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Vitamin D promotes human extravillous trophoblast invasion *in vitro*

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Word count: 3075

Funding: This study was supported by Action Medical Research (#1949 to MDK, SYC).

Competing interest(s): The authors have no conflicts of interest to disclose.

Abbreviations

1,25-D_3, 1,25-dihydroxyvitamin D_3; 25-D_3, 25-hydroxyvitamin D_3; CK-7, cytokeratin 7; CVS, chorionic villous sampling; CYP24A1, 24-hydroxylase; CYP27B1, 1α-hydroxylase; EVT, extravillous trophoblast; FGR, fetal growth restriction; HLA-G, human leukocyte antigen G; MMP, matrix metalloproteinase; PBS, phosphate buffered saline; RT-PCR, reverse transcriptase polymerase chain reaction; uNK, uterine natural killer cells; VDR, vitamin D receptor.

Abstract

Introduction: Incomplete human extravillous trophoblast (EVT) invasion of the decidua and maternal spiral arteries is characteristic of pre-eclampsia, a condition linked to low maternal vitamin D status. It is hypothesized that dysregulated vitamin D action in uteroplacental tissues disrupts EVT invasion leading to malplacentation.

Methods: This study assessed the effects of the active vitamin D metabolite, 1,25-dihydroxyvitamin D_3 (1,25-D_3), and its precursor, 25-hydroxyvitamin D_3 (25-D_3), on primary human EVT isolated from first trimester pregnancies. Expression of EVT markers (cytokeratin-7, HLA-G), the vitamin D-activating enzyme (CYP27B1) and 1,25-D_3 receptor (VDR) was assessed by immunocytochemistry. EVT responses following *in vitro* treatment with 1,25-D_3 (0-10nM) or 25-D_3 (0-100nM) for 48-60h were assessed using quantitative RT-PCR (qRT-PCR) analysis of key target genes. Effects on EVT invasion through Matrigel® were quantified alongside zymographic analysis of secreted matrix metalloproteinases (MMPs). Effects on cell viability were assessed by measurement of MTT.

Results: EVT co-expressed mRNA and protein for CYP27B1 and VDR, and demonstrated induction of mRNA encoding vitamin D-responsive genes, 24-hydroxylase (CYP24A1) and cathelicidin following 1,25-D_3 treatment. EVT could respond to 1,25-D_3 and 25-D_3, both of which significantly increased EVT invasion, with maximal effect at 1nM 1,25-D_3 (1.9-fold; $p<0.01$) and 100nM 25-D_3 (2.2-fold; $p<0.05$) respectively compared with untreated controls. This was accompanied by increased pro-MMP2 and pro-MMP9 secretion. The invasion was independent of cell viability, which remained unchanged.

Discussion: These data support a role for vitamin D in EVT invasion during human placentation and suggest that vitamin D-deficiency may contribute to impaired EVT invasion and pre-eclampsia.

Key words

Vitamin D; pre-eclampsia; placenta; extravillous trophoblast; cell invasion

Introduction

Vitamin D-deficiency, defined as a serum concentration of 25-hydroxyvitamin D (25-D_3; the main circulating form of vitamin D) less than 50nM, and vitamin D-insufficiency (25-D_3<75nM) are especially prevalent in pregnancy. These complicate at least 67% of pregnancies, particularly in women with darker skin pigmentation, in various geographical locations around the world [1-4]. A recent meta-analysis of observational studies noted associations between vitamin D-deficiency in pregnancy with increased risk of pre-eclampsia, gestational diabetes, preterm birth and small for gestational age infants; with pre-eclampsia showing the strongest association with an odds ratio of 2.09 (95%CI 1.50-2.90) [5].

Pre-eclampsia, a syndrome of maternal hypertension, proteinuria and endothelial dysfunction, affects up to 8% of pregnancies and remains a leading cause of maternal and perinatal morbidity and mortality [6]. In one study, maternal serum concentrations of 25-D_3 in prospectively collected samples in early pregnancy were found to be significantly lower in women who subsequently developed pre-eclampsia [7]. However, the pathogenic mechanisms linking low vitamin D levels with pre-eclampsia are not understood and a causative link between the two remains controversial. The prevalence of vitamin D insufficiency and incidence of pre-eclampsia are both increased in Black and South Asian women, which may implicate potential confounding variables associated with ethnicity.

