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The Ontogeny of 25-Hydroxyvitamin D_3 1α-Hydroxylase Expression in Human Placenta and Decidua

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In addition to its classical calciotropic effects, the active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is a potent anti-proliferative/immunomodulatory secosteroid. The enzyme that catalyzes the synthesis of $1,25(OH)_2D_3$, 1α-hydroxylase (1α-OHase), is expressed in many human tissues, highlighting its possible role as an autocrine/paracrine activator of vitamin D. Immunohistochemical and RNA analyses were used to characterize the ontogeny of 1α-OHase expression in human placenta and decidua. Protein for 1α-OHase was detectable in trophoblast and decidua; the latter being stronger in decidualized stromal cells than macrophages, with no staining of lymphocytes. Quantitative reverse transcriptase-polymerase chain reaction was used to assess changes in mRNA expression for 1α-OHase at different gestations: first (mean, 9.1 ± 1.5 weeks); second (mean, 14 ± 1.8 weeks), and third trimester (mean, 39.3 ± 2.5 weeks). 1α-OHase expression in decidua was ~1000-fold higher in first (95% confidence limits, 611 to 1376) and second (95% confidence limits, 633 to 1623) trimester biopsies when compared with the third trimester (95% confidence limits, 0.36 to 2.81) (both $P < 0.001$). In placenta, 1α-OHase expression was 80-fold higher in the first (range, 42 to 137) and second (range, 30 to 199) trimester when compared with third trimester biopsies (0.6 to 1.6) (both $P < 0.001$). Similar results were obtained by semiquantitative IHC. Parallel analysis of the receptor for $1,25(OH)_2D_3$ (vitamin D receptor) indicated that, as with 1α-OHase, highest levels of expression occurred in first trimester decidua. However, changes in vitamin D receptor mRNA expression across gestation were less pronounced than 1α-OHase. These spatiotemporal data emphasize the potential importance of 1α-OHase during early feto-placental life and, in particular, suggest an autocrine/paracrine immunomodulatory function for the enzyme. ***(Am J Pathol 2002, 161:105–114)***

The active form of vitamin D [1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)], plays a pivotal role in mineral homeostasis by facilitating calcium and phosphate uptake in the gut and by modulating bone cell development and function.[1] However, in recent years, studies *in vivo* and *in vitro* have shown that $1,25(OH)_2D_3$ is able to influence a wide range of functions not immediately linked to calcium homeostasis. In particular, potent anti-proliferative and immunosuppressive responses to $1,25(OH)_2D_3$ have led to the use of vitamin D analogs as therapy for diseases such as psoriasis and leukemia.[2–4] The importance of $1,25(OH)_2D_3$ as a pleiotropic modulator of tissue function is strengthened by the presence of key components of vitamin D metabolism and function at these sites. The cognate nuclear receptor for $1,25(OH)_2D_3$, the vitamin D receptor (VDR), is ubiquitous in proliferating cells. Its function is therefore dependent primarily on the local availability of ligand. Synthesis of $1,25(OH)_2D_3$ from the major circulating form of vitamin D, 25-hydroxyvitamin D_3 ($25(OH)D_3$), is catalyzed by the enzyme 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase) that is located in the kidney,[5,6] and at several key extra-renal sites.[2,7,8] Thus, the widespread co-expression of VDR and 1α-OHase emphasizes a putative role for $1,25(OH)_2D_3$ as an autocrine/paracrine prodifferentiation and immunosuppressive agent, with diverse physiological functions.

Despite the fact that renal and extra-renal synthesis of $1,25(OH)_2D_3$ is differentially regulated, the expression of 1α-OHase in different tissues seems to be because of the same gene product.[9–11] Based on this observation we have developed novel polyclonal antisera to the renal 1α-OHase that have allowed us to define for the first time the precise cellular distribution of the enzyme along the nephron[5] and within key peripheral tissues.[7] Our data have confirmed the immunolocalization of 1α-OHase in

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Table 1. Number of Individual Samples of Placenta or Decidua Tissue in Each Trimester and Intrauterine Growth Restriction (IUGR)

Trimester (mean gestational age ±SD)	Placenta	Decidua
First (9.1 ± 1.5 wks)	35	27
Second (14.0 ± 1.8 wks)	67	10
Third (39.3 ± 1.8 wks)	33	10
IUGR (30.8 ± 1.8 wks)	13	
IUGR gestation-matched controls (30.6 ± 2.6 wks)	5	

Mean gestational age in weeks ± standard deviation (SD).

tissues such as lymph nodes and skin but also indicate that 1α-OHase is expressed in placentae from normal pregnancies.[7] In view of this, and the fact that reproduction in females is markedly diminished in states of vitamin D deficiency,[12–14] we have postulated that local synthesis of $1,25(OH)_2D_3$ may play a role in implantation and/or placentation. To investigate further the role of local $1,25(OH)_2D_3$ production in fetal-placental development we have performed immunohistochemical and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses of 1α-OHase using placental and decidual tissue from different gestational ages and from cases of intrauterine growth restriction (IUGR).

