Dentin Matrix Protein 1 induces membrane expression of VE-cadherin on endothelial cells and inhibits VEGF-induced angiogenesis by blocking VEGFR-2 phosphorylation

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DMPI interferes with VEGF-induced angiogenesis

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ABSTRACT

Dentin matrix protein 1 (DMP1) is a member of the Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGs) family, a group of proteins initially described as mineralized extracellular matrices components. More recently, SIBLINGs have been implicated in several key steps of cancer progression, including angiogenesis. Although pro-angiogenic activities have been demonstrated for two SIBLINGs, the role of DMP1 in angiogenesis has not been addressed yet. We demonstrated that this extracellular matrix protein induced the expression of VE-cadherin, a key regulator of intercellular junctions and contact inhibition of growth of endothelial cells that is also known to modulate VEGFR-2 activity, the major high affinity receptor for VEGF. DMP1 induced VE-cadherin and p27\textsuperscript{Kip1} expression followed by cell cycle arrest in human umbilical vein endothelial cells (HUVEC) in a CD44-dependent manner. VEGF-induced proliferation, migration and tubulogenesis responses were specifically blocked upon DMP1 pre-treatment of HUVEC. Indeed, subsequently to VE-cadherin induction, DMP1 inhibited VEGFR-2 phosphorylation and Src-mediated signaling. However, DMP1 did not interfere with bFGF-induced angiogenesis. \textit{In vivo}, DMP1 significantly reduced laser-induced choroidal neovascularization lesions and tumor-associated angiogenesis. These data enable us to put DMP1 on the angiogenic chessboard for the first time and to identify this protein as a new specific inhibitor of VEGF-induced angiogenesis.

\textbf{Keywords:} DMP1 - VEGF - Angiogenesis - VE-cadherin - Contact inhibition - VEGFR-2 - CD44
INTRODUCTION

Dentin matrix protein 1 (DMP1) is a member of the Small Integrin-Binding Ligand N-linked Glycoprotein (SIBLING) genes family, which also includes osteopontin (OPN), bone sialoprotein (BSP) and dentin sialophosphoprotein (DSPP)\textsuperscript{1, 2}. DMP1 was originally considered to be dentin-specific\textsuperscript{3} but later on, its expression was also detected in bone and in non-mineralized tissues such as salivary glands and kidney\textsuperscript{4, 5}. While its precise biological activities have not been identified, this glycoprotein has been mainly associated with the regulation of extracellular matrix mineralization\textsuperscript{6}. We and others have contributed to demonstrate the expression of SIBLINGs in human tumors with OPN and BSP being the two most studied in relation with cancer progression and metastasis development\textsuperscript{1}. Using cancer profiling arrays, Fisher et al. demonstrated the presence of SIBLING mRNAs in different cancers and found that DMP1 was significantly overexpressed in human breast, uterine, colon and lung cancer tissue when compared to normal\textsuperscript{7}. More recently, immunohistochemical studies allowed us to demonstrate the up-regulation of DMP1 in lung and breast human tumors\textsuperscript{8, 9} and DSPP in prostate cancer\textsuperscript{10}.

Angiogenesis is a multistep process in which activated endothelial cells of existing vessels migrate and proliferate in the perivascular stroma to form capillary sprouts. These sprouting endothelial cells stop proliferating, align, form tubes and deposit a basement membrane to finally yield operational new vessels\textsuperscript{11}. Vascular endothelial (VE)-cadherin, the principal cell-cell junction molecule in endothelial cells\textsuperscript{12}, is required for a normal vasculature development in the mouse embryo and for new vessel formation in the adult\textsuperscript{13}. VE-cadherin engagement is associated with the cessation of proliferation commonly known as contact inhibition of growth\textsuperscript{14} where endothelial cells show a reduced proliferative response to specific factors such as vascular endothelial growth factor (VEGF). Binding of VEGF to
vascular endothelial receptor 2 (VEGFR-2) is the principal extracellular signal triggering an angiogenic response and leads to VEGFR-2 dimerization and autophosphorylation. One of the mechanisms of the contact inhibitory activity of VE-cadherin occurs through the modulation of VEGFR-2 signaling\textsuperscript{15}. Src family kinases (SFKs) are involved in VEGFR-2 signaling and the regulation of angiogenesis\textsuperscript{16}. VE-cadherin interacts with C-terminal Src kinase (Csk), an inhibitor of SFKs in vascular endothelial cells. Upon VEGF stimulation, VE-cadherin-Csk complex is disrupted thus allowing the activation of Src kinase and its downstream signaling\textsuperscript{17}. Endothelial cell-matrix interactions mediated by integrins play a critical role in vascular development and angiogenesis\textsuperscript{18}. One characteristic feature of the SIBLINGs is the presence of a highly conserved RGD motif in their primary structure\textsuperscript{2} that is recognized by endothelial integrins. Indeed, DMP1 promotes $\alpha_v\beta_3$-mediated cell attachment and migration\textsuperscript{19}. DMP1 has been also shown to interact with CD44 cell surface receptor\textsuperscript{20}, detectable both on vascular endothelium \textit{in situ} and on cultured human endothelial cells and that is involved in tumor angiogenesis\textsuperscript{21}. While OPN\textsuperscript{22} and BSP\textsuperscript{23} display pro-angiogenic properties through their interaction with endothelial integrins, the question of a potential influence of DMP1 on the behavior of endothelial cells has never been addressed yet.

In the present work, we investigated whether DMP1 is involved in the multistep process required for the formation of new blood vessels by studying the effects of recombinant human DMP1 on human umbilical vein endothelial cells (HUVEC). Our data are the first demonstration of a CD44-dependent function of DMP1 in endothelial morphogenesis through VE-cadherin induction. We show that DMP1 mediated-VE-cadherin increase is accompanied by an arrest of proliferation in sparse HUVEC thus mimicking the contact inhibition of growth that occurs in endothelial cells cultured at high cell density. Because VE-cadherin expression regulates VEGFR-2 signaling, we investigated further the role of DMP1 on VEGF-induced proliferation, migration and tubulogenesis responses. We demonstrate that
DMP1 interferes with each one of these essential events of the angiogenic process most notably by inhibiting VEGFR-2 phosphorylation and modulating Src activity.

