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1   **Vitamin D promotes human extravillous trophoblast invasion *in vitro***

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24 **Abbreviations**

25 $1,25\text{-}D_3$, 1,25-dihydroxyvitamin D_3; $25\text{-}D_3$, 25-hydroxyvitamin D_3; CK-7, cytokeratin

26 7; CVS, chorionic villous sampling; CYP24A1, 24-hydroxylase; CYP27B1, 1α-

27 hydroxylase; EVT, extravillous trophoblast; FGR, fetal growth restriction; HLA-G,

28 human leukocyte antigen G; MMP, matrix metalloproteinase; PBS, phosphate

29 buffered saline; RT-PCR, reverse transcriptase polymerase chain reaction; uNK,

30 uterine natural killer cells; VDR, vitamin D receptor.

31

32   **Abstract**

33   **Introduction:** Incomplete human extravillous trophoblast (EVT) invasion of the

34   decidua and maternal spiral arteries is characteristic of pre-eclampsia, a condition

35   linked to low maternal vitamin D status. It is hypothesized that dysregulated vitamin

36   D action in uteroplacental tissues disrupts EVT invasion leading to malplacentation.

37   **Methods:** This study assessed the effects of the active vitamin D metabolite, $1,25$-

38   dihydroxyvitamin D_3 ($1,25$-D_3), and its precursor, 25-hydroxyvitamin D_3 (25-D_3), on

39   primary human EVT isolated from first trimester pregnancies. Expression of EVT

40   markers (cytokeratin-7, HLA-G), the vitamin D-activating enzyme (CYP27B1) and

41    $1,25$-D_3 receptor (VDR) was assessed by immunocytochemistry. EVT responses

42   following *in vitro* treatment with $1,25$-D_3 (0-10nM) or 25-D_3 (0-100nM) for 48-60h

43   were assessed using quantitative RT-PCR (qRT-PCR) analysis of key target genes.

44   Effects on EVT invasion through Matrigel® were quantified alongside zymographic

45   analysis of secreted matrix metalloproteinases (MMPs). Effects on cell viability were

46   assessed by measurement of MTT.

47   **Results:**   EVT co-expressed mRNA and protein for CYP27B1 and VDR, and

48   demonstrated induction of mRNA encoding vitamin D-responsive genes, 24-

49   hydroxylase (CYP24A1) and cathelicidin following $1,25$-D_3 treatment. EVT could

50   respond to $1,25$-D_3 and 25-D_3, both of which significantly increased EVT invasion,

51   with maximal effect at 1nM $1,25$-D_3 (1.9-fold; $p<0.01$) and 100nM 25-D_3 (2.2-fold;

52    $p<0.05$) respectively compared with untreated controls. This was accompanied by

53   increased pro-MMP2 and pro-MMP9 secretion. The invasion was independent of cell

54   viability, which remained unchanged.

55   **Discussion:** These data support a role for vitamin D in EVT invasion during human

56   placentation and suggest that vitamin D-deficiency may contribute to impaired EVT

57   invasion and pre-eclampsia.

58   **Key words**

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

60

61 **Introduction**

62 Vitamin D-deficiency, defined as a serum concentration of 25-hydroxyvitamin D (25-

63 D_3; the main circulating form of vitamin D) less than 50nM, and vitamin D-

64 insufficiency ($25\text{-}D_3 < 75$nM) are especially prevalent in pregnancy. These complicate

65 at least 67% of pregnancies, particularly in women with darker skin pigmentation, in

66 various geographical locations around the world [1-4]. A recent meta-analysis of

67 observational studies noted associations between vitamin D-deficiency in pregnancy

68 with increased risk of pre-eclampsia, gestational diabetes, preterm birth and small for

69 gestational age infants; with pre-eclampsia showing the strongest association with an

70 odds ratio of 2.09 (95%CI 1.50-2.90) [5].

71 Pre-eclampsia, a syndrome of maternal hypertension, proteinuria and endothelial

72 dysfunction, affects up to 8% of pregnancies and remains a leading cause of maternal

73 and perinatal morbidity and mortality [6]. In one study, maternal serum

74 concentrations of $25\text{-}D_3$ in prospectively collected samples in early pregnancy were

75 found to be significantly lower in women who subsequently developed pre-eclampsia

76 [7]. However, the pathogenic mechanisms linking low vitamin D levels with pre-

77 eclampsia are not understood and a causative link between the two remains

78 controversial. The prevalence of vitamin D insufficiency and incidence of pre-

79 eclampsia are both increased in Black and South Asian women, which may implicate

80 potential confounding variables associated with ethnicity.

81 The human hemochorial placenta is an extra-renal tissue with high expression of the

82 vitamin D-activating enzyme 1α-hydroxylase (CYP27B1), which converts $25\text{-}D_3$ to

83 active 1,25-dihydroxyvitamin D_3 ($1,25\text{-}D_3$). Both CYP27B1 and the receptor for 1,25-

84 D_3 (VDR) are expressed in human decidua and the villous placenta, with higher

85 expression during the first and second trimesters of pregnancy. This suggests a role

86 for vitamin D in decidualisation and uteroplacental remodelling [8].

87 Cytotrophoblast within the villous placenta differentiates into extravillous trophoblast

88 (EVT), which has an invasive phenotype. EVT invades the decidua and maternal

89 spiral arteries from the first trimester until 24 weeks of gestation. This invasion is

90 critical to maternal spiral artery remodelling and promotion of maternal placental

91 blood flow to establish effective maternal-fetal exchange. Impairment of this process

92 predisposes a pregnancy to uteroplacental insufficiency and a significantly increased

93 risk of pre-eclampsia and fetal growth restriction (FGR).

