Universidade de Aveiro Departamento de Química

Ano 2015

João Manuel Fernandes Neto

Improvement of antiangiogenic therapies in colorectal cancer

Melhoramento de terapias antiangiogénicas em cancro coloretal

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia – ramo Biotecnologia Industrial e Ambiental, realizada sob a orientação científica do Doutor Rodrigo Leite de Oliveira, Investigador do Netherlands Cancer Institute, e da Doutora Luisa Alejandra Helguero Shepherd, Investigadora Auxiliar da Universidade de Aveiro.

Dedicada à minha falecida avó Alice, que sempre me quis ver formado.

o júri

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agradecimentos

acknowledgements

Em primeiro lugar quero agradecer ao meu orientador Rodrigo Leite de Oliveira por ter acreditado em mim desde o início e me ter proporcionado uma oportunidade única, que me permitiu aprender imenso, viver uma realidade que me era desconhecida e que me abriu portas para o futuro. Obrigado ainda por todo o acompanhamento, motivação e, sobretudo, pela amizade.

A special thanks to Professor René Bernards for accepting me in your laboratory, which allowed me to grow as a person and as a scientist.

To all the students in the lab I want to thank for all the good moments and for always keeping up a good spirit even when everything failed.

To Bastiaan Evers I want to thank for all the excellent advices throughout the year, for your special humor that always made me laugh no matter what and, above all, for your friendship.

To Loredana Vecchione I want to thank for all the help, sympathy and for the opportunity to work together with you.

To the whole Molecular Carcinogenesis division of the Netherlands Cancer Institute I would like to thank for the helpful advices, fruitful discussions and for all the good moments we lived throughout this year.

Aos meus professores na Universidade de Aveiro agradeço por todo apoio e aprendizagem que me proporcionaram ao longo destes cinco anos.

A todos os meus amigos, um muito obrigado pela presença calorosa e apoio constante ao longo de todos estes anos em tudo o que faço.

Aos meus pais, por todo o apoio incondicional que me deram ao longo da minha vida, e em especial este ano, que sei que foi muito difícil para vocês, devido à distância que nos separou, por me terem sempre dado força para continuar e nunca desistir dos meus objectivos. Estou-vos eternamente agradecido, por tudo!

palavras chave hipóxia, angiogénese, terapias antiangiogénicas, resistência, letalidade sintética e cancro coloretal.

resumo

A angiogénese é essencial à progressão tumoral. As terapias antiangiogénicas bloqueiam a angiogénese e causam regressão dos vasos sanguíneos, o que leva a um aumento da hipóxia nos tumores. A hipóxia é responsável por diversos efeitos na biologia tumoral, entre os quais, a seleção de células cancerígenas mais agressivas e mais resistentes às terapias.

Com este projeto pretendemos descobrir o mecanismo molecular envolvido na resistência à combinação de bevacizumab e cetuximab e também encontrar interações de letalidade sintética com hipóxia.

Os nossos resultados mostram que: a hipóxia induz resistência à inibição de EGFR em células WT4 de cancro coloretal; o HIF1α não é responsável pelo fenótipo de resistência; a hipóxia ativa RAS em células WT4 de cancro coloretal; os inibidores de MEK aumentam a sensibilidade aos inibidores de EGFR em hipóxia e as citoquinas parecem estar envolvidas na ativação de RAS em hipóxia. Identificámos ainda quatro genes que são potenciais candidatos a terem letalidade sintética com hipóxia.

Estes resultados têm uma grande importância clínica e biológica e podem conduzir a melhores terapias combinatórias, contribuindo para melhorar os atuais tratamentos de pacientes com cancro coloretal e podem ainda levar à descoberta de biomarcadores de resposta a terapias antiangiogénicas.

keywords hypoxia, angiogenesis, antiangiogenic therapies, resistance, synthetic lethality and colorectal cancer.

abstract

Angiogenesis is essential for tumor progression. Antiangiogenic therapies block angiogenesis and cause vessel regression, which leads to an increase of tumor hypoxia. Hypoxia is responsible for many effects in tumor biology, among which, the selection of cells that are more aggressive and more resistant to cancer therapies.

