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(54) Title: DENTIN MATRIX PROTEIN 1 (DMP1) FOR USE IN PHARMACEUTICAL COMPOSITIONS

(57) Abstract: The present invention refers to Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1, for use for reducing and/or preventing angiogenesis and for use in the preventive and/or therapeutic treatment of angiogenesis-related diseases.



Dentin Matrix Protein 1 (DMP1) for use in pharmaceutical compositions

The present invention refers to Dentin Matrix Protein 1 (DMP1) for use in pharmaceutical compositions, in particular for use for inhibiting angiogenesis.

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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). DMP1 was originally considered to be dentin-specific but later on, its expression was also detected in bone and in non-mineralized tissues such as salivary glands and kidney. While its precise biological activities have not been identified, this glycoprotein has been mainly associated with the regulation of extracellular matrix mineralization. It was known that SIBLING proteins are expressed in human tumors with OPN and BSP being the two most studied SIBLINGs in relation with cancer progression and metastasis development. Using cancer profiling arrays, the presence of SIBLING mRNAs in different cancers was shown and it was found that DMP1 was significantly overexpressed in human breast, uterine, colon and lung cancer tissue when compared to normal. More recently, immunohistochemical studies demonstrated the up-regulation of DMP1 in lung and breast human tumors and DSPP in prostate cancer.

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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. A family of membrane receptors called cadherins are specialized in the control of cell interaction with neighbouring cells. Vascular endothelial (VE)-cadherin, the principal junctional molecule in endothelial cells, is required for a normal vasculature development in the mouse embryo and for new vessel formation in the adult. The engagement of VE-cadherin has been mainly associated with the cessation of proliferation commonly known as contact inhibition of growth. Indeed, contact-inhibited endothelial cells have a reduced proliferative response to specific growth factors such as vascular endothelial growth factor (VEGF). VEGF is essential for angiogenic processes both in normal and pathological conditions.

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Binding of VEGF to vascular endothelial receptor 2 (VEGFR-2) is the principal extracellular signal triggering an angiogenic response. This binding 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. VE-cadherin expression in confluent endothelial cells is accompanied by a significant reduction of VEGFR-2 phosphorylation notably through to the recruitment of the high cell density-enhanced phosphatase 1 (DEP-1). Src family kinases (SFKs) are involved in VEGFR-2 signaling and have been implicated in the regulation of angiogenesis. More recently, VE-cadherin has been shown to be basally associated with Src and C-terminal Src kinase (Csk) in vascular endothelial cells. Upon VEGF stimulation, VE-cadherin-Csk complex is disrupted thus allowing the activation of Src kinase and its downstream signaling. Endothelial cell-matrix interactions mediated by integrins also play a critical role in vascular development and angiogenesis. In particular, $\alpha\beta3$ and $\alpha\beta5$ integrins are expressed at low levels on quiescent endothelial cells, while they are strongly induced on endothelial cells of angiogenic vessels. One characteristic feature of the SIBLINGs is the presence of a highly conserved RGD motif in their primary structure that is recognized by endothelial integrins. DMP1 has been also shown to interact with the CD44 cell surface receptor, detectable both on vascular endothelium *in situ* and on cultured human endothelial cells and that is involved in tumor angiogenesis. While OPN and BSP bear 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.

The object of the present invention was to provide a new active ingredient for inhibiting angiogenesis and for use in preventive and/or therapeutic treatment of angiogenesis-related diseases.

Summary of the present invention

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The present invention provides a Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1, for use for reducing and/or preventing angiogenesis.

The present invention further provides a Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1, for use in the preventive and/or therapeutic treatment of angiogenesis-related diseases.

- 5 In a preferred embodiment of the Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1 for use for reducing and/or preventing angiogenesis or for use in the preventive and/or therapeutic treatment of angiogenesis-related diseases, said angiogenesis is VEGF-induced angiogenesis. Still further preferred, said angiogenesis or said angiogenesis-
10 related disease is of postnatal individuals.

- In a particularly preferred embodiment of the Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1 for use for reducing and/or preventing angiogenesis or for use in the preventive and/or therapeutic treatment
15 of angiogenesis-related diseases, said angiogenesis is induced by VEGF via binding of VEGF to VEGF-receptor 2 (VEGFR-2). The Dentin Matrix Protein 1 (DMP1) does not inhibit the VEGF action which occurs by binding of VEGF to VEGF-receptor 1 (VEGFR-1).

- 20 In a still further embodiment the angiogenesis-related diseases are selected from the group consisting of malignant tumors, angiofibroma, arteriovenous malformations, arthritis, such as rheumatoid arthritis, atherosclerotic plaques, corneal graft neovascularization, delayed wound healing, proliferative retinopathy such as diabetic retinopathy, macular degeneration, granulations such as those
25 occurring in hemophilic joints, inappropriate vascularization in wound healing such as hypertrophic scars or keloid scars, neovascular glaucoma, ocular tumor, uveitis, non-union fractures, Osier-Weber syndrome, psoriasis, pyogenic glaucoma, retrolental fibroplasia, scleroderma, solid tumors, Kaposi's sarcoma, trachoma, vascular adhesions, chronic varicose ulcers, leukemia, and
30 reproductive disorders such as follicular and luteal cysts, choriocarcinoma, cancerous diseases, and retinal neovascularization.

In a another preferred embodiment the Dentin Matrix Protein 1 (DMP1) is selected from the group consisting of: the sequence SEQ ID NO: 1, the sequence SEQ ID

NO: 2, or the mature protein thereof having the amino acid sequence of position 17 to 513 of SEQ ID NO: 1, or the mature protein thereof having the amino acid sequence of position 17 to 497 of SEQ ID NO: 2.

5 Further, the present invention provides a Dentin Matrix Protein 1 (DMP1), a fragment or derivative thereof, for use for reducing and/or preventing angiogenesis or for use in the preventive and/or therapeutic treatment of angiogenesis-related diseases, selected from any one of (a) to (e):

- (a) a protein having the sequence shown in SEQ ID NO: 1 or SEQ ID NO: 2;
- 10 (b) a protein having the amino acid sequence of position 17 to 513 of SEQ ID NO: 1, or having the amino acid sequence of position 17 to 497 of SEQ ID NO: 2;
- (c) a fragment of (a) or (b) having the activity to inhibit angiogenesis;
- (d) a protein having at least 50% identity to (a) or (b) or (c) having the activity to
15 inhibit angiogenesis;
- (e) a protein having an amino acid sequence comprising a deletion, substitution, insertion and/or addition of one or more amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 2, or with respect to the protein of (b) or (c) or (d).

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In addition, derivatives of the above mentioned proteins are provided for the inventive use, which derivatives have at least 50% identity, preferably at least 60% identity, further preferred at least 70% identity, still further preferred at least 80% identity, particularly preferred at least 90% identity, more preferred at least
25 95% identity and most preferred at least 98 % identity to SEQ ID NO: 1 or SEQ ID NO: 2, respectively.

In addition, derivatives of the above mentioned proteins are provided for the inventive use, said derivative being represented by a protein having an amino acid
30 sequence comprising a deletion, substitution, insertion and/or addition of one or more amino acids with respect to the amino acid sequence shown in in SEQ ID NO: 1, SEQ ID NO: 2, or with respect to the the mature protein thereof having amino acid sequence of position 17 to 513 of SEQ ID NO: 1, or the mature protein thereof having amino acid sequence of position 17 to 497 of SEQ ID NO: 2.

The scope of "one or more" in the phrase "an amino acid sequence comprising a deletion, substitution, insertion and/or addition of one or more amino acids" used herein is not particularly limited in the present patent application. It means
5 preferably 1 to 200, preferred 1 to 100, more preferred 1 to 50, further preferred 1 to 25, still further preferred 1 to 20, even further preferred 1 to 10 and most preferred 1 to 5 amino acids. The term "addition" means N-terminal or C-terminal addition of amino acid residues in respect to above mentioned sequences, and in respect to the term "addition" the term "one or more" means preferably 1 to 200,
10 preferred 1 to 100, more preferred 1 to 50, further preferred 1 to 25, still further preferred 1 to 20, even further preferred 1 to 10 and most preferred 1 to 5 amino acids.

In addition, fragments of the above mentioned proteins having amino acid
15 sequence SEQ ID NO: 1 or SEQ ID NO: 2, respectively, are provided for the inventive use, having the activity to inhibit angiogenesis, wherein preferably the fragment contains at least 200, further preferred at least 300 and particularly preferred at least 400 and most preferred at least 450 consecutive amino acids of the amino acid sequence SEQ ID NO: 1 or SEQ ID NO: 2, respectively, or their
20 derivatives as defined above.

In a preferred embodiment the activity of Dentin Matrix Protein 1 (DMP1) to reduce or to inhibit angiogenesis, e.g. in angiogenesis-related diseases, involves any one or more of the following properties:

- 25 - inhibition of VEGF induced proliferation, migration and tubulogenesis of endothelial cells;
- inhibition of VEGF-induced VEGFR-2 phosphorylation;
- inhibition of VEGF-induced modulation of Src-activity;
- DMP1 mediates HUVEC attachment primarily through molecular interaction with
30 $\alpha v \beta 3$ integrin;
- induction of phosphorylation of p27^{Kip1} (Ser10);
- induction of expression of VE-cadherin;
- induction of expression of VEGFR-2;
- induction of expression of Csk;

- counteracting VEGF-induced Src activation.

The present invention also provides a polynucleotide for use for reducing and/or preventing angiogenesis or for use in the preventive and/or therapeutic treatment
5 of angiogenesis-related diseases encoding the protein of any of (a) to (e):

- (a) a protein having the sequence shown in SEQ ID NO: 1 or SEQ ID NO: 2;
- (b) a protein having the amino acid sequence of position 17 to 513 of SEQ ID NO: 1, or having the amino acid sequence of position 17 to 497 of SEQ ID NO: 2;
- 10 (c) a fragment of (a) or (b) having the activity to inhibit angiogenesis;
- (d) a protein having at least 50% identity to (a) or (b) or (c) having the activity to inhibit angiogenesis;
- (e) a protein having an amino acid sequence comprising a deletion, substitution, insertion and/or addition of one or more amino acids with respect to
15 the amino acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 2, or with respect to the protein of (b) or (c) or (d).

The present invention also provides a medicament comprising the Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding it. The active ingredient Dentin
20 Matrix Protein 1 (DMP1) of this medicament according to the present invention has the activity to reduce and/or to prevent angiogenesis, and is useful for preventive and/or therapeutic treatment of angiogenesis-related diseases

The present invention further provides a method for reducing and/or preventing
25 angiogenesis comprising the administration of an effective dose of Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1 to an individual.

In addition the present invention provides a method for the preventive and/or
30 therapeutic treatment of angiogenesis-related diseases comprising the administration of an effective dose of Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1 to an individual.

The Dentin Matrix Protein 1 (DMP1) may be provided in substantially pure form or in a pharmaceutical formulation with pharmaceutically acceptable carriers. The polynucleotide encoding Dentin Matrix Protein 1 (DMP1), a fragment or derivative thereof may be represented by a linear or circular DNA molecule, a plasmid, a vector, a viral vector etc., wherein the gene encoding Dentin Matrix Protein 1 (DMP1) is operatively linked to regulatory sequences, such as promoter, enhancer, in order to express Dentin Matrix Protein 1 (DMP1), a fragment or derivative thereof in a host cell or tissue or in an individual.

- 10 The present inventors 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). The data presented here are the first demonstration of a CD44-dependent function of DMP1 in endothelial morphogenesis through VE-cadherin induction.
- 15 The studies of the present invention 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 signalization, further the role of DMP1 on VEGF-induced proliferation, migration and tubulogenesis responses was investigated. It was demonstrated that DMP1
- 20 interferes with each one of these essential events of the angiogenic process most notably by inhibiting VEGFR-2 phosphorylation and modulating Src activity. Finally, the use of the laser-induced choroidal neovascularization (CNV) model allowed the demonstration of an anti-angiogenic role of DMP1 *in vivo*.

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Description of the figures

- Figure 1** shows that DMP1 mediates the adhesion through $\alpha_v\beta_3$, promotes the migration and stimulates the differentiation of HUVEC *in vitro*. (A) Cells were
- 30 plated onto increasing concentrations of DMP1. Vitronectin was used as a positive control. 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 $\pm$ SD of 6 replicates of a representative experiment (n=2). (B) Prior to the adhesion assay, cells were incubated 1 hour in the presence of 10 μ g/ml of blocking antibodies

directed against $\alpha_v\beta_3$ (LM609), $\alpha_v\beta_5$ (P1F6) and CD44 (BU75) or IgG used as control. The use of the anti- $\alpha_v\beta_3$ antibody significantly impaired the adhesion of endothelial cells. Error bars represent the mean $\pm$ SD of 6 replicates of a representative experiment (n=3). ***, $p \leq 0.0001$ versus control. n.s. indicates not significant. (C) Modified Boyden chamber chemotaxis assays were performed on HUVEC treated with DMP1 (100 nmol/L). DMP1 was 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 $\pm$ SD of total number of migrated cells within 4 replicates (n=2). (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 (40x) and 24 hours (100x). Phase contrast microscopy photomicrographs were taken at each time. Shown below, the quantification of the assay was realized by counting the branching vessels from 2 representative fields from 2 replicates (n=3). **, $p \leq 0.005$ versus control. n.s. indicates not significant.

