 6000 IU/day during breast feeding to protect infants from deficiency [1, 97, 103]. Furthermore, VD supplementation can be used to prevent and treat preeclampsia by diminishing oxidative stress and the surplus of progesterone caused by CYP11A1 [98]. Vitamin D supplementation is also indicated for women who are obese, have insulin resistance or a small ovarian reserve [1].

## 2.3. Genomic and non-genomic actions of Vitamin D

### *2.3.1. Vitamin D receptor (VDR) structure*

VD signals through the VDR, which is composed of an N-terminal domain, a DNA binding domain with well-conserved zinc finger motifs, a flexible, immunogenic hinge region, and a C terminal domain crucial for ligand binding, translocation into the nucleus and protein-protein interactions with other cofactors (*Figure 3*) [75, 77, 104-110]. Although the three-dimensional configuration of the receptor is what ultimately dictates ligand interactions, the VDR has an inner binding cleft with high affinity for VD3 [105, 111] and may use the activation function 2 (AF-2) domain in the c-terminal to “lock” the

ligand into the binding cleft [112]. Once bound, VD3 can stimulate phosphorylation of the VDR within 15 minutes [113], initiating conformational changes that will release corepressors and allow heterodimerization with retinoid X receptor (RXR) in the cytoplasm [18, 77, 78, 108].

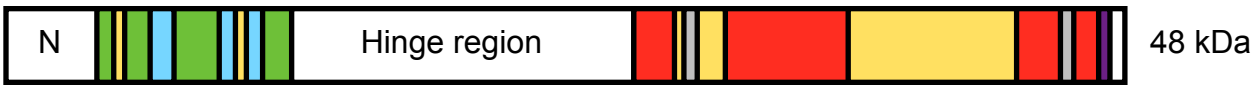


*Figure 3*: **Functional domains of the vitamin D receptor (VDR) protein.** The full length VDR protein (48 kDa) has 5 functional domains. [From left to right] The short N-terminal is followed by the DNA binding domain (green) which contains two zinc finger motifs. Nuclear localization signals (blue) allow translocation into the nucleus. The flexible hinge domain interacts with corepressors. The ligand binding domain (red) has high affinity for VD3 and may use the AF-2 domain (purple) to “lock” the ligand into place. Furthermore the dimerizaton domain (yellow) is required for interaction with RXR and transactivation (grey) by other coactivators. Adapted from [105, 109, 112, 114].

### *2.3.2. VDR-mediated gene transcription*

Once transactivated, the VDR/RXR complex translocates into the nucleus, where it binds to the vitamin D response elements (VDREs) on promotors of VD target genes, and then recruits other proteins that help acetylate histones, decondense chromatin, and ultimately, initiate transcription [5, 9, 77, 110, 115-117]. VDR directly regulates 200 human genes, and has been shown to indirectly regulate several others through distal enhancer regions [3, 77]. In fact, chromatin immunoprecipitation (ChIP) assays have demonstrated that DNA looping, of the transcription start site to distant gene regions, is used to optimize VD3 signalling by maximizing VDRE synergy for transactivation [2, 4].

### *2.3.3. Subcellular localization of the VDR*

Classically, members of the steroid/thyroid nuclear hormone receptor superfamily, were thought to be strictly nuclear (eg. estrogen and progesterone receptors) or cytoplasmic (eg. androgen and glucocorticoid receptors), however, unliganded VDR has also been observed in other subcellular locations, such as the mitochondria and the plasma membrane [78, 106, 108, 118-120]. Carlberg and Campbell (2013) also suggest that unliganded VDRs may be “stored” in less efficient binding elements, and upon VD3 ligation, quickly redistribute themselves to much less frequent DR3-type VDREs to exert their effects. In parallel, approximately 40% of the cytoplasmic VDRs will clump along the nuclear fibrils within 30 seconds of VD3 ligation, and within 3 minutes, all the VDRs will be translocated into the nucleus [118]. Interestingly, some nuclear hormone receptors, including the VDR, have demonstrated the ability to shuttle in and out of the nucleus within 1-8 hours [116], but this may be slowed down by RXR [116] which is an essential to provide efficient DNA binding [121].

### *2.3.4. VDR variants*

Under normal circumstances, mRNA translation is terminated when a ribosome reaches one of the three possible stop codons (UAG, UAA, UGA). However, the ribosomal machinery may “readthrough” these codons approximately 5% of the time, if they are followed by motifs such as CUAG [122]. Using this stop-codon readthrough mechanism, translation of the mRNA sequence may non-canonically continue until the next stop codon (*Figure 4*) [123]. For example, mRNA for the human VDR was found to have 6.7% readthrough efficiency, resulting in a 67 amino acid extension to the multi-purpose C-terminal of the VDR [122]. Loughgran et al. (2018) named the resulting protein extended VDR (VDRx), and showed that the extension does not affect subcellular localization (VDRx can be found in the cytoplasm and nucleus, like VDR). However, it does significantly reduce its ability to bind VD3, heterodimerize with RXRα and recognize VDREs. Furthermore, they also suggested that VDRx might antagonize the VDR by forming heterodimers with it, adding to the negative regulation of VD target gene transcription.

Additionally, one of the three possible isomers of the human VDR, VDRB1, is 50 amino acids longer on the N-terminal domain and reacts differently to VD3 and lithocholic acid in different tissues [124]. Other post-transcriptional and post-translational modifications to the VDR (such as micro RNA (miRNA) regulation and phosphorylation, sumoylation and ubiquitination) have been reviewed in [112]. However, more research is required to fully understand their implications to VDR activity. Nonetheless, it is interesting to note that VD3-binding can inhibit VDR ubiquitination, seemingly elevating VDR expression [125].

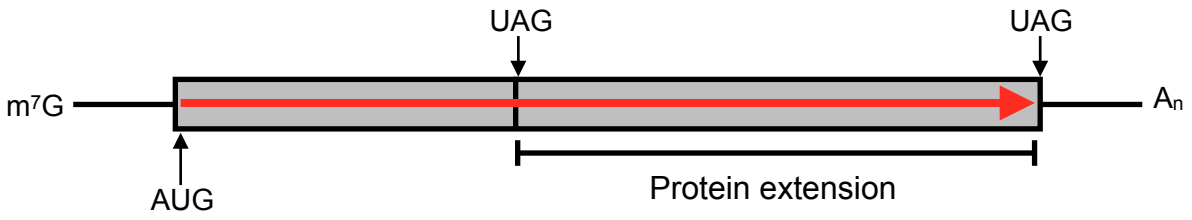


*Figure 4*: **Stop codon readthrough is a mechanism for non-canonical termination of translation.** Eukaryotic translation of an mRNA sequence (with a 5′ cap structure (m $^{7}$G) and a 3′ poly(A) tail) begins at a start codon (AUG) and usually continues until a stop codon (eg. UAG). However, translation of multiple open reading frames can be optimized when ribosomes “read through” a stop codon (straight red arrow) and continue elongation of the polypeptide until the next stop codon. Adapted from [123].

### *2.3.5. Non-genomic Vitamin D actions*

Evidence for non-genomic actions of VD come from studies like those from Costa et al. [7], which show that despite 85-90% VDR knockdown with small interfering RNA (siRNA) in MCF7 cells, VD treatment still hindered proliferation. Consequently, these authors suggested that VD can act through the residual VDR or through other membrane receptors [7]. Consistent with this hypothesis, other groups have found that VD3 can also bind to GPCRs, regulate cAMP and cGMP, as well as activate PLC, Ras, PI3K, and PKA pathways, which, as is discussed herein are important during oogenesis, fertilization and embryo development [6, 8].

## 2.4. Vitamin D and ovarian function

Vitamin D deficiency has a global impact on all age groups, and plays an important role in reproduction [1, 30, 76]. Although the exact mechanisms by which VD functions in this context are still being investigated, VD is essential for full gonadal function in both sexes [126]. VDR is consistently expressed in almost all types of follicular cells in the murine ovary, and consequently, VDR knockout mice and VD-deprived rats were found to have hypoplastic uteri, reduced aromatase gene expression (which impaired folliculogenesis by affecting estrogen biosynthesis) and gonadal insufficiency [81, 126-129].

### *2.4.1. Vitamin D promotes gap junction communication*

Gap junctions are composed of connexin (Cx) proteins that bridge the cytoplasm between adjacent cells, allowing for intercellular exchange of nutrients or small molecules (<1 kDa) [130]. Specifically, connexin 43 (Cx43) plays a key role in the communication between GCs [131]. Although there is no VDRE in the promotor region of the Cx43 gene, VD3 treatment (100nM) can increase Cx43 protein [132] and also rescue testosterone-induced Cx43 decrease in GCs of PCOS rats [133, 134], promoting cellular communication.

