Hypoxia-regulated over expression of soluble VEGFR2 controls angiogenesis and inhibits tumor growth

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Abstract

Vascular endothelial growth factors (VEGFs) are found at high levels in hypoxic tumors. As major components directing pathologic neo-vascularisation, they regulate stromal reactions. Consequently, novel strategies, targeting and inhibiting VEGF over-production upon hypoxia offer considerable potential for modern anti-cancer therapies controlling rather than destroying tumor angiogenesis.

Here we report the design of a vector expressing the soluble form of VEGF receptor-2 (sVEGFR2) driven by a hypoxia responsive element (HRE)-regulated promoter. To enable in vivo imaging by infrared visualization, mCherry and IFP1.4 coding sequences were built into the vector. Plasmid construction was validated through transfection into embryonic human kidney HEK293 and murine B16F10 melanoma cells. sVEGFR2 was expressed in hypoxic conditions only, confirming that the gene was regulated by the HRE-promoter.

sVEGFR2 was found to bind efficiently and specifically to murine and human VEGF-A, reducing the growth of tumor and endothelial cells as well as impacting angiogenesis in vitro. The hypoxia-conditioned sVEGFR2 expression was shown to be functional in vivo: tumor angiogenesis was inhibited and, on stable transfection of B16F10 melanoma cells, tumor growth was reduced. Enhanced expression of sVEGFR2 was accompanied by a modulation in levels of VEGF-A. The resulting balance reflected the effect on tumor growth and on the control of angiogenesis. A concomitant increase of intra-tumor oxygen tension also suggested an influence on vessel normalization.

The possibility to express an angiogenesis regulator as sVEGFR2, in a hypoxia-conditioned manner, significantly opens new strategies for tumor vessel-controlled normalization and the novel design of adjuvants for combined cancer therapies.
Introduction

Tumor growth is dependent on nutrients and oxygen supplied by blood vessels. As growth continues, the tumor mass develops hypoxic zones, that regulate transcription factors sensing hypoxia, such as the HIFs (1), and these in turn induce higher levels of proangiogenic proteins to accumulate (2). As such, vascular endothelial growth factors (VEGFs) are known to be over-expressed in tumors and are major inducing agents of neo-angiogenesis (3). Released in the tumor microenvironment, the VEGFs bind to vascular endothelial growth factors receptors (VEGFRs) on neighboring endothelial cells and promote growth and development of new blood vessels (4).

Whilst the pioneering work of Judah Folkman (5) has prompted the development of new antiangiogenic cancer therapies (6-10), a continuing issue is that most of the treatments in use currently lead to the selection of highly aggressive and resistant stem-like cancer cells (11). This subpopulation arises from selection pressures brought about by the anoxic microenvironment. Tumors adapt well to harsh hypoxia and low pH through rescue mechanisms, such as using anaerobic metabolism via the glycolysis pathway, as well as inducing multidrug resistance receptors. In these ways the cells enter a dedifferentiation process which confers a stem-like and highly aggressive phenotype (11-13). As a means of avoiding these problems, strategies to treat cancer no longer aim to totally inhibit angiogenesis, but rather are designed to promote blood vessel normalization (14-16) and as such have led to a considerable interest in VEGF-based therapies.

Since VEGF-A is also known to be a strong vascular permeabilization factor (VPF) (4, 17), increased levels in the tumor make the blood vessels leaky and increase edema. Vessels become incapable of ensuring efficient blood flow, thereby maintaining the hypoxic character of the tumor (18) and leading to continuous VEGFs production. This continuous cycle of events inevitably leads to tumor progression (11-13). To counteract this outcome, vessel normalization strategies aim to reduce hypoxia-induced angiogenesis, allow maturation of the vessels in order to reduce cell spreading (19)
and restore efficient blood flow thus enhancing oxygen supply. These strategies benefit radiotherapy (14) as well as drug delivery during chemotherapy (20). Consequently, modulation of VEGF expression rather than total inactivation is the preferred route for vessel normalization [21].

Gene therapy is a promising new approach to achieve blood vessel normalization. For example, a tumor-selective promoter enables site-specific expression of molecules regulated by the HIF/HRE elements (Hypoxia Inducible Factor/Hypoxia Response Elements) (21). Since a hypoxic environment is the hallmark of solid tumors, HIF/HRE elements are highly useful for targeted gene therapies. The HRE-inducible sequence allows a tight regulation of events, switching-on at low oxygen tension but, more importantly, switching-off under reoxygenation. This reversibility in the expression of a conditioned angiogenesis inhibitor can be advantageously used in vessel normalization strategies bypassing the need for strictly anti-angiogenic treatments.

In this study, we have designed a conditional gene therapy based on a soluble form of the VEGF receptor-2 (sVEGFR2) (8, 22, 23) to trap and neutralize VEGFs that are pathologically overproduced. The VEGFRs are a family of tyrosine kinase receptors (TKRs) composed of seven extracellular immunoglobulin (Ig)-like domains, a hydrophobic transmembrane region and a cytoplasmic section containing the tyrosine kinase domain (TKD) (24). Binding of dimerized VEGFs on the VEGFRs allows the TKD transphosphorylation, downstream signaling and activation of survival, proliferation, matrix remodeling and cell migration (25). VEGFR-2 is directly involved in angiogenesis, since it mediates the growth of endothelial cells and their permeability to cells and molecules upon binding of VEGFs. In contrast to VEGFR2, VEGFR-1 has been described to have a regulatory role in angiogenesis and, VEGFR-3 is thought to act mainly during embryogenesis and lymphangiogenesis (26, 27).

Amongst the VEGFRs, VEGFR-2 can bind to VEGF-A, C, D, and E, but not to B. Since proteolytic hydrolysis of the membrane form of VEGFR-2 is a natural regulatory mechanism (28), its soluble
form constitutes a potent VEGF-trap, providing its occurrence is limited to the pathological site. New tools such as specific cell carriers, to ensure selective and conditional expression of VEGFR genes, remain a requirement to fully realize the potential of this strategy in cancer therapy.

