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Thérapie génique de l'angiogenèse tumorale ciblée par des cellules endothéliales immatures

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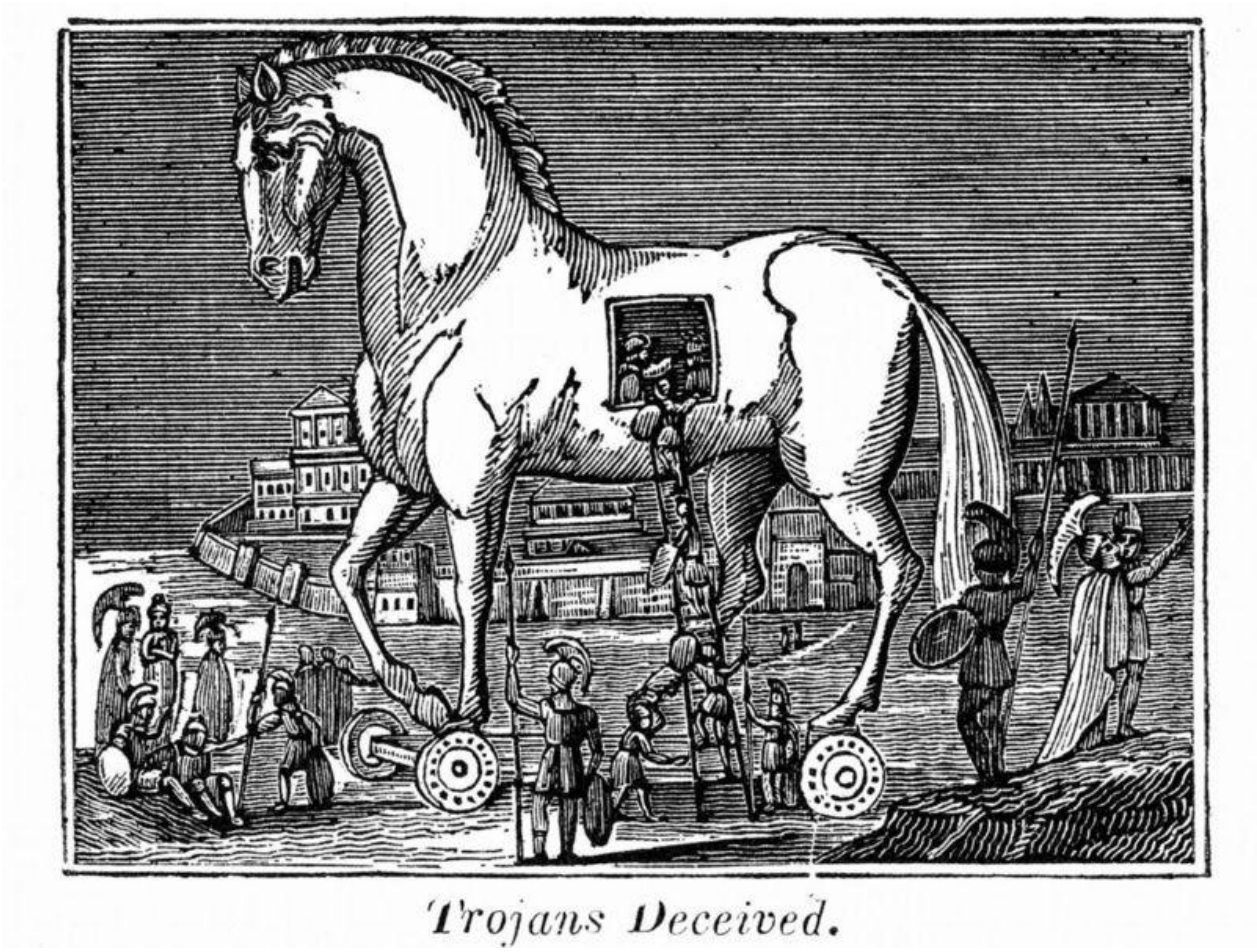

Trojans Deceived.

http://michaelgraeme.wordpress.com/2011/12/29/linkedin-and-the-trojan-horse-how-to-leave-linkedin/

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(in revision in "Molecular Cancer Therapeutics", 2012).

Collet G, Szade K, Nowak W, Grillon C, Sugiyama D, Klimkiewicz K, Mazan A, Szczepanek K, Weglarczyk K, Lamerant N, Guichard A, Fasani F, El Hafny-Rahbi B, Jozkowicz A, Sarna T, Dulak J and Kieda C. Murine endothelial precursor cell lines as models able to target neoangiogenic sites.
(in preparation)

Moreover, I am a co-author of the following research papers:

Original articles:

Kieda C, El Hafny-Rahbi B, **Collet G**, Lamerant-Fayel N, Grillon C, Guichard A, Dulak J, Jozkowicz A, Kotlinowski J, Fylaktakidou K.C, Même S, Vidal A, Auzeloux P, Miot-Noirault E, Beloeil J.C, Lehn J.M, Nicolau C. Stable tumor vessel normalization with pO2 increase and endothelial PTEN activation by inositol tris pyrophosphate brings novel tumor treatment.
(in revision in "Journal of Molecular Medicine", 2012).

K. Skrzypek, M. Tertil, S. Golda, K. Weglarczyk, **G. Collet**, A. Guichard, J. Boczkowski, H. Was, M. Ciesla, A. Loboda, T. Gil, J. Kuzdzal, A. Jozkowicz, C. Kieda, J. Dulak. Interplay between heme oxygenase-1 and miR-378 affects non-small cell lung carcinoma growth, vascularization and metastasis.
(under review in "Antioxidants & Redox Signaling", 2012).

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(under review in "Free Radical Biology and Medicine", 2012)

Review papers:

Collet G, Skrzypek K, Grillon C, Matejuk A, El Hafni-Rahbi B, Lamerant-Fayel N, Kieda C. Hypoxia control to normalize pathologic angiogenesis: Potential role for endothelial precursor cells and miRNAs regulation. Vascul Pharmacol. 2012 May; 56(5-6):252-61. Epub 2012 Mar 8.

Matejuk A, **Collet G**, Nadim M, Grillon C, Kieda C. MiRs and tumor vasculature normalization: impact on anti-tumor immune response.
(in revision in « *Archivum Immunologiae et Therapiae Experimentalis* », 2012).

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To my father…

Abstract

Vascular endothelial growth factors (VEGFs) are over-expressed upon hypoxia in solid tumors. Major actors directing pathologic neo-vascularisation, they regulate the stromal reaction. Novel strategies that target and inhibit VEGF bring promise to modern anti-cancer therapies. They aim to control, rather than destroy, tumor angiogenesis. Consequently, the challenge is to selectively trap VEGFs, over-produced upon hypoxia, in the tumor microenvironment. The thesis presented in this manuscript focuses on the design of a novel cell-based targeting strategy, so-called "Trojan Horse", combining in the same engineered entity, a targeting unit and a specific drug/gene delivery system. To address the therapy to cancer cells without affecting healthy cells, a model of endothelial precursor cell (EPCs) was used as targeting cell able to reach specifically the tumor site. EPCs were "armed" to express a therapeutic gene to inhibit VEGF. Trapping was attempted based on the production of a soluble form of the VEGF receptor-2 (sVEGFR2) as a candidate inhibitor. Hypoxia, a hallmark of developing solid tumors, was chosen to turn on/off the sVEGFR2 expression and secretion by introducing, upstream of the therapeutic gene, a hypoxia response element (HRE) regulating sequence. Properly addressed by the EPCs to the tumor site, such angiogenesis regulator as the soluble form of VEGFR2 is, was chosen to be expressed in a hypoxia-conditioned and reversible manner. This opens new strategies for a stably controlled normalization of tumor vessels in view of adjuvant design for combined therapies.

Key words: Tumor angiogenesis, hypoxia, EPCs targeting, normalization, VEGF-trap

Résumé

Les facteurs de croissance endothéliaux (VEGFs) sont produits par les tumeurs qui sont hypoxiques. Principaux responsables de la néo-vascularisation pathologique, ils régulent le stroma tumoral. Les nouvelles stratégies qui ciblent et inhibent le VEGF ouvrent vers la thérapie anti-cancéreuse moderne. Elles ont pour but de contrôler l'angiogenèse tumorale plutôt que la détruire. Le défi est donc de piéger sélectivement le VEGF produit en excès, dans le microenvironnement tumoral, sous l'effet de l'hypoxie. La thèse présentée dans ce manuscrit est consacrée à la réalisation d'une nouvelle stratégie ciblante par l'intermédiaire de cellules, aussi appelée « Cheval de Troie ». Elle combine dans la même entité, une unité de ciblage et un systême de délivrance spécifique d'un gène/molécule thérapeutique. Dans le but d'adresser la thérapie aux cellules cancéreuses sans affecter les cellules saines, un modèle de cellules endothéliales de type précurseur (CEPs) a été utilisé comme cellules ciblantes capables d'atteindre spécifiquement le site tumoral. Les CEPs ont été « armées » pour exprimer un gène thérapeutique chargé d'inhiber le VEGF. La neutralisation a été obtenue par la production d'une forme soluble du récepteur-2 du VEGF (VEGFR2 soluble), agissant comme inhibiteur. Caractéristique des tumeurs solides se développant, l'hypoxie a été choisie pour déclencher/éteindre l'expression et la sécretion du VEGFR2 soluble, en introduisant, en amont du gène thérapeutique, une séquence régulatrice : HRE. Adressé au site tumoral par les CEPs, le régulateur de l'angiogenèse qu'est la forme soluble du VEGFR2, est exprimé de manière conditionnée et réversible, à l'hypoxie. Ceci ouvre à de nouvelles stratégies de normalisation contrôlée et stable des vaisseaux tumoraux en vue de l'utilisation de thérapies combinées.

Mots clés: angiogenèse tumorale, hypoxie, ciblage des EPCs, normalisation, piège à VEGF

Table of contents

Abbreviations:

A
ACE: angiotensin converting enzyme
AcLDL: acetylated low-density lipoprotein
AGM: aorta-gonad-mesonephros
AKT: kinase B protein
ANGPT: angiopoietin
APC: allophycocyanine
ARNT1: aryl hydrocarbon receptor nuclear translocator
ATP: adenosine triphosphate
A.U.: arbitrary units

B
bFGF: basic fibroblast growth factor
bHLH-PAS: basic helix-loop helix / Per-ARNT-SIM
BM: bone marrow
BMDC: bone marrow derived cell
BSA: bovine serum albumin
BP: band pass
Bp: base pair
BrDU: 5-bromo-2'-deoxyuridine

C
CAF: carcinoma/cancer-associated fibroblast
CBP: CREB Binding Protein
CCD: charge-coupled device
CDC42: cell division cycle 42
cDNA: complementary deoxyribonucleic acid
CDxx: Cluster of differentiation xx
CNREEA: Comité national de réflexion éthique sur l'expérimentation animale
CSC: cancer stem-like cell
CSF1: colony-stimulating factor 1
CMV: cytomegalovirus
Cp: crossing point
CpG: cytosine-phosphorothioate-guanine
Ct: cycle threshold
Cy7: cyanine 7

D
DAG: diacylglycerol
DAAP : double antiangiogenic protein
DIC: differential interference contrast
DCs: dendritic cells
DMEM: Dulbecco's modified Eagle's medium
DNA: Deoxyribonucleic acid
Dpc: days post conception
DSS: distal splice site

E
EBS: Egr-1-binding site
ECG: electrocardiogram
ECM: extracellular matrix
EC: Endothelial cells
EDTA: ethylene diamine tetraacetic acid
ELISA: enzyme-linked immunosorbent assay
EMCCD : electron multiplying charge-coupled device
eNOS: endothelial nitric oxide synthase
EPC: endothelial precursor cell
EPO: erythropoietin
EP: endothelial progenitor cell

F
FACS: fluorescence-activated cell sorting
FBS: fetal bovine serum
FDA: Food and Drug Administration
FGF: fibroblast growth factor
FIH: factor inhibiting hypoxia inducible factor
FITC: fluorescein isothiocyanate
Flk1: fetal liver kinase-1 (VEGFR2)
Flt1: FMS-like tyrosine kinase-1 (VEGFR1)
Flt4: FMS-like tyrosine kinase-4 (VEGFR3)
FTC: fluorescein thiocarbamyl

G
GCV: ganciclovir
GFP: green fluorescent protein

H
H: heparin
HAS: hypoxia inducible factor-1 ancillary sequence
HBS: hypoxia inducible factor-binding Site
HEK293: human embryonic kidney 293E cell
hESC: human embryonic stem cell
HIF: hypoxia inducible factor
HLF: hypoxia inducible factor-like factor
HMEC: human microvascular endothelial cell
HO-1 hemoxygenase-1
HRE: hypoxia response element
HRF: hypoxia inducible factor-related factor
HS: heparan sulfate
HSPG: heparin sulfate proteoglycan
HSP90: heat shock protein-90
H&E: hematoxylin and eosin

I
IARC: international agency for research on cancer
ICAM: intercellular cell adhesion molecule
IF: intensity of fluorescence

IFP1.4: infrared fluorescent protein 1.4
IGF: insulin-like growth factor
IgG: immunoglobulin G
ILx: interleukin x
IPAS: inhibitory PAS
IP3: inositol 1,4,5-trisphosphate
IRES: internal ribosome entry site

J
JAB1: jun activation domain binding protein-1
JAK: janus kinase
jet-PEI: jet-polyethyleneimine
JMD: juxtamembrane domain

K
KD: dissociation constant
KDa: kilo Dalton
KDR: kinase insert domain receptor
KID: kinase insert domain

L
LED: light-emitting diode
LOX : lysyl oxydase
LP: long pass

M
MAgEC: murine endothelial cells from AGM region
MCs: mast cells
MDSC: myeloid-derived suppressor cell
MDM2: murine double minute 2
MEK: MAPK/ERK kinase
miRs: miRNA: micro ribonucleic acid
MLuMEC,FVB: murine lung microvascular endothelial cells, of FVB mice
MMP: matrix metalloproteinase
MOP2: member of the PAS superfamily 2
MRE: metal-response element
mRNA: messenger ribonucleic acid
MSC: mesenchymal stem cell
mTOR: mammalian target of rapamycin

N
NFAT: nuclear factor of activated T-cell
NIS: natrium-iodide symporter
NO: nitric oxide
NRP: neuropilin receptor

O
ODD: oxygen-dependent degradation domain
OptiMEM: opti-minimum essential medium

P
PAS: per arnt sim domain
PBSc: complete phosphate buffer saline
PCR: polymerase chain reaction
PE: phycoerythrin
PECAM: platelet endothelial cell adhesion molecule
PFA: paraformaldehyde
PI3 kinase: phosphoinositide 3 kinase
PIP2: phosphatidylinositol 4,5-bisphosphate
PHD: prolyl hydroxylase
PKB: protein kinase B
PlGF: placenta growth factor
PLT: platelet
PO_2: dioxygen partial pressure
PODXL: Podocalyxin
DNA Pol II: DNA polymerase II
PPIA: peptidylprolyl isomerase A
pVHL protein : von Hippel Lindau protein

Q
qPCR: quantitative polymerase chain reaction

R
RACK1: receptor for activated C-kinase 1
RBX1: Ring-Box 1
RGP: radial-growth-phase
ROS: reactive oxygen species
RPMI: Roswell Park Memorial Institute
RT-qPCR: reverse transcriptase quantitative polymerase chain reaction
RU: Resonance Units

S
SD: standard deviation
SDF1-α: stromal cell-derived factor α (=CXCL12)
SEM: standard error of the mean
sFlt1: soluble Flt1
SHP2: SH2-domain-containing protein tyrosine phosphatase 2
siRNA: small interfering ribonucleic acid
SLCC: stem-like cancer cell
SOS: Son of sevenless
SPR: Surface Plasmon Resonance
STAT: signal transducer and activator of transcription.
sVEGFR2: soluble VEGF receptor-2

T
TAD: transactivation domain
TAF: tumor-associated fibroblast
TAM: tumor-associated macrophage
TAN: tumor-associated neutrophil
TGF-beta: tansforming growth factor-beta

TKD1: tyrosine kinase domain 1
TMD: transmembrane domain
TRAIL: factor-related apoptosis-inducing ligand
Treg: regulatory T-cell
TRITC: tetra methyl rhodamine iso thio cyanate
TSS: transcriptional start site

U
UEA-1: ulex europaeus agglutinin-1
UV: ultraviolet
UTR: untranslated region

V
VCAM: vascular cell adhesion molecule
VE-Cadherin: vascular endothelial-cadherin
VEGF: vascular endothelial growth factor
VEGFR1, 2, 3: vascular endothelial growth factor receptor 1, 2, 3
VGP: vertical-growth-phase
VPF: vascular permeability factor
vWf: von Willebrand factor

W
WHO: World Health Organization

3D: three-dimensional
ΔCp: difference in Cp (crossing points)
ΔIF: difference in intensity of fluorescence

Figures:

Introduction

Cancer, the second major cause of deaths worldwide is an extremely complex disease characterized by massive growth of abnormal cells. Cancer cell growth can give rise to a solid tumor that depends on nutrients and oxygen supply carried by blood vessels. During the course of its development, tumor mass develops hypoxic zones, that turn on transcription factors sensing hypoxia such as HIFs (hypoxia inducible factors) [1] further inducing proangiogenic proteins [2]. Vascular endothelial growth factors (VEGFs) are then expressed in the tumor environment and are major actors leading to neo-angiogenesis [3], with VEGF-A being the main isoform expressed. Thus, VEGFs bind to vascular endothelial growth factors receptors (VEGFRs) on the neighbor endothelial and perivascular cells to promote growth and development of blood vessels [4].

Judah Folkman's pioneer works [5] prompted antiangiogenic therapies for cancer treatment. These approaches are continuously improving [6-10]. Nevertheless, most of antiangiogenic treatments lead to the selection of highly aggressive and resistant stem-like cancer cells (SLCCs) [11]. SLCCs can be classified as a subpopulation that results from a selection pressure due to the anoxic microenvironment. Tumor adapts to harsh hypoxia and low pH through rescue mechanisms. Tumor cells use anaerobic metabolism via glycolysis pathway and resistance tools inducing multidrug receptors. Thus they enter a dedifferentiation process which leads to stemness and highly aggressive phenotype [11-13].

To avoid such pitfalls, anticancer treatment strategies no longer aim at total angiogenesis inhibition but may favor blood vessel normalization [14-16]. Such requirement determines the VEGF-based therapeutic strategies. Indeed, VEGF-A is also a strong vascular permeability factor (VPF) [4, 17]. Its overexpression in the tumor makes the blood vessels leaky and increases edema. Vessels are no longer insuring efficient blood flow thus maintaining the hypoxic character in tumors [18] and keeping continuous VEGFs production. This vicious circle contributes largely to the tumor progression [11-13]. Consequently, vessel normalization strategies are designed to counteract hypoxia-induced angiogenesis, to allow the maturation of the vessels in order to reduce cell spreading [19] and to restore efficient blood flow thus enhancing oxygen supply. This brings a considerable benefit for radiotherapy [14] as well as drug delivery during chemotherapy [20]. Consequently, the modulation of

VEGFs expression rather than total inactivation is to be reached for vessel normalization purposes [21]. Gene therapy is a promising approach in blood vessel normalization strategies.

The thesis project presented in this manuscript gives a new insight to such strategies aiming to normalize the tumor angiogenesis.

To achieve such strategy, a very old stratagem coming from the Greek Mythology, the Trojan Horse Myth, was revisited by the "cell recognition and glycobiology" team. Ulysse'stratagem can be easily transposed to cancer therapy where the tumor becomes the place to reach and to defeat. Still it remains to be as ingenious as Ulysse to build a good Trojan horse. The goal is to combine in the same engineered entity, a targeting unit and a specific drug/gene delivery system, in order to address the therapy to cancer cells without affecting healthy cells. With biology and biotechnology combined, such construction was made feasible.

In the presented manuscript, we are focusing on a novel cell-based targeting strategy. A model of endothelial precursor cell (EPCs) was designed and used as targeting cell to reach the pathologic organ. EPCs were chosen because they were shown to be able to target specifically the tumor site after systemic injection. Indeed, among bone marrow recruited cells in response to stimuli emitted by a given microenvironment, EPCs are considered to reach preferentially the areas where neo-angiogenesis as well as vascular remodeling are occurring. Identified in the 1990s [22], EPCs contribute to postnatal physiological and pathological neovascularization as well [23]. Well adapted tool for tumor targeting [24], "armed" EPCs, expressing a therapeutic gene, were thought to be used as a gene carrier for tumor specific delivery [25-27].

The second part of the system is the expression vector for the therapeutic gene of interest that is designed to "arm" the endothelial precursor cell targeting the pathological angiogenic site. In the case of new approaches to treat pathological angiogenesis, the products resulting from the expression of this gene needed first to be able to normalize the blood vessels. Because tumor-expressed VEGF is responsible for the intra tumor angiogenesis (chaotic, leaky vessels) the approach towards normalization was devoted to VEGF regulation. Consequently, a VEGF-trapping was attempted based on the production of a soluble form of the VEGF receptor-2 (sVEGFR2) [8, 28, 29] as a candidate inhibitor.

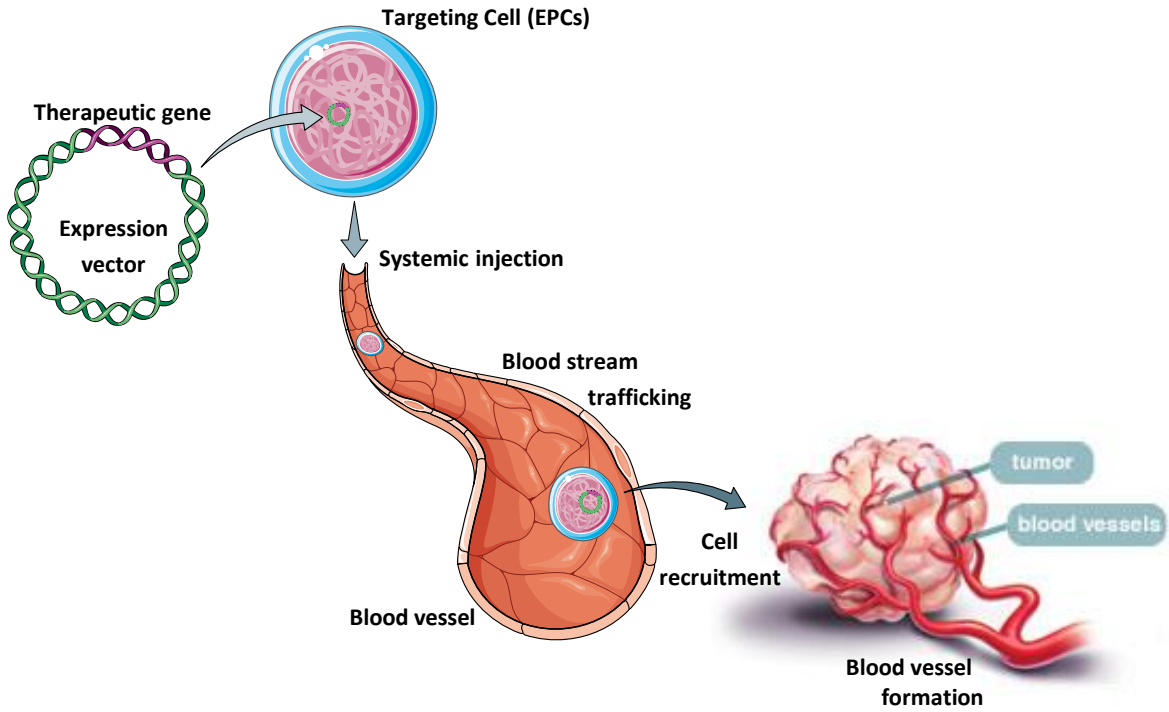
Moreover, our gene therapy strategy, besides an efficient utilization of the gene of interest, was focusing on its controlled expression. Because, an absolute targeting remains a

challenge the approach tried to take advantage of both preferential localization and condition-regulated expression in order to favor a tumor restricted expression of the gene. Hypoxia, a hallmark of all solid tumors was chosen to be the criterion turning on/off the expression of sVEGFR2 and its secretion. Such hypoxia-dependent expression was made possible by targeting the HIF/HRE axis (Hypoxia Inducible Factor/Hypoxia Response Elements) [30]. A HRE sequence was introduced upstream of the therapeutic gene. Specifically, the HRE-inducible sequence allows a tight regulation, switching-on at low oxygen tension but, more importantly, switching-off upon reoxygenation. Thanks to reversibility, the expression of a conditioned angiogenesis inhibitor should be advantageous for vessel normalization strategies as compared to strictly antiangiogenic treatments. Hypoxia induced promoters should reduce toxic side effects and improve the therapeutic efficiency through the precise control of the gene expression.

Indeed, this approach should help overpass the above mentioned problems resulting from antiangiogenesis-restricted therapies. Although a number of such therapies are already approved for clinical use, including administration of VEGF-A blocking monoclonal antibodies (bevacizumab) or VEGFR2 inhibitors (Sunitinib) [11, 13], they suffer of serious drawbacks. Among them the dangerous selection of resistant, dedifferentiated cancer stem-like cells, should be cited [11, 13]. It is now largely admitted that these problems come from the formation of inadequate vessels upon antiangiogenic treatments and from the difficulty to define the therapeutic windows that correspond to a transient step during which vessels are normalized [31]. This state is to be reached to insure efficient drug delivery and to improve radiotherapy [21].

VEGFRs production by tumor cells is a natural regulatory process. Targeting VEGF by VEGFRs gene therapy may thus additionally affect tumor proliferation by depleting a growth factor from its environment [14-16]. Therefore, the benefit of such regulated anti-VEGF approach is the simultaneous action on the endothelium and the tumor cells, improving the efficacy of the treatment. If successful, this setting should suit to Jain's hypothesis about angiogenesis normalization rather than destruction, as the future of cancer therapy [32].

Last part of the vector construction is devoted to allow imaging and tracking the "therapeutic" cells. A near infrared fluorescent reporter, a sequence encoding the IFP1.4 protein [33], was integrated into the vector as *in vivo* tracker, to allow the permanent cell visualization [33-35].



<u>Figure 1:</u> Scheme of the Trojan Horse approach: EPCs as carrier for targeted gene therapy for pathologic angiogenesis

The goal of this work is the construction of the described cell-based gene therapy of cancer. The first part of this manuscript introduces cancer in a general way to further describe the study model, the melanoma. Angiogenesis process is then described first at cellular level and molecular levels. The pathologic features of angiogenesis and some therapeutic approaches are further presented. Results are provided in three parts: first the design and test of a plasmid coding the soluble form of the VEGFR2 in a construct allowing reversible hypoxia-driven expression and imaging tracker; the second part concerns the endothelial precursor cell model, its validation for tumor targeting, and the third part describes the combination of the tools obtained as results from both previous parts: a cell carrier "loaded" with the therapeutic vector. Finally, data will be discussed and conclusions presented with the perspectives that this work opens.

1-Bibliographic overview

1.1-Cancer

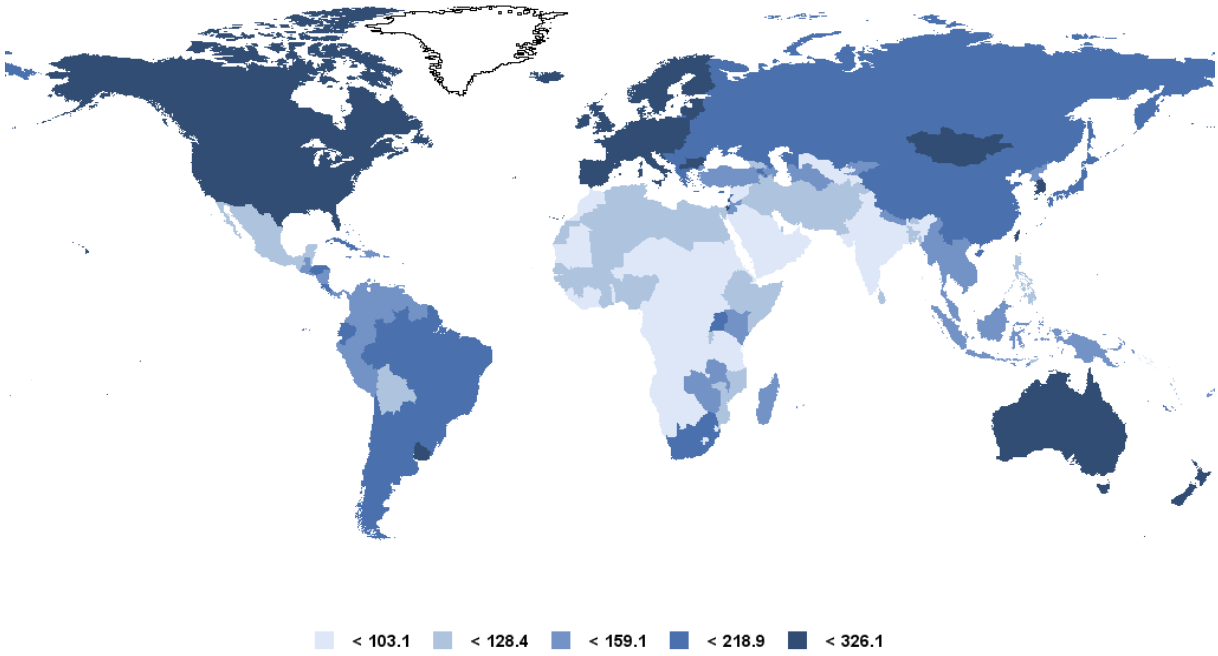
1.1.1-Generalities

1.1.1.1-Cancer in numbers

Announced as the plague of the 21st century, cancer killed around 7.5 million people per year still in 2008. The actual question about cancer appearance is no longer "who" but is rather "when" and "where".

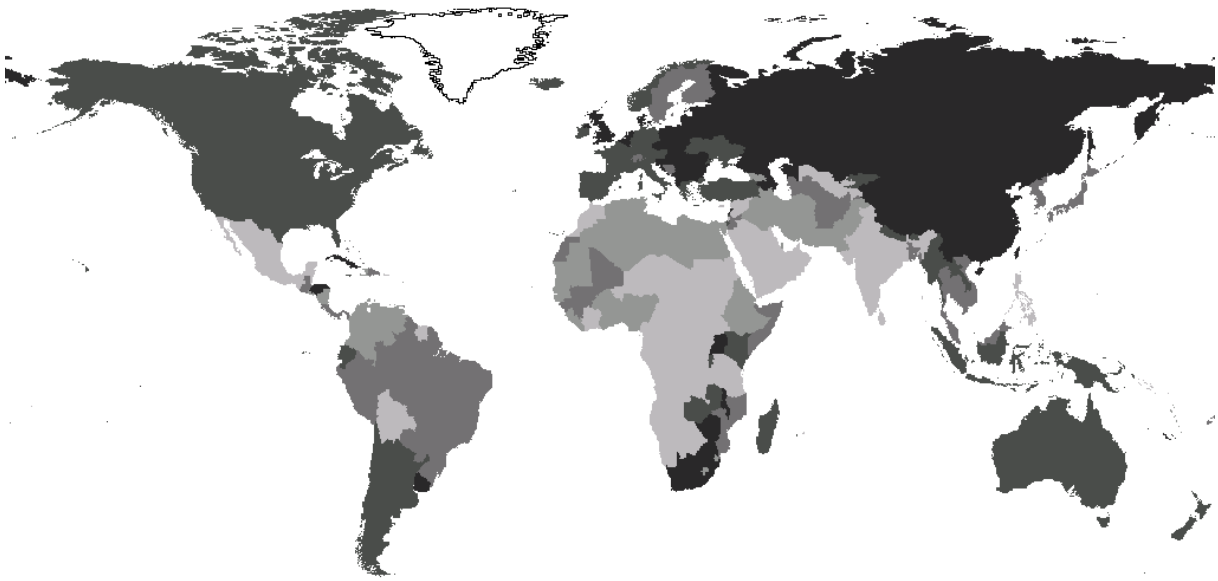
Indeed, cancer stays the main cause of death in numerous countries and will become a major cause of morbidity and mortality in the next decades all over the world [36]. The latest world statistics from 2008 published by the IARC (International Agency for Research on Cancer) show that Europe, USA and Australia are equally affected (Figure 2A). Although cancer mortality is lower in Europe, it remains a serious health problem and is tightly linked to the quality of medical treatment to patients (Figure 2B).

A



Estimated age-standardized incidence rate per 100,000.
All cancers excluding non-melanoma skin cancer: both sexes, all ages (in 2008).

B



Estimated age-standardized mortality rate per 100,000.
All cancers excluding non-melanoma skin cancer: both sexes, all ages.
(in 2008)

<u>Figure 2:</u> Cancer incidence and mortality around the world.
Provided by the World Health Organization (WHO), centralized data coming from 2008 (CANCER*Mondial* website) and statistics about cancer incidence and mortality for 27 cancers worldwide (184 countries) are studied with GLOBOCAN database which generates the above maps. The blue map (A) shows the estimated age-standardized incidence per 100,000 persons for all cancers excluding the non-melanoma skin cancer, for both males and females, and all ages. The black map (B) similarly shows the mortality.

Statistics, calculated over the last decades, indicate that cancer occurrence is gradually increasing. In a statistical analysis published by the WHO, authors estimated that if the cancer-specific and sex-specific estimated trends continue, the incidence of overall cancer incidence will be increased from 12,7 million new cases in 2008 to 21,2 million by 2030 [37].

Taking into account the actual cancer evolution, the demographic changes and population aging, GLOBOCAN calculated the extrapolated incidences and mortalities. Results are presented on the Figure 3 showing an exponential progression over the time of both, incidence and mortality.

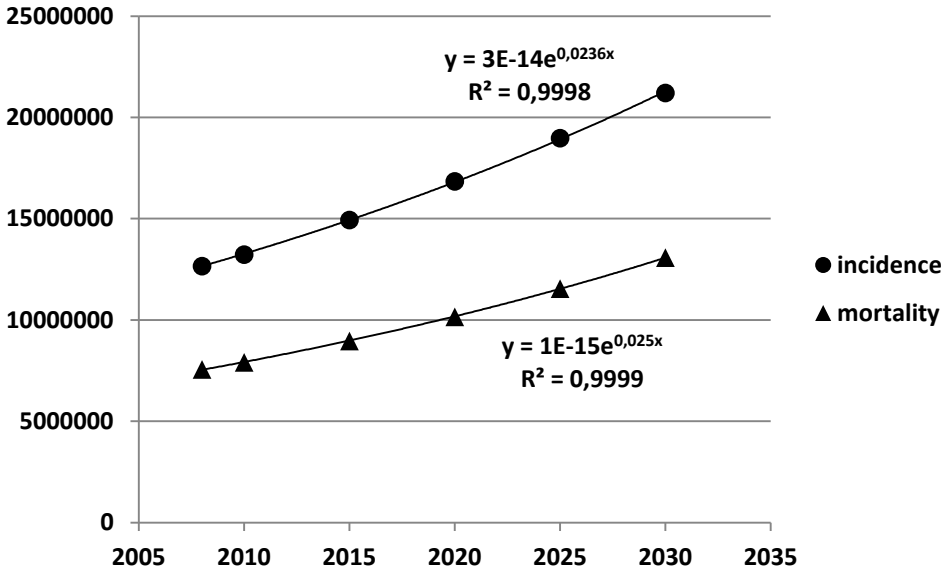


Figure 3: Time related evolution of cancer incidence and mortality.
Centralized and analyzed by GLOBOCAN using already known data of cancer evolution, demographic growth, and populations aging, predictions were obtained in matter of incidence and mortality. The predictions are done from the worldwide cancer data (184 countries included) and with all cancers excluding the non-melanoma skin cancer (27 cancers included), for both sexes, males and females, and for all ages.

Although results are minimized because of incomplete coverage of the population from which data on incidence and mortality are registered or because of insufficient disease/death information, they constitute a first overview of the actual and future situation and highlight how crucial it is to work on the development of new cancer therapies.

1.1.1.2-Cancer in science

In a normal organism, cells are growing at different rates. Along divisions, cell DNA is submitted to many causes of errors that are usually repaired thanks to the adequate cell machinery. However, dysfunctions in the cell regulation mechanism allow the start of an anarchical cycle of cell division. Cell continues to divide quickly resulting in a tumor mass (Figure 4). When such event concern blood cells and bone marrow cells, no solid tumor develop but cells are spread.

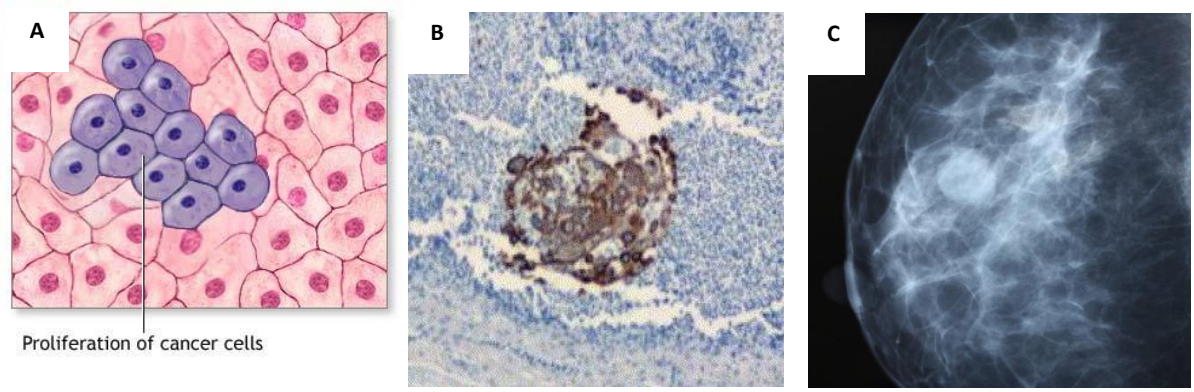


Figure 4: Cancer development.
(A) Schematic drawing of tumor development. By successive and anarchical mitosis, abnormal cells (dark blue) continue to proliferate into healthy tissue (pink) giving a cell mass. More advanced, this mass called tumor will be responsible of the cancer disease. (B) Immunohistochemistry of tumor cell colored in brown. (C). X-ray imaging of breast carcinoma. The tumor mass is visible as white area.
(from http://www.pratt.duke.edu/node/2614/ ; www.cryosites.com/images/cell_nq0pqb5, and [38]

Cancer is characterized by abnormal cell proliferation into a normal tissue leading to the compromised survival of the organism. During the development of the disease, some cells are able to acquire invasive features and to escape from the place they are produced to invade a new tissue and to form metastasis. The metastases are secondary sites or secondary tumors, by opposition to primary site making reference to the place where the tumor first occurred.

Cancer initiation is linked to causes that are endogenous or exogenous to the organism. Various genes are described to induce tumor formation when mutated or to enhance tumor formation when overexpressed. Environmental factors such as car pollution, radiation, gas factories, over exposition to UV radiations, certain chemicals or unbalanced food consumption are examples among tumor inducers.

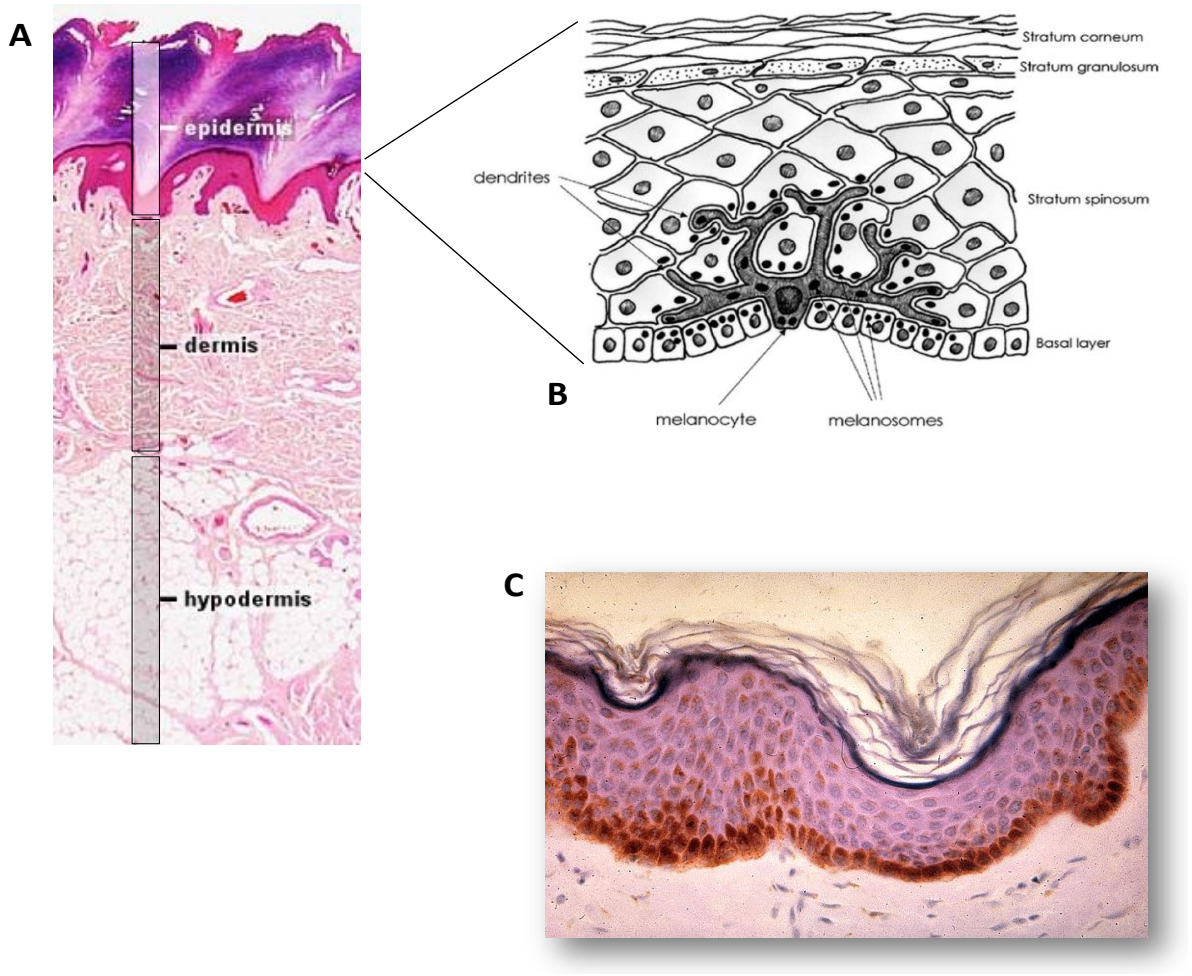
Although causes may differ, cancer is not a recent disease. First cases of cancer were already described in Egyptian writings around -3500 before J.C., reported from the Ebers papyrus, the older medical manuscript found till now. Hippocrate gave the first definition of “carcinoma” (a variety of cancer) or “squire” (hard tumor without pain forming from some part of the body): a hard tumor, non-inflammatory, with a tendency to recur and to generalize till death.

Since the first steps of medicine, cancer continues to mobilize the search for new treatments.

1.1.2-Melanoma

Among skin cancer types, melanoma is less frequent than baso-cellular and the spino-cellular carcinomas but is often lethal. Indeed, melanoma counts 101 807 new cases per year around the world (in 2008) and represents 80% of death linked to skin cancer.

Melanoma comes from the deregulation of the melanocyte located at the basement of epidermis. Figure 5, schematically shows the skin composition and architecture of the three layers: the epidermis, dermis and hypodermis.



http://www.udel.edu/biology/Wags/histopage/colorpage/cin/cin.htm
http://php.med.unsw.edu.au/embryology/index.php?title=Foundations_-_Histology_Epithelia_and_Skin

Figure 5: Skin representation with its 3 layers: epidermis, dermis, and hypodermis.
A thick slice of skin is presented with hematoxylin and eosin (H&E) staining (A). Subdivided in 3 parts with epidermis, dermis, and hypodermis, a schematic of human epidermis is presented on (B) (picture from [39]). On the basal membrane are the melanocytes. Responsible for skin pigmentation, melanocytes produce the melanin in (brown) (C)

Epidermis is the external layer which protects the whole body from sun radiation, a major factor leading to skin cancer. Melanocytes produce melanin, a pigment screen to UV (ultraviolet) radiations. These cells are controlled by keratinocytes for their proliferation, melanin production, motility and survival. Mutations involving genes that drive the cell cycle, growth factors production or cell adhesion can lead to the loss of melanocytes control by keratinocytes [40]. Proliferating melanocytes lead to nevus formation. Some nevi can be dysplasic and harbor atypical melanocytes (Figure 6).

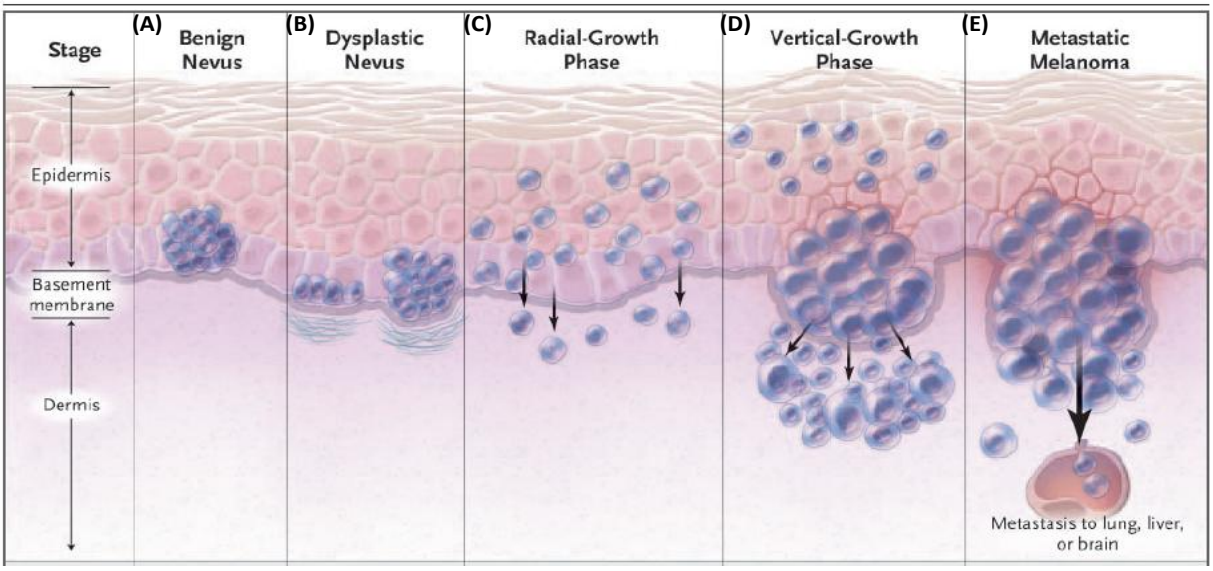


Figure 6: Progression of melanocyte transformation. [41]
(A) A benign nevus, (B) a dysplastic nevi, (C) a radial-growth-phase (RGP) corresponding to melanoma phase I where some cells are invading the dermis, (D) a vertical-growth-phase (VGP) that is the step from which one the cells become metastatic, and (E) a metastatic melanoma where the cells reach the vasculature and allow spreading in organs.

Nevi are often benign lesions but they can progress towards radial-growth-phase (RGP) where cells multiply and propagate, both in the superior part of epidermis (pagetoïd invasion) and from micro-invasions into the dermis. This step is considered as the phase I of melanoma.

RGP phase cells can progress to vertical-growth-phase (VGP), during which one cell becomes metastatic and can disseminate to form nodules invading the dermis. It is the first step directly leading to metastatic malignant melanoma, with tumor cells infiltration into vascular and lymphatic networks [42].

Tumor cells detection in surrounding sentinel lymph nodes indicates that the tumor is in an invasive state and that angiogenesis already occurred (see part 1.3.2, angiogenesis). Consequently, tumor cells may have spread and colonized other organs.
Up to now, an efficient treatment to cure patients with metastatic melanoma does not exist and more than 90% of patients die due to metastasis.

Indeed, melanoma cells are highly migratory and this is similar to their embryonic state as singly migrating melanoblast. Mechanically, metastasis can occur by non-specific trapping of tumor cells in the first encountered capillary bed. But a tropism can be demonstrated and characterized as preferential metastatic sites. In human, lymph nodes, lung, brain, liver and bone are the most common sites of melanoma metastasis [43] but other organs were also documented to host melanoma secondary metastasis. Single organ metastasis was extremely uncommon in malignant melanoma of cutaneous origin and multiple organ metastases occur in 95 per cent of the patients. [44]

In non-invasive melanoma, surgery remains the best therapy. This highlights the need of imaging methods to detect early metastasis to further design the best therapy protocols for both primary and metastasis sites.

The critical step in melanoma, for patient survival, depends closely on neovascularization, thus on angiogenesis and its major trigger: hypoxia. This tumor phenotype modification is called angiogenic switch [45]. Using the new formed blood vessels created from the host vasculature, the cancer cells can spread in other organs and all over the organism.

1.2-Hypoxia

1.2.1-Hypoxia description and introduction to physioxia

Homeostasis is the maintenance of relatively stable internal physiological conditions under fluctuating environmental parameters. It was defined by Claude Bernard and published in 1865 [46] Oxygen tension is one parameter under such tight regulation. Physioxia, describing the physiological oxygen value, is stable in the body as a whole and distinct in organs and tissues [47]. Hypoxia describes the state of insufficient oxygenation, below physioxia.

Indeed, because of this concept of physioxia, different degrees of hypoxia should be considered. The first level concerns the whole body, such as during strenuous physical

exercise or when people ascend to high altitude. Feeling the lower amount of oxygen, the body adapts with, for example, an increase of heart frequency, a hyperventilation, a vasoconstriction of lung's blood vessels combined to vasodilatation in most other tissues of the body. In such situation addressed to the whole organism, the triggers able to detect hypoxia are chemoreceptors localized in the carotid body.

Secondly, hypoxia can be considered at the tissue or organ level, for example after ischemia, infraction, lesions or diabetes. In such situations, hypoxia is characterized by a too low partial oxygen pressure, but this low oxygen tension will be called hypoxia relatively to the oxygen tension characteristic of the organ or tissue in its physiological context. Conscious that all organs and tissues have their own partial oxygen pressure, the term physioxia was introduced [47] to describe this physiological oxygen value.

Consequently, a tissue or organ will be considered in hypoxia when the ambient oxygen partial pressure is inferior to the physioxia of the considered organ or tissue.