In this project we aim to get some molecular insight on the mechanism(s) underlying the resistance to the combination of bevacizumab and cetuximab and to find synthetic lethal interactions with hypoxia.

Our results show that: hypoxia induces resistance to EGFR inhibition in WT4 CRC cell; HIF1α is not driving the resistance phenotype; hypoxia activates RAS in WT4 CRC cells; MEK inhibitors increase the sensitivity to EGFR inhibitors in hypoxia and cytokines seem to be involved in the activation of RAS in hypoxia. We also identified four genes as potential candidates to be synthetic lethal with hypoxia.

Our findings are of great clinical and biological significance and may lead to better combination therapies, improving current treatments for CRC patients and may also lead to the discovery of biomarkers of response to antiangiogenic therapies.

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List of abbreviations

AA	antiangiogenic
APC	adenomatous polyposis coli
Asn	asparagine
ATP	adenosine triphosphate
BRAF	Raf murine sarcoma viral oncogene homolog B
CRC	colorectal cancer
DNA	deoxyribonucleic acid
DMOG	dimethyloxaloylglycine
e.g.	for example
ECM	extracellular matrix
ECs	endothelial cells
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERBB	family of four receptor tyrosine kinases (EGFR/Her1; Her2; Her3; Her4)
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FIH	factor inhibiting HIF-1
GDP	guanosine diphosphate
GEJ	gastro-esophageal junction
GTP	guanosine triphosphate
HIF	hypoxia inducible factor
HRE	hypoxia responsive element
i.e.	that is
IL-8	interleukin 8
KD	knockdown
KRAS	kirsten rat sarcoma viral oncogene homolog
mCRC	metastatic colorectal cancer
MMPs	matrix metalloproteinases
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells

NRAS	neuroblastoma RAS viral oncogene homolog
NSCLC	non-small cell lung cancer
O_2	oxygen
OS	overall survival
PDGF	platelet-derived growth factor
PHD	prolyl hydroxylase domain protein
PlGF	placental growth factor
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
pVHL	von Hippel–Lindau tumor suppressor protein
RCC	renal cell carcinoma
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
TILs	tumor-infiltrating lymphocytes
USA	United States of America
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WT	wild type
WT4	quadruple (KRAS, NRAS, BRAF and PIK3CA) wild type

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1. Introduction

1.1. Cancer

1.1.1. Cancer basics

Cancer is a complex collection of distinct genetic diseases. It is characterized by an abnormal, continued, uncontrolled and damaging growth of cells, with the potential to invade or spread to other parts of the body, that differ structurally and functionally from the normal cells from which they developed.[1–4]

Cancer is the result of genetic and epigenetic alterations in the DNA, specifically those that cause mutations in proto-oncogenes and tumor suppressor genes.[1,5,6] Many different factors initiate changes in cells that lead to cancer, but they are usually divided in two groups: environmental and lifestyle conditions (e.g., chemicals, radiation, viruses, pollution, smoking, diet, alcohol, sun exposure, stress and physical inactivity) – accounting for 90 to 95% of cases; and inherited genetic defects – accounting for 5 to 10% of cases.[1,6–9]

Consistent with Darwinian principles, cancer evolves through a series of accumulated random mutations followed by the clonal selection of cells that can survive and proliferate under circumstances that would normally lead to apoptosis.[4] This process, called carcinogenesis, leads to the acquisition of a set of characteristics, common to most cancers, called the hallmarks of cancer.[10–12] Carcinogenesis is usually a very slow process that can take many years from the first mutation until the formation of the tumor.[12,13]

During the early stages of cancer, tumors are typically benign and remain confined within the normal boundaries of a tissue. But, as tumors grow and become malignant, they gain the ability to break through these boundaries and invade adjacent and/or distant tissues – a process called metastasis.[14–17]

