CALCITRIOL TREATMENT SUPPRESSES CONTRACTION-ASSOCIATED GENE EXPRESSION IN PREGNANT MICE NEAR TERM

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INTRODUCTION

Figure 1. Preterm Birth is a Global Health Problem

Preterm Birth Rates

Globally, ~15 million babies are born prematurely each year leading to death or lifetime disabilities. Preterm birth (PTB) is the leading cause of infant mortality worldwide, contributing to more than one million deaths annually. PTB poses an enormous medical cost to society, exceeding $26 billion annually in the U.S. alone. The overall rate of PTB in the U.S. is ~12%; however, this is much higher in African-Americans who have a PTB rate that approaches 18%.

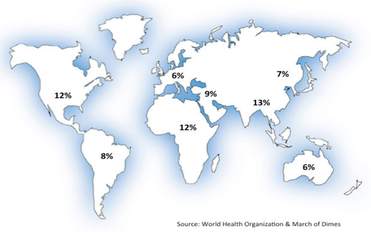
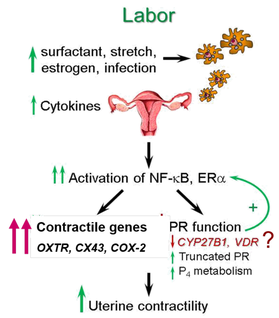


Figure 2. Mechanisms for P_4/PR Regulation of Uterine Quiescence During Pregnancy and for Induction of Uterine Contractility in Term and Preterm Labor



Progesterone, a steroid hormone, is responsible for maintaining myometrial quiescence throughout pregnancy. Increased progesterone (P_4) and progesterone receptor (PR) levels during pregnancy mediate anti-inflammatory actions, which silence expression of pro-inflammatory mediators and contraction-associated protein (*CAP*) genes (i.e. connexin-43 (CX-43), oxytocin receptor (OXTR)). Term and preterm labor are associated with increased levels of pro-inflammatory cytokines within maternal reproductive tissues, where they activate inflammatory transcription factors (e.g. *NF-κB)*, which enhance expression of CAP genes, leading to parturition.

Figure 3. Vitamin D3 Metabolism and VDR Signaling

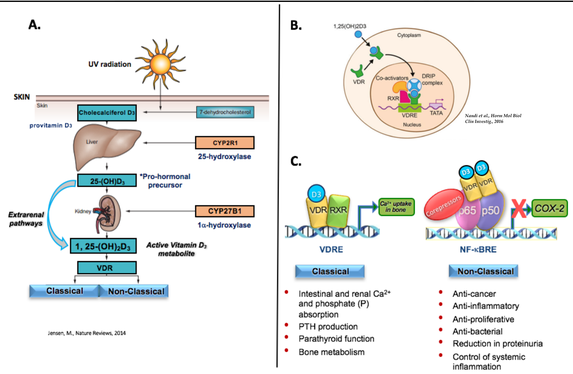
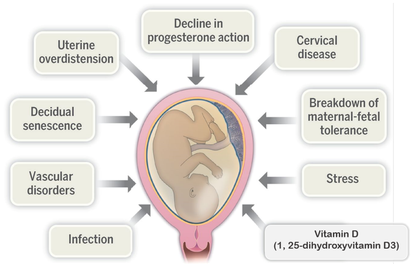