Figure 2 shows that DMP1 decreases the proliferation of HUVEC in vitro, blocks the cell cycle in G1 through CD44 ligation and does not induce apoptosis. (A) Proliferation was assessed using HUVEC treated with DMP1 (1 nmol/L to 100 nmol/L) during 24 and 48 hours was measured and scattered as described in Materials and Methods. Error bars represent the mean $\pm$ SD of 3 replicates of a representative experiment (n=3). **, $p \leq 0.001$ and ***, $p \leq 0.0001$ versus control. n.s. indicates not significant. (B) Annexin-V/Propidium iodide (PI) assay of HUVEC treated with DMP1 (50 nmol/L) during 24 hours. One replicate out of 3 from a representative experiment (n=3) is shown. (C) Cells were treated with increasing concentrations of DMP1 during the indicated time intervals. Lysates from both floating and adherent HUVEC were immunoblotted with an antibody to PARP. Lysates from IGROV-1 cells, treated with cisplatin to induce apoptosis (CT+), showed the expected apoptotic fragment of 89 kDa corresponding to PARP cleavage. This fragment is not observed in DMP1-treated HUVEC lysates. The experiment was repeated 2 times. (D) Cell cycle analysis of serum starved HUVEC treated with DMP1 (50 nmol/L) and mimosine (200 μ mol/L). Mimosine and non serum released cells were used to assess for G1 arrest. Error bars represent the mean $\pm$ SD of 3 replicates of a representative experiment (n=3). **, $p \leq 0.001$ and ***, $p \leq 0.0001$ versus control. n.s. indicates not significant.

$p \leq 0.001$ and $***$, $p \leq 0.0005$ versus control serum released cells. n.s. indicates not significant. (E) S-phase cell cycle analysis of serum starved HUVEC incubated with blocking antibodies to CD44 and $\alpha_v\beta_3$, and IgG used as control followed by treatment with DMP1 (50 nmol/L) as mentioned in Materials and Methods. Error bars represent the mean $\pm$ SD of 3 replicates of a representative experiment (n=2). $**$, $p \leq 0.001$ versus control or DMP1 condition. n.s. indicates not significant.

Figure 3 shows that DMP1 modulates the expression of cell cycle-related proteins and induces p27^{Kip1} through CD44 ligation. (A) Western blot analysis with an antibody to p27^{Kip1} using total lysates from HUVEC treated with increasing concentrations of DMP1. (B) Western blot analysis with antibodies to p21^{Cip1}, pRb and to PCNA using total lysates from DMP1-treated cells. (C) Proliferation was assessed using HUVEC transfected during 48 hours with two siRNAs targeting p27^{Kip1} (siRNA p27#1 and siRNA p27#2) or with no siRNA and siRNA EGT used as negative controls. During the last 24 hours of transfection, cells were treated with DMP1. Error bars represent the mean $\pm$ SD of 3 replicates of a representative experiment (n=2). $*$, $p \leq 0.01$ versus control. n.s. indicates not significant. (D) Western blot analysis with an antibody to p27^{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 μ g/mL of anti-CD44 and anti- $\alpha_v\beta_3$ blocking antibodies or IgG used as control. (E) Western blot analysis with antibodies to p27^{Kip1} and P-p27^{Kip1}(Ser¹⁰) using total lysates from DMP1-treated HUVEC. All Western blotting results were evaluated by densitometric scanning, corrected with respect to β -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- β -actin immunoblotting.

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Figure 4 shows that 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. (B) Western blot analysis with antibodies to VE-cadherin and p27^{Kip1} using total lysates from DMP1-treated HUVEC. (C) Flow cytometry analysis using antibodies to VE-cadherin, ZO-1 and PECAM-1 of cells treated with DMP1 (50 nmol/L) during 24 hours. (D) Western blot analysis with antibodies to p27^{Kip1} 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. (E) 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. (F) Western blot analysis with antibodies to VE-cadherin and p27^{Kip1} 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. (G) 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.

Figure 5 shows that 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). **, $p \leq 0.005$ and ***, $p \leq 0.0005$ 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 $\pm$ SD of 3 replicates of a representative experiment (n=3). ***, $p \leq 0.0005$ 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 $\pm$ SD of 3 replicates of a representative experiment (n=3). **, $p \leq 0.005$ 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 = 400 μ m.

Figure 6 shows that DMP1 affects VEGFR-2, and not VEGFR-1, phosphorylation, induces subsequent Src inactivation and inhibits VEGF-mediated VE-cadherin activation and expression. (A) Western blot analysis with antibodies to VEGFR-2 and VEGFR-1 and their phosphorylated forms using 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 cells treated with DMP1 and VEGF as in C. All Western blotting results were evaluated by densitometric scanning (in italics below the lanes). Western blots were realized 3 times with similar results. Equal protein loading was assessed by anti- β -actin or anti-HSC 70 immunoblotting. (E) Western blot analysis with antibodies to VE-cadherin and to 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 blotting results were evaluated by densitometric scanning (in italics below the lanes). Western blots were realized

3 times with similar results. Equal protein loading was assessed by anti- β -actin immunoblotting.

Figure 7 shows that DMP1 impairs *in vivo* angiogenesis in the CNV model. (A) Mice were injured by laser shots onto the retina (areas within the dotted lines) and were then subjected with intravitreal injection of DMP1 (500 nmol/L). Eyes were removed after 7 days and the angiogenic response was measured as described in Materials and Methods. Shown below, the quantification represents the measure of total vessel fluorescence surface or each impact. Error bars represent the mean $\pm$ SD of 20 impacts of a representative experiment (n=2). *, $p \leq 0.05$ versus control. (B) Model for a role of DMP1 in VEGF-induced signaling. In presence of VEGF, VEGFR-2 is activated through phosphorylation. Src is subsequently phosphorylated on Tyr416 while it is dephosphorylated on Tyr527 resulting in its activation. Active Src thereby inactivates VE-cadherin function at the cell-cell adherens junction through phosphorylation of its intracytoplasmic domain tyrosines. In presence of VEGF and 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. On the other hand, active VE-cadherin is able to impede VEGFR-2 activation notably through the inhibition of its phosphorylation. 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.

Detailed description of the present invention

As mentioned above the present invention provides a Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1, for use for reducing and/or preventing angiogenesis, or for use in the preventive and/or therapeutic treatment of angiogenesis-related diseases.

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 in 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. The present inventors show that the integrin $\alpha v \beta 3$ mediates the adhesion of endothelial cells to DMP1 and 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 p27^{Kip1}. The ligation of CD44, by either a specific monoclonal antibody or its preferential ligand hyaluronan, has previously been associated with cell cycle control via p27^{Kip1} regulation in leukemic cells. It is noteworthy that DMP1-mediated up-regulation of p27^{Kip1} in HUVEC is subsequent to specific VE-cadherin induction. This observation is in accordance with previous reports showing that E- and N-cadherin mediate anti-proliferative effects, in the context of contact-induced inhibition of cell growth, through p27^{Kip1} up-regulation. DMP1 increases the surface expression of VE-cadherin 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 function. 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). DMP1-treated HUVEC demonstrate a precocious and sustained morphogenesis which is in good correspondence with (a) a reduced cell division and (b) an enhanced attachment and migratory responses observed in presence of DMP1.

The exploration of DMP1-induced functional responses of endothelial cells 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 inhibitor of VEGF-induced angiogenesis. The role for VE-cadherin in modulating downstream signaling of VEGF has been largely recognized, and in turn, VEGF-activated Src

kinase phosphorylates VE-cadherin and makes it inactive in the control of cell-cell adherens junctions (Figure 7B, left panel).

A particular interesting finding of this study is the specific inhibition of VEGFR-2
5 activity as a principal mediator of VEGF-dependent angiogenesis. Indeed, in
presence of DMP1 (Figure 7B, right panel), HUVEC do not respond anymore to
VEGF stimulus. Although VEGFR-2 expression is increased probably as a
consequence of VE-cadherin induction, its phosphorylation does not occur in
presence of VEGF. Therefore, VEGF-induced Src kinase activation is
10 counteracted. This repression of Src is dependent on Csk, which was found
indeed highly induced in presence of DMP1. Interestingly, Csk, that has been
previously involved in cadherin-driven proliferation arrest at high density, appears
in this study expressed at high level both in confluent cells and in DMP1-treated
ones. This observation adds weight to the mimicry of contact inhibition of growth
15 mechanism discussed above.

Prior to the studies made according to the present invention, in
immunohistochemical studies the expression of DMP1 in human breast cancer
tumors was revealed. SIBLING proteins expression being generally associated
20 with bad prognosis and poor survival for cancer patients. Surprisingly patients with
tumors expressing high levels of DMP1 presented with a better survival than
patients with low DMP1-expressing tumors. In light of the new data found during
the studies of the present invention and considering that angiogenesis is
indispensable for tumor growth, high-DMP1 expressing tumors may be associated
25 with limited neovessels formation as tumor-secreted DMP1 could favor endothelial
cells differentiation at the expense of their proliferation. Arguing for this possibility
is the observation in the same study that high-DMP1 expressing tumors were
consistently small sized.

30 Finally, the evaluation of *in vivo* angiogenesis in the CNV model is in favor of an
anti-angiogenic role of DMP1 through VEGFR-2. Indeed, while in this model both
VEGFR-1 and VEGFR-2 are required for the VEGF-dependent retinal
neovascularization, the role of VEGFR-1 seems to be predominant. DMP1
specifically affects VEGFR-2 without affecting VEGFR-1 activity.

In conclusion, the present inventors demonstrated for the first time that DMP1 is implicated in endothelial cell morphogenesis *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 interferes with VEGF-induced signaling in HUVEC and that the mechanism for this effect mainly includes the up-regulation of VE-cadherin at adherens junctions as well as VEGFR-2 inactivation.

10 **Angiogenesis, Angiogenic Conditions and Angiogenic Diseases.** The protein of the invention and the respective pharmaceutical compositions and preparations which are capable of inhibiting angiogenesis are useful for preventing or treating any disease or condition which is associated with or results in or from angiogenesis. Such diseases include formation of malignant tumors, angiofibroma, arteriovenous malformations, arthritis, such as rheumatoid arthritis, 15 atherosclerotic plaques, corneal graft neovascularization, delayed wound healing, proliferative retinopathy such as diabetic retinopathy, macular degeneration, granulations such as those occurring in hemophilic joints, inappropriate vascularization in wound healing such as hypertrophic scars or keloid scars, 20 neovascular glaucoma, ocular tumor, uveitis, non-union fractures, Osier-Weber syndrome, psoriasis, pyogenic glaucoma, retrolental fibroplasia, scleroderma, solid tumors, Kaposi's sarcoma, trachoma, vascular adhesions, chronic varicose ulcers, leukemia, and reproductive disorders such as follicular and luteal cysts and choriocarcinoma, among others.

25

Given its anti-angiogenic activity, the protein of the invention is also suitable for use in a method of inhibiting mammalian cell proliferation and organization that depends on vascularization, including the selective inhibition of vascularization of tumors, tumor size reduction and elimination. Examples of tumors undergoing 30 angiogenesis include but are not limited to angiofibroma, arteriovenous malformations, ocular tumors, all solid tumors, Kaposi's sarcoma, trachoma and choriocarcinoma.

Production of the proteins of the present invention

The proteins of the current invention can, for example, be synthesized, prepared from purified proteins, or produced using recombinant methods and techniques known in the art. Although specific techniques for their preparation are described
5 herein, it is to be understood that all appropriate techniques suitable for production of these peptides are intended to be within the scope of this invention.

Generally, these techniques include DNA and protein sequencing, cloning, expression and other recombinant engineering techniques permitting the construction of prokaryotic and eukaryotic vectors encoding and expressing each
10 of the proteins of the invention.

The proteins may be prepared by peptide synthesis according to method described in *Biotechnology and Applied Biochem.*, 12:436 (1990) or by methods described in *Current Protocols in Molecular Biology*, Eds. Ausubel, F.M., et al, John Wiley & Sons, N.Y. (1987).

15 The proteins of the invention may be produced by expression of a nucleic acid encoding the protein of interest, or by cleavage from a longer length polypeptide encoded by the nucleic acid. Expression of the encoded polypeptides may be done in bacterial, yeast, plant, insect, or mammalian hosts by techniques well known in the art.

20 In an embodiment, the protein of the invention is obtained by cloning the DNA sequence into a Vector starting with a DNA codon for methionine inserted upstream 5' to the first DNA codon of the desired protein sequence and modifying the DNA codon corresponding to the last amino acid of a desired protein to a stop codon by mutagenesis techniques known in the art. A host cell is transformed with
25 the modified nucleic acid to allow expression of the encoded protein.

Examples of mutagenesis techniques include, for example, methods described in *Promega Protocols and Applications GWde*, Promega Corp, Madison, WI, p. 98 (1891) or according to *Current Protocols in Molecular Biology*, supra.

If the protein is to be synthesized via a prokaryotic vector, the DNA sequence encoding a protein preferably does not contain a signal peptide sequence. In addition, a DNA codon for methionine (Met) is typically inserted upstream of 5' to the first DNA codon of the coding sequence.

