Role of the Vitamin D Receptor in the Regulation of Early Porcine Embryonic Development

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Abstract

Recently, there has been a major demand and expansion of embryo-related technologies applied to both human and animal reproduction. This demand requires new technologies that may mitigate fertility issues within human reproductive clinics as well as within livestock production. To achieve this, a deeper understanding of the factors influencing fertility and the success rate of these technologies is imperative. A known metabolic player involved in various reproductive processes is vitamin D (VD3) and its receptor, the vitamin D receptor (VDR). VDR plays several roles in various signaling pathways and acts as a transcriptional regulator. Yet, despite its significance, the exact role of VD and VDR during early embryo development, a critical stage when many embryos fail to develop correctly, remains unclear. The primary objective of this study was to investigate the role of VDR in early porcine embryonic development using *in vitro* culture as the research model. First, qRT-PCR was used to characterize the mRNA expression profile of *VDR* in oocytes and during early stages of embryo development. These experiments revealed that *VDR* mRNA was expressed in porcine oocytes and embryos at different developmental stages, and its relative abundance was consistent throughout all developmental stages. Second, knockdown of *VDR* mRNA using microinjection of dicer-substrate short interfering RNAs (DsiRNAs) into MII stage oocytes was applied to establish if *VDR* mRNA had an important regulatory role in early embryonic development. Results indicated that *VDR* mRNA attenuation did not lead to any changes in embryo cleavage, blastocyst development, or embryo quality, as assessed by the total number of cells per blastocyst. Finally, the effects of supplementing 100 nM VD3 during *in vitro* culture (IVC) of both *VDR*-attenuated and control (non-attenuated) embryos was

investigated. Findings revealed that VD3 supplementation did not have a significant impact on embryo cleavage, development to the blastocyst stage, or embryo quality. Collectively, findings from this study suggest that neither VD nor *VDR* play a critical regulatory role required for early development of porcine embryos.

Resumé

Récemment, il y a eu une demande et une expansion majeures des technologies liées à l'embryon appliquées à la fois à la reproduction humaine et animale. Cette demande nécessite de nouvelles technologies susceptibles d'atténuer les problèmes de fertilité dans les cliniques de reproduction humaine ainsi que dans les chaînes de production animale. Pour y parvenir, une compréhension plus approfondie des facteurs influençant la fertilité et le taux de réussite de ces technologies est impérative. Un acteur métabolique connu impliqué dans divers processus de reproduction est la vitamine D (VD3) et son récepteur, le récepteur de la vitamine D (VDR). Le VDR joue plusieurs rôles dans diverses voies de signalisation et agit comme un régulateur transcriptionnel. Pourtant, malgré son importance, le rôle exact du VD et du VDR au cours du développement précoce de l'embryon, une étape critique où de nombreux embryons ne se développent pas correctement, reste incertain. L'objectif principal de cette étude était d'étudier le rôle du VDR dans le développement embryonnaire porcin précoce en utilisant la culture *in vitro* comme modèle de recherche. Premièrement, la qRT-PCR a été utilisée pour caractériser le profil d'expression de l'ARNm du *VDR* dans les ovocytes et au cours des premiers stades du développement de l'embryon. Ces expériences ont révélé que l'ARNm de *VDR* était exprimé dans les ovocytes et les embryons porcins à différents stades de développement, et que son abondance relative était constante à tous les stades de développement. Deuxièmement, l'inactivation de l'ARNm de *VDR* à l'aide de la micro-injection d'ARN interférents courts (DsiRNA) dans des ovocytes au stade MII a été appliquée pour déterminer si le *VDR* avait un rôle régulateur important dans le développement embryonnaire précoce. Les résultats ont indiqué que l'atténuation de

l'ARNm du *VDR* n'entraîné aucun changement dans le clivage de l'embryon, le développement du blastocyste ou la qualité de l'embryon, tel qu'évalué par le nombre total de cellules par blastocyste. Enfin, les effets de la supplémentation de 100 nM VD3 pendant la culture *in vitro* (IVC) des embryons atténués par VDR et contrôle (non atténués) ont été étudiés. Les résultats ont révélé que la supplémentation de VD3 n'avait pas d'impact significatif sur le clivage de l'embryon, le développement jusqu'au stade de blastocyste ou la qualité de l'embryon. Pris ensemble, les résultats de cette étude suggère que le VD et le VDR ne semblent pas jouer un rôle régulateur essentiel requis pour le développement précoce des embryons porcins.

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Vilceu Bordignon designed the experiments, reviewed the thesis and supervised the primary author. Vanessa Guay conducted the experiments, analyzed the data and wrote the thesis. Werner G. Glanzner trained Vanessa Guay. Mariana Priotto de Macedo, Karina Gutierrez, Luke Currin, Maria Elena Carrillo Herrera, and Zigomar Da Silva helped with oocyte collections, some training and media preparation on a weekly basis.

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Abbreviations List

AKT: Serine/threonine protein kinase; protein kinase B (PKB)

AMH: Anti-Müllerian hormone

ATG16L1: Autophagy related 16 like 1

BSA: Bovine serum albumin

CaMKII: Calmodulin-dependent protein kinase II

cAMP: Cyclic adenosine monophosphate

CC: Cumulus cell

CDK: Cycline-dependant kinase

cDNA: Complementary DNA

cKO: conditional knockout

c-Myc : Myc proto-oncogene

COC: Cumulus-oocyte complex

CYP19A1: Cytochrome P450 Family 19 Subfamily A Member 1

CYP2R1: Cytochrome P450 Family 2 Subfamily R Member 1

CYP27B1: Cytochrome P450 Family 27 Subfamily B Member 1

DAPI: 4,6-Diamidino-2-phenylindole

DNA: Deoxyribonucleic acid

DsiRNA: Dicer-substrate short interfering RNAs

E2: Estradiol

EGA: Embryonic genome activation

EGF: Epidermal growth factor

ER: Endoplasmic reticulum

ERK: Extracellular-signal-regulated kinase

FBS: Fetal bovine serum

FF: Follicular fluid

pFF: Porcine follicular fluid

FoxO: Forkhead box O

FSH: Follicle stimulating hormone

GC: Granulosa cell

GV: Germinal vesicle

ICM: Inner cell mass

IVC: *In vitro* culture

IVF: *In vitro* fertilization

IVM: *In vitro* maturation

LH: Luteinizing hormone

MI/MII: Meiosis I/II

MAPK: Mitogen activated protein kinase

mRNA: Messenger RNA

mTOR: Mammalian target of Rapamycin

P4: Progesterone

p21: Cyclin dependant kinase inhibitor 1A

p27: Cyclin dependant kinase inhibitor 1B

PA: Parthenogenic activation

PBS: Phosphate-buffered saline

PCOS: Polycystic ovary syndrome

PI3K: Phosphatidylinositol 3-kinase

PKB: Protein Kinase B (AKT)

PKC: Protein Kinase C

PKD: Protein Kinase D

PLA2: Phospholipase A2

PLC ζ: Phospholipase C zeta

PTEN: Phosphatase and tensin homolog

PZM-3: Ca^{2+}-free porcine zygote medium

qPCR: Quantitative Polymerase Chain Reaction

Rb: Retinoblastoma

RFP: Red fluorescent protein

rpm : Revolutions per minute

RNA: Ribonucleic acid

RXR: Retinoid X receptor

siRNA: Small interfering RNA

sncRNA: Small non-coding RNA

sVD: Serum vitamin D

TCM199: Tissue culture media 199

TF: Transcription factor

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

Wnt: Wingless-type MMTV integration site family

ZP: Zona pellucida

CHAPTER 1: INTRODUCTION

Currently, embryo-related technologies have become a focal expansion point in regards to their use in both human and animal reproduction. This expansion in the use of new technologies aims to mitigate fertility issues in human reproductive clinics, as well as accelerate livestock selection and production. To develop these novel and more efficient technologies, a better understanding of the factors that regulate embryonic development and limit fertility is essential. One important player in various reproductive processes is vitamin D (VD) and its functional receptor, the vitamin D receptor (VDR) (Ozkan et al., 2010; Luk et al., 2012; Garbedian et al., 2013; Paffoni et al., 2014; Neville et al., 2016). VDR is known to regulate numerous genes and plays diverse roles in various signaling pathways, modulating transcription, and controlling cell cycle progression. VDR has been associated with over 3000 genes in the human genome (Haussler et al., 2011), including several relating to the regulation of cell cycle progression (Brennan et al., 1987; Sheikh et al., 1995; Liu et al., 1996; Wang et al., 1996; Freedman, 1999; Accili and Arden, 2004; Adler, 2010; An et al., 2010; Pan et al., 2010; Toropainen et al., 2010; Matsuda et al., 2011; Salehi-Tabar et al., 2012; Yang et al., 2013; Li et al., 2014; Al-Hendy et al., 2016; Yu and Cui, 2016; Chen et al., 2018; Liao et al., 2018; Bhoora and Punchoo, 2020; Shariev et al., 2022). Additionally, VDR has anti-proliferative (Logan and Nusse, 2004; MacDonald et al., 2009; Ma et al., 2010; Vuolo et al., 2012; Feldman et al., 2014; Johnson et al., 2015; Al-Hendy et al., 2016; Tribulo et al., 2017; Zheng et al., 2018; Muralidhar et al., 2019; Rubin et al., 2020), pro-differentiative (Wang et al., 2009), and apoptotic functions (Narvaez and Welsh, 2001; Guzey et al., 2002; Swami et al., 2003; Guzey et al., 2004a; Guzey et al., 2004b; Wood et al., 2004; Suzuki et al., 2006; González-Pardo

et al., 2014; Shariev et al., 2022). Yet, the exact roles of VD and VDR during early embryonic development remain to be fully elucidated.

This research aimed to investigate the role of VD and VDR during early embryonic development using well-established porcine *in vitro* culture as the research model. Firstly, the expression profile of *VDR* mRNA in oocytes and during various stages of early embryonic development was characterized. Secondly, the study examined the impact of attenuating *VDR* mRNA on embryo cleavage, development and quality. Subsequently, an improved method for *VDR* attenuation was developed based on the co-injection of dicer-substrate short interfering RNAs (DsiRNAs) and red fluorescent protein (RFP) mRNA. Furthermore, supplementation of VD3 during embryo culture was used to explore its impact on *VDR* expression, embryo cleavage, development, and quality. Additionally, VD3 supplementation was tested in embryos with attenuated *VDR* mRNA to investigate the impact on embryo development and quality.

This thesis provides a comprehensive literature review on fertilization and early embryonic development, along with insights into VD and VDR functions and signalling. This is followed by methodology, research findings and a discussion.

CHAPTER 2: LITERATURE REVIEW

2.1 Oogenesis, Fertilization, Embryonic Genome Activation and Embryo Development

The oocyte undergoes multiple maturation steps prior to fertilization, including recruitment from the ovarian reserve, selection, cytoplasmic and nuclear maturation, and finally, ovulation of the mature oocyte at the metaphase II stage (MII). In mammals, the oocyte is released into the fallopian tube, whereby the uterine tract is also where sperm becomes active, and capacitated (Singh, 2014; Li and Winuthayanon, 2017). In the ampulla of the fallopian tube, the sperm reaches the ovulated oocyte(s) and releases enzymes as to pass through the oocyte's layered protective surrounding of granulosa cells (GCs) (Lin et al., 1994). These "nurse" cells form multiple layers that encompass the oocyte, whereby the inner most layer is connected to the oocyte via transzonal projections, supplying the developing oocyte with essential factors as well as producing hormones (Carlson, 2018). Once bound to the ooplasmic membrane, the sperm releases phospholipase C zeta (PLC ζ) into the oocyte, thereafter triggering calcium oscillations downstream, as calcium channels located in the endoplasmic reticulum (ER) become activated, which allows for meiosis resumption (Sanders et al., 2016; de Macedo et al., 2019). Similarly, chemicals can be used to induce this same calcium-based mechanism and allow for parthenogenic activation (PA) to be induced without the need for sperm, and other chemicals can also be used to inhibit second polar body release, allowing for a diploid parthenote to be produced (Cha et al., 1997; Macháty and Prather, 1998; Che et al., 2007; Lee et al., 2015; de Macedo et al., 2019). After fertilization, the oocyte will extrude a second polar body, and the haploid chromatins of the sperm and oocyte will

decondense and form the paternal and maternal pronuclei (Kim et al., 2011; Singh, 2014). After DNA replication, the membranes of the two pronuclei are disassembled, thus allowing their materials to be conjoined, consequently restoring the diploid chromosome number (Palermo et al., 1997). From this point, the paired chromosomes will be split in the first mitotic division forming a 2-cell embryo (Kim et al., 2011; Singh, 2014). This first division is termed cleavage. Early embryonic development continues past cleavage, whereby the 2-cell embryo eventually becomes a 4-cell embryo and so on as it continues mitotic divisions (Singh, 2014). During this period, the embryo will undergo what is known as embryonic genome activation (EGA), a time whereby the embryo ceases to rely on proteins, RNA, and mRNA transcripts that were provided by the mother in the ooplasm of the oocyte, and begins to transcribe mRNA from its own genome (DeRenzo and Seydoux, 2004; Lee et al., 2014b). In pigs, EGA occurs on approximately day three of development, or between the 4-8 cell stage, which is similar to that of humans (Braude et al., 1988; Oestrup et al., 2009). This transition is a critical period, whereby failure will lead the embryo to its death. A plethora of factors have been identified to regulate this moment in embryo development including epigenetic marks, such as histone acetylation and methylation, and their regulators (Latham, 1999; Aoshima et al., 2015; Duan et al., 2019; Rissi et al., 2019; Glanzner et al., 2020; Bilmez et al., 2021), DNA methylation (Duan et al., 2019), small non-coding RNAs (sncRNAs) (Hamazaki et al., 2015; Bouckenheimer et al., 2017; Karlic et al., 2017; Deng et al., 2018; Cuthbert et al., 2021; Zhang et al., 2022), and several transcription factors (TFs).

Multiple transcription factors are vital for the activation of EGA-associated genes. This includes double homeobox (*Dux*) (De Iaco et al., 2020), and its regulators,

developmental pluripotency associated 2 (*Dppa2*) and 4 (*Dppa4*) (Eckersley-Maslin et al., 2019; Li et al., 2021). Specifically, *DUX4* regulates multiple other TFs that affect gene expression and EGA, one of these being the zinc finger and SCAN domain-containing 4 (*Zscan4*) (De Iaco et al., 2017; Wakabayashi et al., 2021), which is involved in the regulation of genome stability, telomere elongation and cell developmental potency, and is fundamental to EGA processes (Zalzman et al., 2010; Amano et al., 2013; Wakabayashi et al., 2021). The eukaryotic translation initiation factor 1A, X-chromosomal (EIF1AX) is another important regulator of EGA as it is essential for the binding of translational machinery to RNA and proper protein synthesis (Johnson et al., 2017). Another TF that is critical to EGA is *Myc* as it controls ribosomal RNA transcription in early embryos (Pan et al., 2014; Gambini et al., 2020).

Progression of early embryonic development depends on normal EGA regulation which enables subsequent cell cleavages and cell compaction at the morula stage. At this stage, the cells are still contained within the zona pellucida (ZP). The first cell lineage specification becomes evident when embryos reach the blastocyst stage. At this stage, cells found at the inner portion are termed the inner cell mass (ICM) and will give rise to the fetal cells (embryo proper), whilst those found on the outer layer are termed cells of the trophoblast, a structure that will give rise to the fetal membranes (Singh, 2014).

2.2 Vitamin D and the Vitamin D Receptor

Vitamin D (VD), a secosteroid, is known to play a plethora of roles within the human and animal body (Pérez-López, 2007). Although it is most well-known for its role in calcium and phosphorus homeostasis as well as skeletal metabolism, VD participates in

the modulation of innate and adaptive immune function, inflammation, and regulation of other cellular mechanisms including apoptosis, cellular differentiation, and cellular proliferation (Gallieni et al., 2009; Swami et al., 2013; Khammissa et al., 2018).

VD can be found in two different forms, these being ergocalciferol (VD2) and cholecalciferol (VD3). While cholecalciferol can be uptaken via various dietary sources, it is mainly (80%) produced in the epidermis within keratinocytes. When produced by the body, the skin uses solar ultraviolet B radiation for photoconversion in order to transform the inactive 7-dehydrocholesterol, a cholesterol precursor, to VD3 (Chen et al., 2007; Pérez-López, 2007; Gallieni et al., 2009; O'Mahony et al., 2011; Khammissa et al., 2018). Ergocalciferol, the form of VD that has fungal origin, is less common and represents the second form of VD (Gallieni et al., 2009). VD in the aforementioned forms, however, is not active, and is considered a pro-hormone (Pérez-López, 2007). Thus, both VD2 and VD3 require transformation before they become biologically active.

