Characterisation of novel mechanisms for the regulation of trophoblast function by vitamin D

by

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Abstract

Introduction: Vitamin D deficiency is common during pregnancy but its impact on maternal and foetal health is still unclear. The placenta is a key target for vitamin D: the vitamin D activating enzyme 1α-hydroxylase (CYP27B1), vitamin D receptor (VDR) and vitamin D binding protein (DBP) are all expressed by trophoblast cells early in pregnancy. This PhD aimed to identify new effects of the vitamin D system on trophoblast cell function.

Hypothesis: We hypothesised that vitamin D plays a key role in trophoblast behaviour and that this may be important for healthy placental development.

Methods: *In vitro* studies were carried out using trophoblast cell lines JEG3, BeWo and HTR8, with thyroid cancer cells (TPC) as a non-trophoblast comparator. Expression of mRNA and protein was compared for cells grown on plastic and extra-cellular matrix. Cells were assessed for motility (wound closure assay), proliferation (BrdU assay) and matrix invasion (Boyden chambers). Protein and mRNA expression were assessed following treatments using an ERK1/2 blocker (U0126), a megalin blocker (RAP), *VDR* siRNA, and DBP free serum. Pregnancy serum samples from 1st trimester healthy pregnancy patients and 1st trimester pre-eclampsia patients were assessed for DBP concentration and *in vitro* matrix invasion of trophoblast cells.

Results: In plasticware cultures, JEG3, BeWo and HTR8 cells showed weak expression of VDR and no induction of the vitamin D-response gene *CYP24A1* by 1,25-dihydroxyvitamin D (1,25D). TPC cells showed strong nuclear VDR expression and

potent 1,25D-mediated induction of *CYP24A1*. Conversely, for matrix-grown trophoblast cells there was abundant intracellular VDR and DBP, with apparent nuclear localisation of both, in the presence of 1,25D, but no induction of *CYP24A1*. Treatment with 1,25D significantly enhanced the invasion of Matrigel^{TM} by trophoblast cells. Cells cultured on matrix with serum from DBP knockout mice (DBP-/-) or in the presence of an inhibitor of megalin-mediated endocytosis (RAP) showed no intracellular DBP, indicating uptake of exogenous DBP by these cells. These treatments also resulted in low matrix invasion by trophoblast cells. Trophoblast and TPC cells showed non-genomic induction and nuclear localisation of phospho-ERK1/2 (pERK1/2) in response to 1,25D, and inhibition of pERK1/2 blocked DBP uptake and trophoblast matrix invasion. DBP is also an actin-binder and decreased cellular uptake of DBP resulting in impaired matrix invasion were associated with elevated intracellular levels of G-actin and concomitant lower F-actin. Levels of DBP and 1,25D from 1st trimester pregnancy serum samples significantly correlated with JEG3 matrix invasion, suggesting that this mechanism may be important for normal healthy pregnancies. In 1st trimester serum samples, the concentration of DBP was lower in women who subsequently went on to develop pre-eclampsia.

Discussion: These data suggest that 1,25D enhances cellular uptake of serum DBP by pERK-mediated signalling and that this is important for maintaining homeostasis of intracellular actin. We, therefore, propose that optimal trophoblast function and healthy placentation requires the actions of maternal 1,25D and DBP. Both factors should be measured in studies of vitamin D and pregnancy.

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Abbreviations	
25D2	25-hydroxyvitamin D2
25D3	25-hydroxyvitamin D3
25D	25-hydroxyvitamin D
1,25D2	1,25-dihydroxyvitamin D2
1,25D3	1,25-dihydroxyvitamin D3
1,25D	1,25-dihydroxyvitamin D
DBP	vitamin D binding protein
VDR	vitamin D receptor
VDRE	vitamin D response elements
CYP27A1	vitamin D- 25-hydroxylase enzyme (cytochrome P450 27A1)
CYP2R1	vitamin D- 25-hydroxylase enzyme (cytochrome P450-2R1)
CYP27B1	vitamin D- 1α-hydroxylase enzyme (cytochrome P450 27B1)
CYP24A1	vitamin D- 24-hydroxylase enzyme (cytochrome P450 24A1)
UVB	Ultraviolet B
EVT cells	extra-villous cytotrophoblast cells
TPC-1 cells/TPC cells	thyroid papillary carcinoma-1 cells
mRNA	messenger-ribonucleic acid
siRNA	short interfering RNA (or silencing RNA)
cDNA	complementary deoxyribonucleic acid
FBS	foetal bovine serum
BSA	bovine serum albumin
PBS	phosphate-buffered saline
PBS-Tween	phosphate-buffered saline with Tween^{TM} 20
PTH	parathyroid hormone
ELISA	enzyme-linked immunosorbent assay
RT-qPCR	real-time quantitative polymerase chain reaction
Ct value	threshold cycle value
µg	microgram
µL	microlitre
IU	International Unit
nM	nano-Molar or nanomole/litre
ng/mL	nanogram/millilitre
µg/mL	microgram/millilitre
pg/mL	picogram/millilitre
mg/L	milligram/litre

ABPs	actin binding proteins
ERK	extracellular signal-regulated kinase
pERK	phospho-extracellular signal-regulated kinase
APGAR	Appearance, Pulse, Grimace, Activity, and Respiration
HCG	human chorionic gonadotropin
rpm	rotations per minute
BrdU assay	bromodeoxyuridine/ 5-bromo-2'-deoxyuridine assay
MTT assay	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
BCA assay	bicinchoninic acid assay
Na-K-ATPase	sodium-potassium adenosine triphosphatase
RAP	receptor-associated protein
LRP2	LDL Receptor Related Protein 2 (also known as *megalin* gene)
MMP2	matrix metalloproteinase 2
TIMP1	tissue inhibitor of metalloproteinase 1
T75	non-pyrogenic, non-cytotoxic polymer tissue culture Flask with 75cm^2 cell growth area
ECM	extracellular matrix
GFR Matrigel^{TM}	growth factor reduced Matrigel^{TM}
OD	optical density
SDS-PAGE	sodium dodecyl sulphate- polyacrylamide gel electrophoresis
DAB	chromogen diaminobenzidine
IgG	Immunoglobulin G
G-actin	globular actin
F-actin	filamentous actin
EGF	epidermal growth factor
HGF	hepatocyte growth factor
rpm	rotation per minute

Chapter 2:

INTRODUCTION

2.1 Sources of vitamin D and its physiology

Vitamin D is a unique molecule in biology in that it involves environmental science, nutrition, and endocrinology. The action of sunlight on the skin is the primary source of vitamin D for humans but even the most primitive organisms on earth such as phytoplankton can produce vitamin D when exposed to sunlight [1]. Ultraviolet radiation type B (UVB; λ290-315 nm) photoisomerises 7-dehydro-cholesterol (7DHC) in the epidermis to pre-vitamin D3 [2] as shown in **Figure 2.1**, which subsequently forms vitamin D3 (cholecalciferol) on thermal isomerisation, and enters the circulation to transform into its active form, 1,25D (1,25-dihydroxyvitamin D), which subsequently results in various cellular functions of vitamin D via VDR (vitamin D receptor). The rate of dermal synthesis of vitamin D also depends on skin pigmentation, as increased melanin reduces the absorption of UVB and therefore decreases pre-vitamin D production [3]. Studies have shown that when people with dark coloured skin are exposed to the same amount of sunlight as people with light coloured skin they produce less vitamin D [3]. Similarly, the use of sunscreen on the skin reduces the absorption of UVB light and the production of vitamin D [4]. Effective UV radiation on 1000 cm^2 skin induces adequate levels of vitamin D synthesis with three to seven minutes of exposure, three times per week [5]. However, the absorption of UVB rays by the epidermis is also dependent on other factors, including the amount of ozone that the solar radiation has to pass through, geographical location, the latitude of the location, the season of the year and time of day [6-8]. In this way, vitamin D synthesis can be considered to be dependent on environmental factors.

The other source of vitamin D is via food (**Figure 2.1**). Animal sources including wild salmon [9], eel, herring, egg yolk and liver [10, 11], and the vitamin D gained from sunlight, is termed cholecalciferol (vitamin D3). Non-animal sources (Ergocalciferol, vitamin D2) include mushrooms and alfalfa [12]. Milk has been demonstrated to contain an inadequate amount of vitamin D in the US and Canada, unless fortified [11, 13]. Thus, butter, margarine, infant formula milk and cereals are frequently fortified with vitamin D for human consumption in the UK and US [14, 15]. Vitamin D3 is absorbed efficiently by human intestine [16]. With these observations in mind, vitamin D is considered to be a nutritional factor and the name of vitamin D stems from the fact that it was originally thought to be obtained exclusively from food sources. The following section describes some of the considerations required to maintain optimal vitamin D levels through nutrition.

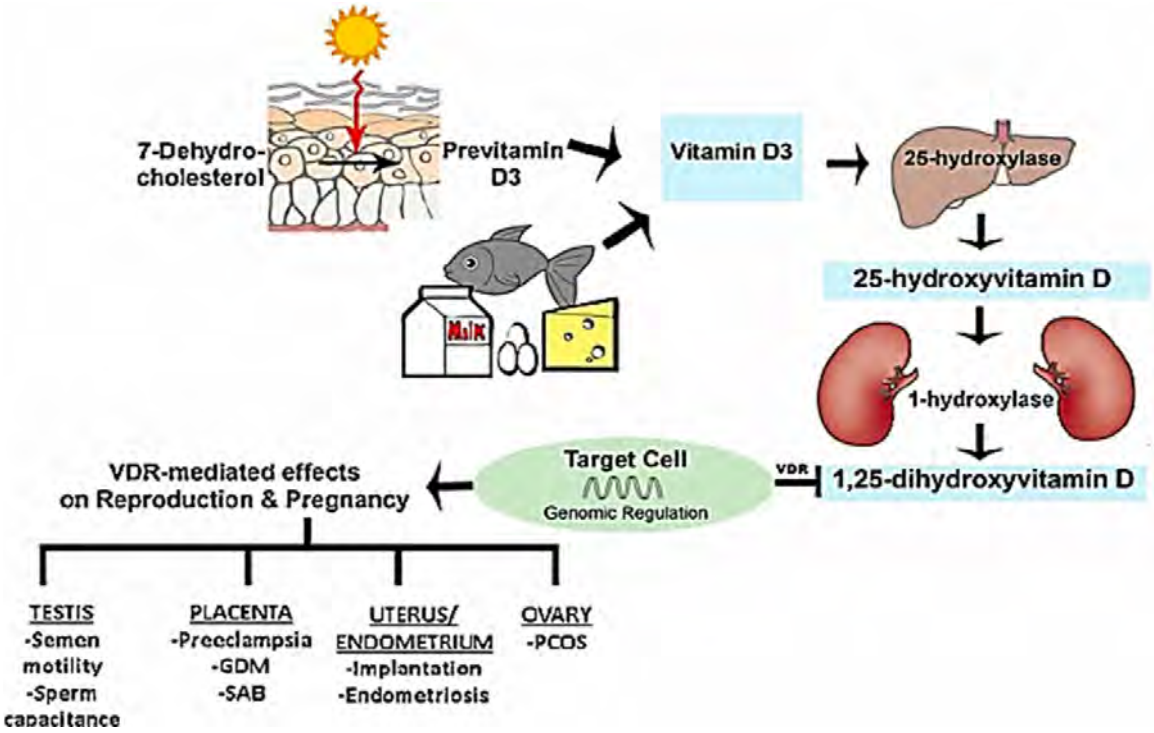


Figure 2.1 Vitamin D pathway in human.
Image adapted from Pacis MM. et. al. [17], showing sources of vitamin D and its metabolic pathway in the human body, ultimately its effect on reproductive organs.

2.2 Dietary vitamin D recommendations

Recommended vitamin D (25-hydroxyvitamin D or 25D) intake varies in different countries (**Table 2.1**), depending on various factors such as sun exposure, latitude, sunscreen use, diet, age and race, so there is likely to be significant intra-individual variation. The average dietary intake for British adults (up to 64 years) is 2-4 µg/day (80-160 IU/day) which is lower than the UK recommended dietary intake of 10 µg/day (400 IU/day) [18]. Similarly, citizens of Poland, Denmark and Finland, consume less vitamin D than recommended [19], highlighting the significance of global vitamin D deficiency**.** Thus, supplementation or food fortification may be required for many countries around the globe. However, supplementation recommendations are not uniform worldwide and vary by country and academic society (**Table 2.1**) [20]. The British Scientific Advisory Committee on Nutrition (SACN) recommends 10 µg/day (400 IU/day) for all age groups [18]. In contrast, the National Academy of Medicine recommendation, USA (previously known as Institute of Medicine, USA) [21] recommends 10 µg/day up to 1 year of age, followed by 15 µg/day (600 IU/day) up to 70 years age and this is increased to 20 µg/day (800 IU/day) in elderly. There is a 1 ng/mL (2.5 nmol/L) increase in serum 25D levels for every 2.5 µg (100 IU) of ingested vitamin D [22]. Different countries and scientific societies of the world show disparity in their dietary vitamin D recommendations (**Table 2.1**), establishing the need for a uniform global recommendation.

Table 2.1 Recommended dietary intake of vitamin D in different countries for adequate 25D serum levels shown as µg/day and (IU/day).

Country	Up to 1 year	>1year to 50years	>50 years to 70years	>70years
European countries [23]	10-25 (400-1000)	10 (400)	10 (400)	10 (400)
Nordic countries [24]	10 (400)	7.5 (300)	10 (400)	10 (400)
US [25-27]	5 (200)	5 (200)	10 (400)	15 (600)
National Academy of Medicine recommendation, USA [21]	10 (400)	15 (600)	15 (600)	20 (800)
British Scientific Advisory Committee on Nutrition (SACN) [18]	10 (400)	10 (400)	10 (400)	10 (400)
US Endocrine Society [28]	10 (400)	15 (600)	15 - 20 (600–800)	15 - 20 (600-800)

2.3 Synthesis of 25-hydroxyvitamin D (25D)

Parental vitamin D from sunlight or diet must undergo a series of metabolic conversions to produce an active, functional form of vitamin D (**Figure 2.1**). The initial step occurs primarily in the liver, and involves hydroxylation at carbon 25 position to form 25-hydroxyvitamin D3 (25D3) and 25-hydroxyvitamin D2 (25D2 [diet derived only]) (**Figure 2.4**) [29]. For the remainder of this thesis, 25D2 and 25D3 will be referred collectively as 25D. Liver synthesis of 25D from vitamin D is catalysed by vitamin D-25-hydroxylase enzyme, involving two possible enzymes, cytochrome P450 27A1 (CYP27A1) [30, 31] and cytochrome P450 2R1 (CYP2R1) [32, 33].

Mitochondrial enzyme CYP27A1 is one of the essential enzymes for 25D hydroxylation as evident from the skeletal deformations observed in a rare genetic disorder resulting from an abnormal *CYP27A1* gene causing cerebrotendinous xanthomatosis (CTX) [34, 35]. This enzyme is found in the mitochondria at various locations such as liver, intestine, endothelial cells, lungs and central nervous system [36]. 25D hydroxylation is also mediated via another enzyme, CYP2R1, which is located in the endoplasmic reticulum (ER) [37, 38]. Mutation of *CYP2R1* gene results in complete elimination of vitamin D 25-hydroxylase enzyme activity leading to 25D deficiency symptoms such as bone pain, muscle weakness, respiratory symptoms and cognitive impairment in older adults [32]. These data suggest the significance of both enzymes in the synthesis of 25D in humans.

25D is the major circulating form of vitamin D, which has a half-life of 14-20 days [39] and is, therefore, the most commonly measured vitamin D metabolite, and the one that is used to define vitamin D status [40]. Like vitamin D and all its metabolites, 25D circulates in serum bound to vitamin D binding protein (DBP) or albumin, but a very small amount of 25D is present in an unbound or free form [41]. Recommended optimal serum 25D status for healthy individuals varies across countries and organisations (**Table 2.2**). The American National Academy of Medicine classified vitamin D "sufficiency" as a serum 25D level of 20 to 50 ng/mL (50-125 nmol/L) [21]. In 2016, the Vitamin D Workshop published an agreement stating that serum 25D levels <20 ng/mL (<50 nmol/L) could be classified as "insufficient", and <12 ng/mL (<30 nmol/L) serum 25D, as "deficient" [42]. The US Endocrine Society defines "sufficient" as 25D level of 30 to 50 ng/mL (75-125 nmol/L), which is higher than the American National Academy

of Medicine's set values [28]. In contrast to the rest of the world, the UK Scientific Advisory Committee on Nutrition (SACN) has only reported a "sufficient" value of >10 ng/mL (>25 nmol/L) serum 25D level [18], however, compared to the rest of the world, the UK has failed to determine the "insufficient" and "toxic" values for 25D level. This is probably because the UK has identified the lowest adequate serum 25D level of 10 ng/mL (25 nmol/L), that is required to prevent vitamin D deficiency complications. Keeping the cut-off at a low level helps to maintain a standard serum 25D level, making recommending of additional supplementation easier.

Table 2.2 Definitions of vitamin D status by country and scientific organisation based on 25D level in blood shown in ng/mL and (nmol/L).

Study group	Sufficient	Insufficient	Deficient	Toxic
American National Academy of Medicine (2011) [21]	20-50 (50-125)	12 – 19.99 (30-49.99)	<12 (<30)	>50 (>125)
US Endocrine Society [43]	30-50 (75-125)	21-29 (52.5-74.9)	<20 (<50)	>50 (>125)
Vitamin-D workshop 2016 [44]	-	<20 (<50)	<12 (<30)	-
UK Scientific Advisory Committee on Nutrition (SACN) [18]	>10 (>25)	-	<10 (>25)	-
Nordic population[24]	>20 (>50)	5 – 10 (12.5-25)	<5 (<12.5)	-
British population [45]	>20 (>50)	11 – 20 (27.5-50)	<11 (<27.5)	-
American population [46]	32- 100 (80-250)	11- 32 (27.5-80)	<10 (<25)	>100 (>250)
Pre-IOM classification (2007) [47]	>29 (>72.5)	21 – 29 (52.5-72.5)	<20 (<50)	>150 (>375)

2.4 Transport of 25D and vitamin D metabolites by DBP

25D is the inactive circulating pre-hormone form of vitamin D (**Figure 2.1**). 25D circulates in serum bound to a dedicated serum binding globulin, vitamin D binding protein (DBP), or albumin; but may also exist as an unbound 'free' form. Studies have shown that around 85% of 25D is bound to DBP, 10% to albumin and <1% stays as free 25D [41, 48, 49] (**Figure 2.2**). Bioavailable 25D refers to the 25D which is non-bound to DBP, that is the 25D which is bound to albumin and the free-25D [50]. The

affinity of 25D to DBP is 1000-fold higher than for another potential less specific serum binding proteins such as albumin [51]. However, albumin is present in much higher concentrations in the serum (approximately 650 µM) relative to DBP (approximately 5 µM) and, consequently up to 10% of all 25D may circulate bound to albumin [41]. The non-bound, free form of 25D is highly lipophilic and may thus diffuse across the lipid-rich membranes of cells. A similar mechanism exists for free 1,25D (1,25D-hydroxyvitamin D) in serum [52]. As 25D has been shown to have a higher binding affinity for DBP compared to 1,25D, it has a greater dependency for DBP than 1,25D. As such, the mechanisms by which DBP and its cargo are imported into cells are much more important for 25D uptake than 1,25D. This is discussed in greater detail later in this section.

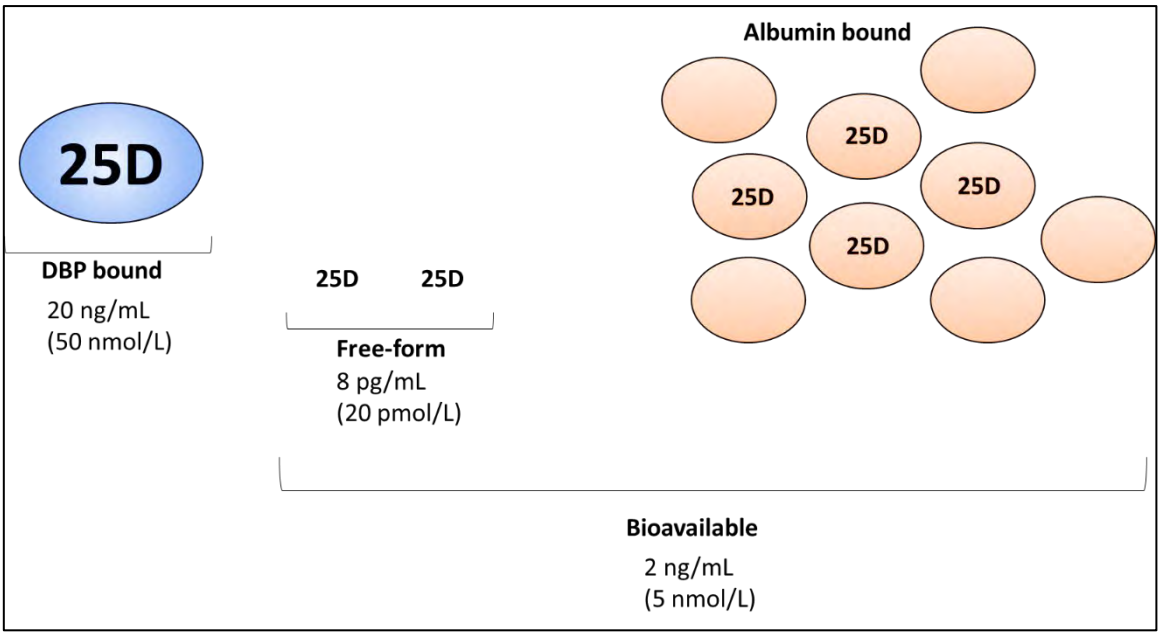


Figure 2.2 Three forms of serum 25D: DBP bound, albumin bound and free- 25D. Schematic showing the different forms of 25D in the circulation. Approximate serum concentrations of DBP-bound, bioavailable 25D and free-25D are shown as ng/mL (nmol/L) and pg/mL (pmol/L). The higher binding affinity of DBP for 25D is shown by the larger DBP shape and bold lettering.

DBP is a 58 kDa glycosylated α-globulin also known as Group-Specific Component (GC), composed of 474 amino acid residues that fold into a disulphide bonded triple-domain structure [53], with a serum concentration of approximately 5 μM in humans [54]. Its half-life is around 1.7 days which is shorter than the half-life of 25D (approximately 15 days) [39]. This is considered as the primary carrier protein of the lipophilic vitamin D metabolites in serum [54]. An estimated daily DBP production in an adult human is 700-900 mg/day, which is lower than the total albumin production of 280 g in a normal healthy adult [55]. However, only around 40% of albumin is in the serum with 60% in interstitial spaces in tissues, with a similarity in distribution observed for DBP in human [56] and rabbit [57]. The liver is the major production site of DBP, however, it is also expressed in kidney, adipose tissue, yolk sac and testis [58]. DBP is partially filtered in the glomerulus of the kidney and then reabsorbed in proximal convoluted tubules of the kidney by a carrier receptor called megalin [55]. This is discussed in greater detail later in the section (**Section 2.5**). The reference range for DBP in women is shown to be 200–550 mg/L [70], and this level increases to between 510 mg/L and 700 mg/L during pregnancy [71], suggesting an increased requirement of DBP during pregnancy.

DBP is a highly polymorphic protein, and based on its mobility on electrophoresis gels, DBP protein has been subdivided into three broad phenotypes: GC-1F (faster migration); GC-1S (slower migration); and GC2. These three classical forms of DBP are the most common genetic variations in humans [59]. African-Caribbean populations demonstrate a higher concentration of the GC1F allele [60], compared to Caucasian populations showing GC1S variation [61]. A recent study suggested that

circulating DBP levels were lower in African-Americans because a higher proportion of these subjects had the GC1F allele combination [62]. Because of these apparent low levels of DBP in African-Americans, it was proposed that even though these subjects had low serum levels of 25D, the actual levels of unbound or free-25D were the same as white Americans. However, subsequent studies have shown that lower measurement of DBP in African-Americans was due to the monoclonal antibody to DBP used in the assay to measure the serum DBP in these subjects, which may not have fully recognised epitopes on the GC1F form of DBP, and thus levels were underestimated [63, 64]. The functional impact of genetic variations in DBP has been studied extensively [65]. In particular, the single nucleotide polymorphisms (SNPs) that contribute to the GC1F, GC1S and GC2 phenotypes are the key genetic determinants of circulating 25D levels [66]. This may be due to effects on DBP transport of 25D or possibly the megalin-mediated recovery of DBP and 25D in the proximal tubules of the kidney.

In contrast to the apparent importance of DBP genetic variations for vitamin D status, complete knockout of the DBP (*GC*) gene in mice did not lead to any major physiological effect [67]. 1,25D has been demonstrated to be a potent promoter of absorption of dietary calcium, resulting in increased calcium in circulation [68]. Along with calcium, parathyroid hormone (PTH) are interdependent with 1,25D to regulate bone resorption, also contributing to increased circulating calcium level and bone formation [69]. Interestingly, global DBP knockout mice have extremely low serum levels of 25D and 1,25D but appear to have no abnormal calcium or PTH (parathyroid hormone) function, and present with normal skeletal function. The DBP knockout mice

only showed abnormal bone and calcium physiology when they were fed a vitamin D3 deficient diet [67]. These observations suggest that many normal functions of vitamin D are due to the small proportion of 25D that is unbound to DBP, in other words, the free-25D. Similarly, mice fed with only vitamin D2 diet showed improved bone status compared to mice fed only vitamin D3 diet [70]. Relative to 25D3, 25D2 shows a lower binding affinity for DBP and thus a greater proportion of this form of vitamin D is free.

In addition to its ability to bind vitamin D metabolites, DBP also has a potent actin-binding capacity [71], allowing DBP in the circulation to regulate surplus extracellular levels of globular-actin (G-actin), which can be polymerised to filamentous-actin (F-actin) after tissue injury [72]. Actin is a conserved protein which forms a tight molecular complex with serum-DBP [71]. Actin is also known to be associated with other proteins such as DNase1 and profilin, and DBP can form bonds with DNAse1, indicating that actin has multiple binding sites for both these proteins [71]. An intracellular, cytoplasmic form of actin exists in nucleated cells which binds DBP, shown by DNase1 binding to cytosolic actin and not serum DBP [71].

2.5 Conversion of 25D to 1,25D

25D largely bound to DBP circulates in the blood and reaches renal tissues, where it is filtered in the glomerulus. This is then followed by reabsorption of the DBP-25D complex from the glomerular filtrate via the membrane receptor megalin and its co-receptor cubilin which are expressed by cells within the proximal convoluted tubule [73-75]. The subsequent endocytic internalisation of the megalin-DBP-25D complex, and the intracellular disruption of this complex, enables delivery of 25D for further

metabolism. In the proximal tubules, 25D is converted to its hormonally active form, 1α,25-dihydroxyvitamin D (1,25D), by hydroxylation of 25D at the carbon 1 position by the 25-hydroxyvitamin D3-1α-hydroxylase enzyme (CYP27B1) [31, 76]. This mechanism is only common in tissues expressing megalin, like kidney [77], placenta [78], breast and prostate [79]. Non-megalin mediated 25D transport occurs in cells not expressing megalin such as monocytes and macrophages [80], where unbound, free-25D is thought to diffuse into the cell through the cellular lipid bilayer. This mechanism is further explained in **Figure 2.3**.

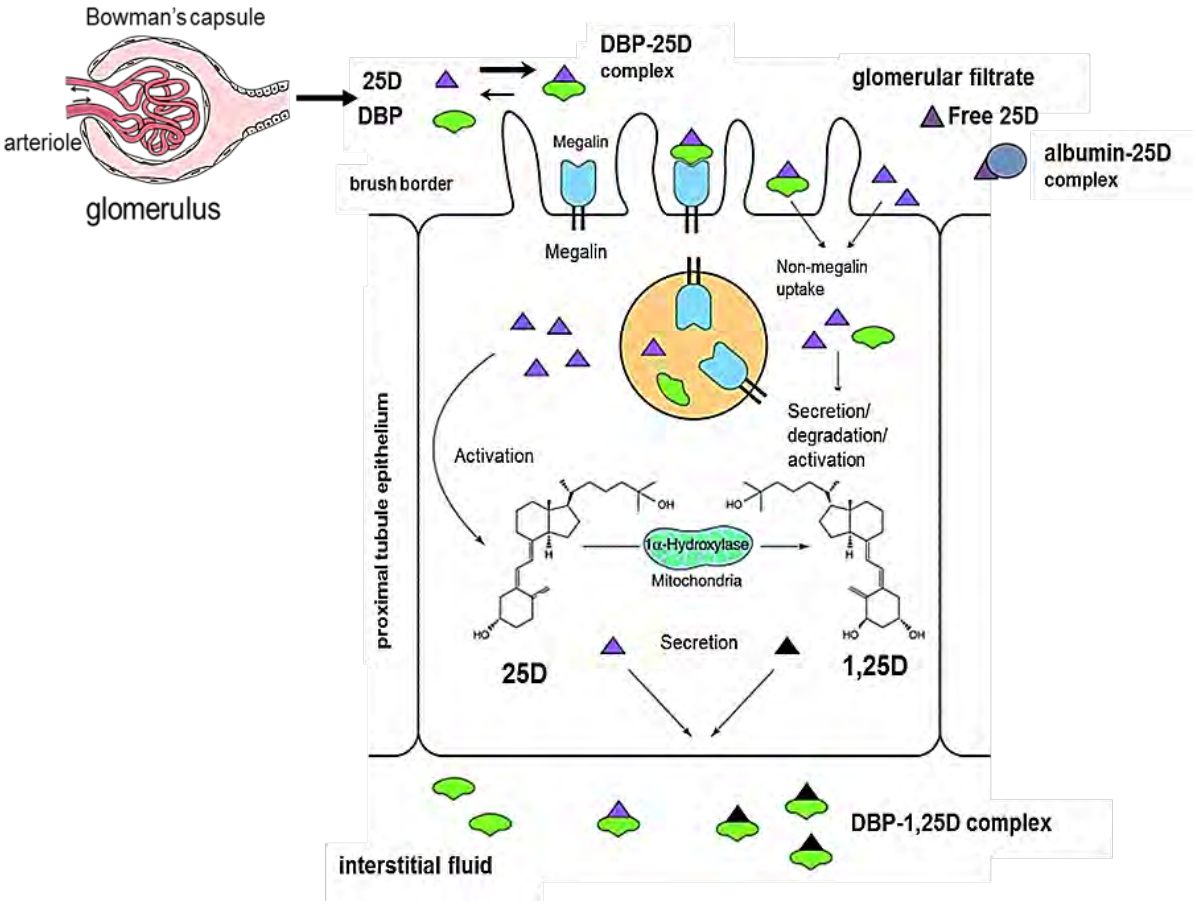


Figure 2.3 Megalin mediated and non-megalin mediated renal reabsorption of DBP and conversion of 25D to 1,25D.
Image adapted from Chun *et al*. manuscript on "Vitamin D and DBP: the free hormone hypothesis revisited" [50]. Schematic representation showing the mechanism of megalin receptor-mediated and non-megalin mediated endocytosis of 25D-DBP complex and free-25D, in proximal convoluted tubule cells of the kidney.

Megalin (glycoprotein 330 or LRP2) is a 600 kDa transmembrane protein that forms part of an endocytic transport mechanism situated at the plasma membrane of epithelial cells [73]. Megalin belongs to the low-density lipoprotein receptor superfamily (LDLR) [81], and its overall structure is similar to LDLR-related protein 1 (LRP1), thus giving it the name of LRP2 [81]. It has a large extracellular region, a transmembrane region and a small intracellular region [82]. This extracellular region can bind to multiple ligands with more than 50 identified thus far, including DBP [83], insulin, insulin-like growth factor and albumin [73, 84]. It has a long half-life and fast recycling, making it ideal for reabsorption. Megalin and cubilin are co-expressed in tissues such as intestine [85], kidney [86], lung [87], neurons [88], endometrium [88, 89], placental cytotrophoblasts [88, 90], thyroid and parathyroid glands [91, 92], breast and prostate [79]. Kidney diseases (such as pre-eclampsia and diabetes) and genetic mutations alter megalin expression and function, resulting in further exacerbation of the pathology. Megalin secretion in urine increases with albuminuria in early type 2 diabetes mellitus [93], indicating that megalin is a potential biomarker for diabetic nephropathy. One of the factors shown to lower abnormal megalin expression is angiotensin II [94].

Conversion of 25D to its active form 1,25D within the kidney is coordinated by serum levels of PTH, fibroblast growth factor 23 (FGF23), calcium, and phosphate [31, 68, 95]. This is involved in many metabolic processes including regulation of bone integrity, calcium homoeostasis as well as extra-skeletal actions [96, 97]. Intracellular conversion of 25D to 1,25D is catalysed by the cytochrome P450 enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) located in the inner mitochondrial

membrane which hydroxylates the 1-carbon position of 25D [98]. 1,25D is the active, hormonal form of vitamin D which mediates its functional activity. Production of 1,25D in the kidney is tightly regulated via the action of parathyroid hormone, and regulated by serum phosphorous and calcium levels [99]. In patients with kidney diseases, CYP27B1 is reduced, resulting in lowering of 1,25D production and ultimately leading to reduced serum calcium levels [100]. On the contrary, in diseases involving dysregulated and dominant extra-renal production of CYP27B1, serum 1,25D mediated hypercalcaemia is observed overriding the renal and parathyroid calcium-lowering functions [101]. This suggests that the CYP27B1 enzyme has a significant role in maintaining adequate serum levels of the active form of vitamin D, resulting in its various target functions.

Although vitamin D can exert direct effects on pregnancy via VDR-mediated responses within the placenta that are the focus of this PhD project, it is also possible that effects of vitamin D on pregnancy can also occur indirectly as a consequence of its potent classical actions on calcium homeostasis. One of the most well-established actions of 1,25D in the human body is the promotion of absorption of dietary calcium from the intestinal lumen and its subsequent accumulation in the circulation. 1,25D increases the calcium-binding proteins within intestinal cells, also increasing the permeability of the intestinal brush border to calcium [68]. Parathyroid hormone and 1,25D also synergistically regulate bone resorption which also contributes to increased circulating calcium level in blood [69]. During calcium deficiency state, 1,25D induces bone absorption, simultaneously inhibiting bone mineralisation thus maintaining circulatory calcium homeostasis. Various changes in calcium and

vitamin D metabolism are observed in the elderly, including less dietary vitamin D intake, reduced renal vitamin D production, decreased VDR level, resulting in reduced renal calcium absorption, ultimately leading to bone loss [102].

The most well-recognised feature of low serum calcium associated with vitamin D-deficiency is the bone disease rickets [40]. However, vitamin D and calcium may also be linked to pregnancy and pregnancy disorders, as reviewed extensively by Kovacs and colleagues [103]. During pregnancy, vitamin D and calcium combined supplementation has been shown to reduce the risk of late pregnancy complications like pre-eclampsia [104]. Nevertheless, vitamin D alone can reduce both early and late pregnancy complications (pre-term birth, pre-eclampsia, low birth weight among others). However, other studies have shown that supplementation with both calcium and vitamin D increases the incidence of pre-term birth [104]. During pregnancy, calcium has been shown to help fetal bone formation, and fetal development [103]. However, vitamin D has a multi-faceted function during pregnancy, including decidual immune cells regulation, development of maternal fetal immunity, and inducing trophoblast functions beginning from early to late pregnancy [105]. Thus, although vitamin D may play a key role in calcium homeostasis during pregnancy, the ability of vitamin D to modulate multiple non-calciotropic mechanisms suggests that it plays a more diverse function in the placenta and pregnancy. Some of these mechanisms form the basis of my PhD project.

1,25D is the active circulating form of vitamin D, nonetheless, it is not routinely measured in serum or plasma to determine vitamin D levels. This is partly because the half-life of the circulating 1,25D is only 4-6 hours implying that the concentration level varies significantly, and its serum level (measured in pg/mL) is almost 1000-fold less than 25D levels (measured in ng/mL) [106]. Besides, the circulating levels of 1,25D are not simply defined by vitamin D status but are also dependent on PTH and FGF23 levels [68]. Thus, there is not necessarily a direct correlation between serum 25D and serum 1,25D levels, and therefore analysis of circulating levels of 1,25D tends to be restricted to abnormalities of CYP27B1 or CYP24A1 expression [107]. The reference range for 1,25D in non-pregnant women, is 5 pg/mL to 100 pg/mL (12 to 240 pmol/L) and for pregnant women, 102 pg/mL to 312 pg/mL (244.8 to 748.8 pmol/L) [108]. 1,25D has been shown to fluctuate with the menstrual cycle, with a rise in 1,25D level on day 15 of the cycle [109].

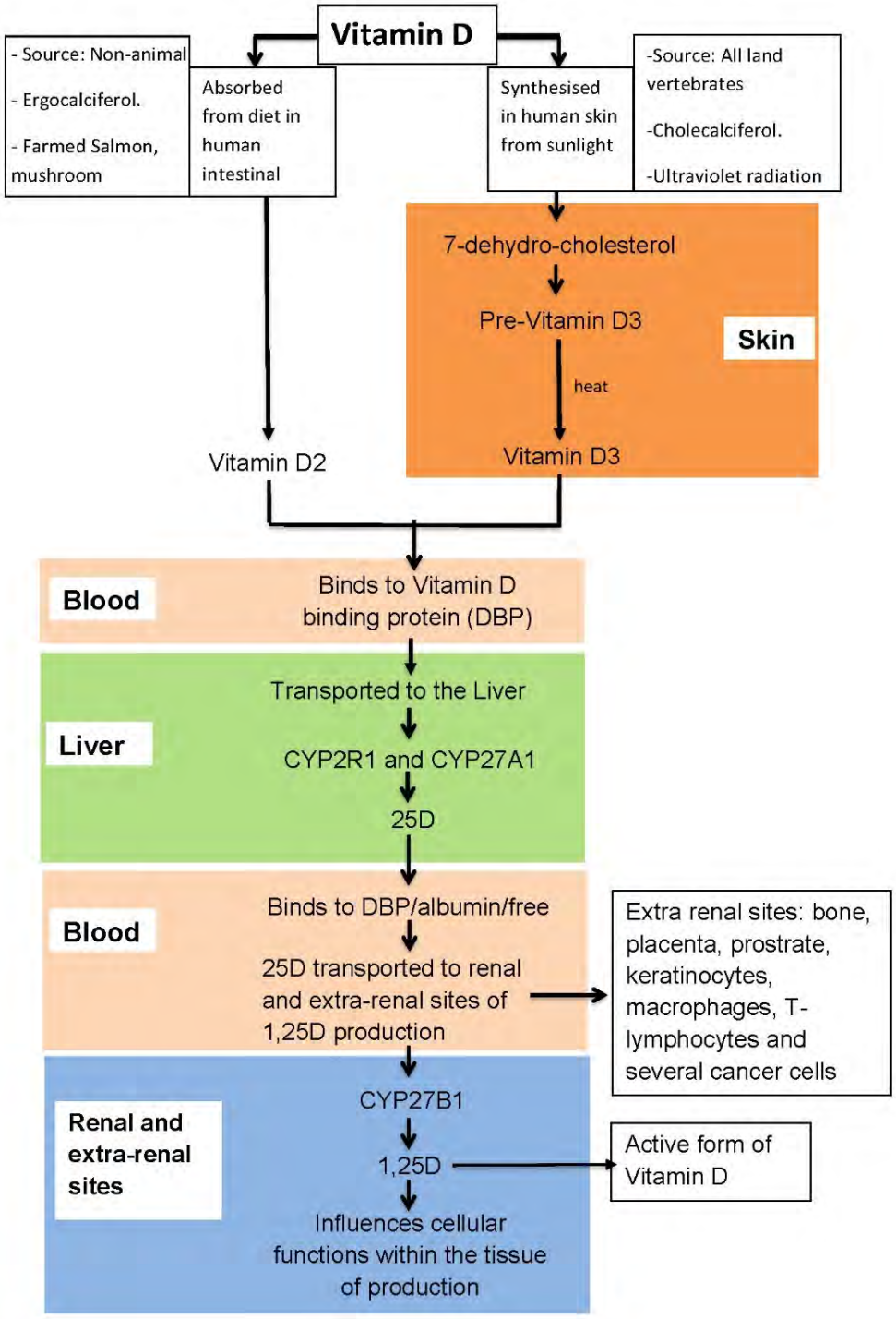


Figure 2.4 Vitamin D synthesis, metabolism, and key physiological actions. Schematic showing a summary of the vitamin D pathway from its precursor to its active form (1,25D), depicting all the major organs involved in the pathway.

2.6 Catabolism of 1,25D

Because of its function as a potent steroid hormone, the production and action of 1,25D have the potential to over-stimulate some cellular responses such as calcium uptake, leading to potential hypercalcaemia. Because of this, levels of 1,25D are tightly regulated by further metabolism to its less-active catabolites.

Catabolism or degradation of vitamin D occurs via two different pathways, the lactone pathway and oxidation pathway (**Figure 2.5**). In the lactone pathway, the active form, 1,25D, is degraded to 1α,23,25-trihydroxyvitamin D (23,24D) by the 24-hydroxylase enzyme (CYP24A1). This product is excreted into bile and urine as 1α,25-dihydroxy vitamin D3-23,26 lactone (1,25D 23-26 lactone) [31, 110]. The end-product of the vitamin D oxidation pathway is calcitroic acid. In this pathway, the active form of vitamin D, 1,25D, is degraded to 1α,24,25-trihydroxyvitamin D3 (1,24,25D) by CYP24A1. This form is excreted in urine and bile as calcitroic acid [31, 110]. This network of catabolic pathways for vitamin D is expressed by most target cells for 1,25D. Within the placenta (the main target tissue for this PhD thesis), CYP24A1 is expressed by trophoblasts, yolk sac, and decidua, [111-113]. Interestingly, some researchers found that the *CYP24A1* mRNA had a low level of expression compared to *CYP27B1* and vitamin D receptor (*VDR)* mRNA, in 1st and 2nd trimester of pregnancy in the placenta and maternal decidua [105]. This is thought to be due to epigenetic gene silencing via methylation of the placental *CYP24A1* to maximise the availability of 1,25D in this tissue [114].

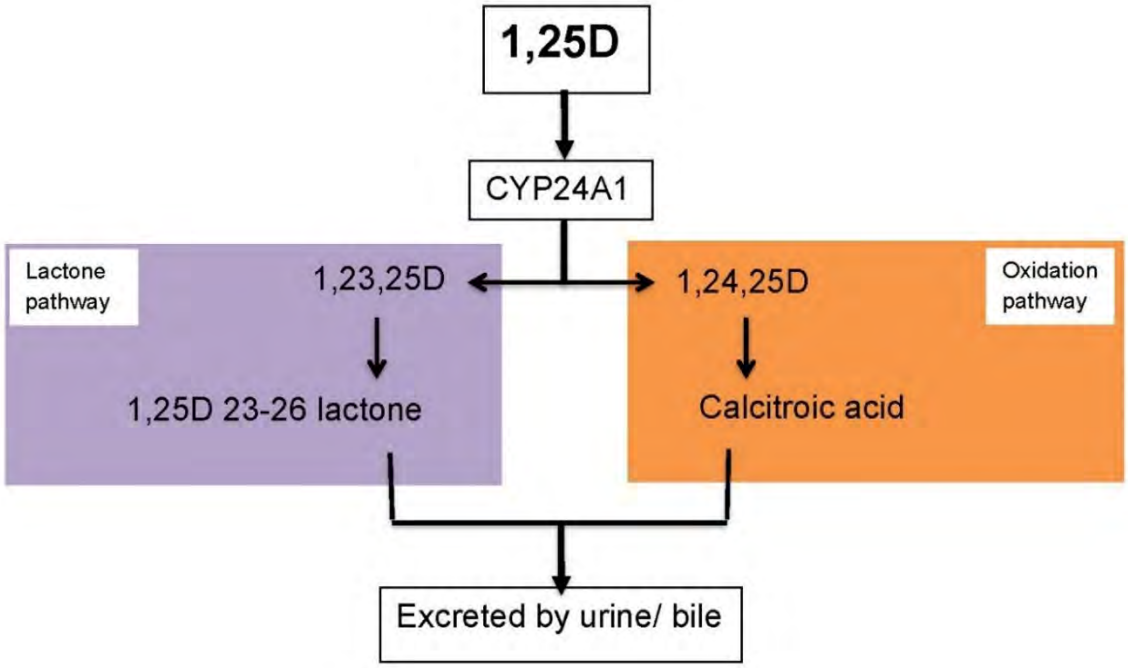


Figure 2.5 Catabolism of 1,25D to less active catabolites.
Schematic showing the catabolic pathways of 1,25D to its key components, which regulate serum 1,25D concentration.

2.7 Signalling mechanisms for 1,25D

The endocrine nature of vitamin D is in part represented by how the other hormones regulate the synthesis of the hormonal form 1,25D in an endocrine organ (the kidney) that acts on distant tissues expressing vitamin D receptors (VDR) for 1,25D (endocrine function). 1,25D can also bind to VDR in cells near those which produced it (paracrine function) or within the cells that also synthesise 1,25D (intracrine function) [115]. Regardless of its site of production, 1,25D acts as the predominant ligand for binding to VDR, although other vitamin D metabolites can also bind to VDR with much lower affinity than 1,25D. VDR belongs to the same family of intracellular receptors for hormones such as oestrogens, androgens, glucocorticoids and thyroid hormone [116],

suggesting that 1,25D achieves its effects via signalling mechanisms that are similar to other hormones, notably the steroid hormones.

Extensive studies have shown that 1,25D achieves most of its physiological effects by binding to VDR in the nuclei of target cells [117]. The major effects of 1,25D involve a key role in the regulation of bone mineral homeostasis via various biological processes in the intestines, bone and kidney [118]. 1,25D also influences mature cell functions including intestinal xenobiotic degradation, regulation of immune system components, modulation of endothelial cells, skin cells differentiation and also in cardiovascular biology [119-121]. 1,25D has also shown potential to influence cellular differentiation, survival and proliferation, suggesting a possible significant role in reducing cancer risk by acting as an anti-tumour agent in human populations [119, 122, 123].

VDR is a ligand-dependent, nuclear transcription factor belonging to the superfamily of nuclear receptors, which when bound to 1,25D, can regulate the expression of multiple genes involved in various physiological functions [124]. In the absence of 1,25D, VDR is primarily shown to be distributed in the cytoplasm [125]. Following 1,25D production/endocytosis into the cells, VDR translocates into the nucleus [125, 126]. Beside gene regulation by VDRE binding (Vitamin D Response Element), VDR can also influence and inhibit gene expression by suppressing several cytokines [127-129]. However, many genes that are regulated by 1,25D do not appear to contain VDRE. It has been suggested that regulation of these genes by 1,25D may occur post-transcriptionally via epigenetic mechanisms such as the regulation of microRNA (miRNA) [130, 131]. This, in turn, can influence gene expression by modulating the

translation of mRNA to protein [132-134]. Finally, 1,25D may regulate gene expression at a post-translation level via changes in protein phosphorylation [135] or protease activity which alters protein stability [136]. Along with the nuclear form of VDR, a plasma membrane form of VDR has also been identified which triggers various signalling pathways more rapidly [137]. The next two sections describe in more detail the genomic and non-genomic pathways that can mediate the actions of 1,25D.

Genomic responses to 1,25D

In common with other members of the nuclear steroid hormone receptor family, VDR mediates actions of 1,25D by regulating gene expression at a transcriptional level [138]. After it binds to 1,25D, the perinuclear VDR forms a heterodimer with another steroid hormone receptor, the retinoic acid X receptor (RXR), and this action facilitates binding to specific cis-acting VDREs in the nucleus [139]. The conventional motif for VDRE is direct repeat (DR) hexamer with a 3 nucleotide spacing (DR3) [68]. The core DNA sequences that make up the VDRE motif can vary from gene to gene, but there appears to be consistency in transcriptional responses to the 1,25D-VDR-RXR complex [140]. The ability of the 1,25D-VDR-RXR complex to regulate transcription is not only dependent on the VDRE motif but also depends on the recruitment of accessory proteins to the transcriptional complex [141, 142]. This includes the VDR co-activators DRIP205 and NcOA1 [143], and the nuclear receptor co-repressors NcoR1 and NcoR2 [144]. The overall effect of these interactions is to facilitate remodelling of chromatin [145] to allow interaction of the 1,25D-VDR-RXR complex with VDRE and enable regulation of gene transcription. **Figure 2.6** summarises the

genomic actions of vitamin D. The genomic pathway for 1,25D signalling is extensively reviewed by Pike *et al*. [146], and Haussler *et al*. [147].

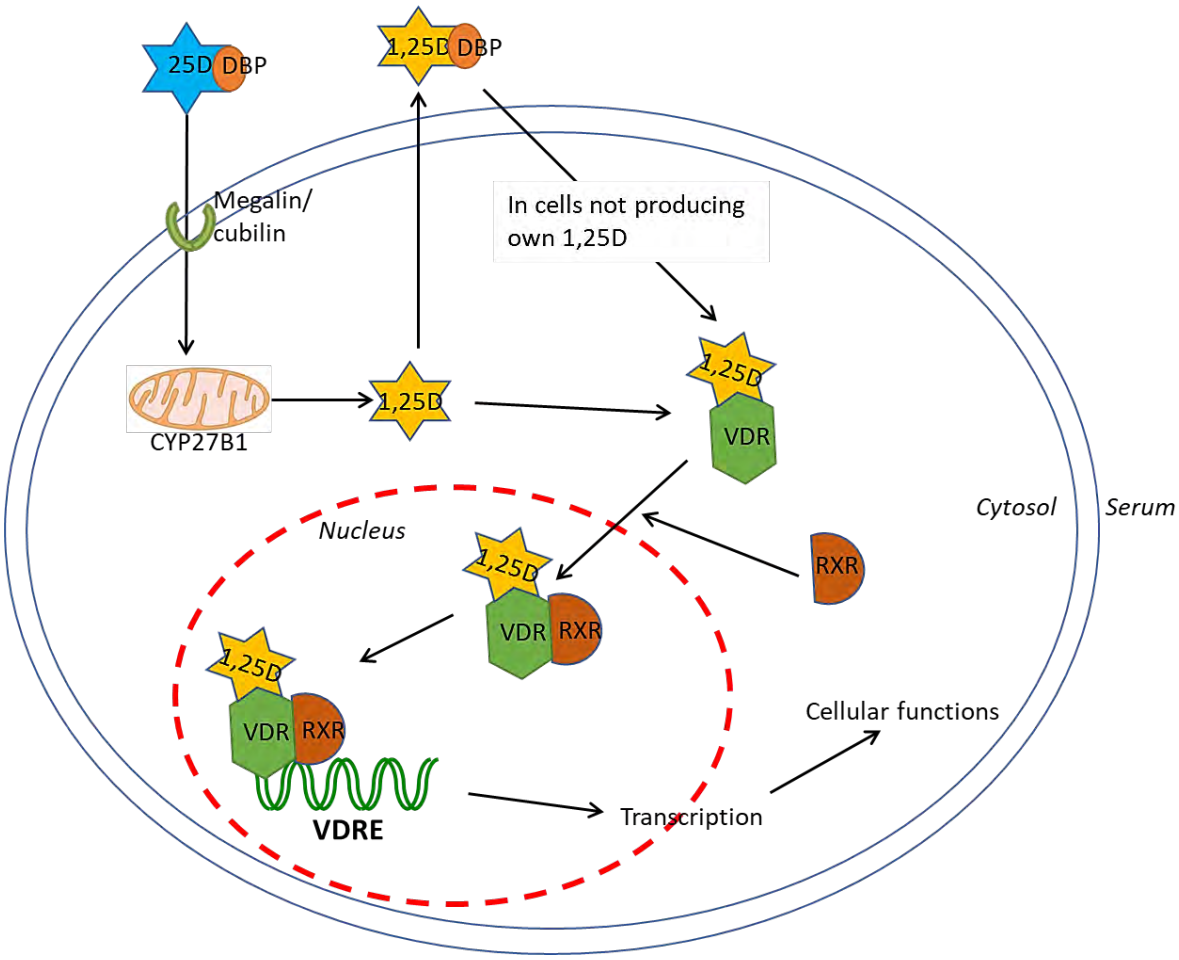


Figure 2.6 Genomic responses to 1,25D.
Schematic showing intracellular transport of 25D-DBP complex via megalin, leading to 1,25D production, which binds to nuclear VDR-RXR complex and VDRE, resulting in transcriptional changes which cause cellular functions.

Non-genomic responses to 1,25D

1,25D is also able to achieve effects via signalling actions that are too rapid to involve a classical nuclear receptor pathway. This response to 1,25D is referred to as the non-genomic actions of 1,25D, and this mechanism is present in chondrocytes of the growth plate [148], keratinocytes in the skin [149] and intestinal cells [150]. Mode of action of

1,25D has been identified via VDR, but in a different configuration to enable binding of non-genomic VDR agonists [151] and membrane-associated rapid response steroid-binding protein (MARRS), also known as ERp57/GRp58/ERp60 [150].

MARRS (mVDR or plasma membrane VDR) receptors located in the plasma membrane [152, 153] follow the non-genomic pathway, involving 1,25D-MARRS-complex binding with caveolae and subsequently stimulating protein kinase C, mitogen-activated protein kinase (MAPK), phospholipase A2, and phospholipase C [154, 155], which influences cellular invasion, proliferation and differentiation (**Figure 2.7**). Some recent additions to the list of pathways activated by 1,25D include MAPK and ERK pathway [156, 157] and PI3K signalling pathway [158]. The various proteins involved in these pathways are activated by phosphorylation of tyrosine residues induced by mitogens [159].

1,25D has been demonstrated to target non-classical sites like lungs [160], cancer cells, immune cells, pancreatic ß-cells [151, 161]. VDR is also expressed in both female and male reproductive tissues [162, 163]. Most prominent among these non-classical sites is placenta (decidual cells and trophoblast cells). This is considered a major extra-renal site for conversion of 25D to 1,25D [164]. To further confirm the 1,25D production in the placenta, placental tissues have been shown to express megalin [165], as well as CYP27B1 and VDR [154]. Correlation between placental tissues and vitamin D metabolites has been explained in **Section 2.12.** To date, unlike the effects of various metabolites of vitamin D, the vitamin D binding protein (DBP) has not been considered to have any role in trophoblast function. A study discovered the

downregulation of DBP in pre-eclamptic pregnancy placenta, however, the authors were unable to explain the mechanism [166].

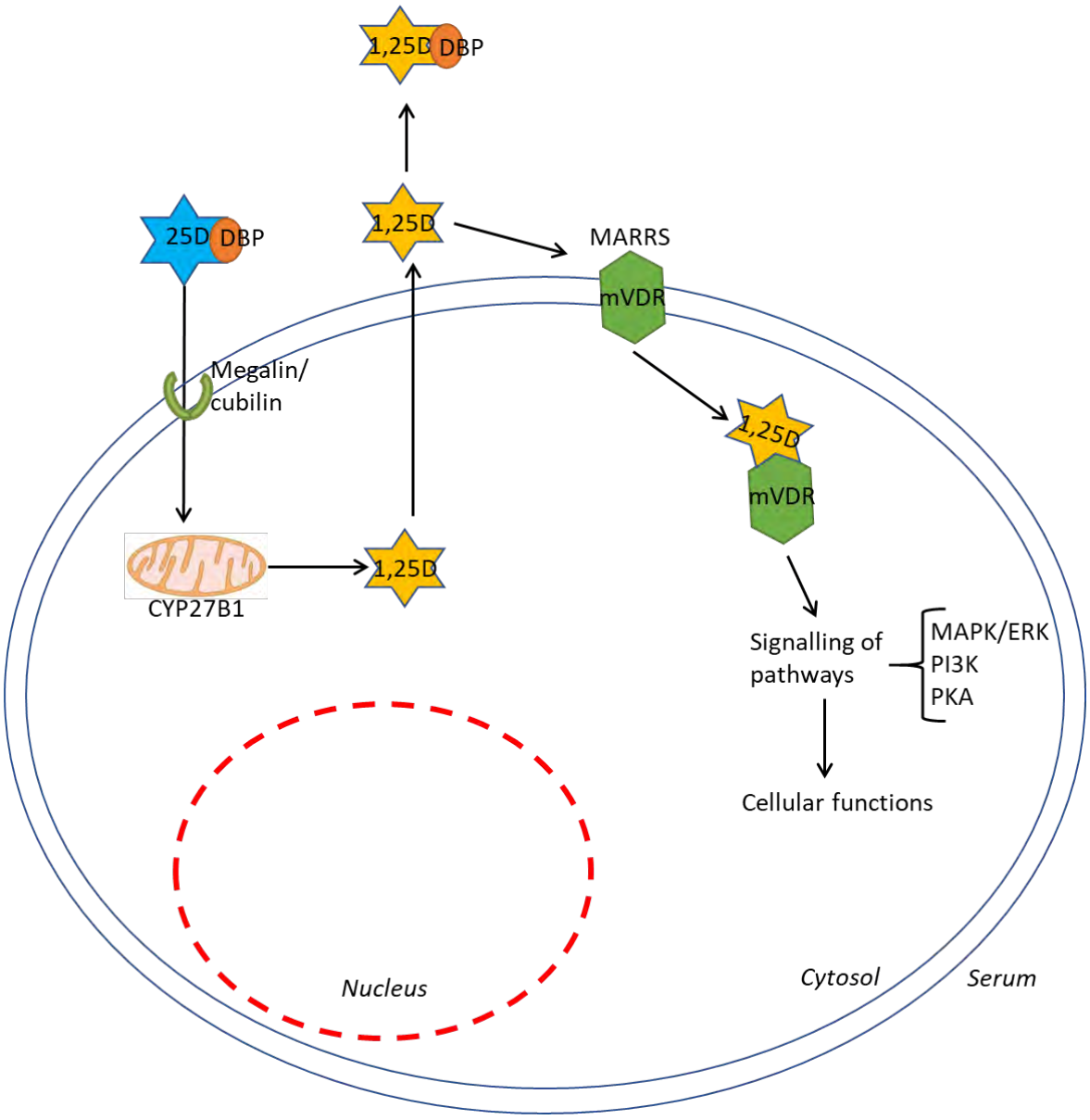


Figure 2.7 Non-genomic responses to 1,25D.
Schematic showing intracellular transport of 25D-DBP complex via megalin, leading to 1,25D production, which binds to membrane VDR, leading to activation of various pathways, resulting in cellular functions.

2.8 Vitamin D in pregnancy

Although vitamin D deficiency is common across the globe [40], low serum 25D appears to be particularly prevalent in pregnant women [167-169]. In several recent reports, 20% of pregnant women in the UK [45, 170], 25% in the UAE [171], 80% in Iran [172], 45% in northern India [173], 60% in New Zealand [174] and 60–84% of pregnant non-western women in the Netherlands [175] were reported to have serum 25D level less than 10 ng/mL (25 nmol/L). Thus, vitamin D status does not depend on the economic conditions of the country (developed or developing) [176]. These findings suggest that impaired function of vitamin D may also be common in pregnant women, underlying the importance of administration of vitamin D during pregnancy [177]. In particular, the effects of vitamin D on placental development and function are likely to be influenced by low serum availability of vitamin D metabolites. The aim of the current PhD project is to better understand these placental effects of vitamin D.

