Hypoxia-Regulated Overexpression of Soluble VEGFR2 Controls Angiogenesis and Inhibits Tumor Growth

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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 hypoxia-inducible factors (HIF; ref. 1), and these, in turn, induce higher levels of proangiogenic proteins to accumulate (2). As such, VEGFs are known to be overexpressed in tumors and are major inducing agents of neoangiogenesis (3).

VEGFs are found at high levels in hypoxic tumors. As major components directing pathologic neovascularization, they regulate stromal reactions. Consequently, novel strategies targeting and inhibiting VEGF overproduction upon hypoxia offer considerable potential for modern anticancer 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 control of angiogenesis. A concomitant increase of intratumor 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 design of adjuvants for combined cancer therapies.

Abstract

VEGFs are found at high levels in hypoxic tumors. As major components directing pathologic neovascularization, they regulate stromal reactions. Consequently, novel strategies targeting and inhibiting VEGF overproduction upon hypoxia offer considerable potential for modern anticancer 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 control of angiogenesis. A concomitant increase of intratumor 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 design of adjuvants for combined cancer therapies.

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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 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/hypoxia-responsive elements (HRE; ref. 21). As 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 antiangiogenic treatments.

In this study, we have designed a conditional gene therapy based on a soluble form of the VEGF receptor-2 (sVEGFR2; refs. 8, 22, 23) to trap and neutralize VEGFs that are pathologically overproduced. The VEGFRs are a family of tyrosine kinase receptors (TKR) composed of 7 extracellular immunoglobulin (Ig)-like domains, a hydrophobic transmembrane region, and a cytoplasmic section containing the tyrosine kinase domain (TKD; ref. 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 activation of survival, proliferation, matrix remodeling, downstream signaling and TKD transphosphorylation, downstream signaling and activation of survival, proliferation, matrix remodeling, and cell migration (25). VEGFR-2 is directly involved in angiogenesis, as 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 having a regulatory role in angiogenesis and lymphangiogenesis (26, 27).

Among the VEGFRs, VEGFR-2 can bind to VEGF-A, C, D, and E but not to B. As 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 pathologic 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 shows 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 sVEGFR2 by the inclusion of HREs (29, 30) driven by a minimal cytomegalovirus (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 msVEGFR2 with its ligand was shown and quantified. Antiangiogenic as well as antitumor growth properties were estimated. Moreover, it was found in vivo, that both effects toward 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 in 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 (WittGas mixer, Witt France) 94% N₂/5% CO₂/1% O₂ (Air Liquide) in an automated PROOX in vitro chamber (C-174; BioSpherix) 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 (K.Y. Tsien, UCSF; ref. 31) were used to generate pIFP1.4-HREmsVEGFR2 vector for msVEGFR2. To develop the construct, several additional vectors were created (Supplementary Fig. 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 and controlled by transient cell transfection in cell lines: HEK293 and B16F10. Transfections were conducted...
using the jetPEI (Polyplus Transfection) according to manufacturer’s instructions. msVEGFR2 expression was obtained by stable transfection of HEK293 and B16F10 cells with the plFP1.4-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, plFP1.4-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) and expanded. Clones were screened on the basis of their msVEGFR2 secretion in hypoxia (1% O2) using ELISA (R&D DY1558B). plFP1.4-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. Eight- to 10-week-old females C57Bl/6 mice (Janvier S.A.S) were used for the studies. Tumors were established by subcutaneous injection of 10^5 cells in 200 μL 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 minutes, 10,000 g) to conduct ELISA measurements.

**Quantification of msVEGFR2**

The secreted msVEGFR2 was analyzed by ELISA duoset R&D kit (DY1558B, R&D Systems; see detailed procedures in Supplementary Data).

**Surface plasmon resonance assays**

Surface plasmon resonance (SPR) analyses were conducted with a Biacore 3000 (Biacore AB). mVEGF (493MV/CF, R&D Systems) and hVEGF (293VE/CF, R&D Systems) were immobilized on CM5 biosensor chip (GE Healthcare) according to the manufacturers’ instructions. For binding studies, mouse sVEGFR2 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 bromodeoxyuridine (BrdUrd) incorporation according to the manufacturer’s instructions (Cell Proliferation ELISA, BrdUrd; Roche) and detailed in Supplementary Data.

