Supplementary data

Cell culture

MLuMEC,FVB and MBrMEC,FVB (3) two models of mature endothelial cells isolated from FVB mouse lung and brain were cultured in OptiMEM with Glutamax-I (Gibco Invitrogen) supplemented with 2 % (vol:vol) fetal bovine serum –FBS (BioWest, Nuaillé, France), 40 µg/ml gentamycin and 0.05 µg/ml fungizone. Human microvascular endothelial cells HMEC-1 (ATCC) were cultured in MCDB 131 medium (Gibco Invitrogen), 10 % (vol:vol) FBS, L-glutamine 2 mM, epidermal growth factor 10 ng/ml, hydrocortisone 1 µg/mL, 50 U/ml penicillin, 50 µg/ml streptomycin, and fungizone 0.05 µg/ml. B16F10 melanoma cells (ATCC, CRL-6475) and HEK293 cells (human embryonic kidney 293E cells, ATCC, CRL-10852) were cultured in DMEM high glucose medium (Gibco Invitrogen), supplemented with 2 % (vol:vol) FBS, penicillin 100 U/mL, streptomycin 100 µg/mL, fungizone 0.05 µg/ml. ATCC supplied cell lines were not further authentified. Lack of mycoplasma infection was confirmed by "MycoAlert Detection kit" (Lonza). Cells were routinely cultured at 37 °C in a humidified incubator in a 95 % air/5 % CO_2 atmosphere and passaged by detaching cells with 0.25 % trypsin-0.05 % EDTA (w/v) solution (Gibco Invitrogen).

Generation of pIFP1.4-HREmsVEGFR2 and pHREmsVEGFR2 vector

From pBLAST45-msFlk1(s7) vector the msFlk1/msVEGFR2 coding sequence was cut by EcoRI restriction enzyme. This fragment was subcloned into the EcoRI opened pAAV-MCS vector (Stratagene). The resulting construct pAAV-MCS-msVEGFR2 (step1) was further used to generate msVEGFR2 sequence flanked by ClaI restriction site upstream and XhoI restriction site downstream. Then the XhoI-ClaI fragment from the intermediary vector (step1, Supplementary Figure S1A), was introduced into the pdAAV-HRE-minCMV-HO1-SV40-pA vector. ClaI and SalI double digestion allows to replace the HO-1 coding sequence by the XhoI-ClaI msVEGFR2 fragment leading to the intermediary vector step 2 named pdAAV-HRE-minCMV-msVEGFR2-SV40pA (XhoI and SalI generate compatible cohesive ends). As vector step 2 was shown to be leaky, minCMV promoter was shortened by digestion with SacI restriction enzyme leading to 104 nucleotides excision from the 3'

side. The purified vector was ligated back to a vector designated as pdAAV-HRE-minCMV-msVEGFR2-SV40pA (short promoter) described in Supplementary Figure S2 and used for all cloning steps. SspI-SspI fragment from the pdAAV-HRE-minCMV-msVEGFR2-SV40pA vector was cloned into the IFP1.4_pcDNA3.1H.ape vector kindly provided by Pr R. Y. Tsien (University of California, San Diego, USA) and double digested by SspI and BstZ17I to get the pIFP1.4-HREmsVEGFR2 vector. To obtain the pHREmsVEGFR2 vector, the pIFP1.4-HREmsVEGFR2 vector was digested by PmeI and re-ligated leading to the excision of IFP1.4-IRES-mCherry region (Supplementary Figure S1B).

Quantification of msVEGFR2 protein production

The msVEGFR2 was secreted in the supernatant by transfected or stably expressing msVEGFR2 cells growing in FBS-free medium. Conditioned supernatants were collected 24h or 48h after incubation in hypoxia (1 % O_2) and normoxia (18.75 % O_2), and analyzed by ELISA according to instructions using the mouse sFlk1 ELISA duoset R&D kit (DY1558B, R&D systems, USA).