### *2.4.2. Vitamin D promotes folliculogenesis*

The TGFβ superfamily of cytokines, which includes TGFβ1-3, anti-Müllerian hormone (AMH) and several bone morphogenic factors (BMFs), locally regulates cell development, proliferation and synthesis of ECM proteins [135]. Their basic signalling pathway is highly conserved throughout the animal kingdom, and involves 4 components to transmit the signal to the nucleus: an extracellular TGFβ ligand, transmembrane TGFβ type I and II receptors and SMAD transcription factors [136]. VD induces TGF-β1 expression, which consequently prompts phosphorylation of SMAD3, and in turn co-activates the VDR to synergistically regulate common target genes that contain both VDRE and SMAD-binding motifs [137]. In this manner, VD3 can indirectly stimulate the differentiation of monocytes [138] while VDR can bind SMAD3 or compete

for its binding elements to inhibit synthesis of fibrotic components like collagen [139]. Furthermore, VD3 has demonstrated the ability to decrease phosphorylation and nuclear localization of SMAD 1/5/8 in human GCs [140].

AMH prevents the depletion of the ovarian reserve of follicles by inhibiting the ability of primordial follicles to respond to FSH [141]. As such, by impeding with AMH signalling, VD could promote the differentiation and development of human GCs [76, 140, 142]. Although an excessive single oral dose of VD3 (50,000 IU) can significantly increase the levels of serum VD and AMH in women [141], serum VD levels do not correlate with follicular AMH or FSH [143]. In fact, VD3 supplementation can decrease AMH while increasing FSHR and VDR mRNA expression in GCs of developing hen follicles [144]. Similarly, another group reported that 60 pM VD3 increased preantral primate follicle survival while 0.24 nM VD3 improved antral follicle growth and promoted oocyte growth [145]. Consistently, Lee et al. (2014), found that VD3 and testosterone favoured growth of larger follicles [134].

### *2.4.3. Vitamin D regulates steroidogenic enzymes*

Steroidogenic enzymes play a key role in follicular development. In particular, aromatase is responsible for converting androgens into estradiol for the GCs. Investigators have reported that VD directly and indirectly represses aromatase transcription by respectively binding to its VDRE upstream of the gene and reducing prostaglandins, which regulate inflammation by promoting cell proliferation and resistance to apoptosis) [146-148]. Although VD3 had no effect on testosterone in purified GC cultures [149], VD3 decreased mRNA and protein expression of other steroidogenic enzymes, such as CYP11A1, StAR, and 3β-HSD, in GCs of mice with Polycystic Ovarian Syndrome (PCOS) [150] and in women without PCOS [151]. On the contrary, it has been proposed that VD3 has the ability to enhance aromatase expression in osteoblasts [146].

### *2.4.4. Vitamin D increases estradiol*

Larger follicles respond better to FSH, produce more estradiol, and consequently have a better chance to be selected for ovulation. While some investigators reported that women with VD3-deficient FF (~40 nM) produced larger follicles and had higher levels of estradiol following ovarian stimulation [152], others found that VD and estradiol levels have been positively correlated in GCs in vitro [149] and in women *in vivo* [153]. In fact, Corduk et al. (2012) suggest that VD/VDR signalling increases both estrogen and its receptors (ERα/β), and this consequently has a more profound effect on SMAD protein decrease in adult rats versus newborn rats [154].

## 2.5. Vitamin D and VDR as regulators of survival, growth and differentiation

### *2.5.1. Vitamin D reorganizes the cytoskeleton architecture*

#### *2.5.1.1. Vitamin D depolarizes actin*

Actin is one of the most abundant cytoskeleton proteins in eukaryotic cells, and is responsible for mediating polar body extrusion during oocyte maturation and exocytosis of cortical granules (containing calcium) at fertilization [155]. As such, 24 hour VD3 treatment (100 nM) of endometrial cells resulted in depolarization of actin (via decreased function of ras-related C3 botulinum toxin substrate 1 (*RAC1*) and p21 protein-activated kinase 1 (*PAK1*)) [156]. This pathway consequently downregulated fibroblast growth factor (Fgf23) [157], and in turn, this biomarker has shown to improve oocyte maturation and fertilization rates in humans [158].

#### *2.5.1.2. Vitamin D interferes with Wnt signalling*

VD3 has been shown to upregulate e-cadherin which cytoplasmically stabilizes β-catenin in a non-phosphorylated form, and VDR further impedes with Wnt signalling by directly associating with β-catenin (through the AF-2 domain) in the nucleus, limiting its transcriptional effects [17, 18, 20, 21]. In leukemic cells, inhibition of β-catenin nuclear

signalling inhibited proliferation, whereas redistribution of β-catenin to the cytoskeleton potentiated apoptosis by facilitating apototic body formation [159]. Interfering with Wnt signalling is required for stem cell development [160-162]. In fact, the reduction in β-catenin also leaves the T-cell factor-3 (Tcf3) transcription factor (also known as E2A, VDR-Interacting Repressor and Negative VDRE-Binding Protein) free and active to repress pluripotency genes, in particular, Oct4 and Nanog in embryos (*Figure 5*) [163-166]. Furthermore, chemical inhibition of β-catenin-Tcf3 interaction during IVM of porcine oocytes improved COC expansion, oocyte maturation, development to the blastocyst stage and cell number [167].

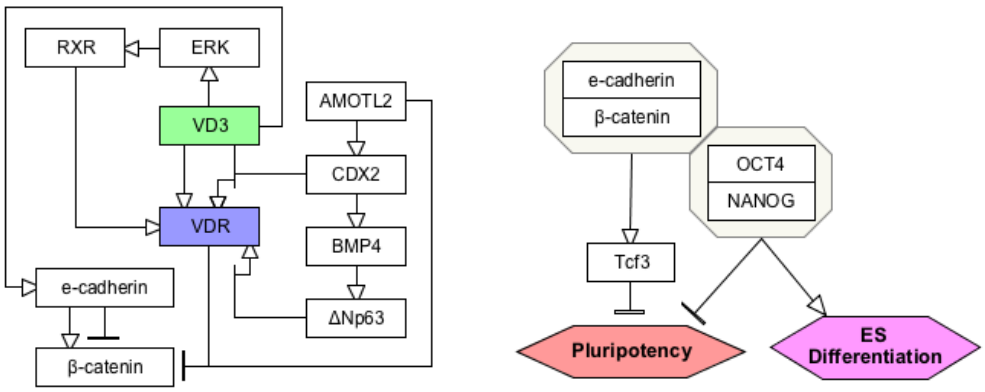


*Figure 5*: **Summary of VD3/VDR signalling as potentially relevant to inhibiting pluripotency and contributing to differentiation of embryonic stem cells.** Diagram created with PathVisio software [168,169], using the molecular interaction map (MIM) notation [170].

Tcf3 has also demonstrated the ability to be a co-factor for Smad2/3 transcription in frog embryos in vivo [171], and in turn, Smad3 can promote transcriptional activation of e-cadherin, positively reinforcing this system [172]. During porcine embryogenesis, e-cadherin can be found on cell surface and interblastomere contacts but then from morula compaction on, more evident at the latter [71]. VDR has also been found to colocalize with e-cadherin in the mouse kidney [173]. However, e-cadherin is not necessary for VD-induced β-catenin inhibition [18].

In addition, VD3 is also able to induce expression of DICKKOPF-1 (DKK-1), an extracellular Wnt inhibitor, in colon cancer cells [19]. Supplementing porcine oocytes with DKK-1 during IVM improved maturation to the MII stage and increased cell number in blastocysts [174]. On the other hand, treating beef cow embryos with this molecule *in vitro* had no effect on cleavage or blastocyst rates, but it did reduce birth weight of calves [175].

### *2.5.1.3. CDX2 induces VDR transcription*

Angiomotin-like protein 2 (AMOTl2) is a linker protein between the VE-cadherin/β-catenin complex and F-actin filaments, and plays a crucial role in maintaining junctional force between neighbouring cells [176]. Consequently, AMOTl2 also regulates TE cell geometry and volume, to ultimately create the mechanical forces necessary for blastocyst hatching [176-178]. Interestingly, AMOTl2 suppresses expression of CDX2 in ICM cells, while induces it expression in polarized TE progenitor cells, through nuclear translocation of the transcriptional co-activator YAP [72, 179, 180]. CDX2 is an extensively studied VDR transcription factor that is essential for cytoskeletal architecture of TE cells as well as polarity, survival and proliferation of blastocysts in pigs [181]. Furthermore, a recent study has shown that different CDX2 polymorphisms alter VDR transcription, which has repercussions on the therapeutic efficacy of VD treatment on breast cancer proliferation [182].

### *2.5.1.4. VDR mediates ezrin expression*

Ezrin is an adherens junction protein that associates with actin and plays a role in the microvilli of polarized blastomeres [183]. Lee et al. (2017) reported that VD supplementation of VK2/E6E7 cells increased VDR which in turn activated ezrin and RhoA, but ablation of VDR attenuated these effects [184]. In contrast, Kühne et al. (2016) found that VDR knockout mice had higher ezrin expression [185]. Nonetheless ezrin expression declines when blastomeres reorganize their cytoskeletons during compaction [183], but at the blastocyst stage, ezrin proteins are post-translationally modified to stabilize polarity of ICM microvilli in mice embryos [186].