Moreover, VEGFRs are often expressed by tumor cells in addition to endothelial cells. Targeting VEGF by VEGFRs gene therapy may thus additionally affect tumor proliferation by depleting a growth factor from its environment (14-16). This work demonstrates the beneficial effect of such regulated anti-VEGF approach by its simultaneous action on the endothelium and the tumor cells, improving the efficacy of the treatment through vasculature normalization rather than destruction, as the future of cancer therapy (14).

Here, we have taken advantage of the hypoxic tumor environment and regulated the expression of soluble VEGFR2 by the inclusion of Hypoxia Response Elements (HRE) (29, 30) driven by a minimal CMV promoter. Moreover, to confirm the selectivity of our construct, a near infrared reporter was integrated into the vector. It encoded the IFP1.4, an intrinsic fluorescent protein enabling real time cell visualization (31).

For the first time, such a vector permits efficient and tumor specific expression of msVEGFR2, modulated by tumor microenvironmental conditions. The mechanism of interaction of msVEGR2 with its ligand was shown and quantified. Anti-angiogenic as well as anti-tumor growth properties were estimated. Moreover, it was found in vivo, that both effects towards tumor cells and endothelial cells occurred through the control of VEGF-A resulting in the inhibition of cancer cell proliferation and in vessel normalization.
Materials and methods

Cell culture and oxygen regulation

MLuMEC,FVB and MBrMEC,FVB (32) are mature endothelial cell models from FVB mouse lung and brain cultured as published (32). HMEC-1 (33), B16F10 cells (ATCC, CRL-6475) and HEK293 cells (ATCC, CRL-10852) were cultured high glucose containing medium (Gibco Invitrogen). Supplied cell lines were not further authenticated. Details for cell culture are given in supplementary data.

Hypoxia was obtained by introducing a stabilized gas mixture (Witt Gas mixer, Witt France) 94 % N₂/5 % CO₂/1 % O₂ (Air Liquide, Paris, France) in an automated PROOX in vitro chamber (C-174; BioSpherix, Redfield, NY) under control of a PROOX sensor-model 110 (BioSpherix).

Generation of pIFP1.4-HREmsVEGFR2 and pHREmsVEGFR2 vector

DNA manipulations used standard procedures and restriction enzymes, T4 DNA ligase, buffers according to manufacturer’s instructions (NEB and Promega). The vectors pBLAST45-msFlk1(s7) (Invivogen), pAAV-MCS (Stratagene), pdAAV-HRE-minCMV-HO1-SV40-pA (34), IFP1.4_pcDNA3.1H.ape (R. Y. Tsien, UCSD, USA) (31), were used to generate pIFP1.4-HREmsVEGFR2 vector for msVEGFR2. To develop the construct, several additional vectors were created (Supplementary Figure S1A) for which details of the cloning and constructs are given in supplementary data.

Vectors validation by cell lines transfection

All vectors were sequenced by GeneScript Company (Piscataway, USA) and controlled by transient cell transfection in cell lines: HEK293 and B16F10. Transfections were performed using the jetPEI™ (Polyplus Transfection, France) according to manufacturer’s instructions. msVEGFR2 expression was obtained by stable transfection of HEK293 and B16F10 cells with the pIFP1.4-HREmsVEGFR2 vector and hygromycin selection. Control lines were established with the same vector IFP1.4_pcDNA3.1H.ape coding for fluorescent proteins.
Selection of stable cell lines containing vectors.

Stable pHREmsVEGFR2, pIFP1.4-HREmsVEGFR2, and IFP1.4_pcDNA3.1H.ape transfected HEK293 and B16F10 cells, were selected by hygromycin (Invitrogen). Hygromycin resistant colonies were single-cell cloned by a FACS DIVA cell sorter (Becton and Dickinson, Sunnyvale, USA) and expanded. Clones were screened on the basis of their msVEGFR2 secretion in hypoxia (1 % O2) using ELISA (R&D DY1558B). pIFP1.4-HREmsVEGFR2 cells and IFP1.4_pcDNA3.1H.ape, hygromycin-resistant cells were further cloned by cell sorting on IFP1.4 fluorescence basis (ex: 633 nm, em: BP 712/21 nm).

Animal models

All animal experiments were approved by the CNREEA 03 ethics committee. 8 to 10-weeks old females C57Bl/6 mice (Janvier S.A.S, Le Genest-St-Isle, France) were used for the studies. Tumors were established by subcutaneous injection of 10^5 cells in 200 μL of Matrigel™ diluted 2 times in saline into the legs of anesthetized mice. Mice were sacrificed 4 weeks after tumor graft. Tumors were excised and weighed. For further protein measurement, tumor parts were crushed in liquid nitrogen in PBS with proteases inhibitors (complete EDTA-free protease inhibitor cocktail, Roche). The lysates were cleared by centrifugation (5 min., 10000 g) to perform ELISA measurements.

Quantification of msVEGFR2

The secreted msVEGFR2 was analyzed by ELISA duoset R&D kit (DY1558B, R&D systems, USA) (see detailed procedures in Supplementary data).

Surface Plasmon Resonance (SPR) assays

SPR analyses were performed with a Biacore 3000 (Biacore AB, Uppsala, Sweden). mVEGF (493MV/CF, R&D systems, USA) and hVEGF (293VE/CF, R&D systems, USA) were immobilized on CM5 biosensor chip (GE Healthcare) according to the manufacturers’ instructions. For binding
studies, mouse soluble VEGFR2 was obtained from HEK-IFPmsVEGFR2 cell supernatant. Details of the method are given in Supplementary data.

**Cell Proliferation Assay**

MLuMEC,FVB and B16F10 cell proliferation and effect of conditioned supernatants was measured by BrdU incorporation according to the manufacturer’s instructions (Cell Proliferation ELISA, BrdU, Roche) and detailed in Supplementary data.

