Calcitriol affects hCG gene transcription in cultured human syncytiotrophoblasts

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Abstract

Background: In pregnancy, maternal serum concentrations of calcitriol significantly rise as a result of increased renal and placental contribution in order to assure calcium supply for the developing fetus. Considering that placenta is a site for vitamin D activation, and the versatility and potency of calcitriol, it is feasible that this hormone participates in fetal/placental development and physiology. In the present work we studied calcitriol actions upon human chorionic gonadotropin (hCG) secretion and expression in cultured trophoblasts, as well as vitamin D receptor (VDR) and CYP27B1 immunolocalization in placental villi.

Methods: Quantification of hCG in culture media was performed by immunoassay. Expression studies were carried out by real time PCR. Analysis of CYP27B1 and VDR localization in placental slides were performed by immunohistochemistry. Statistical significance was established by one way ANOVA using Tukey test for comparisons.

Results: Calcitriol regulated hCG in a time-dependent manner: at 6 h the secosteroid stimulated hCG, whereas longer incubations (24 h) showed opposite effects. Interestingly, calcitriol stimulatory effects on hCG were accompanied by an increase in intracellular cAMP content and were abolished by pre-incubation of the cells with a selective protein kinase A inhibitor. Immunohistochemical techniques showed differential VDR localization in the syncytiotrophoblast layer or in the vascular smooth muscle cells depending on the epitope to which the antibodies were raised (specific for the carboxy- or amino-terminal regions, respectively). CYP27B1 was immunolocalized in the syncytiotrophoblast layer of placental villi.

Conclusion: The presence and location of the vitamin D activating enzyme CYP27B1 as well as the specific receptor for vitamin D were shown in placental sections. The latter, together with findings demonstrating specific effects of calcitriol acting through the VDR and the cAMP/PKA signaling pathway upon hCG expression and secretion, indicate that there is a functional vitamin D endocrine system in the placenta, and recognize calcitriol as an autocrine regulator of hCG.
Background
Vitamin D is metabolized to the steroid hormone 1,25-
dihydroxyvitamin D₃ or calcitriol, which regulates cal-
cium homeostasis, modulates the immune response, and
promotes cellular differentiation, among other actions.
Calcitriol, the most active vitamin D metabolite, exerts its
biological effects by binding to the vitamin D receptor
(VDR), which is a ligand-activated transcription factor
that recognizes cognate vitamin D response elements
(VDREs) in target genes, and can also elicit rapid
responses mediated by membrane receptors [1]. Placenta
is a source and target of calcitriol [2]. In a similar manner
to the renal process, placental production of calcitriol is
catalyzed by the mitochondrial CYP27B1 [3]. In early
reproductive events, calcitriol has shown to evoke specific
biological effects such as regulation of the decidualization
and implantation processes [4,5]. In addition, calcitriol
regulates placental lactogen expression as well as proges-
terone and estradiol secretion in cultured human syncyti-
ottrophoblasts [6,7]. Regarding other molecules that are
regulated by calcitriol in the placenta, Evans et al showed
that calcitriol acts in an autocrine/paracrine fashion to
regulate both acquired and innate immune responses,
decreasing synthesis of cytokines such as granulocytemacrophage colony stimulating factor 2, tumor necrosis
factor, and interleukin 6, but increasing expression of
mRNA for the cathelicidin antimicrobial peptide [8].
Since human chorionic gonadotropin (hCG) is a pivotal
hormone for pregnancy maintenance, the aim of the pre-
sent work was to broaden the knowledge of calcitriol
actions in the placenta, focusing in the study of its effects
upon hCG expression and secretion in cultured human
syncytiotrophoblasts. The data presented herein display a
functional vitamin D endocrine system present in human
placenta and suggest its involvement in regulating placent-
al physiology.