Finally, we were able to demonstrate that DMP1 interferes with two in vivo angiogenesis models, one involving wound healing and the other tumor development.
MATERIALS and METHODS

**Cell culture and recombinant proteins.** Human umbilical vein endothelial cells (HUVEC) were isolated and maintained in culture as previously described\(^2^4\). Cells were cultured in MCDB131 medium (Gibco) supplemented with 20% FBS, 2mmol/L L-glutamine, 50µg/ml heparin and 50µg/ml Endothelium Cell Growth Supplement (ECGS) referred as complete medium thereafter. Recombinant human DMP1, VEGF\(^{165}\) and bFGF were purchased from R&D Systems.

**Antibodies.** Anti-p27\(^{Kip1}\) and anti-pRb were from BD Pharmingen. Anti-phospho-p27\(^{Kip1}\)(Ser\(^{10}\)) was from Zymed Laboratories. Anti-Hsc 70, anti-p21, anti-VEGFR-2, anti-phospho-VEGFR-2 and anti-phospho-VEGFR\(^1\) were from Santa Cruz Biotechnology. Anti-VEGFR-1, anti-β-actin were from Sigma-Aldrich. Anti-VE-cadherin, anti-phospho-VE-cadherin(Y685), anti-Csk, anti-PARP and anti-α\(^{\gamma}\)-integrin were from BD Biosciences. Anti-PECAM-1 was from Dako. Anti-phospho-VE-cadherin(Y731)/(Y658) were from Invitrogen. Anti-ZO-1, anti-phospho-Src(Y416)/(Y527) were from Cell Signaling. Anti-DMP1 was from Takara. For blocking experiments, HUVEC were incubated with RGD peptide (GRGDS, Calbiochem), anti-α\(^{\gamma}\)β\(^3\) (LM609), anti-α\(^{\gamma}\)β\(^3\) (P1F6) (Chemicon), anti-CD44 (BU75, Ancell) and mouse purified IgG (Serotec) prior to DMP1 treatment.

**Western blotting.** Equal amounts of proteins were resolved by SDS-PAGE, blotted and membranes were first probed with the indicated antibodies and re-probed with β-actin or Hsc 70 antibodies. Scanned bands were quantified using ImageJ (http://rsb.info.nih.gov/ij/).

**RNA interference.** HUVEC (15x10\(^3\), 6-well plates) were grown overnight in complete medium. Cells were transfected with 100nmol/L of small interfering RNAs (siRNAs) using calcium phosphate precipitation method and cultured for 48h or treated during the last 24h of
transfection in complete medium without ECGS. VE-cadherin siRNAs SMARTpool and control non-targeting siRNAs SMARTpool were purchased from Dharmacon.

**Adhesion Assay.** Bacteriological 96-well plates (Greiner) were coated with DMP1 or vitronectin (Dako) and HUVEC were seeded at 2x10\(^4\) cells per well. After 2h, attached cells were stained with crystal violet and the incorporated dye was measured by reading the absorbance at 560nm.

**Migration assay.** To assess the migration towards DMP1, HUVEC (12x10\(^5\)) were tested in modified Boyden chambers (Neuroprobe Inc), as previously described\(^{23}\). For the other migration assays, HUVEC (10\(^5\)) in serum-free DMEM containing 0.1% BSA were seeded into the upper part of a transwell filter (Becton-Dickinson) and the lower compartment was filled with DMEM containing 1% BSA, 1% FBS and VEGF (2ng/ml) where indicated. After overnight incubation, migrating cells were counted in 3 random fields per well and expressed as the average number of cells per field. Three wells per condition were counted.

**Tubulogenesis assay.** HUVEC (3x10\(^4\), 24-well plates) were seeded on Matrigel (Chemicon) in 2% serum complete medium without ECGS. For VEGF-induced or bFGF-induced tubulogenesis, HUVEC (3x10\(^3\) cells per well) were seeded on Matrigel in 15-well µ-Slide Angiogenesis plates (Integrated BioDiagnostics) in 0,2% serum complete medium without ECGS and treated with the mentioned growth factor in presence of DMP1 where indicated. Tubulogenesis was determined by counting vessels in 2 random fields per well and expressed as the average number of vessels per field.

**Proliferation assay.** HUVEC (2x10\(^4\), 24-well plates) were treated with DMP1 in complete medium without ECGS and grown up to 48h. For VEGF-induced or bFGF-induced proliferation, sparse (2x10\(^4\), 24-well plates) and confluent cells (4x10\(^5\), 24-well plates) were treated with DMP1 in complete medium without ECGS during 24h and then treated with the mentioned growth factor in presence of DMP1 during another 24h in 2% serum complete medium.
medium without ECGS. Fluorimetric DNA titration was performed with Spectramax Gemini XS using SOFTmax PRO software.

**Cell Cycle Analysis.** In order to have better control in the study of the cell cycle arrest, all experiments were performed with synchronized cultures of HUVEC arrested in G0/G1 by serum starvation. Briefly, cells \((15 \times 10^4, 6\text{-well plates})\) were grown overnight in complete medium and were then serum starved during 48h in 2\% serum complete medium without ECGS. During the last 4h of serum starvation, cells were treated with DMP1 or mimosine (Sigma-Aldrich). Cells were serum-released during 20h either with complete medium without ECGS or with VEGF where indicated. The relative percentage of cells in each stage of the cell cycle was analyzed using the Cycle TEST Plus DNA Reagent Kit (Becton-Dickinson).

**Immunocytochemistry.** For double labeling experiments, sparse HUVEC \((3 \times 10^4, 24\text{-well plates})\) were grown on glass coverslips in complete medium. After overnight incubation, cells were treated with DMP1 in complete medium without ECGS during 3 and 24h. Cells were incubated with anti-VE-cadherin followed by Alexa-488-conjugated antibody and TOPRO-3 (Molecular Probes). Images were obtained under a LEICA TCS SP laser scanning confocal microscope (Leica). For BrdU incorporation, sparse or confluent HUVEC prepared as above were treated with DMP1. At 28h from the beginning of DMP1 treatment, BrdU \((20 \mu\text{mol/L})\) (Sigma-Aldrich) was added, and the incubation continued for another 20h. After incubation with anti-VE-cadherin and its secondary antibody, anti-Phalloïdine-Alexa-633 (Molecular Probes) was added. Cells were fixed and subjected to DNA denaturation followed by incubation with an anti-BrdU-Alexa-543 (Sigma-Aldrich). Images were obtained under a laser scanning confocal microscope (Olympus).