94 We hypothesized that vitamin D insufficiency during pregnancy may lead to

95 dysregulation of placental morphological development, and thus the development of

96 malplacentation disorders including pre-eclampsia and FGR. Vitamin D has been

97 demonstrated to regulate inflammation in human decidual uterine natural killer (uNK)

98 cells [9], which in turn is postulated to impact on the invasion of fetal-derived EVT in

99 a paracrine manner [10]. In this study, we have now investigated the direct effects of

100 $1,25\text{-}D_3$ and $25\text{-}D_3$ upon isolated human first trimester primary EVT *in vitro*.

101 **Methods**

102 *Ethical approval*

103 Human samples were collected with informed written consent and with the approval

104 of the South Birmingham Research Ethics committee (Reference: 06/Q2707/12) and

105 the Research and Development office of the Walsall Manor Hospitals NHS Trust

106 (Project code: 2007013OG(W); approval number: 11070745).

107 *Sample collection*

108 Placental samples were obtained from women undergoing elective surgical

109 termination of apparently uncomplicated pregnancies. Samples were collected from 8-

110 11 completed weeks of gestation as determined by ultrasound measurement of crown

111 rump length prior to pregnancy termination. The fetuses were not known to have

112 abnormal karyotypes nor structural anomalies.

113 *Cell isolation and culture*

114 Following collection, placental tissues were dissected free and washed three times

115 with PBS. Primary EVTs were isolated using a method of enzyme digestion followed

116 by percoll separation as previously described [11]. Characterization of these cells by

117 immunocytochemistry for EVT markers using anti-cytokeratin 7 (Novocastra,

118 Newcastle-upon-Tyne, UK; 1:20) and anti-HLA-G (Serotec, Oxford, UK; 1:200) with

119 an avidin-biotin peroxidase method (Vectastatin Elite kit, Vector Laboratories,

120 Peterbotough, UK) confirmed 95% purity (Figure 1A-C). Cells were cultured on

121 growth factor-reduced Matrigel® matrix (BD Biosciences, Erembodegem, Belgium) in

122 DMEM:F12 medium containing 10% FBS, 1000U/ml penicillin, 1 mg/ml

123 streptomycin, 2 mM L-glutamine and 1.5 μg/ml amphoteracin B (all reagents from

124 Gibco Life Technologies, Paisley, UK) in standard 5% CO_2 in an air incubator at

125 37°C. These cells were treated with 1,25-D_3 (Enzo Life Sciences, Exeter, UK) at

126 concentrations of 0-10 nM or with 25-D_3 (Enzo Life Sciences) at 0-100 nM.

127 Incubations were for 48h or 60h as defined in previously reported studies [12].

128 Inhibition of CYP27B1 was carried out by pre-treatment for 2h with the pan-

129 cytochrome P450 inhibitor, ketoconazole (Sigma-Aldrich, Dorset, U.K), at 10^{-5}M,

130 before culture with or without 25-D_3 treatment for 60h.

131 *Immunofluorescence microscopy*

132 Isolated EVT cells were grown (60h) on chamber slides coated with poly-D-lysine

133 (100µl/well of 0.1mg/ml) and immunofluorescent-stained as described previously

134 [13]. Cells were co-stained for VDR (clone D-6, Santa Cruz Biotechnology) and

135 CYP27B1 (Clone H-90, Santa Cruz Biotechnology) (Figure 1). VDR and CYP27B1

136 were detected using anti-mouse Alexa Fluor 488 (Green) and anti-rabbit Texas Red

137 (Red) secondary antibodies (Life Technologies) respectively. All antibodies were

138 used at 1:100 dilution. Slides were mounted with Vectashield™ containing DAPI

139 (Vector Laboratories Inc., Peterborough, UK) and examined using a fluorescent

140 microscope (Carl Zeiss, Hertfordshire, UK).

141 *Quantitative RT-PCR*

142 Total RNA was extracted using TRI reagent (Sigma-Aldrich, Dorset, U.K.) following

143 recovery of cells from Matrigel Matrix using BD cell recovery solution (BD

144 Biosciences, Bedford, UK) according to the manufacturer's instructions. Total RNA

145 (1µg) was reverse transcribed using Avian Myeloblastosis Virus (AMV) reverse

146 transcriptase (Promega, Southampton, UK) following the manufacturer's guidelines.

147 Expression of mRNA encoding CYP27B1 (Hs01096154_m1), CYP24A1

148 (Hs00167999_m1), VDR (Hs00172113_m1) or cathelicidin (Hs01011707_g1) was

149 determined and normalized to the expression of 18S rRNA (4319413E), an internal

150 control in multiplex reactions, using the ABI PRISM 7500 Sequence Detection

151 System (ABI, Foster City, USA). All primer and probes were produced by Applied

152 Biosystems, Paisley, UK. Quantification of gene expression was determined using the

153 ΔCt method described previously [14]. Relative mRNA expression for each sample

154 was compared with the mean gene expression at the lowest vitamin D dose at which

155 expression was detectable, with this being assigned the arbitrary value of 1 within

156 each experiment.

157 *Invasion assays*

158 Primary EVT were seeded in cell culture inserts for 24-well plates [8µm membrane

159 pore size (BD Biosciences)] coated with growth factor-reduced Matrigel® matrix

160 (10µl; BD Biosciences). Invaded cells (assessed in duplicate) were fixed in ethanol,

161 stained with Mayer's hematoxylin and eosin (Sigma-Aldrich, Dorset, UK), and

162 counted across the entire membrane at 20X magnification using a light microscope

163 [11]. The invasion index was expressed as the ratio of invaded cells in the

164 experimental group relative to the control group (0nM) within each experiment.