#### *2.5.1.5. Vitamin D induces synthesis of alkaline phosphatases*

VD induces *de novo* synthesis of alkaline phosphatase (AP) in microvilli of intestinal epithelial cells, consequently increasing calcium intake [187]. In mice, embryonic AP genes have been detected after genome activation and remain active until the blastocyst stage [188]. Engorged TE cells (called “zona breakers”) located across from the ICM pole have interlocking microvilli and tonofilaments that interact with the ZP during hatching [189]. Thus, it is possible that the APs are expressed to help in this process. Although this embryonic isoenzyme is expressed in pluripotent ICM, it expressed binding sites for Nanog, but not Oct4 or Sox2 [190].

#### *2.5.1.6. VDR interacts with SOC channels*

When ER calcium is depleted, VD3 stimulates VDR insertion into the plasma membrane to interact with store-operated calcium (SOC) channels to elicit a calcium influx [6]. This influx stabilizes e-cadherin/β-catenin complexes on the membrane and ultimately activates PKC, which in turn phosphorylates Raf and continues to transduce the signal through the MAPK/ERK1/2 cascade to provide rapid, non genomic cellular responses [6, 8, 111, 191]. In pigs, the SOC channels are largely responsible for sustaining the repetitive calcium oscillations during the fast polyspermy block at fertilization [192]. However, in keratinocytes, activation of PKC by sustained levels of intracellular calcium also prompted differentiation [191].

### *2.5.2. Vitamin D hinders proliferation and favours differentiation*

#### *2.5.2.1. VDR regulates FoxO proteins*

Downstream of the PI3K/AKT pathway are the FoxO transcription factors. In the presence of growth factors, FoxO proteins will be Akt-phosphorylated and inactive in the cytoplasm [11, 72]. However, under stress conditions, they will be dephosphorylated and translocated into the nucleus to initiate transcription of many genes involved in cell differentiation and survival [11, 72]. Chen et al (2016) reported that treating myotubes with VD3 induced cytoplasmic translocation of FoxO1, thereby inactivating the protein

[193]. On the contrary, other studies report that VD/VDR can directly activate FoxO3 and FoxO4 in less than 4 hours, to control common target genes, such as p27, which can in turn induce apoptosis or promote quiescence at the G1/S or G2/M cell cycle checkpoints [10, 11, 194]. Similarly, when responding to TGFβ, these proteins complex with Smad3/4 on the p21 promotor, possibly prompting cell cycle arrest to allow time for DNA repair [11]. FoxOs can also positively reinforce quiescence or cell death through transcriptional enhancement of PTEN [11] and downregulation of cyclin D [195]. Although the presence of FoxO1/3/4 proteins has been characterized in mice and pig oocytes and preimplantation embryos, their roles have not yet been elucidated during this crucial developmental period [73, 196].

#### *2.5.2.2 VDR inhibits c-Myc*

Also among the contributors of embryonic proliferation is the proto-oncogene c-Myc. This transcription factor is co-localized with RNA polymerase II (within small nuclear "speckles") and changes its distribution dynamically throughout oogenesis and early embryo development [197]. As oocytes grow, several small speckles form aggregates that may be involved in pre-mRNA splicing [197]. High concentrations of c-Myc continue to promote proliferation until fertilized embryos reach the morula stage, but the absence of its expression at the blastocyst stage has an opposing effect [197]. Since c-Myc is a potent regulator of at least 15% of genes, and drives cell cycle progression and differentiation, it is highly susceptible to post-translational modification, proteasomal degradation and opposition by tumor suppressing FoxO proteins [14, 198]. Interestingly, VD treatment can significantly decrease Myc transcription and inhibit its function [13, 14, 17, 199]. In consequence, VD could presumably reverse c-Myc repression of p21, and promote cell cycle arrest until the morula stage [200, 201].

#### *2.5.2.3. Vitamin D promotes differentiation via MAPK/ERK signalling*

VD3/VDR signalling hinders stem cell proliferation through reduction of β-catenin and furthermore diminishes pluripotency by repressing Oct4 [74, 166, 202, 203]. When ESCs differentiate, MAPK/ERK signalling results in Oct4 ubiquination and degradation [74, 204]. Interestingly, VD3 activation of the MAPK/ERK pathway amplifies VDR

expression (through activation of RXR isoforms), and this may further amplify the differentiation prompts [20, 23, 205]. In fact, VD3 analogues have been able to induce differentiation of breast cancer cell lines in vitro, as measured by lipid droplet formation [22].

### *2.5.3. Vitamin D induces cell cycle arrest and promotes cell death*

#### *2.5.3.1. Vitamin D targets cell cycle components*

VD3 can inhibit proliferation by targeting many components of the G1/S transition of the cell cycle (ex. c-Myc, CDK inhibitors (CDKIs) like p21 and p27, as well as cyclin proteins), and consequently, can induce G1 cell cycle arrest (*Figure 6*) within a couple hours for MCF7 cells [12, 22, 182] and RWPE-1 cells [15]. In fact, MCF7 cell proliferation was reduced by 50% when treated with VD for 72 hours [7]. Additionally, breast cancer cell lines also respond VD3 supplementation (50 nM) by upregulating genes involved in DNA repair (such as RAD23) and apoptosis [206]. On the contrary, when subcutaneous adipocytes were supplemented VD3 (10 nM), microarray analysis revealed upregulation of genes involved in oxidative stress (MAPK13), cell cycle progression, proliferation, cytoskeleton architecture and downregulation of genes involved in apoptosis [205, 207].

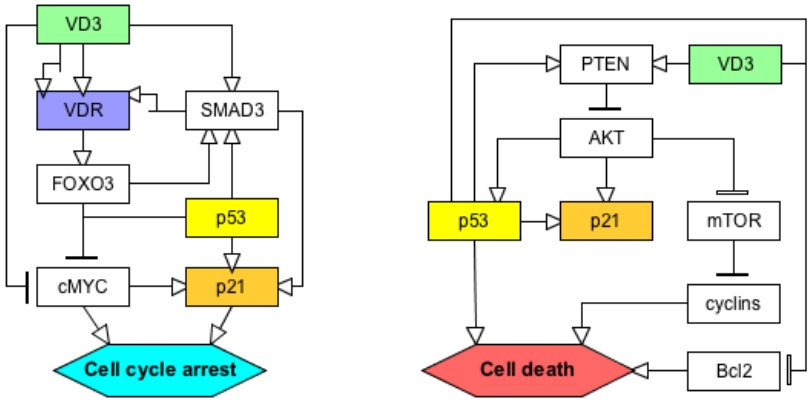


*Figure 6*: **Summary of VD3/VDR signalling that potentially induces responses to cell stress, resulting in cell cycle arrest or cell death, in oocytes and embryos.** Diagram created with PathVisio software [168, 169], using the molecular interaction map (MIM) notation [170].

### *2.5.3.2. Vitamin D and p53-mediated cytostasis*

VD treatment increases p53 transcripts, however, p53 is not able to directly induce expression of VDR in response to DNA damage [206, 208]. On the other hand, p53 family members, p63 and p73, have been shown to increase VDR transcript and protein levels, consequently sensitizing cells to VD3 [208]. It is also possible that p53 increases VDR expression by cooperating with Smad2/3, since p53 is known to regulate TGF-β target genes like p21 [209, 210]. p53 also represses c-Myc and, consequently, increases p21 to induce cell cycle arrest [211]. In order to protect the genome from potential DNA damage, cytostasis occurs relatively quick; VD3 treated RWPE-1 cells were able to induce expression of p21 within 1 hour [15].

### *2.5.3.3. Vitamin D inhibits mTOR*

As previously mentioned, VD3 contributes to sustained elevated levels of intracellular calcium, and this contributes to cell cycle arrest and/or programmed cell death. PTEN is a protein that negatively regulates the AKT/mTOR signalling pathway, which is crucial for differentiation and pluripotency during embryonic development [212]. In response to stress, p53 transactivates PTEN, which consequently inhibits mTOR complex 1 (mTORC1), preventing the translation of cyclins required for cell cycle progression, hindering ribogenesis, and ultimately, inducing autophagy [213-215]. VD3/VDR signalling in gastric cells can induce PTEN expression resulting in apoptosis [16]. Interestingly, inhibition of mTOR in murine COCs impedes with cumulus cell development and survival [216], and compromises oocyte competence by elevating chromosomal aneuploidy rate in MI and MII stage oocytes [60].