Firstly, when VD enters blood circulation, it binds to its associated binding protein, the vitamin D binding protein (VDBP), where it then travels to the liver (Khammissa et al., 2018). In the case of dietary origin, the VD is absorbed through the intestinal walls and is transported to the liver via chylomicrons within the lymph vessels (Bouillon et al., 1998). Once in the liver, CYP2R1 (vitamin-D-25-hydroxylase) hydroxylates the incoming VD into 25(OH)D, which is transported to the kidney where CYP27B1 (25(OH)D-1alpha hydroxylase) hydroxylates this version into its biologically active form, 1,25(OH)2D3 or calciferol (Khammissa et al., 2018). It is important to note that the second hydroxylation reaction is not reserved to the kidney, however, as other tissues like brain, mammary, hepatic, immune, reproductive (particularly the ovary), and bone tissues also express

CYP27B1. Consequently, it has been suggested that all of these tissues can locally activate the 25(OH)D into its active form (Dimitrov et al., 2014; Bikle et al., 2018). The active form of VD3 circulates throughout the body bound to VDBP and disassociates from this protein once it has reached its target cell, so it can enter (De Pascale and Quraishi, 2014; Martucci et al., 2017). In this way, it can act biologically to induce various responses, often through its related nuclear receptor, the vitamin D receptor (VDR).

The VDR, a ligand-inducible transcription factor, is ubiquitously expressed in almost all tissues and all nucleated cells (Pike, 1991; Bouillon et al., 2008). Subcellularly, VDR can be found in the nucleus, as well as in the cytoplasm of the cell (Prüfer et al., 2000). It is also found in the mitochondria of some cells (Silvagno et al., 2010). Once its ligand binds, the VDR is rapidly phosphorylated, which thereby allows surface conformation changes and reconfiguration, which enables release of corepressors. It then recruits its favoured dimer, the retinoid X receptor (RXR), that it heterodimerizes with in the cytoplasm. This complex is very stable and can direct itself into the nucleus and binds to the vitamin D response element (VDRE) located in the promotor region of genes (Freedman et al., 1994; Haussler et al., 1998). The RXR is required for high affinity binding to the VDRE, as single and homodimerized VDR have quite low affinity for binding (Dowd and MacDonald, 2013). Once bound to the VDRE, the high-affinity transcription factor dimer recruits and interacts with other nuclear proteins like chromatin remodelers and modifiers (Wei et al., 2018), including histone acetyltransferases (Herdick and Carlberg, 2000), histone deacetylases (Herdick and Carlberg, 2000), lysine demethylases (Pereira et al., 2011), histone methyltransferases (Pike and Meyer, 2012), or other co-regulatory protein complexes that interact with the transcriptional machinery and recruit

RNA polymerase II (Lonard and O'Malley, 2006). Therefore, VDR can act to decondense and modulate chromatin, and induce or repress transcription of its target genes (Jin and Pike, 1996; Glass and Rosenfeld, 2000).

VDRE-containing genes can be grouped into multiple categories, including inflammation, immune function, apoptosis, cell differentiation and proliferation (Karras et al., 2018); VDR has been shown to directly induce the expression of more than 200 human genes (NCBI, 2020). The ligand binding domain of VDR is a member of the nuclear receptor superfamily. Impressively, it has been suggested that up to 3% of the human or mouse genome is affected by VD3, either directly or indirectly (Kriebitzsch et al., 2009; Bouillon et al., 2014). Furthermore, VDR can regulate multiple other genes indirectly as it can act through proximal and distal enhancers of such genes (Meyer et al., 2010; Pike et al., 2016). In fact, it has been shown that DNA-bound VDR can function at a distance up to 75kb to regulate gene expression (Kim et al., 2006; Meyer et al., 2010). Consequently, VDR can regulate over 3000 human genes (Haussler et al., 2011). In addition, VDR can act non-genomically, via its ability to mobilize intracellular calcium, and activate various intracellular secondary signalling molecules including adenylyl cyclase, PKC, PKD, PI3K, MAP kinases, CaMKII and PLA2 (Dicken et al., 2012; Hii and Ferrante, 2016). VDR can be found in a plethora of tissues including bones, immune and reproductive tissues (Dicken et al., 2012; Gocek et al., 2012; Eelen et al., 2013). Accordingly, VD sufficiency status is vital to maintain the normal functions in these tissues. However, it has been reported that the prevalence of deficiency (below 50 nmol/L) is 37% in Canada, and 24% in pregnant Canadian women (Palacios and

Gonzalez, 2014). This marks concern as VDR is essential for basic control of cell-life functions.

In many cell types, VDR acts anti-proliferatively by promoting the expression of antiproliferative and proapoptotic genes (Swami et al., 2003; Guzey et al., 2004b; Wood et al., 2004; Suzuki et al., 2006). For example, VDR is shown to modulate FoxOs (Brennan et al., 1987; Accili and Arden, 2004; Adler, 2010; An et al., 2010; Matsuda et al., 2011; Yang et al., 2013; Li et al., 2014), c-Myc (Toropainen et al., 2010; Salehi-Tabar et al., 2012; Liao et al., 2018; Bhoora and Punchoo, 2020), p21 and p27 (Sheikh et al., 1995; Liu et al., 1996; Wang et al., 1996; Freedman, 1999; Bhoora and Punchoo, 2020), PTEN/AKT/mTOR (Pan et al., 2010; Al-Hendy et al., 2016; Yu and Cui, 2016; Chen et al., 2018; Shariev et al., 2022) and Wnt signalling (Logan and Nusse, 2004; MacDonald et al., 2009; Ma et al., 2010; Vuolo et al., 2012; Feldman et al., 2014; Johnson et al., 2015; Al-Hendy et al., 2016; Tribulo et al., 2017; Zheng et al., 2018; Muralidhar et al., 2019; Rubin et al., 2020). VDR can also promote cell differentiation via increasing MAPK/ERK signalling (Wang et al., 2009). In addition, VDR is involved in pro-apoptotic functions and cell death (Narvaez and Welsh, 2001; Guzey et al., 2002; Guzey et al., 2004a; González-Pardo et al., 2014; Shariev et al., 2022).

Since VD and VDR play a multitude of roles in various tissues and cellular processes, it is evidently of interest to better the understanding of its exact roles in various systems including the female reproductive system. Considering that VDR has been identified within multiple female reproductive tissues (e.g., placenta, uterus, and ovary) (Yao et al., 2017), and ablation of the receptor or deficiency of the vitamin has

demonstrated startling results (Kinuta et al., 2000; Dicken et al., 2012), it is important to further characterize its roles in reproductive functions.

2.2.1 VD deficiency and VDR null studies

Multiple studies have attempted to identify the consequences of VD deficiency or VDR nullification on female reproduction, this having been done in a handful of species thus far. For example, mice fed VD deficient diets have demonstrated severe effects on female reproductive capacity, which included arrested follicular development and prolonged estrous cycles, as well as less oocytes collected from their oviducts after administering hormonal stimulation (Dicken et al., 2012). In rats, while females kept on lifelong VD deficient diets were able to reproduce, they experienced a detrimental reduction in fertility (75%) and their litter sizes were significantly smaller than VD replete controls (Halloran and Deluca, 1980).

VDR null mice were shown to experience uterine hypoplasia in addition to impaired folliculogensis, however, simply VD deficient mice did not display uterine hypoplasia (Yoshizawa et al., 1997). Moreover, low uterine weight was corrected by estradiol supplementation, in this case demonstrating a clear link between VD deficiency and estrogen deficiency (Yoshizawa et al., 1997). In another case of *VDR* null mice, these had extremely low aromatase expression, which was correlated with a reduced ovarian activity representing 24% when compared to control mice (Kinuta et al., 2000). In the mutated mice, estrogen biosynthesis was disrupted, which impaired follicular development, thus causing ovarian insufficiency. Interestingly, supplementation of VD improved aromatase activity, bringing it to 60% of normal levels, therefore suggesting that

VD likely signals through something in addition to VDR to affect aromatase expression (Kinuta et al., 2000). Nevertheless, this indicates a vital role of the VDR for normal regulation of female reproductive functions. It has been suggested that the consequences observed in *VDR* null mice may be due to the accompanying hypocalcaemia that results from *VDR* nullification, and this has been confirmed to be a major factor explaining such observed results (Johnson and DeLuca, 2001). In normo-calcemic *VDR* null mice, fertility rates ranged from 86%-100% while their hypo-calcemic counterparts had a 14% fertility rate (Johnson and DeLuca, 2001). Nevertheless, some studies have indicated that fertility and litter size were hindered regardless of serum concentration of calcium in VD deficient female rats, thus suggesting that the effect on fertility is not entirely due to calcium status (Halloran and Deluca, 1980).

2.2.2 VD supplementation and female reproductive functions

The effects of supplementing VD have long been investigated and thought to have potential beneficial effects on female reproductive functions and health considering that deficiency has proven negative consequences (Thys-Jacobs et al., 1999; Merhi et al., 2012; Xu et al., 2018). Moreover, in a study that evaluated VD supplementation on cultured macaque ovarian tissue, it was shown that such supplementation allowed for the improvement of follicle survival/growth as well as promoting oocyte maturation in addition to increased estradiol, growth hormone and Anti-Müllerian hormone (AMH) production (Xu et al., 2018). This study provided strong evidence that VD plays an important role in the regulation of follicle development likely by acting in granulosa cells, which are responsible for hormone production.

VD and VDR have proven important for inhibition of proliferation and promoting apoptosis of GCs (Verlinden et al., 1998; Yao et al., 2017; Kong et al., 2019; Yao et al., 2020). In addition, GCs of various species, including pigs, seem to require VD/VDR for proper steroidogenesis function (Smolikova et al., 2013; Hong et al., 2016; Hong et al., 2017; Yao et al., 2017). Supplementation of VD modulated the expression of genes involved in progesterone (P4) and estrogen biosynthesis (Hong et al., 2016; Hong et al., 2017). Indeed, the CYP19A1 gene, which encodes aromatase, the enzyme responsible for aromatizing and thus converting androgens to estrogens, contains a VDRE in its promotor (Sun et al., 1998; Muscogiuri et al., 2017). In addition to affecting the production of sex steroid hormones, VD/VDR are believed to participate in the regulation of proper sexual development of pigs and other livestock (Hong et al., 2016; Hong et al., 2017). It was proposed that VD/VDR play a role in regulating AMH signalling pathways and maturation of follicles via dampening the effects of AMH (Merhi et al., 2014; Jamil et al., 2016; Muscogiuri et al., 2017; Yao et al., 2017). Supplementation of VD was also found to uprise testosterone driven aromatase activation and increase estradiol secretion in rat GCs cultured *in vitro* (Lee et al., 2014a; Muscogiuri et al., 2017). Furthermore, VD supplementation may affect the transcription of a multitude of genes in female reproductive cells (Makieva et al., 2021). Based on these studies, it is now evident that VD and VDR affect steroidogenesis, GC luteinization and other ovarian functions, thus contributing to the maintenance of healthy female reproductive physiology (Yao et al., 2017).

2.2.3 VD and VDR in reproductive conditions and efficiency of assisted reproductive technologies

There appears to be a link between VD status, VDR and polycystic ovary syndrome (PCOS). This endocrine disorder, which affects 4-18% of women of reproductive age (Azziz et al., 2004; March et al., 2010; Muscogiuri et al., 2017), is characterized by chronic anovulation, hyperandrogenism, and polycystic ovaries (Muscogiuri et al., 2017). Women who suffer from this condition tend to have greater prevalence of VD deficiency (Thomson et al., 2012; He et al., 2015). Levels of VD within follicular fluid and the VDR gene expression in GCs of PCOS women and overweight women are decreased in comparison to healthy women (Aghadavod et al., 2017). It has been demonstrated that low levels of serum VD (sVD) can exacerbate the symptoms of PCOS (He et al., 2015). However, the role VD and VDR play in calcium homeostasis may also play a major role in the link between VD status and PCOS (Thys-Jacobs et al., 1999). Nevertheless, the importance of VD/VDR may be due to their roles in GC function, including AMH signalling and P4 production (Merhi et al., 2014; Hong et al., 2016; Jamil et al., 2016; Muscogiuri et al., 2017; Yao et al., 2017). In line with this, several studies demonstrated that VD supplementation increased spontaneous ovulation in women suffering with PCOS (Luciano et al., 1994; Bonakdaran et al., 2012; Dehghani Firouzabadi et al., 2012; Pal et al., 2012; Irani et al., 2014; Fang et al., 2017; Muscogiuri et al., 2017; Behmanesh et al., 2019; Kuyucu et al., 2020; Masjedi et al., 2020).

In multiple instances, a positive correlation has been demonstrated between levels of female sVD and fertilization rates in IVF trials (Abadia et al., 2016; Wu et al., 2018; Liu et al., 2019). These findings suggested the importance of VD sufficiency in the success

of reproductive technologies, however, conflicting results have been published. For example, it was shown that low follicular fluid (FF) VD levels correlated with greater fertilization rates, thus indicating a negative effect of VD sufficiency (Ciepiela et al., 2018). Moreover, another study demonstrated that low VD in FF was linked to higher estradiol (E2) serum concentrations (Antunes et al., 2018). Additionally, other studies have indicated that there is no link between VD status in the serum and *in vitro* fertilization rates (Rudick et al., 2012; Firouzabadi et al., 2014; Banker et al., 2017). Inconsistency was also observed between oocyte retrieval rates and sVD levels (Rudick et al., 2012; Banker et al., 2017; Wu et al., 2018). Finally, it also seems that a link between FF and serum VD and implantation and pregnancy outcomes post-IVF treatment have been shown. Particularly, in one study, it was highlighted that with each ng/mL increase in FF VD level, clinical pregnancy rate was increased by 6% (Ozkan et al., 2010). Nevertheless, like all findings in this realm of study, many studies have not been consistent with the finding that VD sufficiency is important to implantation and pregnancy rates following IVF treatments (Anifandis et al., 2010; Aleyasin et al., 2011; Firouzabadi et al., 2014; Shahrokhi et al., 2016). Consequently, the importance of VD in the success of reproductive technologies remains unclear and requires more investigation (Xu et al., 2021).

Considering the effects of VD and the actions fulfilled by VDR in GCs and in various reproductive conditions and technologies, it has been suspected that this pair may also play a role in embryo development and implantation, although this has not been heavily investigated yet. For example, VD may play an important role in blastocyst implantation, as suggested in a 1991 study where mice had active VD injected into one uterine horn, which resulted in a significant increase in uterine weight and induced endometrial cell

differentiation by inducing differentiation to decidual cells (Halhali et al., 1991). The decidual reaction is an important reaction for embryo implantation as it promotes the formation of the placenta, the maternal and fetal interface, and regulates the production of various factors involved in remodelling of the mother's arteries in addition to refereeing invasiveness of the trophoblast cells (Kurjak and Chervenak, 2015). Additionally, it has been identified that the human *ATG16L1* gene contains a VDRE (Sun, 2016). While this has been demonstrated in intestinal cells, it may be interesting in identifying its control in the embryo and female reproductive organs and cells, as this gene has been demonstrated to be vital for proper decidualization and thus is important for implantation (Oestreich et al., 2020). In fact, in a study where conditional knockout (cKO) of the gene in the reproductive tract of mice was established, the mice had significantly decreased fertility due to decreased implantation rates. Moreover, the embryonic stem cells from the cKO mice were not able to properly decidualize, hence leading to less blastocysts implanting. Thus, this is another gene of interest that may demonstrate an important link between VD status and potential blastocyst implantation success rates, although this direct link has yet to be evaluated (Oestreich et al., 2020). Interestingly however, in terms of VD status, no link has been found between this and implantation rates in human embryo transfer studies (Franasiak et al., 2015; van de Vijver et al., 2016; Cai et al., 2021).