During pregnancy, minerals and nutrients are transferred across the placenta to maintain foetal growth. A significant increase in maternal calcium absorption was observed due to increased foetal demand which may, in turn, result in significant changes in vitamin D metabolism during pregnancy [178]. This is also evident from the increased circulating 1,25D and DBP levels in early to late pregnancy, with highest levels of 1,25D and DBP during late pregnancy, as compared to non-pregnant women [179, 180]. Research has also shown that maternal 25D levels usually remain unchanged throughout pregnancy unless there is severe vitamin D deprivation, or if there is elevated 25D in the setting of vitamin D supplementation [181]. However, there

have been some reports of increased maternal serum 25D levels in the 1st trimester, and reaching a peak in the 3rd trimester [182, 183].

Low vitamin D status during pregnancy has been correlated with maternal hypertension (pre-eclampsia) [108, 184], gestational diabetes mellitus [185, 186], and preterm birth [187, 188]. It has been suggested that vitamin D deficiency in early pregnancy, leads to the abnormal placental formation, which results in pre-eclampsia [189]. Foetal effects of low maternal vitamin D status during pregnancy may include low birth weight, foetal malformation, abnormal organ development, abnormal immune functions, reduced bone density, abnormal foetal brain development and respiratory infections [46, 190-193].

Animal studies have shown that deficiency in vitamin D can reduce fertility in female rats [194]. The authors showed that when rats were fed with a vitamin D deficient diet, the animals were capable of reproduction, however, there were higher chances of failure of implantation in the vitamin D-deficient animals [194]. Interestingly, 1,25D administration [195], along with diets rich in calcium, phosphate and lactose [196] could help induce fertility in animals. Another study using *VDR* knockout (*Vdr−/−*) and *CYP27B1* knockout (*Cyp27b1−/−*) mice further highlighted the significance of the vitamin D pathway in mouse fertility [197, 198]. The authors demonstrated that female *Vdr−/−* and *Cyp27b1−/−* mice developed uterine hypoplasia, infertility, and growth retardation. This suggests that vitamin D metabolic and signalling pathways have a role in blastocyst implantation, which is the primary step for pregnancy.

Collectively, these studies indicate that vitamin D supplementation in vitamin D-deficient pregnant women may have benefits at different stages of pregnancy. However, a vitamin D supplementation regime in pregnancy usually differs from that used for normal adult supplementation. USA recommended dose of 25 µg – 50 µg (1000 IU to 2000 IU), with the highest dosage of 100 µg (4000 IU) is considered safe during pregnancy [199, 200]. However, in France, recommendations suggest a single oral dose of 5000 µg (200,000 IU) in the seventh month of pregnancy (2nd trimester) to maintain a successful pregnancy [201]. In India, recommendations suggest administering 15,000 µg (600,000 IU) at the seventh and the eighth month of gestation for a successful pregnancy [202].

Vitamin D supplementation for pregnant women in the United Arab Emirates suggests that monthly dosages are more effective compared to daily dosages [203]. However, other studies have recommended daily dosages for the entire gestational period [204]. In particular, the supplementary dosage of 100 µg per day (4000 IU) from 12 to 16 weeks until the end of pregnancy appears to be more potent at inducing adequate 25D and 1,25D levels [200]. Keeping in mind all the above studies include women recruited after their pregnancies were confirmed, none of the studies done so far includes the use of vitamin D supplementation for women pre-conception. Analysis of the effects of pre-conception vitamin D supplementation is likely to be important for future research to improve our understanding of the potential benefit of vitamin D on conception, embryo implantation and placentation.

2.9 Vitamin D and pre-pregnancy (pre-conception)

In the pre-pregnancy state, female 25D status has been demonstrated to be of significance for *in vitro* fertilisation (IVF) procedures [205]. Here the authors showed that women with less than 30 ng/mL (75 nmol/L) of serum 25D level, presented with a polycystic ovarian syndrome, decreased ovarian reserve and infertility, which led to the failure of IVF treatment procedure. Women with more than 30 ng/mL (75 nmol/L) 25D level showed an increased oocyte retrieval rate, higher follicular fluid level, a higher rate of implantation, and thus, a higher rate of established clinical pregnancy. The implications from these studies are that a 25D rich endometrium leads to successful implantation of an embryo. 25D and 1,25D have been shown to regulate several endometrial genes which help in implantation. One among these is the *HOXA10* gene [206], the expression of which reaches its peak during implantation [207]. Thus, vitamin D may impact the basic transcriptional regulation of implantation.

Vitamin D has also been shown to influence folliculogenesis, endometrium response to trophoblast, and formation of the embryo in humans [208]. On increasing endometrial receptivity, sufficient maternal serum 25D level results in the induction of trophoblast invasion by inducing epithelial-mesenchymal transition [209]. Maternal 25D transforms into active 1,25D in the decidua by CYP27B1 [164]. This supports trophoblast implantation [210], angiogenesis during placental formation [211], and immunomodulatory embryo protection [212]. From all the above studies, it is evident that pre-conception vitamin D is necessary to maintain a sufficient 25D level in maternal blood, and this, in turn, may be beneficial for conception and healthy pregnancy. However, many women may be unaware of the beneficial effects of vitamin

D, or do not discuss vitamin D supplementation with their physician [213, 214]. This creates a difficult situation for physicians when prescribing 25D supplements to women wishing to conceive. Nonetheless, it is recommended to prescribe vitamin D supplements as a strategy for improved pre-conception health [215].

Vitamin D has also been shown to be beneficial for paternal fertility. Studies have demonstrated the presence of nuclear VDR in sperm [216]. Men with oligospermia (reduced sperm count) and asthenozoospermia (reduced sperm motility) showed reduced 25D level (<20 ng/mL or <50 nmol/L) [217, 218], this phenomenon was not mediated by effects of testosterone [219]. Interestingly, the initial 10 amino acids of IgG from the anti-sperm antibody, are similar in structure to the structure of DBP [220]. Thus, lower 25D and DBP levels in men may contribute to paternal causes of infertility, and we can propose that both maternal and paternal serum 25D sufficiency is important for a successful formation and implantation of an embryo.

2.10 Vitamin D and pregnancy complications

Several studies have shown that one out of every three women giving birth has <20 ng/mL (<50 nmol/L) serum 25D level [221]. This suggests extensive vitamin D deficiency observed in pregnant women which may, in turn, contribute to pregnancy complications. Lowered maternal vitamin D status has been associated with multiple adverse pregnancy outcomes including early pregnancy complications such as: implantation failure, miscarriage, and spontaneous abortion. Low vitamin D may also contribute to delayed pregnancy complications such as: pre-eclampsia, gestational

diabetes mellitus, preterm birth, small for gestational age, maternal bacterial vaginosis and emergency caesarean section [207, 222-225].

Emergency caesarean section

Women with serum levels of 25D less than 15 ng/mL (<37.5 nmol/L) showed 4-times higher risk of undergoing caesarean delivery [225], suggesting that vitamin D deficiency might influence musculoskeletal aspects of female pelvis resulting in poor uterine contractile performance (by uterine smooth muscles) [226] and narrow pelvis [227], leading to emergency caesarean delivery [225]. The authors showed that the common reasons for opting for emergency caesarean delivery were a failure of progress of labour, malpresentation of foetus, cephalopelvic disproportion and variable foetal heart rate. This is similar to another study which explained the correlation between maternal vitamin D deficiency with increased chances of caesarean sections due to uterine atony and postpartum haemorrhage [228]. Uterine atony has been associated with failure of progression of labour. Nonetheless, there are studies which found no effect of early pregnancy 25D on the mode of delivery [229]. However, this study does demonstrate that foetuses from mothers with low 25D levels (<12.8 ng/mL or <32 nmol/L), developed respiratory distress during labour, which led to emergency caesarean section. Foetal distress occurs due to various reasons, one of which is placental insufficiency. Abnormal placental angiogenesis leads to placental insufficiency, foetal distress and foetal mortality [230].

Miscarriage

The procedure of placentation involves invasion of extravillous trophoblast cells into the uterine decidua, resulting in a transformation of uterine spiral arteries by loss of the fibroelastic vascular media and replacement by trophoblast fibrinoids [231, 232]. This is an essential and extensively researched area of normal pregnancy, underlining the significance of the invading trophoblast in a vascular nutrient environment for the growing foetus and developing placenta. Miscarriage or early pregnancy loss is defined by the Royal College of Obstetricians and Gynaecologists (RCOG), UK, as "the spontaneous loss of pregnancy before the foetus reaches viability", beginning from conception until 24 weeks of gestation [233]. Early pregnancy loss usually results due to embryonic chromosomal abnormality (in 50% cases), uterine malformations (37% cases), maternal antiphospholipid antibodies, maternal abnormal uterine natural killer cells, abnormal implantation and or abnormal/shallow placentation [234-236]. Miscarriage after 12 weeks of gestation affects 2% of pregnancies [237] and is less common than early miscarriage (60% of pregnancies) [238]. Early miscarriage is usually caused due to an abnormality in placentation or abnormal fertilised embryo. Whereas late miscarriage is usually caused due to anatomic or chromosomal abnormality in the growing foetus [239, 240] or maternal cervical incompetence [236].

Studies have shown that abnormal placental vasculature early in pregnancy may lead to subsequent pregnancy complications such as pre-eclampsia [241, 242], and miscarriage [243]. In a large prospective cohort study, lower 1st trimester maternal serum 25D levels were associated with more than two-fold increase in miscarriage [244] and also correlated with 2nd trimester miscarriage [245]. Similarly, reduced serum

1,25D concentrations are also observed in miscarriage possibly due to lowered CYP27B1 in trophoblast villous cells and decidua, with serum 25D remaining constant [246]. This data is consistent with Schneuer *et al.,* who reported no significant changes in 25D levels across gestation in 5000 recruited pregnant women [247]. However, the authors failed to measure 1,25D concentration in the participants.

Pre-eclampsia

National Institute for Health and Care Excellence (NICE) guidelines defines pre-eclampsia as a new-onset hypertensive disorder (≥140/90 millimetres of mercury) in pregnancy after 20 weeks of gestation, associated with or without albuminuria (urinary excretion of ≥300 mg of albumin in 24 hours) [248]. Pre-eclampsia is a very common pregnancy hypertensive disorder originating in the placenta and leading to maternal and foetal morbidity and mortality [249]. Around 7% of first pregnancies are affected [184] and is known to be more common in women from ethnic groups. For example, women of African-Caribbean origin are more likely to develop severe pre-eclampsia resulting in death, compared to white women [250, 251]. Reported pathogenesis of pre-eclampsia includes factors directly or indirectly influenced by vitamin D, including placental formation, implantation, abnormal placental angiogenesis, immune dysfunction leading to placental inflammation and hypertension [105, 252-254]. Maternal vitamin D status has been shown to correlate with pre-eclampsia [108]. However, studies on maternal vitamin D status before the development of pre-eclampsia symptoms have been scarce. Bodner *et al.* reported vitamin D deficiency status (<20 ng/mL of serum 25D) in 1st trimester pregnant women who subsequently developed pre-eclampsia [184]. The study explained a similar maternal serum vitamin

D deficiency even at the time of delivery. Interestingly, a seasonal pattern of occurrence of pre-eclampsia has been reported with the lowest occurrence in summer [255].

Early pregnancy deficiency of angiogenic factors leads to abnormal placentation and ultimately pregnancy complications like pre-eclampsia. Pro-angiogenic factors such as placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) are involved in vascular formation at maternal-foetal interface [256, 257]. *In vitro* research demonstrates vitamin D can induce VEGF expression in vascular smooth muscle cells [258]. It has been proposed that 25D binds to VDR which behaves as a transcription factor to VEGF promoter, resulting in increased VEGF expression, thus influencing angiogenic balance [258]. A similar observation was made on a pregnancy-induced hypertension rat model, where vitamin D supplementation could restore angiogenesis [259]. From all the above-published literature, it can be concluded that vitamin D significantly helps prevent pre-eclampsia by promoting angiogenesis.

Small for gestational age

The Royal College of Obstetricians and Gynaecologists (RCOG), UK, defines small for gestational age (SGA) foetuses as “infants born with a birth weight less than the 10th percentile” [260]. The causes of SGA pregnancies have been categorised as constitutional, non-placental causes (for example, foetal infection, chromosomal abnormality, an inborn error of metabolism), and placental causes [260]. Conditions affecting maternal health, placental implantation, and placental vascularity (for

example, pre-eclampsia, thrombophilia, renal diseases, diabetes) results in small for gestational age foetus.

Maternal vitamin D supplementation was shown to improve small for gestational age outcome in a British double-blind randomised trial [261]. In the study, 59 participants were included from an Asian background with low serum 25D levels, and these women were given 25 µg per day (1000 IU) oral vitamin D2 during their 3rd trimester of pregnancy [261]. This intervention showed improvement in maternal weight and lowered the delivery risk of SGA infants. In another study including 449 pregnant women from Iran, a cohort of 34% women receiving vitamin D3 supplements showed a greater length of the new-born baby and a higher 1-min APGAR (Appearance, Pulse, Grimace, Activity, and Respiration) score [262]. Also, since new-born weight and length varies depending on maternal BMI and paternal height, adequate maternal 25D is just one of the potential factors that can influence foetal growth [263]. Not all studies showed a link between vitamin D and SGA foetuses. A Gambian study reported no effect of maternal 25D level on new-born birth weight and length [264]. The study participants from Gambia were only given calcium supplements, and none showed vitamin D deficiency (<20 ng/mL or <50 nmol/L) according to the North American Institute of Medicine (IOM) [21].

Gestational diabetes mellitus (GDM)

Abnormal oral glucose tolerance test (OGTT) with the onset of hyperglycaemia during pregnancy, with or without symptoms, is termed as gestational diabetes mellitus (GDM) [265]. Epidemiological studies reveal 9% of native Americans, Asians, Hispanics, and African-American women living in the USA [266] and 3 to 21% women

in Asian countries [266] are affected with GDM. Interestingly, GDM has been shown to vary according to the season with a higher incidence of GDM in winters, compared to the summer season [267]. This suggests a possible role for vitamin D (and sunlight) in the pathophysiology of GDM. Various pregnancy complications have been associated with GDM, such as shoulder dystocia, macrosomia and higher rates of Caesarean delivery [268]. In addition, GDM mothers can subsequently develop type 2 diabetes mellitus (T2DM) [269], cardiovascular disease [270], hypertension and metabolic syndromes [271] after pregnancy. Following delivery, neonatal complications associated with GDM include neonatal hypoglycaemia, development of obesity and T2DM [268].

GDM pregnancies are associated with lowered serum 25D levels [272-274]. Large systematic meta-analysis studies have confirmed the inverse association of maternal serum 25D and GDM [275, 276]. They also suggest that supplementation with vitamin D in these pregnant women could help lower the GDM cases. Similarly, a meta-analysis of randomised control clinical trials concluded that vitamin D supplementation of pregnant women could promote glycaemic control, thus reducing the incidence of GDM [277]. The study involved a total of 173 participants and 153 control participants from 5 clinical trials, showing reduced fasting blood glucose, reduced glycosylated haemoglobin, and reduced serum insulin concentration with vitamin D supplementation of 25–119.05 µg/day (1000-4762 IU/day), compared to the control group [277].

Newborns from women with GDM and vitamin D deficiency have also been reported to have a higher incidence of hypoglycaemia and SGA requiring ICU management

[278]. Within the placenta, VDR is highly upregulated in placental tissue from women with GDM, especially at the maternal-foetal interface [279], suggesting a more explicit role of vitamin D, which requires further research.

Preterm birth (PTB)

The WHO defines PTB as any birth before 37 weeks of gestation, which is again divided into 3 categories- extremely preterm (<28 weeks), very preterm (28 - <32 weeks) and preterm (>32 weeks and <37 weeks) [280]. In 2010, it was estimated that 15 million babies were born preterm leading to various complications including the premature death of 1 million of these newborns [281]. The survival rate of these pre-term babies depends on the economy of the country, with high-income countries showing a higher rate of survival than low-income countries [280]. Causes of PTB are outlined as multiple pregnancies, pregnancy infections (bacterial vaginosis), and maternal chronic diseases like diabetes or hypertension [280].

Vitamin D-deficiency has been linked to PTB [282]. Lowered maternal vitamin D status has been associated with pre-term birth in some studies [283, 284], but not in others [247, 285-289]. The reason for such disparity between study results might be due to certain factors such as location, ethnicity, gestational age, and test method applied, which might not have been adjusted to the results obtained. PTB is primarily observed in women living in higher altitude with low serum vitamin D concentration, leading to a possible explanation that location and sunlight exposure has a significant effect on pregnancy outcome [290]. Studies of vitamin D supplementation during pregnancy have shown that raising maternal serum 25D may have a significant effect in reducing

the risk of PTB [291]. However, a meta-analysis of vitamin D supplementation studies in pregnancy was less conclusive [292], suggesting that further studies are required to investigate the benefits of vitamin D in protecting against PTB.

2.11 Placental physiology

The placenta forms the connection between the mother and foetus, supplying the foetus with oxygen and nutrients, whilst excreting waste products. The main functions of the placenta can be broadly termed as: transport and metabolism, protection and endocrine [293]. The complex architecture of the placenta (**Figure 2.8**), bounded by the maternal aspect (basal plate) and the foetal aspect (chorionic plate), houses the fundamental functional unit of the placenta, the chorionic villi, which play a major role in the transfusion of oxygen from the maternal blood to the foetal blood vessels. As well as facilitating blood supply to the foetus, the placental immune cells also act to protect the foetus from xenobiotic materials and infectious agents from entering the foetal blood [293]. The placenta also acts as an endocrine organ during pregnancy, mediating the production of various hormones which are important for decidualisation, placental development, angiogenesis, embryo implantation and attachment, endometrial receptivity and foetal development [294]. These hormones are primarily secreted by syncytiotrophoblast cells of the placenta, directly into the maternal circulation, making them potential biomarkers for predicting pregnancy complications [294].

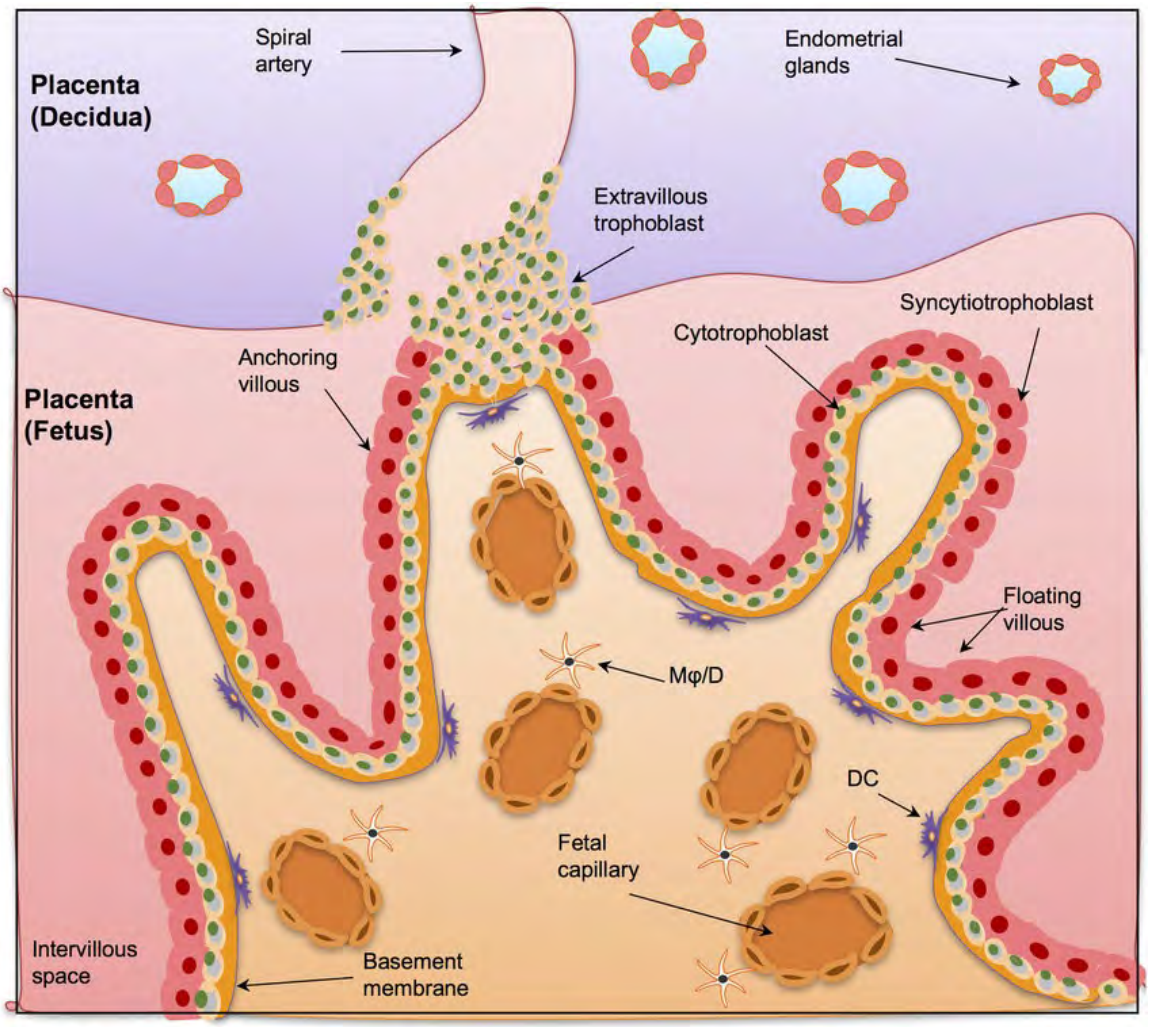


Figure 2.8 Schematic showing the maternal fetal interface with various layers of trophoblasts and maternal decidua.
Adapted from Vazquez-Martınez et al. [295], the image explains the various layers of cells involved in the maternal-fetal interface, which helps in spiral artery formation and gaseous exchange.

Maternal secretory endometrium is transformed into decidua following implantation of the embryo along with various hormonal and immunological influences. Decidua is the innermost uterine layer which forms a base for the embryo attachment, helping in the formation and anchoring of placenta. Trophoblast infiltration in the decidua subsequently leads to the development of villous vasculature connecting mother and the foetus. Trophoblasts from the developing embryo consist of cytotrophoblasts and

syncytiotrophoblasts. Cytotrophoblasts differentiate to form nuclear aggregates, called the syncytiotrophoblast layer (the basal layer of placental villi). Syncytiotrophoblast nuclei are irregularly dispersed and appear aggregated to form multinucleated syncytium from the villous surfaces known as syncytial knots. Syncytial knots are primarily seen in mature villi [296]. Extra-villous cytotrophoblasts invade the uterine decidua to form placental villous structures (the functional unit of the placenta) [297].

Invasion of trophoblasts creates open endings in the maternal vessels forming passageways for maternal blood to circulate into the intervillous spaces. Placental villi comprise an inner mononuclear cytotrophoblast and an outer multinuclear syncytiotrophoblast. Placental chorionic villi are responsible for oxygen transfer from maternal to foetal blood. Excessive or shallow invasion of trophoblasts in the decidua leads to various maternal and foetal complications. A shallow encroachment of trophoblasts results in fragile placental anchoring, which leads to placental detachment in early or late pregnancy leading to placental insufficiency, miscarriage, foetal distress and ultimately foetal death. An excessive invasion of trophoblasts leads to abnormal placentation, foetal death and ultimately hysterectomy (removal of the uterus). Some of the abnormal placentation includes placenta accrete (pathological encroachment of trophoblast up to myometrium), placenta increta (trophoblast encroachment into the myometrium), and placenta percreta (trophoblast invasion through the myometrium and into the adjacent tissues) [298]. Foetal development requires nutrients from the maternal blood, which is in direct contact with the villous ends of the placenta. Thus, during unavailability of sufficient nutrients, foetal hormones signal the trophoblasts to

invade further to reach deeper maternal blood vessels. This might explain the abnormal invasion by trophoblasts on rare occasions.

The successful development of the placenta involves two distinct mechanisms: implantation of the blastocyst into the maternal endometrium to initiate placental attachment, and invasion of foetal trophoblast into the maternal decidua to facilitate maternal-foetal blood supply. Placental attachment is poorly understood and has been studied primarily using animal models [299, 300]. The current PhD project aims to provide an overview of trophoblast invasion during placental development, with a particular emphasis on the potential role of vitamin D as a regulator of placental development through effects on trophoblast cells.

Early development of the chorionic villi plays a pivotal role in establishing the functional architecture of the placenta. The first foetal-placental villi develop as trophoblast sprouts. Allantoic mesoderm invades these structures, to form secondary villi subsequently undergoing vasculogenesis (formation of new blood vessels), into tertiary villi. Human embryogenesis takes place in a hypoxic environment, and between 8-12 weeks of gestation, the placental barrier to maternal blood is gradually breached, due to the invasion of placental bed uteroplacental spiral arteries by the extravillous trophoblast (EVT). Placental oxygen tension thus increases, leading to angiogenesis until 24 weeks of gestation [301, 302]. Placental formation thus primarily involves blood vessel development within the developing intermediate villi. During this phase, the placenta expands to produce 10–16 generations of stem villi. Once the foetus attains viability (24 weeks of gestation), a developmental switch occurs to form innumerable gas-exchanging terminal villi by non-branching angiogenesis in mature intermediate

villi. Several growth factors, including vascular endothelial growth factor (VEGF), placental growth factor (PlGF), angiopoietins, and angiostatin are produced within the villi and act locally, via their receptors, to control angiogenesis [302, 303].

Invasion of foetal EVT into the maternal decidua is a key process in placentation and consequently, this process is also essential for a successful pregnancy. Abnormal placentation due to the extensive or shallow encroachment of EVT causes important maternal and neonatal complications, such as maternal mortality, early abortion, pre-eclampsia, and intrauterine growth restriction, prematurity or even maternal or foetal death [304-306]. Adequate invasion of trophoblast stem cells (cytotrophoblast cells) up to the placental decidual layer and the spiral arteries influences placental perfusion. Cytotrophoblast cells reside in two types of chorionic villi known as floating and anchoring villi. Floating villi, which represent the vast majority of chorionic villi, are bathed in maternal blood and primarily perform gas and nutrient exchange for the developing embryo.

During the early stage of placentation, cytotrophoblast cells in the floating villi proliferate and differentiate by fusing to form the multinucleated syncytiotrophoblast layer. However, cytotrophoblast cells in the anchoring villi either fuse to form the syncytiotrophoblast layer or break through the syncytium (outer layer of the placental villi which is in direct contact with maternal blood) at selected sites and form multi-layered columns of non-polarised EVT cells. These cells form the layer connecting the embryo to the maternal uterine wall. Due to this morphological transformation, syncytiotrophoblast cells acquire specific endocrine functions, including amplification

of human chorionic gonadotropin (hCG) [307]. EVT cells invade into the uterine wall, disturbing the normal endothelium and smooth muscle layer, and replacing the vascular wall, leading to the transformation of the narrow uterine arteries into distended uteroplacental arteries. This allows an adequate supply of oxygen and nutrients to the growing foetus. The invasive activity of EVT cells is at its maximum during the 1st trimester of gestation, peaking at around 10-12 weeks and declining thereafter [302]. Dysregulation of the trophoblast invasion process often leads to adverse pregnancy outcomes such as pre-eclampsia [302], which often results in intrauterine foetal growth restriction (IUGR), foetal anatomical abnormality [308, 309] and stillbirth [310]. Correspondingly, unrestricted invasion by EVT cells is associated with premalignant conditions, such as invasive mole, and malignant choriocarcinoma [302, 311].

Several factors have been identified as regulators of trophoblast function and EVT invasion [312]. I have proposed that vitamin D plays a crucial role in placental development and function by targeting multiple placental cell types [313, 314], with notable responses including induction of antibacterial [315] and anti-inflammatory [316] responses by trophoblast cells. In addition to effects on placental immune function, I have also reported that vitamin D promotes EVT invasion, in agreement with Chan et. al. study [317], suggesting a more fundamental role in modulating placental development.

Abnormality in both maternal decidua formation and placentation has a significant adverse impact on pregnancy outcomes [318, 319]. However, for this thesis, I will focus on the placental trophoblast and pregnancy progression only. Placental malformation

leads to pre-eclampsia [320], foetal growth restriction [321], and miscarriage [322], thus adequate placental development is important for the progression of pregnancy.

2.12 Vitamin D and trophoblast cell function

The maternal-foetal interface comprises trophoblast cells and maternal decidua, along with various modulatory factors such as immunological and hormonal factors. Components of the maternal-foetal interface differentially express elements of the vitamin D system, and various vitamin D related proteins. Some of which has been explained in a recently published literature review by our group [323]. The review shows that vitamin D metabolites expressed significantly in decidua are 1,25D and VDR [323]. Similarly, EVT expresses DBP and VDR [323]. **Figure 2.9** shows the expression of vitamin D related proteins in various components of the maternal-foetal interface [323].

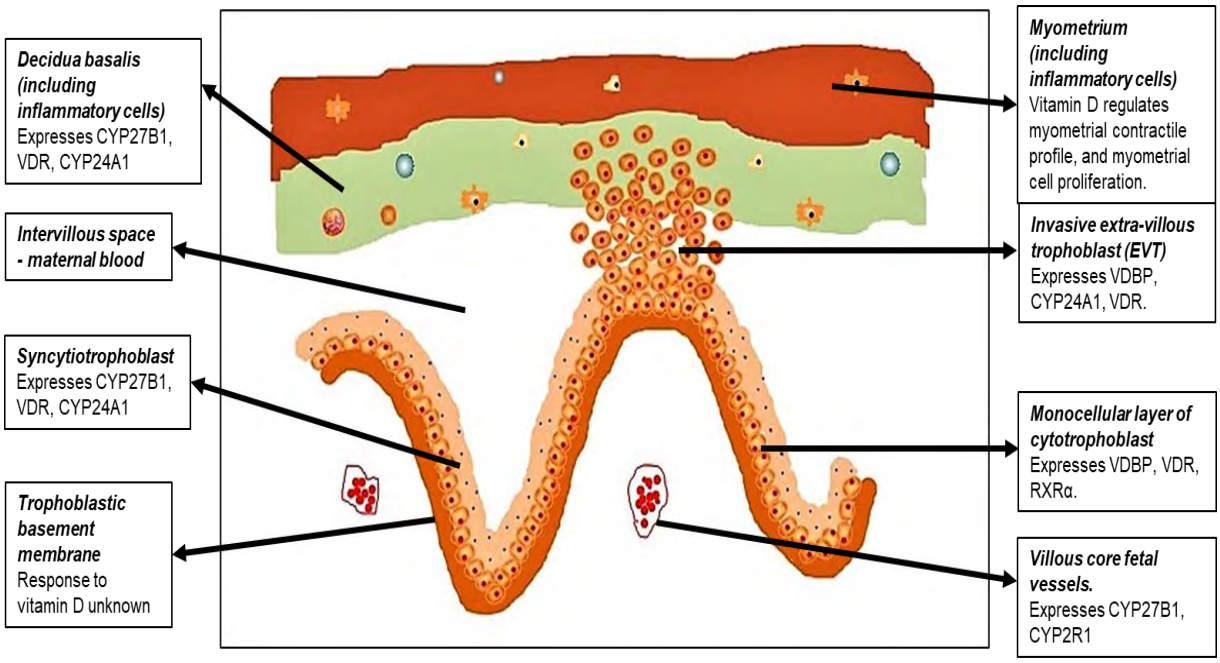


Figure 2.9 Schematic representation of the maternal-foetal interface showing the effects of vitamin D and expression of the vitamin D system within its constitutive cells/layers.
Adapted from a literature review by our Birmingham group [323].

Vitamin D metabolites such as 1,25D and 25D may help extra-villous trophoblast invasion [324]. Lowered expression of VDR and CYP27B1 from early gestation may also result in abnormal trophoblast invasion associated with abnormal spiral artery formation [325] and consequently, disorders such as pre-eclampsia [154, 326, 327]. 1,25D, acting via VDR regulates placental hormone secretion, especially ß-HCG by syncytiotrophoblast cells [328, 329]. *VDR* inactivation or decreased *CYP27B1* mRNA expression resulted in enhanced expression of the apoptosis marker, *TP53* [330, 331], which may lead to placental insufficiency and foetal complications. Similarly, VDR has been observed to act as a protective factor for placental insufficiency by preventing apoptosis [332].

2.13 *In vitro* strategies for investigating the effects of vitamin D on placental function

As a large organ, the placenta provides abundant tissues for analysis *ex vivo.* This includes explant [333], and organ perfusion studies [334] using selected tissue from the placenta or decidua at different stages of gestation. However, the complexity of the cellular composition of the placenta, and the changes in its composition at different stages of pregnancy mean that they may not be an appropriate model for many types of study, particularly in the context of cell and molecular analysis. Cells can also be isolated from both the placenta and decidua to carry out primary short-term cultures. In previous studies, we have used this approach to assess the effects of vitamin D on immune cells from the decidua [323], and extra-villous trophoblasts [317]. Although these primary culture strategies provided important new information, analysis of these cells is highly restricted by the availability of material and the large variations in cell function between donors. This is particularly true with the stage of gestation used to isolate the cells. For example, 1st trimester tissue is required for studies of decidual natural killer cells, as these cells are much more abundant at this stage, but this material is only available as a consequence of the termination of pregnancy. Conversely, term placenta is readily available after birth but may not accurately represent trophoblast function at earlier stages of gestation. We observed that primary cultures of trophoblast cells show substantial variability between donor samples, reflecting the heterogeneity of cyto- and syncytiotrophoblast [335-337]. Thus, it has become common practice to use established cell lines for many aspects of trophoblast function because of their consistency and well-defined phenotypes, although there are currently no equivalents for primary cell culture

Trophoblast cells lines, including JEG3, BeWo, HTR8 and JAR have been extensively used for studying trophoblast functions. Choriocarcinoma cells, such as JEG3, BeWo and JAR cells are the preferred *in vitro* trophoblast cell model for various experiments such as matrix invasion [338, 339], migration [340, 341] and gene expression [342, 343]. The characteristics of these cells are described in greater detail in the Materials and Methods (**Table 3.1A and B**). Some 1st trimester primary trophoblast cells have been transfected with SV40 virus to generate SGHPL (Saint George's Hospital Placental) cell Line, derived from primary human 1st trimester EVTs) and HTR8/SVneo cell line. Both group of cell lines have tested positive for HLA-G and vimentin [335], which are commonly used markers for primary extra-villous cytotrophoblast, but not syncytiotrophoblast cells. Thus, SGHPL cell lines are preferred cells for studying cytotrophoblast behaviour.

JEG3, BeWo and JAR cells express hCG similar to trophoblast cells of the placenta [344]. In contrast, JEG3 and BeWo have been reported to show significant dissimilarity in gene expression determined by microarray analysis [345]. Data suggest that JEG3 cells show high expression of HLA-G and HLA-E transcripts, similar to the EVT cells [335]. However, BeWo cells express hCG, placental growth hormone, and human placental lactogen (hPL) [346], leptin receptor [347], syndecan [348] and 17β-hydroxysteroid dehydrogenase type 1 [349], all of which are consistent with a syncytiotrophoblast phenotype.

It has been suggested that without *in vivo* factors, *in vitro* cultured cells are unable to behave as normal *in vivo* cells [350]. However, when cells are grown on the extracellular matrix, they show similar characteristics to *in vivo* environment [351, 352]. Kolahi *et al.* explained the significant differences observed from mouse embryo cells grown on extracellular matrix to that of polystyrene surface (plastic surface) of conventional culture dishes and flasks [351]. The authors showed consistent improvement in percent development of the embryo, increased hatching frequency of the blastocyst, and an increased trophectodermal cell number when the embryos were cultures on extracellular matrix. Similarly, another study showed that cellular differentiation, gene expression and hCG secretion by choriocarcinoma cells were greatly increased following culture on extracellular matrix [353]. However, the vitamin D pathway is different in JEG3/BeWo cells as compared to primary trophoblast cells [354]. A study by Avila *et al*., explained that by demonstrating less 1,25D production and low CYP27B1 protein secretion by JEG3 cells, compared to primary syncytiotrophoblast cells [355]. These aberrant expression levels may be due to the cells being grown on plastic surfaces, thus showing a different phenotype to that found in the *in vitro* environment.

Even after extensive research in the field of vitamin D and pregnancy, the precise mechanisms of action for vitamin D in early pregnancy remain unanswered, especially for implantation and placentation. My project has tried to address these points to better understand the effects of vitamin D on placental development and early pregnancy events.

HYPOTHESIS AND AIMS

Vitamin D metabolism and signalling system undergoes major changes during pregnancy. Maternal serum levels of the active form of vitamin D, 1,25D, increase dramatically in the 1st trimester and remain elevated throughout pregnancy. Similarly, maternal (decidual) and foetal (trophoblast) tissues show increased expression of VDR and the vitamin D-activating enzyme CYP27B1 early in pregnancy, suggesting that both circulating and local tissue levels of vitamin D can impact pregnancy health. Added to this, clinical studies have linked the deficiency of vitamin D with various adverse events in pregnancy. However, despite all of these observations, it is still not clear how vitamin D contributes to placental function and pregnancy health. Studies from our group in Birmingham have shown that some effects of vitamin D on maternal decidua involve actions on immune cells, but the impact of vitamin D on foetal trophoblast cells is less clear. The overall objective of this PhD project was to address this issue.

Hypothesis: My PhD hypothesis was that vitamin D plays an important role in regulating trophoblast cell function through a novel, as yet unrecognised mechanisms. To test this hypothesis, the project had three main aims:

Aim 1: Characterise the vitamin D system in human placenta tissue and trophoblast cells.

Aim 2: Identify the key trophoblast cell target actions of vitamin D *in vitro.*

Aim 3: Relate trophoblast cell actions of vitamin D *in vitro* to human pregnancy samples and data, including adverse events in pregnancy.

Chapter 3: MATERIALS AND METHODS

3.1 Cell lines

Placental trophoblast cells have been compared with cancer cells at multiple instances because of their invasive characteristics [356, 357]. Trophoblast cells migrate and invade the maternal decidua to anchor the placenta, this allows the formation of chorionic villous vessels (angiogenesis). This characteristic is similar to tumour growth and invasion [358]. However, in normal pregnancy, the trophoblast cells show restricted invasion up to a certain area (decidua) and for a certain length of time (length of pregnancy) [357], whereas tumour cells do not have a limiting factor while invading. However, in adverse pregnancies, placental trophoblast cells show unrestricted invasion in cases such as placenta accrete, placenta increta and placenta percreta [359, 360]. Similar to tumour cells, fertilised ova can travel to the peritoneum and attach at an aberrant location, such as the abdominal wall, and invade this tissue. This is termed an ectopic pregnancy.

The aim of studies carried out in this PhD project was to assess the effects of vitamin D and components of the vitamin D system on the function of trophoblast cells. In particular, experiments were focused on the ability of trophoblast cells to invade matrix *in vitro* to mimic trophoblast invasion of the decidua *in vivo*. Four cell lines were used for this project as shown in **Tables 3.1 and 3.2**. Choriocarcinoma trophoblast cell lines JEG3 [361] and BeWo [361] were compared to the non-neoplastic trophoblast cell line HTR8 [362] and the neoplastic non-trophoblast thyroid papillary cancer cell line (TPC-1) [363]. TPC-1 cells will be referred to as TPC cells in the rest of the thesis. Culture media and constituents for the growth of these cells are detailed in **Table 3.1**.

Table 3.1 Cell lines

	JEG3	BeWo	TPC	HTR8
Passage number	137	6	8	19
Supplied by	European Collection of Authenticated Cell Cultures (ECACC)	European Collection of Authenticated Cell Cultures (ECACC)	Kindly donated by Prof McCabe, University of Birmingham	Kindly donated by Dr Gross, Aston University
Growth mode	Adherent	Adherent	Adherent	Adherent
Morphology	Epithelial	Epithelial	Epithelial	Epithelial-mesenchymal transition
Tissue of Origin	Human Placental (Choriocarcinoma)	Human Placental (Choriocarcinoma)	Human Thyroid (Papillary thyroid carcinoma)	Human Placental (Extra-villous trophoblast cells)
Subculture routine	1:3 to 1:4	1:4 to 1:6	1:25 to 1:30	1:5 to 1:8

3.1.2 Cell culture conditions

Cells were cultured and passaged in their respective culture media as shown in **Table 3.2**. All cells were cultured in T75 flasks with 75 cm^2 area (Corning, CLS430725U) until

70% confluency. Following this, cells were passaged and seeded for experiments; 6 well plates (Sigma, CLS3595), 24 well plates (Sigma, CLS3527), and 96 well plates (Sigma, CLS3516) were used for seeding cells depending on the experiment procedure.

Table 3.2 Growth media used for cell culture

	JEG3	BeWo	TPC	HTR8
Culture media	Minimum Essential Media Eagle (Sigma Aldrich, RNBG2336)	DMEM/F12 Ham (1:1) with HEPES (Thermo Fisher Scientific, 1869010)	RPMI-1640 Media W/L-Glutamine (Thermo Fisher, 11879020)	RPMI-1640 Media W/L-Glutamine (Thermo Fisher, 11879020)
Foetal bovine serum (FBS) (10%)	Thermo Fisher Scientific, 10082147	Thermo Fisher Scientific, 10082147	Thermo Fisher Scientific, 10082147	Thermo Fisher Scientific, 10082147
1% L-Glutamine	Thermo Fisher Scientific, 25030081	Thermo Fisher Scientific, 25030081	--	--
1% Penicillin and Streptomycin	Sigma Aldrich, P4333	Sigma Aldrich, P4333	Sigma Aldrich, P4333	Sigma Aldrich, P4333

For cell passage, the cells were briefly washed with PBS, followed by addition of 2 mL trypsin-EDTA (0.25%) per flask (1X, Thermo Fisher Scientific, 25200056). The flasks were then incubated for 5 mins at 37 °C with 5% CO_2 until the cells had all detached. This was confirmed under a microscope (Leica DM ILM inverted DFC 290). Immediately, 8 mL of complete media was added to inhibit the effect of trypsin. This was then well mixed and transferred to a labelled 15 mL centrifuge tube (Corning,

CLS430791). The tubes were briefly centrifuged at 1200 rpm for 5 mins. The supernatant was carefully removed and discarded. Following this, 6 mL of complete media was added again to the cell pellet in the tube, mixed well using a pipette, and added to a fresh flask with complete media for further growth. The cell residue was added to the fresh complete media in the fresh flask, in a specific ratio (as explained in subculture routine in **Table 3.1**).

For experimental purposes, following centrifugation and removal of the supernatant from the tube, fresh culture media was added to the cells and mixed gently. The cells were then counted (**explained in Section 3.4.1**) and seeded at varying concentrations, according to the protocol of the experiments. Experimental treatments were carried out using either control (no treatment) or specific treatment. Cells were then incubated according to the experimental protocol (0 – 72 hours) at 37 °C and 5% CO_2.

3.1.3 Use of serum with variable levels of DBP

Conventional cultures of trophoblast and TPC cells were maintained using 10% FBS-supplemented media for routine passage of cells and 2% FBS-supplemented media for experimental Matrigel™ culture, with FBS in both cases containing endogenous levels of DBP. To vary levels of DBP in Matrigel™ studies, three strategies were used:

1) Supplementation of media using 0.2% FBS as a 'low serum' comparison with 2% FBS (similar to strategies used by Hertzig *et al.*) [364];

2) Supplementation of media using 2.0% serum from wild type (DBP+/+) and DBP knock-out (DBP-/-) mice (similar to strategies used by Lee *et al.* and Chun *et al.*) [365, 366];

3) Supplementation of media with 2% human serum from individual donor pregnant and non-pregnant women (a similar strategy used by Hertzig *et al.*) [364].

3.1.4 Cell culture using growth factor reduced Matrigel™

Routine passage of cells and some initial experiments were carried out using conventional cell culture in plasticware flasks or dishes. However, other experiments were carried out using cells cultured on an artificial matrix using the commercially available product growth factor reduced Matrigel™ (Matrigel, Corning 354230). In designated experiments, Matrigel™ was added manually to cell culture wells to produce growth surfaces coated with a thin layer of matrix. To do this, Matrigel™ was diluted (1:20) in respective serum-free cell culture media and then added as 50 µL/cm^2 of well area (**Table 3.3**) according to the manufacturer's instructions. The Matrigel™ was then allowed to solidify for 2 hours inside a sterile incubator (37 °C and 5% CO_2). Excess media was then removed, and the wells were briefly washed with PBS. Cells were then seeded immediately in these wells to eliminate drying of the matrix. Manual coating of wells with Matrigel™ was used for the following experiments: – 1) mRNA extraction experiments; 2) protein expression by immunofluorescence assay; 3) BrdU proliferation assays; 4) *VDR* siRNA knockdown experiments. For matrix invasion assays, Matrigel™ pre-coated transwell plates were used (Corning® BioCoat™ Matrigel™8.0 micron, 354480) as explained in **Section 2.6**.

Table 3.3 Volume of Matrigel™ used for each plate.

Wells	Area (cm$^{2)}$	50 µL/cm^2 of Matrigel™ (for thin coat only)	Tot. volume per plate (of Matrigel™)
6 well plate	9.6 per well	480 µL per well * 6	2880 µL
24 well plate	1.9 per well	95 µL per well *24	2280 µL
96 well plate	0.32 per well	16 µL per well *96	1536 µL

3.2 Cell culture treatment reagents

Various treatments have been used for this project:

1. Control (only cells with the culture media).

2. 1,25D, used at a concentration of 10 nM (4166 pg/mL) and 100 nM (41,660 pg/mL) (Enzo Lifesciences, BML-DM200-0050), with 0, 24- and 48-hours incubation (where 1 nM = 416 pg/mL of 1,25D);

3. ERK inhibitor/U0126 (Cell Signalling, 9903), with 2 hours incubation, used at a concentration of 10 µM from 10,000 µM (10 mM) stock solution.

4. Exogenous DBP (East Coast Bio, LA166, 2 µM treated for 24 and 48 hours) used at a concentration of 2 µM from a 200 µM stock solution.

5. RAP (BML-SE552-0100, Enzo Life Sciences) was added to cell cultures at a concentration of 1 µM (from 16 µM stock) at varying time points as indicated by other researchers [367, 368].

DBP concentrations in culture media were also modified by adding 2% and 0.2% foetal bovine serum, 2% harvested male mouse serum (DBP+/+ and DBP -/-), and 1st and 3rd trimester human pregnancy serum to cell cultures (at 2% concentration).

To examine the effect of blocking megalin-mediated uptake of DBP protein by cells, megalin inhibitor-receptor associated protein (RAP) was used in cell cultures. RAP blocks LRP2- ligand interactions, which is also known as a megalin receptor [369].

3.3 Human placental tissues

Surgical termination of pregnancy (TOP) placental tissues (from 1st trimester and early 2nd trimester patients) were collected from Birmingham Woman's Hospital. This was done to study the expression of vitamin D related genes in the placenta in early pregnancy.

3rd trimester (≥37 weeks) uncomplicated pregnancy placental tissues (following caesarean or normal vaginal delivery) were also collected using the same ethics. This was done to compare the placental protein expression in early pregnancy with the later stage of pregnancy.

The tissues were collected by Dr. Jennifer Tamblyn following appropriate consent from patients. Ethics: 14/WM/1146, obtained from West Midlands - Edgbaston Research Ethics Committee.

These tissues were processed, paraffin wax embedded and stored at room temperature for further studies. The tissues were then sectioned and mounted onto slides at a thickness of 5 µm, for immunostaining procedures.

3.4 Analysis of cell proliferation

Three different assays for measuring cell proliferation were used: 1) manual counting of live cells using the dye trypan blue to identify dead cells; 2) cellular incorporation of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) to stain for metabolically active respiring cells; 3) cellular incorporation of bromodeoxyuridine / 5-bromo-2'-deoxyuridine (BrdU) to assess DNA replication. Initial studies comparing trophoblast cell proliferation on plasticware versus Matrigel™-coated cultures, employed all three methods of analysis of cell proliferation, but subsequent studies utilised BrdU-incorporation as the most reproducible methodology.

3.4.1 Manual counting of viable/non-viable cells

Cells were initially grown in T75 plastic culture flasks. Once cells reached 70% confluency, they were trypsinised, washed and centrifuged in PBS and the resultant cell pellets resuspended in serum-free medium at a concentration of 100,000 cells/mL. From this suspension, 50,000 cells were seeded on 6 well plates with 2 mL of culture medium. After designated time points (24 hours and 48 hours), the cells were trypsinised, washed and centrifuged in PBS and resuspended in the complete culture medium. From this solution, 100 µL were aliquoted into a fresh 1.5 mL centrifuge tube, and 400 µL of 0.4% trypan blue was added (1:5 dilution). This was mixed gently. 10 µL from this mixture was applied on a haemocytometer for counting the number of cells under a microscope (Leica DM ILM inverted DFC 290). The clear cytoplasm cells were counted as “viable”, while blue-stained cells were considered to be “non-viable”. The total number of cells (viable and non-viable) were counted from five large squares from

cell counting chamber (Fast-Read® 102) using duplicate samples to produce a mean number of cells. **Figure 3.1** demonstrates the optimisation assay data.

This represented the number of cells in 0.5 µL of suspension, and the total cells in each suspension could then be calculated based on this value.

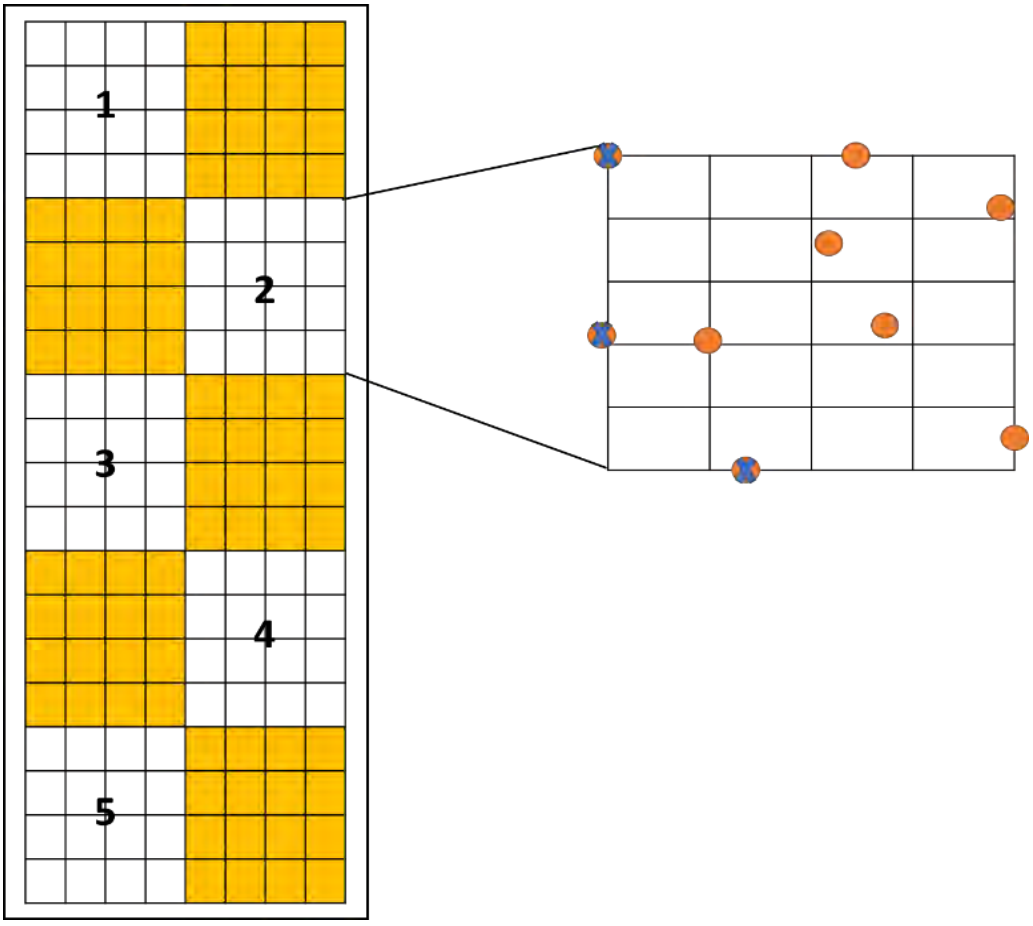
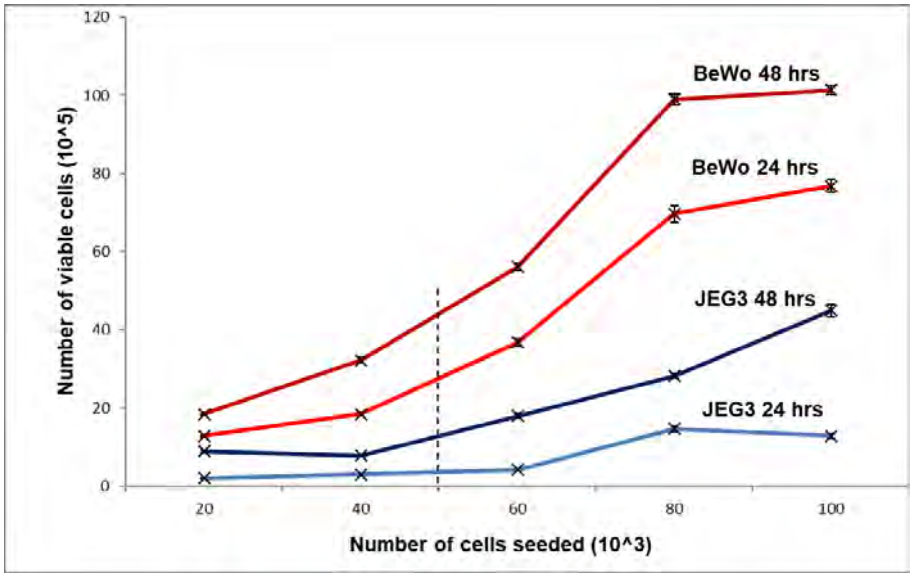


Figure 3.1 FAST-READ® 102 cell counting method.
The chamber structure showing five large squares for cell counting. The figure on the right shows a magnified image of one square with cell counting procedure. The crossed cells on the left border and the lower border of the squares were not considered.

Optimisation data for manual counting of viable and non-viable cells are shown in **Figure 3.2.**

A



B

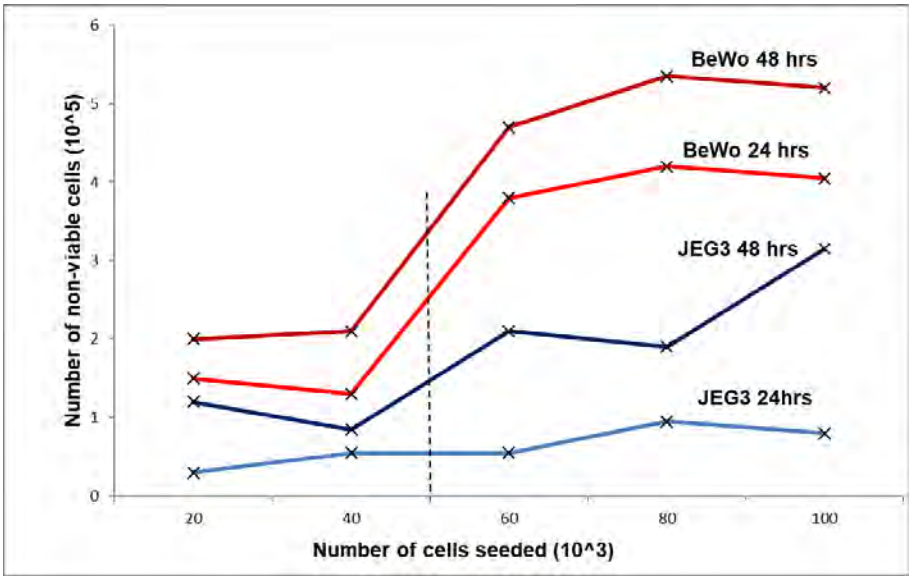


Figure 3.2 Optimisation of manual counting of cell proliferation.
A. viable cell numbers. **B.** non-viable cell numbers, in 24- and 48-hours cultures of JEG3 and BeWo cells. The dotted line shows the $50*10^3$ cells that were considered for further experiments. n=3.

3.4.2 Analysis of cell proliferation by MTT incorporation

The MTT assay kit (Promega, G3582) detects cellular metabolic activity. The MTT reagent detects the changes in NADPH level while cells proliferate.

For optimisation (the standard curve), cells were seeded in 96 well plates at a concentration of 0 to 15,000 cells per well in 6 rows. From the standard curve obtained (**Figure 3.3**), a cellular concentration of 5000 cells was chosen for further treatments with 1,25D. 10 nM and 100 nM 1,25D were added to the wells with 5000 cells. 10% and 2% foetal bovine serum (FBS) was added to the culture medium (100 µL in each well) used in this experiment. This was done to understand the effect of reduced serum on cellular proliferation.

The plates were then incubated at 37 °C, with 5% CO_2 for 48 hours. After 48 hours, 20 µL of cell-titre 96 aqueous one solution reagent (MTT solution) was added. The plates showed colour change following the introduction of the substrate solution, and this was assayed for optical density (OD) values using a plate reader (Perkin Elmer Victor3 V 1420-041 Multilabel Plate Counter) and the plate reader software (Wallac Victor 3 Software 3.00) at 0 hours. Following this, plates were incubated at 37 °C with 5% CO_2, for 1 to 4 hours, with the plates examined and analysed for absorbance levels (OD values) every hour. The absorbance was recorded at every reading, at 490 nm for 1 second.

The data obtained showed 6 different OD values at each time point for each “seeded cell concentration” (**Figure 3.3**).

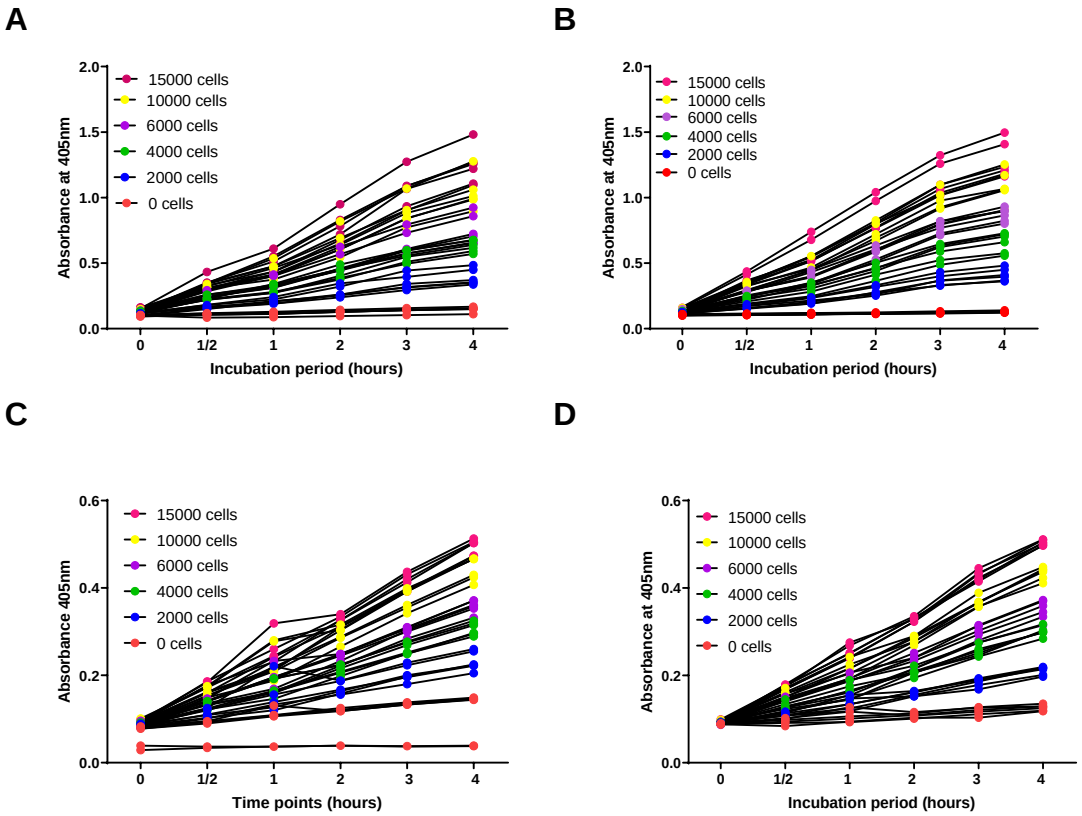


Figure 3.3 Optimisation of MTT assay for cellular proliferation with the various serum concentrations and time points.
A. JEG3 cells (10% FBS). **B.** JEG3 cells (2% FBS). **C.** BeWo cells (10% FBS). **D.** BeWo cells (2% FBS). n=1 with 6 replicates for each cell line.

