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Mechanisms in Decorin Regulation of Vascular Endothelial Growth Factor-Induced Human Trophoblast Migration and Acquisition of Endothelial Phenotype¹

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ABSTRACT

Extravillous trophoblast (EVT) cells of the human placenta invade the uterine decidua and utero-placental arteries to establish an efficient exchange of key molecules between maternal and fetal blood. Trophoblast invasion is stringently regulated in situ both positively and negatively by a variety of factors at the fetal-maternal interface to maintain a healthy utero-placental homeostasis. One such factor, decorin, a transforming growth factor (TGF)- β binding, leucine-rich proteoglycan produced by the decidua, negatively regulates EVT proliferation, migration, and invasiveness independent of TGF- β . We reported that these decorin actions were mediated by its binding to multiple tyrosine kinase receptors, including vascular endothelial growth factor receptor (VEGFR)-2. The present study explores the mechanisms underlying decorin antagonism of VEGF (VEGF-A) stimulation of endovascular differentiation of EVT using our EVT cell line, HTR-8/SVneo. We observe that decorin inhibits VEGF-induced EVT cell migration and endothelial-like tube formation on matrigel. VEGF activates MAPKs (p38 MAPK, MEK3/6, and ERK1/2) in EVT cells, and the activation is blocked in both cases by decorin. Employing selective MAPK inhibitors, we show that both p38 and ERK pathways contribute independently to VEGF-induced EVT migration and capillary-like tube formation. VEGF upregulates the vascular endothelial (VE) markers VE-cadherin and beta-catenin in EVT and endothelial cells, and this upregulation is blocked by decorin and MAPK inhibitors. These results suggest that decorin inhibits VEGF-A stimulation of trophoblast migration and endovascular differentiation by interfering with p38 MAPK and ERK1/2 activation. Thus decorin-mediated dual impediment of endovascular differentiation of the EVT and angiogenesis may have implications for pathogenesis of pre-eclampsia, a hypoinvasive trophoblast disorder in pregnancy.

β -catenin, decorin, endovascular differentiation, extravillous trophoblast, migration, mitogen-activated protein kinases, placenta, vascular endothelial growth factor, VE-cadherin

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INTRODUCTION

The human placenta is a highly invasive organ that invades the uterine endometrium and its vasculature to establish sufficient exchange of oxygen and other key molecules between the maternal and fetal circulations [1]. This organ develops primarily through the proliferation and differentiation of extra-embryonic trophoblast cells derived from the trophoctoderm of the pre-implantation blastocyst [2]. Further differentiation of cytotrophoblast stem cells within the chorionic villi results in a subpopulation of cells known as the extravillous trophoblast (EVT). These cells proliferate to aggregate into cell columns and migrate out of the villous confinement to invade the decidualized endometrium (interstitial invasion). During endovascular invasion, EVT cells, either interstitial in origin or recruited from the cytotrophoblastic shell, surround spiral arteries of the maternal vasculature, migrate into the lumen, acquire an endothelial-like phenotype and replace the endothelial cells lining the vessels [3, 4]. During this process the spiral arteries within the endometrium and a part of the myometrium are modified into flaccid, low-resistance, high-flow tubes allowing unhindered blood flow [5].

It has been well established that trophoblast invasion is influenced by a multiplicity of regulating factors such as cytokines and growth factors, adhesion molecules, proteases, matrix-derived components, and oxygen tension [6, 7]. While a number of factors, both autocrine (trophoblast derived) and paracrine (decidua derived), are known to stimulate trophoblast proliferation, migration, and invasiveness [8, 9], only a few molecules have been shown to inhibit these trophoblast functions [9–13]. Of the latter, transforming growth factor (TGF)- β and the TGF- β -binding proteoglycan, decorin, were identified as two key decidua-derived inhibitors of normal EVT cell proliferation, migration, and invasiveness [11–16]. Immunohistochemical analysis revealed that decorin colocalizes with TGF- β in first-trimester decidual extracellular matrix in situ [15]. This was postulated as a mechanism of localizing TGF- β in an inactive form until its activation by trophoblast-derived proteases at the invasive front to control hyperinvasion. Surprisingly, however, decorin on its own was found to inhibit proliferation, migration, and invasiveness of a primary EVT cell line HTR-8 in a TGF- β -independent manner [16]. The antiproliferative action was due to decorin-mediated upregulation of p21 expression. Furthermore, JAR choriocarcinoma cells were shown to be resistant to the negative regulatory effects of decorin [16]. Recently, by employing EVT cell outgrowths from first-trimester chorionic villus explants on matrigel and the immortalized human first-trimester EVT cell

line HTR-8/SVneo, decorin-mediated control of EVT cell functions were shown to involve multiple tyrosine kinase receptors [17]. The antiproliferative action was mediated primarily by epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR)-2, and the antimigratory action on fibronectin substrate was mediated primarily by insulin-like growth factor receptor (IGFR)-1. However, in the absence of fibronectin, VEGFR-2 binding also contributed to the antimigratory action of decorin [18].

Decorin was shown to negatively regulate a variety of cellular functions, either by binding to extracellular matrix molecules or cell surface receptors. For example, decorin was reported to inhibit angiogenesis *in vitro* by blocking migratory [19–21] and adhesive [20, 21] functions of endothelial cells by interacting with fibronectin [20, 21] and thrombospondin [19, 21]. Furthermore, purified GAG-free decorin and its 26-residue leucine-rich repeat, LRR5, were shown to inhibit vascular endothelial growth factor (VEGF)-A and serum-induced proliferation, migration, and tube formation by endothelial cells *in vitro* [22, 23]. Recently using recombinant VEGFR-2-Fc chimera, as well as VEGFR-2 protein expressed by EVT cells and endothelial cells, we documented the binding of VEGFR-2 with decorin core protein with an affinity that was 7- to 10-fold lower than VEGF-E or VEGF-A. Furthermore, we established that certain amino acid sequences within the LRR5 region of decorin presented as the most avid and functional VEGFR-2 binding site [18], explaining the strong anti-angiogenic function of the LRR-5 peptide reported above [22, 23].

The precise signaling mechanisms underlying decorin antagonism of VEGF-A action on EVT cells remain to be characterized. Members of the VEGF family are important regulators of cellular functions (such as proliferation, migration, survival, and permeability) involved in vascular development and angiogenesis under physiological and pathological conditions by binding to a number of receptors [24–26]. Of the three receptors (VEGFR-1, R-2, and R-3), VEGFR-2 is the principal mediator of most physiological and pathological effects of VEGF-A, including cell survival and migration. We hypothesized that decorin inhibits VEGF-A-induced EVT cell migration and acquisition of an endothelial phenotype by antagonizing VEGFR-2 signaling. Several signaling molecules have been implicated in VEGFR-2-dependent cytoskeletal changes and cell migration, including endothelial nitric oxide synthase (eNOS), focal adhesion kinase (FAK), p38 mitogen-activated protein kinase (p38 MAPK), and extracellular signal-regulated kinase 1/2 (ERK1/2 or p44/42 MAPK) [27–30]. VEGF-A and VEGFR-2 interaction promotes survival of vascular endothelial cells involving cytoplasmic tail of vascular endothelial (VE)-cadherin that binds with cytoskeletal actin via β -catenin [31]. The VE-cadherin/ β -catenin complex has also been implicated in endothelial tube formation *in vitro* [32, 33]. In the study presented here, we examined the effects of decorin on VEGF-A-induced migration and acquisition of an endothelial phenotype by EVT cells (as indicated by endothelial-like tube formation and upregulation of VE-cadherin/ β -catenin) and the possible signaling mechanisms underlying these VEGF actions, which are blocked by decorin.

MATERIALS AND METHODS

Reagents

RPMI-1640 medium, fetal bovine serum (FBS), Dulbecco phosphate-buffered saline (DPBS), 0.25% trypsin-ethylenediaminetetraacetic acid, and penicillin/streptomycin used in the cell culture were purchased from Invitrogen. BD Falcon cell culture flasks (75 cm²), 6-well plates, 24-well plates, Transwell inserts (24-well plate, 6.5-mm diameter, 8- μ m pore size), growth factor-reduced

(GFR) matrigel, and anti-FAK, anti-phospho-FAK (pY397), anti-phospho-eNOS (pS1177), and anti-eNOS/NOS type-III antibodies were from BD Biosciences. Anti-phospho-ERK1/2 (Thr-202/Tyr-204) and anti-p44/42 MAPK antibodies, and U0126 (p44/42 MAPK or ERK inhibitor) were from Cell Signaling. Anti-p38, anti-phospho-p38 (Thr-180/Tyr-182), anti-MEK3/6 (SC-133230), anti-phospho-MEK3/6 (Ser-189) (SC-7994), and anti- β -tubulin (G-8) antibodies were from Santa Cruz Biotechnology. Mammalian protein extraction reagent (M-PER), HALT protease inhibitor cocktail, and restore-plus Western blot stripping buffer were from Pierce. Goat anti-rabbit immunoglobulin G (IgG) and goat anti-mouse IgG secondary antibodies were from Bio-Rad. Bovine articular cartilage-derived decorin and bovine serum albumin were purchased from Sigma. SB203580, a p38 MAPK inhibitor, was from Calbiochem, and recombinant human VEGF₁₂₁ was from R&D Systems. ELISA kits for VE-cadherin (DYC938-2) and human β -catenin (DYC1329-2) were from R&D Systems. Quantitative RT-PCR primers were designed using primer-3 site and were synthesized at the University of Western Ontario Oligo Factory. RNeasy Mini Kit was from Qiagen, and qScript cDNA Synthesis Kit and PerfeCTa Green SuperMix were from Quanta Biosciences.

Cell Culture

The EVT cell line, HTR-8/SVneo (passages 75–90) was grown in RPMI-1640 medium supplemented with 10% FBS, 50 units (U)/ml penicillin and 50 μ g/ml streptomycin, unless specified otherwise, at 37°C and 5% CO₂. This cell line was produced by introducing the gene encoding simian virus (SV) 40 large T-antigen into a short-lived first-trimester primary EVT cell line, HTR-8, followed by neomycin selection [13]. The parental cell line was produced by propagation of EVT cells migrating out of first-trimester chorionic villus explants. Both the parental (HTR-8) and immortalized (HTR-8/SVneo) cell lines were morphologically and functionally similar [13]. HTR-8/SVneo cells (henceforth interchangeably called EVT cells in describing the results) express all the markers of the highly migratory EVT cells *in situ*: cytokeratin 7, 8, and 18; placental-type alkaline phosphatase; high-affinity urokinase-type plasminogen activator receptor; IGF-II mRNA and protein; integrins α 1, α 3, α 5, α v, β 1, and the vitronectin receptor α v β 3/ β 5; HLA framework antigen w6/32 [14, 34–36]; and HLA-G [18, 37, 38]. During the current study, we revalidated HLA-G and cytokeratin 7 expression by these cells. Human umbilical vein endothelial cells (HUVEC) were obtained from ATCC and grown in medium 200 with low serum growth supplement kit (Invitrogen).