## 2.6. Summary

Due to the widespread effects of VD deficiency on many tissues and organ systems and its association with many diseases, research on the secosteroid has augmented in the last 30 years. Although VD's implications in calcium homeostasis in nonskeletal tissues has sparked interest in its role in reproduction, current literature has yet to elucidate mechanisms by which VD actions at this level. Classically, it was thought that VD signals solely through the VDR, however, investigators are finding that VD3 signalling acts through several levels of complexity, both genomically and non-genomically. VD/VDR directly and indirectly regulates expression of many genes and furthermore VD3 and VDR individually are involved in crosstalk between multiple signalling pathways, producing a plethora of cellular responses. For example, VD plays a role in regulating cell cycle progression (through c-Myc, p21, PTEN/AKT/mTOR), promotes differentiation (by inhibiting Wnt signalling and amplifying MAPK/ERK) and potentially mediates the mechanical forces necessary for blastocyst hatching (through ezrin and AMOTl2). VD3 also seems to have a positive impact on oocyte maturation by promoting gap junction communication between GCs, interfering with AMH signalling, mediating steroidogenesis and estradiol to stimulate folliculogenesis. Furthermore, VD reorganizes cytoskeleton molecules such as actin, e-cadherin and β-catenin, which facilitates meiotic maturation or apoptotic body formation. By contributing to sustained elevated levels of intracellular calcium (through APs and SOC channels), VD3 can potentially induce stress responses which may quickly result in cell cycle arrest to allow for repair of DNA damage and/or programmed cell death to protect the genome.

# CHAPTER 3: RATIONALE, HYPOTHESIS AND OBJECTIVES

Although many investigators have studied VD3 and VDR as regulators of growth, differentiation and survival in various other contexts, to our knowledge, their effects on oocyte maturation and early embryo development have not been investigated. For this reason, the objectives of this study were to (1) characterize VDR expression in porcine oocytes and early embryos, (2) determine if VD3 supplementation regulates VDR expression during oocyte maturation or *in vitro* embryo development, (3) investigate whether VD3 supplementation during IVM or IVC affects development of porcine embryos to the blastocyst stage, and finally (4) elucidate if knocking down the VDR alters these effects. We hypothesize that the VDR is present is porcine COCs and developing embryos, VD3 supplementation (100 nM) during both IVM and IVC would upregulate the VDR and improve maturation and embryo development respectively, and lastly, knocking down the VDR would impede development. For our supplementation experiments, we chose a VD3 concentration (100 nM) that was previously used in GC culture studies *in vitro* [140] and is in the top range of physiological levels for humans [217].

# CHAPTER 4: ARTICLE

Manuscript in preparation for submission to Molecular Reproduction and Development

## Attenuation of VDR hinders pre-implantation development of porcine embryos *in vitro*

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keywords: Vitamin D supplementation, vitamin D receptor, VDR, oocytes, embryos

## 4.1. Abstract

The active form of vitamin D, ($1\alpha,25(OH)_2D_3$; VD3) and the vitamin D receptor (VDR) are well known to regulate growth, development and survival in various cell types. However, to our knowledge, the role of VD3 and its receptor, VDR, has not been characterized during oocyte maturation and early embryo development. Therefore, this study aimed at evaluating the effect of VD3 supplementation and VDR attenuation during *in vitro* development of porcine embryos. Immunostained VDR protein was observed around the nuclear envelope of immature oocytes and in their surrounding cumulus cells (CCs). VDR mRNA expression in cumulus-oocyte complexes (COCs) was evaluated by qRT-PCR, and was not significantly altered before or after (46 hours) *in vitro* maturation (IVM), nor after VD3 supplementation (100 nM). Under our conditions, VD3 supplementation did not improve oocyte developmental potential in vitro. Meanwhile, on day 3-5 of embryonic development, VDR mRNA expression decreased by 42% ($p<0.01$) and the VDR protein was detected in both the cytoplasm and the nucleus. Whereas on day 7, the VDR was predominantly nuclear localized. VD3 supplementation (100 nM) during *in vitro* culture (IVC) upregulated VDR mRNA expression by 1.6 fold ($p<0.01$), but reduced blastocyst development by 15% ($p<0.02$) compared to controls. Targeted knockdown of the VDR by DsiRNA hindered blastocyst development (by 46%; $p<0.01$), reduced the average number of cells per embryo (by 33%; $p<0.04$) and reduced expansion compared to controls. Taken together, these findings indicate that VD3 supplementation does not benefit oocyte competence or embryo development, but the VDR is important for regulation of pre-implantation embryo development in swine.

## 4.2. Introduction

Due to the widespread effects of VD deficiency on various tissues and its association with several diseases, research on this secosteroid has augmented in the last 30 years. It has been demonstrated that VD regulates cell cycle progression (e.g., through FoxOs [10, 11], c-Myc [12-14], p21 [15], and PTEN/AKT/mTOR [16]), hinders cell proliferation (e.g., by inhibiting Wnt signalling [17-21]), and promotes cell differentiation (e.g., by amplifying MAPK/ERK signalling [20, 22, 23]) in various cell types. However, regarding

potential roles in the reproductive system, it remains controversial whether VD deficiency is associated with pregnancy outcomes [1, 24-29].

VD3 has high affinity for the inner binding cleft of the vitamin D receptor (VDR) [105, 111], and can stimulate its phosphorylation within 15 minutes [113]. Activation of the VDR initiates conformational changes that release corepressors and allows heterodimerization with retinoid X receptor (RXR) in the cytoplasm [18, 77, 78, 108]. Following transactivation, the VDR/RXR complex translocates into the nucleus, where it binds to the vitamin D response elements (VDREs) on promotors of VD target genes, and subsequently recruits other proteins that help acetylate histones, decondense chromatin, and ultimately, initiate transcription [5, 9, 77, 110, 115-117]. Unlike other steroid hormone receptors, which are strictly nuclear or cytoplasmic, the VDR was also found linked to the PKC and voltage gated $Ca^{2+}$ channels in the mitochondria and the plasma membrane [78, 106, 108, 118-120]. This suggests involvement in rapid, non-genomic cellular responses [111], such as calcium influxes and changes in intracellular signalling pathways regulating cAMP, cGMP, PKA, PKC, PLC and PI-3, which are important during oogenesis, fertilization and embryonic development [8].

VD/VDR can quickly activate FoxO3/4 transcription factors, controlling common target genes such as p27, which promote cell cycle arrest or induce apoptosis [10, 11, 194]. In addition, FoxO proteins [14, 198], VD [13, 14, 17, 199] and p53 [211] oppose c-Myc, amplifying these responses by reversing repression of p21 [200, 201]. In response to stress, transcriptional enhancement of PTEN by FoxOs [11], VD3/VDR [16] and p53 [213-215], negatively regulates AKT/mTOR signalling. Since this pathway is a key mediator of cell cycle progression, it is not surprising that inhibition of mTOR disrupted murine CC development and survival [216], and compromised oocyte competence by elevating chromosomal aneuploidy rates in MI/II stage oocytes [60].

It has also been shown that VD3/VDR signalling hinders stem cell proliferation through reduction of β-catenin and furthermore diminishes pluripotency by repressing Oct4 [74, 166, 202, 203]. VD3 can upregulate e-cadherin, which cytoplasmically stabilizes β-

catenin in a non-phosphorylated form, while VDR binds β-catenin in the nucleus, limiting its transcriptional effects [17, 18, 20, 21]. The reduction in β-catenin leaves the T-cell factor-3 (Tcf3;E2A) transcription factor free to repress pluripotency genes, in particular, Oct4 and Nanog in embryos [163-166]. Moreover, VD3 can activate the MAPK/ERK pathway, enhancing VDR transactivation (through activation of RXR isoforms) [20, 23, 205], and contributing to ESC differentiation by degrading Oct4 [74, 204]. Interestingly, inhibition of Wnt signalling during IVM of porcine oocytes, by blocking β-catenin-Tcf3 interaction [167], improved oocyte maturation, blastocyst development and increased cell numbers in blastocysts.

Moreover, VD has proven to be essential for full ovarian function [126]. In fact, the VDR is consistently expressed in almost all types of follicular cells in the murine ovary, and VDR knockout mice and VD-deprived rats were found to have hypoplastic uteri, reduced aromatase gene expression, which impaired folliculogenesis by affecting estrogen biosynthesis, and gonadal insufficiency [81, 126-129]. Similarly, clinical data showed that women with less VDR are more likely to lose the pregnancy during the first trimester [95], and VD deficiency further increased the risk of pregnancy complications (e.g., preeclampsia and premature birth) [97, 98].

Since current literature has yet to elucidate the roles of VD3/VDR on oocyte maturation and early embryo development, the objectives of this study were to: (1) characterize VDR expression in porcine oocytes and early-stage embryos; (2) determine if VD3 supplementation regulates VDR expression during oocyte maturation or *in vitro* embryo development; (3) investigate whether VD3 supplementation during IVM or IVC affects development of porcine embryos to the blastocyst stage, and (4) assess if knocking down the VDR effects early embryo development and quality.

## 4.3. Materials and Methods

### *4.3.1. Chemicals*

Unless otherwise indicated, chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON).