Materials and Methods

Tissue Samples

The study had the approval of the local hospital ethics committee. Placenta and decidua were either collected from elective first or second trimester termination of pregnancy (in accordance with the Polkinghorne report[15]) or from elective cesarean sections. For immunohistochemistry (IHC) a total of 58 placental and 25 decidual samples were fixed in 10% neutral buffered formalin for 24 to 48 hours, routinely processed, and embedded in paraffin wax. For RNA extraction placental and decidual samples were collected, snap-frozen in liquid nitrogen (−80°C), and grouped as shown in Table 1. Thirteen cases of IUGR were diagnosed prospectively, having at least three of the four following characteristics determined from ultrasound examination: 1) fetal abdominal circumference (AC) ≤ third centile for gestational age; 2) abnormal fetal growth velocity (ΔAC < 1.5 SD throughout 14 days); 3) severe oligohydramnios (amniotic fluid index ≤ third centile for gestational age); 4) absent or reversed velocities in umbilical artery Doppler waveforms. The IUGR group ranged from 25 to 38 weeks of gestational age and was gestationally matched to a group of pregnancies with appropriately grown babies born between 27.3 and 34 weeks but delivered for maternal reasons and not associated with preterm labor ($n = 5$). Placental biopsies were taken from up to five randomly allocated areas; in previous studies we have shown relatively little variation in the expression of genes such as 11β-hydroxysteroid dehydrogenase within individual placentae.[16,17] Decidua was identified from termination samples with both macroscopical and histological confirmation performed. In third trimester samples decidua was mechanically scraped from the uterine cavity at cesarean section. Again, histological confirmation was obtained. Semiquantitative grading of immunohistochemical staining was performed blind by a single individual using randomly selected areas of each section.

Immunohistochemistry

1α-OHase

Immunohistochemical (IHC) analysis of 1α-OHase expression was performed using previously described methods.[5,7] Briefly, dewaxed and rehydrated paraffin-embedded sections were processed in 0.01 mol/L of sodium citrate buffer (pH 6.0) in a pressure cooker at 103 kPa for 2 minutes for antigen retrieval. Slides were then incubated with methanol-hydrogen peroxide (1:100) to block endogenous peroxidase activity, and then washed in Tris-buffered saline, pH 7.6. The slides were then incubated with 1α-OHase antiserum (1:150) in 10% normal swine serum for 45 minutes at room temperature (25°C). After rinsing with Tris-buffered saline for 15 minutes, donkey anti-sheep IgG peroxidase conjugate (1:100; The Binding Site, Birmingham, UK) was added to sections for 45 minutes. Staining was developed using 3,3′-diaminobenzidine (2.5 mg/ml) followed by counterstaining with Mayer's hematoxylin. Negative control sections included: 1) omission of primary antibody; 2) use of primary antibody preabsorbed with a 100-fold excess of immunizing peptide. Immunolabeled slides were assessed semiquantitively blind to the gestational age of the samples. Reactivity was graded as negative, weak, moderate, or strong, assessed against a known strongly positive sample (kidney) that was included in each staining run.

Double-Immunohistochemical Characterization of 1α-OHase-Positive Cells

Selected first and second trimester decidual samples ($n = 10$) were subjected to double-immunoenzymatic labeling to further characterize the 1α-OHase immunoreactive cells. Firstly, sections were labeled for 1α-OHase using an indirect immunoperoxidase method as described above, except that the reaction was developed in aminoethylcarbazole (Vector NovaRed; Vector Laboratories, Peterborough, UK) rather than in 3,3′-diaminobenzidine to give a red-brown reaction product. Sections were then incubated for 60 minutes with monoclonal antibodies directed against CD3 (T lymphocytes, NCL-CD3-PS1 1/20; Novocastra Laboratories, Newcastle on Tyne, UK), CD56 (NCAM, natural killer cells including endometrial granulated lymphocytes, NCL-CD56–1B6 1/50; Novocastra), CD14 (monocytes/macrophages, NCL-CD14–223; Novocastra), CD68 (monocytes/macrophages, KP1 1/50; DAKO, Ely, UK), and CD10 (endometrial stromal cells, NCL-CD10–270 1/40; Novocastra). The second antibody reaction was detected using an avidin-biotin-alkaline phosphatase technique (Vectastain al-

kaline phosphatase kit, Vector Laboratories) and the reaction was developed for 20 minutes in the dark at room temperature with the alkaline-phosphatase substrate kit III (Vecta Blue, Vector Laboratories). The reaction was stopped with excess water. Sections were not counterstained and were initially mounted in Supermount (Biogenix, San Ramon, CA), air-dried overnight, and then mounted in synthetic resin (DPX; Raymond Lamb, London, UK). Negative controls included omission of the first and second primary antibody in turn to exclude nonspecific binding and spurious double labeling.

RNA Extraction

RNA was extracted from placental and decidual samples using the TRI reagent mRNA extraction method (Sigma, Poole, UK) or the StrataPrep total RNA miniprep kit (Stratagene, Amsterdam, Netherlands). An aliquot (2 μg) of RNA from each sample was reverse-transcribed (RT) using AMV reverse transcriptase (Promega, Madison, WI) and random hexamers in 40-μl reaction volumes according to the manufacturer's instructions.

Quantitative RT-PCR Analysis of 1α-OHase mRNA Expression

1α-OHase mRNA levels were analyzed using an ABI 7700 sequence detection system (PE Biosystems, Warring, UK). Amplification of 1α-OHase cDNA was performed in 25-μl volumes on 96-well plates, in a reaction buffer containing TaqMan Universal PCR Master Mix, 3 mmol/L $Mn(Oac)_2$, 200 μmol/L dNTPs, 1.25 U AmpliTaq Gold polymerase, 1.25 U AmpErase uracil-N-glycosylase (UNG), 150 nmol TaqMan probe, 900 nmol primers, and 50 ng cDNA. All reactions were multiplexed with the housekeeping gene 18S rRNA, provided as a preoptimized control probe (PE Biosystems) enabling data to be expressed in relation to an internal reference to allow for differences in sampling and RT efficiency. Data were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) according to the manufacturer's guidelines, and used to determine ΔCt values (ΔCt = Ct of the target gene − Ct of the housekeeping gene). Measurements were performed in triplicate and data were reported as the mean of three values from separate experiments. All target gene probes were labeled with five-carboxy fluorescein (FAM), and the housekeeping gene with VIC. All PCR reactions were performed with the primers and probes outlined below using the following reaction conditions: 50°C for 2 minutes, 95°C for 10 minutes, 44 cycles of 95°C for 15 seconds, 60°C for 1 minute.