The results (**Figure 3.3**) were unable to show any significant change in proliferation when compared with 2% and 10% serum groups. Treatment with 10 nM and 100 nM 1,25D showed similar proliferation as compared to control cells. Low serum media (2% FBS), when compared to very low serum media (0.2% FBS), showed similar proliferation data (not shown). To confirm this proliferation data further, BrdU assay was performed. BrdU assay has been further explained in the results chapter.

3.4.3 Analysis of cell proliferation by BrdU incorporation

The BrdU assay kit (Cell Signalling Technology, 6813) detects 5-bromo-2-deoxyuridine (BrdU) in cellular DNA while they proliferate. Thus, only viable and proliferating cells were detected using BrdU. Optimisation of this method was done for 3 cell lines (JEG3, BeWo and TPC). In the actual experiment, all 4 cell lines were seeded in 96 well plate at a concentration of 5000 cells per well (triplicate wells) as described for specific experiments.

At designated time points, 10 µL of primary labelled antibody (labelling solution) was added in each well with 4 hours incubation at 37 °C with 5% CO_2. After removing the labelling media, cells were fixed with 200 µL of 100% ethanol followed by 30 mins incubation at room temperature. Following fixation, an antibody conjugate was added (100 µL per well, incubated for 1 hour at room temperature) and DNA denatured. The substrate solution was then added (incubated for 20 mins at room temperature). The substrate binds to the denatured DNA and the absorbance was measured on a plate reader at 405 nm for 1 sec (Perkin Elmer Victor3 V 1420-041 Multilabel Plate Counter). Each experiment was done in triplicate and it was repeated three times.

The method had to be optimised for timepoint for treatment with BrdU labelling solution and for treatment with substrate solution. **Figure 3.4** shows the optimisation method with the standard curve for JEG3, BeWo and TPC cells. From the optimisation, 4 hours' time point for treatment with BrdU labelling solution and 20 mins time point for treatment with substrate solution was preferred for further experiments.

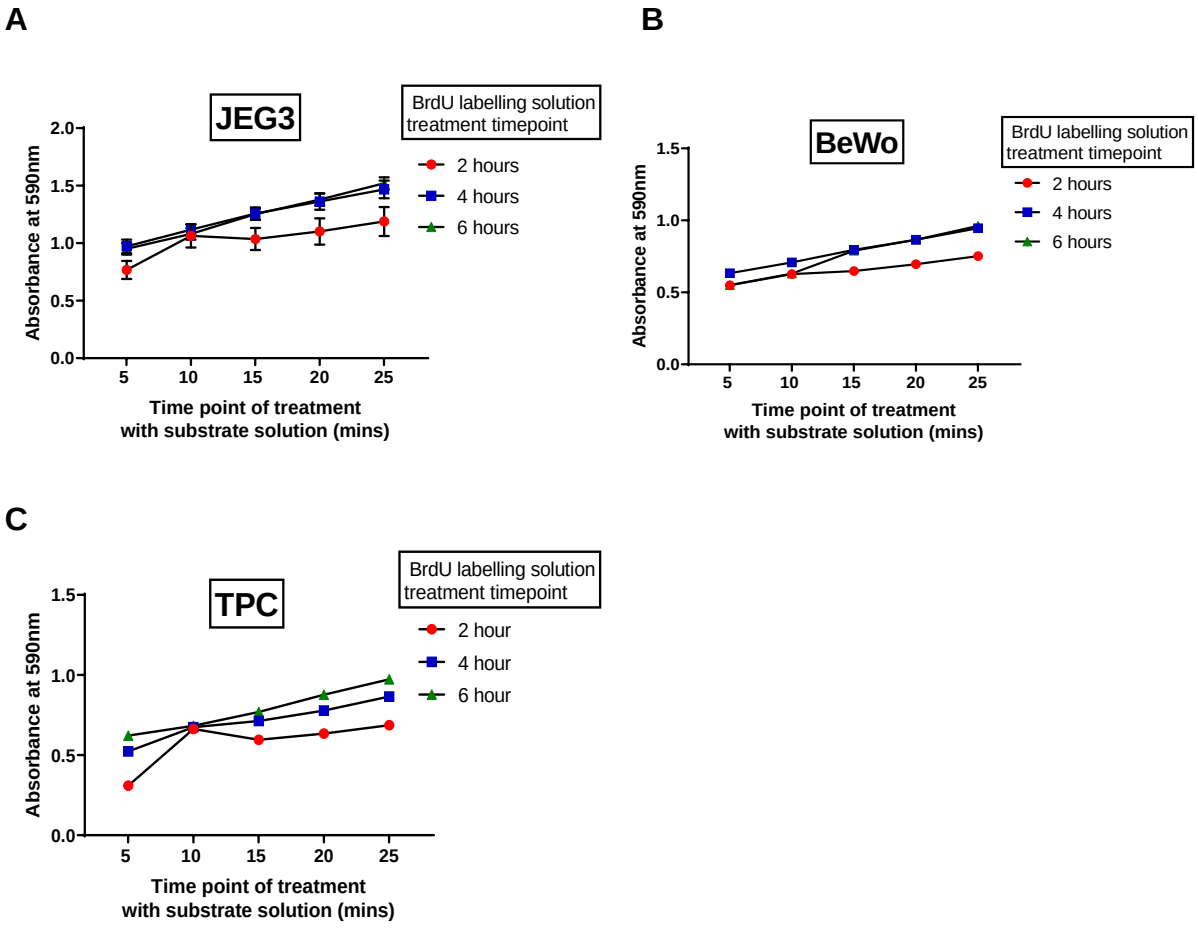


Figure 3.4 Optimisation analysis of BrdU cellular proliferation assay for timepoint of treatment with BrdU labelling solution and duration of treatment with substrate solution.
A. JEG3. **B**. BeWo. **C**. TPC.

3.5 Analysis of cell migration

Cell migration or motility was assessed using a “wound closure” method. Cells were seeded at $8x10^4$ cells/well in a 6 well plate with complete media (10% FBS) and incubated until 90% confluency. A cross ‘wound’ was then drawn on the bottom of each well using a P200 pipette tip, the culture media changed, and fresh culture medium added immediately containing 1,25D (100 nM). This was compared with non-treated wells (controls). Wound distance was measured four times at a fixed distance around the cross point of each wound, and this was done for 3 wells for each cell (n=3). I used 10x magnification of a light microscope (Leica DM ILM inverted DFC 290) and ImageJ software at 0, 24 and 48 hours to determine the rate of wound closure (µm/hr). The rate of wound closure (µm/hr) for each cell culture image was determined by multiple measures of wound distance at 0, 24 and 48 hours of culture. The mean 0-hour wound distance for one well was then subtracted from mean values for 24 hr and 48 hr cultures, the results obtained were further divided by 24 and 48 (hours) respectively and the rate of wound closure calculated as the movement of cells (µm/hr) (see **Figure 3.5**).

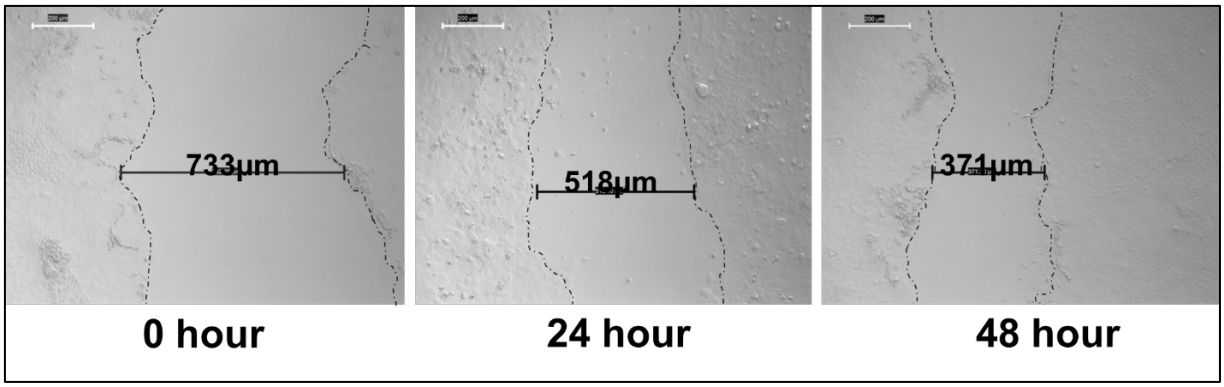


Figure 3.5 Example of cell migration assay using wound closure technique.
The central bar shows a single measurement for each time point. The white scale bar on the top left corner shows 200 µm. A decrease in wound distance can be observed from 0 hour to 48 hours.

3.6 Analysis of matrix cell invasion

Studies show that trophoblast cells require attachment to the components of maternal extra-cellular matrix (ECM) for promoting invasion and migration through the maternal decidua [370, 371]. In this study, we used a growth factor reduced extra-cellular matrix coated wells to study the invasive properties of trophoblastic cells.

Capacity for cell invasion was evaluated using growth-factor-reduced matrix coated transwell assay (Corning® BioCoat™ Matrigel™ 8.0 micron, 354480). All 4 cell lines were treated with pre-warmed culture medium containing 2% FBS for 24 hours before passage. Cells were then trypsinised, resuspended in 2% FBS culture medium and $5x10^4$ cells per well seeded onto transwell inserts in the upper chamber of each well (see **Figure 3.6A**). Complete media (with 10% FBS) was added to the lower chamber of each transwell. Immediately after seeding, cells were treated with 1,25D and incubated for a further 48 hours. A similar method was followed for control cells; however, no treatment was added for control wells. Following 48 hours incubation, the

lower surface of the transwell inserts was then washed with PBS, followed by washing with 95% ethanol. The lower surface of the transwell membrane was then stained with haematoxylin (Sigma, MHS16), followed by washing with Scott's water (2 g sodium bicarbonate, 20 g magnesium sulphate, and 1 L of deionised water), and further staining with Eosin-Y (VWR chemicals, 10047001). After further washes with 70% ethanol and 99% ethanol, the transwell inserts were dried at room temperature. Once dried the lower surface of each insert was imaged using a microscope (Leica DM ILM inverted DFC 290) at 10x magnification. Under blinded conditions, each transwell was imaged 5 times at 5 different quadrants (**Figure 3.6B**). Cells in the upper chamber (low serum chamber) invade into the lower chamber (high serum chamber) through a porous membrane coated with matrix, and the number of cells migrating to the lower chamber reflects the invasive capacity of cells for each culture treatment. Each experiment was done in triplicate and it was repeated multiple times.

An optimisation method was carried out to detect an optimum seeding concentration to achieve a distinct invasion of each cell. This is further explained in **Figure 3.8.**

A **B**

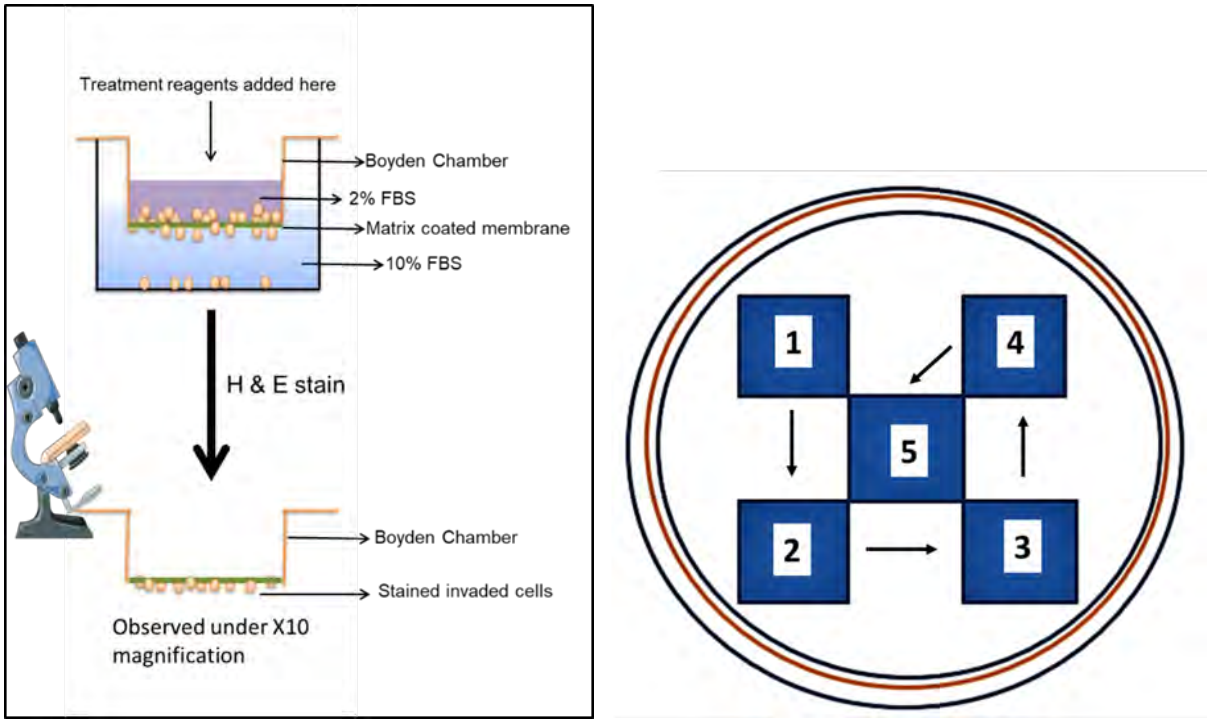


Figure 3.6 Procedure of analysis of cell invasion by Matrigel™ transwell assays. **A**. Schematic showing the principles of cellular matrix invasion using a Boyden chamber. **B**. Schematic showing the method of visualisation of each transwell under a microscope for counting invaded cells.

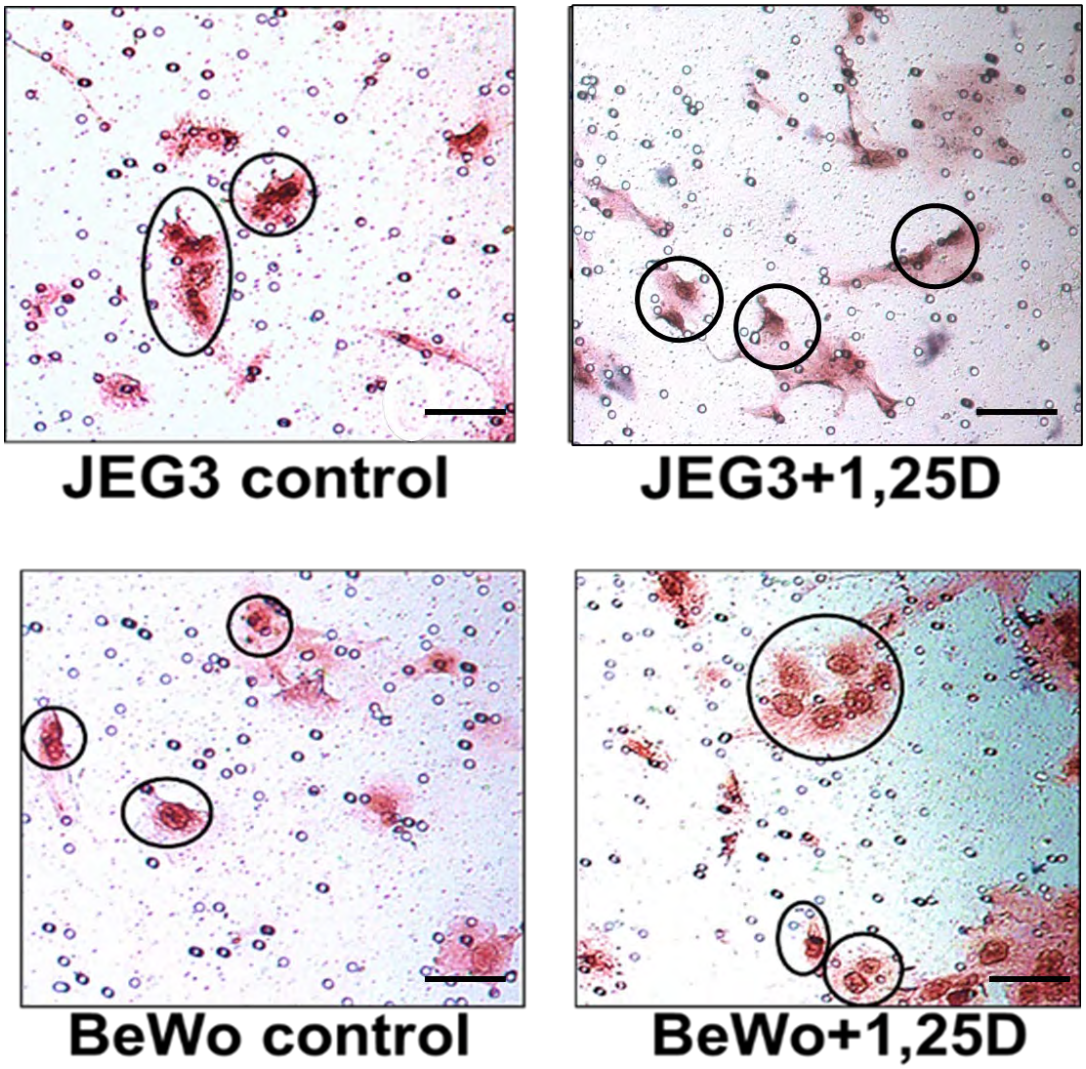
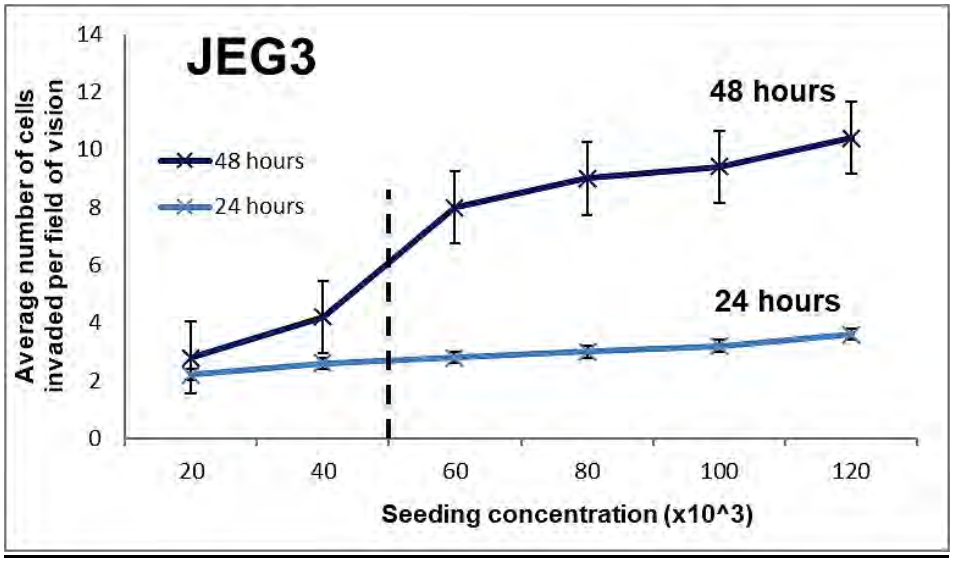


Figure 3.7 Images of invaded cells following invasion through Matrigel™ transwell.
Example images of stained invaded cells as seen under a microscope with 10x magnification. Scale bar is 200 µm.

Each invaded cell was denoted by pink cytoplasm with a blue nucleus (**Figure 3.7**). Total numbers of invaded cells were counted for each of 5 images and data were determined as the mean of these 5 values. For each experiment mean cells per image were determined from triplicate values, and overall median and 95 CI (confidence interval) determined from multiple separate experiments. **Figure 3.8** shows

optimisation experiments for Matrigel invasion by JEG3 and BeWo cells after 24 and 48 hours of incubation.

A.



B.

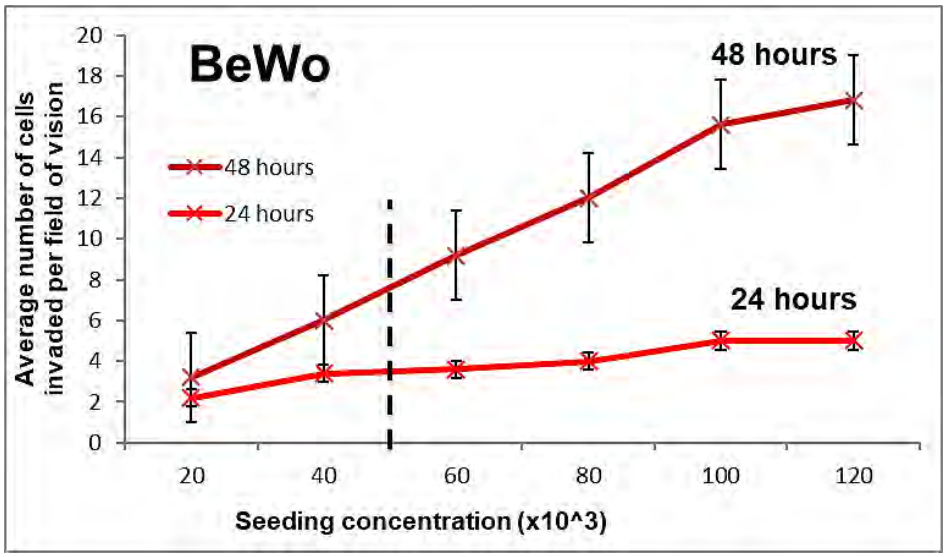


Figure 3.8 Optimisation of cellular matrix invasion using transwell assays.
A. JEG3 and **B**. BeWo cells based on time point (24 and 48 hours) and the number of cells seeded in each well. 50,000 cells/well and 48 hours culture were shown to be optimal for subsequent assays. The dotted line shows the seeded cell count (50,000 cells) considered for further experiments. Each small cross represents mean and the error bars represent 95% confidence interval.

3.6.1 Quantification of Matrigel^{TM} Invasion assays

Quantification of invaded cells in the Matrigel^{TM} transwell assays was carried out using Crystal violet (V5265, Sigma) to stain the invading cells, and acetic acid (1000631011, Sigma) to solubilise the 1% Crystal violet stain. Cell seeding and incubation was carried out as described previously in **Section 3.6**. Invaded cells on the transwell were washed twice with PBS, then stained with 1% Crystal violet (diluted in PBS) for 10 mins, then washed again with PBS. Following this, the transwell inserts were left to dry at room temperature. Images of each insert were taken for counting of invaded cells (5 quadrants per transwell). Transwell inserts were then immersed in 400 µL 30% acetic acid (in 24 well plate) and shaken for 10 mins at room temperature. Following this, 100 µL of the blue stained acetic acid solution was pipetted into triplicate wells in a 96 well plate and absorbance measured at 590 nm and 405 nm (Optical density value or OD values) using an ELISA (enzyme-linked immunosorbent assay) plate reader (SpectraMax ABS, Molecular devices, San Jose). Each experiment was repeated multiple times as indicated. Optimisation data for Crystal violet staining are shown in **Figure 3.9**.

Firstly, I opted for manual counting of invaded cells. This gave the exact number of cells invaded through and the morphology of cells invading. However, analysis of this assay is subject-specific. Thus, to further establish the observed invasion assay data, I also performed a quantification experiment for the same. This gave a more definitive and quantifiable number for the percentage of cell invaded. This assay gave a spectrophotometric analysis data of the percentage of cells dissolved in the acetic acid eluent.

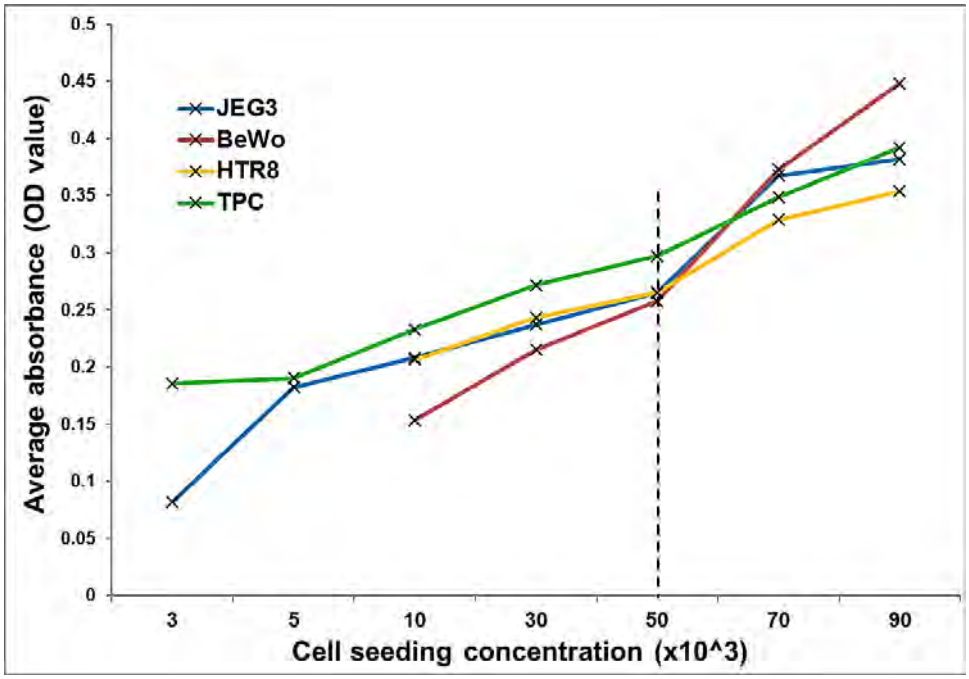


Figure 3.9 Quantification of cellular matrix invasion in transwell assays.
OD values for crystal violet staining (y-axis) of matrix invading control JEG3, BeWo, HTR8 and TPC cells in transwell cultures at increasing seeding densities (x-axis). The dotted line shows the optimal seeding density used for subsequent studies.

3.7 RNA extraction

Total RNA was extracted from cells cultured on plasticware or matrix-coated wells using TRI Reagent (Sigma, T9424) as indicated by the manufacturer. The TRI Reagent consists of phenol, guanidine isothiocyanate, and other components which induce total RNA isolation from a variety of cells and tissues. TRI Reagent maintains the integrity of RNA by inhibiting RNAse activity while disrupting the cells. The RNA extraction procedure was carried out according to the manufacturer's instructions. 500µL of TRI-Reagent was used for each well in 6 well plate. The extracted RNA was stored in sterile tubes at -20 °C overnight, for increased RNA yield.

The following day, samples were thawed, and 100 µL of chloroform ($CHCl_3$) was added to each sample, mixed well and incubated at room temperature for 15 mins. These samples were centrifuged at 13,000 rpm (rotations per minute) for 15 mins at 4 °C. This separates the samples into 3 different layers; top transparent aqueous layer (containing total RNA), middle white layer (containing DNA), and bottom pink layer (containing proteins and lipids). This is further explained in **Figure 3.10**. The top aqueous layer was extracted carefully and transferred into a fresh tube containing 250 µL isopropanol solution (each sample separately). This was mixed well and incubated at room temperature for 15 mins. The samples were then centrifuged at 13,000 rpm for 15 mins at 4 °C. Following this, the supernatant was removed, and 75% ethanol was added very slowly to wash the precipitated RNA. The tubes were centrifuged briefly again at 13,000 rpm for 5 mins at 4 °C. The supernatant ethanol with impurities was then removed, and the tubes were centrifuged again to remove residual ethanol and centrifuge tubes containing RNA pellets were left to dry at room temperature.

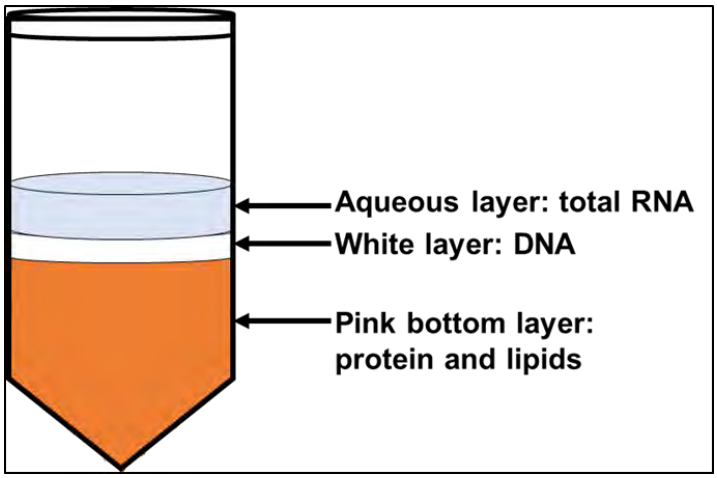


Figure 3.10 DNA, RNA and lipid/protein layers obtained following centrifugation with chloroform in the RNA extraction method using Tri-reagent.

Once dried, 10 µL of nuclease-free water was added to each sample and mixed well. The concentration of total RNA extracted from each sample was quantified using a spectrophotometry (NanoDrop) technique (1 µL of each sample) by measuring absorbance at 260 nm.

3.8 Reverse transcription

Following quantification of RNA, each sample was diluted to 100 ng/µL, with nuclease-free water and converted to complementary-DNA (cDNA) for long term storage. This was carried out using cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368814) (10x buffer, 10x random primers, dNTPs, RNase inhibitors, reverse transcriptase) in a thermocycler (GeneAmp PCR System 2700, ThermoFisher Scientific). The reagents were added in the following volumes for each 10 µL of sample:

- 2 µL 10x buffer
- 2 µL 10x random primers
- 0.8 µL dNTPs
- 1 µL RNase inhibitors
- 1 µL reverse transcriptase
- 3.2 µL nuclease-free water (NFW) for a total volume of 20 µL.

The resulting reverse transcription reaction mix was incubated at:

- 25 °C for 10 mins,
- 37 °C for 120mins,
- 85 °C for 5 mins.

The cDNA obtained was then incubated at 4 °C until ready to be stored at -20 °C until further use.

3.9 PCR amplification of cDNA

To quantify expression levels of specific mRNA from the obtained cDNA samples, real-time quantitative polymerase chain reaction (RT-qPCR) assays were carried out to amplify specific target genes. **Table 3.4** shows the specific primer-probe kits used for the analysis of : vitamin D receptor (*VDR*); 24-hydroxylase (*CYP24A1*); tissue inhibitor of metalloproteinase 1 (*TIMP1*); matrix metalloproteinase 2 (*MMP2*); matrix metalloproteinase 9 (MMP9); matrix metalloproteinase 14 (*MMP14*); low-density lipoprotein-related protein 2 (*LRP2*); transforming growth factor-beta 1 (*TGF-ß1*).
RT-qPCR reactions were carried out using TaqMan Universal qPCR MasterMix (ThermoFisher, 4304437) using qPCR machine (Applied Biosystems 7300/7500

Real-Time PCR System reaction conditions as follows:

- 50 °C for 2 mins
- 95 °C for 10 mins
- 95°C for 15 secs
- 60°C for 1 min

} 40 cycles

Three reference genes were assessed for normalisation of RT-qPCR gene expression data (**Table 3.5**). As shown in **Figure 3.11**, β-actin showed the least variation with mean as- 15.61, and 95% CI- 15.42 and 15.80; GAPDH- mean-19.07, 95%CI- 18.52 and 19.62; 18S- Mean- 13.18. 95% CI- 12.70 and 13.66. β-actin (ThermoFisher) was

selected as the reference gene in comparison with GAPDH (ThermoFisher) and 18S rRNA (ThermoFisher). All PCR experiments were carried out with negative control (non-1,25D treated samples) and measured using a thermocycler (ABI 7500 qPCR machine (Applied Biosystems)). The resulting Ct values were used for statistical analysis of gene expression and visually represented as 1/δCt, where δCt= Ct target gene – Ct reference gene. Each experiment was carried out in triplicate and repeated multiple times as indicated.

Table 3.4 Primers used for RTqPCR.

Gene symbol	Title	Catalogue number	Chromosomal localisation
VDR	Vitamin D receptor	Thermo Fisher, Hs00172113_m1	Chr.12: 47841537 - 47905031 on Build GRCh38
GC	Vitamin D binding protein (DBP)	Thermo Fisher, Hs00167096_m1	Chr.4: 71741693 - 71805520 on Build GRCh38
CYP24A1	24-hydroxylase	Thermo Fisher, Hs00167999_m1	Chr.20: 54145731 - 54173985 on Build GRCh38
TIMP1	Tissue inhibitors of metalloproteinase 1	Thermo Fisher, Hs01092512_g1	Chr.X: 47582291 - 47586791 on Build GRCh38
MMP2	Matrix Metalloproteinase 2	Thermo Fisher, Hs01548727_m1	Chr.16: 55478830 - 55506691 on Build GRCh38
MMP9	Matrix Metalloproteinase 9	Thermo Fisher, Hs00957562_m1	Chr.20: 46008908 - 46016561 on Build GRCh38
MMP14	Matrix Metalloproteinase 14	Thermo Fisher, Hs01037003_g1	Chr.14: 22836533 - 22847600 on Build GRCh38
LRP2	Low-density lipoprotein-related protein 2	Thermo Fisher, Hs00189742_m1	Chr.2: 169127109 - 169362613 on Build GRCh38
TGF-β1	Transforming growth factor-beta 1	Thermo Fisher, Hs01086000_m1	Chr.14: 75958097 - 75982022 on Build GRCh38

Table 3.5 Reference genes used for RTqPCR.

Gene symbol	Catalogue number	Chromosomal localisation
β-actin	Hs01060665_g1	Chr.7: 5527148 - 5530601 on Build GRCh38
GAPDH	Hs02758991_g1	Chr.12: 6534405 - 6538375 on Build GRCh38
18S rRNA	Hs99999901_s1	N/A

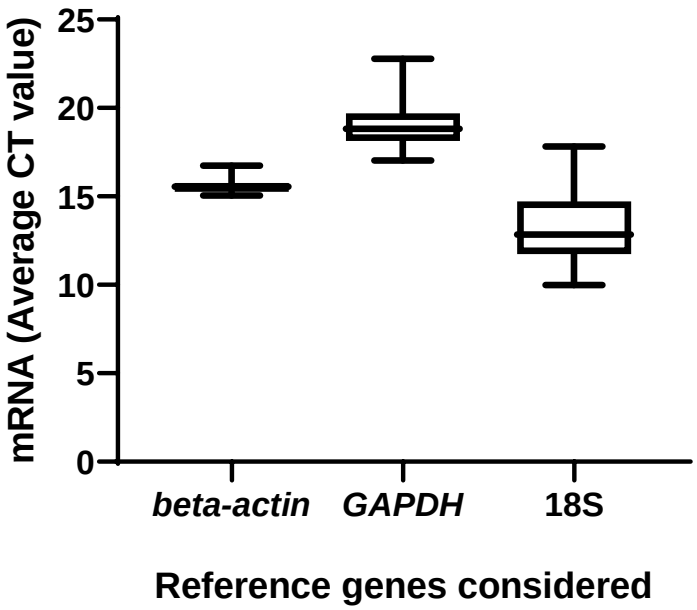


Figure 3.11 Comparison of different reference genes as internal standards for RT-qPCR assays.
Data shows mean and 95% confidence for three reference genes under consideration.

3.10 Western blot analysis of protein expression

Western Blot analysis was carried out using whole-cell protein lysates, and fractionated proteins (nuclear and cytoplasmic proteins) using previously published protocols [372, 373]. Whole-cell protein lysates were extracted using radioimmunoprecipitation assay (RIPA) buffer (with Tris-EDTA) with protease inhibitor (Sigma, P8340) and

phosphatase inhibitor (Sigma, P0044). Cytoplasmic and nuclear proteins were fractionated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, 38835). **Table 3.6** explains various components of the protein lysates used.

Protein extraction

Whole-cell lysate extraction- Culture plates were kept on ice and washed with PBS. Followed by the addition of RIPA buffer with protease and phosphatase inhibitor to the culture well. The plates were then incubated for 15 mins at -20°C. This was followed by scraping of the culture wells and collecting the content in a pre-labelled eppendorf. This was followed by centrifugation of the eppendorf at 4°C for 20 mins at a speed of 13000g. Following this, the supernatant was collected, and the pellet was discarded.

Nuclear and cytoplasmic lysate extraction- The method was done according to the manufacturer's instructions. The culture plates were constantly kept on ice. These were washed with PBS, followed by trypsinisation, and then adding the lysates in a labelled eppendorf. This was followed by centrifugation of the eppendorfs at 500 g for 5 mins at 4°C. Supernatant removed, and PBS was added for washing the pellet and this solution was transferred into a new eppendorf and then this was centrifuged again at 500 g for 5 mins at 4°C. Then the PBS was removed completely, and the pellet was dried. This was followed by addition of ice-cold CER1 solution (200 µl) per eppendorf. The eppendorfs were vortexed for 15mins secs and incubated in ice for 10mins. This was followed by addition of ice-cold CER2 solution (11 µl) per Eppendorf. Followed by vortexing for 5 mins and then 1 min of incubation in ice, and it was vortexed again for

5 secs. The samples were then centrifuged at 16,000 g for 5 mins at 4°C. The supernatant obtained is the cytoplasmic protein lysate. This was then separated in a pre-labelled eppendorf. The pellet in the original eppendorf was resuspended in NER solution (100 µl per eppendorf). Followed by 15 secs of vortex and 10mins incubation on ice. These samples were then centrifuged at 16,000 g for 5 mins at 4°C, and the supernatant obtained was nuclear proteins. These proteins were kept in a pre-labelled eppendorfs in -80 °C for long term storage.

Protein quantification

Extracted proteins were quantified using a BCA assay kit (Thermo Scientific, 23225) with 540 nm wavelength using a plate reader (SpectraMax ABS, Molecular devices, San Jose, California). A protein standard curve was generated using samples with known protein concentrations.

Running the proteins on SDS-PAGE gels

The quantified samples were diluted with loading buffer (437.5 µl per Eppendorf) with beta-mercaptoethanol (62.5 µl) added to the loading buffer, for the volume of protein per 50 µg. The proteins were then denatured at 95°C for 5mins for small proteins. Following this, the samples (50 µg) and the ladder (10 µl) were placed in the SDS-polyacrylamide (10%) gel. Proteins were separated using SDS-polyacrylamide gel, using 100V for 15-20 mins (until the sample has run through the stacking gel), followed by 140 V for 1 hour (or until the sample has run to the end of the resolving gel). The gel was then cold transferred onto nitrocellulose membranes (0.2 µm, Bio-Rad), (after

soaking in 100% methanol for 2 mins) (**Figure 3.12**). This was done with 360 mA current for 1 hour and 15 mins.

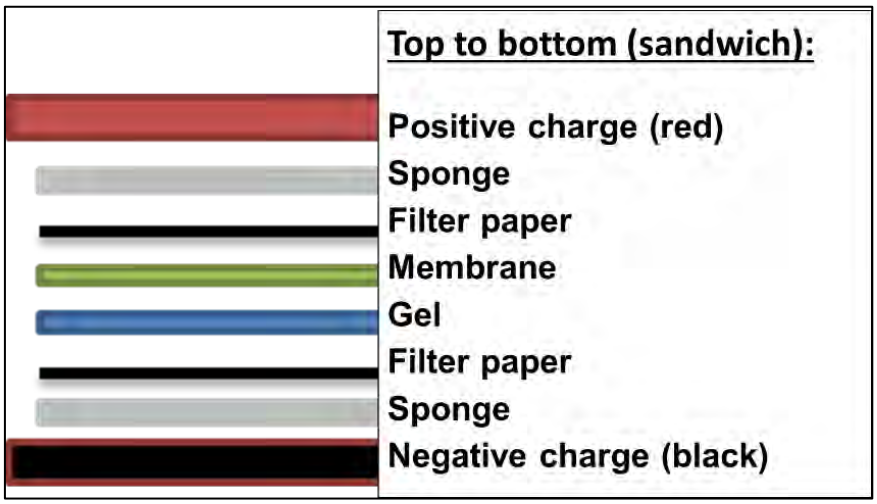


Figure 3.12 Sandwich arrangement for gel with nitrocellulose membrane for the transfer procedure.

Ingredients of each reagent and buffers used:

Preparation of RIPA Buffer (200ml)

- 1.22g Tris
- 160ml water
- 1.8g NaCl
    - pH 7.4 with HCl
- 2ml Igepal
- 5ml 10% sodium deoxycholate
- 2ml 100mM EDTA (3.722g in 100ml)

Preparation of 5X Protein Loading Buffer (20ml) (stored at -20°C)

- 2.5ml of 2M Tris-HCl solution with pH 6.8 (250mM) + 2gm of SDS (10%) + 0.004gm bromophenol blue (0.02%).
- The above solution was mixed well and 10ml glycerol was added (50%).
- Double distilled water was added to bring the total volume of the above solution to 17.5ml.
- 437.5µl of the above solution was aliquoted per Eppendorf and stored at -20ºC.
- When required, an aliquot of the above sample is taken out of -20C, and 62.5µl β-mercaptoethanol was added and mixed well by gently turning the eppendorf upside down. This constitutes the final loading buffer.

Preparation of 10x Running Buffer

- 30g Tris
- 144g glycine
- 10g SDS
    - Make up to 1L with distilled water

Preparation of Transfer Buffer

- 6.06g Tris
- 28.8g glycine
- 400ml methanol
- 1.6L water

Preparation of Resolving gel (15ml for 2 gels) (10%)

- Water - 5.935ml
- 1.5M Tris-HCl pH 8.8 - 3.75ml
- Acrylamide - 5ml
- 10% SDS - 150µl
- TEMED - 15µl
- 10% APS - 150µl

Preparation of Stacking Gel (5% for 2 gels)

- 2.71ml water
- 1.25ml 0.8M TrisHcl pH6.8
- 833µl acrylamide
- 100µl 10% SDS
- 10µl TEMED
- 100µl 10% APS

Blocking of the membrane included 2 hours of incubation with TBS-T (15mls) and powdered milk (0.75gms) solution mixture, with the membrane placed on a rocker. This was followed by the addition of primary antibodies diluted in the same concentration of TBST-milk solution. This was incubated overnight at 4°C on a rocker. Membranes were probed with various antibodies using chemiluminescence (Pierce ECL Plus, Thermo Fisher Scientific, 32132). The primary antibodies used for quantification using western blot technique were: vitamin D receptor (Santa Cruz, D-6: sc-13133) (1:500), CYP24A1 (24-hydroxylase) (Abcam, ab175976) (1:1000), vitamin D binding protein (Abcam,

ab65636) (1:500), ERK1/2 (ThermoFisher, MA5-15134, K.913.4) (1:1000), pERK1/2 (ThermoFisher, MA5-15173, S.812.9) (1:1000). Beta-actin (Abcam, ab8227) (1:10,000) was used as a reference control protein for whole-cell lysates and cytoplasmic proteins and Lamin B1 (Abcam, ab133741) (1:5000) was used as a control protein for nuclear lysates. After incubation with the primary antibody, the membranes were washed with TBST-T solution for 10 mins, for 3 times. Then the secondary antibodies were added. These were goat anti-mouse HRP (Abcam, ab6789), and goat anti-rabbit HRP (Abcam, ab97051). Incubation with secondary antibi was for 1 hour. This was followed by washing with TBST-T for 10 mins each time on a rocker for 3 times. Then the membranes were incubated with ECL-plus solution for 10mins. This was immediately followed by imaging of these membranes on x-ray films using ECOMAX™ X-ray Processor. Each experiment was carried out in triplicate.

Table 3.6 Protein lysate and cell fractions used for western blot analysis.

	Whole-cell lysates	Cytoplasmic protein lysates	Nuclear protein lysates
Plasma membrane proteins	✓	**X**	**X**
Organelle and cytosolic proteins	✓	✓	**X**
Organelle membrane proteins (mitochondria, endoplasmic reticulum)	✓	✓	**X**
Nuclear proteins	✓	**X**	✓

3.11 Analysis of MMP activity by gelatin zymography

The gelatinolytic activities of MMPs (matrix metalloproteinases) were detected by gelatin zymography as described previously [374, 375]. All 4 cell lines were grown in FBS free culture media as serum contains various endogenous MMPs and also TIMP (tissue inhibitor of matrix metalloproteinases), that may influence gelatin zymography values [376, 377]. After 24 hours, conditioned culture media from each cultured cell line was collected and dead cells were precipitated by centrifugation at 3000 rpm, 10 mins, 4 °C. From the supernatant, 500 µL was transferred to the top chamber of the centrifugal device containing a filter (Merck Amicon® Ultra-0.5 Centrifugal Filter Devices, UFC510024). This was centrifuged at 13,000 rpm, 15 mins, 4 °C. Following this step, the filtration device with the precipitated proteins was reversed in a fresh centrifugation tube, and centrifuged at 3000 rpm for 2 mins, to transfer the precipitate into a tube. 25 µL of nuclease-free water was then added to each sample tube to dilute the protein concentration.

10 µL of samples were mixed with SDS gel sample buffer (Life Technologies, LC2676) at a 1:1 ratio for 10 mins. 10 µL from this mixture was loaded on a 7.5% SDS-PAGE gel in the presence of 0.1% gelatin (Sigma, G-8150), and run with 1x running buffer (10x stock = 1 L of 0.25 M Tris base and 1.92 M glycine, pH 8.3) (Life Technologies, LC2675) at 150 V for 1 hour and 10 mins until good band separation. The gels were then incubated at 4 °C in 1x renaturing buffer (10x stock = 200 mL of 25% v/v Triton X-100 in dH2O) (Life Technologies, LC2670) and 1X developing buffer (10x stock= 1 L of 500 mM Tris-HCl, pH 7.8; 2 M NaCl, 50 mM CaCl2; and 0.2% Brij 35), (Life Technologies, LC2671), both for 30 mins at room temperature, followed by overnight

incubation in fresh developing buffer at 37 °C. The gels were then washed (x3) in deionised water and stained with brilliant blue R staining solution (Invitrogen, LC6060), followed by destaining in a solution containing 30% methanol and 10% acetic acid. Images of gels were taken, and the intensity of the bands was determined using Image J (NIH, USA). Each experiment was carried out in triplicate using separate cell culture preparation.

3.12 Immunohistochemistry

1st trimester and 3rd trimester placental tissue samples (obtained as part of the CHHIP ethics agreement described above) were wax blocked and sectioned at 5 microns (5 µm) thickness using a microtome. The resulting tissue sections were analysed by immunohistochemistry using the following steps:

Dewaxing and rehydration: Slides with tissue sections were warmed at 60 °C for 10 mins and then dewaxed in histoclear-I (1st container) for 2 mins, followed by 2 mins in histoclear-II (2nd container) and then 2 mins in histoclear-III (3rd container) [378]. The racks were then transferred between solutions: 100% ethanol-I 2 mins, 100% ethanol-II 2 mins, 70% ethanol 2 mins, 40% ethanol 2 mins, and then placed under running tap water for 5 mins.

Sodium citrate antigen retrieval: A pre-made solution of sodium citrate (2.94 g) (Sigma Aldrich, 1613859), diluted with distilled water (1 L), was poured into a beaker to allow immersion of the tissue sections on the slides, the beaker was covered with aluminium foil and immersed in the water bath at 95 °C. The beaker with the slides

was kept in the water bath for 30 minutes. The sections were then washed in ice-cold phosphate-buffered saline (PBS) (Gibco Life Technologies, 18912014) for 5 mins to wash off all the sodium citrate.

Blocking step: A humidified chamber was set up with damp tissue paper at the bottom of the chamber. Tissue sections on the slide were then circled with a hydrophobic pen (PAP pen) – and the sections then covered with PBS. Quenching of endogenous peroxidase in tissue sections was done with 100 µL hydrogen peroxide for 15 mins (Sigma Aldrich, H1009) (3% hydrogen peroxide in methyl alcohol = 1:10 dilution). The sections were then washed with PBS-Tween (Calbiochem, 524653) for 5 min. Subsequently, a drop of avidin peroxide (Vector Laboratories, SP2001) was placed on each section and incubated for 15 mins at room temperature. Sections were then rinsed again with PBS. Followed by a drop of biotin (Vector Laboratories, SP2001) on each section, and incubated at room temperature for 15 mins. Avidin makes detection of biotin-labelled protein easier, as the avidin-biotin complex is a strong molecule, and withstands extreme temperatures, pH and denaturing reagents. Biotin is added to an already biotin-rich cell (trophoblast cells) to initiate the blocking mechanism for all the cell receptors. The sections were then rinsed with PBSTween (0.6%) for 5 mins and covered with 50 µL of non-immune blocking buffer for 1 hour at room temperature.

The components of the non-immune block were:

1. 5% kit serum (depending on antibody species) (VECTASTAIN® Elite® ABC-HRP Kit, Vector Labs)
2. 1% bovine serum (BSA) (Invitrogen,15561020)

3. PBS

Following the 1 hr incubation at room temperature, sections were dabbed at the edges to remove excess liquid and the primary antibody then applied immediately.

Primary antibody: The primary antibodies **(Table 3.7)** were diluted to the appropriate concentration in blocking buffer. 50 µL of primary antibody solution was required per section slide.

Table 3.7 Primary and secondary antibodies used for immunohistochemistry.

Proteins examined (primary antibody)	Product details	Dilution used	secondary antibodies	Product details (secondary antibody)	DAB exposure time
CYP27B1	Abcam, ab95047	1:50	Biotinylated anti-rabbit IgG	Vector Lab, PK-6101	45 secs
VDR	Santa Cruz Biotechnology, sc-13133	1:50	Biotinylated anti-mouse IgG	Vector Lab, PK-6102	30 secs
CYP24A1	Abcam, ab175976	1:20	Biotinylated anti-rabbit IgG	Vector Lab, PK-6101	40secs

For negative control, the primary antibody was substituted with blocking buffer at the same working concentration as the primary antibody. The positive and negative antibodies were added to sections and incubated at 4 °C, overnight.

Post antibody washes: PBSTween (0.6%) was put on each section and left for 5 mins. This was repeated 3 times. Sections were then dabbed with tissue at the edges to remove excess liquid.

Secondary antibody - Secondary antibody (biotinylated) was diluted with blocking buffer (preparation mentioned before). 50 µL was applied per section. The sections were subsequently incubated for 30 mins at room temperature. **Table 3.7** explains the secondary antibodies used for each primary antibody for this project.

Washes: The sections were subsequently washed with PBSTween (0.6%) and dabbed with tissue paper to remove excess liquid.

Signal amplification: Vectastatin Elite-ABC reagent ready-to-use (Vector Laboratories, PK7200) was applied as one drop on each section, directly from the container, followed by 30 mins incubation at room temperature. The sections were then washed with PBS-Tween (0.6%). The process was repeated 3 times, lasting 5 mins each. The sections were then rinsed with distilled water for 5 mins and dabbed at the edge to remove excess liquid.

Colour development: Chromogen diaminobenzidine (DAB) (Vector Laboratories, ImmPACT DAB Peroxide Substrate Kit, SK-4105) was taken out of the fridge (stored at 4 °C, highly photosensitive and a suspected carcinogen) 5 mins before use. The container was covered with aluminium foil and kept in ice. 1 drop of DAB was added to 1 mL of kit diluent before application. 100µL of this solution was applied to each section. Colour development (brown colour) was monitored under the microscope (Leica DM ILM inverted DFC 290). **Table 3.7** shows the time taken for the brown colour development for each protein. DAB was then washed off with distilled water and covered with tap water on the slide. The sections were then incubated for 5 mins.

Counterstaining of tissue sections was carried out using filtered Harris's haematoxylin (VWR Chemicals, 10047007) for 5 mins. The sections were placed in running tap water for 5 mins to wash off excess stain. Sections were subsequently dipped in acid alcohol (0.5% concentrated HCL + 70% Ethanol) for 2-3 secs, then rinsed immediately under running tap water. This was followed by dipping in hot tap water for 5 mins. This was done to retain the blue colour of the haematoxylin stain. The staining was checked under the microscope (10x magnification). Haematoxylin or acid alcohol was repeated if required.

Tissue mounting: Excess water was shaken off before transfer. The racks were dabbed before transfer between solutions: 40% ethanol 2 mins, 70% ethanol 2 mins, 100% ethanol-I 2 mins, 100% ethanol-II 2 mins. The sections were then cleared in histoclear I, II and III; 2 mins, 3 mins, and 5 mins respectively. The sections were then mounted with DPX and coverslips applied. The slides were then left to dry under a fume hood for 24 hours.

3.13 Immunofluorescence

For analysis of protein expression in cultured trophoblast and TPC cells, immunofluorescence was used. For these analyses, cells were either cultured on coverslips or Matrigel^{TM} transwells. After the specific culture period for experiments, coverslips or transwell inserts were washed three times with PBS and cells fixed for 20 mins in 3% paraformaldehyde (dissolved in distilled water) at room temperature. Immediately after, cells were incubated for 10 mins with chilled 100% methanol or Triton X-100 (Sigma, T8787) according to the antibody used (Triton-X was

recommended specifically for megalin, G-actin and F-actin). This was followed by washing with PBS and blocking with 10% FBS (N4637, Sigma) for 30 mins. Immediately followed by incubation with primary antibodies (**Table 3.8**) constituted with 1% BSA (12133C, Merck) and PBS for 1 hour at room temperature. The cells were then washed three times with PBS and incubated for 1 hour with secondary antibodies and Höechst stain for nuclei (Invitrogen, 33258) at 1:1000 dilution, mixed with 1% FBS and 1% BSA. Secondaries used were Alexa Fluor 488 -conjugated goat anti-mouse IgG (ThermoFisher, A21235) and Alexa Fluor 594 -conjugated goat anti-rabbit IgG (ThermoFisher, A11037) at 1:250 dilution, for each coverslip and Matrigel™ transwell. Following this, the conjugated antibodies were mixed with 1% FBS and 1% BSA, and the samples were incubated for 1 hour with these antibodies. This was followed by washing 3 times with PBS. Coverslips and the Matrigel™ transwell base were then mounted on Thermo Fisher ProLong™ Diamond Antifade Mountant media (ThermoFisher, P36965). The round base of transwells was cut and mounted with mounting media and slides imaged with Confocal Microscope (Zeiss LSM 780), at x40 magnification (using Carl Zeiss™ Immersol™ water immersion, 100707) and 100x magnification (using Carl Zeiss™ Immersol™ Immersion Oil, 111130). These images were analysed and quantified using Zen-blue software (Carl Zeiss) and ImageJ Fiji software (NIH, USA).

Table 3.8 Primary antibodies used for immunofluorescence.

Primary antibodies	Manufacturer	Dilution used
anti-Vitamin D receptor (VDR)	Santa Cruz, D-6: sc-13133	1:50
anti-Vitamin D Binding protein	Abcam, ab65636	1:50
anti-LRP2 (Megalin)	Abcam, ab236244	1:50
anti-pERK1/2	Cell signalling, 9101L	1:50
Deoxyribonuclease-1. Alexa Fluor 594 Conjugate (anti-G-actin)	ThermoFisher, D12372	1:500
Phalloidin-. Alexa Fluor 488 conjugate (anti-F-actin)	Abcam, ab176753	1:500
anti-Sodium Potassium ATPase antibody-Plasma Membrane Marker Alexa Fluor 488 conjugate	Abcam, ab197713	1:100
Hoechst Solution (nuclear stain)	Thermo Fisher, 62249	1:1000

3.14 Knockdown of *VDR* expression using siRNA

VDR siRNA was used to knockdown *VDR* mRNA expression in JEG3 and TPC cells cultured on Matrigel™ transwells. JEG3 cells were specifically chosen for this experiment as these cells have similar morphology to cytotrophoblast cells and extra-villous cytotrophoblast cells [335], and I wanted to observe the effect of knockdown of VDR on these cells.

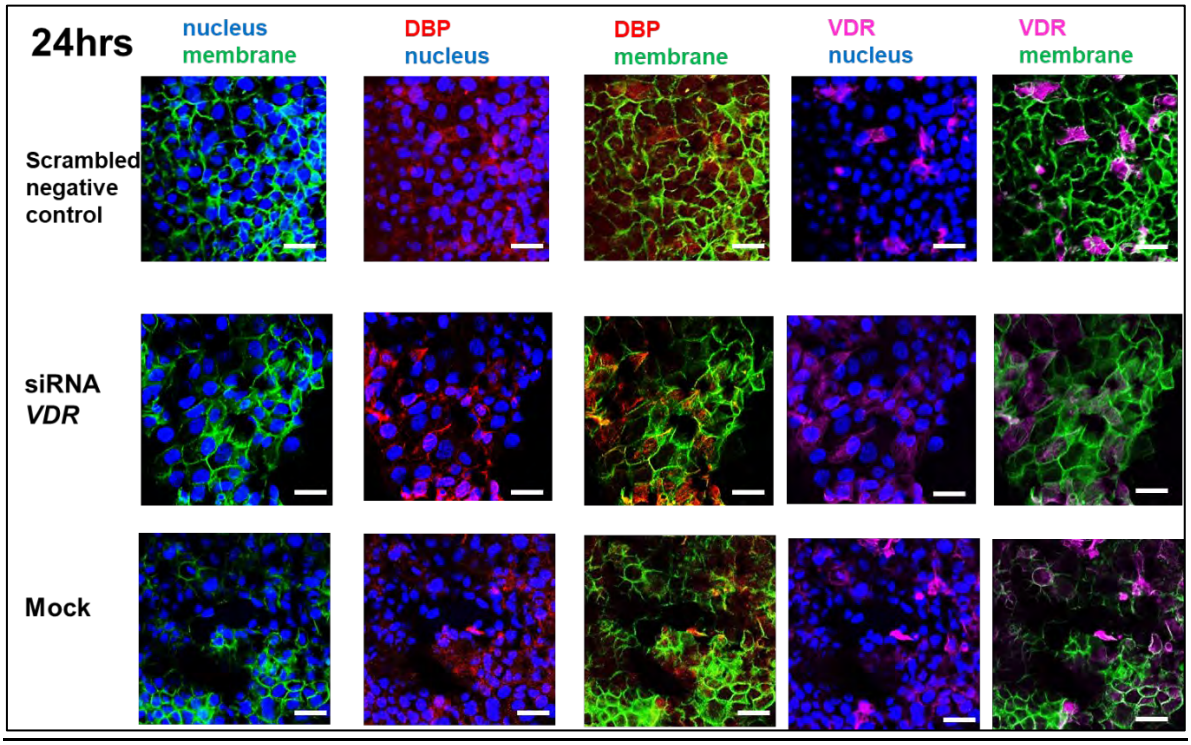
ON-TARGETplus Human *VDR* (7421 siRNA, Dharmacon, L-003448-00-0010) was used for *VDR* knockdown at a concentration of 100 nM. The efficacy of siRNA knockdown was tested after 24, 48 and 72 hours of culture (see **Figure 3.13**). 48 hours were selected because it showed maximum VDR knockdown and less cell death compared to 72 hours. A scrambled sequence siRNA (Ambion, 4390843) was used at a similar concentration of 100 nM as a negative control. As an additional control, mock-

transfected cells were treated with only Optimem reduced serum media (Gibco ThermoFisher, 31985070) and Lipofectamine RNAiMax transfection reagent (ThermoFisher, 13778075) only. Transfections were performed in transwells (24 wells) coated with Matrigel^{TM}.