Figure 3. **(A)** Vitamin D3 (VD3) is obtained from dietary sources or synthesized in skin by UV light conversion of 7-dehydrocholesterol to cholecalciferol (provitamin D3). Provitamin D3 is transported to the liver where it is metabolized by 25-hydroxylase, encoded by CYP2R1. This produces the prohormonal precursor of VD3: 25-hydroxyvitamin-D3, which is transported to the kidneys, or extrarenal sites, where it is metabolized by 1α-hydroxylase, the enzyme encoded by Cyp27b1, to form 1,25-dihydroxyvitamin D3, which binds with high affinity to the vitamin D receptor (VDR), a member of the nuclear receptor family. **(B)** Once $1,25(OH)_2D_3$ reaches the target cell, it binds to the cytoplasmic vitamin D receptor (VDR). VDR bound $1,25(OH)_2D_3$ is transported into the nucleus where it heterodimerizes with orphan nuclear receptor, retinoid X receptor (RXR), binds to the VD response element (VDRE) and recruits coactivators to the promoter regions of target genes, to regulate transcription. **(C)** By classical mechanisms, VDR:RXR binds to promoter regions of target genes involved in calcium uptake and bone homeostasis. Recently, non-classical actions have been described, including anti-cancer, anti-inflammatory, anti-proliferative, and anti-bacterial effects. One possible mechanism for these actions may be by the binding of VDR to proinflammatory transcription factors, such as NF-kB, promoting the recruitment of corepressors and inhibiting expression of proinflammatory genes, such as COX-2.

Figure 4. Proposed Mechanisms Implicated in Preterm Labor



- There are many proposed mechanisms implicated in preterm labor.
- Vitamin D deficiency has been found in several studies to be associated with preterm labor; however, thus far, clear evidence showing a link between the two has yet to be uncovered.

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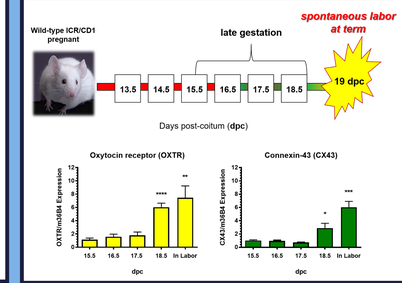
OBJECTIVE

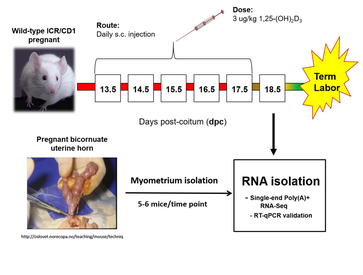
- **To evaluate the effects of calcitriol (1,25-dihydroxyvitamin D_3; $1,25(OH)_2D_3$) treatment on contraction-associated protein (*CAP*) gene expression in mouse myometrium during late gestation.**

METHODS

Model: Spontaneous Labor in Mice

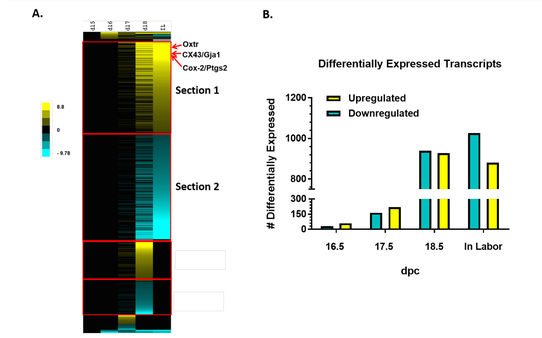


1, 25-dihydroxyvitamin D_3 Treatment



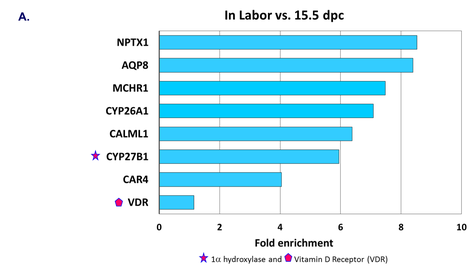
RESULTS

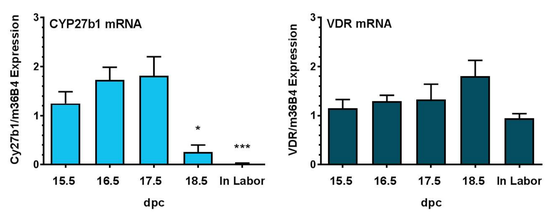
Figure 5. RNA Sequencing (RNA-seq) of the Mouse Myometrium Transcriptome During Late Pregnancy and Active Labor



(A) Heatmap showing differential expression of mRNA transcripts in mouse myometrium between 15.5 and 18.5 dpc and in-labor (IL). Shown are distinct clusters of highly upregulated, in yellow, and downregulated, in turquoise, mRNA transcripts across late gestation and during active labor, as compared to 15.5 dpc levels. **(B)** The number of differentially expressed transcripts (compared to values at 15.5 dpc) demonstrate an equivalent number of up- and downregulated mRNA transcripts across all timepoints.