Primers and Probes for PCR Reactions

PCR primer and probe sequences for 1α-OHase were as follows: forward primer, CACCCGACACGGAGACCTT; reverse primer, TCAACAGCGTGGACACAAACA; and TaqMan probe, TCCGCGCTGTGGGCTCGG. For cytokeratin-8 (CK-8): forward primer, 5′-ATGTGGATGAAGCATACATGAACA-3′; reverse primer, 5′-TCCCGGATCTCCTCTTCAT-3′; and TaqMan probe, 5′-CCGACGAGATCAACTTCCTCAGGCA-3′. For VDR: forward primer, 5′-CTTCAGGCGAAGCATGAAGC-3′; reverse primer, 5′-CCTTCATCATGCCGATGTCC-3′; and TaqMan probe, 5′-AAGGCACTATTCACCTGCCCCTTCAA-3′.

Data Analysis

To exclude potential bias because of averaging data that had been transformed through the equation 2^{-ddCt}, all statistics were performed at the ΔCt stage. Statistical analysis was performed using one-way analysis of variance, with posthoc testing using Student-Newman-Keuls multiple comparison posttest (Instat version 2.04a computer program; GraphPad Software, Inc., San Diego, CA).

Results

Tissue Distribution of 1α-OHase

Analysis of 1α-OHase expression by IHC demonstrated the presence of the enzyme in both placental and decidual tissue (Figure 1). Strong reactivity was observed in villous syncytiotrophoblast from first and second trimester placentae with less intense immunostaining of the villous cytotrophoblast (Figure 1A). Cytotrophoblast columns and cytotrophoblast islands showed comparable staining with villous cytotrophoblast. Lower (moderate) levels of 1α-OHase expression were detected in third trimester villous syncytiotrophoblast (Figure 1B). Third trimester villous cytotrophoblast was generally scanty but was weakly positive for 1α-OHase. Extravillous trophoblast cells in the basal plate were weakly positive. Relatively high levels of 1α-OHase protein were also observed in most samples of first trimester decidua (Figure 1C), with reduced expression in decidua basalis from third trimester samples (Figure 1D). Parallel analysis of cell surface markers for trophoblast (cytokeratin) and decidualized stromal cells (vimentin) confirmed that 1α-OHase was expressed by both cell types (data not shown). In addition, further IHC analyses showed that 1α-OHase was also expressed in glandular tissue. Figure 1E shows double-labeling IHC for 1α-OHase (red-brown staining) and the decidual stromal cell marker CD10 (blue staining), with glands expressing 1α-OHase only and stromal cells showing co-expression of the enzyme with CD10 (purple staining).

Expression of 1α-OHase in Decidual Cell Subgroups

In view of the relatively high levels of 1α-OHase expression in early pregnancy, double-labeling studies were performed to determine the specific decidual cell subgroups associated with the enzyme (Figure 2). Both decidualized and nondecidualized endometrial stromal cells showed immunoreactivity for CD10, although staining was more intense in areas without prominent decidual change in stromal cells. Reactivity for 1α-OHase was more intense in decidualized stromal cells that showed weak CD10 immunostaining, whereas strongly CD10-positive nondecidualized or poorly decidualized endo-