The human hemochorial placenta is an extra-renal tissue with high expression of the vitamin D-activating enzyme 1α-hydroxylase (CYP27B1), which converts 25-D_3 to active 1,25-dihydroxyvitamin D_3 (1,25-D_3). Both CYP27B1 and the receptor for 1,25-D_3 (VDR) are expressed in human decidua and the villous placenta, with higher

expression during the first and second trimesters of pregnancy. This suggests a role for vitamin D in decidualisation and uteroplacental remodelling [8].

Cytotrophoblast within the villous placenta differentiates into extravillous trophoblast (EVT), which has an invasive phenotype. EVT invades the decidua and maternal spiral arteries from the first trimester until 24 weeks of gestation. This invasion is critical to maternal spiral artery remodelling and promotion of maternal placental blood flow to establish effective maternal-fetal exchange. Impairment of this process predisposes a pregnancy to uteroplacental insufficiency and a significantly increased risk of pre-eclampsia and fetal growth restriction (FGR).

We hypothesized that vitamin D insufficiency during pregnancy may lead to dysregulation of placental morphological development, and thus the development of malplacentation disorders including pre-eclampsia and FGR. Vitamin D has been demonstrated to regulate inflammation in human decidual uterine natural killer (uNK) cells [9], which in turn is postulated to impact on the invasion of fetal-derived EVT in a paracrine manner [10]. In this study, we have now investigated the direct effects of 1,25-D_3 and 25-D_3 upon isolated human first trimester primary EVT *in vitro.*

Methods

Ethical approval

Human samples were collected with informed written consent and with the approval of the South Birmingham Research Ethics committee (Reference: 06/Q2707/12) and the Research and Development office of the Walsall Manor Hospitals NHS Trust (Project code: 2007013OG(W); approval number: 11070745).

Sample collection

Placental samples were obtained from women undergoing elective surgical termination of apparently uncomplicated pregnancies. Samples were collected from 8-11 completed weeks of gestation as determined by ultrasound measurement of crown rump length prior to pregnancy termination. The fetuses were not known to have abnormal karyotypes nor structural anomalies.

Cell isolation and culture

Following collection, placental tissues were dissected free and washed three times with PBS. Primary EVTs were isolated using a method of enzyme digestion followed by percoll separation as previously described [11]. Characterization of these cells by immunocytochemistry for EVT markers using anti-cytokeratin 7 (Novocastra, Newcastle-upon-Tyne, UK; 1:20) and anti-HLA-G (Serotec, Oxford, UK; 1:200) with an avidin-biotin peroxidase method (Vectastatin Elite kit, Vector Laboratories, Peterbotough, UK) confirmed 95% purity (Figure 1A-C). Cells were cultured on growth factor-reduced Matrigel® matrix (BD Biosciences, Erembodegem, Belgium) in DMEM:F12 medium containing 10% FBS, 1000U/ml penicillin, 1 mg/ml streptomycin, 2 mM L-glutamine and 1.5 μg/ml amphoteracin B (all reagents from Gibco Life Technologies, Paisley, UK) in standard 5% CO_2 in an air incubator at 37°C. These cells were treated with 1,25-D_3 (Enzo Life Sciences, Exeter, UK) at concentrations of 0-10 nM or with 25-D_3 (Enzo Life Sciences) at 0-100 nM. Incubations were for 48h or 60h as defined in previously reported studies [12]. Inhibition of CYP27B1 was carried out by pre-treatment for 2h with the pan-cytochrome P450 inhibitor, ketoconazole (Sigma-Aldrich, Dorset, U.K), at 10^{-5}M, before culture with or without 25-D_3 treatment for 60h.

Immunofluorescence microscopy

Isolated EVT cells were grown (60h) on chamber slides coated with poly-D-lysine (100µl/well of 0.1mg/ml) and immunofluorescent-stained as described previously [13]. Cells were co-stained for VDR (clone D-6, Santa Cruz Biotechnology) and CYP27B1 (Clone H-90, Santa Cruz Biotechnology) (Figure 1). VDR and CYP27B1 were detected using anti-mouse Alexa Fluor 488 (Green) and anti-rabbit Texas Red (Red) secondary antibodies (Life Technologies) respectively. All antibodies were used at 1:100 dilution. Slides were mounted with Vectashield™ containing DAPI (Vector Laboratories Inc., Peterborough, UK) and examined using a fluorescent microscope (Carl Zeiss, Hertfordshire, UK).