Preparation of the transfection reagents was carried out as follows (**Table 3.9**). In an Eppendorf, 250 µL of Opti-MEM reduced serum media and 6 µL Lipofectamine RNAiMAX were combined and incubated for 5 mins at room temperature. Following this, 2.5 µL of siRNA per well was added to the above solution and incubated for 20 mins. 500 µL of the above final solution was then added to each well (100 nM siRNA concentration, from a stock solution of 40 µM) and cell incubated for 48 hours at 37 °C, 5% CO_2. This experiment follows a similar method as shown by Costa *et al*. [380]. All transfections were performed with 10,000 cells seeded and grown on Matrigel^{TM} transwell inserts for 48 hours. Following transfection, the transfection media was replaced with respective regular cell culture media for control or experimental treatment and immunofluorescence and/or invasion assay. Each experiment was carried out in triplicate and was repeated multiple times. *VDR* knockdown with siRNA was optimised for different siRNA transfection time points (see **Figure 3.13**). The optimal concentration for *VDR* siRNA had been determined earlier by other laboratory groups (Birmingham McCabe laboratory group), thus 100 nM was used as the preferred transfection dose.

Table 3.9 Reagents used for each siRNA experiment (volume for 24 well transwell plates).

	Optimem (250 µL)	Transfection reagent (1.5 µL)	Negative siRNA 100 nM (0.625 µL)	Positive siRNA 100 nM (0.625 µL)
Mock transfection	+	+		
Scrambled Negative siRNA control	+	+	+	
siRNA product	+	+		+



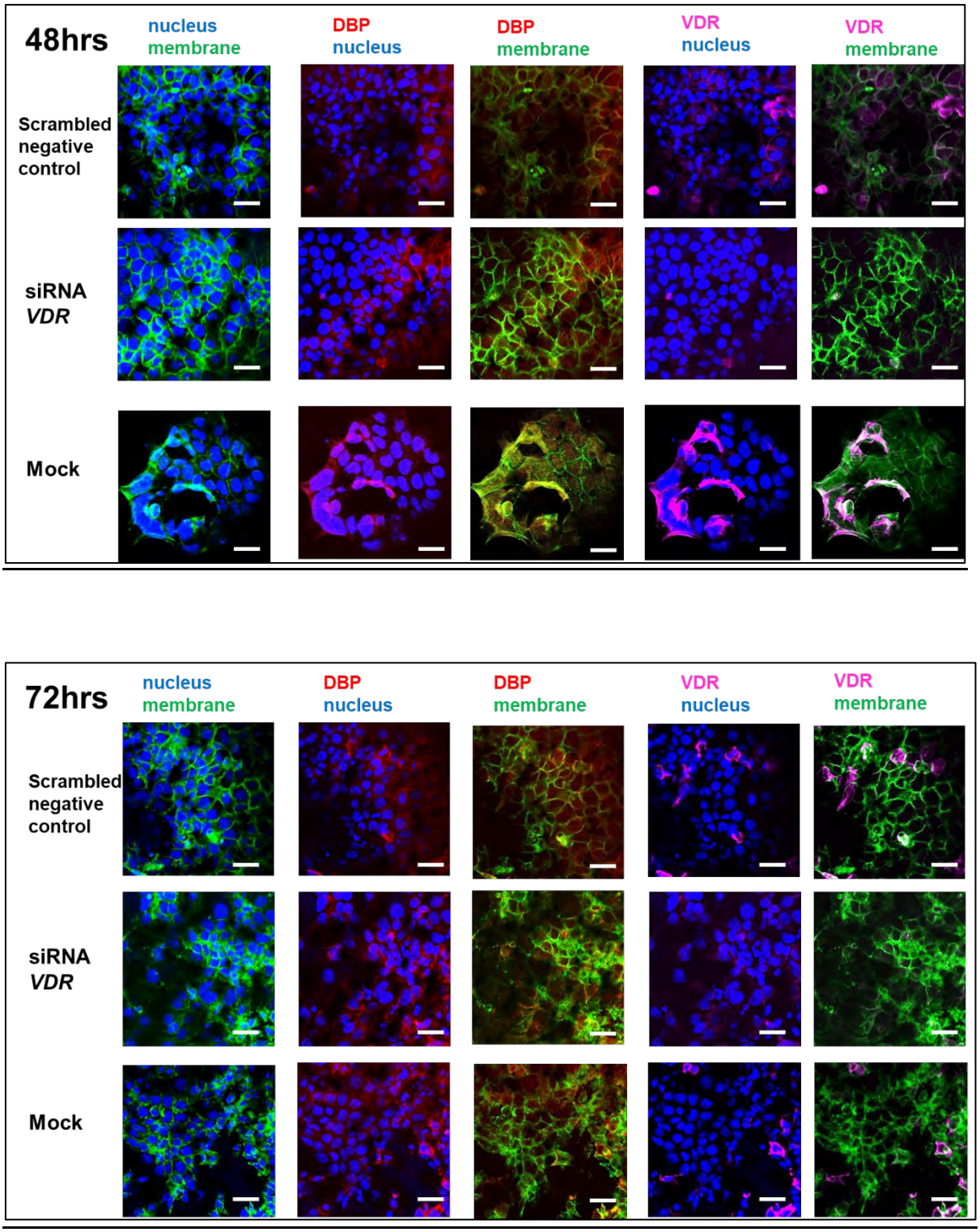


Figure 3.13 Optimisation of siRNA knockdown of *VDR*.
Immunofluorescence images of JEG3 cells. The above images show the various time-points studied to determine the optimal period for VDR knockdown, in comparison to scrambled negative control and mock treatment. Scale bar is 20 µm. Colours: red- DBP. pink- VDR, blue- Hoechst (nucleus) and green- NaKATPase (cell membrane).

3.15 Analysis of DBP concentrations in human serum

Human serum samples from pregnant women were obtained from two sources. The first set of samples were obtained as part of previous studies of maternal serum and placental/decidual concentrations of vitamin D metabolites and DBP in 1st, 2nd and 3rd trimester pregnancies (Ethics: 14/WM/1146, obtained from West Midlands - Edgbaston Research Ethics Committee) [381]. For these samples, serum and placental decidual DBP concentrations were already determined [381], and serum samples were used to prepare patient-specific JEG3 Matrigel^{TM} invasion assays. The second set of samples were obtained from Cork, Ireland, as part of a study to assess vitamin D metabolite concentrations in serum and urine from pregnant women at 1st trimester of pregnancy, 50% of whom went on to develop pre-eclampsia [382]. For these samples, vitamin D related proteins, but no DBP serum measurements, were available. Thus, analysis of serum DBP levels was carried out for these 1st trimester samples.

An ELISA (enzyme-linked immunosorbent assay) Kit (K2314, Immundiagnostik, Bensheim) was used to quantify serum concentrations of DBP as previously reported [381]. Briefly, before use, each assay-well from the 96 well plate was washed x5 with wash buffer and any residual buffer removed. Serum samples were diluted to 1:40,000 (according to manufacturer's instructions) with the sample buffer provided by the manufacturer and then plated at 100 µL per well on the pre-coated ELISA plate, with a separate row of wells for standards of known DBP concentration. This was followed by covering up of the plate with aluminium foil and incubation at room temperature for 1 hour on a shaker with 550 rpm (rotations per minute) and an orbit of 2 mm. The samples were then washed with the wash buffer (as provided by the manufacturer) x5.

This was followed by firmly tapping the plate on tissue paper to ensure the removal of any residual buffer from the wells.

100 µL of the diluted conjugate solution (100 µL conjugate solution provided + 10 mL of diluted wash buffer) was added to each well according to manufacturer's instructions. This was then incubated for 30 mins at room temperature with aluminium foil on the plate with shaking at 550 rpm and an orbit of 2 mm. The contents were then discarded following the previous step and the wells were washed with wash buffer x5. The plate was once more tapped firmly on a tissue paper to remove wash buffer from the wells. This was followed by the addition of 100 µL of substrate solution (provided by the manufacturer). The plate was kept in the dark at room temperature until the blue colour development on the standards (15 mins). "STOP" solution (as provided by the manufacturer) was added immediately to each well and mixed with a pipette.

Following the development of colour, absorption in each well of the ELISA plate was then quantified using an ELISA plate reader (SpectraMax ABS, Molecular devices, San Jose, California) at 620 nm and 405 nm (OD value). Manufacturer's instructions suggest reading the samples at 2 OD values to normalise the OD values obtained for the readings for all the unknown samples. This helps to subtract any background reading for the samples. For each sample, OD values obtained at 620 nm was subtracted from the 405 nm values (reference value). This was then plotted on a graph according to the known concentration of the given standards (**Figure 3.14**). A similar method was also done for all the unknown samples. From the resultant OD value of each sample, DBP concentrations of each human serum sample were obtained from

the ELISA standard curve using the following Excel formula "=trend (known x, known y, known OD value)". The resulting concentration was then multiplied by 40,000 (dilution factor) to get the final DBP concentration in ng/mL. This value was again divided by 1000 to get a concentration in mg/L.

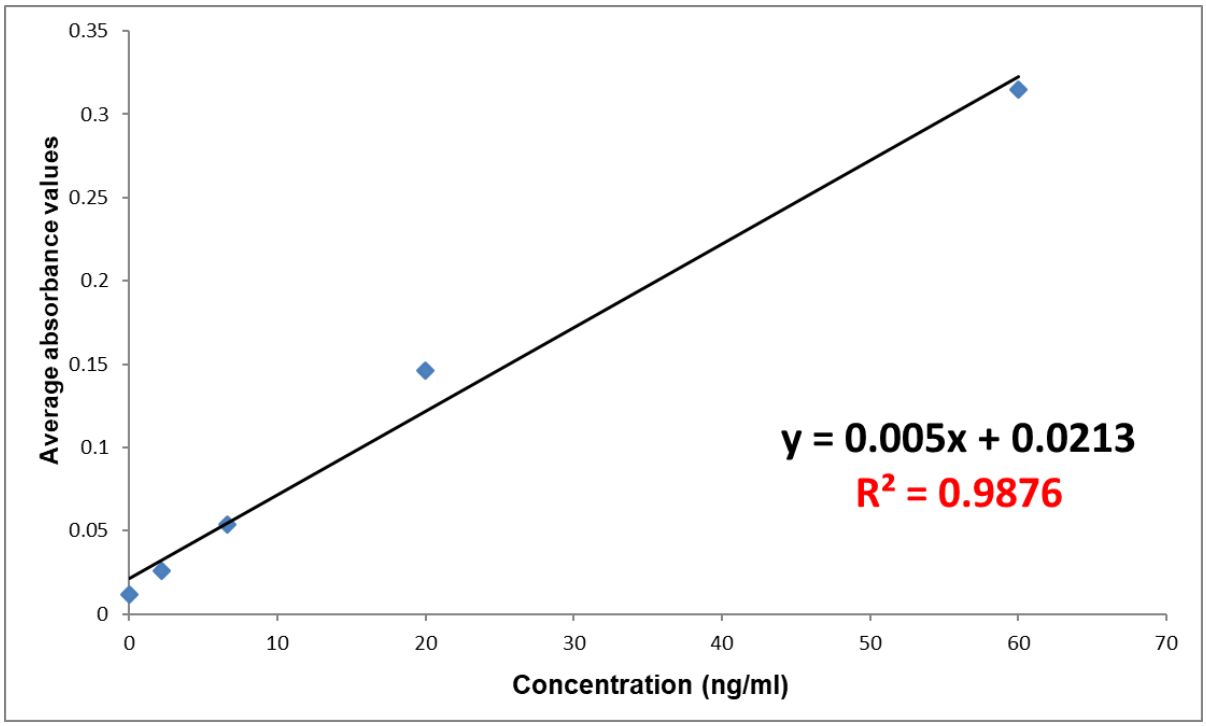


Figure 3.14 Standard curve of OD values with manufacturer DBP standards. Individual values (blue dots) shown are the average of the triplicate values from each sample. R^2 value was 0.9876. This graph was used to calculate DBP concentrations from unknown samples as outlined above.

3.16 Statistics

All experiments were carried out in duplicate or triplicate and repeated multiple times as indicated. For two data sets, unpaired student's *t*-test (for parametric data) and Mann-Whitney U-test (for non-parametric data) were used to determine mean values with 95% confidence interval. For three or more data sets, one-way ANOVA was

preferred. The Dunn-Sidak posthoc test was used for statistically significant One-way ANOVA test results, for comparing means between groups. Research shows that normality (Gaussian) distribution analysis is unproductive [383, 384], thus for three or more data sets, Gaussian distribution analysis was not done. Software used for statistical analysis was GraphPad PRISM (Version 8.3.1, La Jolla, CA). A *p*-value of <0.05 or lower was considered statistically significant.

Chapter 4: RESULTS

4.1 Expression of components of the vitamin D system in human placental tissue

4.1.1 Introduction

A key observation linking vitamin D with pregnancy is that various components of the vitamin D metabolism and signalling system are expressed in the maternal decidual and foetal trophoblast tissues that make up the placenta [385-388]. This includes the intracellular vitamin D receptor (VDR) for 1,25-dihydroxyvitamin D (1,25D), the 1,25D/VDR-inducible catabolic feedback control enzyme CYP24A1, the vitamin D activation enzyme CYP27B1, and the vitamin D binding protein (DBP) described in greater detail in the introduction to the thesis. The overall objective of this PhD project was to investigate the role of these vitamin D system components in modulating the function of foetal trophoblast cells. Therefore, before initiating *in vitro* cell culture studies, initial work was carried out to determine the expression of key factors, in trophoblast tissue from human placentas. Tissue from 1st trimester and 3rd trimester placentas was sectioned and analysed by immunohistochemistry for expression of VDR, CYP24A1 and CYP27B1 protein.

4.1.2 Immunohistochemical analysis of components of the vitamin D system in human placenta

For this study, 1st and 3rd trimester human placental tissues were wax blocked and immunohistochemistry was performed on these tissues to assess levels of expression and specific histological location of VDR, CYP27B1 and CYP24A1 within villous trophoblast tissues. Expression of these proteins was compared with a positive control protein, S100P, which is known to be abundantly expressed in placental tissues [389]. All sections were compared with their respective negative controls (**Figure 4.1**). Total

of 5 images was obtained per sample, and the image shown are representative images.

Immunohistochemical analysis for each target protein is shown in **Figure 4.1**. There was a strong expression of VDR and CYP27B1 but no CYP24A1 in 1st trimester. In 3rd trimester all three proteins were strongly expressed. Colocalisation of VDR and CYP27B1 (in 1st trimester placenta and 3rd trimester placenta), and CYP24A1 (only in 3rd trimester), were observed in the syncytiotrophoblast. Interestingly, CYP24A1 was observed to show reduced expression in 1st trimester compared to 3rd trimester.

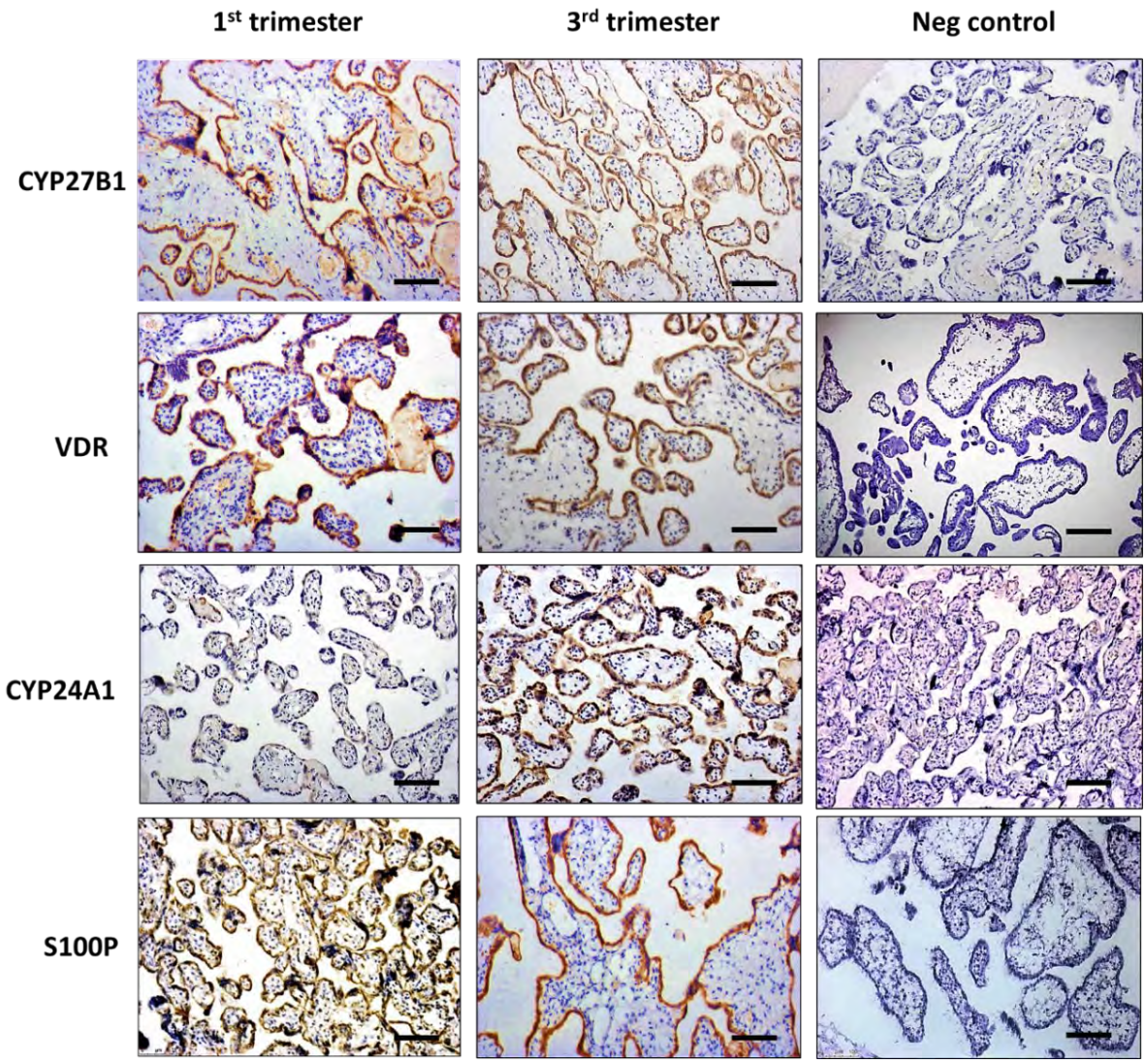


Figure 4.1 Expression of vitamin D system proteins in 1st trimester and 3rd trimester placental tissue.
Sample images are compared to a positive control (S100P) and negative control. The dark brown colour shows the localisation of CYP27B1, VDR, CYP24A1 and S100P in placental villous tissues. n=5, the above images are representative images only. Each image was taken with 10x magnification. The scale bar is 50 µm.

4.1.3 Discussion

Analyses in this part of the thesis were carried out to understand the expression of components of the vitamin D system in trophoblasts in both early and later pregnancy. Results showed colocalisation of VDR and CYP27B1 in the villous area of the trophoblast of 1st trimester (early pregnancy) and 3rd trimester (term) placenta. Both

VDR and CYP27B1 were primarily expressed in the villous membrane (early pregnancy) and around foetal vessels (late pregnancy). Similar findings have also been reported previously [387]. The significant presence of CYP27B1 and VDR in early pregnancy suggests that vitamin D may be involved in early events of pregnancy such as conception, implantation, and subsequent progression of placental development and pregnancy [354, 387, 390]. However, CYP24A1 was not observed in the placenta of early pregnancy, as compared to the placenta of later stage pregnancy. This may reflect an increased demand for the active form of vitamin D (1,25D) during the early stage of pregnancy, so that low levels of CYP24A1 in trophoblasts will minimise catabolism of 1,25D via this enzyme and maintain higher levels of 1,25D for the regulation of early trophoblast function. Previous studies reported that *CYP24A1* was undetectable in trophoblast cells, which correlates with non-mutational gene inactivation (or epigenetic silencing of a gene) due to methylation of the *CYP24A1* gene in human placenta [391]. Again, the suggestion from this observation was that trophoblasts are a crucial target for 1,25D and therefore these cells minimise 24-hydroxylation of 1,25D to 1,24,25-trihydroxyvitamin D (1,24,25D). The absence of an important 1,25D catabolic enzyme (CYP24A1), suggest that vitamin D operates in a non-conventional way in the trophoblasts, as compared to the other VDR-expressing cells, where CYP24A1 expression is abundant and sensitively induced by 1,25D in a VDR-dependent manner [107].

As previously reported, both VDR and CYP27B1 are prominently expressed in placental tissues across pregnancy [386-388]. In some studies 1st trimester placental CYP27B1 expression has been reported to be more pronounced than 3rd trimester

placental expression, suggesting that conversion of 25D to 1,25D is an important feature of early trophoblast function [388]. The presence of both CYP27B1 and VDR in the 1st and 3rd trimester placental tissue in the current study, suggests that activation of 25D to 1,25D and subsequent VDR responses occurs across pregnancy. However, the higher level of CYP24A1 expression in 3rd trimester samples may indicate that catabolic inaction of 1,25D predominates later in pregnancy. Conversely, the higher expression of CYP24A1 later in pregnancy may reflect enhanced sensitivity of trophoblast cells to 1,25D, resulting in increased induction of *CYP24A1* gene expression by VDR-dependent mechanisms. Previous studies have also confirmed the expression of VDR throughout pregnancy, with significantly higher expression during the later stages of pregnancy [392]. This study also showed that VDR expression was primarily concentrated in glycogen rich cells and labyrinth cells of the placenta [392]. It is also important to recognise that although immunohistochemistry presented here suggests that trophoblast cells express an intact vitamin D system capable of synthesising and responding to 1,25D, it is also possible that 1,25D is generated by adjacent decidual cells. Previous studies have suggested that the decidua is the major reservoir of 1,25D in the placenta relative to trophoblast tissue [381].

Based on these observations, I then progressed to the next phase of the project, which was to investigate the functional impact of 1,25D on trophoblast cells. This involved using three different types of trophoblast cell lines, cultured *in vitro* using different methodologies.

4.2: Effects of vitamin D in trophoblast cells

4.2.1 Introduction

Trophoblast cell lines have been a traditional method for studying the cellular and molecular mechanisms associated with the healthy development of the placenta [393], and adverse events in pregnancy [394]. The choriocarcinoma origin of some trophoblast cell lines such as JEG3 and BeWo means that trophoblast cells have been used as an *in vitro* model for studying trophoblast proliferation, differentiation and apoptosis, in a similar fashion to other cancer cell line [395]. Trophoblast cells are similar to cancer cells because both can invade and migrate through tissue, notably invading and migrating through the maternal decidua to facilitate the development of the healthy placenta [396]. Choriocarcinoma cell lines, such as JEG3 and BeWo possess invasive and migratory properties similar to placental trophoblast cells *in vivo* [397-400], and primary placental cells and trophoblast cell lines share a similarity in gene and protein expression for factors such as matrix metalloproteinases (MMP), tissue inhibitor of metalloproteinases (TIMP) that are involved in modulating matrix invasion.

Not all trophoblastic cell lines are neoplastic. The immortalised non-tumour extravillous cytotrophoblast (EVT) cell line, HTR8 is an effective model for studying trophoblast function [401], particularly the role of trophoblast cells in the formation of placental blood vessels [402]. This latter study also highlighted the differential patterns of gene expression when HTR8 cells were cultured on conventional plastic surfaces relative to culture on an artificial matrix to mimic the environment of trophoblast cells *in vivo*, with 481 genes being differentially expressed when HTR8 cells were grown on matrix, as compared to conventional plasticware cultures of HTR8 [402]. In this case, the artificial

matrix used to mimic decidua was Matrigel™ which has also been used as an invasion model for other trophoblast cells [403, 404]. Previous studies by our group have shown that the active form of vitamin D, 1,25D, can promote the invasion of Matrigel™ by primary cultures of EVT [317]. This process involved induction of MMPs but the mechanism by which 1,25D achieved this effect was unclear. In particular, the role of the intracellular vitamin D receptor (VDR) in coordinating trophoblast responses to 1,25D is uncertain [317]. To date, this is the only study that has investigated the effects of 1,25D and the vitamin D system on trophoblast cells grown on matrix. Other studies have explored the ability of 1,25D to regulate matrix invasion by tumour cells [405, 406]. In each case, treatment with 1,25D suppressed tumour cell invasion, consistent with the general anticancer effects of vitamin D [407]. Therefore, when cultured on matrix, vitamin D exerts completely different effects on trophoblast compared to other cells. With this in mind, the overall objective of the next part of my PhD project was to determine how vitamin D promotes invasive responses in trophoblast cells, and how this may influence the development of the healthy placenta. Specific aims were to:

1) Characterise expression and function of the vitamin D system in different trophoblast cell models *in vitro*

2) Characterise expression and function of the vitamin D system in trophoblast cell models *in vitro* when cultured on plastic or Matrigel™

Initial experiments involved multiple cell lines to determine the consistency of 1,25D function, with subsequent experiments focused on fewer cell models.

4.2.2 Expression of the vitamin D system in trophoblast cells

Initial studies were carried out using JEG3, BeWo and TPC cells cultured in a conventional fashion on plasticware. Results in **Figure 4.2A** shows that all three cells expressed mRNA for *VDR,* but no mRNA for DBP *(GC)*, only HTR8 cells expressed *CYP24A1* and not the other trophoblast cells. There was extremely low *CYP24A1* expression in all 3 trophoblast cells. Treatment with 1,25D did not affect *VDR* mRNA expression but resulted in a significant induction of *CYP24A1* in TPC cells. This effect was not observed in JEG3, BeWo and HTR8 cells even though these cells expressed low-level *VDR.*

Immunofluorescence analysis of JEG3 and TPC cells confirmed the expression of VDR in both cell lines, with higher levels and more nuclear localisation observed in TPC cells (**Figure 4.2B**). DBP was not detected in either cell line. Quantification of VDR expression in immunofluorescence images was carried out by measuring Corrected Total Cell Fluorescence (CTCF): “area of fluorescence colour” subtracted from “Integrated density”, then multiplied by “average background area”. This analysis showed that VDR protein levels were significantly higher (arbitrary units) in untreated JEG3 cells (6.9×10^6, 95% CI = 5.8×10^6 and 8.1×10^6) relative to TPC cells (2.9×10^6, 95% CI = 2.5×10^6 and 3.3×10^6). TPC cells also showed an increase in VDR expression following treatment with 1,25D (5.7×10^6, 95% CI= 5.1×10^6 and 6.3×10^6), whereas 1,25D suppressed expression of VDR in JEG3 cells (6.6×10^6 , 95% CI= 5.2×10^6 and 8×10^6). This is probably because of the increased surface area for VDR

expression across the cytoplasm of JEG3 cells, whereas for TPC cells, area of expression of VDR is only the nucleus.

Western blot analysis of subcellular fractions of JEG3, BeWo and TPC cells indicated that all three cell lines expressed protein for CYP24A1 in the cytoplasm but VDR protein was only detectable in the nuclear fraction of TPC cells and not the trophoblast cells (**Figure 4.3**). Similar to mRNA data, DBP protein was undetectable in all three cell lines by western blots.

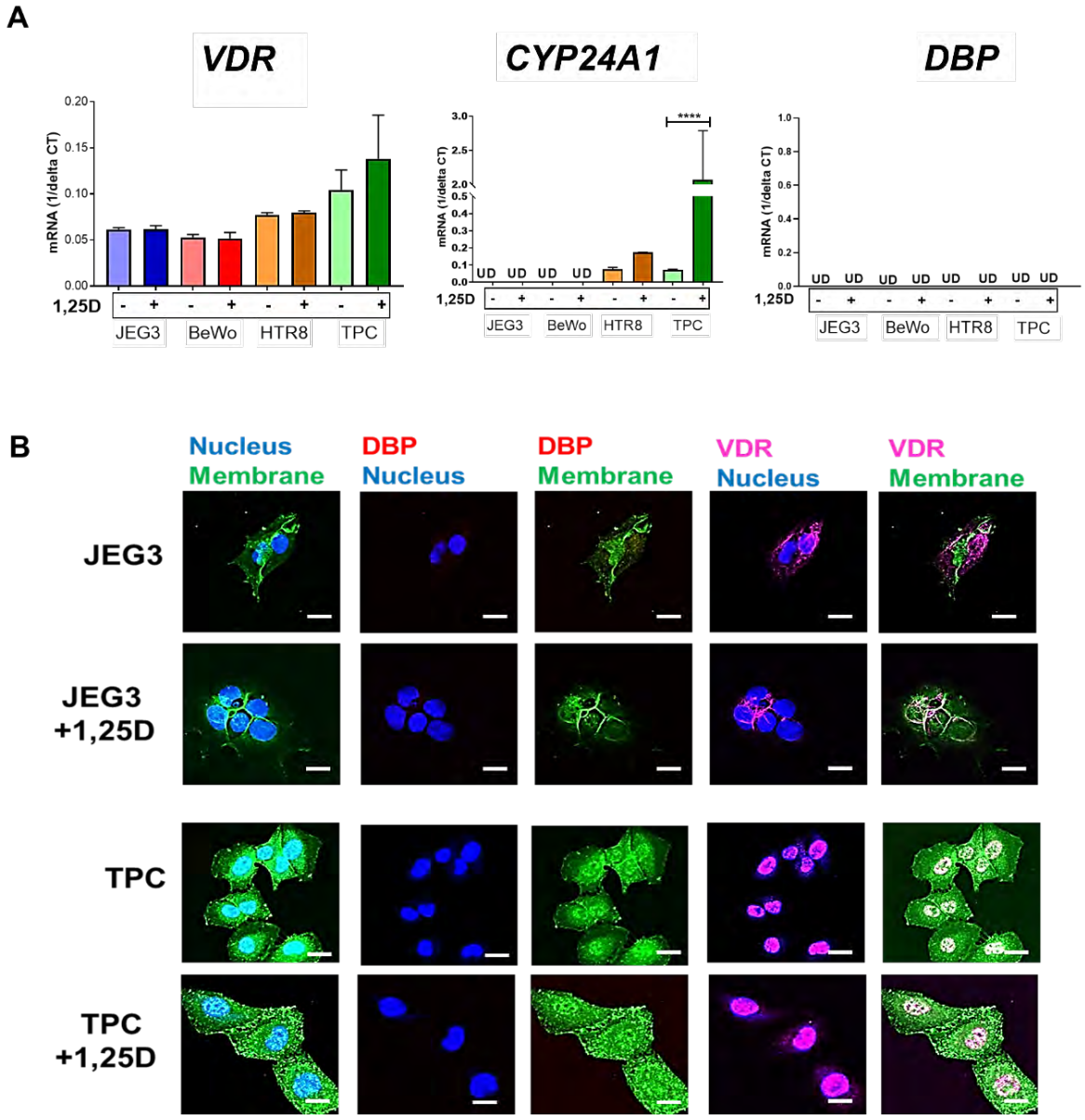


Figure 4.2 Expression of the vitamin D metabolic and signalling system in trophoblast and thyroid cells cultured on plastic.

A. Expression of mRNA for the vitamin D receptor (*VDR*), *CYP24A1* and DBP (*GC*) in JEG3, BeWo, and TPC cells cultured on plastic in the presence/absence of 1,25D (100 nM, 48 hours). Data are mean with 95% CI of 1/delta ct value for n=3 separate experiments with triplicates each time. p=**** = <0.0001. **B.** Immunofluorescence analysis of VDR (pink) and DBP (red) in JEG3 and TPC cells. Nucleus (Hoechst, blue) and membrane (NaKATPase, green) are also shown. Images were taken with 40x magnification. Scale bar is 20 µm.

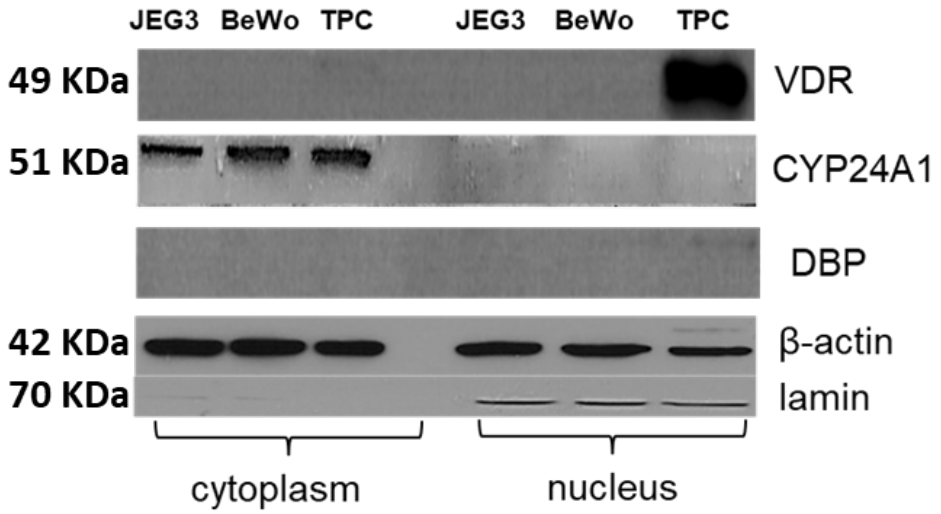


Figure 4.3 Expression of the vitamin D metabolic and signalling system proteins in trophoblast and thyroid cells cultured on plastic.
Western blot analysis of cytoplasmic and nuclear protein for VDR, CYP24A1 and DBP in JEG3, BeWo, and TPC cells cultured on plastic in the presence or absence of 1,25D (100 nM, 48 hours).

Further experiments were then carried out using cells cultured on Matrigel™. As shown in **Figure 4.6**, in JEG3, BeWo and TPC cells cultured on matrix, levels of expression of *VDR* mRNA were similar to plasticware cultures of these cells (**Figure 4.2**), and these were unaffected by treatment with 1,25D. However, unlike plasticware cultures, JEG3 and BeWo cells cultured on Matrigel™ showed expression of *DBP* mRNA, although this was at a relatively low level and was unaffected by treatment with 1,25D (**Figure 4.6A**). In a similar fashion to plasticware cultures, JEG3 and BeWo cells cultured on Matrigel™ showed no expression of *CYP24A1* mRNA, and this was not induced by treatment of these cells by 1,25D. By contrast, *CYP24A1* mRNA showed similar basal and 1,25D-induced levels in TPC cells, suggesting that VDR signalling in these cells was unaffected by matrix versus plasticware culture (**Figure 4.4A**).

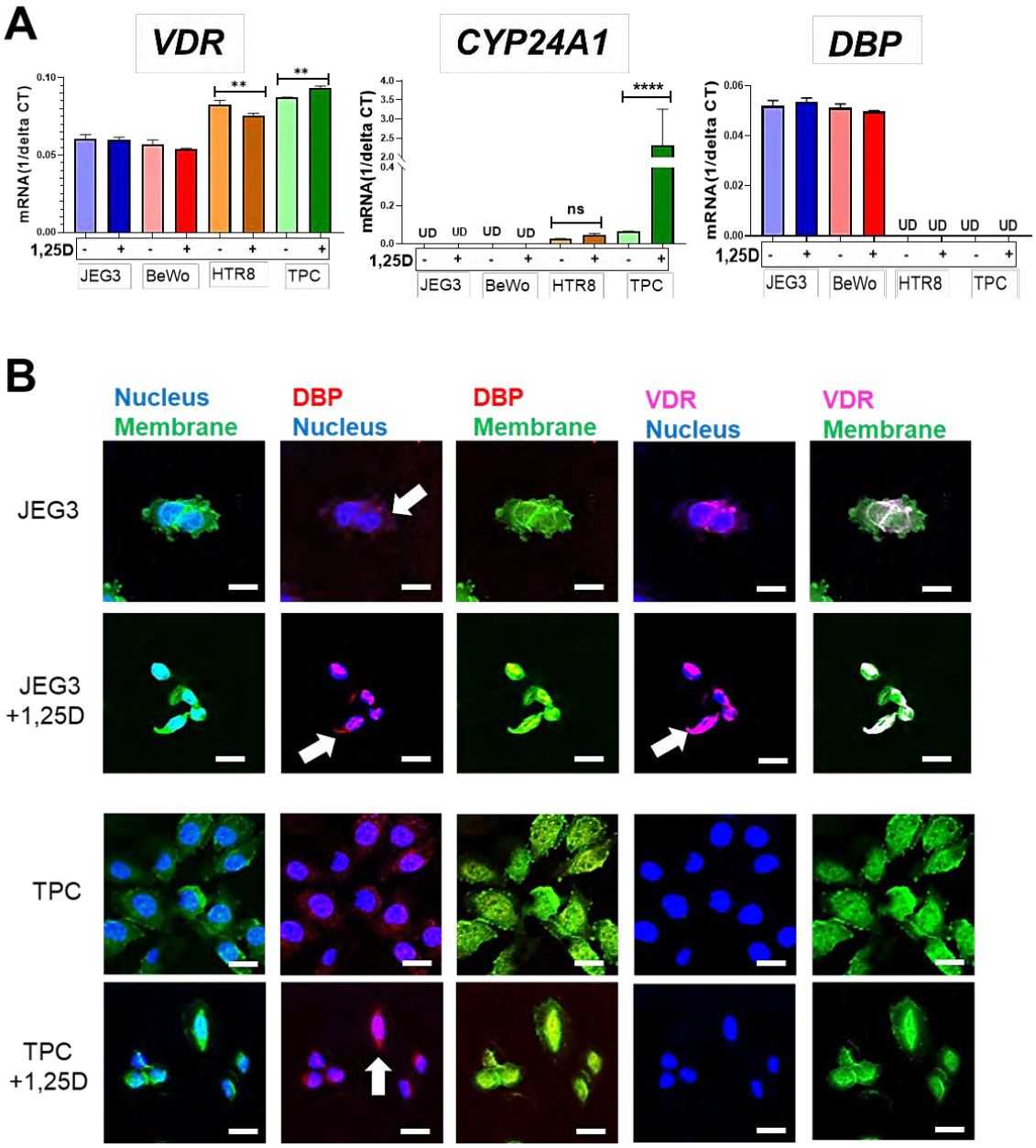


Figure 4.4 Expression of the vitamin D metabolic and signalling system in trophoblast and thyroid cells cultured on Matrigel™.
A. Expression of mRNA for *VDR*, *CYP24A1* and DBP (*GC*) in JEG3, BeWo, and TPC cells cultured on matrix in the presence/absence of 1,25D (100 nM, 48 hours). Data shows mean with 95% CI of 1/delta ct value for n=3 separate experiments with triplicates each time. p=**** = < 0.0001, ** = 0.0019 and 0.0038. **B.** Immunofluorescence analysis of VDR (pink) and DBP (red) in JEG3 and TPC cells. Nucleus (Hoechst, blue) and membrane (NaKATPase, green) are also shown. Scale bar is 20 µm. Images were taken with 40x magnification. White arrow highlights DBP staining.

Immunofluorescence analysis of untreated JEG3 cells cultured on Matrigel™ showed similar levels of VDR expression to plasticware cultures (**Figure 4.4B**). However, on matrix, VDR showed stronger nuclear localisation in JEG3 cells following treatment with 1,25D. By contrast, TPC cells cultured on matrix showed lower expression of VDR relative to plasticware-cultured TPC cells maybe because of post-translational modification of protein (**Figure 4.4B**). Distinct from the plasticware cultures, both JEG3 and TPC cells showed expression of DBP protein when cultured on Matrigel™. DBP was localised in the cytoplasm in untreated controls but with apparent nuclear localisation in cells treated with 1,25D (**Figure 4.4B**).

Analysis of other trophoblast cell lines BeWo and HTR8 showed similar patterns of VDR and DBP protein expression to JEG3 cells when cultured on Matrigel™ (**Figure 4.5**). In BeWo and HTR8, VDR was peri-nuclear in both control- and 1,25D-treated cells. In BeWo, DBP (red) was weakly expressed in controls but showed stronger nuclear localisation (pink staining) following treatment with 1,25D. In control HTR8, DBP (red) showed mainly cytoplasmic localisation but with stronger nuclear staining (pink) in 1,25D-treated HTR8 (**Figure 4.5**).

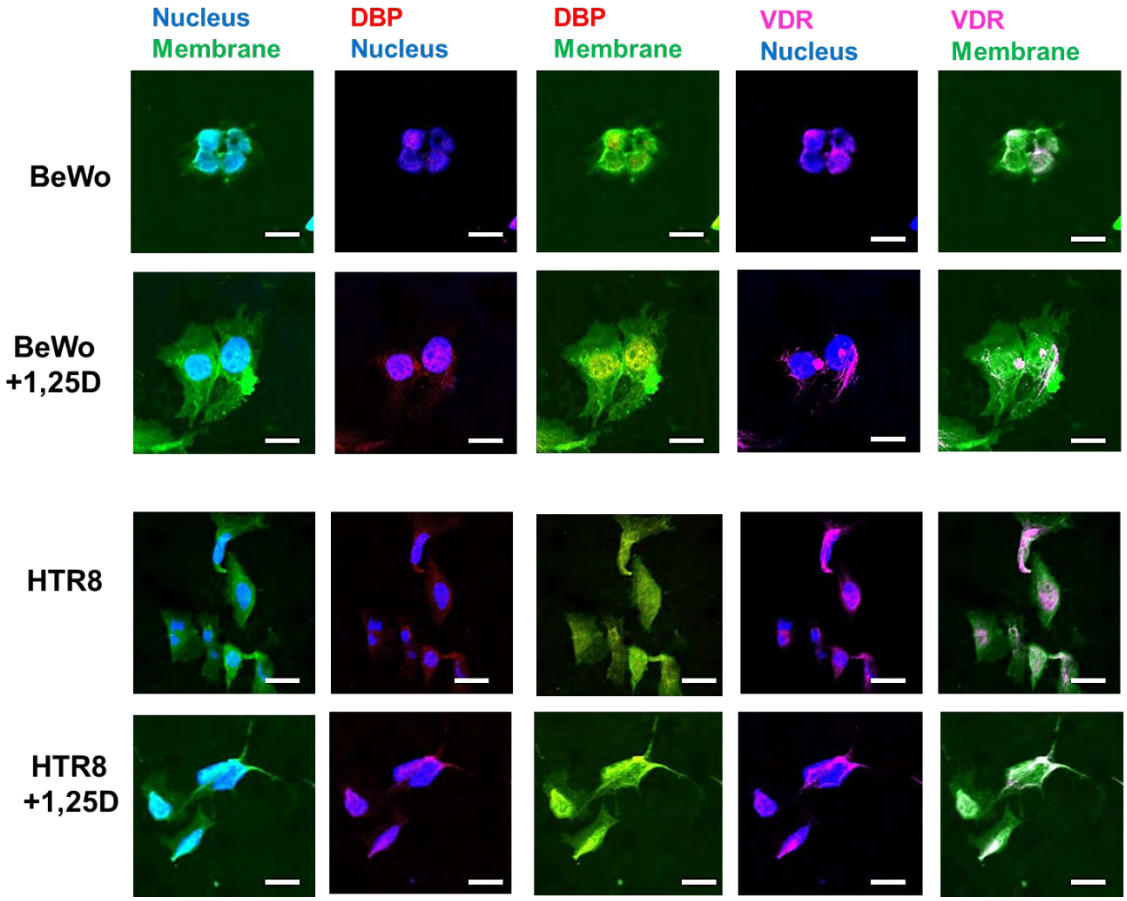


Figure 4.5 Expression of DBP and VDR in BeWo and HTR8 cells cultured on Matrigel™.
Immunofluorescence analysis of expression of protein for VDR (pink) and DBP (red) in BeWo and HTR8 cells cultured the presence or absence of 1,25D (100 nM, 48 hours. Nucleus (Hoechst, blue) and membrane (NaKATPase, green) are also shown. Scale bar is 20 µm. Images were taken with 40x magnification.

Because Matrigel™ cultures were carried out in 2% FBS-supplemented medium compared to the 10% FBS used in conventional plasticware culture, additional experiments were carried out to compare patterns of gene expression in 2% FBS and 10% FBS cultures of JEG3, BeWo, HTR8 and TPC cell. Data in **Figure 4.6** shows that *VDR* and *CYP24A1* remained unchanged in all 3 trophoblast cells (JEG3, BeWo and HTR8) in both 10% and 2% FBS environment. 1,25D treatment did not seem to have any effect on its expression. In contrast, TPC showed consistently high *CYP24A1*

following 1,25D treatment in both 10% and 2% FBS environment. Consistent with previous mRNA data (**Figure 4.4A),** a very low *DBP* mRNA expression was observed in 2% FBS environment, however, not in 10% FBS environment, suggesting a possible feedback control mechanism for DBP uptake by trophoblast cells.

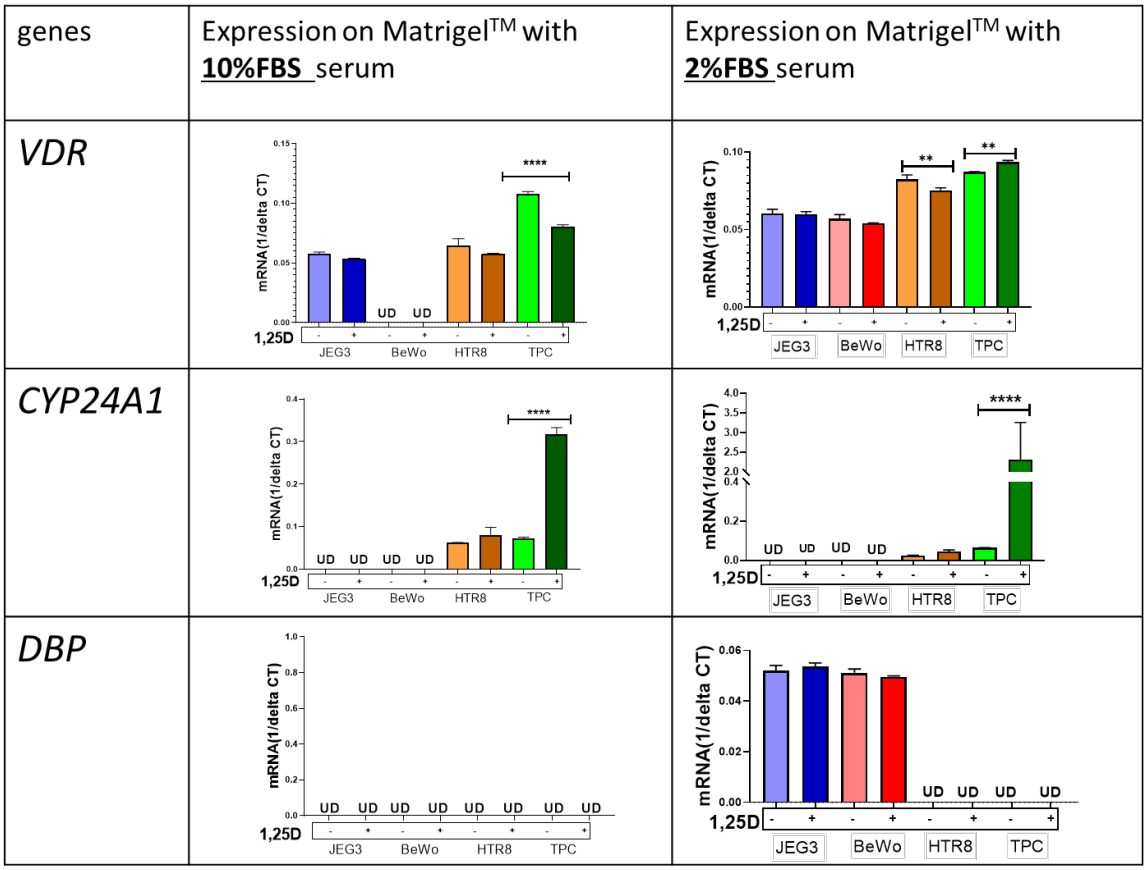


Figure 4.6 Expression of the vitamin D metabolic and signalling system in trophoblast and thyroid cells cultured on 2% FBS and 10% FBS Matrigel™. Expression of mRNA for *VDR*, *CYP24A1* and DBP (*GC*) in JEG3, BeWo, HTR8 and TPC cells cultured on matrix in the presence or absence of 1,25D (100 nM, 48 hours). Data for mRNA expression are mean with 95% CI of 1/delta ct value for n=3 separate experiments with triplicates each time. p= **** = < 0.0001, ** = 0.004 and 0.005.

Quantification of VDR and DBP immunofluorescence in 2% FBS Matrigel™ cultures showed similar levels of VDR in all three trophoblast cell lines in the presence or absence of 1,25D (**Figure 4.7**). Untreated TPC cells showed lower levels of VDR

relative to trophoblast cells, but this increased significantly following treatment with 1,25D. Expression of DBP was similar for all untreated trophoblast cells, with TPC cells showing lower baseline expression of DBP. However, following treatment with 1,25D, all cells showed a significant increase in levels of DBP expression.

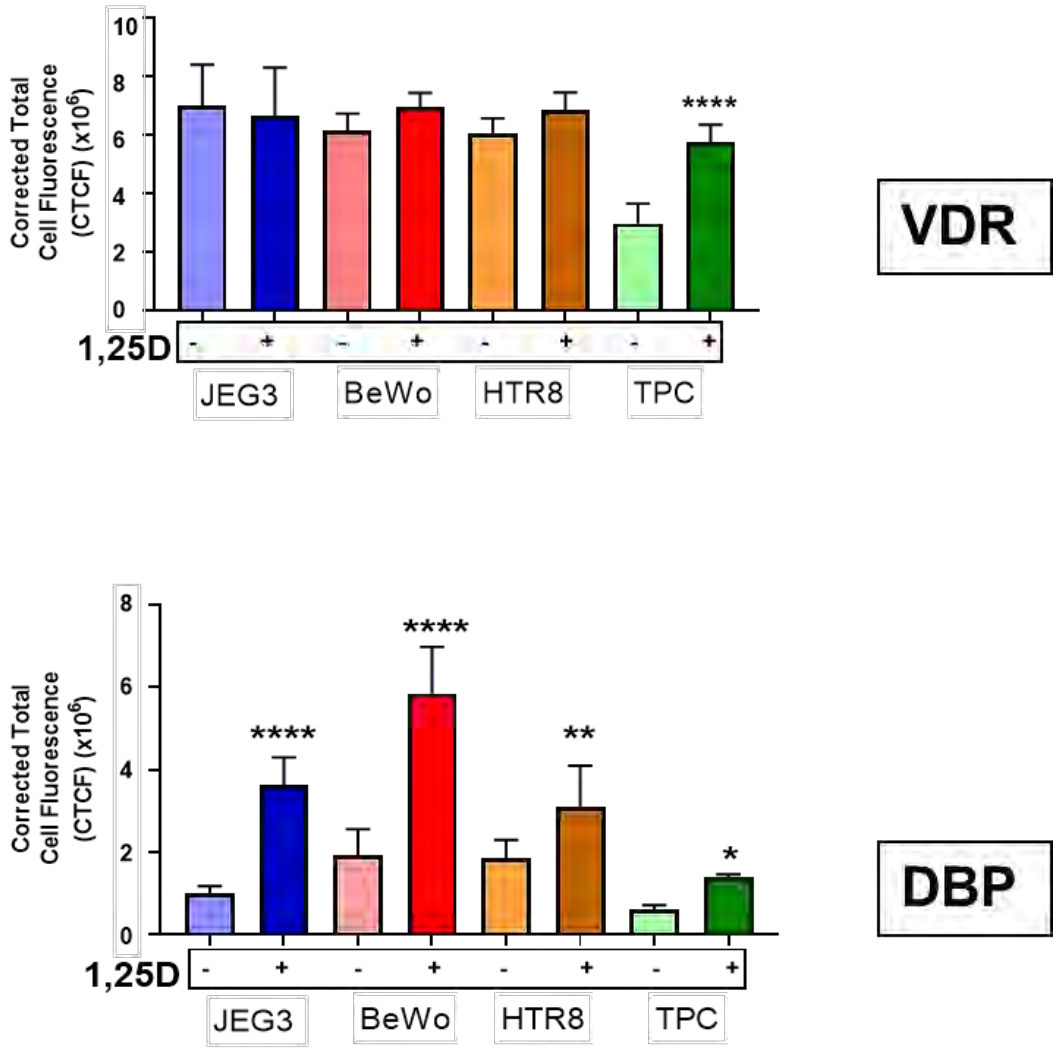


Figure 4.7 Expression of VDR and DBP protein in Matrigel™ cultured cells. Quantification of VDR expression from immunofluorescence images. Data shows mean with 95% CI. p= statistically different from control =* = 0.0258, ** = 0.0063 and **** = <0.0001. n=3 separate experiments with duplicates images each time.

Summary

Data in the initial experiments indicated that trophoblast cells cultured on matrix express both DBP and VDR, but do not appear to exhibit conventional 1,25D-mediated induction of *CYP24A1*. The next series of experiments aimed to determine what, if any, the functional response of trophoblast cells is to 1,25D.

4.2.3 Effects of 1,25D on proliferation and migration

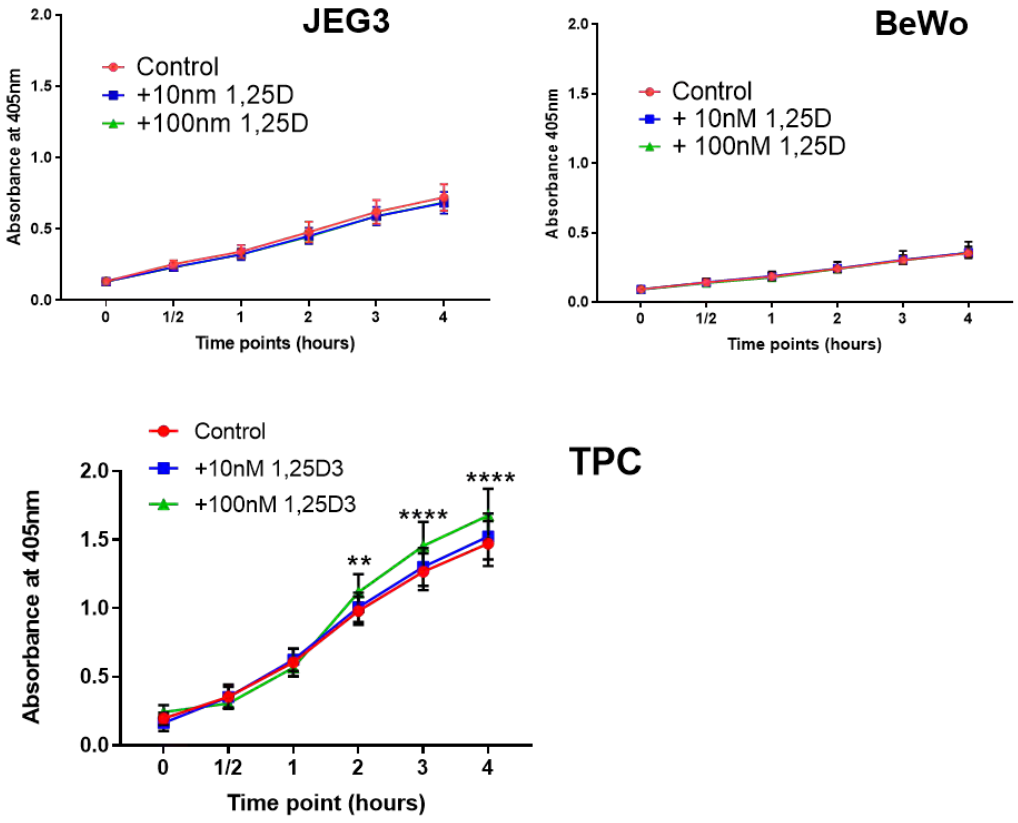
Trophoblast cell function can be broadly classified into proliferation, migration and invasion, with each significant for placental formation and attachment. The next stage of my project aimed to assess the impact of 1,25D on these functions. In the first instance, cell proliferation and migration were assessed using JEG3, BeWo, HTR8 and TPC cells cultured on plastic. Proliferation and invasion were then assessed on cells cultured on Matrigel™.

Cell proliferation

For analysis of cell proliferation, 2 tests were performed, MTT (abbreviation for the dye compound 3-(4,5-Dimethylthiazol-2-yl)-pr-2,5-diphenyltetrazolium bromide) analysis of cell metabolism and analysis of BrdU (Bromodeoxyuridine 5-bromo-2'-deoxyuridine) incorporation into cellular DNA of viable cells. In JEG3, BeWo cells cultured on plastic, 1,25D had no effect on proliferation levels at any time point. However, TPC cells showed increased levels of proliferation following treatment with 100 nM 1,25D, but 10 nM 1,25D did not affect TPC proliferation (**Figure 4.8**). Similar results were obtained using BrdU incorporation to assess cell proliferation on plastic, with only TPC cells showing an anti-proliferative response to treatment with 1,25D (**Figure 4.9**). Further proliferation analysis was carried out using cells cultured on Matrigel™. In this instance, the cells were cultured in 10% FBS-supplemented medium to be consistent with cells cultured on plastic, and proliferation was analysed using BrdU incorporation. Again, only TPC cells showed an anti-proliferative response to 1,25D at both 48 hours and 96 hours (**Figure 4.10A**). However, with 2% FBS culture media, there was no change observed in all 4 cells with and without 1,25D treatment at 48 hours (**Figure**

4.10B). These data indicated that in the same way that TPC cells showed potent induction of *CYP24A1* in responses to treatment with 1,25D, they also showed inhibition of cell proliferation. By contrast, none of the trophoblast cells showed induction of *CYP24A1* or suppression of cell proliferation with 1,25D treatment.

A



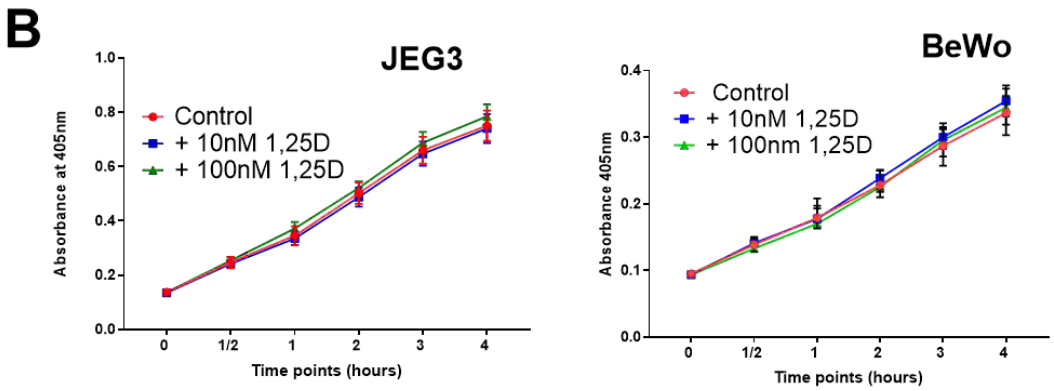


Figure 4.8 Effect of different concentrations of 1,25D on cells grown on plasticware with low FBS (2%) and high FBS (10%) media.
A. Effects of 10 nM and 100 nM 1,25D on trophoblast and thyroid cell proliferation on plasticware cultures with **10% FBS** culture media. **B.** Effects of 10 nM and 100 nM 1,25D on trophoblast proliferation on plasticware cultures with **2% FBS** culture media. Quantification of MTT was done in JEG3, BeWo, HTR8 and TPC cells cultured on plastic at 0 hours, 30 mins, 1 hr, 2 hr, and 3 hr of culture. Data are the mean with 95% CI for n=3 separate experiments with triplicates each time. p= statistically significant data compared to its control=****=<0.0001 and ** = 0.002.