165 *Matrix metalloproteinase (MMP) quantification by zymography*

166 Levels of secreted MMP2 and MMP9 protein, which are key gelatinases in the

167 degradation of the basement membrane by EVT during invasion, were assessed using

168 gelatin zymography as described previously [15]. Briefly, total protein (16-20 µg)

169 from EVT-conditioned medium was resolved by electrophoresis in a 12% SDS-PAGE

170 gel and then incubated (30 min) in zymograph-renaturing buffer followed by

171 zymograph-developing buffer (Invitrogen, Renfrew, UK) overnight at 37°C before

172 staining with Coomassie Brilliant Blue R250. Dried gels were then scanned and

173 densitometry of pro-MMP9 and pro-MMP2 performed using Image J software.

174 Although active MMP2 was detectable, poor resolution of bands precluded

175 quantification by relative densitometry.

176 *MTT assays*

177 Cells were seeded in 96 well plates and experiments performed with three or four

178 replicates of each dose using the quantitative colorimetric 3-(4,5-dimethyldiazol-2-

179   yl)-2,5 diphenyl Tetrazolium Bromide (MTT; Sigma-Aldrich, Dorset, U.K) assay of

180   mitochondrial metabolism, as described previously [16]. Within each experiment, the

181   absorbance values were normalized to the values obtained with no vitamin D

182   treatment (0nM), which was given an arbitrary value of 100%.

183   *Statistical analysis*

184   Data were analyzed using the SigmaStat v3.1 statistical software (Systat Software Inc,

185   California, USA). Repeated measures one-way analysis of variance was performed

186   followed by Tukey all pairwise multiple comparisons post-hoc tests. Data sets passed

187   the normality test except for the quantitative RT-PCR and pro-MMP2 data, which

188   required logarithmic transformation prior to statistical analysis. Statistical significance

189   was taken as $p<0.05$.

190   **Results**

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

192   ***25-D_3***

193   Isolated EVT from first trimester placentae demonstrated coincident protein

194   expression of the vitamin D-activating enzyme CYP27B1 and the intracellular

195   receptor for 1,25-D_3, VDR (Figure 1D-G), confirming previous quantitative RT-PCR

196   and immunohistochemistry findings in intact decidual tissue sections [8]. Expression

197   of mRNA encoding CYP27B1 and VDR (Figure 2A-B) in isolated EVT was

198   unaffected by treatment with 25-D_3 or 1,25-D_3, consistent with previous reports in

199   primary cultures of first trimester human chorionic villous sampling (CVS) specimens

200   [17]. The co-expression of CYP27B1 and VDR indicate the presence of a potential

201   EVT vitamin D intracrine system.

202   Expression of mRNA encoding the vitamin D feedback catabolic enzyme CYP24A1,

203   which attenuates vitamin D responsiveness by converting $1,25\text{-}D_3$ and $25\text{-}D_3$ to less

204   active metabolites, was undetectable in untreated EVT (Figure 2C). This is consistent

205   with the CYP24A1 gene being methylation-silenced as previously reported in term

206   villous placenta and in first trimester cytotrophoblast [17]. However, CYP24A1

207   mRNA expression was induced in EVT treated with 1nM $1,25\text{-}D_3$. Treatment with a

208   higher concentration of $1,25\text{-}D_3$ (10nM) resulted in a 9-fold increase in CYP24A1

209   mRNA expression compared to 1nM-treated EVT ($p<0.01$; Figure 2C). This

210   magnitude of response in EVT is similar to the 10-fold increase reported in CVS cells

211   treated with 100nM 1,25-D3, but this magnitude is still significantly less than those in

212   the hundreds reported in cells in which CYP24A1 is not methylation-silenced [17].

213   Evidence that human EVT is vitamin D-responsive was further demonstrated by a

214   dose-dependent induction of mRNA encoding the vitamin D–responsive antibacterial

215   protein, cathelicidin, by $1,25\text{-}D_3$ (ANOVA $p<0.001$; Figure 2D). Messenger RNA

216   encoding cathelicidin increased by 217-fold and 457-fold with 1nM and 10nM of

217     $1,25\text{-}D_3$ treatment respectively (both $p<0.001$). These data are consistent with

218   previous findings in human term placental explants and isolated primary

219   cytotrophoblast treated with $1,25\text{-}D_3$ [18].

220   *Effects of $1,25\text{-}D_3$ and $25\text{-}D_3$ on EVT invasion*

221   Treatment of human primary EVT with $1,25\text{-}D_3$ significantly increased directly

222   quantified cell invasion into Matrigel® (ANOVA $p<0.01$; Figure 3A). A peak invasion

223   response was demonstrated at 1nM $1,25\text{-}D_3$, with a 1.9-fold increase in the number of

224   invaded cells compared with untreated controls ($p<0.01$). Compared with untreated

225   EVT, a statistically significant increase in invasion by 1.7-fold ($p<0.05$) was also

226   noted with a lower dose of 0.1nM $1,25\text{-}D_3$ but not at a higher dose of 10nM $1,25\text{-}D_3$.

227   The diminished response at 10nM $1,25\text{-}D_3$ could be due to preceptor regulation and

228   inactivation of $1,25\text{-}D_3$ by increased CYP24A1 expression.

229   Primary EVT also responded to $25\text{-}D_3$, confirming the efficacy of the CYP27B1/VDR

230   intracrine system. There was a dose-dependent increase in EVT invasion with rising

231    $25\text{-}D_3$ concentrations (ANOVA $p < 0.05$; Figure 3B). A similar magnitude in the peak

232   response, as seen with $1,25\text{-}D_3,$ of a 2.2-fold rise in the number of invaded cells at

233   100nM $25\text{-}D_3$ compared to controls ($p < 0.05$) suggests that both forms of vitamin D

234   use a similar response pathway in the promotion of EVT invasion.