Surface Plasmon Resonance (SPR) assays

SPR analyses on Biacore 3000 (Biacore AB, Uppsala, Sweden) at 25 °C used CM5 sensor chips (GE Healthcare) immobilized mouse VEGF (493MV/CF, R&D systems, USA) and human VEGF (293VE/CF, R&D systems, USA) by amine coupling according to the manufacturers' instructions. Briefly, dextran surface of the sensor chip, was activated by flowing a mixture of 0.05 M N-hydroxysuccinimide and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride in a PBS-P20 0.005% (vol/vol) at a flow rate of 5 µl/min for 7 min. Then, mouse VEGF or human VEGF (8 µg/mL), diluted in 10 mM Na acetate (pH 5.0) was bound to the activated surface of the sensor chip at a flow rate of 5µl/min for 6min. Remaining functions on the surface of the sensor chip, were neutralized by ethanolamine hydrochloride solution (1 M, pH 8.5) injected at a flow rate of 5 µl/min for 7 min. Both immobilizations estimated by the amount of Resonance Units reached 3000 RU. Mouse soluble VEGFR2 concentrated from HEK-IFPmsVEGFR2 cell supernatant by centrifugation with a 50 KDa cut-off membrane (Millipore), was diluted in running buffer (PBSc-P20 0.005 %) and

flowed over the chip surface at a flow rate of 30 µl/min. Mouse soluble Flk-1 solution in running buffer (PBSc-P20 0.005 %) was flowed over the chip surface at 30 µl/min. After each injection and dissociation phase, the surface was regenerated by injection of 90 µl 2M $MgCl_2$ (flow rate 30 µl/min) chaotropic agent. The response data were analyzed with the BIAevaluation program version 3.2. A Langmuir binding model with a stochiometry of 1:1 was used to analyze the association rate constant k_{on}, the dissociation rate constant k_{off} (M–1s–1 and s–1, respectively), and the dissociation constant, $KD=k_{off}/k_{on}$.

Cell Proliferation Assay

Cells were seeded in 96-well culture plates at a density of 2.10^3 cells per well in complete OptiMEM medium (GIBCO). Once adhered (6 hours), cells were starved in empty OptiMEM for 12 hours. Then conditioned supernatants were added for 48 hours. Media were removed and a cell proliferation based on the BrdU incorporation (Cell Proliferation ELISA, BrdU, Roche). Absorbance was measured in each well using a spectrophotometric plate reader at wavelength of 450 nm. BrdU incorporation was calculated by the following equation: BrdU incorporation = [(absorbance of treated well) - (absorbance of non-BrdU labeled well)]/[(absorbance of control well) - (absorbance of non-BrdU labeled well)].

In Vitro Angiogenesis Assay

HMEC-1 cells angiogenesis, in the presence of msVEGFR2-conditioned medium from HEK293-IFP-msVEGFR2 cell line or control medium from HEK293-IFP cell line, was performed on 96-well plates coated with 50 µL of Matrigel™ (BD Biosciences) diluted at ½ in OptiMEM.

MLuMEC,FVB and MBrMEC,FVB angiogenesis assays were performed after a 48h pre-incubation in the presence of msVEGFR2-conditioned medium from the two msVEGFR2 expressing clones (13.3 and 16.4) or control medium from B16F10wt cells (wild type), in normoxia (21 % O_2). Then, cells were seeded in 96-well plates coated with 50 µL of Growth Factors Reduced Matrigel™ (BD Biosciences) diluted at ½ in OptiMEM.

In both cases, $1.5X10^4$ cells per well were seeded and the plate was introduced into the incubation chamber of the video Zeiss Axio Observer Z1 fluorescence inverted microscope (Zeiss) equipped with an ORCA-R2 high-resolution CCD camera linked to a computer driven-acquisition software Axiovision (Zeiss) to control time-lapse acquisitions (30 min.) over 24h. Tube-like and network structures were analyzed by Wimasis Images Analysis.

***In vivo* angiogenesis assay**

Isoflurane (2.5%, Aerrane, Baxter S.A.S, Maurepas, France) anesthetized female C57Bl/6Tyr^{C2-j}Orl mice, 6-8 weeks old (CDTA-CNRS Orleans, France) were subcutaneously injected with 250 µL of Matrigel™ supplemented with 500 ng/mL of bFGF (R&D Systems) mixed with saline (1:1 v/v) containing 10^5 cells HEK-IFP-msVEGFR2 or HEK-IFP. Matrigel plug remained in the animals for 10 days before sacrifice, surgery and imaging. 100 µL of a FTC-dextran (MW 2000000, FD2000S, Sigma) solution (10 mg/mL in saline) was intravenously injected in the tail vein for angiogenesis visualization. For *in vivo* fluorescence macroscopy, a Nikon AZ100 Multizoom was used, equipped with an EMCCD Evolve 512 photometric camera and driven by the NIS Element BR software. Acquisitions were done on reversed skin. Epifluorescence illumination system used an Intensilight HGFIE HG, precentered fiber illuminator (130 W mercury). The fluorescence channels were set with filters for FITC: λex 482/35 nm, beam splitter 506 nm, λem 536/40 nm; for TRITC channel: λex 543/22 nm, beam splitter 562 nm, λem LP561 nm (Semrock, Rochester, New York, USA).