Migration Assay

Migration assays with EVT cells were performed in 24-well Transwell inserts, as reported earlier [34–36, 39, 40] for 24 h (when migration peaked to a plateau, followed by a decline at 36–48 h) with minor modifications. Near confluent cells were serum starved overnight and pretreated with varying concentrations of decorin (0, 10, 50, and 100 nM), p38 MAPK inhibitor (SB203580 at 0, 0.5, 5, and 25 μ M), or ERK1/2 inhibitor (U0126 at 0, 0.5, 5, and 10 μ M) in serum-free medium (SFM) for 1 h at room temperature. After the treatment, 50 000 cells were plated onto the top chamber of the cell culture inserts. VEGF₁₂₁ (20 ng/ml or 166 nM) was added to the lower chamber. The assembled cell culture insert chamber was then incubated at 37°C in 5% CO₂. On completion of the 24-h incubation, the upper surfaces of the membranes were wiped gently with cotton swabs to remove nonmigratory cells. The membranes were then fixed, stained with 0.1% crystal violet in methanol, and the absolute number of migrant cells was scored visually using a Leitz Laborlux K light microscope at \times 400 magnification. For each treatment, three independent experiments were performed with each condition being tested in triplicate.

Endothelial-Like Tube-Formation Assay

Growth factor-reduced matrigel diluted 1:1 with basal RPMI-1640 media was added to 24-well plates and incubated for 30 min at 37°C to solidify. EVT cells in SFM were seeded on the GFR matrigel-coated plates (50 000 cells per plate) for the tube-formation assay conducted for 4–24 h. Under native serum-free conditions, very low levels of tube formation occurred with EVT cells at 4 h, gradually increasing with time (peaking between 12 and 18 h) and beginning to degenerate at 24 h. Pilot studies on the temporal kinetics of tube formation by EVT cells in the presence of VEGF₁₂₁ revealed that tubulogenesis was accelerated and maximally stimulated with 20–50 ng/ml (166–413 nM) VEGF₁₂₁ at 4 h followed by a plateau for 4–18 h. To study the actions of decorin and MAPK inhibitors on VEGF-stimulation of tube formation, EVT cells were treated for 1 h with varying concentrations of bovine articular cartilage-derived decorin (0, 5, 10, 50, and 100 nM), the pharmacological inhibitor of p38 MAPK (SB203580 at 0, 0.5, 5, and 25 μ M), or the inhibitor of ERK1/2 (U0126 at 0, 0.5, 5, and 10 μ M), and then stimulated with 20 or 50 ng/

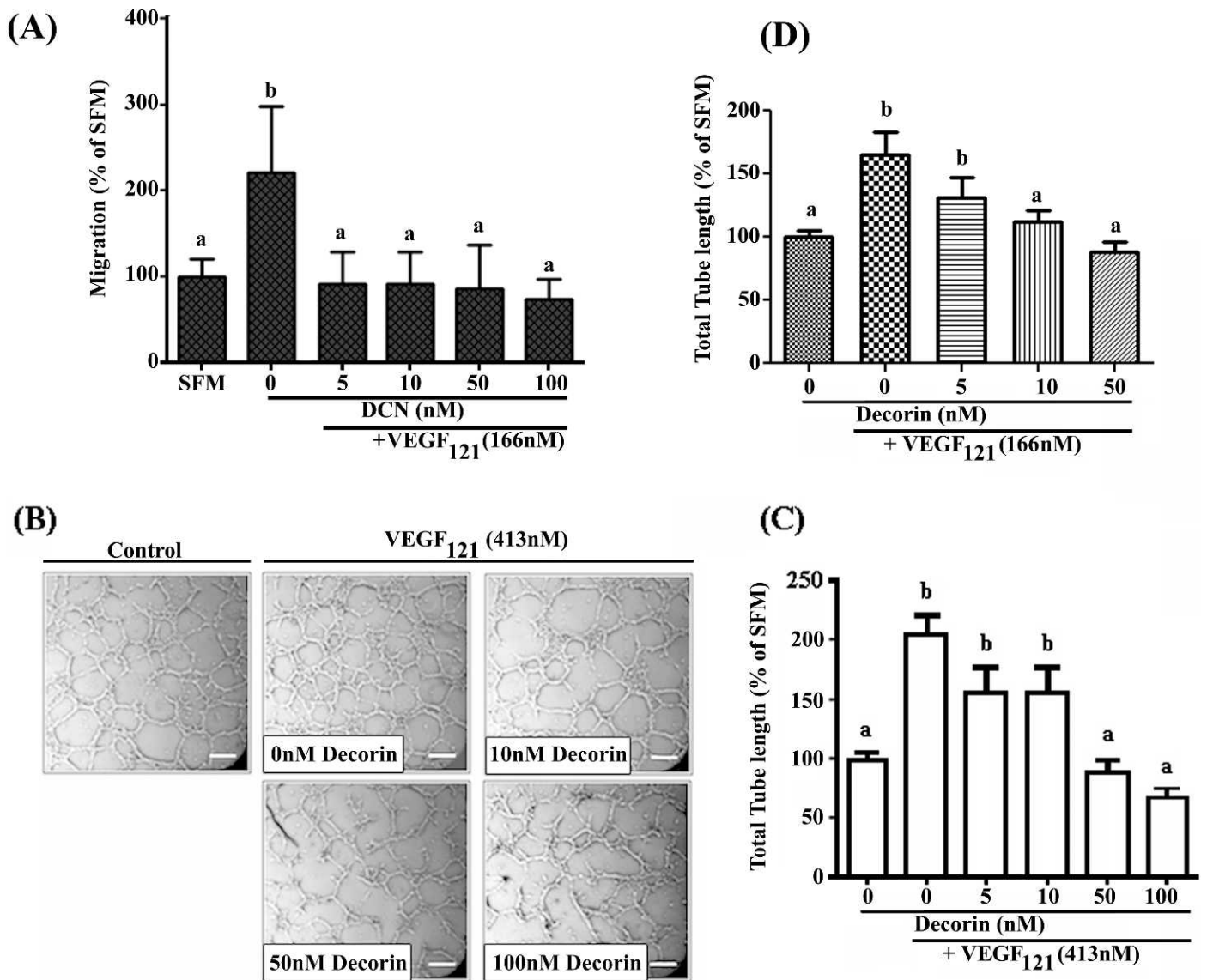


FIG. 1. Decorin inhibits VEGF-induced EVT cell migration and endothelial-like tube formation. For both migration (at 24 h, **A**) and tube formation (at 4 h, **B**, **C**, and **D**) assays, EVT cells were pretreated with varying concentrations of decorin (0, 10, 50, and 100 nM) for 1 h prior to stimulation with VEGF₁₂₁ (20–50 ng/ml). Data for both assays are presented as means ± SEM for three independent experiments with each condition being tested in triplicates. Significant differences ($P < 0.05$) between means are indicated by different lowercase letters. In **B**, the scale bar = 194 μm. In **C** and **D**, the data represent total tube length per field, measured with the NIH ImageJ software. Analysis given by the number of branching points per field (data not shown) produced identical results. Decorin at 10 nM completely abrogated 166 nM VEGF-stimulated migration (**A**). VEGF-induced tube formation (413 nM, **C**; and 166 nM, **D**) was blocked with 50 nM decorin.

ml VEGF₁₂₁. Cells were then subjected to the tube-formation assay by seeding on GFR matrigel and incubated at 37°C and 5% CO₂ for 4–18 h; pictures were taken of three random fields of view using a Leica EC3 camera. The number of branching points and total tube length were quantified using the National Institutes of Health (NIH) ImageJ software. Branching points were considered as a point from which two or more tubes branched.

Western Blot Analysis

EVT cells were grown to near confluence in six-well plates and placed in SFM overnight. Serum-starved cells were treated with 10 ng/ml (83 nM) of VEGF₁₂₁ for varying times (0, 5, 10, 15, 30, and 60 min) or pretreated with decorin (0, 1, 5, 10, and 100 nM), 0.5 μM SB203580, or 5 μM U0126 for 10 min followed by 10 ng/ml (83 nM) VEGF stimulation. This VEGF concentration was guided by pilot experiments in which activation of MAPKs reached a maximum at 10ng/ml. After VEGF treatment, cells were washed with ice-cold DPBS (including 10 nM NaF and 1 mM Na₃VO₄) and lysed in M-PER lysis buffer supplemented with HALT protease inhibitor cocktail, 10 mM NaF, and 1 mM Na₃VO₄. Cell lysates were centrifuged, and the supernatant protein

was quantified using the BCA protein assay kit. Equal amounts of protein (25 μg) were separated on 12% SDS-PAGE gels and transferred to a polyvinylidene fluoride membrane. Membranes were blocked in 5% nonfat milk in TBS (20 mM Tris, 0.14 M NaCl, pH 7.8) containing 0.05% Tween-20 for 1 h at room temperature and probed for phospho-p38 (1:200), total p38 (1:200), phospho-MEK3/6 (1:1000), total MEK3/6 (1:1000), phospho-ERK1/2 (1:1000), and total ERK1/2 (1:1000) at 4°C overnight. The membranes were washed with TBS containing 0.05% Tween-20 and incubated in horseradish peroxidase-conjugated rabbit or mouse secondary antibodies (1:5000; Bio-Rad) for 1 h at room temperature. Peroxidase activity was detected with enhanced chemiluminescence reagent (Pierce). NIH ImageJ was used to determine the average density of each band, and the results were expressed as a ratio of phosphorylated protein to total protein in three independent experiments.