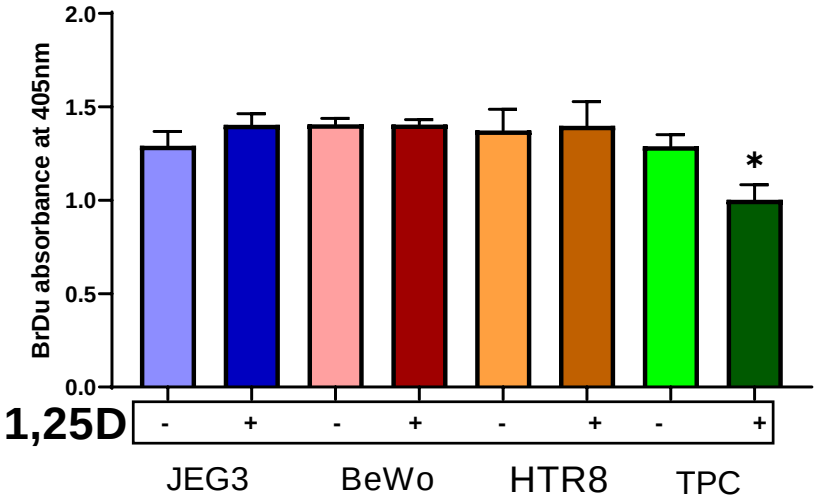


Figure 4.9 Effects of 100nM 1,25D on trophoblast and thyroid cell proliferation for plasticware cultures with 10% FBS culture media.
BrdU incorporation in JEG3, BeWo, HTR8 and TPC cells cultured on plastic at 48 hours of culture. Data shows mean and 95 CI for n=3 separate experiments with triplicates each time. The statistical difference is shown as p=*= 0.033.

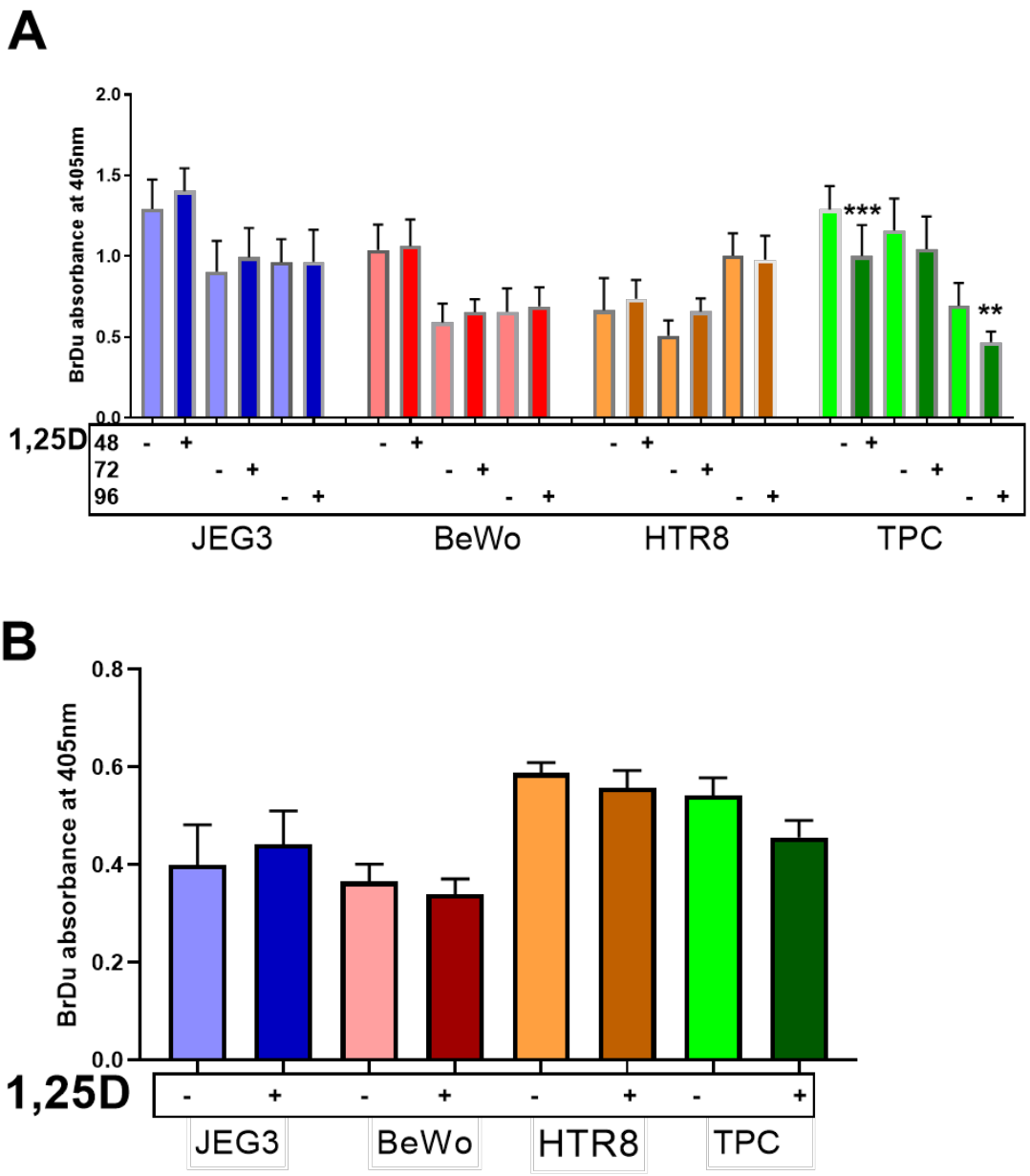


Figure 4.10 Effect of 1,25D (100 nM) on cells grown on matrix with low FBS (2%) and high FBS (10%) culture media.
A. BrdU incorporation in JEG3, BeWo, HTR8 and TPC cells cultured on Matrigel™ at 48, 72 and 96 hours with **10% FBS** culture media. **B.** BrdU incorporation in JEG3, BeWo, HTR8 and TPC cells cultured on Matrigel™ at 48 hours with **2% FBS** culture media. Data shows mean and 95% CI for n=3 with triplicates each time. Statistical difference shown as compared to respective controls, p= *** = 0.0008, ** = 0.0054.

Cell migration

To determine if 1,25D can modulate the ability of trophoblast and TPC cells to migrate, assays were carried out to determine the rate of wound closure in monolayers of each cell type cultured on plastic. Data in **Figure 4.11** showed that each of the trophoblast cells demonstrated similar migration rates when cultured, and this was unaffected by treatment with 1,25D (100 nM, 48 hours). In contrast, TPC cells showed a higher baseline rate of migration, but this was profoundly suppressed by treatment with 1,25D (**Figure 4.11**).

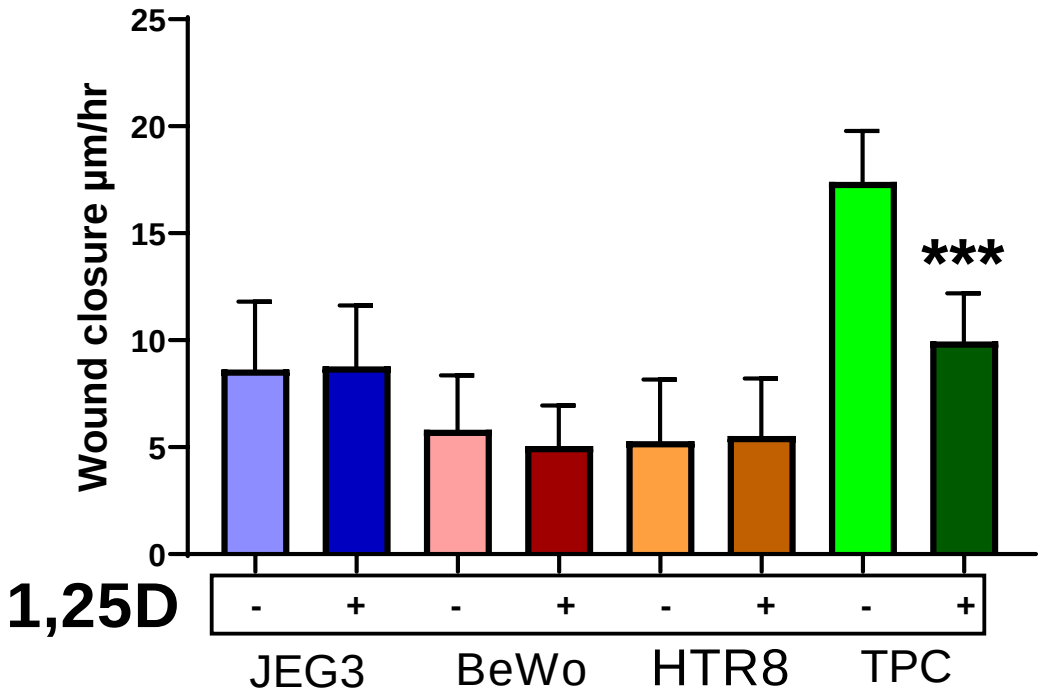


Figure 4.11 Effects of 1,25D on trophoblast and thyroid cell migration for plasticware cultures.
Rates (µm/hr) of wound closure for monolayers of JEG3, BeWo, HTR8 and TPC cells cultured on plastic for 48 in the absence (-) or presence (+) of 1,25D (100 nM). Data shows mean and 95% CI for n=3 separate experiments with triplicates each time. p= statistically different from controls= *** = 0.0002.

4.2.4 Effects of 1,25D on matrix cell invasion

Analysis of matrix invasion by trophoblast and thyroid cells was performed using transwells with 0.8-micron pores and coated with growth factor reduced Matrigel™, which allowed cells to invade through the matrix to the other side of the transwell membrane for detection and quantification. Cells were cultured on matrix with 2% FBS-supplemented media and, to facilitate chemoattraction of cells, the media in the bottom of the transwell was supplemented with 10% FBS.

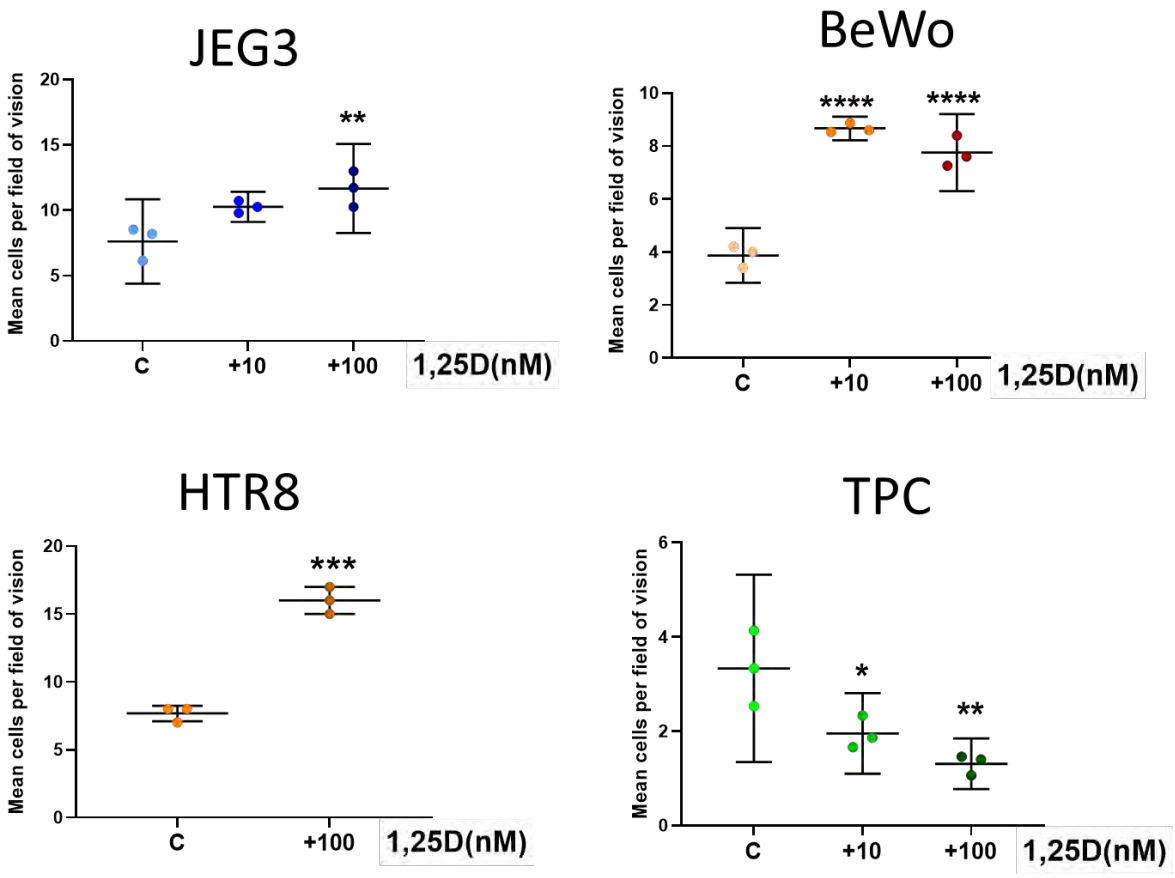


Figure 4.12 Effect of 1,25D on matrix invasion by trophoblast and thyroid cells. Number of JEG3, BeWo, HTR8 and TPC cells invading Matrigel™ in the absence (control (C)) or presence of 1,25D (10/100 nM, 48 hours). Data shows mean and 95% CI for multiple fields of vision from n= 3 separate experiments with triplicates each time for all 4 cells. Each dot represents a replicate field of vision of each well. p=statistically different from control = ****= <0.0001, ***= 0.0003, ** p=0.0012, *= 0.01**.**

Data in **Figure 4.12** indicated that consistent with previous studies of cancer cells on Matrigel™, TPC cells showed significantly decreased matrix invasion when treated with 1,25D. By contrast, all three trophoblast cell lines showed significantly *increased* Matrigel™ invasion following treatment with 1,25D, even at the lower concentration of 10 nM (**Figure 4.12**). These studies were carried out by blinded manual counting of invaded cells on Matrigel™. However, to validate these data, further studies were carried out to quantify the number of matrix-invading cells (see **Section 3.6.1**). Data using this method confirmed that 1,25D potently suppressed matrix invasion by TPC but stimulated invasion by JEG3, BeWo and HTR8 cells (**Figure 4.13)**.

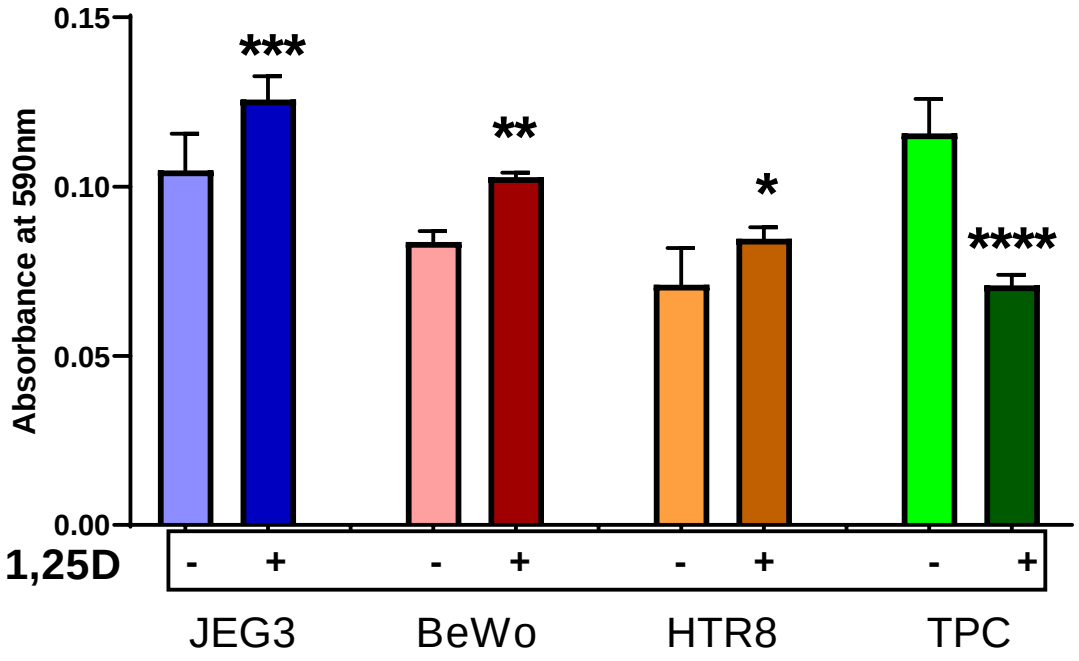


Figure 4.13 Effect of 1,25D on matrix invasion by trophoblast and thyroid cells. Quantification of Crystal Violet-stained JEG3, BeWo, HTR8 and TPC cells invading through Matrigel™ in control (C) and following treatment with 1,25D (100 nM, 48 hours). Data represented after subtracting the background absorbance data of 0.040 nm. Data shows mean with 95% CI for n=4 separate experiments with duplicates. p= statistically different from control =****= <0.0001, ***= 0.0002, **= 0.0013, *=0.0295.

4.2.5 A role for MMPs in the pro-invasive effects of 1,25D on trophoblast cells

Matrix invasion by cells is associated with induction of matrix-metalloproteinase proteins (MMP) and downregulation of the counterpart, tissue inhibitor of metalloproteinases (TIMP) [408]. Although MMPs and TIMPs are known to be involved in pathogenic processes such as tumour invasion, they are also important for normal physiological events, notably development of the healthy placenta [409]. MMP2 and MMP9 are crucial for trophoblast invasion of decidua during placental development [397], and our laboratory group have shown previously that MMPs are involved in pro-invasive effects of 1,25D on primary cultures of EVT [317]. We, therefore, carried out experiments to assess the role of MMPs and TIMPs in stimulating the invasion of Matrigel^{TM} by JEG3, BeWo and HTR8 cells.

Data in **Figure 4.14A** showed that mRNA expression for *MMP2* is significantly increased in Matrigel^{TM} cultures of JEG3, BeWo and HTR8 cells following treatment with 1,25D. By contrast, 1,25D significantly suppressed *MMP2* expression in TPC cells. Conversely, for *TIMP1*, 1,25D suppressed mRNA expression in JEG3, BeWo and HTR8 cells, but was increased in TPC cells (**Figure 4.14A**). MMP activity was assessed by gelatin zymography, an example zymography gel showing increased MMP2 activity (63 kDa) is shown in **Figure 4.14B**. Densitometric analysis of multiple gelatin zymography plates confirmed elevated MMP2 expression in trophoblast cells treated with 1,25D, whilst no similar effect was observed with TPC cells (**Figure 4.14C**).

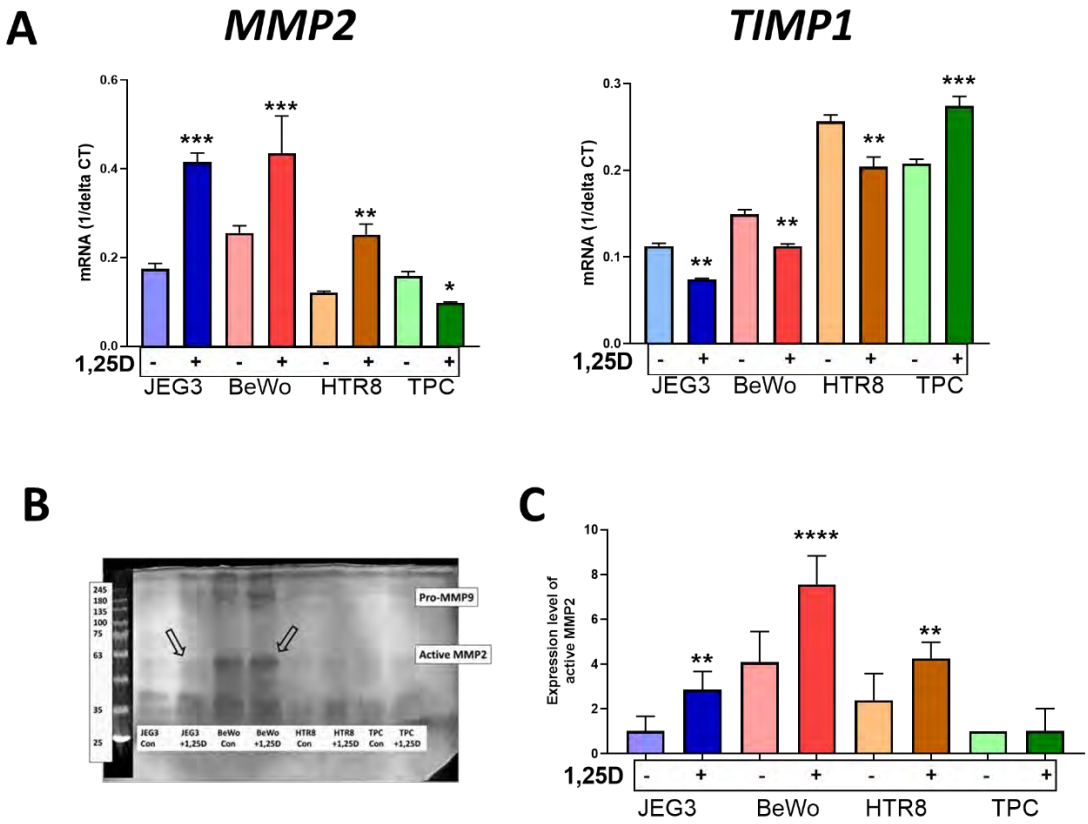


Figure 4.14 Effect of 1,25D on *MMP2* and *TIMP1* in trophoblast and thyroid cells. **A.** mRNA expression levels of *MMP2* and *TIMP1*, represented as 1/delta ct values. Data shows mean with 95% CI. For *MMP2*, p=*= 0.03, **= 0.007, ***= 0.006, 0.004. For *TIMP1*, p= **= 0.0043, 0.007, 0.0064, ***=0.0004. n=3 separate experiments with triplicates each time. **B.** Representative gelatin zymography plate showing active MMP2 bands. **C.** Quantification of expression level of gelatin zymography active MMP2 band**.** n=5 separate experiments with 1 replicate each time. Data represent mean with 95% CI. p= statistically different from control = **= 0.0030, ****= <0.0001, **=0.0027.

Data presented in **Sections 4.2.2** to **4.2.5** show that trophoblast cells, JEG3, BeWo and HTR8 cells respond to 1,25D differently to thyroid cells. However, TPC shows conventional 1,25D anti-proliferation, anti-migration and anti-matrix invasion responses as well as induction of *CYP24A1* mRNA that are consistent with nuclear signalling via the intracellular VDR [29]. In contrast, the trophoblast cells showed no classical responses to 1,25D but instead confirmed previously reported matrix invasion responses [317]. The role of VDR in mediating this trophoblast action of 1,25D is

unclear, so in the next part of the PhD project I investigated how suppression of *VDR* expression in one of the trophoblast cells (JEG3) could affect the function of these cells in response to 1,25D.

4.2.6. Role of VDR in trophoblast responses to 1,25D

To understand the effect of VDR on trophoblast cell function, particularly matrix invasion, responses to 1,25D, siRNA for *VDR* was used to suppress VDR protein expression. Optimisation experiments (see **Section 3.14**) confirmed the efficacy of *VDR* siRNA transfection in 48-hour incubations of Matrigel^{TM}-cultured JEG3 cells. JEG3 cells were specifically chosen for this experiment as these cells have similar morphology to cytotrophoblast cells and extra-villous cytotrophoblast cells [335]. Data in **Figure 4.15** show that at this time point (48 hours) *VDR* siRNA potently suppressed VDR immunofluorescence protein expression whilst no effect on DBP protein expression or expression of the cell membrane or nuclear markers was observed. Quantification of VDR immunofluorescence indicated that levels of VDR in control (scrambled) siRNA transfections were $6.1x10^6$, and 95% CI of $5x10^6$ and $7.2x10^6$, and in JEG3 cells transfected with *VDR* siRNA was $0.9x10^6$, 95% CI of $0.8x10^6$ and $1.1x10^6$.

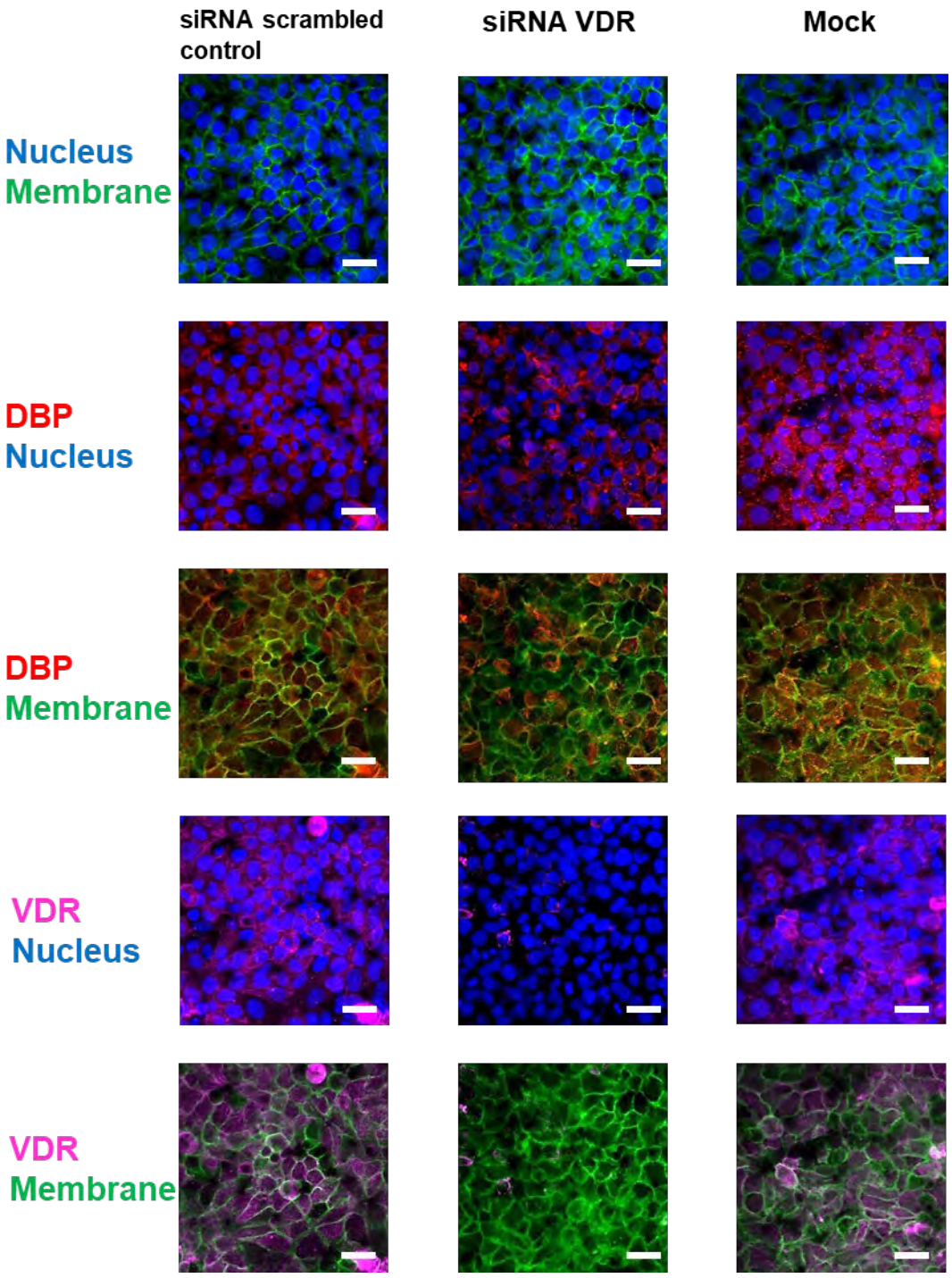


Figure 4.15 Effect of *VDR* siRNA on the expression of VDR and DBP in JEG3 cells.

Immunofluorescence analysis of VDR (pink) and DBP (red) in JEG3 cells following 48 hours of mock transfection; scrambled control siRNA; *VDR* siRNA. Nucleus (Hoechst, blue) and membrane (NaKATPase, green) are also shown. Scale bar is 20 µm. Images were taken with 40x magnification.

Studies were then carried out to determine the effect of siRNA knockdown of *VDR* gene in JEG3 and TPC cells on Matrigel™ invasion. Data in **Figure 4.16** shows that *VDR* knockdown had no effect on matrix invasion by JEG3 in the absence of 1,25D and did not affect the increased matrix invasion by JEG3 cells treated with 1,25D. In TPC cells, knockdown of *VDR* gene decreased matrix invasion even in the absence of 1,25D, and treatment with 1,25D did not suppress this any further.

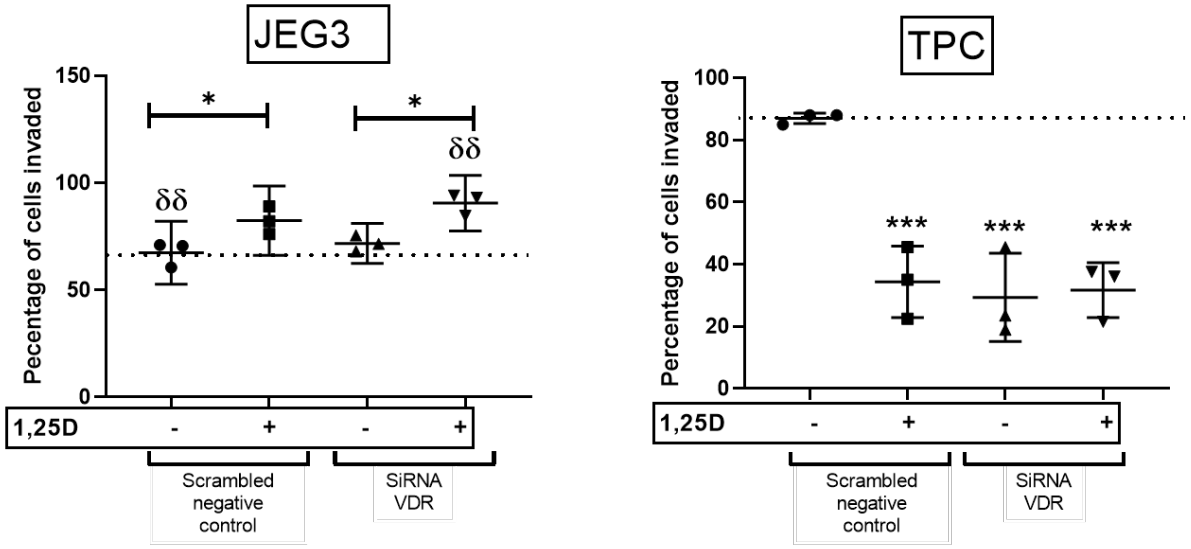


Figure 4.16 Effect of siRNA knockdown of *VDR* gene on matrix invasion by JEG3 and TPC cells.
Both graphs represent mean and 95%CI. JEG3 cells, p=*=0.0479 and 0.0142, p= δδ =0.0041. TPC cells p=***= <0.001. n=3 with duplicates each time.

These data indicate that VDR expression is not required for JEG3 matrix invasion or the stimulation of JEG3 matrix invasion by 1,25D. By contrast, knockdown of VDR had a potent effect on matrix invasion by TPC cells, underlining the differences in the vitamin D system between JEG3 and TPC cells. In the next part of the project, I investigated other mechanisms by which 1,25D could regulate matrix invasion by trophoblast cells.

4.2.7 A role for DBP in trophoblast cell function

Data in **Section 4.2.6** indicate that, when cultured on Matrigel™, trophoblast and TPC cells express two proteins which can bind vitamin D – VDR and DBP. Data in **Figure 4.16** indicate that *VDR* does not play a role in either baseline or 1,25D-induced matrix invasion by JEG3 cells. Therefore, in the next part of the project, experiments were carried out to investigate a possible role for DBP in mediating matrix invasion effects of 1,25D. The first of these experiments was designed to determine the relative importance of exogenous DBP (DBP in the FBS present in cell culture medium) versus endogenous DBP (protein translated from the DBP *(GC)* mRNA detected in Matrigel™ cultures of cells).

JEG3 cells were cultured on Matrigel™ using medium supplemented with either 2% serum from wild type DBP-expressing (DBP+/+) or 2% serum from mice with knockout of the DBP *(GC)* gene (DBP-/-). Immunofluorescence analysis showed that, under these conditions, no DBP was detectable in cells cultured in DBP-/- serum (**Figure 4.17**). Unlike previous studies, immunofluorescence analysis was also carried out for the membrane endocytic receptor for DBP, megalin (gene name *LRP2*). Expression of megalin was detected on JEG3 cells and this was increased in cells cultured without DBP. These studies suggested that the expression of DBP by JEG3 cells cultured on Matrigel™ was dependent on exogenous DBP in the serum supplement of culture medium and that intracellular uptake of DBP could take place via its endocytic receptor, megalin. To investigate megalin-mediated uptake of DBP by JEG3 cells, further experiments were carried out using an inhibitor of megalin-mediated endocytosis.

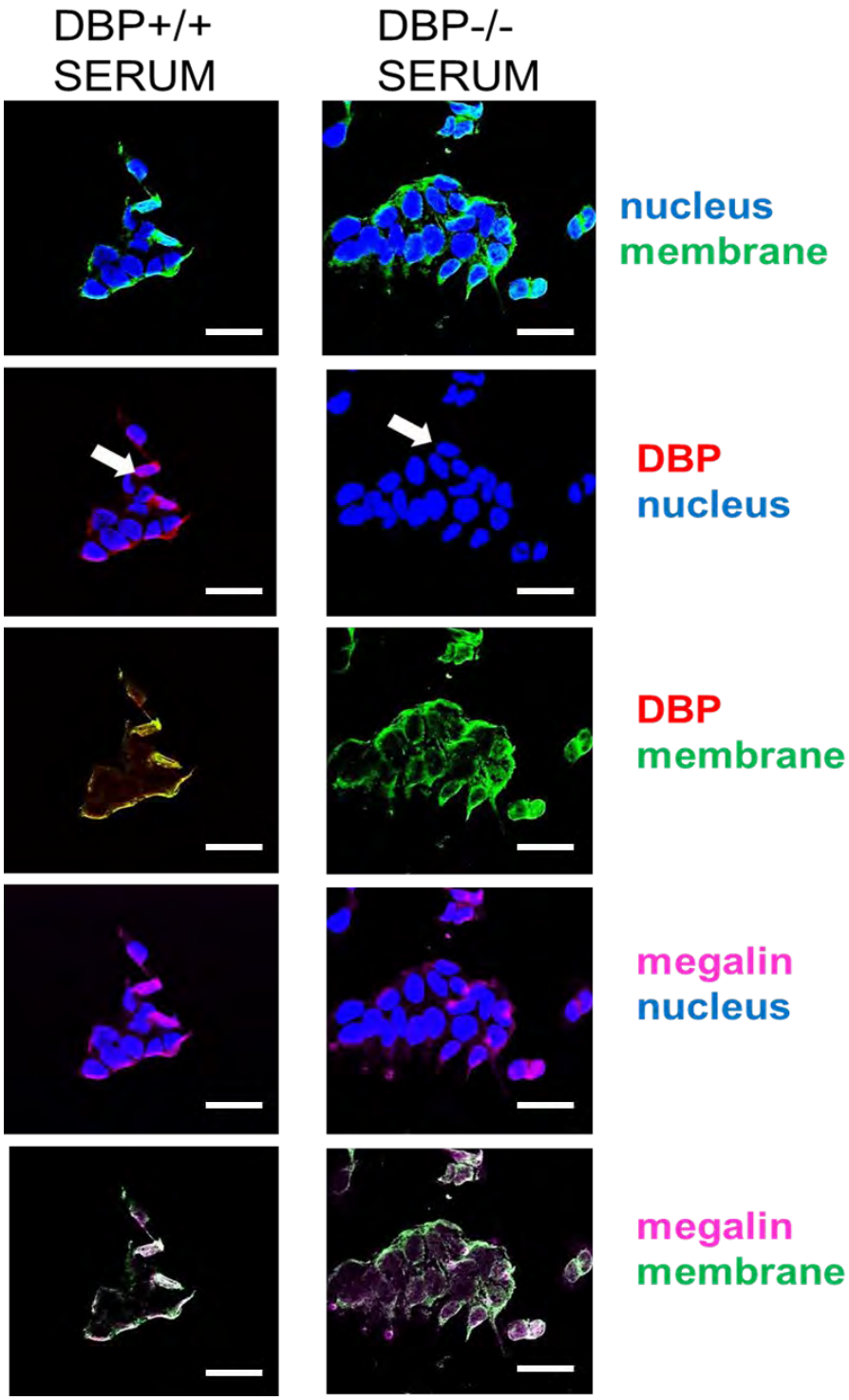


Figure 4.17 Effect of serum DBP on megalin and DBP expression on Matrigel™ cultured JEG3 cells.
Immunofluorescence analysis of DBP (red) and megalin (pink) in JEG3 cells following 48 hours culture with DBP+/+ or DBP-/- serum. Nucleus (Hoechst, blue) and membrane (NaKATPase, green) are also shown. Scale bar is 20 µm. Images were taken with 40x magnification. White arrow highlights DBP staining showing the presence and absence of DBP.

Receptor-associated protein (RAP) was used to inhibit the binding of DBP to megalin and thus preventing megalin-mediated endocytosis. Data in **Figure 4.18** showed that RAP completely blocked DBP expression by JEG3 cells. As well as blocking DBP endocytosis, RAP also inhibited membrane megalin expression.

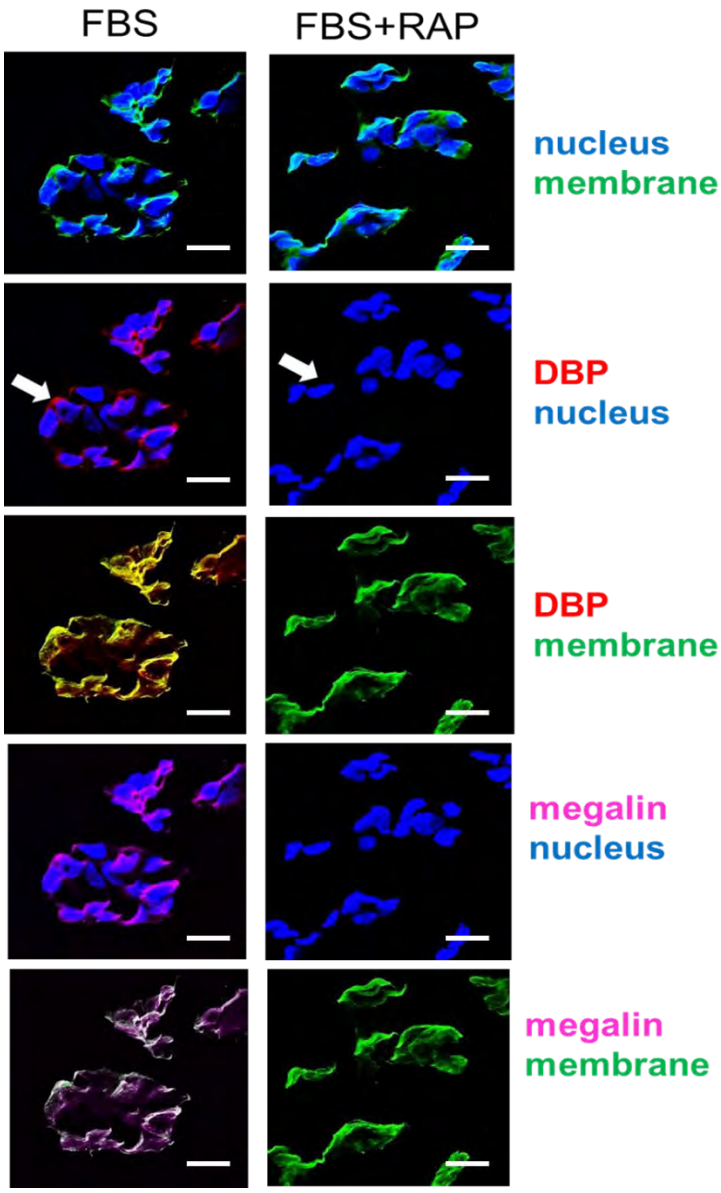


Figure 4.18 Effect of 1 μM of RAP on DBP and megalin expression in Matrigel™ cultured JEG3 cells.
Immunofluorescence analysis of DBP (red) and megalin (pink) in JEG3 cells following culture with and without the megalin inhibitor (RAP). Nucleus (Hoechst, blue) and membrane (NaKATPase, green) are also shown. Scale bar is 20 μm. Images were taken with 40x magnification. White arrow shows the presence and absence of DBP.

Quantification of immunofluorescence images confirmed that both DBP-/- serum and RAP completely inhibited DBP expression in JEG3 cells cultured on Matrigel™ (**Figure 4.19**). Interestingly, cells cultured in medium supplemented with serum lacking DBP (DBP-/-) showed elevated expression of megalin, suggesting a possible feedback mechanism for DBP uptake by trophoblast cells.

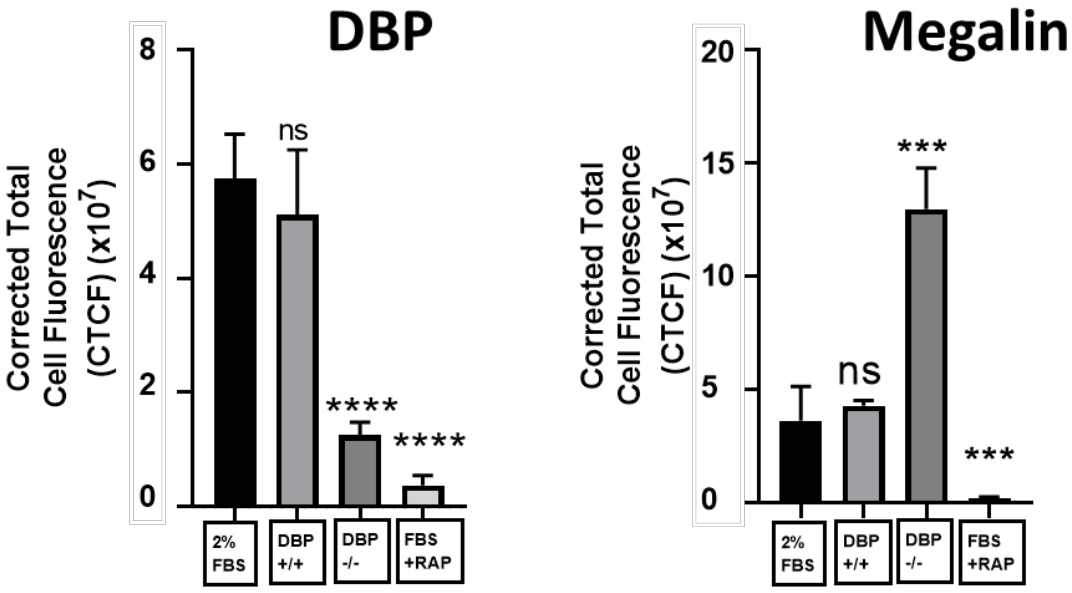


Figure 4.19 Quantification of DBP and megalin in JEG3 cells cultured with mouse serum or 1 µm RAP.
Quantification of immunofluorescence images. Data shows mean with 95% CI. For DBP, p=****= <0.0001. For megalin, p=***= 0.0001 and 0.0004. n=3 separate experiments with duplicate images for each experiment.

Collectively these studies showed that unlike cells cultured on plastic, cells cultured on Matrigel™ actively take up DBP from exogenous serum via a megalin-mediated pathway. Subsequent studies aimed to determine the role that DBP uptake in trophoblast matrix invasion and the effects of 1,25D on matrix invasion. Data in **Figure 4.20** show the effect of culture media containing DBP or no DBP on JEG3 cell Matrigel™ invasion and mRNA expression, in response to 1,25D.

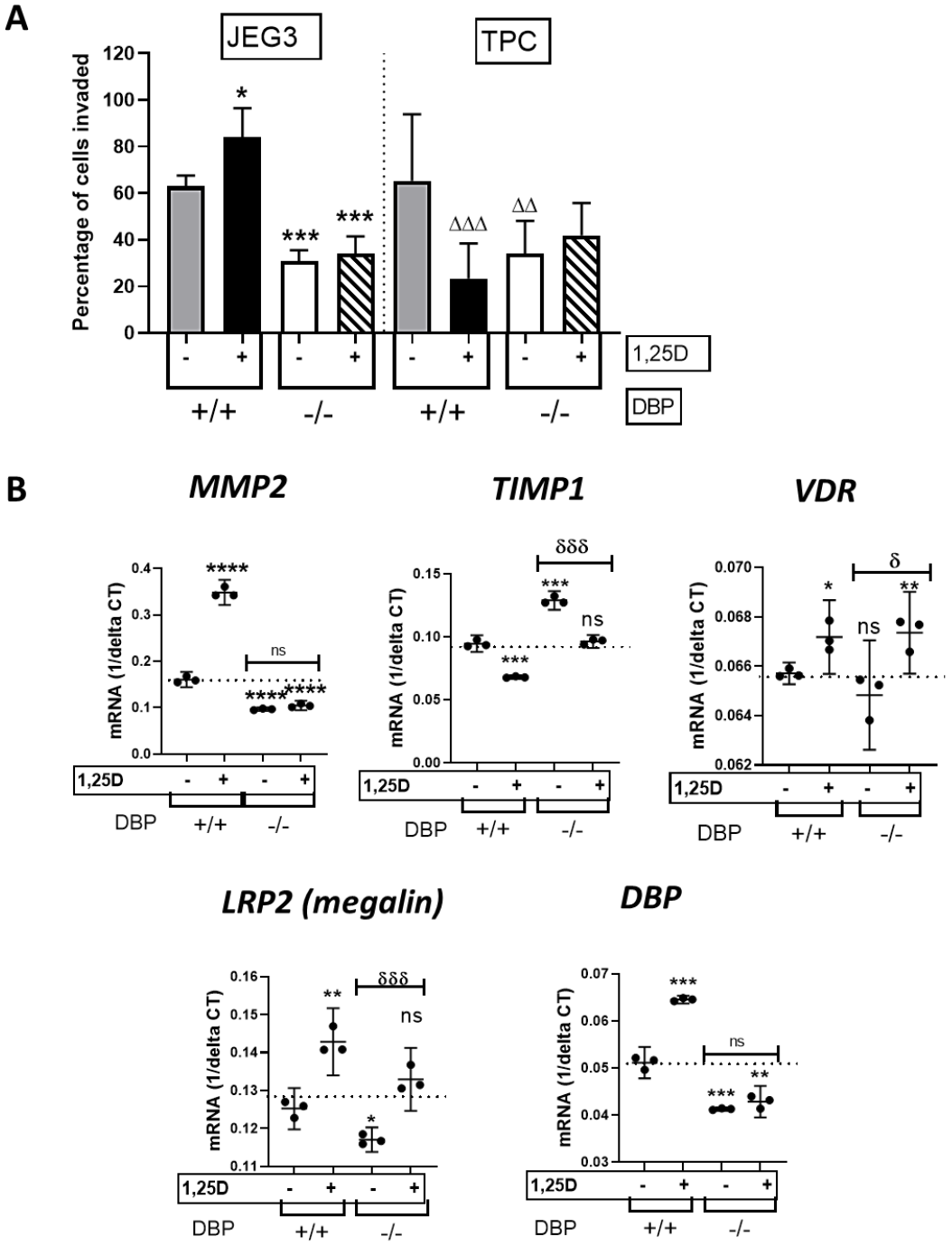


Figure 4.20 Effect of serum DBP on JEG3 matrix invasion and mRNA expression. **A.** Matrigel^{TM} invasion and **B.** *MMP2, TIMP1, VDR,* DBP*(GC)* and *LRP2* (megalin*)* mRNA expression by JEG3 cells cultured in DBP+/+ or DBP-/- serum-supplemented medium in the presence or absence of 1,25D (100 nM, 48 hours). Data shows mean with 95% CI for n=3 with duplicates each time. For MMP2, p=****= <0.0001. For TIMP1, p=***=0.0003, 0.0001, and p= δδδ= 0.0004. For VDR, p=*= 0.03, **= 0.0052 and δ= 0.02. For megalin, p=δδδ= 0.0002, **=0.0063, *= 0.0260. For DBP, p=***=0.0004 and 0.0002, **= 0.0045. "ns" =non-significant.

The most notable observation was that in controls, JEG3 invasion of Matrigel™ was significantly suppressed when cells were cultured in the absence of DBP, even when these cells were treated with 1,25D (**Figure 4.20A**). The net effect was that for cells treated with 1,25D, Matrigel™ invasion was approximately 3-fold higher for JEG3 cells in the presence of DBP relative to DBP absence. Similar observations were also made for *MMP2* mRNA which was also suppressed in JEG3 cells cultured in the absence of DBP, with 1,25D showing no effect on induction of *MMP2* in the absence of DBP (**Figure 4.20B**). By contrast, *TIMP1* mRNA was increased in cells cultured in the absence of DBP, but this effect was counteracted by treatment with 1,25D. Levels of mRNA for DBP*(GC)* showed a significant increase by the presence of DBP in the JEG3 culture medium, suggesting exogenous DBP is entering the cells. There was a small but significant increase in *VDR* and *megalin* mRNA following treatment with 1,25D in both the absence and presence of DBP (**Figure 4.20B**). This might suggest an alternative pathway for 1,25D action.

Data from serum DBP knockout experiments suggested that DBP plays a crucial role in supporting matrix invasion by trophoblast cells such as JEG3. To confirm that this effect involved cellular uptake of DBP from culture medium into the trophoblast cells, further experiments were carried out using the megalin inhibitor RAP to block endocytic uptake of DBP by JEG3 cells. Data in **Figure 4.21** showed that similar to culture with DBP-/- serum, treatment of JEG3 cells with RAP significantly suppressed invasion of Matrigel™ in the presence or absence of 1,25D.

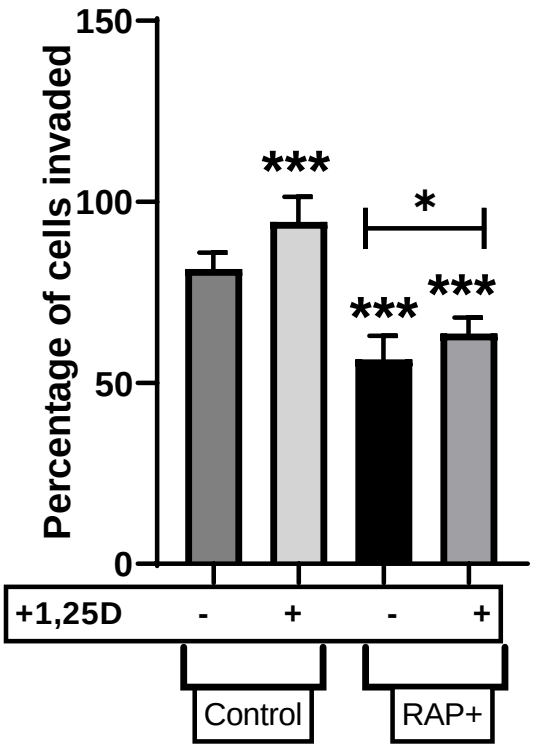


Figure 4.21 Effect of inhibition of megalin (by 1 μm RAP) on JEG3 matrix invasion.
Matrigel^{TM} invasion by JEG3 cells cultured in the presence and absence of RAP supplemented medium, in the presence or absence of 1,25D (100 nM, 48 hours). Data shows mean with 95% CI for n=3 separate experiments with duplicates each time. p= *** = 0.0004, 0.0006, 0.0005, * = 0.0195.

The overall conclusion from these studies (**Figure 4.22**) is that both DBP and 1,25D act to promote matrix invasion by trophoblast cells. This effect involves intracellular uptake of DBP protein from exogenous serum but the mechanism by which DBP promotes cellular invasion was unclear. The mechanism by which 1,25D enhanced trophoblast matrix invasion was also unclear, but did not appear to involve conventional signalling via the VDR.

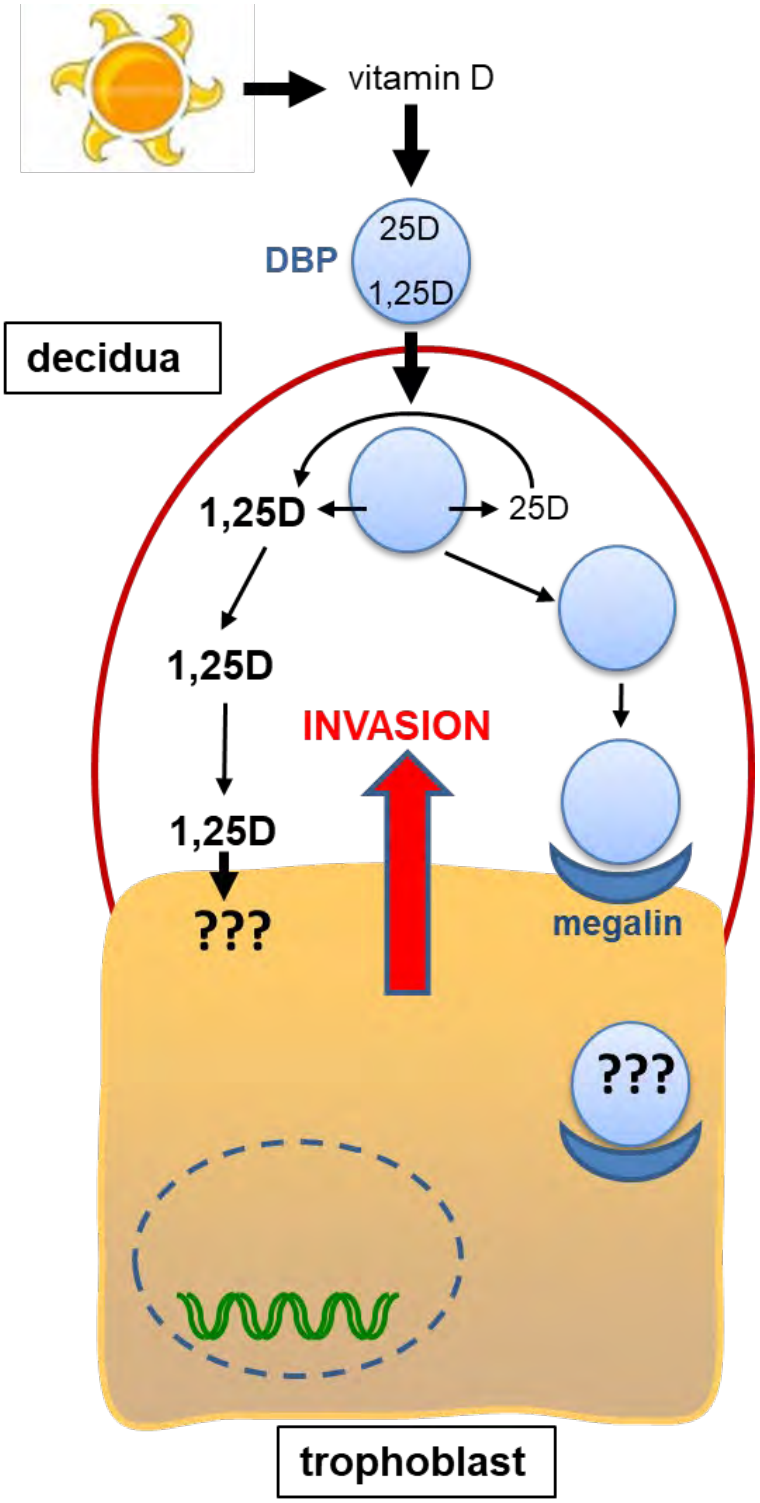


Figure 4.22 Schematic representation of observations from studies of the vitamin D system and matrix invasion in trophoblast cells cultured on Matrigel™.

4.2.8 A role for ERK kinase in mediating matrix invasion effects of DBP and 1,25D in trophoblast cells

Data from **Sections 4.2.2** to **4.2.7** indicate that although trophoblast cells express mRNA and protein for VDR, this receptor does not appear to promote classical responses to 1,25D in these cells, and knockdown of VDR had no effect on matrix invasion effects of 1,25D on trophoblast cells. Based on this, further studies were carried out to investigate alternative mechanisms for the action of 1,25D on trophoblast cells. Previous studies have shown that enhanced matrix invasion by trophoblasts following treatment with 1,25D involves activation of the intracellular ERK signalling pathway [410]. Further studies were therefore carried out to determine if ERK phosphorylation was associated with the effects of 1,25D and DBP on trophoblast Matrigel^{TM} invasion.

Because of the relative inefficiency of recovering cellular protein from Matrigel^{TM} cultures, initial experiments were carried out using JEG3, BeWo and TPC cells cultured in conventional plasticware. Data in **Figure 4.23** showed that treatment of JEG3 and BeWo cells with 1,25D rapidly (30 mins) stimulated nuclear localisation of pERK with no effect on ERK itself. This rapid response was not observed for TPC cells. However, at 48 hours, JEG3, BeWo and TPC cells showed a significant shift from cytoplasmic to nuclear pERK, with this response involving pERK1 and pERK2.

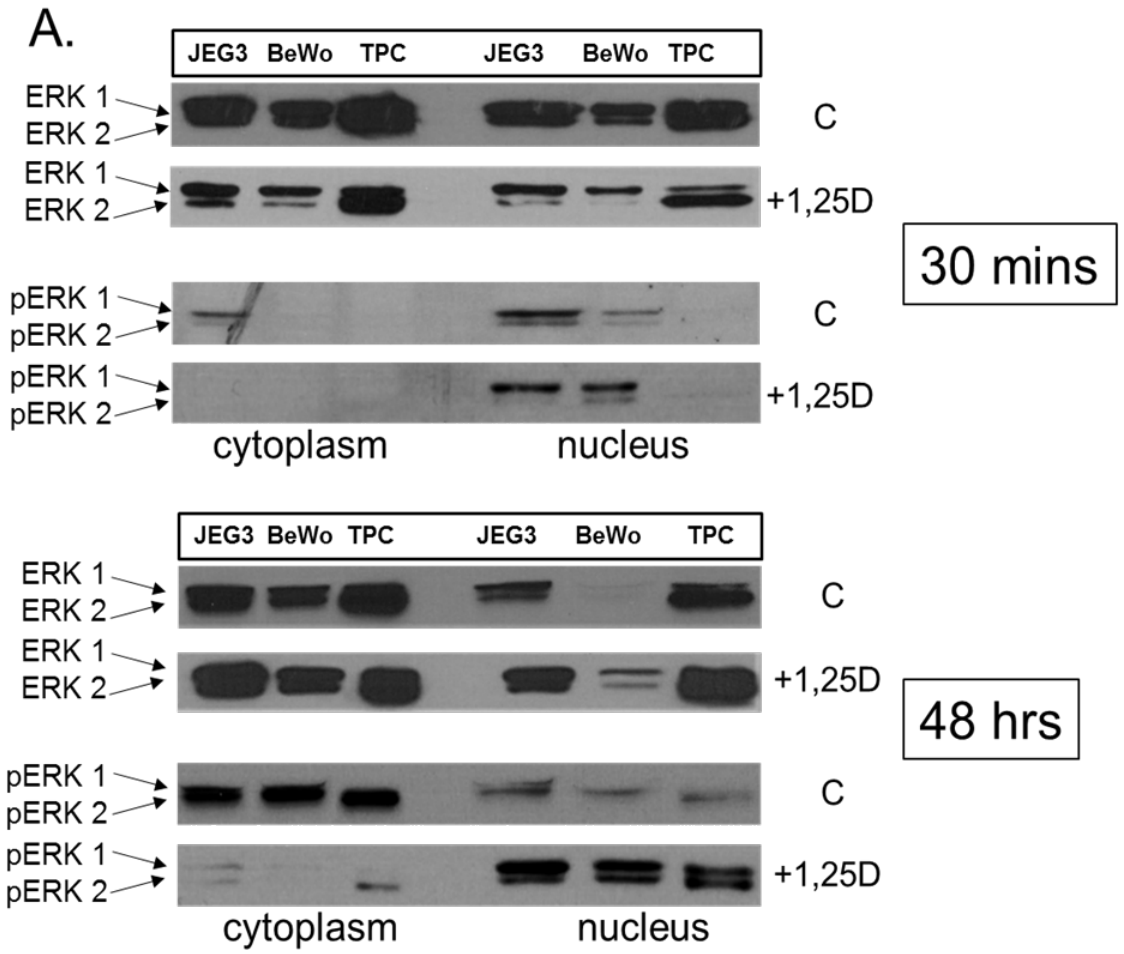


Figure 4.23 Regulation of ERK pathway by 1,25D in trophoblast cells.
Western blot analysis of ERK and pERK in cytoplasmic and nuclear fractions of JEG3, BeWo and TPC cells treated with and without 1,25D (100 nM) for 30 mins or 48 hours.