1.2.2-Hypoxia in tumor

As previously described, the tumor is a mass of neoplasic cells growing inside a tissue or organ. Because of the limited oxygen diffusion from blood vessels into a tissue (100µm), tumor cells become rapidly too distant and consequently poorly oxygenated. Then, a hypoxic core takes place in the center of the growing tumor. This results into a milieu characterized by a very low oxygen partial pressure, close to zero, which goes together with an acidic pH due to lactic acid production. These very harsh conditions will enhance the selection of highly resistant and metastatic cells designated cancer stem-like cells (CSCs). Moreover, felt by the surrounding tissues, this too low oxygen tension will lead to the establishment of a repair mechanism to perfuse the tumor tissue: the angiogenesis. This mechanism is initiated to answer to oxygen deprivation by the formation of new blood vessels for reoxygenation, till recovery of tissue physioxia.

1.3-Blood vessels and angiogenesis

1.3.1-Blood vessels and oxygen delivery

Blood vessels are a network of connected tubules in charge of blood borne cells and molecules carried to all parts of the body. They supply oxygen and nutrients to all organs and tissues, and evacuate wastes (figure 7).

Endothelium is the thin layer of cells that lines the interior surface of blood vessels and lymphatic vessels forming an interface between circulating blood or lymph in the lumen and the rest of the vessel wall.

(http://kids.britannica.com/elementary/art-89227/Lymph-vessels-and-blood-vessels-both-carry-white-blood-cells)

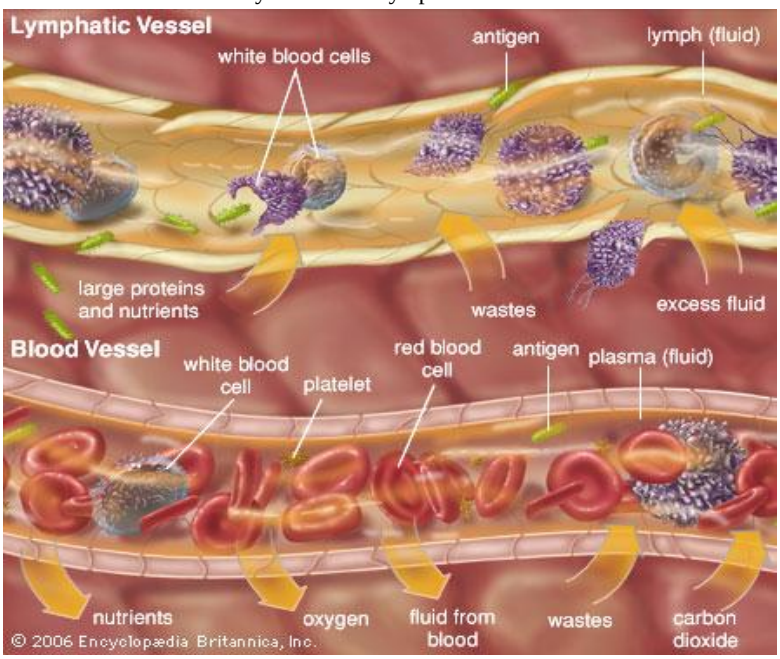


Figure 7: Lymphatics and blood vessels
Cells are carried by the lymphatics and the blood vessels. The red blood cells are carried only by the blood vessels insuring tissue oxygen supply.

Long considered as an inert barrier to separate blood from underlying tissues, endothelial cells which compose the endothelium are now known as dynamic players of key roles in physiological and pathological processes [48]. Covering the total vascular system endothelium is approximately composed of 1 to 6.10^{13} cells in the adult human. This represents around 1kg and a surface from 1 to 7 m^2. Endothelium controls the passage of small molecules as well as cells in specific and tightly regulated conditions [49].

Among the various cells carried by the blood stream in the vascular network, 40 to 52% are red blood cells (volume percentage) and are the oxygen suppliers as hemoglobin

carriers. A failure in oxygen delivery leads to hypoxia and triggers reparatory mechanisms for the reperfusion of the damaged hypoxic tissue.

Oxygen is able to diffuse from a blood vessel or capillary up to 100 µm into a tissue. Beyond this distance the tissue starts to be oxygen deprived (hypoxia). This shows the important role of blood vessels to maintain oxygen supply and how their defect leads to pathological situation.

1.3.2-Angiogenic process

Angiogenesis is often described as the development of new blood vessels network from a preexisting one and can occur in physiological as well as pathological contexts.

Historically, the term angiogenesis was first used to describe the growth of endothelial sprouts from preexisting venules. More recently, this term has been used to generally designate the growth and remodeling process of the primitive network into a complex network [50].

This physiological mechanism restores a properly adapted oxygenation of the deprived tissue or organ. As mediator of this process, pro-angiogenic factors are secreted by the cells which become hypoxic. Reaching the neighborhood blood vessels, these pro-angiogenic factors act directly on existing vessels to induce their sprouting toward the hypoxic area. This first described mechanism of angiogenesis, involves matrix degradation, tissue remodeling and endothelial cell proliferation.

The secreted pro-angiogenic factors can be carried by the blood flow in the whole body. Acting in a paracrine manner on the bone marrow, they induce the mobilization of bone marrow derived cells (BMDC) among which the endothelial precursor cells (EPCs). These EPCs are specifically attracted by the factors emitted at the tumor level and are carried by the blood stream to reach the neo-angiogenesis site to participate to new blood vessels establishment. This second mechanism of participation to angiogenesis, involves the highly specific and tightly regulated recruitment process of EPCs. This targeting ability has a strong potential for therapeutic applications through newly forming vessels.

Although less known, other mechanisms can also occur to make new blood vessels, such as intussusceptions (splitting angiogenesis), vascular mimicry by surrounding pathologic cells, or vessel co-option described in several pathologies including cancer.

Altogether these mechanisms of building new vessels from pre-existing ones allow tissue re-perfusion. This physiological mechanism is observed in embryogenesis, hair growth, menstruation and wound healing.

Various diseases are characterized by angiogenesis initiation in order to expand as shown largely in cancer development, inflammation or auto-immune diseases. Angiogenesis can be by itself the main pathologic factor as in diabetic retinopathy, macular degeneration, endometriosis [31, 45].

1.3.3-Tumor angiogenesis

1.3.3.1-Introduction

When a tumor reaches 1–2 mm in diameter, the passive diffusion for gas exchange (together with nutrients and metabolic waste) is no longer possible. This leads to a hypoxic microenvironment that induces the angiogenesis process to perfuse and irrigate the tumor tissue.

Because angiogenesis is a highly complex, dynamic process regulated by a number of pro- and antiangiogenic molecules, the “angiogenic switch” occurs when the tumor cannot grow more, limited by the oxygen and nutrient supply and must initiate new vessels formation [51]. It characterizes the transition from non-angiogenic phenotype to angiogenic one. Angiogenesis is highly controlled by a tight equilibrium between pro- and anti-angiogenic factors that can immediately stimulate or inhibit the process. Tumor hypoxia destabilizes this equilibrium in favor of pro-angiogenic factors. The acquisition of angiogenic phenotype is considered as a hallmark of the malignant process whereby proangiogenic mechanisms circumvent negative regulators [52].

Angiogenesis around tumors was first observed a century ago [53]. The first evidence about production of any angiogenic substance by tumors was shown in 1968 [54, 55].

In 1971, Judah Folkman proposed first that tumor growth and metastasis could be dependent of angiogenesis [5]. His subsequent hypothesis was that if a tumor could be stopped from growing its own blood supply, it would wither and die. Disregarded by most experts in the field this hypothesis became, later on, widely accepted and exploited for angiogenesis-based cancer therapies and vascular pathologies such as retinopathy.

A new degree of consideration was brought by Rakesh Jain revisiting Folkman's postulate. In a review: "Molecular regulation of vessel maturation" [16] was introduced the innovative concept of "normalization". The challenge is to revert to normal the chaotic, disorganized and non functional blood vessels which characterize tumor angiogenesis and other vascular pathologies, in order to facilitate the drug delivery and to improve radio-therapies. This might lead to hypoxia compensation and provide new avenues for cancer combinatory treatments.

1.3.3.2-Angiogenesis cell mechanisms

In order to vascularize a hypoxic tissue, different mechanisms take place. Depending on the context, the various processes can occur in parallel, independently and during *in vivo* physiological /pathological situations.

Cell migration and remodeling occur in all mentioned mechanisms for which the priming step is commonly due to a low oxygen partial pressure, inferior to organ or tissue physioxia.

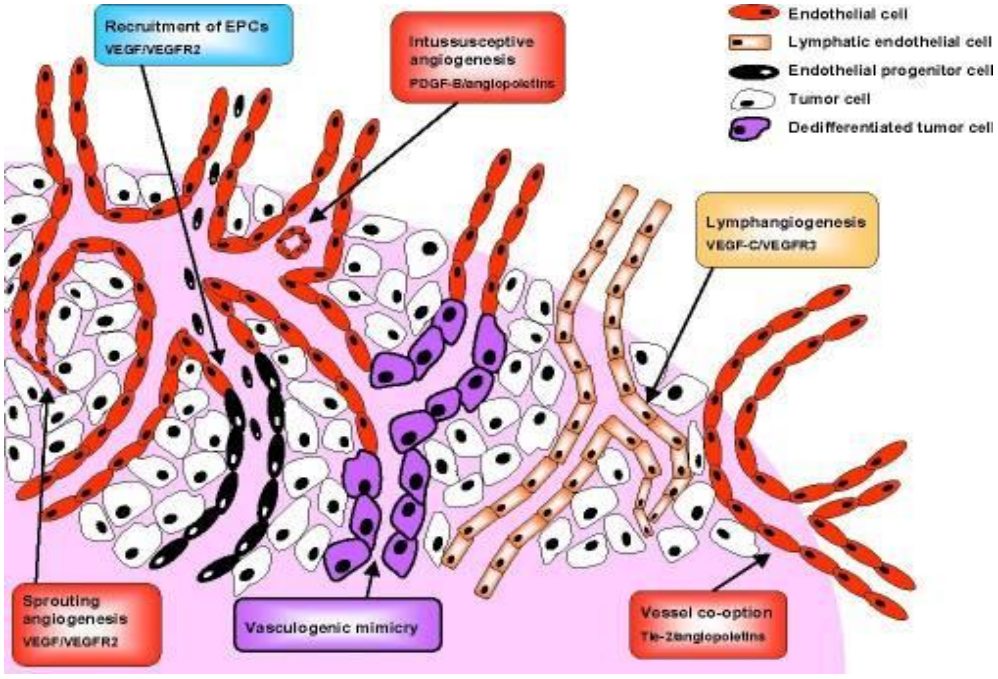


Figure 8: Mechanisms of tumor vascularization [56]
Six different types of vascularization observed in solid tumors: sprouting and intussusceptive angiogenesis, recruitment of endothelial progenitor cells-vasculogenesis, vessel co-option, vasculogenic mimicry and lymphangiogenesis.

Initially, the term “angiogenesis” was introduced to define the process of new blood vessels formation. This definition was quickly revisited for a more clear description, introducing new terms added to angiogenesis: vasculogenesis, sprouting, intussusception, vascular mimicry and lymphangiogenesis, presented in Figure 8.

1.3.3.2.1-Sprouting

First described and considered as the “true angiogenesis”, sprouting mechanism is defined as the formation, from pre-existing vessels, of thin-walled endothelium-lined structures with muscular smooth muscle wall and pericytes surrounding.

Triggered by hypoxia, cancer cells release pro-angiogenic factors which diffuse in the surrounding tissue to reach the endothelial cells of a neighboring blood vessel and bind their corresponding surface receptors. Among these signals, the vascular endothelial growth factor (VEGF) is a key regulator of this process and is well known to promote endothelial cell growth, migration, and survival from pre-existing vasculature [52]. Moreover, also known as vascular permeability factor (VPF), its involvement in the vessel permeability has been associated with malignant effusions [57].

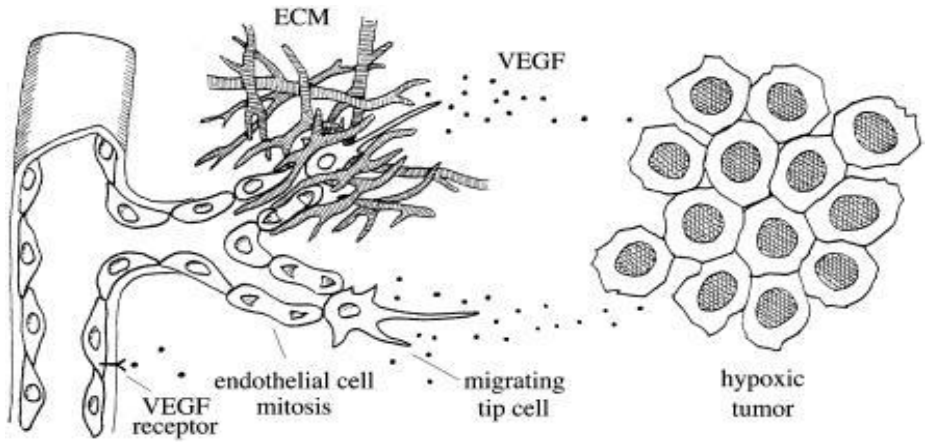


Figure 9: Sprouting angiogenesis [58]
In hypoxia, tumor cells produce other cytokines and growth factor than VEGF to activate the endothelial cells and to induce the sprouting mechanism. These factors signal to endothelial cells leading to basement membrane degradation, endothelial cell migration and invasion into the extracellular matrix. The tip cell leads sprout extension oriented toward the tumor. After initiation, maturation occurs stabilize vessel making it functional to blood flow, by basement membrane synthesis and pericytes recruitment. Reaching the tumor, the newly formed vessels should be able to irrigate the tumor.

Pro-angiogenic signals prompt ECs (endothelial cells) to degradation of basal membrane and surrounding matrix by released proteases. This participates to allow endothelial cells escape from the original vessels and to the oriented migration of endothelial cells towards the tumor as a source of signals, (figure 9) [59, 60].

This is followed by a proliferative phase where endothelial cells into the surrounding matrix multiply and differentiate in capillary to create new blood vessels. Altogether the oriented endothelial cell migration combined to proliferation lead to sprout extension. That involves matrix remodeling, specific adhesion molecules such as integrins [61] and highly specialized cells.

Among them, the "tip cells" guides the developing blood vessels in their orientation, sampling the gradient of pro-angiogenic factors as the VEGF [62]. Indeed, the tip cell is able to "sense" the VEGF gradient to adapt the cell alignment and using filopodia and cytoskeleton organization to migrate towards higher VEGF concentration. To go through the matrix, the tip cell use "tools" like proteolytic enzymes to digest extracellular matrix for progression [63]. Following the tip cell into the extracellular matrix, stalk cells proliferate elongating the capillary sprout and making the trunk of the newly formed blood vessels. Newly formed sprouts are immature and not quite functional [64]. To support blood flow, the extended sprouts should present a lumen, stop invading the matrix, build a new basement membrane and recruit specialized cells such as the pericytes and smooth muscle cells. The latter cells are essential for vessel stability and maturation, they contribute to inhibit endothelial cell proliferation and to promote basement membrane synthesis, resulting in fully mature and functional vessel [65].

1.3.3.2.2-Vasculogenesis

Vasculogenesis defines the formation of vascular structures from circulating or tissue-resident endothelial stem cells (angioblasts), which proliferate into *de novo* blood vessels to build their walls (figure 10). This relates to the *in utero* embryonic development of the vascular system.

The progenitor for endothelial cells has been proposed to be common with hematopoietic cells progenitor, and termed the hemangioblast. This was based on the observation that endothelial and hematopoietic cells emerge from blood islets in close

proximity and at a similar time during embryonic development. Definitive hematopoietic stem cells (HSCs) arise in the aortagonad-mesonephros (AGM) region in the vicinity of the dorsal aorta around embryonic day 10.5 (E10.5) [66, 67].

Endothelial and hematopoietic cells share a number of common markers during the course of their development, including Flk1, Flt1, and Tal1, providing further evidence in favor of the existence of the hemangioblast [66].

VE-cadherin is a cell adhesion molecule expressed exclusively in endothelium leading to its use by a number of laboratories as an endothelial marker. Other endothelial markers have been identified, including PECAM-1 (CD31), CD34, Tie2, and endoglin, although they are also expressed in other tissues. The expression of an individual marker but rather a combination of EC markers by a particular cell is necessary to support evidence for a cell to be classified as endothelial or of endothelial origin. Functional or morphologic characteristics are also necessary [68].

In addition to anatomic data showing that hematopoietic cells bud from the luminal wall of the ventral side of dorsal aorta, they have been shown to express CD34, Tie2, and PECAM-1[68]. Nishikawa et al [69] found that lymphohematopoietic cells are derived from VE-cadherin–expressing cells. PECAM-1 and VE-cadherin are expressed in fetal HSCs. Thus, it has been proposed that definitive hematopoietic cells have a close developmental relationship with ECs and that they originate from endothelial precursors; although it remains controversial [68].

In the adult life endothelial precursors were identified in the blood. Asahara showed that a purified population of CD34-expressing cells isolated from the blood of adult mice could purportedly differentiate into endothelial cells *in vitro* [22], that this endothelial progenitor cells (EPCs) mobilization depends on signals like VEGF [70, 71] and that EPCs contribute to postnatal physiological and pathological neovascularization as well [23]. For their capacity to reach a neovascularization area, these EPCs were thought as potential carriers for targeted therapies and for the delivery of anti- or pro-angiogenic agents to pathologic angiogenesis [25, 72].

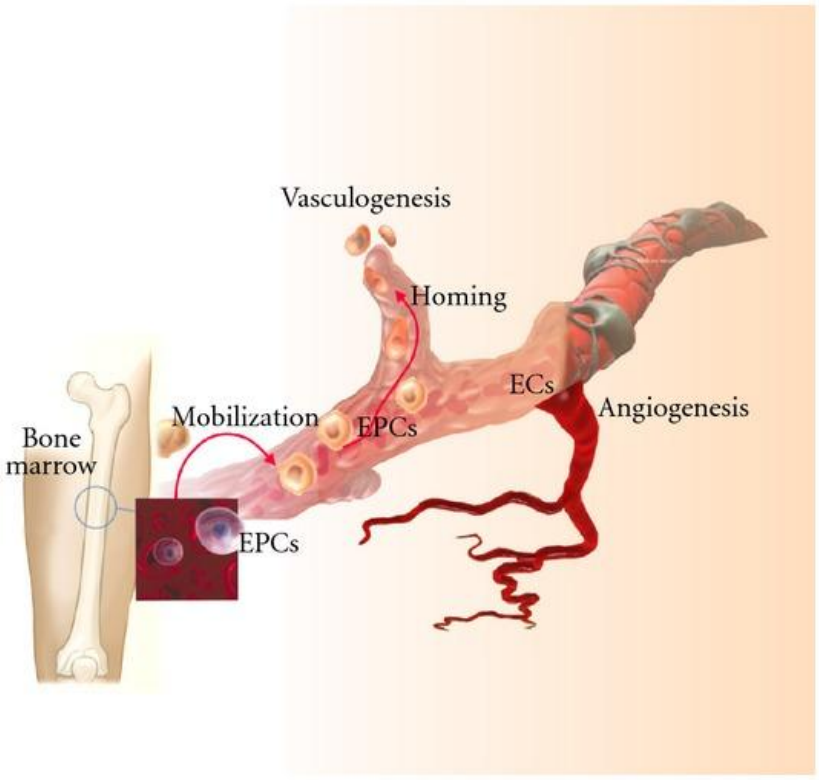


Figure 10: Schematic representation of postnatal neovascularization [73]
Recruitment of diverse bone marrow-derived cell populations, such as EPCs, to the tumor microenvironment plays a critical role in the regulation of postnatal angiogenesis and vasculogenesis. They are mobilized from the bone marrow to the peripheral blood through secretion of cytokines and chemokines by tumor and stromal cells, and have the potential to home and incorporate into the neoangiogenesis site.

Circulating EPCs are recruited in angiogenic sites thanks to surface adhesion molecules as CD54/ICAM-I or CD106/VCAM-I. Recruitment mechanism is very close to the inflammatory one, with leukocytes specifically interacting to inflamed endothelial cells. As during inflammation, the process is highly specific and tightly regulated. Deregulation occurs in cancer.

Circulating EPCs, shed from the vessel wall or mobilized from bone marrow, are a population of rare cells that circulate in the blood stream. Nevertheless, they reflect biological states. They are used in trials for regenerative medicine of heart infarctions and ischemia, retinopathy, tumor and endometriosis where 37% of the microvascular endothelium of the ectopic endometrial tissue originates from EPCs [74].

1.3.3.2.3-Intussusception

Also known as splitting angiogenesis, for intussusception vessel formation, one capillary wall extends into the lumen to split a single vessel in two (Figure 11). This mechanism makes more blood vessels by reorganization of existing ones without affecting the number of endothelial cells (cells divisions and recruitments). Moreover, it requires only 4–5 h for completion allowing rapid adaptation to milieu changes [75].

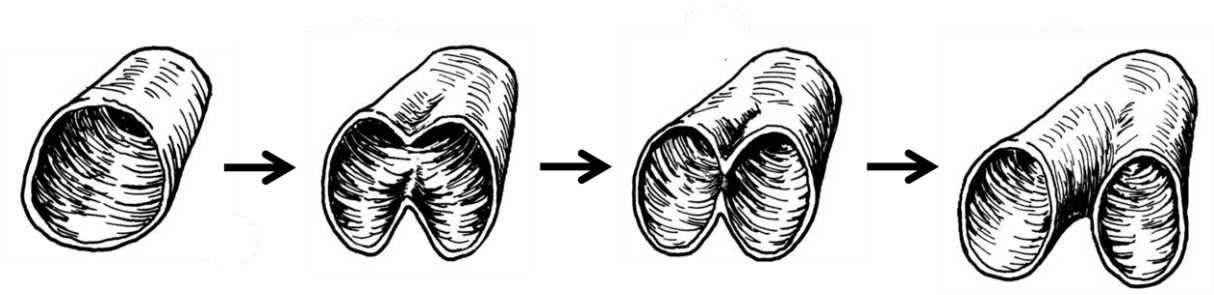


Figure 11: Scheme of intussusception process (adapted from [76])
Represented in three dimensions and in cross section, the process begins with protrusion of opposing endothelium wall/activated endothelial cells into the capillary lumen. Their contact leads to the physical separation of the vessel in two smaller vessels.

This type of angiogenesis was shown during embryogenesis and postnatal development but also in the adult, in different organs and either in pathological or physiological context. It may represent the unique means in some mechanisms of vascular tree formation and vascular remodeling.

Nevertheless, this angiogenesis mechanism remains poorly understood compared to sprouting angiogenesis and vasculogenesis but shear stress and increasing of blood flow play a major role, [77, 78].

Implication in pathologies was described as an adaptive response to restore the hemodynamic, structural properties of the vasculature and the oxygen supply to the tumor. In this context, shear stress and intermittent blood flow, that are typical of the abnormal tumor vasculature, might activate tumor intussusceptive microvascular growth [75].

1.3.3.2.4-Vascular mimicry

Among other angiogenesis mechanisms identified, vasculogenic mimicry is found in the tumor vasculature only and does not involve endothelial cells. This process describes highly aggressive tumor cells which can form vessel-like structures themselves, due to their high plasticity (figure 12). Initially observed by Maniotis *et. al.* [79] in melanomas, vascular mimicry was later reported from various others cancers including breast, ovarian, prostate, Ewing sarcoma, lung and clear cell renal carcinoma.

Dedifferentiation of melanoma cells towards endothelial-like leads to the formation of *de novo* vasculogenic-like matrix-embedded networks (i.e. vascular-like structures) containing plasma and red blood cells and ultimately contributing to blood circulation.

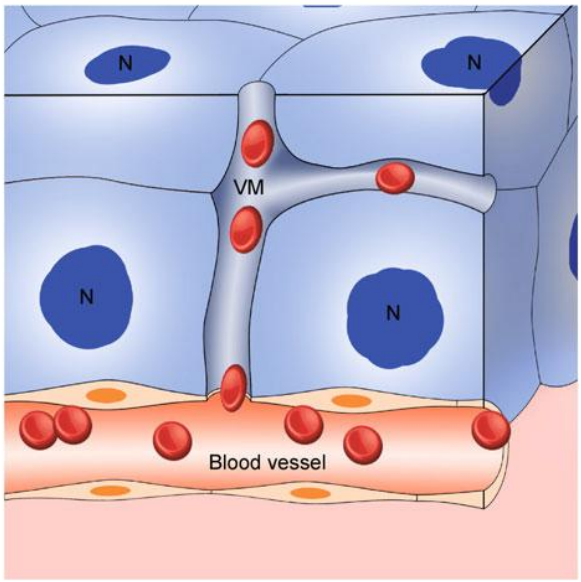


Figure 12: Scheme of vascular mimicry [80]
The cancer stem cells of vascular mimicry differentiate/transdifferentiate and line up to form branching lumens to provide nutrition for tumor mass. The tubes formed by cancer stem cells progeny extend and merge with vessels from angiogenesis or vascularization, and conduct red blood cells.

Various hypotheses co-exist concerning the cancer stem cells and their endothelial phenotype which would allow cell arrangement into tube-like structures. Indeed, cells capable of vascular mimicry display a high degree of plasticity and stem features. This stem-like differentiation plasticity of cancer cell involved in the vascular mimicry makes the tumor cells aggressive and is directly correlated to malignancy and poor prognosis for patient [81].

1.3.3.2.5-Vessel co-option

Vessel co-option phenomenon describes a mechanism by which one tumor cell grows along pre-existing vasculature in the host tissue and benefits of oxygen supply and nutrients (Figure 13).

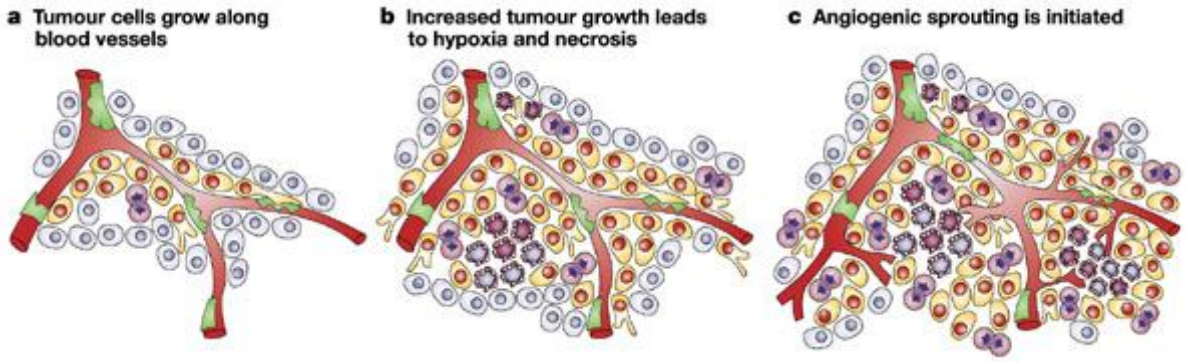


Figure 13: Scheme of vessel co-option during tumorigenesis [82]
Cancer cell represented in yellow on the draw (a) first acquire their blood supply by co-opting existing normal blood vessels without the necessity to initiate angiogenesis. Limited by the diffusion of oxygen into the growing tumor tissue, hypoxic and necrotic area appears (draw b). This condition of too low oxygen value is the trigger inducing angiogenesis mechanism such as sprouting angiogenesis, illustrated on the draw (c).

This process was observed for some tumors and especially in the early stage of tumorigenesis, when the tumor can grow without evoking an angiogenic response [56]. Nevertheless, it may persist during the entire period of primary or metastatic tumor growth.

Moreover, it occurs mainly in well-vascularized tissues where early tumor vessels appear similar to normal vessels in caliber and heterogeneity.

Vessel co-option was described first in the brain, one of the most densely vascularized organs. Thus, brain tumors may develop without the need of an angiogenic switch. [83]

1.3.3.2.6-Arteriogenesis

Arteriogenesis refers to the remodelling and enlargement of existing vessels and increase of luminal diameter, resulting in higher blood flow (figure 14). Enlargement implies wall remodeling, reorganization and modification of endothelial cells, smooth muscle cells and fibroblasts of the vessels [76].

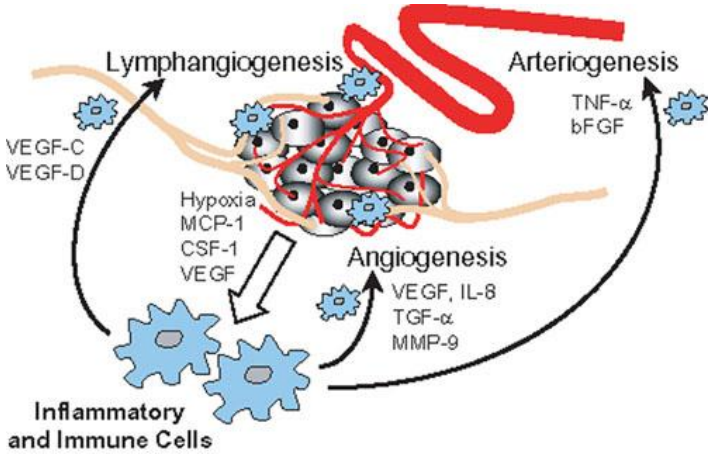


Figure 14: Scheme of arteriogenesis [84]
The diameter of the blood vessel supplying the tumor is enlarged increasing blood flow capacity to downstream vascular elements. This draw shows that inflammatory cells are recruited by the tumor which plays a supporting role during tumor progression, promoting tumor expansion by stimulating angiogenesis and arteriogenesis.

This mechanism is observed in various pathologies such as arteriole occlusion, heart disease, ischemia as well as tumor development.

Arteriogenesis is closely linked to maturation and stability of blood vessels insured by specialized cells such as pericytes, smooth muscle cells and fibroblasts [50]. Various signals are able to trigger arteriogenesis including cellular mechanism of cell invasion (e.g. monocytes), molecular mechanism including HIF-1 contribution, factors as VEGF and physical stimuli as shear stress [50, 85]. In ischemic tissues, arteriogenesis was positively correlated with higher levels of circulating stem/progenitor cells suggesting their supportive role [86].

1.3.3.2.7- Lymphangiogenesis

While “hemangiogenesis” is the formation of blood vessel, lymphangiogenesis refers to the formation of lymphatic vessels (Figure 15).

Part of the vascular circulatory system, the lymphatic network regulates tissue fluid homeostasis by draining, collecting antigens and other macromolecules from surrounding tissues. It controls trafficking of immune cells to lymphatic organs such as spleen or lymph nodes. Lymphatic vessels are lined with a single cell layer of endothelial cells without tight junctions and with a discontinuous basement membrane deprived of pericytes and smooth

muscle cells, making these vessels "open" and highly permeable for interstitial fluid and immune cells.

Invasive tumor cells can take advantage of this high permeability for spreading thus actively contributing to tumor metastasis in other organs and tissues (Figure 14).

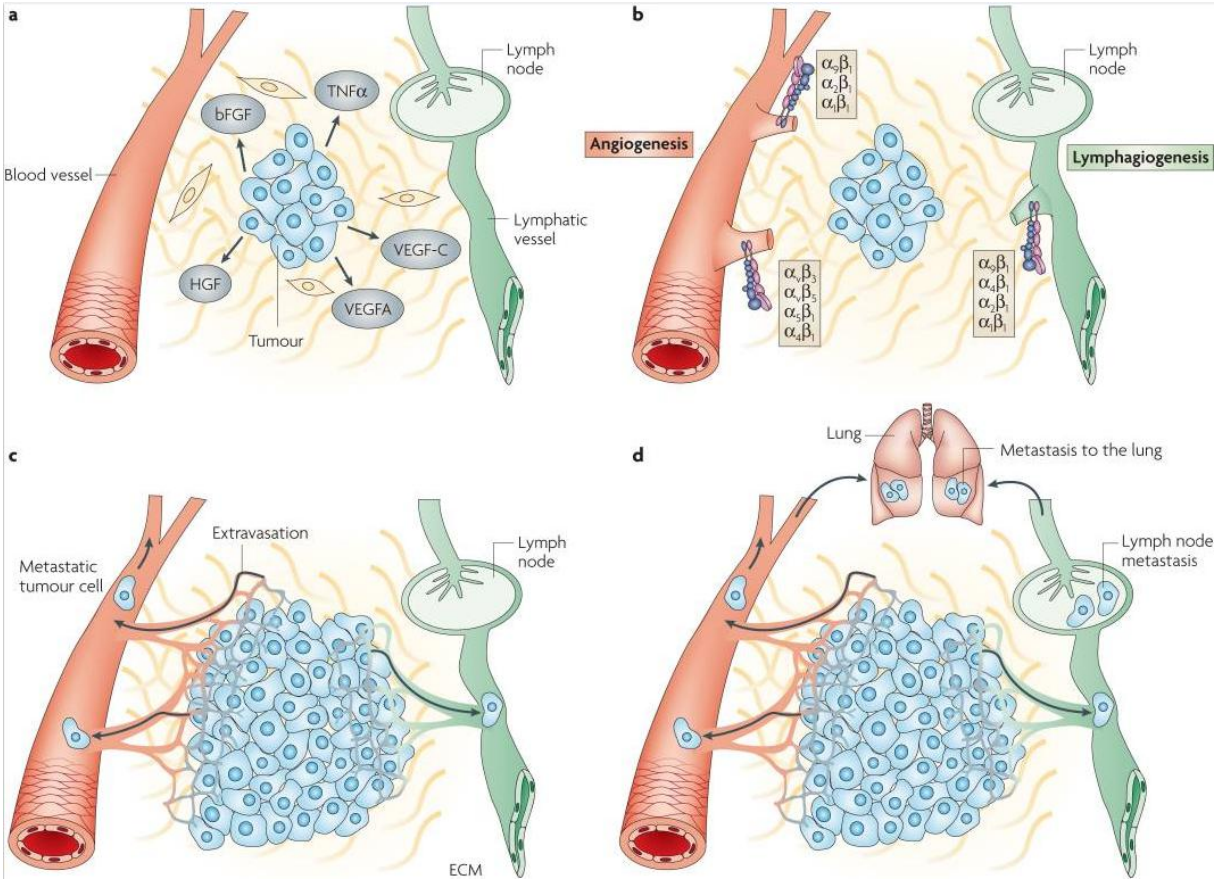


Figure 15: Scheme of tumor lymphangiogenesis and angiogenesis [87]
Time related evolution of (a, b, c and d) of tumor parallel lymphangiogenesis and angiogenesis. The secretion of growth factors and chemoattractants, i.e. VEGF, bFGF, stimulates endothelial cells to induce sprouting on both lymphatic and blood vessels (a, b). (b) Shows integrin-mediated vessel extension towards and into the tumor mass. Vessel perfusion of the tumor allows waste products removal and nutrient/oxygen supply. It provides means for cell spreading and tumor metastasis formation in vicinal lymph nodes via lymphatics and distant organs via both lymphatics and blood vessels (c and d).

Tumor microenvironment induces lymphangiogenesis and breaks physiological quiescence. Similarly to blood vessels formation, a "lymphangiogenic switch" can be described [88].

1.3.4-Physiological and pathological angiogenesis

1.3.4.1-Healthy vasculature

Endothelial cell proliferation is described as one of the slower. Endothelial cells are mostly quiescent, except in very specific situations such as angiogenesis.

A partial overlapping between cells create a continuous structure, impermeable to blood and molecules which does not exceed 75000Da.

Endothelial cells are anchored to the underlying extracellular matrix and interact with one another by cell junctions involving adhesion molecules such as VE-Cadherin/CD144 and PECAM-1/CD31. Along vessels, mural cells consolidate the junctions between endothelial cells and stabilize the vessel structure. Altogether, tight junctions, matrix anchorage and mural cells, make the vasculature hermetic and adapted to carry the blood in the whole body.

1.3.4.2-Physiologic *versus* pathologic angiogenesis

Angiogenesis is primarily a repair mechanism responding to oxygen defect (hypoxia). Both physiologic and pathologic angiogenesis react to the perfusion need to compensate hypoxia.

Although sharing many common features, vessels formed in physiological conditions are distinguishable from pathologic ones. Physiological angiogenesis such as during embryogenesis or wound healing is complete and functional to perfuse/oxygenate tissues with vessels set hierarchically (arteries, capillaries and veins) [89]. Under adequate oxygen tension, the equilibrium between pro- and anti-angiogenic factors favors the anti-angiogenic factors maintaining the control on VEGF level. The resulting newly formed vessels appear structurally normal, mature and functional, thanks to the coordinated activation of mediators and response of endothelial and mural cells [16].

In tumor angiogenesis and other diseases with vascular disorders, vessels are unable to allow a proper perfusion and oxygenation. The pathologic angiogenic cascade is persistent and unresolved and becomes driven by the pathological condition [90]. To adapt to fast tumor growth and increased needs in oxygen and nutrients, the tumor vasculature is perpetual activated. This leads to pathologic vessels, chaotic, with many abnormalities, including irregular diameter, tortuosity, fragility, lack of pericytes and a propensity for bleeding and exudation [91, 92].

Disorganized and morphologically abnormal vasculature is a hallmark of solid tumors (figure 16). Tumors display a poor blood flow participating to harsh hypoxic conditions, pro-angiogenic environment and selection of cancer stem-like cells and ultimately impairing the drug delivery.

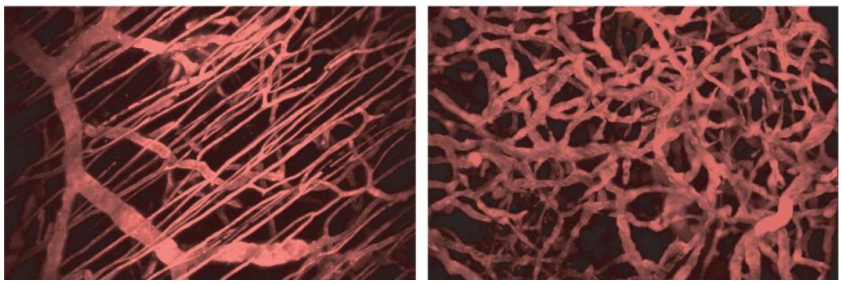


Figure 16: Physiologic and pathologic vascular network [16]
Intravital microscopy pictures of a normal arterio-venous network and capillary bed of skeletal muscle (left) and tumor vasculature (right).

1.3.4.3-Collateral actors

First concerned (*cf* part 1.3.3.2), endothelial cells are the main actors of the angiogenesis process. But, some other cells are able to directly or indirectly participate and modulate the process. Some of these cells are already present in the region where angiogenesis takes place, while others are recruited upon the secretion of numerous chemoattractants.

In the pathological context of cancer, all these cellular actors play important roles in amplifying pathological angiogenesis, deeply modifying the microenvironment by forming new vessels [90].

1.3.4.3.1-Pericytes and smooth muscle cells

Small blood vessels consist only of endothelial cells, whereas larger vessels are surrounded by mural cells: pericytes in medium-sized vessels and smooth muscle cells in large vessels. Pericytes play a role in the vessel stabilization by contact with endothelial cells reinforcing vascular structure and regulating microvascular blood flow. These mural cells are direct actors modulating the vessel stability by contact with endothelial cells. Their

association with newly formed vessels or remodeling vessels regulates endothelial cell proliferation, survival, migration, differentiation, stabilization, vascular branching, blood flow and vascular permeability. Covering the vessels, they form their own basement membrane and are circumferentially arranged, closely packed and tightly associated with the endothelium. Reported from pathological context such as tumors, dropout or insufficient recruitment of mural cells results in endothelial cells growth, permeability, fragility, vessel enlargement, bleeding, impaired perfusion and favored hypoxia [18] (Figure 17).

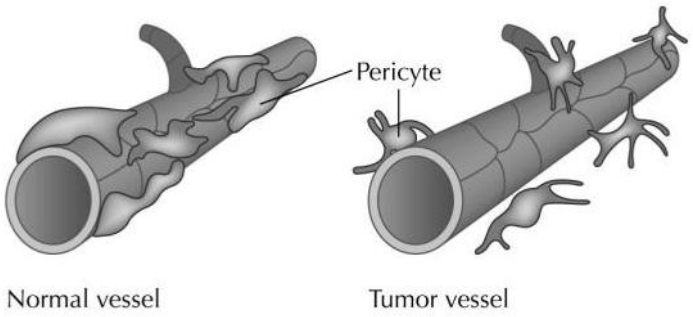


Figure 17: Pericytes localization in vasculature [93].
Mature blood vessels have uniform pericyte coverage offering stabilization (left). Tumor blood vessels have poorly attached pericytes leading to leaky vessels (right).

The ratio between the number of endothelial cells and pericytes seems to be highly controlled. Regulators include soluble factors as PIGF, acting in an autocrine and/or paracrine manner, mechanical forces secondary to blood flow and blood pressure, as well as homotypic and heterotypic cell contacts [94].

Accordingly, pericytes are recruited by differentiation from surrounding mesenchymal precursors or by migration from the mural wall of the adjacent vessel [95].

Pericyte deficiency could be partly responsible for vessel abnormalities in tumor blood vessels [96] and partial dissociation of pericytes contributes to increase tumor vascular permeability [97].

1.3.4.3.2-Tumor-associated fibroblasts

Normal stroma consists of various connective tissues that act like a supportive framework for tissues and organs. Among stromal components, fibroblasts are essential to synthesize, deposit the extracellular matrix (ECM) and for remolding. Fibroblasts are

necessary for the formation of the basement membrane which separates the epithelium from the stroma. Fibroblasts are the source of various soluble paracrine and autocrine growth factors [98].

In various cancers, a specialized group of fibroblasts called carcinoma/cancer-associated fibroblasts (CAFs) or tumor-associated fibroblasts (TAFs), are described to play an active role in tumorigenesis and metastasis by providing a unique tumor microenvironment (figure 18). This is mainly due to extracellular matrix remodeling, secretion of proangiogenic factors as VEGF and FGF [99] and chemoattractants as SDF-1α for the recruitment of bone marrow–derived progenitors [100].

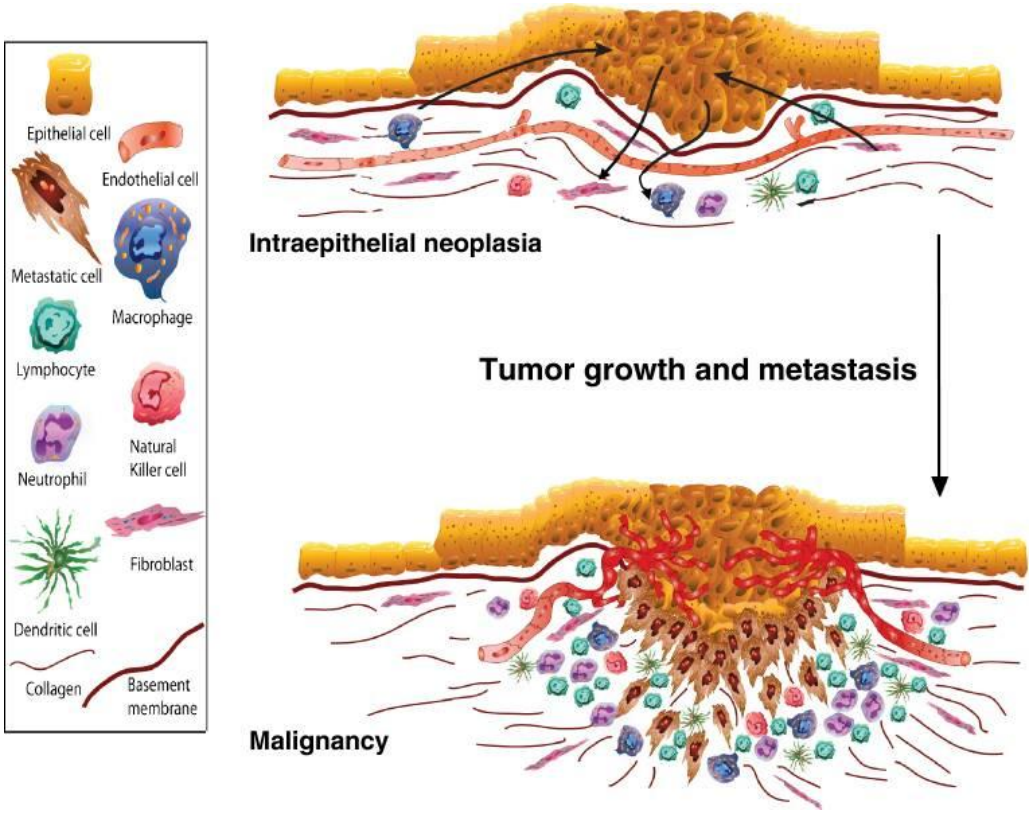


Figure 18: Tumor-infiltrating cells [101]
Schematic drawing of the tumor microenvironment showing the tumor-infiltrating cells and their influence on tumor development (model of epithelial tumor). Tumor microenvironment is associated with massive infiltration of deregulated immune cells and promotes tumor growth, angiogenesis and metastasis. Tumor-infiltrating cells predominantly include tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), CD4 T-cells, CD8 T-cells, CD4 regulatory T-cells (Tregs), mesenchymal stem cells (MSCs), cancer-associated fibroblasts (CAFs), endothelial progenitor cells (EPs), mast cells (MCs) and platelets (PLTs). Cells maintain tumor associated inflammation, angiogenesis and immunosuppression, which promotes tumor growth and metastasis

During the progression of cancer, tumor cells are able to alter the characteristics of the adjacent stroma to create a supportive microenvironment. This notion is strongly supported by the recent evidence that over 80% of the fibroblasts display an activated phenotype in breast cancer. The close relationship between cancer cells and CAFs indicates that development of cancer cannot be dissociated from its microenvironment. However, neither the origin of CAFs nor the criteria to distinguish CAFs from normal fibroblasts has been well established. They may originate from normal fibroblasts or from cancer cells, mensenchymal cells and even endothelial cells, all tightly regulated by the tumor microenvironment [98].

1.3.4.3.3-Tumor-associated macrophages

Macrophages are important immune cells that protect the host against injury and infection. Extensive animal and clinical studies now indicate that these cells also infiltrate most solid human tumors (Figure 18).

Tumor-associated macrophages (TAMs) can stimulate growth. Their density is associated with adverse outcome and shorter survival in several cancers, including breast cancer, Hodgkin lymphoma and lung adenocarcinoma.

Many tumor-derived chemoattractants, including VEGF-A, PlGF, colony-stimulating factor 1 (CSF1), CCL2 and CCL5 recruit monocytes [102]. The established tumor microenvironment directs polarization and activation of recruited monocytes from a proimmunogenic and antitumor phenotype (M1) to an immunosuppressive phenotype (M2). The latter is proangiogenic, induces tissue remodeling and tumor promotion [102].

Producing NO• in the tumor microenvironment, TAMs (as well as tumor and endothelial cells) are responsible of vascular hyperpermeability. NO was shown to be directly involved in endothelial cells functions by decreasing PECAM-1 expression and adhesion with endothelial and immune cells [103].

Tumor-associated macrophages (TAMs), which phenotypically resemble M2 macrophages, accumulate preferentially in the poorly vascularized regions of tumors, with low oxygen tension, where they cooperate with tumor cells to produce angiogenesis stimulators such as VEGF-A, bFGF and CXCL8 [102].

Therefore, macrophage recruitment together with tumor-derived proangiogenic factors, amplify angiogenesis. [90].

1.3.4.3.4-Tumor-associated neutrophils

Neutrophils are immune cells involved in host protection. Like macrophages in cancer (Figure 18), tumor-associated neutrophils (TANs) can promote the progression of primary tumors. Indeed, the recruitment of mature myeloid cells as neutrophils have been demonstrated in various human tumors, including gastric, colon and bronchioloalveolar carcinoma and correlate with increased tumor vascular density and poor prognosis [104].

Neutrophils are a major source of MMPs within the tumor microenvironment [105]. These enzymes mediate the release of proangiogenic factors bound to HSPG (heparin sulfate proteoglycan) in the extracellular matrix as bFGF or VEGFs (i.e.VEGF-A206, a splice isoform of VEGF-A). Moreover, activated neutrophils release proangiogenic factors including VEGF-A to amplify tumor angiogenesis [106].

In vivo, tumor-associated neutrophils might be polarized similarly to TAMs, exhibiting either an antitumor (N1) or a protumor (N2) phenotype [107].

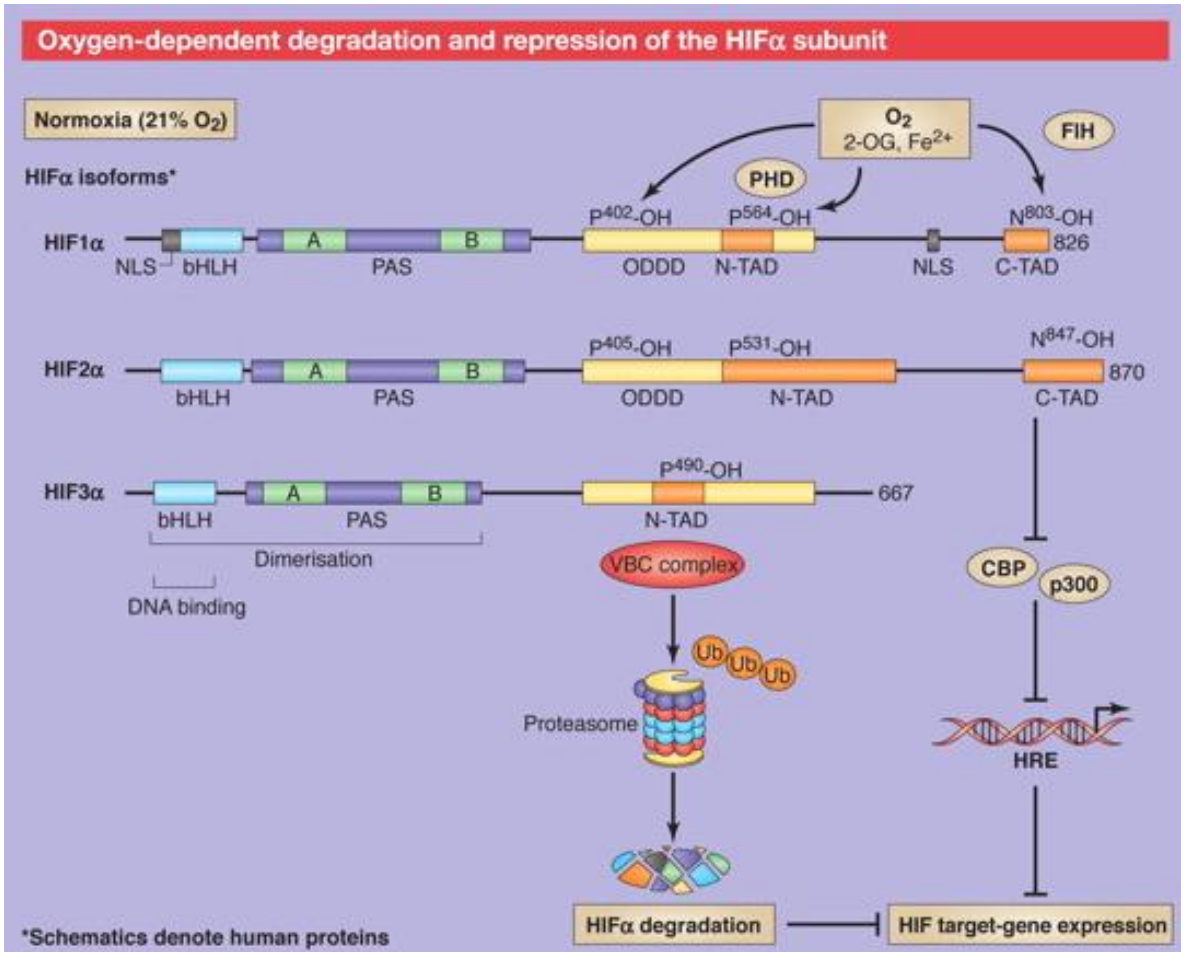
The sustained levels of such recruitment and factors, either tumor- or stromal cell–derived, maintain a protumor-growth environment. Future therapeutic strategies to improve current antiangiogenic therapies need elucidation of the proangiogenic pathways [90].