**In vitro angiogenesis assays**

*In vitro* tests for angiogenesis were adapted from the work of Carreau and colleagues (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 basic fibroblast growth factor (bFGF; R&D Systems) mixed with saline (1:1 v/v) containing 10^5 cells HEK-IFP-msVEGFR2 or HEK-IFF. Ten days postinjection, angiogenesis was visualized by intravenous injection of a FITC-dextrin (MW 2,000,000, FD2000S, Sigma) solution (10 mg/mL) in saline. A Nikon AZ100 Multizoom microscope was used for fluorescence imaging. Detailed method is described in Supplementary Data.

**Flow cytometric assays**

Flow cytometric analyses for B16F10 wt cells (wild-type) and msVEGFR2-expressing clones 13.3, 16.4, and MLuMEC, FVB endothelial cells, were conducted on FACSLSR 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 pO2 measurement was conducted with a fiber optic oxygen-sensing device (OxyLite 4000, Oxford Optronix). On the basis of 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 pO2 measurement. Each OxyLite probe was calibrated by the manufacturer before its delivery and used according to the manufacturer’s instructions.

**Immunohistochemistry**

Tumor crossections were fluorescently labeled 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 Colibiri LED illumination at 555 nm was used for mCherry and TRITC imaging (beam splitter 570 nm, emission filter 605/70 nm). Fluorescein isothiocyanate (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 and colleagues (31) and detailed in Supplementary section.

In vivo fluorescence imaging

IVIS Kinetic (Caliper) fluorescent images were acquired to visualize in vivo subcutaneous tumor in C57BL/6J-Tyr<sup>2</sup>Yrt<sup>2</sup>Orl mice injected with 10<sup>9</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 conducted 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 the 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 Fig. S1A and shows the HRE-minCMV-msVEGFR2 expression cassette and the hygromycin phosphotransferase encoding sequence, introduced downstream the IFP1.4 promoter encoding cassette for eukaryotic cell selection and the hygromycin phosphotransferase expressing cassette and stable cell line establishment. The IFP1.4 cassette for in vivo imaging by using near-infrared 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.

On the basis of 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 Fig. S1B and schematically presented on Fig. 1A. The sequence of the vector was confirmed by restriction endonuclease 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 with normoxia (18.75% oxygen). Transient transfection was conducted on HEK293 cells and the supernatant was conditioned for 48 hours. 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 as a basal level of msVEGFR2 (13 ng/mL/10<sup>5</sup> 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 Fig. S2 and Fig. 1A, left). The shortened promoter substantially reduced leakiness in terms of msVEGFR2 production in normoxia (5 ng/mL/10<sup>5</sup> cells, up to 60% reduction; Fig. 1A, left). Thus, the "short minCMV" promoter was used to design the following 2 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 shown (36, 37), and in our hands as well, the CMV promoter would be stronger in human than murine cells (Fig. 1A, right). This reduced efficacy of the CMV promoter highlighted the differences in the levels of msVEGFR2 expression observed in hypoxia versus normoxia, in murine cells where hypoxia induced a 6.0-fold increase as compared with a 1.7-fold increase in human cells.

Engineered cell lines for msVEGFR2 production

A nonviral 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-IFP1.4-HREmsVEGFR2 cells [Fig. 1B (c) and (d)]. These cells produced up to 70 ng/mL/10<sup>5</sup> cells of msVEGFR2 in hypoxia. As a control, the same transfection and selection process was conducted with the "empty" vector pIFP1.4 (31), coding for the IFP1.4 and mCherry proteins only, providing lines called HEK-IFP cells [Fig. 1B (a) and (b)]. Thus, transfection by all used vectors could be controlled in fluorescence microscopy for mCherry detection [Fig. 1B (a) and (b)].