Data in **Figure 4.23** indicates that 1,25D stimulated ERK phosphorylation in JEG3, BeWo and TPC cells, with the trophoblast cells showing a more rapid nuclear localisation of pERK. Although these studies were carried out using cells cultured on plastic, the data were used as a basis for experiments to investigate possible effects of ERK inhibition on trophoblast function. Preliminary western blot experiments using plasticware cultures confirmed the efficacy of U0126 in inhibiting pERK and its nuclear accumulation (**Figure 4.24A**). However, analysis of JEG3 cells cultured on Matrigel^{TM}

confirmed that U0126 also completely inhibited pERK when cells were cultured on matrix (**Figure 4.24B**).

Further experiments were then carried out to assess the functional impact of pERK inhibition on matrix invasion by trophoblast cells. Initial experiments showed that treatment with U0126 completely inhibited DBP expression in JEG3 (**Figure 4.25**), BeWo (**Figure 4.26**) and HTR8 (**Figure 4.26**) however did not affect VDR expression in these cells. Co-treatment with 1,25D partially restored DBP expression in JEG3 and BeWo cells, but levels were still significantly lower than in cells cultured without the pERK inhibitor. 100X magnified images in **Figure 4.25** also showed that while expression of VDR was perinuclear even in the presence of 1,25D, DBP (red) appeared to show nuclear localisation (pink staining) when JEG3 cells were treated with 1,25D.

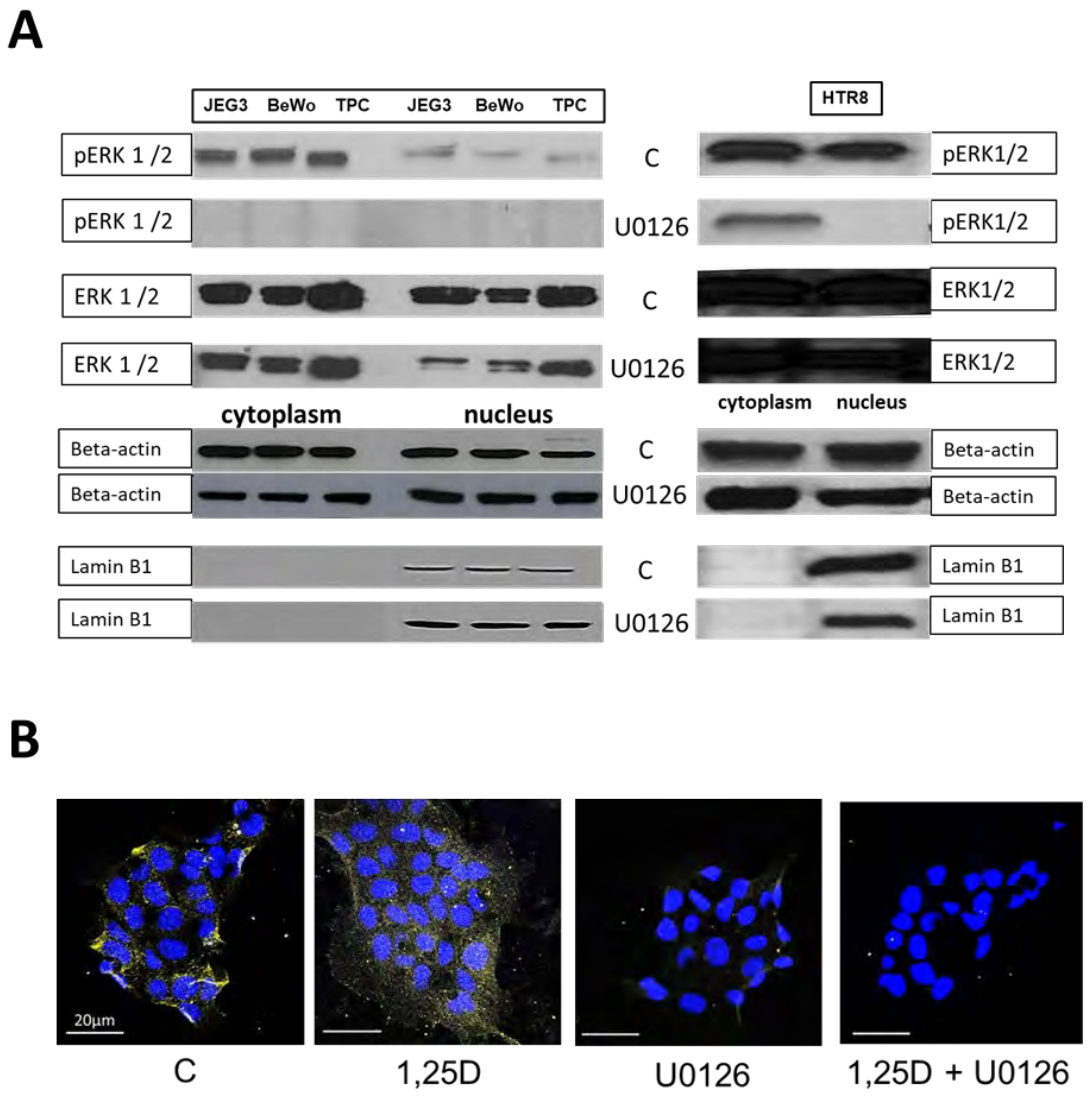


Figure 4.24 Inhibition of pERK by 10 µM U0126.
A. Western blot analysis of ERK and pERK in cytoplasmic and nuclear fractions of JEG3, BeWo, TPC cells cultured on plastic and treated with and without U0126 for 48 hours. Cellular (β-actin) and nuclear (lamin B1) controls are also shown. **B.** Effect of 1,25D (100nM) and U0126 on pERK immunofluorescence in JEG3 cells cultured on Matrigel^{TM}. Scale bar is 20 µm. Images were taken with 40x magnification.

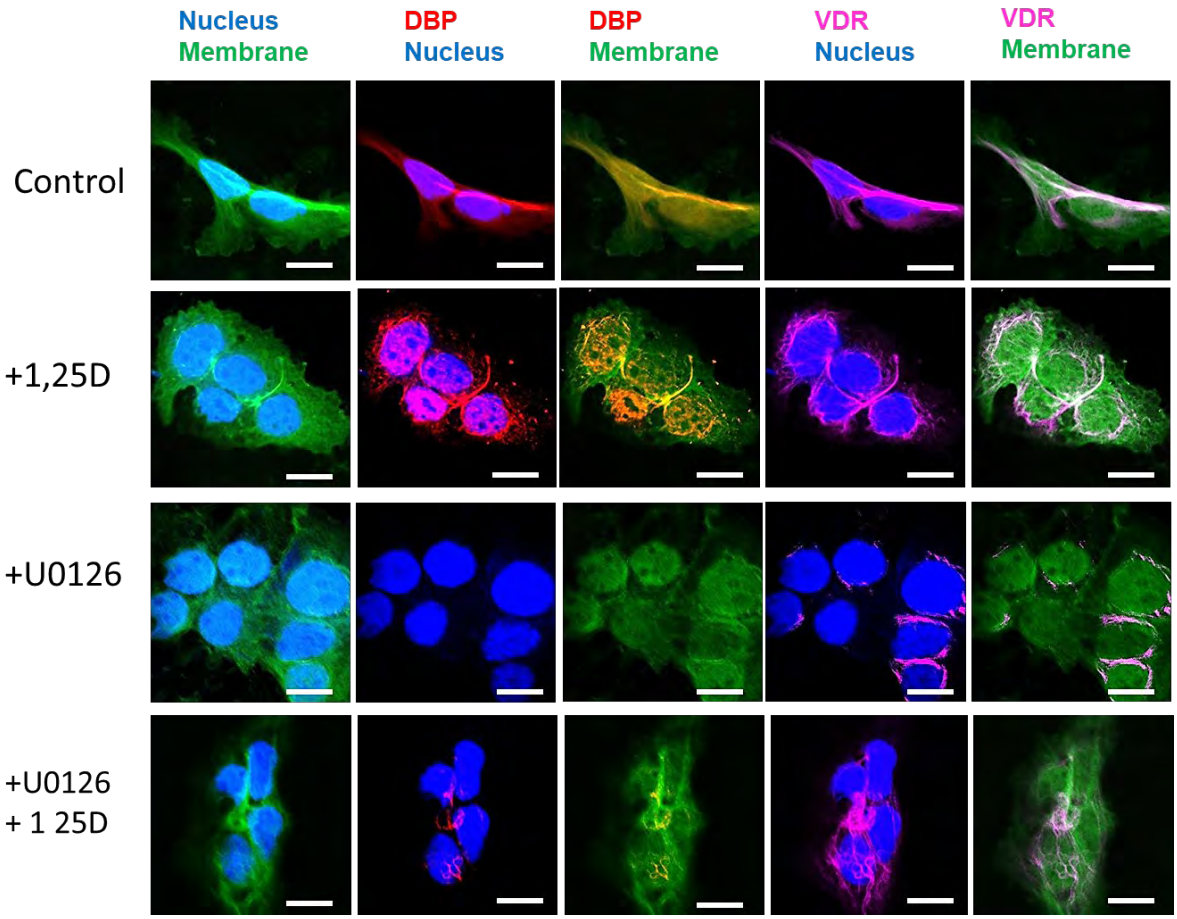
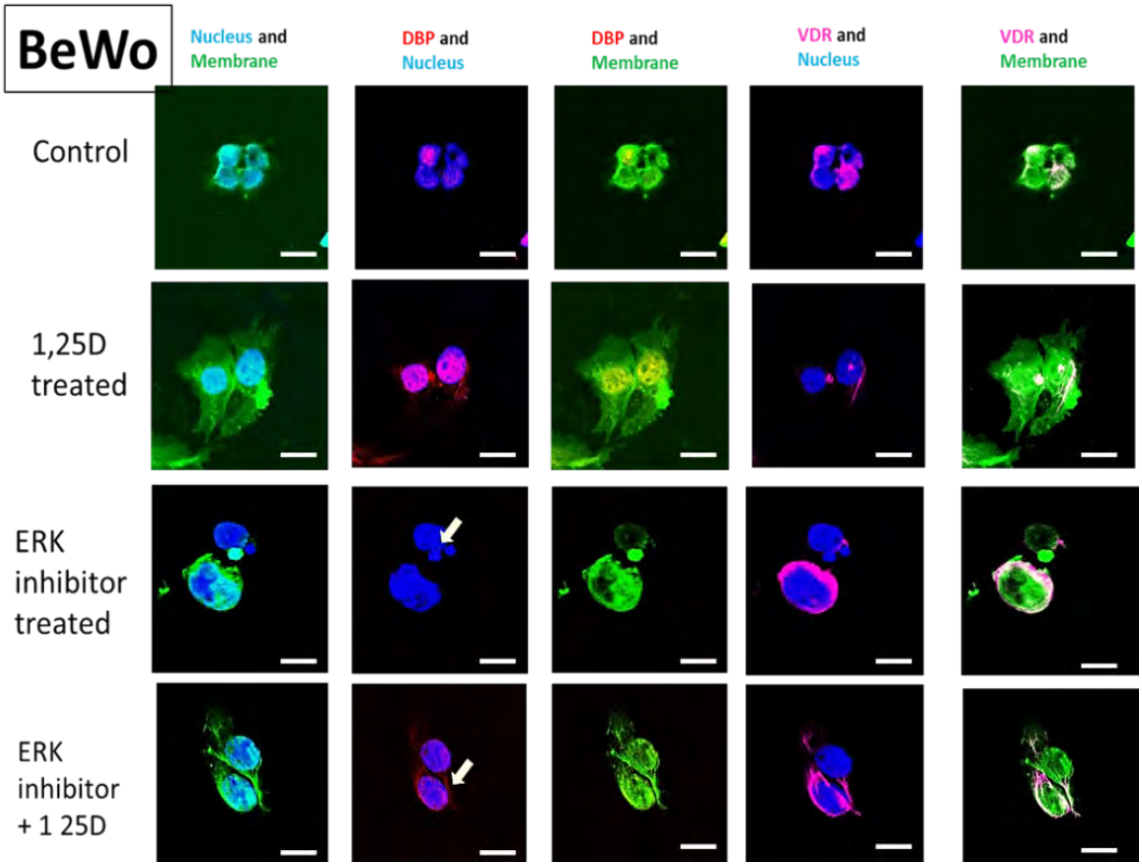


Figure 4.25 Effect of 1,25D treatment and pERK inhibition on DBP and VDR expression in JEG3 cells.
Immunofluorescence analysis of expression of protein for DBP (red) and VDR (pink) in JEG3 cells cultured the presence or absence of 1,25D (100 nM, 48 hours) without or with the pERK inhibitor U0126. Nucleus (Hoechst, blue) and membrane (NaKATPase, green) are also shown. Scale bar is 75 µm. Images were taken with 100x magnification.


BeWo
Nucleus and Membrane
DBP and Nucleus
DBP and Membrane
VDR and Nucleus
VDR and Membrane
Control
1,25D treated
ERK inhibitor treated
ERK inhibitor + 1 25D

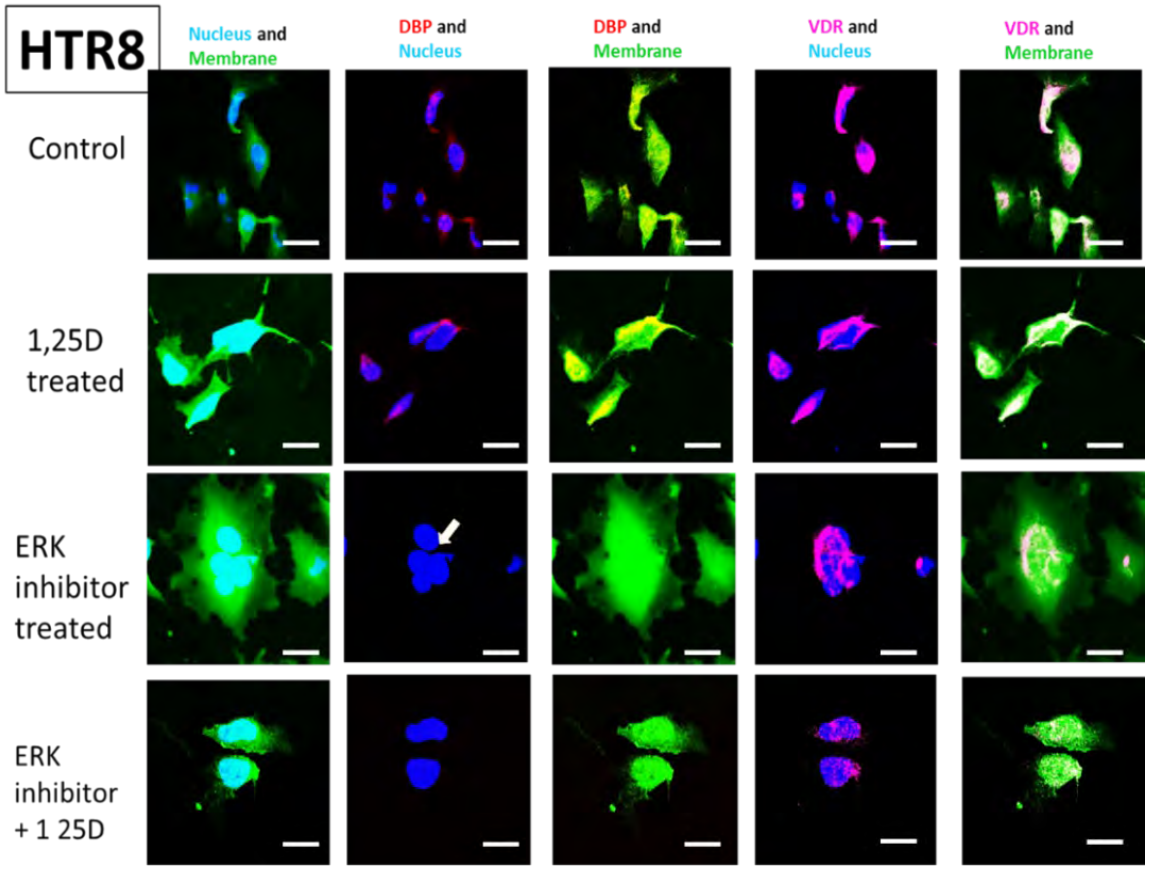


Figure 4.26 Effect of pERK inhibition on DBP and VDR expression in BeWo and HTR8 cells.
Immunofluorescence analysis of expression of protein for DBP (red) and VDR (pink) in BeWo and HTR8 cells cultured in presence and absence of 1,25D (100 nM, 48 hours) with and without pERK inhibitor U0126. Nucleus (Hoechst, blue) and membrane (NaKATPase, green) are also shown. Scale bar is 20 µm. Images were taken with 40x magnification. The white arrow marks DBP expression.

Inhibition of pERK was similar to serum DBP knockout and megalin inhibition in blocking cellular expression of DBP. Further studies were therefore carried out to determine if inhibition of pERK also affected trophoblast matrix invasion.

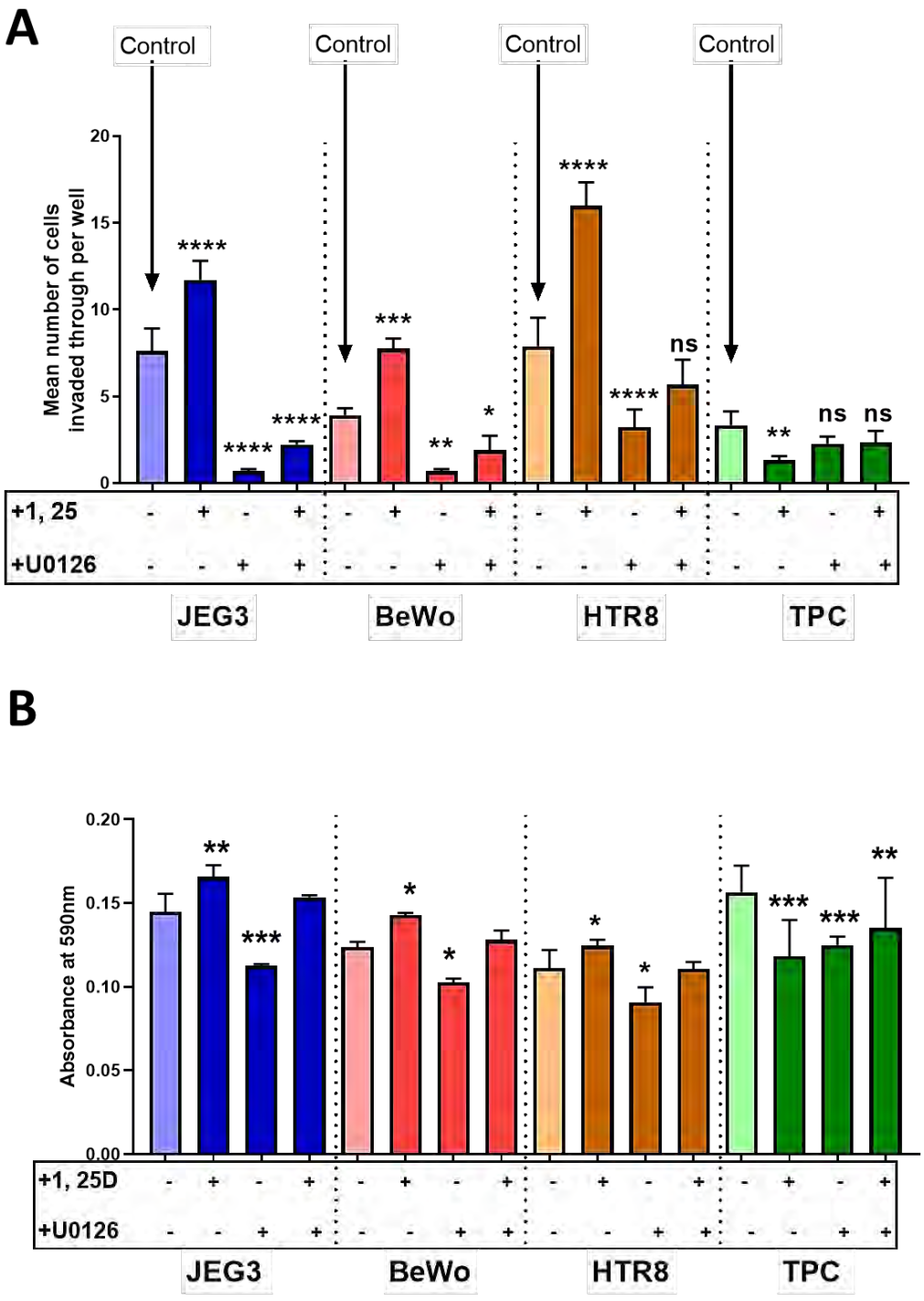


Figure 4.27 Effect of pERK inhibition on matrix invasion by trophoblast and thyroid cells.

A. Manual counting of invaded cells on Matrigel™, **B.** Quantification with crystal violet staining by JEG3, BeWo, HTR8 and TPC cells treated with 1,25D (100 nM) for 48 hours in the presence or absence of the pERK inhibitor U0126. p=**** = <0.0001, *** = <0.001, ** = <0.01 and * = <0.05., ns= non-significant. n=3 with triplicates each time for both experiments.

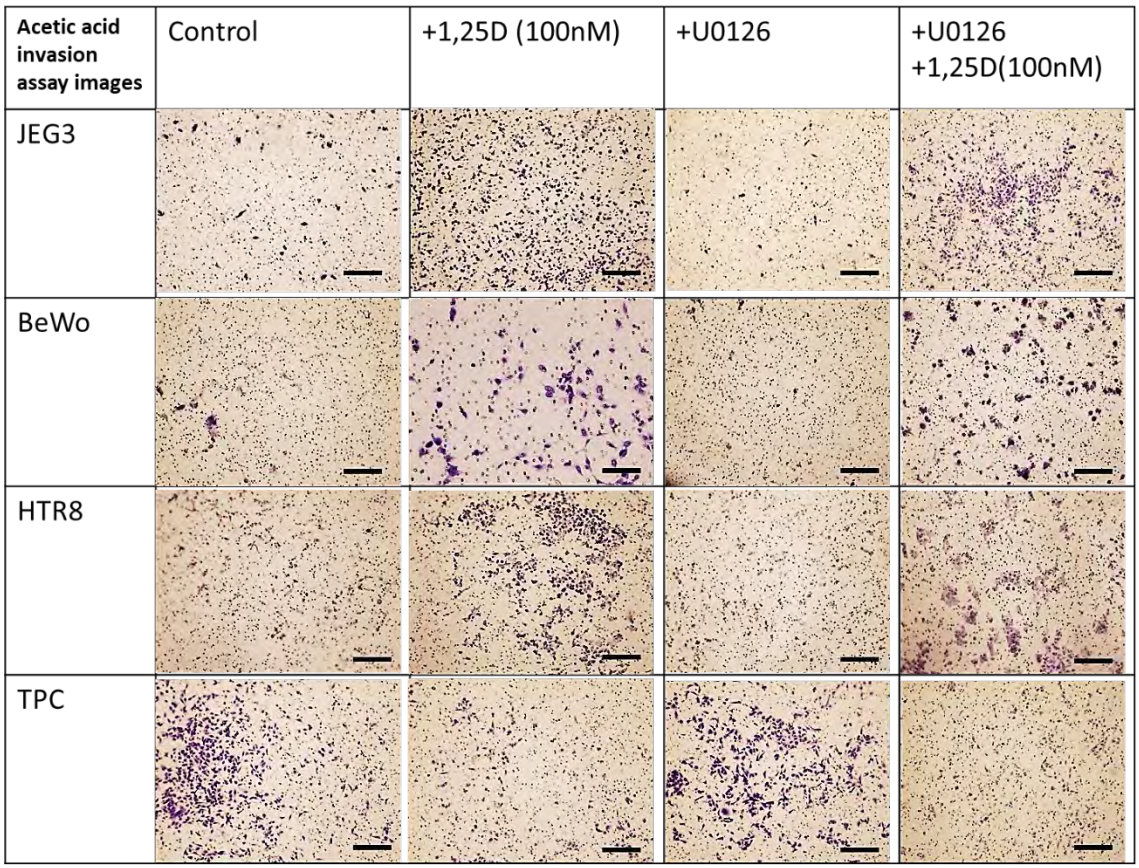


Figure 4.28 Microscopic images of Matrigel^{TM} wells showing the effect of pERK inhibition on matrix invasion by trophoblast and thyroid cells.
Microscopic images of cells stained with crystal violet showing matrix invading of JEG3, BeWo, HTR8 and TPC cells treated with and without 1,25D (100 nM, 48 hours) in the presence or absence of the pERK inhibitor U0126.
Scale bar is 200 µm. Each image was taken in 10x magnification.

Data in **Figure 4.27** and **Figure 4.28** showed that in all three trophoblast cell lines treated with U0126 suppressed Matrigel^{TM} invasion significantly. As shown previously, treatment with 1,25D stimulated matrix invasion by trophoblast cells, and co-treatment with 1,25D partially alleviated suppression of trophoblast invasion by U0126. Treatment with 1,25D suppressed invasion by TPC cells.

Inhibition of pERK by U0126 led to a decreased invasion of Matrigel™ by all three trophoblast cell lines. However, it is also possible that the suppression of nuclear pERK could influence matrix invasion by suppressing cell proliferation. Further experiments were therefore carried out to determine if 1,25D and/or U0126 altered proliferation of cells when cultured on Matrigel™. In this study, I also carried out additional treatment to determine if exogenously added DBP could also change the proliferation of cells. Data in **Figure 4.29** shows that inhibition of pERK or addition of DBP did not affect cell proliferation. The only change in proliferation was observed for TPC cells which showed suppressed proliferation with 1,25D treatment.

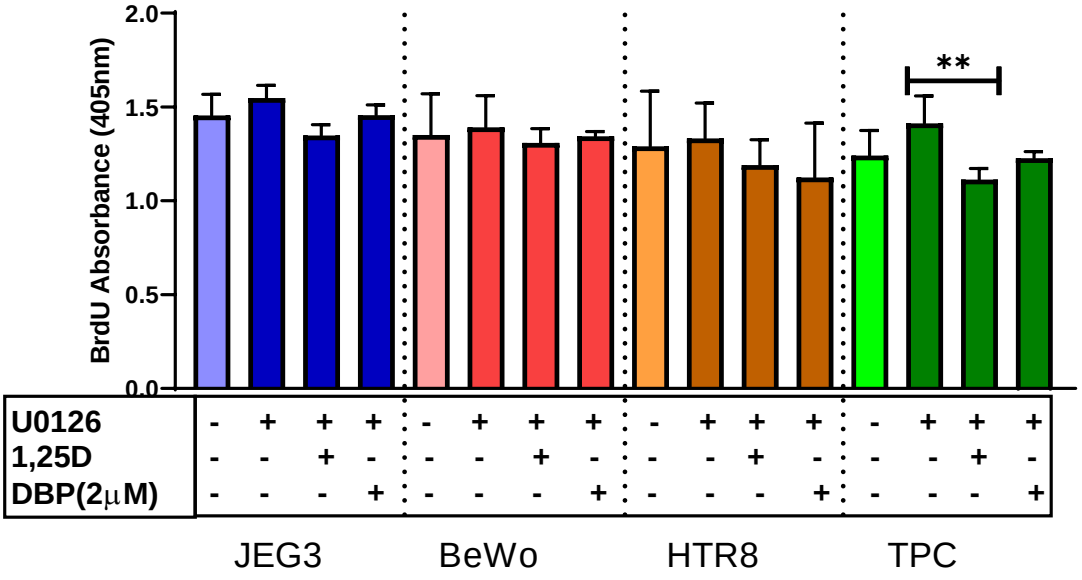


Figure 4.29 Effect of treatment with U0126 and DBP (2 μM) on trophoblast and thyroid cell proliferation.
BrdU incorporation analysis of JEG3, BeWo, HTR8 and TPC cells treated with 1,25D (100 nM, 48 hours) in the presence and absence of the pERK inhibitor U0126. Cells were also treated with exogenously added DBP (2 μM). Data shows mean with 95% CI. p=**= 0.0032. n=4 separate experiments with duplicates each time.

4.2.9 Effects of vitamin D binding protein uptake on intracellular actin in trophoblast cells

In addition to its central function as a serum carrier of vitamin D metabolites, and the uptake and metabolism of vitamin D in the kidney, DBP is also known to exhibit several other properties that may be independent of vitamin D [411]. These include a role for DBP as a macrophage-activation factor [412], and in fatty acid transport [413]. DBP also binds the monomeric, globular, form of actin (G-actin) with high affinity, allowing DBP to compete with other established actin-binding factors such as gelsolin that act to incorporate G-actin into filamentous actin (F-actin) [414]. In this way, DBP can also function as an actin-scavenger, with a potential role in protecting against tissue damage due to systemic F-actin accumulation [415]. Although the DBP-actin complex may also fulfil a pro-inflammatory role as a neutrophil chemotactic factor [416]. This latter function of DBP as an actin scavenger has, to date, been restricted to effects in the general circulation. In the current project, DBP appears to be important to trophoblast cells as an intracellular factor. I, therefore, carried out studies to determine if DBP also functions as an actin-scavenger at an intracellular level in trophoblast cells.

Results in **Figure 4.30** showed that when JEG3 or HTR8 cells were cultured on Matrigel^{TM} in the absence of serum DBP (DBP-/-) there was decreased intracellular DBP, but also decreased expression of F-actin and a concomitant increase in G-actin, with the latter appearing to be located in the nucleus. Quantification of changes in F-actin and G-actin are shown in **Figure 4.31**.

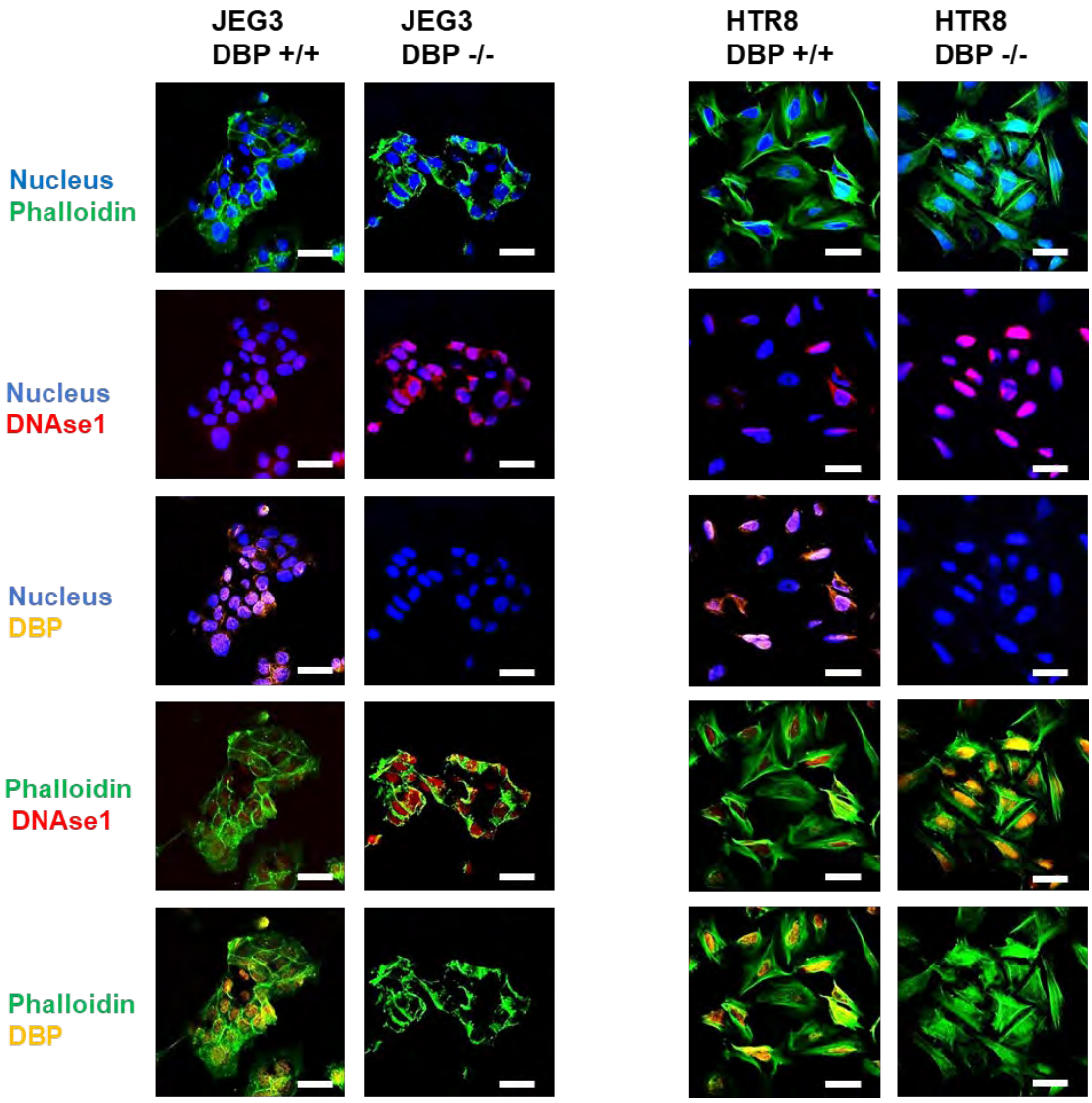


Figure 4.30 Effect of serum DBP on F-actin (Phalloidin) and G-actin (DNase1) in JEG3 and HTR8 cells.
Immunofluorescence images for protein expression of DBP (yellow), Phalloidin (green), DNase1 (red) in JEG3 and HTR8 cells cultured in medium supplemented with DBP-containing (DBP+/+) or DBP-free (DBP-/-) serum. Nucleus (Hoechst, blue) is also shown. Scale bar is 20 µm. Each image was taken with 40x magnification and viewed with Carl Zeiss water immersion liquid.

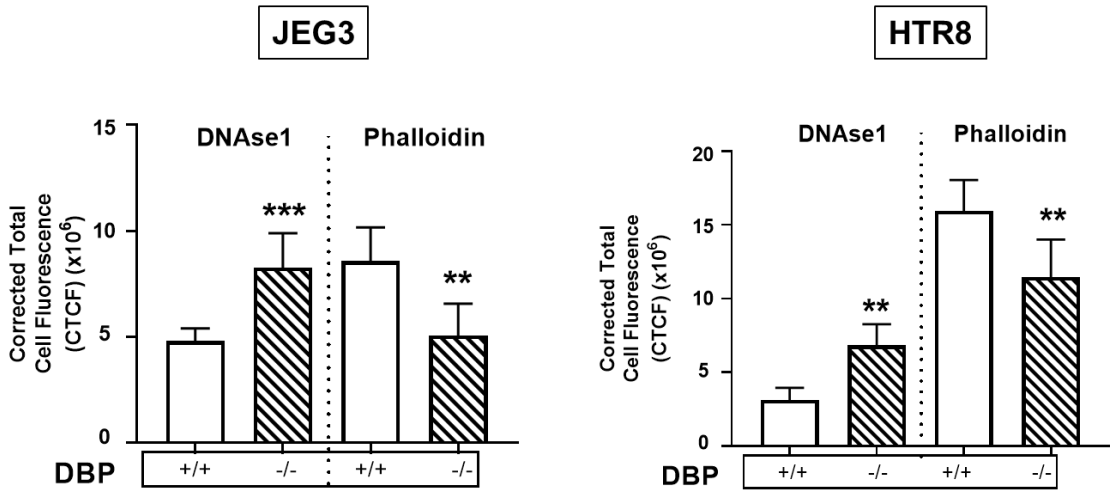


Figure 4.31 Effect of serum DBP on protein expression of F-actin (Phalloidin) and G-actin (DNase1) in JEG3 and HTR8 cells.
Quantification of immunofluorescence for F-actin and G-actin in JEG3 and HTR8 cells cultured in DBP+/+ and DBP-/- cell culture environment. n=3 separate experiments each carried out in duplicate. Data shows mean with 95% CI. p=***=0.0006, **= 0.0013, 0.0017 and 0.0027. n=3 separate experiments with duplicate images each time.

Data in **Figures 4.30** and **4.31** indicate that intracellular DBP plays a key role in controlling intracellular levels of both F- and G-actin. To confirm this association, further experiments were carried out using the pERK inhibitor U0126 to suppress intracellular DBP. Data in **Figure 4.32A** and **4.32B** showed that treatment with U0126 did not alter the total levels of G-actin in JEG3 cells but rather increased the nuclear localisation of G-actin (pink staining). Treatment with 1,25D suppressed G-actin levels but this effect was blocked by co-treatment with U0126.

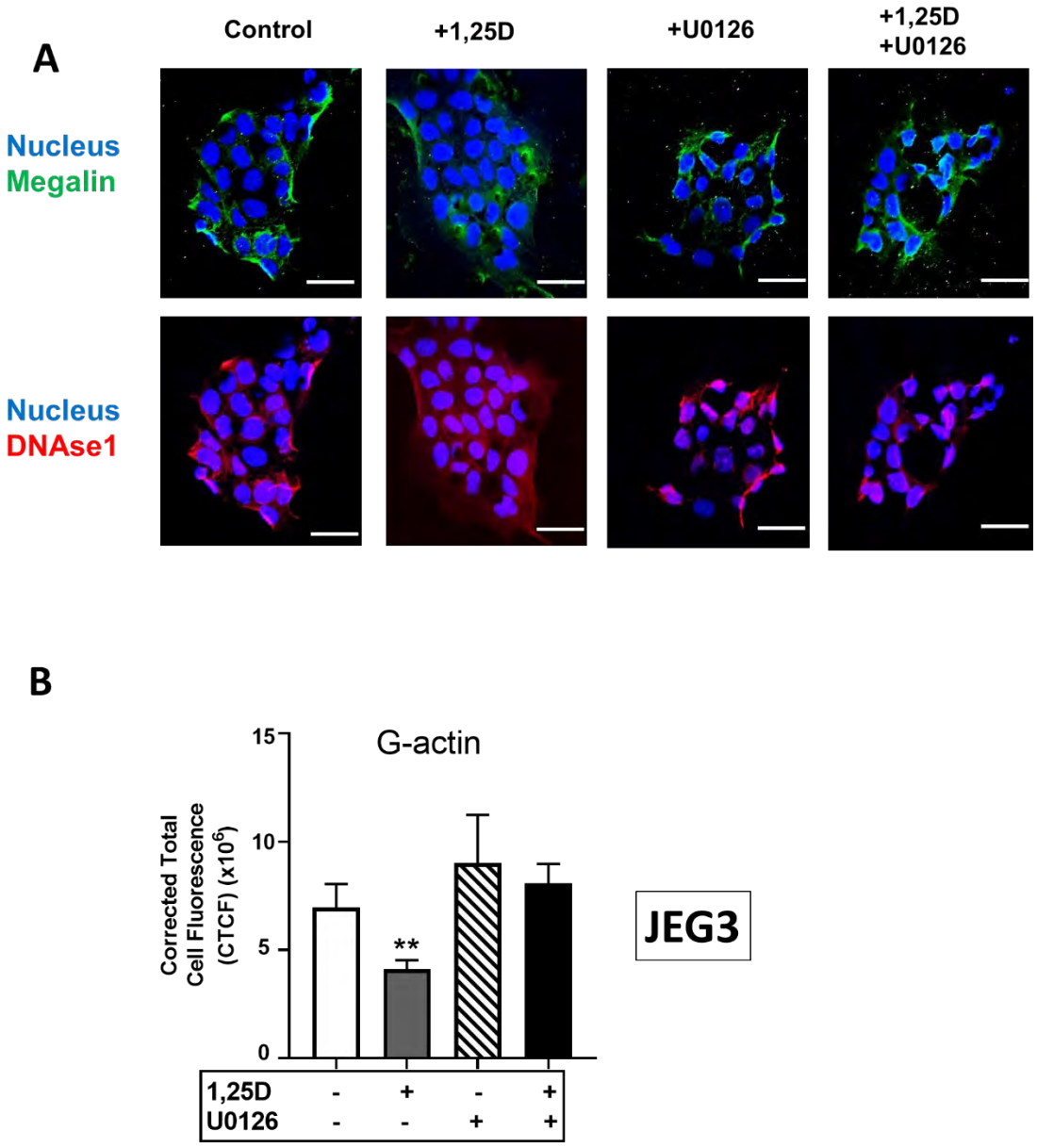


Figure 4.32 Effect of serum 1,25D and U0126 on Megalin and G-actin protein expression in JEG3 and HTR8 cells.

A. Immunofluorescence images for protein expression of G-actin (red) and megalin (green) in JEG3 cells treated with 1,25D (100 nM, 48 hours) in the presence or absence of U0126. Nucleus (Hoechst, blue) is also shown. Scale bar is 20 µm. Each image was taken with 40x magnification with a Carl Zeiss water immersion liquid. **B.** Quantification of immunofluorescence for G-actin in JEG3 cells. Data shows mean with 95% CI. n=3 separate experiments with duplicate images each time. p=**= 0.01.

4.2.10 Conclusion

The overall conclusion from the studies in this section of the project is that exogenous (serum) DBP is internalised by trophoblast cells using a megalin-mediated pathway that is linked to ERK phosphorylation. This, in turn, has a significant effect on intracellular G-actin levels, which also affects intracellular F-actin levels. Collectively these changes appear to contribute to an increased capacity of trophoblast cells for matrix invasion (**Figure 4.33**). Conversely, lack of serum DBP, inhibition of megalin-mediated endocytosis, or inhibition of pERK all suppressed intracellular DBP and trophoblast invasion of the matrix.

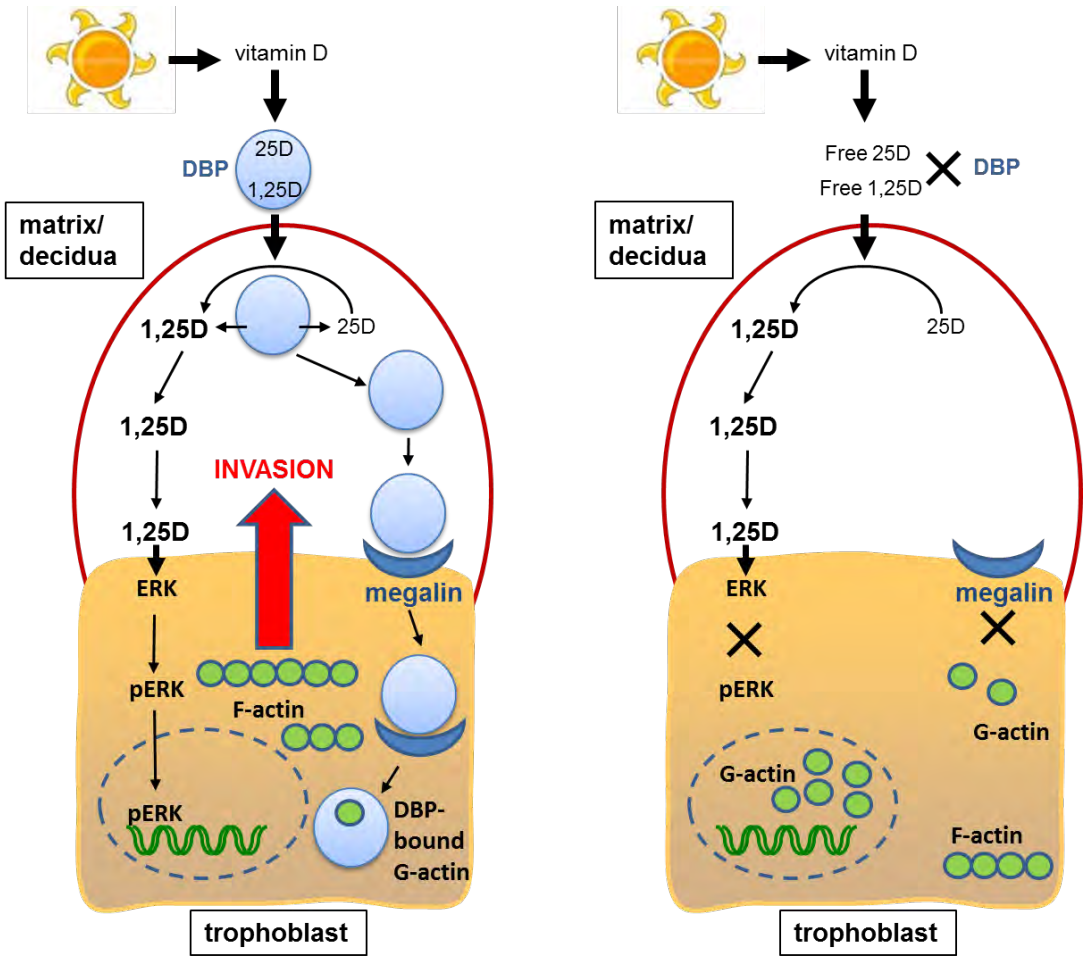


Figure 4.33 Schematic representation of observations from studies of the vitamin D and matrix invasion in trophoblast cells cultured on Matrigel™.

DBP is known to bind G-actin, with the DBP α helix binding to a cleft in actin [414]. Monomeric G-actin is incorporated into polymeric F-actin by factors such as Wiskott-Aldrich syndrome protein (WASP) and Cdc42 [417]. However, DBP can influence the formation of polymeric F-actin by sequestering monomeric G-actin, while other actin-binding factors such as gelsolin suppress F-actin by severing the F-actin polymer [418]. Thus, changes in intracellular DBP levels may act to alter the cellular equilibrium of G- and F-actin within cells. The data presented in this part of my thesis suggest that this equilibrium between G- and F-actin plays an important role in the ability of trophoblast cells to invade matrix.

In the final part of the PhD project, I aimed to investigate whether the data generated in **Sections 4.2.2** – **4.2.9**, and the deduced mechanism outlined in **Figure 4.33** could be applied to samples taken from human pregnancies.

4.3: Role of serum DBP as a determinant of trophoblast function and pregnancy health in the human population

4.3.1 Introduction

Variations in serum vitamin D metabolites [419, 420] and SNPs for *DBP* [421, 422] have been linked to adverse events in pregnancy such as the hypertensive disorder pre-eclampsia but the mechanisms underpinning these associations remain unclear. In particular, although DBP and vitamin D metabolites are abundant in the placenta even early in pregnancy [381, 387], their role in placental development has yet to be defined. The aim of the final part of this PhD project was to investigate the first hypothesis that:

Serum concentrations of DBP during pregnancy define trophoblast DBP levels and determine matrix invasion by trophoblast cells

To test this hypothesis, I used 1st trimester sera from normal healthy pregnancies as a cell culture supplement to determine the effects of normal variations in serum DBP and vitamin D metabolite levels on trophoblast matrix invasion.

The second hypothesis tested in this part of the project was:

Serum concentrations of DBP may be a marker of placental health in adverse events of pregnancy such as pre-eclampsia

To test this hypothesis, I measured serum concentrations of DBP in a cohort of pregnant women at 1st trimester of pregnancy in which 50% of women went on to develop pre-eclampsia later in pregnancy, while the others had normal healthy pregnancies.

4.3.2 Effect of DBP levels in serum from pregnant women on matrix invasion by trophoblast cells

Serum from 14, 1st trimester uncomplicated pregnancies was used for this experiment. All samples had known concentrations of DBP, 1,25D and 25D previously reported. Each serum sample was used to produce 2% donor-specific cell culture medium for use in Matrigel™ transwell invasion assays as previously described for the analysis of matrix invasion in the presence of mouse serum (**Figure 4.23A**). Recruitment of pregnant women and collection of serum samples was part of a previous study [381], according to Ethical Approval 14/WM/1146 (obtained from West Midlands - Edgbaston Research Ethics Committee), which included permission to use the samples in the invasion assays used in this study. The demographics of serum donors are shown in **Table 4.1**.

Table 4.1 Data for 1st trimester pregnant women serum donors.

Patient recruitment details	**1st trimester pregnancy patients (n=14)**
Maternal age, median (25th-75th interquartile range [IQR])	27.0 (21-33)
BMI, median (25th-75th IQ range), unit	24.4 (21.3-33.8)
Vitamin D supplementation	0
Positive smoking status,	0
Gestational age at delivery, median (25th-75th IQR), week	10.6 (8.70-11.4)
Ethnicity (White: BAME)	14: 0

The serum concentration of vitamin D metabolites and DBP for the 14 serum samples from pregnant women are shown in **Table 4.2**

Table 4.2 Serum concentrations of DBP, 1,25D and 25D in 14 pregnant women. Data are shown for serum DBP (mg/L), serum 1,25D (pg/mL) and serum 25D (ng/mL). Corresponding Matrigel™ invasion data for JEG3 cells cultured in medium containing the 14 donor serum samples are shown as Crystal Violet absorbance data (AU) and % invasion values. Data are the mean of duplicate assays.

Sample	DBP (mg/L)	1,25D (pg/mL)	25D (ng/mL)	Invasion absorbance (AU)	% invasion
PL143	266.4	87.5	9.2	0.299	77.9
PL141	221.2	33.3	8	0.250	60.6
PL146	183.3	33.3	3.2	0.236	55.8
PL140	165.0	33.3	10.4	0.219	49.6
PL134	163.9	50.0	8	0.229	53
PL147	122.3	20.8	3.6	0.210	46.5
PL161	118.0	75.0	19.6	0.248	59.9
PL164	89.9	37.5	11.6	0.213	47.3
PL163	77.9	45.8	34.4	0.201	43.1
PL173	70.3	87.5	18	0.243	58.1
PL125	70.3	150.0	18.8	0.265	66
PL150	42.1	41.7	11.2	0.201	43.1
PL160	41.5	25.0	11.6	0.195	41
PL171	35.6	145.8	9.6	0.217	49

When used in Matrigel^{TM} transwell invasion assays with identical numbers of cells seeded in each well, JEG3 cells showed varying levels of matrix invasion (see **Table 4.2**). These differing levels of trophoblast invasion were related to donor serum concentrations of DBP, 1,25D and 25D. Data in **Figure 4.34** showed that serum DBP was correlated with JEG3 matrix invasion (R=0.6466, p =0.0125). Serum 1,25D showed a trend towards increased invasion but serum 25D showed no correlation to matrix invasion by JEG3 cells. Because previous findings in this project indicated that 1,25D could enhance JEG3 matrix invasion, it was proposed that both serum DBP and 1,25D could contribute to the response of JEG3 cells. A serum sample with high levels of DBP might nevertheless have a low level of 1,25D, and *vice versa*. To address the combined effects of DBP and 1,25D, serum concentrations of DBP were adjusted to normalise to the highest level of 1,25D in the current cohort of samples to determine what effects would occur if all samples had the same level of 1,25D but variable DBP. Data in **Figure 4.34** showed that when serum samples were normalised for 1,25D there was a very close correlation between serum DBP levels and matrix invasion by JEG3 cells (R=0.9299, P<0.0001). By contrast, if DBP levels were normalised to 25D, the correlation was lost (R=0.4867, p=0.0776).

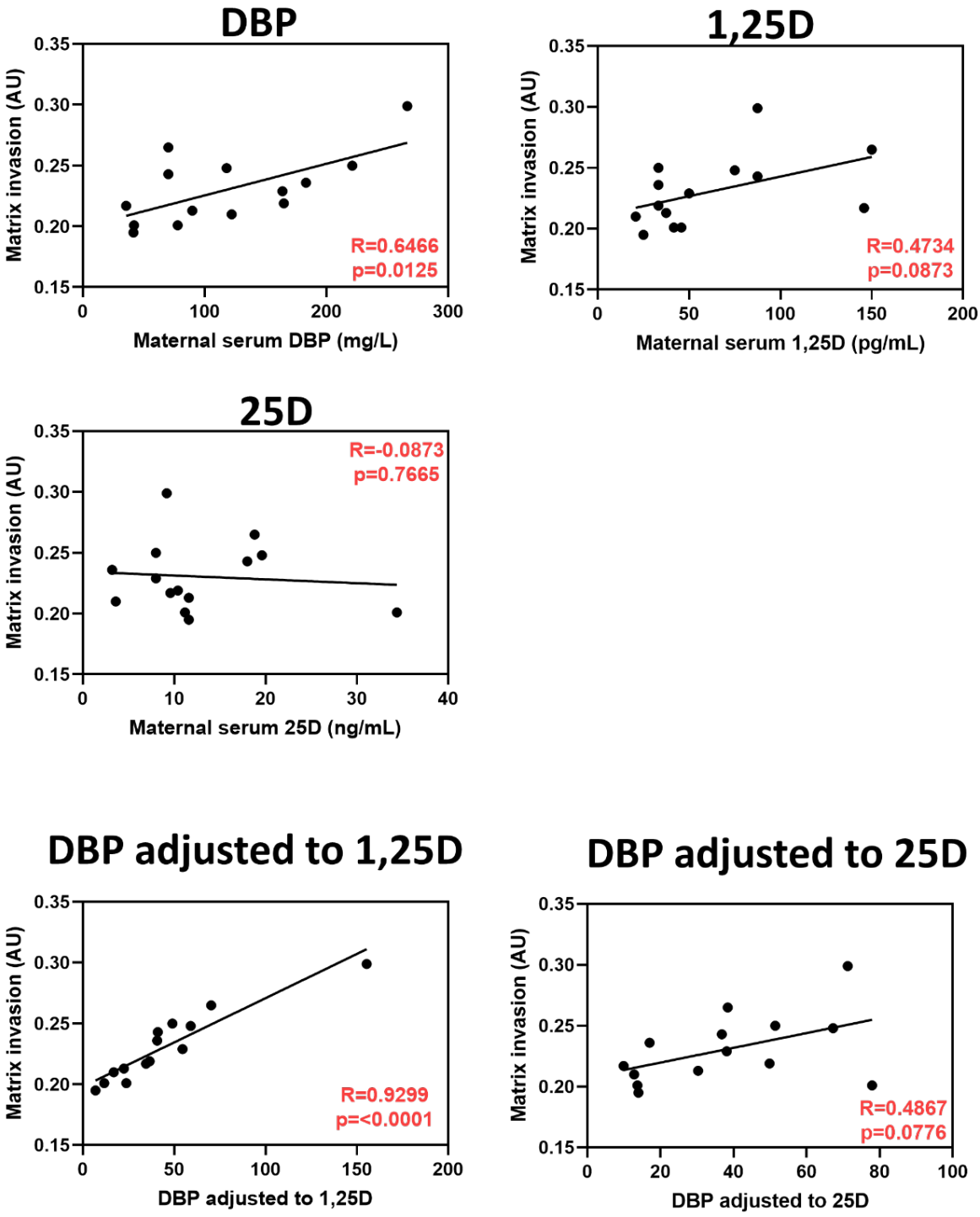


Figure 4.34 Relationship between pregnancy serum DBP, 1,25D and 25D concentrations and matrix invasion by JEG3 cells.
Correlation of serum DBP, 1,25D, 25D, DBP adjusted for 1,25D and DBP adjusted for 25D with invasion on Matrigel™ by JEG3 cells for n=14 pregnancy serum samples. R and p-value are written corresponding to the graphs.

4.3.3 Relationship between serum DBP levels of 1st trimester pregnant women and the risk of pre-eclampsia

To further investigate the possible effect of DBP on human pregnancy, I investigated whether circulating DBP levels were different in women at an early stage of pregnancy who later developed an adverse event in pregnancy associated with impaired placental development. In this case, 48 donor serum samples from women at 1st trimester of pregnancy were used, 24 of whom went on to have normal healthy pregnancies, and 24 of whom went on to have pregnancies complicated by pre-eclampsia.

Matched serum and urine samples were purchased from the SCOPE (Screening for Pregnancy Endpoints) Ireland study (Clinical Research Ethics Committee [REC] of the Cork Teaching Hospital: ECM5 [05.02.08 approval]). The SCOPE study was conducted according to the Declaration of Helsinki guidelines. Appropriate Health Research Authority - West Midlands, Edgbaston REC (14/WM/1146 RG_14-194 [09.12.2016 approval]) and material transfer agreement (MTA) (15.04.2016 15-1386) approvals were acquired by the University of Birmingham (UoB) prior to shipment (June 2016). As with the previous part of the project, serum levels of 1,25D and 25D had already been reported by our group [108]. However, these serum samples had not been analysed for DBP, and this was therefore carried out as part of my project.

Patient details have been adapted from Tamblyn et al., paper [108] as shown in **Figure 4.35.**

Figure 4.35 Showing the demographics of the study participants for the 1st trimester serum samples from healthy and pre-eclamptic pregnancies.

	Healthy n=24	Pre-eclampsia n=24
Maternal age, years (range)	30.5	31
	(24.0 – 38.0)	(22.0 – 36.0)
Body mass index, median (25th-75th IQR), unit	26.2	25.5
	(22.9-29.2)	(22.9- 29.7)
Ethnicity white Caucasian, frequency (%)	25 (100)	25 (100)
Mean arterial blood pressure, median (25th-75th IQR), unit	92.7	117.3****
	(89.3 – 96.7)	(113.8 – 124.8)
Vitamin D supplementation (400iu daily); pre-pregnancy		
total (%), 1st trimester total (%)	10 (40.0)	5 (20.0)
Season at recruitment (15 weeks): summer, total (%); winter, total (%)	9 (36.0)	3 (12.0)
Positive smoking status at 15w, total (%)	10 (40.0) 15 (60.0)	11 (44.0) 14 (56.0)
Gestation at PET diagnosis, mean (range), week	2 (8.0)	4 (16.0%)
Term PET (gestation ≥ 37w), frequency (%)	-	37 (31-41)
Preterm PET (gestation < 37w), frequency (%)	-	14 (56.0 %)
Severe preterm PET (gestation < 34w), frequency (%)	-	11 (44.0)
Gestational age at delivery, mean (25th-75th IQR), weeks	-	1 (4.0)
Fetal birthweight, median (25th-75th IQR), grams:	41.0 (40.0-41.0)	39.0 **** (37.0-40.0)
Fetal small for gestational age, frequency (%)	3650 (3275 – 4040)	3030 ** (2580 – 3535)
Stillbirth, frequency (%)	0 (0)	1 (4.0)

Table 4.3 1st trimester pregnancy serum samples with DBP, 25D and 1,25D values, from healthy pregnancies.
n=24 samples, each sample quantified in duplicate.