### *4.3.2. Oocyte retrieval and in vitro maturation (IVM)*

Ovaries from pre-pubertal gilts were excised in a local abattoir (Olymel; St. Esprit, QC) and transported to the laboratory in warm (32-35ºC) saline solution (0.9% NaCl;S5886) supplemented with 100 IU/mL penicillin and 10 mg/mL streptomycin (P4333). Follicular contents from follicles 3-6 mm in diameter were aspirated using a 20G needle and a 10 mL syringe, and centrifuged at 300 revolutions per minute (rpm) for 3 minutes. Twice, the supernatant was discarded and replaced with warm HEPES-buffered TCM 199 (Life Technologies, Burlington, ON) supplemented with 1% porcine follicular fluid (pFF), then centrifuged to pellet the COCs. Groups of 25 COCs were cultured in 90 µL IVM1 drops, under mineral oil (equilibrated to 5% $CO_2$ and 95% air at 38.5ºC), for 22h. This maturation media consisted of TCM 199 supplemented with 0.1 µg/mL cystein, 0.91mM sodium pyruvate, 3.05 mM D-glucose, 10 ng/mL epidernal growth factor (Life Technologies), 20 µL/mL gentamicin (Life Technologies), 20% pFF, 0.5 µg/mL follicle stimulating hormone (FSH; SIOUX Biochemical Inc., IA, United States)), 0.5 µg/mL luteinizing hormone (LH; SIOUX Biochemical Inc.) and 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP). COCs were then incubated in equilibrated 90 µL IVM2 drops (without FSH, LH or dbcAMP), under mineral oil, for another 24 hours.

### *4.3.3. Parthenogenic activation (PA)*

Oocytes were denuded by vigorous mixing in HEPES-buffered TCM 199 supplemented with 0.1% hyaluronidase (H3506), for 2-3 mins, then rinsed in manipulation media (consisting of TCM 199 supplemented with 2 mg/mL fatty acid free bovine serum albumin (BSA-FAF; A6003) and 2 µL/mL gentamicin). To execute parthenogenic activation (PA), oocytes were first exposed to 15 µM ionomycin (I0634; in TCM 199

supplemented with 2 mg/mL BSA and 2 µL/mL gentamicin) for 5 minutes, rinsed in TCM 199 supplemented with 3 mg/mL BSA-FAF and 2 µL/mL gentamicin, and finally incubated in $Ca^{2+}$-free porcine zygote medium (PZM-3) [218], supplemented with 10 nM strontium chloride (255521), 7.5 µg/mL cytochalasin B (C6762), and 10 µg/mL cyclohexamide (C1988), for 4 hours, as previously described [219].

### *4.3.4. Embryo culture*

Following PA, embryos were rinsed in equilibrated IVC media (composed of PZM-3 supplemented with 5 mM hypotaurine (H1384), 1 mM glutamine (G8540) and 3 mg/mL BSA-FAF), and incubated in equilibrated 60 µL IVC drops under mineral oil, in 5% $CO_2$ and 95% air at 38.5ºC. Embryo cleavage was assessed at 48 hours. On day 5, embryos were “fed” by replacing 30 µL of each IVC drop with 30 µL of fresh IVC supplemented with a final concentration of 10% fetal bovine serum.

### *4.3.5. Vitamin D3 supplementation*

VD metabolites can be found in FF, therefore to eliminate the possibility of other VD metabolites competing for the VDR, the concentration of VD3 present in the IVM media was completely defined by substituting the pFF with 0.05% polyvinyl alcohol (PVA; P8136) when supplementing COCs with VD3 during IVM (46 hours). Matured oocytes were then subjected to PA and standard IVC (as previously described). On the other hand, the embryos supplemented with VD3 during IVC (7 days) were incubated in standard IVM media (as previously described), subjected to PA and then supplemented during IVC. These embryos received fresh VD3 supplementation when “fed” on day 5 (as previously described). Vitamin D3 (D1530) was diluted in dimethyl sulfoxide (DMSO; D5879) and serially diluted to 100 nM with IVM or IVC. Control media was supplemented with DMSO (vehicle) diluted 1:1000 in IVM or IVC.

### *4.3.6. RNA extraction*

Total RNA was extracted from groups of 60 COCs, CCs and oocytes (also from groups of 60 COCs) using TRIzol® (Life Technologies, 15596026) according to manufacturer’s instructions for cells grown in suspension. The quality and quantity of RNA from each

sample was measured using a DS-11 FX+ spectrophotometer (DeNovix, Wilmington, DE, USA), and 250 ng of total RNA from each sample was treated with DNase (Life Technologies, 18068015) and reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, 1708891). Total RNA was extracted from groups of 10 embryos on D3, D5 and D7 of development using the PicoPure RNA isolation kit (Life Technologies, KIT0202). The entire volume of RNA extracted was treated with DNase (Qiagen, Hilden, Germany, 79254) and reverse transcribed using the Superscript® VILO™ cDNA Synthesis Kit (Life Technologies, 11754050).

### *4.3.7. Quantitative real-time PCR (qRT-PCR)*

Reactions were carried out in a final volume of 10 µL (containing 250 nM of each primer (*Table 1*) and SYBR (Wisent Bioproducts, St-Bruno, QC) using a CFX384 Real-Time detection system (Bio-Rad). The qRT-PCR was performed with a hot-start denaturation step at 95°C for 5 minutes, followed by 40 cycles at 95°C for 15 seconds and 62°C for 30 seconds. Samples were run in duplicates and melting-curve analysis was used to verify the specificity of reaction products. The ΔΔCt method was used to determine the abundance of mRNA for each gene, and expression was normalized to the mean abundance of β actin or H2A housekeeping genes. Values are expressed as a mean of the values from 3-4 biological replicates ± SEM. All reactions used for quantification had efficiency levels between 92% and 112% (coefficient of determination [$R^2$] ≥ 0.98) and slope values from −3.6 to −3.1. The PCR products were loaded onto a 1.5% agarose gel and a UV transilluminator was used to visualize the ethidium bromide staining.

| Target gene | Primer Sequence | Accession Number |
|---|---|---|
| VDR | F: 5'-AAG-GCA-GGC-AGG-AGA-AAT-AG-3'<br>R: 5'-GAA-GAA-GGA-AGA-TCC-CAC-CAG-3' | XM_021091108.1<br>XM_021091109.1 |
| Beta actin | F: 5'-GCA-GAT-GTG-GAT-CAG-CAA-GC-3'<br>R: 5'-GAA-TGC-AAC-TAA-CAG-TCC-GCC-3' | XM_003124280.5 |
| H2A | F: 5'-GGT-GCT-GGA-GTA-TCT-GAC-CG-3'<br>R: 5'-GTT-GAG-CTC-TTC-GTC-GTT-GC-3' | XM_001927727.2 |

*Table 1*: **Primers used for quantitative real-time PCR.** Forward (F) and Reverse (R) primers were each diluted to 2.5 µM and combined for each target gene.

### *4.3.8. Immunofluorescence analysis*

Immunofluorescence staining was used to characterize the localization of VDR protein in porcine COCs, maturing oocytes, and developing embryos. Samples were fixed in 4% formalin (HT501128) for 15 minutes, permeabilized in 1% Triton X-100 (T8787) in PBS (Life Technologies, Carlsbad, CA, USA, 21600) and stored at 4°C until immunofluorescence analyses could be performed on all replicates simultaneously, to reduce experimental variability in fluorescence staining. Oocytes and embryos were then rinsed in a blocking solution (consisting of 3% BSA (fraction 5; Roche, Basel, Switzerland, 10775835001) with 0.5% Tween-20 (P1379) in PBS; 3 x 30 minutes), and incubated overnight at 4ºC with a primary antibody against rabbit VDR (abcam; ab134826; diluted 1:50). The following morning, samples were rinsed in blocking solution (3 x 30 minutes), and subsequently incubated in the dark with anti-rabbit Alexa Fluor 488 (Life Technologies, A-11008; diluted 1:2000) secondary antibody for 50 minutes. Samples were then rinsed in blocking solution (2 x 30 minutes), counterstained with 10 µL/mg DAPI (Life Technologies, D3571) for 20 minutes, rinsed in blocking solution for 30 minutes, and finally, mounted on microscope slides using a drop of warm Mowiol® (10852). Fluorescence was detected using a Nikon Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY, USA) and images were captured at 200x magnification using a Retiga 2000R monochrome digital camera (Qimaging, Surrey, BC) and Simple PCI Imaging Software (Compix, Inc., Sewickly, PA, USA)