**In Vitro Angiogenesis Assays**

In vitro tests for angiogenesis were adapted from (35) as reported in supplementary data.

**In vivo angiogenesis assay**

Mice 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 10⁵ cells HEK-IFP-msVEGFR2 or HEK-IFP. Ten days post-injection, angiogenesis was visualized by intravenous injection of a FTC-dextran (MW 2000000, FD2000S, Sigma) solution (10 mg/mL) in saline. A Nikon AZ100 Multizoom macroscope was used for fluorescence imaging. Detailed method is described in Supplementary data.

**Flow cytometry assays**

Flow analyses for B16F10wt cells (wild type) and msVEGFR2 expressing clones 13.3, 16.4, and MLuMEC,FVB endothelial cells, were performed on FACS-LSR flow cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences) after labeling with anti VEGFR1,2,3 antibodies. Detailed procedure is described in Supplementary data.

**In situ oxygen tension measurement**

Tumor tissue $pO_2$ measurement was performed with a fiber-optic oxygen-sensing device (OxyLite™4000, Oxford Optronix, Oxford, UK). Based on the principle of oxygen-induced quenching
of the fluorescent light emitted by the ruthenium, the partial oxygen pressure in the probe tip was immediately determined to give a $pO_2$ measure in several sites of the tumor (depending of the tumor size). Each OxyLite probe was calibrated by the manufacturer prior to its delivery, and used according to the manufacturer’s instruction.

**Immunohistochemistry**

Tumor cryosections were fluorescently labelled by specific lectins and monoclonal antibodies and observed by fluorescence microscopy (Zeiss observer). Methods are detailed in Supplementary data.

**Plasma collection**

Plasma samples from B16F10 melanoma bearing mice were obtained after anesthesia with isoflurane, followed by heart puncture as detailed in Supplementary section.

**Fluorescence microscopy**

Zeiss Axio Observer Z1 fluorescence inverted microscope (Zeiss) equipped with the Colibri LED illumination at 555 nm was used for mCherry and TRITC imaging (beam splitter 570 nm, emission filter 605/70 nm). FITC was imaged with the 470 nm diode (beam splitter 510 nm, emission filter 525/50 nm). IFP1.4 was imaged with the 625 nm diode (beam splitter 660 nm, emission filter 690/50 nm).

**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 Shu et al. (31) and detailed in Supplementary section.
In vivo fluorescence imaging

IVIS Kinetic (Caliper, USA), fluorescent images were acquired to visualize in vivo subcutaneous tumor in C57BL/6JTyr<sup>cr2-j</sup>Orl mice injected with 10<sup>5</sup> pHREmsVEGFR2-IFP1.4 B16F10 cells, mCherry protein imaging used excitation BP=570/35 nm, emission BP=620/20 nm filters. IFP1.4 imaging was performed with excitation BP=675/35 nm, emission BP=720/20 nm filters. Complementary details are in Supplementary data.

Statistical analysis

All values were expressed as means ± SD. Statistical analysis was conducted by Student t test. Group differences resulting in p = 0.05 by student t test were considered statistically significant.

Results

Construction of pIFP-HRE-msVEGFR2 Vector and pHRE-msVEGFR2

The structure of the plasmid is reported in Supplementary Figure S1A, and shows the HRE-minCMV-msVEGFR2 expression cassette and the hygromycin phosphotransferase encoding cassette for eukaryotic cell selection and stable cell line establishment. The IFP1.4 cassette for in vivo imaging by using near infra red excitation and emission was also inserted. The expression of mCherry protein encoding sequence, introduced downstream the IFP1.4 via IRES insertion, allowed classical fluorescence cell imaging.

Based on the same template, a second vector was designed from the pIFP-HRE-msVEGFR2 after deletion of the IFP1.4 cassette leading to pHRE-msVEGFR2 as presented in Supplementary Figure S1B and schematically presented on Figure 1A. The sequence of the vector was confirmed by restriction endonucleases digestion and sequencing.

Validation and optimization of hypoxia-driven msVEGFR2 expression
The first expression cassette was controlled by ELISA for measurement of msVEGFR2 secretion in the medium by transfected cells when placed under hypoxia (<1 % oxygen) compared to normoxia (18.75% oxygen). Transient transfection was performed on HEK293 cells and the supernatant was conditioned for 48h. Figure 1A (left) shows that msVEGFR2 production was indeed induced upon hypoxia, confirming that hypoxia-regulation of msVEGFR2 expression is efficient. Some leakage was observed since a basal level of msVEGFR2 (13 ng/mL/10^5 cells) was produced by transfected cells in normoxia. As a consequence, a shorter minCMV promoter was constructed by restriction endonuclease digestion leading to 3’ promoter DNA excision (Supplementary Figure S2 and Figure 1A, left). The shortened promoter substantially reduced leakiness in terms of msVEGFR2 production in normoxia (5ng/mL/10^5 cells, up to 60% reduction) (Figure 1A, left). Thus, the “short minCMV” promoter was used to design the following two vectors: pIFP-HRE-msVEGFR2 and pHRE-msVEGFR2, used in the subsequent experiments. The final construct pIFP-HREmsVEGFR2 was transfected into human HEK293, as well as into murine B16F10 melanoma cells, to assess its efficacy and its hypoxia-dependent regulation in human and murine species. As previously demonstrated (36, 37), and in our hands as well, the CMV promoter was stronger in human than murine cells (Figure 1A, right). This reduced efficacy of the CMV promoter highlighted the differences in the levels of msVEGFR2 expression observed in hypoxia vs normoxia, in murine cells where hypoxia induced a 6.0 fold increase as compared to a 1.7 fold increase in human cells.