Methods
Reagents
Culture media, fetal bovine serum (FBS) and Trizol were
from Invitrogen (NY, USA). TaqMan Master reaction, Taq-
Man probes and the transcriptor RT system were from
Roche (Roche Applied Science, IN, USA), calcitriol
(1α,25-dihydroxycholecalciferol) was kindly donated
from Hoffmann-La Roche Ltd (Basel, Switzerland). 3-Isob-
uty1-1-methykanthine (IBMX). 8-Bromo cAMP (8-Br-
cAMP), H-89 and the enzymes used for cell cultures were
from Sigma-Aldrich (MO, USA). Immunoassay for hCG
was from Immunometric Ltd. (London, UK). CYP27B1
antibody (sheep anti-murine 25-hydroxyvitamin D-1α-
hydroxylase) was from The Binding Site (Birmingham,
UK). The VDR antibodies (rabbit polyclonal anti-VDR N-
20 sc-1009 and anti-VDR C-20 sc-1008), as well as the sec-
ondary antibodies rabbit anti-sheep-horseradish peroxi-
dase, and mouse anti-rabbit IgG-HRP were purchased
from Santa Cruz Biotechnology (CA, USA). DAB (3,3’-
diaminobenzidine tetrahydrochloride) was from Zymed
Laboratories Inc. (CA, USA).

Immunohistochemistry
This study was approved by the Institutional Human Eth-
ical Committee (Hospital de Gineco-Obstetricia “Luis
Castelazo Ayala”, IMSS, México), and written informed
consents forms were obtained from each placental donor.
Term placentae (37-42 weeks of gestation) were acquired
from uncomplicated pregnancies.

Fresh placental tissue from 5 term placentas was embed-
ded in paraffin after fixation in 4% paraformaldehyde in
0.1 M phosphate buffer, pH 7.4. Serial sections (7 μm)
were obtained according to standard procedures. Slides
were treated with methanol-hydrogen peroxide in order
to block the endogenous peroxidase activity. Normal rab-
tirin serum and 1% BSA were used as blockers to decrease
non-specific signal. Slides were then incubated with pri-
mary antibodies (anti-CYP27B1, anti-VDR-N and anti-
VDR-C) during 45 minutes at room temperature, fol-
lowed by further washing and incubation with secondary
antibodies for another 45 minutes. Staining was devel-
oped using DAB substrate and the chromogen was con-
tasted with Mayer’s hematoxylin. Immunolabeling
specificity was tested by omitting the primary antibody.

Trophoblast cell culture
Trophoblasts were cultured as previously described
[3,9,10]. Briefly: Villous cytotrophoblasts were obtained
by enzymatic dispersion and cells were separated on den-
sity Percoll gradients. Trophoblasts were plated at a den-
sity of 8 × 10⁵ cells/mL in supplemented medium
[(DMEM) 100 U/ml penicillin, 100 mg/ml streptomycin,
0.25 mg/ml Fungizone], containing 20% heat-inactivated
FBS. Incubations were performed in humidified 5% CO₂-
95% air at 37°C. The morphological aspects of cells were
examined daily, secreted hCG was measured by immu-
noassay (EIA) following manufacturer instructions and
results were normalized against total protein content. Pro-
tein was determined by the method of Bradford [11].

Calcitriol effects on hCG secretion
Two days-cultured trophoblasts were incubated in the
presence of different concentrations of calcitriol or etha-
nol as vehicle, in serum-free DMEM-F12 during 6 h or 24
h. Additional experiments were performed incubating the
cells with a selective protein kinase A inhibitor (H-89).
Incubations were stopped by media collection, cell lysis
with RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7
mM monobasic sodium phosphate, 150 mM NaCl, 1%
Nonidet P-40, 0.1% SDS, pH 7.4) was used for protein
determination and hCG was quantified in culture media.
Calcitriol effects on hCG expression

For expression studies, 3 x 10^6 cells were plated in 25 cm² cell culture flasks and subjected to the same treatments as stated above. Total RNA was extracted using Trizol and 1 μg was reverse transcribed using the transcriptor RT system. Real-time PCR was carried out using the LightCycler 2.0 from Roche (Roche Diagnostics, Mannheim, Germany), according to the following protocol: activation of Taq DNA polymerase and DNA denaturation at 95 °C for 10 min, proceeded by 45 amplification cycles consisting of 10 s at 95 °C, 30 s at 60 °C, and 1 s at 72 °C. The primer pair was targeted to the β subunit of the hCG mRNA and the sequences were as follows: GCTCACCCCGACGCATCAT and CAGGACCAACAGCAGCAG. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also amplified as an internal control, using the primers: AGCCACATCGTACGACAC and GCCAAATACGACAAATCC. The sizes of the resulting amplicons were 131 bp and 66 bp, and the probes utilized were # 79 and # 60 (human universal probe library), for hCG and GAPDH, respectively. The expression of CYP24A1 was used as a control for calcitriol effects was evaluated using the following sense and anti-sense primers: CATCATGCGCATCAAAACAA and GCAGCTCGACTGGGCT and the probes utilized were # 79 and # 60 (Roche human universal probe library).