### *4.3.9. VDR silencing via RNA interference*

To verify the role of the VDR during early embryo development, we used RNA interference to knockdown its expression from the MII stage. To optimize target binding efficiency, the porcine VDR mRNA sequence (NCBI Reference Sequence: NM_001097414.1) was transcribed and translated into an amino acid sequence (using an online tool available at: http://www.attotron.com/cybertory/analysis/trans.htm) and then compared to the porcine VDR protein (NCBI Reference: NP_001090883.1) to determine the protein coding region (sequence positions 127-1440). Dicer-substrate short interfering RNAs (DsiRNAs) were designed using the Custom DsiRNA Design Tool and synthesized by Integrated DNA Technologies (Coralville, IA, USA). Specificity was

confirmed using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD, USA). Matured oocytes were microinjected with approximately 10 pL of 25 μM diluted sense and antisense DsiRNAs that targeted positions 742-767 and 1258-1283 within the mRNA of VDR (VDR1/2 respectively) or a negative control (scrambled sequences; Table 2). Microinjection was performed in manipulation media (previously described) using an inverted Nikon microscope (Nikon, Tokyo, Japan) that was equipped with a micromanipulator system (Narishige International, Long Island, NY, USA) and FemtoJet 4i (Eppendorf, Hamburg, Germany). For each treatment, 150 oocytes were injected, incubated for 75 mins, subjected to PA, and cultured *in vitro*. Cleavage was assessed at 48 hours. On day 3, 10 embryos were collected to assess VDR knockdown efficacy by qRT-PCR (using the primers described in *Table 1*). Embryos were “fed” on day 5 and cultured until day 7 to evaluate blastocyst development and count cell numbers. Three independent replicates were conducted.

| Target | Sense (5’-3’) | Antisense (5’-3’) |
|---|---|---|
| VDR (1) | GAUCUGAGCGAAGAAGACUCUGATG | CAUCAGAGUCUUCUUCGCUCAGAUCCA |
| VDR (2) | CUGCUCUACGCCAAGAUGAUCCAGA | UCUGGAUCAUCUUGGCGUAGAGCAGGU |
| Negative Control | CGUUAAUCGCGUAUAAUACGCGUAT | AUACGCGUAUUAUACGCGAUUAACGAC |

*Table 2*: **List of DsiRNAs used for knockdown experiments.**

### *4.3.10. Statistical analyses*

Statistical analyses were carried out using JMP, version 13.0 (SAS institute Inc.). VDR expression and cell number data was analyzed by one-way ANOVA, followed by the Student-t test, whereas cleavage and blastocyst rates were analyzed by Chi-Square followed by LS means. $P<0.05$ was considered statistically significant.

## 4.4. Results

### *4.4.1. VDR expression in porcine cumulus-oocyte complexes*

To verify if VDR is expressed during porcine oocyte maturation, we evaluated VDR transcripts by qRT-PCR, and protein presence and localization in COCs by immunofluorescence staining (Fig. 1). The relative mRNA abundance of VDR did not significantly change during chemically defined IVM (46 hours) of porcine COCs (Fig. 1A). In addition, immunofluorescent signals revealed the presence of VDR protein in the CCs and on the nuclear envelope of immature porcine oocytes (Fig. 1B).

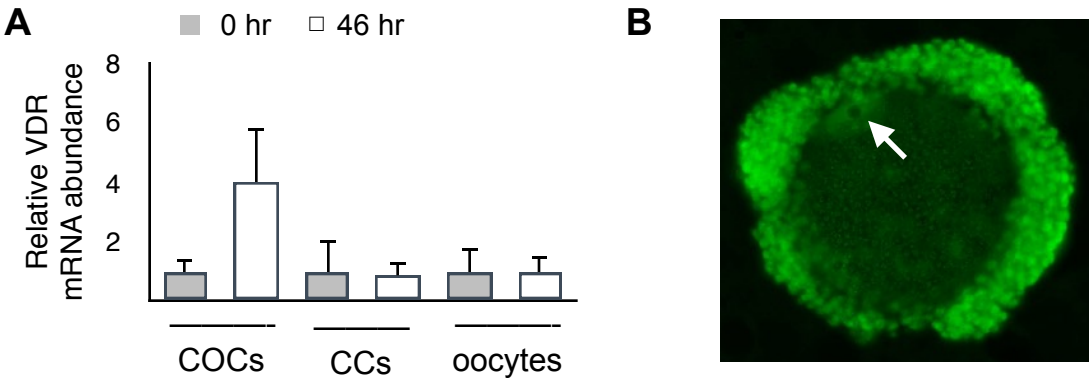


*Figure 1:* **VDR expression in porcine cumulus-oocyte complexes (COCs). (A)** Relative mRNA expression was compared between samples (n = 60/treatment) collected before (0 hours) and after (46 hours) *in vitro* maturation (IVM) of porcine cumulus oocyte complexes (COCs) in a chemically defined media. Oocytes were denuded of their cumulus cells (CCs) either before or after IVM and samples were processed separately. Values are presented as means of VDR mRNA expression corrected to beta-actin housekeeping gene expression from 3 replicates ± SEM. For each group (COCs, CCs, oocytes) the mRNA abundance after IVM is shown as the relative change from before IVM. **(B)** Representative immunofluorescence staining of a COC with clear VDR protein expression in CCs surrounding the oocyte and around the nuclear envelope in the oocyte (arrow).

### *4.4.2. Effect of VD3 supplementation on VDR expression during oocyte maturation and embryo culture*

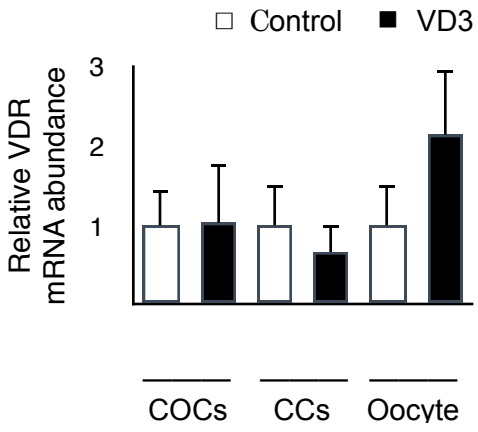


*Figure 2:* **Effect of VD3 supplementation on VDR expression during *in vitro* maturation (IVM) of porcine oocytes.** VDR mRNA expression following 100 nM VD3 or vehicle (control) supplementation during *in vitro* maturation (IVM; 46 hours) in a chemically defined media (n = 60/ treatment). Oocytes were denuded of their CCs at sample collection and the different cell samples were processed separately. Values are presented as means of VDR mRNA expression, corrected to beta-actin housekeeping gene expression, from 3 replicates ± SEM. For each group (COCs, CCs, oocytes), the mRNA abundance after VD3 supplementation is shown as the relative change from control.

To evaluate if VD3 regulates VDR expression during oocyte maturation and embryo development, the relative mRNA abundance of VDR was assessed by qRT-PCR, in oocytes and embryos that were cultured *in vitro* in the absence or presence of 100 nM VD3 supplementation. Under chemically defined conditions (without VD metabolites present in the follicular fluid), there was no change in VDR expression following 100 nM VD3 supplementation during IVM (46 hours; Fig. 2). When we evaluated VDR mRNA expression during embryo development (D3-7), there was a significant (42%) decrease in VDR abundance between D3-D5 control embryos. On the other hand, VD3 supplementation significantly enriched VDR expression by 1.6 fold in D7 embryos (Fig. 3A). The latter seems to be in line with our immunofluorescence staining results, which confirm that VDR protein is found in both the cytoplasm and the nucleus on D3 and D5 of development, but is predominantly nuclear localized in D7 embryos (Fig. 3B).