**Engineered cell lines for msVEGFR2 production**

A non-viral transfection strategy was chosen to establish cell lines in view of optimizing cell delivery for future clinical applications. HEK293 cells were stably transfected by the pIFP1.4-HREmsVEGFR2 vector and cloned. The clones were selected on the basis of their ability to produce msVEGFR2 in hypoxia, taking into account the lowest leakage in normoxia, and designated HEK-IFP-msVEGFR2 cells (Figure 1B, c, d). These cells produced up to 70 ng/mL/10^5 cells of msVEGFR2 in hypoxia. As a control, the same transfection and selection process was performed with the “empty” vector pIFP1.4 (31), coding for the IFP1.4 and mCherry proteins only, providing lines called HEK-IFP cells (Figure
Thus transfection by all used vectors could be controlled in fluorescence microscopy for mCherry detection (Figure 1B, b, d).

**msVEGFR2 expression switches-on by low pO2 level and switches-off upon reoxygenation.**

The oxygen threshold value necessary to turn-on msVEGFR2 expression was analyzed at various oxygen partial pressures: 21 %, 5 %, 3 % and 1 %. msVEGFR2 levels were estimated by ELISA in supernatants from HEK-IFP-msVEGFR2 cells after a 48h incubation in hypoxia. As shown in Figure 1C (top), the hypoxia regulated promoter was switched-on only when oxygen tension decreased to 3 %. This threshold ensured that expression of msVEGFR2 would be restricted to cells within hypoxic microenvironments, such as those encountered in solid tumors. The validation of the switch-off control of msVEGFR2 expression by pO2 was performed upon cells reoxygenation after hypoxia and measurement of the msVEGFR2 production. As shown on Figure 1C (bottom) the production of msVEGFR2 by HEK-IFPmsVEGFR2 cells was totally reversed after a 96h reoxygenation phase.

**Soluble mVEGFR2 binds to its ligand**

We anticipated that the soluble VEGF receptor-2 (msVEGFR2) encoded by the pHRE-msVEGFR2 vector would recognize and bind to VEGF. This activity was assessed and quantified by a surface plasmon resonance assay with murine and human VEGF-A using hypoxia-conditioned supernatant from HEK-IFP-msVEGFR2 cells compared to the HEK-IFP control cell line. The data clearly demonstrated that the recombinant msVEGFR2 was able to bind to either murine or human VEGF-A (Figure 2A). Clear interspecies cross recognition was illustrated by the affinity constant values: KD =4,5 nM for msVEGFR2 to mVEGF (Figure 2A, left) and KD =7,8 nM to hVEGF (Figure 2A, right).

**Proliferation of Endothelial and Melanoma Cells is inhibited by msVEGFR2**

msVEGFR2-conditioned supernatant was able to inhibit VEGF-induced proliferation of tumor cells and angiogenesis in terms of endothelial cell growth. This was assessed on the B16F10 murine melanoma cells and on mature murine endothelial cells MLuMEC,FVB (32). Each of these VEGF-
responsive cell line was incubated with a 48h hypoxia-conditioned supernatant containing msVEGFR2 (60 ng/mL) from HEK-IFP-msVEGFR2 and compared to an HEK-IFP control. Figure 2B shows that both tumor and endothelial lines displayed a strongly reduced proliferation by 60%. This indicates that expressed msVEGFR2 was active to recognize, bind and neutralize the mVEGFs (Figure 2B). As the above data assume the presence of VEGF receptors on the tested cells, this was assessed in flow cytometry experiments reported in Figure 2C. VEGFR1, R2 and R3 presence and modulation by hypoxia were investigated at the single cell surface level. Figure 2C (top a, b) shows that B16F10 cells express the VEGF receptors 1 and 2 in normoxia. This expression is clearly increased on the cell surface upon hypoxia (Figure 2C top, d, e) while VEGFR3 was absent in both conditions (Figure 2C top, c, f) (38). Figure 2C (bottom) validates the above data, by reporting on MLuMEC,FVB endothelial cells the expression of VEGF receptors and their modulation by hypoxia, as positive control.

**msVEGFR2 decreases VEGF-induced angiogenesis in vitro**

Anti-angiogenic potential of the msVEGFR2 protein was assessed in a Matrigel™ angiogenesis assay. The msVEGFR2 activity was evaluated in a first attempt after incubation of the conditioned supernatants with HMEC-1 human endothelial cells. As shown in Figure 3A, the organized pseudo-vessel network was drastically reduced when endothelial cells had been exposed to msVEGFR2 (70 ng/mL) containing supernatant compared to control HEK-IFP cell supernatant (Figure 3A, right).

**neo-angiogenesis is inhibited in vivo by msVEGFR2 expression**

To determine whether the msVEGFR2 can modulate angiogenesis in vivo, a Matrigel™ plug model was used. Matrigel™ plugs containing either HEK-IFP-msVEGFR2 (Figure 3B, right) or HEK-IFP (Figure 3B, left) cells were injected as described in Materials and Methods. Mice bearing control Matrigel plugs containing HEK-IFP cells displayed an extensive vascularization (Figure 3B, left) while plugs containing msVEGFR2 producing cells appeared to be non angiogenic (Figure 3B, right). Plug limits could be clearly delineated by mCherry fluorescence (Figure 3B). The reduction of
vascularization in plugs containing msVEGFR2 producing cells was confirmed by fluorescent imaging of blood vessels reported by FTC-labelled dextran, intravenously injected to trace the vasculature (Figure 3C). FTC-dextran angiography shows that Matrigel did induce neo-angiogenesis (Figure 3B and C, a, b) as compared to control skin (Figure 3C, c). Confirming the above data, plugs containing msVEGFR2-secreting cells displayed reduced neo-angiogenesis in terms of vessel numbers (Figure 3C, b) compared to controls (Figure 3C, a, white arrows). Angiogenesis appeared less chaotic and vessels looked better formed in the presence of msVEGFR2 secreting cells (Figure 3C, b, white arrow). These results prove that msVEGFR2 is active in vivo to decrease neo-angiogenesis by VEGF trapping and apparent pruning (Figure 3C, b) suggests blood vessels normalization.