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 48-hour incubation in hypoxia. As shown in Fig. 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 conducted upon cells reoxygenation after hypoxia and measurement of the msVEGFR2 production. As shown on Fig. 1C (bottom), the production of msVEGFR2 by HEK-IFPmsVEGFR2 cells was totally reversed after a 96-hour reoxygenation phase.
Figure 1. Demonstration of the hypoxia-dependent regulation of mSVEGFR2 expression from the constructed vector. A, description and optimization 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% O$_2$) or hypoxia (1% O$_2$). 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% O$_2$) or hypoxia (1% O$_2$). 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% O$_2$) or hypoxia (1% O$_2$). Production was measured by ELISA. The results are reported to 10$^5$ cells. Values are mean ± SD (n = 3), *; P < 0.05. a–d, HEK-IFP cells (a and b) and HEK-IFPmsVEGFR2 cells (c and d) observed under fluorescence microscopy, in visible light (a and c) or red fluorescence (λ ex 530 nm, λ em 608/75 nm) for mCherry observation (b and d). Scale bars, 50 μm. C, validation of pO$_2$ 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 conducted by ELISA in the supernatants after 48 hours. Results are expressed as an mSVEGFR2 relative expression with the hypoxia production (1% O$_2$) 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% O$_2$) during 48 hours to switch-on the hypoxia-dependent promoter (corresponding to t$_0$), then cultured in normoxia (21% O$_2$) during 48 and 96 hours. Quantification of the mSVEGFR2 was conducted by ELISA in the supernatants. Results are expressed as mSVEGFR2 relative expression with the normoxia production (21% O$_2$) set at 1. Values are mean ± SD (n = 3), *; P < 0.05.
Soluble mVEGFR2 binds to its ligand

We anticipated that msVEGFR2 encoded by the pHRE-msVEGFR2 vector would recognize and bind to VEGF. This activity was assessed and quantified by an SPR assay with murine and human VEGF-A using hypoxia-conditioned supernatant from HEK-IFP-msVEGFR2 cells compared with the HEK-IFP control cell line. The data clearly showed that the recombinant msVEGFR2 was able to bind to either murine or human VEGF-A (Fig. 2A). Clear interspecies cross-recognition was illustrated by the affinity constant values: $K_D = 4.5$ nmol/L for msVEGFR2 to mVEGF (Fig. 2A, left) and $K_D = 7.8$ nmol/L to hVEGF (Fig. 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

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**Figure 2.** Binding properties of msVEGFR2 to murine and human VEGF and its antiproliferation effects in vitro. A, binding quantification assessed by SPR. 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 BrdUrd incorporation. Results are expressed in percentage of the control after 48 hours 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–c) and hypoxia (d–f) were labeled with selected antibodies as described in Materials and Methods, for VEGFR1 (a and d), VEGFR2 (b and e), and VEGFR3 (c and f) labeling. Histogram overlays: empty histograms are isotypic controls and filled histograms are antibody-labeled cells.

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incubated with a 48-hour hypoxia-conditioned supernatant containing msVEGFR2 (60 ng/mL) from HEK-IFP-msVEGFR2 and compared with 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 (Fig. 2B). As the above data assume the presence of VEGF receptors on the tested cells, this was assessed in flow cytometric experiments reported in Fig. 2C. VEGFR1, R2, and R3 presence and modulation by hypoxia were investigated at the single-cell surface level. Figure 2C (top, a and b) shows that B16F10 cells express the VEGFR1 and VEGFR2 in normoxia. This expression is clearly increased on the cell surface upon hypoxia [Fig. 2C (d) and (e)] whereas VEGFR3 was absent in both conditions [Fig. 2C (c) and (f); ref. 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**

Antiangiogenic 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 Fig. 3A, the organized pseudovessel network was drastically reduced when endothelial cells had been exposed to msVEGFR2 (70 ng/mL) containing supernatant compared with control HEK-IFP cell supernatant (Fig. 3A, right).

**Neoangiogenesis is inhibited in vivo by msVEGFR2 expression**

To determine whether the msVEGFR2 can modulate angiogenesis in vivo, a Matrigel plug model was used.