Flow cytometry

After 48h incubation either in normoxia or hypoxia, B16F10wt cells (wild type) and msVEGFR2 expressing clones, 13.3, 16.4, as well as MLuMEC,FVB endothelial cells were washed twice with complete phosphate-buffered saline (PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$; cPBS) containing 0.5 % bovine serum albumin and 0.1 % NaN_3 (cPBS/BSA). Cells were detached by collagenase (Invitrogen). After two washings with cPBS/BSA solution, cells were incubated for 1h at 4 ^{o}C with directly labelled antibody or corresponding isotype: rat anti-mFlt1-PE IgG2B (R&D FAB4711P), rat anti-Flk1-PE $IgG2a_{kappa}$ (eBioscience 12582182). The goat anti-Flt4 IgG polyclonal (R&D AF743)

was revealed, after washing, by the secondary antibody rabbit anti-goat-PE (Santa Cruz BioTechnologies sc-3755) incubated for 30 min at 4 °C. Cells were then washed and their fluorescence was detected using a FACS-LSR flow cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences). Results are expressed either by the relative fluorescence intensity ΔIF (the difference between the relative fluorescence intensity of antibody labeled cells and corresponding isotype).

Immunohistochemistry

Tumors were embedded in optimal cutting temperature compound (OCT Tissue-Tek, Sakura 4583), Frozen sections (8 µm) were collected on SuperFrost Plus slides (Fisher Scientific), dried for 2h at room temperature. OCT compounds were removed by a 30 min. incubation at 37°C in PBS. Blood vessels were labeled using lectin from *Ulex europaeus* conjugated with FITC (UeA-1-FTC, Sigma Aldrich, L-9006) used at 100 µg/mL and incubated for 45 minutes. Then slices were washed 3 times with PBS. For pericyte detection, a second labeling for α-SMA (α-smooth muscle actin) was performed. Briefly, slices were fixed with 1 % PFA (paraformaldehyde) for 30 minutes and washed with PBS to avoid detachment of the UeA-1 lectin. Then slices were incubated 10 min. in -20°C pre-cooled acetone. After 3 washings with PBS, blocking was performed by a 5% FBS solution in PBS for 30 minutes prior incubation for 1h with rabbit anti α-SMA antibody (Abcam, ab5694, 2 µg/mL). A Isotypic control was polyclonal rabbit IgG (Sigma, I-5006). Immunodetection of primary antibody, was performed by 1h incubation with a goat anti-rabbit TRITC-conjugated antibody (sigma, T6028, 2 µg/mL). Washed slides were mounted with the anti-fading mounting medium for fluorescence (Vectashield, Vector Laboratories, H-1200) containing DAPI and were examined with a fluorescent microscope.

Plasma collection

Plasma samples from B16F10 melanoma bearing mice were obtained after anesthesia with isoflurane, followed by heart puncture to collect 0.5 to 1 mL of blood for coagulation, centrifugation at 10000 g

for 2 min. to get the serum. mVEGF and msVEGFR2 concentration in serum were estimated by ELISA following the manufacturer's protocol (mouse sVEGFR2 Duoset kit DY1558B, and mouse VEGF Duoset kit DY493, R&D Systems, USA).

Spectrofluorimetry

IFP1.4 and mCherry spectra were measured from cells lysates using HEK293 empty or transfected cells (pHREmsVEGFR2-IFP1.4 vector) as well as IFP1.4 expressing B16F10 cells adapted from Chiu et al. (4). Briefly, cells were detached with the non-enzymatic Cell Dissociation Solution (C5789 from Sigma) and washed twice with PBS prior to be mechanically disrupted by successive passages through a 29-gauge insulin needle in 1 mL of PBS. After clearing the resulting lysates by centrifugation (5 min., 10000 g), 100 µL of protein in solution in the clear lysate were introduced into a 3x3 mm quartz cuve for reading. Excitation and emission spectra were performed with a Jobin Yvon–Horiba Fluoromax-2 spectrofluorimeter equipped with a R1527P Hamamatsu detector for visible and a 150 W Xenon lamp.