5 Methods for cloning DNA into a vector and for inserting, deleting and modifying polynucleotides and for site directed mutagenesis are described, for example, in *Promega Protocols and Applications Guide, supra*. Cells or bacteria may be transfected with a vector, preferably with an expression vector, having the desired DNA sequence attached thereto, by known techniques including heat shock,
10 electroporation, calcium phosphate precipitation and lipofection, among others. The proteins may then be extracted and purified by, for example, high pressure liquid chromatography (HPLC), ion exchange chromatography or gel permeation chromatography. However, other methods and techniques known in the art of conducting the different steps or combinations of these steps necessary to derive
15 the peptide of this invention or equivalent steps are contemplated to be within the scope of this invention.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial
20 identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, or may comprise a complete cDNA or gene sequence.

Optimal alignment of sequences for aligning a comparison window may, for
25 example, be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. U.S.A.* 85:2444 (1988), or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA,
30 and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI).

- As applied to polypeptides, the terms "substantial identity" or "substantial sequence identity" mean that two polypeptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity or more. "Percentage amino acid identity" or "percentage amino acid sequence identity" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids. For example, "95% amino acid identity" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have 95% amino acid identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to effect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.
- "Homologous" amino acid residues as used herein refer to amino acid residues which have similar chemical properties concerning hydrophobicity, charge, polarity, steric features, aromatic feature etc. Examples for amino acids which are homologous to each other include in terms of positive charge lysine, arginine, histidine; in terms of negative charge: glutamic acid, aspartic acid; in terms of hydrophobicity: glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine; in terms of polarity serine, threonine, cysteine, methionine, tryptophan, tyrosine, asparagine, glutamine; in terms of aromaticity: phenylalanine, tyrosine, tryptophan; in terms of chemically similar side groups: serine and threonine; or glutamine and asparagines; or leucine and isoleucine.
- Conservative amino acid substitutions: Conservative amino acid substitutions usually have minimal impact on the activity of the resultant protein. Such substitutions are described below. Conservative substitutions replace one amino acid with another amino acid that is similar in size, hydrophobicity, charge, polarity, steric features, aromaticity etc.. Such substitutions generally are conservative when it is desired to finely modulate the characteristics of the protein.

Examples of amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions include: Ser for Ala; Lys for Arg; Gln or His for Asn; Glu for Asp; Ser for Cys; Asn for Gln; Asp for Glu; Pro for Gly; Asn or Gln for His; Leu or Val for Ile; Ile or Val for Leu; Arg or Gln for
5 Lys; Leu or Ile for Met; Met, Leu or Tyr for Phe; Thr for Ser; Ser for Thr; Tyr for Trp; Trp or Phe for Tyr; and Ile or Leu for Val.

The phrase "substantially purified" or "isolated" when referring to a peptide or protein, means a chemical composition which is essentially free of other cellular
10 components. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified. Generally, a substantially
15 purified or isolated protein will comprise more than 80% of all macromolecular species present in the preparation. Preferably, the protein is purified to represent greater than 90% of all macromolecular species present. More preferably the protein is purified to greater than 95%, and most preferably the protein is purified to essential homogeneity, wherein other macromolecular species are not detected
20 by conventional techniques.

Nucleic Acids of the Invention

Also provided herein are isolated nucleic acids that comprise DNA or RNA sequences (polynucleotides) encoding the peptides of the invention. The nucleic acids of the invention may further comprise vectors for expression of the peptides
25 of the invention. In some embodiments the DNA may comprise cDNA sequences encoding the protein of the present invention. It is understood by one of ordinary skill in the art that because of degeneracy in the genetic code, substitutions in the nucleotide sequence may be made which do not result in changes in the encoded amino acid sequence. Thus, "substantially identical" sequences as defined herein
30 are included in the scope of the invention. It is further understood by one of

ordinary skill in the art that both complementary strands of any DNA molecule described herein are included within the scope of the invention.

The terms "substantial identity" or "substantial sequence identity" as applied to nucleic acid sequences and as used herein denote a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, and more preferably at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence.

15 **Treatment Protocols**

The method for inhibiting angiogenesis or for preventive and/or therapeutic treatment of angiogenesis-related diseases comprises administering to a patient an angiogenesis-inhibitory amount of the DMP1 protein of the invention. As used herein, the term "treatment" is intended to refer to the prevention, amelioration, or reduction in severity of a symptom of angiogenesis-related disease. Similarly, an angiogenesis inhibitory effective dose of a DMP1 of the invention is a dose sufficient to prevent, ameliorate, or reduce the severity of a symptom of angiogenesis.

The proteins of the invention may be administered singly or in combination with other, particularly angiogenesis and/or cancer inhibitory agents. Typically, the proteins of the invention are administered in an amount of about 5 to 10,000 µg/kg per day, preferably 8 to 3,000 µg/kg per day, and more preferably about 20 to 1,500 µg/kg per day preferably once or twice daily. However, other amounts, including substantially lower or higher amounts, may also be administered. The proteins of the invention are administered to a human subject in need of the

treatment intramuscularly, subcutaneously, intravenously, intratumorally, by any other acceptable route of administration.

Gene Therapy

Gene therapy utilizing recombinant DNA technology to deliver nucleic acids (polynucleotides) encoding DPM1 proteins according to the invention into patient
5 cells or vectors which will supply the patient with gene product in vivo is also contemplated within the scope of the present invention.

Gene therapy techniques have the potential for limiting the exposure of a subject to a gene product, such as polypeptide, by targeting the expression of the
10 therapeutic gene to a tissue of interest, such as skeletal muscle, myocardium, vascular endothelium or smooth muscle, or solid or circulating tumor cells. For example, PCT patent application publication No. WO 93/15609 discloses the delivery of interferon genes to vascular tissue by administration of such genes to areas of vessel wall injury using a catheter system. In another example, an
15 adenoviral vector encoding a protein capable of enzymatically converting a prodrug, a "suicide gene", and a gene encoding a cytokine are administered directly into a solid tumor.

Other methods of targeting therapeutic genes to tissues of interest include the three general categories of transductional targeting, positional targeting, and
20 transcriptional targeting (for a review, see, e.g., Miller et al. FASEB J. 9:190-199 (1995)). Transductional targeting refers to the selective entry into specific cells, achieved primarily by selection of a receptor ligand. Positional targeting within the genome refers to integration into desirable loci, such as active regions of chromatin, or through homologous recombination with an endogenous nucleotide
25 sequence such as a target gene. Transcriptional targeting refers to selective expression attained by the incorporation of transcriptional promoters with highly specific regulation of gene expression tailored to the cells of interest.

Examples of tissue-specific promoters include a liver-specific promoter (Zou et al., Endocrinology 138:1771-1774 (1997)); a small intestine-specific promoter
30 (Oliveira et al., J. Biol. Chem. 271:31831-31838 (1996)); the promoter for creatine kinase, which has been used to direct of dystrophin cDNA expression in muscle

and cardiac tissue (Cox et al., *Nature* 364:725-729 (1993)); and immunoglobulin heavy or light chain promoters for the expression of suicide genes in B cells (Maxwell et al., *Cancer Res.* 51:4299-4304 (1991)). An endothelial cell-specific regulatory region has also been characterized (Jahroudi et al., *Mol. Cell, Biol.* 14:999-1008 (1994)). Amphotrophic retroviral vectors have been constructed carrying a herpes simplex virus thymidine kinase gene under the control of either the albumin or alpha-fetoprotein promoters (Huber et al., *Proc. Natl. Acad. Sci. U.S.A.* 88:8039-8043 (1991)) to target cells of liver lineage and hepatoma cells, respectively. Such tissue specific promoters can be used in retroviral vectors (Hartzoglu et al., *J. Biol. Chem.* 265:17285-17293 (1990)) and adenovirus vectors (Friedman et al., *Mol. Cell. Biol.* 6:3791-3797 (1986)) and still retain their tissue specificity.

Other elements aiding specificity of expression in a tissue of interest can include secretion leader sequences, enhancers, nuclear localization signals, endosmolytic peptides, etc. Preferably, these elements are derived from the tissue of interest to aid specificity.

Viral vector systems useful in the practice of the instant invention include but are not limited to adenovirus, herpesvirus, adeno-associated virus, minute virus of mice (MVM), HIV, sindbis virus, and retroviruses such as Rous sarcoma virus, and MoMLV. Typically, the nucleic acid encoding the therapeutic polypeptide or peptide of interest is inserted into such vectors to allow packaging of the nucleic acid, typically with accompanying viral DNA, infection of a sensitive host cell, and expression of the polypeptide of interest.

Similarly, viral envelopes used for packaging the recombinant constructs of the invention can be modified by the addition of receptor ligands or antibodies specific for a receptor to permit receptor-mediated endocytosis into specific cells (e.g., WO 93/20221, WO 93/14188; WO 94/06923). In some embodiments of the invention, the DNA constructs of the invention are linked to viral proteins, such as adenovirus particles, to facilitate endocytosis (Curiel et al., *Proc. Natl. Acad. Sci. U.S.A.* 88:8850-8854 (1991)). In other embodiments, molecular conjugates of the instant invention can include microtubule inhibitors (WO 94/06922); synthetic peptides mimicking influenza virus hemagglutinin (Plank et al., *J. Biol. Chem.*

269:12918-12924 (1994)); and nuclear localization signals such as SV40 T antigen (WO 93/19768).

The nucleic acid can be introduced into the tissue of interest *in vivo* or *ex vivo* by a variety of methods. In some embodiments of the invention, the nucleic acid is introduced into cells by such methods as microinjection, calcium phosphate
5 precipitation, liposome fusion, or biolistics. In further embodiments, the nucleic acid is taken up directly by the tissue of interest. In other embodiments, nucleic acid is packaged into a viral vector system to facilitate introduction into cells.

In some embodiments of the invention, the compositions of the invention are administered *ex vivo* to cells or tissues explanted from a patient, then returned to
10 the patient. Examples of *ex vivo* administration of gene therapy constructs include Asteaga et al., *Cancer Research* 56(5):1098-1103 (1996); Nolte et al., *Proc. Natl. Acad. Sci. USA* 93(6):2414-9 (1996); Koc et al., *Seminars in Oncology* 23 (1):46-65 (1996); Raper et al., *Annals of Surgery* 223(2):116-26 (1996);
15 Dalesandro et al., *J Thorac. Card. Surg.* 11(2):416-22 (1996); and Makarov et al., *Proc. Natl. Acad. Sci. USA* 93(1):402-6 (1996).

Means of Administration

The form of the vector introduced into a host or host cell can vary, depending in part on whether the vector is being introduced *in vitro* or *in vivo*. For instance, the
20 nucleic acid can be closed circular, nicked, or linearized, depending on whether the vector is to be maintained extragenomically (*i.e.*, as an autonomously replicating vector), integrated as a provirus or prophage, transiently transfected, transiently infected as with use of a replication-deficient or conditionally replicating virus, or stably introduced into the host genome through double or single
25 crossover recombination events. Prior to introduction into a host, a vector containing the polynucleotide of the present invention can be formulated into various compositions for use in therapeutic and prophylactic treatment methods. In particular, the vector can be made into a pharmaceutical composition by combination with appropriate pharmaceutically acceptable carriers or diluents,
30 and can be formulated to be appropriate for either human or veterinary applications.

Thus, a pharmaceutical composition can comprise one or more of the aforementioned vectors, preferably in combination with a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well-known to those skilled in the art, as are suitable methods of administration. The choice of carrier
5 will be determined, in part, by the particular vector, as well as by the particular method used to administer the composition. One skilled in the art will also appreciate that various routes of administering a composition are available, and, although more than one route can be used for administration, a particular route can provide a more immediate and more effective reaction than another route.
10 Accordingly, there are a wide variety of suitable formulations of the composition of the present invention.

A composition comprised of a vector containing the polynucleotide of the present invention, alone or in combination with other anti-angiogenic or other anti-cancer compounds, can be made into a formulation suitable for parenteral administration,
15 preferably intraperitoneal administration. Such a formulation can include aqueous and nonaqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and nonaqueous sterile suspensions that can include suspending agents, solubilizers, thickening
20 agents, stabilizers, and preservatives. The formulations can be presented in unit dose or multidose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneously injectable solutions and suspensions can be prepared from
25 sterile powders, granules, and tablets, as described herein.

An aerosol formulation suitable for administration via inhalation also can be made. The aerosol formulation can be placed into a pressurized acceptable propellant, such as dichlorodifluoromethane, propane, nitrogen, and the like.

The dose administered to an animal, particularly a human, in the context of the
30 present invention should be sufficient to induce a therapeutic response in the infected individual over a reasonable time frame. The dose will be determined by the potency of the particular vector employed for treatment, the severity of the

disease state, as well as the body weight and age of the infected individual. The size of the dose also will be determined by the existence of any adverse side effects that can accompany the use of the particular vector employed. It is always desirable, whenever possible, to keep adverse side effects to a minimum.

- 5 The dosage can be in unit dosage form, such as a tablet or capsule. The term "unit dosage form" as used herein refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of a vector, alone or in combination with other therapeutic agents, calculated in an amount sufficient to produce the desired effect in
10 association with a pharmaceutically acceptable diluent, carrier, or vehicle. The specifications for the unit dosage forms of the present invention depend on the particular compound or compounds employed and the effect to be achieved, as well as the pharmacodynamics associated with each compound in the host. The dose administered should be an "anti-angiogenesis effective amount" or an
15 amount necessary to achieve an "effective level" in the individual patient.