1.3.5-Molecular mechanisms of angiogenesis

1.3.5.1-The triggers: HIF transcription factors family

The cell response to oxygen is directly linked to pathophysiology of cancer, heart infarction and stroke among ischemia related pathologies. The mechanism which leads to angiogenesis is coordinated by Hypoxia Inducible Factors (HIFs), the key element in oxygen sensing and response. Hypoxia Inducible Factors are transcription factors in all tissues, regulated by the oxygen partial pressure. They are composed of a regulated oxygen-destructible α-subunit and a constitutively expressed oxygen-indestructible β-subunit. Three isoforms of the α-subunit (HIF-1α, HIF-2α, and HIF-3α) and two isoforms of the β-subunit (HIF-1β and HIF-2β) are thought to be involved in the in vivo response to hypoxia. The α-subunits activities are mainly regulated in a post-translational degree and all of them contain at least one nuclear localization signal to reach the nucleus and exert their functions [108].

Structurally, both alpha and beta subunits are parts of the large family of transcription factors called bHLH-PAS (basic helix-loop helix / Per-ARNT-SIM). The HLH domain of the protein acts on the dimerization of two subunits, while the basic part acts in the specific binding to DNA (Figure 19). Alpha subunits harbor an oxygen-dependent degradation domain (ODD) enabling its proteolysis in normoxia. Moreover, this subunit contains also transactivation domains (TAD) located in the N- and C-terminal for HIF-1α and 2α. It is responsible for the transcriptional activity of this factor while HIF-3α contains only the N-TAD domain. HIF-1β and 2β present a single domain in the C-terminal TAD that is not required for transcriptional activity. These TAD domains are also responsible for binding with co-activators such as p300/CBP [109].



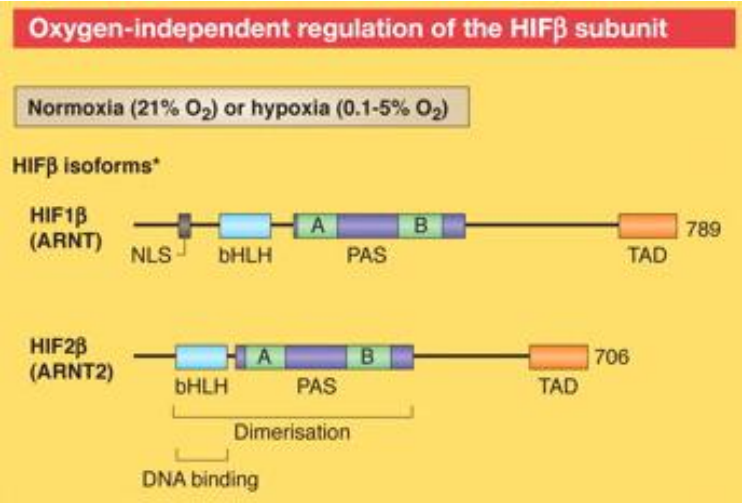


Figure 19: Structure of the three HIF-α and the two HIF-β isoforms [108].
NLS, nuclear localization signal; bHLH, basic helix loop helix domain; PAS, per arnt sim domain subdivided into PAS A and PAS B; ODDD, oxygen dependent degradation domain; TAD, transactivation domain.HIF 1 and HIF 2 have two distinct TAD, in the C (C TAD) and N (N TAD) terminal domains. The PAS and bHLH domains are dedicated to dimerization and recognition of target DNA sequences.

1.3.5.2-HIFs members presentation

1.3.5.2.1- HIF-1

HIF-1 factor is the predominant member and the best characterized one. This factor was discovered in 1992 by Gregg Semenza [110] while working on the erythropoietin (EPO) gene. He showed that, in hypoxic conditions, this protein was able to specifically recognize and bind a sequence located in the 3' noncoding region of the EPO promoter, called HRE for hypoxia response element.

Up to date, more than 200 genes were described as HIF-1α regulated genes [111]. The encoded proteins participate in homeostatic responses to hypoxia by modulation of apoptosis/survival pathways, metabolism and angiogenesis. The latter effect is the best documented because of its implication in solid tumors. Expression of HIF-1α gene is constitutive and controlled mainly by the transcription factor Sp1. Other binding sites for transcription factors like AP-1 and 2, NF-1 and NF-kappaB are present in the HIF-1α promoter sequence. The translation of HIF-1α is constitutive. However, this protein is extremely labile and its half life is less than 5 minutes in normoxia.

The beta subunit HIF-1β, also called ARNT1 (aryl hydrocarbon receptor nuclear translocator), is constitutively expressed in the cell nucleus, under the influence of transcription factor sp1 and is involved in cell detoxification.

The heterodimerization of the two subunits 1α and 1β compose the HIF-1 factor which acts as a transcription factor upon binding to the specific DNA sequence HRE thus allowing the transcription of downstream target genes. Highly regulated, this factor is controlled by either hypoxia dependent or independent pathways.

HIF-1α is ubiquitously expressed in human and mouse tissues and has a general role in multiple physiological responses to hypoxia, as erythropoiesis and glycolysis, which counteract oxygen deficiency and angiogenesis [112].

1.3.5.2.2- HIF-2

Shortly after the cloning of HIF-1α, a closely related protein, HIF-2α [also termed endothelial PAS protein, HIF-like factor (HLF), HIF-related factor (HRF) was identified as a member of the PAS superfamily 2 (MOP2)] and cloned [113]. HIF-2α shares 48% amino acid sequence identity with HIF-1α and accordingly shares a number of structural and biochemical similarities with HIF-1α such as heterodimerization with HIF-1β and binding HREs (Figure 18). In contrast to ubiquitously expressed HIF-1α, though, HIF-2α is predominantly expressed in the lung endothelium and carotid [112, 113]. In tissues where HIF-1 and HIF-2 are present, their transcriptional targets seem to be common and distinct; their roles are not completely redundant. Their respective inactivation does not lead to the same phenotype.

1.3.5.2.3- HIF-3

Discovered later, HIF-3α is also expressed in a variety of tissues. It dimerizes with HIF-1β, and binds to HREs [114] (figure 18). In addition, a splice variant of HIF-3α, inhibitory PAS (IPAS), which is predominantly expressed in the Purkinje cells of the cerebellum and corneal epithelium, was discovered [115]. IPAS possesses no endogenous transactivation activity; it interacts with the amino-terminal region of HIF-1α and prevents binding to DNA, as such it is acting as a dominant-negative regulator of HIF-1 [115].

However, IPAS can also be induced by hypoxia in the heart and lungs, contributing to a negative feedback loop for HIF-1 activity in these tissues [112].

Structurally, HIF-3 differs from the other HIFs by the absence of transactivation domain in the C-terminal part. Comparably to HIF-2, its distribution is tissue-restricted.

The HIF-3 regulation mechanisms are still under investigations. A protective role of HIF-3 has been reported in pulmonary epithelial cells as well as a long term induction of HIF-3 in prolonged hypoxia

Recently, a study demonstrated the presence of a HRE sequence in one of the variant of the HIF-3 gene. Hypoxia was reported to induce HIF-3 in a HIF-1 dependent manner [116].

1.3.5.3-Hypoxia sensing and regulation

The activities of three well-described HIFα isoforms (HIF1α, HIF2α and HIF3α) are regulated by post-translational modifications.

Very unstable in normoxia, the alpha subunit is hydroxylated in presence of oxygen at one of two proline sites (residues 402 and 564) within the ODD domain (oxygen-dependent degradation domain) [117]. These chemical modifications occur in the cytosol and are achieved by three oxygen-dependent prolyl hydroxylases (PHD1, PHD2, and PHD3) that display specific activities. Once hydroxylated, HIF-α is bound by pVHL protein (von Hippel Lindau protein), a tumor suppressor protein which induces ubiquitylation leading to proteosomal degradation. pVHL is a component of E3 ubiquitin ligase complex that includes Elongin-B, Elongin-C, Cul2, RBX1 (Ring-Box 1) and an ubiquitin-conjugating enzyme (E2) [118]. This complex, together with an ubiquitin-activating enzyme (E1), adds ubiquitin motifs to HIF-α. Then, ubiquitinilated HIF is addressed to the proteasome to be degraded.

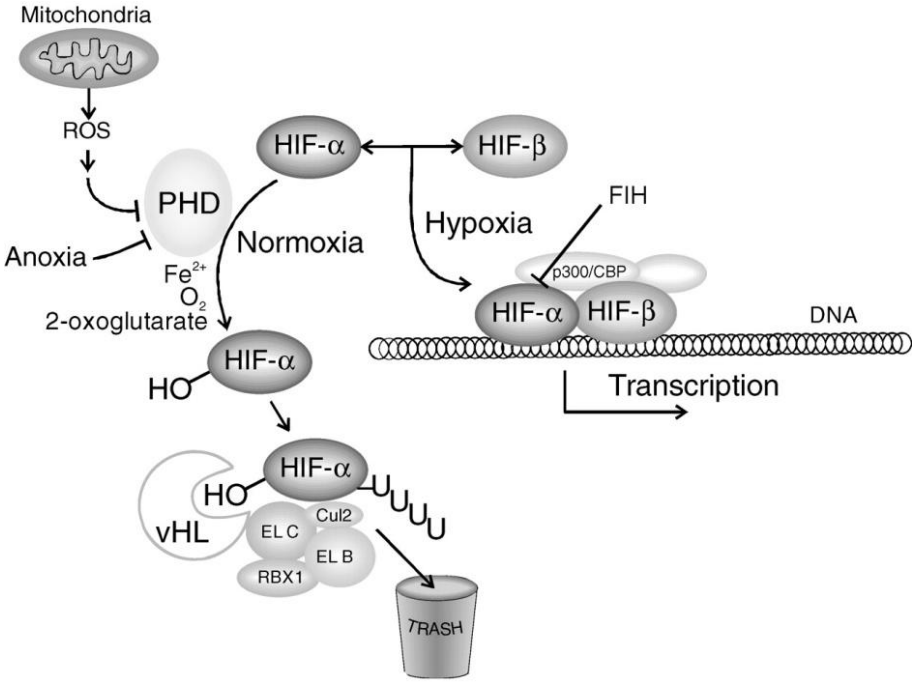


Figure 20: Regulation of HIF in normoxia and hypoxia [119]
Schematic drawing of the regulation of stability and transcriptional activity of HIF-α. In the presence of O_2 and cofactors Fe^{2+} and 2-oxoglutarate, PHDs hydroxylate HIF-α, allowing its recognition by vHL protein which is followed by the ubiquitylation complex. Thus ubiquitylated, HIF-α is degraded in the proteasome mentioned as trash. In the absence of O_2, PHDs are inactivated, non-hydroxylated HIF-α translocates to the nucleus, dimerizes with HIF-β, recruits p300/CBP, and induces the expression of its target genes upon binding to the HRE. (HIF, hypoxia inducible factor; HRE, hypoxia-response element; PHDs, prolyl hydroxylases; U, ubiquin; vHL, von Hippel-Lindau protein).

In hypoxia, the alpha subunit gets stabilized, as it is no longer hydroxylated by PHDs. Indeed, HIF-α is stabilized when oxygen partial pressure reaches 5% down to complete anoxia. PHDs activity decreases when oxygen, one of the substrates of these enzymes, is in too low concentration. Moreover, below 1.5% oxygen, PHDs are reversibly inhibited by reactive oxygen species (ROS) such as H_2O_2. Consequently, non hydroxylated HIF-α no longer binds pVHL protein thus being preserved from proteosomal degradation. Accumulated α subunit is translocated into the nucleus where it binds to the beta subunit forming a heterodimer together with cofactors: CBP (CREB Binding Protein)/p300 and the Pol II (DNA polymerase II) complex. Bound to the HRE sequences (Hypoxia Responsive Element), the complex thus formed allows the transcription of downstream target genes.

HIF-α regulation described above becomes more elaborated by several transcriptional, translational and post-translational modifications that are important in regulation of HIF-1 activity. Among these modifications: hydroxylation (i.e. FIH, factor inhibiting HIF), acetylation, phosphorylation and SUMOylation occur. Interestingly, additional hydroxylation

by the FIH protein (factor inhibiting HIF, asparagyl hydroxylase) at the end of the C-terminus of the HIF1α and HIF2α subunits abrogates HIF activation by inhibiting the binding of co-activators such as p300 and its paralogue CREB-binding protein (CBP).

Not connected to oxygen sensing, the protein kinases A and C stimulate the transcription of Sp1, AP-1 and AP-2 genes increasing the HIF-1α transcription. At the translation regulation level, cell stimulation by growth factors, cytokines and hormones can lead to increased translation through kinase PI3 and its effectors mTOR and p70S6 kinase. Thus, higher translation than degradation enhances HIF-regulated genes expression [108].

Hypoxia induces p53 protein accumulation which directly interacts with HIF-1α and allows the recruitment of the MDM2 ubiquitin ligase. MDM2-mediated ubiquitinylation of HIF-1α leads to its proteasomal degradation [120]. HIF-1α degradation mediated by p53 in hypoxic condition is inhibited by direct interaction with the JAB1 (Jun Activation domain Binding protein-1) and the ODD domain that block the interaction with p53. HIF-1α also associates with the molecular chaperone HSP90 (Heat Shock Protein-90). HSP90 antagonists also inhibited HIF-1α transcriptional activity and dramatically reduced both hypoxia-induced accumulation of VEGF mRNA and hypoxia-dependent angiogenic activity [121].

Indirectly connected to HIF-α, co-activators as p300/CBP, involved in the HIF driven expression can undergo modifications like phosphorylation, affecting *in fine* the HIF activity. The HIF mediated hypoxia sensing was shown recently to be submitted to new degree of regulation by the microRNAs (miRs). Hypoxia dependent, a specific category of miRs was described by Loscalzo, called hypoxamir [122]. Among reported pathways, some miRs are induced by HIF and are targeting directly HIF or proteins involved in its regulation.

1.3.5.4-HRE sequence

Second part of the hypoxia triggering system, the Hypoxia-Responsive Element (HRE) sequence is the minimal cis-regulatory element mediating trans-activation. This DNA sequence is present in regulatory sequences of hypoxia targeted genes. The specific binding of HIFs on HRE mediates the recruitment of the transcriptional machinery leading to downstream mRNAs production and protein expression after translation.

HREs are regulatory elements, comprising the conserved HBS, for HIF-Binding Site, with a core A/GCGTG sequence and highly variable flanking sequences (Figure 21).

Figure 21: HRE sequence pattern [111]
(A) Numbering of the nucleotide positions in HBS (the core sequence in bold); (B). Schematic outline of HBS and HAS in the EPO HRE.

Wenger *et al.* [123] reviewed 70 HIF target genes modulated by the HBS core sequence and microarray experiments indicate more than 200 HIF target genes might exist. Thus, the HBS sequence was described as necessary but not sufficient for efficient gene activation in response to hypoxia highlighting the importance of flanking sequences as regulatory regions.

Epigenetic mechanisms bring another degree of regulation which can occur directly in HBS sequence as well as in flanking regulatory regions. Methylation of CpG dinucleotide contained in the HBS sequence occurs by DNA methyltransferases as in erythropoietin HBS sequence where methylation abolishes the HIF-1 binding [124]. Oxidative DNA damage by ROS represents another epigenetic modification regulating HRE accessibility. ROS induced in response to hypoxia oxidize particular bases within specific DNA sequences of the HIF target gene VEGF. The modified nucleotide in the terminal guanine of the VEGF core HRE (ACGTGGG) leads to an increased binding of HIF-1 and Ref-1 (also known as Ape1) transcription factors, increasing gene expression [125]. Furthermore, in some genes, the HBS cooperates with the HIF-1 ancillary sequence (HAS, Figure 20), a functionally poorly characterized non-conservative regulatory element adding to the regulation degrees [111].
Understanding of the HREs sequences and functions facilitates the design of new hypoxic enhancers making possible constructions of hypoxia-inducible vectors for therapeutic use [126]. Multimers of HREs of various lengths from other genes were used to generate hypoxia-sensing transgenes, most frequently EPO and VEGF. To date, published hypoxia-inducible constructs are based on multimers of naturally occurring (endogenous) HREs. New promoting sequences sensitive to hypoxia were developed, combining known HREs to the (Egr-1)-binding site (EBS) from the Egr-1 gene and to the metal-response element (MRE) from the metallothionein gene [127].

1.3.6-Hypoxia-regulated genes expression

The expression of more than 60 gene products is increased by HIF [128]. One of the best characterized is the gene that encodes vascular endothelial growth factor A (VEGF-A), which induces vascular endothelial tip cells to migrate to hypoxic areas and promotes blood vessel growth: angiogenesis [129]. Other HIF-induced genes are involved in metabolism, vasodilation, erythropoiesis, pH homeostasis, oxygen sensing and autophagy [128]. In addition, the expression of a broad range of gene products can be repressed by HIF [130].

Given that cells and organs need to adapt to changes in oxygen supply, it would not be surprising if a significant number of HIF-1 target genes were regulated in a tissue-specific manner. The list of hypoxia regulated genes is growing: more than 200 HIF-1 downstream genes identified as direct target [111]. Moreover, DNA microarrays showed that more than 2% (till 5%) of all human genes are regulated by HIF-1 in arterial endothelial cells [111, 130].

1.3.6.1-Molecular effectors of angiogenesis

The regulation of gene transcription by hypoxia-inducible factor 1 (HIF-1) represents the best-defined molecular mechanism for maintaining O_2 homeostasis in metazoans. For that, hypoxic cells secrete active molecules able to diffuse to various distances (autocrine, juxtacrine and paracrine).

Among these pro-angiogenic molecules NO (nitric oxide) produced by ECs in hypoxia, or TAMs, plays a central role in angiogenesis. Vasodilatator, NO is able to initiate the ECs proliferation of healthy blood vessels and to enhance the blood flow toward the center of the tumor [131].

Working at a different level, matrix modifying enzymes are produced and secreted like matrix metalloproteases (MMPs) or lysyl oxydases (LOXs). These proteins remodel the extracellular matrix by degradation of collagen, laminin, fibronectin, vitronectin, aggrecan, entactin, tenascin, elastin and proteoglycans. Their action facilitates the cell motility and organization which will help angiogenesis. Moreover, the extracellular matrix degradation can release some growth factors such as VEGFs isoforms. It can also modulate cell surface receptor as well as chemokines receptors [132].

Hypoxic cells are known to secrete growth factors and cytokines. Angiopoietins 1 and 2 (ANGPT1 and ANGPT2), antagonizing one another, they work directly on ECs.

Angiogenesis is enhanced when ANGPT2 binds the Tie-2 receptor promoting the sprouting and ANGPT1 binding promotes the maturation [133].

bFGF (basic fibroblast growth factor) was the first pro-angiogenic growth factor described [5], the PlGF (placental growth factor), the EGF (epithelial growth factor), the IGF (insulin-like growth factor), the chemokines such as the CXCL12/SDF-1 and interleukins like IL-6 or Il-8 are cooperative angiogenic factors.

1.3.6.2-The vascular endothelial growth factors: VEGFs

1.3.6.2.1-Presentation and history

Overproduced by hypoxic cells, the VEGFs (vascular endothelial growth factors) are a family of growth factors, around 45kDa when dimerized, secreted and involved in the growth of vascular endothelium and in physiological as well as pathological angiogenesis.

Historically, H.F. Dvorak with his collaborators reported in 1983 the identification of a factor found in tumor cell culture supernatant, responsible of vascular leakage and called it VPF for vascular permeability factor [134]. In 1989, N. Ferrara identified a mitogenic factor active on endothelial cells and called it VEGF for vascular endothelial growth factor [135, 136]. Further isolated and cloned, the VEGF sequence was elucidated describing 3 isoforms (VEGF-121, -165 and -189). Later on D. T. Connolly's team elucidated the VPF sequence [137] proving VEGF/VPF identity.

VEGF-A is the best characterized member of these homodimeric glycoproteins VEGFs family that includes placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D. Naturally occurring heterodimers of VEGFA and PlGF have been described.

For over a decade, the role of VEGF (currently VEGF-A) in the regulation of angiogenesis was the object of intense investigation for physiological and pathological angiogenesis associated with tumor growth [129].

1.3.6.2.2- VEGF activities

VEGF promotes the growth of vascular endothelial cells (ECs) from arteries, veins and lymphatics, in angiogenesis as well as lymphangiogenesis.

VEGF exerts its effects by binding to its receptors and signaling promotes EC proliferation, filopodia extension, degradation of the extracellular matrix and chemotaxis. Hence, VEGFA signaling induces the motile and invasive behavior that drives tip cells and activates the angiogenic switch [138]. VEGF modulates protein expression, endothelial cells migration and apoptosis.

Above described effects of VEGF are essentially autocrine and juxtacrine and sometimes paracrine. VEGF (overproduced in pathological context) reaches the blood compartment and is carried in the blood flow up to the bone marrow where it induces EPCs mobilization and promotes their differentiation [71, 139, 140].

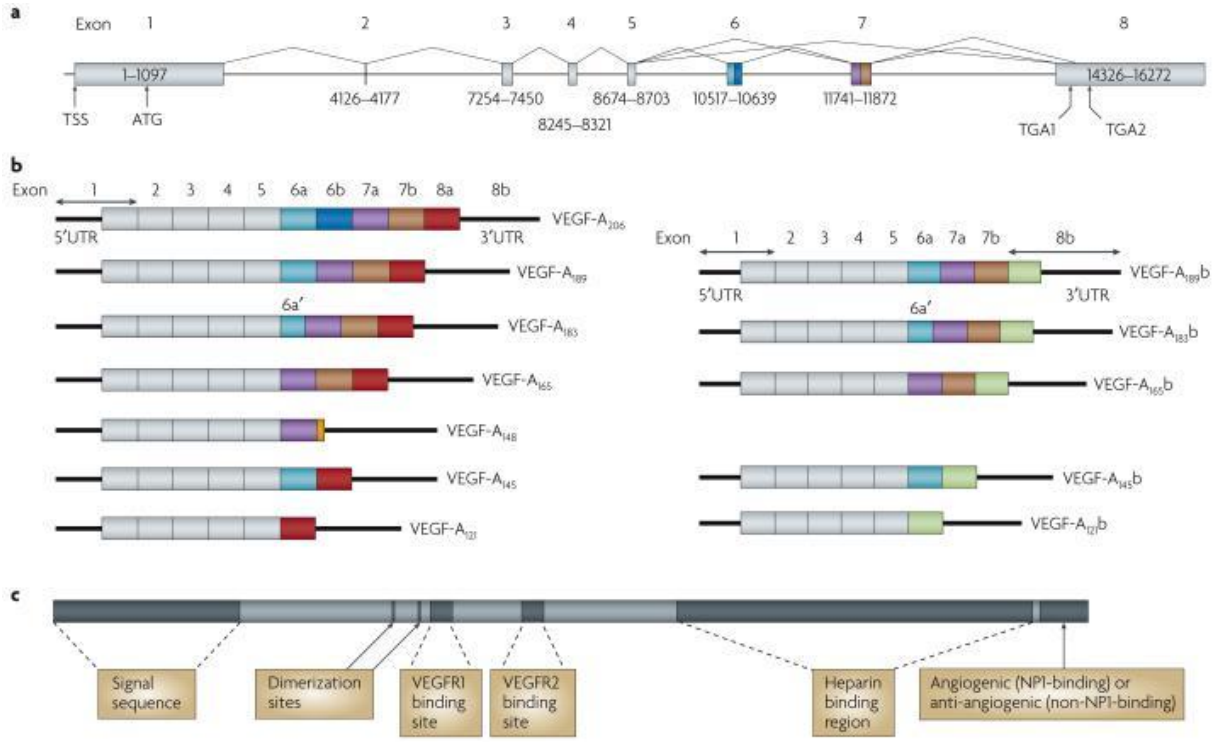
VEGF is also a vascular permeability factor. It results in a vascular leakage [134, 141]. Such permeability-enhancing activity underlies significant effects in inflammation and pathological circumstances. Consistent with vascular permeability, VEGF induces endothelial fenestration [57].

Although ECs are the main VEGF targets, mitogenic effects on non-EC types of cells as cancer cells are shown [142]. This suggests autocrine VEGF signaling in various cancer cells and provides the basis to explain variability of the clinical responses to antiangiogenic therapies.

1.3.6.2.3- VEGF-A isoforms

In general three out of four human genes are spliced to generate two or several proteins. They may display different, even antagonistic properties, localization and degradation potential. Splicing is a highly regulated process controlled by external stimuli, hormones, immune response and stress. Detailed mechanisms of splicing regulation remain to be elucidated for most genes.

VEGF-A exists in multiple isoforms with variable exon content and strikingly contrasting properties and expression patterns. This product diversity from the 8-exon VEGF-A gene on chromosome 6 complicates the understanding of VEGF-A biological effects (Figure 22). In cancer, alterations in isoform expression may be essential for malignancy as well as angiogenesis.



<u>Figure 22:</u> Protein and mRNA products of human VEGF-A [143]
(a). Gene structure of human VEGF-A. VEGF-A spans 16,272 bp of chromosome 6p12 and consists of eight exons. Alternate 5′ and 3′ splice site selection in exons 6, 7 and 8 generate multiple isoforms. (b). Alternative splicing of the VEGF gene gives rise to multiple variants with differing affinities for heparin binding (dependent upon the inclusion or exclusion of exons 6 and 7). Proximal splice-site selection in the terminal exon 8 produces the proangiogenic family, $VEGF_{xxx}$, whereas distal splice-site selection 66 bp downstream gives rise to the antiangiogenic family, $VEGF_{xxx}b$. (c). Protein structure of VEGF-A containing the dimerization sites and binding sites for heparin, VEGF-A receptor 1 (VEGFR1; encoded by exon 3) and VEGFR2 (encoded by exon 4), which are present in all isoforms. The six amino acids at the extreme carboxyl terminus of the protein can be either pro-angiogenic (CDKPRR, encoded by exon 8a) or anti-angiogenic (SLTRKD, encoded by exon 8b).
UTR, untranslated region; TSS, transcriptional start site

The first VEGF-A isoform described, $VEGF\text{-}A_{165}$ [136], has been extensively investigated for its function, signaling, expression and roles in cancer. Other isoforms including $VEGF\text{-}A_{121}$, $VEGF\text{-}A_{145}$, $VEGF\text{-}A_{148}$, $VEGF\text{-}A_{183}$, $VEGF\text{-}A_{189}$ and $VEGF\text{-}A_{206}$ (numbers indicate the number of amino acids in the human polypeptides), are generated by alternative splicing. This produces motives that bind to the highly negatively charged heparin and other glycosaminoglycans molecules with different degrees of affinity. Indeed, $VEGF_{121}$ is an acidic polypeptide that does not bind heparin. $VEGF_{189}$ and $VEGF_{206}$ are highly basic and bind to heparin with high affinity. Whereas $VEGF_{121}$ is a freely diffusible protein, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the extracellular matrix (ECM).

$VEGF_{165}$ has intermediary properties, as it is secreted but a significant fraction remains bound to the cell surface and ECM. This sequestration in the ECM highlights the role of secreted proteases, as LOXs or MMPs, to release the linked factors [129].

In 2002, an additional isoform was identified [144]. Called VEGF-A_{165}b, this isoform was generated by exon 8 distal splice site (DSS). Therefore that VEGF-A mRNA splicing generates two families of proteins, which differ by their six C' terminal amino acids (figure 22). They are termed VEGF-A_{xxx} (pro-angiogenic) and VEGF-A_{xxx}b (anti-angiogenic), xxx denoting the amino acid numbers of the mature protein [143].

VEGF-A_{165} binding, dimerizes the receptor, re-positions the kinase domain inside the dimer and induces tyrosine autophosphorylation. By contrast, VEGF-A_{165}b is predicted not to achieve rotation, thus autophosphorylation is not efficient.

Functional VEGF-A is a dimer. The theoretical formation of heterodimers of paired isoforms (for example, VEGF-A_{165}-VEGF-A_{165}b) or non-paired isoforms (for example, VEGF-A_{121}-VEGF-A_{189}b), increases the complexity of the mechanism. Heterodimerization of VEGF-A and PlGF, or VEGF-C and –D has been documented as well. These should be taken into account for new therapeutic designs in anti-angiogenesis based tumor therapies [143].

1.3.6.2.4- VEGF gene expression regulation

The VEGF transcription is triggered by hypoxia or by oncogenes and growth factors. Indeed, the previously mentioned HIF/HRE couple (part 1.3.5.1) induces the VEGF mRNA expression in low oxygen tension. Moreover, independently of oxygen partial pressure, but acting on the same pathway, some mutations are reported to interfere in this regulation such as in case of VHL-deficient renal carcinoma cell lines [145].

Furthermore, several major growth factors, including the epidermal growth factor, TGF-beta, keratinocyte growth factor, insulin-like growth factor-1, FGF and platelet-derived growth factor, upregulate VEGF mRNA expression, suggesting that paracrine or autocrine release of such factors cooperates with hypoxia in regulating VEGF release in the microenvironment. In addition, inflammatory cytokines such as IL-1beta and IL-6 induce expression of VEGF in several cell types, including synovial fibroblasts, mediating angiogenesis and permeability in inflammatory disorders [129]. Specific transforming events also result in induction of VEGF gene expression such as the oncogenic mutations or amplification of Ras which lead to VEGF upregulation [146].

1.3.7-Response to angiogenic signals: cells receptors

1.3.7.1- A variety of receptors and signals

Tumor and its environment are responsible for the production of signals including pro-angiogenic ones that are received and transmitted by cell surface receptors among which FGFs, EGF, IGFs, VEGFs, PlGF receptors as well as Tie-1 and Tie-2, the receptors of angiopoietin-1 and -2, the interleukin receptors and chemokine receptors.

1.3.7.2- VEGFRs as example

In mammals VEGF-A, -B, -C, -D and PlGF control the vascular development during embryogenesis, the blood vessel and lymphatic vessel functions in the adult, including tumor angiogenesis. They act through three receptor tyrosine kinases and signaling is modulated through neuropilin, VEGF co-receptors. Moreover, Heparan sulfate and integrins are also important modulators of VEGF signaling.

In mammals, three structurally related VEGFRs (VEGF receptor tyrosine kinases) have been identified, namely VEGFR1, VEGFR2 and VEGFR3 (Figure 23).

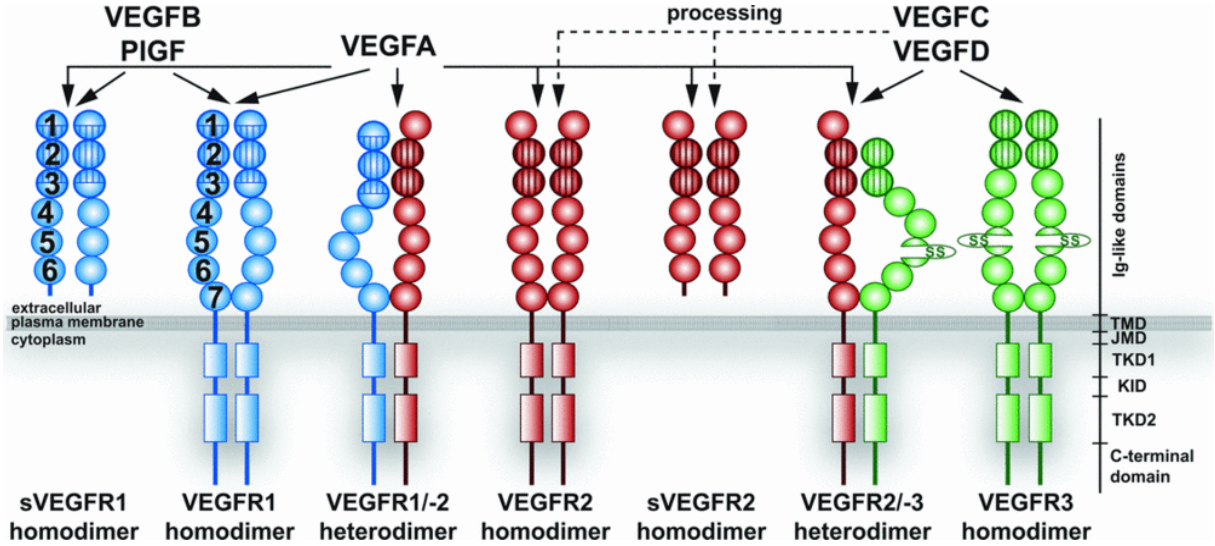


Figure 23: VEGF binding specificities and VEGFR signaling complexes [147]
Schematic outline of the five molecules (VEGFs, VEGFA, VEGFB, VEGFC, VEGFD and PlGF) binding with different affinities to three VEGFRs, initiating VEGFR homo- and hetero-dimer formation. Proteolytic processing of VEGFC and VEGFD allows binding to VEGFR2. VEGFR Ig-like domains involved in VEGF binding are indicated by hatched circles. Soluble VEGFRs (sVEGFR1 and sVEGFR2) lack the seventh Ig-like domain.
JMD, juxtamembrane domain; KID, kinase insert domain; TMD, transmembrane domain; TKD1, ATP-binding domain; TKD2, phosphotransferase domain.

The VEGFRs molecules display similarities: an extracellular ligand-binding domain composed of immunoglobulin-like loops, a transmembrane domain, a juxtamembrane domain, a split tyrosine kinase domain and a C-terminal tail.

Binding of VEGF to its VEGFR can occur *in cis*, e.g. by freely diffusible VEGF or by presentation of VEGF through co-receptors expressed on the same cell as the VEGFR, or *in trans*, e.g. by presentation through co-receptors expressed on adjacent cells [147].

1.3.7.2.1- VEGFR1/Flt1

VEGFR1, or murine Flt1, is a 180–185 kDa glycoprotein that is activated in response to binding of VEGFA, VEGFB and PlGF (Figure 24).

VEGFR1 is expressed in vascular endothelial cells at relatively high levels throughout development and in the adult [148]. In addition, a wide range of non-endothelial cells, such as monocytes and macrophages, human trophoblasts, renal mesangial cells, vascular smooth muscle cells, dendritic cells and different human tumor cell types, express VEGFR1 [147].

VEGFR1 expression is regulated by hypoxia through a hypoxia-inducible enhancer element in the VEGFR1 promoter [149].

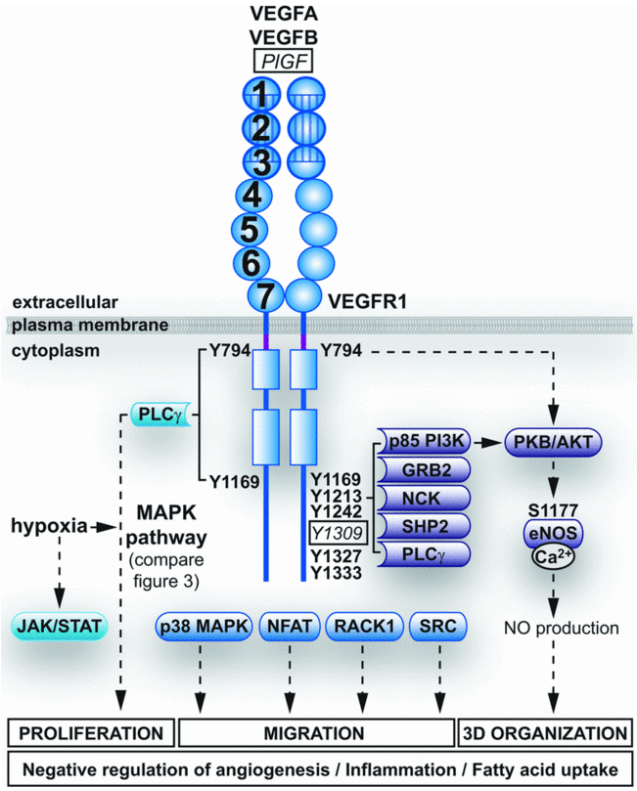


Figure 24: VEGFR1 receptor signal transduction [147]
Schematic outline of activated and dimerized VEGFR1 with downstream signaling pathways. Hatched circles represent ligand-binding domains. A repressor sequence (purple) in the juxtamembrane domain is one possible mechanism for the weak kinase activity of VEGFR1, another being the lack of phosphorylation of tyrosine residues in the kinase activation loop. Certain signaling pathways are not yet reported to connect to a particular phosphotyrosine site in VEGFR1 and detailed information on signaling pathways may be lacking (broken arrows).
3D, three-dimensional; JAK, Janus kinase; NFAT, nuclear factor of activated T-cells; PKB, protein kinase B; RACK1, receptor for activated C-kinase 1; SHP2, SH2-domain-containing protein tyrosine phosphatase 2; STAT, signal transducer and activator of transcription.

Although VEGFR1 binds VEGFA with higher affinity than VEGFR2 does, VEGFR1 tyrosine kinase activity is only weakly induced by its ligands. Several underlying mechanisms have been suggested. First, Gille et al. [150] identified a repressor sequence in the juxtamembrane domain of VEGFR1. Secondly, structural properties of the activation loop of VEGFR1, including the lack of positive regulatory tyrosine residues, contribute to the poor kinase activity [147].

The exact role for VEGFR1 in endothelial cells, apart from serving for VEGF binding, is disputed. Several studies imply that VEGFR1 is dispensable for proliferation or migration of endothelial cells *in vitro*. VEGFR1-neutralizing antibodies demonstrated that VEGFR1 in endothelial cell mediates actin reorganization for cell migration [151]. Endothelial cell differentiation and organization into vascular tubes may involve VEGFR1-dependent activation of PI3K/Akt [152]

Moreover, VEGFR1 cross talks with VEGFR2, through dimerization. VEGFR1–VEGFR2 heterodimers would form as a consequence of VEGFA binding, but not PlGF or VEGFB binding, since these latter ligands bind to VEGFR1 only [147].

In monocytes, VEGFR1-specific ligands VEGFB and PlGF induce signaling pathways regulating monocyte chemotaxis. Vascular smooth muscle cells may respond to PlGF via VEGFR1, in particular under hypoxia, inducing proliferation. VEGFR1 also transduces signals for migration and invasion of several cancer cell lines [147].

1.3.7.2.2- VEGFR2/Flk1

VEGFR2, also known as KDR in the human and Flk1 (fetal liver kinase-1) in the mouse, is a 210–230 kDa glycoprotein that binds VEGFA with a 10-fold lower affinity than VEGFR1. In addition to VEGFA, VEGFR2 binds the proteolytically processed VEGFC and VEGFD (Figure 25).

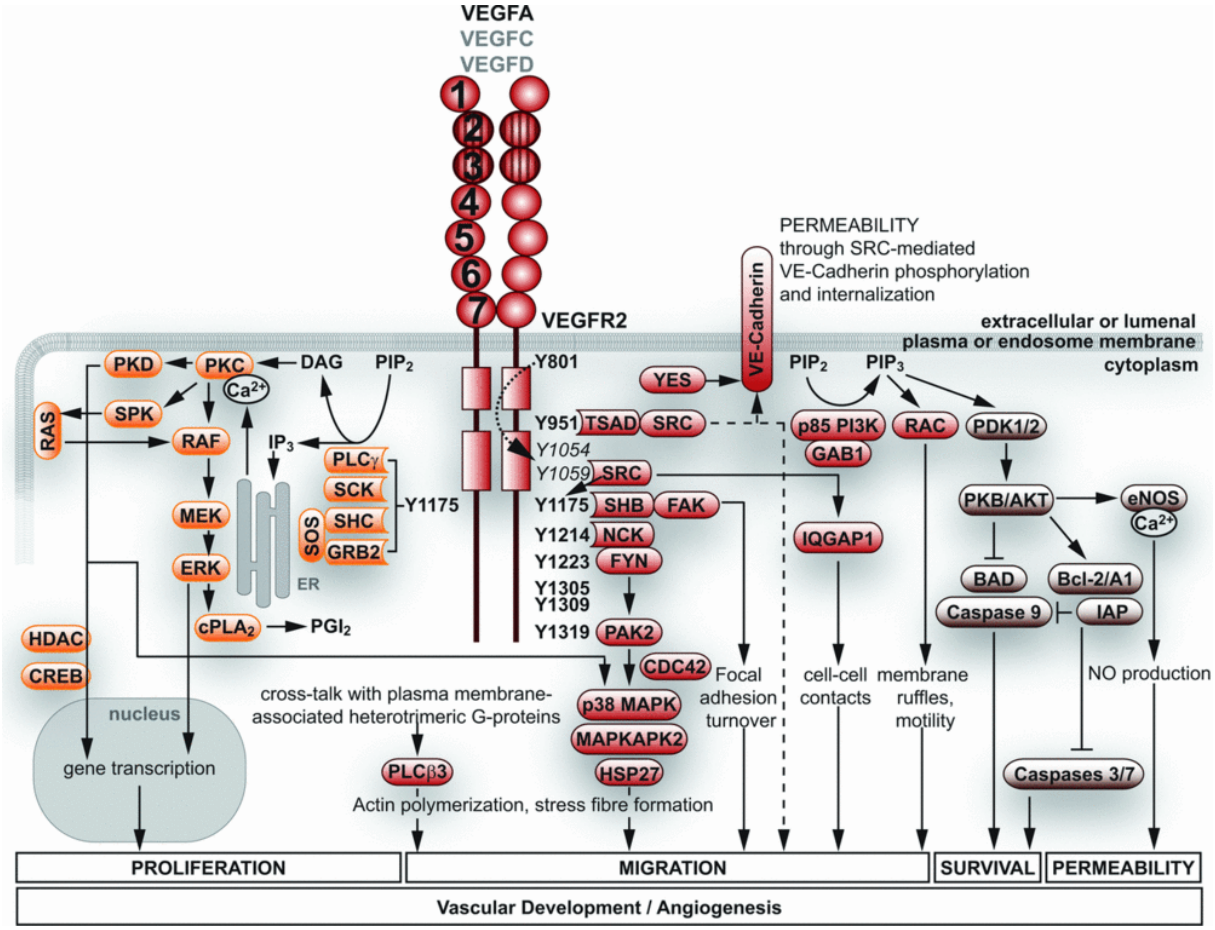


Figure 25: VEGFR2 receptor signal transduction [147]
Schematic outline of activated and dimerized VEGFR2. Intracellular signal transduction results in biological responses: proliferation, migration, survival and permeability (bottom boxes), which are all required for the co-ordinated arrangement of endothelial cells in three dimensions to form and maintain vascular tubes.
CDC42, cell division cycle 42; DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate; MEK, MAPK/ERK kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; SOS, Son of sevenless.

VEGFR2 is expressed most prominently in vascular endothelial cells and their embryonic precursors, with highest expression levels during embryonic vasculogenesis and angiogenesis [153, 154]. VEGFR2 is also found in a range of non-endothelial cells such as pancreatic duct cells, retinal progenitor cells, megakaryocytes and haemopoietic cells.VEGFR2 expression is induced in conjunction with active angiogenesis, such as in the uterus during the reproductive cycle and in pathological processes associated with neovascularization, such as cancer. VEGFR2 expression on tumor cells has been noted for melanoma and haematological malignancies [155].

VEGFR2 transduces the full range of VEGF responses in endothelial cells, i.e. regulating endothelial survival, proliferation, migration and formation of vascular tubes. All

EC responses are directly involved and required for angiogenesis. As far as vessel permeabilisation by VEGF is concerned which is a very important feature in cancer, fenestrae can be induced by VEGFA when it binds to the VEGFR2, leading to the extravasation of proteins or cells. Two major mechanisms have been implicated in vascular permeability: creation of transcellular endothelial pores and transient opening of paracellular endothelial junctions. Furthermore, VEGF-induced permeability also involves eNOS (endothelial nitric oxide synthase)-mediated generation of NO. The leaky vessels are characteristic for pathological angiogenesis directly connected to local excess of VEGF in the vessel environment [147].

1.3.7.2.3- VEGFR3/Flt4

VEGFR3, also called Flt4, is synthesized as a precursor protein of 195 kDa. The precursor is proteolytically cleaved in the fifth Ig-like domain, generating an N-terminal peptide, which remains linked to the precursor protein by disulfide-bond (Figure 26). VEGFR3 is activated by binding of VEGFC and VEGFD. Proteolytic processing of VEGFC and VEGFD further increases affinity for binding to both VEGFR2 and VEGFR3 [147].

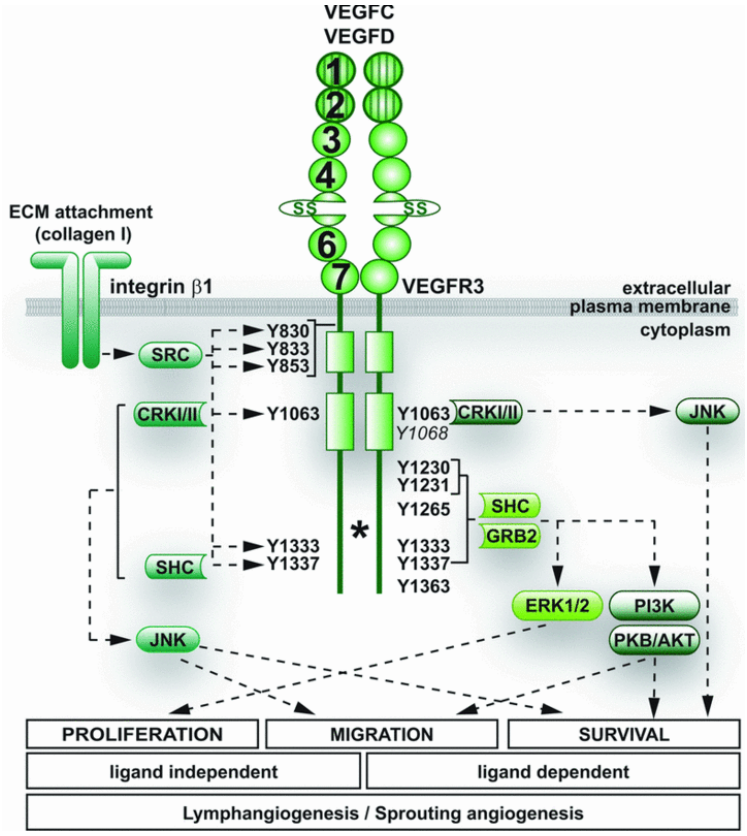


Figure 26: VEGFR3 receptor signal transduction [147]
Activated and dimerized VEGFR3 contributes to proliferation, migration and survival of lymphendothelial cells through ligand-dependent or -independent mechanisms. *ECM, extracellular matrix.*

VEGFR3 is essential present in lymphatic endothelial cells. Its expression is induced in endothelial cells in conjunction with active angiogenesis [156] as in the tumor vasculature or in endothelial tip cells of angiogenic sprouts in the developing retina [157]. It contributes to proliferation, migration and survival of lymphatic endothelial cells through ligand-dependent or ligand-independent mechanisms.

VEGFR3 is also expressed in non-endothelial cells such as osteoblasts, neuronal progenitors and macrophages. Whether VEGFR3 is expressed in tumor cells is disputed.

Independently to ligand binding, integrins can induce VEGF-independent phosphorylation of VEGFR3 mediating its effects on cell survival and migration. Moreover, there are several studies describing heterodimers between VEGFR2 and VEGFR3 [158] (Figure 22). Heterodimers appear to be functionally different from homodimers of each receptor. Indeed, VEGFR3 needs to be associated with VEGFR2 to induce at least certain VEGFC and VEGFD-dependent cellular responses. VEGFR3 homodimers have been

implicated in three-dimensional organization of endothelial cells and lumen formation [159]. VEGFR2–VEGFR3 heterodimers are also active in angiogenesis, as shown by approaches of treatment utilizing receptor-neutralizing antibodies [147].

1.3.7.2.4- sVEGFR1/sFlt1

Alternative splicing of VEGFR1 results in the generation of sVEGFR1 (soluble VEGFR1), also called sFlt1 (soluble Flt1), encompassing the N-terminal six extracellular Ig-loops as presented on the Figure 23 [147].

Overexpression of sFlt1 has been implicated in the etiology of pregnancy-induced hypertension (pre-eclampsia) [160]. Plasma levels of sFlt1 are also elevated in other diseases, such as cancer and ischaemia [161, 162].

Naturally produced or linked to pathology, this soluble form allows the regulation of the VEGFR1 activity, acting as pro or anti-angiogenic according to the context and effects of this interaction on angiogenesis.

Furthermore, sFlt1 has important physiological functions like vascular maturation [163] and the maintenance of corneal avascularity [164].

Because of its high affinity for VEGF-A, the soluble form of the VEGFR1 was used in therapeutic application against cancer. This is based on its ability to sequester overproduced VEGFA from signaling receptors or by formation of non-functional heterodimers with VEGFR2. This resulted in reduction of tumor growth and number of microvessels [165-168].

1.3.7.2.5- sVEGFR2/sFlk1

Following the anti-angiogenic approach using the naturally sFlt1 mentioned above and, based on the same scheme, a soluble form of the VEGFR2 was engineered (Figure 23) [147].

The recombinant sVEGFR-2, encoding for the extracellular domain of the full-length receptor, has shown similar characteristics to sVEGFR-1 such as the ability to bind to VEGF and VEGF/placental growth factor heterodimers as well as mediating antitumor effects [165, 169-172].

Ebos *et al.* reported in 2004 a naturally occurring form of the soluble VEGFR2 in the serum. They found it also in the conditioned media of mouse and human endothelial cells *in vitro*, thus showing its secretion as sVEGFR-1. The mechanism could be alternative splicing or proteolytic cleavage from the cell surface [173].

The soluble VEGFR2 form could be used to regulate pathological angiogenesis linked to overproduced VEGF [174-176].