Patient ID	DBP (mg/L)	1,25D (pg/mL)	25D (ng/mL)
5457	692.8	111.3	2.8
7703	902.3	104.1	44.84
7811	997	140.1	22.2
5804	907.4	305.4	6.28
5836	854	205.2	3.96
5838	766.7	287.1	7.24
5051	742.7	92.1	17.88
5966	819.4	100.5	10.96
6280	701.8	175.5	25.56
6434	775.7	189.0	8
6454	651.1	117.3	23.44
8210	771.4	210.9	20.28
6581	1113.4	66.0	21.96
6695	863.4	41.1	13.56
8320	1124.4	Not-known	3.48
6846	917.6	119.7	30.12
7428	942	212.7	25.28
6856	865.8	221.4	8.32
7445	854.7	300.0	14.88
5447	791.8	113.1	18.4
8497	925.5	197.1	1.76
8509	1089.8	83.1	5.6
8558	879.1	126.3	26.32
8538	831.9	140.4	33.48

Table 4.4 1st trimester pregnancy serum samples with DBP, 25D and 1,25D values, from pre-eclampsia pregnancies.
n=24 samples, each sample quantified in duplicate.

Patient ID	DBP (mg/L)	1,25D (pg/mL)	25D (ng/mL)
7887	779.3	126.3	18.92
7810	703.4	174.9	20.28
7886	546.2	226.8	24.36
5971	811.1	165.6	22
7526	686.5	142.8	11.32
7527	640.1	123.0	17.24
7836	776.1	131.1	18
7979	491.5	159.3	18
6187	704.2	147.3	21.96
6165	900	136.8	13.24
6210	730.9	171.0	26.72
6666	673.5	245.4	7.96
6711	662.5	211.8	22.92
6822	527.3	101.4	6.52
5185	596.1	232.2	20.32
6816	796.6	374.6	12.44
5445	778.9	Not-known	4.24
6916	644	184.8	8.52
6994	844.5	108.6	16.12
8483	679.8	170.1	14.36
8516	671.2	114.0	6.28
7246	710.1	162.0	11.4
7217	517.5	166.8	5.72
7354	721.5	205.5	8.44

Serum concentrations of DBP were significantly lower in women who went on to have pregnancies complicated by pre-eclampsia relative to those who went on to have a normal healthy pregnancy (**Figure 4.36**). The mean DBP level for healthy pregnancy outcome was 865.9 mg/L, (95% CI 812.7 and 919.1). For pre-eclampsia pregnancy outcome, the mean DBP level was 691.4 mg/L (95% CI 647.2 and 735.6).

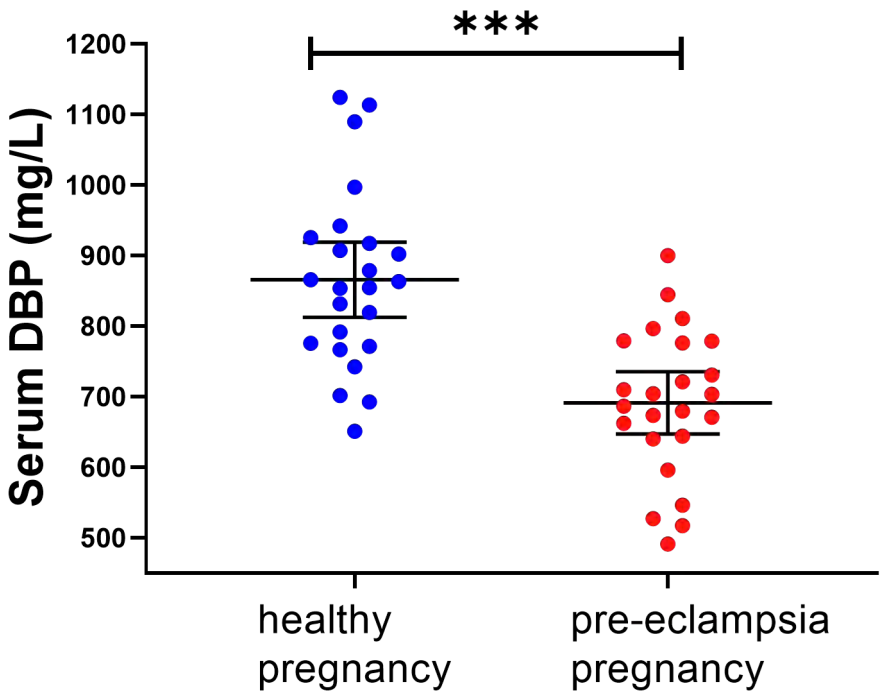


Figure 4.36 1st trimester serum DBP level in subsequent 3rd trimester healthy pregnancy delivery and pre-eclampsia pregnancy delivery.

Concentrations of DBP in serum from 1st trimester pregnant women who went on to either have a healthy pregnancy or develop pre-eclampsia. n=24 in each group. Each group shows mean with 95% CI. p=***=0.0002.

4.3.4 Conclusion

Serum DBP has been consistently observed as a potential key marker for pregnancy health. For years, research has focussed only on 25D levels in pregnant women, however, even with sufficient 25D levels during pregnancy, some studies could not establish a correlation between adverse pregnancy outcomes and 25D levels [289]. Higher serum DBP and serum 1,25D has been observed to influence increased invasion by trophoblast cells grown on matrix (**Figure 4.34**), which further anchors the placenta to the decidua, preventing placental detachment and miscarriage.

According to American Mayo clinic, severe vitamin D deficiency (based on 25D concentrations) is demarcated by serum levels lower than 10 ng/mL (25 nmol/L) [423]. When **Table 4.3** is studied separately, there are nine patients with very low 25D levels (<10 ng/mL) classifying them as severe vitamin D deficient [423]. However, when the DBP level is studied for the same patients, these values are among the highest in the list. And, interestingly, every patient in **Table 4.3** had a successful pregnancy. A similar observation can be made in **Table 4.4.** 25D level was less than 10 ng/mL in seven patients in **Table 4.4**.

Figure 4.36 establishes that DBP might have a significant role from the beginning of pregnancy (1st trimester), which further determines the placental growth and pregnancy complications. A similar result has been shown by Sorensen *et al.* [424], that lowered 3rd trimester DBP levels were an accurate predictor for babies who later developed type 1 diabetes. This study also showed a direct correlation of 25D levels with pregnancy complications in established low serum vitamin D patients [425]. In the

following discussion section of the thesis, a detailed explanation connecting DBP and early pregnancy development will be elaborated to further clarify this theory.

Chapter 5: DISCUSSION

5.1 Vitamin D system expression in placental tissues

Our laboratory has previously shown that 1st trimester decidua is abundant in the expression of *CYP27B1* and *VDR*, compared to 1st trimester placenta [105]. However, decidual *CYP24A1* remained unaltered when compared to placental *CYP24A1* expression [105]. My data contributed to this study by showing the colocalisation of both CYP27B1 and VDR proteins in placental villous syncytiotrophoblast layer. In 1st trimester placenta, CYP24A1 protein was shown to be less expressed compared to 3rd trimester placenta. This was interesting because it suggests that there is lowered catabolism of 1,25D in 1st trimester of pregnancy. However, since we have not measured the expression of 1,24,25D in these placental tissues, we could not confirm this hypothesis. Lowered CYP24A1 is probably a necessary step to ensure an abundance of 1,25D is retained for decidual and trophoblast function initiating from 1st trimester, which leads to peak levels of 1,25D in the 3rd trimester at maternal-foetal interface [426]. Foetal serum 1,25D levels are low, compared to maternal serum 1,25D levels, which returns to normal post-delivery of the baby [427]. Thus, with CYP24A1 protein being low/absent in trophoblasts, the maternal abundance of 1,25D in placenta might help in placental and foetal development and also immunomodulatory functions [428, 429]. Multiple studies have shown placenta as a primary extra-renal site for 1,25D production [430], demonstrating the significance of vitamin D in pregnancy.

My data showed that Similar to mRNA expression of *CYP24A1,* protein expression of CYP24A1 (measured as Corrected Total Cell Fluorescence (CTCF), arbitrary units) also showed a trend towards low 1st trimester expression (mean 25.33, 95% CI of 19.60 and 31.07), compared to 3rd trimester protein expression (mean 28.67, 95% CI of 13.69

and 43.64), which is similar to findings by other researchers [105] [166]. VDR expression was observed to be reduced in 3rd trimester compared to 1st trimester, similar to previously reported studies [105]. This may demonstrate the increased requirement of VDR at the beginning of pregnancy, for vitamin D functions. However, in contrast to a previous study [105], CYP27B1 protein expression remained unchanged across gestation, suggesting that conversion of 25D to 1,25D is required throughout the pregnancy for foetal development and pregnancy progression. Due to the unprecedented circumstances posed by the COVID-19 pandemic, I was unable to observe DBP protein expression on placental tissues. Given the previously performed experiments highlighting the importance of DBP for trophoblast invasion, it would have been interesting to observe if there was any change in DBP protein expression across gestation. A previous study showed DBP is expressed in placenta throughout gestation in normal pregnancies [166]. The authors also showed lowered expression of CYP24A1 in 1st trimester pregnancies, which is similar to my observed data. However, pre-eclampsia pregnancies expressed lowered DBP, lowered VDR and raised CYP24A1 expression [166].

5.2 Vitamin D system expression in trophoblast cells

Trophoblast cells cultured *in vitro* for this project were- JEG3, BeWo and HTR8. JEG3 and BeWo are trophoblast cells (choriocarcinoma cells) and HTR8 are extravillous cytotrophoblast cells. To explain the vitamin D system in these cells, we carried out an extracellular matrix culture. Studies have shown that Matrigel grown cell culture is more reflective of the *in vivo* environment than 2D monolayer plastic cell culture [431-433].

These studies also report that cells grown on matrix culture differ morphologically and physiologically, compared to plastic culture.

A mechanism of VDR action on trophoblast functions remains unclear. VDR has been reported to be expressed in human placenta at both mRNA [434] and protein levels [435]. VDR expression is also linked to regulation of human chorionic gonadotropin (hCG) secretion, placental lactogen expression, calcium transport and calbindin-D28k expression in the placenta [435-437]. *VDR* mRNA expression has also been detected in cultured human cytotrophoblasts, and in choriocarcinoma cell lines- JEG3 and BeWo [435-438]. Similar to my observation, relatively low expression of VDR has been reported for choriocarcinoma cells [439]. This may be due to epigenetic suppression of VDR, and VDR expression is restored by interfering with epigenetic gene suppression, by treatment with sodium butyrate and with 5-deoxy-3′-azacytidine, to inhibit DNA methylation and histone deacetylation [440].

On western blot and immunofluorescence analyses, VDR protein was not observed inside the nuclei of trophoblasts (both plastic and matrix grown cells). And this remained unchanged after 1,25D treatment. However, TPC showed a distinct nuclear VDR in both western blot and immunofluorescence, as reported by most other studies in various cancer cells [441-443]. *VDR* mRNA expression in trophoblast cells has been reported to be epigenetically suppressed when grown on the plastic environment [354]. The same study also showed the ability of 1,25D to activate the ERK pathway, suggesting that 1,25D does not function via a conventional VDR pathway in these cells.

While research has shown that nuclear VDR is vital for vitamin D's endocrine functions [444], other studies have highlighted non-nuclear VDR responses to 1,25D [147, 445].

A possible role for non-genomic responses to 1,25D was underlined by analysis of CYP24A1 expression. Unlike *VDR*, the *CYP24A1* gene is highly methylated in JEG3, BeWo and human placental trophoblast cells [391]. In the current study, *CYP24A1* mRNA expression was undetectable but CYP24A1 protein was detected by western blot. The explanation for this is unclear but it is possible that *CYP24A1* mRNA is present at very low levels and translation of this to protein is very stable. More important was the observation that even following treatment with 1,25D there was no induction of *CYP24A1* mRNA. In most cells that express VDR, induction of *CYP24A1* is a classical target cell transcriptional response to 1,25D [29]. This reflects the organisation of vitamin D response elements (VDRE) within the *CYP24A1* gene promoter [446] and provides a sensitive mechanism for limiting VDR exposure to 1,25D, which is catabolised to less active 1,24,25-trihydroxyvitamin D by 24-hydroxylase activity [107]. The lack of 1,25D-induced *CYP24A1* in trophoblast cells on plastic or Matrigel™ suggested that these cells have a completely different response to 1,25D. It could be postulated, that rather than use genomic VDR signalling, trophoblast cells respond to 1,25D via non-genomic mechanisms. This is discussed later with the induction of trophoblast ERK phosphorylation by 1,25D.

The lack of 1,25D-induced *CYP24A1* mRNA expression was restricted to trophoblast cells. However, expression of DBP in Matrigel™ cultures was common to all cells, including the TPC cells. This suggests that conventional plasticware cultures lack a

key component of vitamin D function. Previous studies have shown the importance of cellular uptake of DBP in both renal [74] and extra-renal actions of vitamin D [447]. However, it is important to recognise that most studies of vitamin D *in vitro* are carried out in the absence of matrix and therefore may not fully reflect the actions of vitamin D and components of the vitamin D system. This may be particularly important for the actions of vitamin D on trophoblast cells where a key function is to invade the decidua. In future studies, it will be interesting to determine the broader differences between plastic and Matrigel™ cultures of trophoblast cells. Recent studies have used transcriptomic technology such as RNA sequencing (RNAseq) to characterise trophoblast cells in different tissue sites [448-450]. These studies showed single cells trophoblast RNA-sequencing to understand the maternal-foetal RNA based communication. The authors differentiated the trophoblasts into cytotrophoblast, syncytiotrophoblast and invasive trophoblast (EVTs) for RNA-sequencing, and different gene expression was observed for EVT cells [450]. It would be interesting to carry out similar analyses using plastic and Matrigel™ cultures in the presence or absence of 1,25D.

In Matrigel™ cultures of cells (including TPC), the most obvious difference with plasticware cultures was the presence of DBP protein. DBP is present in the serum used to supplement cell culture growth medium for both plastic and Matrigel™ cultures. This initially suggested that the DBP in Matrigel™ cultures could have been due to endogenous *DBP* gene expression. However, the levels of mRNA for *DBP* were very low and Matrigel™ culture of cells cultured with DBP-/- serum completely eliminated cellular expression of DBP. This suggested that the DBP in Matrigel™ cultures is due

to cellular adhesion or uptake of DBP when cells are cultured on a matrix. The results also suggested two possible explanations. Firstly, it is possible that in Matrigel™ cultures, DBP simply adheres to cells better. The second possibility is that DBP is actively taken up by cells cultured on the matrix. The data presented in this thesis suggest that the latter is correct. When treated with 1,25D, DBP appeared to localise predominantly around the nuclei of cells (see **Figure 4.4**) indicating that DBP is not simply restricted to the outside of cells, and also that translocation of DBP is a dynamic process that is stimulated by 1,25D (see **Figure 4.7**). Most important of all, when the megalin receptor for DBP was blocked using RAP, this eliminated DBP expression in cells. RAP has been used previously to block uptake of DBP and vitamin D metabolites by breast cancer cells [451], and the data in this thesis show for the first time that a similar mechanism occurs in trophoblast cells. Taken together, these observations demonstrate two new mechanisms for vitamin D function in trophoblast cells: 1) when cultured on matrix, trophoblast cells take up DBP from surrounding serum; 2) this process is stimulated by 1,25D. Subsequent experiments were aimed at investigating firstly the cellular impact of DBP uptake and, secondly, the mechanism by which 1,25D influences DBP uptake and function.

5.3 Cellular proliferation, migration, and invasion

Initial characterisation of trophoblast cell responses focused on fundamental proliferation and migration responses when cells were cultured on plasticware or Matrigel™. TPC cells showed decreased proliferation and migration following 1,25D treatment, which is consistent with other studies of 1,25D and cancer cells [452-454], and consistent with classical nuclear VDR signalling. By contrast, when cultured on

either plastic or matrix, trophoblast cells showed no anti-proliferation or migratory response to 1,25D. This was consistent with the lack of *CYP24A1* induction described earlier. Reports have suggested that VDR expression is epigenetically suppressed in choriocarcinoma cells, which results in a lowered classical nuclear activity of VDR in these cells [439]. Instead, it is possible that in trophoblast cells 1,25D may signal via non-genomic mechanisms, as initially proposed by Nemere *et al*. [455]. Here the authors showed a membrane-associated protein with high affinity for 1,25D, which mediates rapid non-genomic effects of vitamin D. These non-genomic actions of 1,25D may occur even in the absence of a nuclear VDR [456], thus speculating that another form of VDR exists which mediates non-genomic action. Data in this thesis have expanded these results by suggesting that a similar non-genomic vitamin D pathway exists in trophoblast cells. Immunofluorescence showed a distinct non-nuclear VDR protein in all three choriocarcinoma cells (compared to TPC which showed distinct nuclear VDR). VDR protein was observed outside the nucleus in both plastic grown, and matrix grown trophoblast cells.

In addition to its classical nuclear form and VDRE-mediated transcriptional actions [29, 147], VDR has also been shown to support 1,25D mediated activation of p38, ERK1/2 and PI3K/Akt pathways [457]. This study was done on skeletal muscles, which does not produce 1,25D, and expresses nuclear VDR. Thus, VDR function varies on specific tissues, depending on its location. VDR has also been shown to be present at the cellular membrane as mVDR (also known as MARRS- membrane-associated rapid response steroid), this protein is located at the plasma membrane and binds to caveolin-1 and phospholipase A2 [155, 458]. Previous reports have shown that 1,25D

can cause activation of various signalling pathways including MAP kinases-extracellular signal-regulated protein kinase (ERK1/2, ERK5), c-Jun N-terminal Kinase (JNK1/2), phosphatidylinositol 3-kinase (PI3K), phospholipase A2 (PLA2), p21ras and protein kinase C [459-464]. In the current project, we specifically studied the ERK pathway as the placenta has been shown to express ERK1 and 2 in the villous cytotrophoblast cells. However, their activated forms (phospho-ERK 1/ 2) are present only until the 12th week of gestation, implying the significance of phospho-ERK in early pregnancy [465]. Data presented in this thesis show that 1,25D actively stimulates ERK phosphorylation and nuclear localisation of pERK in trophoblast and TPC cells. However, the trophoblast cells were distinct from the TPC cells in that this nuclear translocation of pERK is initiated at a much earlier stage (30 mins). Further work is required to determine the precise timescale for ERK phosphorylation in the different cell types, and also whether other kinases are involved in trophoblast responses to 1,25D. For these studies, unbiased kinase arrays would be an important future strategy. Nevertheless, based on the observations from this project, my thesis suggests that trophoblast and TPC cells have different signalling responses to 1,25D. TPC cells show classical nuclear VDR and delayed ERK kinase signalling which leads to anti-proliferative and anti-migration responses. By contrast, trophoblast cells show non-nuclear VDR action and a rapid ERK pathway signal, and no proliferation or migration response to 1,25D. The next experiments used matrix cultures to determine if these differences in 1,25D signal also affected cell invasion.

Trophoblast invasion is modulated by multiple factors like oxygen concentration [466], transforming growth factor-ß (TGF-ß) with IGF-II and IGFBP-1 [467, 468], epidermal

growth factor (EGF) [469], and hepatocyte growth factor (HGF) [470]. Data from this project indicate that 1,25D is also a potent stimulator of trophoblast invasion. Similar results have been described previously by our group [317] and others [410], but the current study is the first to contrast the invasive effects of 1,25D on trophoblast cells versus TPC cells. This showed that the classical VDR genomic effects observed in TPC cells resulted in the suppression of Matrigel™ invasion, similar to the inhibitory effects described previously for other cells [406, 471]. It was therefore interesting to observe the opposite effect of 1,25D on matrix invasion by trophoblast cells. In contrast to TPC cells, the effects of 1,25D on trophoblast invasion did not appear to involve normal VDR signalling, as knockdown of *VDR* expression had no effect on the pro-invasion effects of 1,25D in JEG3 cells. In previous studies Yamauchi *et al.,* demonstrated that *VDR* knockout had no effect on ERK (MAPK) pathway in response to 1,25D [456]. In contrast, a study showed that cellular oestrogen level regulates VDR expression via activated ERK1/ 2 (phospho-ERK 1/ 2) [472]. There is a VDR independent pathway for regulating *calbindin-D28k* and *CYP27B1* gene expression in primary placental trophoblast cells and JEG3 cells [437, 473]. JEG3 cells were used for the siRNA *VDR* experiment in this project because these cells represent EVT endocrinologically and functionally [474]. Because of the induction of trophoblast ERK phosphorylation by 1,25D, we, therefore, hypothesised that the matrix invasion effects in trophoblast cells involved pERK.

Other kinase pathways such as the MAP kinase pathway have been suggested to play a crucial role in trophoblast migration. HTR8 cells have shown migratory effect by induction of insulin-like growth factor-II (IGF-II) [475] and (insulin-like growth factor

binding protein-1) IGFBP-1 via phosphorylation of ERK1/ 2 [476]. These studies showed that blocking the ERK pathway (by U0126) in these cells reduced cellular migration and abolished the effects of IGF-II and IGFBP-1. A similar observation was made with EGF and HGF which promoted HTR8 migration by activating the ERK kinase pathway, and inhibition of the pathway resulted in reduced motility of cells [477-479]. In choriocarcinoma cells, angiotensin-II and VEGF influences trophoblast proliferation via ERK-dependent signalling pathway [480-482].

Data from this project shows that blocking the ERK pathway with U0126, resulted in inactivation of pERK and reduced trophoblast invasion. However, it is important to note that inhibition of pERK potently suppressed matrix invasion even in the absence of 1,25D, with 1,25D having a very modest effect in stimulating U0126-suppressed invasion. Based on this we concluded that ERK kinase is required for 1,25D invasion responses in trophoblast cells, but it also has a more fundamental role in promoting matrix invasion by cells in general. Further studies indicated that this effect is mediated via cellular uptake of DBP and this will be assessed later in the discussion.

Cellular matrix invasion is associated with changes in various factors, which are either upregulated or downregulated to achieve invasion. Some of these genes are matrix metalloproteinases (*MMPs*) [209], vimentin [483], urokinase-type plasminogen activator *(uPA)* [484], migration and invasion enhancer 1 (MIEN1) proteins [485]. TIMP1 is downregulated in invasive cells [209]. Thus, to clarify our observation of increased invasion of JEG3, BeWo and HTR8 cells following 1,25D addition, these cells were tested for *MMP2* and *TIMP1*. *MMP2* was observed to be highly upregulated

and *TIMP1* was significantly downregulated following 1,25D addition in trophoblast cells. However, TPC showed the opposite, which correlated with lower invasion in these cells after 1,25D treatment. MMP2 expression was further clarified with gelatin zymography. Expression levels and activation of MMP proteins has been widely studied by gelatin zymography [486] because MMP proteins are potent gelatin degrading compounds, which gives near accurate data of its activity. Our study showed that active MMP2 is highly expressed in trophoblast cells, with higher activity in BeWo cells. This is similar to a previous study which showed BeWo cells to be a significant positive control for MMP2 activity [487].

Trophoblast JEG3 cells continued to show increased Matrigel^{TM} invasion following the addition of 1,25D, despite blocking of *VDR* expression with siRNA. In contrast, TPC cells showed a reduction in matrix invasion with *VDR* siRNA. This demonstrates that blocking the conventional VDR pathway in TPC cells causes a reduction in the invasive capability of this cancer cell. A similar mechanism has been observed in various other cancer cell studies like breast cancer [488-490], prostate cancer [491], oral cancer [492], and colon cancers [493, 494]. A study using a mouse model [495], showed that knocking down *Vdr* (*Vdr* -/-) in the female mouse, resulted in reduced ability to conceive, less number of viable foetus with lower body weight, and increased placental inflammation [496]. Another study showed that female mice with vitamin D deficient diet showed reduced placental size [497]. These data suggest that the maternal decidua may mediate the impact of low vitamin D levels on trophoblast cells during pregnancy, in turn reducing placental development and impaired foetal growth [497, 498]. Collectively, these observations suggest that although VDR does not appear to

affect the trophoblast model studied in this PhD, it may still be important for other aspects of implantation and placental development, possibly through effects within the maternal decidua.

5.4 Vitamin D binding protein (DBP) in trophoblast cells

Data from this project indicate that DBP plays an important role in trophoblast invasion. Although this part of the vitamin D system has not previously been studied with placental development, nonetheless studies have shown the presence of DBP in trophoblast cells isolated from human placenta [499]. DBP, along with vitamin D metabolites has been detected in both primary placental tissues and trophoblast cell lines [177, 500-502]. In placental trophoblast cells, DBP is commonly expressed on the cell surface during normal human pregnancy [501]. This observation led to the explanation of increased maternal serum DBP level during pregnancy, which could be the result of highly proliferating trophoblasts which are in direct contact with maternal blood [50]. Similar to my data, researchers have also demonstrated an intracellular vitamin D binding protein (iDBP) which is entirely distinct to DBP [503]. Cell surface DBP is a cysteine-rich steroid-binding protein which is principally confined to the extracellular domain [504, 505]. However, iDBP is cysteine poor and is not confined to the cytoplasm [505]. Both of the above types of DBP have been shown to bind to 25D but, unlike DBP, iDBP appears to preferentially bind and transport 1,25D [506]. My study has shown that DBP, a serum vitamin D binding globulin, can also act at an intracellular level and influence placental trophoblast functions. It would have been interesting to determine the precise amounts of DBP internalised from serum used in the culture medium. Previous studies have shown that this can be carried out using

fluorescently-tagged DBP [366]. However, due to lack of time and earlier than expected termination of laboratory work (because of the COVID-19 pandemic), I was unable to perform this analysis.

DBP can also influence expression levels of placental aminotransporters which regulate amino acid transfer to the foetus, via the microvillous and subsequently through the syncytiotrophoblast during foetal development [507, 508]. Animal studies have shown that abnormal amino acid transfer results in pregnancy complications and foetal abnormalities [509]. This suggests that delivery of vitamin D and DBP to the foetus contributes to the transport of other factors across the placenta. In addition to this role, placental origin DBP forms a target for maternal auto-antibodies formed in pre-eclamptic women [510], suggesting that maternal serum DBP could be a marker of pregnancy complications. This was explored in further detail in the final part of the project. However, the key objective of this PhD was to investigate the cellular impact of vitamin D and DBP on trophoblast cells.

5.5 DBP, megalin and trophoblast cell function

The ‘free hormone hypothesis’ suggests that, due to its lipophilic property, only non-bound/free serum 25D is capable of entering cells and initiating biological functions [49]. However, a mechanism has been identified which enables cellular uptake of DBP and the delivery of its 25D/1,25D cargo to its receptor, VDR. This mechanism involves the receptor combination of megalin/cubilin, which was discovered by Nykjaer A *et al.,* in 1999 [77]. They showed that 25D bound to DBP is carried inside the proximal convoluted tubules of the kidney via the endocytic receptor, megalin. Endocytosis of

25D-DBP is necessary to mediate the conversion to its active form 1,25D and thereby increasing its local availability [77]. They also found a significant loss of 25D and DBP in the urine of megalin knockout mice, demonstrating that the 25D-DBP complex was not endocytosed in the absence of megalin. The mice also developed vitamin D deficiency symptoms [77]. Lowered megalin expression in human syncytiotrophoblast cells due to placental malarial disease, was reported to result in low birth weight deliveries [511]. Placental malaria resulting in placental inflammation is similar to Heymann nephritis in the kidney, which leads to shedding of the megalin exodomain [512]. This might suggest a significant role of megalin in pregnancy health. Blocking megalin with RAP involves inhibition of megalin binding to all its ligands including DBP, resulting in lowered expression of megalin at the cell membrane [513]. Literature shows that RAP results in lowering of more than 90% of megalin expression, which was demonstrated using immunohistochemistry, immunoblotting and chromatography [514] the mechanism is largely unknown. This PhD project (**Figure 4.18**) shows a similar finding of absence of megalin expression after treatment with RAP, demonstrated using immunofluorescence study. Beginning from the 8-cell stage of embryo development, megalin-cubilin is expressed between the inner cells mass and the trophectoderm [515]. Following implantation, this receptor complex is expressed in visceral endoderm [516] which subsequently becomes the yolk sac, providing nutrients to the foetus for development of the digestive system [515, 517]. Animal studies have shown that megalin knockout mice produced litters with brain malformation, neural defects [518, 519], proteinuria, low bone density and rickets [77]. These data suggest that megalin is an important factor in the placenta and foetal development but, until now, the assumption was that megalin acted to transport vitamin D metabolites or other

cargoes across the placenta. Data from this PhD project suggest that DBP may be an equally important cargo for megalin in the placenta.

Experiments in this project used two different approaches to confirm DBP uptake by trophoblasts. Serum from DBP knockout mice and, in DBP abundant FBS cultures we blocked trophoblast DBP uptake using the megalin inhibitor RAP. Interestingly, both these methods blocked trophoblast matrix invasion. The use of serum from DBP knockout mice was similar to another study which used monocytes culture [366]. Here the authors showed that lack of serum DBP enhanced monocyte responses to 25D and 1,25D because it appears that these cells utilise unbound free-25D [520]. In contrast to trophoblast cells, monocytes do not express megalin or cubilin and do not show endocytic uptake of DBP [366]. Therefore, the culture of trophoblast cells on Matrigel^{TM} provides a model that is quite distinct from other cell model targets for vitamin D. Confirmation of cellular uptake of DBP was also carried out by inhibiting megalin function using RAP. This completely blocked cellular expression of DBP and, like DBP knockout serum, resulted in decreased trophoblast Matrigel^{TM} invasion. The importance of DBP for matrix invasion was further underlined by the actions of the ERK kinase inhibitor U0126 which also blocked DBP uptake and matrix invasion. This allowed us to produce the mechanistic model shown in **Figure 4.22**, which proposes a cooperative function for DBP and 1,25D in promoting trophoblast matrix invasion. In this case, megalin-mediated uptake of DBP is a fundamental driver of trophoblast invasion with 1,25D non-genomic induction of pERK action to enhance this process. Based on this model, the next objectives of the project were: 1) to determine how

intracellular DBP impacts trophoblast matrix invasion; 2) to determine how serum DBP and 1,25D could influence human pregnancy health.

5.6 DBP and intracellular actin binding

Although DBP is the principal serum carrier of vitamin D metabolites, it also fulfils several other functions including possible vitamin D-independent functions. These include a role for DBP as a macrophage-activation factor [412], and in fatty acid transport [413]. DBP also binds with the monomeric, globular, form of actin (G-actin) with high affinity [521, 522], allowing DBP to out-compete other established actin-binding factors such as gelsolin that act to incorporate G-actin into filamentous actin (F-actin) [414]. In this way, DBP can also function as an actin-scavenger, with a potential role in protecting against tissue damage due to systemic F-actin accumulation [415], although the DBP-actin complex may also fulfil a pro-inflammatory role as a neutrophil chemotactic factor [416]. Based on these previous studies, we hypothesised that the uptake of DBP by trophoblast cells could be linked to the balance between G- and F-actin not only in the circulation but also at an intracellular level. Both G- and F-actin interact with numerous proteins, and this is known to be crucial for the maintenance of cell structure and changes in cell conformation [523], as well as facilitating cell motility [524-526].

Some of the proteins required to induce actin polymerisation to form F-actin are, Wiskott-Aldrich syndrome protein (WASP), CDC42, profilin, and thymosin [523], while capping protein, gelsolin and cofilin, terminate the polymerisation process. F-actin can also undergo depolymerisation which involves dissociation of the γ phosphate,

ADF/cofilin proteins, leading to debranching of F-actin [523]. F-actin polymerisation continuous filament growth leading to membrane protrusion at a rate of 1-25 μm/min [527]. The barbed growth end of filaments at the leading edge is fed by subunits provided by repeated depolymerisation of filaments from the rear end of the lamellipodial extension [528]. This rear end remains at a constant length and acts as propulsion for cellular motility [529]. The continuous polymerisation reflects the rapid turnover of actin filaments which helps cellular movement. To move the cellular membrane forward at a speed of 10 μm/min, the individual barbed ends has to recruit 100 subunits [527]. This is 200-fold higher than the *in vitro* non-motile state F-actin turnover rate [527]. Along with F-actin formation, actin-binding proteins (ABPs) have also been shown to be important for trophoblast invasion through decidua [530]. Transgelin2 (TAGLN2) is one such ABPs which binds to F-actin, resulting in cellular migration and cytoskeletal stabilising properties, and is found in trophoblast cells [531]. Absence of TAGLN2, resulted in alteration and lowering in F-actin formation in trophoblasts, and failure of implantation in the mouse model [530].

Along with cellular motility and cellular interactions, actin also participates in non-cytoskeletal processes, such as transcription and chromatin modelling, however, this function is a poorly researched area [532]. One such example is actin mediated control of MAL/MRTF-A (myocardin-related transcription factor A) and MKL2/MRTF-B, which are myocardin family transcriptional coactivators, resulting in transduction of Rho GTPase signals to the transcription factor- serum response factor (SRF) [533, 534]. G-actin binds to the MRTF N-terminus, inhibiting its activity by suppressing transcriptional activation by MRTF-SRF complex [533, 535]. Actin has also been

shown to be of significance in trophoblast adhesion and invasion through decidua [530].

The various factors associated with actin polymerisation and depolymerisation are shown in **Figure 5.1.**

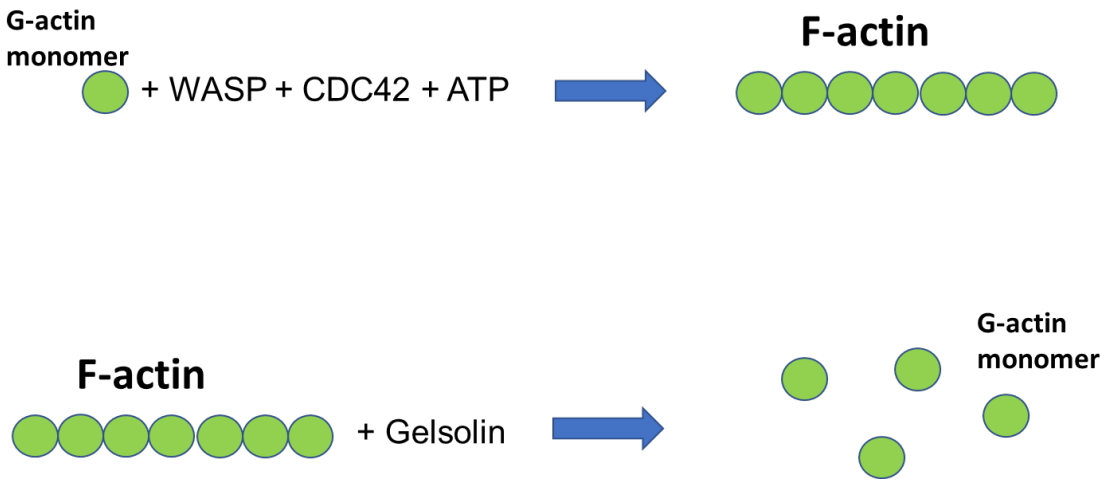


Figure 5.1 Mechanisms for actin polymerisation and depolymerisation.

Cytoplasmic F-actin has a very high affinity for phalloidin, whereas G-actin has a high affinity for DNase1 [71]. Consequently, phalloidin staining is used as a standard marker for intracellular F-actin, whilst DNase1 staining is used to identify G-actin. Previous studies have described dynamic changes in phalloidin and DNase1 staining during trophoblast cell fusion and differentiation [536]. In this study, treatment of BeWo cells with forskolin resulted in an overall decrease in cellular actin, but with a proportionally higher level of G-actin compared to F-actin [536]. In this project, the initial observation was that in JEG3 and HTR8 cells cultured on Matrigel^{TM} in serum-containing DBP (DBP+/+), F-actin was much more abundant than G-actin, with F-actin appearing to be

extra-nuclear. In the absence of DBP (DBP-/-), F-actin levels were decreased, with a concomitant increase in G-actin, principally within the nucleus. Thus, it can be concluded that G-actin can exist at multiple locations of cells. This has also been supported by studies showing that numerous proteins reside in more than one cellular compartment and are engaged in different compartment-related activities (also known as moonlighting proteins) [537-539]. Recently, a similar theory has been applied for G-actin and its nuclear location [540-542]. Studies have previously shown the free movement of G-actin between nucleus and cytoplasm [543]. However, some authors have explained this nuclear actin as an isomeric form of the actin-binding protein, which does not stain for phalloidin [544].

In view of the data from this project, we propose that in the presence of serum DBP, trophoblast intracellular DBP binds to cytoplasmic G-actin, which appears to limit nuclear localisation of DBP and increases its sensitivity to binding to ATP, leading to increased filament formation for F-actin. This appears to facilitate the trophoblast matrix invasion as described previously for invasive cancer cells [545]. Conversely, in the absence of DBP, G-actin is more able to translocate to the nucleus, resulting in less cytoplasmic monomeric G-actin, leading to lowered sensitivity to ATP. Thus, reducing the formation and expression of F-actin. However, it is also possible that the presence of actin within the nucleus may have direct effects on nuclear function in trophoblast cells. Studies using other cell types have shown that nuclear actin exerts distinct effects on transcription which may affect cell differentiation and function independent of cytoplasmic cell function [546, 547]. It is, therefore, possible that changes in cellular DBP have a significant effect on trophoblast invasion of the matrix

by either altering G-actin binding and cytoplasmic F-actin formation or by modulating nuclear function by regulating nuclear access of G-actin. Previous studies have underlined the importance of G-actin/F-actin homeostasis in trophoblast differentiation [548] and implantation [549]. Data from my PhD project suggest that DBP may be a master regulator of this process in trophoblast cells.

Trophoblast intracellular actin homeostasis was strongly influenced by the presence of DBP in the cell culture medium. However, even in the presence of exogenous DBP, if this is unable to be internalised by the trophoblast cells the eventual effect is similar, with increased nuclear G-actin. Thus, the ERK kinase inhibitor U0126, which blocks DBP uptake, also enhanced nuclear G-actin in trophoblast cells, further underlining the pivotal role of DBP in regulating intracellular actin homeostasis and cell invasiveness. In the current project, I was unable to investigate G-and F-actin responses in cells treated with RAP to block megalin-mediated DBP uptake. Nevertheless, it can be predicted that RAP would also promote increased nuclear localisation of G-actin, with concomitant effects on matrix invasion. Previous studies have shown that decreased expression of trophoblast megalin may be a key contributor to low birth weight pregnancies in malaria infection [550]. In this case, it is not clear whether the pathological impact of decreased megalin is due to impaired DBP uptake or altered trophoblast invasion. Other studies have shown that *LRP2* gene (megalin) mRNA expression is elevated in placentas from preterm pregnancies, suggesting a role of megalin in pregnancy which requires future research.

As shown in **Figure 4.33**, the data from my PhD thesis, indicate that several components of the vitamin D system contribute to the capacity for matrix invasion by

trophoblast cells: 1) maternal serum concentration of DBP; 2) maternal serum concentration of 1,25D; 3) trophoblast ERK expression; 4) trophoblast megalin expression. In the final part of this PhD project, I explored how some of these components are involved in human pregnancy.

5.7 DBP and human pregnancy

The purpose of this PhD project was to understand the functions of various vitamin D metabolites and components of the vitamin D system on placental development, specifically the trophoblast function. *In viv*o, trophoblast cells are exposed to vitamin D metabolites in serum and within the decidual environment. Previous research by our group at Birmingham has shown that maternal serum DBP correlates with healthy pregnancy placental DBP [551]. This work also showed that correlation between maternal and placental DBP was disturbed in the pregnancy complication pre-eclampsia [551]. Based on the idea that serum levels of DBP directly impact placental (trophoblast) DBP, we designed an experiment where serum from normal healthy 1st trimester pregnancies was related to JEG3 invasion of Matrigel^{TM}. The significant correlation between serum DBP levels and JEG3 invasion suggests that DBP *in vivo* may be a predictor of the invasive potential of trophoblast cells in the placenta. The other variables in serum that could also affect trophoblast invasion are 1,25D and its precursor 25D. Serum 25D appeared to have no link to trophoblast invasion. This is not surprising as 25D is an inactive form of vitamin D and requires conversion to 1,25D via CYP27B1. Although CYP27B1 expression has been reported for trophoblast tissues [386, 387], this does not appear to be true for JEG3, BeWo or HTR8 cells. Thus, they would not be expected to synthesise 1,25D and achieve an effect. However,

it is possible that primary cultures of trophoblast cells have significantly higher levels of CYP27B1 and may respond to differences in serum 25D levels. In the current project, serum 1,25D showed a trend towards correlation with invasion. This was not statistically significant but by adjusting the DBP levels to incorporate both DBP and 1,25D concentrations, an improved correlation with JEG3 matrix invasion was produced. This suggests that DBP and 1,25D act together on trophoblast cells to optimise invasion potential as shown in **Figure 4.33**.

This project was carried out using only JEG3 cells, thus, megalin expression remained uniform for all the human serum experiments, suggesting the effect of variable DBP level in healthy and complicated pregnancy. 25D circulates in the serum bound to DBP, which vary significantly in individuals [64, 552]. There is also evidence that serum 25D levels may vary depending on DBP genotype [553], which varies according to race and country of residency [64]. Another study concluded that measuring 25D should not be the primary criterion for determining vitamin D status and that 25D function largely varies with DBP binding [554]. DBP could either prolong the half-life of 25D by acting as a reservoir or modulate its biological functions by altering free or bioavailable 25D [67, 555]. In clinical conditions, some individuals can be misclassified as vitamin D sufficient or insufficient by measuring total 25D serum level, with an undiagnosed DBP level. An example of a similar phenomenon can be observed in cirrhotic patients with lowered DBP production [554, 555]. These patients showed sufficient serum 25D levels, however, with lowered serum DBP level, the 25D-DBP complex was unable to enter the cells and perform its actions. Some researchers have suggested measuring

available 25D serum concentration combined with serum DBP concentration [554, 555]. Thus, DBP is a significant component while determining vitamin D status.

During pregnancy, bioavailable 25D concentrations are lowered as the pregnancy progresses with an increase in DBP values [551, 556, 557]. However, there is an increase of both 1,25D and DBP throughout pregnancy, beginning from the 1st trimester [556, 558]. The 1st trimester of pregnancy is associated with extra-villous cytotrophoblast invasion in the maternal decidua, which helps to anchor the placenta, for foetal and placental development and growth. A Scandinavian study measured DBP in healthy controls and compared resultant levels with pregnant women, cancer patients and chronic liver disease patients [552]. The authors found that in healthy controls, median DBP levels were around 305 mg/L, with a higher DBP level in women (332 mg/L) compared to men (288 mg/L). It was significantly increased during pregnancy, with median DBP level of 542 mg/L in 1st trimester and 574 mg/L in 2nd trimester [552]. These findings are similar to another study which also investigated 3rd trimester DBP values, which were 693 mg/L [559]. Thus, there is a consistent rise of serum DBP throughout pregnancy, initiating from the first trimester. In comparison, our study showed a mean of 866 mg/L DBP concentration in uncomplicated (healthy) 1st trimester pregnancy serum, compared to 691 mg/L in 1st trimester pregnancies that went on to develop pre-eclampsia. Our group in Birmingham had previously analysed multiple serum vitamin D metabolites in the same 1st trimester samples, but none of these metabolites was able to predict subsequent pre-eclampsia as effectively as DBP [382]. We, therefore, propose that DBP is an important new marker of a healthy

pregnancy, with higher levels of DBP in pregnant women being a possible indicator of pregnancy success.

Other than the data presented in this thesis, any alternative functions of DBP in pregnancy are unclear. Bouillon *et al.* found a positive correlation of 1,25D with DBP in women with progesterone supplements [558]. A significant increase in the progesterone level is observed in an uncomplicated (healthy) pregnancy, which might correlate with its protective role towards the embryo and the foetus [560]. Therefore, it can be concluded that in this situation, as with my study, DBP and 1,25D may act together to enhance pregnancy health. Recent studies have also shown that maternal DBP levels are linked with the health status of an ongoing pregnancy, and can predict foetal birth parameters or any adverse pregnancy outcomes [561]. Sorensen *et al.* demonstrated that low DBP status in 3rd trimester pregnancy, resulted in diabetes mellitus in the delivered foetus [562], the same way, maternal serum DBP is associated with gestational diabetes mellitus [563]. Other studies have suggested that DBP may be a potential serum biomarker for early-onset pre-eclampsia [564, 565] and idiopathic foetal growth restriction [566]. Development of the intrauterine environment for healthy foetal growth and subsequently to an uneventful delivery has also been correlated with DBP levels [567].

From the above studies, it can be proposed that DBP is an important pregnancy protective component and it can be considered as a potential biomarker for placental dysfunction and pregnancy complications. However, until this PhD project, the mechanism of action of DBP on trophoblast cells and placental health was unclear.

This PhD project has explained the mechanisms by which, both 1,25D and DBP exert their actions on placental cells. Both vitamin D components act in a coordinated fashion, even though serum 1,25D does not correlate with either serum DBP or 25D levels [556, 558].

5.8 DBP and infertility

Although this PhD project describes the relationship between DBP, vitamin D metabolites and trophoblast cell function, with a focus on pre-eclampsia, it is also possible that the mechanisms described here may affect other adverse events in pregnancy. For example, DBP has been linked to the pathogenesis of unexplained infertility. A study comparing 39 infertile (pre-menopausal) women with 29 fertile women, identified that DBP levels were lowered in the infertile group (400 mg/L) compared to a fertile group (530 mg/L) [568]. However, total serum 25D remained unchanged in both groups. This shows that DBP is important beginning from the pre-conception stage and it has potential as a biomarker for infertility.

A study by Pop *et al*. demonstrated that oestradiol is a significant regulator of DBP [569]. The authors observed that DBP and oestrogen were highly expressed in pre-menopausal women, as compared to post-menopausal women. They showed that serum oestradiol is independently associated with serum DBP level. This observation is significant because low oestradiol is associated with infertility, and may also reflect lowered DBP, although in women undergoing IVF treatment, changing oestradiol concentration throughout the menstrual cycle did not have any effect on serum DBP concentration [509]. Thus, measurement of vitamin D components such as DBP and

1,25D may help to predict pregnancy outcomes at an early stage and help healthcare professionals to make better arrangements for the progression of the pregnancy.

It is also interesting to note a recent report of the first family showing a mutation in the DBP *(GC)* gene, which manifested in a complete absence of the DBP protein in a homozygous member, and a reduction to 50% its normal concentration in heterozygous members [570]. The homozygous patient showed undetectable total serum 25D levels, whereas the free 25D concentration was similar to the heterozygous patients and other family members. The authors concluded that it was probably the free-25D which influences cellular functions and not the bound 25D-DBP component. This endorses the free-hormone hypothesis mentioned previously. The homozygous DBP mutant patient in this study was a female, who was not reported to have had any pregnancies or children. This is the only reported incidence of a human abnormality in DBP expression, but it will be interesting to determine if similar abnormalities are associated with dysregulation of placental development and pregnancy health.

A further study of DBP absence mouse model (DBP-/- mouse) will be interesting to understand the effect of an absence of DBP in the maternal genome, in pregnancy health and foetal health. Detailed future work is further explained in the next chapter

Chapter 6: FUTURE WORK

The focus of this thesis was to explain the significance of vitamin D on placentation and placental growth. DBP and 1,25D emerged as the key factors responsible for healthy placental development and healthy pregnancy. Following on from this, I would like to propose a series of future experiments to help further our knowledge of the effects of vitamin D and its metabolites on placental function and pregnancy.

Laboratory-based experimental design:

Project 1: In the current PhD I have observed significant differences in vitamin D-related protein and gene expression for plastic- versus matrix-cultured choriocarcinoma trophoblast cell lines (**Figures 4.2 and 4.4**). An initial series of studies I would like to carry out is to do an unbiased genome-wide analysis of the different genes that are regulated by 1,25D in trophoblast cells cultured on plastic or matrix. I would also like to propose similar experiments for primary trophoblast cells. That is, culturing primary trophoblast cells on plastic and matrix, and assessing possible differences in expression levels for DBP and VDR, and functional responses to 1,25D. Previous studies from our group have shown 1,25D-induced invasion of Matrigel^{TM} by primary cultures of human trophoblast cells [317]. It, therefore, seems likely that in primary trophoblast cells, there will be similar effects of 1,25D on cellular DBP uptake, interaction with intracellular actin and associated matrix invasion as observed in this thesis for trophoblast cell lines. In these studies, it would also be interesting to assess the effects of donor serum DBP and 1,25D, on donor primary trophoblast matrix invasion.

Project 2: The current PhD project showed that lowered DBP in cell culture medium leads to lowered trophoblast matrix invasion (**Figure 4.21A**). *In vivo,* this effect of lowered DBP availability could have a detrimental effect on placental development by suppressing the potential for trophoblast invasion of the decidua. Therefore, it would be interesting to study the effects of DBP *in vivo* by investigating mice with a DBP null phenotype (DBP-/-). To determine whether DBP from the mother is taken up by cells from the foetus, two types of breeding could be carried out. Firstly, if wild-type (DBP+/+) female mice were mated with DBP null (DBP-/-) male mice then all of the foetuses would be exposed to maternal DBP, and all of the foetuses would have one allele of DBP themselves, so this would be essentially a normal placenta and pregnancy (**Figure 6.1A**). In the second type of mating, DBP null females would be mated with wild-type male mice. Here all of the foetuses would have one DBP allele as before, but **none** of the foetuses would have been exposed to maternal DBP (**Figure 6.1B**). We would predict that the second type of mating would result in dysregulated placental development because of a lack of maternal DBP function.

Studies would include histological analysis of placentas at different stages of gestation, but it might also be interesting to investigate any loss of foetuses or early births in the absence of maternal DBP. Normal litters could be born to DBP-/- mothers, but this would be under pre-eclamptic conditions, with subsequent detrimental effects on the offspring. In this scenario, baseline measurements of maternal blood pressure, proteinuria and offspring health would be important as described previously [571]. These studies are complicated by the fact that mice are unable to show the full spectrum of human pregnancy disorders [572]. Besides, DBP knockout mice are

characterised by extremely low levels of serum 25D and 1,25D. This could adversely affect maternal pregnancy health, but for all other aspects of physiology, DBP null mice are considered physiologically normal because they still retain a circulating pool of free 25D and 1,25D [573]. This means that some cells may still be able to acquire 25D and 1,25D despite the absence of DBP. However, because megalin appears to be essential for the trophoblast uptake of DBP, it would be interesting to determine if mice with megalin knockout within the foetuses show similar effects to DBP knockout in the mother. However, this experiment is complicated by the fact that megalin knockout mice are infertile [574], also because megalin is also involved in the transport of factors other than simply DBP [575].

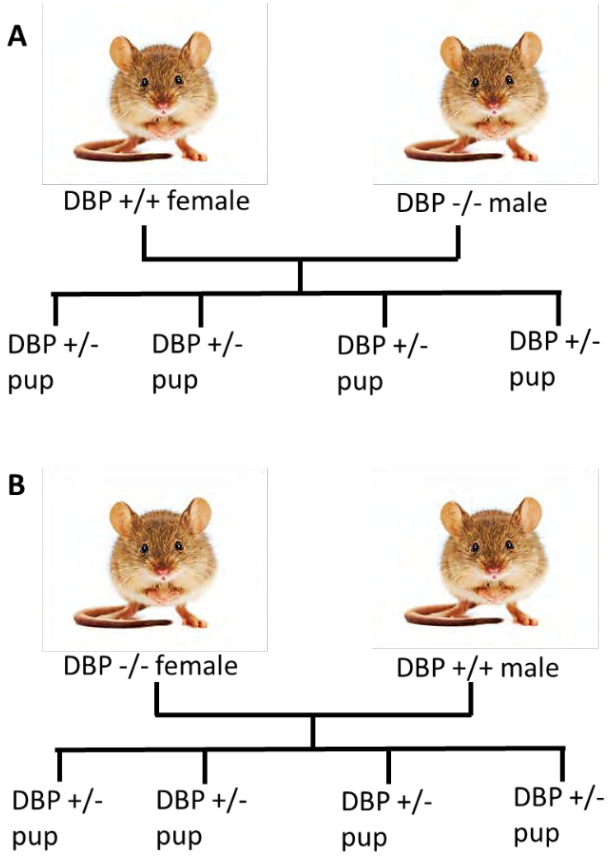


Figure 6.1 Schematic showing DBP knockout mouse breeding to be used for Project 2.

Project 3: Immunofluorescence images from this project show that DBP is internalised into the trophoblast cytoplasm, followed by translocation to the nucleus (**Figures 4.4, 4.26, 4.27, 4.33A**). In future studies, it will be interesting to observe and confirm the translocation of DBP from the extracellular to the intracellular environment using fluorescent-labelled DBP added to the culture media of cells [366]. To do this, the cells will need to be cultured in DBP free serum, and fluorescent dye labelled DBP will be added to the media. This needs to be constantly monitored at 0 hours, 24 hours, and 48 hours to measure the rate of DBP internalised at each time point as described previously [366]. This experiment could also potentially be extended to include *in vivo* injection of pregnant mice with fluorescent-tagged DBP to monitor the DBP transfer from the maternal circulation to the foetal trophoblast cells at the maternal-foetal interface. However, research has shown that fluorescent tagging of DBP results in the loss of DBP-25D binding [366]. This might, therefore, result in abnormal vitamin D metabolite transfer. However, the primary objective for **Project 3** would be to better understand the translocation of DBP and not to quantify vitamin D metabolites.

Project 4: Data in this PhD thesis shows distinct cell membrane megalin expression in trophoblast cell lines grown on matrix (**Figure 4.18 – 4.20**). Results also showed distinct expression of CYP27B1, CYP24A1 and VDR in 1st trimester and 3rd trimester healthy pregnancy placental tissues (**Figure 4.1**). However, due to time constraints, we were unable to study DBP or megalin expression in these tissues. It will be interesting in future projects to study both DBP and megalin expression, and possible variations in megalin expression in placenta tissue from different stages of pregnancy, and in healthy placentas compared to placentas from complicated pregnancy such as

pre-eclampsia and miscarriage. My project has also shown that DBP is expressed intracellularly in trophoblast cell lines grown on matrix (**Figures 4.4, 4.26, 4.27, 4.33A**). It will, therefore, be interesting to characterise cellular localisation of DBP in primary placental tissues.

Clinical experimental design:

Project 5: The *in vitro* experiments in this thesis suggest a role for DBP in early pregnancy, as a serum marker of pregnancy health and pregnancy complications (**Figures 4.35 and 4.36**), which is similar to the observations of Karras *et al.* [576]. However, data also showed that serum DBP and 1,25D cooperate to promote optimal matrix invasion by trophoblast cells (**Figure 4.35**). In future studies I would like to take this forward by employing novel mass-spectrophotometry methods [577] to measure both serum DBP, 1,25D and serum 25D in pregnant women (with no associated comorbidities) beginning from the time of the first obstetric visit to the doctor (in the 1st trimester), followed by subsequent follow-up visit once every month, until the delivery of the baby. This study would demonstrate the longitudinal changes in serum levels of vitamin D metabolites and DBP across gestation, and the relationship between these and healthy/complicated deliveries. Although this particular PhD project is focused on miscarriage as an adverse event in pregnancy, it would also be interesting to correlate vitamin D metabolites and DBP with other pregnancy complications, such as pregnancies showing symptoms of placental abnormality, foetal abnormality, pre-eclampsia, preterm birth, gestational diabetes mellitus, small for gestational age foetus and stillbirth or intrauterine foetal death. Since severe vitamin D deficiency is observed

in pregnant women [46], it will be interesting to observe the serum concentration of DBP and 1,25D across gestation for healthy and complicated pregnancies.

Project 6: Data from this PhD suggest that low serum level of DBP in 1st trimester pregnancy is a potential marker of subsequent development of pre-eclampsia. (**Figure 4.36**). This may also be true for other abnormalities of placentation. However, as well as being a marker of pregnancy health, DBP could potentially be used to improve pregnancy health. Measurement of DBP in pregnant women on their first obstetric visit to the doctors could be used to determine those with 'low serum DBP'. These patients could be given DBP infusion to maintain 'normal' DBP levels (>600 mg/L) [55]. As the pregnancy progresses, serum DBP concentration would be reassessed and the following observations would need to be made via ultrasound scans: placental health, placental blood flow, foetal health and development. I would hypothesise that establishing sufficient DBP levels in the maternal circulation would improve the chances of healthy placental development and healthy pregnancy. This pharmacologic approach to DBP has been proposed previously for control of circulating actin and hypercoagulation [578]. Data from this study indicate that administration of DBP in humans is safe and my conclusion from my PhD project is that DBP may provide benefits for the prevention or treatment of adverse events in pregnancy.

Chapter 7: ETHICS

Re-issue 2017.01.10: correction to attendees list

NHS
Health Research Authority

West Midlands - Edgbaston Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

09 December 2016

Dr Sean Jennings
Research Support Group
[redacted]
University of Birmingham
B15 2TT

Dear Dr Jennings

Study title:	**Understanding the role of cellular, humoral and hormonal factors in the control of immunity in healthy human pregnancy, and pregnancies complicatated by malplacentation disorders**
REC reference:	**14/WM/1146**
Protocol number:	**RG_14-194**
Amendment number:	**3**
Amendment date:	**24 November 2016**
IRAS project ID:	**155401**

The above amendment was reviewed on 05 December 2016 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Re-issue 2017.01.10: correction to attendees list

Document	*Version*	*Date*
Notice of Substantial Amendment (non-CTIMP) [AmendmentForm_CHHIP_IRAS Project ID_155401_24.11.2016]	3	24 November 2016
Other [2016.11.30 Email from Jennifer Tamblyn confirmation midwives part of care team]		30 November 2016
Other [chhip+clinical+sample form_Amniotic Fluid_final version]	1.0	
Other [chhip+clinical+sample form_Urine_final version]	1.0	
Research protocol or project proposal [CHHIP Protocol Final Amended Version 1.5.]	1.5	05 September 2016
Research protocol or project proposal [CHHIP Protocol Final Amended Version 1.4_tracked suggestions 1.5 version.]	1.5	05 September 2016

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/WM/1146:	**Please quote this number on all correspondence**

Yours sincerely

Professor John Marriott
Chair

E-mail: 

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Ms Kelly Hard, R&D Manager Birmingham Women's Hospital Foundation Trust*
Professor Mark David Kilby

Re-issue 2017.01.10: correction to attendees list

West Midlands - Edgbaston Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 05 December 2016

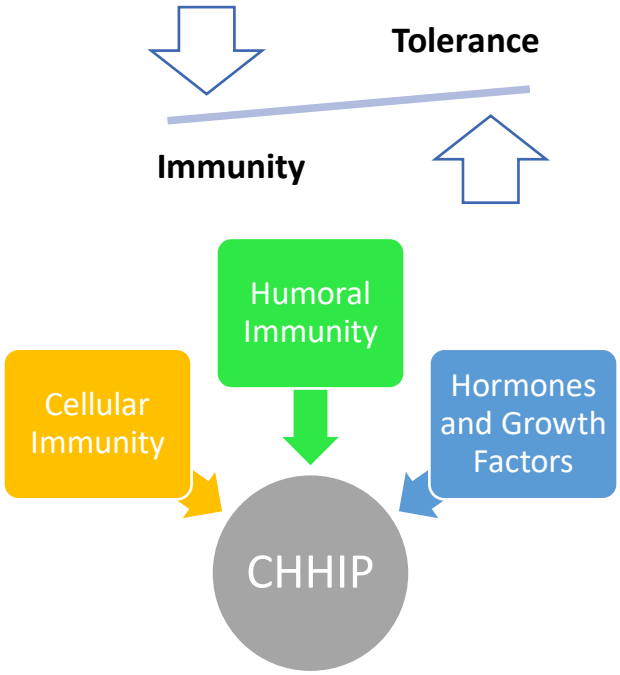
Committee Members:

Name	*Profession*	*Present*	*Notes*
Paul Hamilton	Parish Administrator	Yes	
Professor John Marriott (Chair)	Pharmaceutical Chemist/Academic Pharmacist	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Mr Adam Garretty	REC Assistant

Figure 1 - CHHIP 'Cellular Humoral and Hormonal Control of Immunity in Pregnancy' Study Summary



The CHHIP study represents the collaboration of 3 research groups at the University of Birmingham and Birmingham Women's Foundation Hospital Trust. The overarching aim of the study is to advance our current understanding of materno-fetal tolerance and immunity in both healthy human pregnancy and those complicated by adverse pregnancy outcomes. The benefit of the groups working together collaboratively is that this will facilitate recruitment of patients, minimise repeat approaches for participation being made to individuals, and mean maximal benefit can be gained from the donated tissues as collected tissue can be shared amongst the collaborating research groups.