Calcitriol effects on cAMP accumulation

Cells were incubated in the presence of calcitriol or its vehicle in DMEM-F12 supplemented with IBMX (0.05 mM). Incubations were terminated after 10 minutes by media collection and homogenization of the cells in RIPA buffer. Samples were boiled during 5 min for phosphodiesterases inactivation and intracellular cAMP was measured by specific radioimmunoassay (RIA) as previously described [12]. Results were normalized against total protein content and expressed as fmol cAMP/mg protein.

Statistical analysis

Data are presented as the mean ± S.D. Statistical significance among groups was established by one way ANOVA using Tukey test. A P value ≤ 0.05 was considered statistically significant.

Results

Immunohistochemical studies

Analysis of sequential placentatissue sections indicated the presence of immunoreactive CYP27B1 in the syncytiotrophoblast layer (Fig. 1A). A similar immunostaining pattern for VDR was visualized using an anti C-terminus specific antibody, immunostaining was also identified in vascular smooth muscle cells (VSMC, Fig. 1C). Interestingly, the use of an anti N-terminus VDR specific antibody disclosed intense immunostaining in the VSMC, and weak signal in the syncytiotrophoblast layer was observed (Fig. 1E). Control incubations without primary antibodies are shown in figure 1B, D and 1F. These results demonstrated the presence in the placenta of two important components of the vitamin D endocrine system. Considering these data, we searched for a marker of placental function. Since trophoblast cell culture has proven to be a good model to study placental physiology and hCG is an important marker of placental functionality, we decided to use this system in order to evaluate calcitriol regulatory actions at the placental level.

Calcitriol effects on hCG in cultured trophoblasts

Cultured purified cytotrophoblasts aggregated and formed syncyti in a time dependent manner. Cell culture viability was evaluated by measurements of hCG in the presence or absence of 8-Br-cAMP, an analog of cAMP, a well known regulatory factor of hCG expression [13,14]. Basal secretion of hCG into the culture media increased during the cytodifferentiation process, and cells cultured in the presence of 8-Br-cAMP secreted significantly more hCG than controls (Fig. 2A). Similarly, hCG mRNA increased with the same pattern observed under basal conditions (Fig. 2B), and in the presence of 8-Br-cAMP the highest hCG β gene expression was observed on day 2, which preceded the maximal hCG secretion on day 3. These data, in addition to morphological cell evaluation, further confirmed the functional integrity of the primary culture system.

In the presence of calcitriol, hCG secretion increased significantly after 6 hours of incubation (Fig. 3A). Calcitriol also upregulated hCG mRNA (Fig. 3B). Since hCG is highly regulated by the cAMP/PKA pathway, and calcitriol has shown to induce cAMP accumulation [12,15], we investigated the participation of this second messenger upon hCG-upregulation by calcitriol. For this purpose, cAMP was quantified in calcitriol-incubated cells. Results indicated that after 10 minutes of treatment, the seconderoid significantly increased intracellular cAMP content in a dose-dependent manner (Fig. 4A). Preincubation of cells with a selective inhibitor of PKA (H-89) reduced hCG expression below basal levels and prevented the calcitriol-dependent protein and gene hCG-stimulation detected at the 6 h incubation period (Fig. 4B and 4C, respectively). The same results were obtained in 72 h cultures (data not shown).

Calcitriol long-term effects upon hCG were also studied. The stimulatory effects observed at 6 h were no further evident after 24 h (data not shown), and when cells were incubated in the presence of calcitriol during 2 consecutive days, the effects were rather inhibitory (Fig. 5A). Inhibition was evident at the mRNA level after 24 hours treatment (Fig. 5B), preceding the observed response in hCG protein. This repressive calcitriol effect could not be
Immunolocalization of CYP27B1 and VDR in placental chorionic villi. Placental sections were incubated with specific antibodies in order to localize important components of the vitamin D endocrine system. CYP27B1 protein was located in the syncytiotrophoblast layer (A). The use of anti N-terminus VDR specific antibody disclosed intense immunostaining in the VSMC (E), whereas a VDR-C-terminus antibody preferentially stained the syncytiotrophoblast layer (C). Figure shows representative pictures of 5 different placentas. Negative controls in the absence of first antibodies are shown in B, D and F. SC = syncytiotrophoblast layer, VSMC = vascular smooth muscle cells, RBC = red blood cells. (200×).

