Vitamin D Receptor Gene Ablation in the Conceptus Has Limited Effects on Placental Morphology, Function and Pregnancy Outcome

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Abstract

Vitamin D deficiency has been implicated in the pathogenesis of several pregnancy complications attributed to impaired or abnormal placental function, but there are few clues indicating the mechanistic role of vitamin D in their pathogenesis. To further understand the role of vitamin D receptor (VDR)-mediated activity in placental function, we used heterozygous Vdr ablated C57Bl6 mice to assess fetal growth, morphological parameters and global gene expression in Vdr null placentae. Twelve Vdr+/− dams were mated at 10–12 weeks of age with Vdr+/− males. At day 18.5 of the 19.5 day gestation in our colony, females were euthanised and placental and fetal samples were collected, weighed and subsequently genotyped as either Vdr+/+, Vdr+/− or Vdr−/−. Morphological assessment of placentae using immunohistochemistry was performed and RNA was extracted and subject to microarray analysis. This revealed 25 genes that were significantly differentially expressed between Vdr+/+ and Vdr−/− placentae. The greatest difference was a 6.47-fold change in expression of Cyp24a1 which was significantly lower in the Vdr−/− placentae (P < 0.01). Other differentially expressed genes in Vdr−/− placentae included those involved in RNA modification (Snord123), autophagy (Atg4b), cytoskeletal modification (Shroom4), cell signalling (Piscr1, Pex5) and mammalian target of rapamycin (mTOR) signalling (Deptor and Prr5). Interrogation of the upstream sequence of differentially expressed genes identified that many contain putative vitamin D receptor elements (VDREs). Despite the gene expression differences, this did not contribute to any differences in overall placental morphology, nor was function affected as there was no difference in fetal growth as determined by fetal weight near term. Given our dams still expressed a functional VDR gene, our results suggest that cross-talk between the maternal decidua and the placenta, as well as maternal...
Introduction

Normal fetal development is undoubtedly underpinned by normal placental function. The placental vascular network provides an interface between the fetus and mother for the exchange of gases, nutrients and wastes [1]. Additionally, the placenta acts as an endocrine organ responsible for the production of numerous hormones which maintain pregnancy and orchestrate maternal adaptation to pregnancy [2]. Maternal nutrient status underlies the availability of nutrients being transferred to the fetus to support optimal growth. Placental research is increasingly focused on how the organ adapts to support adequate fetal growth in a potentially sub-optimal nutrient available environment [3].

The prevalence of vitamin D deficiency and insufficiency in pregnant women is increasing worldwide [4, 5] and accumulating evidence associates vitamin D deficiency with a range of pregnancy complications including preeclampsia [6, 7], gestational diabetes mellitus [8] and preterm birth [9]. Additionally, maternal vitamin D deficiency increases the chance of delivering a baby who is small for gestational age [10, 11] and has also been linked to the development of asthma [12], autism [13], and reduced bone mineral accrual [14, 15] in the offspring. During human pregnancies, serum levels of the active form of vitamin D₃ (1,25(OH)₂D₃) increase by 2 to 5-fold [16–18] suggesting an important role for vitamin D in supporting the pregnancy and fetal development. While vitamin D supplementation has been reported to help neonatal outcomes [19], the lack of high quality intervention data to confirm a causal role for vitamin D in pregnancy outcomes [20] and a description of the underlying mechanisms are lacking.

While the secosteroid hormone, 1,25(OH)₂D₃, is widely associated with calcium and phosphate homeostasis [21], other functions for 1,25(OH)₂D₃ activity have been identified such as in modulation of immune [22] and vascular [23] function, brain [24] and muscle [25] development, and bone remodelling [26–28]. In general, the actions of 1,25(OH)₂D₃ are broadly associated with regulating cell proliferation and differentiation [29, 30]. The effects of 1,25(OH)₂D₃ are mediated through the vitamin D receptor (VDR), a predominantly nuclear receptor, expressed in numerous tissues including the placenta [31, 32]. The liganded VDR, together with retinoid X receptor (RXR) in a dimer complex, binds to genomic vitamin D responsive elements (VDREs), located primarily in upstream flanking regions of genes, and recruit a cell-specific transcription factor complex which regulates the expression of numerous genes [33, 34]. Local synthesis and metabolism of 1,25(OH)₂D₃ within the placenta is likely to occur given that placental cells expresses both CYP27B1, which encodes the enzyme to produce 1,25(OH)₂D₃, and CYP24A1, which encodes for enzyme responsible for the catabolism of 1,25(OH)₂D₃ [35].

Although vitamin D activity in the decidua is suggested to regulate immune tolerance during pregnancy [22], the evidence that supports the link between VDR expression, placental growth, function and fetal outcome is lacking. Previously, Vdr knockout (Vdr⁻/⁻) dams have been shown to exhibit both a reduction in the rate of conception and reduced fetal weights when compared to heterozygous (Vdr⁺/⁻) dams [36]. However, such studies are unable to discern whether the ablation of Vdr in the placenta contributes to these outcomes. Studies on vitamin D and placental function are limited and have focused on immune function within the maternal decidua of Vdr knockout mice [37] or on placental morphometry in dietary vitamin...
D restricted animals [38]. Thus, we used heterozygous matings of Vdr knockout mice to investigate the effects of Vdr ablation specifically in the conceptus by characterising placental morphology, fetal growth and global placental gene expression measures near term. The study design specifically excluded confounding effects of perturbed Vdr signalling in the mother to elucidate placenta specific effects. We chose late gestation as a first step in elucidating the role of vitamin D signalling in placental structural and functional development as this corresponds most closely to the time at which placentas could be sampled from women.

**Methods**

**Animals**

Ethics approval was obtained from both the SA Pathology/Central Northern Adelaide Health Service Animal Ethics Committee and the University of Adelaide Animal Ethics Committees with all animal work complying with the Australian Code of Practice for the Care and Use of Animals. Global Vdr ablated C57Bl6 mice (strain B6.129S4-VDRtm1Mbd/J, Jackson Laboratory JAX Mice Services) were generated as previously described [39]. At weaning, 12 virgin Vdr+/- females were fed a standard rodent diet containing 0.8% calcium and 0.7% phosphorus (Specialty Feeds), water ad libitum and were maintained on a 12:12 light-dark cycle. Females at 10–12 weeks of age were mated with a Vdr+/- male to generate offspring of all three genotypes (Vdr-/-, Vdr+/-, Vdr+/+). The day of copulatory plug detection was designated day 0.5 of pregnancy. On day 18.5 of the 19.5 day pregnancy in our colony, females were anaesthetised with an intraperitoneal injection of Avertin (20 mg/mL) to collect blood and then killed via cervical dislocation. Fetuses and placentae were collected and weighed. The placentae were bisected mid-sagittally with half stored RNAlater and subsequently at -80°C for gene expression analyses, while the remaining half was fixed for histological analyses.