**Direct effect of hypoxia driven msVEGFR2 expression by B16F10 melanoma cells: influence on cell proliferation and angiogenesis**

Stable cell lines of B16F10 melanoma were established to express the extracellular soluble murine VEGFR2 receptor. Two clones were selected on the basis of their ability to produce msVEGFR2 upon induction by hypoxia: clone 16.4 and clone 13.3 (12 ng/mL/10^6 cells) as shown on Figure 4A (left). In these in vitro conditions, native B16F10 cells produced very low amounts of the soluble form of VEGFR2 (Figure 4A, left). msVEGFR2 production did not impair the expression of VEGF-A by the transfected clones (supplementary Figure S3A, C). Indeed, its detection by ELISA was not affected by the presence of msVEGFR2 in the supernatants and revealed comparable expression levels of VEGF-A by B16F10 cells and both transfected clones.

The direct effect of msVEGFR2 on melanoma cell growth was demonstrated by the observed reduction, by 30 to 40%, of the growth rate of the clones upon hypoxia induction of msVEGFR2 as compared to B16F10 wild cells and reported on Figure 4A (right).

To elucidate the msVEGFR2 effect on cell growth, its available concentration to further treated cells, was assessed taking into account the VEGF-A concentration, both produced in hypoxia-conditioned supernatants from B16F10 wild and transfected clones (supplementary Figure S3B, C). A high production of msVEGFR2 as displayed by clone 13.3 (Figure 4A, left and supplementary Figure S3A)
might be counteracted by the high secretion of VEGF-A in hypoxia (Figure 4A, left and supplementary Figure S3B). This is clearly illustrated by the effects of msVEGFR2 production on B16F10 melanoma cell growth (Figure 4B, left) as well as murine endothelial MLuMEC cell growth (Figure 4B, right). Both tumor and endothelial cells growth rates were increased by B16F10 VEGF-containing supernatant upon induction by hypoxia. This was clearly inhibited when supernatants contained msVEGFR2 as produced by the transfected clones. Indeed, hypoxia-dependent msVEGFR2-containing supernatants did reduce tumor and endothelial cell growth in proportion related to the level of msVEGFR2 and VEGF-A produced by the tested tumor and endothelial cells respectively (Bielawska Pohl et al.2010).

Hypoxia-induced tumor angiogenesis is achieved by endothelial cells growth and tube formation which is largely monitored by tumor cell derived VEGF. msVEGFR2 trapping activity was assessed in an in vitro angiogenesis experiment. Figure 4C, shows that when murine mature endothelial cells from lung (MLuMEC,FVB) and brain (MBrMEC,FVB) were incubated in the presence of msVEGFR2 containing supernatants from both clones, the hypoxia-dependent VEGF-induced angiogenesis was inhibited up to 50%, in terms of number of vessel-like structures (Figure 4C, top right) and to 20%, in terms of tube length (Figure 4C, bottom right).

**Hypoxia driven msVEGFR2 expression reduces melanoma tumor growth in vivo and restores vessel functionality.**

The in vivo effect of msVEGFR2 production was assessed three weeks after subcutaneous implantation of tumor cells. Tumor size was estimated by weight showing that both 16.4 and 13.3 clones displayed drastic tumor growth reduction compared to the native B16F10 cells (Figure 5A and inserts a, b) demonstrating msVEGFR2-mediated inhibition of VEGF-A-dependent tumor growth. This is corroborated by the expression of VEGFR1 and 2 on the surface of msVEGFR2 expressing clones (supplementary Figure S4 A, B).

As VEGF-A overproduction is regulated by its receptor cleavage and secretion, their levels were quantified in vivo. The amount of msVEGFR2 was higher inside the tumors induced by the 16.4 and
13.3 clones as compared to the B16F10 melanoma tumors (supplementary Figure S5, D) and in the serum (supplementary Figure S5 B). Although the level of VEGF-A inside the msVEGFR2 expressing tumors is higher (supplementary Figure S5 C), it seems to be retained locally since its level is lower in the corresponding sera (supplementary Figure S5 A). Such data corroborate the in vitro results reported on Figure 4 and on supplementary Figure S3. Moreover this effect is confirmed by the in vivo kinetics measurement of tumor growth, which is clearly slower when B16F10 cells express the msVEGFR2 (supplementary Figure S5 E).

These data suggest an effect on tumor angiogenesis. Thus vessels structure was observed and function assessed. Consequently, pericyte recruitment and vessel structure were studied by histochemical staining of tumors frozen and fixed sections. Figure 5B displays FTC-UeA+ endothelial cells (green arrows) and localization of TRTC-SMA+ pericytes (red arrows) that are spread over the B16F10wt tumors (Figure 5B), while pericytes appear to be recruited close by the vessel structures and colocalized with endothelial cells in clones 13.3- and 16.4-derived tumors (yellow arrows). As this is a criterion for vessel normalization (39) it should correspond to an increase in intratumor oxygen tension. Figure 5C shows that pO2, measured by Ru fluorescence quenching was increased in msVEGFR2 expressing melanoma tumors.

As kinetics of tumor growth (supplementary Figure S5E) indicated heterogeneity among tumor groups, pO2 was assessed in tumors of similar volume. Independently of size, tumors from B16F10wt were more hypoxic than tumors from the msVEGFR2 producing clones 13.3 and 16.4. Since the effect on tumor oxygenation is not due to the tumor size, it means that decrease in VEGF production may result from the normalization of the vasculature upon pO2 increase as previously shown (39).