- Since the "effective level" is used as the preferred endpoint for dosing, the actual dose and schedule can vary, depending on interindividual differences in pharmacokinetics, drug distribution, and metabolism. The "effective level" can be defined, for example, as the blood or tissue level desired in the patient that
20 corresponds to a concentration of one or more vector(s) containing the polynucleotide according to the invention, which inhibits angiogenesis, in an assay predictive for clinical anti-angiogenic activity of chemical compounds. The "effective level" for compounds of the present invention also can vary when the compositions of the present invention are used in combination with known anti-
25 angiogenic compounds.

- One skilled in the art can easily determine the appropriate dose, schedule, and method of administration for the exact formulation of the composition being used, in order to achieve the desired "effective level" in the individual patient. One skilled in the art also can readily determine and use an appropriate indicator of the
30 "effective level" of the compounds of the present invention by a direct (*e.g.*, analytical chemical analysis) or indirect (*e.g.*, with surrogate indicators of viral

infection) analysis of appropriate patient samples (e.g., blood and/or tissues) or the use of reporter proteins.

The pharmaceutical composition can contain other pharmaceuticals, in conjunction with a vector according to the invention, when used to therapeutically
5 treat angiogenesis-related disease. These other pharmaceuticals can be used in their traditional fashion. Further representative examples of these additional pharmaceuticals that can be used in addition to those previously described, include immunomodulators, immunostimulants, antibiotics, and other agents and treatment regimes (including those recognized as alternative medicine) that can
10 be employed to treat angiogenesis-related diseases. Immunomodulators and immunostimulants include, but are not limited to, various interleukins, CD4, cytokines, antibody preparations, blood transfusions, and cell transfusions. Antibiotics include, but are not limited to, antifungal agents, antibacterial agents.

Formulations and Pharmaceutical Compositions

15 The compositions of the invention will be formulated for administration by manners known in the art acceptable for administration to a mammalian subject, preferably a human. In some embodiments of the invention, the compositions of the invention can be administered directly into a tissue by injection or into a blood vessel supplying the tissue of interest. In further embodiments of the invention the
20 compositions of the invention are administered "locoregionally", i.e., intravesically, intralesionally, and/or topically. In other embodiments of the invention, the compositions of the invention are administered systemically by injection, inhalation, suppository, transdermal delivery, etc. In further embodiments of the invention, the compositions are administered through catheters or other devices to
25 allow access to a remote tissue of interest, such as an internal organ. The compositions of the invention can also be administered in depot type devices, implants, or encapsulated formulations to allow slow or sustained release of the compositions.

In order to administer therapeutic agents based on, or derived from, the present
30 invention, it will be appreciated that suitable carriers, excipients, and other agents

may be incorporated into the formulations to provide improved transfer, delivery, tolerance, and the like.

A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences, (15th Edition, Mack Publishing Company, Easton, Pennsylvania (1975)), particularly Chapter 87, by Blaug, Seymour, therein. These formulations include for example, powders, pastes, ointments, jelly, waxes, oils, lipids, anhydrous absorption bases, oil-in-water or water-in-oil emulsions, emulsions carbowax (polyethylene glycols of a variety of molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax.

Any of the foregoing formulations may be appropriate in treatments and therapies in accordance with the present invention, provided that the active agent in the formulation is not inactivated by the formulation and the formulation is physiologically compatible.

The quantities of active ingredient necessary for effective therapy will depend on many different factors, including means of administration, target site, physiological state of the patient, and other medicaments administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the active ingredients. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, for example, in *Goodman and Gilman's the Pharmacological Basis of Therapeutics*, 7th Edition (1985), MacMillan Publishing Company, New York, and *Remington's Pharmaceutical Sciences 18th Edition*, (1990) Mack Publishing Co, Easton Penn. Methods for administration are discussed therein, including oral, intravenous, intraperitoneal, intramuscular, transdermal, nasal, iontophoretic administration, and the like.

The compositions of the invention may be administered in a variety of unit dosage forms depending on the method of administration. For example, unit dosage forms suitable for oral administration include solid dosage forms such as powder, tablets, pills, capsules, and dragees, and liquid dosage forms, such as elixirs,

5 syrups, and suspensions. The active ingredients may also be administered parenterally in sterile liquid dosage forms. Gelatin capsules contain the active ingredient and as inactive ingredients powdered carriers, such as glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium
10 stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like. Examples of additional inactive ingredients that may be added to provide desirable color, taste, stability, buffering capacity, dispersion or other known desirable features are red iron oxide, silica gel, sodium lauryl sulfate, titanium dioxide, edible white ink and the like. Similar diluents can be used to make
15 compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric-coated for selective disintegration in the gastrointestinal tract. Liquid dosage forms for oral
administration can contain coloring and flavoring to increase patient acceptance.

The concentration of the compositions of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode
20 of administration selected.

The compositions of the invention may also be administered via liposomes. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the composition of the invention to be delivered is incorporated as
25 part of a liposome, alone or in conjunction with a molecule which binds to a desired target, such as antibody, or with other therapeutic or immunogenic compositions. Thus, liposomes either filled or decorated with a desired composition of the invention can delivered systemically, or can be directed to a tissue of interest, where the liposomes then deliver the selected
30 therapeutic/inununogenic peptide compositions.

Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a

sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety in, e.g., Szoka et al. Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369,
5 incorporated herein by referece.

A liposome suspension containing a composition of the invention may be administered intravenously, locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the composition of the invention being delivered, and the stage of the disease being treated.

10 For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally
15 employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more compositions of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the compositions of the invention are preferably supplied in finely divided form along with a surfactant and propellant. Typical
20 percentages of compositions of the invention are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with
25 an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1 %-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

The compositions of the invention can additionally be delivered in a depot-type system, an encapsulated form, or an implant by techniques well-known in the art. Similarly, the compositions can be delivered via a pump to a tissue of interest.

5 The compositions of the invention are typically administered to patients after the onset of symptoms, although treatment can also be prophylactic in some embodiments. Typically, treatment with direct administration of polypeptides is done daily, weekly, or monthly, for a period of time sufficient to reduce, prevent, or ameliorate symptoms. Treatment with the nucleic acids of the invention is typically done at intervals of several months. In some embodiments, administration of the
10 compositions of the invention is done in utero.

The composition of the invention may also be provided in the kit as a slow-release composition such as a daily, weekly, monthly unit provided as a sponge, dermal patch, subcutaneous implant and the like in a wrapping or container as described above. In this case, the patient may release a unit of the composition from the
15 container and applies it as indicated in the kit instructions. The composition may then be replaced at the end of the specified period by a fresh unit, and so on.

The present composition may also be administered by means of injection, as indicated above. Typically, the peptide may be administered by itself, or, for instance, in the case of a diabetic, in a composition also comprising insulin. The
20 same is true for the slow-release forms of the composition. Similarly, the peptide of the invention may be administered in a composition that also comprises another drug.

The following examples are given for the purpose of illustrating various embodiments of the present invention and are not meant to limit the present
25 invention in any fashion. A person skilled in the art will appreciate readily that the present invention is able to generate the objects and obtain the advantages mentioned, as well as those objects and advantages inherent herein.

Examples

Example 1: DMP1 mediates the adhesion of HUVEC through $\alpha_v\beta_3$ integrin and not CD44

To investigate the possibility that DMP1 could serve as an adhesion substrate for endothelial cells, their ability to adhere to various concentrations of DMP1 was tested with vitronectin used as a positive control. HUVEC adhered to DMP1 in a dose-dependent manner in the same range of concentrations and to a similar extent as observed with vitronectin (Figure 1A). DMP1 contains an RGD motif, which has been identified as a common integrin recognition site mediating cell-ECM interactions. Both $\alpha_v\beta_3$ integrin and CD44 receptor have been described as potential receptors for DMP1.

To identify which cell surface receptors might be implicated in the adhesion of HUVEC to DMP1, blocking studies were performed with the use of monoclonal antibodies specifically directed against these receptors. Because activated endothelial cells express both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, the effect of a monoclonal antibody directed against $\alpha_v\beta_5$ on the adhesion of HUVEC to DMP1 was also tested. Incubation of HUVEC with anti- $\alpha_v\beta_3$ integrin blocking antibody almost completely inhibited their adhesion to immobilized DMP1 (Figure 1B). No significant effect was observed when HUVEC were pre-incubated with anti- $\alpha_v\beta_5$ and anti-CD44 blocking antibodies or with mouse IgG used as control. These data indicate that DMP1 mediates HUVEC attachment primarily through molecular interaction with $\alpha_v\beta_3$ integrin.

Example 2: DMP1 promotes the migration and stimulates the differentiation of HUVEC in vitro

Next, it was investigated whether DMP1 had an impact on HUVEC migration *in vitro* by using a modified Boyden chamber assay. When placed in the lower chamber, DMP1 (100nmol/L) stimulated HUVEC migration (Figure 1C). There was no migration to BSA in the lower chamber (data not shown). To determine the importance of a concentration gradient for the observed migratory response, cell migration was also evaluated when the protein 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 in this experiment did not totally abolish cell migration, it was considered
5 that DMP1 exhibits chemokinetic ability towards endothelial cells.

Then, the impact of DMP1 on the capacity of HUVEC to form capillary-like structures in a standard Matrigel *in vitro* model was tested. As early as one hour after seeding, it was observed that DMP1-treated endothelial cells organized
10 differently on Matrigel. The counting of branching vessels after 4 hours demonstrated that DMP1-treated HUVEC rapidly formed a tubular network still maintained after 24 hours incubation when compared to control cells (Figures 1D), suggesting that DMP1 could act as a pro-differentiating factor for endothelial cells that contributes to the organization and/or stability of developing endothelial
15 tubular networks.

Example 3: DMP1 decreases the proliferation of HUVEC in vitro and blocks the cell cycle in G1 through CD44 ligation

20 Further, the impact of DMP1 on HUVEC proliferation was tested. As result is was observed that DMP1-treated HUVEC were less proliferative than the control cells (Figure 2A). The potential effect of DMP1 treatment on apoptosis was examined by Annexin-V assay. No significant modulation in the percentage of apoptotic cells in DMP1-treated cells compared to control cells was found (Figure 2B). To confirm
25 that DMP1 did not induce apoptosis, lysates from DMP1-treated cells were immunoblotted with antibodies directed against poly-ADP-ribose-polymerase (PARP) which cleavage occurs at the onset of apoptosis. DMP1-treated HUVEC extracts did not show the expected cleavage fragment when compared to cisplatin-treated IGROV-1 cell extract used as positive control (Figure 2C).
30 Together these experiments demonstrated that DMP1 effect on HUVEC proliferation was not related to apoptosis induction.

Then is was investigated whether the decrease of HUVEC proliferation was due to an arrest of the cell cycle. Synchronized HUVEC were released with culture

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 2D).

To investigate whether the effect of DMP1 on the cell cycle was mediated through an interaction with $\alpha_v\beta_3$ integrin or CD44, HUVEC was incubated with anti- $\alpha_v\beta_3$ and anti-CD44 blocking antibodies prior to DMP1 treatment and cell cycle analysis. 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- $\alpha_v\beta_3$ integrin blocking antibody or control IgG (figure 2E). These results demonstrated that the inhibitory effect of DMP1 on the cell cycle is mediated, at least in part, through CD44 receptor and not $\alpha_v\beta_3$ integrin.

Example 4: DMP1 modulates the expression of cell cycle-related proteins and induces p27^{Kip} through CD44 ligation

The cyclin dependent kinase (CDK) inhibitors p21^{Cip1} and p27^{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^{Kip1} was induced by DMP1 in a dose-dependent manner (Figure 3A) whereas the expression of p21^{Cip1} was unaffected (Figure 3B). The retinoblastoma gene encodes a phosphoprotein, pRb, that arrests cells in the G1-phase. pRb is phosphorylated and dephosphorylated during the cell cycle; the phosphorylated (inactive) form predominates in proliferating cells, whereas the unphosphorylated (active) form is generally more abundant in quiescent or differentiating cells. Consistent with the cell cycle arrest observed in DMP1-treated HUVEC, these cells showed a significant decrease of the phospho-pRb form in favor of the unphosphorylated form. Additionally, the expression of the proliferating cell nuclear antigen (PCNA), which is synthesized as a phospho-pRb-mediated gene product in the early G0/G1 and S phases, was

decreased following DMP1 treatment in HUVEC (Figure 3B). Thus, the results of the present studies indicate that DMP1 treatment induced HUVEC cell cycle arrest in G1, which is sustained by both the increase of p27^{Kip1} and the decrease of the related G1/S-checkpoint protein, phospho-pRb.

