RESEARCH ARTICLE

Curcumin stimulates angiogenesis through VEGF and expression of HLA-G in first-trimester human placental trophoblasts

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Abstract

Curcumin has a protective role in placental diseases like preeclampsia and preterm birth. Very little is known about its functional effects on growth, angiogenesis, and epigenetic activities of human first trimester placenta. HTR8/SVneo trophoblasts were used as model for human first trimester placenta. Effects of curcumin (≥80%) in these cells were investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), radioactive thymidine uptake, quantitative real-time polymerase chain reaction (qRT-PCR), promoter DNA methylation, qRT-PCR array, tube formation, wound healing, and immunoblot assays. PC3 (prostate cancer), JEG-3 (trophoblast), and HMEC-1 (endothelial) cells were used as control in various experiments. Unlike in PC3 cells, curcumin stimulated growth, proliferation, and viability in HTR8/SVneo cells. Curcumin increased tube formation, and messenger RNA (mRNA) expression of angiogenic factors such as vascular endothelial growth factor A (VEGFA) and protein expression of proangiogenic factor VEGF receptor-2 and fatty acid-binding protein-4 (FABP4) in these cells. Curcumin-stimulated tube formation was associated with an increased expression of VEGFR2 and FABP4. The stimulatory effects of curcumin were inhibited by VEGFR2 (SU5416) and FABP4 (BMS309403) inhibitors. Curcumin also significantly increased both mRNA and protein expression of HLA-G in HTR8/SVneo cells. Curcumin increased mRNA expression of DNMT3A and NOTCH signaling system whereas down-regulated mRNA expression of HSD11B2. Curcumin enhanced hypomethylation of gene promoters against oxidative stress and DNA damage pathway mediators. Curcumin promotes cell growth, migration, and thus angiogenic potential of these cells. Increased expression of HLA-G by curcumin, hitherto unknown, is a novel finding since HLA-G not only favors the immune environment for invasive trophoblasts but also positively modulates angiogenesis.

Keywords: 3H-Thymidine assay; curcumin; DNA methylation tube formation; EpiTect methyl PCR arrays; FABP4; HLA-G; HTR8/SVneo; PC-3 cells; real-time PCR-array; trophoblasts; VEGFA; VEGFR2; wound healing

Introduction

Curcumin (diferuloylmethane) isolated from the dried powder of rhizome of Curcuma longa Linn (Zingiberaceae) (Akbar et al., 2018), has been shown to have beneficial properties in vitro and in vivo studies (Akbar et al., 2018; Mokhtari-Zaer et al., 2018). Curcumin modulates cell growth and inhibits angiogenesis in cancer cells (Fan et al., 2016; Huang et al., 2017; Chan et al., 2018). The anti-cancer effects of curcumin are mediated partly via down-regulation of angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), and upregulation of expression of anti-metastatic proteins and the tissue inhibitor of metalloprotease-2 (TIMP2). Its anti-angiogenic effect is mediated via inhibition of VEGF and FAK/P-38 MAPK-signaling pathways (Buhrmann et al., 2014; Huang et al., 2017; Hosseini et al., 2019). The anti-cancer effects of curcumin are reported against various forms of cancers (Fadus et al., 2017; Patel et al., 2019). Curcumin also protects the reproductive cells owing to its anti-inflammatory, anti-apoptotic, and antioxidant effects (Devi et al., 2015; Zhang et al., 2018; Patel et al., 2019). Curcumin rescued the preeclampsia-like phenotype in the rat model by decreasing inflammation related to the TLR4 signaling pathway (Gong et al., 2016). Curcumin activates...
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Akt phosphorylation and IGFII/Akt signaling pathways, as these processes are down-regulated in IUGR rat models (Keim et al., 2018). There are few studies reported the effects of curcumin on reproductive cells though used high concentrations (Lim et al., 2013; Devi et al., 2015; Hong et al., 2016). However, there are very few data available on the effects of curcumin on early human placental growth and development.

Optimum growth and development of the placenta is the key to the optimal fetal outcome (Duttaroy, 2009; Jung et al., 2016). Placental growth depends on placental angiogenesis, vascularization, and blood flow. Early placenta is associated with extensive growth and remodeling of maternal uterine vasculature by invasive trophoblasts (Khong and Brosens, 2011; Shainker et al., 2017). Therefore, migration, invasion, and angiogenesis of placental trophoblasts are the critical events in the early placenta (Shainker et al., 2017). Inadequate placentation compromises placental size and nutrients transport activity and consequently leading to constrained fetal growth in utero (Duzyj et al., 2018). Placental angiogenesis mimics metastatic tumor growth and similarities exist between the invasiveness of tumor and trophoblast cells during embryo implantation, vascularization, and placentaion (Duzyj et al., 2018). Therefore, migration, invasion, and angiogenesis of placental trophoblasts are the critical events in the early placentaion (Shainker et al., 2017). Inadequate placentation compromises placental size and nutrients transport activity and consequently leading to constrained fetal growth in utero (Duzyj et al., 2018).

Here we investigated the effects of curcumin on cellular growth, migration, angiogenesis, and epigenetic functionality of the first-trimester human placental cells.

This paper reports for the first time that curcumin at very low concentrations promotes cellular growth, migration, and tube formation in the first trimester placental trophoblast cells, and influences epigenetic modifications by DNA methylation.