Interestingly for anti-angiogenic applications, sVEGFR2 (like the natural full length receptor) is able to bind all VEGFs except B type, restricted to VEGFR1. Thus, it makes it a tool of choice to inhibit or regulate angiogenesis as well as lymphangiogenesis. Indeed, Albuquerque *et al.* reported that sVEGFR2 binds VEGFC and prevents binding to VEGFR3, consequently inhibiting lymphatic endothelial cell proliferation [177].

Moreover, sVEGFR2 may also contribute to vessel maturation by regulating mural cell migration and vessel coverage [163].

1.3.7.2.6- sVEGFR3/sFlt4

Comparably, but less documented, a soluble form of the VEGFR3 was designed to act on the lymphangiogenesis process [178].

In the tumor context, the use of a sVEGFR3 to sequester VEGF-C and VEGF-D inhibited both tumor-associated lymphangiogenesis and the development of lymph node metastases [179-181]

Parallel to the designed antagonist of VEGF-C/-D inhibiting VEGFR-3 mediated signaling, a natural soluble VEGFR3 was found in the blood of patients with tumor and used as biomarker to assess therapy efficiency [182-184].

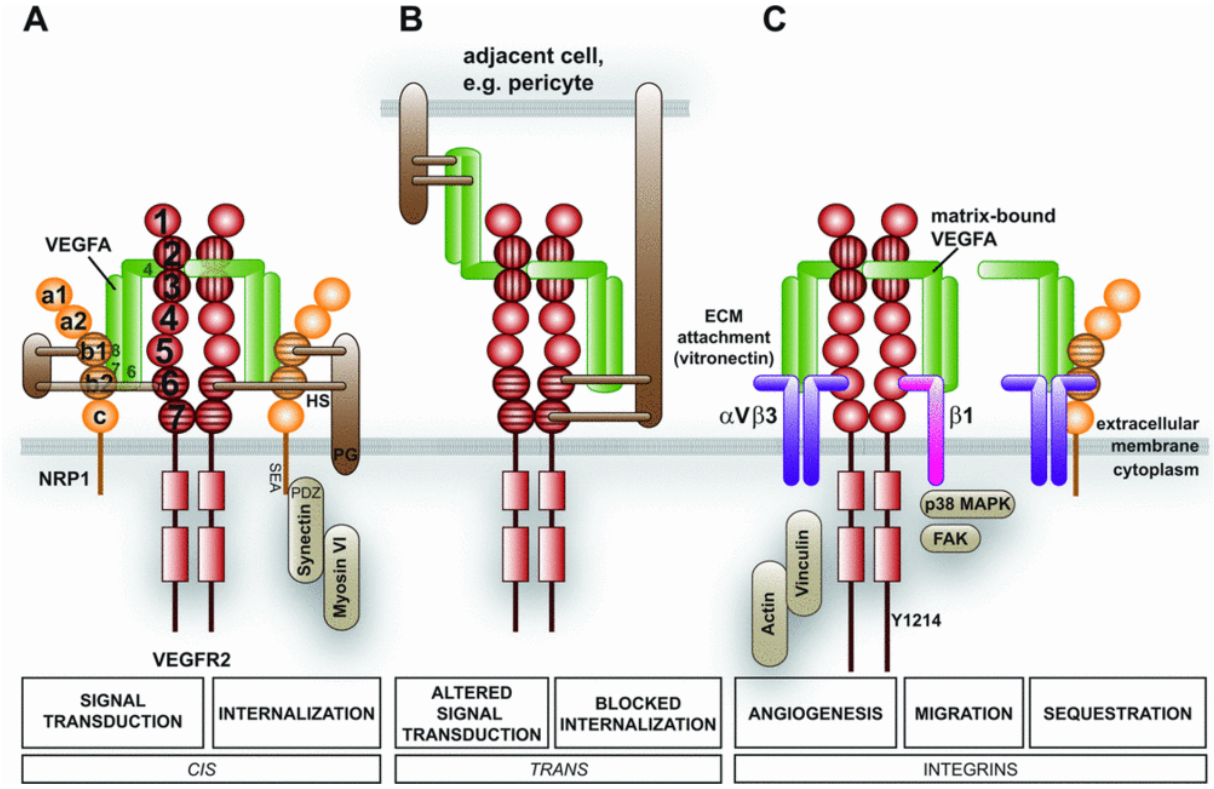
1.3.7.2.7- VEGF co-receptors

VEGF co-receptors are defined as VEGF-binding cell-surface-expressed molecules that are devoid of intrinsic catalytic activity but which modulate the signal transduction output downstream of VEGFRs. However, it cannot be categorically excluded that signals may be transmitted downstream of co-receptors also independently of VEGFRs.

Heparan sulfate (HS) and heparin (H) modulate VEGF by binding the molecule itself but also its receptors and co-receptors (Figure 27A). VEGFA isoforms except VEGFA121 and the VEGFAxxxb forms bind HS/H. VEGFR1 and VEGFR2 have also been shown to directly interact with HS/H [185], as well as the neuropilin receptor-1 (NRP1) but not the -2 (NRP2). Moreover, presentation of VEGFA165 to VEGFR2 *in trans*, by HSPGs (HS proteoglycans) expressed on adjacent cells such as pericytes, further increases the signaling amplitude and duration [186] (Figure 27B), most probably by blocking internalization of the receptor. HS serves as a reservoir for growth factors and controlled release allows formation of growth-factor gradients. Thus tip cells of sprouting blood vessels migrate in response to VEGFA164 gradients, and these gradients are shaped by interactions with HSPG [147, 187].

There are two neuropilin receptors (NRP), NRP1 and NRP2, which were firstly identified as receptors for class 3 semaphorins, a family of soluble molecules with neuronal guidance functions. NRP1 was later shown to bind VEGFA isoforms such as VEGFA165 (figure 27A). Moreover, heparin-mediated VEGFR2–NRP1 complex formation was reported. Distinct interactions are documented with different affinity between various members including VEGFRs, NRPs, HS, and the VEGFs and PlGF ligands [188]. It modulates VEGFR signaling, enhancing its effects on cell migration, survival [189] and permeability [190]. Soluble forms that may serve to sequester VEGF have been identified for both neuropilins [147].

Integrins are also involved in VEGF regulation. As transmembrane heterodimers they mediate cell to matrix adhesion by specific binding to extracellular matrix components, such as collagen, fibronectin, vitronectin and laminin. Induced by the VEGF, a complex between VEGFR2 and the $\alpha V\beta 3$ integrin (Figure 27C) is formed in which one integrin subunit stabilized the complex [191]. VEGFR2–$\alpha V\beta 3$-integrin association is important for VEGFR2 activity through signal transduction by recruitment of the essential trigger molecules [192]. Comparably, $\beta 1$-integrin can be involved in the regulation of VEGFRs. Integrins αVβ3 may also sequester NRP1 to prevent interaction with VEGFR2 [147].



<u>Figure 27:</u> Schematic outline of the interactions of VEGFR2 with its co-receptors HS/H, NRP1 and integrins [147]
(**A**). VEGFA (green) bridges VEGFR2 (red) and NRP1 (yellow). The VEGFA interacts with HS/H (brown) and is involved in binding VEGFR2. HS/H binds VEGFA, VEGFR2 and NRP1. Proteoglycans (brown) can be soluble or plasma membrane anchored. The intracellular signalization of NRP1 mediates internalization of the signaling complex via myosin VI. (**B**). Alternatively, VEGFA can be presented to VEGFR2 *in trans* through HSPGs located on adjacent cells, possibly leading to altered signaling and arrest of the signaling complex in the plasma membrane. (**C**). Integrin αVβ3 (purple) can bind VEGFR2 in a VEGFA-dependent manner and support the attachment of the cell to the extracellular matrix (ECM) through vitronectin. Matrix-bound VEGFA prolongs VEGFR2 activation dependent by β1 integrin (pink) association. Integrin αVβ3 may also sequester NRP1 to prevent interaction with VEGFR2.

1.4-Cancer Therapy

Progress of the scientific knowledge in cancer mechanisms has, along the last 10 years, changed the way to approach the problem of cancer. Newly highlighted mechanisms constitute potential therapeutic targets. Standard validity is continuously questioned thanks to which new cancer therapies are being developed and rise new hopes to defeat this disease.

New therapies aim to overtake previous limitations and pitfalls: to maximize treatment efficacy and to minimize systemic toxicity. These approaches are now focusing on the tumor

microenvironment in addition to the tumor cells themselves. This had led to the development of new methods for therapeutics delivery to the targeted tumor vasculature and providing promising results against the tumor with minimal systemic toxicity.

1.4.1- To consider the tumor like an organ: importance of cell populations and microenvironment

The endothelial cell biology has recently pointed the importance of the interactions between blood vessels and other stromal components that guide vascular remodeling during development, healing and pregnancy. In cancer, the same mechanisms are exploited for tumor stroma setting, the developing vessels and other stroma components respond to signals that participate to tumor development and dissemination.

Already mentioned, angiogenic signals are induced by tumor hypoxic conditions. Critical parameter of the tumor microenvironment, hypoxia controls endothelial/tumor cell interactions and is the key to tumor angiogenesis development. Under hypoxic stress, tumor cells produce factors that promote angiogenesis, vasculogenesis, tumor cell motility, metastasis and cancer stem cell selection. Endothelial cells (ECs) get activated to grow and detach from the neighboring cells by splitting their junctions. This permits EC progression towards pro-angiogenic factors thus distinguishing the leading tip cells from the stalk cells of the new vessel. The forming tubes need to recruit pericytes to get matured and remodeled into a functional network [193]. Neovascularization also relies on the signals that tumor cells provide to distant sites as bone marrow which efficiently contributes to the evolution of the tumor vessels by mobilization of endothelial precursor cells (EPCs). It has been recently shown that this process not only depends on tumor cell signals but also on angiocrine factors from tumor endothelial cells attracting endothelial precursors towards the site of angiogenesis [194, 195].

The molecular cross-talk between tumor cells and host cells has profound implications for the understanding of stromal reactions and for any further anti-tumor approach.

Consequently, tumors are no longer considered as tumor cells only but as a tissue. This comprises a stroma made of a matrix intimately interacting with tumor-associated and cooperating cells as fibroblasts, myeloid inflammatory cells and infiltrating lymphocytes. In addition to the continuously growing tumor cells, these stromal cells are contributing to raise the angiogenic response [196]. Tumor and stromal cells cross-talk enhances tumor growth,

metastasis and alters the response to anticancer therapy [197]. Thus, anti-tumor therapies should take into account this complex structure and be addressed to the whole tumor organ, not restricting their action to the cancer cells.

1.4.2- From anti-angiogenesis to tumor vessel normalization: J.Folkman's hypothesis revisited

As a result of the fundamental observation by J. Folkman in 1971 on angiogenesis as a necessity for tumor survival and development [5, 198], the main antitumor targeted strategies were focused to the efficient destruction of pathologic vasculature. Extensively used, antiangiogenic agents have produced very interesting results. However, because of their efficacy, these treatments showed that total destruction of the vessels leads to the failure of treatment by raising new pitfalls. Vessels become inadequate and tumor cells are located in areas of complete hypoxia and harsh pH conditions. They are submitted to strong pressure to select resistant cancer stem-like cells (CSCs) that display high aggressiveness and invasiveness [199, 200].

Although submitted to the tumor influence, the endothelial cells in tumor vessels are not transformed (except in the angiogenesis formed by vascular mimicry). Non-malignant, they are genetically stable and less likely to evolve into drug resistant phenotypes. Thus new avenues opened for antiangiogenic strategies were based on the features distinguishing pathologic tumor angiogenesis from normal vasculature.

Indeed, deregulation of the vasculature is now a hallmark of cancer progression. It results from a vicious circle in which the production of proangiogenic factors due to hypoxic conditions in the tumor leads to the growth response of the endothelial cells to finally produce abnormal vessels (Figure 28). Those appear pathologic in terms of size, dilatation, and tortuousness of the network as well as hyper permeability. Consequently, tumor oxygen delivery is irregular and inefficient. These parameters, together with heterogeneous blood flow and increase of interstitial fluid pressure inside the tumor, are contributing to cancer progression. The mechanistic pressures impair drug delivery, reduce chemotherapy and radiotherapy efficacy but also immunotherapy benefits. Altogether, they favour the immune tolerance to cancer [201-203].

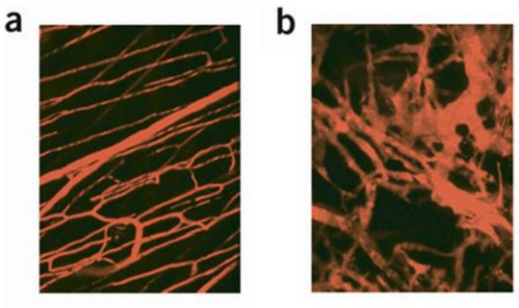


Figure 28: Comparison of normal and abnormal vessels [16].
(**a**) Normal capillary bed (dorsal skin and striated muscle). (**b**) Tumor vasculature (human tumor xenograft). Images were obtained using a two-photon microscope.

Consequently, new developments in anticancer strategies pay deep attention to the balance between tumor pro-angiogenic *vs* anti-angiogenic actions and favor therapeutic normalization rather than destruction of the vasculature. This concept is now taken into account [14, 16] for the design of new therapies to restore functionality from the chaotic and inefficient tumor vessels. Counteracting the hypoxia-induced angiogenesis, it allows the maturation of the vessels in order to reduce cell spreading [19] and gain efficient blood flow with enhanced oxygen supply (Figure 29).

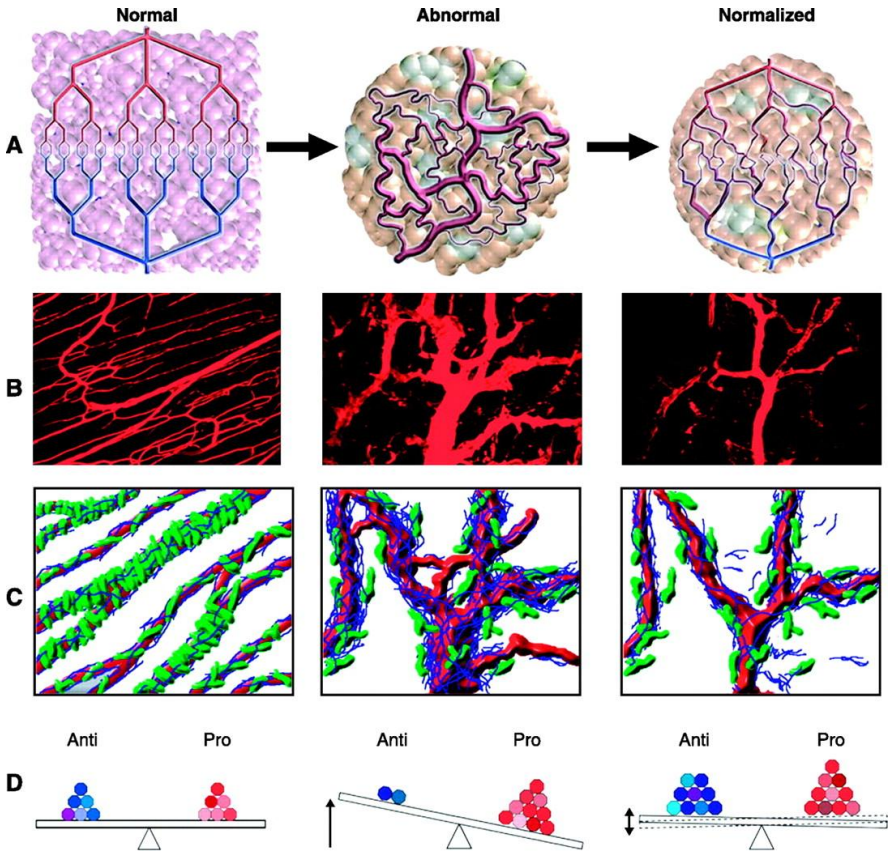
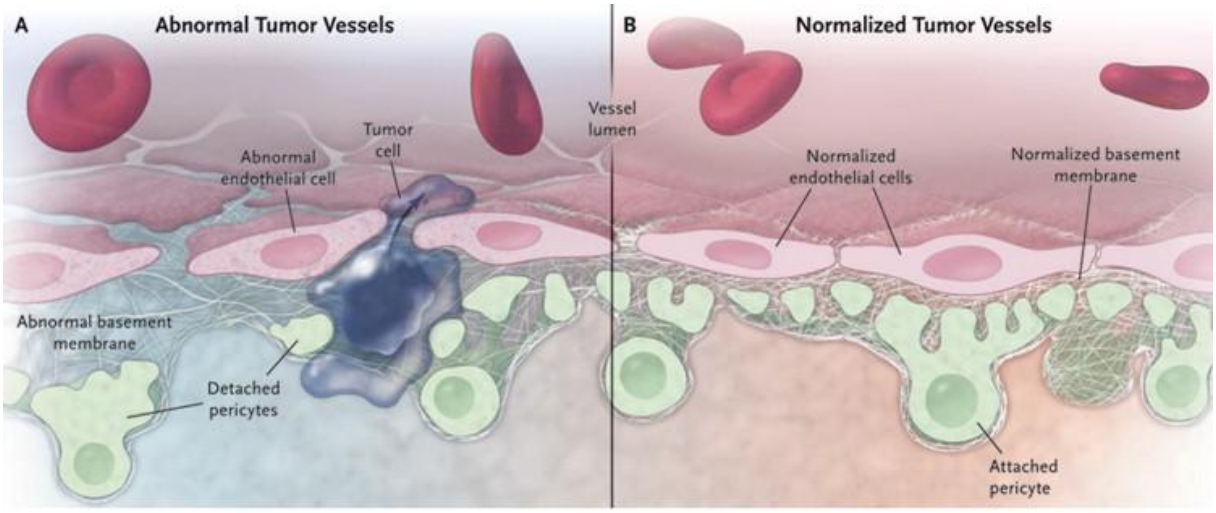


Figure 29: Normalization of tumor vasculature [14]
Proposed effects of tumor vessel normalization in response to antiangiogenic therapy. (A) Tumor vasculature is structurally and functionally abnormal. It is described that antiangiogenic therapies initially improve both the structure and the function of tumor vessels. However, sustained or aggressive antiangiogenic regimens may eventually prune away these vessels, resulting in a vasculature that is both resistant to further treatment and inadequate for the delivery of drugs or oxygen. (B) Dynamics of vascular normalization induced by VEGFR2 blockade. On the left is a two-photon image showing normal blood vessels in skeletal muscle; subsequent images show human colon carcinoma vasculature in mice at day 0, day 3, and day 5 after administration of VEGR2-specific antibody. (C) Diagram depicting the concomitant changes in pericyte (red) and basement membrane (blue) coverage during vascular normalization. (D) These phenotypic changes in the vasculature may reflect changes in the balance of pro- and antiangiogenic factors in the tissue.

The advantages of tumor vessel normalization is first of all mediated by hypoxia compensation which stops the HIFs pathways and consequently all the downstream signaling leading to angiogenesis, cell proliferation or cell migration. This accompanies the changes in the cross talk and signals between endothelial cells and other cells of the tumor stroma. Significant changes in the vessel structure and properties are observed. The recovery from permeability, activity of VE cadherin and CD31 expression [103] as well as recruitment of pericytes/mural cells [204] make the vessels to be functional. The resulting decrease of vascular permeability reduces cancer cell spreading and metastasis formation (Figure 30).



<u>Figure 30:</u> Normalizing Blood Vessels in Tumors: view at cell level [205].
The microvasculature of solid tumors demonstrates a number of structural abnormalities when compared to healthy tissues. (A) Endothelial cell aberrations in shape and loose junctions. In addition, there are fewer pericytes, which are often poorly attached to endothelial cells and lie within a basement membrane that is either abnormally thin or thick. The widened endothelial junctions, coupled with the more tenuous vascular investment by pericytes, promote vascular hyperpermeability and facilitate the intravasation of tumor cells into the circulation, such that they can disseminate to form distant metastases. Tumor microvessels create a hostile microenvironment that fuels tumor growth, metastasis, and resistance to therapies. (B) Preclinical and clinical data support anti-angiogenic therapies mediated "normalization" of tumor vasculature, restoring the structural and functional aberrations of vessels towards a more normal state.

Such observations allowed the definition of therapeutic normalization windows, i.e. time periods during which vessels are transiently normalized [14]. It is then possible to apply chemotherapeutic drugs which display improved efficacy because of better tumor penetration and improved accessibility of the tumor cells [201, 206]. It enhances also the effect of radiotherapy in increasing the tumor blood flow and oxygenation [31].

It is noticeable that changes in hypoxia-mediated signalling result in deep changes in circulating cells recruitment. This has important effects on the immune reaction against the tumor (cells, immuno modulatory cytokines, and chemokines) [202]. Indeed, endothelial cells constitute the interface between circulating blood cells, tumor cells and the extracellular matrix, thereby they control leukocyte recruitment, tumor cell behavior and metastasis formation.

Thus, endothelial cells play a key role in shaping tumor microenvironment and controlling tumor development through angiogenesis [207]. Targeting tumor vessel

endothelial cells should provide survival advantages to patients with advanced cancers [208]. This approach confirms the benefits of considering tumor microenvironment as a therapeutic target.

Consequently, future therapeutic strategies might be addressed to modulation of several pathways. Indeed, it appears that blocking a single pathway may have opposing effects according to the cancer type, considering the variety of target cell types [201].

It should be possible to take advantage of the knowledge about bone marrow-derived endothelial precursor cells, their tissue-specific homing, their active recruitment and repair activity. In addition, the fact that they are "normal" cells entering a pathologic site where they can express various therapeutic genes, opens new perspectives to manipulate the tumor microenvironment. This should help making a step forward normalization of the vasculature to help cancer treatments.

1.4.3-VEGF and tumor therapies

The Vascular Endothelial Growth Factors (VEGFs) produced [134, 135] by the hypoxic growing tumor mass result from the stabilization of the HIF-1α transcription factor. Directly involved in the tumor angiogenesis process and responsible for the vascular permeability, these main angiogenic factors constitute the best targets for antiangiogenic treatments together with the regulation of the VEGF receptor 2 [209].

Because VEGFA is the key factor responsible for the vicious circle that maintains angiogenesis pathologically activated and continuously growing, a large body of work devoted to the production of anti-VEGF antibodies, as Bevacizumab [6] and inhibitors of VEGF Receptor phosphorylation as Sorafenib and Sunitinib, have brought an invaluable breakthrough in angiogenesis-related treatments.

As described above this led to the transient normalization of tumor vessels during the course of the cure [16] but the further "success" of anti-VEGF treatment would lead to inadequate vessels with a destroyed structure. Extreme hypoxia appears then, to be a main characteristic of the microenvironmental state that induces tumor cells to adapt by setting a rescue process and selects the most resistant cells to such harsh conditions in terms of lack of oxygen and low pH values [50].

Moreover, secreted by cancer cells VEGF acts as an immunosuppressive cytokine. The major role played by VEGF in the immune response resides in the efficient chemo-attraction

of inflammatory cells [210], macrophages, neutrophils, dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs). The latter secrete immunosuppressive mediators and induce T-cells dysfunction [211] by which tumor cells directly down-regulate the antitumor immune response [212]. As such, tumor angiogenesis causes non proper recruitment of immune cells, helping tumor progression.

Normalization should reverse this dysfunction. Indeed, effects of tumor vessel normalization and hypoxia regulation by lowering VEGF production should stop the recruitment of tumor favoring cells and suppressors that help tumor progression. Indeed, Tregs and myeloid derived suppressor cells invasion is considerably reduced [203]. Such data pointing to new therapeutic applications of vessel normalization are mechanistically illustrated by the chemokines and receptors balance.

Finally, recent data show the expression of VEGFR2 by some cancer cells as reported in cases of glioma [213]. In such case, an anti-VEGF therapy will be addressed to both endothelial cells as well as cancer cell, in reducing their proliferation.

Modulation of the VEGF signaling pathway deeply affects the tumor blood vessels. It confirms VEGF as an interesting target for cancer therapy to normalize the tumor vasculature. However, these observations highlight the need of regulation mechanisms controlling the therapeutic gene enough for the vessels to reach a normalized state and avoid overtaking it.

1.4.5- Overview of therapeutic approaches

Currently, three main approaches of cancer treatment are: surgery, radiotherapy and chemotherapy. Nevertheless numerous new methods are in development such as the techniques that use ultrasounds or very recently, cold plasma as examples of biophysics-derived techniques. However, the most dynamically developing is for sure chemotherapy and other systemically applied treatments.

Chemotherapy acts preferentially on dividing cells as cancer cells are. It is used alone or in combination with other modalities. Tumor heterogeneity in cell populations, as well as in microenvironmental conditions (hypoxia) renders this approach difficult. Noticeably, resting populations resistant to drugs as the stem like cancer cells are a result of tumor heterogeneity and make the treatments fail.

New therapies include anti-metastatic, differentiation and anti-angiogenic agents. They are not restricted to dividing cells. They include molecules which interfere with cell

signalization pathways or surface receptors, protein ligands, soluble receptors, traps, peptides binding or receptor blockers, antibodies, nucleic acids (DNA, RNA, interfering RNA - siRNA and miRNA), viruses such as oncolytic viruses or modified cells.

1.4.6- Cancer gene therapy and Trojan Horse approaches

Gene therapy was born 50 years ago thanks to Dr W. Szybalski and Dr E. Szybalska experiments [214] reporting for the first time a gene transfer. This approach has widespread applications in treatment of diseases among which cancer takes a prominent place. The main limitation remains a major challenge for gene therapy: the proper targeted delivery of therapeutic genes to tissues.

Biotechnologies allow emerging new strategies that improve and succeed to change the means of cancer treatments. Among elaborated approaches of gene therapies, the image of the “Trojan horse” is largely used to describe the combination of targeting unit and a specific drug/gene delivery system. Inspired from the Greek Mythology, the Trojan Horse was the source of inspiration for the presented approaches and revisited for numerous applications. The Trojan horse is an engineered targeting tool which can be a designed molecule, a liposome, an exosome, a specialized cell or a modified virus in order to specifically reach the tumor site. Ulysse’s army can be replaced by various therapeutic genes or interfering RNAs using all available described data in matter of therapeutic genes.

Nucleic acids which are anionic and hydrophilic macromolecules carry the instructions that encode all biological molecules, allowing transmission and expression of genetic information. In gene therapy, proteins encoded by these nucleic acids are modulated either by exogenous DNA or mRNA delivery giving rise to specific protein expression or by small RNAs (siRNAs, microRNAs) inhibiting or modulating endogenous protein translation. The therapeutic molecule being often unstable in the blood stream, the Trojan Horse assures a protection during the trafficking to the target site where they can exert their therapeutic effects.

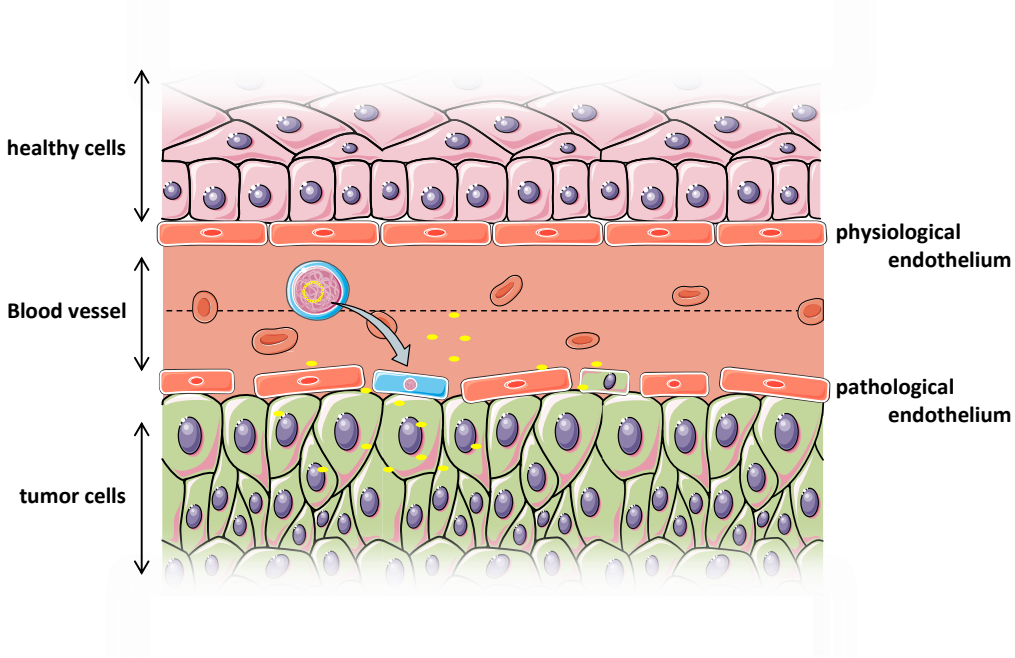
Based on the Trojan Horse strategy, various elaborated approaches were designed including the use of cationic polymers, carbon nanotubes, liposomes, exosomes, cells or viruses.

Although very interesting, all these approaches are not involved in this work and consequently, will be not discussed in the manuscript.

1.4.7- Cell-mediated gene therapy

Many cells are known to be able to target specifically well-defined parts of the organism. Endothelial precursor cells (EPCs) are among these specific cells and reach selectively neoangiogenesis areas as well as vascular remodeling regions. In the 1990s Asahara and colleagues reported the existence of CD34-expressing cells, isolated from the blood of adult mice, which could differentiate *in vitro* into endothelial cells [22]. EPCs mobilization depends of signals such as vascular endothelial growth factor (VEGF) [71] and EPCs contribute to postnatal physiological and pathological neovascularization as well [23]. They are thus a perfectly adapted tool for tumor targeting [24].

Few years later, the Trojan horse was making sense one more time, revisiting the Asahara's pioneer works with EPCs thanks to the possibility, to "arm" cells with therapeutic genes prior systemic injection [25-27] (Figure 31).



<u>Figure 31:</u> Cell-mediated gene delivery approach revisiting the Trojan Horse strategy for cancer gene therapy.
In the upper part, a blood vessel in a physiological context, harboring a continuous and well organized endothelium. The lower part refers to a blood vessel in cancer context, showing disorganized endothelium, with tumor cells in the vessel wall, achieving vascular mimicry. Leaky and chaotic tumor vessels give poor access of therapy to cancer cells. In this aim, it is possible to use the homing property of specific cells like EPCs or MSCs able to target specifically neoangiogenic sites. Following systemic application, transfected cells are recruited to the tumor environment where they will express the therapeutic gene acting on tumor (Figure produced using Servier Medical Art).

Alternatively to EPCs, mesenchymal stem cells (MSCs) represent another category of stem cells [215]. The "homing" of MSCs toward tumors has led to extensive research for their use to achieve cancer-specific gene delivery [216, 217]. This approach was extended to neural stem cells [218], macrophages [219] or neutrophils homing as well [104] and to cells able to reach specifically a targeted site [27, 220].

Various methods and vectors can be used to engineer cells and express therapeutic transgenes. Not excluding the use of non-viral methods, the viral constructs are often preferred. Adenoviruses, retroviruses and adeno-associated viruses have been reported [221-225] and more recently, baculoviral vectors [218]. The choice is governed by advantages *vs* disadvantages as integration into the genome of the recipient cell, ability to transduce the carrier cells, immunogenic potential and level of transgene expression as well as duration of expression.

Once the cell vehicle is identified, the gene to transfere (cDNA) or the small RNA (i.e. siRNA or miRNA) should fulfill the requested conditions. Therapeutic genes have been reported including the prodrug-activating enzymes (cytosine deaminase, carboxylesterase, thymidine kinase) [218, 226-229], interleukins (IL-2, IL-4, IL-12, IL-23) [230-232], interferon-β [233, 234], apoptosis-promoting genes such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [235, 236], soluble Flk1 receptor as VEGF-trap [237], natrium-iodide symporter (NIS) for ^{131}Iodide-based therapy [238, 239] and chemokines as fractalkine (CX3CL1) [240].

In order to improve the cell-mediated approach, innovative targeted anticancer gene therapies were designed. Zhao *et al.* described the use of neural stem cells to target glioma, previously "armed" by baculoviral vector to introduce the herpes simplex virus TK suicide gene. Then, the TK gene product, combined with the prodrug ganciclovir (GCV), produces a potent toxin which affects replication and inhibits tumor growth [218, 226]. Advantages of baculoviral vectors reside first in their non-integrating and transient transgene expression in human cells, on both dividing and non-dividing cells including human embryonic stem cells (hESCs) and MSCs [226, 241]. On the other hand, they are presented as a safe class of gene delivery vectors because of their inability to replicate or cause toxicity in mammalian cells [242, 243].

To improve the regulation specificity, Conrad *et al.* and Niess *et al* reported engineered MSCs successfully expressing the therapeutic gene (TK-ganciclovir couple) under the selective control of Tie2 promoter/enhancer [227, 244]. Actively recruited to growing

tumor vasculature, the construction drives the therapeutic gene expression only in the context of angiogenesis. This restricted expression resulting in a tumor-specific toxic environment confers another degree of control making the approach safer.

For validation, some authors use close or intratumor injections of engineered cells. The most promising strategy for clinical application remains the systemic injection of cells using their targeting potential toward the tumor site [229, 233, 245, 246].

Based on the acquired knowledge on the mechanism of tumor angiogenesis formation and its regulation, we shall describe:

- in a first chapter, the design of a vector encoding the soluble form of the VEGFR2 combined with a hypoxia-regulated promoter. The purpose of such construction is to allow a conditional expression which will be validated in this part;

- in a second chapter, the establishment of a model of endothelial precursor cells and its validation *in vitro* and *in vivo* as a tool for tumor targeting. Its sensitivity for the chosen tumor model, the melanoma, will be evaluated in this chapter;

- in a third and last chapter of results, the data obtained by the combination of the vector with the targeting cells mentioned above. Thus expressed by the cell carried-vector, the soluble VEGFR2 production and its regulation will be evaluated.

Results will be then discussed in view of the wide perspectives that the strategy opens.

2-Vector design

2.1-Introduction

Because vascular endothelial growth factors (VEGFs) are over-expressed in hypoxic tumors, they are major actors directing pathologic neo-vascularisation and regulate stromal reaction. Novel strategies that target and inhibit VEGF bring promise to modern anti-cancer therapies. They aim to control rather than destroy tumor angiogenesis. Consequently, the challenge is to trap selectively VEGFs overproduced upon hypoxia in the tumor microenvironment.

Involved in the Trojan Horse approach as described in part 1.4.6, a therapeutic vector was designed to be *in fine* loaded into tumor targeting cells. However, this vector was required to fulfill with various specifications making it specific and safer for the patient.

Here we report the design and construction of such vector expressing the soluble form of VEGF receptor-2 (sVEGFR2) driven by a hypoxia responsive element (HRE)-regulated promoter. Indeed, among VEGFRs, VEGFR-2 is able to bind almost all VEGFs (VEGF-A, C, D, and E, except B). As a consequence, its soluble form constitutes a potent VEGF-trap to neutralize the pathologically overproduced VEGFs [8, 28, 29]. Thanks to the well known HIF/HRE couple used to design a hypoxia-selective promoter, this parameter was the trigger allowing the transgene expression [247, 248].

Moreover, to allow *in vivo* imaging by near-infrared visualization, mCherry and IFP1.4 coding sequences were built into the vector to allow the permanent cell visualization [33]. Despite considerable limitations of its optical properties, IFP1.4 has been chosen for its *in vivo* imaging properties [33-35].

This first part of results presents the vector which was designed to satisfy with the above expectations and its validation as a regulatory tool of the overexpressed VEGF.

2.2-Materials and methods

2.2.1-Generation of pIFP1.4-HREmsVEGFR2 and pHREmsVEGFR2 vector

DNA manipulations were done by standard procedures using restriction enzymes, T4 DNA ligase, and buffers according to manufacturer's instructions (New England BioLabs and Promega). The vectors pBLAST45-msFlk1(s7), pAAV-MCS (Stratagene), pdAAV-HRE-minCMV-HO1-SV40-pA [249], IFP1.4_pcDNA3.1H.ape (kindly provided by R. Y. Tsien (University of California, San Diego, USA) [33]), were used to generate the pIFP1.4-HREmsVEGFR2 vector. To develop the construct, several additional vectors were created (see later Figure 32A). From pBLAST45-msFlk1(s7) vector (Invivogen) the msFlk1/msVEGFR2 coding sequence was cut by EcoRI restriction enzyme. This fragment was subcloned into the EcoRI opened pAAV-MCS vector (Stratagene). The resulting construct pAAV-MCS-msVEGFR2 (step1) was further used to generate msVEGFR2 sequence flanked by ClaI restriction site upstream and XhoI restriction site downstream. Then the XhoI-ClaI fragment from the intermediary vector (step1, Figure 32A), was introduced into the pdAAV-HRE-minCMV-HO1-SV40-pA vector. ClaI and SalI double digestion allows to replace the HO-1 coding sequence by the XhoI-ClaI msVEGFR2 fragment leading to the intermediary vector step 2, named (XhoI and SalI generate compatible cohesive ends). As pdAAV-HRE-minCMV-msVEGFR2-SV40pA vector step 2 was shown to be leaky, minCMV promoter was shortened by digestion with SacI restriction enzyme leading to 104 nucleotides excision from the 3' side. The purified vector was ligated back to a vector designated as pdAAV-HRE-minCMV-msVEGFR2-SV40pA (short promoter) described in Figure 34 and used for all cloning steps. SspI-SspI fragment from the pdAAV-HRE-minCMV-msVEGFR2-SV40pA vector was cloned into the IFP1.4_pcDNA3.1H.ape vector kindly provided by Pr R. Y. Tsien (University of California, San Diego, USA) and double digested by SspI and BstZ17I to get the pIFP1.4-HREmsVEGFR2 vector. To obtain the pHREmsVEGFR2 vector, the pIFP1.4-HREmsVEGFR2 vector was digested by PmeI and re-ligated leading to the excision of IFP1.4-IRES-mCherry region (Figure 32B).

2.2.2-Cell culture and oxygen regulation

MLuMEC,FVB [250] a model of mature endothelial cells were cultured in OptiMEM with Glutamax-I (Gibco Invitrogen) supplemented with 2 % (vol:vol) fetal bovine serum – FBS (BioWest, Nuaillé, France), 40 µg/ml gentamycin and 0.05 µg/ml fungizone. Human microvascular endothelial cells HMEC-1 were cultured in MCDB 131 medium (Gibco Invitrogen), 10 % (vol:vol) FBS, L-glutamine 2 mM, epidermal growth factor 10 ng/ml, hydrocortisone 1 µg/mL, 50 U/ml penicillin, 50 µg/ml streptomycin, and fungizone 0.05 µg/ml. B16F10 melanoma cells and HEK293 cells (human embryonic kidney 293E cells, ATCC, CRL-10852) were cultured in DMEM high glucose medium (Gibco Invitrogen), supplemented with 2 % (vol:vol) FBS, penicillin 100 U/mL, streptomycin 100 µg/mL, fungizone 0.05 µg/ml. Lack of mycoplasma infection was confirmed by "MycoAlert Detection kit" (Lonza). Cells were routinely cultured at 37 °C in a humidified incubator in a 95 % air/5 % CO_2 atmosphere and passaged by detaching cells with 0.25 % trypsin-0.05 % EDTA (w/v) solution (Gibco Invitrogen). Hypoxia was obtained by introducing a stabilized gas mixture (Witt Gas mixer, Witt France) 94 % N_2/5 % CO_2/1 % O_2 (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).

2.2.3-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', 10000 g) to perform ELISA measurements.

2.2.4-Vectors validation by cell lines transfection

All produced vectors were sequenced by the GeneScript Company (Piscataway, USA) and controlled by transient cell transfection in cell lines: human embryonic kidney 293E cells (HEK293E, ATCC CRL-10852) and B16F10 murine melanoma cells. Transfections were performed using the jetPEI^{TM} (Polyplus Transfection, France) as DNA complexing agent according to manufacturer's instructions. Moreover msVEGFR2 expression was obtained by stable transfection of the HEK293cells and B16F10 melanoma cells with the pIFP1.4-HREmsVEGFR2 vector and selection by hygromycin. Control lines were established with the same vector IFP1.4_pcDNA3.1H.ape coding for fluorescent proteins only.

2.2.5-Quantification of msVEGFR2 protein production

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

2.2.6-Selection of stable cell lines containing the vectors.

Stable pHREmsVEGFR2, pIFP1.4-HREmsVEGFR2, and IFP1.4_pcDNA3.1H.ape transfected HEK293 and B16F10 cells, were selected in hygromycin (Invitrogen). Hygromycin concentration was specifically adapted to each cell line and adjusted according to control cells. 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 % O_2) using ELISA method (R&D DY1558B). pIFP1.4-HREmsVEGFR2 cells and IFP1.4_pcDNA3.1H.ape, hygromycin resistant cells were further cloned by cell sorting on the basis of the IFP1.4 fluorescence (ex: 633 nm, em: BP 712/21 nm). Highly fluorescent clones were selected and secondly screened for their msVEGFR2 production.

2.2.7-ELISA-based binding assay

The assay was adapted from Craig, T.J.*et al* [251] (Figure 37). Mouse VEGF164 (50 ng in PBS) (493MV/CF, R&D systems, USA) and human VEGF165 (293VE/CF, R&D systems, USA) were coated on 96-well plate. After washing by 0.05 % Tween20 solution in PBS, well saturation was performed by PBS-BSA 1 % (w/vol). 48h-conditioned msVEGFR2 cell supernatants were incubated during 2h and the detection of VEGF-bound msVEGFR2 was performed using the mouse sFlk1 ELISA duoset R&D kit (DY1558B, R&D systems, USA).

2.2.8-Surface Plasmon Resonance (SPR) assays

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

Mouse soluble VEGFR2 concentrated from HEK-IFPmsVEGFR2 cell supernatant by centrifugation with a 50KDa cut-off membrane (Millipore), was diluted in running buffer (PBSc-P20 0.005 %) and flowed over the chip surface at a flow rate of 30 µl/min.

Mouse soluble VEGFR2 solution in running buffer (PBSc-P20 0.005 %) was flowed over the chip surface at 30 µl/min. After each injection and dissociation phase, the surface was regenerated by injection of 90 µl 2M $MgCl_2$ (flow rate 30 µl/min) chaotropic agent. The response data were analyzed with the BIAevaluation program version 3.2. A Langmuir binding model with a stochiometry of 1:1 was used to analyze the association rate constant kon, the dissociation rate constant koff (M–1s–1 and s–1, respectively), and the dissociation constant, KD=koff/kon.

2.2.9-Cell proliferation assay

MLuMEC, and B16F10 cells were seeded in 96-well culture plates at a density of 2.10^3 cells per well in complete OptiMEM medium (GIBCO). Once adhered (6 hours), cells were starved in empty OptiMEM for 12 hours. Then conditioned supernatants were added for 48 hours. Media were removed and a cell proliferation based on the BrdU incorporation was measured according to the manufacturer's instructions (Cell Proliferation ELISA, BrdU, Roche). Absorbance was measured in each well using a spectrophotometric plate reader at wavelength of 450 nm. BrdU incorporation was calculated by the following equation: BrdU incorporation = [(absorbance of treated well) - (absorbance of non-BrdU labeled well)]/[(absorbance of control well) - (absorbance of non-BrdU labeled well)].

2.2.10-*In vitro* angiogenesis assay

Angiogenesis was performed on 96-well plates coated with 50 µL of Matrigel™ (BD Biosciences, San José, CA) diluted at ½ in OptiMEM. After polymerization at 37 °C, $1{,}5.10^4$ cells per well were seeded in the presence of msVEGFR2 (70 ng/mL) conditioned medium or control medium and the plate was introduced into the incubation chamber of the video Zeiss Axio Observer Z1 fluorescence inverted microscope (Zeiss, Le Pecq, France) equipped with an ORCA-R2 high-resolution CCD camera linked to a computer driven-acquisition software Axiovision (Zeiss) to control time-lapse acquisitions (30 min.) over 24h. Tube-like and network structures were analyzed by Wimasis Images Analysis.

2.2.11-*In vivo* angiogenesis assay

Female C57Bl/6Tyr$^{C2\text{-}j}$Orl mice 6-8 weeks old (CDTA-CNRS Orleans, France) were anesthetized by isoflurane 2.5 %/air 97,5 % (Aerrane, Baxter S.A.S, Maurepas, France). 250 µL of Matrigel^{TM} supplemented with 500 ng/mL of bFGF (R&D Systems) was mixed with saline (1:1 v/v) containing 10^5 cells HEK-IFP-msVEGFR2 or HEK-IFP. The mixture was introduced subcutaneously in the abdominal region using a 21-gauge needle. To allow cell rearrangement in the surrounding tissue and neovascularization, Matrigel plug remained in the animals for 10 days before sacrifice, surgery and imaging. 100 µL of a FTC-dextran (MW 2000000, FD2000S, Sigma) solution at 10 mg/mL in saline was intravenously injected in the

tail vein for angiogenesis visualization. For *in vivo* fluorescence macroscopy, a Nikon AZ100 Multizoom was used, equipped with an EMCCD Evolve 512 photometric camera and driven by the NIS Element BR software. Acquisitions were done on reversed skin of sacrificed mice. For fluorescence imaging, the epifluorescence illumination system used an Intensilight HGFIE HG, precentered fiber illuminator (130 W mercury). The fluorescence channels are set with filter (Semrock, Rochester, New York, USA) combinations for FITC: λex 482/35 nm, beam splitter 506 nm, λem 536/40 nm; for TRITC channel: λex 543/22 nm, beam splitter 562 nm, λem LP561 nm.

2.2.12-Flow cytometry

After 48h incubation either in normoxia or hypoxia, B16F10 cells were washed twice with complete phosphate-buffered saline (PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$; cPBS) containing 0.5 % bovine serum albumin and 0.1 % NaN_3 (cPBS/BSA). Cells were detached by collagenase (Invitrogen). After two washings with cPBS/BSA solution, cells were incubated for 1h at 4 ^{o}C with directly labelled antibody or corresponding isotype: rat anti-mFlt1-PE IgG2B (R&D FAB4711P), rat anti-Flk1-PE $IgG2a_{kapa}$ (eBioscience 12582182). The goat anti-Flt4 IgG polyclonal (R&D AF743) was revealed with the secondary antibody rabbit anti-goat-PE (Santa Cruz BioTechnologies sc-3755) incubated 30 min at 4 °C after washing. After incubations, cells were washed and their fluorescence was detected using a FACS-LSR flow cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences). Results are expressed either by the relative fluorescence intensity ΔIF (the difference between the relative fluorescence intensity of antibody labeled cells and corresponding isotype).

2.2.13-*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.

2.2.14-Serum collection

Serum samples from B16F10 melanoma bearing mice were obtained after anesthesia with isoflurane, followed by heart puncture to collect 0.5 to 1 mL of blood for coagulation, centrifugation at 10000g for 2 mins to get the serum. mVEGF and msVEGFR2 concentration in serum were estimated by ELISA following the manufacturer's protocol (mouse sVEGFR2 Duoset kit DY1558B, and mouse VEGF Duoset kit DY493, R&D Systems, USA).

2.2.15-Fluorescence microscopy

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

2.2.16-Spectrofluorimetry

IFP1.4 and mCherry spectra were measured from cells lysates using either HEK293 empty or transfected cells (pHREmsVEGFR2-IFP1.4 vector) adapted from Chiu *et al.* [252].

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

2.2.17-*In vivo* fluorescence imaging

An IVIS Kinetic (Caliper, USA), acquiring fluorescent images, was used to visualize *in vivo* subcutaneous tumor. C57BL/6JTyr$^{c2\text{-}j}$Orl mice were injected with 10^5 pHREmsVEGFR2-IFP1.4 positive B16F10 cells, subcutaneously in the hind leg in 100 µL saline. 10 days after injection fluorescence images were obtained, after fur removal. Mouse

was anesthetized (Aerrane®, Maurepas, France) with 2% isoflurane in air/O_2. Acquisition for mCherry protein imaging used using an excitation filter BP= 570/35 nm and an emission BP= 620/20 nm. IFP1.4 imaging was performed with excitation filter BP=675/35 nm and emission filter BP=720/20 nm. Acquisition settings (binning and duration) were adapted to the tumor size and number of living cells. Pictures were obtained with binning 8 for 2 s exposure time. Autofluorescence reduction and signal enhancement were achieved by spectral filter scanning (emission from 620 nm to 680 nm) and spectral unmixing algorithms included in the device analysis tools.

2.2.18-Statistical analysis

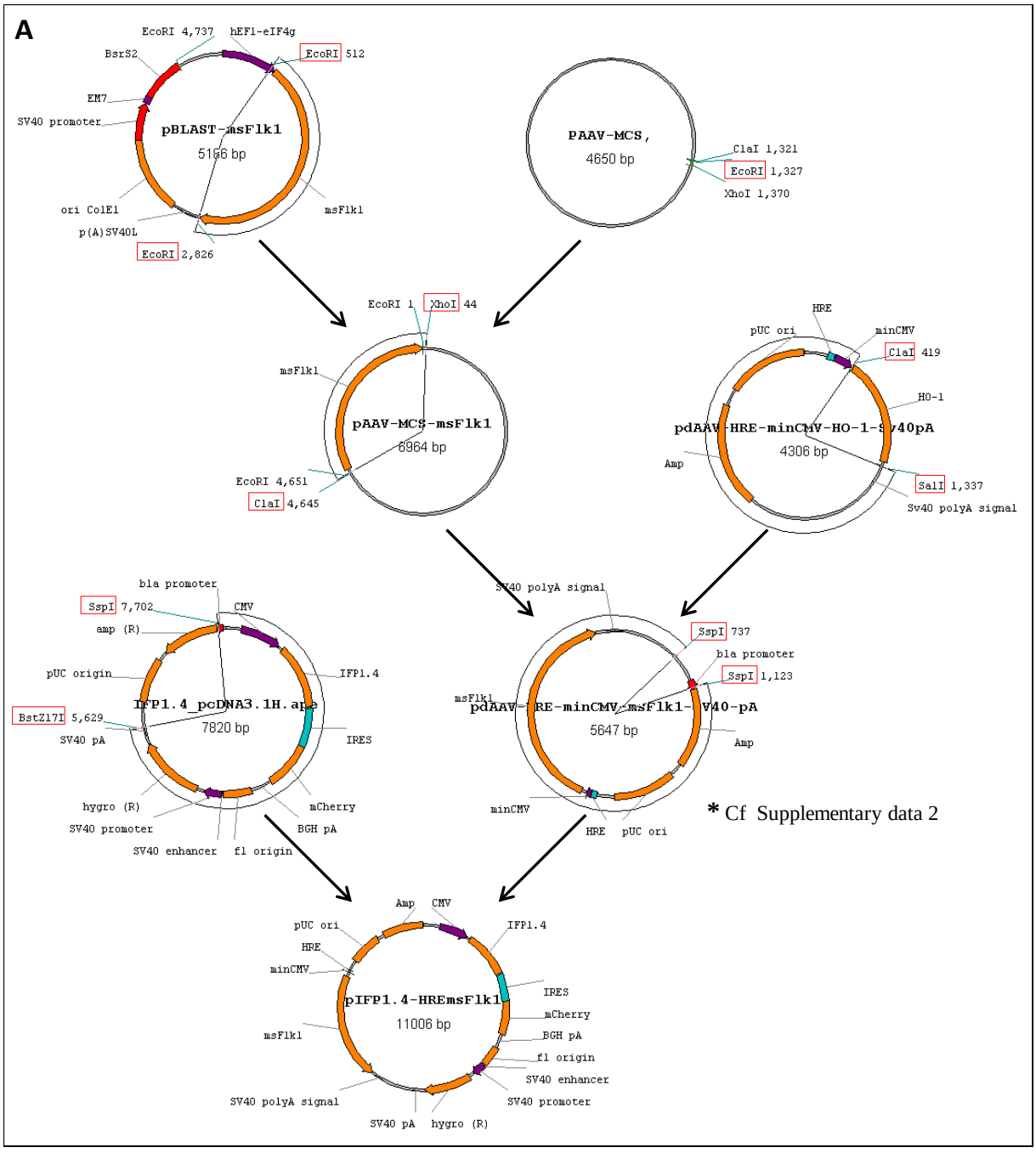
All values were expressed as means ± SD. Statistical analysis was conducted by Student *t* test. Group differences resulting in $p < 0.05$ by student *t* test were considered statistically significant.