Quantitative Real Time (RT)-PCR for VE-Cadherin and β-Catenin mRNA

Total RNA was extracted from EVT and HUVEC cells after specific treatments as detailed in the *Results* section using the RNeasy Mini Kit. The

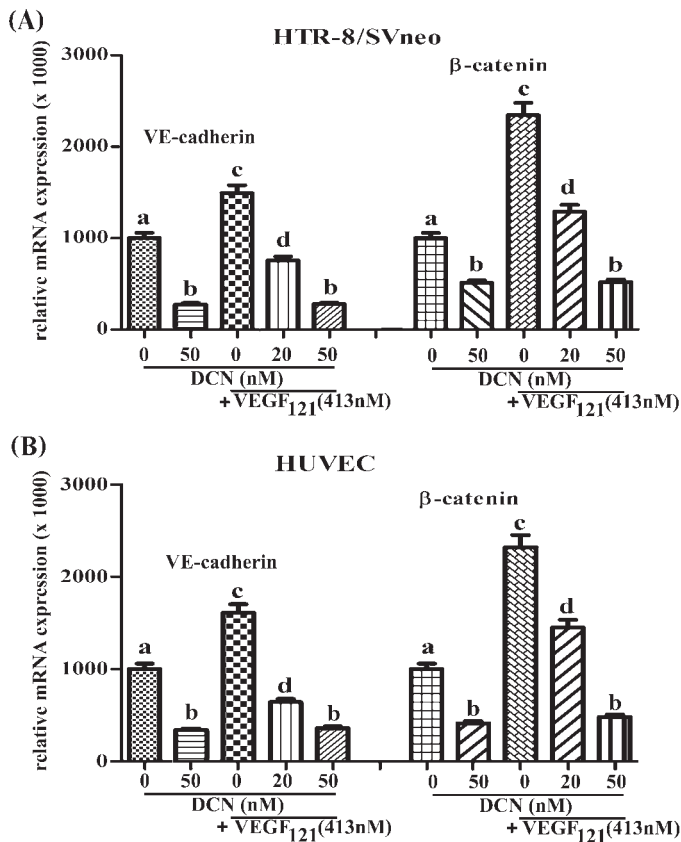


FIG. 2. Decorin inhibits VEGF-A-induced upregulation of VE-cadherin and β -catenin mRNAs in EVT and HUVEC cells. EVT and HUVEC cells were grown to 60% confluency, washed with PBS, and incubated with serum-free media for 12 h. VEGF₁₂₁ or decorin or their combination (decorin added 10 min prior to VEGF₁₂₁) were added at specified concentration to each cell line and incubated for another 12 h. Cells were washed twice with PBS; total RNA was isolated, and the cDNA was synthesized and quantified with qPCR for the mRNA of GAPDH, β -catenin, VE-cadherin in each of the treated cell lines. The obtained β -catenin and VE-cadherin values were normalized relative to GAPDH levels and expressed as a ratio of the values for the untreated cells. Data for EVT cells are presented in **A** and HUVEC cells in **B**. Each treatment was done in triplicate in each of two experiments. Data represent means \pm SEM (n = 6). Significant differences ($P < 0.01$) are indicated by different lowercase letters.

respective cDNA was synthesized using qScript cDNA Synthesis Kit, and real-time quantitative PCR analysis was performed with an iCycler iQ detector (Bio-Rad thermocycler) using PerfectA Green SuperMix; the data was analyzed using CFX Manager software (Bio-Rad) for GAPDH, VE-cadherin, and β -catenin gene expression. The program started at 95°C for 3 min and amplification included 39 cycles of two steps of 0.10 min at 95°C and 0.30 min at 55°C. The fluorescent product was detected at the last step of each cycle. To determine the relative levels of gene expression, the comparative threshold cycle method (Δ Ct) was used [41]. The final mRNA levels were normalized according to their Ct values from the standard curves and expressed in relation to the respective GAPDH level. The following primer pairs were used: GAPDH forward (5-gag atc cct cca aaa tca atg-3), GAPDH reverse (5-tga tga tct tga ggc tgt tg-3), VE-cadherin forward (5-gct gta ctg agc act gaa ccac-3), VE-cadherin reverse (5-ctg agg agt ctg cca gca aggt-3), β -catenin forward (5-att tga tgg agt tgg aca ttgc-3), and β -catenin reverse (5-gag gaa gag gat gtg gat acc tcc-3). The formed amplicons were verified by running a DNA gel.

Knockdown of VEGFR-2 in EVT Cells

VEGFR2 small interfering RNA (siRNA) consisted of pools of three to five target specific 19–25 nucleotide siRNAs designed to knock down human VEGFR2 gene expression (SC-29318; Santa Cruz Biotechnology), and the negative control siRNA (SC-37007; Santa Cruz Biotechnology) was used as the

VEGFR2-scramble siRNA. HTR-8/SVneo cells were incubated in a 6-well culture plate, at a count of 5×10^4 cells/well, and the cells were transfected with experimental and negative control siRNAs in separate wells using JetPrime Polyplus DNA transfection reagent (VWR) in RPMI-1640 medium with 10% FBS and incubated for 48 h. Both the experimental and negative control siRNA-treated cells were washed with PBS and incubated for 12h in serum free RPMI-1640 medium containing VEGF₁₂₁ or decorin or both. Cells were then washed with PBS. The total RNA from the respective treated cells were extracted with the RNeasy Mini kit, and cDNA was synthesized with the qScript cDNA synthesis kit. Transcript levels for GAPDH, VE-cadherin, and β -catenin for each set of cDNA was quantified using the respective primers as described above. The mRNA levels of VE-cadherin and β -catenin following treatment of the cells were expressed relative to GAPDH using the comparative threshold cycle method (Δ Ct) [41].

ELISA for VE-Cadherin and β -Catenin

ELISA was performed with protein extracts of the cell lysates following the procedures specified in the respective VE-cadherin and β -catenin ELISA kits (R&D Systems). In brief, EVT and HUVEC cells were grown in their respective media to reach 60% confluency, washed with PBS, and incubated in SFM for 12 h. Then VEGF₁₂₁ or decorin or their combinations (decorin pretreatment for 10 min followed by VEGF treatment) were added at concentrations specified in the *Results* section, and the cells were incubated for another 12 h. The choice of this time was based on pilot experiments that indicated a maximal stimulation by VEGF. Cells were washed with PBS twice, centrifuged, resuspended in the media, and the cell number counted. Cells were centrifuged to obtain the cell pellet that was lysed with radioimmunoprecipitation assay lysis buffer (Millipore) and protein extracted. The protein extract for each of the conditions (in triplicate) was incubated in the ELISA plates, and the respective ELISA was performed for β -catenin and VE-cadherin with their respective standards. The experiment was replicated twice.

Statistical Analysis

The data are presented as mean \pm SEM for each treatment compared to the control. Data were analyzed by a one-way ANOVA followed by a post-hoc TUKEY test or Dunnett multiple comparison test (for densitometry data) using Graph Pad Prism 5 software. Differences between two treatment groups were accepted as significant at $P < 0.05$.

RESULTS

Decorin Blocks VEGF-A Stimulation of EVT Cell Migration and Endothelial-Like Tube Formation

To examine the effect of decorin on migration or tube formation, serum-starved EVT cells were treated with varying concentrations of decorin for 1 h before stimulation with 20–50 ng/ml (166–413 nM) VEGF₁₂₁. VEGF₁₂₁ lacks in the heparin-binding domains of VEGF-A, thus excluding any confounding effects of heparin binding. In the presence of 20ng/ml VEGF₁₂₁ alone, EVT cell migration was enhanced to 221% of control level (Fig. 1A). This stimulation was blocked completely and nearly equally in the presence of 10–100 nM decorin.

The effect of decorin on the ability of EVT cells to adopt an endothelial phenotype was examined by two approaches: 1) formation of endothelial-like tubes after seeding EVT cells on GFR matrigel and 2) expression of the vascular endothelial marker VE-cadherin and its downstream partner β -catenin at the mRNA and protein levels. When plated on GFR matrigel, low levels of capillary-like networks formed at 4 h even under serum-free conditions. However, in the presence of 50 ng/ml (413 nM) VEGF₁₂₁, tube formation was stimulated to 204% of serum-free control level (Fig. 1, B and C). Pretreatment with decorin blocked this stimulation, showing a concentration-dependent trend. Abrogation of VEGF stimulation was complete at 50 nM decorin. When tube formation was stimulated with 20 ng/ml (166 nM) VEGF₁₂₁, it reached to 165% of serum-free control level, and a complete abrogation of the stimulation was noted with 10–50 nM decorin (Fig. 1D).