Materials and methods

Materials

Human placental trophoblast cell line HTR8/SVneo was kindly gifted by Dr. C.H. Graham, Queen’s University, Canada. Human prostate cancer (PC3), human microvascular endothelial (HMEC-1), and placental choriocarcinoma JEG-3 cell lines were purchased from American Type Culture Collection (ATCC), USA. FABP4 inhibitor BMS309403 (#341310) was obtained from Calbiochem, UK. Matrigel (#356230) was procured from BD Biosciences, USA. Thymidine (H]- (#NET027 X250UC) was purchased from PerkinElmer, USA. Curcumin (#C7727), methylthiazolyl diphenyl-tetrazolium bromide (MTT#5655), trypsin-ethylendiaminetetraacetic acid (EDTA) (#T3924), penicillin-streptomycin solution (#P4458), RPMI-1640 (#R0883), fetal bovine serum (FBS; #F7524), L-glutamine (#G7513), VEGF inhibitor SU5416 (#S8442), calcine AM (#C1430) were purchased from Sigma Aldrich, Germany.

Methods

Cell culture

Human first trimester trophoblast cells, HTR8/SVneo

Cells were cultured in RPMI, supplemented with 5% FBS, 2 mM L-glutamine and 1% antibiotics (50 U/mL penicillin and 50 mg/mL streptomycin) (Basak et al., 2017). The cells were routinely maintained (passage 3–10) at 37°C in a 5% CO₂ chamber and sub-cultured using a trypsin-EDTA solution to resuspended the cells. Curcuminoid was dissolved in dimethyl sulfoxide (DMSO) and stored in dark glass container. The stock solution was diluted with working media prior to their treatment to the cells in final concentration of DMSO (0.1% v/v).

Human placental choriocarcinoma cells, JEG-3

JEG-3 choriocarcinoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS. The culture medium was changed every 2–3 days and cells were passaged 2–10 using trypsin-EDTA solution. JEG-3 cells were used as a positive control for human leukocyte antigen-G (HLA-G) expression (Kovats et al., 1990).

Human microvascular endothelial cells, HMEC-1

HMEC-1 transformed cells contain SV-40 viral DNA sequences. The cell was cultured in MCDB131 by adding 10 ng/mL epidermal growth factor (EGF), 1 µg/mL hydrocortisone, 10 mM glutamine, FBS to a concentration of 10%.

Human prostate cancer cells, PC3

PC3 cells were cultured (passage 2–12) in RPMI with 10% FBS media. PC3 cells secreted condition media (PC3-CM) was used in tube forming activities as done previously (Chen et al., 2011).

Cell growth and viability by MTT assay

Cell growth and viability assay were performed in a 96-well plate using MTT assay. Cells (5 × 10^3/well/100 µL) were suspended in 5% FBS-RPMI and seeded homogeneously using 12-channel pipette and loading reservoir. The assay was performed using the protocol described before.
(Basak et al., 2018b) except incubation with assay media for 24 h. Optical density (OD) was recorded at 562 nm using Synergy H1 hybrid multimode reader (Biotek Instrument, USA).

**3H-Thymidine incorporation assay to estimate cell proliferation**

Tritiated thymidine incorporates into the new DNA that is synthesized when the cells proliferate. On the basis of this principle, cellular proliferation was measured in HTR8/SVneo in the presence of assay media by adopting the protocol described previously (Basak et al., 2017).

**Angiogenesis assay by measuring tubule formation**

Cellular angiogenesis was measured in vitro by measuring tube length as the index of tube formation capacity in the presence of assay media by following the protocol described previously except a few modifications (Basak et al., 2018b). Both HTR8/SVneo and HMEC-1 cells were pre-starved in FBS free, growth factor free RPMI, and MCDB131 media, respectively for 48 h prior to their seeding in matrigel containing complete media. Cells were stained with fluorescent Calcein AM dye (final concentration 0.1 µg/mL) prior to adding an assay media. Tube length was measured by ImageJ 1.50i (NIH, USA) and expressed as centimeter. Tubular length was calculated as a percentage of tube length over control. Each experimental groups consider tube length of five fields per image derived from repeated independent experiments performed in duplicates.

**Measurement of cellular migration by wound healing scratch assay**

Pre-starved (24 h) HTR8/SVneo cells (3 × 10^5 cells/mL) were cultured until 70–80% confluent. Cell monolayers were scratched by finer F10 pipette tips from one to another end with the 90° position. Perpendicular to the first scratch, the second scratch was made in order to get across a junction. Wound areas were captured using ×4 magnifications (Nikon TS100F, Japan) at 0 and 24 h. Cell migration rates were determined by measuring wound healed areas at the scratch region over initial time point and expressed as a percentage change of wound recovery of the surface area between 0 and 24 h by using Image 1.50i (NIH).

**Gene expression analysis by quantitative real-time polymerase chain reaction (qRT-PCR)**

Gene expression was measured by quantifying mRNA expression of the target genes and housekeeping gene (TBP) using the ABI7900HT system as described before (Basak et al., 2018b). Predesigned SYBR green I primers (#KSPQ12012; Sigma) were used (Table S1) for this purpose.

**Analysis of gene expression-pathways by qRT-PCR array**

HTR8/SVneo cells were stimulated without or with curcumin (5 µM) for 24 h. Total RNA (1 µg) was purified from the cell lysate as per the guideline provided by the manufacturer (Qiagen, Cat# 74104 and Cat# 330401). Real-time PCR was performed in ABI 7900HT using RT2 Profiler PCR array of a human NOTCH signaling pathway (Qiagen Cat# PAHS-059YA) according to the instruction of the supplier. Change in the mRNA folds expression difference was calculated by ΔΔCt method where 2(-ΔΔCt) is the normalized mRNA expression of 2(-ΔCt) of the curcumin induction over normalized mRNA expression 2(-ΔCt) of the control. The minimum cut off expression difference was considered ≥3 folds.