235   To confirm that this observed increase in EVT invasion is mediated by vitamin D,

236   EVT were pre-treated with a cytochrome P450 inhibitor, ketoconazole [19], prior to

237    $25\text{-}D_3$ treatment. Ketoconazole by itself did not inhibit invasion of EVT, but when

238   CYP27B1 activity was blocked by ketoconazole the pro-invasive effects of $25\text{-}D_3$ was

239   significantly attenuated (ANOVA $p < 0.01$; Figure 3C), suggesting that intracellular

240   EVT metabolism of $25\text{-}D_3$ mediates EVT invasion.

241   Enhanced Matrigel® invasion by EVT with vitamin D treatment was paralleled by

242   increased secretion of pro-MMP2 and pro-MMP9 (Figures 4A-4D). Pro-MMP2

243   increased significantly (ANOVA $p < 0.001$; Figure 4C) with $1,25\text{-}D_3$ at 1nM ($p < 0.001$)

244   and 10nM ($p < 0.001$), and pro-MMP9 was increased significantly (ANOVA $p < 0.05$;

245   Figure 4D) with 100nM $25\text{-}D_3,$ compared with untreated EVT ($p < 0.05$).

246   *Effects of $1,25\text{-}D_3$ and $25\text{-}D_3$ on EVT cell viability*

247   To confirm that the observed increase in EVT invasion with vitamin D reflected

248   increased invasive capability rather than enhanced cell proliferation and/or survival,

249   we assessed EVT cell viability. Data from MTT analyses showed no significant

250 change in EVT cell viability following treatment with $1,25\text{-}D_3$ or $25\text{-}D_3$ (Figures 4E-

251 F).

Discussion

253 Vitamin D deficiency in pregnancy has been associated with an increased risk of pre-

254 eclampsia [7, 20], but the underlying mechanisms are unclear. We have demonstrated

255 that the vitamin D metabolites, $1,25\text{-}D_3$ and $25\text{-}D_3$, have a direct pro-invasive effect

256 on isolated human EVT *in vitro*, highlighting an entirely novel action for vitamin D

257 in the placenta. Furthermore, pro-invasive responses to vitamin D suggest that

258 attenuated EVT invasion of uterine decidua and vasculature, may be one of the

259 mechanisms by which vitamin D deficiency contributes to the increased risk of pre-

260 eclampsia and FGR.

261 The pathogenesis of pre-eclampsia is proposed to be a two-stage process: the first

262 stage occurring in the first and early second trimesters of pregnancy involving

263 impaired EVT invasion and maternal spiral artery remodelling (malplacentation), and

264 the second stage occurring after 20 weeks of gestation when the clinical syndrome of

265 hypertension and proteinuria manifests associated with vascular endothelial

266 dysfunction [21]. Maternal factors (genetic, behavioural, environmental) interact with

267 events at both stages, and also influence the link between the first and second stages,

268 leading to variable pre-eclampsia phenotypes, which are likely to require different

269 preventive strategies and treatment [21].

270 Studies where maternal circulating $25\text{-}D_3$ was measured at a time coinciding with the

271 beginning of the critical maternal vascular remodelling process, have shown

272 conflicting results, with one study associating low $25\text{-}D_3$ with subsequent

273 development of pre-eclampsia [7], but with two other studies showing no association

274 [22, 23]. However, one of these latter studies did report a significant association

275 between low circulating $25\text{-}D_3$ at 24-26 weeks gestation with pre-eclampsia in a

276 predominantly white population with pre-existing risk factors for pre-eclampsia [22].

277 These discrepancies may be due to studies using different assay methodology,

278 different populations of various ethnic mix and risk factors for pre-eclampsia, being

279 underpowered, to the lack of differentiation between the various manifestations of

280 pre-eclampsia and failure to account for disruptions in vitamin D metabolism within

281 the local uteroplacental environment.

282 Malplacentation, which is characteristically associated with pre-eclampsia, may also

283 result in fetal growth restriction (FGR). Interestingly, women with severe early onset

284 pre-eclampsia who also delivered small for gestational age babies had lower

285 circulating $25\text{-}D_3$ compared with pre-eclamptic women with appropriately grown

286 babies [24]. Furthermore, independent of maternal hypertension, FGR has also been

287 associated with lower maternal serum $25\text{-}D_3$ concentrations [25]. All of this supports

288 the hypothesis that vitamin D deficiency is an etiological factor in the first stage of

289 pre-eclampsia pathogenesis and in malplacentation.

290 Furthermore, given the relatively high expression of the activating CYP27B1 in the

291 placenta [8], local uteroplacental concentrations of the active metabolite, $1,25\text{-}D_3$,

292 may not reflect the prevailing concentration of $25\text{-}D_3$ in the maternal circulation. In

293 pre-eclampsia there is additional disruption of the placental vitamin D system with

294 reports of reduced CYP27B1 activity in primary villous trophoblasts [26], thus

295 potentially exacerbating the effects of low maternal circulating vitamin D

296 concentrations or contribute to pre-eclampsia risk despite normal circulating $25\text{-}D_3$

297 concentrations. Vitamin D may also be implicated in the development of the second

298 stage of pre-eclampsia pathophysiology as vitamin D deficient rodents display

299 endothelial vasodilator dysfunction and hypertension [27, 28] although data from

300 human studies are conflicting [29, 30].