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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 (left) or HEK-IFPmsVEGFR2 cells (right). Tube-like structures network was analyzed after 10-hour incubation in normoxia with Wimasis Image Analysis. Scale bars, 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, FITC-dextran angiography was acquired by fluorescence microscopy. mCherry-expressing cells were detected with the TRITC combination filters. The blood vessels were visible using FITC combination filters for FITC-dextran labeling of endothelium. a, control plug containing HEK-IFP cells; b, plug containing HEK-IFPmsVEGFR2 cells; and c, a control skin without Matrigel plug. White arrows show the blood vessels. Dot lines delineate the Matrigel plug borders. Scale bars, 5 mm.
Matrigel plugs containing either HEK-IFP-msVEGFR2 (Fig. 3B, right) or HEK-IFP (Fig. 3B, left) cells were injected as described in Materials and Methods. Mice bearing control Matrigel plugs containing HEK-IFP cells displayed an extensive vascularization (Fig. 3B, left), whereas plugs containing msVEGFR2 producing cells appeared to be nonangiogenic (Fig. 3B, right). Plug limits could be clearly delineated by mCherry fluorescence (Fig. 3B). The reduction of vascularization in plugs containing msVEGFR2-producing cells was confirmed by fluorescent imaging of blood vessels reported by FITC-labeled dextrin, intravenously injected to trace the vasculature (Fig. 3C). FITC-dextrin angiography shows that Matrigel did induce neoangiogenesis [Fig. 3B and C (a) and (b)] as compared with control skin [Fig. 3C (c)]. Confirming the above data, plugs containing msVEGFR2-secreting cells displayed reduced neoangiogenesis in terms of vessel numbers [Fig. 3C (b)] compared with controls [Fig. 3C (a), white arrows]. Angiogenesis appeared less chaotic and vessels looked better formed in the presence of msVEGFR2-secreting cells [Fig. 3C (b), white arrow]. These results prove that msVEGFR2 is active in vivo to decrease neoangiogenesis by VEGF trapping, and apparent pruning [Fig. 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/10⁶ cells) as shown in Fig. 4A (left). In these in vitro conditions, native B16F10 cells produced very low amounts of the soluble form of VEGFR2 (Fig. 4A, left). msVEGFR2 production did not impair the expression of VEGF-A by the transfected clones (Supplementary Fig. S3A and S3C). 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 shown by the observed reduction, by 30% to 40%, of the growth rate of the clones upon hypoxia induction of msVEGFR2 as compared with B16F10 wild cells and reported in Fig. 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 Fig. S3B and S3C). A high production of msVEGFR2 as displayed by clone 13.3 (Fig. 4A, left and Supplementary Fig. S3A) might be counteracted by the high secretion of VEGF-A in hypoxia (Fig. 4A, left, and Supplementary Fig. S3B). This is clearly illustrated by the effects of msVEGFR2 production on B16F10 melanoma cell growth (Fig. 4B, left) as well as murine endothelial MLuMEC cell growth (Fig. 4B, right). Both tumor and endothelial cell 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.

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 (Fig. 4C, top right) and to 20%, in terms of tube length (Fig. 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 3 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 with the native B16F10 cells [Fig. 5A and insets (a) and (b)] showing 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 Fig. S4A and S4B).

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 with the B16F10 melanoma tumors (Supplementary Fig. S5D) and in the serum (Supplementary Fig. S5B). Although the level of VEGF-A inside the msVEGFR2-expressing tumors is higher (Supplementary Fig. S5C), it seems to be retained locally, as its level is lower in the corresponding sera (Supplementary Fig. S5A). Such data corroborate the in vitro results reported on Fig. 4 and in Supplementary Fig. 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 Fig. S5E).