**Analysis of promoter DNA methylation of metabolic and oxidative stress-responsive genes by 384-well real-time PCR (qRT-PCR) array**

Promoter DNA methylation of genes associated with human metabolic and oxidative stress was performed using Epitect PCR array kit (#335222 EAHS-3580ZE; Qiagen) as described before (Basak et al., 2018b). In this method, each sample with an equal amount of genomic DNA (2 µg) was digested with restriction enzymes in four separate setups consists of mock (control) digest (Mo), methylation-sensitive (Ms), methylation-dependent (Md), and double digests (Msd). The method quantified remaining digested DNA of each gene by real-time PCR using pre-designed primers that flanked with the promoter (gene) region of interest. Each 96-genes of the 384 well arrays were amplified with equal amount of four digested DNA (Mo, Ms, Md, and Msd) with high specificity and amplification efficiency to detect the methylation status of the promoter region of the genes.

**Immunoblotting assay**

Cells were lysed by adding 200 µL of radioimmunoprecipitation assay buffer (Sigma Aldrich). The lysates were sonicated for 1 min (Vibra-Cell sonicator) and centrifuged. The soluble protein levels were estimated by BCA protein assay (Pierce, USA). A total 10 µg of proteins/lane were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%) prior to their transfer to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp.). After blocking, blots were immunoblotted with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5,000, #PA534847), anti-FABP4 (1:5,000, #PA-530591), VEGFR2/KDR (1:5,000, #PA116613) (Thermo Fisher Scientific, USA) and rabbit monoclonal anti-AKT.
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(1:5,000, #4685S), rabbit monoclonal, phospho-AKT (1:5,000, #4060S) antibodies (Cell Signaling Technology, USA), and mouse monoclonal anti-HLA-G antibody (1:5,000, #11-394C025; Exbio C2). The blots were detected by using a chemiluminescence substrate (Cat-32132; Pierce, USA). Immunoblot signals were captured by Storm860 phosphor-imager and quantified by Image Quant software (GE Healthcare). Data of protein expression are reported as fold expression after normalizing the expression with GAPDH from the repeated independent (n = 3) experiments.

Statistical analysis

Data were evaluated by unpaired Student’s t test to compare group over control using GraphPad Prism 4. One-way analysis of variance was used to compare multiple groups followed by Bonferroni post hoc test (SPSS 16, SPSS Inc., USA). Statistical significance was obtained when P < 0.05. Results are tabulated from repeated independent experiments as indicated in the text, or figure legend and data are expressed as mean ± standard error of the mean.

Results

Growth of the first-trimester trophoblast (HTR8/SVneo) and human prostate cancer (PC3) cells in the presence of curcumin

In order to investigate whether curcumin affects the similar way in reproductive and cancer cells, we performed a comparative assessment on the cellular growth using equal numbers of first-trimester trophoblast (HTR8/SVneo) and human prostate cancer (PC3) cells in the absence and presence of curcumin (1–10 µM) for 24 h. At 1–10 µM concentration, growth of HTR8/SVneo trophoblast cells was increased significantly by 25–30% over control as measured by increased cellular viability (control vs. 1 µM, 5 µM and 10 µM curcumin: 101.1 ± 1.09% vs. 134.9 ± 6.69%, 131.6 ± 7.04 and 126.2 ± 8.61%). The concentration of (1–5 µM) curcumin was considered for the subsequent assays as a mid-value of the growth effects (Figure 1). While curcumin (1–10 µM) exhibited increased cellular growth in HTR8/SVneo trophoblast cells; however, growth of the prostate cancer PC3 cells did not alter to a significant extent as compared with control cells (Figure 1).

Curcumin stimulated proliferation of trophoblast cells by the increased rate of new DNA synthesis with concomitant activation in the Akt phosphorylation of trophoblast HTR8/SVneo cells

To investigate whether curcumin affects proliferation of first-trimester trophoblast cells HTR8/SVneo cells, active DNA synthesis of the growing cells was measured by labeling proliferative cells with radioactive thymidine [3H] after incubating the cells with curcumin (5–10 µM) for 24 h. FABP4 (100 ng/mL) was used as a positive control for the cellular proliferation of HTR8/SVneo cells (Basak et al., 2018a). Compared with control, [3H]thymidine incorporation was significantly increased by ~29–32% (control vs. 5 µM and 10 µM curcumin: 25,300 ± 147.6 vs. 32,680 ± 144.8 and 33,830 ± 1,338 cpm) when these cells were treated with curcumin 5–10 µM for 24 h (Figure 2A). Curcumin-stimulated proliferation was comparable with FABP4 (control vs. FABP4 100 ng/mL: 25,300 ± 147.6 vs. 33,480 ± 825 cpm). Since protein kinase B/Akt, plays a key role in cellular proliferation; therefore, its activation was measured in HTR8/SVneo cells in the presence of curcumin. Curcumin (5 µM) increased the Akt phosphorylation by 48% (fold expression: control vs. curcumin 5 µM: 1.05 ± 0.05 vs. 1.560 ± 0.09) as compared with control after 24 h (Figure 2B). These data suggest that curcumin stimulates proliferation of trophoblast cells by increasing the rate of new DNA synthesis with concomitant activation of the Akt phosphorylation in the trophoblast HTR8/SVneo cells.