5

The importance of p27^{Kip1} in DMP1-induced effect on HUVEC growth was further demonstrated by the use of specific siRNAs directed against p27^{Kip1}. Indeed, it was shown that DMP1 treatment failed to inhibit the proliferation of p27^{Kip1} silenced-HUVEC when compared to either mock- or irrelevant siRNA transfected cells (Figure 3C). Next, it was demonstrated that p27^{Kip1} induction was abolished when cells were treated with anti-CD44 blocking antibody prior to DMP1 treatment while significant inductions were still observed with cells pre-treated with anti- $\alpha_v\beta_3$ integrin blocking antibody or control IgG (Figure 3D). The data provided in the present studies 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 p27^{Kip1} expression in HUVEC. Regarding p27^{Kip1}, it has been shown that the phosphorylation on serine 10 increases its stability. DMP1 treatment of HUVEC rapidly induced a significant increase of phospho-p27^{Kip1}(Ser¹⁰) while total p27^{Kip1} level was unchanged (Figure 3E). After 24 hours, the level of phospho-p27^{Kip1}(Ser¹⁰) was still increased in treated HUVEC when compared to untreated cells. However, the total p27^{Kip1} level increased, suggesting its accumulation over time. The extent of Ser¹⁰ phosphorylation of p27^{Kip1} has been shown to be increased in cells in the G0-G1-phase of the cell cycle in comparison with cells in S- or M-phase. The present inventors showed that DMP1 induced p27^{Kip1}(Ser¹⁰) phosphorylation which could explain its stabilization and accumulation over time.

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Example 5: DMP1 induces CD44-dependent VE-cadherin expression and mediates inhibition of growth in sparse HUVEC

The endothelial-specific cadherin, vascular endothelial-cadherin (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. The expression of VE-cadherin was evaluated using immunofluorescence in HUVEC treated with DMP1 during 3 and 24 hours. DMP1 treatment significantly induced the

30

expression of VE-cadherin at the cell membrane already after 3 hours (Figure 4A). Western blot analysis showed a twofold increase of VE-cadherin expression level in HUVEC treated with DMP1, as well as a p27^{Kip1} increase assessed on the same cell extracts (Figure 4B). FACS analysis confirmed DMP1-induction of VE-cadherin expression at the cell surface (Figure 4C) whereas DMP1 did not affect other endothelial cell-cell junction protein expression such as ZO-1 and PECAM-1.

It has previously been reported that N-Cadherin and E-cadherin mediated signaling is involved in contact inhibition of growth by inducing cell cycle arrest at the G1-phase and elevation of p27^{Kip1} levels. However, no direct evidence of an effect of VE-cadherin on p27^{Kip1} expression in endothelial cells has been reported to this date. To explore this possibility, siRNAs specifically directed against VE-cadherin were transfected in HUVEC prior to their treatment with DMP1. VE-cadherin-silenced cells did not show an increased p27^{Kip1} expression upon DMP1 treatment when compared to cells transfected with non-targeting siRNAs used as control (Figure 4D), indicating that VE-cadherin expression is essential to DMP1-mediated p27^{Kip1} induction and subsequent growth control of HUVEC.

Then the involvement of CD44 on the observed increase of VE-cadherin expression upon DMP1 treatment was evaluated. As reported in figure 4E, DMP1-mediated induction of VE-cadherin was abolished when HUVEC were treated with anti-CD44 blocking antibody prior to DMP1 treatment. DMP1 induction of VE-cadherin expression was still visible in presence of IgG used as control. These results pointed to CD44 as a major actor of VE-cadherin expression increase, p27^{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. To address the question of a contact inhibition of growth signal mimicry occurring upon DMP1 treatment of HUVEC, the expression of VE-cadherin and p27^{Kip1} in sparse and confluent cultured cells was studied. It was shown that DMP1 induced the expression of VE-cadherin and p27^{Kip1} in sparse

cells while, as expected, long confluent cells did not show any modulation of expression of both proteins (Figure 4F). Then VE-cadherin staining by immunofluorescence in parallel with BrdU incorporation in sparse and confluent HUVEC was evaluated. As shown in Figure 4G, a large fraction of sparse HUVEC progressed through G1 and entered S-phase under control condition, whereas only a minor fraction of confluent cells progressed through the cell cycle under identical conditions. DMP1-treated sparse cells showed a strong positive VE-cadherin staining and less BrdU incorporation than the control sparse cells, a pattern similar to that of control or DMP1-treated confluent cells. Since confluent HUVEC establish VE-cadherin-dependent junctions and undergo 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 confirmed that DMP1 induced contact inhibition of growth signal mimicry on sparse endothelial cells.

15

Example 6: DMP1 counteracts VEGF-induced angiogenesis

Further, the effects of DMP1 on VEGF-induced angiogenesis were investigated. First the effect of DMP1 on VEGF-induced endothelial cell proliferation was studied. Sparse and confluent cells were treated with DMP1 or with PBS during 24 hours and were then treated with VEGF during 24 hours. It was observed that DMP1 pre-treatment impaired VEGF-induced sparse cell proliferation in a dose-dependant manner when compared to control VEGF-treated sparse cells (Figure 5A), while it did not affect confluent cells.

Next the impact of DMP1 on VEGF-induced cell cycle progression was studied. Synchronized HUVEC treated with DMP1 were released with 2% serum culture medium containing VEGF (50ng/ml). As expected, HUVEC responded to VEGF by a significant entry in S-phase when compared to control cells released with complete medium (Figure 5B). The S-phase cell population in DMP1-treated cells corresponded to that of non released cells. Indeed, DMP1 treatment significantly impaired VEGF release, with a decrease of HUVEC in the S-phase cell population of two fold.

The migration of endothelial cells is a critical step in forming new vessels. Therefore, the impact of DMP1 on VEGF-mediated migration was tested by testing the ability of DMP1-treated endothelial cells to migrate through fibronectin-coated inserts towards VEGF. It was shown that DMP1 impaired migration of cells
5 towards VEGF when compared to control cells (Figure 5C).

To finally assess the effect of DMP1 on VEGF-induced angiogenesis, the impact of DMP1 on the VEGF-induced capillary-like structures in a standard Matrigel *in vitro* model was tested. After 6 hours, it was observed that DMP1-treated
10 endothelial cells impaired VEGF-induced tubular network on Matrigel (Figure 5D). Together, these results demonstrated that DMP1 blocked crucial steps of VEGF-induced angiogenic process.

**Example 7: DMP1 inhibits VEGF-dependent activities through blockade of
15 VEGFR-2 phosphorylation**

At the surface of endothelial cells, the VEGF receptor 2 (VEGFR-2) has been identified as the major mediator of VEGF-dependent signaling and cellular activities. In the present studies the hypothesis was tested according to which DMP1 treatment could affect VEGFR-2 expression and its phosphorylation status
20 in HUVEC. As shown in Figure 6A, VEGFR-2 expression level was significantly increased while its phosphorylated form was completely inhibited upon DMP1 treatment. Under the same conditions, DMP1 did not show any significant effect on VEGFR-1 expression nor activation (Figure 6A).

**Example 8: DMP1 affects VEGFR-2 phosphorylation through the induction
25 of VE-cadherin expression**

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, the present inventors postulated during their
30 studies 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, VE-cadherin expression in HUVEC was inhibited using a pool of specific siRNAs prior to the addition of DMP1. Indeed, VE-cadherin-silenced cells did not show any significant decrease of VEGFR-2 phosphorylation

upon DMP1 treatment when compared to cells transfected with control non-targeting pool of siRNAs (Figure 6B) indicating that VE-cadherin expression is indispensable to DMP1-mediated VEGFR-2 phosphorylation inhibition.

5 **Example 9: DMP1 blocks VEGF-induced VEGFR-2 phosphorylation**

VEGFR-2 receptor is phosphorylated upon activation by its ligands. Therefore, the next test related to the question if DMP1 could impair VEGF-induced modulation of VEGFR-2 expression and phosphorylation. In this experiment, endothelial cells were treated with DMP1 during 24 hours and challenged with VEGF (50ng/ml) during 10 minutes. As depicted in Figure 6C, DMP1 pretreatment not only induced VEGFR-2 expression but completely impaired its phosphorylation in presence of VEGF.

Example 10: DMP1 inhibits VEGF-mediated Src activation

15 Src family kinases (SFKs) are involved in VEGFR-2 signaling and have been involved in the regulation of vascular permeability and angiogenesis. Src possess two sites of tyrosine (Tyr) phosphorylation that are critical to the regulation of its kinase activity. Autophosphorylation on an activation loop Tyr residue (Tyr416, chicken c-Src numbering) increases its catalytic activity, while phosphorylation of a C-terminal Tyr (Tyr527) inhibits its activity. Thus, VEGF induces dephosphorylation of phospho-Tyr527 and increases Src kinase activity. Here, it was shown that DMP1 impaired both VEGF-induced dephosphorylation of Src on Tyr527 and slightly impairs VEGF-induced phosphorylation on Tyr416 (Figure 6D), indicating that DMP1 counteracts VEGF-triggered Src activation.

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Example 11: DMP1 inhibits VEGF-mediated VE-cadherin down-regulation at the cell junctions and induction of tyrosine 685 phosphorylation

It was known that VEGF induces a decrease of cell surface VE-cadherin through endocytosis. Remarkably, DMP1 significantly inhibited the VEGF-induced decrease in VE-cadherin expression as shown in Figure 6E. VEGF regulates VE-cadherin activity by inducing its phosphorylation on Tyr residues. Therefore the effect of DMP1 treatment on VEGF-induced VE-cadherin phosphorylation was studied. As expected, VEGF induced the phosphorylation of Tyr731, 658 and 685 known to be VEGF-dependent, while DMP1 alone did not affect their

phosphorylation status. Interestingly, DMP1 treatment specifically impaired VEGF-induced VE-cadherin phosphorylation on Tyr685 (Figure 6E).

It has been demonstrated that Src phospho-Tyr527 level is dictated by the activities of C-terminal Src Kinase (Csk) which is recruited at the membrane by VE-cadherin. Accordingly, the present inventors 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 6F).

Finally, it was 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 6G). This ability of DMP1 to block VEGF-triggered Src activation through the induction of Csk expression is consistent with the potent antagonistic effect of DMP1 on VEGF-induced angiogenesis.

Example 12: DMP1 inhibits angiogenesis in the choroidal neovascularization model in mice

To further investigate the anti-angiogenic effect of DMP1, the laser-induced choroidal neovascularization (CNV) model was used in which VEGF has been shown to play a major role in the development of subretinal angiogenesis. In this experiment, mice were injected intravitreally with 500 nanomoles/liter DMP1 or with the solvent control on the day of the laser injuries. After 7 days, the experiments were stopped and whole-mount choroids were stained in order to reveal neoformed blood vessels. As shown in Figure 7A, DMP1 significantly limited the size of a representative neovascularized ocular lesion when compared to control lesion. Quantification of the lesions showed a 30% decrease upon DMP1 treatment in good accordance with its VEGF blocking activity (Figure 7A).

30 MATERIALS and METHODS

Cell culture and recombinant Protein. Human umbilical vein endothelial cells (HUVEC) were isolated and maintained in culture as previously described. 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 culture medium thereafter. Recombinant human DMP1 and VEGF 165 were purchased from R&D System.

5 **Antibodies.** Anti-p27^{Kip1} and anti-pRb antibodies were from BD Pharmingen. Anti-phospho-p27^{Kip1} (Ser¹⁰) antibody was from Zymed Laboratories. For detection of apoptosis, anti-PARP antibody was from BD Biosciences Clontech. Anti-p21, anti-PCNA, anti-VEGFR-2, anti-phospho-VEGFR-2 and anti-phospho-VEGFR1 antibodies were from Santa Cruz Biotechnology. Anti-VEGFR-1 antibody was
10 from Sigma-Aldrich. Anti-VE-cadherin, anti-phospho-VE-cadherin(Y685), anti-phospho-VE-cadherin(Y731) and anti-Csk antibodies were from BD Transduction laboratories. PECAM-1 antibody was from Dako. ZO-1 antibody was from Cell Signaling. Anti-phospho-VE-cadherin(Y658) antibody was from Upstate. Anti-phospho-Src(Y416) and anti-phospho-Src(Y527) antibodies were from Cell
15 signaling. Anti-β-actin antibody was from Sigma-Aldrich. Anti-HSC 70 antibody was from Santa Cruz Biotechnology. For blocking experiments, cell suspensions were incubated with anti-α_vβ₃ (LM609, Chemicon), anti-α_vβ₅ (P1F6, Chemicon), purified IgG (Serotec) and anti-CD44 (BU75, Ancell) each used at 10µg/ml during 1h prior to the adhesion assay or prior to DMP1 treatment.

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RNA interference. HUVEC were seeded at a density of 15x10⁴ cells per wells in 6-well plates and grown overnight in complete culture medium. Cells were transfected with 100nmol/L of small interfering RNA (siRNA) using calcium phosphate precipitation method and cultured for 48h or treated during the last 24h
25 of transfection in complete culture medium without ECGS. Specific siRNAs directed against p27^{Kip1}: #1, 5'-GGA-GCA-AUG-CGC-AGC-AAU-AUU-3' and #2, 5'-CGA-CGA-UUC-UUC-UAC-UCA-AUU-3' and VE-cadherin siRNAs SMARTpool were purchased from Eurogentec and Dharmacon, respectively. Non-targeting siRNA (EGT) (Eurogentec), mock siRNA (no siRNA) and non-targeting siRNA
30 SMARTpool (Dharmacon) were used as control.

Western blotting. Equal amounts of proteins were resolved by SDS-PAGE. Membranes were probed with primary antibodies followed by HRP-conjugated secondary antibodies and developed using a chemiluminescence detection

system. Films were scanned and bands were quantified using ImageJ (<http://rsb.info.nih.gov/ij/>). Membranes were re-probed with β -actin or HSC 70 antibodies used as a control for equivalent protein loading.