**Figure 1**
Figure 2
Temporal pattern of hCG secretion (A) and expression (B) in cultured human trophoblasts. Cytotrophoblasts were plated in the absence (•) or presence (■) of 8-Br-cAMP (1.5 mM). Two scale bars were used in order to show all data [stimulated (▲) vs. non stimulated (▼)] in the same graphic. Media was changed every day. A) Secretion of hCG in culture media was measured daily and results were expressed as mIU/mg protein. B) Real time PCR analysis of hCGβ expression in different culture days. Results were normalized against GAPDH mRNA. Vehicle data were arbitrarily given a value of 1. Basal hCG secretion and expression increased significantly compared with day 1. Note that hCGβ mRNA increased considerably on day 2 in the presence of 8-Br-cAMP (B), which was reflected on hCG secretion on day 3 (A), showing an important protein synthesis activity between day 2 and 3 of the cell culture. Data are presented as the mean ± S.D. of three different cell cultures. *p < 0.05 vs. day 1; **p < 0.05 vs. control.

attributed to decreased cell viability, since under the same conditions, calcitriol upregulated CYP24A1 gene expression (Fig. 5C).

Discussion
Serum concentrations of biologically active hCG depend on the rate of synthesis of its specific β subunit; whereas at the cellular level multiple factors modulate hCG produc-

Figure 3
Stimulatory effects of calcitriol on hCG secretion and gene expression in cultured syncytiotrophoblasts. A) Hormone secretion was determined by EIA after 6 hours incubation in the presence of increasing concentrations of calcitriol or its vehicle (-). B) Real time PCR analysis of hCGβ gene expression of calcitriol-treated cells. Results were normalized against GAPDH mRNA. Vehicle data were arbitrarily given a value of 1. Each bar represents the mean ± S.D. of triplicate cultures. *p < 0.05 vs. control.
signal transduction cascade impaired the ability of calcitriol to elicit transcriptional induction of hCGβ gene, as well as hCG secretion into the culture media. Rapid cAMP generation induced by calcitriol has been previously reported in other cell types [15,22], and may be the result of its interaction with membrane-VDR or other surface proteins. In addition, since it has been demonstrated that calcium ion channels are involved in GnRH dependent-hCG secretion [23], calcitriol could also release stored
The fact that [3H]25-hydroxyvitamin D bioconversion into hCG through promoting a rapid calcium entry into the cell. Further studies are needed in order to clarify this matter.

The concentration of hCG was also measured after 12, 24 and 48 h of calcitriol treatment, but the results reported in the present study were only those that differed significantly when compared with the vehicle alone. The stimulatory effects observed at 6 h were no further evident after 12 or 24 h, and when cells were incubated in the presence of calcitriol during 2 consecutive days, the effects were rather inhibitory. Inhibition was evident at the mRNA level after 24 hours treatment, preceding the observed response in hCG protein. These data are probably more likely to be reflective of the true biological situation. Indeed, our results that calcitriol inhibited hCG were in line with previous data from this and other laboratories where low serum calcitriol and high serum hCG levels were found in preeclampsia [24-26], that conjointly with the fact that [3H]25-hydroxyvitamin D bioconversion into [3H]1,25-dihydroxyvitamin D was significantly reduced in preeclamptic placentas [9], may suggest a direct regulatory effect of calcitriol on hCG production. Regarding the inhibitory effects of calcitriol on hCG, it is likely that a secondary metabolic C23/C24 calcitriol oxidation pathway might play a role, since the resulting trihydroxylated metabolite is considered biologically inactive [1]. Alternatively, since calcitriol has been shown to stimulate progesterone secretion [6] and in turn this hormone inhibits hCG secretion [27], this mechanism could additionally participate in calcitriol long term inhibitory effects in placenta. In any case, the demonstration in this study of genomic mediated effects of calcitriol on hCG suggested the presence of VDR dependent regulatory regions on hCG promoters. Indeed we found five putative VDR/RXR heterodimer binding sites in the hCGβ-5 gene promoter [28], which probably may be acting as calcitriol dependent-transcriptional regulatory regions. Nevertheless, the sole presence of the VDREs in the hCGβ-5 promoter is not sufficient to indicate transcriptional function; therefore, functional evaluation of the putative VDREs deserves further investigation.