**Choroidal Neovascularization (CNV).** Two months old C57bl6 mice (5 per condition) were maintained in a 12h-light–dark cycle with free access to food and water. All animal experiments were performed in compliance with the ARVO Statement for the Use of Animals
in Ophthalmic and Vision Research. CNV was induced by laser burns as described previously\textsuperscript{25}. Eyes were locally anesthetized and subjected to intravitreal injection of DMP1 (500nmol/L) or PBS used as vehicle. At day 7 post-laser burn, mice were intravenously injected with FITC-dextran (2,000,000 Da, Sigma) before sacrifice. Eyes were fixed in paraformaldehyde at room temperature. Retinae were discarded and the choroids were flat mounted in Vectashield medium (Vector Laboratories) for epifluorescence microscopy analysis. Quantitation was realized by measuring total vessel fluorescence surface (ImageJ) as we previously described\textsuperscript{26}.

**Experimental glioma model.** Human glioma cells U87-MG were maintained in MEM with 10% FBS, 2mM L-glutamine, 1% non essential amino acid (NEAA) and 1mM sodium pyruvate. Cells (45x10\textsuperscript{5}, petri dishes) were grown overnight and transfected with 1µg/ml of plasmid using Lipofectamine\textsuperscript{TM} 2000 (Invitrogen) according to the manufacturer’s instructions during 24h before implantation on CAM. DMP1 expression plasmid (pDMP1) and its control empty plasmid (pEmpty) were purchased from GeneCopoeia. Transfected U87-MG cells (5x10\textsuperscript{6}) were deposited on the CAM of fertilized day 10 eggs and tumors developed after 5 days were harvested as we described previously\textsuperscript{27}. Tumor volumes were calculated as described previously\textsuperscript{28}. Tumors were fixed with 4% paraformaldehyde, and processed for cryo-sectioning. Tissue sections were stained with hematoxylin and eosin. The vessels were stained using fluorescein-coupled *Sambucus nigra* Lectin SNA-1 (FL-1301, Vector). For determination of DMP1 protein expression by CAM tumors, control and DMP1 tumors at day 5 (pool of 3-5 tumors/condition) were homogenized in lysis buffer (Sigma-Aldrich) and extracts were submitted to western blot analysis.

**Statistical Analysis.** Student’s $t$ test was used to compare differences between experimental conditions. A $p$ value $< 0.05$ was considered as statistically significant. The analyses were carried out using the Statistica software (Statsoft).
RESULTS

DMP1 induces HUVEC adhesion, migration and differentiation but inhibits proliferation

HUVEC adhered to DMP1 to a similar extent than to the other RGD-containing protein, vitronectin, while they did not adhere to control BSA. The addition of an RGD peptide completely inhibited adhesion to DMP1, suggesting that DMP1 mediates HUVEC attachment through molecular interaction with integrins (Figure 1A). When placed in the lower chamber of a modified Boyden chamber, DMP1 stimulated HUVEC migration (Figure 1B). To determine the importance of a concentration gradient for the observed migratory response, cell migration was evaluated when DMP1 was placed either in the top chamber only or in both chambers. DMP1 has chemotactic properties, inasmuch as placing this molecule in both chambers at the same concentration reduced maximal migration by 34%. However, because the absence of a concentration gradient did not totally abolish cell migration, we can also consider that DMP1 exhibits chemokinetic ability towards endothelial cells. We next showed that DMP1-treated HUVEC were less proliferative than control cells (Figure 1C). We assessed by Annexin-V assay (Figure S1A) and a western blot to PARP (Figure S1B) that DMP1-treated cells were not apoptotic.

Then, we tested the impact of DMP1 on tubulogenesis in a Matrigel in vitro assay. As early as 1h after seeding, we observed that DMP1-treated endothelial cells spread and attached on Matrigel when compared to control. Vessels counting after 4h demonstrated that DMP1-treated HUVEC rapidly formed a tubular network still maintained after 24h (Figure 1D). Altogether these data indicate that DMP1 could act as a pro-differentiating factor for HUVEC and contributes to the organization and/or stability of developing endothelial tubular networks.

DMP1 blocks the cell cycle in G1, modulates the expression of cell cycle-related proteins and induces p27Kip1 through CD44 ligation
We next investigated whether the decrease of HUVEC proliferation was due to an arrest of the cell cycle. Synchronized HUVEC were released with medium containing 20% serum in presence of DMP1 or mimosine, an inhibitor of DNA replication leading to mammalian cell cycle arrest in G1-phase used as control. DMP1 treatment significantly impaired cell cycle progression after serum release, with an accumulation of HUVEC in the G1-phase from 70 to 82% and a decrease of S-phase cell population from 17 to 5% (Figure 2A).

To investigate whether the DMP1 effect on the cell cycle was mediated through an interaction with $\alpha_{\text{V}}\beta_3$ or CD44, we used specific blocking antibodies. In DMP1-treated cells, the S-phase population was decreased to 28% when compared to the control S-phase cell population arbitrarily set as 100%. However, cells pre-treated with anti-CD44 proved to be able to enter the S-phase, with the population of S-phase cells being significantly increased to 74%. No significant changes were observed with cells pre-treated with anti-$\alpha_{\text{V}}\beta_3$ or control IgG (Figure 2B). Thus, the inhibitory effect of DMP1 on the cell cycle is mediated, at least in part, through CD44 receptor and not $\alpha_{\text{V}}\beta_3$.

The cyclin dependent kinase (CDK) inhibitors p21$^{\text{Cip1}}$ and p27$^{\text{Kip1}}$ can bind and inhibit the kinase activities of several cyclin-CDK complexes and arrest cell growth at G1/S boundary. The expression of p27$^{\text{Kip1}}$ was induced by DMP1 in a dose-dependent manner (Figure 2C) whereas the expression of p21$^{\text{Cip1}}$ was unaffected (Figure 2D). Consistent with the cell cycle arrest observed in DMP1-treated HUVEC, these cells showed a significant decrease of phospho-pRb (Figure 2E). The importance of p27$^{\text{Kip1}}$ in DMP1-induced effect on HUVEC growth was further demonstrated by the use of specific siRNAs. We found that DMP1 treatment failed to inhibit the proliferation of p27$^{\text{Kip1}}$ silenced-HUVEC when compared to either mock- or irrelevant siRNA transfected cells (Figure S2).