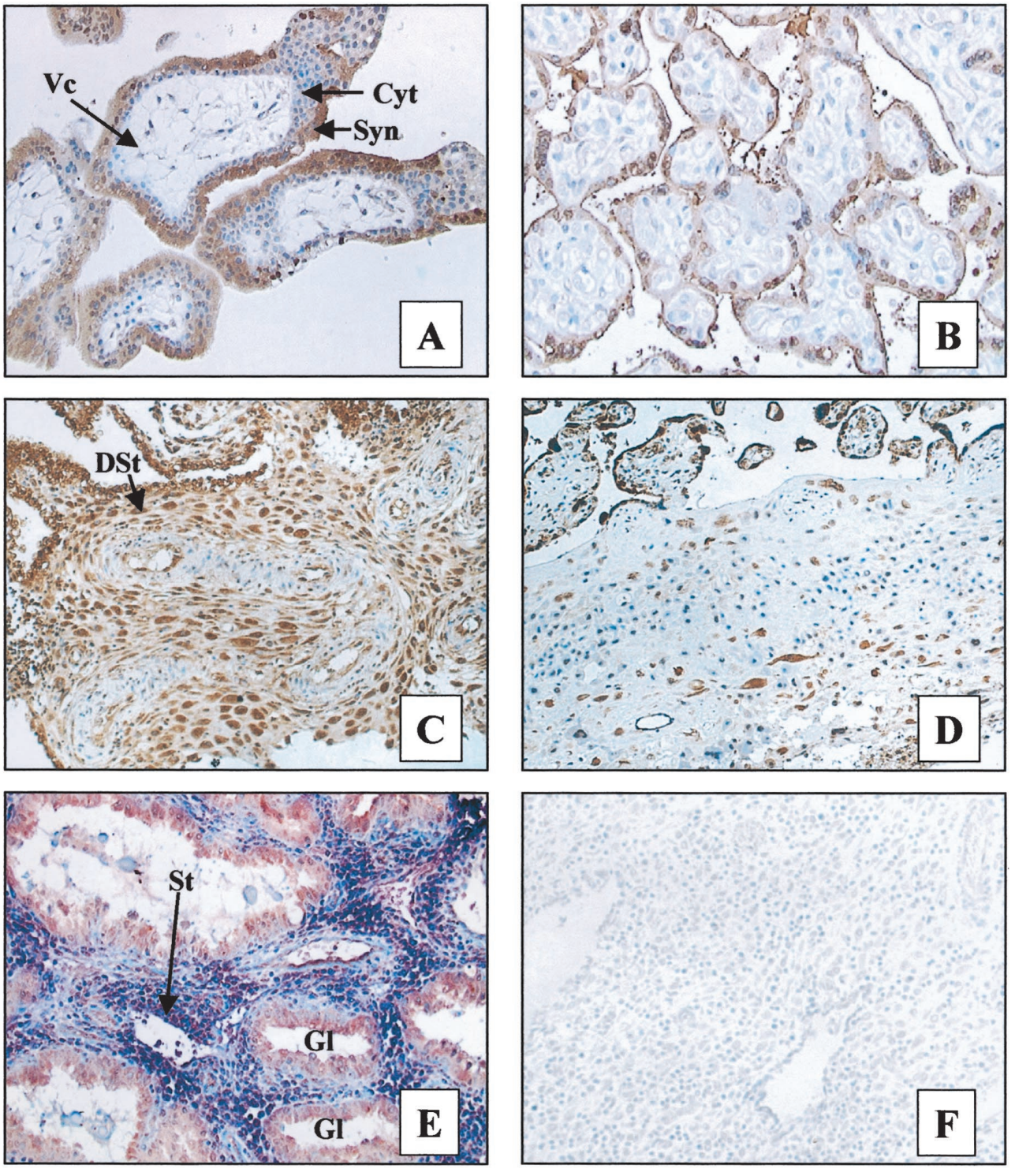


Figure 1. Immunohistochemical analysis of 1α-OHase expression in human placenta and decidua (brown staining). **A:** First trimester placenta. **B:** Third trimester placenta. **C:** First trimester decidua. **D:** Third trimester decidua. **E:** Double labeling of decidua with 1α-OHase antiserum (brown staining) and antibody to the stromal cell marker CD10 (blue staining). **F:** Negative control for specificity of 1α-OHase expression (preabsorption of antiserum with 200-fold excess of immunizing peptide). Tissue features: Syn, syncytiotrophoblast; Cyt, cytotrophoblast; Vc, villous core; St, stromal cells; DSt, decidual stromal cells; GL, gland. Original magnifications: ×200 (**A**, **B**, **E**, **F**); ×150 (**C**, **D**).

metrial stromal cells showed weaker reactivity for the enzyme (Figures 1E and 2B). The second major decidual leukocyte population, macrophages, showed double labeling for 1α-OHase with either CD14 (Figure 2C) or CD68 (data not shown). In all first and second trimester decidua studied, up to 25% of the CD14-positive and CD68-positive population co-expressed the enzyme. CD68 was also expressed, albeit less intensely, by de-