Quantitative RT-PCR

Total RNA was extracted using TRI reagent (Sigma-Aldrich, Dorset, U.K.) following recovery of cells from Matrigel Matrix using BD cell recovery solution (BD Biosciences, Bedford, UK) according to the manufacturer's instructions. Total RNA (1µg) was reverse transcribed using Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega, Southampton, UK) following the manufacturer's guidelines. Expression of mRNA encoding CYP27B1 (Hs01096154_m1), CYP24A1 (Hs00167999_m1), VDR (Hs00172113_m1) or cathelicidin (Hs01011707_g1) was determined and normalized to the expression of 18S rRNA (4319413E), an internal control in multiplex reactions, using the ABI PRISM 7500 Sequence Detection System (ABI, Foster City, USA). All primer and probes were produced by Applied Biosystems, Paisley, UK. Quantification of gene expression was determined using the ΔCt method described previously [14]. Relative mRNA expression for each sample was compared with the mean gene expression at the lowest vitamin D dose at which expression was detectable, with this being assigned the arbitrary value of 1 within each experiment.

Invasion assays

Primary EVT were seeded in cell culture inserts for 24-well plates [8µm membrane pore size (BD Biosciences)] coated with growth factor-reduced Matrigel® matrix (10µl; BD Biosciences). Invaded cells (assessed in duplicate) were fixed in ethanol, stained with Mayer's hematoxylin and eosin (Sigma-Aldrich, Dorset, UK), and counted across the entire membrane at 20X magnification using a light microscope [11]. The invasion index was expressed as the ratio of invaded cells in the experimental group relative to the control group (0nM) within each experiment.

Matrix metalloproteinase (MMP) quantification by zymography

Levels of secreted MMP2 and MMP9 protein, which are key gelatinases in the degradation of the basement membrane by EVT during invasion, were assessed using gelatin zymography as described previously [15]. Briefly, total protein (16-20 µg) from EVT-conditioned medium was resolved by electrophoresis in a 12% SDS-PAGE gel and then incubated (30 min) in zymograph-renaturing buffer followed by zymograph-developing buffer (Invitrogen, Renfrew, UK) overnight at 37°C before staining with Coomassie Brilliant Blue R250. Dried gels were then scanned and densitometry of pro-MMP9 and pro-MMP2 performed using Image J software. Although active MMP2 was detectable, poor resolution of bands precluded quantification by relative densitometry.

MTT assays

Cells were seeded in 96 well plates and experiments performed with three or four replicates of each dose using the quantitative colorimetric 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl Tetrazolium Bromide (MTT; Sigma-Aldrich, Dorset, U.K) assay of mitochondrial metabolism, as described previously [16]. Within each experiment, the

absorbance values were normalized to the values obtained with no vitamin D treatment (0nM), which was given an arbitrary value of 100%.

Statistical analysis

Data were analyzed using the SigmaStat v3.1 statistical software (Systat Software Inc, California, USA). Repeated measures one-way analysis of variance was performed followed by Tukey all pairwise multiple comparisons post-hoc tests. Data sets passed the normality test except for the quantitative RT-PCR and pro-MMP2 data, which required logarithmic transformation prior to statistical analysis. Statistical significance was taken as $p<0.05$.

Results

EVT express a functional vitamin D intracrine system and respond to 1,25-D_3 and 25-D_3

Isolated EVT from first trimester placentae demonstrated coincident protein expression of the vitamin D-activating enzyme CYP27B1 and the intracellular receptor for 1,25-D_3, VDR (Figure 1D-G), confirming previous quantitative RT-PCR and immunohistochemistry findings in intact decidual tissue sections [8]. Expression of mRNA encoding CYP27B1 and VDR (Figure 2A-B) in isolated EVT was unaffected by treatment with 25-D_3 or 1,25-D_3, consistent with previous reports in primary cultures of first trimester human chorionic villous sampling (CVS) specimens [17]. The co-expression of CYP27B1 and VDR indicate the presence of a potential EVT vitamin D intracrine system.