We next demonstrated that p27$^{\text{Kip1}}$ induction was abolished when cells were treated with anti-CD44 prior to DMP1 treatment while significant p27$^{\text{Kip1}}$ induction was still
observed in cells pre-treated with anti-αβ3 or control IgG (Figure 2F). Our data are consistent with CD44 being the key receptor involved in mediating the observed effects of DMP1 on cell cycle arrest and on the increase of p27Kip1 expression in HUVEC. Regarding p27Kip1, it has been shown that the phosphorylation on serine 10 increases its stability. DMP1 treatment of HUVEC rapidly induced a significant increase of phospho-p27Kip1 (Ser10) while total p27Kip1 level was unchanged (Figure 2G). After 24h, total p27Kip1 level also increased, suggesting its accumulation over time.

**DMP1 induces CD44-dependent VE-cadherin expression and mediates inhibition of growth in sparse HUVEC**

VE-cadherin is a major homophilic cell-to-cell adhesion molecule involved in the control of blood vessel formation and contact inhibition of endothelial cell growth. Using immunofluorescence, we demonstrated that DMP1 treatment significantly induced the expression of VE-cadherin at the cell membrane already after 3h (Figure 3A). Western blot performed on membrane extracts showed a two-fold increase of VE-cadherin expression level in DMP1-treated HUVEC (Figure 3B). FACS analysis confirmed this increase whereas DMP1 did not affect surface expression of other endothelial cell-cell junction protein expression such as zonula-occludens-1 (ZO-1) and PECAM-1 (Figure S3).

It has been previously reported that N-Cadherin and E-cadherin mediated signaling is involved in contact inhibition of growth by inducing p27Kip1 expression and cell cycle arrest at the G1-phase. However, no direct evidence of an effect of VE-cadherin on p27Kip1 expression in endothelial cells has been reported to this date. To explore this possibility, we transfected VE-cadherin siRNAs in HUVEC prior to their treatment with DMP1. VE-cadherin-silenced cells did not show an increased p27Kip1 expression upon DMP1 treatment when compared to cells transfected with non-targeting siRNAs (Figure 3C), indicating that VE-cadherin expression is essential to DMP1-mediated p27Kip1 up-regulation and subsequent
growth control of HUVEC. Then, we evaluated the involvement of CD44 on the observed DMP1-induced VE-cadherin up-regulation. As shown in Figure 3D, VE-cadherin induction is abolished when HUVEC were treated with anti-CD44 prior to DMP1 treatment, but not with control IgG. Our data pointed to CD44 as a major actor of VE-cadherin expression increase, p27$^{\text{Kip1}}$ induction and cell cycle arrest induced by DMP1 in HUVEC.

VE-cadherin induction has been shown to control contact inhibition of growth in endothelial cells$^{14}$. Using sparse and confluent HUVEC, we addressed the question of a contact inhibition of growth signal mimicry occurring upon DMP1 treatment. We showed that DMP1 induced the expression of VE-cadherin and p27$^{\text{Kip1}}$ in sparse cells while, as expected, high density cells did not show any modulation of expression of both proteins (Figure 3E). We then evaluated VE-cadherin staining by immunofluorescence in parallel with BrdU incorporation in sparse and confluent HUVEC. A large proportion of sparse HUVEC were BrdU positive when compared to confluent cells (Figure 3F). DMP1-treated sparse cells showed a positive VE-cadherin staining and less BrdU incorporation than control sparse cells, a pattern similar to that of control or DMP1-treated confluent cells. Since confluent HUVEC established VE-cadherin–dependent junctions and underwent growth arrest, they did not show any modulation of their VE-cadherin staining intensity and the proportion of BrdU-positive cells was averagely the same in both control and DMP1-treated conditions. These observations indicate that DMP1 induced contact inhibition of growth signal mimicry on sparse endothelial cells.

**DMP1 counteracts VEGF-induced angiogenesis**

Knowing that VEGF is essential for angiogenic processes both in normal and pathological conditions$^{33}$ and based on our previous observations, we decided to investigate the effects of DMP1 on VEGF-induced proliferation, migration and tubulogenesis. Sparse and confluent cells were treated with DMP1 during 24h and then with VEGF another 24h. We observed that
DMP1 pre-treatment impaired VEGF-induced sparse cell proliferation in a dose-dependant manner when compared to VEGF-treated sparse cells (Figure 4A) while it did not affect confluent cells. We next studied the impact of DMP1 on VEGF-induced cell cycle progression. Synchronized HUVEC responded to VEGF release by a significant entry in S-phase when compared to control cells released with serum. However, the S-phase cell population corresponded to that of non released cells upon DMP1 treatment (Figure 4B). The migration of endothelial cells is a critical step during vessel formation. We showed that DMP1 impaired migration of cells towards VEGF when compared to control cells (Figure 4C).

To finally assess the effect of DMP1 on VEGF-induced angiogenesis, we tested the impact of DMP1 on VEGF-induced tubulogenesis. After 6h, we observed that DMP1 treatment impaired VEGF-induced tubular network formation on Matrigel (Figure 4D). Interestingly, these DMP1 effects appear to be specific to VEGF-induced angiogenesis, as DMP1 did not interfere with bFGF-induced proliferation (Figures S4A) and tubulogenesis (Figure S4B).

**DMP1 inhibits VEGFR-2 phosphorylation through the induction of VE-cadherin expression**

At the surface of endothelial cells, VEGFR-2 has been identified as the major mediator of VEGF-dependent signaling and cellular activities. VEGFR-2 expression level was significantly increased while its phosphorylation was completely inhibited upon DMP1 treatment. Under the same conditions, DMP1 did not show any significant effect on VEGFR-1 expression nor phosphorylation (Figure 5A).

VE-cadherin has been shown to control contact inhibition of endothelial cell growth by inhibiting VEGFR-2 phosphorylation, notably through the recruitment of specific phosphatases. Therefore, we postulated that the observed decrease of VEGFR-2 phosphorylation could be subsequent to the induction of VE-cadherin expression following
DMP1 treatment. To test this hypothesis, we inhibited VE-cadherin expression in HUVEC prior to the addition of DMP1. VE-cadherin-silenced cells did not show any significant decrease of VEGFR-2 phosphorylation upon DMP1 treatment when compared to cells transfected with non-targeting siRNAs (Figure 5B) indicating that VE-cadherin expression is indispensable to DMP1-mediated VEGFR-2 phosphorylation inhibition.