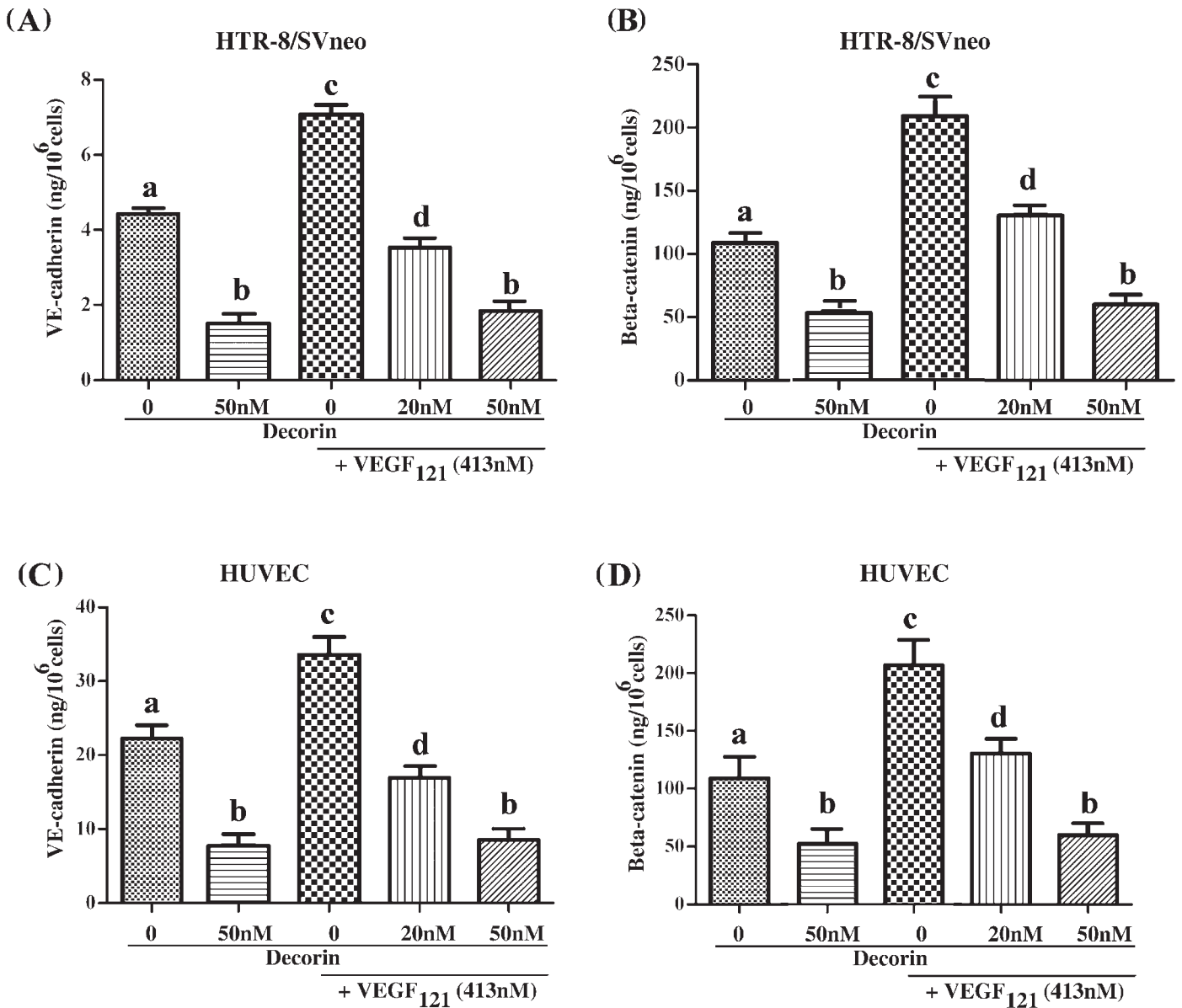


FIG. 3. Decorin inhibits VEGF-A-induced upregulation of VE-cadherin and β -catenin proteins in EVT and HUVEC cells. The details for performing ELISA with the protein extracts of EVT and HUVEC cell lysates are given in the *Materials and Methods* section. Each treatment was done in triplicate for each experiment, and the experiments were conducted twice. The levels of VE-cadherin or β -catenin proteins are expressed per unit number of cells. The data for EVT cells are presented in **A** and **B** and HUVEC cells in **C** and **D** as means \pm SEM ($n = 6$). Significant differences ($P < 0.01$) between the means are indicated by different lowercase letters.

Decorin Blocks VEGF-A-Induced VE-Cadherin and β -Catenin Upregulation in EVT and HUVEC Cells

Quantitative PCR and ELISA were performed to quantify the relative levels of VE-cadherin and β -catenin mRNAs and protein, respectively, in EVT cells and HUVEC cells (used as positive control) under native conditions (serum-starved cells), VEGF₁₂₁ treatment, decorin treatment, or a combination (decorin pretreatment for 10 min followed by VEGF) thereof. VEGF₁₂₁ treatment with 50 ng/ml (413 nM) significantly upregulated VE-cadherin (to 1.5- to 1.6-fold) and β -catenin (to 2- to 2.2-fold) both at the mRNA (Fig. 2, A and B) and protein (Fig. 3A–D) levels in both EVT and HUVEC cells in a nearly identical manner. Decorin pretreatment (at 20 or 50 nM) blocked the VEGF-induced stimulation of mRNAs (Fig. 2, A and B) and proteins (Fig. 3A–D) in both cell types. At 50 nM

decorin followed by VEGF treatment, the values decreased to below the native levels, equivalent to the values noted with decorin treatment alone.

VEGF-A-Induced VE-Cadherin and β -Catenin Upregulation in EVT cells Is VEGFR-2 Dependent

Because VEGF-A action on EVT cells can theoretically be mediated by VEGFR-1 as well as VEGFR-2, both of which are expressed by EVT cells, we tested the effects of siRNA-mediated knockdown of VEGFR-2 on VEGF-A-induced upregulation of VE-cadherin and β -catenin. As shown in Figure 4, VEGFR-2 siRNA treatment led to highly significant (to 35% of the initial level) downregulation of VEGFR-2 in EVT cells. This was associated with an equally significant downregulation of VE-cadherin (to 20%) and β -catenin (to

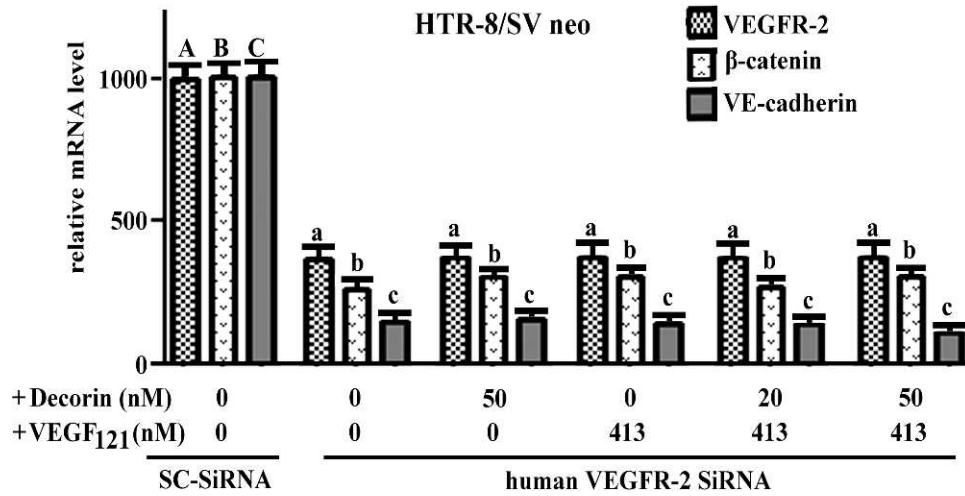


FIG. 4. VEGF-A-induced upregulation of VE-cadherin and β -catenin is VEGFR-2 dependent. EVT cells were transfected with scrambled (SC) siRNA (controls) or VEGFR-2 siRNA (48 h) followed by treatments with VEGF₁₂₁ (413 nM), decorin (20 or 50 nM), or their combination (for 12 h) for the determination of VEGFR-2, VE-cadherin, and β -catenin mRNAs by qPCR. The results are presented relative to controls normalized to GAPDH mRNA. The data represent means of three experiments \pm SEM. A significant decrease ($P < 0.005$) in mRNA levels of all the respective genes (A vs. a, B vs. b, and C vs. c) by VEGFR-2 siRNA treatment remained unaffected by further treatments with VEGF₁₂₁ or decorin or their combination.

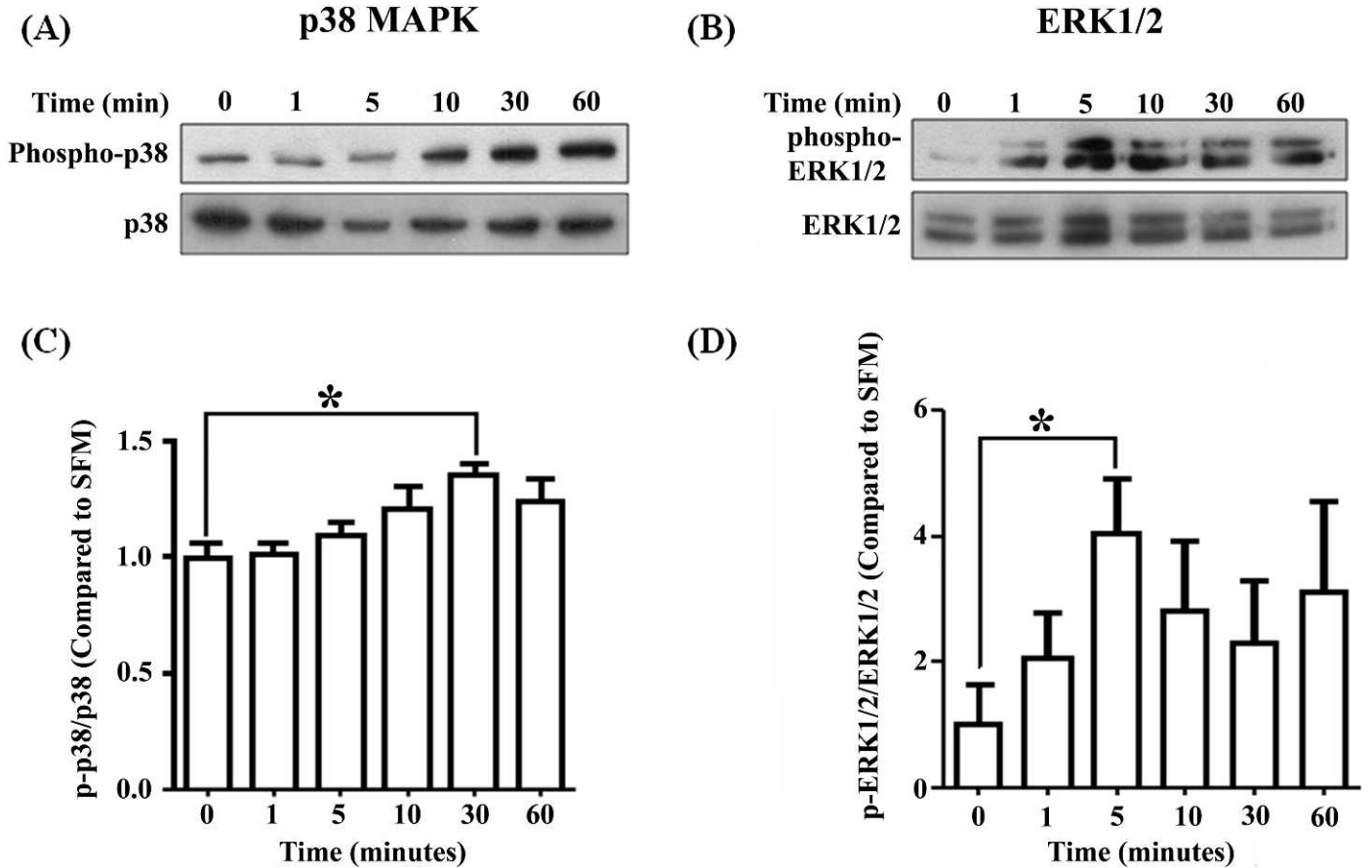


FIG. 5. VEGF stimulates p38 and ERK1/2 activity in EVT cells. EVT cells were serum starved for 24 h before stimulation with VEGF₁₂₁ (10 ng/ml) for varying lengths of time (0, 1, 5, 10, 30, and 60 min). Cell lysate protein was analyzed by Western blot for p38 activity (Thr-180/Tyr-182; **A**) and ERK1/2 activity (Thr-202/Tyr-204; **B**). Densitometry for p38 (**C**) and ERK1/2 (**D**) was performed and expressed as the ratio of phosphorylated to total p38 or ERK, respectively. Maximum activation of p38 and ERK1/2 occurred after 30 and 5 min, respectively. Densitometry data are presented as means \pm SEM for three independent experiments ($n = 3$) with each time point being compared to phosphorylation under serum-free conditions (0 min). Asterisks (*) indicate significant differences ($P < 0.05$) between treatment and control (0 min). Untreated controls at 1–60 min time points were not different from 0 min (data not shown).