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...
Figure 4. msVEGFR2-producing B16F10 melanoma cells: hypoxia-driven regulation. A, effect of msVEGFR2. Left, production measured in supernatants of 2 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 2 clones 16.4 and 13.3 cultured for 48 hours in hypoxia (1% O₂) compared with normoxia (21% O₂). Proliferation was assessed by BrdUrd incorporation. Results are expressed in percentage of control representing a 48-hour incubation in normoxia. Values are mean ± SD (n = 3). *P < 0.05. B, proliferation assay of B16F10 cells (left) and MLuMEC, FVB cells (right), cultured for 48 hours in conditioned supernatants from the 2 clones 16.4 and 13.3, obtained after a 48-hour incubation in hypoxia (1% O₂) compared with normoxia (21% O₂). Proliferation was assessed by BrdUrd incorporation. Results are expressed in percentage of control representing a 48-hour incubation in normoxia. Values are mean ± SD (n = 3). *P < 0.05. C, MBrMEC, FVB (a–c) and MLuMEC, FVB endothelial cells (d–f) angiogenesis. Cells were seeded on growth factor-reduced Matrigel after a 48-hour preincubation in conditioned supernatants from the B16F10 wt (a and d) and the 2 clones 13.3 (b and e) and 16.4 (c and f), cultured for 48 hours in normoxia (21% O₂). Tube-like structure networks were analyzed after 3-hour incubation for the MBrMEC, FVB cells and 6-hour incubation for the MLuMEC, FVB cells, both in normoxia, with Wimasis Image Analysis. A representative picture of each condition is presented. Scale bars, 200 μm. Right, angiogenesis quantification of the mean tube numbers (top) and tube length (bottom) by Wimasis Image Analysis. Results are expressed in % of the control. Values are mean ± SD (n = 3). *P < 0.05.
localization of TRTC-SMA+ pericytes (red arrows) that are spread over the B16F10wt tumors (Fig. 5B), whereas 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 Fig. 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. As 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 in Fig. 6A were used to evaluate in vitro IFP1.4 (excitation and emission maxima at 684 and 708 nm, respectively) protein expression and its
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, 30 μm. E, normalized spectra conducted in cell lysates obtained from the stably transfected HEK by the pIFP1.4-HREmsVEGFR2 vector with the following settings: emission fluorescence spectra, λ:ex 560 nm (red) of mCherry in PBS and corresponding excitation spectra λ:em 610 nm (black). Emission spectra of IFP1.4 in PBS: λ:ex 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, 15 μm. E, normalized spectra conducted in cell lysates obtained from the stably transfected B16F10 by the pIFP1.4-HREmsVEGFR2 vector with the following settings: emission fluorescence spectra, λ:ex 560 nm (red) of mCherry in PBS and corresponding excitation spectra λ:em 610 nm (black). Emission spectra of IFP1.4 in PBS: λ:ex 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 conducted 10 days after implantation.
reporter protein mCherry (excitation and emission maxima at 587 nm and 610 nm) by fluorescence microscopy [Fig. 6B (a–d) and C (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 [Fig. 6B (a) and (c) and C (a) and (c)]. This allows cell tracking in non–near-infrared optical settings and reporting of the IFP1.4. Figure 6B (a–d) and C (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 with theoretical spectra in HEK cells [Fig. 6B (e) and in B16F10 [Fig. 6C (e)]. In vivo imaging of stably transfected B16F10 melanoma cells expressing both IFP1.4 and mCherry fluorescent proteins were conducted. As presented in Fig. 6D, the fluorescent tumor cell distribution could be unambiguously detected by an IVIS Kinetic instrument (Caliper). 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 protein expression (Supplementary Fig. 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 SPR. The kinetics constants qualified it as a VEGF-trap comparable to the previously described DAAP (10). The biologic 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 3 × HRE combination pattern that appears an optimized enhancer cassette to modulate the truncated CMV activity (minCMV promoter; ref. 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 and colleagues (8), such soluble form of the VEGFR2 was efficient to trap the VEGF and acts as a natural cell regulator (40).

Physioxia (39, 41, 42), the partial oxygen tension characteristic of a tissue/organ in physiologic context, has deep implications for hypoxia-mediated therapies. Indeed, the hypoxia-driven expression was dependent on 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 shown here by the Matrigel plug assay. As such matrix contains proangiogenic factors, msVEGFR2 activity on angiogenesis is minimized and thus msVEGFR2 works out the proangiogenic effect of the Matrigel.

This was validated in vivo, using a tumor model for msVEGFR2 expression showing tumor reduction and angiogenesis normalization, with pericyte recruitment and restoration of vessels function illustrated by intratumor pO2 increase (39, 43).

This regulated and targeted therapy brings an important breakthrough into antiangiogenesis strategies by overtaking their limitations. Indeed, antiangiogenic therapies able to act via vessel normalization are more promising than vessels destruction, which is achieved hereby the hypoxia-regulated and reversible sequence. This strategy will help fighting cancer resistance, stem-like cancer cell selection and enhance the efficacy of combinatorial chemotherapeutics and radiotherapy by hypoxia compensation (39, 42, 44). Moreover, the designed vector is applicable in vivo imaging, as 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 (45, 46).

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, 47).

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 (48). 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 with U.S. Food and Drug Administration (FDA) approved and clinically used bevacizumab
(Avastin; Genentech Inc.; refs. 49, 50), 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 (51). As msVEGFR2 is a part of the VEGFR2, it should naturally recognize and bind all VEGF-A, C, D, and E forms. As endothelium is the main VEGF target, this strategy may be developed independently of the tumor cell 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 window (52–54).

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 overexpressed VEGFs in the tumor area, suppressing in vitro and 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 toward a microenvironment-regulated gene therapy for the future use of an integrated gene cell carrier model (55, 56) that provides new avenues for normalization-based combinatorial therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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