**Genotyping**

To determine Vdr genotype and fetal sex, DNA was extracted from fetal tails using the salting-out procedure detailed in [40]. Following DNA quantification, samples were diluted to 20 ng/μL in TE buffer and used in PCR for Vdr genotyping (Table A in S1 File) [41] or Sry detection (Table B in S1 File) [42], respectively. Final PCR reactions were performed on 10 ng/μL of DNA in a 20 μL reaction containing 10 μL SsoFast EvaGreen Supermix (BioRad) and 10 μM Vdr primers or 200 nM Sry primers. Outcomes of the PCR were validated using gel electrophoresis on a 2% and 2.7% agarose gel for Vdr and Sry, respectively (Fig A in S1 File).

**Placental histology**

Histological analyses were performed on all placentae from Vdr+/+ dams to capture all genotypes. Bisected placentae were washed twice in PBS over 2 hours to remove RNAlater then fixed in 10% neutral buffered formalin (Australian Biostain). Samples were subsequently washed in three changes of PBS and stored in 70% ethanol prior to paraffin embedding. 5 μm thickness full-face sections were stained with Masson’s Trichrome following standard protocols in order to determine mid sagittal labyrinth and junctional zone cross sectional areas or subjected to immunohistochemistry (IHC) as previously described [43].

Fetal capillaries and trophoblast cells in the placental labyrinth were identified by double-label IHC with anti-vimentin (#M7020, Dako, Agilent Technologies; 1/5 dilution) and anticytokeratin antibodies (#MAB3412, Merck Millipore; 1/100 dilution), respectively [43]. Briefly, antigen retrieval was performed with 0.3 mg/mL Pronase (P8811, Sigma-Aldrich) in PBS, with a Mouse-on-Mouse IHC kit (Abcam) used to prevent non-specific binding. Chromogen
diaminobenzidine (DAB, Sigma Aldrich) was used to form a brown precipitate for anti-cyto-keratin labelling and with 2% nickel II sulphate (Sigma Aldrich) to form a black precipitate for anti-vimentin labelling. Sections were counterstained with haematoxylin and eosin (Sigma Aldrich).

Immunohistochemically labelled slides were analysed by point counting ten fields per placenta to estimate volume densities and volumes of fetal capillaries, trophoblasts and maternal blood space and intercept counting to estimate the surface density and thickness of trophoblast for exchange, previously described in [43]. The coefficient of variation was <5%.

**RNA extraction, microarray preparation and qPCR**

Placental tissue was homogenised using a Powerlyzer with ceramic 1.4 mm beads (Mo Bio Laboratories, Inc) before total RNA was extracted using Trizol (Invitrogen) following the manufacturer’s instructions. RNA integrity was determined using the Experion (BioRad) system.

For microarray, eight Vdr<sup>+/+</sup> and eight Vdr<sup>-/-</sup> placentae from six heterozygous dams were analysed. Biotinylated cRNA was prepared according to the standard Affymetrix protocol from 250 ng total RNA following the Manual Target Preparation Guidelines for GeneChip Whole Transcript (WT) Expression Arrays. RNA with RQI > 9 was sent to the Ramaciotti Centre for Genomics, Sydney, Australia, where 3.5 μg of fragmented and labelled single-stranded cRNA was hybridised on Affymetrix MoGene 2.1 ST arrays and washed and stained following the Manual Target Preparation Guidelines for GeneChip Whole Transcript (WT) Expression Arrays. Arrays were scanned using the Affymetrix GeneChip scanner.

For microarray validation, extracted RNA from 17 Vdr<sup>+/+</sup> and 16 Vdr<sup>-/-</sup> placentae was DNase treated using TURBO DNA-free (Ambion) as per the manufacturer’s instructions. PCR and subsequent agarose gel confirmed the absence of genomic DNA (Table C in S1 File). 500 ng of each sample was then reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Each cDNA sample was diluted 1:20 before performing quantitative PCR (qPCR) in triplicate by real time PCR using TaqMan Gene Expression assays (Table D in S1 File). All qPCR gene expression data were normalised to Hbms.

**Microarray differential expression**

Affymetrix Mouse Gene 2.1 ST array data were pre-processed, background subtracted and quantile normalised using the RNA method in the Oligo package. Array probes were annotated using the Bioconductor Affymetrix mogene21 annotation data package, with all unannotated probes subsequently removed from the dataset. Testing for differential expression between groups was performed using linear models and Empirical Bayes methods, with contrasts between groups incorporating the mother as a blocking factor using the Limma package [44]. All P-values were corrected for multiple testing by calculating the false discovery rate (FDR).

Microarray data have been deposited to NCBI GEO under accession GSE61583 and analysis code is included with the S2 File. Data analyses were performed in R version 3.1.1.

**VDRE enrichment analysis**

The top up- and down-regulated genes between Vdr<sup>-/-</sup> and Vdr<sup>+/+</sup> placentae as determined in the microarray (>1.5 fold-change, p < 0.01) were analysed for presence of putative vitamin D responsive elements (VDRE’s) that potentially bind the RXRA::VDR transcription factor complex using oPOSSUM and the JASPAR vertebrate core profile for RXRA::VDR (MA0074.1) [45, 46]. For each gene, we searched for RXRA::VDR motifs in the 10 kb upstream and downstream sequences from the transcription start site using a conservation cut-off of 0.4, a matrix score threshold of 75% and a minimum specificity of 8-bits.
Statistics
To test for morphological differences between Vdr genotypes, weighted mixed-effects linear models were fitted to the data and included fetal sex as a covariate and were weighted by litter size using the lme function in the nlme package in R v3.1.1. Gene expression differences were assessed by the Mann-Whitney test to calculate exact P-values. Results are reported as mean normalised expression ± standard error.

Results
To examine the role of Vdr signaling in the placenta and the effects on fetal and placental growth and development, Vdr+/− females were mated with Vdr+/− males and sacrificed on day 18.5 of pregnancy. A standard Mendelian 1:2:1 ratio distribution of genotypes for Vdr was observed when accounting for and excluding resorptions. Of the 12 pregnancies, 77 fetuses were collected and analysed, with Sry genotyping revealing 45 female and 32 male fetuses (Table 1).