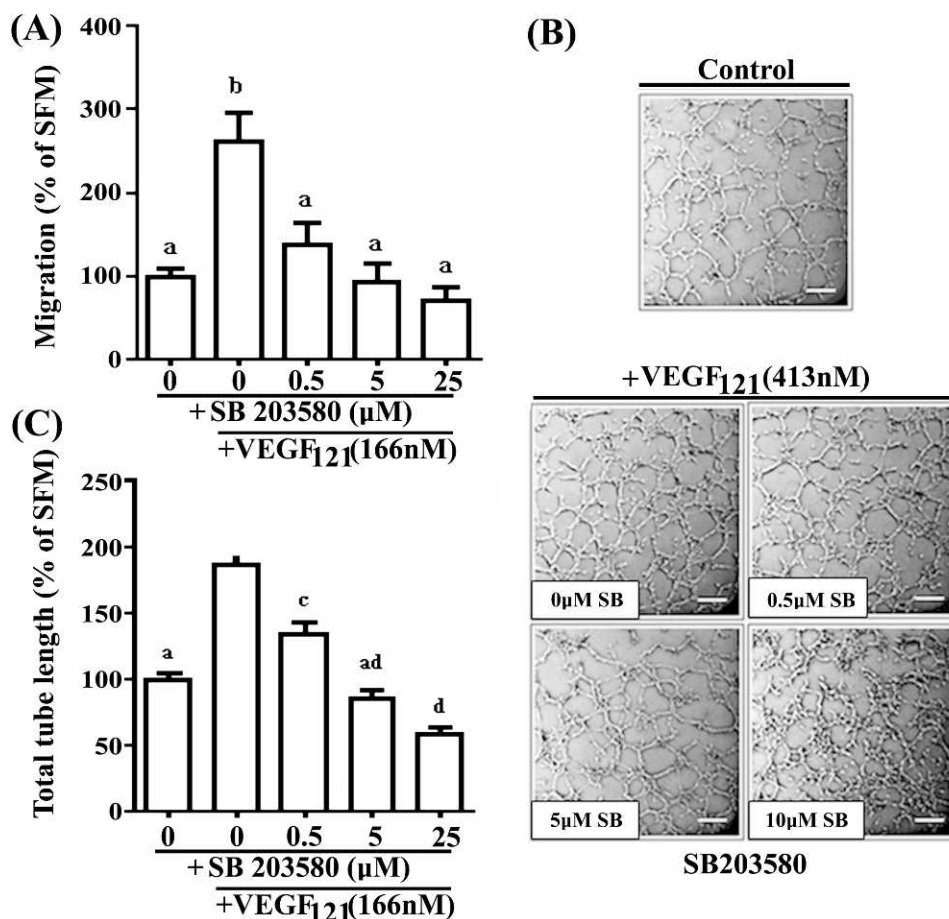


FIG. 6. VEGF-stimulated cell migration and endothelial tube formation require p38 MAPK. EVT cells were pretreated with varying concentrations of SB203580 (0, 0.5, 5, and 25 μM) for 1 h prior to stimulation with 20 ng/ml (166 nM) VEGF₁₂₁ for the 24-h migration assay (A) or stimulation with 50 ng/ml (413 nM) of VEGF₁₂₁ for the 4-h tube-formation assay (B, C). Bars in B = 194 μm . C represents total tube length per field. Migration and tube-formation data are presented as a mean \pm SEM for three independent experiments ($n = 3$) with each condition being tested in triplicates. Significant differences ($P < 0.05$) in the means are indicated by different lowercase letters.

15%) that could not be rescued by the addition of VEGF-A. Treatment with decorin at 20 or 50 nM in combination with VEGF-A had no further effect on the mRNA levels of either molecule. The data clearly reveal that VEGF-A-induced upregulation of VE-cadherin and β -catenin noted in Figure 2A is VEGFR-2 dependent and decorin effects in blocking VEGF-A-induced upregulation of these molecules (Fig. 2A) is also VEGFR-2 dependent.

VEGF-A Induces p38 MAPK and ERK1/2 (p44/p42 MAPK) Activation in EVT Cells

Because MAPKs have been implicated in VEGF-stimulated migration in a variety of cell types [29, 30, 42, 43], EVT cells stimulated with 10 ng/ml (83 nM) VEGF₁₂₁ were harvested and processed for Western blots. Very low levels of phospho-p38 MAPK and phospho-ERK1/2 (p44/p42 MAPK) were observed in untreated, serum-starved EVT cells (Fig. 5, A and B). Densitometry analysis revealed that p38 MAPK was significantly activated after 30 min of treatment with 10 ng/ml VEGF₁₂₁ compared to untreated cells (Fig. 5C). Phosphorylation of both ERK1 and ERK2 were induced by 10 ng/ml (83 nM) VEGF₁₂₁ and reached a maximum after 5–10 min of treatment compared to untreated cells (Fig. 5D). Because both MAPK pathways were activated after exposure to VEGF₁₂₁, we next tested whether or not pharmacological inhibitors of

p38 and ERK, SB203580 and U0126, respectively, influenced EVT cell migration and endothelial-like tube formation.

VEGF-A-Induced EVT Cell Migration and Endothelial-Like Tube Formation Depend on Activation of p38 MAPK and ERK1/2 (p44/p42 MAPK)

VEGF₁₂₁ stimulation of migration and endothelial-like tube formation by EVT cells was compromised when cells were pretreated with the p38 MAPK inhibitor SB203580 (Fig. 6) or the ERK1/2 inhibitor U0126 (Fig. 7) in a concentration-dependent manner. These inhibitors were nontoxic at all the tested concentrations in cell viability assays. The presence of VEGF₁₂₁ alone at 20 ng/ml (166 nM) for migration and 50 ng/ml (413 nM) for tube formation caused 2- to 2.5-fold stimulation of these events. VEGF stimulation of EVT cell migration (Figs. 6A and 7A) and tube formation (Figs. 6, B and C, and 7, B and C) were completely blocked when cells were pretreated with 5.0 μM of the either inhibitor SB203580 or U0126, attesting to the need of both MAPK pathways for VEGF stimulation of these events. These findings prompted us to examine whether decorin blocked activation of these pathways by VEGF.

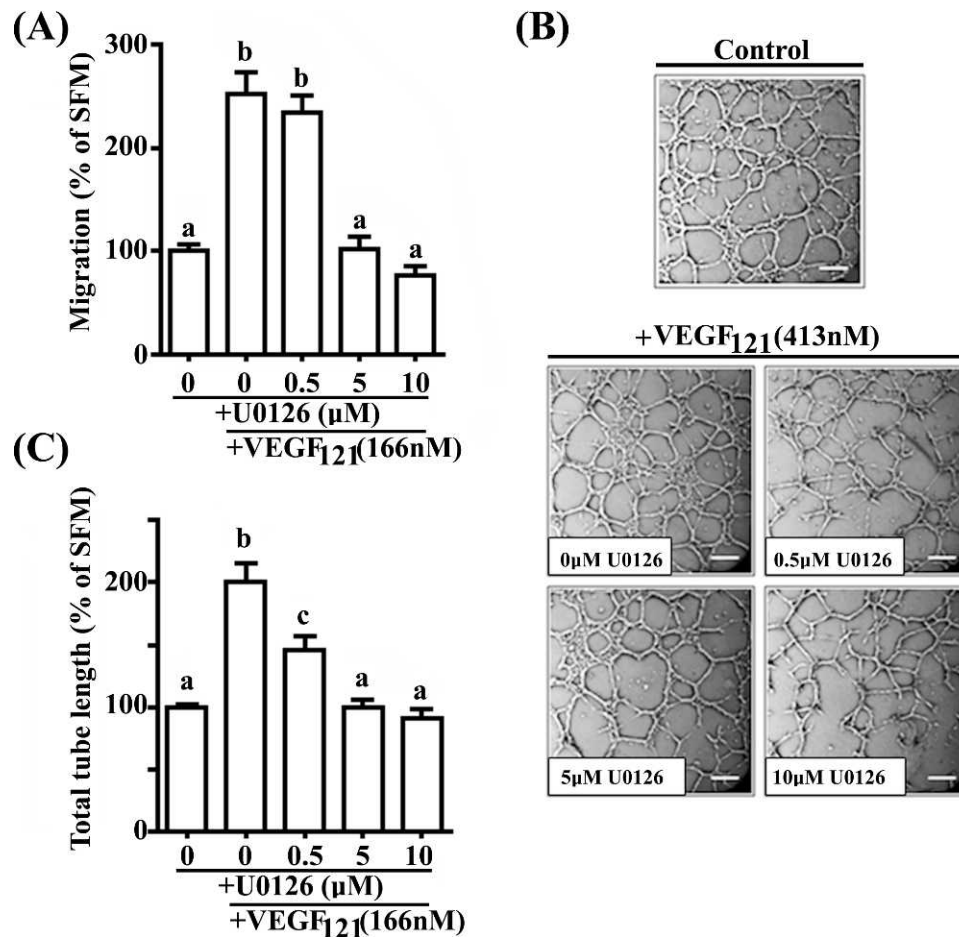


FIG. 7. ERK1/2 is required for VEGF-stimulated cell migration and endothelial-like tube formation. EVT cells were pretreated with varying concentrations of U0126 (0, 0.5, 5, and 10 µM) for 1 h and stimulated with 20 ng/ml (166 nM) VEGF₁₂₁ for the 24-h migration assay (A) or with 50 ng/ml (413 nM) VEGF₁₂₁ for the 4-h tube formation assay (B). Bars in B = 194 µm; C represents total tube length per field. Migration and tube-formation data are presented as means ± SEM for three independent experiments with each condition being tested in triplicates. Significant differences ($P < 0.05$) in the means are indicated by different lowercase letters.

Decorin Blocks VEGF-A-Induced Activation of p38 MAPK and ERK1/2 (p44/p42 MAPK) in EVT Cells

When EVT cells were stimulated with 10ng/ml (83nM) VEGF after pretreatment with decorin at various concentrations (1–100 nM), we observed a decrease in p38 activation as measured by Western blots (Fig. 8A). Densitometry analysis revealed that VEGF-A-induced p38 activation was blocked at all the concentrations of decorin, but more so at lower concentrations (Fig. 8C). In spite of the fact that VEGF₁₂₁ stimulated both ERK1 (p44) and ERK2 (p42) (Figs. 5 and 8D), this stimulation was higher for ERK1 than ERK2 (Fig. 8, E and F), and decorin at all the concentrations blocked VEGF₁₂₁-induced phosphorylation of ERK1 but not ERK2 (Fig. 8, B and D). Taken together, these findings suggest that decorin at low concentrations abrogates VEGF-stimulated signaling via p38 MAPK and ERK1 pathways in EVT cells.