In couples undergoing IVF or intracytoplasmic sperm injection procedures, it has been suggested that VD rates are not linked to the cleavage or blastocyst development rates and it has also been suggested that VD has no effect on early embryo quality (Rudick et al., 2012; Banker et al., 2017; Jiang et al., 2019). It has been suggested that

the threshold for an effect on this reproductive event is extremely low, thus even individuals with deficient levels still have enough to experience the same outcomes in terms of cleavage and blastocyst rates as individuals with a sufficient VD status (Jiang et al., 2019). It is worth noting, however, that studies which have attempted to identify a correlation between VD status and embryo quality or development, lacked the ability to truly isolate VD status and therefore VDR's roles in this process, as the individuals undergoing such procedures may face an innumerable number of other factors that contribute to such results. Hence, it remains imperative to identify the singular role of VD and VDR in early embryonic development.

Conclusively, VD and VDR are essential for proper female reproductive physiology and particularly in ovarian function via their roles in steroidogenesis and follicular development. Moreover, VDR acts in cell functions via regulating aspects of the cell cycle, cell differentiation, proliferation, and apoptosis. However, establishing how these functions may affect the early developing embryo, and how *solely* VDR acts, regulates and participates in early embryonic development, remains to be determined. Therefore, this study aimed to elucidate the importance of the VDR in regulating early embryonic development by using a well described porcine model. Pigs have numerous physiological similarities to humans (Gutierrez et al., 2015). In terms of embryonic development, it is important to highlight that critical events for normal development, such as EGA, occur at the same cell stage (4-8 cell stage) between humans and pigs (Braude et al., 1988; Oestrup et al., 2009).

CHAPTER 3: RATIONALE, HYPOTHESIS AND OBJECTIVES

Considering that VD and its receptor, VDR, play important roles in various reproductive functions like steroidogenesis and folliculogenesis, in addition to their roles in cell-life control (proliferation, differentiation, apoptosis), it is of interest to identify how this receptor may function and contribute to early embryonic development. Consequently, we hypothesize that *VDR* mRNA is present in early-stage embryos and is important in the regulation of embryo development. Hence, this research project aimed to determine whether or not *VDR* has an important role in early embryo development. By using a well-established porcine *in vitro* culture model, the specific research goals were to: 1) determine how *VDR* mRNA is expressed during early embryonic development; 2) evaluate the impact of *VDR* mRNA attenuation on early embryonic development; and 3) assess if supplementing VD3 in culture media impacts early embryonic development.

CHAPTER 4: METHODOLOGY, RESEARCH FINDINGS AND DISCUSSION

4.1 Methodology

4.1.1 Chemicals and reagents

All chemicals and reagents were purchased from MilliporeSigma (Sigma-Aldrich, Oakville, Ontario, Canada), unless indicated otherwise.

4.1.2 Oocyte collection and in vitro maturation

Ovaries from pre-pubertal gilts were obtained from a nearby slaughterhouse (CBCo, Les Cèdres, Quebec, Canada) and placed into a thermos containing a warm (30ºC-35ºC) saline solution (0.9% NaCl). Ovaries were then transported to the laboratory, and washed with saline solution containing 100 IU/mL penicillin and 10 mg/mL streptomycin (P4333). Follicles ranging in diameter size from 3-6 mm had their contents aspirated using a 20G needle and 10mL syringe. The contents were then centrifuged at 300 revolutions per minute (rpm) for 3 minutes, two or three times. The supernatant was removed and the pellet was resuspended with warm HEPES-buffered TCM 199 (Life Technologies, Burlington, Ontario, Canada) supplemented with 1% porcine follicular fluid (pFF), then centrifuged to pellet the COCs. The COCs were then collected by searching petri dishes.

Collected COCs were first selected for IVM based on having at least 3 layers of cumulus cells and a homogenous ooplasm, then washed in HEPES-buffered TCM 199 before being transferred by groups of 30 into 90 µL IVM1 drops covered with mineral oil

(equilibrated to 5% CO_2 and 95% air at 38.5ºC). The COCs were then cultured in the incubator at 38.5ºC for 22 hours. The IVM1 media consisted of TCM-199 (Life Technologies) supplemented with 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP), 10 ng/mL epidermal growth factor (EGF; Life Technologies), 0.91 mM sodium pyruvate, 3.05 mM d-Glucose, 0.5 UI/mL hCG (Chorulon®; Merck Animal Health, Kirkland, Quebec, Canada), 10 µg Armour std./mL FSH (Folltropin-V®; Vétoquinol, Lavaltrie, Quebec, Canada) and 20 µg/mL gentamicin. Following IVM1, the COCs were then transferred into equilibrated 90 µL IVM2 drops, this media being identical to IVM1 except lacking the hCG, FSH, and dbcAMP, and incubated under mineral oil for 24 hours.

4.1.3 In vitro fertilization

COCs were first denuded by vortexing the them in HEPES-buffered TCM 199 supplemented with 0.1% hyaluronidase (H3506), for 2 minutes. They were then washed with manipulation media (TCM 199 supplemented with 2 mg/mL fatty acid free bovine serum albumin (BSA-FAF; A6003) and 2 µL/mL gentamicin) and consequently washed with pre-stabilized IVF media. The IVF media consisted of sterile ultrapure water supplemented with 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2$ $2H_2O$, 20 mM TRIZMA base, 11 mM D-Glucose, 0.1% phenol red, 5 mM sodium pyruvate, 2 mg/mL FAF-BSA, and 2 mM caffeine. Then the oocytes were placed, in groups of 30, into equilibrated 90 µL IVF drops under mineral oil. The oocytes were co-incubated for 5 hours with sperm, using 2 x 10^5 sperm/mL in the IVF drops. Following this, oocytes were denuded of the remaining sperm attached to their zona pellucida by vortexing them in IVF media, for 30

seconds. They were then washed with the same manipulation media mentioned in the previous section.

4.1.4 Parthenogenic activation

COCs were first denuded by vortexing them in HEPES-buffered TCM 199 supplemented with 0.1% hyaluronidase (H3506), for 2 minutes. They were then washed with manipulation media (TCM 199 supplemented with 2 mg/mL fatty acid free bovine serum albumin (BSA-FAF; A6003) and 2 µL/mL gentamicin). For the parthenogenic activation (PA), the oocytes were first washed in TCM2 (I0634; in TCM 199 supplemented with 2 mg/mL BSA and 2 µL/mL gentamicin), then transferred to 15 µM ionomycin (I0634; in TCM 199 supplemented with 2 mg/mL BSA and 2 µL/mL gentamicin) for 5 minutes, then washed thoroughly in TCM3 (TCM199 supplemented with 3 mg/mL BSA-FAF and 2 µL/mL gentamicin), then placed in 200 µM TPEN (in PZM-3) for 15 minutes. Finally, oocytes were washed in PZM-3 before being placed in PZM-3 supplemented with 7.5 µg/mL cytochalasin B (C6762) for 4 hours.

4.1.5 In vitro culture of embryos

After IVF or PA, the embryos were washed with equilibrated IVC media (consisting of PZM-3 supplemented with 5 mM hypotaurine (H1384), 1 mM glutamine (G8540) and 3 mg/mL BSA-FAF). They were then transferred into equilibrated 60 µL drops under mineral oil and were incubated in 5% CO_2 and 95% air at 38.5ºC. Cleavage was verified after 48 hours and non-cleaved embryos were removed. On day 5 of development, each IVC drop had 30 µL of media removed and replaced with fresh IVC that also was

supplemented with 20% fetal bovine serum (FBS), thus the final concentration in the drops included 10% FBS. Blastocyst development was assessed at 168 hours of development.

4.1.6 Knockdown of VDR via RNA interference and co-injection

Interference RNA was used to knockdown *VDR* mRNA by microinjecting at the MII stage. As to ensure proper target binding, the mRNA sequence of the porcine VDR (NCBI Reference Sequence: NM_001097414.1) was translated into an amino acid sequence and compared to the porcine VDR protein (NCBI Reference: NP_001090883.1) as to determine the protein coding region (sequence positions 127-1440). Thereafter, DsiRNAs were designed by using the Custom DsiRNA Design Tool. Subsequently, these were synthesized by Integrated DNA Technologies (Coralville, Iowa, United States). Verification of specificity was accomplished using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, Maryland, United States). An inverted Nikon microscope (Nikon, Tokyo, Japan) equipped with a micromanipulator system (Narishige International, Long Island, New York, United States) and FemtoJet 4i (Eppendorf, Hamburg, Germany) was used to microinject the oocytes. Microinjection was conducted in manipulation medium. MII 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 1*). For each treatment, 125-160 oocytes were injected, incubated for an hour, activated, and subsequently cultured *in vitro*. Cleavage was assessed at 48 hours. On the third day of embryo development, 10

embryos were collected to assess *VDR* mRNA knockdown efficiency via qRT-PCR (using the primers described in *Table 1*). In the case of co-injection with RFP mRNA, this being a non-embryo-lethal and non-autofluorescent marker to be used as an indicator of effective injection, 10 pL of 25 µM siVDR was injected alongside 20 ng/µL of RFP mRNA, whereby negative controls were also injected with 10 pL of 25 µM scrambled sequences alongside 20 ng/µL of RFP mRNA as well. The mRNA for RFP was synthetically synthesized by first using primers to amplify the coding sequence of turboRFP from the pRFP-C-RS plasmid; this amplicon was then used to produce the final mRNA using the mMESSAGE mMACHINE T7 Transcription Kit (Invitrogen, Life Technologies).

Table 1: List of DsiRNAs used for knockdown experiments.

Target	**Sense (5'-3')**	**Antisense (5'-3')**
VDR (1)	GAUCUGAGCGAAGAAGACUCUGATG	CAUCAGAGUCUUCUUCGCUCAGAUCCA
VDR (2)	CUGCUCUACGCCAAGAUGAUCCAGA	UCUGGAUCAUCUUGGCGUAGAGCAGGU
Negative control	CUUCCUCUCUUUCUCUCCCUUGUGA	UCACAAGGGAGAGAAAGAGAGGAAGGA

4.1.7 Vitamin D supplementation

Embryos were supplemented with vitamin D3 (D1530) for the seven days duration of culture. VD3 was diluted in 95% ethyl alcohol and serially diluted to 10 nM or 100 nM with IVC. Control media was supplemented with ethyl alcohol (vehicle) diluted

1:500 in IVC. Moreover, embryos were supplemented with fresh VD3 when “fed” on day 5 of development (as previously described).

4.1.8 RNA extraction

Total RNA was extracted from groups of 10, immature COCs, immature oocytes, mature oocytes, 30h embryos, 45h embryos, 60h embryos, 75h embryos (day 3 embryos), and 90h embryos, or groups of 5 blastocysts (168h), using the PicoPure RNA isolation kit (Life Technologies, KIT0202). The entire volume of extracted RNA was treated with DNase (Qiagen, Hilden, Germany, 79254) and reverse transcribed using the Superscript® VILO™ cDNA Synthesis Kit (Life Technologies, 11754050).

4.1.9 Quantitative Reverse Transcriptase PCR

Reactions were carried out in a final volume of 10 µL (containing 250 nM of each primer (*Table 2*) and SYBR (Wisent Bioproducts, St-Bruno, Quebec, Canada) using a CFX384 Real-Time detection system (Bio-Rad). The qRT-PCR started with a hot-start denaturation step at 95°C for 5 minutes, followed by 40 cycles beginning with 95°C for 15 seconds and followed by 62°C for 30 seconds. Samples were run in duplicates and melting-curve analysis was evaluated to ensure specificity of reaction products. The ΔΔCt method was used to evaluate the relative abundance of mRNA for each gene; expression was normalized to the mean abundance of the H2A housekeeping gene. Values are expressed as a mean of the values from 3-4 biological replicates ± SEM. All reactions used for quantification had efficiency levels between 80% and 120% (coefficient of determination [R^2] ≥ 0.98).

Table 2: Primers for quantitative real-time PCR.

Target	Primer Sequence
VDR	F: 5'-AAG-GCA-GGC-AGG-AGA-AAT-AG-3'
	R: 5'-GAA-GAA-GGA-AGA-TCC-CAC-CAG-3'
H2A	F: 5'-GGT-GCT-GGA-GTA-TCT-GAC-CG-3'
	R: 5'-GTT-GAG-CTC-TTC-GTC-GTT-GC-3'

Forward (F) and Reverse (R) primers were both diluted to 2.5 µM and then mixed for each target gene.

4.1.10 Staining and fluorescent microscopy

Subsets of blastocysts were fixed in 4% paraformaldehyde (HT501128) for 15 minutes and then permeabilized in 1% Triton X-100 (T8787) in PBS (Life Technologies) containing 0.3% BSA. Samples were consequently stored at 4°C prior to fluorescent microscopy analysis being executed on all replicates simultaneously. Thereafter, samples were stained with 10 µg/mL 4',6-diamidino-2-phenylindole (DAPI; Life Technologies, D3571) for 20 minutes, and then rinsed in the same PBS-BSA-Triton permeabilization solution for 20 minutes. Samples were then mounted on microscope slides using a drop of warm Mowiol® (10852). Fluorescence was detected using a Nikon Eclipse 80i microscope (Nikon Instruments Inc., Melville, New York, United States) and images were taken at 200x magnification using a Retiga 2000R monochrome digital camera

(Qimaging, Surrey, British Columbia, Canada) and Simple PCI Imaging Software (Compix, Inc., Sewickly, Pennsylvania, United States).

4.1.11 Statistical analysis

Statistical analyses were completed with JMP, version 15.0 (SAS institute Inc.). The relative expression of *VDR* mRNA, cleavage rates, blastocyst rates, and cell number data were analyzed by either Student-t test or a one-way ANOVA, followed by Tukey HSD test. $P < 0.05$ was considered statistically significant.

4.2 Research Findings

4.2.1 Expression of VDR mRNA in oocytes and embryos

The primary objective of this study was to investigate the expression and regulation of *VDR* mRNA during oocyte maturation and early development of porcine embryos. The results obtained through qRT-PCR analyses indicated that *VDR* mRNA was expressed at all developmental stages, starting from immature oocytes up to blastocyst stage embryos (Fig. 1). Notably, there were no significant changes in the relative mRNA abundance of *VDR* throughout oocyte maturation and embryonic development. However, a slight decrease was observed at 60 hours of culture (Fig. 1). These findings suggest that *VDR* mRNA might be stored in the oocytes during oogenesis and then newly synthesized by developing embryos following EGA.

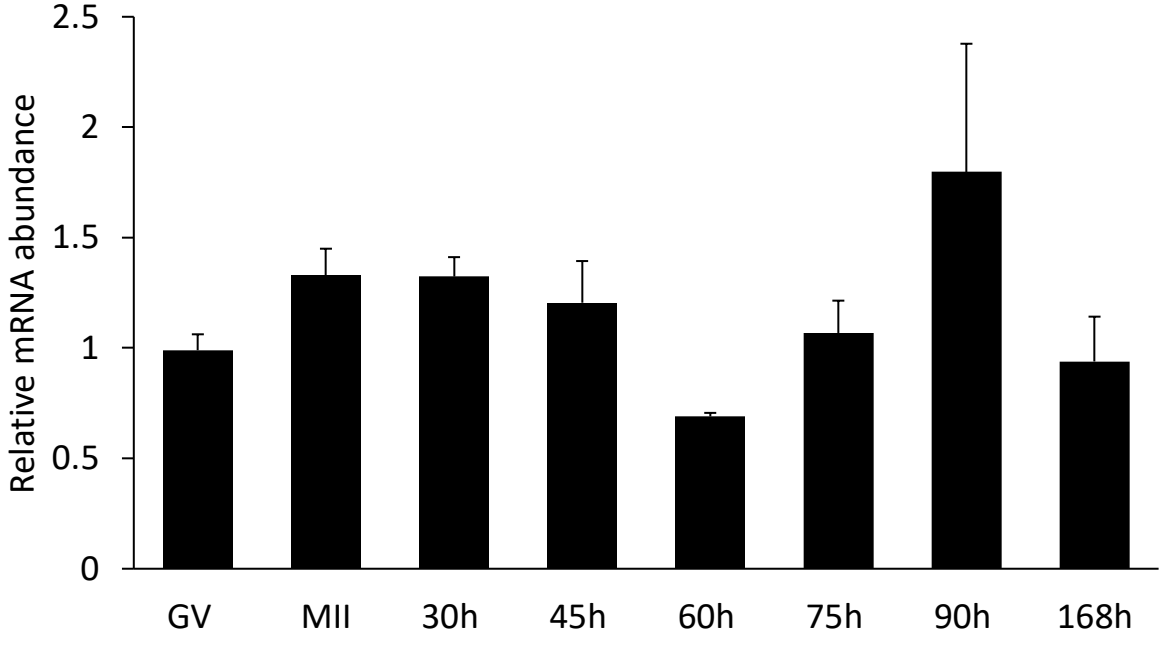


Figure 1: Relative mRNA expression of *VDR* in porcine oocytes and embryos.