Impact of curcumin on in vitro angiogenesis (tube formation) in reproductive (HTR8/SVneo), endothelial (HMEC-1), and cancer (PC3) cells

In order to investigate whether curcumin stimulates its angiogenic effects in a similar way in reproductive and cancer cells, tube formation response of the HTR8/SVneo and HMEC-1 cells was compared between curcumin itself,
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Curcumin-treated HTR8/SVneo-CM as well as PC3 cells-CM. Previous study used PC3-CM for angiogenesis in vitro (Gupta et al., 2013). HMEC-1 cell is used as a control of in vitro angiogenesis (tube formation) of HTR8/SVneo cells. Tube length was increased significantly in the presence of curcumin in the placental trophoblast HTR8/SVneo cells (control vs. curcumin 5 µM: 120.9 ± 9.849% vs. 197.2 ± 14.03%). CM from the curcumin incubated HTR8/SVneo cell also increased tube length to a significant extent in human microvascular endothelial HMEC-1 cells (control vs. curcumin 5 µM: 111.8 ± 6.175% vs. 154.3 ± 13.12%). However, curcumin incubated PC3-CM on tube formation of HTR8/SVneo cells remained insignificant (control vs. curcumin 5 µM: 107.4 ± 3.791% vs. 120.3 ± 6.348%, Figure 3). Tube length exhibited stimulation in the presence of curcumin (5 µM) both in placental trophoblast HTR8/SVneo cells and endothelial HMEC-1 cells but not in PC3 cells (Figure 3). Thus, curcumin (5 µM) stimulates tube formation of placental trophoblast HTR8/SVneo cells by increasing tube length, whereas it has no significant effects on tube formation stimulated by prostate cancer PC3 CM incubated with curcumin.

**Curcumin-stimulated tube formation is inhibited by BMS309403 and SU5416 in HTR8/SVneo cells**

In order to understand whether pro-angiogenic factor FABP4 is involved in curcumin-stimulated angiogenesis, HTR8/ SVneo cells were incubated with or without curcumin in the presence or absence of FABP4 inhibitor, BMS309403. In absence of inhibitor, tube formation was increased by 91% and 116% (control – BMS vs. curcumin – BMS and FABP4 – BMS: 101.5 ± 0.8084% vs. 194.1 ± 3.978%, and 219.7 ± 2.363%) in presence of curcumin (5 µM) and FABP4 (100 ng/mL), respectively (Figure 4A). No significant difference was observed between curcumin (5 µM) and FABP4-stimulated tube formation over controls (Figure 4B). In the presence of BMS309403, tube formation was inhibited by 20% (control – BMS309403 vs. control + BMS309403:...
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Curcumin stimulates angiogenesis in the placenta. Curcumin (5 μM) significantly increased the expression of VEGFR2 protein by twofold (fold expression: control – SU5416 vs. curcumin – SU5416: 1.036 ± 0.019 vs. 2.022 ± 0.054; Figures 7A and 7B) and 1.8-fold (fold expression: control – BMS309403 vs. curcumin – BMS 309403: 0.977 ± 0.001 vs. 1.818 ± 0.021; Figures 7C and 7D) while expression of FABP4 protein was significantly increased by 1.5-fold (fold expression: control – SU5416 vs. curcumin – SU5416: 1.008 ± 0.004 vs. 1.534 ± 0.005; Figures 7A and 7B) and twofold (control – BMS 309403 vs. curcumin – BMS 309403: 0.9797 ± 0.010 vs. 1.986 ± 0.012; Figures 7C and 7D) as compared with control. Curcumin-stimulated VEGFR2 (fold expression: control – SU5416 vs. curcumin + SU5416: 2.022 ± 0.054 vs. 1.051 ± 0.010) and FABP4 (fold expression: control – SU5416 vs. curcumin + SU5416: 1.534 ± 0.005 vs. 0.766 ± 0.020) expression were significantly inhibited by 48% and 18% in presence of SU5416 over control (basal) inhibition of the respective proteins (fold expression: VEGFR2: control – SU5416 vs. control + SU5416: 1.036 ± 0.019 vs. 1.087 ± 0.013 and FABP4: control – SU5416 vs. control + SU5416: 1.008 ± 0.004 vs. 0.680 ± 0.014; Figures 7A and 7B). In the presence of BMS 309403 (Figures 7C and 7D), curcumin-stimulated VEGFR2 (fold expression: control – BMS309403 vs. curcumin + BMS 309403: 1.818 ± 0.021 vs. 0.849 ± 0.004) and FABP4 (fold expression: curcumin – BMS 309403 vs. curcumin + BMS 309403: 1.986 ± 0.012 vs. 0.720 ± 0.016) expression were significantly inhibited by 53% and 51% over control (basal) inhibition of the respective proteins (fold expression: control – BMS 309403 vs. control + BMS 309403: VEGFR2: 0.977 ± 0.011 vs. 1.003 ± 0.006; and FABP4: 0.979 ± 0.010 vs. 0.857 ± 0.024; Figures 7C and 7D). These data suggest that curcumin (5 μM) significantly stimulates the expression of VEGFR2 and FABP4 proteins in early...
Unlike VEGFR2, expression of FABP4 was inhibited both at basal (control) and stimulated conditions in the presence of VEGF and FABP4 inhibitors (Figures 7B and 7D). Curcumin stimulated VEGFR2 and FABP4 protein expression were inhibited by both the VEGF receptor inhibitor (SU5416) and FABP4 (BMS 309403) inhibitor in placental HTR8/SVneo cells.