**DMP1 blocks VEGF-induced VEGFR-2 phosphorylation and inhibits Src activation**

VEGFR-2 receptor is expressed and phosphorylated upon activation by its ligands. Therefore, we next evaluated whether DMP1 could impair VEGF influence on VEGFR-2. HUVEC were treated with DMP1 during 24h and challenged with VEGF (50ng/ml) during 10 minutes. DMP1 pretreatment not only induced VEGFR-2 expression but also completely impaired its phosphorylation in presence of VEGF (Figure 5C).

Src family kinases (SFKs) are involved in VEGFR-2 signaling in the context of angiogenesis. Src possesses two sites of tyrosine (Tyr) phosphorylation that are critical to the regulation of its kinase activity. Autophosphorylation of Tyr416 (chicken c-Src numbering) increases its catalytic activity, while phosphorylation of the C-terminal Tyr527 inhibits it. To exert its pro-angiogenic activities, VEGF activates Src through phosphorylation of Tyr416 and dephosphorylation of Tyr527. Upon DMP1 addition, the phosphorylation of Src Tyr416 was reduced to almost basal level and that of Tyr527 was increased (Figure 5D), indicating that DMP1 counteracts VEGF-triggered Src activation.

**DMP1 inhibits VEGF-mediated VE-cadherin down-regulation and phosphorylation**

It is known that VE-cadherin undergoes both a decrease of expression and an inactivation through tyrosine phosphorylation by VEGF. DMP1 impaired VEGF-mediated decrease of VE-cadherin expression (Figure 5E). As expected, VEGF induced the phosphorylation of VE-cadherin on Tyr731, 658 and 685 while DMP1 alone did not affect its phosphorylation status.
Interestingly, DMP1 treatment specifically reversed VEGF-induced VE-cadherin phosphorylation on Tyr685 (Figure 5E).

It has been shown that Src phospho-Tyr527 level is dictated by the activities of C-terminal Src Kinase (Csk)\textsuperscript{37} which is recruited at the membrane by VE-cadherin. Accordingly, we found that DMP1 induced the expression of Csk in HUVEC sparse cultures and as such mimicked the induction of Csk that occurs in confluent cells (Figure 5F). Finally, we observed that, in good accordance with its Src kinase promoting activity, VEGF induced a significant decrease of Csk while DMP1 treatment proved to be able to counteract this effect (Figure 5G). The ability of DMP1 to block VEGF-triggered Src activation through the induction of Csk expression is consistent with its potent antagonistic role on VEGF-induced angiogenesis.

\textbf{DMP1 inhibits angiogenesis in the choroidal neovascularization model in mice}

To further investigate the anti-angiogenic effect of DMP1, we took advantage of the laser-induced choroidal neovascularization (CNV) model in which VEGF has been shown to be a major stimulator of subretinal angiogenesis\textsuperscript{38}. In this experiment, mice were injected intravitreally with DMP1 (500nmol/L) on the day of the laser injuries. After 7 days, the whole-mount choroids were stained in order to reveal neoformed blood vessels. As shown in Figure 6A, DMP1 significantly limited the size of neovascularized ocular lesions when compared to control lesions. Quantification of the lesions showed a 30% decrease upon DMP1 treatment (Figure 6A).

\textbf{Overexpression of DMP1 in glioma tumor cells decreases tumor growth and tumor-angiogenesis in vivo}

Hagedorn et al.\textsuperscript{28} have established a robust and highly reproducible \textit{in vivo} human tumor model that allows fast and precise analysis of the main steps of tumor progression and tumor-associated angiogenesis where human glioma cells U87-MG grafted onto the vascularized...
chicken CAM develop into a tumor within a short period of time. DMP1-overexpressing U87-MG cells developed into tumors that were smaller and less vascularized, as they appeared much more white, than fully vascularized control tumors (Figure 6B). H&E as well as specific vasculature immunostaining confirmed that DMP1-overexpressing tumors were significantly less angiogenic than control tumors (Figures 6C and 6D, respectively). The estimation of tumor volume demonstrated that the experimental gliomas were significantly smaller upon DMP1 overexpression (Figure 6E). DMP1 western blot on glioma tumor extracts validated the overexpression of DMP1 in vivo (Figure 6F).
DISCUSSION

Angiogenesis is the process by which new blood vessels are formed from pre-existing vasculature. The present study reports for the first time the specific functional responses elicited by DMP1 on human endothelial cells and demonstrates a novel biological role for this SIBLING protein during the angiogenic process. Both receptors known to bind DMP1 are expressed on HUVEC and have been implicated in critical endothelial cell functions. Here, we show that CD44 ligation is responsible for DMP1-induced cell cycle blockade. The mechanism by which DMP1 inhibits endothelial cell growth implicates, at least in part, a CD44-dependent up-regulation of p27Kip1. The ligation of CD44, by either a specific monoclonal antibody or its preferential ligand hyaluronan, has previously been associated with cell cycle control via p27Kip1 regulation in leukemic cells39. It is noteworthy that DMP1-mediated up-regulation of p27Kip1 in HUVEC is subsequent to specific VE-cadherin induction. This new finding is in accordance with previous reports showing that E- and N-cadherin mediate anti-proliferative response, in the context of contact-induced inhibition of cell growth, through p27Kip1 up-regulation31, 32, 40. DMP1 increases VE-cadherin surface expression in sparse HUVEC thereby inducing a mimicry of contact inhibition of growth mechanism exemplified further by the entry of the cells in G1 phase of the cell cycle.

Beside the control of contact-induced inhibition, VE-cadherin is also an important player of capillary tube formation, a specialized endothelial cell function41. Indeed, cells lacking VE-cadherin are unable to initiate in vitro morphogenesis, defined here as the process whereby endothelial cells assemble into cell cords in a 2D culture (Matrigel)42. DMP1-treated HUVEC demonstrate a precocious and sustained morphogenesis that is in good correspondence with the reduced cell division and the enhanced attachment and migratory responses observed in presence of DMP1.
Our exploration of endothelial cell functional responses induced by DMP1 in presence of VEGF includes cell migration and proliferation, which are essential for more complex processes such as formation of the endothelial tube network during angiogenesis in vitro and in vivo. Pre-treatment of HUVEC with DMP1 significantly blocks all these responses and let us envisage DMP1 as a new specific inhibitor of VEGF-induced angiogenesis since DMP1 does not interfere with bFGF-induced proliferation nor tubulogenesis.