Decorin Blocks VEGF-A-Induced Activation of MEK 3/6 in EVT Cells

MEK 3/6, also known as p38 MAPKK, are upstream signaling molecules required for activation of p38 MAPK [44]. EVT cells exhibited some constitutive phosphorylation of MEK3/6 as noted with Western blotting even under a serum-

starved state (Fig. 9A). When EVT cells were treated with 10 ng/ml (83 nM) VEGF₁₂₁, a further stimulation was noted. After pretreatment with decorin at various concentrations (0–50 nM), we observed significant abrogation of VEGF-A stimulation of MEK 3/6 activation at decorin concentrations of 20–50 nM (Fig. 9B). When MEK3 and MEK6 blots were individually analyzed (Fig. 9, C and D), MEK 6 was found to be more sensitive to VEGF-A-mediated stimulation as well as decorin antagonism of this activation. Decorin on its own (at 5–50 nM) significantly reduced the constitutive activation of MEK 3/6, indicative of decorin antagonism of unidentified endogenous ligands responsible for this constitutive activation. This was true for both MEK3 and MEK 6 analyzed individually (Fig. 9, C and D).

We also tested whether possible VEGF-mediated activation of eNOS and FAK pathways in EVT cells was involved. We found by Western blot analysis that while eNOS was expressed by HUVEC cells, it was not detectable in EVT cells in the absence or presence of VEGF₁₂₁. Furthermore, FAK (pY397) was constitutively phosphorylated in EVT cells and further stimulation was not significant after VEGF₁₂₁ treatment (data not shown).

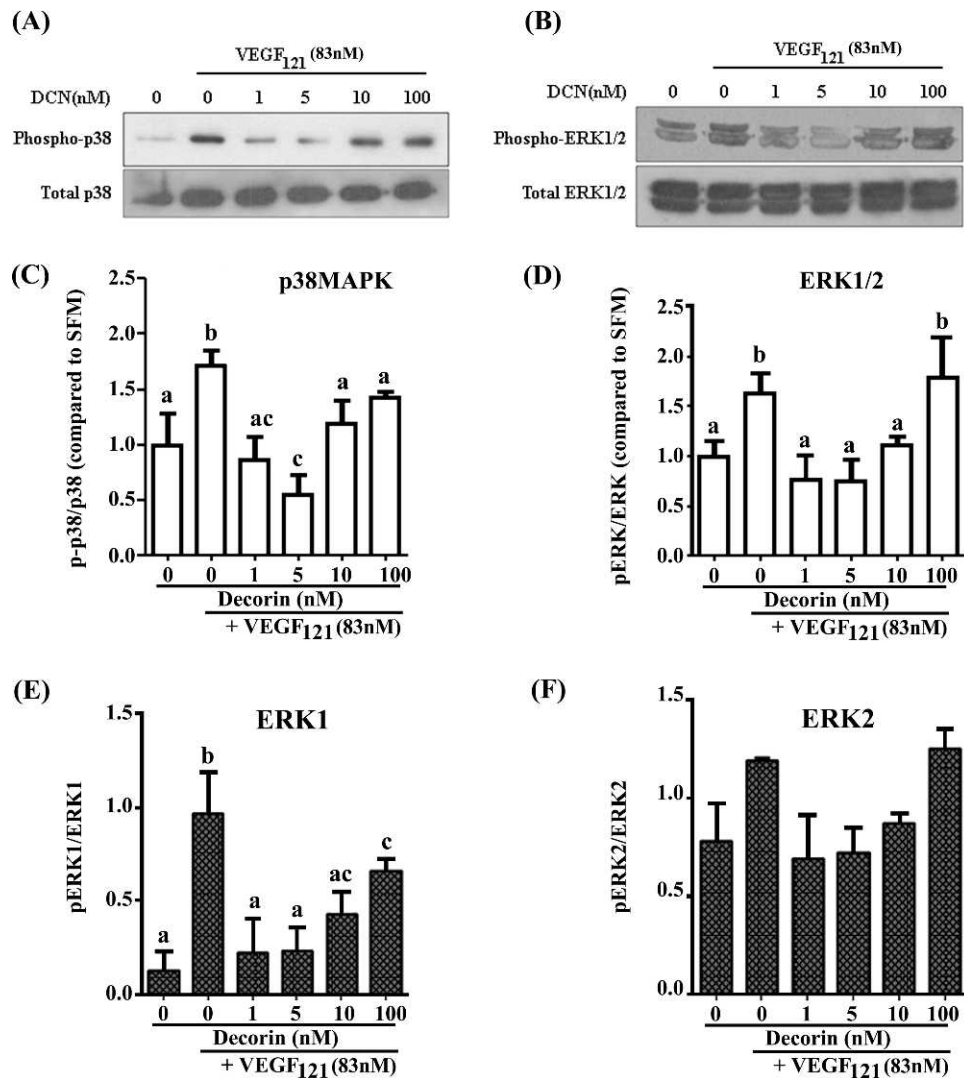


FIG. 8. Decorin inhibits p38 MAPK and ERK1/2 activation in EVT cells. EVT cells were serum starved for 24 h followed by treatment with varying concentrations of decorin (0, 1, 5, 10, or 100 nM). After 1 h, decorin-treated cells were stimulated with 10 ng/ml (83 nM) VEGF₁₂₁. Cell lysate protein was analyzed by Western blot for p38 activity (Thr-180/Tyr-182) in **A** and ERK1/2 activity (Thr-202/Tyr-204) in **B**. Densitometry for p38 MAPK (**C**), ERK1/2 (**D**), ERK1 (**E**), and ERK2 (**F**) was performed. The data for phosphorylated relative to total p38 or ERK are presented as means \pm SEM for three independent experiments with each condition in triplicate. Phosphorylation under no treatment (SFM control), VEGF stimulation alone (0 nM decorin), or decorin treatment followed by VEGF were compared. Data in **C** and **D** were normalized to SFM. Significant differences ($P < 0.05$) in the means are indicated by different lowercase letters.

VEGF-A Activates ERK1/2 and p38 MAPK by Distinct Pathways

Western blot analysis was performed to determine whether VEGF-A-induced p38 and ERK1/2 activation in EVT cells occurred by the same pathway or two distinct pathways. EVT cells treated with the p38 MAPK inhibitor SB203580 showed no stimulation of p38 MAPK phosphorylation in the presence of 10 ng/ml (83 nM) VEGF₁₂₁ (Fig. 10A), whereas phosphorylation of ERK1/2 still occurred in the presence of the SB compound (Fig. 10B). In EVT cells treated with the MEK1/2 inhibitor U0126, VEGF was unable to stimulate ERK1/2 phosphorylation (Fig. 10B); however, no effect was found on stimulation of p38 phosphorylation (Fig. 10A). Densitometry analysis revealed that SB203580 significantly decreased phosphorylation of p38 MAPK under serum-free conditions as well as in the presence of VEGF₁₂₁ (Fig. 10C). ERK1/2 phosphorylation was significantly reduced when cells

were treated with U0126 under stimulatory conditions only (i.e., VEGF₁₂₁ treatment) (Fig. 10D).

ERK1/2 and p38 MAPK Activity Are Required for VEGF-A-Induced Upregulation of VE-Cadherin and β -Catenin in EVT Cells

To test whether VEGF-A-induced upregulation of VE-cadherin or β -catenin in EVT cells is MAPK dependent, serum-starved cells were treated with 50 ng/ml (413nM) VEGF₁₂₁ in the presence (added 10 min earlier) or absence of various concentrations of the p38 MAPK inhibitor (SB203580 at 0.5–25 μ M) or the ERK1/2 inhibitor (UO126 at 0.5–10 μ M) for 12 h prior to conducting qRT-PCR for measuring the levels of VE-cadherin or β -catenin mRNA relative to GAPDH mRNA (Fig. 11, A and B), or the levels of VE-cadherin and β -catenin proteins as measured with ELISA (Fig. 11, C and D). As noted earlier when discussing Figures 2 and 3, VEGF upregulated both molecules at the mRNA and protein levels. At

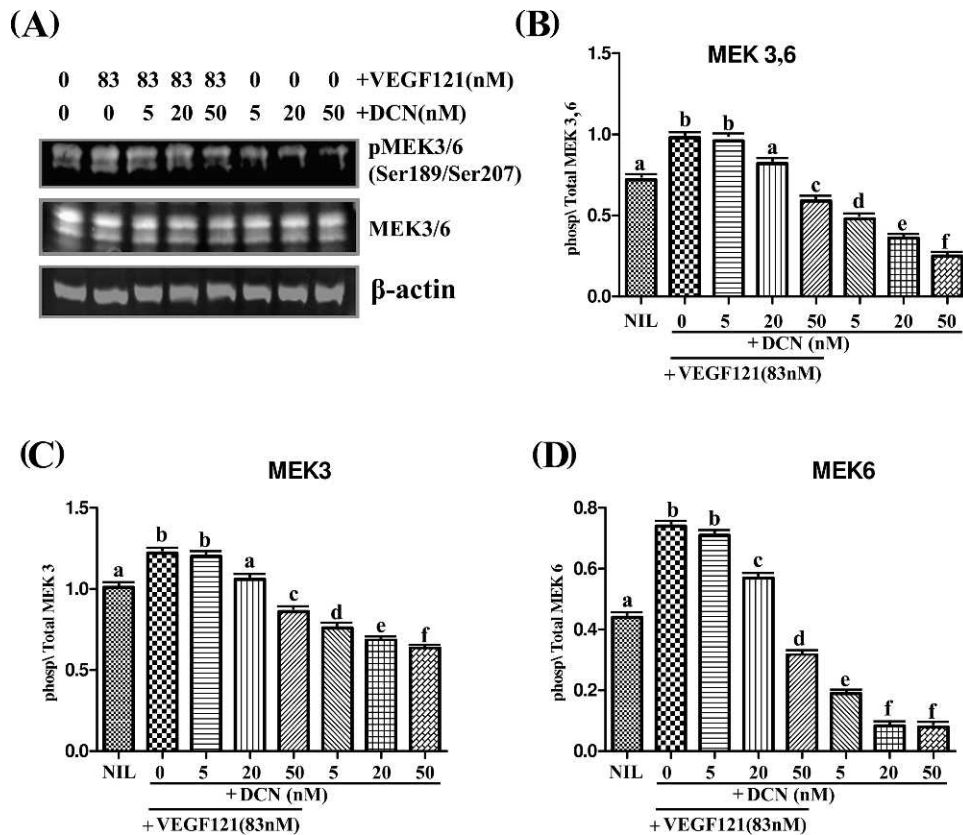


FIG. 9. Decorin blocks VEGF-A-induced activation of MEK 3/6 in EVT cells. EVT cells were serum starved for 24 h followed by treatment with varying concentrations of decorin (0, 5, 20, or 50 nM). After 1 h, decorin-treated cells were stimulated with 10 ng/ml (83 nM) VEGF₁₂₁. Cell lysate protein was prepared and analyzed by Western blot for pMEK3,6 (Ser-189 for MEK3 and Ser-207 for MEK6), MEK 3,6, and β -actin (A). Densitometry for pMEK3,6 relative to MEK 3,6 is shown in B; for pMEK3 relative to MEK 3 in C; and for pMEK 6 relative to MEK 6 in D. In all the cases, the data are presented as means \pm SEM for two independent experiments with each condition in triplicate (n = 6). Significant differences ($P < 0.05$) between the means are indicated by different lowercase letters.

all the inhibitory concentrations of both MAPK inhibitors, the mRNA levels (Fig. 11, A and B) as well as the protein levels (Fig. 11, C and D) of both molecules were brought down to control or below control levels, indicating a complete abrogation of VEGF stimulation. Thus, this VEGF action is also dependent on the two MAPK pathways. That these inhibitors on their own caused a small downregulation of these molecules in some cases indicates that these pathways are not exclusive to VEGF-A action and that some endogenous molecules were responsible for maintaining their constitutive expression in EVT cells.