Curcumin stimulates the expression of HLA-G in the trophoblast HTR8/SVneo cells. Unlike VEGFR2, expression of FABP4 was inhibited both at basal (control) and stimulated conditions in the presence of VEGF and FABP4 inhibitors (Figures 7B and 7D). Curcumin-stimulated VEGFR2 and FABP4 protein expression were inhibited by both the VEGF receptor inhibitor (SU5416) and FABP4 (BMS 309403) inhibitor in placental HTR8/SVneo cells.

Curcumin stimulates the expression of HLA-G in the trophoblast cells, HTR8/SVneo

Expression of HLA-G mRNA was significantly increased by sevenfold and 47-fold (fold expression control vs. curcumin 1 μM and 5 μM: 1.00 ± 0.462 vs. 7.444 ± 1.474 and 47.09 ± 3.778; Figure 8A) after stimulation with 1–5 μM of curcumin in the trophoblast HTR8/SVneo cells. HLA-G protein expression was undetected when HTR8/SVneo cells were cultured in the plastic tissue culture plate but detected in JEG-3 cells under similar experimental condition (Figure S1). HLAG protein expression was detected when HTR8/SVneo cells were cultured on matrigel coated plate as evidenced previously (Kilburn et al., 2000). Curcumin (5 μM) significantly stimulated the expression of HLA-G protein by 1.7–2.0-fold as compared with control (fold expression: control vs. curcumin 5 μM: 0.997 ± 0.004 vs. 1.786 ± 0.012 and 2.032 ± 0.026; Figures 8B and 8C).
Effect of curcumin on the expression of angiogenesis, metabolism, and DNA methylation factors in HTR8/SVneo cells

To understand the mechanism of growth stimulatory effects of curcumin, mRNA expression of angiogenesis, metabolism and DNA methylation were investigated in trophoblast HTR8/SVneo cells. Curcumin (5 µM) significantly upregulated the mRNA expression of VEGFA by 1.9-fold and MMP2 by 3.7-fold over control and downregulated HSD11β2 mRNA expression while expression of other angiogenesis and

Figure 6 Effects of curcumin on the wound healing capacity of the trophoblast HTR8/SVneo cells. Wound healing capacity was determined by measuring the migration rate of the trophoblast cells after 24 h of curcumin (1–5 µM) stimulation as described in the method. (A) Representative image of wound healed areas captured at ×4 magnification at 0 and 24 h. (B) Percentage of area for each group was calculated after comparing the changes in the surface area between 0 and 24 h. Data represent mean ± standard error of the mean versus control (n = 6). *P < 0.05 versus control (Student’s t test).

Figure 7 Effects of curcumin on VEGFR2 and FABP4 protein expression in the presence of SU5416 and BMS309403 in trophoblast HTR8/SVneo cells. (A and B) Cells were pre-incubated with SU5416 (300 nM) for 1 h prior to stimulating with curcumin (5 µM) for 24 h. (C and D) Cells were co-incubated with BMS309403 (50 µM) and curcumin (5 µM) for 24 h. Cellular proteins were harvested, and VEGFR2 and FABP4 proteins were measured by immunoblotting (A and C), and their expressions were measured (B and D) as described in the method. Data of protein expression are reported as fold expression of mean ± standard error of the mean (n = 3) after normalizing expression with glyceraldehyde 3-phosphate dehydrogenase; mean values with different superscript letters are significantly different at P < 0.05 level by one-way analysis of variance with Bonferroni’s multiple comparison tests.
growth factors (VEGFB, VEGFC, ANGPTL4, KRT7, TGFβ1, TGFβ2, and CTGF), invasion and adhesion factors (MMP1, MMP9, MMP14, TIMP1, and ICAM1) remained insignificant (Table 1). Curcumin (5 µM) significantly stimulated the expression of DNA methyltransferase 3 alpha (DNMT3A) by 7.6-fold over control while expression of other DNA methylation-sensitive genes such as DNMT1, DNMT3B remained unaltered (Table 1).

Table 1  Curcumin stimulated expression of genes in the first trimester trophoblast HTR8/SVneo cells.a

| Type of genes                | Gene symbol | Relative mRNA expression (target gene/TBP)b | Fold expression over controlc |
|------------------------------|-------------|---------------------------------------------|-------------------------------|
| Angiogenesis and growth factors | VEGFA       | 1.923 ± 0.028 (+)                            | 1.9*                          |
|                              | VEGFB       | 1.568 ± 0.231 (+)                           | 1.5                           |
|                              | VEGFC       | 1.506 ± 0.26 (+)                            | 1.5                           |
|                              | ANGPTL4     | 1.605 ± 0.149 (+)                           | 1.6                           |
|                              | KRT7        | 1.915 ± 0.455 (+)                           | 1.9                           |
|                              | TGFβ1       | 0.972 ± 0.07 (−)                            | 1.02                          |
|                              | TGFβ2       | 0.860 ± 0.032 (−)                           | 1.2                           |
|                              | CTGF        | 0.688 ± 0.054 (−)                           | 1.4                           |
| Invasion and adhesion factors | MMP1        | 0.837 ± 0.033 (−)                           | 1.2                           |
|                              | MMP2        | 3.738 ± 0.515 (+)                           | 3.7*                          |
|                              | MMP9        | 1.818 ± 0.332 (+)                           | 1.8                           |
|                              | MMP14       | 0.668 ± 0.222 (−)                           | 1.5                           |
|                              | TIMP1       | 1.174 ± 0.024 (−)                           | 1.1                           |
|                              | ICAM1       | 0.577 ± 0.091 (−)                           | 1.7                           |
| Metabolic factors            | HSD11β2     | 0.502 ± 0.068 (−)                           | 1.9*                          |
|                              | FABP4       | 1.123 ± 0.435 (−)                           | 1.1                           |
|                              | COX-2       | 1.487 ± 0.464 (−)                           | 1.4                           |
|                              | ADRP        | 0.755 ± 0.17 (−)                            | 1.3                           |
| DNA methylation              | DNMT1       | 1.632 ± 0.429 (+)                           | 1.6                           |
|                              | DNMT3A      | 7.606 ± 0.547 (+)                           | 7.6*                          |
|                              | DNMT3B      | 1.861 ± 0.405 (+)                           | 1.8                           |