- 5 **Adhesion Assay.** Bacteriological 96-well plates (Greiner) were coated with DMP1 or vitronectin (Dako). HUVEC were seeded at a density of 20×10^3 cells per wells in the precoated wells. Attached cells were stained with crystal violet and the incorporated dye was measured by reading absorbance at 560nm.
- 10 **Boyden chamber assay.** HUVEC suspended in serum-free DMEM containing 0.1% BSA were seeded at a density of 12×10^4 into the top chamber of a modified Boyden chemotaxis chamber (Neuroprobe Inc) and the bottom chamber was filled with DMEM containing 1% BSA and 1% serum. Serum-free DMEM 0.1% BSA was used as negative control. HUVEC that had traversed the filter after overnight
- 15 incubation were stained using Diff Quick Stain Set (Medion Diagnostics). Migration was determined by counting cells in 4 replicates, and the extent of migration was expressed as the average number of cells per condition.

- 20 **Fibronectin inserts migration assay.** HUVEC suspended in serum-free DMEM containing 0.1% BSA were seeded in triplicate at a density of 10^5 into the upper part of a transwell filter (diameter 6.5mm, pore $3 \mu\text{m}$; 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 in the lower surface of the filter were stained with crystal violet. Migration was determined by
- 25 counting cells in 3 random fields per well, and the extent of migration was expressed as the average number of cells per field. Three wells per condition were counted.

- 30 **Tubulogenesis assay.** Tubulogenesis was assessed using Matrigel (Chemicon) as instructed by the manufacturer. HUVEC were seeded at a density of 30×10^3 cells per well on Matrigel in 24-well plates in 2% serum culture medium. For assessment of VEGF-induced angiogenesis, HUVEC were seeded at a density of 3×10^3 cells per well on Matrigel in 15-well μ -Slide Angiogenesis plates (Integrated BioDiagnostics) and treated with VEGF in presence of DMP1 where indicated in

0,2% serum culture medium. Pictures were taken under a phase-contrast microscope. Tubulogenesis was determined by counting branching vessels in 2 random fields per well, and the extent of tubulogenesis was expressed as the average number of branching vessels per field.

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Proliferation assay. HUVEC were seeded at a density of 20×10^3 in 24-well plates in complete culture medium. After overnight incubation, cells were treated with DMP1 in complete culture medium without ECGS and grown up to 48h. For the study of VEGF-induced proliferation, sparse (20×10^3) and confluent cells (40×10^4) were grown in 24-well plates in complete culture medium. After overnight incubation, cells were treated with DMP1 in complete culture medium without ECGS during 24h and then treated with VEGF in presence of DMP1 during another 24h in 2% complete culture medium without ECGS. Fluorimetric DNA titration was performed and used as an indicator of cell density. Spectrofluorimetric measurements were performed with Spectramax Gemini XS using SOFTmax PRO software.

Flow Cytometry. HUVEC were seeded at a density of 15×10^4 cells per wells in 6-well plates and grown overnight in complete culture medium before experimentation. After 24h of treatment, cells were incubated with the mentioned primary antibodies for 30 minutes, and then incubated with Alexa-488-conjugated secondary antibody for another 30 minutes. Cells were then analyzed by flow cytometry using a FACSCantoll cytometer and FACSDiva™ analysis software (BD Biosciences).

25

Synchronization and 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 seeded at a density of 15×10^4 in 6-well plates were grown overnight in complete culture medium and were then serum starved during 48h in medium containing 2% serum. During the last 4h of serum starvation, cells were treated with DMP1. Cells were serum-released during 20h with complete culture medium without ECGS in presence of the indicated treatment. For VEGF-released cell cycle assay, cells were treated with DMP1 during the last 24h of serum starvation. Cells were then

released with VEGF during 24h. The relative percentage of cells in each stage of the cell cycle was analyzed using the Cycle TEST Plus DNA Reagent Kit (Becton-Dickinson) on a FACS Calibur. Mimosine (Sigma-Aldrich) was used as G0/G1-synchronization control.

5

Annexin-V Assay. HUVEC were seeded at a density of 15×10^4 cells per wells in 6-well plates and grown overnight in complete culture medium before experimentation. After 24h of treatment in complete culture medium without ECGS, adhesive cells were pooled with detached cells. After centrifugation and
10 washing steps, the pellet was incubated in presence of Annexin V-Fluos. Propidium iodide (PI) was added before flow cytometric analysis using a FACSCantoll cytometer and FACSDiva™ analysis software (BD Biosciences).

For PARP detection, used as positive control of apoptosis, total adherent and
15 floating cell lysates from IGROV-1 treated with cisplatin (*cis*-diamminedichloroplatinum) $7 \mu\text{M}$ (Sigma-Aldrich) were extracted and analyzed by western blot²⁵.

Immunocytochemistry. For double labeling experiments, sparse HUVEC
20 (30×10^3) were seeded on glass coverslips in 24-well plates in complete culture medium. After overnight incubation, cells were treated with DMP1 in complete culture medium without ECGS during 3 and 24h. Culture medium was then removed, cells were subjected to fixation/permeabilization with ice-cold methanol-acetone during 10 minutes, and incubated with primary antibody against VE-
25 cadherin during 2h. Cells were then incubated with an Alexa-488-conjugated secondary antibody (Molecular Probes) during 1h, then incubated with TOPRO-3 (Molecular Probes) for nuclear counterstaining during 35 minutes and were finally mounted onto microscope slides using Mowiol. Images were obtained by observing slides under a LEICA TCS SP laser scanning confocal microscope
30 (Leica) using 40X objective. For BrdU incorporation staining, sparse (20×10^3) or confluent (40×10^4) were grown onto coverslips. Cells were treated with DMP1 for a total 48h. 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. Cells were then incubated with primary antibody against VE-cadherin, Alexa-488-

conjugated secondary antibody and Phalloïdine-Alexa-633-conjugated antibody (Molecular Probes). Cells were fixed with paraformaldehyde and subjected to DNA denaturation followed by incubation with BrdU-Alexa-543-conjugated antibody (Sigma-Aldrich). Cells were mounted and images were obtained under a
5 laser scanning confocal microscope (Olympus) using 40X objective.

Choroidal Neovascularization. *Laser photocoagulation:* 2 months old C57bl6 mice (five or more in each group) were maintained in a 12h-light–dark cycle with free access to food and water. All animal experiments were performed in
10 compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. CNV was induced by laser burns as described previously. Eyes were locally anesthetized and subjected to intravitreal injection of DMP1 or PBS used as control. Animals were terminated after 7 days. *Flat mount of choroids:* Before exsanguination, mice were injected (iv) with FITC-dextran (2,000,000 Da,
15 Sigma). Eyes were then taken and fixed in paraformaldehyde for 1h at room temperature. Retinae were discarded and the choroid was prepared in Vectashield medium (Vector Laboratories) for epifluorescence microscopy analysis. Quantitation was realized by measuring of total vessel fluorescence surface (ImageJ64 from NIH).

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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 version 8.0 (Statsoft).

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Claims

1. Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1, for use for reducing and/or preventing angiogenesis.
- 5 2. Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1, for use in the preventive and/or therapeutic treatment of angiogenesis-related diseases.
- 10 3. The Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1 according to claim 1 or 2, wherein said angiogenesis is VEGF-induced angiogenesis.
- 15 4. The Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1 according to any one of claims 1 to 3, wherein said angiogenesis is induced by VEGF by binding of VEGF to VEGF-receptor 2 (VEGFR-2).
- 20 5. The Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding Dentin Matrix Protein 1 according to any one of claims 2 to 3, wherein angiogenesis-related diseases are selected from the group consisting of malignant tumors, angiofibroma, arteriovenous malformations, arthritis, such as rheumatoid arthritis, atherosclerotic plaques, corneal graft neovascularization, delayed wound healing, proliferative retinopathy such as diabetic retinopathy, macular degeneration, granulations such as those occurring in hemophilic joints, inappropriate
25 vascularization in wound healing such as hypertrophic scars or keloid scars, neovascular glaucoma, ocular tumor, uveitis, non-union fractures, Osier-Weber syndrome, psoriasis, pyogenic glaucoma, retrolental fibroplasia, scleroderma, solid tumors, Kaposi's sarcoma, trachoma, vascular adhesions, chronic varicose ulcers, leukemia, and reproductive disorders such as follicular and luteal cysts,
30 choriocarcinoma, cancerous diseases and retinal neovascularization.

6. The Dentin Matrix Protein 1 (DMP1) according to any one of claims 1 to 5, selected from any one of (a) to (e):
- (a) a protein having the sequence shown in SEQ ID NO: 1 or SEQ ID NO: 2;
 - (b) a protein having the amino acid sequence of position 17 to 513 of SEQ ID NO: 1, or having the amino acid sequence of position 17 to 497 of SEQ ID NO: 2;
 - (c) a fragment of (a) or (b) having the activity to inhibit angiogenesis;
 - (d) a protein having at least 50% identity to (a) or (b) or (c) having the activity to inhibit angiogenesis;
 - (e) a protein having an amino acid sequence comprising a deletion, substitution, insertion and/or addition of one or more amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 2, or with respect to the protein of (b) or (c) or (d).
7. A polynucleotide for use for reducing and/or preventing angiogenesis encoding the protein of any of (a) to (e) of claim 6.
8. A polynucleotide for use in the preventive and/or therapeutic treatment of angiogenesis-related diseases encoding the protein of any of (a) to (e) of claim 6.
9. A medicament comprising the Dentin Matrix Protein 1 (DMP1) or a polynucleotide encoding it.
10. A method for reducing and/or preventing angiogenesis comprising the administration of an effective dose of Dentin Matrix Protein 1 (DMP1) or of a polynucleotide encoding Dentin Matrix Protein 1 to an individual.
11. A method for the preventive and/or therapeutic treatment of angiogenesis-related diseases comprising the administration of an effective dose of Dentin Matrix Protein 1 (DMP1) or of a polynucleotide encoding Dentin Matrix Protein 1 to an individual.

Figure 1 - S Ptotte et al.

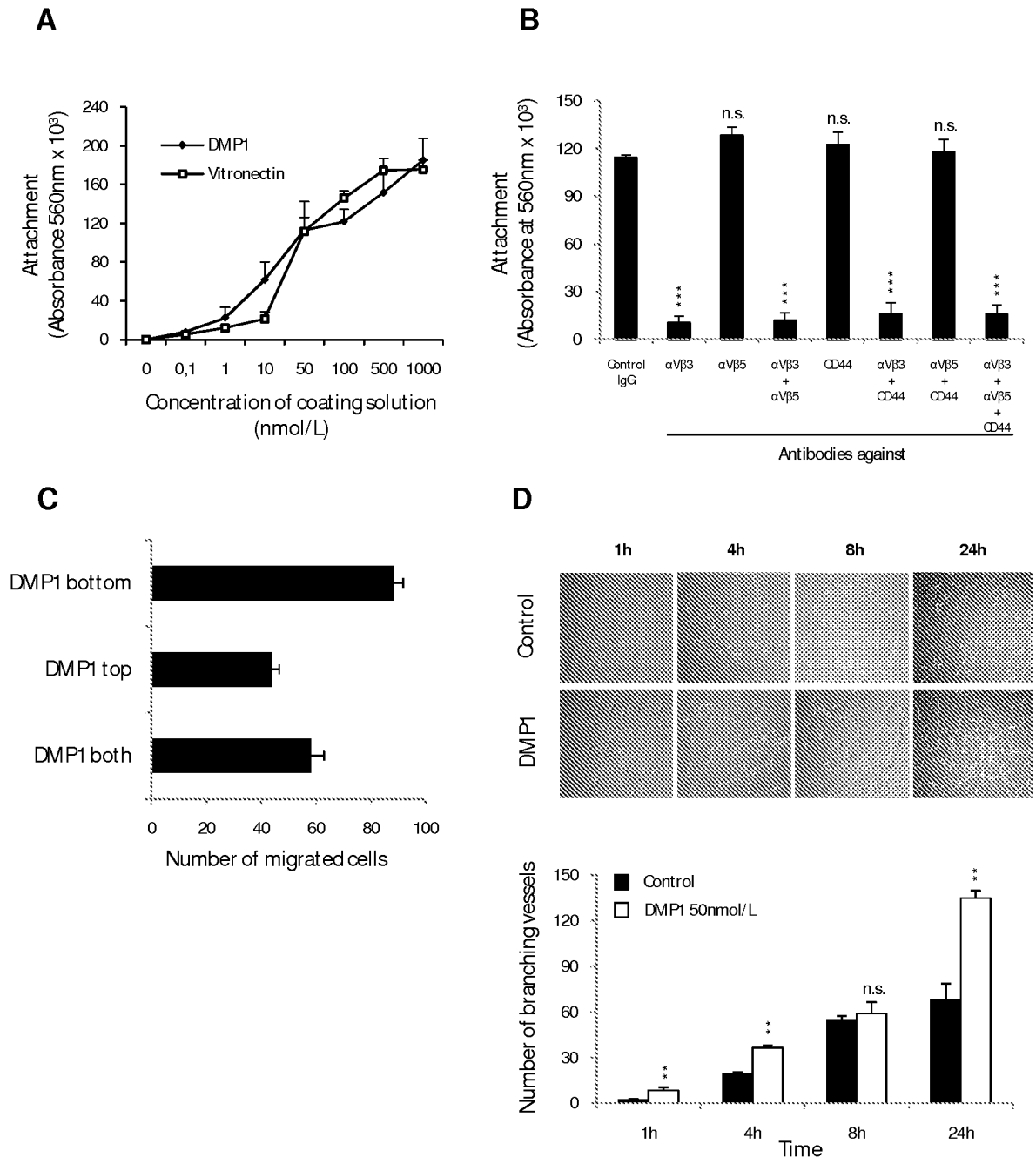


Figure 2 - S Ptotte et al.