The other major finding of this study is the specific inhibition of VEGFR-2 activity by DMP1. Thus, in presence of DMP1 (Figure 7, right panel), HUVEC do not respond anymore to VEGF stimulus. Even though VEGFR-2 expression is increased as a consequence of VE-cadherin induction\textsuperscript{43}, its phosphorylation does not occur anymore and VEGF-induced Src kinase activation is counteracted in DMP1-treated cells. The role for VE-cadherin in modulating downstream signaling of VEGF has been largely recognized\textsuperscript{13, 15, 44}, and in turn, VEGF-activated Src kinase phosphorylates VE-cadherin and makes it inactive in the control of endothelial functions\textsuperscript{36} (Figure 7, left panel). DMP1 specifically impairs VEGF-induced Tyr685 phosphorylation through Src activity which has been indeed exclusively associated with the phosphorylation of this tyrosine\textsuperscript{45}. Supporting this, DMP1 does not affect VEGF-induced VE-cadherin phosphorylation on other tyrosines such as Tyr658 and Tyr731. Concerning the repression of Src, it is known to be dependent on the activity of Csk when the latter is bound to VE-cadherin Tyr685\textsuperscript{17}. It is noteworthy that (a) DMP1 partially reverses VEGF-induced Tyr685 phosphorylation which would still allow Csk binding to VE-cadherin and (b) induces Csk expression that inactivates Src despite the presence of VEGF. We found that Csk, which has also been involved in cadherin-driven proliferation arrest at high density\textsuperscript{17}, is expressed at high level both in confluent and DMP1-treated HUVEC. This observation adds weight to DMP1-induced mimicry of contact inhibition of growth discussed above.
In a previous study, we have investigated DMP1 expression by immunohistochemistry in human breast cancer tumors\(^9\). SIBLING proteins expression being generally associated with bad prognosis and poor survival for cancer patients\(^1\), we have been a little puzzled by the observation that patients with tumors expressing high levels of DMP1 presented with a better survival than patients with low DMP1-expressing tumors. In this study, we observed a striking impact of DMP1 on tumor growth and tumor-related angiogenesis \textit{in vivo}. In fact, DMP1 inhibitory effect on both processes was comparable to that of siRNAs directed against VEGF as assessed using the same \textit{in vivo} model in a previous study\(^27\). In light of these new data, it is tempting to speculate that high-DMP1 expressing tumors may be associated with limited neovessel formation as tumor-secreted DMP1 could favor endothelial cells differentiation at the expense of their proliferation. Arguing for this possibility is the observation that high-DMP1 expressing human tumors were consistently small sized\(^9\).

In conclusion, we demonstrate for the first time that DMP1 is implicated in endothelial cell morphogenesis \textit{in vitro} indicating that secreted ECM proteins are endowed with specific functions that influence the dynamic balance controlling vessel growth. The other picture of this study is that DMP1 induces the up-regulation of VE-cadherin and VEGFR-2 inactivation thus specifically leading to impaired VEGF-mediated wound healing and tumor-associated angiogenesis \textit{in vivo}. 
ACKNOWLEDGEMENTS

S. Pirotte is a Télévie Research Fellow, S. Ormenese is a Logistic Research Worker, D. Mottet is a Research Associate, and A. Bellahcène is a Senior Research Associate, all from the National Fund for Scientific Research (FNRS). This work was supported by grants from FNRS, Télévie, Centre Anti-Cancéreux, Fonds Léon Fredericq, D.G.T.R.E. and SPW-DG06 Convention n°0616476 “NéoAngio” from the “Région Wallonne”, the European Union Framework Program FP7-projects and the University of Liège. The authors acknowledge P. Heneaux, V. Hennequière and N. Maloujahmoum for their expert technical assistance.
AUTHORSHIP CONTRIBUTIONS AND DISCLOSURE OF CONFLICTS OF INTEREST

S. Pirotte designed and performed the research, analyzed the data and wrote the manuscript; V. Lamour designed and performed the CAM model, analyzed the data and reviewed the manuscript; V. Lambert and M-L. Alvarez Gonzalez designed and performed the CNV model, analyzed the data and reviewed the manuscript; S. Ormenese provided microscopy expertise and reviewed the manuscript; A. Noël designed the CNV model and critically reviewed the manuscript; D. Mottet analyzed the data and critically reviewed the manuscript; V. Castronovo designed the research, analyzed the data and critically reviewed the manuscript; A. Bellahcène designed the research, analyzed the data and wrote the manuscript.

The authors declare no competing financial interests.
REFERENCES


FIGURE LEGENDS

Figure 1. DMP1 induces the adhesion, the migration and the differentiation of HUVEC in vitro but decreases their proliferation. (A) Cells were plated onto DMP1 (50 nmol/L) or incubated 1 hour in the presence of 50 nmol/L of RGD peptide and then plated onto DMP1 (50 nmol/L). Vitronectin (50 nmol/L) and BSA 1% were used as positive and negative control, respectively. Cells were allowed to adhere for 2 hours at 37°C and were quantified as described in Materials and Methods. Error bars represent the mean ± SD of 6 replicates of a representative experiment (n=2). **, p ≤ 0.005 versus control. n.s. indicates not significant. (B) Modified Boyden chamber chemotaxis assays were performed on HUVEC with DMP1 (100 nmol/L) placed in the bottom chamber (DMP1 bottom), in the top chamber with the cells (DMP1 top), or in both the top and bottom chambers (DMP1 both). Each bar represents mean ± SD of total number of migrated cells within 4 replicates (n=2). (C) HUVEC were treated with DMP1 (1 nmol/L to 100 nmol/L) and proliferation was followed during 24 and 48 hours and scattered as described in Materials and Methods. Error bars represent the mean ± SD of 3 replicates of a representative experiment (n=3). **, p ≤ 0.001 and ***, p ≤ 0.0001 versus control. n.s. indicates not significant. (D) Capillary tube-like assay using HUVEC treated with DMP1 (50 nmol/L) during 24 hours and then cultured on Matrigel during 1, 4, 8 hours (100x) and 24 hours (40x). Phase contrast microscopy photomicrographs were taken at each time. Shown below, the quantification of the assay was realized by counting the number of vessels from 2 representative fields from 2 replicates (n=3). **, p ≤ 0.005 versus control. n.s. indicates not significant.