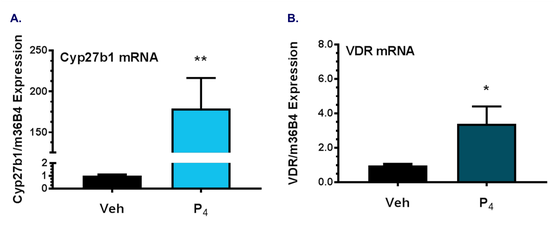
Figure 6. Downregulated mRNA Transcripts in Laboring Myometrium Identified by RNA Sequencing





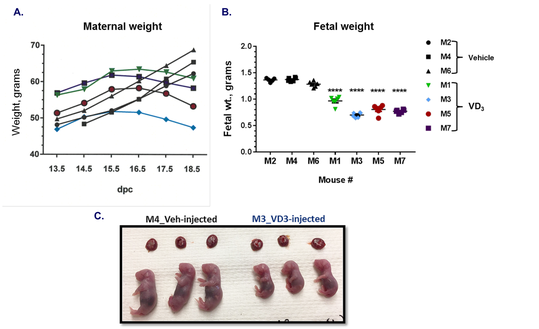
(A) RNA-seq analysis reveals that Cyp27b1 (encodes 1α-hydroxylase) and the vitamin D receptor (VDR) are among the major downregulated mRNA transcripts in mouse myometrium during late gestation and in-labor. **(B, C)** This was validated using RT-qPCR analysis and an independent series of myometrial tissues isolated from mice at 15.5 – 18.5 dpc and during active labor. Cyp27b1 ((**B)** mRNA levels declined markedly and significantly at 18.5 dpc and in-labor compared to earlier gestational timepoints. VDR **(C)** mRNA levels declined by 20% during active labor, relative to 15.5 dpc; however, this was not significant. (*p<0.01; ***p<0.0001). n ≥ 10 mice per time point; S.E.M., one-way ANOVA with Bonferroni's post-hoc test.

Figure 7. Progesterone Treatment Upregulates Cyp27b1 and VDR Expression in Mouse Myometrium During Late Gestation



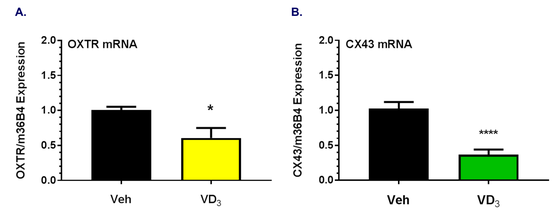
Timed-pregnant wild-type mice were injected daily with either 1 mg of progesterone (P_4) or vehicle (sesame oil) between 15.5 and 17.5 dpc. Myometrial tissue was harvested at 18.5 dpc. mRNA was analyzed by RT-qPCR for **(A)** Cyp27b1 and **(B)** VDR. Data are the mean ± S.E.M; n≥3 samples/time point/group; Student's t-test, *p<0.05; * * p<0.001

Figure 8. Maternal and Fetal Weights Decreased in $1,25(OH)_2D_3$ (Calcitriol)-injected Mice Relative to Controls



(A) Vehicle-injected mice increased in weight as expected across all time points; however, mice treated with calcitriol (VD_3) began to lose weight between 15.5 and 16.5 dpc and continued to decline in mass until 18.5 dpc. Additionally, VD_3-treated mice appeared sick (rough coats, labored breathing). Due to these health concerns, tissues were harvested at 18.5 dpc. **(B,C)** While litter size was not affected by VD_3 treatment, there was a significant difference in the fetal weights of VD_3-treated mice relative to controls.