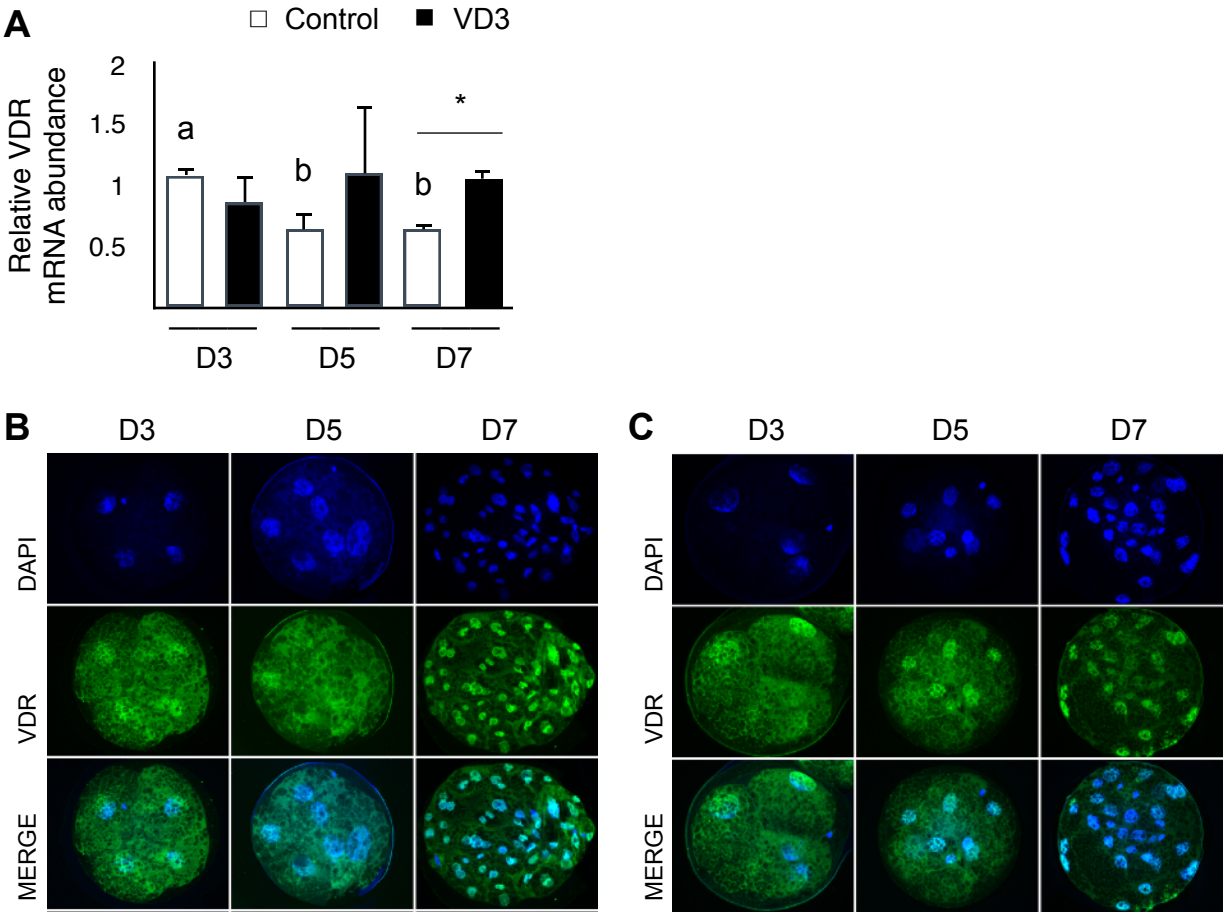


*Figure 3*: **Effect of VD3 supplementation during IVC on VDR mRNA expression and nuclear localization in pre-implantation porcine embryos. (A)** VDR mRNA expression in PA embryos on day 3, 5 and 7 of *in vitro* culture in the absence or presence of VD3 (100 nM). Total RNA was extracted from 10 embryos from each treatment and stage of development. Values are presented as means of VDR mRNA expression corrected to H2A housekeeping gene expression from 3 replicates ± SEM. Different letters (a,b) represent statistical difference between days within the same treatment, whereas the (*) represents statistical difference between treatments in the same day of embryo development. $P<0.05$ was considered statistically significant. **(B)** Representative immunofluorescence staining of embryos supplemented with vehicle (control; DMSO) on D3, D5, and D7. **(C)** Representative immunofluorescence staining of embryos supplemented with VD3 on D3, D5, and D7. Embryos were immunostained to detect VDR and counterstained with DAPI. Original magnification 200x.

### *4.4.3. Effect of VD3 supplementation on pre-implantation embryo development*

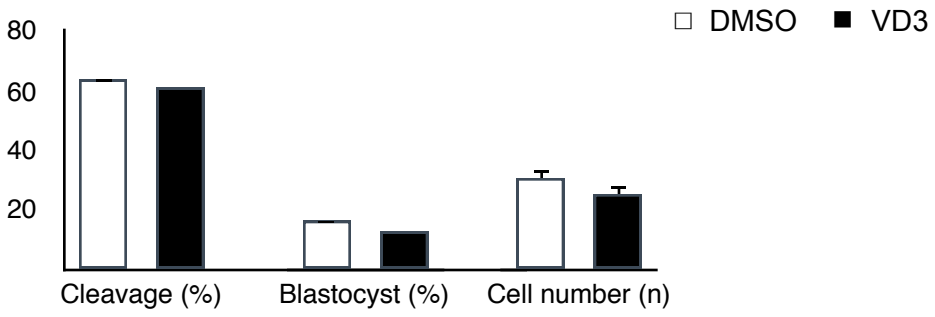


*Figure 4*: **Effect of VD3 supplementation during IVM on pre-implantation embryo development**. Porcine COCs were matured in vitro in a completely defined media with vehicle (DMSO) or VD3 supplementation (100 nM), and then subject to PA. The respective numbers of oocytes and embryos used in the control and VDR-treated groups were 242 and 244 to evaluate cleavage, 153 and 149 to evaluate development to the blastocyst stage, and 33 and 14 to count number of cells. Values are presented as means from 4 replicates ± SEM.

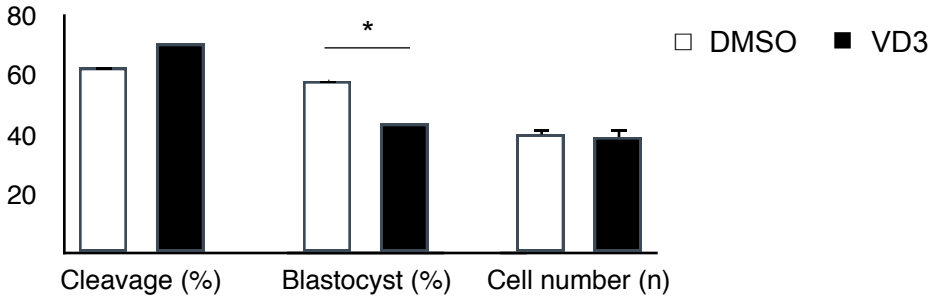


*Figure 5*: **Effect of VD3 supplementation during IVC on pre-implantation embryo development**. Porcine COCs were cultured in standard IVM media, subjected to PA and then cultured in vitro with vehicle (DMSO) or VD3 supplementation (100 nM) for 7 days. The respective numbers of oocytes and embryos used in the control and the VDR-treated groups were 202 and 209 to evaluate cleavage, 125 and 147 to evaluate development to the blastocyst stage, and 63 and 68 to count number of cells. Values are presented as means from 3 replicates ± SEM. The asterisk (*) indicates significant difference ($P<0.02$) between treatments.

To assess whether VD3 supplementation affects embryo development, we subjected mature porcine oocytes to PA, and then evaluated embryo cleavage at 48 hours, and development to the blastocyst stage on day 7. Finally, embryos were fixed to examine embryo quality, by counting the number of cells per embryo. Under our conditions, VD3 supplementation during IVM had no significant effect on any of these parameters (Fig. 4). On the other hand, VD3 supplementation during IVC had no effect on embryo cleavage or quality, but significantly impaired blastocyst development by 15% ($p<0.05$) compared to controls (Fig. 5).

### *4.4.4. Effect of VDR knockdown on embryo development and quality*

To elucidate the involvement of the VDR in early embryo development, we used dicer substrate small interfering RNAs (DsiRNAs) to specifically knockdown its expression. Scramble sequence siRNAs (control; siCT) or VDR siRNAs (siVDR) were injected into the ooplasm of mature oocytes prior to PA, and embryos were cultured *in vitro* for 7 days. We observed 81% knockdown of VDR mRNA by qRT-PCR in day 3 embryos that were injected with siVDR compared to siCT (Fig. 6A). VDR attenuation had no effect on embryo cleavage (at 48 hours), but significantly impaired development to the blastocyst stage by 46% ($p<0.01$), and reduced embryo cell numbers by 33% compared to controls ($p<0.05$; Fig. 6B). The proportion of the cleaved embryos that arrested at earlier stages of development (those presenting less than 8 nuclei) was higher in the siVDR compared to the siCT group, while the proportion of embryos presenting 9-14 and over 15 nuclei were higher in the siCT compared to the siVDR groups (Fig. 6C). Furthermore, blastocysts that developed from siVDR injected embryos were morphologically smaller and less expanded than those from the siCT group (Fig. 6D-E).