Expression of mRNA encoding the vitamin D feedback catabolic enzyme CYP24A1, which attenuates vitamin D responsiveness by converting 1,25-D_3 and 25-D_3 to less active metabolites, was undetectable in untreated EVT (Figure 2C). This is consistent

with the CYP24A1 gene being methylation-silenced as previously reported in term villous placenta and in first trimester cytotrophoblast [17]. However, CYP24A1 mRNA expression was induced in EVT treated with 1nM 1,25-D_3. Treatment with a higher concentration of 1,25-D_3 (10nM) resulted in a 9-fold increase in CYP24A1 mRNA expression compared to 1nM-treated EVT ($p<0.01$; Figure 2C). This magnitude of response in EVT is similar to the 10-fold increase reported in CVS cells treated with 100nM 1,25-D3, but this magnitude is still significantly less than those in the hundreds reported in cells in which CYP24A1 is not methylation-silenced [17]. Evidence that human EVT is vitamin D-responsive was further demonstrated by a dose-dependent induction of mRNA encoding the vitamin D–responsive antibacterial protein, cathelicidin, by 1,25-D_3 (ANOVA $p<0.001$; Figure 2D). Messenger RNA encoding cathelicidin increased by 217-fold and 457-fold with 1nM and 10nM of 1,25-D_3 treatment respectively (both $p<0.001$). These data are consistent with previous findings in human term placental explants and isolated primary cytotrophoblast treated with 1,25-D_3 [18].

Effects of 1,25-D_3 and 25-D_3 on EVT invasion

Treatment of human primary EVT with 1,25-D_3 significantly increased directly quantified cell invasion into Matrigel® (ANOVA $p<0.01$; Figure 3A). A peak invasion response was demonstrated at 1nM 1,25-D_3, with a 1.9-fold increase in the number of invaded cells compared with untreated controls ($p<0.01$). Compared with untreated EVT, a statistically significant increase in invasion by 1.7-fold ($p<0.05$) was also noted with a lower dose of 0.1nM 1,25-D_3 but not at a higher dose of 10nM 1,25-D_3. The diminished response at 10nM 1,25-D_3 could be due to preceptor regulation and inactivation of 1,25-D_3 by increased CYP24A1 expression.

Primary EVT also responded to 25-D_3, confirming the efficacy of the CYP27B1/VDR intracrine system. There was a dose-dependent increase in EVT invasion with rising 25-D_3 concentrations (ANOVA $p<0.05$; Figure 3B). A similar magnitude in the peak response, as seen with 1,25-D_3, of a 2.2-fold rise in the number of invaded cells at 100nM 25-D_3 compared to controls ($p<0.05$) suggests that both forms of vitamin D use a similar response pathway in the promotion of EVT invasion.

To confirm that this observed increase in EVT invasion is mediated by vitamin D, EVT were pre-treated with a cytochrome P450 inhibitor, ketoconazole [19], prior to 25-D_3 treatment. Ketoconazole by itself did not inhibit invasion of EVT, but when CYP27B1 activity was blocked by ketoconazole the pro-invasive effects of 25-D_3 was significantly attenuated (ANOVA $p<0.01$; Figure 3C), suggesting that intracellular EVT metabolism of 25-D_3 mediates EVT invasion.

Enhanced Matrigel® invasion by EVT with vitamin D treatment was paralleled by increased secretion of pro-MMP2 and pro-MMP9 (Figures 4A-4D). Pro-MMP2 increased significantly (ANOVA $p<0.001$; Figure 4C) with 1,25-D_3 at 1nM ($p<0.001$) and 10nM ($p<0.001$), and pro-MMP9 was increased significantly (ANOVA $p<0.05$; Figure 4D) with 100nM 25-D_3, compared with untreated EVT ($p<0.05$).

Effects of 1,25-D_3 and 25-D_3 on EVT cell viability

To confirm that the observed increase in EVT invasion with vitamin D reflected increased invasive capability rather than enhanced cell proliferation and/or survival, we assessed EVT cell viability. Data from MTT analyses showed no significant change in EVT cell viability following treatment with 1,25-D_3 or 25-D_3 (Figures 4E-F).

Discussion

Vitamin D deficiency in pregnancy has been associated with an increased risk of pre-eclampsia [7, 20], but the underlying mechanisms are unclear. We have demonstrated that the vitamin D metabolites, 1,25-D_3 and 25-D_3, have a direct pro-invasive effect on isolated human EVT *in vitro*, highlighting an entirely novel action for vitamin D in the placenta. Furthermore, pro-invasive responses to vitamin D suggest that attenuated EVT invasion of uterine decidua and vasculature, may be one of the mechanisms by which vitamin D deficiency contributes to the increased risk of pre-eclampsia and FGR.