Figure 9. OXTR and CX43 mRNA Expression is Decreased at 18.5 dpc in Myometrium of $1,25(OH)_2D_3$ (3.0 μg/kg) Treated Mice



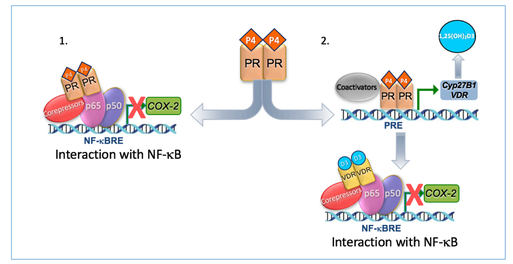
Timed-pregnant mice where serially injected subcutaneously with either 3.0 μg/kg 1,25-dihydroxyvitamin D_3 (VD_3) or vehicle (sesame oil) between 15.5 and 17.5 dpc. Myometrial tissues were harvested at 18.5 dpc. mRNA was isolated and expression of **(A)** OXTR and **(B)** CX43 mRNA was determined by RT-qPCR. N=3 Veh; N=4 VD3.; Students t-test, *p<0.01; *** p<0.0001

CONCLUSIONS

Figure 10. During Pregnancy, Progesterone/P_4 Inhibits Pro-Inflammatory and Contractile Genes in the Myometrium, in part, by Enhancing the VDR Pathway

Based on these findings, we conclude that:

- Expression of Cyp27b1 (1α-hydroxylase), the key regulatory enzyme in the synthesis of the active form of vitamin D_3 (1,25-dihydroxy vitamin D_3), and the vitamin D receptor (VDR) decline significantly in mouse myometrium toward term.
- This suggests that the decrease in Cyp27b1 expression, coupled with the decline in PR function near term, may contribute to increased CAP gene expression, leading to increased myometrial contractility and labor.
- Progesterone (P_4) treatment of timed-pregnant mice from 15.5 – 18.5 dpc, which causes a delay in labor and inhibits CAP gene expression, significantly enhances expression of Cyp27b1 and VDR mRNA in the pregnant mouse myometrium.
- Cyp27b1/VDR may serve as a key progesterone/PR target genes that act cooperatively with P_4/PR to maintain myometrial quiescence via their anti-inflammatory actions.
- Notably, treatment of timed-pregnant mice with 1,25-dihydroxy vitamin D_3 (calcitriol) significantly inhibited expression of the CAP genes, OXTR and CX43.
- Thus, treatment of pregnant women with derivatives of calcitriol that have decreased effects on bone, but maintain their anti-inflammatory activity, may provide excellent therapeutic options for prevention of preterm birth.



We suggest that P_4/PR maintains myometrial quiescence during pregnancy by at least two mechanisms (above):

1. P_4 and PR can tether to NF-κB heterodimers bound to promoters of pro-inflammatory and CAP genes and recruit co-repressors to inhibit their expression.
2. P_4 and PR may bind directly to progesterone response elements in the CYP27B1 and VDR promoters to increase their expression. 1,25-dihydroxyvitamin D_3 bound to VDR can then bind to promoters of CAP and proinflammatory genes, recruit corepressors and inhibit their expression. Near term, the decline in PR function in myometrium results in de-repression of pro-inflammatory and CAP genes by decreased interaction of PR and VDR with NF-κB bound to their promoters, resulting in an upregulation of myometrial contractility, leading to parturition.

FUTURE STUDIES

To learn more about the efficacy of calcitriol in preventing preterm birth, in the future, we will conduct the following studies:

- In light of the apparent toxic effects of calcitriol in pregnant mice (Fig. 8), we will test the effects of subcutaneous administration of calcitriol at lower doses.
- When an effective lower dose of calcitriol is found that exhibits no toxicity and delays normal term labor, we will test its effect to prevent preterm birth induced either by PR antagonist (RU486) or LPS treatment.
- We will test the efficacy of the VDR agonist, Paricalcitol, which has decreased toxicity on kidney and bone, but enhanced anti-inflammatory actions.

ACKNOWLEDGMENTS

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