2.3-Results and discussion

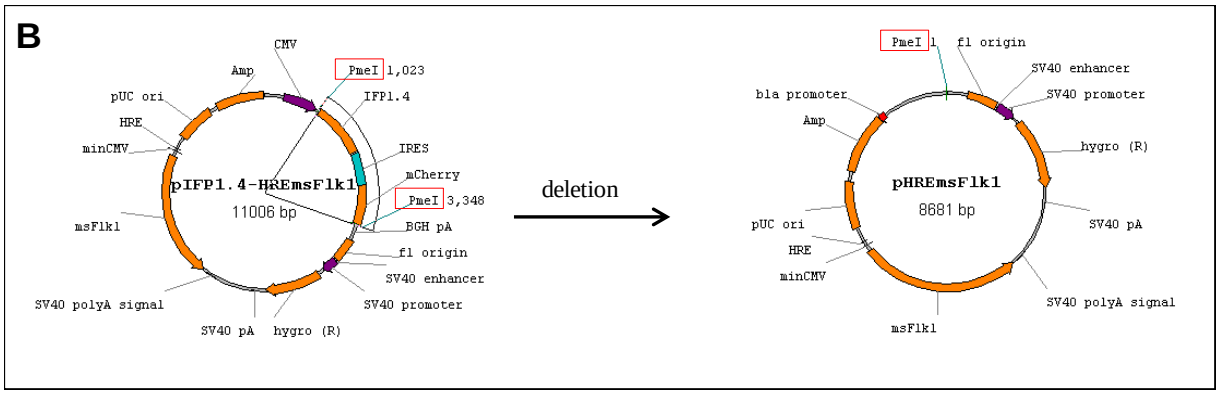
2.3.1-pIFP-HRE-msVEGFR2 vector construction

The structure of the plasmid is reported in Figure 32A and describes the HRE-minCMV-msVEGFR2 expression cassette and the hygromycin phosphotransferase encoding cassette for eukaryotic selection and stable cell line establishment. Moreover, the IFP1.4 cassette for *in vivo* imaging introduction is shown. IFP1.4 was the first near infrared fluorescent protein described [33] which allowed tracking of transfected cell into animals.

The internal ribosome entry site (IRES) has been used to coexpress the mCherry protein together with the IFP1.4 protein from a single bicistronic transcription unit. IRES-driven mCherry expression confirmed IFP1.4 expression and allowed cell imaging with non-infrared optical bench.



* Cf Supplementary data 2



<u>Figure 32:</u> Construction maps for the pIFP1.4-HREmsVEGFR2 and pHREmsVEGFR2 vector

(A) Schematic drawing of the pIFP1.4-HREmsVEGFR2 construction process. (B) Schematic drawing of the DNA excision leading to pHREmsVEGFR2 from the pIFP1.4-HREmsVEGFR2. The msVEGFR2 is denoted msFlk1 on the vector maps.

Based on the same template, a second vector was designed from the pIFP-HRE-msVEGFR2 after deletion of the IFP1.4 expression cassette leading to pHRE-msVEGFR2 as presented in Figure 32B and schematically presented on Figure 33A. Proper cloning was confirmed by restricting endonuclease digestion and sequencing.

2.3.2-Validation and optimization of hypoxia-driven msVEGFR2 expression

The first expression cassette was controlled by ELISA measurement of the msVEGFR2 secretion in the medium of transfected cells under hypoxia (< 1 % oxygen) compared to normoxia (18.75 % oxygen). Transient transfection was performed on HEK293 cells and supernatant was conditioned for 48h. Figure 33B shows that the msVEGFR2 production was indeed induced upon hypoxia, confirming efficient hypoxia-regulation of msVEGFR2 expression. Nevertheless, a leakage was observed since non negligible amount of msVEGFR2 (13 ng/mL/10^5 cells) was produced by transfected cells in normoxia (Figure 33B). Therefore, the shorter minCMV promoter was obtained by restriction of the endonuclease digestion leading to 3' promoter DNA excision (Figure 34 and Figure 33A). Compared to the non-reduced promoter, the leakiness was decreased in the shorter form by 60 % in terms of msVEGFR2 production in normoxia (5 ng/mL/10^5cells) (Figure 33B). Consequently, the short minCMV promoter was used to design the two final vectors pIFP-HRE-msVEGFR2 and pHRE-msVEGFR2. The final construct pIFP-HREmsVEGFR2 was

transfected into human HEK293 as well as murine B16F10 melanoma cells to assess its efficacy under hypoxia regulation in human and murine species. As previously demonstrated [253, 254], experiments conducted in our laboratory showed that the CMV promoter was stronger in human compared to murine cells (Figure 33C). Taking into account the differences in transfection efficacy, this reduced activity of the CMV promoter in murine cells highlighted differences in the levels of msVEGFR2 expression observed in hypoxia *vs* normoxia (Figure 33C).

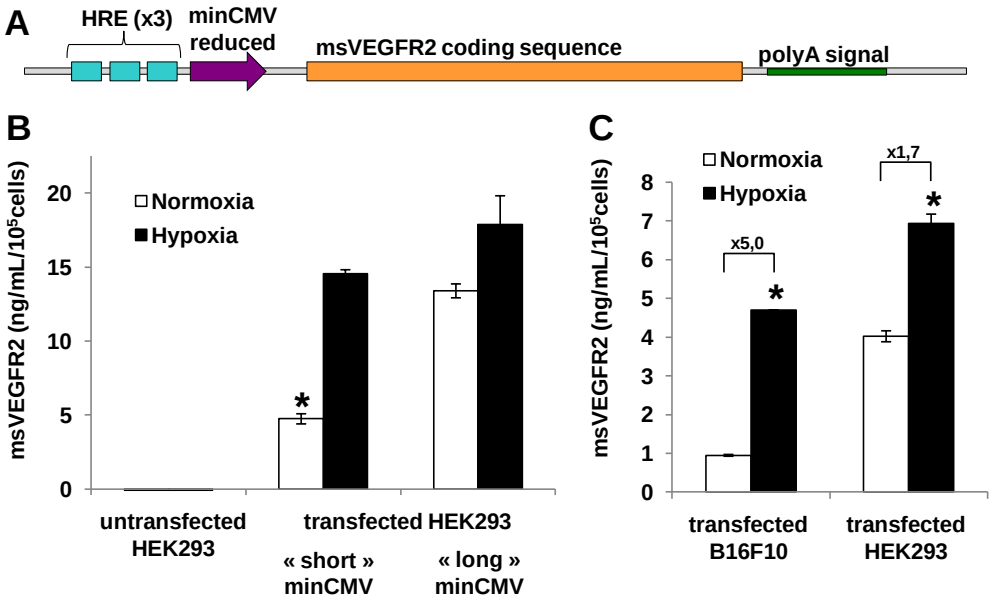


Figure 33: Demonstration of the hypoxia-dependent regulation of msVEGFR2 expression from the constructed vector

(A) Schematic description of the hypoxia driven msVEGFR2 expression cassette. (B) 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. (C) 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.

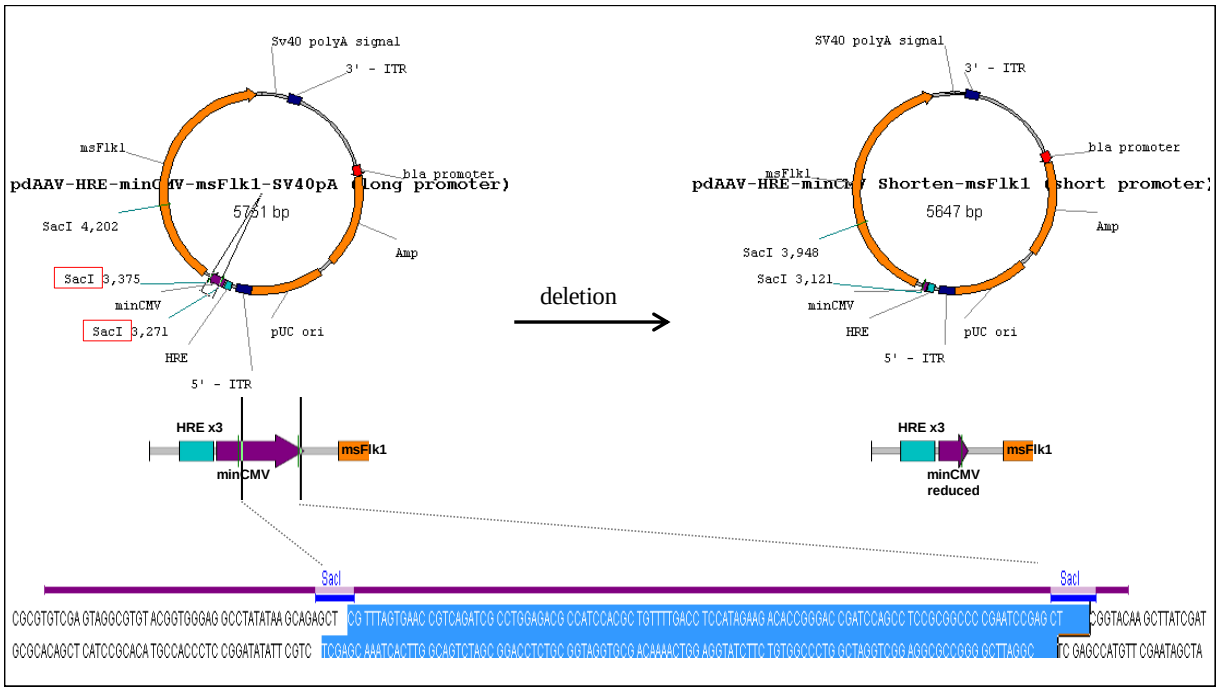


Figure 34: Shortening of the minCMV promoter

Schematic drawing describing the approach used to shorten the minCMV promoter and the relative vectors maps. The msVEGFR2 is denoted msFlk1 on the vector maps.

2.3.3-Engineered cell lines for msVEGFR2 production

Considering the applicability of this attempted alternative approach, non-viral transfection strategy was chosen to establish cell lines. HEK293 cells were stably transfected by the vector pIFP1.4-HREmsVEGFR2 and cloned. The clones were selected on the basis of their ability to produce msVEGFR2 in hypoxia with low leakage in normoxia and designated HEK-IFP-msVEGFR2 cells (Figure 35A, D, E). These cells produced up to 70 ng/mL/10^5 cells msVEGRF2 in hypoxia, leakage being reduced to 0.2 ng/mL/10^5 cells. As a control, the same transfection and selection process was performed with the "empty" vector pIFP1.4 [33], coding only for the IFP1.4 protein, leading to so called HEK-IFP cells (Figure 35D, B, C).

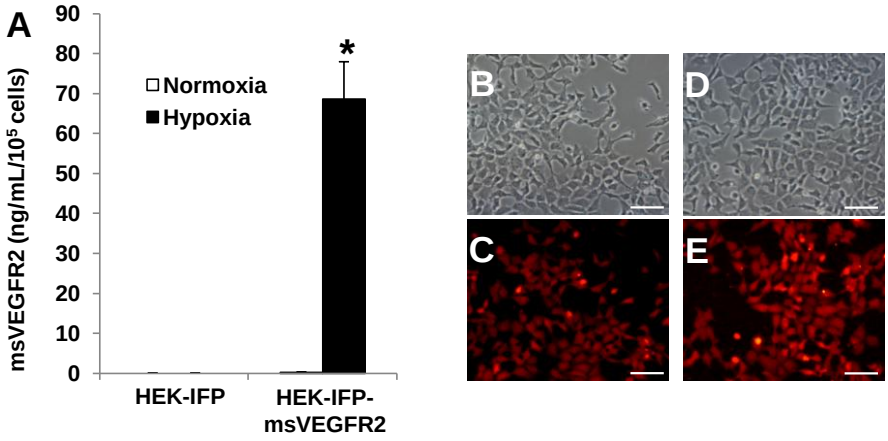


Figure 35: Validation of sVEGFR2 production by HEK293 cell lines transfected with pIFP1.4 or pIFP1.4-HREmsVEGFR2

(A) 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. (B, C, D, E) HEK-IFP cells (B, C) and HEK-IFPmsVEGFR2 cells (D, E) observed under fluorescence microscopy, in visible light (B, D) or red fluorescence (λex 530 nm, λem 608/75 nm) for mCherry observation (C, E). Scale bars represent 50 µm.

2.3.4-mVEGFR2 expression and production switch-on depends on pO_2 level

The oxygen threshold value for msVEGFR2 expression was studied in a semi-quantitative assay, according to the oxygen partial pressure set at 21 %, 5 %, 3 % and 1 %. msVEGFR2 was detected by ELISA, in supernatants from HEK-IFP-msVEGFR2 cells after 48h in hypoxia. As shown in Figure 36, the hypoxia regulated promoter was switched-on when oxygen tension went down to 3 %. This threshold, assures a restricted expression in tissues where oxygen tension is lower than 3 %, limiting the expression to deeply hypoxic microenvironments as in solid tumor.

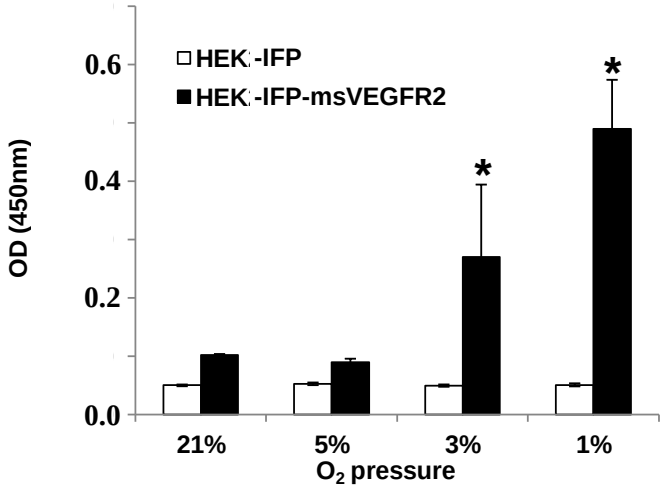


Figure 36: Demonstration of the hypoxia-dependent regulation of mSVEGFR2 expression by the constructed vector

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. Optical densities were measured in a plate reader at 450 nm. Values are mean ± SD (n = 3). *p < 0.05. One representative experiment out of N=3.

2.3.5-Soluble mVEGFR2 binds to its ligand

The soluble VEGF receptor-2 (msVEGFR2) coded by the pHRE-msVEGFR2 vector was expected to recognize and bind VEGF. Its activity was assessed in a binding assay on murine and human VEGF-A using hypoxia conditioned supernatant from HEK-IFP-msVEGFR2 cells compared to HEK-IFP control cell line. ELISA quantification revealed a msVEGFR2 concentration of 35 ng/mL. Binding was studied by semi-quantitative ELISA (Figure 37A, B) and quantified by surface plasmon resonance demonstrating that msVEGFR2 was able to bind both murine and human VEGF-A (Figure 38A, B). Clear interspecies cross recognition was illustrated by affinity constant values: KD = 4,5 nM for msVEGFR2 to mVEGF (Figure 38A) and KD =7,8 nM to hVEGF (Figure 38B).

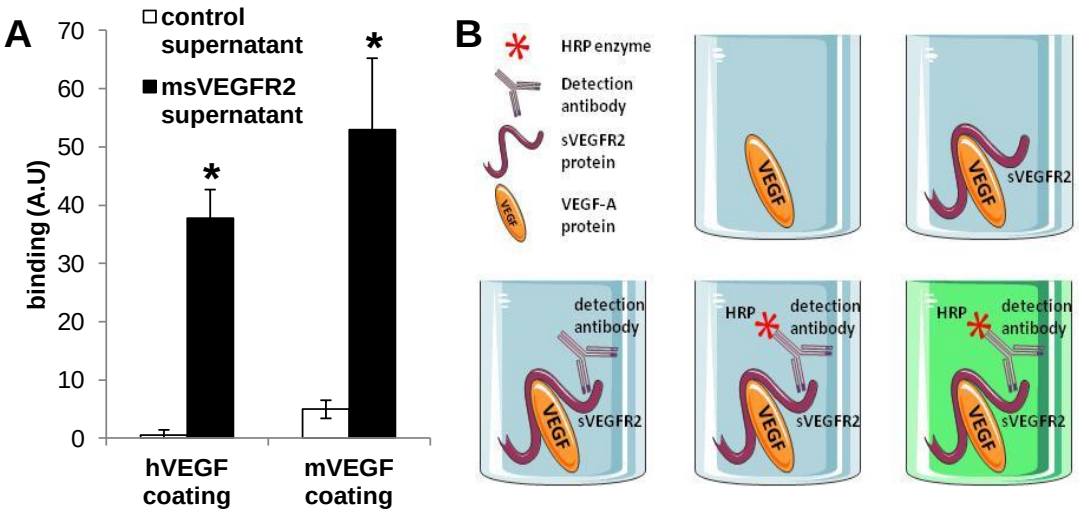


Figure 37: Activity of the produced sVEGFR2: cross reactivity toward immobilized human and murine VEGF-A.

(A) ELISA-based binding assay. Results are expressed in arbitrary units, showing the recognition of mouse VEGF164 and human VEGF165 by msVEGFR2. The hypoxia conditioned supernatant from HEK-IFP cells was compared to HEK-IFPmsVEGFR2. Values are mean ± SD (n = 3). *p < 0.05. (B) Schematic drawing representing the different steps of the ELISA-based binding assay. One representative experiment out of N = 3.

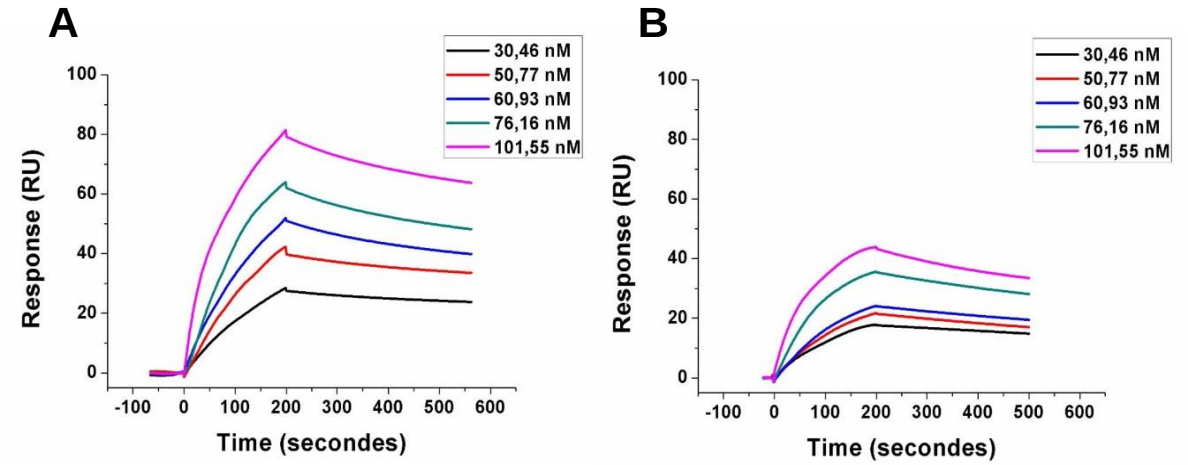
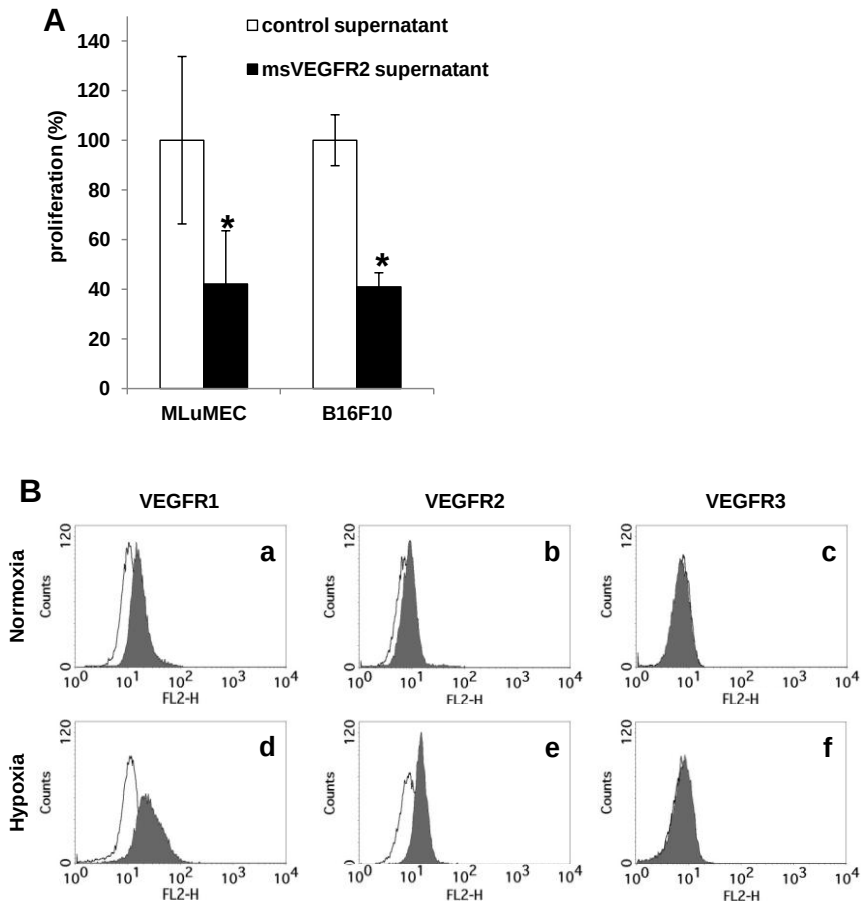


Figure 38: Binding properties of msVEGFR2 to murine and human VEGF

(A, B) 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 (A), or human (B) coated chip. Experiments were done in triplicate.

2.3.6-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. This fact was assessed on the B16F10 murine melanoma cells and on mature murine endothelial cells MLuMEC,FVB [250]. VEGF-A mediated cell activation depend on its binding to VEGFRs, their presence had to be investigated at the single cell level as well as their modulation by hypoxia. Figure 39B (a, b) shows that B16F10 cells express the VEGF receptors 1 and 2 in normoxia. Their expression is clearly increased on the cell surface upon treatment in hypoxia (Figure 39B d,e) as opposed to VEGFR3 which is absent in both conditions (Figure 39B c, f). Each of this VEGF-responsive cell lines was incubated with a 48h hypoxia msVEGFR2-conditioned supernatant (60 ng/mL) from HEK-IFP-msVEGFR2 and compared to an HEK-IFP control. Each line displayed a significantly reduced proliferation. This indicates that expressed msVEGFR2 was able to recognize, bind and neutralize the VEGFs (Figure 39A). It thus inhibited by 60 % the proliferation of MLuMEC, FVB endothelial cells as well as B16F10 melanoma cells. This result confirms the above observed cross recognition of human VEGF by murine sVEGFR2.



<u>Figure 39:</u> *In vitro* anti-proliferative effect of msVEGFR2 and cell hypoxia induced expression.

(A) 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). *$p < 0.05$.

(B) Expression of VEGFRs on B16F10 cells. The cells cultured in normoxia (a, b, c) and hypoxia (d, e, f) were labeled with selected antibodies as described in Materials and Methods, with 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.

2.3.7-msVEGFR2 decreases VEGF-induced Angiogenesis

Anti-angiogenic potential of the msVEGFR2 protein was assessed in a Matrigel™ angiogenesis assay. The msVEGFR2 activity was evaluated after incubation of the conditioned supernatants with HMEC-1 human endothelial cells. As shown in Figure 40A and 40B, the organized pseudo-vessel network was reduced (Figure 40B) when endothelial cells

had been exposed to msVEGFR2 (70 ng/mL) containing supernatant compared to control HEK-IFP cell supernatant (Figure 40A).

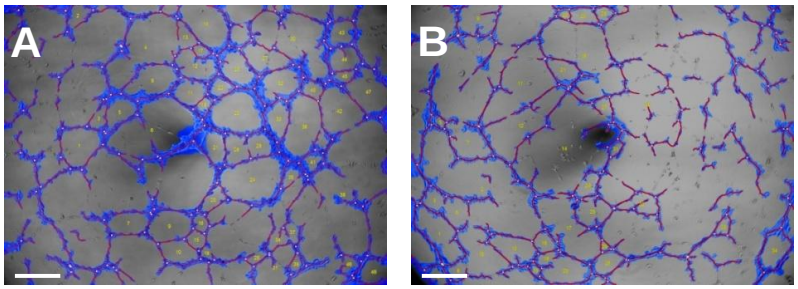


Figure 40: Effect of msVEGFR2 on the angiogenesis process *in vitro*
(A, B) One representative experiment for HMEC-1 endothelial cells seeded on Matrigel in the presence of hypoxia-conditioned medium from HEK-IFP cells in (A) or HEK-IFPmsVEGFR2 cells in (B). Tube-like structures network was analyzed after 10h incubation in normoxia with Wimasis Image Analysis, based on the total tube length parameter, indicated a 10% inhibition in B compared to A. Scale bars represent 300 µm.

2.3.8-*In vivo* neo-angiogenesis is inhibited by msVEGFR2 expression

To determine whether the msVEGFR2 could modulate angiogenesis *in vivo*, a Matrigel plug model was used. Matrigel plugs containing either HEK-IFP-msVEGFR2 or HEK-IFP cells were injected as described in Materials and Methods. Control Matrigel plugs displayed an extensive vascularization (Figure 41A) thus confirming previous experiments [255, 256], while plugs containing msVEGFR2 producing cells appeared to be non angiogenic (Figure 41B). Plug limits were clearly delineated by mCherry fluorescence (Figure 41C, D). The reduction of vascularization in plugs containing msVEGFR2 producing cells was confirmed by fluorescent imaging of blood vessels reported by FTC-labelled dextran, intraveneously injected to trace the vasculature (Figure 41C-E). FTC-dextran angiography shows that Matrigel did induce angiogenesis (Figure 41C and D) as compared to control skin (Figure 41E). Plugs containing msVEGFR2-secreting cells displayed reduced vessel numbers (Figure 41D) compared to controls (Figure 41C, white arrows). Angiogenesis appeared less chaotic and vessels looked better formed in the presence of msVEGFR2 secreting cells (Figure 41D, white arrow). These results prove that msVEGFR2 is active *in vivo* to decrease neo-angiogenesis supposedly by VEGF trapping and indicate the blood vessels pruning occurred (Figure 41D) which is one of the conditions required to reach normalization.

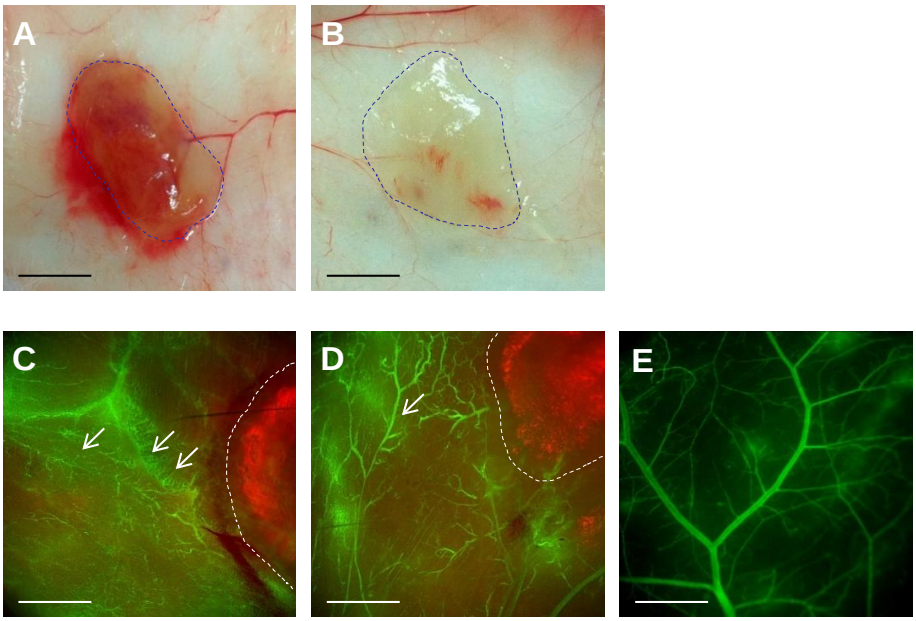


Figure 41: Effect of msVEGFR2 on the angiogenesis process *in vivo*
(A, B) Photography of the reversed mouse skin showing the effect of msVEGFR2 on *in vivo* angiogenesis initiated by a Matrigel™ plug assay. HEK-IFP control cells (A) or producing HEK-IFPmsVEGFR2 (B) were mixed to Matrigel before subcutaneous injection into mice (N=6). Results are presented 10 days after Matrigel™ plug induction. Scale bars = 1 cm. (C, D, E) 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. (C) represents the control plug containing HEK-IFP cells; (D) a plug containing HEK-IFPmsVEGFR2 cells (E) a control skin without Matrigel plug. White arrows show the blood vessels. Scale bars = 5mm.

2.3.9-Hypoxia driven msVEGFR2 expression reduces melanoma tumor growth

As the next step, msVEGFR2 produced was evaluated for its ability to block B16F10 melanoma growth. A stable cell line of B16F10 melanoma cells was established to express the extracellular soluble murine VEGFR2 receptor. Two clones were selected on the basis of their ability to produce msVEGFR2 induced by hypoxia: clone 16.4, a high msVEGFR2-producer (1100 ng/mL/10^6cells), and clone 13.3, moderate producer (180 ng/mL/10^6 cells) as shown on

Figure 42A. In these *in vitro* conditions, native B16F10 cells did not produce detectable amount of the soluble form of VEGFR2 (not shown). Confirmation of the msVEGFR2 effect on B16F10 melanoma cell growth is reported in Figure 42B, showing a significant reduction of the tumor cell proliferation *in vitro*. However, the inhibition of proliferation appears not directly related to the amount of msVEGFR2 produced by the clones. Cross regulation of the released VEGF-A by VEGFR2 might occur. Indeed, in the same conditions, the clones16.4 and 13.3 released different quantities of VEGF (250 ng/mL/10^6 cells and 80 ng/mL/10^6 cells, respectively) comparable to the msVEGRF2 as shown in the Figure 43A. The *in vivo* effect of msVEGFR2 production was then assessed three weeks after the subcutaneous implantation of tumor cells. Tumor size was estimated by weight showing that both 16.4 and 13.3 clones displayed significant tumor reduction when compared to the native B16F10 cells (Figure 42 C and inserts a, b). These data indicate that msVEGFR2 inhibited tumor growth. As regulation of VEGF overproduction is assessed to the receptor cleavage and secretion, its level was quantified *in vivo.* The amount of msVEGFR2 was found to be higher inside the tumors induced by the 16.4 and 13.3 clones as compared to the B16F10 melanoma tumors (Figure 42D). Pointing to the direct effect of msVEGFR2 on the growth of the tumor, Figure 42C also confirms that the effectiveness of soluble receptor produced is modulated *in vivo*.

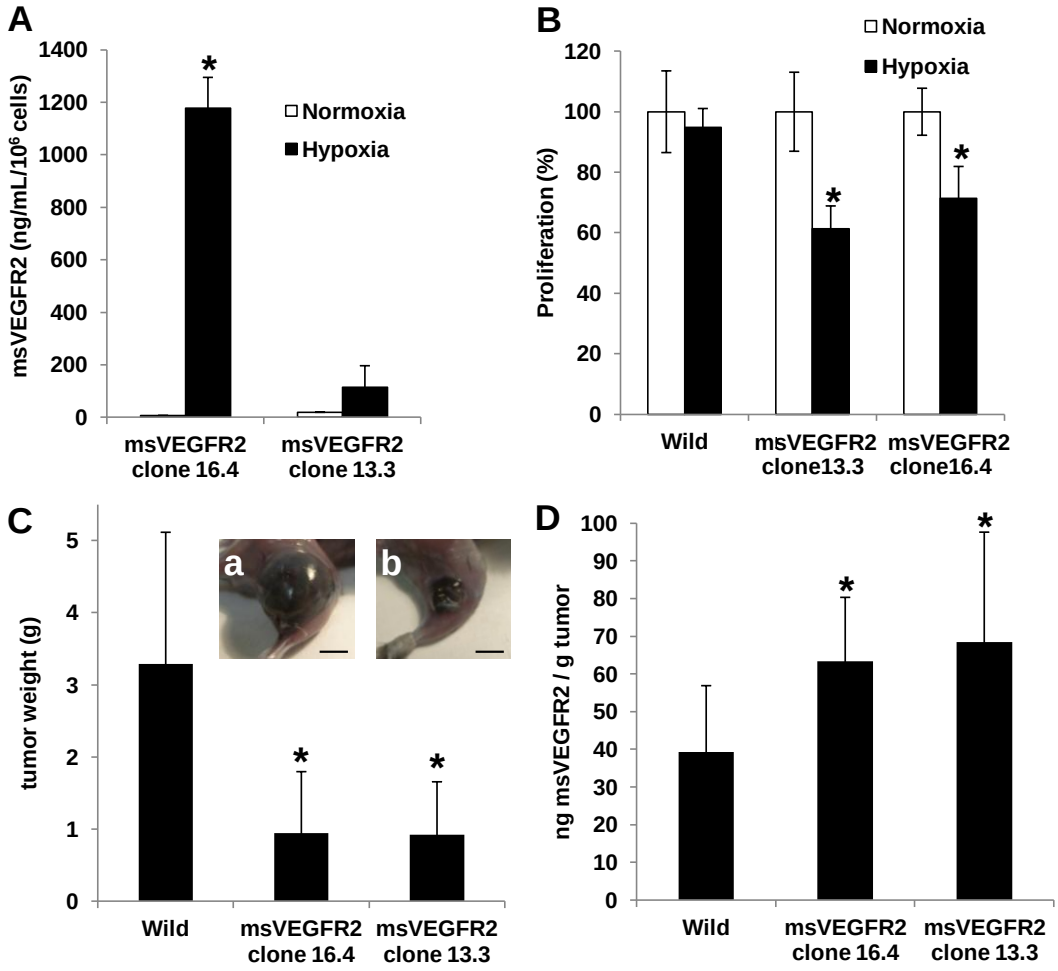


Figure 42: msVEGFR2-producing B16F10 melanoma cells: hypoxia driven regulation
(A) msVEGFR2 production measured in supernatants of two stably-transfected cell lines of B16F10-msVEGFR2: 16.4 and 13.3 clones cultured 48 hours in normoxia (21 % O_2) or hypoxia (1% O_2). Production was measured by ELISA. The results are reported to 10^6 cells. Values are mean ± SD (n = 3). *p < 0.05. (B) Proliferation assay of the two clones 16.4 and 13.3, cultured 48 hours in normoxia (21 % O_2) or hypoxia (1 % O_2). Proliferation was assessed by BrdU incorporation. Results were expressed in % of the control after a 48h incubation. Values are mean ± SD (n = 3). *p < 0.05. (C) 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 control cells and (b) a msVEGFR2 expressing B16F10 cells. (D) Quantification of msVEGFR2 production by growing tumors from cells transfected with msVEGFR2 plasmid (clones 16.4 and 13.3) as compared to control B16F10 cells. ELISA determined values are normalized per gram of tumor. Values are mean ± SD (n = 7). *p < 0.05.

Provided the clonal selection is not deleterious for the cell tumorigenicity, the above data point to an efficient trap effect towards VEGF.

2.3.10-Modulation of msVEGFR2 activity by VEGF-A *in vivo*

Despite distinct msVEGFR2 production *in vitro*, no significant difference was observed between clones *in vivo*, in terms of tumor growth. This observation confirmed the *in vitro* observation on cell proliferation (Figure 42B) and might be due to the variations in the VEGF-A production shown *in vitro* (Figure 43A) and *in vivo* (43B). The calculated ratio expressing the quantities of hypoxia-induced VEGF-A *vs* msVEGFR2 by B16F10 cells (R > 400, not shown) compared to the 13.3 clone (R = 0.70) and the 16.4 (R = 0.20), shown on Figure 44A, corroborated this hypothesis.

Similarly, the levels of msVEGFR2 and VEGF-A measured *in vivo* showed that higher production of the soluble receptor by the 16.4 and 13.3 clones was accompanied by a higher VEGF-A production by the same cells (Figure 43B). Consequently, the ratio expressing the VEGF-A *vs* msVEGFR2 in the tumor was calculated (Figure 44B) and data related to the observed reduction of tumor growth (Figure 42C). This result was corroborated by the concentration values of msVEGFR2 measured in the serum (Figure 44C) and the calculated ratio expressing the circulating VEGF *vs* msVEGFR2 (Figure 44D).

The addition of moderate excess of VEGF-A to *in vitro* experiments will bring the answer to this remaining open question.

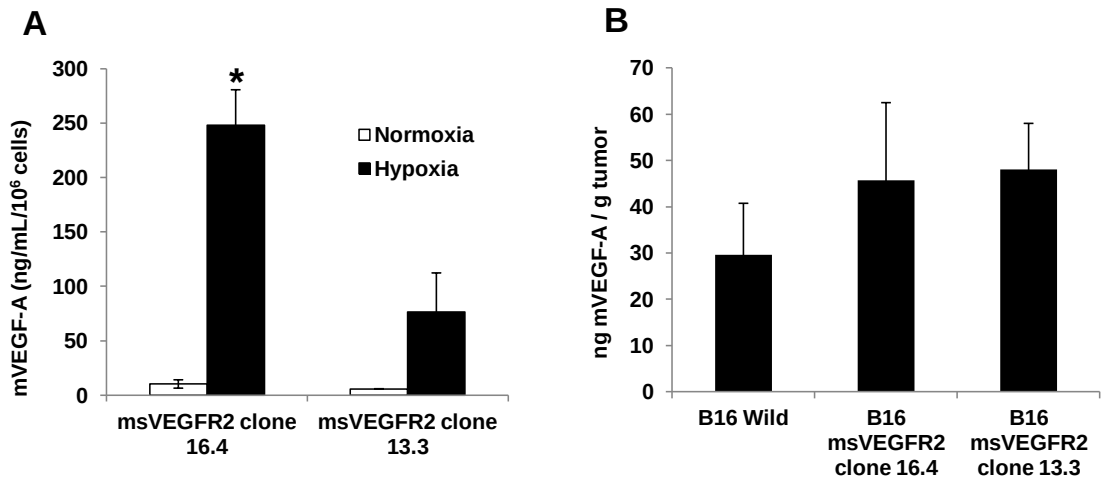
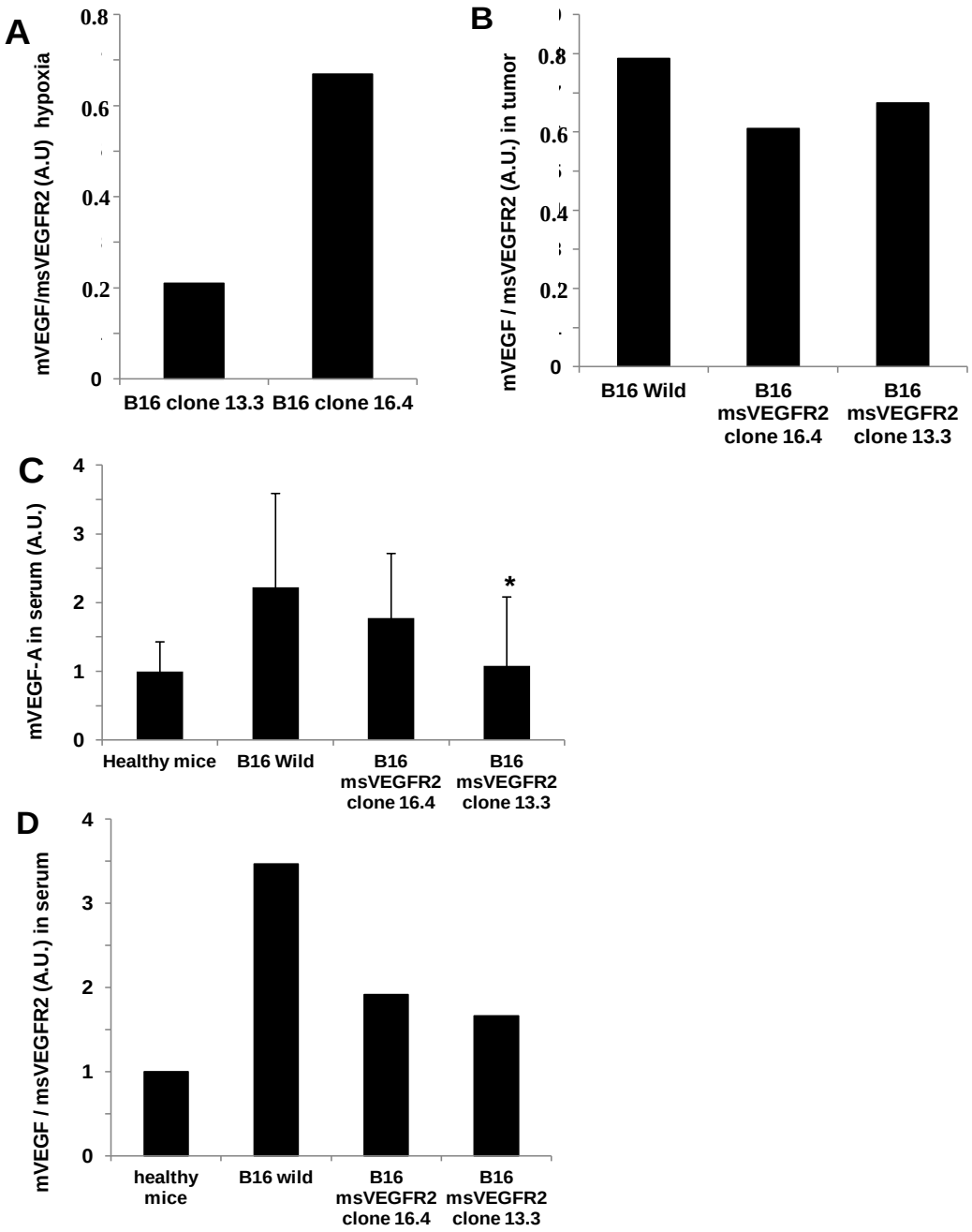


Figure 43: mVEGF-A production by msVEGFR2-producing B16F10 melanoma cells *in vitro* and *in vivo*

(A) mVEGF-A production measured in supernatants of two stably-transfected cell lines of B16F10-msVEGFR2: 16.4 and 13.3 clones cultured 48 hours in normoxia (21 % O_2) or hypoxia (1% O_2). Production was measured by ELISA. The results are reported to 10^6 cells. Values are mean ± SD (n = 2). *p < 0.05.

(B) Quantification of mVEGF-A production measured in the serum of mice bearing tumors from cell lines transfected with msVEGFR2 plasmid (clones 16.4 and 13.3) as compared to control B16F10 cells. ELISA determined values are normalized per gram of tumor. Values are mean ± SD (n = 7). *p < 0.05.



<u>Figure 44:</u> VEGF-A and msVEGFR2 relative production *in vitro* and *in vivo* by msVEGFR2-producing B16F10 melanoma cells

(A) mVEGF/ msVEGFR2 ratio values expressed in arbitrary units (A.U.) and calculated from the 13.3 and 16.4 clones in hypoxia. (B) mVEGF/ msVEGFR2 ratio values expressed in arbitrary units (A.U.) calculated from the 13.3 and 16.4 clones and B16F10 tumors in vivo. (C) mVEGF-A quantified in the serum of mice harboring tumors producing soluble VEGFR2 (clone 16.4 and 13.3) compared to animals bearing wild type B16F10 melanoma. Serum of healthy mice was used as reference. Results are expressed in arbitrary units (A.U.). Values are mean ± SD (n = 10). *p < 0.05 compared to the B16 wild. (D) mVEGF/ msVEGFR2 ratio values expressed in arbitrary units (A.U.) calculated from the serum of 13.3 and 16.4 clones and B16F10 tumor bearing mice. Healthy mice serum was used as reference.

2.3.11-Hypoxia driven msVEGFR2 expression in melanoma controls VEGF production and tumor oxygenation

The trapping effect of msVEGFR2 and its consequence on the down regulation of the VEGF-A release by the tumor is shown on Figure 44C. VEGF-A appears to be reduced in the serum of the mice bearing msVEGFR2 expressing tumors. Indeed, in animals which received clone 13.3, VEGF-A is reduced to the level found in healthy animals while it is 2 fold increased in mice bearing wild type cells. Interestingly, the intratumor oxygen tension measured by Ru fluorescence quenching was increased in msVEGFR2 expressing melanoma clones as reported on Figure 45. According to Jain[14] and data by Holash on VEGF trapping [8, 257, 258] this effect on tumor oxygenation suggest that the decreased VEGF production is the result of the normalization of the vasculature.

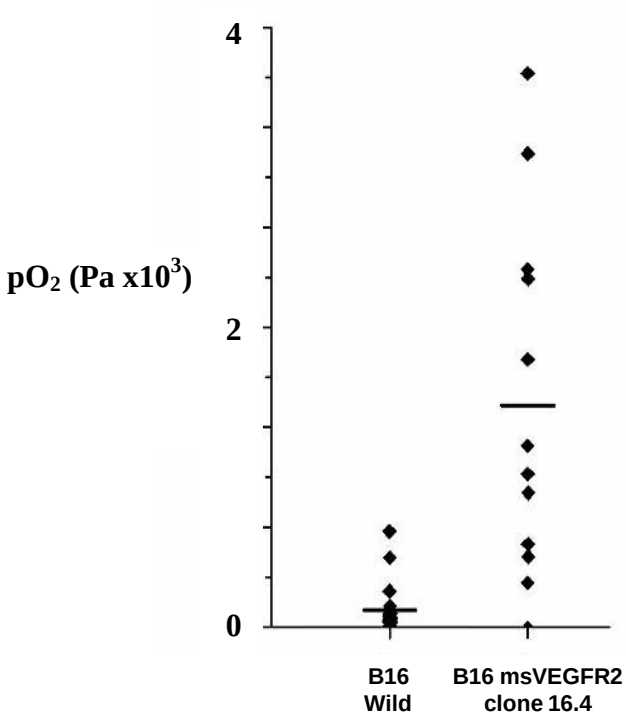


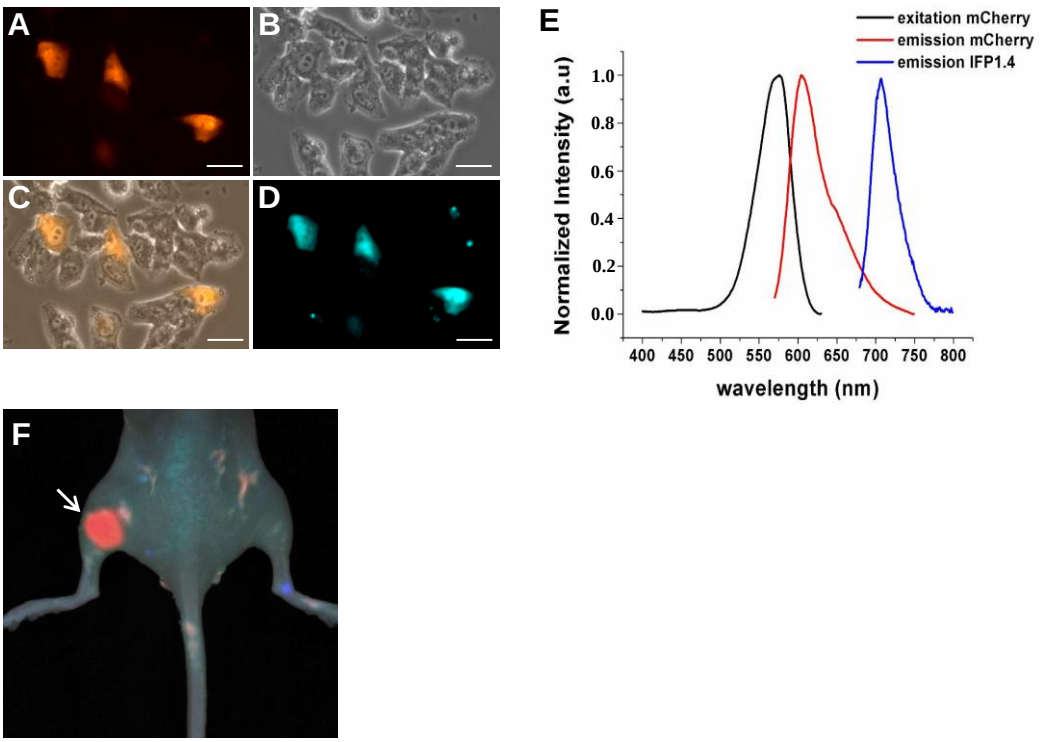
Figure 45: Tumors growing from cells overexpressing msVEGFR2 are better oxygenated than wild type
Representative scatter plot of the oxygen values measured by Oxylite in 3 weeks tumors developed from a B16F10 wild type cells and a B16F10 clone 16.4 producing hypoxia driven msVEGFR2. Results are expressed in mmHg. The average is represented by a small bar. 19 measures were performed with the B16F10 wild tumor bearing mouse, and 12 with the B16F10 clone 16.4.