The pathogenesis of pre-eclampsia is proposed to be a two-stage process: the first stage occurring in the first and early second trimesters of pregnancy involving impaired EVT invasion and maternal spiral artery remodelling (malplacentation), and the second stage occurring after 20 weeks of gestation when the clinical syndrome of hypertension and proteinuria manifests associated with vascular endothelial dysfunction [21]. Maternal factors (genetic, behavioural, environmental) interact with events at both stages, and also influence the link between the first and second stages, leading to variable pre-eclampsia phenotypes, which are likely to require different preventive strategies and treatment [21].

Studies where maternal circulating 25-D_3 was measured at a time coinciding with the beginning of the critical maternal vascular remodelling process, have shown conflicting results, with one study associating low 25-D_3 with subsequent development of pre-eclampsia [7], but with two other studies showing no association [22, 23]. However, one of these latter studies did report a significant association between low circulating 25-D_3 at 24-26 weeks gestation with pre-eclampsia in a predominantly white population with pre-existing risk factors for pre-eclampsia [22]. These discrepancies may be due to studies using different assay methodology, different populations of various ethnic

mix and risk factors for pre-eclampsia, being underpowered, to the lack of differentiation between the various manifestations of pre-eclampsia and failure to account for disruptions in vitamin D metabolism within the local uteroplacental environment.

Malplacentation, which is characteristically associated with pre-eclampsia, may also result in fetal growth restriction (FGR). Interestingly, women with severe early onset pre-eclampsia who also delivered small for gestational age babies had lower circulating 25-D_3 compared with pre-eclamptic women with appropriately grown babies [24]. Furthermore, independent of maternal hypertension, FGR has also been associated with lower maternal serum 25-D_3 concentrations [25]. All of this supports the hypothesis that vitamin D deficiency is an etiological factor in the first stage of pre-eclampsia pathogenesis and in malplacentation.

Furthermore, given the relatively high expression of the activating CYP27B1 in the placenta [8], local uteroplacental concentrations of the active metabolite, 1,25-D_3, may not reflect the prevailing concentration of 25-D_3 in the maternal circulation. In pre-eclampsia there is additional disruption of the placental vitamin D system with reports of reduced CYP27B1 activity in primary villous trophoblasts [26], thus potentially exacerbating the effects of low maternal circulating vitamin D concentrations or contribute to pre-eclampsia risk despite normal circulating 25-D_3 concentrations. Vitamin D may also be implicated in the development of the second stage of pre-eclampsia pathophysiology as vitamin D deficient rodents display endothelial vasodilator dysfunction and hypertension [27, 28] although data from human studies are conflicting [29, 30].

In this study, a significant pro-invasive effect of 25-D_3 was only demonstrable at an optimal maternal circulatory concentration of 100nM although at lower 25-D_3 doses an insignificant trend suggestive of dose-dependent increased EVT invasion with rising 25-D_3 concentrations was observed. The pro-invasive effect of 1,25-D_3 in EVT is in contrast to previous reports of an anti-invasive effect of 1,25-D_3 in several human cancer cell lines including the human breast cancer cell line MDA-MB-231 [31], human prostate cancer cell lines [32], Lewis lung carcinoma cells [33] and murine squamous carcinoma cells [34]. Similarly, 1,25-D_3 inhibited MMP2 and MMP9 activity in human primary uterine fibroid cells and the immortalized HuLM fibroid cell line [35]. The specific differences in human EVT cell characteristics which lead to differential invasion responses to vitamin D treatment are unknown and warrant further investigation. With our methodology, despite the high purity of primary EVT cultures, there remains the possibility that uncharacterized non-EVT invasive cell types could have made a minor contribution to the population of invaded cells. Although increased pro-MMP2 and pro-MMP9 secretion was associated with the EVT invasion promoted by vitamin D, attribution of a direct causative role for MMP in the mechanism of effect requires further study.

In addition to a direct vitamin D effect on EVT themselves, indirect paracrine effects on invasion could also occur through vitamin D regulation of cytokine secretion by neighbouring decidual uNK cells [9] and villous trophoblasts [36]. Thus, *in vivo*, EVT invasion is tightly regulated at multiple levels and the summation of vitamin D effects at all of these levels is an area for further research.

Apart from EVT invasive capacity, vitamin D may also impact on other events in placental development such as angiogenesis [37], immune regulation [9, 36] and enhanced hormone synthesis [38, 39] through autocrine and paracrine mechanisms.