In non pregnant women the physiological concentration of calcitriol fluctuates between 40–100 pM. In the present study the calcitriol doses tested were: 100 pM, 1 nM and 10 nM. The lowest concentration (100 pM) is within the physiological range of circulating calcitriol levels in healthy pregnant women (127 pM and 151 pM) as observed previously [24,29]. The other doses tested were supra-physiological, nevertheless, calcitriol effects upon hCG were evident starting with the lowest concentration.

Placenta is considered not only as a source but also as a target of calcitriol [2]. In order to get insights on calcitriol paracrine/autocrine effects in placenta, we investigated the immunolocalization of VDR and CYP27B1 in placental chorionic villi. In accordance with previous reports [30], CYP27B1 protein was located in the syncytiotrophoblast layer, corroborating that the endocrine phenotype of trophoblasts cells is responsible for vitamin D activation in placenta. To answer where the locally produced calcitriol acts in the placenta, we looked for VDR protein in placental sections. To our knowledge, this is the first report to show immunoreactive VDR in different locations in the placental villi, since VDR expression has been mainly addressed at the mRNA level in placenta [2,31]. The antibodies showed the presence of VDR in the endocrine placental cells and VSMC, suggesting that calcitriol could be involved in regulating hormonal production and vascular remodeling through the VDR. The latter assumption derives from previous studies demonstrating that calcitriol acts in the vasculature promoting VSMC growth and migration [32,33]. Interestingly, the C-terminus antibody intensely stained the syncytiotrophoblast layer and faintly stained the surrounding cells of placental vessels, whereas the N-terminus antibody detected a strong signal in the endothelial and VSMC. These observations may indicate different epitopes recognized by the antibodies depending on the topological position of the VDR. An interesting challenge would be to define specific VDR responses in different placental structures.

Conclusion
In summary, the present study broadens the knowledge of placental vitamin D endocrine system by demonstrating the physiological effects of calcitriol on an important biochemical placental function marker such as hCG. In addition, this is the first report to show immunoreactive VDR in different locations in human placental villi, and opens the field to address important research questions on the role of calcitriol in specific placental structures.

Competing interests
The author(s) declare that they have no competing interests.

Authors’ contributions
DB carried out real time PCR’s analysis, hCG quantification and participated in the design of the study and statistical analysis. EA participated in the design of the study, particularly the molecular studies, performed real time PCR analysis and helped to draft the manuscript. GH performed placenta collection, trophoblast primary cell cultures, RNA extraction and reverse transcription reactions. IM and LG were in charge of all experiments concerning cAMP, including design and analysis of the results. AH contributed in interpretation of data and was involved in drafting the manuscript. FL made substantial contribution to the design of the study, was involved in drafting the
manuscript and revised it critically. AM performed the immunochemical studies and helped to draft the manuscript. LD conceived the study, participated in the design and coordination, structured the manuscript and actively participated in experimental procedures. All authors read and approved the final manuscript.