Figure 12 shows a schematic presentation of the decorin inhibition of VEGF-A mediated signaling in EVT cells responsible for endovascular differentiation. VEGF-A-VEGFR2 interaction stimulated EVT cell migration, endothelial-like tube formation and upregulation of VE-cadherin/ β -catenin, all of which are antagonized by decorin binding to VEGFR2.

DISCUSSION

Successful placental development requires proper remodeling of the spiral arteries to allow for unhindered blood flow to the placenta [2, 45]. During arterial remodeling, EVT cells acquire a phenotype that mimics cells of the vascular system, particularly endothelial cells [46]. This change in phenotype, often referred to as endovascular differentiation, includes expression of vascular endothelial markers such as VE-cadherin, platelet endothelial cell adhesion molecule-1, vascu-

lar cell adhesion molecule-1, and integrin α V β 3 [46], and the ability to mimic the tube-forming behavior of endothelial cells in vitro upon culture on matrigel [37, 47–49].

We had earlier used a primary first-trimester EVT cell line HTR-8 [16] and first-trimester chorionic villus explants [17] to show that decidua-derived decorin negatively regulates migratory function of EVT cells. The present study explored the mechanisms in decorin antagonism of VEGF-A actions on EVT cells. In the absence of a better in vitro model, endovascular differentiation by EVT cell was examined by two different approaches: 1) in vitro tube formation assay with the HTR-8/SVneo EVT cell line on GFR matrigel and 2) measuring the expression of the endothelial marker VE-cadherin and its downstream partner β -catenin in the same cells. Tube formation is a complex process requiring adhesive and migratory functions amongst other undefined cell-cell and cell-matrix interactions. Our findings of tube formation by HTR-8/SVneo cells are consistent with several reports using different EVT cell lines. For example, another human EVT cell line TCL1 formed endothelial-like tubes on GFR matrigel [50–52]. In another report, the present EVT cell line was found to form sparse or no tubes on matrigel when cultured alone; however, in the presence of endothelial cells, they collaborated and synchronized with endothelial cells, forming capillary-like networks [47]. Other studies similar to the present have shown that EVT cell lines have the intrinsic capacity to form tubes on matrigel, which can be stimulated by various decidua-derived factors including EG-VEGF[53], CTGF [54], and products of

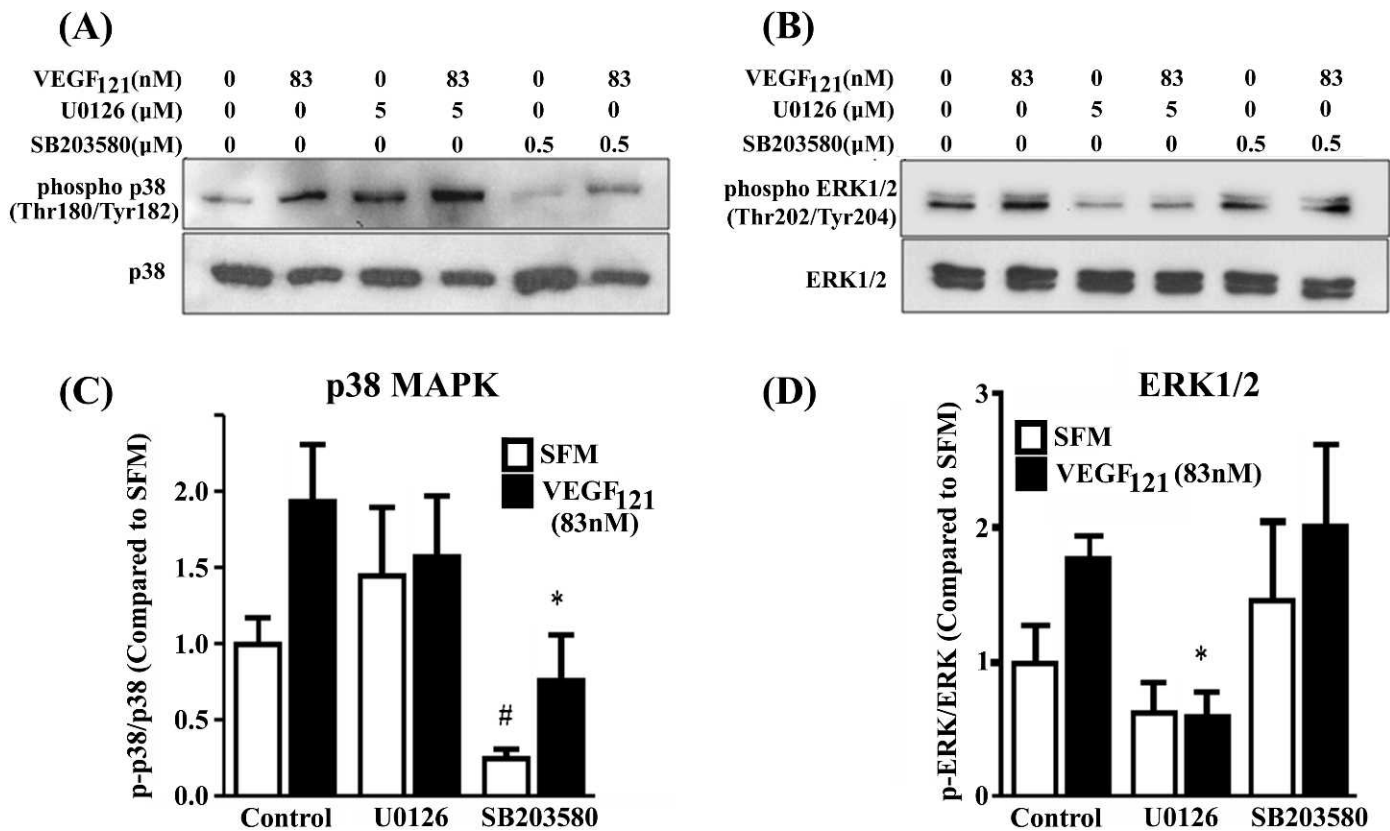


FIG. 10. VEGF stimulation of p38 MAPK and ERK1/2 in EVT cells represents independent pathways. EVT cells were serum starved for 24 h followed by treatment with pharmacological inhibitors SB203580 (0.5 μM) and U0126 (5 μM), for 1 h before stimulation with 10 ng/ml VEGF₁₂₁. Cell lysate proteins were analyzed by Western blot for p38 MAPK activity (A) and ERK1/2 activity (B). Densitometry for p38 activity (C) and ERK1/2 (D) was performed and are presented as means ± SEM for three independent experiments (n = 3). Asterisks (*) indicates significant differences ($P < 0.05$) between the treatment and VEGF control; (#) indicates significant differences ($P < 0.05$) between the treatment and SFM control.

decidual natural killer cells [49] that may include angiopoetins, PlGF, and VEGF-C [55]. Now VEGF-A, another decidual product, can be added to this list. The intrinsic capacity for tube formation by the cells could be due to unknown endogenous stimulants present in GFR matrigel, including components of the basement membrane.

In this study, we demonstrated that VEGF-A stimulated the acquisition of an endothelial phenotype by the EVT cell line, as measured by accelerated tube formation as well as an upregulation of VE-cadherin and β-catenin, and that decorin blocked this stimulation in both cases. VEGFR-2 dependence of VEGF-A-induced upregulation of VE-cadherin/β-catenin was established by knocking down VEGFR-2. That decorin alone at high concentrations (50 nM) suppressed constitutive VE-cadherin/β-catenin levels (Figs. 2 and 3) can be explained by its capacity to antagonize other endogenous ligands, for example, TGFα binding to EGFR expressed by the cells [9]. The present results are similar to the reported anti-angiogenic function of decorin on endothelial cells in vitro. For example, one study revealed that tube formation by endothelial cells was compromised when grown on decorin-coated surfaces [19]. Another laboratory demonstrated that both GAG-free decorin and the LRR5 fragment of the decorin core protein inhibited VEGF-induced tube formation by endothelial cells [22, 23]. This function of the LRR5 peptide is consistent with our findings that certain amino acid sequences within this peptide represent the most avid VEGFR-2 binding site of decorin core protein, antagonizing actions of VEGF-E, an Orf virus-derived member of the VEGF family, that exclusively binds to VEGFR-2 [18].

In the pregnant uterus, VEGF-A is primarily produced by the glandular epithelium and decidual macrophages, and EVT cells expressing VEGF receptors are believed to migrate toward the maternal decidua containing the VEGF-A-producing macrophages [56]. Thus, VEGF-A may be acting as a promoter of migration and endovascular differentiation for EVT cells in situ, and these functions may be spatially regulated by decorin produced by the decidual cells.