***In vivo* fluorescence imaging**

C57BL/6JTyr^{c2-j}Orl mice were injected with 10^5 pHREmsVEGFR2-IFP1.4 positive B16F10 cells, subcutaneously in the hind leg in 100 µL saline. 10 days after injection fluorescence images were obtained, after fur removal. Mouse was anesthetized (Aerrane®, Maurepas, France) with 2% isoflurane in air/O_2. Acquisition settings (binning and duration) were adapted to the tumor size and number of living cells. Pictures were obtained with binning 8 for 2 s exposure time. Autofluorescence reduction and signal enhancement were achieved by spectral filter scanning (emission from 620 to 680 nm) and spectral unmixing algorithms included in the device analysis tools.

Supplementary References

Bizouarne N, Denis V, Legrand A, Monsigny M, Kieda C. A SV-40 immortalized murine endothelial cell line from peripheral lymph node high endothelium expresses a new alpha-L-fucose binding protein. Biol Cell. 1993;79:209-18.

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Supplementary legends

Figure S1. Construction maps for the pIFP1.4-HREmsVEGFR2 and pHREmsVEGFR2 vector

(A) Schematic draw outlining the description of the pIFP1.4-HREmsVEGFR2 construction process.

(B) Schematic draw showing the DNA excision leading to pHREmsVEGFR2 from the pIFP1.4-HREmsVEGFR2.

Figure S2. Shortening of the minCMV promoter

Schematic draw describing the approach used to shorten the minCMV promoter and the relative vectors maps.

Figure S3. Characterization of the transfected clones for msVEGFR2 and mVEGF-A production

(A) ELISA quantification of mVEGF-A produced in the supernatants by B16F10 wt cells and two stably-transfected B16F10-msVEGFR2 clones: 16.4 and 13.3, cultured for 48 hours in normoxia (21 % O_2) or hypoxia (1% O_2). The results are reported to 10^6 cells. Values are mean ± SD (n = 3).

(B, C) msVEGFR2 (B) and mVEGF-A (C) production mentioned in Figure 4A and Figure S3A and expressed as absolute values in pg of protein of interest per mL of supernatant, avoiding normalization to 10^6 cells. Values are mean ± SD (n = 3). *$p < 0.05$.

Figure S4. Expression of VEGFRs on the surface of B16F10-msVEGFR2 melanoma clone 13.3 and 16.4

(A, B) Expression of VEGFRs on B16F10-msVEGFR2 melanoma clone 13.3 (A) and 16.4 (B). The cells cultured in normoxia (a, b, c) and hypoxia (d, e, f) were labeled with selected antibodies as described in Materials and Methods, for VEGFR1 (a, d), VEGFR2 (b, e), and VEGFR3 (c,f). Histogram overlays represent: empty histograms are isotypic controls and filled histograms are antibody-labeled cells.

Figure S5. Characterization of B16F10 wt and msVEGFR2 expressing clones 13.3 and 16.4 tumors *in vivo*

(A, B) mVEGF-A (A) and msVEGFR2 (B) quantified in the serum of mice harboring tumors producing soluble mVEGFR2 (clone 16.4 and 13.3) compared to animals bearing wild type B16F10 melanoma. Serum of B16F10 wt bearing mice was used as reference and set at 1. Results are expressed in arbitrary unit (A.U.). Values are mean ± SD (n = 7).

(C, D) Quantification of mVEGF-A (C) and msVEGFR2 (D) production by growing tumors from cell lines transfected with msVEGFR2 plasmid (clones 16.4 and 13.3) as compared to control B16F10 cells. The amount of protein of interest is normalized as a function of the tumor weight and expressed in arbitrary unit (A.U.). Data of B16F10 wt bearing mice was used as reference and set at 1. Values are mean ± SD (n = 7).

(E) Post-graft *in vivo* tumor volume progression. The tumor size was measured over 13 days comparing the B16F10-msVEGFR2 melanoma clones 16.4 and 13.3 to wild type B16F10. Tumor volumes were calculated as follows: (maximal length)X(perpendicular width)^2/2. Each value is plotted on the graph to allow a linear regression of tumor progression and determination of the regression lines equations. The reported data concern 5 B16F10 wt tumor bearing mice and 6 of each msVEGFR2 expressing tumor (clone 13.3 and 16.4) bearing mice.

Figure S6. Effect of the IFP1.4 and mCherry expression on the B16F10 cells proliferation

The stably transfected B16F10 by the pIFP1.4-HREmsVEGFR2 vector were compared to B16F10 wt for their proliferation rate. The amount of cells is determined by cell numeration at various culture times: 24, 48, 72 and 96h in normoxia (21 % O_2). Values are mean ± SD (n = 5). *$p < 0.05$.