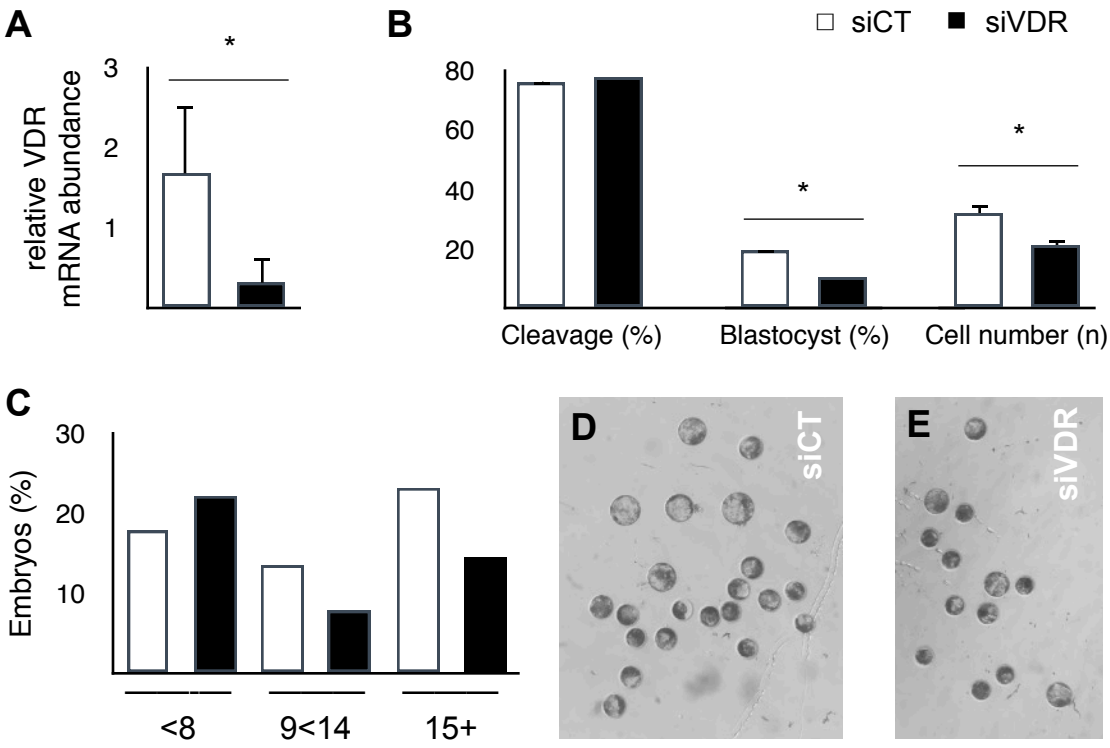


*Figure 6:* **Effect of VDR attenuation on early embryo development. (A)** Relative mRNA abundance of VDR in day 3 embryos following intraooplasmic injection of siRNA. Total RNA was collected from groups of 10 day-3 embryos injected with siCT or siVDR. Values are presented as means of VDR mRNA expression corrected to H2A housekeeping gene expression from 3 replicates ± SEM. **(B)** The respective number of oocytes injected with control (siCT) or VDR (siVDR) DsiRNA sequences and used to evaluate cleavage were 446 and 456, 278 and 293 to evaluate development to the blastocyst stage, and 43 and 27 to count number of cells. Values are presented as the mean from 3 replicates ± SEM. **(C)** Proportion of embryos that had different number of cells (<8 cells, 9<14 cells or 15+ cells) at day 7 of culture, after injection of siCT (n = 102) or siVDR (n = 83) DsiRNA sequences. **(D-E)** Representative images of embryos that developed to the blastocyst stage at day 7 in the siCT and siVDR groups. The asterisks (*) indicate significant difference ( $P < 0.04$) between treatments.

## 4.5. Discussion

VD deficiency has a global impact on all age groups, and plays an important role in reproduction due to its ability to regulate various biological processes [1]. In 2011, 41% of Canadians of reproductive age were VD deficient, and 1 in 3 Canadians were taking VD supplements [31]. To gain additional insights into the role of VD in the reproductive system, this study used a well-defined *in vitro* porcine model to evaluate the effect of VD during oocyte maturation and early embryo development. The expression profile of VDR was initially characterized, at both the mRNA and protein levels, and confirmed that VDR is expressed in cumulus cells, oocytes and in early developing embryos, which suggests that VD may play important roles in regulating oocyte maturation and early embryo development.

This study has next evaluated if exposure, of COCs during IVM or embryos during IVC, alters embryo development and quality. For this, we used a VD3 concentration (100 nM) that was previously used in GC culture studies *in vitro* [140] and is in the top range of physiological levels for humans [217]. Although measuring circulating VD levels may not always reflect bioavailable VD levels in a given tissue since only <1% of VD is free and the remaining ~99% is bound to VD binding protein (VDBP) or to albumin [30], sVD has been positively correlated to VD levels in FF [85, 87, 92]. While some investigators reported that women with VD3-deficient FF (~40 nM) produced larger follicles and had higher levels of estradiol following ovarian stimulation [152], others found that VD and estradiol levels have been positively correlated in GCs *in vitro* [149] and in women *in vivo* [153]. In fact, Corduk et al. (2012) suggest that VD/VDR signalling increases both estrogen and its receptors (ERα/β), and this consequently has a more profound effect on SMAD protein decrease in adult rats versus newborn rats [154]. SMAD transcription factors are downstream of the anti-müllerian hormone (AMH) signalling pathway. By binding directly to SMAD3 and competing for its binding elements [139], VDR can impede with AMH signalling, and as such, VD encourages the differentiation and development of human GCs [76, 140, 142] and increases FSHR mRNA in hen GCs [144]. Similarly, other studies have shown that VD3 promoted follicle survival and growth [134, 145]. Based on those previous reports, we hypothesized that VD

supplementation during IVM would have a positive impact on oocyte developmental potential. However, our findings indicated that under defined conditions used in this study, VD3 supplementation during IVM of porcine COCs had no significant effect on VDR expression, developmental capacity and embryo quality. To eliminate the possibility of other VD metabolites in the FF competing for the VDR, as previously demonstrated in other studies [220], we replaced pFF with PVA [219] to completely define the amount of VD available to the oocytes in the IVM media. However, the use of a completely defined IVM medium may have accounted for the overall low embryo developmental rates observed in the first experiment.

To our knowledge, no previous studies have investigated the effect of VD3 supplementation during embryo culture. It has been demonstrated that women with sufficient VD in their serum and FF are far more likely to achieve and maintain clinical pregnancy following IVF, than VD-deficient women [26, 89, 96, 221]. However, sVD levels at ovulation did not correlate with pregnancy outcomes [28]. In contrast to those clinical observations, our results indicated that supplementing embryos with VD3 during IVC upregulated VDR mRNA expression in D7 blastocysts and reduced embryo development to the blastocyst stage. Although further studies are required to elucidate the mechanism by which VD3 decreased embryo development, many possibilities may be envisaged. For instance, by contributing to sustained elevated levels of intracellular calcium (through alkaline phosphatases [187] and store-operated calcium (SOC) channels [6]), VD3 can potentially induce stress responses which may quickly result in cell cycle arrest to allow for repair of DNA damage and/or programmed cell death to protect the genome. Additionally, VD3 can inhibit proliferation by targeting many components of the G1/S transition of the cell cycle (e.g., c-Myc, CDK inhibitors (CDKIs) like p21 and p27, as well as cyclin proteins), and consequently induce G1 cell cycle arrest [12, 15, 22, 182]. In fact, a previous study showed that MCF7 cell proliferation was reduced by 50% when treated with VD for 72 hours [7].

Finally, this study has evaluated if VDR is required for regulation of embryo development. Although it remains unclear whether the anti-proliferative effects of VD are

lost [9] or maintained [7] when the VDR is absent, there is evidence that VDR ablation significantly increases p53 [222] and overactivates p53 upregulated modulator of apoptosis (PUMA) [223]. Our findings indicated that attenuation of VDR expression by DsiRNAs hindered development to the blastocyst stage and decreased embryo quality, as determined by the reduction in number of cells per embryo. Classically, it was thought that VD signals solely through the VDR, however, there is now evidence that VD3 signalling acts through several levels of complexity, both genomically [2-5] and non-genomically [6-8]. VD/VDR directly and indirectly regulates the expression of many genes, and furthermore, VD3 and VDR individually are involved in crosstalk between multiple signalling pathways, producing a plethora of cellular responses [9].

In conclusion, findings from this study revealed that: i) VDR is present in porcine COCs and throughout early-stages of embryo development; ii) VD3 supplementation (100 nM) during IVM is not toxic for porcine oocytes and does not perturb their developmental capacity; iii) VD3 supplementation (100 nM) during IVC upregulates VDR expression but hinders blastocyst development, and iv) attenuation of VDR expression decreases early embryo development and quality. Further studies are required to elucidate the molecular mechanisms of VD and VDR during early embryonic development.

## 4.7. Acknowledgments

This study was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada. The authors thank Olymel S.E.C./L.P of Saint-Esprit, QC, Canada for providing porcine ovaries for use in these experiments, as well as Drs. Raj Duggavathi and Sarah Kimmins for the use of their laboratory equipment and facilities.

# CHAPTER 5: CONCLUSION

There is growing interest in the role of VD in reproduction, specifically how it impacts success rates of assisted reproductive technologies. This study characterized the role of VD, and its receptor, VDR, during oocyte maturation and embryo development using a well defined *in vitro* porcine model. Findings from this study revealed that: i) VDR is present in porcine COCs and throughout early-stages of embryo development; ii) VD3 supplementation (100 nM) during IVM is not toxic for porcine oocytes and does not perturb their developmental capacity; iii) VD3 supplementation (100 nM) during IVC upregulates VDR expression but hinders blastocyst development, and iv) attenuation of VDR expression decreases early embryo development and quality. Further studies are required to elucidate the molecular mechanisms of VD and VDR during early embryonic development.

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