The effects of VDR depletion on fetal and placental parameters
The effect of Vdr ablation on fetal and placental measures was assessed initially by analyzing fetal and placental weights in 17 Vdr+/+, 54 Vdr+/− and 21 Vdr−/− conceptuses, with no significant differences detected across the genotypes (Fig 1 shows data for 8 Vdr+/+ verses 8 Vdr−/− conceptuses for which microarray analyses were undertaken). Placental structure, examined firstly by Masson’s trichrome staining, revealed no significant differences in morphology between Vdr−/− and Vdr+/+ genotypes. These morphology measures included total mid sagittal cross sectional area, junctional zone and labyrinth zone areas and the proportion of junctional zone to labyrinth zone. In mice, the placental labyrinth is the area in which physiological exchange of nutrients and waste products occurs between fetal and maternal bloodstreams, whereas the junctional zone contains placental stem cells and is involved in hormone production. A larger labyrinth or a higher labyrinth to junctional zone ratio suggests enhanced placental efficiency. Given there were no differences in the proportions of junctional and labyrinth zones, this suggests similar placental efficiency, which corresponds to the similar fetal weights across genotypes (Fig 1).

Further quantification of labyrinth zone structure using double-labelled IHC showed no significant differences between genotypes for volume densities or volumes of trophoblasts, fetal

| Table 1. Pregnancy characteristics of Vdr−/+ dams at gestational day 18.5. Data expressed as mean ± SEM.  |
|-----------------------------------------------|--------------------------|
| Parameter                                      | Vdr−/+ (n = 12)           |
| Percentage conceived                           | 100%                     |
| Weight gain during pregnancy (g)               | 18.13 ± 3.64             |
| % Maternal weight gain during pregnancy        | 89.15 ± 19.89            |
| Viable litter size                             | 6.58 ± 0.54              |
| Number reabsorptions                           | 1.17 ± 0.32              |
| Average Fetal Weight (g)                       | 1.13 ± 0.016             |
| Placental weight (g)                           | 0.78 ± 0.074             |
|                                                | Male (n = 34)            |
|                                                | Female (n = 45)          |
| Fetal weight                                   | 1.16 ± 0.035             |
|                                                | 1.10 ± 0.019             |
| Placental weight                               | 0.12 ± 0.0029            |
|                                                | 0.11 ± 0.0033            |
| P-value                                        | 0.008                    |
|                                                | 0.255                    |

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capillaries and maternal blood space, as well as surface density of trophoblast. Our data suggest feto-placental Vdr ablation does not affect placental composition nor functional capacity.

Fig 1. Comparison of mouse placental morphology measurements between Vdr^{−/−} and Vdr^{+/+} genotypes at day 18.5pc. No significant differences were observed in any of the morphology parameters assessed between the two genotypes (P>0.05) in the 8 Vdr^{+/+} and 8 Vdr^{−/−} placentas analysed by microarray. Horizontal line on each plot represents mean. MBS: maternal blood space; VD: volume density.

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Altogether, analyses of fetal and placental parameters clearly indicated that there were no gross morphological differences that may underpin phenotypic changes such as hypocalcemia, hyperparathyroidism and rickets experienced by \( Vdr^-/- \) pups from weaning \cite{39, 47}. Such changes may however be modulated by placental or fetal gene expression differences.

### The effect of VDR ablation on the placental transcriptome

To test for the effect of \( Vdr \) ablation on gene expression in the placenta, transcriptome profiles of eight placentae per genotype were assessed by microarray. Twenty-five genes were detected as being differentially expressed between \( Vdr^-/- \) and \( Vdr^{+/+} \) placentae with an absolute fold change >1.3 and a false discovery rate (FDR) <0.05 (Table 2). The greatest difference was a 6.47-fold change (FDR = 0.0012) in the expression of \( Cyp24a1 \), which was lower in the \( Vdr^-/- \) placentae. As \( Cyp24a1 \) is directly upregulated through Vdr as part of the vitamin D metabolic pathway, severely reduced placental \( Cyp24a1 \) expression in \( Vdr^-/- \) placentae would be expected.

Other genes that were differentially expressed included genes involved in RNA modification (\( Snord123 \)), autophagy (\( Atg4b \)), cytoskeletal modification (\( Shroom4 \)), cell signaling (\( Plscr1 \), \( Pex5 \), \( Rgs17 \)), and mammalian target of rapamycin (mTOR) signaling (\( Deptor \), \( Prr5 \)). Of these differentially expressed genes, 12 were more highly expressed in \( Vdr^-/- \) placentae and 13 had lower expression levels when compared to \( Vdr^{+/+} \) placentae. No significant differences in gene expression between the sexes within each genotype were detected (data not shown).