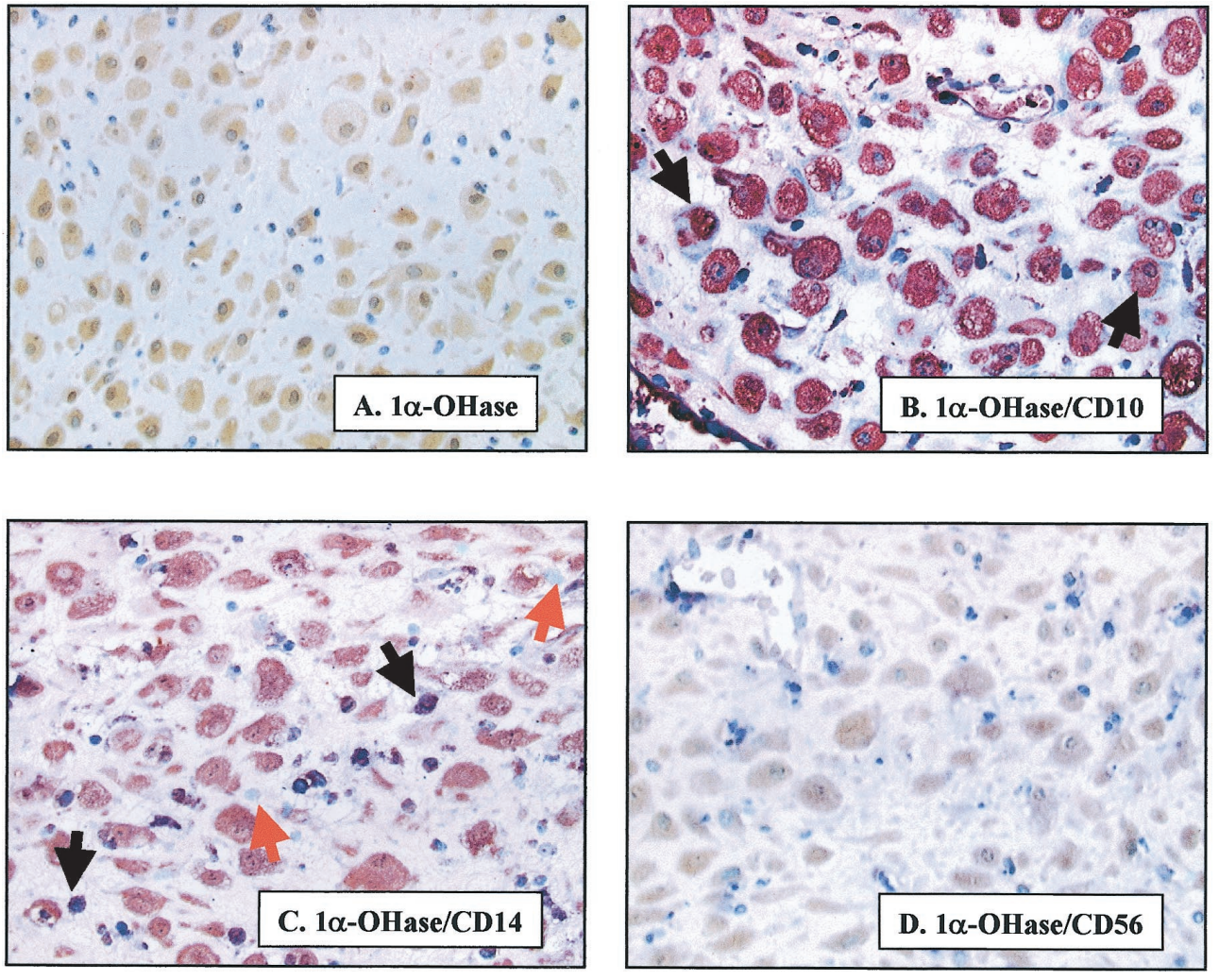


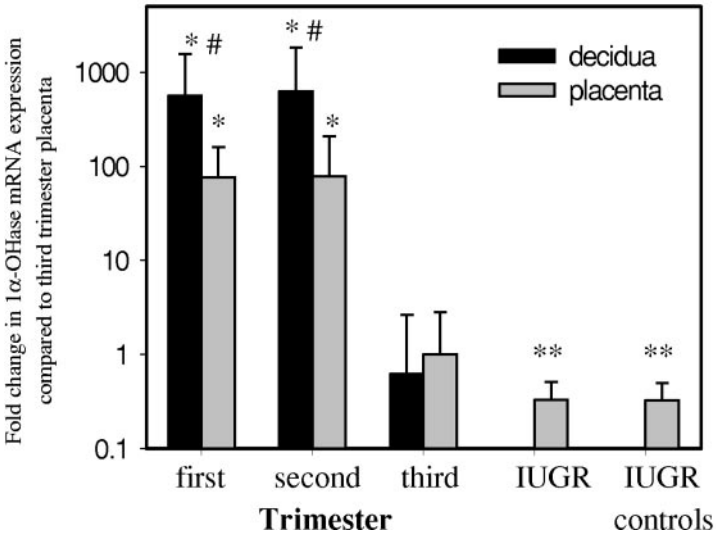
Figure 2. Co-localization of 1α-OHase with markers of stromal cell subtypes in first trimester decidua. **A:** Staining for 1α-OHase protein only (brown staining). **B–D:** Double labeling with 1α-OHase antiserum (brown staining) and antibodies to: stromal cell marker CD10 (blue staining) (**B**); monocyte marker CD14 (blue staining) (**C**); and lymphocyte marker CD56 (blue staining) (**D**). Blue staining only is shown by **red arrows**. Co-localization (purple staining) is shown by **black arrows**. Original magnifications, ×250.

cidualized stromal cells. However, in contrast to CD10, CD68 was detected in decidualized stromal cells that were 1α-OHase-positive, but was not detected in the nondecidualized stromal cells that showed weaker 1α-OHase immunostaining (data not shown). Double-labeling studies using antibodies to CD56 (Figure 2D) and CD3 (data not shown) confirmed that decidual T lymphocytes and endometrial granulated lymphocytes, respectively, did not co-express 1α-OHase.

Quantitative Analysis of Gestational Changes in 1α-OHase mRNA Expression

Data from real-time RT-PCR analyses revealed strong expression of 1α-OHase mRNA in first and second trimester placenta and decidua (Figure 3). Furthermore, decidual samples showed 10-fold higher levels of mRNA when compared to placenta. In both types of tissue, expression of 1α-OHase decreased dramatically in third trimester and IUGR placentae. The latter showed a small but significant decrease in 1α-OHase expression when compared to normal third trimester placenta but IUGR values were not significantly different to the gestationally matched placental samples. To assess the extent to which changes in 1α-OHase expression were because of the altered cellular composition of placental and decidual tissue, further RT-PCR studies were performed for CK-8, a trophoblast/endometrial epithelium cell marker (Figure 4). CK-8 levels in the first and second trimester placenta were not significantly different from third trimester or IUGR samples (including gestational controls). In contrast, decidual CK-8 levels paralleled changes in 1α-OHase with 100-fold lower levels of expression in third trimester samples. Based on these observations, further analysis of 1α-OHase expression was performed after correction for CK-8 mRNA (Figure 5). Data revealed comparable levels of 1α-OHase mRNA in first trimester placenta and decidual tissue, although in both types of tissue expression of 1α-OHase remained lower in third trimester samples. Importantly, after correction for CK-8,

A.



B.

Trimester/ tissue	Fold-change mRNA	95% confidence limit
First decidua	564.38	276.10 – 846.91
First placenta	75.81	42.04 – 136.71
Second decidua	623.71	389.42 – 998.97
Second placenta	77.58	30.22 – 199.13
Third decidua	0.62	0.22 – 1.73
Third placenta	1	0.59 – 1.69
IUGR placenta	0.33	0.20 – 0.54
IUGR controls	0.33	0.28 – 0.35

Figure 3. Expression of 1α-OHase mRNA in human placenta and decidua. **A:** Placental and decidual 1α-OHase mRNA expression normalized for 18S rRNA levels in first, second, and third trimester samples, and placentae from patients with IUGR. All data are reported relative to a third trimester placenta value of 1. *, Significantly different from third trimester placenta, $P < 0.001$; **, significantly different from third trimester placenta, $P < 0.05$; #, significantly different from equivalent placenta, $P < 0.01$. **B:** Data expressed with 95% confidence limits. IUGR controls, gestationally matched placentae.