**Fluorescent imaging properties of the pIFP-msVEGFR2 transfected cells**

Stably transfected HEK293 and B16F10 cells by the expressing sequence reported on Figure 6A, were used to evaluate in vitro IFP1.4 (excitation and emission maxima at 684 and 708 nm respectively) protein expression and its reporter protein mCherry (excitation and emission maxima at 587 nm and 610 nm) by fluorescence microscopy (Figure 6B, a-d, and Figure 6C, a-d). Well adapted
for in vivo imaging (31), IFP1.4 protein can be used as cell tracker to follow the transfected cells
distribution in whole animals with the help of its near infrared emission properties.
Similarly, the expression of mCherry, cloned after IRES downstream the IFP1.4 coding sequence was
detected (Figure 6B, a, c and Figure C, a, c). This allows cell tracking in non near infrared optical
settings and reporting of the IFP1.4. Figure 6B (a-d) and Figure 6C (a-d) display fluorescence
microscopy detection of mCherry and IFP1.4 and show their homogeneous cytosolic distribution in
transfected cells. Both proteins fluorescence spectra in cell extracts were compared to theoretical
spectra in HEK cells (Figure 6B, e) and in B16F10 (Figure 6C, e). In vivo imaging of stably
transfected B16F10 melanoma cells expressing both IFP1.4 and mCherry fluorescent proteins were
performed. As presented on Figure 6D, the fluorescent tumor cell distribution could be unambiguously
detected by an IVIS Kinetic instrument (Caliper, USA). Fluorescent images were recorded 10 days
after a subcutaneous injection showing the in vivo tumor distribution in the entire animal without
surgery. Thus the construct was validated as a valuable tool for in vivo cell tracking although growth
of the transfected cells was affected by the fluorescent proteins expression (supplementary Figure S6).
The low fluorescence yield of the IFP was compensated by the reporter effect provided by the
mCherry in an IFP1.4-IRES-mCherry construct.

Discussion

Given the role of angiogenesis in tumor growth and progression, the strategy that aims at targeting
tumor vasculature is of high interest. VEGFs are the most efficient activators of angiogenesis and
VEGF-A, being the key inducer, is the good candidate to control.
In this study, we have developed a regulated therapeutic gene for cancer treatment by hypoxia-driven
VEGF trapping by the soluble form of its receptor: msVEGFR2. Interaction of the produced
msVEGFR2 with the murine VEGF-A was quantified by surface plasmon resonance. The kinetics
constants qualified it as a VEGF-trap comparable to the previously described DAAP (10). The
biological efficacy was proven by its efficient effect on decreasing angiogenesis in vitro and in vivo, and diminishing tumor cell proliferation. This therapy consequently acts on both tumor and endothelial targets.

The described vector, is mainly adapted for its 3x HRE combination pattern that appears an optimized enhancer cassette to modulate the truncated CMV activity (minCMV promoter) (29). Such combination allows gene expression to be restricted to hypoxic environment. In the present study, we have fused this conditional sequence to msVEGFR2 coding sequence. According to published results from Holash et al. (8), such soluble form of the VEGFR2 was efficient to trap the VEGF and acts as a natural cell regulator (40).

Physioxia (41-43), the partial oxygen tension characteristic of a tissue/organ in physiological context has deep implications for hypoxia-mediated therapies. Indeed, the hypoxia-driven expression was dependent of strong O₂ deprivation. A threshold was determined indicating that the therapeutic gene would be expressed only in hypoxic environment and restricted to it, thus avoiding or limiting side effects as confirmed by the reversibility of the gene expression upon reoxygenation. This confirms that such therapy is a mean to control rather than destroy the angiogenic process leading to a normalization of the vessels (39).

Validating its further use in vivo, the produced msVEGFR2 protein was indeed angiostatic, as demonstrated here by the Matrigel™ plug assay. As such matrix contains pro-angiogenic factors, msVEGFR2 activity on angiogenesis is minimized, and thus msVEGFR2 works out the pro-angiogenic effect of the Matrigel™.

This was validated in vivo, using a tumor model for msVEGFR2 expression showing tumor reduction and angiogenesis normalization, with pericytes recruitment and restoration of vessels function illustrated by intratumor pO2 increase (42, 44).

This regulated and targeted therapy brings an important breakthrough into anti-angiogenesis strategies by overtaking their limitations. Indeed, anti-angiogenic therapies able to act via vessel normalization are more promising than vessels destruction which is achieved here by the hypoxia-regulated and reversible sequence. This strategy will help fighting cancer resistance, stem-like cancer cell selection.
and enhance the efficacy of combinatory chemo- and radio-therapy by hypoxia compensation (39, 43, 45). Moreover, the designed vector is applicable to *in vivo* imaging, since it expresses the Infrared Fluorescent Protein (IFP1.4) and mCherry.

In that respect, our strategy opens opportunities to combine gene- with cell-targeting therapies or viruses-therapies (i.e. oncolytic viruses or gene transfer). Its flexibility presents an invaluable advantage in view of future *in vivo* applications (46, 47).

It is noteworthy that all VEGF-inhibitors (antibodies, decoys, traps and small molecule inhibitors) induce harsh acidic and oxygen-deprived conditions which lead to the selection of resistant tumor cells that explain therapy failures and enhancement of tumor invasiveness and metastasis (11, 13, 48).

Thus a VEGF-trap, combined to hypoxia regulation, will provide an efficient strategy to optimize the control of tumor angiogenesis, inhibit metastasis, vascular leakage, and limit the tumor growth.

Moreover, extended endogenous VEGF-A blockade treatments are correlated with toxicity such as hypertension (49). This might be limited by the described strategy thanks to the localized and regulated gene delivery, its restricted response to low oxygen values and its repressed expression upon hypoxia compensation.

When compared to FDA (Food & Drug Administration) approved and clinically used Bevacizumab (Avastin®; Genentech Inc.) (50, 51), a humanized variant of a VEGF neutralizing monoclonal antibody, the engineered vector can encode for a human sVEGFR2 protein that will not raise any immune response. Hypoxia-regulation assures the reversibility of expression and its long tolerance.