### Table 2. Genes differentially expressed between \( Vdr^-/- \) and \( Vdr^{+/+} \) placentae.

| Gene       | Microarray Fold change | \( Vdr^-/- \) Expression | \( P \)-value | FDR  | qPCR Fold change | \( Vdr^-/- \) Expression | \( P \)-value |
|------------|------------------------|---------------------------|--------------|------|-----------------|---------------------------|-------------|
| Cyp24a1    | 6.47                   | †                         | 5.0E-08      | 0.001| 95.27           | †                         | <0.001      |
| Snord123   | 1.58                   | †                         | 4.3E-06      | 0.027| -               | -                         | -           |
| Atg4b      | 1.33                   | †                         | 4.5E-06      | 0.027| -               | -                         | -           |
| Snora28    | 1.49                   | †                         | 5.5E-06      | 0.027| -               | -                         | -           |
| Snora69    | 1.75                   | †                         | 8.4E-06      | 0.03 | -               | -                         | -           |
| Nmp28      | 1.54                   | †                         | 8.5E-06      | 0.03 | -               | -                         | -           |
| Plscr1     | 1.47                   | †                         | 1.2E-05      | 0.035| 1.59            | †                         | 0.017       |
| Deptor     | 1.54                   | †                         | 1.3E-05      | 0.035| 1.37            | †                         | 0.029       |
| Ep400      | 1.78                   | †                         | 1.4E-05      | 0.035| -               | -                         | -           |
| Shroom4    | 1.41                   | †                         | 1.7E-05      | 0.037| -               | -                         | -           |
| Anp32a     | 1.40                   | †                         | 1.83E-05     | 0.037| -               | -                         | -           |
| Col16a1    | 1.37                   | †                         | 1.95E-05     | 0.037| -               | -                         | -           |
| DSetrd579e | 1.57                   | †                         | 2.32E-05     | 0.039| -               | -                         | -           |
| Pex5       | 1.31                   | †                         | 2.35E-05     | 0.039| -               | -                         | -           |
| A730036I17R| 1.54                   | †                         | 2.60E-05     | 0.039| -               | -                         | -           |
| Ms4a4d     | 2.01                   | †                         | 2.70E-05     | 0.039| -               | -                         | -           |
| Prr5       | 1.36                   | †                         | 2.88E-05     | 0.039| -               | -                         | -           |
| Sdk2       | 1.35                   | †                         | 3.52E-05     | 0.040| -               | -                         | -           |
| Raldgs     | 1.33                   | †                         | 5.42E-05     | 0.049| -               | -                         | -           |
| Tnfl2      | 1.33                   | †                         | 5.52E-05     | 0.049| -               | -                         | -           |
| Mir877     | 1.76                   | †                         | 5.90E-05     | 0.050| -               | -                         | -           |
| Mgp        | 2.75                   | †                         | 6.31E-05     | 0.050| -               | -                         | -           |
| Cd302      | 1.43                   | †                         | 6.70E-05     | 0.050| -               | -                         | -           |
| Snora15    | 1.50                   | †                         | 6.77E-05     | 0.050| -               | -                         | -           |
| Farn69a    | 1.42                   | †                         | 6.81E-05     | 0.050| -               | -                         | -           |
Although only 25 genes were classed as statistically different between $Vdr^{-/-}$ and $Vdr^{+/+}$ groups, unsupervised clustering analysis of the top 50 differentially expressed genes grouped samples together by genotype, and inspection of the standardised z-scores revealed distinct patterns in gene expression between the groups with unknown subsequent effects in offspring (Fig 2A).

**Microarray validation by qPCR**

Independent validation of the microarray results on 17 $Vdr^{+/+}$ and 16 $Vdr^{-/-}$ placentae was performed by qPCR and included additional biological replicates. $Vdr$ expression was virtually
undetectable in \(Vdr^{+/+}\) placentae by both microarray (Fig 2B) and qPCR (Fig 3). Validation by qPCR of the microarray findings eliminates the possibility of significant transcript contamination from the heterozygous maternal tissues, as the wild type allele was not detected. Therefore, it is likely that the \(Vdr\) background levels of expression in the microarray data is the result of non-specific cDNA binding with the Vdr probes. Further expression analysis of \(Cyp24a1\), \(Deptor\) and \(Plscr1\) by qPCR correlated with results obtained by microarray and showed that even changes <1.5 fold, such as with \(Plscr1\), were replicable (Table 2 and Fig 3).

VDRE enrichment analysis

To assess if differential expression between \(Vdr^{+/+}\) and \(Vdr^{-/-}\) placentae was potentially driven by the VDR-RXR transcription factor complex, we searched for the presence of VDR-RXR transcription factor motifs in the 10kb up and down-stream of the transcription start sites of differentially expressed genes. These analyses revealed that genes that were more highly in \(Vdr^{+/+}\) placentae feature more VDR binding motifs in the regions upstream of transcriptional start sites (Fig 4A), with many of these genes having more than one site per gene (Fig 4B). Expression of \(Vdr\) was also positively correlated with the expression of genes with upstream VDRE’s such as \(Cyp24a1\) (\(R^2 = 0.56, P = 2.7e-05\)) and \(Deptor\) (\(R^2 = 0.41, P = 7e-04\)) (Fig 5).
Fig 4. VDRE enrichment analysis of differentially expressed genes between Vdr<sup>−/−</sup> and Vdr<sup>+/+</sup> placenta. (A) Density of predicted VDR transcription factor binding sites in the sequence flanking transcription start sites (TSS) of genes differentially expressed between Vdr<sup>−/−</sup> and Vdr<sup>+/+</sup> samples. Blue curve represents genes more highly expressed in Vdr<sup>+/+</sup> samples, orange curve represents genes more highly expressed in Vdr<sup>−/−</sup> samples. We have used kernel density estimation to model the distribution of VDR transcription factor binding sites. (B) Number of predicted VDR binding sites per gene for genes expressed more highly in Vdr<sup>−/−</sup> placentae (blue bars) and those more highly expressed in Vdr<sup>+/+</sup> placenta (orange bars).

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Although maternal vitamin D deficiency has been implicated in the pathogenesis of several pregnancy complications attributed to impaired or abnormal placental function, there are few clues indicating the mechanistic role(s) of vitamin D in their pathogenesis. Preeclampsia, preterm birth and intrauterine growth restriction have been associated with impaired placental trophoblast invasion, and remodelling, of the uterine vasculature. Vitamin D metabolites have recently been shown to enhance trophoblast invasion \textit{in vitro} \cite{48} and together with the presence of a local placental vitamin D metabolic pathway \cite{22} suggest a direct role for vitamin D in the placenta. To further understand how vitamin D may influence placental development, and thereby pregnancy outcome, we used a \textit{Vdr} gene ablated mouse model with heterozygous matings to assess placental morphological parameters and global gene expression near term without confounding by the absence of maternal vitamin D signalling. Despite analyzing multiple aspects of placental morphology including total cell volume densities, the proportion of labyrinth to junctional zones, trophoblast, fetal capillary and maternal blood space volume densities and volumes and total surface area of trophoblast cells for exchange, no differences were observed between knockout and wild type placentae. Nor were there any observed differences in fetal and placental weights indicating apparently normal function. Previous reports have found that VDR-mediated signaling in the placenta is not required for the transport of calcium to the fetus or for fetal bone mineralization in offspring born to \textit{Vdr}^{+/-} dams \cite{36}. Consistent with these findings, we found no apparent phenotype in the \textit{Vdr}^{-/-} fetus or placenta when gestated in a heterozygous mother with adequate dietary vitamin D and calcium. In contrast, vitamin D-deficient dams carried pregnancies with smaller placentae and reduced fetal capillary diameter \cite{38}. Thus, the effects of maternal vitamin D deficiency on placental structure are likely mediated through the decidua rather than directly via VDR signaling in the placenta.