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

Stably transfected HEK-IFP and HEK-IFP-msVEGFR2 cells were used to evaluate *in vitro* IFP1.4 (excitation and emission maxima at 684 and 708 nm respectively) protein expression by fluorescence microscopy (Figure 46A-D). Well adapted for *in vivo* imaging [33], this fluorescent 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 [34, 35].

Similarly, the expression of mCherry (excitation and emission maxima at 587 nm and 610 nm), cloned after IRES (internal ribosome entry site) downstream the IFP1.4 coding sequence, was detected (Figure 46A, C). This operation allows cell tracking in non near infrared optical settings and the reporting of the IFP1.4 expression. Figure 46A-D, shows fluorescence microscopy detection of mCherry and IFP1.4 and their homogeneous cytosolic distribution in transfected cells. Both proteins expression was detected by fluorescence spectra measurements in cell extracts compared to theoretical spectra (Figure 46E) although mCherry and IFP1.4 excitation spectra overlap making the discrimination by fluorescence impossible. Similar spectra were obtained from B16F10 transfected cells (data not shown).

In vivo imaging of stably transfected B16F10 melanoma cells expressing both IFP1.4 and mCherry fluorescent proteins was performed. As presented on Figure 46F, the fluorescent tumor cell distribution was 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. The low fluorescence yield of the IFP was compensated by the reporter effect provided by the mCherry in an IFP1.4-IRES-mCherry construct.



<u>Figure 46:</u> ***In vitro*** **and** ***in vivo*** **fluorescence imaging of IFP1.4 and mCherry reporter expressing cells**

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

2.4-Conclusion

Given the role of the 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 consequently a good candidate as a control.

In this study, we have developed a regulated therapeutic gene for cancer therapy by hypoxia- driven trapping of VEGF by msVEGFR2. Interaction of the produced msVEGFR2 with the murine VEGF-A was quantified by surface plasmon resonance and provided kinetics data on which allowed us to qualify it as a VEGF-trap, comparable to the previously described DAAP [10]. The biological effect was proven by the efficient decrease of angiogenesis *in vitro* and *in vivo* and diminished tumor cell proliferation. This observation underlines the fact that this therapy may act on both tumor and endothelial targets.

The main advantages of the vector as it is presented here, come from the experiments suggesting that the 3x HRE combination pattern could be an optimized enhancer cassette to modulate the truncated CMV activity (minCMV promoter) [247]. Such combination allows the expression to be restricted to a 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.

Physioxia [47] as described in the introduction and in section 1.2.1. is the oxygen tension characteristic of a tissue/organ in its physiological context. It indeed, has deep implications for hypoxia-dependent therapies. Here, the hypoxia-driven expression of msVEGFR2, was indeed dependent of a strong O_2 deprivation (3% compared to 21%). A threshold could be determined indicating that the therapeutic gene would be expressed only in hypoxic environment and restricted to it, avoiding or limiting the side effects.

The msVEGFR2 protein was indeed anti-angiogenic/angiostatic, validating further use for *in vivo* applications as demonstrated here by the Matrigel™ plug assay *in vivo* for the development of angiogenesis. Moreover, it must be remarked that msVEGFR2 activity on angiogenesis is underestimated in such an assay due to the origin of the matrix that contains pro-angiogenic factors. Thus msVEGFR2 works out the pro-angiogenic effect of the Matrigel™.

Moreover, the presence of VEGF-Receptors 1 and 2 on the surface of murine B16F10 melanoma cells together with an effect of the msVEGFR2 on their proliferation underlined the fact that this therapy may act on both tumor and endothelial cells. Confirmed *in vivo,*

when expressed by the tumor cells themselves, the vector expressing melanoma cells produced smaller tumors and displayed improved reoxygenation.

Furthermore, the designed vector is applicable to *in vivo* imaging, since it expresses the Infrared Fluorescent Protein (IFP1.4) and mCherry.

Satisfying expectations, the vector was successfully constructed combining both therapeutic and imaging modalities. Functional and regulated, the next step of the Trojan Horse is the choice of the carrier cells, able of target the tumor site, in order to be "armed" by the construction.

3-Carrier cell

3.1-Introduction

The above described and vector having been validated and shown to act on tumor cells growth upon expression by other cells. Our pupose is to act on the level of the tumor angiogenesis process. Consequently we chose cells that are known to reach naturally this site and tried to provide ourselves with a cellular model. Thus the vector was to be introduced in a carrier cell able to reach the tumor site (presented in part 1.4.7).

Although controversial [259], EPCs have been found recruited and incorporated into neoangiogenesis, they might thus constitute a simple and well-suited tool to target pathologic sites [260]. The use of EPC for therapeutic applications has been demonstrated as a strategy to enhance endothelium regeneration [72, 261-264] and as a vehicle to reach developing tumors [27].

In this purpose, we undertook to build a cell model of immature endothelial cells. As the first cells restricted to the endothelial lineage appear in aorta-gonad-mesonephros (AGM) region [265], cells were isolated from this region at 10.5 and 11.5 days post conception (dpc), time where these cells diverge from hemangioblasts as described in the section 1.3.3.2.2. Two cell lines (patent pending) could be established, representing different endothelial differentiation steps occurring at 10.5 dpc and 11.5 dpc. They were selected on the basis of their differentiation characteristics and called MAgECs 10.5 and 11.5.

This second part of our results presents the establishment of these cell lines and their validation as carrier cells to be used as experimental targeting tool for cancer therapy mainly for their ability to reach and stay at the tumor site. Although, upon systemic injection, the cells will distribute over the whole organism and mainly in the lungs in a first mechanical step, recirculation is the start of the “homing” process.

3.2-Materials and methods

3.2.1-Isolation of Mouse Aorta-gonad-mesonephros Embryonic Cells (MAgECs)

Embryos were taken from 10.5 dpc and 11.5 dpc pregnant mice. Aorta-gonad-mesonephros **(**AGM) regions were isolated and washed extremely gently in RPMI (Gibco Invitrogen, Villebon sur Yvette, France) supplemented with FBS 15% (PAA, Austria) and 40µg/ml gentamycin (Gibco Invitrogen). Then the AGM derived tissues were cut into very small pieces and cell cultures were started at 37°C in a 5% CO2/95% air atmosphere in plastic culture plates (Falcon, Becton Dickinson, USA) using OptiMEM (Gibco Invitrogen) supplemented with 2% FBS, 40µg/mL gentamicin (Gibco Invitrogen) and fungizone 0.5µg/mL (Gibco Invitrogen). After 1 to 2h, non-adherent cells were removed and submitted to a second adhesion step in other culture plates. Non adherent cells and debris were then totally removed from the cultures.

3.2.2-Immortalization and selection of MAgECs

After 2 days, AGM primary culture from 10.5 dpc and 11.5 embryo were transfected, directly in the culture plates, with pSV40-neoplasmid (previously constructed in the laboratory), which contained the large T-antigen of SV40 early region associated to the neo^R gene (pSV40-neo plasmid). Transfection, using lipofectin reagent (Gibco Invitrogen) and pSV40-neo plasmid, was conducted as previously described [250]. The culture was allowed to grow for several days until appearance of colonies of proliferating cells. The selection of transfected cells was done in the presence of increasing concentrations of geneticin (Gibco Invitrogen, starting at 10µg/mL). Geneticin-resistant cells were cloned by the limiting dilution procedure, amplified and then banked in liquid nitrogen. Among obtained cell lines from 10.5 and 11.5 cells, one representative clone of each was chosen according to their characterizations and properties.

3.2.3-Cell Culture

MAgEC cells and mature lung endothelial cell line were cultured in OptiMEM with Glutamax-I (Gibco Invitrogen) supplemented with 2% fetal bovine serum (BioWest, Nuaille, France), 40 µg/ml gentamycin and 0.05 µg/ml fungizone Cells were maintained at 37 °C in a 5% CO_2/95% air atmosphere. Cell passages were performed by detaching the cells with 0.25% trypsin-0.05% EDTA (w/v) solution (Gibco Invitrogen). The presence of mycoplasma was checked using "MycoAlert Detection kit" (Lonza) and only certified mycoplasma-free cells were used for experiments.
B16F10 melanoma cell culture is performed are described in the part 2.2.2.

3.2.4-Quantitative PCR.

Extraction of cellular mRNA was performed using the RNeasy Plus mini kit (Qiagen) according to the manufacturer's instructions. The hypoxia stimulation of MAgECs 10.5 and 11.5 was stopped after 24 hours with RNA isolation. All extracted mRNAs were eluted in RNase-free water. Absorption spectra were measured on an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) before to be stored at -80°C. RNA were reverse-transcribed to cDNA using "Maxima First Strand cDNA Synthesis kit for RT-qPCR" (Fermentas). 3µg of RNA was used for each sample. The obtained cDNA were stored at -20°C until qPCR was performed. The real-time PCR was then performed on LightCycler 480 (Roche) using the "SYBR Premix Ex Taq (Perfect Real Time)" (Takara) and "QuantiTect Primer Assay" (Qiagen) in white 96-well optical microtiter plate (Roche). 2µL of cDNA were used in a final volume of 20µL by well. Primer sequences, concentrations of forward and reverse primers, and the size of the gene amplicons are given in Annexe 1. All reactions were completed in triplicate and reported as the average. For reference, 7 housekeeping genes were tested. Mean and standard deviation from the 6 samples were calculated and the gene which had the lowest standard deviation was chosen for reference. For each target gene, mean and standard deviation were calculated by cell line (10.5 or 11.5) and condition (normoxia or hypoxia), then normalized by the corresponding value for reference gene (PPIA) to obtain the ΔCp. In a second step, the same method was used to compare hypoxia to normoxia and obtain the ΔΔCp.

3.2.5-Immunocyto chemistry staining of MAgEC cells

Detection of vWf and ACE was performed on fixed and permeabilized cells according to the technique established previously [266]. Briefly, cells were seeded on 8-well gelatin-coated microscope slides (ICN Biomedicals, USA). After 48 h, cells were fixed and permeabilized during 10 min at 37°C in a cPBS (complete-phosphate buffered saline) solution containing PFA (paraformaldehyde) 1% (Merck-Schuchardt, Germany) and saponin 0.2% (Sigma, USA). For VE-cadherin and CD31 staining, cells were only fixed with PFA. Then cells were washed twice and incubated at 37°C during 1h, in the presence of rabbit anti-vWF at 50 µg/mL (Santa Cruz Biotechnology, USA), rabbit anti-ACE at 50µg/mL (Santa Cruz Biotechnology, USA), rat anti-VE-cadherin at 50µg/mL (BD Pharmingen, USA), rat anti-CD31 at 50 µg/mL (eBioscience, USA) in c-PBS-5 % BSA with or without 0,1 % w/v saponin. Control staining was performed in the same way using the corresponding isotype at the same concentration. After two washings with cPBS-0,5% BSA, the secondary labelled antibodies, fluorescein thiocarbamyl (FTC)-anti-rabbit (Sigma) at 12.5 µg/mL or FTC-anti-rat (Zymed,USA) at 10 µg/mL were added for 30 min at 37ºC in the same buffer. Cells were examined using inverted fluorescence microscope Zeiss Axiovert 200 equipped with DIC and results were analyzed using AxioVision 3.1 software.

3.2.6-Identification of progenitor cell markers on MAgECs by flow cytometry analysis

Both MAgEC cells, 10.5 and 11.5, were immunostained with a first set of antibodies for the presence of CD133 (rat IgG1; eBioscience, USA), Tie-2 (rat IgG1; eBioscience, USA), Podocalyxin (PODXL) (rat IgG2b; R&D Systems, USA) and EphB4 (goat IgG; R&D Systems, USA). Cells were washed with c-PBS containing BSA (0.5% w/v, c-PBS-BSA) and NaN_3 (0.1% w/v) and were detached from culture plates with type-1 collagenase (0.5 mg/ml; Gibco Invitrogen) in c-PBS-BSA for 15 minutes at 37ºC. After washing, cells were incubated with monoclonal antibodies against murine antigens for 30 min at 4ºC (0.3µg/3.10^5 cells, 10µg/ml in c-PBS-BSA). Cells were incubated with the corresponding isotypes in the same conditions as control. After washing, secondary labelled antibodies were added and incubated for 30 min: goat FTC-anti-rat IgG (Zymed) or rabbit FTC-anti-goat IgG (Sigma), respectively. Stained cells were analysed by flow cytometry using fluorescence-activated cell

sorter FACS LSR (Becton Dickinson, USA) and analysed using CellQuest Pro software (Becton Dickinson).

A second set of immunostaining was performed with the fluorescently conjugated antibodies or corresponding isotypes: anti-CD29-FITC (biolegend, 102205), anti-CD90.2-APC (biolegend, 105312), anti-CD105-PE-Cy7 (biolegend, 120410), anti-Sca-1-FITC (BD, 553335), anti-c-kit-PE (BD, 553355), CD49e-PE (biolegend, 103905), anti-CD45-APC-Cy7 (BD, 557659), anti-CD31-PE (BD, 553373), anti-CD34-FITC (BD, 560238), and anti-VEGFR2-APC (BD, 560070). Stained cells were collected on LSR II flow cytometer (Becton Dickinson) and analysed using BD Biosciences FACSDiva software.

For both set of experiments, mean fluorescence intensity of the appropriate isotype labeled control cells was set below 10 and this value was used as a threshold for positively stained cells. Results are expressed as the relative fluorescence intensity ΔIF (the difference between the relative fluorescence intensity of antibody labeled cells and corresponding isotype).

3.2.7-Production of VEGF

48h after incubation in hypoxia (1% O_2) and normoxia (21% O_2), the supernatants from MAgECs 10.5 and 11.5 were collected and analyzed by ELISA according to instructions using the mVEGF duoset kit (DY493) (R&D systems, USA).

3.2.8-*In vitro* angiogenesis assay

Angiogenesis was performed as described in part 2.2.10 except that 8.10^3 cells per well were seeded. Data were acquired with a Zeiss Axiovert 200M fluorescence inverted microscope (Zeiss, Le Pecq, France) equipped with an Axiocam high-resolution numeric camera linked to a computer driving the acquisition software Axiovision (Zeiss). Tube-like and network structures were documented after 12 hours of culture. MAgECs cell lines were studied independently, mixed together and mixed with mature endothelial cells to study their behaviour in hybrid angiogenesis.

3.2.9-*In vivo* angiogenesis assay and ultrasound imaging

For *in vivo* validation of the angiogenesis, C57/BL6 mice were subcutaneously injected in the abdomen with 500 µL of Matrigel (BD Biosciences, San Jose, CA) mixed with 5.10^5 MAgECs cells, either 10.5 or 11.5, to be compared with an empty Matrigel plug as control. Ten days after graft, plugs were imaged using a VisualSonics Vevo® 2100 Imaging System (VisualSonics Inc., Toronto, Ontario, Canada) connected to MS500D ultrasound transducers (22-55MHz). Before imaging, alll hairs were removed from the region of interest with depilatory cream. Imaging was performed under anesthesia by a gas mixture injected at 2L/min and composed of air mixed with 2.5 vol % isoflurane. A medical ultrasound acoustic gel was applied to the imaging area surface as a coupling fluid between the transducer and the skin to allow the ultrasound propagation. Biological parameters were constantly monitored with the VisualSonics integrated rail system with physiological monitoring unit to assess the electrocardiograms (ECGs) of the animals and respiratory rate. Body temperature was maintained at 37.5°C. Ultrasound imaging was performed with B-mode to find the Matrigel plug under the skin surface and to position the transducer in a perpendicular axis to mouse body. Then blood flow (vein and artery) was measured using color Doppler mode and fitting it with B-mode imaging. The imaging was driven and computed with VisualSonics Vevo® 2100 software. After imaging, mice were sacrificed, and the plugs were retrieved to take pictures.

3.2.11-Establishement of GFP^+ MAgECs cell lines

Both MAgECs cells, 10.5 and 11.5, were transfected with a GFP coding vector, pdAAV-CMV-GFP designed by J. Stepniewski (Annexe 2). Transfections were performed using the jetPEI™ (Polyplus Transfection, France) as DNA complexing agent according to manufacturer's instructions. After recovery, transfected cells were single-cell cloned by a MoFlo™ cell sorter (MoFlo™, Beckman Coulter, Miami, FL, USA). The higher stably GFP expressing clones, for each MAgECs 10.5 and 11.5, was selected and expanded.

3.2.12-*In vivo* MAgEC recruitment at angiogenic sites

To assess the recruitment of MAgECs cells to newly formed blood vessels, a neo-angiogenic site was induced by Matrigel plug assay into adult female C57Bl/6 mice (6-8 weeks old) (Janvier, France). Briefly, 500 µL of Matrigel containing 500 ng/mL VEGF + bFGF was implanted by subcutaneous injection in the abdominal region. After Matrigel polymerization, 2.10^6 GFP^+-MAgECs 10.5 and 11.5 into 100 µL of saline were intravenously injected in the tail vein. Due to injection method into the vein, the cells are physically retained in the lung. After 24-48h post injection, almost all of them can be considered as circulating in the blood stream. This time allows the initiation of angiogenesis in and around the Matrigel plug. After 10 days following the GFP^+-MAgECs injection, the mice were sacrificed and peripheral blood was collected by cardiac ponction with heparin syringe. After red blood cells lysis, single-cell suspensions were filtered using cell strainer 70 µm just before flow cytometer analysis. A single cell suspension was prepared harvesting the plugs, lungs, and bone marrow. Organs were dissected into approximately 1 to 3 mm^3 fragments and digested with 1000 mg/mL collagenase (Invitrogen) in PBS-FBS 10% for 2 hours at 37°C while shaking. Then, cells were filtered using cell strainer 70 µm and washed twice before analysis on the BD LSR-I flow cytometer (Beckman Coulter). The proportion of GFP^+ cells was investigated in each sample collecting 10^6 events.

3.2.13-Spheroids formation

Spheroids were generated as described [267]. A trypsinized B16F10 cells were labeled with DiO lipidic dye (Invitrogen, Carlsbad, CA) adding 5 µL of probe to a cell suspension of 10^6 cells in 1 mL of OptiMEM without FBS incubated 10 minutes at 37°C. Then, 1000 B16F10 cells were mixed in corresponding culture medium containing 0.25% (w/v) methylcellulose (Sigma, Steinheim, Germany) and seeded in non-tissue culture 96-well microplate with V-shape bottoms (Nunc, 277143). In a final volume of 100 µL per well and under 48h incubation at 37 °C in a 5% CO2/95% air atmosphere, a single spheroid per well is obtained.

Fixed spheroids are obtained after addition of 4% PFA (paraformaldehyde) during 30 minutes followed by washing and a neutralization step by addition of 20 mM urea for 30 minutes.

3.2.14-Migration of MAgECs to tumor cell spheroids

MAgECs were labeled with DiI lipidic dye (Invitrogen, Carlsbad, CA), adding 5 µL of probe to a cell suspension of 10^6 cells in 1 mL of OptiMEM without FBS incubated 10 minutes at 37°C. Performed in a 24 well plate, 2.10^5 labeled MAgECs cells (DiI) are mixed with 225 µL of the collagen/methylcellulose matrix together with 50 B16F10 labeled spheroids (DiO). The matrix is composed of collagen matrix realized according to provider advices (rat tail collagen type I, BD Biosciences, San Jose, CA, 354249) at final concentration of 1.6 mg/mL, and supplemented with methylcellulose to a final concentration of 1,12% (W/V) and with FBS 10% final. Poured in the microplate wells, the matrix containing MAgECs cells and spheroids are observed with the help of fluorescence microscopy allowing time lapse acquisition over 24h (Zeiss Axio Observer Z1 fluorescence inverted microscope - Zeiss, Le Pecq, France).

3.3-Results and discussion

3.3.1-Establishment of immortalized cell lines from AGM region of murine 10.5 dpc and 11.5 dpc embryo

From AGM region of murine 10.5 and 11.5 dpc embryos, we obtained cloned cell lines called respectively MAgEC 10.5 and MAgEC 11.5 (realized by Daisuke Sugiyama). Their doubling time was approximately 24 hours. As reported in figure 47, their morphology was different. MAgEC 10.5 presented a more stellar/dentritic aspect. In comparison, MAgEC 11.5 were less elongated and presented a square shape. When confluent, both MAgEC cell lines presented a typical endothelial cell pavement aspect, so called cobblestone aspect, as visible on the figure 47.

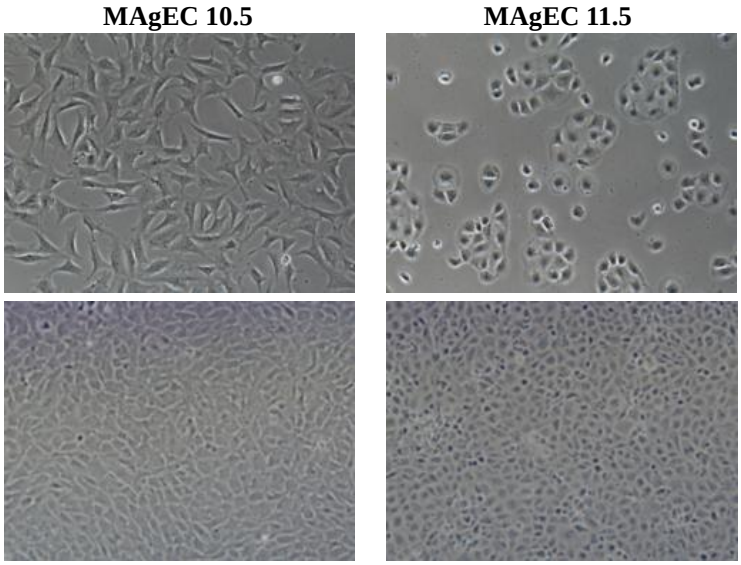


Figure 47: Morphology of MAgECs
Murine AGM-derived progenitor endothelial cells, MAgEC 10.5 and MAgEC 11.5, cultured as monolayers (bottom pictures: confluent cell layer; top: non confluent cell cultures).

3.3.2-Characterization of MAgECs cells

Immortalized cells were characterized for stem, endothelial and mesenchymal markers. Their presence was assessed both at mRNA, for 50 typical genes and also protein level for selected molecules. MAgECs cells expressed mRNAs of usual markers of hematopoietic stem cells but also of mesenchymal stem cells (MSCs) and endothelial

precursor cells (EPCs) as well (Figure 48A). Consequently, various markers were also studied at the protein level.

Confirming the Q-PCR data on Figure 48, where mRNA amounts are expressed as $2^{-\Delta Ct}$, the CD29, CD34, Sca-1and CD49e protein expression levels were high as detected by flow cytometry (Figure 49). Similarly, the CD133 and CD45 mRNA expression were very low and proteins were also hardly detected in flow cytometry. This congruent expression levels between mRNA and the corresponding proteins is found for c-kit, displaying the same preferential expression on MAgECs 11.5 as well as for CD31, showing a moderate expression of mRNA and proteins which was further confirmed by immunocytochemistry. The von Willebrand factor mRNA, although relatively less expressed, is detected at the protein level, as in all endothelial cells. ACE is similarly expressed at the protein level in MAgEC10.5 and 11.5 while its mRNA is predominantly expressed in MAgEC 11.5 cells.

Strongly different expression of proteins, compared to their mRNAs, is found for CD90.2 and VE-cadherin. mRNAs are poorly expressed, although clearly present, while the proteins are easily detectable in flow cytometry for CD 90.2. Interestingly, VE-cadherin is clearly visible in MAgEC 11.5 only, as shown by immunocytochemistry (Figure. 49) while its mRNA is more expressed in MAgECs 10.5. This apparent discrepancy may be due to the modulation of protein expression by cells.

A similar kind of observation can be done for VEGF-A, which is comparably highly expressed as mRNA in both MAgEC lines while the secreted protein could be measured in MAgEC 11.5 only.

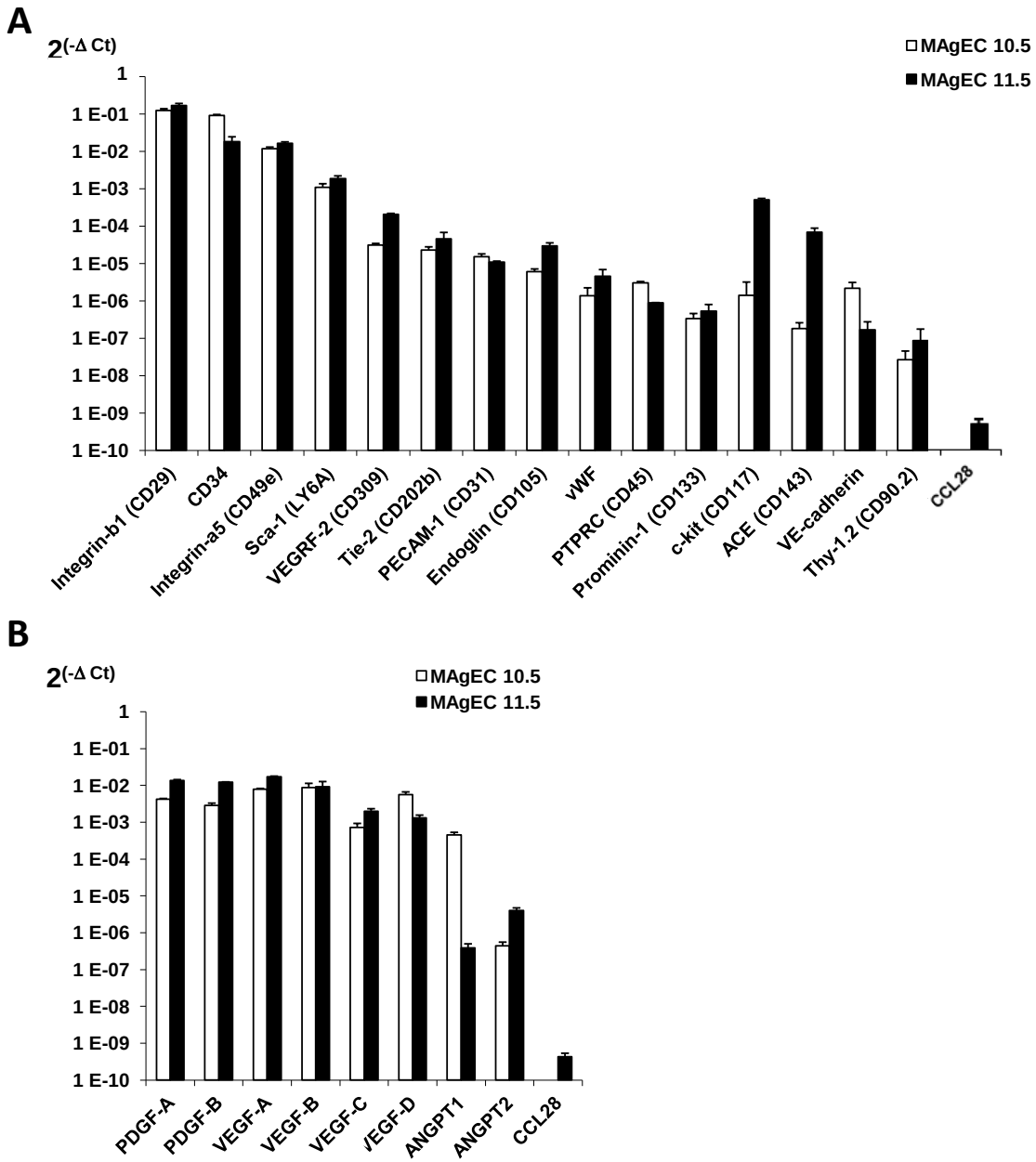
EPCs can express fibroblast growth factor receptor, CD38, c-kit, CD31, CD146, CXCR4, von Willebrand factor (vWF), vascular endothelial cadherin (VE-cadherin), Tie-2/TEK (angiopoietin- 1 receptor precursor or tunica intima EC kinase), and CD133 [268].
As shown in Figure 49A-B reporting the flow cytometry and immunocytochemistry data, both cell lines showed high expression of Sca-1 and CD34 that are found on stem/precursor cells. As other stem cell markers detected at the surface of EPCs, c-kit is also present on both MAgECs 10.5 and 11.5. Moreover, they are $CD45^-$ excluding their hematopoietic commitment. Interestingly, both MAgEC cell lines also express EPCs/mature endothelial cell markers such as PECAM (CD31), von Willebrand factor (vWF), and angiotensin converting enzyme (ACE), Podocalyxin-like protein 1 (PODXL). As previously indicated, these markers

are indicative but not exclusively typical for endothelial cells and their precursors. They should be combined to classify the cells as endothelial or endothelial precursors. In contrast, VE-cadherin (CD144) is a marker of endothelial cells. It is expressed only by the MAgECs 11.5. This distinct expression pattern for the VE-cadherin between the two cell lines may reflect a different degree of differentiation. Thus, the MAgEC 11.5 cell line consists of more mature, endothelial-committed cells. This observation confirms the endothelial functionality of MAgECs. The Ephrin-B4 receptor implicated in angiogenesis, is expressed by the two cell lines showing the potential of these cells to participate to angiogenesis process like EPCs.

Some markers are lacking though to allow any possible classification of MAgECs cells as EPCs such as the VEGFR2 receptor reported by Asahara *et al* [22] or the prominin (CD133).

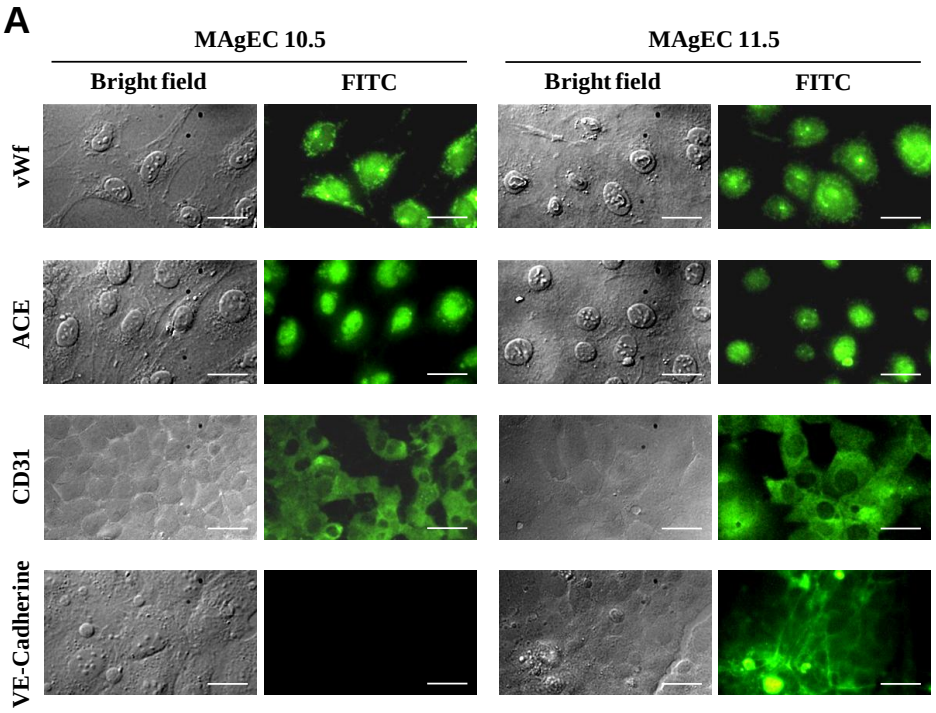
Deeper investigations revealed that MAgECs cells share some common markers with the MSCs. Indeed, both MAgECs express typical surface marker proteins expressed by MSCs like some adhesion molecules such as integrins (CD29, CD49e) or other membrane proteins as CD90, CD105. Nevertheless, they cannot be considered as MSCs because they express markers which should be negative such as CD34.

These data confirm that MagEC lines are distinct in terms of differentiation step but they do not permit to conclude about any categorization.



<u>Figure 48:</u> mRNA level of EPCs, MSCs, and ECs specific genes by the MAgECs 10.5 and 11.5.

Obtained by qPCR experiments, histogram A shows the normalized mRNA expression of various genes expressed by EPCs, MSCs, or ECs. Histogram B concerns normalized mRNA expression of various secreted factors. All results are normalized using the house keeping PPIA gene (peptidylprolyl isomerase A / cyclophilin A) and expressed as $2^{-\Delta Ct}$. Values are mean ± SD (n = 3).



B

	MAgEC 10.5		MAgEC 11.5	
	Δ IF	sem	Δ IF	sem
CD45	1.0	0.2	1.5	0.3
CD31	32.9	0.6	21.8	0.6
CD34	76.7	1.9	82.7	1.8
KDR	3.4	0.3	-2.8	0.6
sca-1	359	13.4	342.4	10.3
CD133	0.3	0.0	0.0	0.0
PODXL	53.7	7.0	142.2	18.5
Tie-2	0.1	0.0	0.1	0.0
CD105	11.2	0.5	13.1	0.4
CD29	315.4	2.8	486.1	0.3
CD90.2	18.8	0.3	26.7	0.3
CD49e	583.6	3.8	790.7	0.3
c-kit	5.8	0.3	33.5	0.4
EphB4	18.0	2.3	17.3	2.3

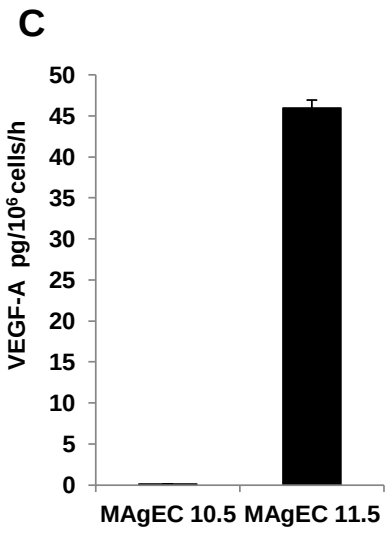


Figure 49: Immunophenotype of MAgECs 10.5 and 11.5 cell Lines

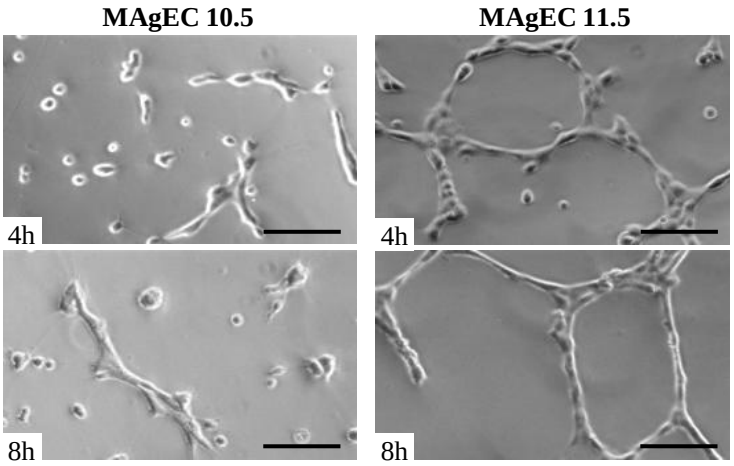
(A) Immunocytochemical staining of MAgECs 10.5 and 11.5 with anti-vWF, anti-ACE, anti-CD31 and anti-VE-Cadherin antibodies (green). Scale bare = 20 µm. (B) Table of flow cytometry results representing the difference of the mean fluorescence intensity between the selected antibodies as described in materials and methods and isotype controls, for the two MAgECs lines 10.5 and 11.5. Results are expressed in ΔIF ± SEM. (D) VEGF-A production by MAgECs 10.5 and 11.5 cells cultured 24 hours under normoxia condition (21 % O_2). Production was measured by ELISA. The results are reported to 10^6 cells and per hour (h). Values are mean ± SD (n = 3), (results from K. Szczepanek, W. Nowak, K. Szade and G. Collet).

MAgECs characterization profile was completed at mRNA level looking for angiogenic factors potentially secreted. It revealed the presence of PDGF-A, -B, VEGF-A, -B, -C, -D, and the angiopoietin-1 and -2 transcripts. These molecules are involved in the blood vessels formation and in the vascular remodeling (Figure 48B). As VEGF-A is one of the most important pro-angiogenic factor it was assessed. As presented on the Figure 49C, its production by the MAgECs cells was estimated by the ELISA method in the supernatant showing a detectable amount found only for the MAgECs 11.5. This observation confirms the mRNA expression levels (Figure 48B) and is also in favor of a more advanced differentiation stage in the endothelial differentiation. Such secretion of pro-angiogenic factors is very important for their involvement in angiogenesis but also in the maturation of precursor cells [269].

Altogether, both cell lines present features of non-hematopoietic mesenchymal like stem cells and endothelial precursor cells as well, without strict belonging to one defined category yet.

3.3.3-MAgECs 10.5 and MAgEC 11.5 differ in their *in vitro* angiogenic potential

To investigate the angiogenic potential of MAgEC, cells were seeded on a Matrigel matrix. MAgEC 11.5 generated tube-like structures and networks within 4-8h as shown in figure 50 (right), confirming that they are more engaged towards the endothelial cell differentiation. On the opposite, MAgECs 10.5 did not form networks as shown in figure 50 (left) where there is no closed structure but only cell contacts and few pseudovessels. This is also in favour of a more immature phenotype.



<u>Figure 50:</u> Tube-like structure formation on Matrigel by MAgECs 10.5 and 11.5 cell lines

Representative picture of tube formation from Matrigel-plated MAgEC cells. As described in materials and methods, MAgEC 10.5 and 11.5 were seeded on Matrigel and incubated for up to 24 h. Scale bars represent 50 µm (results from K. Szczepanek).

Then, a second angiogenesis assay was performed mixing the MAgECs 10.5 and 11.5 together but also with a mature endothelial cell, to assess if they are able to communicate and to interact together. As represented on the figure 51, the MAgECs 10.5 (green) participate to the tube-like structures formed by the MAgECs 11.5 labelled in red.

Consequently MAgECs 10.5 that are not able to achieve the angiogenesis process by themselves, can participate and be incorporated into an ongoing angiogenic network. This has a strong potential for future *in vivo* angiogenesis intervention. MAgECs10.5 cells could thus be recruited and be involved in new vessel formation as shown here *in vitro*.

This result is in favour of a communication between the two kinds of cells and reveals the possibility for the MAgECs 10.5 to cooperate with more differentiated endothelial cells as they do with the MAgECs 11.5 cells.

Similarly, MAgECs cells were mixed with a model of microvascular mature endothelial cell called MLuMEC (established previously from FVB mice lungs). Figure 51, shows that MAgECs 10.5 and 11.5 are able to take place in the tubulogenesis process initiated by the MLuMEC and participate to network formation. Moreover the same observation was done when all 3 cells were mixed which confirms the cross-talks between cells to form the pseudo-vessels.

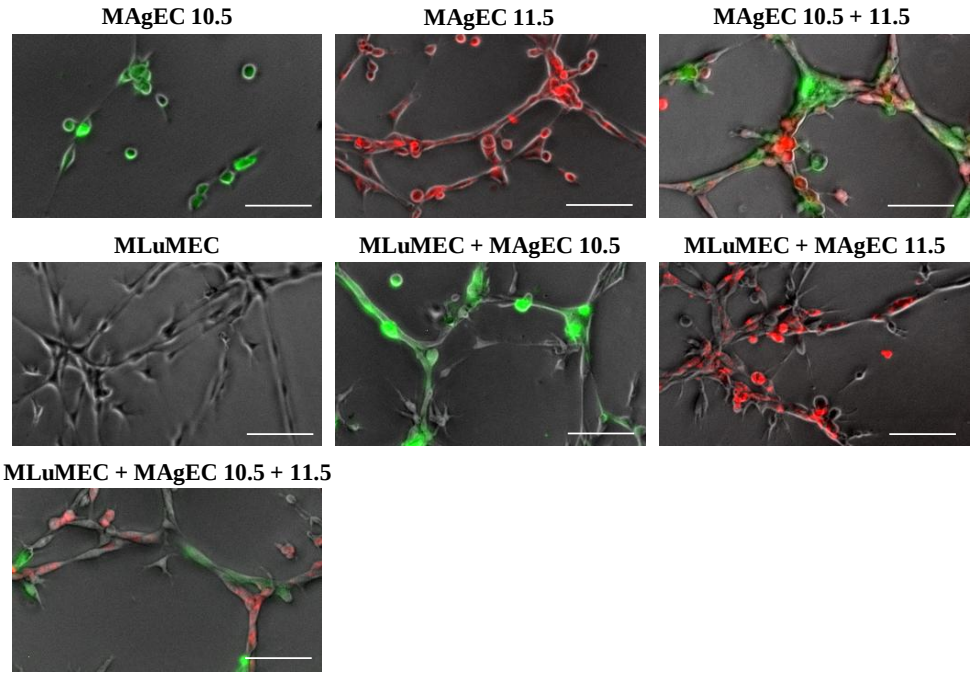


Figure 51: Tube-like structure formation: cooperation between MAgECs 10.5, 11.5 and MLuMEC cell lines
Representative picture of tube formation from Matrigel-plated cells. As described in materials and methods, cells were seeded on Matrigel and incubated for up to 24h. The MAgECs 10.5 (green) and MAgECs 11.5 (red) were labelled with the lipidic dye, DiO and DiI respectively. The MLuMEC were unlabeled. Pictures correspond to best networks formation for each condition. Scale bars represent 50 µm (results from K. Klimkiewicz).

Altogether, these results show the potential that MAgECs cells have to participate in the blood vessel formation. Furthermore, this collaboration to form neo-vessels does not seem to be affected by the degree of differentiation of the MAgECs cells.

3.3.4-Contribution of MAgECs to *in vivo* angiogenesis

After observation of *in vitro* angiogenesis on Matrigel, further investigation was performed to validate MAgEC cells as an EPC cell model and demonstrated their functionality by assessing their capacity to make vessels *in vivo.* To answer this question, a Matrigel-plug assay was performed. MAgECs cells were mixed with Matrigel prior subcutaneous grafting. 10 days later, *in vivo* ultrasound imaging allows appreciating the plug shape under the skin (Figure 52A). Morevover, when coupled to Color Doppler Imaging modality which provides a visual overview of flow within the vessels, the ultrasounds revealed that blood flow was established in the plug mixed with MAgECs 10.5 and 11.5 cells

as well, when compared to empty plug. This method does not provide a quantitative overview which could be obtained with the power Doppler mode. Immediately after imaging, plugs were taken out of mice for pictures (Figure 52B) in order to correlate the *in vivo* imaging with the macroscopic observation of potential blood vessels or traces of blood. Especially in the case of plug mixed with MAgECs 10.5, the Doppler signal fits perfectly with the macroscopic observation.

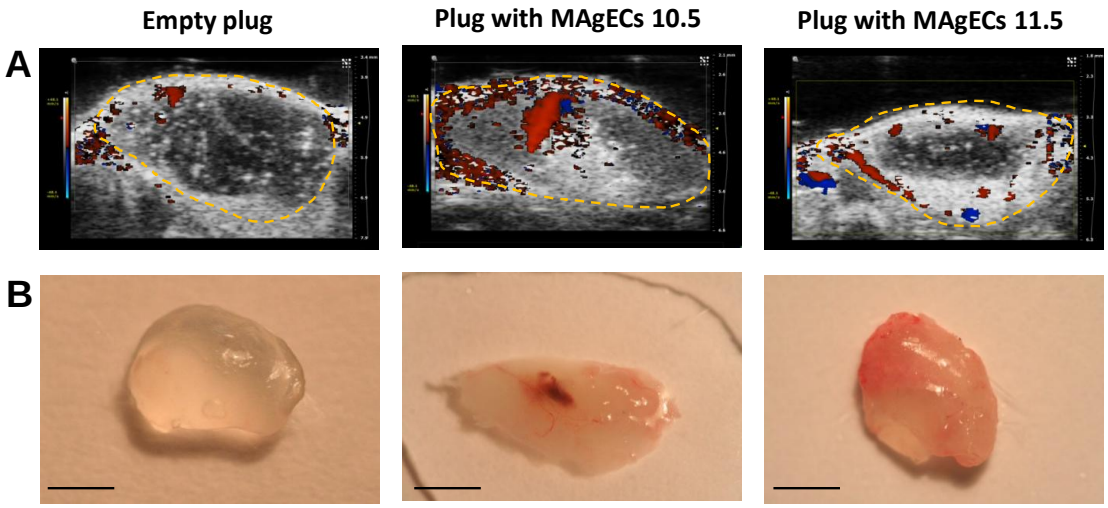


Figure 52: ***In vivo*** **angiogenesis of MAgECs 10.5 and 11.5.**
(A) Representative pictures of ultrasound imaging (echography) combined with Doppler mode of either empty plug, or plug mixed with MAgECs 10.5 or 11.5. The red and blue color allows discriminating if the flow is going away from the transducer (blue) or toward (red). (B) Macroscopic pictures of plug out of mice. Scale bare represents 1cm (Results from K. Szade and G. Collet)

Altogether, these promising results obtained by such model, suggest the efficient contribution of MAgECs cells to *in vivo* angiogenesis. However, the question that remains to be answered is whether the MAgECs takes really part in the new blood vessels that are developing in the Matrigel-plugs or whether increase recruited angiogenic cells by secretion of proangiogenic signals. Histological studies could answer, using a model of MAgECs-GFP cells (10.5 and 11.5), permiting to discriminate MAgECs from other precursor cells.

3.3.5-*In vivo* recruitment of MAgECs in a neo-angiogenic site

For the above described reasons, the MAgECs cells were further evaluated for their potential to be recruited *in vivo,* into a neo-angiogenic site when intravenously injected. To

assess for this targeting, GFP^+-MAgECs 10.5 and 11.5, were established by stable transfection with a GFP-encoding vector, and injected in the blood stream of Matrigel-plug bearing mice (Figure 53 B-E).

Flow cytometry analysis was used to estimate the sites where GFP^+-MAgECs were homing. Analysis of blood borne cells revealed that 10 days post injection, the GFP^+-MAgECs cells had left the blood stream and very few cells, in the range of 0.4-0.5‰ for both MAgECs 10.5 and 11.5, were still detectable, (Figure 53A). The lungs were analyzed to verify the post injection clearance and to confirm the availability of MAgECs cells to enter neo-angiogenic sites induced by the Matrigel plug and their recruitment into newly formed blood vessels. It is admitted that after intravenous injection, cells are stopped in small capillaries of the lungs for 12 to 48h. They can then re-circulate into the blood stream. This clogging, linked to injection, should be taken into account because it could interfere with targeting in decreasing their amount as blood circulating cells. Our data reveal that the two kinds of MAgECs, 10.5 and 11.5, left the lungs since, by the end of the experiment, only 0.1-0.2‰ of the injected cells were found associated to the lungs (Figure 53). This indicates that recirculation from lungs to blood could occur. The bone marrow (BM) was checked because of its fenestrated endothelium which permits the accumulation of injected cells in this compartment. The detection of GFP^+ cells in the bone marrow reveals comparable data to lungs (around 0.1-0.2‰) and confirmed that MAgECs were available for neo-angiogenesis and eventual recruitment into the Matrigel plug. In plugs, quantification of the recruited MAgECs indicated that 15 to 35 fold more GFP^+-MAgECs were found compared to other analyzed compartments, to reach an average of 2.5‰ cells. No significant differences were shown between the MAgECs 10.5 and 11.5, but both appeared to be effective tools for angiogenic site targeting. Moreover, our results are minimized due to the poor accessibility of the Matrigel plug and to the duration of the experiment over 10 days.

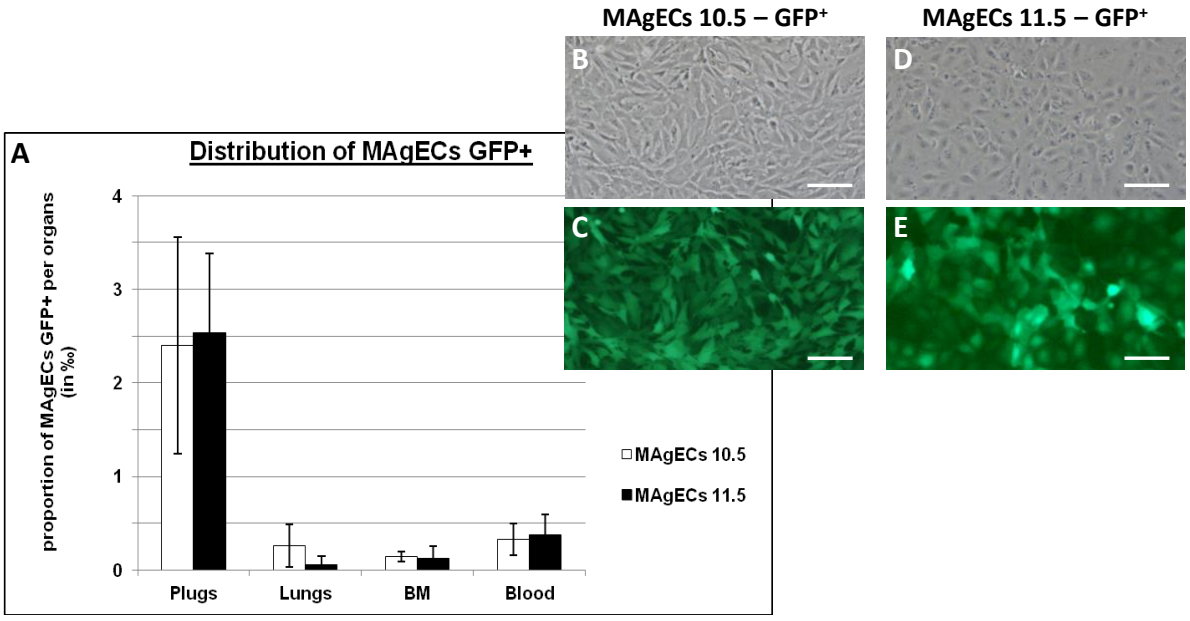


Figure 53: Distribution of systemically administered MAgECs-GFP$^+$ in Matrigel-plug bearing mice.
(A) Flow cytometry data of the percentage of MAgECs-GFP$^+$, 10.5 and 11.5, found in distinct organs of mice and Matrigel-plugs after tissue dilaceration, including plugs, lungs, bone marrow (BM) and blood collection. Results are expressed in ‰ of total cells from each tissue. Values are mean of 3 mice ± SD. (B-E) Representative picture obtained in fluorescence microscopy of GFP expressing stable cell lines of MAgECs 10.5 and 11.5, in bright field (B and D) and green fluorescence (C and E). Scale bare represent 50 µm.

These preliminary data demonstrate that the MAgECs 10.5 and 11.5, are efficient to reach a neo-angiogenic site in adult mice, and that they can be used as carrier cell for specific therapeutic delivery.

Comparatively to previously exposed results, a histological confirmation is required to conclude, showing that GFP$^+$ cells located into the plugs are found in the vessels walls of the newly formed vasculature.