Figure 2. DMP1 blocks the cell cycle in G1, modulates the expression of cell cycle-related proteins and induces p27^{Kip1} through CD44 ligation. (A) Cell cycle analysis of serum
starved HUVEC treated with DMP1 (50 nmol/L) and mimosine (200 µmol/L). Mimosine and non serum released cells were used to assess for G1 arrest. Error bars represent the mean ± SD of 3 replicates of a representative experiment (n=3). **, \( p \leq 0.001 \) and ***, \( p \leq 0.0005 \) versus control serum released cells. n.s. indicates not significant. (B) S-phase cell cycle analysis of serum starved HUVEC incubated with blocking antibodies to CD44 and \( \alpha \nu \beta_3 \), and IgG used as control followed by treatment with DMP1 (50 nmol/L) as mentioned in Materials and Methods. Error bars represent the mean ± SD of 3 replicates of a representative experiment (n=2). **, \( p \leq 0.001 \) versus control or DMP1 condition. n.s. indicates not significant. (C) Western blot analysis with an antibody to \( p27^{\text{Kip1}} \) using total lysates from HUVEC treated with increasing concentrations of DMP1. (D) Western blot analysis with antibodies to \( p21^{\text{Cip1}} \) using total lysates from DMP1-treated cells. (E) Western blot analysis with antibodies to pRb using total lysates from DMP1-treated cells. (F) Western blot analysis with an antibody to \( p27^{\text{Kip1}} \) using total lysates from DMP1-treated cells. Prior to DMP1 treatment (50 nmol/L) during 24 hours, cells were incubated during 1 hour in the presence of 10 µg/mL of anti-CD44 and anti-\( \alpha \nu \beta_3 \) blocking antibodies or IgG used as control. (G) Western blot analysis with antibodies to \( p27^{\text{Kip1}} \) and P-\( p27^{\text{Kip1}}(\text{Ser}^{10}) \) using total lysates from DMP1-treated HUVEC. All Western blotting results were evaluated by densitometric scanning, corrected with respect to \( \beta \)-actin expression, and expressed relative to the value obtained with the corresponding control (arbitrarily set as 1). These relative protein level values are shown in italics below the lanes. Western blots were realized at least 2 times with similar results. Equal protein loading was assessed by anti-\( \beta \)-actin immunoblotting.

**Figure 3.** DMP1 induces CD44-dependent VE-cadherin expression and mediates inhibition of growth in sparse HUVEC. (A) Immunofluorescence microscopy of sparse HUVEC treated with DMP1 (50 nmol/L) during 3 and 24 hours. Nuclei appear blue after
TOPRO-3 staining. Representative confocal fields of one experiment (n=3) are shown (Magnification 40x). (B) Western blot analysis with antibodies to VE-cadherin and αV-integrin using membrane lysates from DMP1-treated HUVEC. (C) Western blot analysis with antibodies to p27Kip1 and VE-cadherin using total lysates from HUVEC transfected during 48 hours with VE-cadherin or non-targeting siRNAs and treated with DMP1 (50 nmol/L) during the last 24 hours of transfection. (D) Western blot analysis with an antibody to VE-cadherin using total lysates from HUVEC incubated during 1 hour in the presence of 10 µg/mL of anti-CD44 blocking antibody or IgG prior to DMP1 treatment (50 nmol/L) during 24 hours. (E) Western blot analysis with antibodies to VE-cadherin and p27Kip1 using total lysates from sparse and confluent treated HUVEC as mentioned in Materials and Methods. All Western blotting results were evaluated by densitometric scanning. These relative protein level values are shown in italics below the lanes. Western blots were realized 3 times with similar results. Equal protein loading was assessed by anti-β-actin immunoblotting. (F) Immunofluorescence of sparse and confluent DMP1-treated HUVEC incubated with BrdU during 20 hours to evaluate the S-phase population as mentioned in Materials and Methods. VE-cadherin, BrdU and phalloïdine are shown in green, red and gray, respectively. As expected, control sparse cells presented with S-phase-positive and VE-cadherin-negative staining when compared with control confluent cells. DMP1-treated sparse cells showed a strong positive VE-cadherin staining and less BrdU incorporation than control cells, similarly to that of control or DMP1-treated confluent cells. Confluent cells did not show any modulation of their VE-cadherin staining intensity or BrdU incorporation upon DMP1 treatment. Representative confocal fields of one experiment (n=3) are shown (Magnification 40x).

Figure 4. DMP1 counteracts VEGF-induced angiogenesis. (A) Proliferation was assessed in sparse and confluent cells treated with DMP1 during 48 hours. During the last 24 hours of
DMP1 treatment, cells were treated with VEGF 50 ng/ml as mentioned in Materials and Methods. Error bars represent the mean ± SD of 3 replicates of a representative experiment (n=3). **, p ≤ 0.005 and ***, p ≤ 0.0005 versus control or versus VEGF. n.s. indicates not significant. (B) S-phase cell cycle analysis of serum starved HUVEC treated with DMP1 (50 nmol/L) and released with either serum or VEGF (50 ng/ml). Error bars represent the mean ± SD of 3 replicates of a representative experiment (n=3). ***, p ≤ 0.0005 versus control. (C) Assessment of HUVEC migration towards VEGF (2 ng/ml) of cells treated with DMP1 (50 nmol/L) during 24 hours and then seeded into the upper compartment of fibronectin coated inserts as mentioned in Materials and Methods. Error bars represent the mean ± SD of 3 replicates of a representative experiment (n=3). **, p ≤ 0.005 versus VEGF condition. (D) Capillary tube-like assay of HUVEC treated with DMP1 (100 nmol/L) during 24 hours and then cultured on Matrigel and treated with VEGF (25 ng/ml). Phase contrast microscopy photomicrographs were taken after 4 hours and representative fields from one replicate out of 2 from one experiment is shown (n=2). Scale bar = 400µm.