In profiling placental transcriptomes by microarray, we detected 25 differentially expressed genes between \textit{Vdr}^{-/-} and \textit{Vdr}^{+/-} placentae, a number of which have been shown to be expressed in the human placenta (\textit{Tinf2} \cite{49}, \textit{Rgs7} \cite{50}, \textit{Plscr1} \cite{51} \textit{Cd302} \cite{52}). The greatest gene expression difference observed between \textit{Vdr}^{-/-} and \textit{Vdr}^{+/-} placentae was for \textit{Cyp24a1} (Table 2), a gene that directly interacts with VDR in the canonical vitamin D signaling pathway.
and plays a key role in the vitamin D endocrine system negative feedback loop [53]. Our results show that expression of Vdr and Cyp24a1 are positively correlated (Fig 4). Cyp24a1 expression is typically induced directly by 1,25(OH)2D3 via a VDR-mediated transcriptional response [54], therefore significant reduction in Cyp24a1 expression in Vdr−/− placentae was expected, and suggests a functional role for VDR in the placenta.

Expression of both Pex5 (which encodes the peroxisome-targeting signal 1 receptor) and Tinf2 (which encodes the TERF1-interacting nuclear factor 2 (Tin2)) was greater in Vdr−/− placentae when compared to Vdr+/+. Pex5 plays a central role in the function of peroxisomes which are present in cells to clear reactive oxygen species (ROS) like hydrogen peroxide [55]. Tin2 is a component of the shelterin telomere protection complex which acts to protect telomeres from DNA damage [56] potentially caused by ROS. Increased expression of both genes within Vdr−/− placentae may be indicative of increased ROS, therefore increased oxidative stress, which has been hypothesized to be an underlying factor in the development of pregnancy complications like preeclampsia [57]. However, further work is required in order to establish whether there is an over-production of oxidative species within Vdr−/− placentae.

Of particular interest, we observed lower Deptor expression in Vdr−/− placentae and higher expression of Prr5 when compared to Vdr+/+. Both genes are components of the mTOR signaling pathway. During pregnancy, placental mTOR signaling plays an important role in the regulation of fetal growth, particularly as a maternal nutrient and growth factor sensor [58]. Furthermore, both DEPTOR and PRR5 have been shown to be highly expressed within the placenta [59, 60]. Deptor is an inhibitor of the mTOR signaling pathway [61] and by directly binding to mTORC1 and mTORC2 it acts to inhibit cell proliferation and protein synthesis. Alternately, Prr5 is a component of the mTORC2 complex that promotes cell growth through its interaction with Rictor [62]. It has been hypothesized that 1,25(OH)2D, through VDR signaling, can suppress downstream mTOR signaling [63]. In Vdr−/− placentae decreased Deptor and increased Prr5 indicates activation of the mTOR pathway. Thus, mTOR activation may explain why there was no difference in fetal weight and placental structure as there would be a drive for growth which may normalize any differences between the genotypes (Fig 1).

Our results indicate that VDR signaling in the placenta is not essential for pregnancy success. This is supported by recent studies assessing placental VDR expression and polymorphisms in complicated pregnancies. VDR polymorphisms do not appear to predispose women to preeclampsia and gestational hypertension [64] and VDR expression is similar between normal placentae and those from pregnancies complicated by gestational diabetes [65]. Furthermore, there is no linear correlation between placental VDR protein expression and birth weight [66]. Cho et al. did, however, observe that 85% of women suffering gestational diabetes were classified as vitamin D deficient (25(OH)D serum level < 20 ng/mL) [65]. Maternal vitamin D deficiency has been associated with pregnancy complications such as preeclampsia, small for gestational age and preterm birth [67] suggesting important roles for vitamin D in maternal tissues.

In this study, we used Vdr−/− dams to assess the effect of vitamin D on placental and fetal development without the confounding factor of poor maternal health seen in Vdr−/− mice [39]. Despite gene expression differences between knockout and wild-type placentae, this did not translate to differences in placental morphology and function with no apparent differences in fetal outcome near term. Our results suggest that maternal vitamin D status may be more crucial in determining pregnancy outcome than VDR signaling in the conceptus alone. This may be due to the presence of non-genomic VDR signaling which has been largely ignored in many studies, as well as genomic signaling in maternal tissues including the decidua. We suggest experiments using homozygous knockout dams will need to be undertaken in order to fully investigate the potential cross-talk between the maternal decidua and the placenta in regards to
VDR signaling. Furthermore, the gene expression differences observed in this study suggest some genes harbour VDRE’s in the placenta (Fig 1B and 1C) highlighting the need for further work to elucidate the role of the vitamin D endocrine pathway in placental function.

Supporting Information
S1 File. Primers and cycling conditions for PCR analyses. Vdr genotyping PCR primers and conditions [41] (Table A). Genotyping of Vdr alleles by PCR and gel electrophoresis for wild-type allele (Figure Aa) and for knockout allele (Figure Ab). Primers and PCR conditions for sex typing of mice [42] (Table B). PCR primers and cycling conditions to validate DNAse treatment of placental RNA extracts (Table C). Quantitative PCR assay and cycling conditions for microarray validation (Table D).

S2 File. Analysis methods and code for the microarray differential expression experiment.

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Author Contributions
Conceived and designed the experiments: SB FS SO TBM PHA CTR. Performed the experiments: RLW SB FS JD JAL SO. Analyzed the data: RLW SB FS SL. Contributed reagents/materials/analysis tools: PHA CTR. Wrote the paper: RLW SB FS TBM PHA CTR.

References
1. Cross JC. Formation of the placenta and extraembryonic membranes. Annals of the New York Academy of Sciences. 1998; 857:23–32. Epub 1999/01/26. PMID: 9917829.
2. Murphy VE, Smith R, Giles WB, Clifton VL. Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus. Endocrine reviews. 2006; 27(2):141–69. Epub 2006/01/26. doi: 10.1210/er.2005-0011 PMID: 16434511.
3. Jansson T, Powell TL. IFPA 2005 Award in Placentology Lecture. Human placental transport in altered fetal growth: does the placenta function as a nutrient sensor?—a review. Placenta. 2006; 27 Suppl A: S91–7. Epub 2006/01/31. doi: 10.1016/j.placenta.2005.11.010 PMID: 16442615.
4. De Laine KM, Matthews G, Grivell RM. Prospective audit of vitamin D levels of women presenting for their first antenatal visit at a tertiary centre. Australian and New Zealand Journal of Obstetrics and Gynaecology. 2013:n/a-n/a. doi: 10.1111/ajo.12052
5. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. The Journal of clinical endocrinology and metabolism. 2011; 96(7):1911–30. Epub 2011/06/08. doi: 10.1210/jc.2011-0385 PMID: 21646368.
6. Bodnar LM, Catov JM, Simhan HN, Holick MF, Powers RW, Roberts JM. Maternal vitamin D deficiency increases the risk of preeclampsia. The Journal of Clinical Endocrinology & Metabolism. 2007; 92(9):3517–22.
7. Baker AM, Haeri S, Camargo CA, Jr., Espinola JA, Stuebe AM. A Nested Case-Control Study of Mid-gestation Vitamin D Deficiency and Risk of Severe Preeclampsia. The Journal of clinical endocrinology
and metabolism. 2010. Epub 2010/08/20. doi: jc.2010-0996 [pii] doi: 10.1210/jc.2010-0996 PMID: 20719829.