Relative mRNA expression was compared between oocytes collected before (0 hours – germinal vesicle or GV) and after (46 hours - MII) IVM, and developing embryos collected at 30, 45, 60, 75, 90 and 168 (blastocysts) hours post-IVF. Values are presented as means ± SEM from 3 replicates.

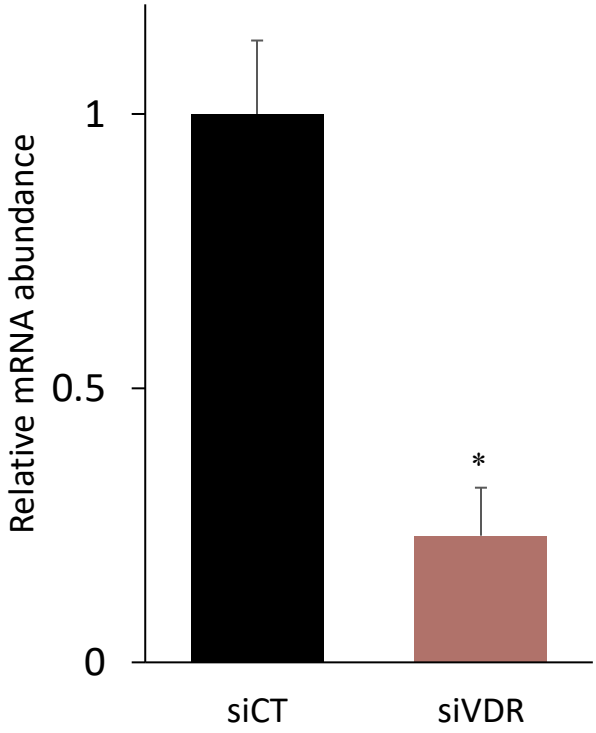
4.2.2 Development of VDR-attenuated embryos

The second objective of this study aimed to investigate whether VDR is essential for the normal development of porcine embryos up to the blastocyst stage. For this, mature oocytes were microinjected with either control DsiRNAs with a scrambled sequence (siCT) or DsiRNAs targeting two sequences of the *VDR* mRNA (siVDR), and then subjected to PA and cultured for 7 days to evaluate their developmental outcomes. The results showed that the knockdown efficiency of *VDR* mRNA in day 3 embryos was 77% (Fig. 2A). Surprisingly, there were no significant differences observed in embryo cleavage and development to the blastocyst stage between control embryos and *VDR*-attenuated embryos (Fig. 2B). This suggests that *VDR* mRNA may not be required for early embryo development in pigs.

To further validate these findings, a method for increasing *VDR* knockdown efficiency was implemented based on the co-injection of either siCT or siVDR with RFP mRNA, which allows for selecting embryos that were effectively microinjected by assessing RFP expression. Following microinjection and PA, cleaved embryos were selected using a fluorescent microscope at 48 hours of development and only RPF positive embryos were used to determine *VDR* knockdown efficiency on day 3 and

blastocyst rates on day 7 of development. This innovative approach significantly increased the *VDR* knockdown efficiency to 95.6% (Fig. 3A). Even with this highly effective attenuation of *VDR* mRNA, there was still no detrimental impact on embryo development up to the blastocyst stage, as well as embryo quality, based on the total number of cells per blastocyst (Fig. 3B).

A.



B.

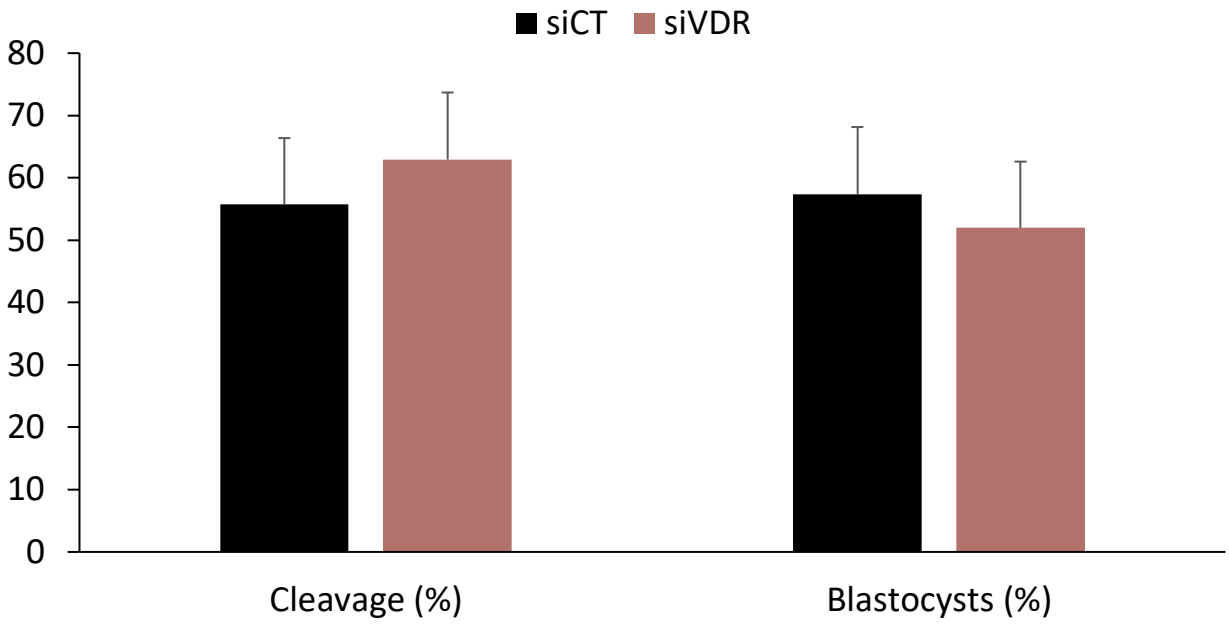
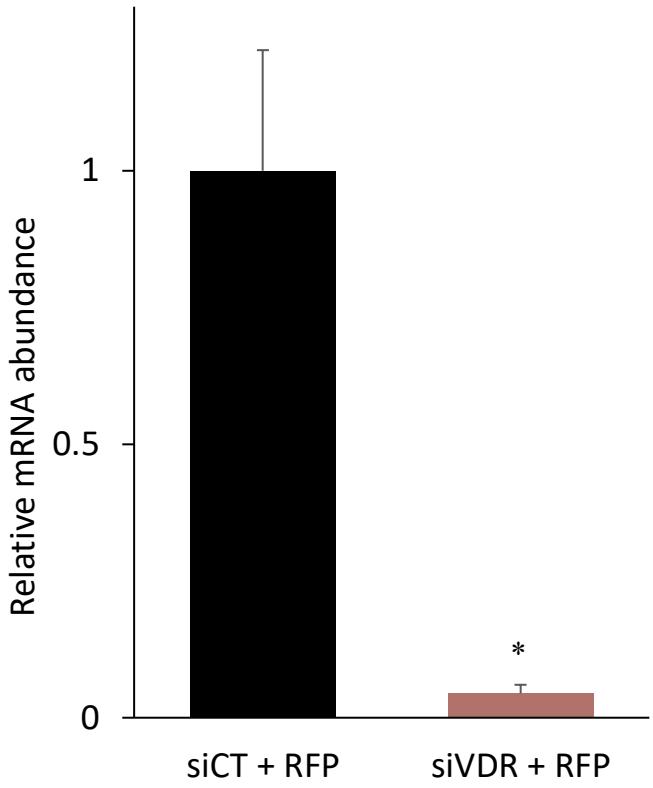


Figure 2: Effect of *VDR* mRNA attenuation on early embryonic development. **A)** Relative mRNA abundance of *VDR* in day 3 embryos that were microinjected with either control DsiRNAs (siCT) or DsiRNAs targeting two sequences of the *VDR* mRNA (siVDR) at the MII stage. Values are presented as means ± SEM of 3 replicates. **B)** Development of control (siCT) or VDR-attenuated (siVDR) embryos. A total number of 223 and 209 oocytes were used to evaluate embryo cleavage, and 97 and 112 cleaved embryos were used to evaluate blastocyst rates, for the siCT and siVDR treatments, respectively. Values are presented as the mean ± SEM from 3 replicates. The asterisk (*) indicates a significant difference ($P < 0.05$) between treatments.

A.


Relative mRNA abundance
1
0.5
0
*
siCT + RFP
siVDR + RFP

B.

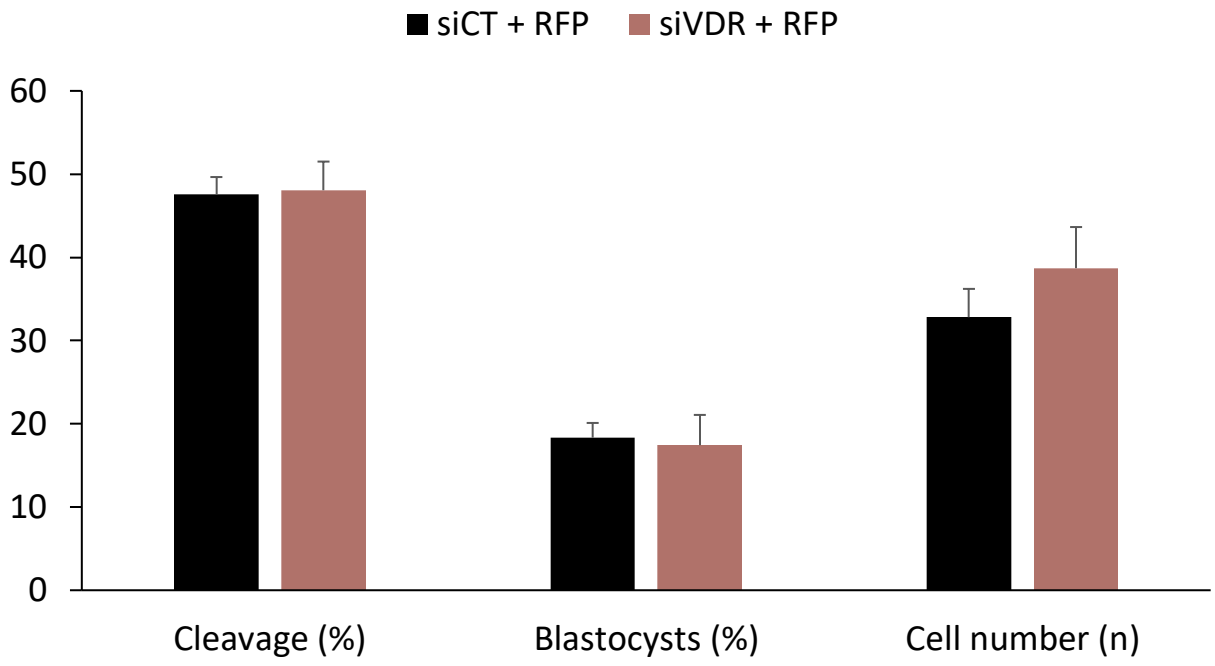

siCT + RFP
siVDR + RFP
60
50
40
30
20
10
0
Cleavage (%)
Blastocysts (%)
Cell number (n)

Figure 3: Use of RFP mRNA for selection of embryos with improved *VDR* attenuation. A) Relative abundance of *VDR* mRNA in day 3 embryos that were selected by RFP expression at 48 hours following microinjection of siCT + RFP mRNA (siCT + RFP) or siVDR + RFP mRNA (siVDR + RFP). Values are presented as means ± SEM of 3 replicates. **B)** Cleavage, development and average number of cells per blastocyst of siCT+RFP and siVDR+RFP embryos. The number of oocytes injected was 783 and 713, the number of cleaved embryos used to determine blastocyst rates was 210 and 204 embryos, and the number of blastocysts used to count cells was 12 and 15, for the siCT + RFP and siVDR + RFP treatments, respectively. Values are presented as the mean ± SEM from 7 replicates. The asterisk (*) indicates a significant difference ($P < 0.05$) between treatments.

4.2.3 Impact of vitamin D supplementation on embryo development

The last objective was to evaluate if VD3 supplementation during culture had an impact on development and quality of control and *VDR*-attenuated embryos. For this, *in vitro* matured oocytes underwent PA, and were then cultured *in vitro* in presence of either 0 nM, 10 nM, or 100 nM VD3. Cleavage was evaluated at 48 hours, and development to the blastocyst stage and total number of cells per blastocyst on day 7. Supplementation of VD3 throughout *in vitro* culture did not have a significant effect on embryo cleavage, blastocyst rates, or the total number of cells per blastocyst (Fig. 4). However, despite not reaching statistical significance, there was a noticeable trend ($p = 0.09$) of decreasing blastocyst rates with increasing concentrations of VD3 in the

culture medium. These results suggest that higher concentrations of VD3 might potentially influence cell pathways crucial for pre-blastocyst development. It is noteworthy that despite the observed trend in blastocyst rates, this negative impact of VD3 supplementation on development was not supported by a decrease in overall embryo quality, as indicated by the similar number of cells per blastocyst (Fig. 4).

Finally, the impact of VD3 supplementation (100 nM) was assessed using siCT and siVDR embryos. The knockdown efficiency of *VDR* mRNA was confirmed to be significantly different (~92%) between the si-CT and si-VDR groups on day 3 of development (Fig. 5A). Supplementation of VD3 during culture did not result in a significant alteration of *VDR* mRNA expression on day 3 of development in either the siCT or siVDR groups (Fig. 5A). Moreover, the supplementation of VD3 throughout the embryo culture period did not significantly affect embryo development, cleavage and blastocyst rates, or the total number of cells per blastocyst (Fig. 5B). However, it is worth highlighting that the VD3 supplemented groups showed a trend towards decreased development to the blastocyst stage, although this difference was not statistically significant. This observation is consistent with the trends observed in the previous experiment (Fig. 5B).