301 In this study, a significant pro-invasive effect of 25-D_3 was only demonstrable at an

302 optimal maternal circulatory concentration of 100nM although at lower 25-D_3 doses

303 an insignificant trend suggestive of dose-dependent increased EVT invasion with

304 rising 25-D_3 concentrations was observed. The pro-invasive effect of 1,25-D_3 in EVT

305 is in contrast to previous reports of an anti-invasive effect of 1,25-D_3 in several

306 human cancer cell lines including the human breast cancer cell line MDA-MB-231

307 [31], human prostate cancer cell lines [32], Lewis lung carcinoma cells [33] and

308 murine squamous carcinoma cells [34]. Similarly, 1,25-D_3 inhibited MMP2 and

309 MMP9 activity in human primary uterine fibroid cells and the immortalized HuLM

310 fibroid cell line [35]. The specific differences in human EVT cell characteristics

311 which lead to differential invasion responses to vitamin D treatment are unknown and

312 warrant further investigation. With our methodology, despite the high purity of

313 primary EVT cultures, there remains the possibility that uncharacterized non-EVT

314 invasive cell types could have made a minor contribution to the population of invaded

315 cells. Although increased pro-MMP2 and pro-MMP9 secretion was associated with

316 the EVT invasion promoted by vitamin D, attribution of a direct causative role for

317 MMP in the mechanism of effect requires further study.

318 In addition to a direct vitamin D effect on EVT themselves, indirect paracrine effects

319 on invasion could also occur through vitamin D regulation of cytokine secretion by

320 neighbouring decidual uNK cells [9] and villous trophoblasts [36]. Thus, *in vivo*, EVT

321 invasion is tightly regulated at multiple levels and the summation of vitamin D effects

322 at all of these levels is an area for further research.

323 Apart from EVT invasive capacity, vitamin D may also impact on other events in

324 placental development such as angiogenesis [37], immune regulation [9, 36] and

325 enhanced hormone synthesis [38, 39] through autocrine and paracrine mechanisms.

326 In conclusion, we present *in vitro* experimental evidence that supports a direct role for

327 vitamin D in human EVT function. We have provided evidence which suggests that

328 improved vitamin D status through supplementation early in pregnancy or prior to

329 conception may therefore be a potential strategy for reducing the risk of pre-eclampsia

330 and FGR through adequate EVT invasion during the critical phase of placentation

331 occurring in the first half of gestation. Indeed, a retrospective study of maternal

332 supplementary intake of vitamin D demonstrated a 27% reduction in the incidence of

333 pre-eclampsia [40] and a pooled analysis of trials suggested protective effects of

334 supplementation on low birth weight [41].

335 **Acknowledgments**

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466

467 **Figure legends**

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

469 (A-C) Immunocytochemistry using an avidin-biotin peroxidase method for the EVT

470 markers (A) cytokeratin 7 (CK-7) and (B) HLA-G, with (C) control performed with

471 omission of primary antibody. (D-K) Immunofluorescence microscopy of (D) the

472 intracellular vitamin D receptor (VDR), (E) the vitamin D-activating enzyme

473 (CYP27B1), (F) DAPI only, with a merged image (G) in primary EVT from first

474 trimester human placentae. Control images are of experiments with omission of the

475 primary antibody with (H) Alexa Fluor 488 (Green) or (I) Texas Red (Red) secondary

476 antibodies, with the corresponding DAPI-stained and merged images (J and K

477 respectively). Images were captured using the Axiovision Software (Carl Zeiss,

478 Hertfordshire, UK).

479 **Figure 2: Effect of $1,25\text{-}D_3$ on expression of mRNA for CYP27A1, VDR,**

480 **CYP24A1 and cathelicidin in primary EVT.** Relative expression of mRNA

481 encoding: (A) CYP27B1; (B) VDR; (C) 24-hydroxylase (CYP24A1); (D) cathelicidin

482 in human first trimester primary EVT. Mean mRNA expression at the lowest vitamin

483 D dose at which expression was detectable was assigned the arbitrary value of 1. Bars

484 represent mean + SEM from three different EVT isolates. Statistical significance are

485 indicated by ** $p < 0.01$, *** $p < 0.001$.

486 **Figure 3: Effect of $1,25\text{-}D_3$ and $25\text{-}D_3$ on Matrigel® invasion by primary EVT.**

487 Effect of treatment with increasing concentrations of: (A) $1,25\text{-}D_3$ for 48 hours; (B)

488 $25\text{-}D_3$ for 60 hours on human first trimester primary EVT. For invasion through

489 growth factor-reduced Matrigel® the number of invaded EVT cells in each experiment

490 was normalized to the average number of invaded cells in the control group (0 nM)

491 and expressed as a percentage of control. (C) Increased invasion of EVT by $25\text{-}D_3$

492 (100 nM) is inhibited by ketoconazole (KC; 10^{-5}M), a cytochrome P450 inhibitor.

493 Bars represent mean data from EVT isolated from eleven (A) or six (B) or three (C)

494 different pregnancies respectively $\pm$ SEM. Statistically significant differences

495 compared to control (0nM) are indicated by *p<0.05, **p<0.01.

496 **Figure 4: Effect of 1,25-D$_3$ and 25-D$_3$ on primary EVT secretion of matrix**

497 **metalloproteinase (MMP) and EVT cell viability.** (A and B) Representative gel

498 zymograph from one experiment showing bands representing pro-MMP9 (92kDa),

499 pro-MMP2 (72kDa) and active MMP2 (63kDa) in conditioned media from culture of

500 primary human EVT following treatment with: (A) 1,25-D$_3$ or (B) 25-D$_3$. (C and D)

501 Relative densitometry of pro-MMP2 and pro-MMP9 following treatment with: (C)

502 1,25-D$_3$ or (D) 25-D$_3$. Results were normalized to their respective controls (0 nM)

503 within each experiment. Bars represent the mean $\pm$ SEM (C: n=5; D: n=6).