**Figure 5. DMP1 affects VEGFR-2, and not VEGFR-1, phosphorylation, induces Src inactivation and inhibits VEGF-mediated VE-cadherin down regulation and phosphorylation.** (A) Western blot analysis with antibodies to VEGFR-2 and VEGFR-1 and their phosphorylated forms using total lysates from HUVEC treated with DMP1 during 24 hours. (B) Western blot analysis with antibodies to P-VEGFR-2 and VE-cadherin using total lysates from HUVEC transfected during 48 hours with VE-cadherin or non-targeting siRNAs and treated with DMP1 (50 nmol/L) during the last 24 hours of transfection. (C) Western blot analysis with antibodies to VEGFR-2 and P-VEGFR-2 using total lysates from HUVEC treated with DMP1 during 24 hours and then pulsed with VEGF (50 ng/ml) for a further 10 minutes. (D) Western blot analysis with specific antibodies to Src, P-Src Tyr 416 and P-Src...
Tyr 527 using total lysates from HUVEC treated with DMP1 and VEGF as in C. (E) Western blot analysis with antibodies to VE-cadherin and P-VE-cadherin Tyr 658, Tyr 731 and Tyr 685 using total lysates from HUVEC treated with DMP1 and VEGF as in C. (F) Western blot analysis with an antibody to Csk using total lysates from sparse and confluent HUVEC treated with DMP1 during 24 hours. (G) Western blot analysis with an antibody to Csk using total lysates from HUVEC treated with DMP1 and VEGF as in C. All Western blots were evaluated by densitometric scanning (in italics below the lanes) and were realized 3 times with similar results. Equal protein loading was assessed by anti-β-actin or anti-Hsc 70 immunoblotting.

Figure 6. DMP1 impairs in vivo angiogenesis in the CNV model and its overexpression decreases glioma tumor growth and tumor angiogenesis in vivo. (A) Mice were injured by laser shots onto the retina (areas within the dotted lines) and were then subjected to intravitreous injection of DMP1 (500 nmol/L). Eyes were removed after 7 days and the angiogenic response was measured as described in Materials and Methods. The quantification represents the measure of total vessel fluorescence surface or each impact. Error bars represent the mean ± SEM of 20 impacts of a representative experiment (n=2). *, p ≤ 0.05 versus control vehicle. (B) The growth of both DMP1-overexpressing (pDMP1) and control (pEmpty) tumors on the chick embryo chorioallantoic membrane (CAM) was documented by biomicroscopy at day 5 after U87-MG cell grafting. A representative tumor for each condition is shown. DMP1 tumors appeared completely white when compared to control, in which blood vessels can be seen under the surface. (C) H&E photos of the tumors shown in B were taken right under the surface of the tumor. Magnification 40x. Numerous irregular and dilated capillaries (black arrows in the enlarged insert) were visible in control pEmptyy tumors but not in DMP1-overexpressing tumors. (D) Specific FITC-lectin staining was used for visualization
of blood vessels in the tumors shown in B confirmed the avascular phenotype of DMP1-overexpressing tumors when compared to control pEmpty tumors. Nuclei appear blue after TOPRO-3 staining. Magnification 40x. (E) Tumor volume was calculated 5 days after grafting for each condition as indicated in Materials and Methods section. DMP1 overexpression induced a significant decrease of tumor volume when compared to control. Results are expressed as a mean ± SD of 6 replicates of a representative experiment (n=3). **, $p \leq 0.005$ versus pEmpty. (F) Western blot analysis with an antibody to DMP1 on tumor lysates allowed to assess that pDMP1 tumors expressed significantly more DMP1 than control pEmpty tumors.

**Figure 7. Model for a role of DMP1 in VEGF-induced signaling.** In presence of VEGF, VEGFR-2 is activated through phosphorylation (bold red P). Src is subsequently phosphorylated on Tyr416 while it is dephosphorylated on Tyr527 which results in its activation. Active Src thereby inactivates VE-cadherin function through phosphorylation of its intracytoplasmic domain tyrosines and more particularly on Tyr685. In presence of VEGF and upon DMP1 binding to CD44, VE-cadherin expression level is increased. This VE-cadherin upregulation induces, on one hand, p27$^{Kip1}$ expression and cell cycle arrest, thus mimicking contact inhibition of growth, and on the other hand, active VE-cadherin sequesters VEGFR-2 and impedes therefore its activation through the inhibition of its phosphorylation. DMP1 pre-treatment partially impairs (light red P) VE-cadherin phosphorylation on its tyrosine 685 which still allows the recruitment of Csk. In turn, DMP1 inactivated-VEGFR-2 is not able to phosphorylate further Src on Tyr416 while it is phosphorylated on Tyr527 by Csk, which expression is induced in presence of DMP1.
Figure 1 - S. Pirotte et al.

A

![Graph showing attachment of different substances](image)

B

![Bar graph showing number of migrated cells](image)

C

![Bar graph showing proliferation](image)

D

![Images of control and DMP1 conditions with bar graph showing number of vessels](image)
Figure 2 - S. Pirotte et al.

A

B

C

D

E

F

G
**Figure 3 - S. Pirotte et al.**

**A**

Control | DMP1
---|---
3h | ![Image](image1)
24h | ![Image](image2)

**B**

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**F**

Control | DMP1
---|---
Sparse Cells | ![Image](image14) | ![Image](image15) |
Confluent Cells | ![Image](image16) | ![Image](image17) |

*VE-cadherin / BrdU / Phalloidine*
Figure 4 - S. Pirro et al.

A

![Graph showing proliferation and fold of S phase cells.](image)

B

![Graph showing fold of S phase cells.](image)

C

![Bar graph showing absorbance at 560 nm.](image)

D

![Images showing control, VEGF, and DMP1 + VEGF.](image)
Figure 5 - S. Pirotte et al.

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Figure 6 - S. Pirotte et al.

A

Control

DMP1

Surface of CNV (µm²)

Control

DMP1 500nmol/L

B

pEmpty

pDMP1

Tumor Volume (mm³)

pEmpty

pDMP1

C

pEmpty

pDMP1

Tumor extracts

DMP1

β-actin

F
**Figure 7 - S. Pirotte et al.**

**In presence of VEGF**

- Active VEGFR-2
- VEGF signaling induction
- Angiogenesis

**In presence of VEGF and DMP1**

- Inactive VEGFR-2
- VEGF signaling blockade
- Impaired Angiogenesis
- Cell Cycle Arrest
  - Contact Inhibition Mimicry

**Inactive VE-cadherin**

- Y416 P
- Y527 P
- P Y685

**Active VE-cadherin**

- CD44
- p27Kip1
- Active Csk
- Csk
- P Y685
- Y416 P
- Y527 P
- S10 P
Dentin Matrix Protein 1 induces membrane expression of VE-cadherin on endothelial cells and inhibits VEGF-induced angiogenesis by blocking VEGFR-2 phosphorylation

Sophie Pirotte, Virginie Lamour, Vincent Lambert, Maria-Luz Alvarez Gonzalez, Sandra Ormenese, Agnes Noël, Denis Mottet, Vincent Castronovo and Akeila Bellahcène