mRNA, messenger RNA; SEM, standard error of the mean. a mRNA expression was measured after incubating cells with curcumin (5 µM) for 24 h. b The level of each mRNA expression of genes was quantified after normalized with endogenous control, TBP, and calculated according to the ΔΔCt method. Data are expressed as mean of relative mRNA fold expression over control ± SEM, n = 3. c Net fold expression are depicted by down (−) or up (+) regulation over control. *P < 0.05 versus control.
Effect of curcumin on the expression of NOTCH signaling mediators in HTR8/SVneo cells

Since curcumin stimulated FABP4 and VEGF expression in HTR8/SVneo cells and FABP4 response to VEGF are dependent on NOTCH pathway (Elmasri et al., 2009) therefore, expression of NOTCH signaling pathway mediators were assessed in HTR8/SVneo cells by using RT<sup>2</sup> Profiler PCR array after stimulation with curcumin (5 µM) (Table 2). Curcumin (5 µM) significantly increased mRNA expression of delta-like 1 (DLL1, 3.5-fold), LIM domain only 2, (LMO2, threefold), pre T-cell antigen receptor alpha (PTCRA, 8.2-fold), actin filament associated protein 1 like 2 (AFAP1L2, 3.2-fold), Hairy/enhancer-of-split related with YRPW motif protein 2 (HEY2, 11-fold), TNF-related apoptosis-inducing ligand (TNFSF10, 4.8-fold), and decreased cyclin E1 (CCNE1, fivefold), serpin peptidase inhibitor clade A member 3 transducing-like enhancer of split 1 (SERPINA3, fourfold) genes that are directly or indirectly involved with NOTCH-signaling pathway (Table 2).

Effects of curcumin on promoter DNA methylation of metabolic and oxidative stress mediators in the first-trimester trophoblast cells, HTR8/SVneo

To investigate whether curcumin has any protective effects on the first-trimester placenta which could be mediated via epigenetic modification, an assay was performed after stimulation of the first-trimester trophoblast HTR8/SVneo cells with curcumin, by measuring promoter DNA methylation of oxidative and metabolic stress genes. Percentage of DNA methylation was compared between the cells without or with curcumin (5 µM) and presented in the heat map (Figure 9). Out of a total of 78 genes analyzed, hypermethylation and differential hypermethylation were observed in 42, and 16 genes in curcumin stimulated cells as compared to 39 and 14 genes in control cells, respectively. Percentage of DNA methylation (CpG) of metabolic and oxidative stress mediators in response to curcumin (5 µM) stimulation of trophoblast HTR8/SVneo cells is presented in Table 3. CpG promoter methylation of HUS1, PCNA, RARA, VCP, and PRKDC genes (cell growth, proliferation, and DNA replication), VEGFA, HIF1A (angiogenic factors), ATM, HSPE1, PRDX3, RAD51 (protection of oxidative stress and DNA damage), CEFP, EIF2AK3 (immune stress and cellular protection) were upregulated while a large number of genes those are associated with the protection of oxidative stress and DNA damage (BCL2L1, CAT, CCS, Ddit3, DnajA1, Gsr, HSPA4, HSPA8, HSPH1, MDM2, MSH2, POR, SOD2, SYVN1, UBE2G2, E2F1, and RAD1) were down-regulated (Table 3). Overall, the percentages of promoter hypermethylation of protective genes against oxidative stress and DNA damage pathway mediators were higher on a number in the presence of curcumin.

Discussion

For the first time, this paper reports, that curcumin at low concentrations increase expression of angiogenic mediators and enhance hypomethylation of gene promoters associated with the protection against oxidative stress and DNA damage. Curcumin may play a role in epigenetic regulation of gene expression which is necessary for the correct establishment of developmental programs. In addition, curcumin stimulated the expression of HLA-G, which plays a key role in the immune regulation during trophoblast invasion. Curcumin upregulated mRNA expression of angiogenesis factors such as VEGFA and MMP2 and protein expression of proangiogenic factor FABP4 in trophoblast cells. All these modulatory effects of curcumin may be involved in promoting the angiogenesis processes in the first-trimester placenta.