3.3.6-MAgECs migration toward B16F10 melanoma spheroids quantification

To evaluate the MAgECs as a targeting tool to reach the tumor, an *in vitro* experiment was developed based on the method published by Beckermann *et al.* in 2008 [270].

In this method, an *in vitro* micro-tumor was made from the tumor cells of our study model: the B16F10 murine melanoma cells. As presented on the Figure 54, these micro-

tumors called spheroids are spherical entities around 250 µm in diameter which are used to mimic a tumor. They are placed in a 3D collagen/methylcellulose matrix and mixed with the cells to study, i. e. the MAgECs cells, as illustrated on the Figure 54. Then, it is possible to evaluate along the time the incidence of the secreted signals on the surrounding MAgECs cells and their behavior. It should allow answering the question whether they are to respond to soluble signals coming from the spheroids and follow the formed gradient to reach the spheroids. In the case of oriented migration toward a spheroid, the distance traveled and the speed can be quantified, as well as the cell recruitment in the spheroid.

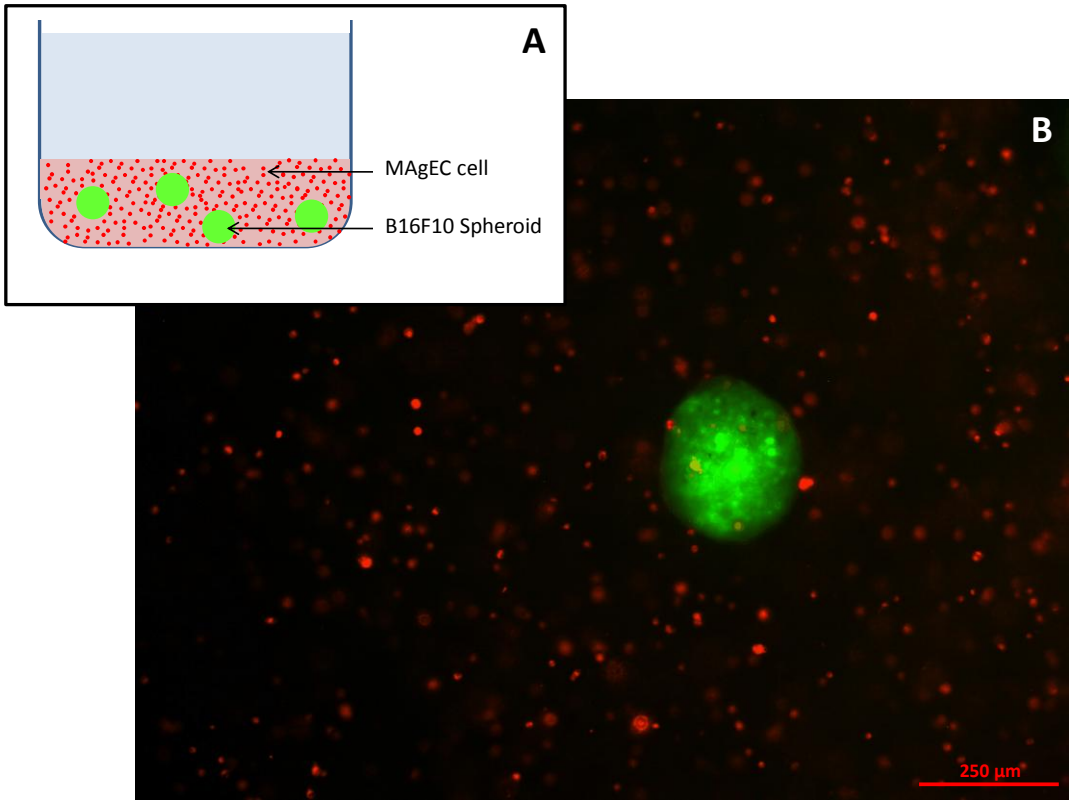


Figure 54: ***in vitro*** **3D migration assay**
(A) Scheme of a transversal view of the collagen/methylcellulose matrix containing B16F10 melanoma spheroid in green and MAgECs in red. (B) Representative picture obtained by fluorescent microscopy of one spheroid (green) included into the matrix with MAgECs cells around (red). Scale bare = 250 µm.

Stained in red with a lipidic dye (DiI), the MAgECs are monitored for 24h in fluorescence microscopy, spheroids are stained in green (DiO).

Results from the MAgECs 11.5 migration (Figure 55) were analyzed to obtain both the distances traveled by the cells toward spheroids and their mean speed (calculation details in material and method). When compared to fixed spheroids which cannot produce factors such

as cytokines or chemokines (negative control), live spheroids secrete chemo-attractant factors as indicated by the migration of MAgECs 11.5 toward the spheroids. Both speed and distance are in favor of B16 melanoma factors being sensed by the MAgECs 11.5 which make them good candidates for B16 melanoma targeting.

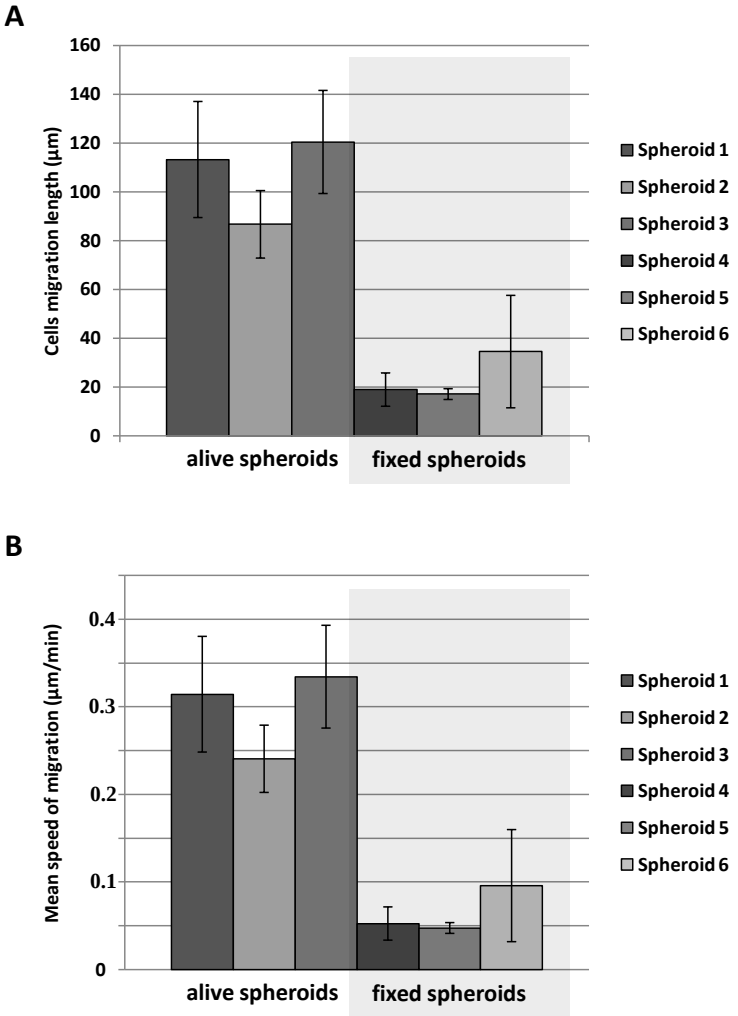


Figure 55: ***In vitro*** **study of the MAgECs 11.5 migration toward B16F10 spheroids**
Analyzed with the "cell tracker" pluggin of ImageJ software, (A) represents the distance travelled by the MAgECs 11.5 in the matrix, and (B) the mean speed. 3 spheroids were analyzed in "alive" condition, compared to "fixed" condition. For each spheroid, 5 cells were tracked along time to determine the presented features. The experiment was recorded during 8 hours (N > 5).

MAgECs cells recruitment on the B16F10 spheroids could be further estimated. The colocalized pixels could be estimated as a function of time between the two fluorescent channels, red and green, respectively MAgECs 11.5 and B16F10 spheroids (details are provided in the material and method). The top panel from the Figure 56 (A, B, and C) shows

the main step of the process with, in A, the merge of the two channels, in B the determination of colocalized pixels which appear in blue, calculated with the ImageJ software and its "RG2B colocalization" pluggin. In C the extracted colocalized pixels are displayed in grey scale to take into account their intensities. Figure 56D presents the results of 3 independents spheroids and for each the integrated density of colocalized pixels at 0, 6 and 12h. As previously explained, alive spheroids are active as opposed to fixed spheroids. Data reveals an efficient MAgECs 11.5 cells recruitment on the B16F10 spheroids, even though important variations exist between the 3 spheroids.

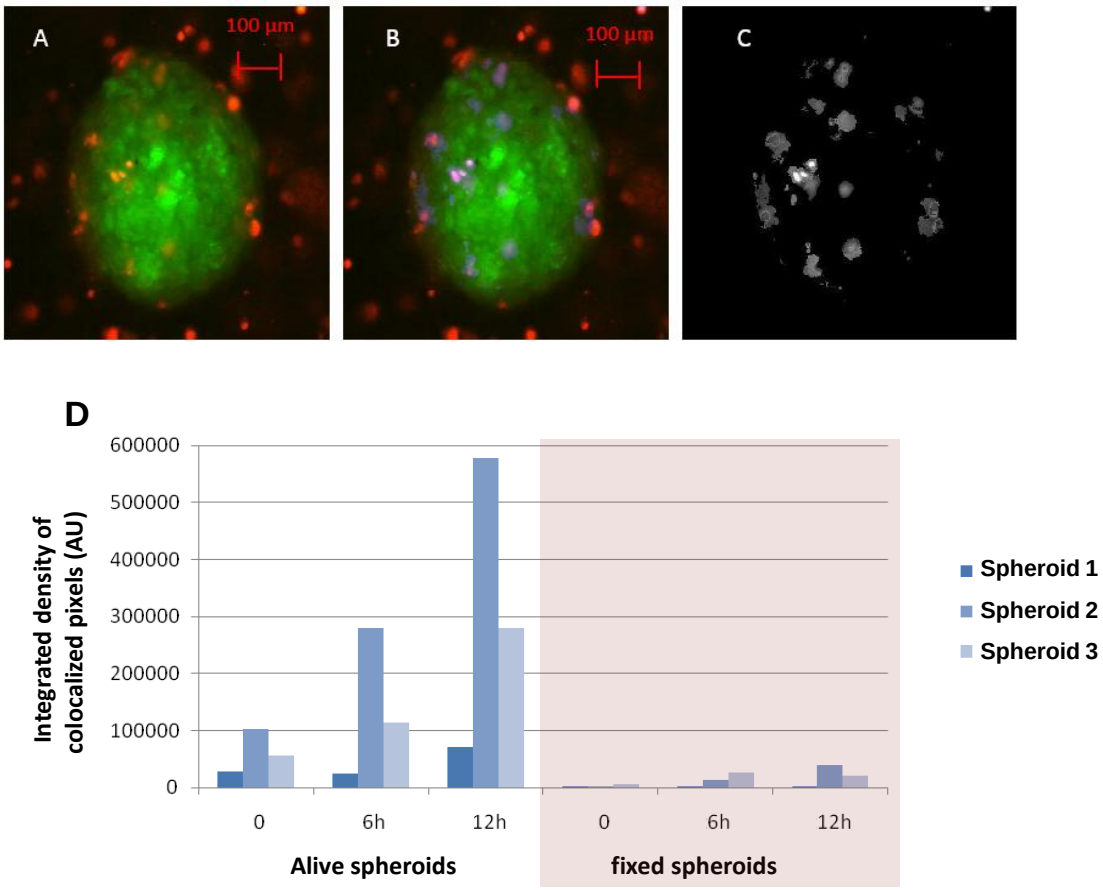


Figure 56: ***In vitro*** **study of the MAgECs 11.5 recruitment on B16F10 spheroids**
Analyzed with the "RG2B colocalization" pluggin of the ImageJ software. (A) is a merge of the pictures of fluorescence microscopy with the MAgECs 11.5 cells labeled with DiI (red) and the B16F10 spheroids labeled with the DiO (green). (B) is the determination of colocalized pixels which appear in blue. (C) represents the extraction of all colocalized pixels displayed in grey scale. (D) represents the results of 3 independents spheroids and for each the integrated density of colocalized pixels at 0, 6 and 12h. 3 spheroids were analyzed for either the "alive" condition, compared to "fixed" condition. (N > 10).

These representative data were repeatedly obtained and analyzed qualitatively. Taken together, these data report that the MAgECs 11.5 are sensitive to B16F10 melanoma secreted signals. Indeed, they are able to “feel” the chemo-attractant factors secreted, to migrate toward the B16F10 spheroids to be recruited by it. Moreover, when compared to fixed spheroids, which are not able to produce chemoattractant factors, almost no migration was observed indicating that the mechanism is active.

Because of huge variations between spheroids, the study should be extended to a bigger number of spheroids for statistics and the same study should be done with the MAgECs 10.5 as well.

3.4-Conclusion

It is now well admitted that EPCs provide a valuable tool for tumor targeting because of their ability to be naturally and specifically recruited to sites where active angiogenesis takes place and of the possibility to make them carry therapeutic drug or gene [27].
Our purpose in selecting a good cellular candidate was to obtain the early precursor committed to endothelial phenotype during embryonic development. This was supposed to allow the determination of the maturation step at which endothelial precursor cell represent the best candidate to control angiogenesis.

As the first cells restricted to the endothelial lineage appear in aorta-gonad-mesonephros (AGM) of mouse embryo [265], cells were isolated from that region [271]. In this aim, cells were isolated from 10.5 and 11.5 days post conception (dpc) embryos, when they diverge from hemangioblasts. Two cell lines were established, called MAgECs 10.5 and MAgECs 11.5. Established cell lines should facilitate their use as cell models for their phenotypic stability as compared to isolated EPCs and MSCs.

Characterization showed that such early precursors could be classified as EPCs although they did not share all described features. They clearly were differently committed towards the endothelial type. They indeed displayed progenitor endothelial characteristics, in terms of phenotype and angiogenic properties, reflecting their distinct maturation stage. They cooperate with mature endothelial cells in the formation of angiogenesis network. They are potentially good candidates for the delivery of therapeutic genes. They should provide an effective cell model of EPC *in vivo* according to data obtained by *in vitro* and *in vivo* investigations reporting their ability to home specifically into neoangiogenic sites.

Moreover, the sensitivity of these cells to B16F10 melanoma-secreted signals was observed *in vitro* with a model of microtumor showing active cell migration toward the tumor site.

Still in progress and requiring complementary experiments, these promising data announce the MAgECs as good model of cell carrier for the expected cell-based gene therapy presented in this project.

4-Construction of the cell-carried therapeutic gene

3.1-Introduction

To complete the Trojan Horse, this last part of results presents the first step leading to assembling the designed vector presented in part 2 and the carrier cell presented in part 3. The purpose was to load the therapeutic vector inside the MAgECs cells as they were shown to represent a potentially good candidate for *in vivo* tumor targeting. Experiments presented below are still in progress.

4.2-Materials and methods

4.2.1-MAgECs transfection for stable cell lines establishment

To introduce vectors (pHREmsVEGFR2, pIFP1.4-HREmsVEGFR2, and IFP1.4_pcDNA3.1H.ape) into MAgECs to establish stable cell lines, various methods were used. Physical methods including electroporation with Neon™ transfection system (Invitrogen) as well as Nucleofector® II (Amaxa Biosystems, Germany) were performed according to producer's advices with recommended technical setting for optimization. For chemical transfection, we used the cationic polymer, jet-PEI, jet-PEI-HUVEC (Polyplus Transfection, France), "superfect" (Qiagen, Chatsworth, CA) and the cationic lipid "lipofectin" and "lipofectamine" (invitrogen, Carlsbad, CA), checking for each different ratio for optimization.

After recovery, hygromycin was applied to select transfected cells. Hygromycin concentration was specifically adapted to each cell line and adjusted according to control cells. 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 % O_2) using ELISA method (R&D DY1558B).

4.2.2-Quantification of msVEGFR2 and VEGF-A protein production

The msVEGFR2 and the VEGF-A were quantified from cell culture supernatant by ELISA as described in part 2.2.5 and 3.2.7.

4.3-Results

4.3.1-MAgECs "arming" by the pIFP1.4-HREmsFlk1 vector

In order to establish stable cell lines of "armed" MAgECs 10.5 and 11.5, cells were transfected by the vector pIFP1.4-HREmsVEGFR2 (Figure 57) coding for the soluble form of the VEGF rececptor 2 with its expression driven by hypoxia and the IFP1.4 and mCherry fluorescent protein for imaging.

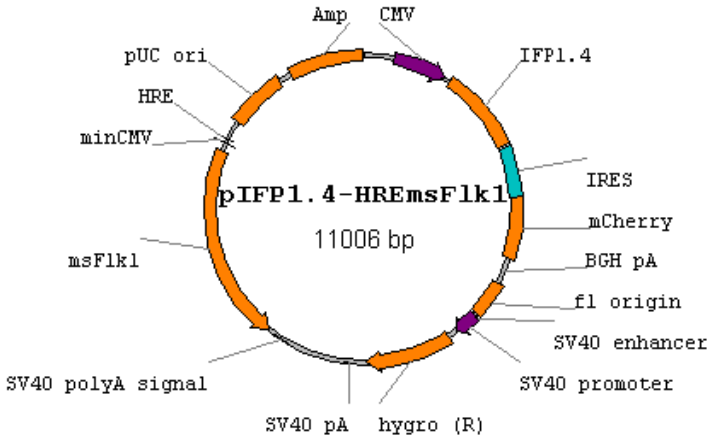
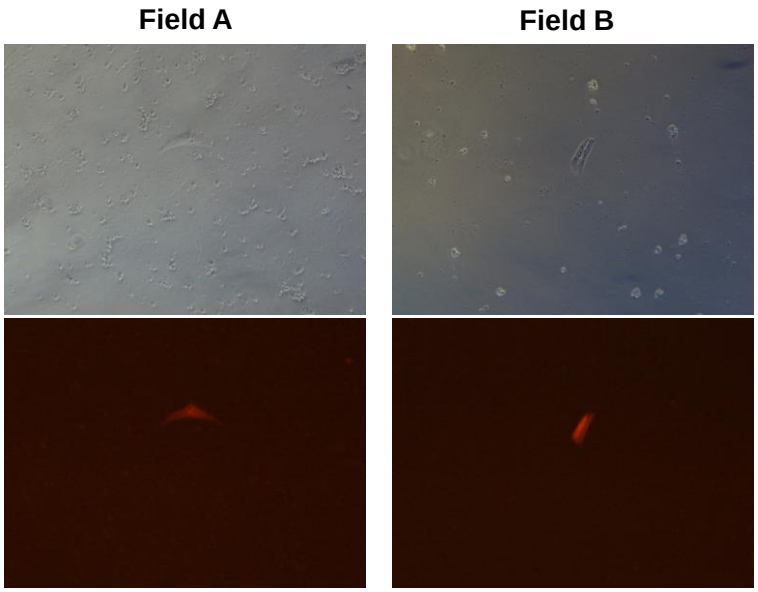


Figure 57: pIFP1.4-HREmsFlk1 vector map.

However, upon completed transfection and selection process, no clone was observed, despite the various transfection techniques employed. Indeed, physical methods including electroporation as well as nucleofection were performed according to producers's advices with various recommended technical setting for optimization. Chemical transfection methods have been assessed too, using the cationic polymer, jet-PEI, jet-PEI-HUVEC described as more adapted for endothelial cells as well as the "superfect" and the cationic lipids "lipofectin" and "lipofectamine" verifying for all of them various ratio DNA/transfection reagent.

The best observations obtained from all enumerated methods are presented on the figure 58. Two representative fields, A and B, show transfected cell expressing the mCherry fluorescent protein. These very rare transfected cells able to survive both transfection and selection, were not dividing any longer upon vector expression.



<u>Figure 58:</u> pIFP1.4-HREmsFlk1 transfected MAgECs cells.
Fluorescence microscopic observation of MAgECs cells after transfection and hygromycin selection. Top panel represent the brightfield pictures whereas the bottom panel represent the mCherry fluorescent channel pictures.

The mCherry toxicity, even weak, can explain the troubles to obtain stable cell lines. The IFP1.4 is not documented at all since its publication in *Science* in 2009 by R.Y.Tsien team [33]. All experiments were performed in normoxia, consequently the potential toxicity brought by sVEGFR2 can be excluded because of the inducibility of the HRE-minCMV construction and the leakage was really low in previous validations presented in the figures 34 and 35 of part 2.3.2 and 2.3.3 respectively.

To discriminate between the influences of the two main vector components, the intermediary vectors pHREmsVEGFR2, and IFP1.4_pcDNA3.1H.ape were added to a new set of transfections. The pHREmsVEGFR2 vector (Figure 59A) is expressing only the

sVEGFR2 when cultured in hypoxia whereas the IFP1.4_pcDNA3.1H.ape vector (Figure 59B) is constitutively expressing both IFP1.4 and mCherry.

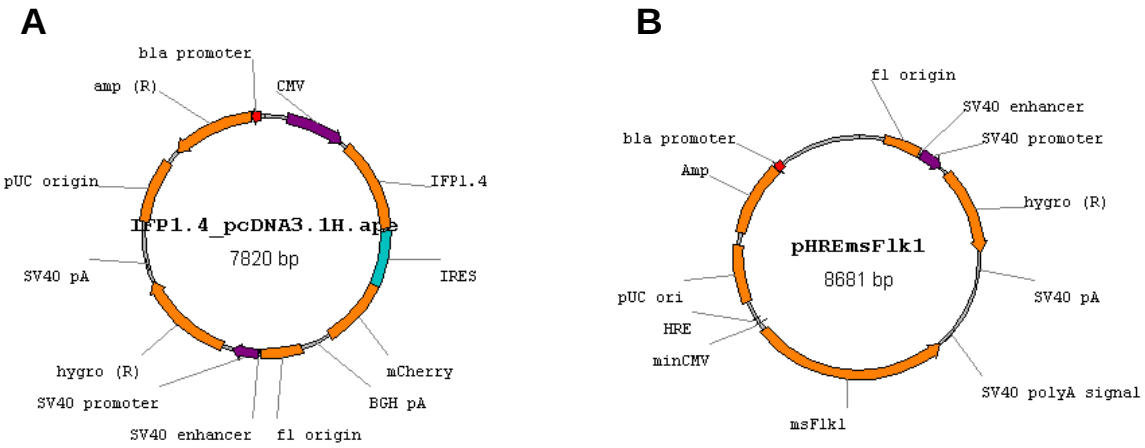


Figure 59: IFP1.4_pcDNA3.1H.ape and pHREmsFlk1 vectors maps.

Not presented here, the transfection results for IFP1.4_pcDNA3.1H.ape vector were comparable to the above described results (Figure 58) without IFP/mCherry^{+} positive cells that could be viable and dividing along the time. This observation suggests clearly a too high toxicity brought by the fluorescent proteins IFP1.4 and mCherry. Cells transfected with the vector pHREmsFlk1 only coding the msVEGFR2 are viable and efficiently transfected.

Efficient on MAgECs transfection and giving satisfactory numbers of transfected cells revealed by hygromycin resistance, the lipofectin reagent was kept for further transfections. Further checked by ELISA, msVEGFR2 produced after 48h of culture in hypoxia (1% O_2) indicated that a detectable amount of msVEGFR2 was produced.

4.3.2-Validation of pHREmsFlk1 vector "armed" MAgECs

Despite the toxicity linked to IFP1.4/mCherry expression, the pHRE-msFlk1 (Figure 59B) remains adapted for MAgECs arming. Loosing the imaging modalities renders the approach less adapted for cell targeting imaging but the therapeutic approach is conserved.
Thus the selected but still heterogeneous cell populations, that were transfected with the pHREmsVEGFR2 and consequently expressed the msVEGFR2 in a hypoxia regulated manner, were submitted to a single cell cloning. Starting from one cell, this process allows establishing a stable cell line.

Consequently a screening was performed for the different clones obtained, both for MAgECs 10.5 and 11.5, quantifying by ELISA the production of msVEGFR2.

Presented on Figure 60, one part of the screening results (others not presented) indicates the best clones among both MAgECs, 10.5 (denoted clone 2.2) and 11.5 (denoted clone 131) transfected cells. Producing the higher amount of msVEGFR2 in hypoxia for a limited leakage in normoxia, these two clones were kept for further validations.

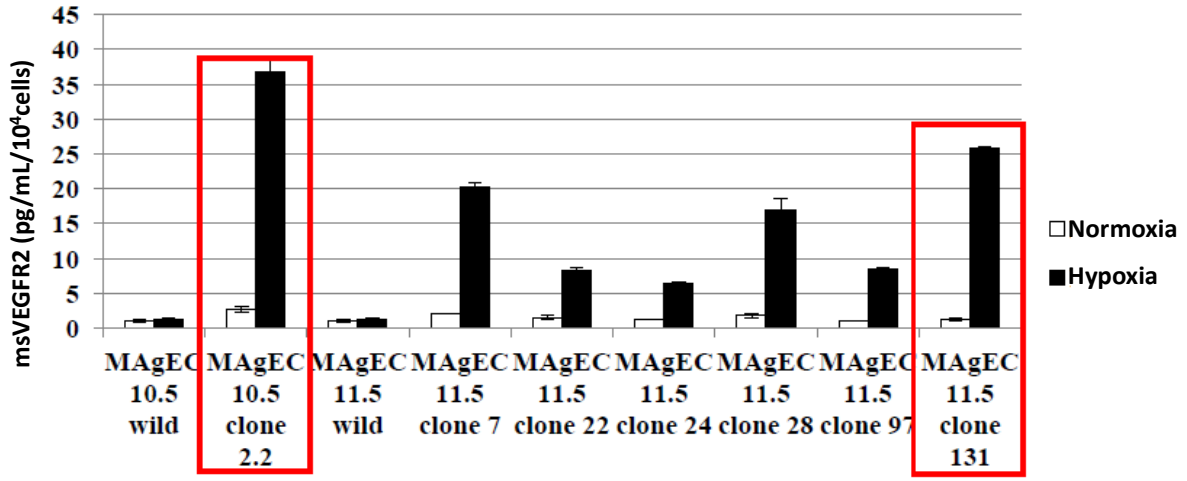


Figure 60: Screening of MAgECs-HREmsVEGFR2 for msVEGFR2 production.
msVEGFR2 production by different clones of pHREmsVEGFR2 stably transfected MAgECs 10.5 and 11.5 cultured 48 hours under normoxia (21 % O_2) or hypoxia (1 % O_2). Production was measured by ELISA. The results are reported for 10^4 cells. Values are mean ± SD (n = 3). Red square indicates the two best MAgECs candidates, 10.5 and 11.5, for hypoxia driven msVEGFR2 expression

Among further validations, the VEGF production should be quantified, using methods that allow discriminating the free among total VEGF, part of which being linked to msVEGFR2. Specific ELISA kit, should allow verifying the free amount of msVEGFR2 and VEGF produced to estimate the trapping efficacy, *in* vivo.

Moreover, these transfected cells should be also validated for their targeting properties to be sure that they are not altered by the transfection process.

4.4-Conclusion

In this last part of results showing that it was possible to combine the therapeutic vector to carrier cells, we describe two cell lines that were successfully established from both MAgECs 10.5 and 11.5 and expressing msVEGFR2 driven by hypoxia. Quantification allowed their validation for the proper regulation by hypoxia (1% O_2) which should favor *in fine* the expected preferential expression in hypoxic tumors.

Further validations are required to secure the fact that the msVEGFR2 production by the MAgECs is not already occupied by their endogenous VEGF and also to confirm that the transfection did not affect the targeting properties.

Too sensitive for IFP1.4 and mCherry expression, the MAgECs were loaded with the vector encoding only the msVEGFR2. Nevertheless, the full vector pIFP1.4-HREmsVEGFR2 can be used for other applications in different cell carrier as proven for HEK and B16 F10 cells. The IFP1.4, encoded near infrared fluorescent reporter, could allow the permanent cells visualization as *in vivo* tracker to follow the cell distribution.

5-Discussion

This work was undertaken on the basis of the demonstration by Asahara (1997) that circulating EPCs can specifically be recruited and participate to angiogenesis development [22] and on the numerous possibilities this offers to therapeutic use [139]. For tumor therapy, EPCs appeared to be a tool of choice for angiogenesis targeting. Their use to reach the tumor site should prevent the lack of specificity observed with classical therapies and consequently should reduce potential side effects. This interesting targeting modality provided by homing cells is explored in this work to take advantage of it to make them carriers that would specifically deliver a therapeutic gene to tumor site. Included in the so-called "Trojan Horse" approaches, the choice of endothelial precursor cells should allow the long term expression of therapeutic genes.

It was decided in this project to use natural angiogenesis process in order to act on pathological angiogenesis: EPCs routing towards tumor to deliver a therapeutic gene. Due to their quiescent character in normal conditions, circulating EPCs, when incorporating into the endothelium, should be good candidates to allow long-term expression of the therapeutic genes.

Overproduced by the tumors, the VEGF is a major actor of neo-vessel formation as well as vessel permeability, thus being a primary target. A soluble form of the VEGFR2 was chosen to neutralize the VEGF excess (VEGF-trapping).

VEGF as a target has been already described for anti-angiogenic therapies and targeted in many therapeutic approaches (antibodies, decoys, traps and small molecule inhibitors). Recent data reveals that, without tight regulation in the therapeutic process, anti-angiogenic molecules lead to adverse effects because of continuous activity destroying the tumor blood vessels and reducing the potential of drug delivery and efficiency of radiotherapy. Inducing harsh acidic and oxygen-deprived conditions, it leads to the selection of highly resistant tumor cells called cancer stem-like cells. This fact may explain some therapy failures, enhancing tumor invasiveness and metastasis [11, 13, 272].

Consequently, new anti-angiogenic therapies were developped with the aim to normalize the vessels, making mature and functional the chaotic and inefficient tumor vessels. It should then improve the drug delivery as well as radiotherapy.

Such approach requires taking into account the specificities of tumor microenvironment and its regulation. Thus, therapeutic genes like traps for angiogenesis inducers such as angiopoietins and VEGFs should be conditionally expressed in the tumor context.

The first part of the manuscript shows the design of a therapeutic vector encoding a VEGF-trap, the msVEGFR2, its functional and regulated expression driven by hypoxia thanks to HRE-controlled promoter. The knowledge of the tumor microenvironment and its hypoxia were used as a safety-lock, using the HRE/HIF regulation system to drive the VEGF-trap expression. Able to be turned-on at low partial oxygen pressure, its expression is reversible in case of re-oxygenation, preventing all over-action and adverse effects and maintaining a long lasting normalized state.

An imaging modality was combined to the therapeutic VEGF-trap by the addition of an *in vivo* fluorescent protein chosen in the near infrared: the IFP1.4. The encoding sequence was joined to the vector to be constitutively expressed making the transfected cells visible via near infrared illumination.

Able to bind and neutralize the VEGF, its anti-angiogenic effects were validated *in vitro* and *in vivo.* Moreover, the presence of VEGF-Receptors 1 and 2 on the surface of murine B16F10 melanoma cells together with an effect of the msVEGFR2 on their proliferation underlined the fact that this therapy may act on both tumor and endothelial targets. Confirmed *in vivo,* when expressed by the tumor cells themselves, the vector leads to smaller tumor with an improved perfusion and efficient reoxygenation.

Moreover, treatments directed to endogenous VEGF-A blockade, are correlated with toxicity such as hypertension [273]. This might be limited by the described strategy due to the localized and regulated gene delivery, its restricted response to very 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.) [274, 275], a humanized variant of a VEGF neutralizing monoclonal antibody, the engineered vector can encode for a human sVEGFR2 protein that will not induce any immune response. Hypoxia-regulation assures the reversibility of expression and should allow its long tolerance. Consequently, it will help define the therapeutic windows for combined therapies. Moreover, some tumors are resistant to anti-VEGF therapies which may explain resistance to Bevacizumab [276]. As it is designed on the basis of the VEGF receptor 2, msVEGFR2 should naturally recognize and bind the

VEGF-A,–C, -D, and –E. Endothelium is the main tissue reacting to VEGF, consequently this strategy may be developed independently of the tumor cells reactivity to VEGF.

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 expression may create the conditions triggering the vessel normalization. Its reversibility would prevent the vascular destruction in normal tissues in case of vector leakage.

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. One should mention that the presented therapy is a different anti-angiogenic approach which, *in fine,* has to be carried to the tumor site for expression. The system is designed to bring the temporal regulation of the therapeutic gene and to be targeted by cells that will insure the spatial control of the gene expression.

Presented in a second part, further studies are dedicated to the appropriate cell carrier, able to target the tumor stroma and other pathologic sites of angiogenesis.

Historically, EPCs were initially identified and isolated in 1997 by Asahara *et al.* [22] on the basis of vascular endothelial growth factor receptor-2 (VEGFR2) and CD34 co-expression. Since this brief cell description and with the ongoing knowledge of stem cells combined to emergence of specific surface markers, numerous distinct stem and progenitor cell populations were identified. However, "specific" markers are chosen to facilitate the isolation and purification of these cells but their increasing number complexifies the definition of "true" EPCs among EPC-like cells. Generally admitted, the term ''EPC'' may encompass a group of cells existing in a variety of stages ranging from primitive haemangioblasts to fully differentiated ECs.

Despite controversial data on the EPC identity, characterization and classification [277-280], circulating endothelial precursors involvement, in the adult organism, in physiological and pathological processes was evidenced [281-286].

Characterization of EPCs remains complex. EPCs cells should express several markers, including VEGF receptor-2 (VEGFR2, KDR, Flk-1), VE-cadherin, CD34, platelet endothelial cell adhesion molecule (PECAM; CD31) and von Willebrand factor (VWF). They should also be able to bind acetylated low-density lipoprotein (AcLDL) and lectins such as BS-1 and ulex europaeus agglutinin-1 (UEA-1) which are usually considered as endothelial-specific markers [139, 287].

To validate a model of carrier cell, a first set of data describes the characterization of early EPCs, called MAgECs 10.5 and 11.5. To obtain early endothelial precursors, cells were isolated from aorta-gonad-mesonephros (AGM) region [265] at 10.5 and 11.5 days post conception (dpc). In this region of the embryo the first cells restricted to the endothelial lineage appear to be diverging from the hemangioblast as common precursor.

Their *in vitro* and *in vivo* effects showed that they behave as "true" EPCs, able to participate to blood vessels formation and to be recruited at neo-angangiogenesis site when systemically administered [22]. Moreover, preliminary *in vitro* results indicate that MAgECs cells are sensitive to secreted signals from B16F10 melanoma cells and that they actively migrate toward micro tumors. Complementary data are required as well as further *in vivo* evidence of tumor targeting. However, this first set of results is encouraging for the future use of such cells as carriers for the expected cell-based gene therapy of this project.

In parallel to deeper investigation on MAgECs cells, the "arming" step was initiated in order to complete the Trojan Horse by assembling cells and the therapeutic vector. For the time being, the MAgECs cells lines, 10.5 and 11.5, were both "armed" by stable transfection and able to produce the msVEGFR2 in response to hypoxia. Further validations will allow *in vivo* trials.

Described as a Trojan Horse approach, this regulated and targeted therapy may bring 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. Thanks to a hypoxia-regulated sequence and reversibility, the presented therapeutic vector should allow for a novel way of tumor angiogenesis normalization which should be durably installed. This achievement should help fighting cancer resistance, stem-like cancer cell selection and enhance the efficacy of combinatory chemo- and radio-therapy by hypoxia compensation [288, 289].

In that respect, our strategy opens opportunities to control pathology–specific and reversible expression of a therapeutic gene. This approach can be combined to cell targeting-based therapies or viruses-based therapies (i.e. oncolytic viruses or gene transfer). The flexibility of this strategy presents an invaluable advantage in view of future therapeutic *in vivo* applications [290, 291].

This work constitutes the basis for a proof of concept towards a microenvironment regulated gene therapy for the future use of an integrated gene-cell carrier model [292, 293]

which would provide new therapeutic avenues for normalization-based combinatorial cancer therapies.

6-General conclusion and perspectives

Over last decades a significant effort has been made in gene therapy approaches targeting tumor angiogenesis with a main focus on VEGF. Part of the so-called Trojan Horse strategies, a cell-mediated gene therapy approach was designed in the challenging purpose of reaching vessel normalization [14, 16]. This was obtained by combining the conditional and reversible HRE promoter with the sVEGFR2 as VEGF-trap [8] to construct the therapeutic vector and EPCs for targeting [24]. This combinatorial therapy should confer advantages on classical therapy by improving the ratio benefits/side effects and giving the possibility to be introduced into protocols for conventional therapies such as chemo and radiotherapy [31].

As presented in this manuscript, our work helps to bring a proof of concept opening new avenues for future therapies. Moreover, such approach can be expanded to other targeting cells such as MSC, macrophages or neutrophils. Regarding human applications, it is possible to think of cells isolated from bone marrow biopsies, and from the cord blood [260] then properly differentiated into targeting cells (EPCs, MSCs, …) and "armed" prior to systemic injection [25-27].

To enhance the therapeutic delivery and its robustness, more advanced approaches are being developed, combining cell-mediated gene therapy with virus-based vectors. Such activity- regulated genes, carried by cells that display selective tissue tropism, are known as very efficient to infect large amounts of cells locally. This combination aims to take the best from various strategies [294, 295]. Thus, even though a small number of cells home into the tumor site, the virus multiplicity considerably increases the therapeutic effect.

A second new option concerns the choice of the proper targets and the possibility to multiply and combine them in a same approach. These strategies affect the tumor cell or its environment from several approaches to improve the therapeutic efficiency. As example, Koh *et al.* engineered double antiangiogenic protein able to trap both VEGF-A and angiopoietins [10].

Ongoing trials are raising new targets such as the Notch receptors for therapies. The Notch pathway is central for controlling cell fate both during angiogenesis and selection of cancer stem cells in several tumors. It is involved in the modulation of cell proliferation, migration, survival/apoptosis, mobilization and differentiation of bone marrow-derived EPCs,

in affecting various cell types, ECs, tumor cells, CSCs, involved in various processes including angiogenesis or CSCs selection.

The exact nature of the role of Notch in angiogenesis is still under investigation. Interestingly, the selection between “tip" and “stalk” cell fate depends on the interplay between VEGF/VEGFRs and Notch pathways. They interact at several levels to generate a highly organized blood vessel network. The switch from “tip” phenotype toward “stalk” includes recruitment of pericytes to promote and stabilize the extending pseudo-vessels as well as lumen formation to allow blood flow. Controlled by Notch signaling, such balance can be directly linked to normalization and should be exploited to make of Notch signaling a tool to help achieving such strategies [296, 297].

In summary, treatments that cover various pathways should be considered in order to treat complex diseases as cancer. By improving our understanding of the tumor microenvironment and the setting of pathologic niches we should be able to design better future therapies.

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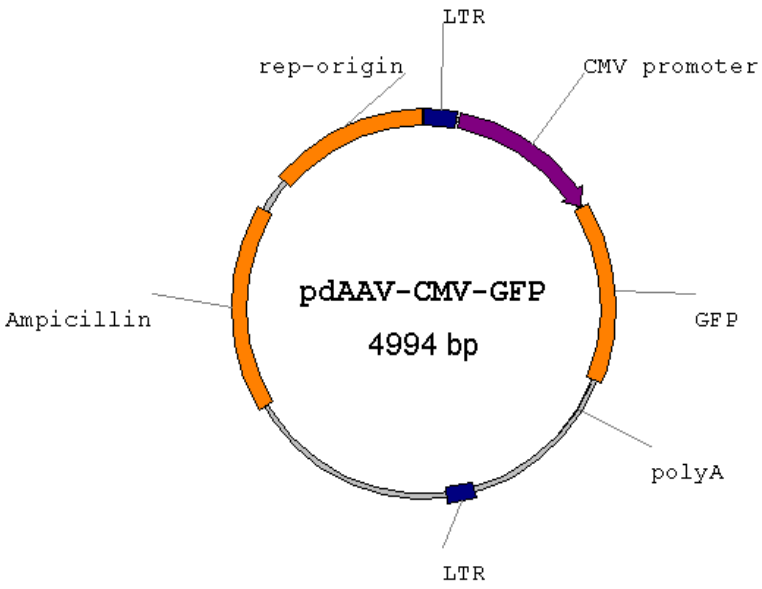
Annex 1

Primers table for qPCR.

gène	QIAGEN ref.	Entrez Gene ID	Detected transcript	Amplicon length
18S	QT01036875	19791	X00686	149 bp
actin B	QT00095242	11461	NM 007393	149 bp
GAPDH	QT01658692	14433	NM 008084	144 bp
PPIA	QT00247709	268373	NM 008907	119 bp
GUSB	QT00176715	110006	NM 010368	108 bp
HPRT1	QT00166768	15452	NM 013556	168 bp
actine B	QT01136772	11461	NM_007393	77 bp
B2M	QT01149547	12010	NM 009735	143 bp
ACE	QT00100135	11421	NM 207624	116 bp
CD34	QT00114107	12490	NM 133654	77 bp
CD31	QT01052044	18613	NM 008816	95 bp
VWF	QT00116795	22371	NM 011708	79 bp
VEGFR2	QT00097020	16542	NM 010612	133 bp
CD133	QT01065162	19126	NM 008935	101 bp
CD105	QT00148981	13805	NM 007932	65 bp
Tie-2	QT00114576	21687	NM_013690	105 bp
VE-cadh	QT00110467	12562	NM 009868	84 bp
VEGF-A	QT00160769	22339	NM 009505	117 bp
ANGPT1	QT00166859	11600	NM 009640	102 bp
ANGPT2	QT00173026	11601	NM 007426	150 bp
CD45	QT00139405	19264	NM 011210	96 bp
Sca-1	QT00293167	110454	NM 010738	90 bp
c-kit	QT00145215	16590	NM 021099	143 bp
CD29	QT00155855	16412	NM 010578	119 bp
CD49e	QT00114611	16402	NM 010577	82 bp
CD90.2	QT00245287	21838	NM 009382	84 bp
VEGF-B	QT01059863	22340	NM 011697	125 bp
VEGF-C	QT00104027	22341	NM 009506	91 bp
VEGF-D	QT00164024	14205	NM 010216	98 bp
PDGFA	QT00197610	18590	NM 008808	108 bp
PDGFB	QT00266910	18591	NM 011057	78 bp

Annex 2

Map of the GFP coding vector used to established the GFP+-MAgECs described in part 3.2.11 (designed by J. Stepniewski).



Annex 3

Current Prospects in Vascular Biology

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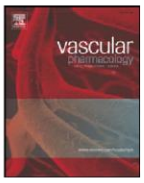

Review

Hypoxia control to normalize pathologic angiogenesis: Potential role for endothelial precursor cells and miRNAs regulation

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a b s t r a c t

Tumor microenvironment is a complex and highly dynamic milieu that provides very important clues on tumor development and progression mechanisms. Tumor-associated endothelial cells play a key role in stroma organization. They achieve tumor angiogenesis, a formation of tumor-associated (angiogenic) vessels mainly through sprouting from locally preexisting vessels and/or recruitment of bone marrow-derived endothelial progenitor cells. This process participates to supply nutritional support and oxygen to the growing tumor.

Endothelial cells constitute the interface between circulating blood cells, tumor cells and the extracellular matrix, thereby controlling leukocyte recruitment, tumor cell behavior and metastasis formation.
Hypoxia, a critical parameter of the tumor microenvironment, controls endothelial/tumor cell interactions and is the key to tumor angiogenesis development. Under hypoxic stress, tumor cells produce factors that promote angiogenesis, vasculogenesis, tumor cell motility, metastasis and cancer stem cell selection.
Targeting tumor vessels is a therapeutic strategy that has lately been fast evolving from antiangiogenesis to vessel normalization as discussed in this review. We shall focus on the pivotal role of endothelial cells within the tumor microenvironment, the specific features and the part played by circulating endothelial precursors cells. Attention is stressed on their recruitment to the tumor site and their role in tumor angiogenesis where they are submitted to miRNAs-mediated de/regulation. Here the compensation of the tumor deregulated angiogenic miRNAs – angiomiRs – is emphasized as a potential therapeutic approach. The strategy is to over express anti-angiomiRs in the tumor angiogenesis site upon selective delivery by precursor endothelial cells as miRs carriers.

Contents

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1. Introduction

The endothelial cell biology has recently pointed the importance of the interactions between blood vessels and other stromal components that guide vascular remodeling during development, healing, and pregnancy. In cancer, the same mechanisms are exploited for tumor stroma setting, the developing vessels and other stroma components respond to various signals that participate to tumor development and dissemination.

As a result of the fundamental observation by J. Folkman in 1971 on angiogenesis as a necessity for tumor survival and development (Folkman, 2002), the main antitumor targeted strategies were focused to the efficient destruction of this pathologic angiogenesis.

Angiogenic signals are induced by tumor hypoxic conditions. Endothelial cells (ECs) get activated to grow and detach from the neigh-boring cells by splitting their junctions. This permits EC progression towards proangiogenic factors thus distinguishing the leading tip cells from the stem cells of the new vessel. The forming tubes need to recruit pericytes to get matured and remodeled into a functional network (Carmeliet and Jain, 2011). Neovascularization also relies on the signals that tumor cells provide to distant sites as bone mar-row, which efficiently contributes to the initiation and evolution of the tumor vessels by mobilization and recruitment of endothelial pre-cursor cells (EPCs). It has been recently shown that this process depends not only on tumor cell signals but also on angiocrine factors from tumor endothelial cells attracting stem cells and endothelial precursors towards the site of angiogenesis (Butler et al., 2010; Lyden et al., 2001).

The active and bi-directional molecular cross-talk between tumor cells and host cells has profound implications for the understanding of stromal reactions and for any further anti tumor approach.

Consequently, tumors are no longer considered as mainly tumor cells but as a tissue comprising a stroma made of a matrix intimately interacting with tumor-associated and cooperating cells as fibroblasts, myeloid inflammatory cells and infiltrating lymphocytes. In addition to the continuously growing tumor cells these stromal cells are contributing to escalate the angiogenic response (Grivennikov and Karin, 2010). Tumor and stromal cells cross-talk enhances tumor growth, metastasis and alters response to anticancer therapy (Hu and Polyak, 2008). The recruitment of endothelial cells by a tumor to achieve angiogenesis is the key to further leukocyte-endothelial cell interactions within tumor microvasculature that mount the host antitumor immune response thus controlling tumor progression. Consequently, endothelial cells play a key role in shaping tumor microenvironment and controlling tumor development through angiogenesis (Kerbel, 2008). Targeting tumor vessels endothelial cells should provide survival advantages to patients with advanced cancers (Ferrara and Kerbel, 2005). This approach confirms the benefits of considering tumor microenvironment as a therapeutic target.

Although submitted to the tumor influence, the endothelial cells in tumor vessels are not transformed. As non-malignant cells they are more genetically stable and less likely to evolve into drug resistant phenotypes. New avenues opened by the antiangiogenic strategies were based on the features distinguishing pathologic tumor angiogenesis from normal vasculature. But, the efficient destruction of neoangiogenesis raised new pitfalls. Vessels become inadequate and tumor cells are located in areas of complete hypoxia and harsh pH conditions. They are submitted to strong pressure to select resistant cancer stem like cells that display high aggressiveness and invasiveness (Henze et al., 2011; Song et al., 2006). Consequently, new developments in anticancer strategies pay deep attention to the balance between tumor pro-angiogenic vs anti-angiogenic actions and favor therapeutic normalization rather than destruction of the vasculature (Jain, 2005). This review will focus on some strategies developed aiming to vessel normalization, paying special attention to the consequences on the tumor immune response. These new cellular and molecular targeting strategies of hypoxia compensation, if properly administered, may help radio- and chemotherapies (Goel et al., 2011). Because bone marrow-derived endothelial precursors cells recruitment at the tumor site of angiogenesis is an 'ideal" natural cell-based tumor targeting, these cells may provide a new tool to reach tumor angiogenesis and regulate it. A new regulation approach would take advantage of the potential control provided by the microRNAs (miRs) that are extensively described as highly active in modulating the angiogenesis-related processes. In the tumor, in response to hypoxia a number of miRs are deregulated and participate to pathologic angiogenesis. Consequently, in a therapeutic purpose, the over expression of miRs able to counteract the tumor angiogenic miRs, when selectively delivered by endothelial precursor cells should provide potent tools to regulate, rather than destroy, angiogenesis.

The advantages of tumor vessel normalization being established, some strategies will be described paying special attention to effects resulting from hypoxia compensation on the immune reaction against the tumor (cells, immuno modulatory cytokines, chemokines) and to the potential role/use of the endothelial precursor cells as carriers for the new regulatory tools that are the non-coding microRNAs.

2. Vessel normalization

2.1. Expected advantages of tumor vasculature normalization

Extensively used, efficient antiangiogenic agents have produced very interesting results. Because of their efficacy, these treatments showed that excess destruction of the vessels leads to the failure of treatment.

The complete review by (Goel et al. 2011) describes how beneficial can vessel normalization strategies be in cancer treatment as well as other diseases like diabetes (Goel et al., 2011). Deregulation of the vasculature is now a hallmark of cancer progression. It builds a vicious circle in which the production of proangiogenic factors due to hypoxic conditions in the tumor leads to the growth response of the endothelial cells to finally produce abnormal vessels. Those appear pathologic in terms of size, dilatation, and tortuousness of the networking as well as hyper permeability. Consequently, tumor oxy-gen delivery is irregular and inefficient. These parameters, together with heterogeneous blood flow and increase of interstitial fluid pres-sure inside the tumor, are contributing to cancer progression. The mechanistic pressures impair drug delivery, reduce chemotherapy and radiotherapy efficacy but also immunotherapy benefits and, altogether, favour the immune tolerance towards cancer (Palazon et al., 2012; Sato, 2011).

The Vascular Endothelial Growth Factors (VEGFs) produced (Leung et al., 1989; Senger et al., 1983) by the hypoxic growing tumor mass result from the stabilization of the HIF-1α transcription factor. These main angiogenic factors constitute the best targets for antiangiogenic treatments together with the regulation of the VEGF receptor 2 (Terman et al., 1992). Because VEGFA is the key factor responsible for the vicious circle that maintains angiogenesis pathologically activated and continuously growing, a large body of work devoted to the production of anti-

VEGF antibodies, as Bevacizumab (Ferrara et al., 2005) and inhibitors of VEGFRec phosphorylation as Sorafenib and Sunitinib, have brought an invaluable breakthrough in angiogenesis-related treatments.

This pointed to the transient normalization of tumor vessels that occurs during the course of the cure (Jain, 2003) but the further "success" of anti-VEGF treatment would lead to inadequate vessels with a destroyed structure. Extreme hypoxia appears then, to be a main characteristic of such microenvironment that induces tumor cells to adapt by setting a rescue process and select the most resistant cells to such harsh conditions in terms of lack of oxygen and low pH values (Carmeliet, 2000).