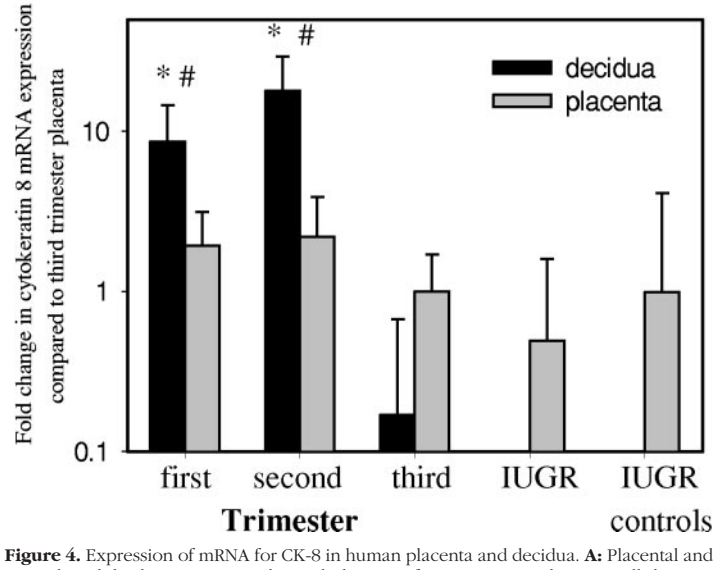
the expression of 1α-OHase in IUGR samples did not differ significantly from normal third trimester placentae or gestationally matched samples.

Further studies were performed to compare spatiotemporal changes in 1α-OHase mRNA levels with gestational variations in VDR expression. Data, again corrected for CK-8 expression (Figure 6), indicated that, as with 1α-OHase, VDR expression is highest in early gestation. However, in contrast to 1α-OHase, changes in VDR expression across gestation were less pronounced with only first trimester decidua and second trimester placenta expressing mRNA at levels that were significantly higher than third trimester placental tissue.

Discussion

Detailed analysis of the nonclassical actions of vitamin D has shown that its active form, $1,25(OH)_2D_3$, is a pluripotent secosteroid with immunomodulatory and anti-proliferative properties. The clinical importance of this is most clearly illustrated by the development of synthetic ana-

A.

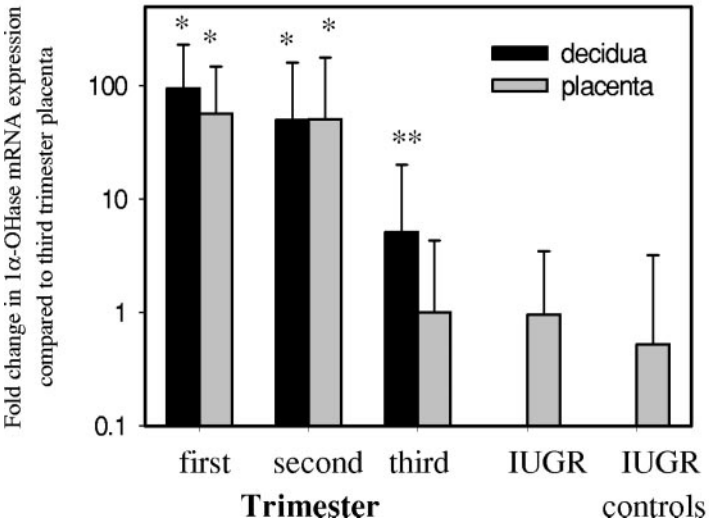


B.

Trimester/ tissue	Fold-change mRNA	95% confidence limit
First decidua	8.56	574 – 12.77
First placenta	1.92	1.21 – 3.04
Second decidua	17.82	10.56 – 30.08
Second placenta	2.19	1.42 – 3.38
Third decidua	0.17	0.05 – 0.65
Third placenta	1	0.59 – 1.70
IUGR placenta	0.49	0.17 – 1.45
IUGR controls	0.99	0.03 – 8.28

Figure 4. Expression of mRNA for CK-8 in human placenta and decidua. **A:** Placental and decidual CK-8 mRNA expression normalized for 18S rRNA levels in first, second, and third trimester samples and placentae from patients with IUGR. All data are reported relative to a third trimester placenta value of 1. *, Significantly different from third trimester placenta, $P < 0.001$; #, significantly different from equivalent placenta, $P < 0.001$. **B:** Data expressed with 95% confidence limits. IUGR controls, gestationally matched placentae.

A.



B.

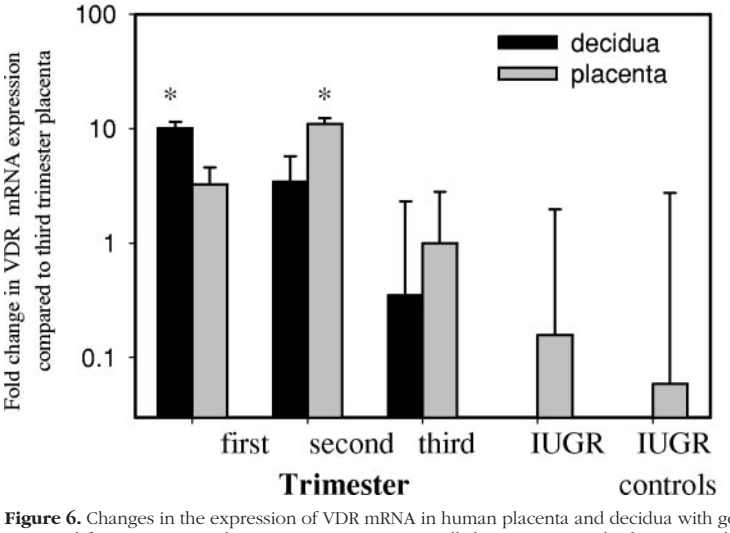
Trimester/ tissue	Fold-change mRNA	95% confidence limit
First decidua	94.20	61.70 – 143.81
First placenta	56.46	42.05 – 75.82
Second decidua	49.99	23.17 – 107.87
Second placenta	50.55	29.38 – 86.97
Third decidua	5.07	1.13 – 22.74
Third placenta	1	0.3 – 3.35
IUGR placenta	0.95	0.34 – 2.65
IUGR controls	0.49	0.02 – 7.46