8. Bodnar LM, Krohn MA, Simhan HN. Maternal vitamin D deficiency is associated with bacterial vaginosis in the first trimester of pregnancy. J Nutr. 2009; 139(6):1157–61. Epub 2009/04/10. doi: jn.108.103168 [pii] doi: 10.3945/108.103168 PMID: 19357214; PubMed Central PMCID: PMC2682987.

9. Perez-Ferre N, Torreon MJ, Fuentes M, Fernandez MD, Ramos A, Bordiu E, et al. Association of low serum 25-Hydroxyvitamin D levels in pregnancy with glucose homeostasis and obstetric and newborn outcomes. Endocrine Practice. 2012; 18(5):676–84. doi: 10.4158/EP12025.OR PMID: 22548949

10. Bodnar LM, Catov JM, Zmuda JM, Cooper ME, Parrott MS, Roberts JM, et al. Maternal Serum 25-Hydroxyvitamin D Concentrations Are Associated with Small-for-Gestational Age Births in White Women. The Journal of Nutrition. 2010; 140(5):999–1006. doi: 10.3945/jn.109.119636 PMID: 20200114

11. Leflèlaar ER, Vrijkotte TG, van Eijden M. Maternal early pregnancy vitamin D status in relation to fetal and neonatal growth: results of the multi-ethnic Amsterdam Born Children and their Development cohort. British Journal of Nutrition. 2010; 104(01):108–17.

12. Grant WB, Soles CM. Epidemiologic evidence supporting the role of maternal vitamin D deficiency as a risk factor for the development of infantile autism. Dermatoendocrinol. 2009; 1(4):223–8. Epub 2010/07/02. PMID: 20592795; PubMed Central PMCID: PMC2835879.

13. Wagner CL, McNeil R, Hamilton SA, Winkler J, Rodriguez Cook C, Warner G, et al. A randomized trial of vitamin D supplementation in 2 community health center networks in South Carolina. American journal of obstetrics and gynecology. 2013; 208(2):137.e1–13. Epub 2012/11/08. doi: 10.1016/j.ajog.2012.10.868 PMID: 23131462.

14. Javaid MK, Crowther SA—Harvey NC, Harvey NC—Gale CR, Gale CR—Dennison EM, Dennison EM—Boucher BJ, Boucher BJ—Arden NK, et al. Maternal vitamin D status during pregnancy and childhood bone mass at age 9 years: a longitudinal study Maternal vitamin D status during pregnancy and bone mass in offspring at 20 years of age: a prospective cohort study. (1474-547X (Electronic)).

15. Zhu K, Whitehouse AJ, Hart PH, Kusel M, Mountain J, Lye S, et al. Maternal vitamin D status during pregnancy and bone mass in offspring at 20 years of age: a prospective cohort study. Journal of bone and mineral research: the official journal of the American Society for Bone and Mineral Research. 2014; 29(5):1088–95. Epub 2013/11/06. doi: 10.1002/jbmr.2138 PMID: 24189972.

16. Kumar R, Cohen WR, Silva P, Epstein FH. Elevated 1,25-dihydroxyvitamin D plasma levels in normal human pregnancy and lactation. The Journal of clinical investigation. 1979; 63(2):342–4. Epub 1979/02/01. doi: 10.1172/jci109308 PMID: 429557; PubMed Central PMCID: PMC371958.

17. Ardawi MS, Nasrat HA, HS BAA. Calcium-regulating hormones and parathyroid hormone-related peptide in normal human pregnancy and postpartum: a longitudinal study. European journal of endocrinology / European Federation of Endocrine Societies. 1997; 137(4):402–9. Epub 1997/11/22. PMID: 9368509.

18. Papapetrou PD. The interrelationship of serum 1,25-dihydroxyvitamin D, 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D in pregnancy at term: a meta-analysis. Hormones (Athens, Greece). 2010; 9(4):44. Epub 2010/08/07. PMID: 20687397.

19. Sablok A, Batra A, Thariani K, Batra A, Bhatti R, Aggarwal AR, et al. Supplementation of Vitamin D in pregnancy and its correlation with feto-maternal outcome. Clinical endocrinology. 2015. Epub 2015/02/11. doi: 10.1111/cen.12751 PMID: 25683660.

20. Moon R, Harvey N, Cooper C. ENDOCRINOLOGY IN PREGNANCY: Influence of maternal vitamin D status on obstetric outcomes and the foetal skeleton. European journal of endocrinology / European Federation of Endocrine Societies. 2015. Epub 2015/04/12. doi: 10.1530/eje-14-0826 PMID: 25862787.

21. Anderson P, May B, Morris H. Vitamin D metabolism: new concepts and clinical implications. Clin Biochem Rev. 2003; 24(1):13–26. Epub 2008/07/25. PMID: 18650961; PubMed Central PMCID: PMC1853332.

22. Tamblyn JA, Hewison M, Wagner CL, Bulmer JN, Kilby MD. Immunological role of vitamin D at the maternal-fetal interface. The Journal of endocrinology. 2015; 224(3):R107–21. Epub 2015/02/11. doi: 10.1530/joe-14-0642 PMID: 25663707.

23. Ni W, Watts SW, Ng M, Chen S, Glenn DJ, Gardner DG. Elimination of vitamin D receptor in vascular endothelial cells alters vascular function. Hypertension. 2014; 64(6):1290–8. Epub 2014/09/10. doi: 10.1161/hypertensionaha.114.03971 PMID: 25201890.

24. Durk MR, Han K, Chow EC, Ahrens R, Henderson JT, Fraser PE, et al. Alphalac.25-Dihydroxyvitamin D3 reduces cerebral amyloid-beta accumulation and improves cognition in mouse models of Alzheimer's
Liu NQ, Ouyang Y, Bulut Y, Lagishetty V, Chan SY, Hollis BW, et al. Dietary vitamin D restriction in...