504 Statistically significant differences compared to control (0nM) are indicated by

505 *p<0.05, ***p<0.001. (E and F) EVT cell viability was assessed using MTT assays.

506 Within each experiment data were compared to no treatment (0 nM), which was given

507 an arbitrary value of 100%. Absorbance is expressed as the difference between

508 absorbance at OD 570nm and 690nm (background). Bars represent the mean $\pm$ SEM

509 (A: n=6; B: n=5). Although the overall ANOVA on the cell viability data for 25-D$_3$

510 was statistically significant (p<0.05), further analysis by post-hoc tests failed to

511 identify any statistically significant differences between the different 25-D$_3$
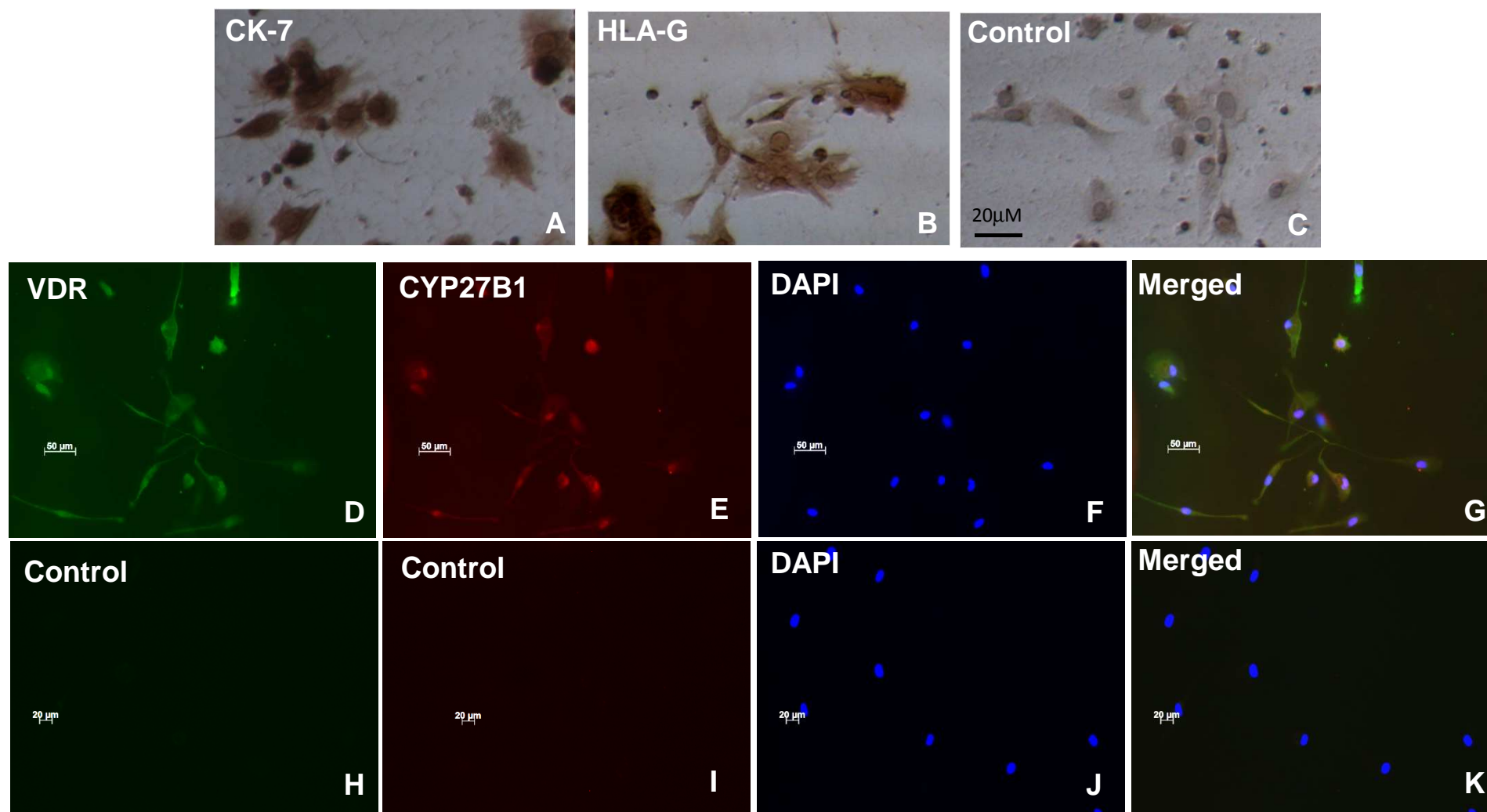
512 concentrations.