Here we define some of the signaling mechanisms responsible for decorin antagonism of VEGF-A-stimulated EVT cell migration and tube formation. We had earlier shown that decorin utilizes multiple tyrosine kinase receptors—EGFR, VEGFR-2, and IGFR-1—to inhibit EVT cell functions [17]. Impediment of native proliferative ability was mediated by EGFR and VEGFR-2, whereas impairment of fibronectin-stimulated EVT cell migration was mediated by IGFR-1 [17]. Based on the above and our recent observations that decorin core protein binds to VEGFR-2 utilizing its LRR5 domain [18], we hypothesized that decorin-mediated inhibition of VEGF-A-stimulated EVT cell migration results from blocking signals downstream of VEGFR-2. VEGF-A has been reported to stimulate cellular migration in other cell types by activating multiple signaling molecules downstream of VEGFR-2, including eNOS [57, 58], FAK [27, 59], p38 MAPK, and ERK1/2 (p44/p42 MAPK) [24, 29, 30]. We have also shown that EVT cell migration stimulated by a variety of other ligands such as IGF-II [35], IGFBP-1 [34], and prostaglandin E2 [39] were mediated through stimulation of ERK1/2.

We found that FAK was constitutively active in our EVT cell line. Furthermore, eNOS was not expressed in these cells

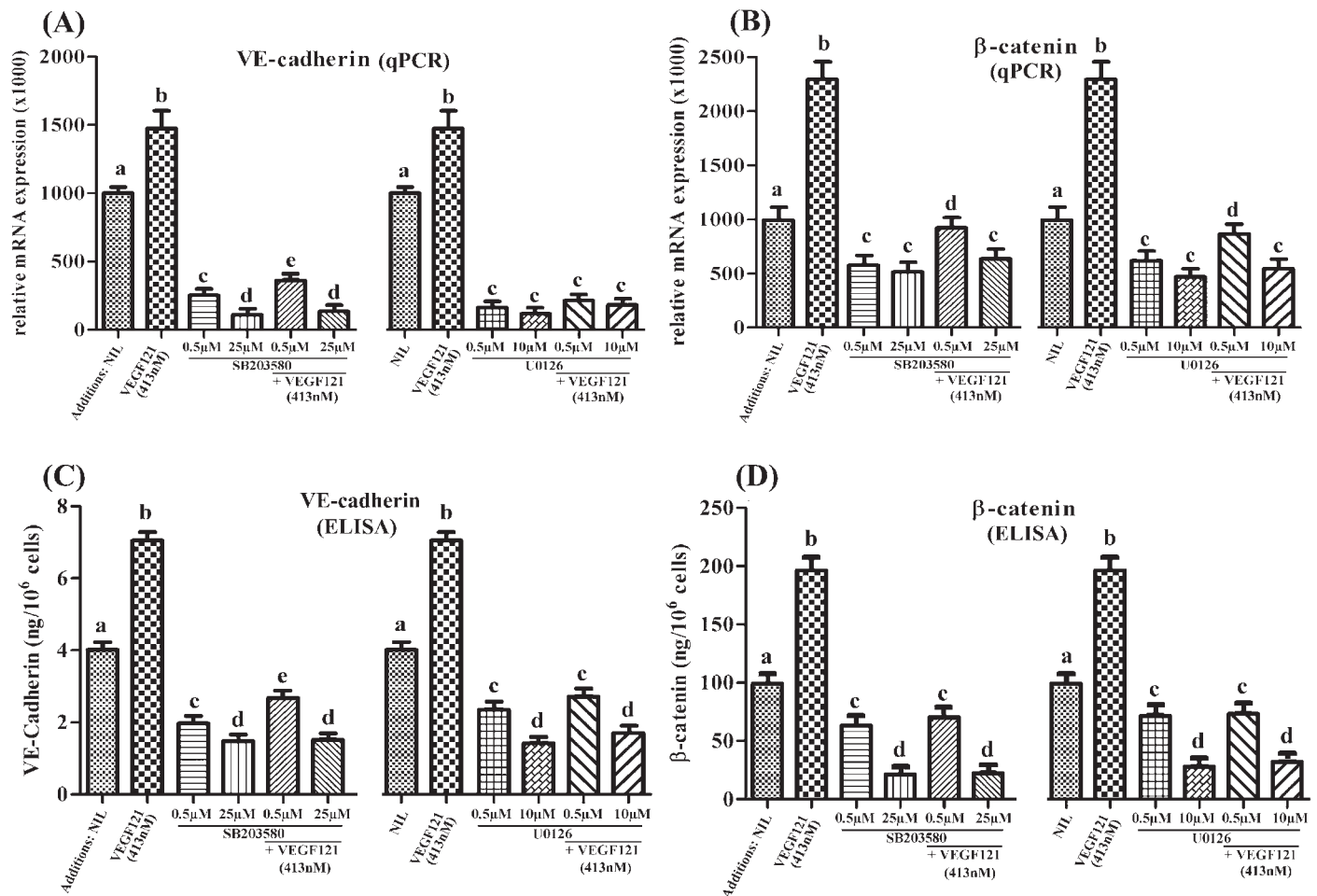


FIG. 11. VEGF-mediated upregulation of VE-cadherin and β -catenin in EVT cells is dependent on p38 MAPK and ERK1/2 pathways. The details of the analyses of RNA by qPCR with RNA and of the protein extracts by ELISA of EVT are given in the *Materials and Methods* section. Each treatment (VEGF-A alone or various concentrations of p38 MAPK or ERK inhibitors followed by VEGF) was done in triplicate for each experiment, and the experiments were conducted twice. The levels of VE-cadherin or β -catenin mRNAs (A and B) relative to GAPDH mRNA were normalized to 1000 for control untreated cells incubated with SFM alone. The protein levels (per unit number of cells) in the treatment groups were expressed relative to control untreated cells (C and D). The data represent mean \pm SEM ($n = 6$). Significant differences ($P < 0.05$) between the means are indicated by different lowercase letters.

under serum-free conditions, and its expression could not be induced by VEGF-A. However, VEGF-A stimulated phosphorylation of MAPK family members p38 MAPK (at Thr-180/Tyr-182), its upstream partner MEK 3/6 (at Ser-189/Ser-207), and ERK1/2 (at Thr-202/Tyr-204) in these cells. We noted that ERK1 was more sensitive than ERK2 during VEGF-A-induced ERK activation in EVT cells. We also showed that blocking p38 MAPK or ERK1/2 using the pharmacological inhibitors SB203580 or U0126, respectively, inhibited VEGF-induced tube formation and upregulation of VE-cadherin/ β -catenin as well as cellular migration. Finally, we established that decorin inhibits VEGF-induced endothelial-like tube formation and upregulation of VE-cadherin/ β -catenin in this EVT cell line as well as their migration, by interfering with p38 MAPK and ERK1/2 activation, and that these two molecules belong to parallel pathways.

Decorin antagonism of ERK activation was primarily mediated through ERK1. Similarly, MEK-6 was more sensitive than MEK3 to both VEGF-A stimulation and decorin antagonism. However, we noted that decorin antagonism of both p38 and ERK1 activation was more efficient at lower than at higher concentrations of decorin. This finding could be due to the possibility that decorin pretreatment at higher concentrations prior to VEGF-A treatment led to enhanced receptor

(VEGFR-2) endocytosis, resulting in a dampened sensitivity for the negative regulation of MAPK phosphorylation. The highly selective p38 MAP inhibitor SB203580 had been reported to block the catalytic activity of p38 MAPK by competing for ATP binding and preventing activation of its downstream substrate without having an effect on p38 phosphorylation at Thr-180/Tyr-182 [60]. In contrast, we noted that this compound reproducibly attenuated VEGF-A-induced p38 phosphorylation (Fig. 10C). We suggest that there is a positive feedback mechanism in regulation of VEGF-induced p38 MAPK activation by some downstream signaling molecule. Nevertheless, our findings of decorin antagonism of VEGF-A-induced activation of both p38 MAPK (Fig. 8) as well as its upstream partner MEK6 (Fig. 9) substantiate the role of this signaling pathway in decorin's action.

The key role of the interaction between VEGF-A and VEGFR-2 in the process of endovascular differentiation in EVT cells was established by the fact that knocking down VEGFR-2 attenuated the VEGF-A-induced upregulation of VE-cadherin/ β -catenin. Based on our results, the signaling pathways involved in the negative regulation of VEGF-A-induced EVT cell migration and endovascular differentiation by decorin are presented schematically in Figure 12.

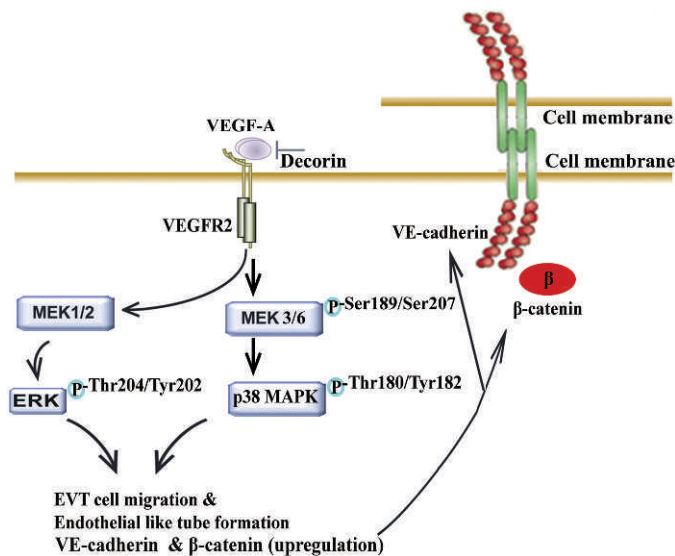


FIG. 12. Schematic illustration of decorin antagonism of pathways in VEGF-A-induced migration and tube formation by EVT cells. Based on our findings of decorin interaction and binding with VEGFR-2 on EVT cells [18], our present findings suggest that decorin, as an antagonistic ligand of VEGFR-2, negatively regulates VEGF-induced migration and tube formation as well as upregulation of VE-cadherin and β -catenin by impeding the activation of p38 MAPK and ERK pathways in parallel.

The present findings of decorin antagonism of VEGF-A-induced EVT cell migration and acquisition of an endovascular phenotype by interfering with VEGF-A-induced activation of MAPKs are relevant to understanding placental physiology and pathology. Preeclampsia is a multifactorial syndrome that affects approximately 2%–3% of pregnant women and is a major cause of maternal and fetal morbidity and mortality [7]. This disease is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype, including VE-cadherin expression [61, 62]. Poor EVT invasion leads to an inadequate remodeling of the endometrial spiral arteries, resulting in poor placental perfusion with maternal blood, which in turn can cause fetal growth restriction and trigger vascular damage in the mother [9]. Increased levels of antiangiogenic molecules such as soluble VEGFR-1 and soluble endoglin [63, 64] have been implicated in the pathogenesis of preeclampsia. Whether decorin overexpression or activity in the decidua may also contribute to this disease by its dual role of antagonizing VEGF actions on both the trophoblast and endothelial cells remains to be investigated.

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