As such any woman (+/- partner) recruited to the CHHIP study will be provide valuable clinical data and samples for each research group. It is anticipated this collaborative approach will also enhance our understanding of how these mechanisms interplay and interact.

Section 1 - CHHIP Background

Viviparous pregnancy, in which the fetus develops within the mother's body, represents a significant challenge for the maternal immune system. It is now widely accepted that a state of functional immunological tolerance for the semi-allogenic fetus persists throughout human haemochorial placentation despite the close juxtaposition between placental villi and the uteroplacental circulation, and intimate cellular contact between maternal and fetal tissue. Furthermore, fetal cells cross the placenta into the maternal circulation[1] and have the capacity to generate a cellular maternal immune response towards paternal-derived antigen[2].

The human decidua, which represents the maternal component of the placenta, is formed in part from stromal scaffolding cells. However, we now understand that a diverse population of immune cells (70% of all cells in the 1st trimester) is in fact predominant, and it is clear fetal survival is not solely reliant upon maternal immune ignorance[3, 4]. In fact a diverse array of immune-modulatory mechanisms co-exists specifically at this placental interface in order to form a unique, protective environment. These pathways include up-regulation of indoleamine 2, 3-dioxygenase (IDO), IFNγ and CTLA-4 expression and T Regulatory (TReg) cell induction(Reviewed in 5)[5]. The importance of maternal natural killer (NK) cells in modulating these maternal-fetal interaction is also well recognised; in particular their importance in facilitating trophoblast infiltration and their impact on overall pregnancy outcome[6].

Of further interest to our groups are the synergistic actions of certain steroid hormones and growth factors, including vitamin D, thyroid hormone, and progesterone. As we know from our own studies these play an important role in regulating implantation and successful placental development. For example, ex-vivo studies have demonstrated that vitamin D may stimulate fetal trophoblastic cells implantation, and acts to suppress pro-inflammatory cytokine production by decidual cells (Reviewed in 7)[7]. How this occurs remains to be understood, in particular the functional impact of vitamin D on specific T cell subtypes and uterine NK cells.

Most importantly, more recent insights suggest that failure to adapt may significantly interfere with fetal development and survival. A breakdown in materno-fetal tolerance is proposed to play a role in the pathogenesis of pregnancy complications including pre-eclampsia, low birth weight and preterm birth. For example, reduced TReg numbers and increased pro-inflammatory cytokines (tumour necrosis factor-α) in human decidua and murine models have been associated with significantly higher fetal loss rates[5]. Low serum concentrations of vitamin D have also been linked to adverse pregnancy outcomes, through malplacentation syndromes (i.e. pre-eclampsia) , as well as possible effects on fetal development/programming and childhood disease[7]. Similarly, altered levels of key proteins associated with steroid hormone and growth factor function (e.g. corticosteroid receptor expression, hepatocyte growth factor, and thyroid hormone receptors) have been reported by our group in IUGR placenta [8]. Indeed, thyroid hormone disorders in women are associated with an increased risk of placental disorders such as IUGR as well as

miscarriages, stillbirths and placental bleeding[9,10, 11]. We have also recently reported that thyroid hormones regulate inflammation in the human decidua during early pregnancy[12]. Mutations in the cortisol−inactivating enzyme, 11 beta−HSD2, have also been associated with low birth weight[8].

Hence the principal objective of CHHIP is to improve our knowledge of the underlying causes behind pregnancy complications, which have been linked to failure of maternal tolerance of the fetus. Numerous pregnancy treatments are being trialled to try to reduce these immune consequences but only by understanding the biology better will we be able to optimise these treatments. The work of this project will lead to a deep understanding of the immunology that governs pregnancy and will enable the targeted development of novel ways of treating pregnancy complications, and identifying pregnancies at risk of complications.

This may in future suggest new, targeted diagnostic or immunomodulatory treatment strategies.

Section 2 – CHHIP Goals

1. To understand the mechanisms by which steroid hormones, growth factors, antibodies and immune cell populations function and interact within the human placenta throughout normal pregnancy

2. To understand how the functional role of steroid hormones, growth factors, antibodies and immune cell populations in the placenta may become deregulated in women who develop malplacentation disorders. Specifically pre-eclampsia, intra-uterine growth restriction, spontaneous miscarriage, preterm birth and late fetal loss

Section 3 – CHHIP Participant Recruitment and Prospective Specimen Collection

Inclusion Criteria

1. Women ≥16 years with a pregnancy not complicated by pre-existing maternal disease or pregnancy complications

2. Women with pregnancy complications that may be associated with malplacentation: pre-eclampsia, fetal growth restriction, gestational diabetes, fetal intrauterine death, preterm delivery, villitis of unknown aetiology

3. Women at risk of complications due to the presence of maternal antibodies that may cause fetal disease including fetal anaemia, fetal thrombocytopenia and fetal heart block

4. Partners of above participants (not compulsory for maternal inclusion)

5. Healthy non-pregnant female controls

Exclusion Criteria

1. Women or their partners who are unwilling or unable to provide informed consent

2. Under the age of 16 years

Participant Identification

In order to help identify study participants with pregnancy complications (as per the outlined inclusion criteria), members of the patient's direct clinical team will access patient records and initially approach regarding their potential recruitment if deemed appropriate. This will be aided by the use of a participant information sheet (enclosed). If they express an interest in participating further information will be obtained by either a specifically trained research midwife or clinician. The research team may make enquiries with the clinical team and refer to the ward handover board in order to help identify potential participants. This will not involve screening of patients personal hospital notes until an interest in participation has been expressed to the clinical team. Extreme caution and care will be taken to ensure those women who have unfortunately experienced an intrauterine death are only approached by their clinical team regarding the study if deemed appropriate to do so. If they express an interest in participating further information will be obtained by either a specifically trained research midwife, nurse, or clinician as for all other participants. Pregnant women booked antenatally for elective (non-emergency) caesarean section may also be identified in the antenatal clinic. The CHHIP research team (clinical researcher or midwife) will refer to the theatre booking information to identify eligible pregnant women. Potential participants will be offered a participant information sheet by the study researcher (midwife or clinician) prior to deciding whether they would like to receive verbal information about the study.

Concerning women specifically approaching the NHS for an elective termination of pregnancy (1967 Abortion Act) (1^{st} – 2^{nd} trimester 'healthy pregnant controls without complication'), these patients will receive routine counselling as per the trusts clinical policies. Only once the procedure has been agreed, if the clinical team deem it appropriate for that patient to be approached, then she will be provided with an information leaflet regarding the study (enclosed). If having read this the woman is interested in participation then she will be approached by a research midwife or clinician who will provide full information and obtain informed consent. Those agreeing to participate will be required to confirm this by signing a consent form (enclosed).

Concerning the identification of male partners for study inclusion, potential participants will be invited to participate antenatally once their female partner is formally recruited. When the direct clinical team initially approach the mother regarding their potential recruitment, they will explain that in some cases the research team invite the partner to participate if happy to do so. This will be aided by the use of a participant information sheet (enclosed). If they express an interest in participating further information may be obtained by either a specifically trained research midwife or clinician. Inclusion in the study will be discussed in detail by a member of the research team specifically trained in obtaining consent for CHHIP, and shall be be aided by the patient information leaflet enclosed. The partner will be reassured prior to making a decision regarding their participation that this will have no effect on their female partner's eligibility in the study.

Regarding our healthy non-pregnant cohort, this group will comprise healthy female volunteers formally recruited by our research team within our designated research / clinical areas in order to provide a 20 ml 'control' blood and / or urine sample following written informed consent.

Post recruitment a unique study code shall be appointed to all participants in order to pseudo-anonymise any clinical data / specimens collected for the study.

Consent Procedures

For pregnant women with complications which may be at risk of causing emotional distress, consent will initially be requested by the direct care team prior to the potential participant being approached by either a research midwife, nurse, or clinician. Informed consent for all participants will be obtained for inclusion in the CHHIP study. Consent will only be sought at an appropriate time when the participant is not in distress or pain therefore it will not be appropriate to approach women in labour. Women +/- their partners will also not be approached outside of the clinical area, and the initial approach regarding inclusion in the study will be by a member of their designated clinical care team as indicated. The partner will also not be approached at these times.

The hospital interpreting or telephone interpreting service will be used if required. If it is not possible to provide full informed consent then the participant will not be recruited. The use of a relative for interpretation will be avoided as this may breech patient confidentiality.

Information regarding the study will be provided and patients / partners will be given the patient information leaflet and as much time as they require to read it and consider their inclusion in the study. The study team member will provide answers to any further questions. If the participant would like to be included then the researcher will go through the consent form with them and answer any further questions. They will then be asked to sign the consent form and be given a copy. A further copy will be kept in the site file and in the patient's notes. A copy of the

participant information leaflet will also be given to the patient and a copy filed in their notes.

All patients recruited to the CHHIP study will be offered the option of receiving a bi-annual update of the CHHIP study's progress in the form of a written newsletter. If they would like to receive the newsletter their name and contact details will be recorded in a separate file which will be stored on a password protected computer in an encrypted format on a University computer. This information will not be linked to any clinical information so that pseudo-anonymisation is preserved and will only be accessible to Professor Kilby, Dr David Lissauer, and Dr Jennifer Tamblyn following entry to the study.

Specimen Collection – Prospective (n=860 total)

Antenatal - A 20ml sample of maternal blood shall be obtained from all women outlined above by a trained phlebotomist, midwife, doctor or anaesthetist. In certain cases serial blood samples may be obtained (≤ 4 antenatally) in order to evaluate gestational changes. In certain cases a maternal urine sample may also be requested for collection at this time (20ml (≤ 4 antenatally). This will be performed during a woman's routine clinical care where possible, and will not require additional visits to hospital to participate. For those women undergoing termination of pregnancy, one 20 ml blood sample shall be obtained for analysis

Postnatal – A matched set of maternal blood, maternal urine (5ml), amniotic fluid (20ml), placenta/decidual and cord tissue, and cord blood samples (where applicable) shall be obtained where applicable from the following pregnancy cohorts:

1. 1st and early 2nd trimester pregnancies following elective termination of pregnancy not complicated by malplacentation associated problems (n=150)
2. 3rd trimester term (≥37 weeks) uncomplicated normal pregnancy (obtained following caesarean section or normal vaginal delivery) (n=150)
3. 1st and early 2nd trimester miscarriage - confirmed as intrauterine death (IUD) on ultrasound (n=150)
4. Late 2nd trimester – 3rd trimester pregnancies complicated by malplacentation syndromes; pre-eclampsia, intra-uterine growth restriction, intra-uterine death, preterm birth, gestational diabetes, and maternal antibody production (n=260)
5. Healthy non-pregnant control group (blood and urine samples) (n=150)

– Fetal cord tissue and blood shall be obtained in all gestational cohorts where applicable (20 ml sample).

- Amniotic fluid shall be collected where applicable at the time of caesarean section by the operating surgeon (20ml sample).
- In women with complications where maternal antibody production has caused fetal disease fetal blood samples may be taken for testing, or fetal blood transfusions performed as part of their clinical care. Consent will also be obtained to retain fetal blood samples which are surplus to those obtained for routine clinical care and diagnostic testing. In routine clinical practice these samples are normally discarded. For women where antibody-related disease is identified later in pregnancy, 1st and 3rd trimester maternal blood samples (plasma) stored by the national blood service from routine antibody screening during the 1st and 3rd trimesters will be obtained for retrospective testing for humoral factors. Similarly, only samples surplus to diagnostic requirement shall be utilised.

Where required, samples will then be divided as appropriate to enable the required individual work packages as outlined below to be carried out.

Existing Samples – Retrospectively collected (n=459)

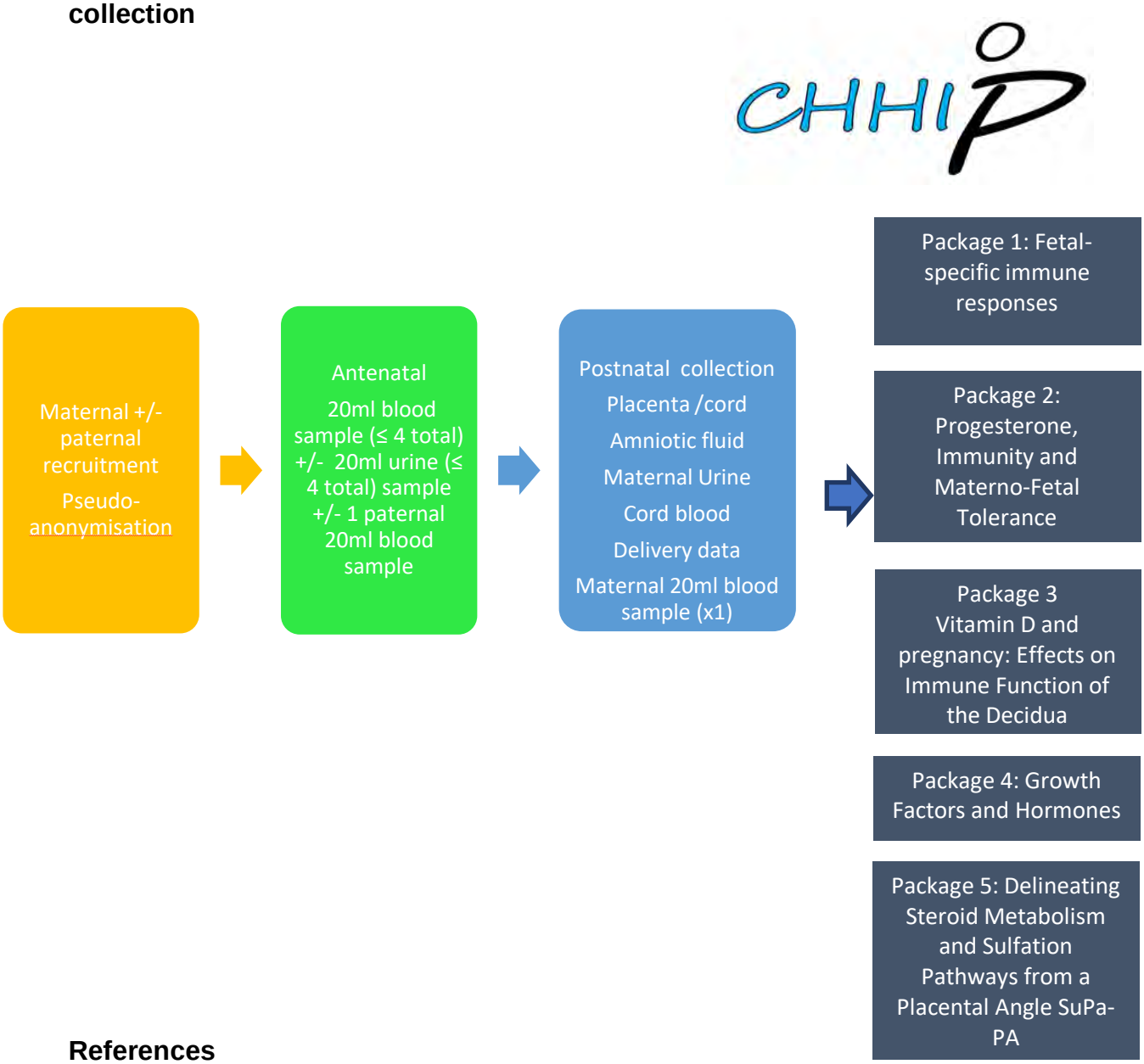
We also have access to 459 previously collected placental samples from 6−41 weeks of pregnancy (weeks) stored in the freezer. These are full thickness biopsies from the middle of the placenta near where the umbilical cord is attached to the placenta). These had been collected [according to Polkinghorne and MRC guidelines and with South Birmingham REC approval (06/Q2707/12 - Dr Shiao Chan - Steroid Hormone and Growth factor action in human placenta and the effects of intrauterine growth restriction)] immediately after surgical termination of pregnancy or elective caesarean section. This includes placental samples (25−38 weeks) from severe IUGR pregnancies (collected according to Polkinghorne and MRC guidelines and with South Birmingham REC approval) prospectively diagnosed with ultrasound.

SCOPE samples – Prospectively collected (n=50)

We will also have access to 50 patient samples purchased from the INFANT (The Irish Centre for Fetal and Neonatal Translational Research) SCOPE Study Biobank (total 5690 samples), University College Cork, Cork University Maternity Hospital, Ireland. These are matched serum (total of 3 250ul aliquots per woman) and urine (1ml aliquot per woman) samples collected from n=50 women. Of these, 25 prospectively received a diagnosis of pre-eclampsia, and 25 were selected to provide a matched normotensive control arm (body mass index (BMI), ethnicity and maternal age). For all cases relevant maternal demographic, obstetric and past medical data were obtained and shall be utilised by our group to facilitate comparative analysis. Additional information regarding disease onset, severity, and materno-fetal

management shall also be collected. These samples have been collected with Cork, Ireland Clinical Research Ethics Committee Approval (ECM 5(10) 05/02/08 – Dr Louise Kenny).

Figure 2 – Summary of CHHIP prospective patient recruitment, data and tissue collection



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Package 3 – Vitamin D and pregnancy: Effects on Immune Function of the Decidua

Principal Investigators

1. Dr Jennifer Tamblyn (Clinical Research Fellow Obstetrics and Gynaecology)
2. Professor Mark Kilby (Professor Fetal Medicine)
3. Professor Martin Hewison (Professor of Molecular Endocrinology)
4. Professor Paul Moss (Professor Haematology)
5. Dr Omi Ohizua (Consultant Obstetrics and Gynaecology)
6. Professor Janesh Gupta (Professor of Obstetrics and Gynaecology)
7. Dr Ankana Ganguly (Dr Obstetrics and Gynaecology, PhD student)

Background

The active form of vitamin D, 1,25D, is a pluripotent seco-steroid, with putative biological actions that extend far beyond its classical effects on calcium homeostasis and bone metabolism. In particular, 1,25D exerts potent effects on cells from both the innate and adaptive immune system that are known to express the nuclear vitamin D receptor (VDR) for 1,25D[1]. These immunomodulatory actions of vitamin D appear to be independent of circulating 1,25D, and instead involve localised synthesis of 1,25D by APCs, catalysed by the enzyme CYP27B1. As a consequence, immune responses to vitamin D are highly dependent on the level of substrate for CYP27B1, namely 25D, the major circulating form of vitamin D and principal marker of human vitamin D 'status'.

Impaired vitamin D status has been linked to numerous immune-related diseases, with effects including aberrant antibacterial activity and de-regulated expression of Treg and T helper (Th) cells sub-types[2]. Recent studies have detailed the ontogeny of VDR and CYP27B1 in human decidua, indicating that these genes are expressed early in pregnancy[3,4,5]. Ex-vivo studies have also shown that 25D stimulates innate antibacterial responses in fetal trophoblastic cells[6], and promotes matrix invasion by EVT[7]. 25D can also act to suppress the production of inflammatory cytokines by decidual cells, but the impact of this on T cell and uNK function in the placenta remains unclear[5].

Deficiency or insufficiency of vitamin D (low serum concentrations of 25D) has also been reported to be common in pregnant women, notably those with darker skin pigmentation where UV light generation of vitamin D in the skin is less efficient[8]. Low serum concentrations of the major circulating form of vitamin D (25-hydroxyvitamin D, 25D) have been linked to adverse pregnancy outcomes, through malplacentation syndromes (i.e. pre-eclampsia)[9], as well as possible effects on fetal development/programming[10] and childhood disease[11, 12]. As such, vitamin D supplementation, which is very cheap, is now routinely advised in all pregnant women but, as yet, the biological functions of vitamin D during pregnancy are largely unknown as there have been no studies to define the mechanisms by which low vitamin D status affect pregnancy.

8. Research Hypothesis

We postulate vitamin D plays a key role in immune-tolerance during pregnancy, and that this effect may be compromised by low maternal 25D. We hypothesise vitamin D is a pivotal component of "leukocyte" interactions within the human decidua, with relative vitamin D deficiency leading to impaired innate and adaptive immune cell function.

Project Aims

This project aims to investigate an immunomodulatory role for vitamin D at the maternal-fetal interface, with decidual leukocytes being the primary target cells. The following main objectives are as follows -

1. To obtain decidua from uncomplicated pregnancies at different gestational stages of human pregnancy in order to assess the relationship between circulating and decidual 25D/1,25D concentrations, and decidual leukocyte populations.

2. To obtain decidua from term and preterm pregnancies in order to assess differential expression of leukocyte cell sub-types associated with malplacentation syndromes, including gestational diabetes, for each gestational cohort, and the potential impact of vitamin D (25D) status on these cells.

3. To investigate the effects of localized conversion of 25D to 1,25D upon leukocyte phenotype

4. To explore a possible paracrine role for fetal cells in directing vitamin D-mediated maternal decidual immune activity (24-36 months).

5. To explore a possible paracrine role for maternal stromal cells in directing vitamin D immune responses.

6. To compare vitamin D status (2nd trimester) in a cohort of matched control pregnancies and those subsequently complicated by pre-eclampsia, collected by the INFANT - SCOPE study.

Plan of Research:

Aims 1 and 2: Localisation and characterisation of specific lymphocyte populations within decidua from normal human pregnancies and those complicated by malplacentation: Association with local and systemic 1,25 (active) and 25 (inactive) vitamin D status.

The overall objective of these studies will be to assess firstly the relationship between maternal vitamin D status, decidual levels of 25D/1,25D and the composition of decidual leukocytes at different stages of gestation. In addition, we will explore the possible impact of vitamin D status and decidual leukocytes on preterm birth.

Human decidual cells obtained from the cohorts outlined shall be assessed in several ways:

1) FACS will be used to assess the leukocyte composition of each decidual sample within cohorts, using previously defined markers.

2) Decidual leukocytes prepared by MACS magnetic bead separation and the resulting purified cells lysed prior to isolation of total RNA. Using qRT-PCR analysis, the expression of key components of the vitamin D system such as VDR, CYP27B1, and the vitamin D catabolic enzyme CYP24A1 shall be measured in these decidual leukocyte populations.

3) Isolated decidual cells to analyse tissue-specific concentrations of 25D and 1,25D (by liquid chromatography–mass spectrometry (LC-MS) of each metabolite.

4) Tissue embedded in paraffin will be used to define the distribution of each leukocyte sub-type within each decidual sample by immune-histochemical analysis.

These data will then be compared to maternal serum, and fetal cord levels of 25D and 1,25D, and their respective peripheral leukocyte compositions.

Aim 3: Ex vivo studies investigating the effect of 25, and 1,25 vitamin D on decidual lymphocyte populations.

The objective of these studies will be to determine whether changes in 25D or 1,25D concentration are sufficient to alter the composition of decidual leukocytes. In particular, these studies will determine whether 25D, the main marker of vitamin D status, is sufficient to alter expression uNK, DC, or T cells via localized synthesis of 1,25D. Similarly, these studies will seek to determine the dominant cell type(s) that respond to added 25D or 1,25D. We expect that added 25D and 1,25D will increase expression of TReg cells and decrease Th17 cells in decidual cell populations. However, if only 25D achieves this effect, this will endorse a DC-mediated effect involving localized synthesis of 1,25D, as T cells do not appear to exhibit CYP27B1 activity[2].

Method

CD45+ decidual leukocyte populations from term pregnancies will be used for ex vivo studies. Cultures of cells maintained in normal RPMI medium supplemented with 10% fetal calf serum (FCS) will be treated for 24-72 hrs with either

- Vehicle (0.1% ethanol) – Control
- 25D (1-100 nM) – Inactive form
- 1,25D (0.1-10 nM) - Active

The resulting cultures will then be analysed by FACS for changes in leukocyte phenotype. After optimization for dose and incubation period, parallel isolation of cell RNA will be carried out to determine total expression of vitamin D signalling system components, such as the VDR.

Aim 4: Functional analysis of fetal EVT cells and their role in vitamin D immune-regulation

These studies will be performed to determine whether EVT can act as potent localised converters of 25D to 1,25D for use by decidual leukocytes. Although previous studies have shown that EVT express CYP27B1 and VDR, and respond to 25D in an intracrine fashion, the possible paracrine role of EVT for vitamin D has yet to be studied. If successful, this will endorse a role for fetal cells in directing vitamin D-mediated immune responses in maternal decidua.

Method

EVT from human placentae will be obtained from uncomplicated pregnancies and those complicated by either miscarriage or a disorder of malplacentation (inclusion criteria; page 6). . After purification of EVT, cells will be co-cultured with CD45+ decidual leukocytes or decidual stromal cells under conditions of varying concentrations of added 25D (1-100 nM) or 1,25D (0.1-10 nM). Control decidual stromal/leukocyte cells will also be cultured in the absence of EVT. The decidual and fetal trophoblast cells will then be assessed to characterise their phenotype and function. .

Aim 5: *Ex vivo studies investigating the* interaction between cultured decidual stromal cells, immune cells and placental cells in the presence of 25OHD and 1,25D.

These studies shall explore the role of decidua derived CD10+ stromal cells in mediating vitamin-D mediated responses in the human placenta. Previous studies by our group have indicated that the stromal cell compartment positively expresses key components of the vitamin D metabolic system. We anticipate these scaffolding cells may mediate vitamin D effects in a paracrine manner, targeting individual leukocyte subsets and promoting an immune-regulatory phenotype and functional role.

Method

CD10+ stromal cells shall be isolated from human placentae obtained from uncomplicated pregnancies and those complicated by either miscarriage or a disorder of malplacentation (inclusion criteria; page 6).. These cells shall be co-cultured with both CD45+ decidual leukocytes and placental trophoblast / EVT cells under conditions of varying concentrations of added 25D (1-100 nM) or 1,25D (0.1-10 nM) or vehicle control. The cells shall be subsequently assessed by FACS for cell phenotype, qRT-PCR for gene expression (including vitamin D metabolic system, angiogenic and immune transcripts) , enzyme-linked immunosorbent assay for functional marker quantification (including key cytokine and angiogenic markers such as IL-6, IFN-y, VEGF and SP100). A genome-wide non-targeted approach, such as RNA sequence analysis, may also be performed to identify other previously unknown vitamin D targets.

Aim 6: To measure serum vitamin D metabolites and FGF23 concentrations (serum and urine) in pregnant women in the 1st or 2nd trimester who were subsequently diagnosed with pre-eclampsia (PET), and compare this to matched normotensive controls (SCOPE).

Vitamin D deficiency is common in pregnant woman and is associated with an increased risk of pre-eclampsia. FGF23 is a known regulator of vitamin D metabolism and more recently has been recognised as a marker of vitamin D deficiency related

disease. To achieve this aim, 2nd trimester vitamin D metabolism and FGF23 levels shall be measured in pregnant women who subsequently developed pre-eclampsia, and compared to normotensive matched controls. These data will provide preliminary evidence regarding the potential association between vitamin D status, FGF23 and pre-eclampsia-onset.

Method

Using banked serum and urine samples from The Irish Centre for Fetal and Neonatal Translational Research (INFANT) – SCOPE Biobank (Cork University Maternal Hospital, Cork, Ireland) (Cork, Ireland Clinical Research Ethics Committee Approval (ECM 5(10) 05/02/08 – Dr Louise Kenny) concentrations of 25D, 1,25D and 24,25D and FGF23 levels shall be measured from a cohort of 50 pregnant women at 15 weeks gestation. Of these women, 25 prospectively received a diagnosis of pre-eclampsia, and 25 were selected to provide a matched normotensive control arm (body mass index (BMI), ethnicity and maternal age). For all cases relevant maternal demographic, obstetric and past medical data has been obtained and shall be utilised to facilitate comparative analysis. Additional information regarding disease onset, severity, and materno-fetal management shall also be collected.

Vitamin D metabolites, including 1,25D, 25D, and 24,25D), shall be measured in maternal serum samples (n=50) using liquid chromatography–tandem mass spectrometry (LC-MS/MS). FGF23 shall be measured in maternal serum and urine samples using an ELISA-based assay at the University of Birmingham.

Data shall be expressed as the mean ± SD. To compare parameters between the two groups a Student's t test will be used. To compare multiple groups, one-way analysis of variance followed by multiple comparisons with the Tukey-Kramer method shall be used. A $p < 0.05$ will be considered significant. We would also like permission from INFANT to combine these analytes with the biomarkers measured in the Alere study published in Hypertension in 2014.

Type(s) and volume of specimens:

Specimen Type (e.g., EDTA plasma)	Gestation of Specimen (15w) –Normal and PET samples	Number of Aliquots per woman*	Total (n=50 pregnant women)
Serum (EDTA)	15w	3	150
Urine	15w	1	50

*All blood specimens - 250ul aliquots, urine - 1 ml aliquot

Conclusion

The overall aim of this project will be to define a link between vitamin D status, maternal 25D, and tolerogenic/anti-inflammatory immunity at the fetal-maternal interface. In this way, the project aims to provide an immunological explanation for the link between vitamin D-deficiency and adverse events in pregnancy. Importantly,

these studies will also provide a functional mechanistic rationale for future vitamin D supplementation trials in pregnant women. Vitamin D is a cheap and relatively safe supplement that may nevertheless help to optimize immune function at the fetal-maternal interface.

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Chapter 8: APPENDICES

7.1 Publications arising from this thesis

- **Ganguly, Ankana**, Jennifer A. Tamblyn, Sarah Finn-Sell, Shiao-Y. Chan, Melissa Westwood, Janesh Gupta, Mark D. Kilby, Stephane R. Gross, and Martin Hewison. "Vitamin D, the placenta and early pregnancy: effects on trophoblast function" (Review). Journal of Endocrinology 236, no. 2 (2018): R93-R103 [579].

- **Ganguly, Ankana**, Shattock Alexandra, Joseph Annsha, Jennifer A. Tamblyn, Janesh Gupta, Stephane R. Gross, and Martin Hewison. "Vitamin D binding protein (DBP) is required for pro-invasion effects of vitamin D on placental trophoblastic cells." – *In preparation.*

REVIEW

Vitamin D, the placenta and early pregnancy: effects on trophoblast function

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Abstract

Pregnancy is associated with significant changes in vitamin D metabolism, notably increased maternal serum levels of active vitamin D, 1,25-dihydroxyvitamin ($1,25(OH)_2D$). This appears to be due primarily to increased renal activity of the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) that catalyzes synthesis of $1,25(OH)_2D$, but CYP27B1 expression is also prominent in both the maternal decidua and fetal trophoblast components of the placenta. The precise function of placental synthesis of $1,25(OH)_2D$ remains unclear, but is likely to involve localized tissue-specific responses with both decidua and trophoblast also expressing the vitamin D receptor (VDR) for $1,25(OH)_2D$. We have previously described immunomodulatory responses to $1,25(OH)_2D$ by diverse populations of VDR-expressing cells within the decidua. The aim of the current review is to detail the role of vitamin D in pregnancy from a trophoblast perspective, with particular emphasis on the potential role of $1,25(OH)_2D$ as a regulator of trophoblast invasion in early pregnancy. Vitamin D deficiency is common in pregnant women, and a wide range of studies have linked low vitamin D status to adverse events in pregnancy. To date, most of these studies have focused on adverse events later in pregnancy, but the current review will explore the potential impact of vitamin D on early pregnancy, and how this may influence implantation and miscarriage.

Key Words
- vitamin D
- pregnancy
- placenta
- trophoblast
- miscarriage

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Introduction

The human placenta is a vital organ without which the mammalian fetus cannot survive. It forms the interface between the mother and fetus, supplying the fetus with oxygen, nutrients, excreting waste products, while protecting against maternal immunologic attack. The main functions of the placenta can be broadly categorized into transport and metabolism, protection and endocrine (Gude *et al.* 2004). The complex architecture of the placenta, bounded by the maternal aspect (basal plate) and the fetal aspect (chorionic plate), houses an abundance of the fundamental functional unit of the placenta, the chorionic villus, where all nutritional-waste exchange between the maternal blood and the fetal circulation occurs. In addition to facilitating a good maternal blood

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supply for nutrition–waste exchange and orchestrating endocrine mediators of pregnancy to maintain maternal physiological changes for an optimal environment for fetal development, the placenta also acts to protect the fetus from xenobiotic materials and infectious agents (Yang 1997, Moore *et al.* 1999, Gude *et al.* 2004, Rudge *et al.* 2009). Successful development of the placenta involves two distinct mechanisms: implantation of the blastocyst, initiated by attachment of the embryo to the maternal endometrial epithelium and invasion of fetal trophoblast cells into the maternal endometrium to facilitate maternal–fetal exchange of nutrients, gases and waste. The diverse mechanisms associated with the regulation of trophoblast invasion have been well documented (Menkhorst *et al.* 2016). The aim of the current review is to provide an overview of these early events in placental development, with particular emphasis on the potential role of vitamin D as a determinant of early placental development through effects on trophoblast cells, particularly via effects of vitamin D on trophoblast invasion.

Vitamin D and pregnancy

Despite its long-standing association with rickets and osteoporosis, vitamin D has become increasingly recognized as a pluripotent regulator of biological functions above and beyond its classical effects on bone and calcium homeostasis. Expression of vitamin D receptor (VDR) for the active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), as well as the 1α-hydroxylase enzyme that synthesizes $1,25(OH)_2D$ (CYP27B1), has been reported for various tissues that can be broadly termed 'barrier sites' (Jones *et al.* 1998, Townsend *et al.* 2005), indicating that localized responses to vitamin D may be a key feature of these tissues. Prominent among these barrier sites is the placenta, acting as the interface between mother and fetus. Historically, the placenta was one of the first extra-renal tissues shown to be capable of synthesizing $1,25(OH)_2D$, with CYP27B1 activity detectable in both maternal decidua and fetal trophoblast (Gray *et al.* 1979, Weisman *et al.* 1979). Initially, this was linked to the rise in maternal serum $1,25(OH)_2D$ that occurs at the end of the first trimester of pregnancy. However, studies of CYP27B1-deficient animals and an anephric pregnant woman indicated that this is not likely to be the case (Kovacs & Kronenberg 1997). Instead, the presence of VDR in the placenta suggests that vitamin D functions in tissue-specific fashion at the fetal–maternal interface (Bruns & Bruns 1983). One possible explanation is that $1,25(OH)_2D$ acts as a regulator of placental calcium transport (Bruns & Bruns 1983), but a placental immunomodulatory function has also been proposed (Liu & Hewison 2012). Moreover, the rapid induction of VDR and CYP27B1 early in pregnancy (Zehnder *et al.* 2002) suggests that vitamin D may play a more fundamental role in the process of conception, implantation and development of the placenta itself.

Vitamin D and implantation

To date, the precise role of vitamin D in the process of implantation remains unclear. Nevertheless, vitamin D has a biologically plausible role in female reproduction and implantation process. $1,25(OH)_2D$ has been shown to regulate expression of the homeobox gene *HOXA10* in human endometrial stromal cells (Du *et al.* 2005*b*). *HOXA10* is important for the development of the uterus during fetal life and, later in adulthood, is essential for endometrial development, allowing uterine receptivity to implantation (Bagot *et al.* 2000). Interestingly, animal studies have shown that vitamin D deficiency reduces mating success and fertility in female rats. Female rats fed with a vitamin D-deficient diet are capable of reproduction, but overall fertility is reduced including the failure of implantation (Halloran & DeLuca 1980). This was shown to be corrected by administration of $1,25(OH)_2D$ (Kwiecinksi *et al.* 1989), but also by use of diets high in calcium, phosphate and lactose (Johnson & DeLuca 2002), suggesting that the fertility effects of vitamin D may be due to indirect effects on mineral homeostasis. Other studies using knockout mouse models have further highlighted the importance of the vitamin D metabolic and signaling system in the process of implantation, with *Vdr*−/− and *Cyp27b1*−/− female mice both presenting with uterine hypoplasia and infertility (Yoshizawa *et al.* 1997, Panda *et al.* 2001). Conversely, injection of $1,25(OH)_2D$ has been shown to increase uterine weight and promote endometrial to decidual differentiation (Halhali *et al.* 1991).

In addition to regulating uterine and decidual development, vitamin D may also influence implantation indirectly via its well-known immunomodulatory actions. Regulation of immune function at the maternal–fetal interface involves a heterogeneous population of innate and adaptive immune cell subsets. Thus, throughout pregnancy, decidual synthesis of $1,25(OH)_2D$ has the potential to influence uterine natural killer cells, dendritic cells, macrophages and T-cells (Evans *et al.* 2004,

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Tamblyn *et al.* 2015). Notable effects include inhibition of Th1 cytokines and promotion of Th2 cytokines (Gregori *et al.* 2001), which are known to play a significant role in the process of implantation (Piccinni *et al.* 2000, Zehnder *et al.* 2002). Purification of decidual cells into non-adherent stromal cells and adherent cells, which include decidual macrophages and uterine natural killer cells, has shown that adherent cells demonstrate a greater capacity for $1,25(OH)_2D$ production (Kachkache *et al.* 1993). Furthermore, first trimester decidual cells treated with either precursor 25-hydroxyvitamin D or $1,25(OH)_2D$ demonstrate significant induction of antibacterial protein cathelicidin and β-defensins (Evans *et al.* 2006, Liu *et al.* 2009). Since similar effects of vitamin D are observed in peripheral monocytes, an equivalent innate antimicrobial responsivity is postulated to exist at the maternal–fetal interface (Liu & Hewison 2012).

Vitamin D metabolism and function in trophoblast cells

The organization of maternal and fetal cells within the developing placenta has been well documented elsewhere (Vigano *et al.* 2003, Oreshkova *et al.* 2012) and is represented schematically in Fig. 1. Both the maternal decidua and fetal trophoblast components of the placenta (including syncytiotrophoblast and invasive extravillous trophoblast (EVT)) express CYP27B1 (Zehnder *et al.* 2002) and are able to produce detectable levels of $1,25(OH)_2D$ (Gray *et al.* 1979, Weisman *et al.* 1979). The resulting tissue concentrations of $1,25(OH)_2D$ appear to be significantly higher in the decidua (Tamblyn *et al.* 2017), but the coincident expression of VDR in trophoblast as well as decidua (Evans *et al.* 2004) means that multiple cell types within the placenta are capable of responding to the locally synthesized $1,25(OH)_2D$, either in an autocrine or paracrine fashion.

To date, studies of the physiological impact of decidual-trophoblast $1,25(OH)_2D$ production have focused primarily on trophoblast cells, using both primary cultures of EVT and trophoblast cells lines. Primary cultures of human syncytiotrophoblast express CYP27B1 and are able to synthesize $1,25(OH)_2D$ (Diaz *et al.* 2000) and also express VDR (Pospechova *et al.* 2009). However, in choriocarcinoma trophoblast cell lines such as BeWo and JEG-3, expression of VDR is low, with analysis of the effects of chromatin remodeling agents suggesting that

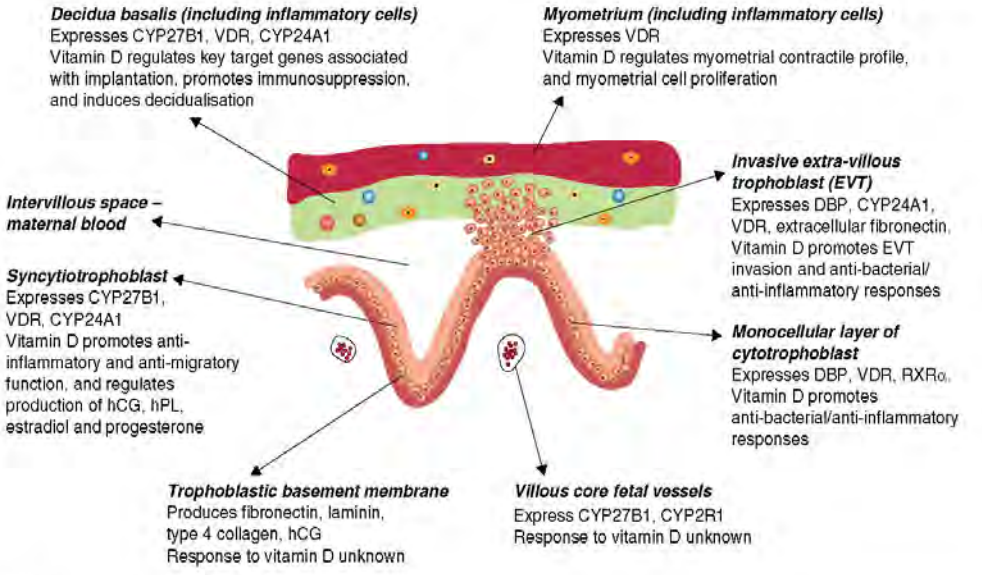

Figure 1
Vitamin D pathway components at the maternal–fetal interface associated with implantation. Schematic showing key cell types involved in implantation and associated expression of components of the vitamin D system: CYP2R1, vitamin D-25 hydroxylase; CYP24A1, vitamin D-24-hydroxylase; CYP27B1, 25-hydroxyvitamin D-1α-hydroxylase; DBP, vitamin D binding protein; hCG, human chorionic gonadotropin; hPL, human prolactin; RXR, retinoid X receptor; VDR, vitamin D receptor.

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this may be due to epigenetic suppression of VDR in these cells (Pospechova *et al.* 2009). Further studies to assess the impact of differentiation of cultured trophoblast cells have been carried out using cyclic AMP (cAMP) to mimic the process of syncytialization (Keryer *et al.* 1998). Expression of hCG is elevated by cAMP in trophoblast cells, and this was associated with decreased expression of CYP27B1, with VDR expression being unaffected (Avila *et al.* 2007), suggesting that presence of the vitamin D metabolic and signaling pathways in the placenta is differentiation sensitive. The JEG-3 trophoblast cell line has also been reported to express CYP27B1, but synthesis of $1,25(OH)_2D$ by these cells appears to be significantly less than that observed with primary trophoblast cells and unaffected by cAMP (Pospechova *et al.* 2009). In addition to cAMP, inflammatory cytokines (Noyola-Martinez *et al.* 2014) and insulin-like growth factor I (Halhali *et al.* 1999) also stimulate trophoblast expression of CYP27B1 and synthesis of $1,25(OH)_2D$.

The vitamin D catabolic enzyme CYP24A1 has been reported to be undetectable in trophoblast cells, consistent with methylation epigenetic silencing of this gene in the human placenta (Novakovic *et al.* 2009). This suggests that synthesis of $1,25(OH)_2D$ by trophoblast cells is not subject to the same catabolic feedback control observed in other VDR-expressing tissues. However, other studies have shown that trophoblast expression of CYP24A1 is increased following treatment with cAMP (Avila *et al.* 2007). In addition, studies using the *Hyp* mouse model, which has elevated circulating levels of the positive regulator of 24-hydroxylase fibroblast growth factor 23 (FGF23), showed elevated placental expression of CYP24A1 mRNA in these mice (Ma *et al.* 2014, Ohata *et al.* 2014). Likewise, direct injection of FGF23 into normal placentas from wild-type mice also induced expression of CYP24A1 (Ohata *et al.* 2014). This appears to be mediated via trophoblast expression of fibroblast growth factor receptor 1 and its co-receptor α-klotho by trophoblast, suggesting that catabolism via CYP24A1 plays an as yet undefined role in mediating trophoblast effects of vitamin D.

Despite a wide range of studies showing regulation and activity of vitamin D metabolic enzymes in primary trophoblast cells and trophoblast cell lines, the principal functional analysis of vitamin D in these cells has centered on responses to $1,25(OH)_2D$. Initial experiments using JEG-3 cells described stimulation of calcium uptake (Tuan *et al.* 1991), and the regulation of the cytosolic calcium-binding protein calbindin-D28K (Belkacemi *et al.* 2005) by $1,25(OH)_2D$, consistent with a role for vitamin D in the endocrinology of placental calcium homeostasis. However, subsequent investigations of trophoblast cells and $1,25(OH)_2D$ have explored other mechanisms associated with placental endocrine function. These reports include the stimulation of human placental lactogen synthesis and release (Stephanou *et al.* 1994), hCG expression (Barrera *et al.* 2008) and the regulation of estradiol and progesterone synthesis (Barrera *et al.* 2007).

In recent years, our perspective on vitamin D and trophoblast function has been expanded to include studies of immunomodulatory function. In primary trophoblast cells and trophoblast cell lines, $1,25(OH)_2D$ has been shown to potently stimulate the expression of the antibacterial protein cathelicidin (Liu *et al.* 2009), while also suppressing inflammatory responses to tumor necrosis factor α (TNFα) (Diaz *et al.* 2009). Similar anti-inflammatory responses to $1,25(OH)_2D$ have also been reported using trophoblasts from women with the inflammatory disorders of pregnancy, preeclampsia (Noyola-Martinez *et al.* 2013) and antiphospholipid syndrome (APS) (Gysler *et al.* 2015). In recent studies, the anti-inflammatory effects of $1,25(OH)_2D$ on trophoblasts have been reported to include attenuation of oxidative stress-induced microparticle release from preeclampsia trophoblastic cells (Xu *et al.* 2017), further underlining the importance of this facet of vitamin D function within the placenta. *In vivo*, studies using *Cyp27b1*−/− and *Vdr*−/− mice have shown that loss of both alleles for either of these genes on the fetal side of the placenta alone was sufficient to dramatically exacerbate anti-inflammatory responses to lipopolysaccharide (LPS) immune challenge (Liu *et al.* 2011). Thus, in addition to the active immune cell function classically observed in the maternal decidua, trophoblast cells also appear to make a major contribution to the regulation of placental inflammation.

A role for vitamin D in EVT invasion?

Controlled invasion of fetal cytotrophoblast and differentiated EVT cells into the maternal decidua and myometrium in the first trimester of pregnancy is a key process in placentation and is essential for successful pregnancy. A complex network of communications among trophoblast, decidual stromal and immune cells is reported to facilitate implantation and maintenance of pregnancy, with key roles in tissue remodeling, cell trafficking and immune tolerance being evident (Oreshkova *et al.* 2012). The mechanisms underpinning these processes have received increasing attention since

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abnormal placentation due to shallow invasion of EVT can cause important pregnancy disorders such as miscarriage (Ball *et al.* 2006), preeclampsia (Caniggia *et al.* 2000), fetal growth restriction, preterm birth and stillbirth (Goldman-Wohl & Yagel 2002, Kaufmann *et al.* 2003, Kadyrov *et al.* 2006, Reddy *et al.* 2006). By contrast, unrestricted invasion resulting from a failure to restrain the invading cytotrophoblast is associated with premalignant conditions such as malignant choriocarcinomas and invasive mole (Ringertz 1970, Caniggia *et al.* 2000) and can lead to aberrant placentation such as pathological adhesion to the myometrium (placenta accreta), extension into the myometrium (placenta increta) or invasion through the myometrium into adjacent organs (placenta percreta) (Khong 2008).

In recent studies, we have shown that human EVT isolated from first trimester pregnancies are a target for both 25(OH)D and $1,25(OH)_2D$ (Chan *et al.* 2015). In *ex vivo* experiments, both vitamin D metabolites promoted the invasion of EVT through Matrigel, with zymographic analysis showing that this effect involves enhanced expression of the matrix metalloproteinases pro-MMP2 and pro-MMP9 (Chan *et al.* 2015). These observations are in direct contrast to previously published studies describing $1,25(OH)_2D$ inhibition of matrix invasion by tumor cells (Bao *et al.* 2006). In this case, the primary mode of action for $1,25(OH)_2D$ was indirect suppression of MMPs via enhanced tissue inhibitor of metalloproteinase-1 (TIMP-1) expression. However, in other reports, low vitamin D status has been shown to be associated with elevated circulating MMP2 and MMP9 (Timms *et al.* 2002). Suppression of a variety of MMPs, including MMP2 and MMP9, by $1,25(OH)_2D$ has also been described for primary cultures of human uterine fibroid cells and uterine fibroid cell lines (Halder *et al.* 2013). Thus, the pro-invasive effects of vitamin D on EVTs appear to be quite distinct to pregnancy and the placenta.

The concept of vitamin D as a regulator of cellular motility and invasion is not novel and has been extensively reported in cancer states (Krishnan *et al.* 2012, Leyssens *et al.* 2014, Ma *et al.* 2016), where effects of vitamin D have been related to modulation of epithelial–mesenchymal transition (EMT) (Fischer & Agrawal 2014, Chen *et al.* 2015, Hou *et al.* 2016). Interestingly, this effect of vitamin D has not been observed in non-pathophysiological states or during embryogenesis. For example, vitamin D is known to inhibit invasion and motility of ovarian cancer and teratocarcinoma cell lines, but does not affect these cellular characteristics in the non-neoplastic ESD3 murine embryonic cell line (Abdelbaset-Ismail *et al.* 2016). The precise molecular mechanisms that mediate migration and invasion regulation by vitamin D remain unclear, although several different pathways have been studied. Notably, vitamin D has been shown to regulate the actin cytoskeleton in numerous cell types. In osteoblast-like cells, vitamin D promotes actin polymerization as part of its transcriptional induction of fibroblast growth factor 23 (Fajol *et al.* 2016). In endometrial cells, vitamin D treatment has also been shown to induce changes in actin architecture, through regulation of the RAc1/Pak1 axis (Zeng *et al.* 2016). It is not clear if such responses are also seen in trophoblast cells during placental development, but vitamin D has been shown to rescue motility defects in fetal endothelial colony-forming cell function of umbilical vein endothelial cells derived from pregnancies complicated by preeclampsia (von Versen-Hoynck *et al.* 2014) and gestational diabetes (Gui *et al.* 2015).

Effects of vitamin D on EVT invasion and migration may also be mediated indirectly via effects on other known EVT regulators. $1,25(OH)_2D$ has been shown to abolish S1P-mediated inhibition of migration via suppression of S1PR2 in trophoblast cell lines Swan-71 and JEG-3 (Westwood 2017). $1,25(OH)_2D$ has also been shown to stimulate hCG expression and secretion via a cAMP/PKA-mediated signaling pathway (Barrera *et al.* 2008). Although hCG is a potent regulator of trophoblast motility and invasion (Chen *et al.* 2011, Evans 2016), it is unclear whether changes in hCG expression are specifically required for effects of vitamin D on trophoblast invasion. In a similar fashion, $1,25(OH)_2D_3$ has been shown to positively regulate progesterone synthesis by human trophoblast cells from term placenta (Barrera *et al.* 2007). In HTR8/SVneo trophoblast cells, which have been reported to consist of a mixed population of cells, progesterone appears to suppress trophoblast motility and invasion (Chen *et al.* 2011). Thus, $1,25(OH)_2D$ may exert indirect effects on trophoblast invasion, although it is still not clear whether these effects are pro-migratory. Indirect actions of vitamin D on EVT function may also stem from effects on placental cell differentiation. Recent studies have shown that inactivation of VDR in trophoblastic BeWo cells resulted in increased trophoblast differentiation and syncytium formation (Nguyen *et al.* 2015). In a similar fashion, vitamin D may also influence EVT invasion and motility indirectly by targeting the development of cells on the maternal side of the placenta. Endometrial stromal cells treated with $1,25(OH)_2D$ have elevated expression of specific genes, including *HOXA10*

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(Du *et al.* 2005*a*), which are known to be involved in the regional development of uterine decidualization and embryo implantation by controlling downstream target genes. The complex circuitry of vitamin D metabolism and function involved in mediating direct or indirect effects on EVT invasion and migration has still to be fully elucidated and is likely to be a key component of future studies of vitamin D in pregnancy.

Vitamin D and trophoblast function: clinical implications

Irrespective of proposed functional targets, vitamin D dysregulation during pregnancy has been linked to adverse effects on placental function and pregnancy in general. In 2010, the Institute of Medicine (IOM) defined vitamin deficiency as serum concentrations of 25(OH)D less than 20 ng/mL (50 nM) (Holick *et al.* 2011). Subsequently, the Endocrine Society issued slightly different guidelines, defining vitamin D insufficiency as being serum 25(OH) D levels below 30 ng/mL (75 nM) (Holick *et al.* 2011). Against this backdrop, several recent publications have highlighted the prevalence of low serum concentrations of 25(OH)D (less than 25 nM) in pregnant women: 20% of pregnant women in the UK (Javaid *et al.* 2006), 25% in the UAE (Dawodu *et al.* 1997), 80% in Iran (Bassir *et al.* 2001), 45% in northern India (Sachan *et al.* 2005), 60% in New Zealand (Eagleton & Judkins 2006) and 60–84% of pregnant non-Western women in the Netherlands (van der Meer *et al.* 2006). It remains unclear if this reflects simply a normal physiological drop in vitamin D concentrations during pregnancy or if pregnancy is a stress test that can exacerbate and unmask pathological vitamin D deficiency.

Vitamin D deficiency in pregnant women has been shown to be associated with increased risk for pregnancy complications (Lewis *et al.* 2010). These include preeclampsia (Bodnar *et al.* 2007*b*), fetal growth restriction, small-for-gestational-age fetus (Bodnar *et al.* 2010), bacterial vaginosis (Bodnar *et al.* 2009) and gestational diabetes mellitus (Maghbooli *et al.* 2008, Zhang *et al.* 2008). Maternal vitamin D deficiency has also been linked to adverse effects in offspring, including reduced bone density (Javaid *et al.* 2006) and childhood rickets (Wagner & Greer 2008), as well as increased risk of asthma (Camargo *et al.* 2007) and schizophrenia (McGrath 2001).

The impact of vitamin D status on early events in pregnancy has also been studied. In northern countries, where there is a strong seasonal contrast in light exposure and UVB-induced vitamin D production in skin, conception rates are decreased during winter months, with rates rising during summer and an increased birth rate in spring (Rojansky *et al.* 1992). Interestingly, ovulation rates and endometrial receptivity also appear to be reduced during long dark winters in northern countries (Rojansky *et al.* 2000), which may be explained in part by seasonal variations in vitamin D levels. With this in mind, several observational studies have investigated the potential impact of vitamin D on *in vitro* fertilization (IVF), albeit with largely conflicting outcomes. In a study of infertile women undergoing IVF, those with higher levels of 25(OH)D in serum and follicular fluid, were more likely to achieve pregnancy following IVF, and high vitamin D levels were also shown to improve the parameters of controlled ovarian hyperstimulation (Ozkan *et al.* 2010). Aleyasin and coworkers found no significant association between 25(OH)D levels in serum and follicular fluid with IVF outcomes (Aleyasin *et al.* 2011). However, this did not include any women with a serum vitamin D level >50 nmol/L. In another study of 100 women undergoing IVF, serum concentrations of 25(OH)D were positively associated with fertilization rate (Abadia *et al.* 2016). However, serum 25(OH)D was unrelated to the probability of pregnancy or live birth after IVF (Abadia *et al.* 2016). Anifandis and coworkers investigated 101 women who received IVF-intracytoplasmic sperm injection (ICSI) ovarian stimulation cycles. In this study, women with vitamin D sufficiency (25(OH)D level >30 ng/mL in follicular fluid) had a lower quality of embryos and were less likely to achieve clinical pregnancy, compared with women with insufficient (follicular fluid 25(OH)D level 20.10–30 ng/mL) or deficient vitamin D status (follicular fluid 25(OH)D level <20 ng/mL) (Anifandis *et al.* 2010).

Elucidation of the immunomodulatory effects of $1,25(OH)_2D$ has led to the suggestion that vitamin D might have a role in protecting against spontaneous abortion (Bubanovic 2004). This was supported by *ex vivo* analyses showing that $1,25(OH)_2D$ is able to suppress inflammatory cytokine production by endometrial cells from women with unexplained recurrent spontaneous abortions (Tavakoli *et al.* 2011). More recently, $1,25(OH)_2D$ has been shown to potently regulate natural killer cells from women with recurrent miscarriage (Ota *et al.* 2015). Considering these observations, the impact of maternal vitamin D status on pregnancy outcome has been studied in several cohorts. In a large prospective cohort study of 1683 pregnant women donating serum before

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gestational week 22, serum concentrations of 25(OH)D less than 50 nM were associated with a >2-fold increase in first miscarriage rate, although no significant effect was observed for second trimester miscarriage (Andersen *et al.* 2015). In a prospective study of pre-conceptual vitamin D, maternal serum 25(OH)D levels were not found to be associated with chances of conceiving or overall risk of miscarriage (Moller *et al.* 2012). However, women with miscarriage in the second trimester had lower first trimester serum concentrations of 25(OH)D than those women who did not miscarry (Moller *et al.* 2012). In a much larger, nested case-control study of over 5000 women did not reveal any adverse effects of low serum 25(OH)D on pregnancy outcomes (Schneuer *et al.* 2014). A recent meta-analysis and systematic review concluded that vitamin D deficiency is not associated with increased risk of spontaneous recurrent abortion (Amegah *et al.* 2017). Thus, the possible impact of sub-optimal vitamin D on implantation and adverse pregnancy outcomes such as miscarriage still remains unclear. Interestingly, in endometrial tissue from women with unexplained recurrent spontaneous abortion, expression of key components in the vitamin D metabolic (CYP27B1/CYP24A1) and signaling (VDR) systems was found to be comparable to endometrial tissue from healthy fertile women (Tavakoli *et al.* 2015). By contrast, recent studies of women with recurrent miscarriage showed that expression of mRNA and protein for CYP27B1 in villous and decidual tissue was lower than in control tissues from normal healthy pregnancies (Wang *et al.* 2016). In future studies it will be important to clarify how variations in the vitamin D system within the placenta and fetal trophoblast cells affect implantation and the maintenance of a successful healthy pregnancy.

A major contributing factor to vitamin D status in pregnant women is obesity, with lower circulating levels of 25(OH)D being reported in in pregnant women with high body mass index (BMI), relative to pregnant women with a normal BMI (Bodnar *et al.* 2007*a*, Karlsson *et al.* 2015). Maternal obesity is associated with adverse health effects for both mother and child, with increased inflammation has been proposed as an important pathological mechanism for the detrimental effects of obesity during pregnancy (Denison *et al.* 2010, Pantham *et al.* 2015). A role of vitamin D in the process is still unclear. However, given the established anti-inflammatory effects of vitamin D at the fetal–maternal interface (Tamblyn *et al.* 2015), it is possible that some pregnancy effects of obesity are mediated via low circulating maternal vitamin D.

Conclusions

Expression of placental CYP27B1 and VDR at early stages of pregnancy suggests an important role for vitamin D in placental physiology. In previous studies, we have hypothesized that placental vitamin D may function, at least in part, to promote antimicrobial and anti-inflammatory immune activity, with both the maternal decidua and fetal trophoblast contributing to these actions. However, analysis of trophoblast cells *ex vivo* and *in vitro* indicates that vitamin D may have a much broader role in placental function, including the regulation of trophoblast differentiation and EVT invasion of the decidua and myometrium (Fig. 1). Thus, effects of vitamin D may occur earlier in pregnancy than previously appreciated, underlining the requirement for adequate vitamin D status across gestation. To date, studies of vitamin D status (maternal serum 25(OH)D) in pregnancy have tended to focus on later stages of pregnancy, and associated adverse events such as preterm birth, gestational diabetes and preeclampsia. Likewise, supplementation trials for vitamin D in pregnancy have focused on women between 10 and 18 weeks of pregnancy. However, the responsiveness of trophoblast cells to $1,25(OH)_2D$, notably effects on EVT invasion, suggests that further studies of vitamin D and adverse events in early pregnancy are required. To date, there have been a limited number of reports of vitamin D deficiency and miscarriage, but these need to be expanded to include more rigorous supplementation trials. The review we present is supportive of early, pre-conceptual, supplementation with vitamin D.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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