Figure 1

Figure 2

A: CYP27B1

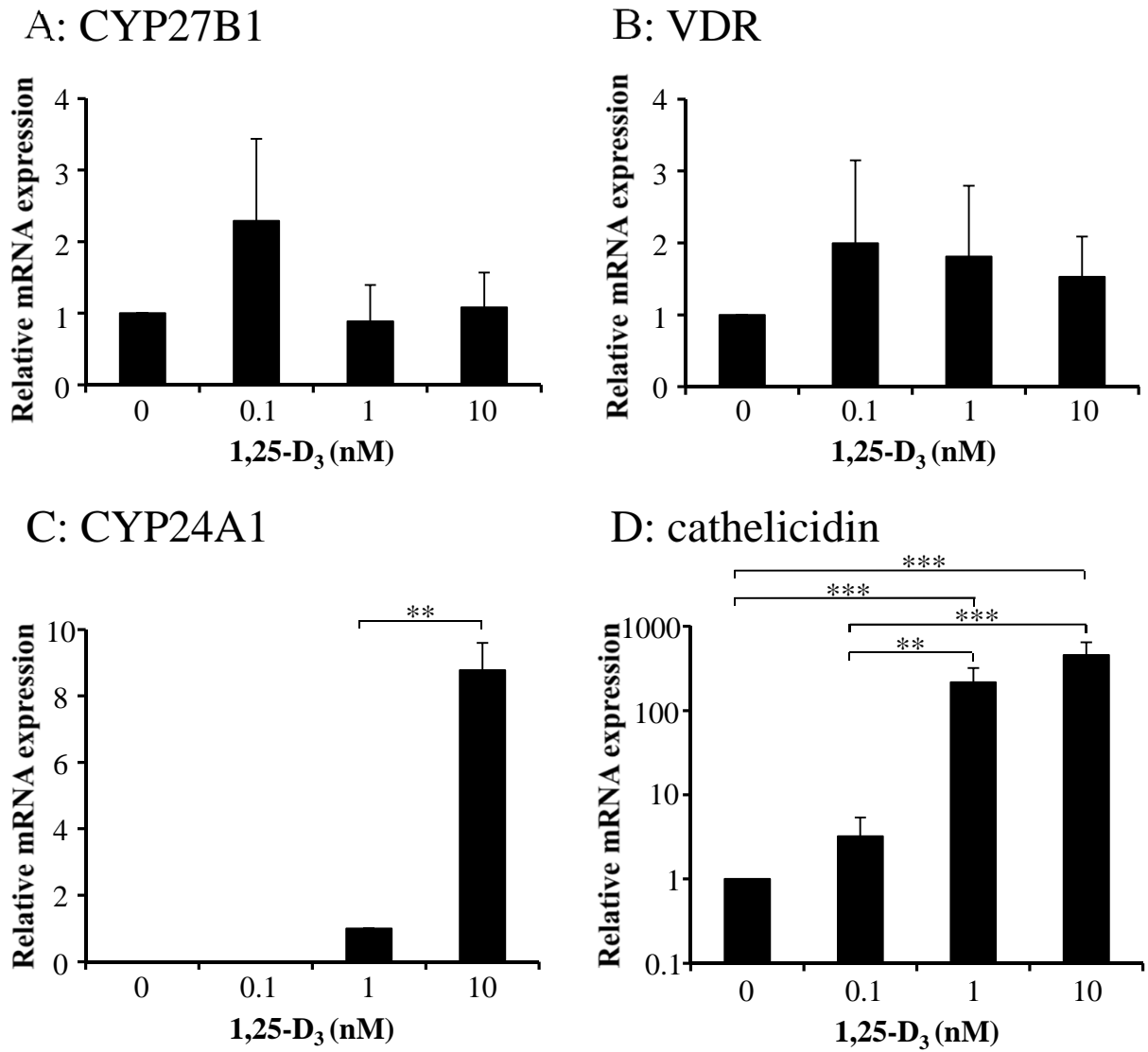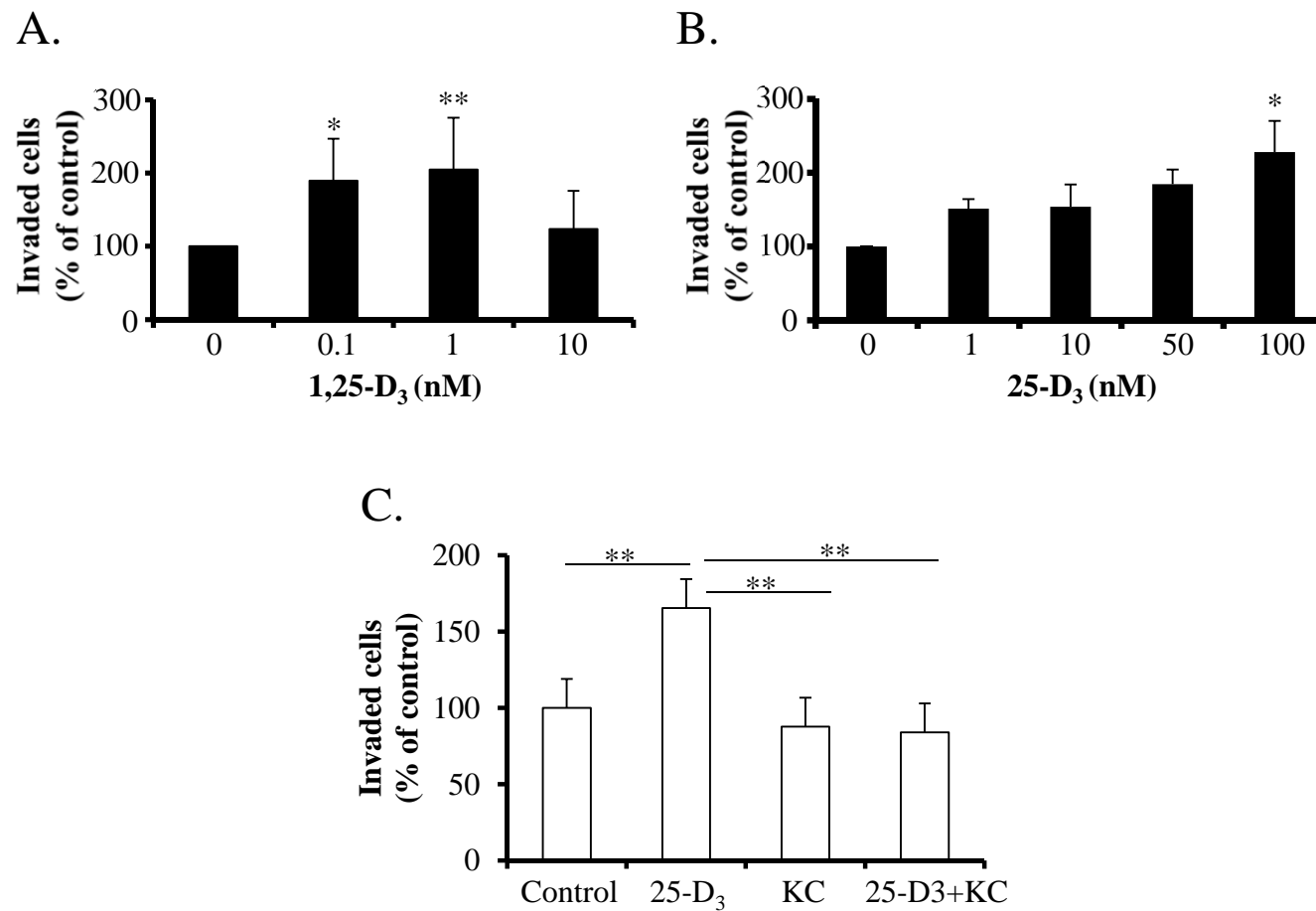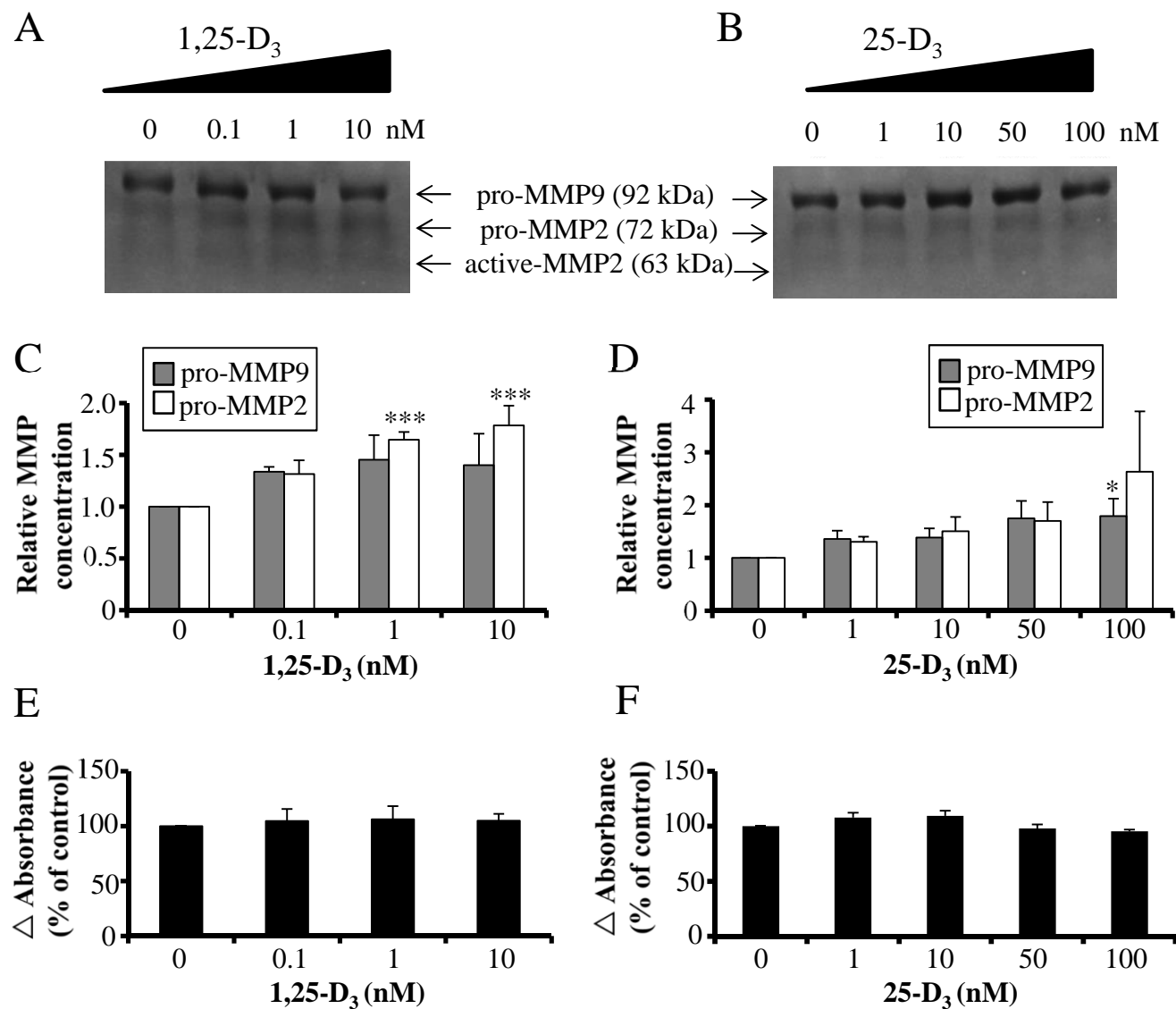B: VDR

C: CYP24A1

D: cathelicidin

Figure 3

Figure 4

Highlights

- Human primary extravillous trophoblast (EVT) is vitamin D responsive.

- Vitamin D directly promotes the invasion of primary EVT through Matrigel®.

- Maternal vitamin D deficiency may thus lead to malplacentation and pre-eclampsia.