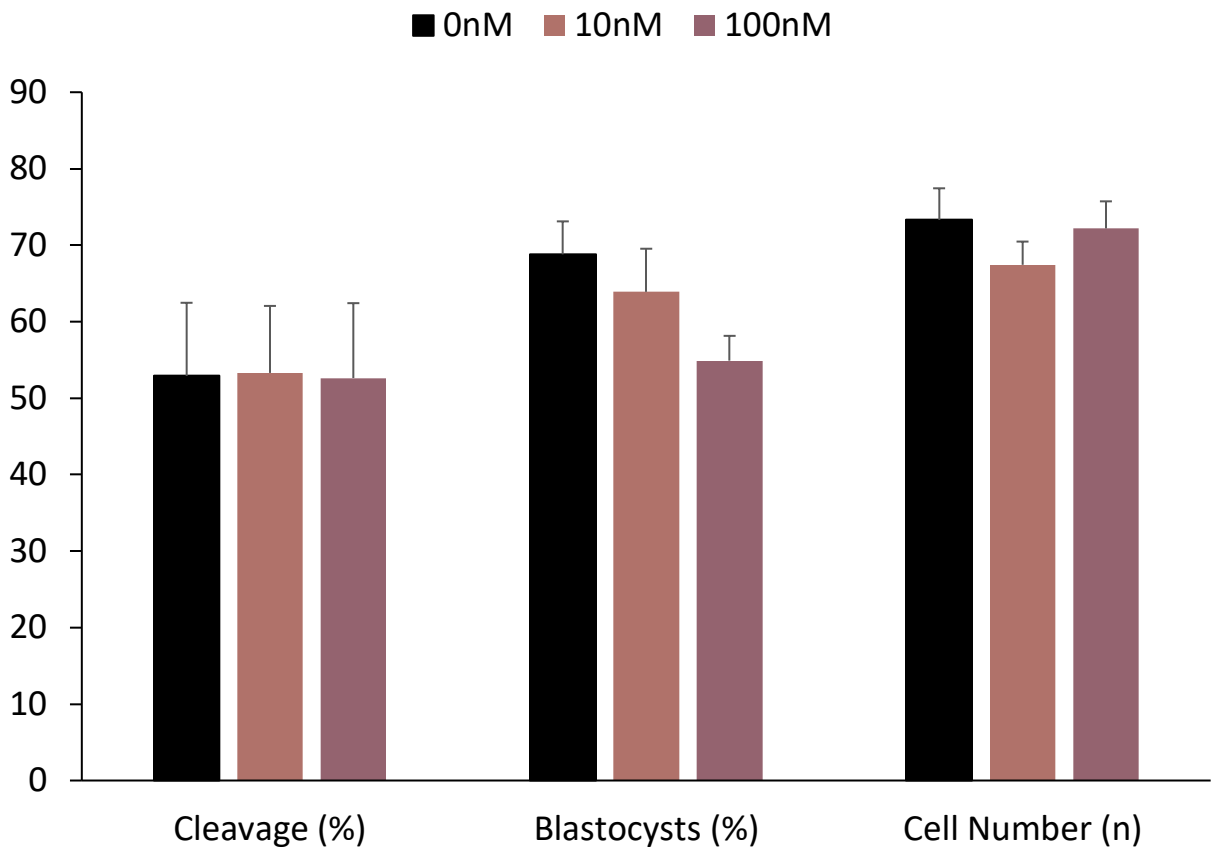
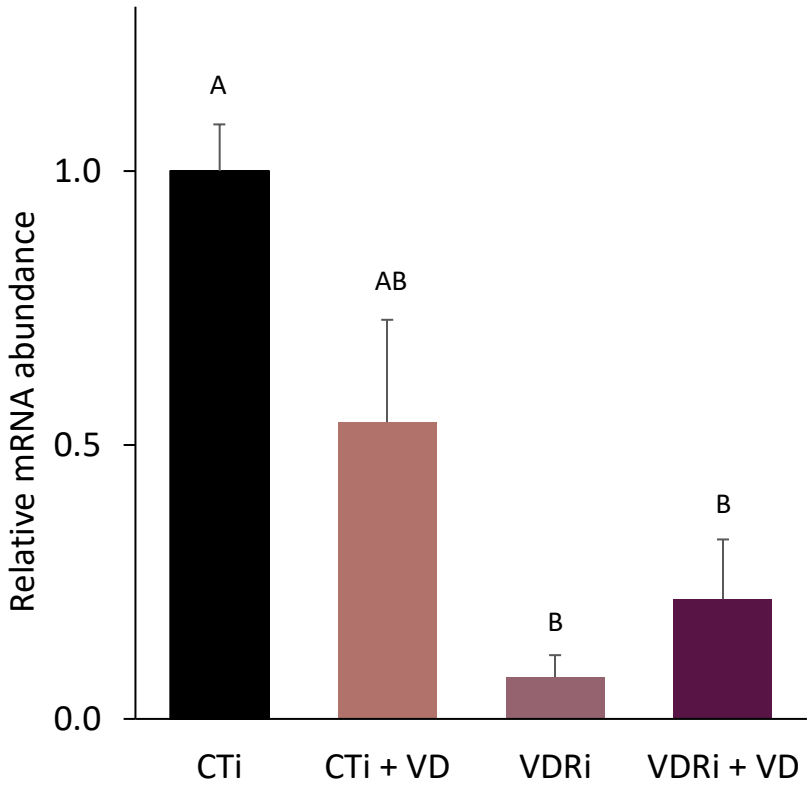


Figure 4: Effect of VD3 supplementation on embryo development. The number of oocytes used to evaluate embryo cleavage was 297, 315 and 290, the number of cleaved embryos to evaluate blastocyst rates was 162, 170 and 153, and the number of blastocysts used to count cells was 91, 127 and 111, for the 0 nM (control), 10 nM and 100 nM VD3 treatments, respectively. Values are presented as means ± SEM from 4 replicates.

A.


Relative mRNA abundance
1.0
0.5
0.0
A
AB
B
B
CTi
CTi + VD
VDRi
VDRi + VD

B.

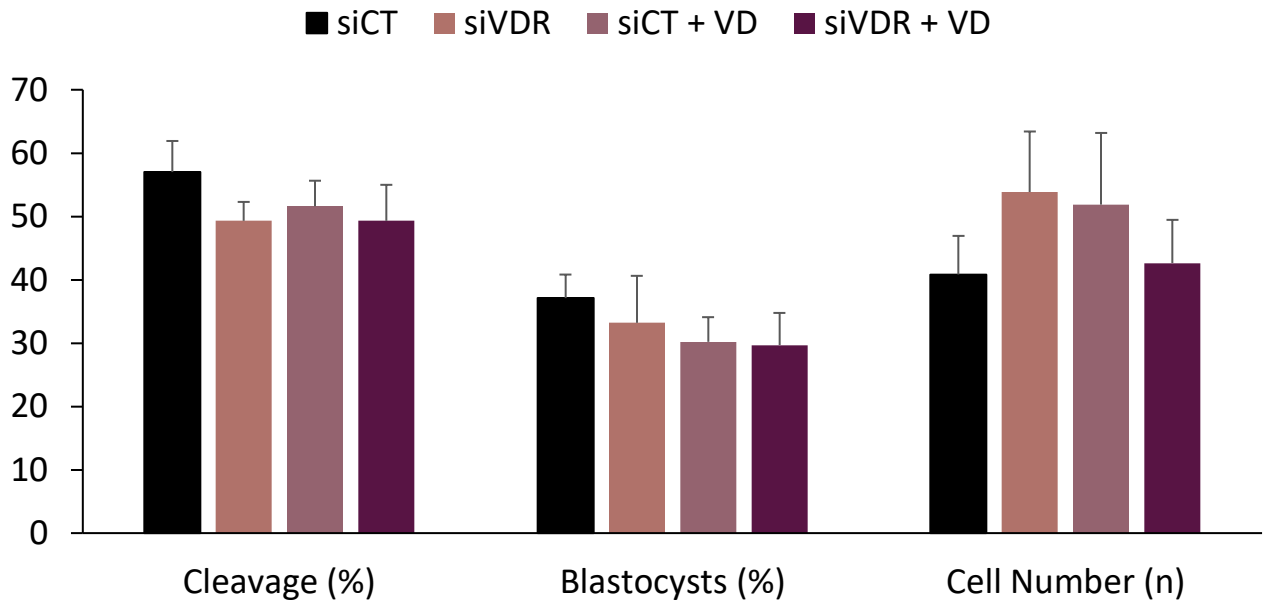

siCT
siVDR
siCT + VD
siVDR + VD
70
60
50
40
30
20
10
0
Cleavage (%)
Blastocysts (%)
Cell Number (n)

Figure 5: Effect of VD3 supplementation on development of *VDR*-attenuated embryos. A) Relative abundance of *VDR* mRNA on day 3 of development in siCT and siVDR embryos either treated or not treated with 100 nM VD3. Values are presented as means ± SEM of 3 replicates. Different letters (A,B) denote statistical differences between treatments ($P < 0.05$). **B)** Embryo development and average number of cells per blastocyst. The number of oocytes injected and used to evaluate cleavage was 396, 382, 388 and 392, the number of cleaved embryos used to evaluate development to the blastocyst stage was 195, 154, 169 and 162, and the number of blastocysts used to count cells was 24, 14, 15 and 14, for the siCT, siVDR, siCT + VD3 and siVDR + VD3, respectively. Values are presented as the mean ± SEM from 6 replicates.

4.3 Discussion

Vitamin D plays varied critical roles in numerous cellular processes throughout the body. However, a considerable proportion of North Americans are deficient in VD. It has been reported that the prevalence of deficiency in regards to sVD (<50 nmol/L) is 37% in Canada, with 7.4% categorized as severely deficient, having less than 30 nmol/L of sVD (Sarafin et al., 2015). In terms of reproductive concerns, a 2014 study indicated that 24% of pregnant Canadian women have sVD levels below the critical 50nmol/L (Palacios and Gonzalez, 2014). Not only this, but when considering that it has been reported in one study that with each ng/mL increase in FF VD level, clinical pregnancy rate increased by 6% (Ozkan et al., 2010), it underlines how deficiency may be having a critical impact on reproductive success and suggests it must be further investigated. Contrastingly, it is worth emphasizing that findings across this realm of study have not been consistent with demonstrating the link between VD status and pregnancy rates, as shown by various research studies (Anifandis et al., 2010; Aleyasin et al., 2011; Firouzabadi et al., 2014; Shahrokhi et al., 2016). This lack of consistency challenges the suggestion that VD sufficiency is a crucial factor influencing implantation and pregnancy rates following IVF treatments. Furthermore, it has already been demonstrated that low FF VD levels correlate with greater fertilization rates, thus indicating a negative effect of VD sufficiency (Ciepiela et al., 2018). Additionally, other studies have indicated that there is no link between VD status in the serum and *in vitro* fertilization rates (Rudick et al., 2012; Firouzabadi et al., 2014; Banker et al., 2017). In addition, it is also noteworthy that these studies often fail to *isolate* the impact of VD status, as a plethora of other factors within the body may simultaneously impact human IVF trials, for example. Hence, these

inconsistencies leave much to be desired in terms of what role VD/VDR may truly play in reproductive technology success rates.

Therefore, to investigate how this issue may impact reproduction, this study aimed to explore the role of VD and its functional nuclear receptor, the VDR, during early embryonic development, using a well-established *in vitro* porcine model. The mRNA expression profile of *VDR* was first evaluated at various time points throughout oocyte maturation and early embryonic development, confirming consistent *VDR* mRNA presence within both the oocyte and embryo and suggesting that *VDR* mRNA likely plays a critical role during late oogenesis and early embryogenesis. However, we were unable to verify protein presence and levels, as the available antibody did not show specificity in the porcine species. Nevertheless, following the mRNA discovery, this study next aimed to evaluate the need of *VDR* mRNA in early embryonic development, by attenuating *VDR* mRNA via microinjection of DsiRNA. Although a significant attenuation of 77% in *VDR* mRNA levels was observed on day three of embryonic development, there was no impact on cleavage and blastocyst development. These findings suggest that *VDR* mRNA may not be indispensable for regulation of pre-blastocyst development in porcine embryos. It is worth highlighting that VD can signal via other pathways, both genomically and non-genomically (Meyer et al., 2010; Haussler et al., 2011). Additionally, it is also possible that VDR-regulated genes involved in embryonic development experience VDR protein binding early on in development, prior to the KD of *VDR* mRNA being fully effective. Furthermore, considering that VDR binding stability to its response elements may vary between genes, it is possible that VDR protein stockpiled in the mature oocyte can bind

long enough to preclude KD impacts on embryo regulation (Freedman and Towers, 1991; Nishikawa et al., 1993; Toell et al., 2000; Shaffer and Gewirth, 2002).

To further confirm the impact of *VDR* attenuation on development, a novel and improved method for effectively detecting microinjected embryos was developed. This method involved co-injecting RNAi targeting *VDR* mRNA along with RFP mRNA. The consequent RFP expression served as an indicator of effective injection, allowing us to select only RFP positive embryos for evaluation of blastocyst development and quality. Importantly, when the injection of RFP mRNA was done alone, and not alongside DsiRNA targeting *VDR* mRNA, it did not affect cleavage nor impede embryonic development (Suppl. Fig. 1), demonstrating that this method was effective and safe for use in oocytes and embryos. By ensuring that every embryo used in the evaluation was indeed attenuated, results can gain in terms of confidence and can be seen as being more robust. This novel methodology holds substantial potential for its use in other attenuation studies whereby KD can be used to study the roles of various other genes that may be critical during early embryogenesis as well. In our study, using this approach, a remarkable >95% KD efficiency was achieved with no detrimental consequences on embryo development and quality. These results further reinforce the assumption that *VDR* mRNA is not required for the regulation of early porcine embryo development.

Findings from this study indicate that supplementing VD3 during *in vitro* embryo culture does not significantly impact porcine embryonic development and quality. To our knowledge, there are no previous studies which have assessed the impact of VD3 supplementation throughout the *in vitro* culture of embryos. While various previous studies suggested different possibilities and outcomes for VD3 supplementation

(Anifandis et al., 2010; Aleyasin et al., 2011; Firouzabadi et al., 2014; Shahrokhi et al., 2016), positive results have indicated a positive correlation between increased levels of VD in follicular fluid and higher pregnancy rates (Ozkan et al., 2010). Results from human IVF trials have also yielded inconsistent conclusions. Some studies reported a positive correlation between sVD levels and fertilization rates (Abadia et al., 2016; Wu et al., 2018; Liu et al., 2019), while another study indicated that low levels of VD in follicular fluid correlated with higher fertilization rates, suggesting a negative effect of VD sufficiency (Ciepiela et al., 2018). Our findings are consistent with this latter observation, as we effectively noticed a trend towards decreased embryo development to the blastocyst stage with VD3 supplementation in the embryo culture medium. While the effect was not statistically significant, the observed tendency supports the idea that VD3 supplementation might have a detrimental impact on embryo development. Considering that VD/VDR are known to regulate a plethora of genes, the effect of these potential alterations in expression may be partially responsible for the observed dampened development rates (Haussler et al., 2011). Additionally, it is worth highlighting that our study was conducted with uniformly selected oocytes which were derived from young, healthy pigs that were fed balanced diets, while most published results from human IVF trials involved patients with infertility issues, different ages, and diets; consequently, the inability to isolate VD status, due to the aforementioned inconsistencies, could explain the varied results among different studies. Since VD3 supplementation during embryo culture has not been tested under such conditions, it is possible that it could have a different impact on embryo development than what was observed in our porcine model.

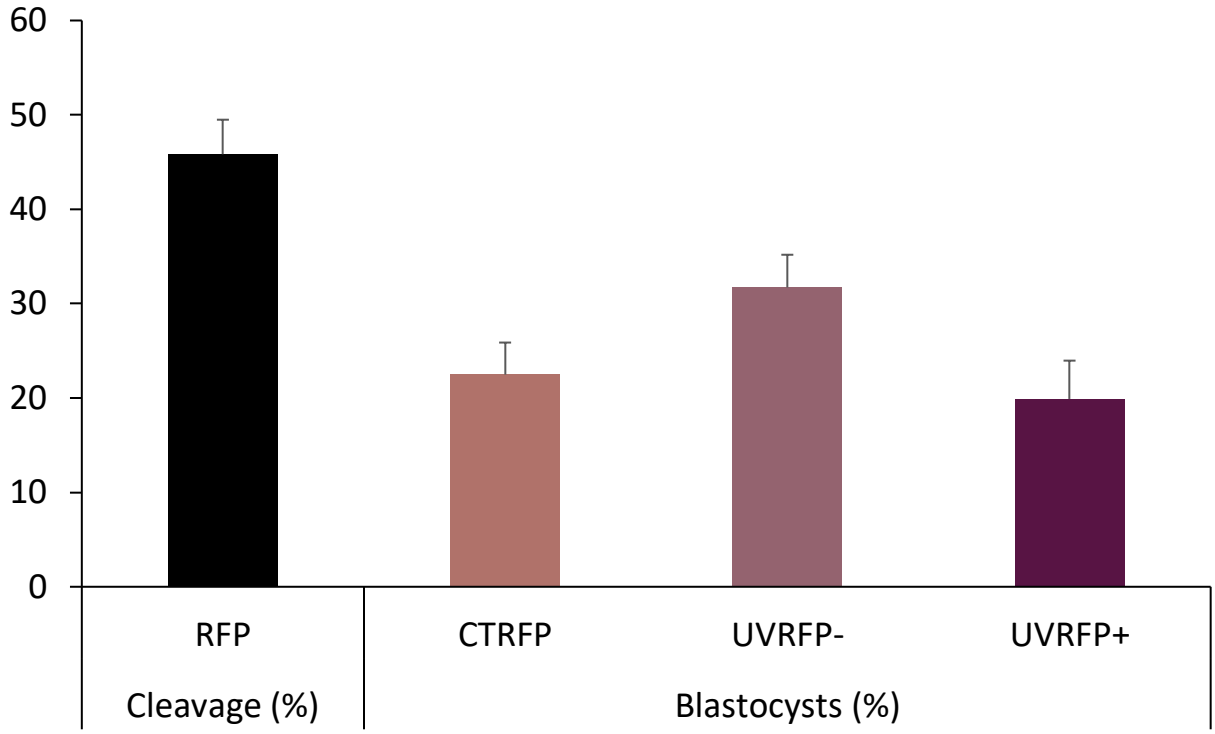
To further investigate whether or not VD3 has a role in early embryonic development, *VDR* attenuated embryos were cultured with 100 nM VD3 supplemented medium. There was no significant effect of VD3 supplementation on cleavage, development to the blastocyst stage, or cell number per blastocyst in *VDR* attenuated embryos. However, it is noteworthy that a similar tendency of VD3 supplementation to reduce blastocyst development was noticed even in *VDR* attenuated embryos, although the difference was not statistically different. These results suggest that the potential detrimental effect of VD3 on early embryo development may not necessarily be dependent on the presence of VDR, and could also potentially function through other non-genomic pathways (Meyer et al., 2010; Haussler et al., 2011). The exact mechanism by which VD3 might influence early embryonic development remains unclear and warrants further investigation. It would be valuable for additional studies to explore whether increased VD3 concentrations could lead to more pronounced effects on early embryo development, not only in porcine, but other species as well. Additional studies should investigate if increased VD3 concentration would further impact early embryo development.