In conclusion, we present *in vitro* experimental evidence that supports a direct role for vitamin D in human EVT function. We have provided evidence which suggests that improved vitamin D status through supplementation early in pregnancy or prior to conception may therefore be a potential strategy for reducing the risk of pre-eclampsia and FGR through adequate EVT invasion during the critical phase of placentation occurring in the first half of gestation. Indeed, a retrospective study of maternal supplementary intake of vitamin D demonstrated a 27% reduction in the incidence of pre-eclampsia [40] and a pooled analysis of trials suggested protective effects of supplementation on low birth weight [41].

Acknowledgments

We thank Laurence Loubiere, Gendie Lash and Ana-Maria Gonzalez for their technical advice. This study was supported by Action Medical Research (#1949 to MDK, SYC).

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Figure legends

Figure 1: Expression of an intracrine vitamin D system in primary EVT.

(A-C) Immunocytochemistry using an avidin-biotin peroxidase method for the EVT markers (A) cytokeratin 7 (CK-7) and (B) HLA-G, with (C) control performed with omission of primary antibody. (D-K) Immunofluorescence microscopy of (D) the intracellular vitamin D receptor (VDR), (E) the vitamin D-activating enzyme (CYP27B1), (F) DAPI only, with a merged image (G) in primary EVT from first trimester human placentae. Control images are of experiments with omission of the primary antibody with (H) Alexa Fluor 488 (Green) or (I) Texas Red (Red) secondary antibodies, with the corresponding DAPI-stained and merged images (J and K respectively). Images were captured using the Axiovision Software (Carl Zeiss, Hertfordshire, UK).

Figure 2: Effect of 1,25-D_3 on expression of mRNA for CYP27A1, VDR, CYP24A1 and cathelicidin in primary EVT. Relative expression of mRNA encoding: (A) CYP27B1; (B) VDR; (C) 24-hydroxylase (CYP24A1); (D) cathelicidin in human first trimester primary EVT. Mean mRNA expression at the lowest vitamin D dose at which expression was detectable was assigned the arbitrary value of 1. Bars represent mean + SEM from three different EVT isolates. Statistical significance are indicated by ** $p<0.01$, *** $p<0.001$.

Figure 3: Effect of 1,25-D_3 and 25-D_3 on Matrigel® invasion by primary EVT. Effect of treatment with increasing concentrations of: (A) 1,25-D_3 for 48 hours; (B) 25-D_3 for 60 hours on human first trimester primary EVT. For invasion through growth factor-reduced Matrigel® the number of invaded EVT cells in each experiment was normalized to the average number of invaded cells in the control group (0 nM) and expressed as a percentage of control. (C) Increased invasion of EVT by 25-D_3 (100 nM)

is inhibited by ketoconazole (KC; 10^{-5}M), a cytochrome P450 inhibitor. Bars represent mean data from EVT isolated from eleven (A) or six (B) or three (C) different pregnancies respectively ± SEM. Statistically significant differences compared to control (0nM) are indicated by *p<0.05, **p<0.01.

Figure 4: Effect of 1,25-D_3 and 25-D_3 on primary EVT secretion of matrix metalloproteinase (MMP) and EVT cell viability. (A and B) Representative gel zymograph from one experiment showing bands representing pro-MMP9 (92kDa), pro-MMP2 (72kDa) and active MMP2 (63kDa) in conditioned media from culture of primary human EVT following treatment with: (A) 1,25-D_3 or (B) 25-D_3. (C and D) Relative densitometry of pro-MMP2 and pro-MMP9 following treatment with: (C) 1,25-D_3 or (D) 25-D_3. Results were normalized to their respective controls (0 nM) within each experiment. Bars represent the mean ± SEM (C: n=5; D: n=6). Statistically significant differences compared to control (0nM) are indicated by *p<0.05, ***p<0.001. (E and F) EVT cell viability was assessed using MTT assays. Within each experiment data were compared to no treatment (0 nM), which was given an arbitrary value of 100%. Absorbance is expressed as the difference between absorbance at OD 570nm and 690nm (background). Bars represent the mean ± SEM (A: n=6; B: n=5). Although the overall ANOVA on the cell viability data for 25-D_3 was statistically significant (p<0.05), further analysis by post-hoc tests failed to identify any statistically significant differences between the different 25-D_3 concentrations.