28. Ormsby RT, Findlay DM, Kogawa M, Anderson PH, Morris HA, Atkins GJ. Analysis of vitamin D metabolism in maternal hypertension and altered placental and fetal development. American journal of physiology Endocrinology and metabolism. 2012; 303(7):E928–E935. PMID: 22871339; PubMed Central PMCID: PMCPmc2945184.

29. Girgis CM, Mokbel N, Cheng K, Gunton JE. Vitamin D signaling regulates proliferation, differentiation, and myotube size in C2C12 skeletal muscle cells. Endocrinology. 2014; 155(2):347–57. Epub 2013/11/28. doi: 10.1210/en.2013-1205 PMID: 24280059.

30. Hu L, Bikle DD, Oda Y. Reciprocal role of vitamin D receptor on beta-catenin regulated keratinocyte proliferation and differentiation. The Journal of steroid biochemistry and molecular biology. 2014; 144 Pt A:110–3. Epub 2013/10/15. doi: 10.1016/j.jsbmb.2013.09.016 PMID: 24120913.

31. Zehnder D, Evans KN, Klity MD, Buhrman KN, Innes BA, Stewart PM, et al. The ontogeny of 25-hydroxyvitamin D(3) 1alpha-hydroxylase expression in human placenta and decidua. The American journal of pathology. 2002; 161(1):105–14. Epub 2002/07/11. PMID: 12107095; PubMed Central PMCID: PMCPmc1850695.

32. Pospechova K, Rozehnal V, Stejskalova L, Vrzal R, Pospisilova N, Jamborova G, et al. Expression and activity of vitamin D receptor in the human placenta and in choriocarcinoma BeWo and Jeg-3 cell lines. Molecular and cellular endocrinology. 2009; 299(2):178–87. Epub 2009/01/10. doi: 10.1016/j.mce.2008.12.003 PMID: 19133314.

33. Haussler MR, Jurutka PW, Mizwicki M, Norman AW. Vitamin D receptor (VDR)-mediated actions of 1alpha,25(OH)(2)D(3): genomic and non-genomic mechanisms. Best practice & research Clinical endocrinology & metabolism. 2011; 25(4):543–59. Epub 2011/08/30. doi: 10.1016/j.beem.2011.05.010 PMID: 21872797.

34. Ramagopalan SV, Heger A, Berlanga AJ, Maugerdi NJ, Lincoln MR, Burrell A, et al. A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. Genome research. 2010; 20(10):1352–60. Epub 2010/08/26. doi: 10.1101/gr.107920.110 PMID: 20736230; PubMed Central PMCID: PMCPmc2945184.

35. Ma R, Gu Y, Zhao S, Sun J, Groome LJ, Wang Y. Expressions of vitamin D metabolic components VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR in placentas from normal and preeclamptic pregnancies. American journal of physiology Endocrinology and metabolism. 2012; 303(7):E928–35. Epub 2012/08/09. doi: 10.1152/ajpendo.00279.2012 PMID: 22871339; PubMed Central PMCID: PMCPmc3469619.

36. Kovacs CS, Woodland ML, Fudge NJ, Friel JK. The vitamin D receptor is not required for fetal mineral homeostasis or for the regulation of placental calcium transfer in mice. American journal of physiology Endocrinology and metabolism. 2005; 289(1):E133–44. Epub 2005/03/03. doi: 10.1152/ajpendo.00354.2004 PMID: 15741244.

37. Liu NQ, Kaplan AT, Lagishetty V, Ouyang YB, Ouyang Y, Simmons CF, et al. Vitamin D and the regulation of placental inflammation. Journal of immunology (Baltimore, Md: 1950). 2011; 186(10):5968–74. Epub 2011/04/13. doi: 10.4049/jimmunol.1003332 PMID: 21482732.

38. Liu NQ, Ouyang Y, Bulut Y, Lagishetty V, Chen SY, Hollis BW, et al. Dietary vitamin D restriction in pregnant female mice is associated with maternal hypertension and altered placental and fetal development. Endocrinology. 2013; 154(7):2270–80. Epub 2013/05/17. doi: 10.1210/en.2012-2270 PMID: 23677931.

39. Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R, et al. Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. Proceedings of the National Academy of Sciences of the United States of America. 1997; 94(18):9631–5. Epub 1997/09/02. PMID: 9275211; PubMed Central PMCID: PMCPmc23277.
40. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic acids research. 1988; 16(3):1215. Epub 1988/02/11. PMID:3344216; PubMed Central PMCID: PMCPmc334765.

41. Panda DK, Miao D, Bolivar I, Li J, Huo R, Hendy GN, et al. Inactivation of the 25-hydroxyvitamin D 1alpha-hydroxylase and vitamin D receptor demonstrates independent and interdependent effects of calcium and vitamin D on skeletal and mineral homeostasis. The Journal of biological chemistry. 2004; 279(16):16754–66. Epub 2004/01/24. doi:10.1074/jbc.M310271200 PMID:14739296.

42. Albay R 3rd, Chen A, Anderson GM, Tatevosyan M, Janusonis S. Relationships among body mass, brain size, gut length, and blood tryptophan and serotonin in young wild-type mice. BMC physiology. 2009; 9:4. Epub 2009/03/27. doi:10.1186/1472-6793-9-4 PMID:19321004; PubMed Central PMCID: PMCPmc2671477.

43. Weibel ER. Stereological Methods Vol 1. Practical Methods for Biological Morphometry. London: Academic Press; 1979.

44. Kwon AT, Arenillas DJ, Worsley Hunt R, Wasserman WW. oPOSSUM-3: advanced analysis of regulatory motif over-representation across genes or ChIP-Seq datasets. G3 (Bethesda, Md). 2012; 2(9):987–1002. Epub 2012/09/14. doi:10.1534/g3.112.013202 PMID:22973536; PubMed Central PMCID: PMCPmc3965086.

45. Mathelier A, Zhao X, Zhang AW, Parcy F, Worsley-Hunt R, Arenillas DJ, et al. JASPAR 2014: an extensively expanded and updated open-access database of transcription factor binding profiles. Nucleic acids research. 2014; 42(Database issue):D142–7. Epub 2013/11/07. doi:10.1093/nar/gkt997 PMID:24194598; PubMed Central PMCID: PMCPmc3965086.

46. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, et al. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nature genetics. 1997; 16(4):391–6. Epub 1997/08/01. doi:10.1038/ng0897-391 PMID:9241280.