The concept of curing tumors by antiangiogenic treatments had then to be revisited and, on the contrary, present strategies are taking advantage of the therapeutic normalization windows, i.e. time periods during which vessels are transiently normalized (Jain, 2005). As shown with Trastuzumab in breast cancer, one of the main advantages of tumor vessel normalization relies on the possibility to take advantages of the therapeutic windows to apply chemotherapeutic drugs which then display improved efficacy because of their better penetration towards tumor together with an improved accessibility of the tumor cells (Jain et al., 2009).

In summary, the expected advantages of vessel normalization (Sato, 2011) are: decrease in permeability, interstitial fluid pressure and oedema which consequently increase tumor blood perfusion and oxygenation, altogether improving drug delivery.

Concomitant to the previously mentioned effects, the cancer cells are less likely to be shed and invasiveness is lowered. Movement and escape of metastasizing cells are blocked thus braking tumor progression and improving the therapeutic outcome.

2.2. Why should hypoxia be compensated in tumors?

Among the above mentioned advantages resulting from elevation of tumor perfusion/oxygenation the increased sensitivity to drugs and to radiations is essential to establish efficient protocols taking into account the therapeutic windows. It is noticeable that when hypoxia-mediated signalling changes, circulating cells recruitment also considerably changes which impacts the immune response towards the tumor.

Blood flow increase directly elevates the oxygen tension in the tumor. This accompanies the changes in the cross talk and signals between endothelial cells and the other cells of the tumor stroma. Deep changes in the vessel structure and properties are observed. The recovery from permeability, activity of VE cadherin and CD31 expression (Carreau et al., 2011b) as well as recruitment of pericytes/ mural cells (Sawamiphak et al., 2010) make the vessels functional. It improves drug delivery, cooperative effects with radiotherapy and results in deep changes in the populations of tumor recruited immune cells (Palazon et al., 2012).

It is generally accepted that besides its organo-specificity (Kieda et al., 2002), endothelium reflects biological reactions thus may help assessment or diagnosis of pathologies (Esposito et al., 2011; Quilici et al., 2004) which are ischemia-related.

In tumors, considered as a wound that does not heal (Dvorak, 1986), angiogenesis is a mechanism using variety of cells for its achievement. Not only does it occur by the activated endothelial cells of committed vessel sprouting as illustrated in Fig. 1, but bone marrow derived cells (BMDC), among which endothelial precursors take also a large part in vessel formation. Moreover tumor cells and especially cancer stem cells, participate actively to the tumor vasculature by vascular mimicry.

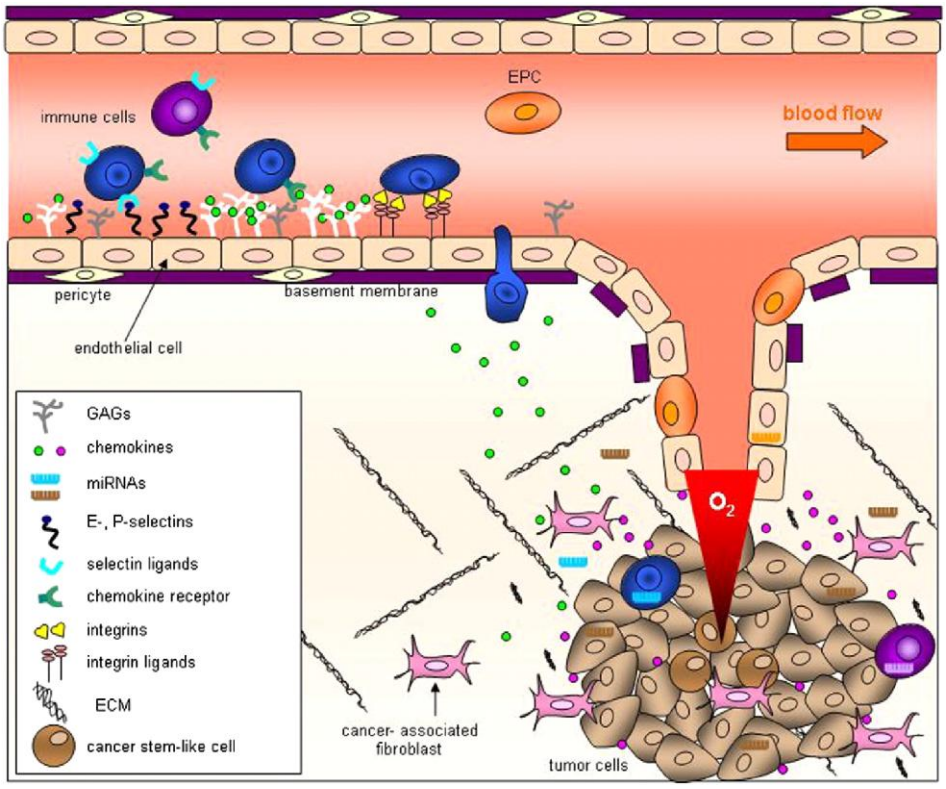

Fig. 1. Tumor angiogenesis-mediated cell and molecular recruitment: hypoxia is the common activation initiating parameter.

Controlling one aspect of this remodelling process results in limited effect. The redundancy and diversity of means by which blood vessels can remodel might account for resistance in antiangiogenic therapies.

It is thus essential to approach the common downstream signal-ling hubs to highlight the potential new therapeutic strategies to re-verse pathologic angiogenesis and suppress tumor progression.

As shown in Fig. 1, it is remarkable that hypoxia is the common parameter that activates selectively a series of targets in tumor cells, stromal cells and in the bone marrow–derived cells that cooperate to potentiate the angiogenic response.

Although little attention is paid to "physioxia" which represents the real oxygen tension inside normal tissues, and differs largely from one organ to another (Carreau et al., 2011a), the oxygen homeostasis is fine-ly tuned by crucial pO2 sensing enzymes, the prolyl hydroxylases 1,2 and 3 (Mazzone et al., 2009) and the factor inhibiting hypoxia (Fig. 2). These enzymes are the main controllers of HIF-1α stability vs degradation (Mazure et al., 2003; Palazon et al., 2012). It is the hypoxia-mediated signalling that covers many strong deleterious effects of can-cer aggressiveness, mainly the cancer stem cell selection and acquisition of resistance to drugs and radiotherapies (Loges et al., 2010). This consequence makes tumor hypoxia compensation the highest type of challenge in treating cancer. This comes with normalization of angiogenesis which is by now recognized as a necessity for future therapies.

Consequently, hypoxia compensation in tumor, leading to normalization of tumor vasculature, is a process that is expected to bring breakthroughs for the design of modern therapies (Jain et al., 2007). Normalization directly acts by reducing interstitial hypertension, peritumor oedema and metastasis while it allows increasing the partial oxygen pressure by blood flow boosting (Jain, 2009).

The molecular mechanism is directly related to HIF-1α stabilization versus degradation (Fig. 2) to control the transcription activity via binding to the hypoxia responsive element (HRE) of the HIF-1α/ HIF-1β heterodimer in hypoxia. Because of the gene cascade initiated by this promoter many strategies aiming to modulate angiogenesis are devoted to the control of the transcription but also to the control of the stability of HIF-1α protein and mRNA (Galban and Gorospe, 2009) as a very promising approach.

Such strategies would bring means to achieve the treatment of tumor hypoxia and reach the objectives raised by the work by (Mazzone et al. 2009) on PHD2 partial silencing demonstrating the benefits of tumor vessels normalization (Mazzone et al., 2009). Confirmation was brought by the curing effects observed with the double antiangiogenic protein (Koh et al., 2010) that is able to neutralize both VEGF-A and angiopoietin through vasculature normalization.

Direct effects of HIF-1α in terms of proangiogenic protein products of hypoxia mediated activation of angiogenesis are also compensated in controlling the PTEN/PI3K/Akt pathway in tumor endothelial cells (Qayum et al., 2009). This permits the blood flow increase and vessel normalization as well as the cooperative effect of chemotherapies (Rodriguez and Huynh-Do, 2012).

Consequently, such benefits are clues to new cancer treatments when used in conjunctions with other approaches within therapeutic windows offered by vasculature normalization.

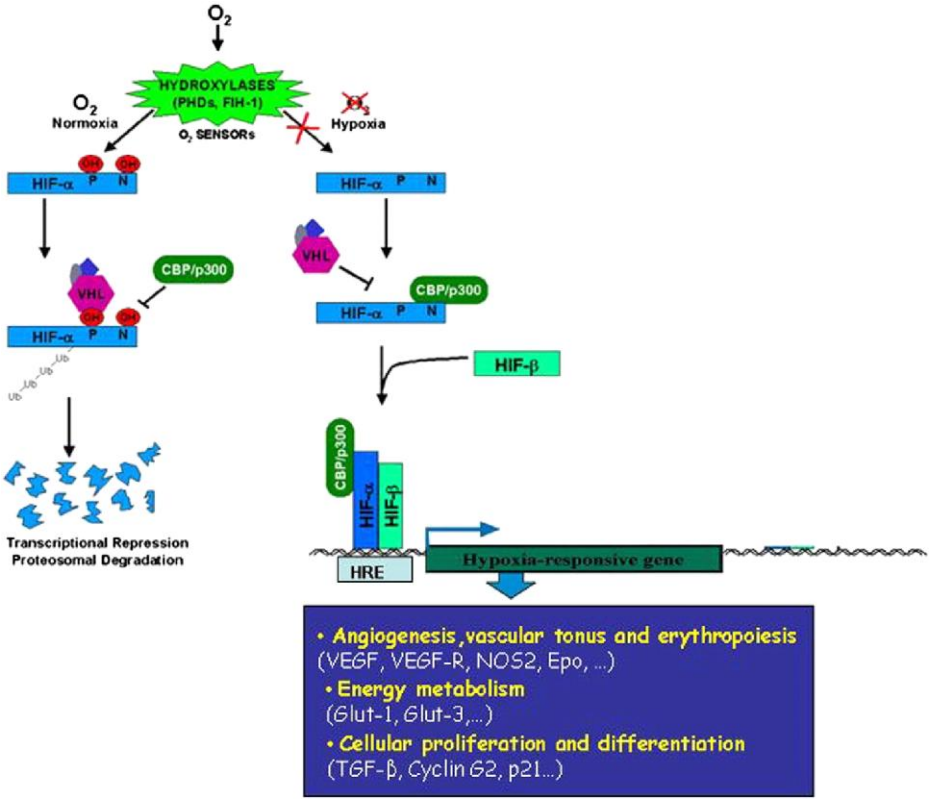

Fig. 2. Oxygen-dependent regulation of HIF-1α protein stability. In the presence of oxygen (normoxia), prolyl hydroxylases (PHDs) and factor inhibiting HIF (FIH) hydroxylate, respectively, proline and asparagine residues on HIF-1α, allowing it to interact with an ubiquitin-protein ligase complex through VHL (von Hippel-Lindau). Ubiquitinylation of HIF-1α targets it for degradation by the proteasome. Under hypoxic conditions, binding of VHL to HIF-1α is inhibited, resulting in the accumulation of HIF-1α and its dimerization with HIF-1β. The heterodimer then translocates to the nucleus and binds to HRE elements in the promoter region of genes, inducing the expression of various hypoxia-responsive genes. (adapted from http://www.adelaide.edu.au/mbs/research/peet).

3. Hypoxia versus normalization of tumor angiogenesis impact the tumor stroma composition

3.1. Hypoxia and VEGF induce immune tolerance

Many cells and molecules participate in tumor angiogenesis mechanism to control the complex interactions between the tumor and vessels that favor tumor progression and metastasis. Hypoxia rules tumor microenvironment by linking angiogenesis with immune tolerance and tumor growth by activating HIF-1α and HIF-2α (Semenza, 1999) and the subsequent genes that enhance vascularity, as VEGF. Secreted by cancer cells VEGF acts as an immunosuppressive cytokine. By binding to its tyrosine-kinase receptor, VEGF-receptor-2 (VEGF-R2, or KDR, Flk-1), VEGF supports proliferation, survival, and motility of endothelial cells. As mentioned above anti-VEGF-R2 agents, are highly effective in blocking tumor growth and angiogenesis (Rafii et al., 2002). The major role played by VEGF in the immune response resides in the efficient chemo- attraction of inflammatory cells (Huang et al., 2008), macrophages, neutrophils, dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs). The latter secrete immunosuppressive mediators and induce T-cells dysfunction (Gabrilovich and Nagaraj, 2009) by which way tumor cells directly down-regulate the antitumor immune response (Oyama et al., 1998).

As such, tumor angiogenesis causes non proper recruitment of immune cells, helping tumor progression. Normalization should reverse this dysfunction. Indeed, effects of tumor vessel normalization and hypoxia regulation by lowering VEGF production should stop the recruitment of tumor favoring cells and suppressors that contribute to stroma composition and help tumor progression. Indeed, Tregs and myeloid derived suppressor cells invasion is considerably reduced (Loges et al., 2010). Such data pointing to new therapeutic applications of vessel normalization are mechanistically illustrated by the chemokines and receptors balance.

3.2. Chemokine/chemokine receptors regulation by hypoxia and compensation

Cells in tumor stroma respond to the chemokine gradient established on the endothelial cell surface (Crola Da Silva et al., 2009). When tumor angiogenesis develops it reflects the hypoxia/normoxia balance. The two main axes that tumor stroma cell populations de-pend on are the CXCL12/CXCR4 and the CCL21/CCR7.

CXCL12/CXCR4 interactions are critical for metastasis setting. CXCL12 - SDF-1 - (Stromal cell derived factor-1) is expressed in a num-ber of tissues including liver, lung, lymph nodes, adrenal glands and bone marrow. Tumor cells are submitted to CXCR4/CXCL12 trail for their metastasis setting (Luker and Luker, 2006). SDF-1 can also bind CXCR7, a second chemokine receptor, which is expressed on endothelial cells, T-cells, dendritic cells, B-cells, chondrocytes, endometrial stromal cells (Balabanian et al., 2005). SDF-1 gradient displays a dual activity, secreted by stromal fibroblasts from the tumor microenvironment, it stimulates cell motility or chemotaxis of tumor cells as they respond to an SDF-1 gradient while, through binding to CXCR7, it enhances tumor growth. Efficiently regulated by hypoxia CXCR4 is used as a marker (Deschamps et al., 2011) which is decisive for the recruitment of antitumor Tregs (Yan et al., 2011). HIF-1α induces the expression of CXCR4 in tumor cells but also in microvascular endothelial cells (Schutyser et al., 2007). The CXCL12-rich organs serve as fertile ground for the $CXCR4^{+}$ tumor cells and link metastasis and angiogenesis through CXCL12-CXCR4 signaling (Righi et al., 2011).

CCL21/CCR7 axis: shown first in breast cancer, this chemokine-chemokine receptor pair plays a key role in the migration of tumor cells into the sentinel lymph nodes in many tumors (Muller et al., 2001). CCL21, in the lymph nodes, is presented to the circulating cells in the lumen of the vessels as a gradient through its binding to glycosaminoglycans of the endothelial cell surface (Crola Da Silva et al., 2009). This allows attracting specific chemokine receptor (CCR7)-bear-ing tumor cells (Folkman and Kalluri, 2004) and plays a fundamental role in the recruitment of immune cells as Tregs (Chen et al., 2010a).

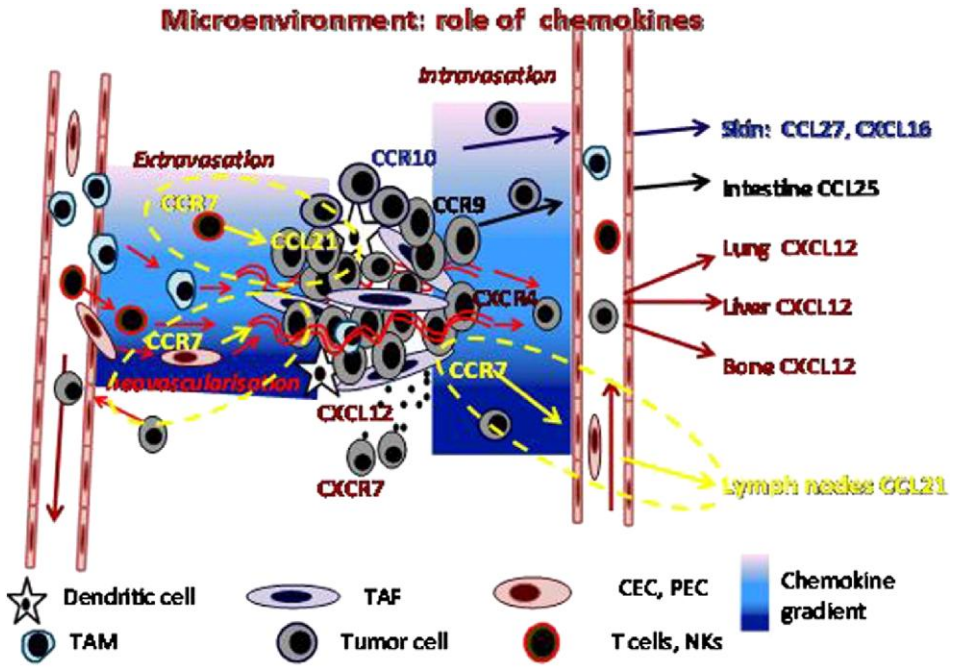

Fig. 3. The role of CCL12/CXCR4 axis and CCL21/CCR7 axis in the tumor recruitment of tumor stromal cells.

and bone marrow derived cells (BMDC) (Zhao et al., 2011) (Fig. 3). Consequently, therapeutic disruption of the CCR7/CCL21 trail may prevent metastases to lymph node (Croci et al., 2007; Issa et al., 2009; Li et al., 2011; Liu et al., 2010). Its regulation is a new therapeutic target as we have shown that its modulation is hypoxia-dependent.

4. Endothelial precursor cells participate to tumor stroma

Among the main BMDCs, the endothelial precursors are early participants to build the tumor stroma and determine the tumor angiogenesis development.

4.1. Tumor progression and specific homing of circulating endothelial precursor cells

Tumor cell factors recruit precursor cells, among which circulating endothelial precursors are key elements of the tumor stroma constitution. Once at the tumor site, precursor cells participate to the cross talk with tumor and other stromal cells. They may differentiate into tumor associated fibroblasts (TAFs) that release the Stromal Derived Factor 1α (SDF1α, CXCL12) which, in turn, enhances recruitment of bone marrow-derived cells, consequently EPCs, resulting in angiogenesis promotion (Spring et al., 2005). Compared to mesenchymal stem (MSC) cells, EPCs express a whole panel of chemokine receptors as CCR7 and Toll-like receptors that impact on stimulation of migration (Tomchuck et al., 2008) and aggressiveness (Albini and Sporn, 2007).

Indeed, to tumor angiogenesis, the circulating endothelial cells are participating, although Dudley et al. stated (Dudley et al., 2010) that bone marrow-derived endothelial cells (BMDEC) constitute only 0,027% of tumor endothelial cells (TEC), tumor endothelial cells ac-count for 0,01-0,04% of total BMDEC and in fact 99% of endothelial cells in the tumor vasculature originate from local vessels. Fig. 4 illustrates the intra tumor localization of precursor endothelial cells (Paprocka et al., 2011) after intravenous injection (Kieda, unpublished data).

These tumor-specific endothelial precursors would be highly use-ful if it is possible to monitor them in subjects at high risk for cancer development or recurrence after therapy (Folkman and Kalluri, 2004). As described above the tumor-lining ECs have long been considered as genetically stable (Kerbel, 1991) in contrast to tumor cells. But, tumor endothelial cells were found to share the same genetic abnormalities as found in cancer cells (Della Porta et al., 2008) which could be due to a common cancer/endothelial cell progenitor (Ergun et al., 2008), to cancer-to-endothelial cell trans-differentiation (Verfaillie, 2008), to fusion between cancer and ECs (Bertolini et al., 2006) or to cancer stem like cells under-going vascular mimicry. Tumor endothelial cells have unique properties (Weis and Cheresh, 2011) suggesting that oncogene-bearing circulating endothelial cells/precursors (CEC/CEPs) might be one of the possible hidden identities of cancer stem cells thus providing a possible explanation for resistance to anti-angiogenic drug therapy of cancer.

4.2. Endothelial precursor cells as carriers of genes regulating angiogenesis

Each of the above cited steps are confirming the hypothesis that endothelial precursor cells constitute a potential tool to carry thera-peutic genes to pathologic sites.

We have shown that endothelial cells home into their organo-specific site of origin. In the case of the tumor the best cell candidates to reach the pathologic site are the precursor endothelial cells mimicking the process of precursor endothelial cell recruitment from the bone marrow (Chouaib et al., 2010). This was demonstrated by direct observation of endothelial cells in the tumor (Fig. 4). The use of a cell model of precursor endothelial cells (Paprocka et al., 2011) showed that such cells are a promising tool to allow long term expression of therapeutic genes. The quiescent character of the endothelium in nor-mal conditions permits the prolonged expression of therapeutic genes in order to modify the proangiogenic activity of over-expressed VEGF (Zhang et al., 2010). Endothelial precursor cells

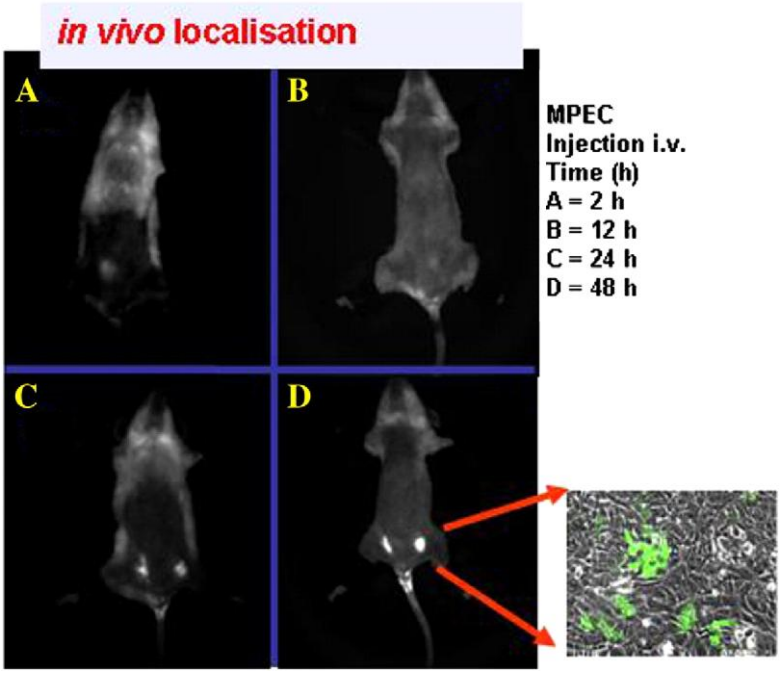

Fig. 4. Endothelial precursor cells are able to home into the tumor site.Fluorescent and bioluminescence detection of EPC cells injected intravenously to melanoma bearing mice. Murine endothelial precursor cells (MEPC) model cells concentrated after 48 hours into the tumor sites.

represent a small proportion of the cells that participate to neo-angiogenesis: these cells are not transformed. They are then good candidates for long term expression of a therapeutic gene. Such approach requires taking into account the specificities of tumor micro-environment and its regulation. Therapeutic genes like traps for angiogenesis inducers such as angiopoietins and VEGFs should be conditionally expressed in the tumor context (Koh et al., 2010). The knowledge of the microenvironment is again the key parameter. Recently a wide field of investigation was opened with the understanding of microRNAs as strong regulators of biological pathways which appears to provide very potent means to treat angiogenesis.

5. MicroRNAs have a decisive role in angiogenesis

MicroRNAs (miRNAs) are small non-coding RNAs that control diverse cellular functions by either promoting degradation of target messenger RNA or inhibition of translation. They are able to control vascular development and repair; their deregulation is a signature of vascular dysfunction (Hartmann and Thum, 2011).

5.1. MicroRNAs are angiogenic switches

MicroRNAs (miRNAs) bind to the 3'untranslated region of mRNAs, blocking translation by a silencing complex (Pillai, 2005). Their activity is balanced by sets of binding proteins regulating their biogenesis, localization, degradation and activity (van Kouwenhove et al., 2011). In angiogenesis the role of miRNAs is largely documented: acting to promote angiogenesis, they are called angiomirs (Wang and Olson, 2009) and define the angiogenic switch (Anand and Cheresh, 2011). By profiling the miR transcriptome, expression signatures of miRs were shown associated with tumorigenesis steps and the acquisition of hallmarks of cancer progression as miR-130a, miR-210 and miR-296. Metastases and a subset of primary tumors shared characteristic miR signatures (Olson et al., 2009). Negative regulation occurs by anti angiomirs as : miR-221 and miR-222 that were shown to block angiogenesis (Fish and Srivastava, 2009). Upregulated in circulating endothelial cells, miR-221/miR-222 are highly committed in diseases involving the targeting of c-kit receptor for stem cell in vascular cells and are statin-dependent (Li et al., 2009) by controlling STAT5 (Dentelli et al., 2010). miR-222 was shown to target ZEB2 in endothelial cells (Chen et al., 2010b) maintaining the cell cycle arrested which provides a useful approach to cancer antiangiogenic therapy.

In angiogenic process miRs control occurs at the level of distinct mechanisms as migration, survival and response to hypoxia.

Indeed, while anti-miR-132 (Anand et al., 2010), suppresses Ras and blocks angiogenesis up to quiescence of vascular endothelial cells, miR-20b modulates vascular VEGF in the stromal context by targeting HIF-1α and the signal transducer and activator of transcription 3 (STAT3) (Cascio et al., 2010), miR-93 promotes tumor growth and angiogenesis by targeting integrin β8 (Fang et al., 2011), miR-107 acts by suppressing HIF-1α expression through tumor suppressor p53 thus suppressing tumor angiogenesis, tumor growth and VEGF expression in mouse tumors (Yamakuchi et al., 2010).

A review of the miRs involved in the vascular biology indicates the strategic functions they regulate (Hartmann and Thum, 2011). Focus-ing to tumor angiogenesis some miRs should be cited (Table 1). Directly acting towards angiogenesis, a set of 25 miRNA has been directly shown. MiR-15a and the miR-17-92 cluster, modulate the endothelial cells growth and activation up to apoptosis, while mir-21 is characterized by its participation to defence during oxidative stress, it is proangiogenic, oncogenic but can be proapoptotic; miR-23a in-hibits endothelial cell growth and is proangiogenic in terms of cell differentiation (Zhou et al., 2011).

While miR-132 was shown angiogenic in pathologies (Anand et al., 2010), miR-145 (Xu et al., 2012) that controls smooth muscle cells differentiation and contractility, inhibits cancer cell growth and

Table 1
microRNAs participating in regulating angiogenesis.

Vascular microRNAs	main molecular targets	Affected functions in angiogenesis
miR-15a	Bcl-2 (block)	block endothelial apoptosis, blood brain barrier integrity
miR 17-92	trombospondin1 connective tissue growth factor integrins α5, αV	anti/pro angiogenic, oncogenic
miR-20b	HIF-1α, STAT3	proangiogenic
mir-21	PTEN, PPARα, SOD	oxidative stress, pro-angiogenic, apoptotic, oncogenic
miR-23a	E2F1	proangiogenic, inhibits endothelial cell growth
miR-93	integrin β8	tumor growth, pro angiogenic
miR-107	HIF-1β, p53	suppress tumor growth, antiangiogenic
miR-126	VEGF, EphrinB2	antiangiogenic, anti-inflammatory, tumor suppressor
	VCAM-1, PIK3R2	restores endothelial functions,
miR-132	P120RasGAP	antiangiogenic
miR-145	OCT4, SOX2, cMyc	antiangiogenic, SMC VSMC differentiation, PSC induction
	insulin receptor, actins	tumor suppression
miR-210	HIF-1α, Ephrin A3, CTGF Death assoc. kinase1	proangiogenic, proapoptotic
miR-221/ 222	c-kit, p27 STAT5, ZEB2	antiangiogenic, tumor suppressor inhibition CAC differentiation,
miR-378	SuFu, Fus-1	proangiogenic, oncogenic

controls the induction of pluripotent stem cells. MiR-126 was largely described as modulating angiogenesis (Wang et al., 2008). It is anti-inflammatory, suppressing endothelial motility and permeability, inducing tubule formation and tumor suppression. It is endothelium specific through epidermal growth factor like domain7 (Nikolic et al., 2010), consequently miR-126 represents a strong candidate for future tumor angiogenesis normalizing treatment (Chen and Zhou, 2011).

Since miRNA signatures are distinct enough to be attributed according to the tumor development stages, they bring a powerful approach in view of manipulating tumor progression. The miRNA modulation approach is promising provided miRNAs or their inhibitory anti-miRs can be optimally targeted. Targeting miRNA signaling pathways in tumor cells as well as in angiogenic endothelial cells opens new therapeutic avenues to suppress pathologic tumor-associated angiogenesis.

5.2. MicroRNAs future in therapeutic applications to control neoangiogenesis

In endothelial cell biology, the action of miRs is quite vast and extensively studied.

To achieve signalling between cells miRs are segregated into exosomes that are transferred between cells insuring paracrine modulation of distant cells. Thus circulating miRNAs (Gupta et al., 2010) (Lorenzen et al., 2011) are now actively studied since their level correlates with vessel dysfunction (Fichtlscherer et al., 2010).

The function of miR-126 is quite remarkable as a tumor suppressor in lung cancer cells. It is down regulated in many lung cancer cell lines and is normally enriched in endothelial cells (Sun et al., 2010). MiR-126 regulates angiogenesis because it presents an inhibitory effect on VEGF expression by targeting a binding site in its mRNA 3′UTR. It was hypothesized that delivery of miR-126 could be a therapeutic intervention in human lung cancer treatment (Liu et al., 2009) by acting both on angiogenesis and tumor expansion (Semenza, 2003). Moreover, miR-126 was shown to direct stem cell differentiation into endothelial cells (Kane et al., 2010), hence strategies to increase miR-126 levels may be beneficial to repair pathological vascularization. The latter is most often characterized by a

hypoxic microenvironment. Among hypoxia-induced miRNA, the miR-210 was constantly upregulated. It functions in cell survival and angiogenesis (Ivan et al., 2008). In endothelial cells miR-210 expression is increased in response to low oxygen tension and leads to up regulation of several angiogenic factors, inhibition of caspase activity and prevention of cell apoptosis (Hu et al., 2010). As targets for miR-210, ephrin-A3 (Fasanaro et al., 2008) that is crucial in vascular remodelling (Kuijper et al., 2007) and protein tyrosine phosphatase 1b (Ptp1b) (Hu et al., 2010), a negative regulator of VEGF signalling in endothelial cells, have been identified. In hypoxia miR-210 is induced to down regulate these targets thus modulating the angiogenic response to ischemia (Hu et al., 2010). Additionally miR-210 target genes have been described for their important roles in angiogenesis-mediated tissue repair and cancer progression; miR-210-based therapeutic intervention was shown beneficial in the treatment of ischemic diseases (Hu et al., 2010).

Other miR-dependent pathways in angiogenesis regulation point to miR-221/miR-222 that strongly down regulate ZEB2 (Chen et al., 2010b), which usually modulates epithelial–mesenchymal transition (Lorenzen et al., 2011). Down regulation of ZEB2 decreases angiogenesis through inhibition of nuclear factor-κB (Patel et al., 2005) and increase of p21WAF/CIP1 (Chen et al., 2007), maintaining the endothelial cells in G0/G1 cell cycle arrest. Targeting of ZEB2 might be useful for an antiangiogenic therapy of cancer and other angiogenic disorders.

The interplay between the pro/anti angiogenic effects of miRs and their oncogenic vs suppressor activity is an interesting feature that could be beneficial for further antitumor strategies based on control of angiogenesis.

As the miRNAs or anti-miRNAs are short RNA sequences that must be expressed in the target cells for efficient therapeutic effect, the miRNA/anti-miRNA approach to block angiogenesis requires new gene delivery methods.

Although this is feasible in preclinical models, translating this ap-proach to humans is more complicated because the used miRNA or anti-miRNA needs to be effectively delivered to the chosen cell and taken up by the relevant cell type in vivo.

As liver is the organ that takes up injected reagents, this would be a natural therapeutic option for liver cancer/metastases (Huynh et al., 2011). Collagen delivery procedure targets miR to the bone (Takeshita et al., 2010) while lung retains miR delivered by neutral lipids (Trang et al., 2011).

Endothelial cells are the main target to aim because of their presence and action in tumor development; the proof of concept is de-scribed by delivery of miR to tumor endothelium using αvβ3-nanoparticles (Anand and Cheresh, 2011).

Another approach aiming to deliver gene(s) and modulators to-wards angiogenesis-related pathologic sites is to take advantage of the active endothelial precursor cells as putative carriers for the miRs-based treatments.

Endothelial precursor cells will be considered as carriers for the miRs chosen to be over expressed at the tumor site where angiogenesis is developing. Reaching the hypoxic site EPCs express hypoxia and/or anti angiogenic miRs. Regulation of the pathologic angiogenesis by counteraction of the HIF-1α/VEGF cascade will allow normalization of the vessel, blocking growth and movement in favour of maturation and quiescence. In such aim, compensation of miRs identified as down regulated upon hypoxia in pathologic vessels, is a highly attractive challenge.

Some miRs like miR-126, that link angiogenic control ability with a tumor suppressor effect could be tentatively over expressed in circulating endothelial precursor model cell that could deliver the regulatory miRs either directly, through tumor specific homing ability, or through their exosomes production and paracrine action onto the developing tumor angiogenesis as presented on the graphical abstract. The reverse mechanism results in overproduced miRs that are angiomirs and oncomiRs, as miR-378, that sustains tumor growth and angiogenesis in vitro and in vivo (Ciesla et al., 2011; Lee et al., 2007). MiRs of this type provide a good model for an antagomir-based strategy of treatment.

6. Perspectives

Targeting angiogenesis by cytotoxic drugs to make a tumor be starving of its blood supply has long been considered as the rational approach to fight cancer progression. But, a tumor successfully manages to set rescue pathways that exploit existing physiological functions and may lead to reversing quiescent cell back to their embryonic state. Moreover the angiogenic signalling in tumors improperly regulates the normal vascular remodelling that occurs during wound healing. This leads to a vicious circle played by proangiogenic signals that stimulate endothelial cells to form new vessels whose poor efficacy causes the endless production of proangiogenic factors.

These pitfalls apply to most therapeutic strategies dedicated to anti angiogenesis factors, antibodies, chemotherapies, immuno therapies and radiotherapies.

Angiogenesis-devoted research has identified hundreds of new therapeutic targets, although they appear difficult to translate into human therapies.

Consequently, future therapeutic strategies might be addressed to modulation of several pathways as it appears that blocking a single pathway may have opposing effects according to the cancer type and considering the variety of targets on different cell types (Sato, 2011).

Combining advances in the knowledge of bone marrow-derived endothelial precursor cells, their tissue-specific homing, their active recruitment effect and repair activity with the fact that they are “nor-mal” cells entering a pathologic site where they express natural regulators as microRNAs, appear as new perspectives to manipulate the tumor microenvironment. The potential ability of these cells to deliver microRNAs through exosomes formation offers also new means to modulate the tumor reactivity and its angiogenic response to hypoxia. Delivery of microRNAs counteracting hypoxic reaction effects would help restore the endothelial cells quiescence and normalize, rather than block, the angiogenic response inside the tumor. This should help making a step towards the normalization of the vasculature and take advantages of the subsequent cooperative effects that are expected to help cancer treatments.

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Annex 4

Stable tumor vessel normalization with pO2 increase and endothelial PTEN activation by inositol tris pyrophosphate brings novel tumor treatment

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Running title: Tumor vasculature stable normalization-based therapy

Abstract

Tumor hypoxia is characteristic of cancer cell growth and invasion, promoting angiogenesis which facilitates metastasis. Oxygen delivery remains impaired because tumor vessels are anarchic and leaky, contributing to tumor cell dissemination. Counteracting hypoxia by normalizing tumor vessels in order to improve drug and radio therapy efficacy and avoid cancer stem-like cell selection is a highly challenging issue. We show here that inositoltris pyrophosphate (ITPP) treatment stably increases oxygen tension and blood flow in melanoma and breast cancer syngeneic models. It suppresses hypoxia-inducible factors (HIFs) and proangiogenic/glycolysis-genes and -proteins cascade. It selectively activates the tumor suppressor PTEN *in vitro* and *in vivo* at the endothelial cell (EC) level thus inhibiting PI3K and reducing tumor AKT phosphorylation. This mechanism normalizes tumor vessels by EC reorganization, maturation, pericytes attraction and lowering progenitor cells recruitment in the tumor. It strongly reduces vascular leakage, tumor growth, drug resistance and eradicates metastasis. ITPP treatment abolishes cancer stem-like cells selection, MDR activation and efficiently enhances chemotherapeutic drugs activity. These data show that counteracting tumor hypoxia by stably restoring healthy vasculature is achieved by ITPP treatment which opens new therapeutic options overcoming hypoxia-related limitations of antiangiogenesis-restricted therapies. By achieving long-term vessels normalization, ITPP should provide the adjuvant treatment required in order to overcome the subtle definition of therapeutic windows for *in vivo* treatments aimed by the current strategies to cure angiogenesisdependent tumors.

Keywords:

Angiogenesis, normalization, oxygen, PTEN, tumor-hypoxia

(in revision in "Journal of Molecular Medicine", 2012).

Annex 5

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

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Running title: Therapeutic vector for angiogenesis normalization therapy.

Abstract

Vascular endothelial growth factors (VEGFs) are over-expressed in hypoxic tumors. Major actors directing pathologic neo-vascularisation, they regulate stromal reaction. Novel strategies that target and inhibit VEGF bring promise to modern anti-cancer therapies. They aim to control rather than destroy tumor angiogenesis. Consequently, the challenge is to trap selectively VEGFs overproduced upon hypoxia in the tumor microenvironment. Here we report the design and construction of a new vector expressing the soluble form of VEGF receptor-2 (sVEGFR2) driven by an hypoxia responsive element (HRE)-regulated promoter. To allow *in vivo* imaging by near-infrared visualization, mCherry and IFP1.4 coding sequences were built into the vector. Plasmid construction was validated upon transfection into embryonic human kidney HEK293 and murine B16F10 melanoma cells. sVEGFR2 was successfully expressed in hypoxia, proving that its synthesis was indeed regulated by the HRE promoter. sVEGFR2 bound specifically murine and human VEGF-A, reduced tumor and endothelial cell growth as well as angiogenesis *in vitro*. Next, the hypoxia-conditioned sVEGFR2 expression was shown to be functional *in vivo*: tumor angiogenesis was inhibited and, upon B16F10 melanoma cells stable transfection, tumor growth was reduced. Enhanced expression of sVEGFR2 was accompanied by VEGF-A modulation. The resulting balance reflected the effect on tumor growth and on the angiogenesis control. The concomitant increase of intra-tumor oxygen tension suggests an influence on vessel normalization. The possibility to express such angiogenesis regulator as the soluble form of VEGFR2, in a hypoxia-conditioned manner, opens new strategies for controlled normalization of tumor vessel in view of adjuvant design for combined therapies.

Keywords

soluble VEGFR-2, hypoxia conditioning, tumor angiogenesis, near infrared imaging

(in revision in “Molecular Cancer Therapeutics”, 2012).

Annex 6

Murine endothelial precursor cell lines as models able to target of neoangiogenic sites

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Running title: Endothelial precursors as tools for therapy

Abstract:

Angiogenesis is necessary for tissue repair after ischemia and in pathologies linked to hypoxia. It recruits endothelial precursor cells (EPCs) that reach specifically sites where neovascularisation is needed. This homing is an ideal natural targeting mechanism which is fundamental and could be mimicked to design drug/gene delivery. Here we describe isolation of EPCs from the AGM region of murine embryos. Series of lines representing the endothelial differentiation steps occurring at 10.5dpc and 11.5 dpc were established (patent pending). Two lines were selected based on their differentiation characteristics: MAgECs10.5 and 11.5. EPCs were characterized by antigen expression profiles at protein and mRNA levels, showing distinct maturation steps. Cytokines, chemokines production, their receptors as well as endothelial functional factors delineated their commitment as endothelial precursors. Functional angiogenesis test determined their biological ability as endothelial precursors. MAgECs clones could be "educated" to acquire an endothelial phenotype in response to chemokines. Validation *in vitro* and *in vivo* in a Matrigel™ mix plug model of angiogenesis, showed that MAgECs achieved efficient vessel formation leading to rapid blood flow establishment.

Moreover, when intraveneously injected, MAgECs actively invaded Matrigel™ empty plugs, mimicking hypoxic pathologic matrix, to develop a functional vascular network.

MAgEcs cells mimic the in vivo endothelail cell recruitment into the angiogenic sites. They were shown in vitro to invade 3D-spheroid models of melanoma. Injected in vivo, Magecs reached specifically the tumor site and were incorporated into the developing angiogenesis.

The MAgecs model will provide a valuable tool to help development of cell carried drug/gene designs to angiogenic sites mainly in response to hypoxia as during tumor progression.

Keywords

endothelial precursor cells, cell targeting, angiogenesis, cell therapy

(in preparation)

Annex 7

MiRs and tumor vasculature normalization: impact on anti-tumor immune response

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Abstract:

Inefficient immune response is a major glitch during tumor growth and progression. Chaotic and leaky blood vessels created in the process of angiogenesis allow tumor cells to escape and extricate anti-cancer immunity. Proangiogenic characteristics of hypoxic tumor microenvironment maintained by low oxygen pressure attract endothelial progenitor cells, drive expansion of cancer stem cells and deviantly differentiate monocyte descendants, which further boost immune tolerance and eventually appoint immunity for cancer advantage. Blood vessel normalization strategies that equilibrate oxygen levels within tumor and fix abnormal vasculature bring exciting promises to future anticancer therapies especially when combined with conventional chemotherapy. Recently a new group of microRNAs (miRs) engaged in angiogenesis called angiomiRs and hypoxamiRs emarged as new therapeutic targets in cancer. Some of those miRs were found to effieciently regulate cancer immunity and their dysregulation efficiently programs abberant angiogenesis and cancer metastasis. The present review highlights new findings in the field of miRs proficiency to normalize aberrant angiogenesis and to restore anti-tumor immune responses.

Keywords

miRs regulation, hypoxia, angiogenesis, cancer, vessels normalization, tumor immune response

(in revision in « *Archivum Immunologiae et Therapiae Experimentalis* », 2012).

Annex 8

Interplay between heme oxygenase-1 and miR-378 affects non-small cell lung carcinoma growth, vascularization and metastasis

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Running title: Interplay between HO-1 and miR-378 in NSCLC

Abstract:

Aims: Heme oxygenase-1 (HMOX1), an antioxidant enzyme can prevent tumor initiation while it has been demonstrated to promote various tumors growth, angiogenesis and metastasis. Here we investigated whether HMOX1 can modulate microRNAs and regulate human lung cancer development. **Results:** Stable HMOX1 overexpression in non-small cell lung cancer NCI-H292 cells enhanced global production of microRNAs and significantly diminished expression of oncomirs and angiomirs, whereas upregulated tumor suppressive microRNAs. The most potently downregulated was miR-378. HO-1 overexpressing cells displayed also upregulated p53, downregulated Ang-1 and MUC5AC, reduced proliferation, migration and diminished angiogenic potential. Carbon monoxide was a mediator of HMOX1 effects on tumor growth. In contrast, stable miR-378 overexpression decreased HMOX1 and p53 while enhanced expression of oncogenic MUC5AC and proangiogenic VEGF, IL-8 and Ang-1 and consequently increased proliferation, migration and stimulation of endothelial cells. Introduction of HMOX1 to miR-378 overexpressing cells reversed miR-378 effect on proliferation and migration of cancer cells. *In vivo*, HMOX1 overexpressing tumors were smaller, less vascularized and oxygenated and less metastatic. Inversely, miR-378 overexpression exerted opposite effects on tumor growth in mice. Accordingly, in patients with NSCLC, HMOX1 expression was lower in metastases to lymph nodes than in primary tumors while miR-378 did not differ significantly. **Innovation:** *In vitro* and *in vivo* data indicate that an interplay between HMOX1 and miR-378 significantly modulates NSCLC progression and angiogenesis and miR-378 may be a new target for therapy. **Conlusion:** To conclude, HMOX1 diminishes, whereas miR-378 enhances tumorigenic and angiogenic potential of human lung cancer.

(under review in "Antioxidants & Redox Signaling", 2012).

Annex 9

Regulation and novel protumoral action of thymidine phosphorylase in non-small cell lung cancer: crosstalk with Nrf2 and HO-1

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Abstract:

Thymidine phosphorylase (TP) is a proangiogenic enzyme playing a dual role in cancer. It is considered as target for antiangiogenesis, but its enzymatic activity is necessary for activation of fluoropyrimidine chemotherapeutic agents. This complicates the use of TP inhibitors in combinatorial approaches and urges the search for mediators of its action. We investigated the regulation and effects of TP overexpression in non-small cell lung cancer.

In NCI-H292 mucoepidermoid carcinoma cell line TP was upregulated in cells overexpressing transcription factor Nrf2 or Nrf2-target gene, heme oxygenase-1 (HO-1), while in cells with HO-1 knockdown TP expression was decreased. Interestingly, overexpression of TP attenuated cell proliferation, migration and expression of MMP-1 and MMP-2 in vitro but enhanced their angiogenic activities, which was accompanied by a better oxygenation of TP-overexpressing tumors in vivo and a higher expression of IL-1β and IL-6. In endothelial cells TP overexpression augmented HO-1 expression and VEGF synthesis. HO-1 inhibition, however, did not affect proangiogenic action of TP products towards either endothelial cells or bone marrow-derived proangiogenic progenitor cells.

Here we show that TP can be upregulated in NSCLC by activation of Nrf2/HO-1 pathway. TP induction attenuates tumorigenic properties of cancer cells in vitro. On the other hand, increased expression of IL-1β and IL-6 in TP-expressing cells together with enhanced proangiogenic effects of TP-expressing NSCLC cells on endothelial cells can contribute to acceleration of tumor growth in vivo.

Keywords

tumor angiogenesis, thymidine phosphorylase, non-small cell lung cancer, heme oxygenase-1; Nrf2 - nuclear factor (erythroid-derived 2)-like 2

(under review in “Free Radical Biology and Medicine”, 2012)

Annex 10

PATENT

Controled Oxygen Pressure in Cell Cultures
Ref : 03235-01

BENEFITS
This novel device allows maintaining the cells of a culture in controlled O2 pressure, for a long time and in non expensive settings, in order to reproduce conditions equivalent to natural milieu in in vitro cell cultures.

Intellectual property :
FR 10 50523: Priority patent of invention filed on: 26/01/2010 entitled: " Dispositif de contrôle et de régulation de la pression partielle en oxygène et son utilisation dans les essais in vitro permettant de respecter la phisioxie"

There will come a time when you believe everything is finished.
Yet that will be the beginning.

Louis L'Amour

Guillaume COLLET

Thérapie génique de l'angiogenèse tumorale ciblée par des cellules endothéliales immatures

Les facteurs de croissance endothéliaux (VEGFs) sont produits par les tumeurs qui sont hypoxiques. Principaux responsables de la néo-vascularisation pathologique, ils régulent le stroma tumoral. Les nouvelles stratégies qui ciblent et inhibent le VEGF ouvrent vers la thérapie anti-cancéreuse moderne. Elles ont pour but de contrôler l'angiogenèse tumorale plutôt que la détruire. Le défi est donc de piéger sélectivement le VEGF produit en excès, dans le microenvironnement tumoral, sous l'effet de l'hypoxie. La thèse présentée dans ce manuscrit est consacrée à la réalisation d'une nouvelle stratégie ciblante par l'intermédiaire de cellules, aussi appelée « Cheval de Troie ». Elle combine dans la même entité, une unité de ciblage et un système de délivrance spécifique d'un gène/molécule thérapeutique. Dans le but d'adresser la thérapie aux cellules cancéreuses sans affecter les cellules saines, un modèle de cellules endothéliales de type précurseur (CEPs) a été utilisé comme cellules ciblantes capables d'atteindre spécifiquement le site tumoral. Les CEPs ont été « armées » pour exprimer un gène thérapeutique chargé d'inhiber le VEGF. La neutralisation a été obtenue par la production d'une forme soluble du récepteur-2 du VEGF (VEGFR2 soluble), agissant comme inhibiteur. Caractéristique des tumeurs solides se développant, l'hypoxie a été choisie pour déclencher/éteindre l'expression et la sécrétion du VEGFR2 soluble, en introduisant, en amont du gène thérapeutique, une séquence régulatrice : HRE. Adressé au site tumoral par les CEPs, le régulateur de l'angiogenèse qu'est la forme soluble du VEGFR2, est exprimé de manière conditionnée et réversible, à l'hypoxie. Ceci ouvre à de nouvelles stratégies de normalisation contrôlée et stable des vaisseaux tumoraux en vue de l'utilisation de thérapies combinées.

Mots clés: angiogenèse tumorale, hypoxie, ciblage des EPCs, normalisation, piège à VEGF

Cell-mediated gene therapy based on endothelial precursor cells to target tumor angiogenesis

Vascular endothelial growth factors (VEGFs) are over-expressed upon hypoxia in solid tumors. Major actors directing pathologic neo-vascularisation, they regulate the stromal reaction. Novel strategies that target and inhibit VEGF bring promise to modern anti-cancer therapies. They aim to control rather than destroy tumor angiogenesis. Consequently, the challenge is to selectively trap VEGFs, over-produced upon hypoxia, in the tumor microenvironment. The thesis presented in this manuscript focuses on the design of a novel cell-based targeting strategy, so-called "Trojan Horse", combining in the same engineered entity, a targeting unit and a specific drug/gene delivery system. Aiming to address the therapy to cancer cells without affecting healthy cells, a model of endothelial precursor cell (EPCs) was used as targeting cell able to reach specifically the tumor site. EPCs were "armed" to express a therapeutic gene to inhibit VEGF. Trapping was attempted based on the production of a soluble form of the VEGF receptor-2 (sVEGFR2) as a candidate inhibitor. Hypoxia, a hallmark of developing solid tumors, was chosen to turn on/off the sVEGFR2 expression and secretion by introducing, upstream of the therapeutic gene, a hypoxia response element (HRE) regulating sequence. Properly addressed by the EPCs to the tumor site, such angiogenesis regulator as the soluble form of VEGFR2 is, was chosen to be expressed in a hypoxia-conditioned and reversible manner. This opens new strategies for a stably controlled normalization of tumor vessels in view of adjuvant design for combined therapies.

Key words: Tumor angiogenesis, hypoxia, EPCs targeting, normalization, VEGF-trap

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