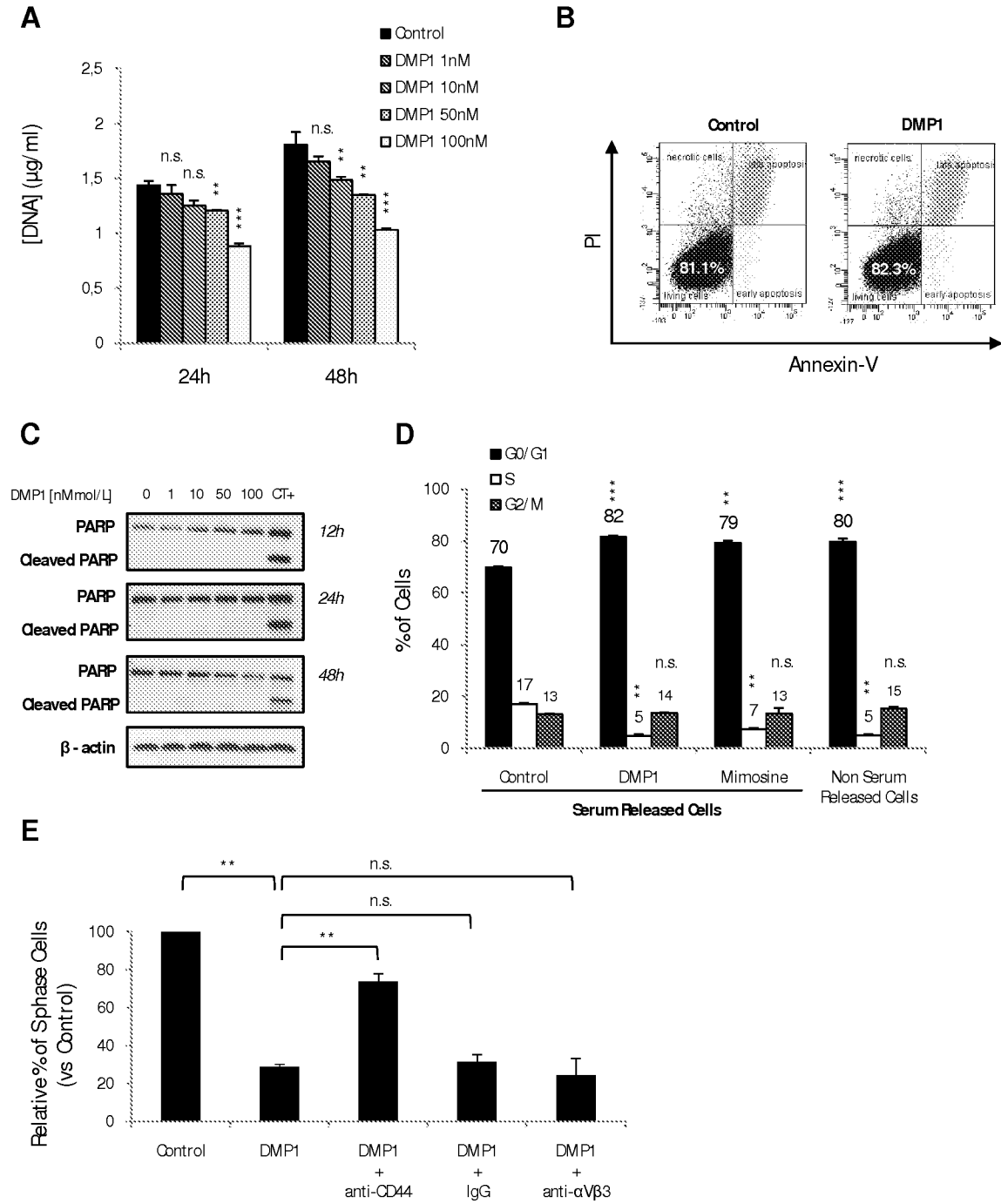


Figure 3 - S Pirotte et al.

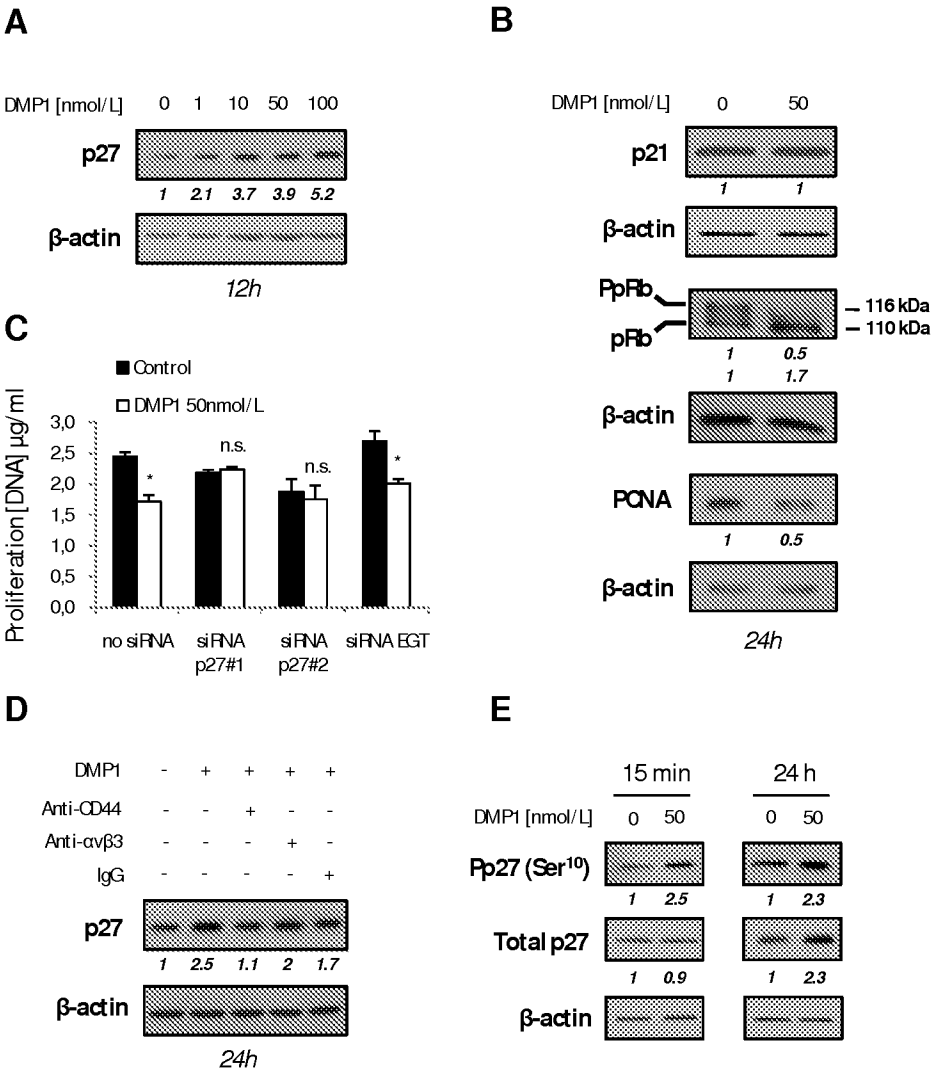


Figure 4 - S Protte et al.

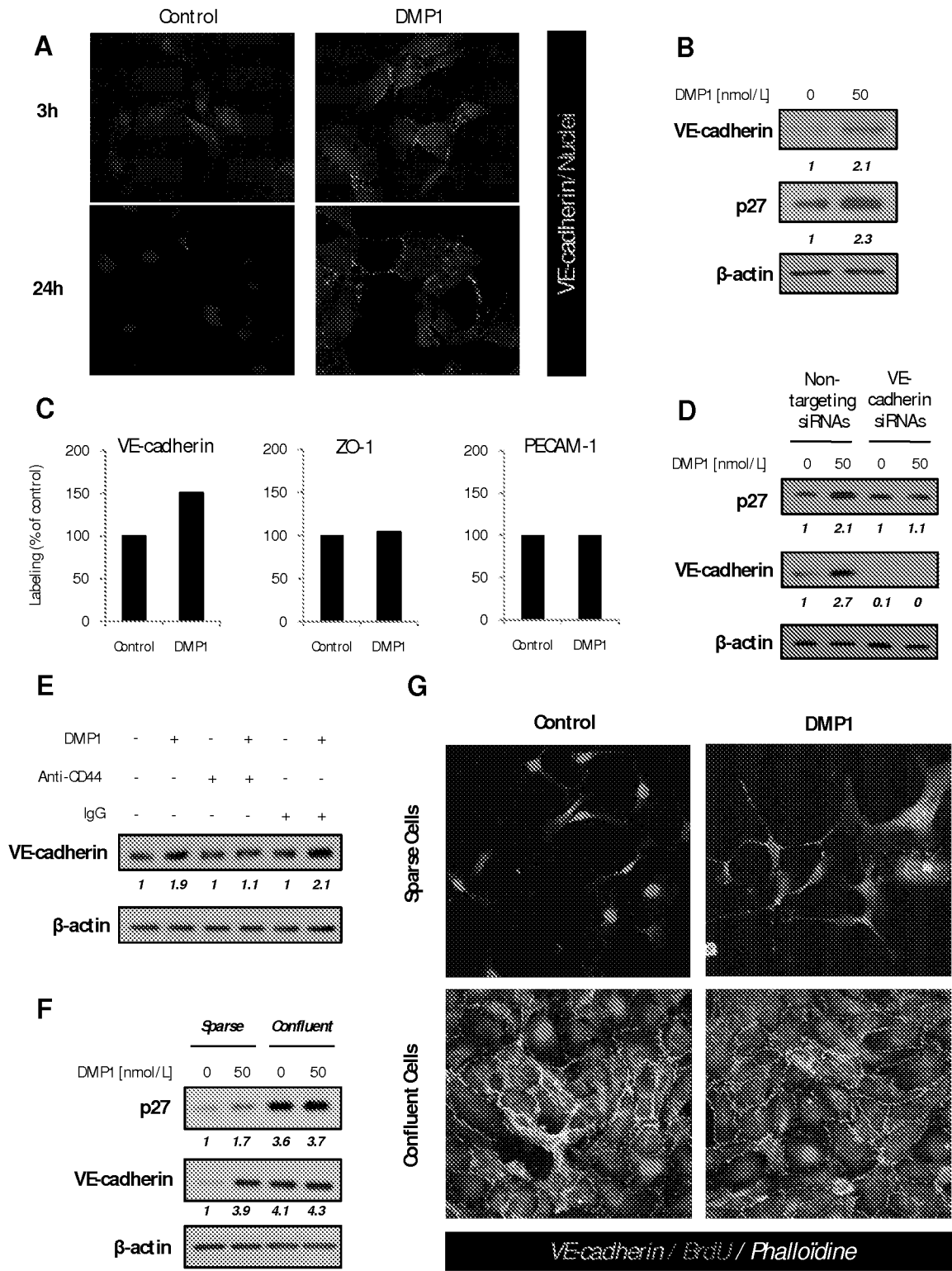


Figure 5 - S Ptotte et al.