Consequently, this approach should help define large therapeutic windows for combined therapies. In addition, some tumors are resistant to anti-VEGF therapies like Bevacizumab (52). As msVEGFR2 is a part of the VEGFR-2 receptor, it should naturally recognize and bind all VEGF-A, –C, -D, and –E forms. Since endothelium is the main VEGF target, this strategy may be developed independently of the tumor cells reactivity to VEGF. Nevertheless, controversial data mentioning the circulating VEGF or soluble VEGFR1 and 2 as reporter of tumor behavior as well as reporter of a therapeutic efficacy should be taken into account to identify the potential therapeutic windows (53-55). Intensively
explored, this research devoted to find such markers did not provide yet an absolute way to follow such windows.

In conclusion, msVEGFR2 is a decoy receptor that binds and blocks over expressed VEGFs in the tumor area, suppressing \textit{in vitro} and \textit{in vivo} angiogenesis and tumor proliferation. The hypoxia-driven, reversible expression may create the conditions to trigger vessel normalization. Reversibility upon reoxygenation should prevent the vascular destruction in normal tissues in case of vector leakage.

Further studies are dedicated to the appropriate cell carrier, able to target specifically the tumor stroma and other pathologic sites of angiogenesis.

This work constitutes a proof of concept towards a microenvironment regulated gene therapy for the future use of an integrated gene-cell carrier model (56, 57) that provides new avenues for normalization-based combinatorial cancer therapies.

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\textbf{References}


Figure legends

Figure 1. Demonstration of the hypoxia-dependent regulation of mSVEGFR2 expression from the constructed vector

(A) Description and optimisation of the hypoxia driven msVEGFR2 expression cassette.
Top: Schematic design of the hypoxia driven msVEGFR2 expression cassette.
Left: Optimization of msVEGFR2 production by shortening of minCMV promoter. msVEGFR2 expressed by transiently transfected HEK293 cells cultured 48 hours under normoxia (21 % O2) or hypoxia (1 % O2). Values are mean ± SD (n = 3). *p < 0.05.
Right: Comparison of the msVEGFR2 production by transiently transfected B16F10 melanoma cells with HEK293 cells, cultured 48 hours under normoxia (18.75 % O2) or hypoxia (1 % O2). Production was measured by ELISA. Values are mean ± SD (n = 3). *p < 0.05.

(B) msVEGFR2 production by HEK-IFPmsVEGFR2 and HEK-IFP cells cultured 48 hours under normoxia (21 % O2) or hypoxia (1 % O2). Production was measured by ELISA. The results are reported to 10^5 cells. Values are mean ± SD (n = 3). *p < 0.05.
(a, b, c, d) HEK-IFP cells (a, b) and HEK-IFPmsVEGFR2 cells (c, d) observed under fluorescence microscopy, in visible light (a, c) or red fluorescence (λex 530 nm, λem 608/75 nm) for mCherry observation (b, d). Scale bars represent 50 μm.

(C) Validation of pO2 dependence of the hypoxia driven sequence.
Top: Screening of the oxygen tension dependence of the promoter-induced msVEGFR2 production. Oxygen values are set to 21, 5, 3, 1 % and quantification of the msVEGFR2 was performed by ELISA in the supernatants after 48h. Results are expressed as an msVEGFR2 relative expression with the hypoxia production (1 % O2) set at 1. Values are mean ± SD (n = 3). *p < 0.05.
Bottom: Reversibility of the hypoxia-dependent promoter. HEK-IFPmsVEGFR2 cells and HEK-IFP control cells were cultured in hypoxia (1 % O₂) during 48h to switch-on the hypoxia dependent promoter (corresponding to t₀), then cultured in normoxia (21 % O₂) during 48h and 96h. Quantification of the msVEGFR2 was performed by ELISA in the supernatants. Results are expressed as msVEGFR2 relative expression with the normoxia production (21 % O₂) set at 1. Values are mean ± SD (n = 3). *p < 0.05.

Figure 2. Binding properties of msVEGFR2 to murine and human VEGF and its anti-proliferation effects in vitro

(A) Binding quantification assessed by surface plasmon resonance. Distinct dilutions of conditioned supernatant containing the msVEGFR2 as described in materials and methods was injected on the surface of a murine VEGF (left), or human (right) coated chip.

(B) Proliferation assay on MLuMEC,FVB and B16F10 cells cultured in presence of hypoxic conditioned medium from HEK-IFP and HEK-IFPmsVEGFR2 cells. Proliferation was assessed by BrdU incorporation. Results were expressed in % of the control after 48h of incubation. Values are mean ± SD (n = 3). *p < 0.05.

(C) Expression of VEGFRs on B16F10 cells (top) and MLuMEC, FVB cells (bottom). 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) labeling. Histogram overlays represent: empty histograms are isotypic controls and filled histograms are antibody-labeled cells.
Figure 3. Effect of msVEGFR2 on the angiogenesis process in vitro and in vivo

(A) HMEC-1 endothelial cells were seeded on Matrigel in the presence of hypoxia-conditioned medium from HEK-IFP cells in (left) or HEK-IFPmsVEGFR2 cells in (right). Tube-like structures network was analysed after 10h incubation in normoxia with Wimasis Image Analysis. Scale bars represent 200 μm.

(B) Photography of the reversed mouse skin showing the effect of msVEGFR2 on in vivo angiogenesis initiated by a Matrigel™ plug assay. HEK-IFP control cells (left) or producing HEK-IFPmsVEGFR2 (right) were mixed to Matrigel before subcutaneous injection into mice. Results are presented 10 days after Matrigel™ plug induction. Dot lines delineate the Matrigel plug borders. Scale bars = 1 cm.