In conclusion, findings from this study showed that: i) *VDR* mRNA was expressed in porcine oocytes and throughout early embryonic development; ii) attenuation of *VDR* mRNA did not affect embryo cleavage, development or embryo cell number; and iii) supplementation of VD3 during *in vitro* embryo culture did not have a significant impact on development and cell number of both normal and *VDR*-attenuated embryos. These results provide robust evidence that both VD3 and VDR may not be indispensable for early embryonic development. As a follow-up to this, further studies are required to

explore the molecular relationship between VD3/VDR and embryonic development, potentially revealing valuable insights applicable to assisted reproductive techniques in both animals and humans.

4.4 Appendix

4.4.1 Effect of RFP mRNA microinjection into oocytes on early embryonic development



Supplemental Figure 1: Effect of RFP mRNA microinjection into oocytes on early embryonic development. Cleavage rate of oocytes injected with RFP mRNA (RFP, n=399). Blastocyst rates of oocytes injected with RFP mRNA (CTRFP, n=96), injected with RFP mRNA and exposed to UV showing negative signal for RFP (UVRFP-, n=34), or positive signal for RFP (UVRFP+, n=70). Values are presented as the mean ± SEM from 4 replicates.

To evaluate the effect of RFP mRNA microinjection on early embryonic development, matured oocytes were injected with RFP mRNA, and were then subjected to PA. At 48 hours after injection, cleavage was evaluated and non-control embryos were briefly exposed to UV for 1-2 seconds to verify RFP status (positive; RFP+, or negative; RFP-), and cleaved embryos were cultured *in vitro* for 7 days. Injection of RFP mRNA did not affect embryo cleavage and injection of RFP mRNA along with verification of its expression through UV exposure did not have a significant effect on development (Supp. Fig. 1). Injection of RFP mRNA and exposure to UV for status verification did not hinder embryo development when compared to the injection of RFP mRNA alone.

CHAPTER 5: CONCLUSION

There is a growing need to better comprehend the role of VD and its receptor (VDR) in female reproduction, especially their potential impact on embryonic development and quality. To address this, our study used a well-established *in vitro* porcine model to investigate the significance of VD and VDR during early embryonic development. Our research yielded several important findings: i) *VDR* mRNA was expressed in porcine oocytes and throughout early embryonic development; ii) attenuation of *VDR* mRNA did not affect embryo cleavage, development or embryo cell number; and iii) supplementation of VD3 during *in vitro* embryo culture did not have a significant impact on the development and cell number of both normal and *VDR* attenuated embryos. While these findings suggest that although *VDR* mRNA is expressed throughout embryonic development, VD and VDR may not play major regulatory roles during early embryonic development, and they also lay the groundwork for future research. Further studies are required to delve deeper into the molecular relationship between VD/VDR and embryonic development. Such studies could potentially reveal how these mechanisms may be exploited to enhance assisted reproductive techniques, benefiting both animal breeding and selection programs, as well as human fertility.

Bibliography

Abadia, L., A. Gaskins, Y. Chiu, P. Williams, M. Keller, D. Wright, I. Souter, R. Hauser, and J. Chavarro. 2016. Serum 25-hydroxyvitamin D concentrations and treatment outcomes of women undergoing assisted reproduction. American Journal of Clinical Nutrition 104:729-735.

Accili, D., and K. C. Arden. 2004. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell 117(4):421-426.

Adler, E. M. 2010. Shedding Light on FoxO Function. Science Signaling 3(141):ec295-ec295.

Aghadavod, E., H. Mollaei, M. Nouri, and H. Hamishehkar. 2017. Evaluation of relationship between body mass index with vitamin D receptor gene expression and vitamin D levels of follicular fluid in overweight patients with polycystic ovary syndrome. International journal of fertility & sterility 11(2):105.

Al-Hendy, A., M. P. Diamond, T. G. Boyer, and S. K. Halder. 2016. Vitamin D3 inhibits Wnt/β-catenin and mTOR signaling pathways in human uterine fibroid cells. The Journal of Clinical Endocrinology & Metabolism 101(4):1542-1551.

Aleyasin, A., M. A. Hosseini, A. Mahdavi, L. Safdarian, P. Fallahi, M. R. Mohajeri, M. Abbasi, and F. Esfahani. 2011. Predictive value of the level of vitamin D in follicular fluid on the outcome of assisted reproductive technology. European Journal of Obstetrics & Gynecology and Reproductive Biology 159(1):132-137.

Amano, T., T. Hirata, G. Falco, M. Monti, L. V. Sharova, M. Amano, S. Sheer, H. G. Hoang, Y. Piao, and C. A. Stagg. 2013. Zscan4 restores the developmental potency of embryonic stem cells. Nature communications 4(1):1-10.

An, B.-S., L. E. Tavera-Mendoza, V. Dimitrov, X. Wang, M. R. Calderon, H.-J. Wang, and J. H. White. 2010. Stimulation of Sirt1-regulated FoxO protein function by the ligand-bound vitamin D receptor. Molecular and cellular biology 30(20):4890-4900.

Anifandis, G. M., K. Dafopoulos, C. I. Messini, N. Chalvatzas, N. Liakos, S. Pournaras, and I. E. Messinis. 2010. Prognostic value of follicular fluid 25-OH vitamin D and glucose levels in the IVF outcome. Reproductive Biology and Endocrinology 8(1):1-5.

Antunes, R. A., A. C. Mancebo, M. W. Reginatto, V. A. Deriquehem, P. Areas, E. Bloise, M. I. Chiamolera, G. C. Ribeiro, A. R. Carvalho, and M. C. Souza. 2018. Lower follicular fluid vitamin D concentration is related to a higher number of large ovarian follicles. Reproductive biomedicine online 36(3):277-284.

Aoshima, K., E. Inoue, H. Sawa, and Y. Okada. 2015. Paternal H3K4 methylation is required for minor zygotic gene activation and early mouse embryonic development. EMBO reports 16(7):803-812.

Azziz, R., K. S. Woods, R. Reyna, T. J. Key, E. S. Knochenhauer, and B. O. Yildiz. 2004. The prevalence and features of the polycystic ovary syndrome in an unselected population. The Journal of Clinical Endocrinology & Metabolism 89(6):2745-2749.

Banker, M., D. Sorathiya, and S. Shah. 2017. Vitamin D deficiency does not influence reproductive outcomes of IVF-ICSI: a study of oocyte donors and recipients. Journal of Human Reproductive Sciences 10(2):79.

Behmanesh, N., A. Abedelahi, H. N. Charoudeh, and A. Alihemmati. 2019. Effects of vitamin D supplementation on follicular development, gonadotropins and sex hormone concentrations, and insulin resistance in induced polycystic ovary syndrome. Turkish journal of obstetrics and gynecology 16(3):143.

Bhoora, S., and R. Punchoo. 2020. Policing cancer: Vitamin D arrests the cell cycle. International Journal of Molecular Sciences 21(23):9296.

Bikle, D. D., S. Patzek, and Y. Wang. 2018. Physiologic and pathophysiologic roles of extra renal CYP27b1: Case report and review. Bone reports 8:255-267.

Bilmez, Y., G. Talibova, and S. Ozturk. 2021. Dynamic changes of histone methylation in mammalian oocytes and early embryos. Histochemistry and Cell Biology:1-19.

Bonakdaran, S., Z. M. Khorasani, B. Davachi, and J. M. Khorasani. 2012. The effects of calcitriol on improvement of insulin resistance, ovulation and comparison with metformin therapy in PCOS patients: a randomized placebo-controlled clinical trial. Iranian journal of reproductive medicine 10(5):465.

Bouckenheimer, J., S. Assou, S. Riquier, C. Hou, N. Philippe, C. Sansac, T. Lavabre-Bertrand, T. Commes, J.-M. Lemaitre, and A. Boureux. 2017. Long non-coding RNAs in human early embryonic development and their potential in ART. Human Reproduction Update 23(1):19-40.

Bouillon, R., G. Carmeliet, E. Daci, S. Segaert, and A. Verstuyf. 1998. Vitamin D metabolism and action. Osteoporosis international 8:S13.

Bouillon, R., G. Carmeliet, L. Lieben, M. Watanabe, A. Perino, J. Auwerx, K. Schoonjans, and A. Verstuyf. 2014. Vitamin D and energy homeostasis—of mice and men. Nature Reviews Endocrinology 10(2):79-87.

Bouillon, R., G. Carmeliet, L. Verlinden, E. Van Etten, A. Verstuyf, H. F. Luderer, L. Lieben, C. Mathieu, and M. Demay. 2008. Vitamin D and human health: lessons from vitamin D receptor null mice. Endocrine reviews 29(6):726-776.

Braude, P., V. Bolton, and S. Moore. 1988. Human gene expression first occurs between the four-and eight-cell stages of preimplantation development. Nature 332(6163):459-461.

Brennan, A., D. R. Katz, J. Nunn, S. Barker, M. Hewison, L. J. Fraher, and J. L. O'Riordan. 1987. Dendritic cells from human tissues express receptors for the immunoregulatory vitamin D3 metabolite, dihydroxycholecalciferol. Immunology 61(4):457.

Cai, S., J. Li, S. Zeng, L. Hu, Y. Peng, S. Tang, S. Zeng, C. Chu, F. Gong, and G. Lin. 2021. Impact of vitamin D on human embryo implantation—a prospective cohort study in women undergoing fresh embryo transfer. Fertility and Sterility 115(3):655-664.

Carlson, B. M. 2018. Human embryology and developmental biology. Elsevier Health Sciences.

Cha, S., N.-H. Kim, S. Lee, C. Baik, H. Lee, and K. Chung. 1997. Effect of cytochalasin B and cycloheximide on the activation rate, chromosome constituent and in vitro development of porcine oocytes following parthenogenetic stimulation. Reproduction, fertility and development 9(4):441-446.

Che, L., A. Lalonde, and V. Bordignon. 2007. Chemical activation of parthenogenetic and nuclear transfer porcine oocytes using ionomycin and strontium chloride. Theriogenology 67(7):1297-1304.

Chen, C.-Y., J. Chen, L. He, and B. L. Stiles. 2018. PTEN: tumor suppressor and metabolic regulator. Frontiers in endocrinology 9:338.

Chen, T. C., F. Chimeh, Z. Lu, J. Mathieu, K. S. Person, A. Zhang, N. Kohn, S. Martinello, R. Berkowitz, and M. F. Holick. 2007. Factors that influence the cutaneous synthesis and dietary sources of vitamin D. Archives of biochemistry and biophysics 460(2):213-217.

Ciepiela, P., A. J. Dulęba, E. Kowaleczko, K. Chełstowski, and R. Kurzawa. 2018. Vitamin D as a follicular marker of human oocyte quality and a serum marker of in vitro fertilization outcome. Journal of assisted reproduction and genetics 35(7):1265-1276.

Cuthbert, J. M., S. J. Russell, I. A. Polejaeva, Q. Meng, K. L. White, and A. D. Benninghoff. 2021. Dynamics of small non-coding RNAs in bovine scNT embryos through the maternal-to-embryonic transition. Biology of Reproduction 105(4):918-933.

De Iaco, A., E. Planet, A. Coluccio, S. Verp, J. Duc, and D. Trono. 2017. DUX-family transcription factors regulate zygotic genome activation in placental mammals. Nature genetics 49(6):941-945.

De Iaco, A., S. Verp, S. Offner, D. Grun, and D. Trono. 2020. DUX is a non-essential synchronizer of zygotic genome activation. Development 147(2):dev177725.

de Macedo, M. P., W. G. Glanzner, V. B. Rissi, K. Gutierrez, L. Currin, H. Baldassarre, and V. Bordignon. 2019. A fast and reliable protocol for activation of porcine oocytes. Theriogenology 123:22-29.

De Pascale, G., and S. A. Quraishi. 2014. Vitamin D status in critically ill patients: the evidence is now bioavailable! Critical Care 18(4):1-2.

Dehghani Firouzabadi, R., A. Aflatoonian, S. Modarresi, L. Sekhavat, and S. MohammadTaheri. 2012. Therapeutic effects of calcium & vitamin D supplementation in women with PCOS. Complementary therapies in clinical practice 18(2):85-88.

Deng, M., Z. Liu, C. Ren, G. Zhang, J. Pang, Y. Zhang, F. Wang, and Y. Wan. 2018. Long noncoding RNAs exchange during zygotic genome activation in goat. Biology of reproduction 99(4):707-717.

DeRenzo, C., and G. Seydoux. 2004. A clean start: degradation of maternal proteins at the oocyte-to-embryo transition. Trends in cell biology 14(8):420-426.

Dicken, C. L., D. D. Israel, J. B. Davis, Y. Sun, J. Shu, J. Hardin, and G. Neal-Perry. 2012. Peripubertal vitamin D3 deficiency delays puberty and disrupts the estrous cycle in adult female mice. Biology of Reproduction 87(2):51, 51-12.

Dimitrov, V., R. Salehi-Tabar, B.-S. An, and J. H. White. 2014. Non-classical mechanisms of transcriptional regulation by the vitamin D receptor: insights into calcium homeostasis, immune system regulation and cancer chemoprevention. The Journal of steroid biochemistry and molecular biology 144:74-80.

Dowd, D. R., and P. N. MacDonald. 2013. Vitamin D Receptor. In: W. J. Lennarz and M. D. Lane, editors, Encyclopedia of Biological Chemistry (Second Edition). Academic Press, Waltham. p. 540-544.

Duan, J., L. Zhu, H. Dong, X. Zheng, Z. Jiang, J. Chen, and X. Tian. 2019. Analysis of mRNA abundance for histone variants, histone-and DNA-modifiers in bovine in vivo and in vitro oocytes and embryos. Scientific reports 9(1):1-13.

Eckersley-Maslin, M., C. Alda-Catalinas, M. Blotenburg, E. Kreibich, C. Krueger, and W. Reik. 2019. Dppa2 and Dppa4 directly regulate the Dux-driven zygotic transcriptional program. Genes & development 33(3-4):194-208.

Eelen, G., L. Verlinden, M. B. Meyer, R. Gijsbers, J. W. Pike, R. Bouillon, and A. Verstuyf. 2013. 1, 25-Dihydroxyvitamin D3 and the aging-related forkhead box O and sestrin proteins in osteoblasts. The Journal of steroid biochemistry and molecular biology 136:112-119.

Fang, F., K. Ni, Y. Cai, J. Shang, X. Zhang, and C. Xiong. 2017. Effect of vitamin D supplementation on polycystic ovary syndrome: A systematic review and meta-analysis of randomized controlled trials. Complementary therapies in clinical practice 26:53-60.

Feldman, D., A. V. Krishnan, S. Swami, E. Giovannucci, and B. J. Feldman. 2014. The role of vitamin D in reducing cancer risk and progression. Nature reviews cancer 14(5):342-357.

Firouzabadi, R. D., E. Rahmani, M. Rahsepar, and M. M. Firouzabadi. 2014. Value of follicular fluid vitamin D in predicting the pregnancy rate in an IVF program. Archives of gynecology and obstetrics 289(1):201-206.

Franasiak, J. M., T. A. Molinaro, E. K. Dubell, K. L. Scott, A. R. Ruiz, E. J. Forman, M. D. Werner, K. H. Hong, and R. T. Scott Jr. 2015. Vitamin D levels do not affect

IVF outcomes following the transfer of euploid blastocysts. American journal of obstetrics and gynecology 212(3):315. e311-315. e316.

Freedman, L., V. Arce, and R. Perez Fernandez. 1994. DNA sequences that act as high affinity targets for the vitamin D3 receptor in the absence of the retinoid X receptor. Molecular Endocrinology 8(3):265-273.

Freedman, L. P. 1999. Transcriptional targets of the vitamin D3 receptor–mediating cell cycle arrest and differentiation. The Journal of nutrition 129(2):581S-586S.