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References

1. Dusso AS, Brown AJ, Slatopolsky E: Vitamin D. Am J Physiol Renal Physiol 2005, 288:F278-28.
2. Avila E, Díaz L, Halhali A, Larrea F: Regulation of 25-hydroxyvitamin D3 1alpha-hydroxylase and vitamin D receptor gene expression by 1,25-dihydroxyvitamin D3 in cultured human syncytiotrophoblast cells. J Steroid Biochem Mol Biol 2004, 85:2543-2549.
3. Diaz L, Sanchez I, Avila E, Halhali A, Vilchis F, Larrea F: Identification of a 25-hydroxyvitamin D3 1alpha-hydroxylase gene transcription product in cultures of human syncytiotrophoblast cells. J Clin Endocrinol Metab 2000, 85:2543-2549.
4. Halhali A, Acker GM, Garabedian M: 1,25-Dihydroxyvitamin D3 induces in vivo the decidualization of rat endometrial cells. J Reprod Fertil 1991, 91:59-64.
5. Du H, Daftary GS, Lalwani SI, Taylor HS: Expression of the human chorionic gonadotropin beta subunit (CG beta) gene: regulation of transcriptionally active CG beta gene by cyclic AMP. Mol Cell Bio 1988, 8:5100-5107.
6. Halhali A, Boland RI: Studies suggesting the participation of protein kinase A in 1,25(OH)2-vitamin D3-dependent protein phosphorylation in cardiac muscle. J Mol Cell Cardiol 1998, 30:225-233.
7. Petraglia F, Santuz M, Florio P, Simoncini T, Luisi S, Plano L, Genazzani AR, Genazzani AD, Volpe A: Paracrine regulation of human placenta: control of hormonogenesis. J Reprod Immunol 1998, 39:21-23.
8. Licht P, Cao H, Lei ZM, Rao CV, Merz WE: Novel self-regulation of human chorionic gonadotropin biosynthesis in term pregnancy human placenta. Endocrinology 1993, 133:3014-3025.
9. Milsted A, Cox RP, Nilson JH: Cyclic AMP regulates transcription of the genes encoding human chorionic gonadotropin with different kinetics. DNA 1987, 6:213-219.
10. Ringer GE, Kao LC, Miller WL, Strauss JF 3rd: Effects of 8-bromo-cAMP on expression of chorionic gonadotropin and human trophoblast cells. Regulation of specific mRNAs. Mol Cell Endocrinol 1989, 61:1-21.
11. Jameson JL, Hogenhout AN: Regulation of chorionic gonadotropin gene expression. Endocr Rev 1993, 14:203-221.
12. Johnson W, Albamante C, Handwerger S, Williams T, Pestell RG, Jameson JL: Regulation of the human chorionic gonadotropin alpha- and beta-subunit promoters by AP-2. J Biol Chem 1997, 272:15405-15412.
13. Fleet JC: Rapid, membrane-initiated actions of 1,25 dihydroxyvitamin D3: what are they and what do they mean? J Nutr 2004, 134:3215-3218.
14. Sharma SC, Rao AJ: Effect of calcium ion channel antagonists on chorionic gonadotropin secretion. Biochem Mol Int 1997, 43:101-1106.
15. Halhali A, Tovar AR, Torres N, Bourges H, Garabedian M, Larrea F: Preeclampsia is associated with low circulating levels of insulin-like growth factor I and 1,25-dihydroxyvitamin D in maternal and umbilical cord compartments. J Clin Endocrinol Metab 2000, 85:1828-1836.
16. August P, Marcaccio B, Gartner JM, Druzin ML, Resnick LM, Laragh JH: Abnormal 1,25-dihydroxyvitamin D metabolism in preeclampsia. Am J Obstet Gynecol 1992, 166:1295-1299.
17. Basarir Z, Barat S, Hajihamidi M: Serum beta human chorionic gonadotropin levels and preeclampsia. Saudi Med J 2006, 27:1001-1004.
18. Yamamoto T, Matsumoto K, Kurachi H, Okamoto Y, Nishio Y, Sakata M, Tasaka K, Murata Y: Progesterone inhibits transcriptional activation of human chorionic gonadotropin-alpha gene through protein kinase A pathway in trophoblast cells. Mol Cell Endocrinol 2001, 182:215-224.
19. Genomatix [http://www.genomatix.de/]
20. Halhali A, Villa AR, Madraza E, Soria MC, Mercado E, Diaz L, Avila E, Garabedian M, Larrea F: Longitudinal changes in maternal serum 1,25-dihydroxyvitamin D and insulin like growth factor I levels in pregnant women who developed preeclampsia: comparison with normotensive pregnant women. J Steroid Biochem Mol Biol 2004, 89-90:353-356.
21. Zehnder D, Bland R, Williams MC, McNinch RW, Howie AJ, Stewart PM, Hewison M: Extraenal expression of 25-hydroxyvitamin d(3)-1alpha-hydroxylase. J Clin Endocrinol Metab 2001, 86:888-894.
22. Zehnder D, Evans KN, Kilby MD, Bulmer JN, Innes BA, Stewart PM, Hewison M: The ontogeny of 25-hydroxyvitamin D(3) 1alpha-hydroxylase expression in human placenta and decidua. Am J Pathol 2002, 161:105-114.
23. Rebsamen MC, Sun J, Norman AW, Liao JK: 1alpha,25-dihydroxyvitamin D3 induces vascular smooth muscle cell migration via activation of phosphatidylinositol 3-kinase. Circ Res 2002, 91:17-24.
24. Mitsuhashi T, Morris RC Jr, Ives HE: Isolation and characterization of the human chorionic gonadotropin beta subunit (CG beta) gene cluster: regulation of transcriptionally active CG beta gene by cyclic AMP. Mol Cell Bio 1988, 8:5100-5107.