Previous research has established an anti-cancer effects of curcumin by inhibiting cell viability (breast cancer cell lines, prostate cancer cells, including PC-3 cells), promoting apoptosis (ovarian cancer), and potentiating chemosensitivity (gastric cancer cells). This study compares the previously documented effect(s) of curcumin on PC-3 cell viability and angiogenesis to that of curcumin’s effect on

Table 2 Expression of NOTCH-signaling pathway mediators in HTR8/SVneo cells after curcumin stimulation.<sup>a</sup>

| Gene symbol | Gene name               | Up   | Down |
|-------------|-------------------------|------|------|
| DLL1        | Delta-like 1            | 3.51 |      |
| LMO2        | LIM domain only 2       | 3.05 |      |
| PTCRA       | Pre T-cell antigen receptor alpha | 8.22 |      |
| AFAP1L2     | Actin filament associated protein 1 like 2 | 3.25 |      |
| HEY2        | Hairy/enhancer-of-split related with YRPW motif protein 2 | 11.0 |      |
| TNFSF10     | TNF-related apoptosis-inducing ligand | 4.79 |      |
| CCNE1       | Cyclin E1               | 5.0  |      |
| SERPINA3    | Serpin peptidase inhibitor clade A member 3 | 4.05 |      |

<sup>a</sup>Messenger RNA (mRNA) expression was measured after stimulating HTR8/SVneo cells with curcumin (5 µM) for 24 h. Fold expression difference was calculated by ΔΔ<sup>Ct</sup> method where 2<sup>-ΔΔCt</sup> is the normalized mRNA expression of 2<sup>-ΔΔCt</sup> of the curcumin induction over normalized mRNA expression 2<sup>-ΔΔCt</sup> of the control. Minimum cut-off expression difference was considered ≥3 folds.
the HTR8 trophoblastic cells. Anti-angiogenic, antiproliferative and pro-apoptotic effects of curcumin have been shown in a wide variety of cancer cells and animal models (Fadus et al., 2017). The curcumin-increased tube formation (a measure of angiogenesis in vitro) in HMEC-1 and HTR8/SVneo cells contributes to angiogenesis, which is associated with early placentation development. The in vitro tube-like formation represent migration and differentiation of first trimester trophoblasts toward an invasive phenotype, a physiological critically important step in early placentation (Waddell et al., 2011). First-trimester placental trophoblasts interact with maternal immune cells during the invasion of the maternal uterine wall (Moffett and Loke, 2006). These cross-talks are regulated by finely tuned interactions of HLA-G-expressing invading trophoblasts with maternal innate immune cells and other adjoining cells. First trimester placental trophoblasts contain HLA such as HLA-C and HLA-G. HLA-G is important for a successful pregnancy as it is responsible for maternal immune tolerance by inhibiting NK cytotoxicity and cytokine production (Moffett and Loke, 2006; Chen et al., 2010). HLA-G is an important regulator of trophoblast invasion in an autocrine way, but the mechanism is not known at present. In addition to the immunoregulatory functions, HLA-G regulates cell proliferation, migration, and tubule formation in endothelial cells (Liu et al., 2013). In fact, soluble HLA-G locally influences uterine vascular remodeling (Le Bouteiller et al., 2007). In preeclampsia, placental HLA-G transcription levels were significantly decreased (O’Brien et al., 2001). A defect in placental HLA-G expression was associated with placental vascular defects (McMaster et al., 1995; Le Bouteiller et al., 2003). The exact mechanism of HLA-G action in trophoblast invasion is yet to be deciphered. Curcumin by inducing HLA-G expression may promote normal placental function. Further work on HLA-G functions would provide us with important new insights into the immunological and placental trophoblasts function.

Expression of VEGFA by curcumin in these cells is a novel finding. In addition to curcumin, VEGFA mRNA is also induced by several growth factors and cytokines (Basak and Duttaroy, 2013). The mechanisms responsible for the increased VEGFA expression in placental trophoblast cells by curcumin are not known at present. In addition to VEGFA, curcumin also induced VEGFR2 expression in these cells. Expression of VEGFR2 is associated with cell growth and survival in endothelial cells (Meissner et al., 2011). Increased expression of VEGFR2 by curcumin may thus represent a part of the pro-angiogenic mechanisms resemble with endothelial cells by which curcumin effects are mediated in trophoblast cells.

Curcumin stimulated the expression of a significant number of genes associated with NOTCH-signaling pathways mediators such as PTCRA, HEY2, and FABP4 in HTR8/SVneo cells. FABP4 response to VEGFA is dependent on DLL-NOTCH pathway. Since NOTCH signaling regulates angiogenesis, thus curcumin may stimulate angiogenesis by increasing expression of signaling mediators. DLL1 gene of a NOTCH signaling pathway is dysregulated in preeclampsia. DLL1 expressions in villous trophoblast are significantly lowered in the early-onset preeclamptic placenta. Curcumin may, therefore, play a protective role in preeclampsia by stimulating DLL1 gene. Upregulation of FABP4 expression by curcumin may stimulate the angiogenesis in these cells. FABP4 is a potent regulator of the angiogenic process in endothelial cells (Elmasri et al., 2009), and also mediates such process by fatty acids, leptin or VEGFA in trophoblast cells (Basak et al., 2011). pPTCRA protein regulates early T-cell development that may modulate the immune system in early placentation development. TNFSF10 gene encodes a protein cytokine that belongs to the TNF ligand family. The protein also designated as TNF-related apoptosis-inducing ligand (TRAIL), is a cytokine that is produced and secreted by most normal cells. The TRAIL protein expressed at a significant level in most normal tissue may control cellular maintenance and plays a protective role in the normal...
Table 3 Changes in promoter DNA (CpG) methylation of metabolic and oxidative stress genes in trophoblast cells, HTR8/SVneo by curcumin.