Figure 5. Changes in the expression of 1α-OHase in human placenta and decidua with gestation are independent of variations in trophoblast/endometrial epithelial cell status. **A:** Expression of 1α-OHase mRNA in human placenta and decidua corrected for 18S rRNA and CK-8 mRNA expression. All data are reported relative to a third trimester placenta value of 1. *, Significantly different from third trimester placenta, $P < 0.001$; **, significantly different from third trimester placenta, $P < 0.05$. **B:** Data expressed with 95% confidence limits. IUGR controls, gestationally matched placentae.

logs of $1,25(OH)_2D_3$ as therapy for autoimmune disease, psoriasis, and cancer.[2–4,18] In contrast, our understanding of the impact of $1,25(OH)_2D_3$ on reproduction is much less clear despite evidence linking vitamin D status with reproductive function. Spermatogenesis is compromised by vitamin D deficiency but this can be corrected by calcium supplementation, suggesting an indirect effect of $1,25(OH)_2D_3$ on male reproduction.[19] Female fertility also seems to be markedly reduced in vitamin D deficiency but may involve both calcium-dependent or -independent actions depending on the animal model used. In vitamin D-deficient rats decreased fertility and litter size seems to be independent of calcium status.[12–14] Conversely female reproductive efficiency in VDR-null mice seems to be a direct consequence of altered calcium status.[20,21] In either model the precise mechanism for this has yet to be determined, although ovarian cells express VDR and are an established target tissue for $1,25(OH)_2D_3$.[22–24] Recent studies have suggested that $1,25(OH)_2D_3$ is able to influence ovarian function by modulating local estrogen synthesis. VDR-null mice show decreased expression of the estrogenic enzyme aromatase in ovaries and testis, although this seemed to be because of, in part, altered calcium homeostasis.[21]

Previous reports have also implicated $1,25(OH)_2D_3$ in fetoplacental development. In particular, maternal circu-

A.



B.

Trimester/ tissue	Fold-change mRNA	95% confidence limit
First decidua	10.11	0.52 – 25.81
First placenta	3.26	0.11 – 11.79
Second decidua	3.44	0.03 – 9.25
Second placenta	10.93	0.66 – 22.31
Third decidua	0.35	0.13 – 18.38
Third placenta	1	0.02 – 17.88
IUGR placenta	0.16	0.02 – 1.36
IUGR controls	0.06	0.01 – 0.23

Figure 6. Changes in the expression of VDR mRNA in human placenta and decidua with gestation. **A:** Expression of VDR mRNA in human placenta and decidua corrected for 18S rRNA and CK-8 mRNA expression. All data are reported relative to a third trimester placenta value of 1. *, Significantly different from third trimester placenta, $P < 0.05$. **B:** Data expressed with 95% confidence limits. IUGR controls, gestationally matched placentae.

lating levels of $1,25(OH)_2D_3$ more than double with advancing gestation, resulting in a concomitant increase in calcium uptake by the maternal intestine.[25] Although $1,25(OH)_2D_3$ is the principal stimulator of calcium uptake in the gut, its role in placental calcium handling has yet to be clearly defined. Perfusion studies in animals using pharmacological doses of $1,25(OH)_2D_3$ have demonstrated an increase in fetal blood calcium, placental calcium transport, and fetal calcium status.[26] Furthermore, previous nephrectomy of animals has been shown to result in decreased placental calcium transport.[27] However, these observations are further complicated by the fact that decidual and placental tissues are themselves a key site for peripheral $1,25(OH)_2D_3$ production. Both decidua and trophoblast cells express VDR as well as 1α-OHase, suggesting an autocrine or paracrine role for $1,25(OH)_2D_3$ within these tissues.[28–33] In particular, we have postulated that local activation of vitamin D may influence implantation, either through the established immunomodulatory effects of $1,25(OH)_2D_3$ or via the regulation of specific target genes associated with implantation. To test this hypothesis we have characterized spatiotemporal variations in 1α-OHase expression in human decidua and placenta.

IHC data showed that 1α-OHase protein is expressed by both syncytio- and cytotrophoblast layers of the placental chorionic villus. These observations are similar to previous reports of 1α-OHase mRNA expression.[31,33] However, the intensity of immunoreactivity (as measured using a blinded semiquantitative scoring system), seemed to be greater in first and second trimester pregnancies. In particular, decidual samples from early pregnancy showed strong immunolocalization of 1α-OHase within the decidualized endometrial stromal cells. Although there are no specific markers for these cells, CD10 and CD68 were both detectable in these cells, with the intensity of expression seeming to be related to the degree of decidualization. Lymphocytes were negative for 1α-OHase but double-labeling IHC studies demonstrated reactivity for 1α-OHase in a substantial proportion of decidual macrophages. Macrophages are less abundant than granulated lymphocytes in the first half of pregnancy but they persist throughout gestation and are often closely associated with extravillous trophoblast in the decidua basalis.[34]

The presence of 1α-OHase in decidual macrophages is analogous to that previously described for lymphatic tissues in diseases such as sarcoidosis and tuberculosis, in which local synthesis of $1,25(OH)_2D_3$ has the potential to influence several elements of the local immune system. The hormone may act in an autocrine/intracrine manner to enhance cellular immune function, including cytokine production,[35,36] antigen presentation,[37] and cytotoxic function.[38] Local production of $1,25(OH)_2D_3$ by macrophages may also influence lymphocyte development and function,[39] as well as T-cell-mediated B-cell immunoglobulin production.[40] Modulation of specific T-cell subsets may also occur. Specifically, $1,25(OH)_2D_3$ promotes a shift away from cell-mediated (Th1-type) responses toward humoral immunity (Th2-type) with accompanying changes in cytokine expression.[41] In this way, local activation of vitamin D seems to act by maximizing the ability of the host to control the activating antigen while minimizing the potentially self-destructive effects of unregulated lymphocyte activity. The immunological nature of decidual tissue suggests that the synthesis $1,25(OH)_2D_3$ by decidual cells may act in a paracrine manner to modulate local lymphocyte activity. Evidence is accumulating from both mouse and humans that successful pregnancy is associated with a bias toward Th2-type cytokines, Th1-type responses being suppressed both systemically and locally.[42] We can therefore postulate that vitamin D may play a role in promoting this shift away from cell-mediated immune responses in pregnancy. It would be of interest to examine levels of 1α-OHase in pregnancy disorders such as recurrent miscarriage and primary infertility that have been associated with abnormalities of this Th1/Th2 shift.