47. Chan SY, Susarla R, Canovas D, Vasilopoulou E, Ohizua O, McCabe CJ, et al. Vitamin D promotes human extravillous trophoblast invasion in vitro. Placenta. 2015. Epub 2015/01/19. doi:10.1016/j.placenta.2014.12.021 PMID:25596923.

48. Kim SH, Kaminker P, Campisi J. TIN2, a new regulator of telomere length in human cells. Nature genetics. 1999; 23(4):405–12. Epub 1999/12/02. doi:10.1038/70508 PMID:10581025.

49. Nunn C, Mao H, Chidiac P, Albert PR. RGS17/RGSZ2 and the RZ/A family of regulators of G-protein signaling. Seminars in cell & developmental biology. 2006; 17(3):390–9. Epub 2006/06/13. doi:10.1016/j.semcdb.2006.04.001 PMID:16765607.

50. Berghold VM, Gauster M, Hemmings DG, Moser G, Kremshofer J, Siwetz M, et al. Phospholipid scramblase 1 (PLSCR1) in villous trophoblast of the human placenta. Histochemistry and cell biology. 2014. Epub 2014/11/05. doi:10.1007/s00418-014-1294-y PMID:25362260.

51. Kato M, Khan S, d’Aniello E, McDonald KJ, Hart DN. The novel endocytic and phagocytic C-Type lectin receptor DCL-1/CD302 on macrophages is colocalized with F-actin, suggesting a role in cell adhesion and migration. Journal of immunology (Baltimore, Md: 1950). 2007; 179(9):6052–63. Epub 2007/10/20. PMID:17947679.

52. Jones G, Prosser DE, Kaufmann M. 25-Hydroxyvitamin D-24-hydroxylase (CYP24A1): its important role in the degradation of vitamin D. Archives of biochemistry and biophysics. 2012; 523(1):9–18. Epub 2011/11/22. doi:10.1016/j.abb.2011.11.003 PMID:22100522.

53. St-Arnaud R. CYP24A1: Structure, Function, and Physiological Role. In: Feldman D, Pike JW, Adams DL, editors. Vitamin D. Third ed. London: Elsevier; 2012. p. 43–56.

54. Miyata N, Fujiki Y. Shuttling mechanism of peroxisome targeting signal type 1 receptor Pex5: ATP-independent import and ATP-dependent export. Molecular and cellular biology. 2005; 25(24):10822–32. PMID:16314507.

55. Savage SA, Giri N, Baerlocher GM, Orr N, Lansdorp PM, Alter BP. TINF2, a Component of the Shelterin Telomere Protection Complex, Is Mutated in Dyskeratosis Congenita. The American Journal of Human Genetics. 2008; 82(2):501–9.

56. Burton GJ, Jauniaux E. Placental oxidative stress: from miscarriage to preeclampsia. Journal of the Society for Gynecologic Investigation. 2004; 11(6):342–52. Epub 2004/09/08. doi:10.1016/j.jsgi.2004.03.003 PMID:15350246.

57. Roos S, Powell TL, Jansson T. Placental mTOR links maternal nutrient availability to fetal growth. Biochemical Society transactions. 2009; 37(Pt 1):295–8. Epub 2009/01/16. doi:10.1042/bst0370295 PMID:19143650.
59. Foster HA, Davies J, Pink RC, Turkcigdem S, Goumenou A, Carter DR, et al. The human myometrium differentially expresses mTOR signalling components before and during pregnancy: evidence for regulation by progesterone. The Journal of steroid biochemistry and molecular biology. 2014; 139:166–72. Epub 2013/04/02. doi: 10.1016/j.jsbmb.2013.02.017 PMID: 23541542; PubMed Central PMCID: PMCPmc3855612.

60. Johnstone CN, Castellvi-Bel S, Chang LM, Sung RK, Bowser MJ, Pique JM, et al. PRR5 encodes a conserved proline-rich protein predominant in kidney: analysis of genomic organization, expression, and mutation status in breast and colorectal carcinomas. Genomics. 2005; 85(3):338–51. Epub 2005/02/19. doi: 10.1016/j.ygeno.2004.11.002 PMID: 15718101.

61. Peterson TR, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, et al. DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell. 2009; 137(5):873–86. Epub 2009/05/19. doi: 10.1016/j.cell.2009.03.046 PMID: 19446321; PubMed Central PMCID: PMCPmc2758791.

62. Woo S-Y, Kim D-H, Jun C-B, Kim Y-M, Vander Haar E, Lee S-i, et al. PRR5, a novel component of mTOR complex 2, regulates platelet-derived growth factor receptor β expression and signaling. Journal of Biological Chemistry. 2007; 282(35):25604–12. PMID: 17599906

63. Lisse TS, Hewison M. Vitamin D: a new player in the world of mTOR signaling. Cell cycle (Georgetown, Tex). 2011; 10(12):1888–9. Epub 2011/05/12. PMID: 21558808; PubMed Central PMCID: PMCPmc3154412.

64. Rezende VB, Sandrim VC, Palei AC, Machado L, Cavalli RC, Duarte G, et al. Vitamin D receptor polymorphisms in hypertensive disorders of pregnancy. Molecular biology reports. 2012; 39(12):10903–6. Epub 2012/10/12. doi: 10.1007/s11033-012-1988-y PMID: 23053984.

65. Cho GJ, Hong SC, Oh MJ, Kim HJ. Vitamin D deficiency in gestational diabetes mellitus and the role of the placenta. American journal of obstetrics and gynecology. 2013; 209(6):e1–8. Epub 2013/08/21. doi: 10.1016/j.ajog.2013.08.015 PMID: 23954530.

66. Young BE, Cooper EM, McIntyre AW, Kent T, Witter F, Harris ZL, et al. Placental vitamin D receptor (VDR) expression is related to neonatal vitamin D status, placental calcium transfer, and fetal bone length in pregnant adolescents. FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2014; 28(5):2029–37. Epub 2014/02/22. doi: 10.1096/fj.13-246736 PMID: 24558197.

67. Aghajafari F, Nagulesapillai T, Ronksley PE, Tough SC, O’Beirne M, Rabi DM. Association between maternal serum 25-hydroxyvitamin D level and pregnancy and neonatal outcomes: systematic review and meta-analysis of observational studies. BMJ (Clinical research ed). 2013; 346:f1169. Epub 2013/03/28. doi: 10.1136/bmj.f1169 PMID: 23533188.