Freedman, L. P., and T. L. Towers. 1991. DNA binding properties of the vitamin D3 receptor zinc finger region. Molecular Endocrinology 5(12):1815-1826.

Gallieni, M., M. Cozzolino, G. Fallabrino, S. Pasho, L. Olivi, and D. Brancaccio. 2009. Vitamin D: physiology and pathophysiology. The International journal of artificial organs 32(2):87-94.

Gambini, A., P. Stein, V. Savy, E. J. Grow, B. N. Papas, Y. Zhang, A. C. Kenan, E. Padilla-Banks, B. R. Cairns, and C. J. Williams. 2020. Developmentally programmed tankyrase activity upregulates β-catenin and licenses progression of embryonic genome activation. Developmental cell 53(5):545-560. e547.

Garbedian, K., M. Boggild, J. Moody, and K. E. Liu. 2013. Effect of vitamin D status on clinical pregnancy rates following in vitro fertilization. Canadian Medical Association Open Access Journal 1(2):E77-E82.

Glanzner, W. G., K. Gutierrez, V. B. Rissi, M. P. de Macedo, R. Lopez, L. Currin, N. Dicks, H. Baldassarre, L. B. Agellon, and V. Bordignon. 2020. Histone lysine demethylases KDM5B and KDM5C modulate genome activation and stability in porcine embryos. Frontiers in cell and developmental biology 8:151.

Glass, C. K., and M. G. Rosenfeld. 2000. The coregulator exchange in transcriptional functions of nuclear receptors. Genes & development 14(2):121-141.

Gocek, E., H. Baurska, A. Marchwicka, and E. Marcinkowska. 2012. Regulation of leukemic cell differentiation through the vitamin D receptor at the levels of intracellular signal transduction, gene transcription, and protein trafficking and stability. Leukemia Research and Treatment 2012

González-Pardo, V., A. Suares, A. Verstuyf, P. De Clercq, R. Boland, and A. R. de Boland. 2014. Cell cycle arrest and apoptosis induced by 1α, 25 (OH) 2D3 and TX 527 in Kaposi sarcoma is VDR dependent. The Journal of steroid biochemistry and molecular biology 144:197-200.

Grzesiak, M., M. Tchurzyk, M. Socha, A. Sechman, and A. Hrabia. 2022. An Overview of the Current Known and Unknown Roles of Vitamin D3 in the Female Reproductive System: Lessons from Farm Animals, Birds, and Fish. International Journal of Molecular Sciences 23(22):14137.

Gutierrez, K., N. Dicks, W. G. Glanzner, L. B. Agellon, and V. Bordignon. 2015. Efficacy of the porcine species in biomedical research. Frontiers in genetics 6:293.

Guzey, M., D. Jukic, J. Arlotti, M. Acquafondata, R. Dhir, and R. H. Getzenberg. 2004a. Increased apoptosis of periprostatic adipose tissue in VDR null mice. Journal of cellular biochemistry 93(1):133-141.

Guzey, M., S. Kitada, and J. C. Reed. 2002. Apoptosis Induction by 1α, 25-Dihydroxyvitamin D3 in Prostate Cancer 1 Supported by the International Union Against Cancer [International Cancer Technology Transfer (ICRETT) and American Cancer Society Beginning Investigator (ACSBI) awards], the

Department of Defense, and Cap-CURE. 1. Molecular cancer therapeutics 1(9):667-677.

Guzey, M., J. Luo, and R. H. Getzenberg. 2004b. Vitamin D3 modulated gene expression patterns in human primary normal and cancer prostate cells. Journal of cellular biochemistry 93(2):271-285.

Halhali, A., G. Acker, and M. Garabedian. 1991. 1, 25-Dihydroxyvitamin D3 induces in vivo the decidualization of rat endometrial cells. Reproduction 91(1):59-64.

Halloran, B. P., and H. F. Deluca. 1980. Effect of vitamin D deficiency on fertility and reproductive capacity in the female rat. The Journal of nutrition 110(8):1573-1580.

Hamazaki, N., M. Uesaka, K. Nakashima, K. Agata, and T. Imamura. 2015. Gene activation-associated long noncoding RNAs function in mouse preimplantation development. Development 142(5):910-920.

Haussler, M. R., P. W. Jurutka, M. Mizwicki, and A. W. Norman. 2011. Vitamin D receptor (VDR)-mediated actions of 1α, 25 (OH) 2vitamin D3: genomic and non-genomic mechanisms. Best practice & research Clinical endocrinology & metabolism 25(4):543-559. doi: 10.1016/j.beem.2011.05.010

Haussler, M. R., G. K. Whitfield, C. A. Haussler, J. C. Hsieh, P. Thompson, S. H. Selznick, C. E. Dominguez, and P. W. Jurutka. 1998. The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. Journal of bone and mineral research 13(3):325-349.

He, C., Z. Lin, S. W. Robb, and A. E. Ezeamama. 2015. Serum vitamin D levels and polycystic ovary syndrome: a systematic review and meta-analysis. Nutrients 7(6):4555-4577.

Herdick, M., and C. Carlberg. 2000. Agonist-triggered modulation of the activated and silent state of the vitamin D3 receptor by interaction with co-repressors and co-activators. Journal of molecular biology 304(5):793-801.

Hii, C. S., and A. Ferrante. 2016. The non-genomic actions of vitamin D. Nutrients 8(3):135.

Hong, S.-H., J.-E. Lee, S.-M. An, Y. Y. Shin, D. Y. Hwang, S. Y. Yang, S.-K. Cho, and B.-S. An. 2017. Effect of vitamin D3 on biosynthesis of estrogen in porcine granulosa cells via modulation of steroidogenic enzymes. Toxicological Research 33(1):49-54.

Hong, S.-H., J.-E. Lee, H. S. Kim, Y.-J. Jung, D. Hwang, J. H. Lee, S. Y. Yang, S.-C. Kim, S.-K. Cho, and B.-S. An. 2016. Effect of vitamin D3 on production of progesterone in porcine granulosa cells by regulation of steroidogenic enzymes. Journal of biomedical research 30(3):203.

Irani, M., H. Minkoff, D. B. Seifer, and Z. Merhi. 2014. Vitamin D increases serum levels of the soluble receptor for advanced glycation end products in women with PCOS. The Journal of Clinical Endocrinology & Metabolism 99(5):E886-E890.

Jamil, Z., S. S. Fatima, K. Ahmed, and R. Malik. 2016. Anti-mullerian hormone: above and beyond conventional ovarian reserve markers. Disease markers 2016

Jiang, L., L. Ji, J. Song, and K. Qian. 2019. The effect of serum vitamin D levels in couples on embryo development and clinical outcomes. Reproductive biomedicine online 38(5):699-710.

Jin, C. H., and J. W. Pike. 1996. Human vitamin D receptor-dependent transactivation in Saccharomyces cerevisiae requires retinoid X receptor. Molecular Endocrinology 10(2):196-205.

Johnson, A. L., G. M. Zinser, and S. E. Waltz. 2015. Vitamin D3-dependent VDR signaling delays ron-mediated breast tumorigenesis through suppression of β-catenin activity. Oncotarget 6(18):16304.

Johnson, C. P., I. K. Kim, B. Esmaeli, A. Amin-Mansour, D. J. Treacy, S. L. Carter, E. Hodis, N. Wagle, S. Seepo, and X. Yu. 2017. Systematic genomic and translational efficiency studies of uveal melanoma. PLoS One 12(6):e0178189.

Johnson, L. E., and H. F. DeLuca. 2001. Vitamin D receptor null mutant mice fed high levels of calcium are fertile. The Journal of nutrition 131(6):1787-1791.

Karlic, R., S. Ganesh, V. Franke, E. Svobodova, J. Urbanova, Y. Suzuki, F. Aoki, K. Vlahovicek, and P. Svoboda. 2017. Long non-coding RNA exchange during the oocyte-to-embryo transition in mice. Dna Research 24(2):129-141.

Karras, S. N., C. L. Wagner, and V. D. Castracane. 2018. Understanding vitamin D metabolism in pregnancy: From physiology to pathophysiology and clinical outcomes. Metabolism 86:112-123. doi: 10.1016/j.metabol.2017.10.001

Khammissa, R., J. Fourie, M. H. Motswaledi, R. Ballyram, J. Lemmer, and L. Feller. 2018. The biological activities of vitamin D and its receptor in relation to calcium and bone homeostasis, cancer, immune and cardiovascular systems, skin

biology, and oral health. BioMed research international 2018doi: 10.1155/2018/9276380

Kim, A. M., M. L. Bernhardt, B. Y. Kong, R. W. Ahn, S. Vogt, T. K. Woodruff, and T. V. O'Halloran. 2011. Zinc sparks are triggered by fertilization and facilitate cell cycle resumption in mammalian eggs. ACS chemical biology 6(7):716-723.

Kim, S., M. Yamazaki, L. A. Zella, N. K. Shevde, and J. W. Pike. 2006. Activation of receptor activator of NF-κB ligand gene expression by 1, 25-dihydroxyvitamin D3 is mediated through multiple long-range enhancers. Molecular and cellular biology 26(17):6469-6486.

Kinuta, K., H. Tanaka, T. Moriwake, K. Aya, S. Kato, and Y. Seino. 2000. Vitamin D is an important factor in estrogen biosynthesis of both female and male gonads. Endocrinology 141(4):1317-1324. doi: 10.1210/endo.141.4.7403

Kong, F., C. Du, and Y. Wang. 2019. MicroRNA-9 affects isolated ovarian granulosa cells proliferation and apoptosis via targeting vitamin D receptor. Molecular and cellular endocrinology 486:18-24.

Kriebitzsch, C., L. Verlinden, G. Eelen, B. K. Tan, M. Van Camp, R. Bouillon, and A. Verstuyf. 2009. The Impact of 1, 25 (OH) 2D3 and its structural analogs on gene expression in cancer cells-a microarray approach. Anticancer research 29(9):3471-3483.

Kurjak, A., and F. A. Chervenak. 2015. Textbook of perinatal medicine: 2 volume set. JP Medical Ltd.

Kuyucu, Y., L. Sencar, Ö. Tap, and U. Ö. Mete. 2020. Investigation of the effects of vitamin D treatment on the ovarian AMH receptors in a polycystic ovary

syndrome experimental model: an ultrastructural and immunohistochemical study. Reproductive Biology 20(1):25-32.

Latham, K. E. 1999. Mechanisms and control of embryonic genome activation in mammalian embryos. International review of cytology 193:71-124.

Lee, C.-T., J.-Y. Wang, K.-Y. Chou, and M.-I. Hsu. 2014a. 1, 25-Dihydroxyvitamin D3 increases testosterone-induced 17beta-estradiol secretion and reverses testosterone-reduced connexin 43 in rat granulosa cells. Reproductive biology and endocrinology 12(1):1-12.

Lee, K., A. Davis, L. Zhang, J. Ryu, L. D. Spate, K.-W. Park, M. S. Samuel, E. M. Walters, C. N. Murphy, and Z. Machaty. 2015. Pig oocyte activation using a Zn2+ chelator, TPEN. Theriogenology 84(6):1024-1032.

Lee, M. T., A. R. Bonneau, and A. J. Giraldez. 2014b. Zygotic genome activation during the maternal-to-zygotic transition. Annual review of cell and developmental biology 30:581-613.

Li, H., C. Long, J. Xiang, P. Liang, X. Li, and Y. Zuo. 2021. Dppa2/4 as a trigger of signaling pathways to promote zygote genome activation by binding to CG-rich region. Briefings in Bioinformatics 22(4):bbaa342.

Li, L., S.-Y. Ji, J.-L. Yang, X.-X. Li, J. Zhang, Y. Zhang, Z.-Y. Hu, and Y.-X. Liu. 2014. Wnt/β-catenin signaling regulates follicular development by modulating the expression of Foxo3a signaling components. Molecular and cellular endocrinology 382(2):915-925.

Li, S., and W. Winuthayanon. 2017. Oviduct: roles in fertilization and early embryo development. J Endocrinol 232(1):R1-R26.

Liao, Y.-J., Y.-S. Chen, J.-X. Lee, L.-R. Chen, and J.-R. Yang. 2018. Effects of Klf4 and c-Myc knockdown on pluripotency maintenance in porcine induced pluripotent stem cell. Cell Journal (Yakhteh) 19(4):640.

Lin, Y., K. Mahan, W. F. Lathrop, D. G. Myles, and P. Primakoff. 1994. A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. The Journal of cell biology 125(5):1157-1163.

Liu, M., M.-H. Lee, M. Cohen, M. Bommakanti, and L. P. Freedman. 1996. Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes & development 10(2):142-153.

Liu, X., W. Zhang, Y. Xu, Y. Chu, X. Wang, Q. Li, Z. Ma, Z. Liu, and Y. Wan. 2019. Effect of vitamin D status on normal fertilization rate following in vitro fertilization. Reproductive Biology and Endocrinology 17(1):1-10.

Logan, C. Y., and R. Nusse. 2004. The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. 20:781-810.

Lonard, D. M., and B. W. O'Malley. 2006. The expanding cosmos of nuclear receptor coactivators. Cell 125(3):411-414.

Luciano, A., A. Pappalardo, C. Ray, and J. Peluso. 1994. Epidermal growth factor inhibits large granulosa cell apoptosis by stimulating progesterone synthesis and regulating the distribution of intracellular free calcium. Biology of Reproduction 51(4):646-654.

Luk, J., S. Torrealday, G. Neal Perry, and L. Pal. 2012. Relevance of vitamin D in reproduction. Human reproduction 27(10):3015-3027.

Ma, Y., D. L. Trump, and C. S. Johnson. 2010. Vitamin D in combination cancer treatment. Journal of Cancer 1:101.

MacDonald, B. T., K. Tamai, and X. He. 2009. Wnt/β-catenin signaling: components, mechanisms, and diseases. Developmental cell 17(1):9-26.

Macháty, Z., and R. S. Prather. 1998. Strategies for activating nuclear transfer oocytes. Reproduction, Fertility and Development 10(8):599-614.

Makieva, S., M. Reschini, S. Ferrari, F. Bonesi, E. Polledri, S. Fustinoni, L. Restelli, V. Sarais, E. Somigliana, and P. Viganò. 2021. Oral Vitamin D supplementation impacts gene expression in granulosa cells in women undergoing IVF. Human Reproduction 36(1):130-144.

March, W. A., V. M. Moore, K. J. Willson, D. I. Phillips, R. J. Norman, and M. J. Davies. 2010. The prevalence of polycystic ovary syndrome in a community sample assessed under contrasting diagnostic criteria. Human reproduction 25(2):544-551.

Martucci, G., F. Tuzzolino, A. Arcadipane, T. R. Pieber, C. Schnedl, T. Urbanic Purkart, G. Treiber, and K. Amrein. 2017. The effect of high-dose cholecalciferol on bioavailable vitamin D levels in critically ill patients: a post hoc analysis of the VITdAL-ICU trial. Intensive care medicine 43(11):1732-1734.

Masjedi, F., S. Keshtgar, F. Zal, T. Talaei-Khozani, S. Sameti, S. Fallahi, and M. Kazeroni. 2020. Effects of vitamin D on steroidogenesis, reactive oxygen species production, and enzymatic antioxidant defense in human granulosa cells of

normal and polycystic ovaries. The Journal of steroid biochemistry and molecular biology 197:105521.

Matsuda, F., N. Inoue, A. Maeda, Y. Cheng, T. Sai, H. Gonda, Y. Goto, K. Sakamaki, and N. Manabe. 2011. Expression and function of apoptosis initiator FOXO3 in granulosa cells during follicular atresia in pig ovaries. Journal of Reproduction and Development 57(1):151-158.

Merhi, Z., A. Doswell, K. Krebs, and M. Cipolla. 2014. Vitamin D alters genes involved in follicular development and steroidogenesis in human cumulus granulosa cells. The Journal of Clinical Endocrinology & Metabolism 99(6):E1137-E1145.

Merhi, Z. O., D. B. Seifer, J. Weedon, O. Adeyemi, S. Holman, K. Anastos, E. T. Golub, M. Young, R. Karim, and R. Greenblatt. 2012. Circulating vitamin D correlates with serum antimüllerian hormone levels in late-reproductive-aged women: Women's Interagency HIV Study. Fertility and sterility 98(1):228-234.