| Gene pathways                                      | Gene symbol | Control | Curcumin (5 µM) | Fold changea | Gene function                                                                 |
|---------------------------------------------------|-------------|---------|-----------------|--------------|-------------------------------------------------------------------------------|
| Cell growth, proliferation and DNA replication    | HUS1        | 6.1     | 50.0            | (+) 8.2      | Involved in the cell-cycle arrest in response to DNA damage                    |
|                                                   | PCNA        | 0.2     | 2.13            | (+) 10.65    | Component of the replication and repair machinery                             |
|                                                   | RARA        | 0.04    | 0.26            | (+) 6.5      | Regulation of cellular development, differentiation                           |
|                                                   | VCP         | 0.21    | 4.25            | (+) 20.24    | Regulation of the cell cycle                                                  |
|                                                   | EZF1        | 0.37    | 0.04            | (−) 9.25     | Cell cycle regulation or DNA replication                                      |
|                                                   | RAD1        | 1.77    | 0.39            | (−) 4.54     | Checkpoint in cell cycle progression in response to DNA damage                |
|                                                   | PRKD1       | 0.13    | 0.47            | (+) 3.62     | DNA-dependent protein kinase                                                   |
| Angiogenic factors                                | HIF1A       | 0.02    | 6.5             | (+) 325.0    | Embryonic vascularization, angiogenesis, hypoxia                              |
|                                                   | VEGFA       | 0.21    | 1.38            | (+) 6.57     | Vascular endothelial growth factor required for angiogenesis                  |
| Protection of oxidative stress and DNA damage    | ATM         | 0.01    | 0.55            | (+) 55.0     | DNA damage response gene                                                      |
|                                                   | HSPE1       | 2.9     | 50.0            | (+) 17.24    | Chaperon facilitates the correct folding of imported proteins                 |
|                                                   | PRDX3       | 0.01    | 0.06            | (+) 6.0      | Encodes a mitochondrial protein with antioxidant function                      |
|                                                   | RAD51       | 0.81    | 7.76            | (+) 9.58     | Repair of DNA double-strand breaks                                             |
|                                                   | UNG         | 0.55    | 7.52            | (+) 13.67    | Prevent mutagenesis by eliminating uracil from DNA                            |
|                                                   | BCL2L1      | 8.07    | 0.83            | (−) 9.72     | Potent inhibitor of cell death                                                 |
|                                                   | CAT         | 2.22    | 0.1             | (−) 22.2     | Key antioxidant enzyme in defense                                              |
|                                                   | CCS         | 15.43   | 0.15            | (−) 102.87   | Copper chaperone for superoxide dismutase to display misfolding and aggregation |
|                                                   | DDIT3       | 50      | 3.23            | (−) 15.48    | Transcription factor in ER stress response                                    |
|                                                   | DNAJA1      | 8.72    | 1.34            | (−) 6.51     | Heat shock protein prevent protein aggregation                                |
|                                                   | GSR         | 41.01   | 6.54            | (−) 6.27     | Cellular antioxidant defense                                                  |
|                                                   | HSPA4       | 5.88    | 0.21            | (−) 48.0     | Chaperone-mediated protein complex assembly                                    |
|                                                   | HSPA8       | 1.87    | 0.48            | (−) 3.9      | Facilitates the proper folding of newly translated and misfolded proteins     |
|                                                   | HSPH1       | 0.64    | 0.02            | (−) 32.0     | Facilitates the proper folding of newly translated and misfolded proteins     |
|                                                   | MDM2        | 1.02    | 0.22            | (−) 4.64     | Encodes protein can promote tumor formation by targeting tumor suppressor proteins |
|                                                   | MSH2        | 3.29    | 0.41            | (−) 8.02     | Component of the post-replicative DNA mismatch repair system                   |
|                                                   | POR         | 0.16    | 0.04            | (−) 4.0      | Cytochrome P450 oxidoeductase                                                  |
|                                                   | SOD2        | 0.3     | 0.06            | (−) 5.0      | Antioxidant and DNA damage response                                           |
|                                                   | SYVN1       | 5.74    | 0.03            | (−) 191.33   | Encoded protein removes unfolded proteins                                     |
|                                                   | UBE2G2      | 6.15    | 0.41            | (−) 15.0     | Targeting abnormal or short-lived proteins for degradation                    |
| Immune stress and cellular protection            | CEBPB       | 0.06    | 50.0            | (+) 833.33   | Regulating the expression of genes involved in immune and inflammatory responses |
|                                                   | EIF2AK3     | 2.74    | 11.08           | (+) 4.04     | Metabolic-stress sensing protein kinase                                        |

*aFold change in percentage promoter methylation are depicted by down (−) or up (+) regulation over control.
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In conclusion, the present study demonstrates that curcumin at low concentrations positively modulates gene expression and angiogenesis that favor the development of the placental trophoblast cells. Further work is required to ascertain if curcumin can be used as positive modulator of placental angiogenesis processes that may protect impaired feto-placental growth and development.

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Author contribution

S.B. and A.K.D.R. were responsible for the concept and study design, and provided critical input; S.B. was responsible for laboratory experiments, data collection; data analysis and interpretation and for drafting the
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manuscript; and A.K.D.R. provided critical review of the manuscript. V.S. and A.M. performed lab experiments.

Conflict of interest

The authors declare that there are no conflict of interests.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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