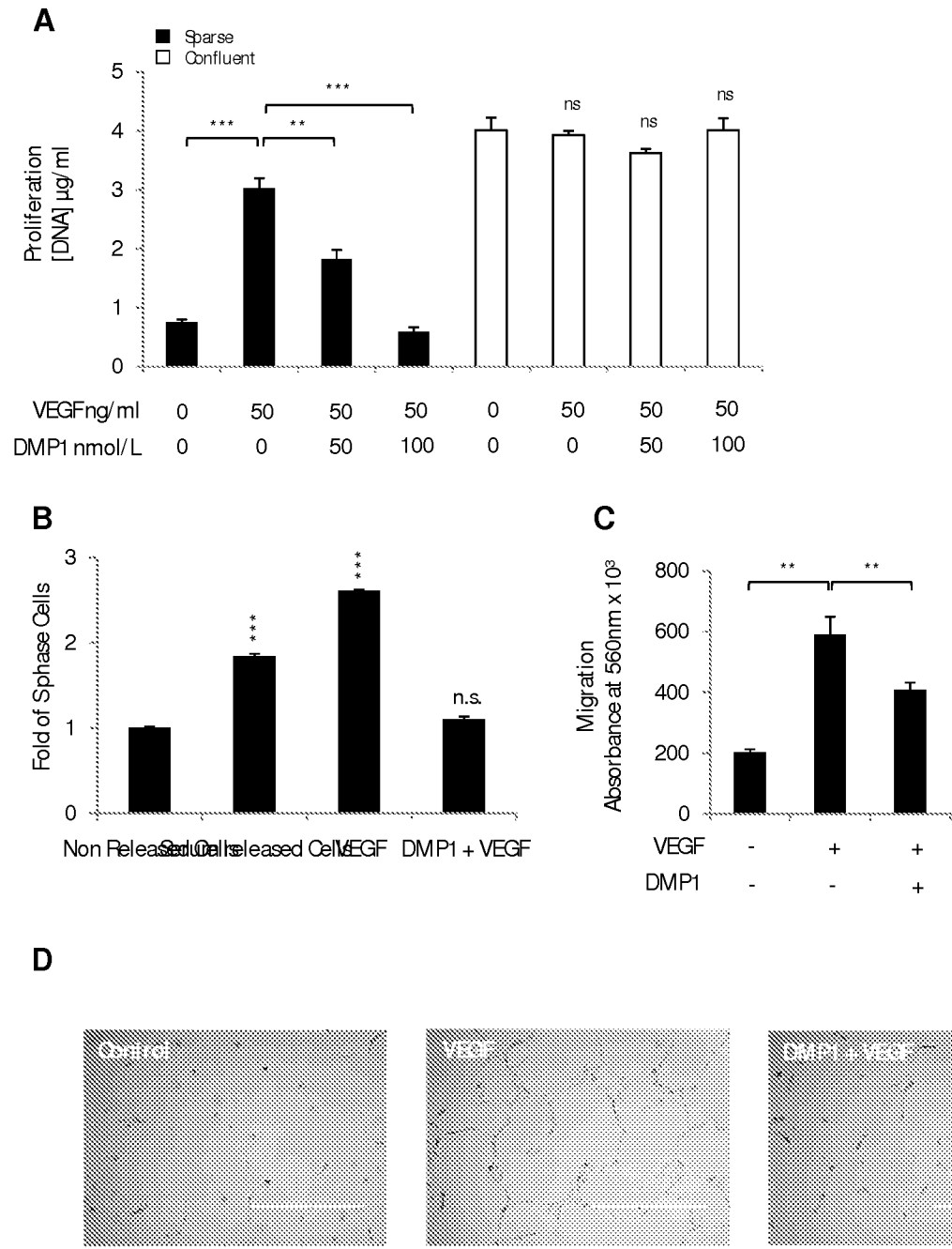


Figure 6 - S Flotte et al.

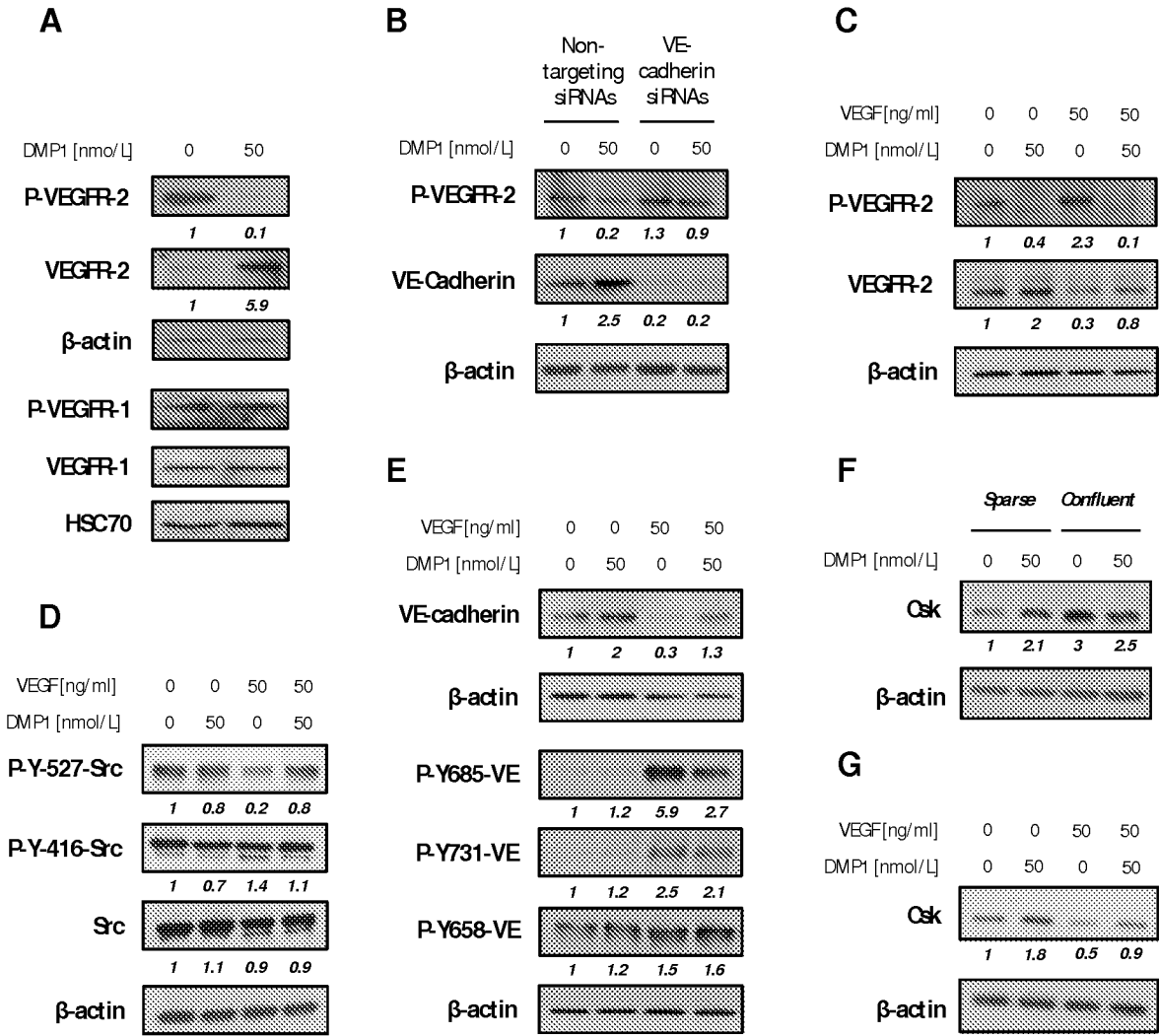
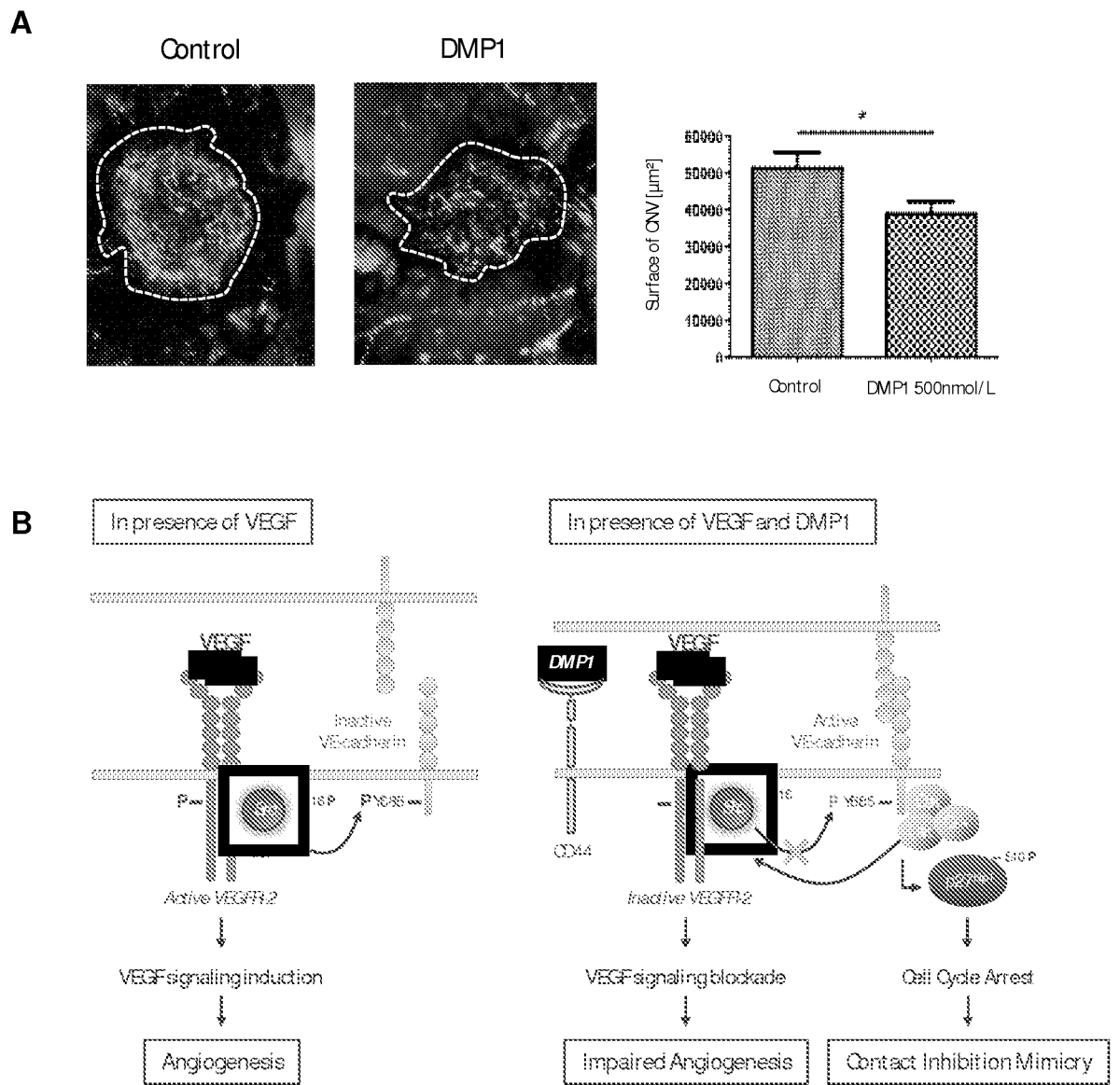


Figure 7- S Protte et al.



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/061154

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/17 A61P35/00 A61P19/02 A61P9/10 A61P17/02
A61P27/00 A61P37/00 A61P17/06 A61P19/00 A61P15/00
A61P27/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPQ-Internal, WPI Data, BIOSIS, COMPENDEX, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/43415 A1 (ST JUDE CHILDRENS RES HOSPITAL [US]) 20 November 1997 (1997-11-20) page 38, line 13 - page 39, line 30	1-11
X	DATABASE WPI Week 200357 Thomson Scientific, London, GB; AN 2003-601822 XP002606068 & JP 2003 116560 A (TEIJIN LTD) 22 April 2003 (2003-04-22) * abstract	1-11

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

21 October 2010

Date of mailing of the international search report

05/11/2010

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Authorized officer

Pilling, Stephen

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/061154

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 200464 Thomson Scientific, London, GB; AN 2004-662362 XP002606069 & WO 2004/078217 A1 (KOKEN KK) 16 September 2004 (2004-09-16) * abstract	1-11
X	HASSAN ALI H ET AL: "Use of bone morphogenetic protein-2 and dentin matrix protein-1 to enhance the osteointegration of the Onplant system." CONNECTIVE TISSUE RESEARCH 2003 LNKD- PUBMED:12945802, vol. 44, no. 1, 2003, pages 30-41, XP9140245 ISSN: 0300-8207 * abstract	1-11
X	DATABASE WPI Week 200629 Thomson Scientific, London, GB; AN 2006-277086 XP002606071 & JP 2006 089395 A (KOKEN KK) 6 April 2006 (2006-04-06) * abstract	1-11
X	MALLAKIN ALI ET AL: "The Arf-inducing transcription factor Dmpl encodes a transcriptional activator of amphiregulin, thrombospondin-1, JunB and Egr1." INTERNATIONAL JOURNAL OF CANCER. JOURNAL INTERNATIONAL DU CANCER 15 MAR 2010 LNKD- PUBMED:19816943, vol. 126, no. 6, 15 March 2010 (2010-03-15), pages 1403-1416, XP9140238 ISSN: 1097-0215 * abstract	1-11
X	MALLAKIN ALI ET AL: "Mutually exclusive inactivation of DMP1 and ARF/p53 in lung cancer" CANCER CELL, vol. 12, no. 4, October 2007 (2007-10), pages 381-394, XP002606073 ISSN: 1535-6108 * abstract	1-11

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/061154

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YE L ET AL: "Dmpl-deficient mice display severe defects in cartilage formation responsible for a chondrodysplasia-like phenotype" JOURNAL OF BIOLOGICAL CHEMISTRY 20050218 AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY INC. US, vol. 280, no. 7, 18 February 2005 (2005-02-18), pages 6197-6203, XP9140241 DOI: DOI:10.1074/JBC.M412911200 * abstract page 6203, column 1, line 33 - line 42 -----	1-11

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2010/061154

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)
☐ on paper
☒ in electronic form
 - b. (time)
☒ in the international application as filed
☐ together with the international application in electronic form
☐ subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2010/061154

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9743415	A1	20-11-1997	AU 3132397 A	05-12-1997
JP 2003116560	A	22-04-2003	NONE	
WO 2004078217	A1	16-09-2004	NONE	
JP 2006089395	A	06-04-2006	NONE	