Meyer, M. B., P. D. Goetsch, and J. W. Pike. 2010. A downstream intergenic cluster of regulatory enhancers contributes to the induction of CYP24A1 expression by 1α, 25-dihydroxyvitamin D3. Journal of Biological Chemistry 285(20):15599-15610.

Muralidhar, S., A. Filia, J. Nsengimana, J. Poźniak, S. J. O'Shea, J. M. Diaz, M. Harland, J. A. Randerson-Moor, J. Reichrath, and J. P. Laye. 2019. Vitamin D–VDR Signaling Inhibits Wnt/β-Catenin–Mediated Melanoma Progression and Promotes Antitumor Immunity. Cancer Research 79(23):5986-5998.

Muscogiuri, G., B. Altieri, C. de Angelis, S. Palomba, R. Pivonello, A. Colao, and F. Orio. 2017. Shedding new light on female fertility: the role of vitamin D. Reviews

in Endocrine and Metabolic Disorders 18(3):273-283. doi: 10.1007/s11154-017-9407-2

Narvaez, C. J., and J. Welsh. 2001. Role of mitochondria and caspases in vitamin D-mediated apoptosis of MCF-7 breast cancer cells. Journal of Biological Chemistry 276(12):9101-9107.

NCBI. 2020. Conserved Domain Database. https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=132731 2023).

Neville, G., F. Martyn, M. Kilbane, M. O'Riordan, M. Wingfield, M. McKenna, and F. M. McAuliffe. 2016. Vitamin D status and fertility outcomes during winter among couples undergoing in vitro fertilization/intracytoplasmic sperm injection. International Journal of Gynecology & Obstetrics 135(2):172-176.

Nishikawa, J.-i., M. Matsumoto, K. Sakoda, M. Kitaura, M. Imagawa, and T. Nishihara. 1993. Vitamin D receptor zinc finger region binds to a direct repeat as a dimer and discriminates the spacing number between each half-site. Journal of Biological Chemistry 268(26):19739-19743.

O'Mahony, L., M. Stepien, M. J. Gibney, A. P. Nugent, and L. Brennan. 2011. The potential role of vitamin D enhanced foods in improving vitamin D status. Nutrients 3(12):1023-1041.

Oestreich, A. K., S. B. Chadchan, P. Popli, A. Medvedeva, M. N. Rowen, C. S. Stephens, R. Xu, J. P. Lydon, F. J. Demayo, and E. S. Jungheim. 2020. The autophagy gene Atg16L1 is necessary for endometrial decidualization. Endocrinology 161(1):bqz039.

Oestrup, O., V. Hall, S. Petkov, X. Wolf, S. Hyldig, and P. Hyttel. 2009. From zygote to implantation: morphological and molecular dynamics during embryo development in the pig. Reproduction in domestic animals 44:39-49.

Ozkan, S., S. Jindal, K. Greenseid, J. Shu, G. Zeitlian, C. Hickmon, and L. Pal. 2010. Replete vitamin D stores predict reproductive success following in vitro fertilization. Fertility and sterility 94(4):1314-1319.

Paffoni, A., S. Ferrari, P. Viganò, L. Pagliardini, E. Papaleo, M. Candiani, A. Tirelli, L. Fedele, and E. Somigliana. 2014. Vitamin D deficiency and infertility: insights from in vitro fertilization cycles. The Journal of Clinical Endocrinology & Metabolism 99(11):E2372-E2376.

Pal, L., A. Berry, L. Coraluzzi, E. Kustan, C. Danton, J. Shaw, and H. Taylor. 2012. Therapeutic implications of vitamin D and calcium in overweight women with polycystic ovary syndrome. Gynecological Endocrinology 28(12):965-968.

Palacios, C., and L. Gonzalez. 2014. Is vitamin D deficiency a major global public health problem? The Journal of steroid biochemistry and molecular biology 144:138-145.

Palermo, G. D., L. T. Colombero, and Z. Rosenwaks. 1997. The human sperm centrosome is responsible for normal syngamy and early embryonic development. Reviews of Reproduction 2:19-27.

Pan, L., A. F. Matloob, J. Du, H. Pan, Z. Dong, J. Zhao, Y. Feng, Y. Zhong, B. Huang, and J. Lu. 2010. Vitamin D stimulates apoptosis in gastric cancer cells in synergy with trichostatin A/sodium butyrate-induced and 5-aza-2′-deoxycytidine-induced PTEN upregulation. The FEBS journal 277(4):989-999.

Pan, X., D. Kong, L. Liu, F. Gao, X. Zhang, B. Tang, and Z. Li. 2014. Development block of golden hamster ICSI embryos is associated with decreased expression of HDAC1, HSPA1A and MYC. Cell Biology International 38(11):1280-1290.

Pereira, F., A. Barbachano, J. Silva, F. Bonilla, M. J. Campbell, A. Munoz, and M. J. Larriba. 2011. KDM6B/JMJD3 histone demethylase is induced by vitamin D and modulates its effects in colon cancer cells. Human molecular genetics 20(23):4655-4665.

Pérez-López, F. R. 2007. Vitamin D: the secosteroid hormone and human reproduction. Gynecological endocrinology 23(1):13-24.

Pike, J. W. 1991. Vitamin D3 receptors: structure and function in transcription. Annual review of nutrition 11(1):189-216.

Pike, J. W., and M. B. Meyer. 2012. The vitamin D receptor: new paradigms for the regulation of gene expression by 1, 25-dihydroxyvitamin D3. Rheumatic Disease Clinics 38(1):13-27.

Pike, J. W., M. B. Meyer, N. A. Benkusky, S. M. Lee, H. S. John, A. Carlson, M. Onal, and S. Shamsuzzaman. 2016. Genomic determinants of vitamin D-regulated gene expression. Vitamins & Hormones 100:21-44.

Prüfer, K., A. Racz, G. C. Lin, and J. Barsony. 2000. Dimerization with retinoid X receptors promotes nuclear localization and subnuclear targeting of vitamin D receptors. Journal of Biological Chemistry 275(52):41114-41123.

Rissi, V. B., W. G. Glanzner, M. P. De Macedo, K. Gutierrez, H. Baldassarre, P. B. D. Gonçalves, and V. Bordignon. 2019. The histone lysine demethylase KDM7A is

required for normal development and first cell lineage specification in porcine embryos. Epigenetics 14(11):1088-1101.

Rubin, B., C. Pilon, R. Pezzani, A. Rebellato, and F. Fallo. 2020. The effects of mitotane and 1α, 25-dihydroxyvitamin D3 on Wnt/beta-catenin signaling in human adrenocortical carcinoma cells. Journal of Endocrinological Investigation 43(3):357-367.

Rudick, B., S. Ingles, K. Chung, F. Stanczyk, R. Paulson, and K. Bendikson. 2012. Characterizing the influence of vitamin D levels on IVF outcomes. Human reproduction 27(11):3321-3327.

Salehi-Tabar, R., L. Nguyen-Yamamoto, L. E. Tavera-Mendoza, T. Quail, V. Dimitrov, B.-S. An, L. Glass, D. Goltzman, and J. H. White. 2012. Vitamin D receptor as a master regulator of the c-MYC/MXD1 network. Proceedings of the National Academy of Sciences 109(46):18827-18832.

Sanders, J., E. Bateson, M. Nomikos, A. Lai, and K. Swann. 2016. PLCz-induced Ca2+ oscillations are enhanced after germinal vesicle breakdown during mouse oocyte maturation. Reproduction Abstracts 3

Sarafin, K., R. Durazo-Arvizu, L. Tian, K. W. Phinney, S. Tai, J. E. Camara, J. Merkel, E. Green, C. T. Sempos, and S. P. Brooks. 2015. Standardizing 25-hydroxyvitamin D values from the Canadian health measures survey. The American journal of clinical nutrition 102(5):1044-1050.

Shaffer, P. L., and D. T. Gewirth. 2002. Structural basis of VDR–DNA interactions on direct repeat response elements. The EMBO journal 21(9):2242-2252.

Shahrokhi, S. Z., F. Ghaffari, and F. Kazerouni. 2016. Role of vitamin D in female reproduction. Clinica chimica acta 455:33-38.

Shariev, A., N. Painter, V. E. Reeve, N. K. Haass, M. S. Rybchyn, F. A. Ince, R. S. Mason, and K. M. Dixon. 2022. PTEN: a novel target for vitamin D in melanoma. The Journal of Steroid Biochemistry and Molecular Biology:106059.

Sheikh, M. S., H. Rochefort, and M. Garcia. 1995. Overexpression of p21WAF1/CIP1 induces growth arrest, giant cell formation and apoptosis in human breast carcinoma cell lines. Oncogene 11(9):1899-1905.

Silvagno, F., E. De Vivo, A. Attanasio, V. Gallo, G. Mazzucco, and G. Pescarmona. 2010. Mitochondrial localization of vitamin D receptor in human platelets and differentiated megakaryocytes. PLoS One 5(1):e8670.

Singh, I. 2014. Human embryology. JP Medical Ltd.

Smolikova, K., A. Mlynarcikova, and S. Scsukova. 2013. effect of 1α, 25-dihydroxyvitamin D. Endocrine Regulations 47:123-131.

Sun, J. 2016. VDR/vitamin D receptor regulates autophagic activity through ATG16L1. Autophagy 12(6):1057-1058.

Sun, T., Y. Zhao, D. J. Mangelsdorf, and E. R. Simpson. 1998. Characterization of a region upstream of exon I. 1 of the human CYP19 (aromatase) gene that mediates regulation by retinoids in human choriocarcinoma cells. Endocrinology 139(4):1684-1691.

Suzuki, T., H. Tazoe, K. Taguchi, Y. Koyama, H. Ichikawa, S. Hayakawa, H. Munakata, and M. Isemura. 2006. DNA microarray analysis of changes in gene expression

induced by 1, 25-dihydroxyvitamin D3 in human promyelocytic leukemia HL-60 cells. Biomedical research 27(3):99-109.

Swami, S., A. V. Krishnan, L. Peng, J. Lundqvist, and D. Feldman. 2013. Transrepression of the estrogen receptor promoter by calcitriol in human breast cancer cells via two negative vitamin D response elements. Endocrine-Related Cancer 20(4):565-577.

Swami, S., N. Raghavachari, U. R. Muller, Y. P. Bao, and D. Feldman. 2003. Vitamin D growth inhibition of breast cancer cells: gene expression patterns assessed by cDNA microarray. Breast cancer research and treatment 80(1):49-62.

Thomson, R. L., S. Spedding, and J. D. Buckley. 2012. Vitamin D in the aetiology and management of polycystic ovary syndrome. Clinical endocrinology 77(3):343-350.

Thys-Jacobs, S., D. Donovan, A. Papadopoulos, P. Sarrel, and J. P. Bilezikian. 1999. Vitamin D and calcium dysregulation in the polycystic ovarian syndrome. Steroids 64(6):430-435.

Toell, A., P. Polly, and C. Carlberg. 2000. All natural DR3-type vitamin D response elements show a similar functionality in vitro. Biochemical Journal 352(2):301-309.

Toropainen, S., S. Väisänen, S. Heikkinen, and C. Carlberg. 2010. The down-regulation of the human MYC gene by the nuclear hormone 1α, 25-dihydroxyvitamin D3 is associated with cycling of corepressors and histone deacetylases. Journal of molecular biology 400(3):284-294.

Tribulo, P., J. I. Moss, M. Ozawa, Z. Jiang, X. C. Tian, and P. J. Hansen. 2017. WNT regulation of embryonic development likely involves pathways independent of nuclear CTNNB1. Reproduction 153(4):405-419.

van de Vijver, A., P. Drakopoulos, L. Van Landuyt, A. Vaiarelli, C. Blockeel, S. Santos-Ribeiro, H. Tournaye, and N. P. Polyzos. 2016. Vitamin D deficiency and pregnancy rates following frozen–thawed embryo transfer: a prospective cohort study. Human reproduction 31(8):1749-1754.

Verlinden, L., A. Verstuyf, R. Convents, S. Marcelis, M. Van Camp, and R. Bouillon. 1998. Action of 1, 25 (OH) 2D3 on the cell cycle genes, cyclin D1, p21 and p27 in MCF-7 cells. Molecular and cellular endocrinology 142(1-2):57-65.

Vuolo, L., A. Faggiano, and A. A. Colao. 2012. Vitamin D and cancer. Frontiers in endocrinology 3:58.

Wakabayashi, M., S. Tamura, S. Kanzaki, M. Kosugi, Y. Yoshimura, T. Ito, K. Nagata, K. Sato, S. Takada, and Y. Sekita. 2021. Five multicopy gene family genes expressed during the maternal-to-zygotic transition are not essential for mouse development. Biochemical and Biophysical Research Communications 534:752-757.

Wang, J., Y. Zhao, M. A. Kauss, S. Spindel, and H. Lian. 2009. Akt regulates vitamin D3-induced leukemia cell functional differentiation via Raf/MEK/ERK MAPK signaling. European journal of cell biology 88(2):103-115.

Wang, Q. M., J. B. Jones, and G. P. Studzinski. 1996. Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D3 in HL60 cells. Cancer Res 56(2):264-267.

Wei, Z., E. Yoshihara, N. He, N. Hah, W. Fan, A. F. Pinto, T. Huddy, Y. Wang, B. Ross, and G. Estepa. 2018. Vitamin D switches BAF complexes to protect β cells. Cell 173(5):1135-1149. e1115.

Wood, R. J., L. Tchack, G. Angelo, R. E. Pratt, and L. A. Sonna. 2004. DNA microarray analysis of vitamin D-induced gene expression in a human colon carcinoma cell line. Physiological genomics 17(2):122-129.

Wu, L., J. Kwak-Kim, R. Zhang, Q. Li, F. T. Lu, Y. Zhang, H. Y. Wang, L. W. Zhong, and Y. S. Liu. 2018. Vitamin D level affects IVF outcome partially mediated via Th/Tc cell ratio. American Journal of Reproductive Immunology 80(6):e13050.

Xu, F., S. Wolf, and J. Xu. 2021. Vitamin D in follicular development and oocyte maturation. Reproduction 161(6):R129-R137.

Xu, J., M. S. Lawson, F. Xu, Y. Du, O. Y. Tkachenko, C. V. Bishop, L. Pejovic-Nezhat, D. B. Seifer, and J. D. Hennebold. 2018. Vitamin D3 regulates follicular development and intrafollicular vitamin D biosynthesis and signaling in the primate ovary. Frontiers in Physiology:1600.

Yang, C., E. C. Wright, B. J. Hale, A. F. Keating, and J. W. Ross. 2013. FOXO3 expression and function in the pig oocyte and embryo. Iowa State University Animal Industry Report 10(1)

Yao, X., Z. Wang, M. El-Samahy, C. Ren, Z. Liu, F. Wang, and P. You. 2020. Roles of vitamin D and its receptor in the proliferation and apoptosis of luteinised granulosa cells in the goat. Reproduction, Fertility and Development 32(3):335-348.

Yao, X., G. Zhang, Y. Guo, E.-S. Mohamed, S. Wang, Y. Wan, L. Han, Z. Liu, F. Wang, and Y. Zhang. 2017. Vitamin D receptor expression and potential role of vitamin D on cell proliferation and steroidogenesis in goat ovarian granulosa cells. Theriogenology 102:162-173.

Yoshizawa, T., Y. Handa, Y. Uematsu, S. Takeda, K. Sekine, Y. Yoshihara, T. Kawakami, K. Arioka, H. Sato, and Y. Uchiyama. 1997. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nature genetics 16(4):391-396.

Yu, J. S., and W. Cui. 2016. Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. Development 143(17):3050-3060.

Zalzman, M., G. Falco, L. V. Sharova, A. Nishiyama, M. Thomas, S.-L. Lee, C. A. Stagg, H. G. Hoang, H.-T. Yang, and F. E. Indig. 2010. Zscan4 regulates telomere elongation and genomic stability in ES cells. Nature 464(7290):858-863.

Zhang, Q., W. Li, P. Feng, Y. Liu, B. Chu, J. Zhao, Y. Li, Y. Zhang, and J. Liu. 2022. Lnc5926 is essential for early embryonic development in goats through regulation of ZSCAN4 and EIF1AX. Theriogenology 180:87-93.

Zheng, W., B. Duan, Q. Zhang, L. Ouyang, W. Peng, F. Qian, Y. Wang, and S. Huang. 2018. Vitamin D-induced vitamin D receptor expression induces tamoxifen sensitivity in MCF-7 stem cells via suppression of Wnt/β-catenin signaling. Bioscience Reports 38(6)