(C) FTC-dextran angiography was acquired by fluorescence macroscopy. mCherry expressing cells were detected with the TRITC combination filters. The blood vessels were visible using FITC combination filters for FTC-dextran labeling of endothelium. (a) Represents the control plug containing HEK-IFP cells; (b) a plug containing HEK-IFPmsVEGFR2 cells (c) a control skin without Matrigel plug. White arrows show the blood vessels. Dot lines delineate the Matrigel plug borders. Scale bars = 5 mm.

Figure 4. msVEGFR2-producing B16F10 melanoma cells: hypoxia driven regulation

(A) Effect of msVEGFR2.
Left: production measured in supernatants of two stably-transfected cell lines of B16F10-msVEGFR2: 16.4 and 13.3 clones cultured for 48 hours in normoxia (21 % O₂) or hypoxia (1% O₂). Production was measured by ELISA. The results are reported to 10⁶ cells. Values are mean ± SD (n = 3).
Right: proliferation assay of the two clones 16.4 and 13.3, cultured for 48 hours in hypoxia (1 % O₂) compared to normoxia (21 % O₂). Proliferation was assessed by BrdU incorporation. Results were
expressed in % of the control representing a 48h incubation in normoxia. Values are mean ± SD (n = 3). *p < 0.05.

(B) Proliferation assay of B16F10 cells (left) and MLuMUC, FVB cells (right), cultured for 48h in conditioned supernatants from the two clones 16.4 and 13.3, obtained after a 48 hours incubation in hypoxia (1 % O₂) compared to normoxia (21 % O₂). Proliferation was assessed by BrdU incorporation. Results were expressed in % of the control representing a 48h incubation in normoxia. Values are mean ± SD (n = 3). *p < 0.05.

(C) MBrMEC,FVB (a, b, c) and MLuMEC,FVB endothelial cells (d, e, f) angiogenesis. Cells were seeded on Growth Factor Reduced Matrigel after a 48h pre-incubation in conditioned supernatants from the B16F10 wt (a, d) and the two clones 13.3 (b, e) and 16.4 (c, f), cultured for 48 hours in normoxia (21 % O₂). Tube-like structure networks were analyzed after 3h incubation for the MBrMEC,FVB cells and 6h incubation for the MLuMEC,FVB cells, both in normoxia, with Wimasis Image Analysis. A representative picture of each condition is presented. Scale bars represent 200 μm. Right: angiogenesis quantification of the mean tube numbers (top) and tube length (bottom) by Wimasis Image Analysis. Results were expressed in % of the control. Values are mean ± SD (n = 3). *p < 0.05.

Figure 5. msVEGFR2-induced regulation of proangiogenic VEGF-A production and tumor reoxygenation

(A) Effect of msVEGFR2 on tumor growth in vivo. The tumor size was measured 3 weeks after graft, comparing the B16F10-msVEGFR2 melanoma clone 16.4 and 13.3 to wild type B16F10. Values are mean ± SD (n = 10). Representative pictures are presented for (a) B16F10 wt control cells and (b) hypoxia driven msVEGFR2 expressing B16F10 cells.
(B) Histological section of B16F10-msVEGFR2 melanoma clone 16.4 and 13.3, and B16F10 wt melanoma cells grown in mice during 3 weeks. Cryostat section were double labeled with UeA-1-FTC (green) and with the α-SMA revealed by a TRITC-conjugated secondary antibody (red). A counterstaining was performed with DAPI (blue). Scale bars = 20 μm.

(C) In vivo measurement of the oxygen partial pressure by Oxylite into tumor developed from the B16F10-msVEGFR2 melanoma clone 16.4 and 13.3 compared to wild type B16F10. Several measurements were performed per tumor (3-6) at different time of the tumor growth. Results are classified into two ranges of size, from 0 to 1.5 cm³, and from 1.5 to 6 cm³. Results are expressed in mmHg. The box chart shows the 25–75 percentile with 2 standard deviations of the mean delineated by error bars. The average is represented by a black square and the median by a small bar. 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 6. In vitro and in vivo fluorescence imaging of IFP1.4 and mCherry reporter expressing cells

(A) Schematic description of the IFP1.4 and mCherry expression cassette.

(B) HEK293 cells transiently transfected by the pIFP1.4-HREmsVEGFR2 vector and observed by fluorescence microscopy, in red fluorescence (λex 530 nm, λem 608/75 nm) for mCherry observation (a), visible light (b) and near infrared fluorescence (λex 625 nm, λem 690/50 nm) for IFP observation (d). Merged image of red fluorescence and visible light is shown in (c). Scale bars represent 30 μm.

c) Normalized spectra performed in cell lysates obtained from the stably transfected HEK by the pIFP1.4-HREmsVEGFR2 vector with the following settings: emission fluorescence spectra, λex: 560
nm (red) of mCherry in PBS and corresponding excitation spectra λem 610 nm (black). Emission spectra of IFP1.4 in PBS: λex: 650 nm (blue).

(C) B16F10 cells stably transfected by the pIFP1.4-HREmsVEGFR2 vector and observed by fluorescence microscopy, in red fluorescence (λex 530 nm, λem 608/75 nm) for mCherry observation (a), visible light (b) and near infrared fluorescence (λex 625 nm, λem 690/50 nm) for IFP observation (d). Merged image of red fluorescence and visible light is shown in (c). Scale bars represent 15 μm.

(e) Normalized spectra performed in cell lysates obtained from the stably transfected B16F10 by the pIFP1.4-HREmsVEGFR2 vector with the following settings: emission fluorescence spectra, λex: 560 nm (red) of mCherry in PBS and corresponding excitation spectra λem 610 nm (black). Emission spectra of IFP1.4 in PBS: λex: 650 nm (blue).

(D) In vivo imaging with an IVIS Lumina imager of a mice harboring a B16F10 tumor stably transfected with the pIFP1.4-HREmsVEGFR2 vector. Acquisition was performed 10 days after implantation.
Figure 1
Figure 2
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Figure 5
Hypoxia-regulated over expression of soluble VEGFR2 controls angiogenesis and inhibits tumor growth

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