The importance of local $1,25(OH)_2D_3$ production in early decidual function is emphasized by the variations in 1α-OHase and VDR expression across gestation. Levels of 1α-OHase mRNA were maximal in first and second trimester placentae and deciduae, compared with the third trimester, even after adjustment for changes in the cellular population (as determined by CK-8 levels). VDR expression also seems to be relatively high in early gestation although cross-gestational changes in mRNA for the receptor were less pronounced than for 1α-OHase. Collectively, these observations suggest that in early gestation decidual cells are primed for both synthesis of and response to $1,25(OH)_2D_3$. This is consistent with a more localized function for the hormone with 1α-OHase being the most likely rate-limiting mechanism. During early pregnancy the extravillous trophoblast invades uterine decidua, myometrium, and spiral arteries, establishing the uteroplacental blood supply. This invasive trophoblast expresses the nonclassical HLA-G class I MHC antigen as well as HLA-C.[43] At this stage of pregnancy the uterine decidua contains an abundant population of granulated lymphocytes, capable of natural killer cell activity, as well as CD8-positive T lymphocytes.[44] The mechanisms that allow the potentially semiallogeneic fetally derived extravillous trophoblast cells to migrate through maternal uterine tissues remain to be established but suggest a local modification of immune responses.

In addition to its potent immunosuppressive actions $1,25(OH)_2D_3$ may influence implantation by regulating key developmental target genes, such as homeobox (HOX) genes. In particular, the HOXA10 gene has been shown to be intimately associated with implantation.[45] Female homozygotes with HOXA10 knock outs ovulate and fertilize normally but demonstrate embryo lethality at days 2.5 to 3.5. This coincides spatially and temporally with expression of maternal HOXA10 in distal oviductal and uterine epithelium.[46] These results indicate a role for HOX genes in female fertility and suggest that maternal HOXA10 is required to regulate the expression of a factor that affects the viability of preimplantation embryos.[47] Human endometrium shows up-regulation of HOXA10 expression at the time of implantation, indicating a conserved role in women.[48] Recent reports have highlighted a link between vitamin D and HOXA10. Studies *in*

vitro using myeloid leukemic cells have shown that $1,25(OH)_2D_3$ stimulates a dose-dependent increase in HOXA10 expression.[49] This seems to be because of direct regulation of transactivation as other reports have documented a functional vitamin D response element in the promoter of the HOXA10 gene.[50]

Temporal data indicate that 1α-OHase expression is much stronger in the first and second trimesters. However, the presence of detectable levels of the enzyme in third trimester placenta emphasizes that 1α-OHase may have functions across gestation. This is consistent with previous studies that have characterized 1α-OHase mRNA expression in human syncytiotrophoblast cells.[33] To investigate the possible impact of $1,25(OH)_2D_3$ synthesis on later stages of gestation, we assessed 1α-OHase expression in placentae from IUGR pregnancies. IUGR is characterized by abnormal placentation and remains a major cause of perinatal morbidity and mortality. The specific cohort of IUGR pregnancies with uteroplacental failure described in this article have a particularly poor prognosis. The villous placentae of these pregnancies are significantly smaller than normal, with reduced cytotrophoblast proliferation and abnormal vasculature. Also, in common with preeclampsia, there is a paucity of extravillous trophoblast invasion of the maternal spiral arteriolar circulation. However, in contrast to previous studies of preeclampsia that have highlighted an association with decreased circulating levels of $1,25(OH)_2D_3$,[51–53] relatively little is known about the impact of vitamin D on IUGR. Our observations indicated that 1α-OHase levels were lower in IUGR samples compared to normal third trimester placentae. However, this was not statistically significant after normalization for changes in CK-8 expression and, more importantly, the IUGR samples showed no difference in 1α-OHase expression compared to gestationally matched controls.

Data presented here indicate that both mRNA and protein for 1α-OHase are abundantly expressed in placenta and decidua from first and early second trimester pregnancies. The relatively high levels of 1α-OHase in decidua raises the possibility that the enzyme plays a local role in human implantation and/or placentation. In particular, expression of the enzyme by decidualized stromal cells and macrophages suggests an autocrine/paracrine modulatory role for $1,25(OH)_2D_3$ within this immune privileged site. A potential role for vitamin D at the maternofetal interface was first postulated by Rebut-Bonneton and Demingnon[54] who showed that $1,25(OH)_2D_3$ inhibited the proliferation of allogenically-stimulated lymphocytes and cytotoxic T cells *in vitro*. Analysis of placental/decidual 1α-OHase expression suggests that, *in vivo*, the effects of $1,25(OH)_2D_3$ are more likely to be a consequence of locally synthesized hormone rather than circulating levels. In this respect it is important to recognize that, unlike its kidney counterpart, extra-renal 1α-OHase is not subject to tight autoregulation.[2,8] As such the amount of $1,25(OH)_2D_3$ synthesized by placenta and decidua will primarily be a reflection of the availability of substrate; in other words dietary/environmental access to precursor vitamin D. We can therefore postulate that the decreased fertility associated with vitamin D deficiency[12–14,19] is because of suboptimal synthesis of $1,25(OH)_2D_3$ in placenta/decidua, with concomitant loss of immune regulation in these tissues. Further analysis of implantation and placentation in the recently reported 1α-OHase knockout mouse[55] will help to clarify this mechanism.

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