In primary tumors several subclones coexist, and although some expand, others remain dormant or become extinct. Metastases can originate from either a major clone in the primary tumor or from minor clones. Metastases can also undergo clonal evolution (figure 1).[18]

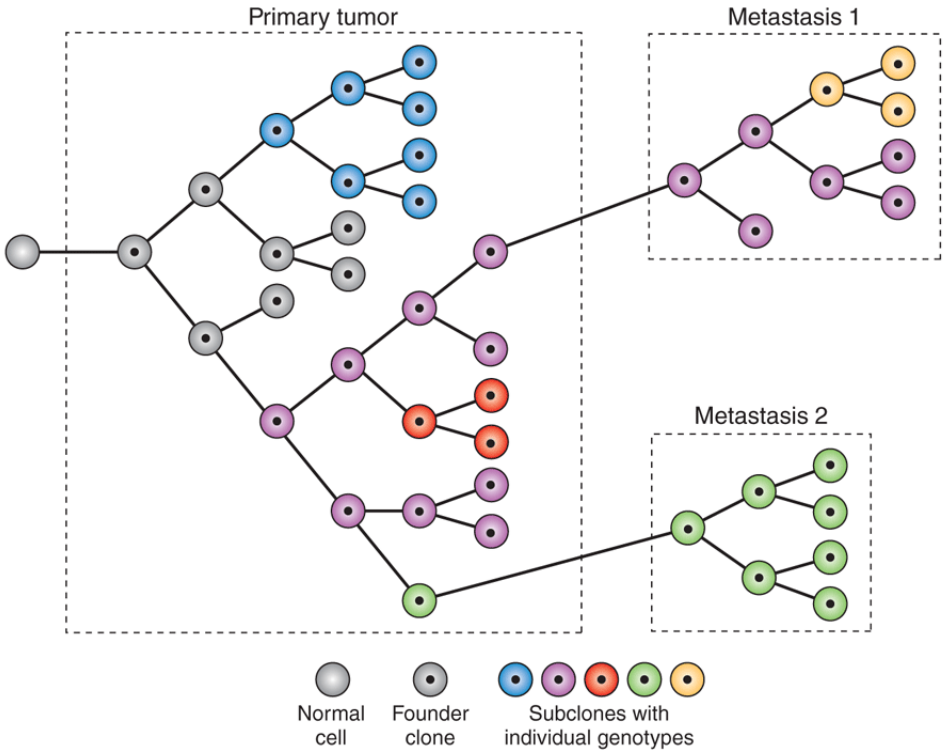

Figure 1: Clonal evolution in primary tumors and metastases.
The grey circle represents a normal cell and the central dot depicts the initiating somatic mutation that drives the founder clone in the tumor. The different colored circles represent subclones that have accumulated successive mutations. Note that in the primary tumor several subclones coexist, and although some expand, others remain dormant or become extinct. Metastases can originate from either a major clone in the primary tumor (metastasis 1), or from minor clones (metastasis 2). Metastases can also undergo clonal evolution (as shown in metastasis 1).[18]

Surgical resection and adjuvant therapy can cure well confined primary tumors, however, metastatic disease is largely incurable because of its systemic nature and the resistance of disseminated tumor cells to existing therapeutic agents. This explains why more than 90% of mortality from cancer is attributable to metastases and not the primary tumors from which these malignant lesions arise.[14–17]

Metastasis is a complex succession of cell-biological events (figure 2) – collectively termed the invasion-metastasis cascade – whereby epithelial cells in primary tumors: (1) invade locally through surrounding extracellular matrix (ECM) and stromal cell layers, (2) intravasate into the lumina of blood vessels, (3) survive the rigors of transport through the vasculature, (4) arrest at distant organ sites, (5) extravasate into the parenchyma of distant tissues, (6) initially survive in these foreign microenvironments in order to form micrometastases, and (7) reinitiate their proliferative programs at metastatic sites, thereby generating macroscopic, clinically detectable neoplastic masses.[14–17,19]

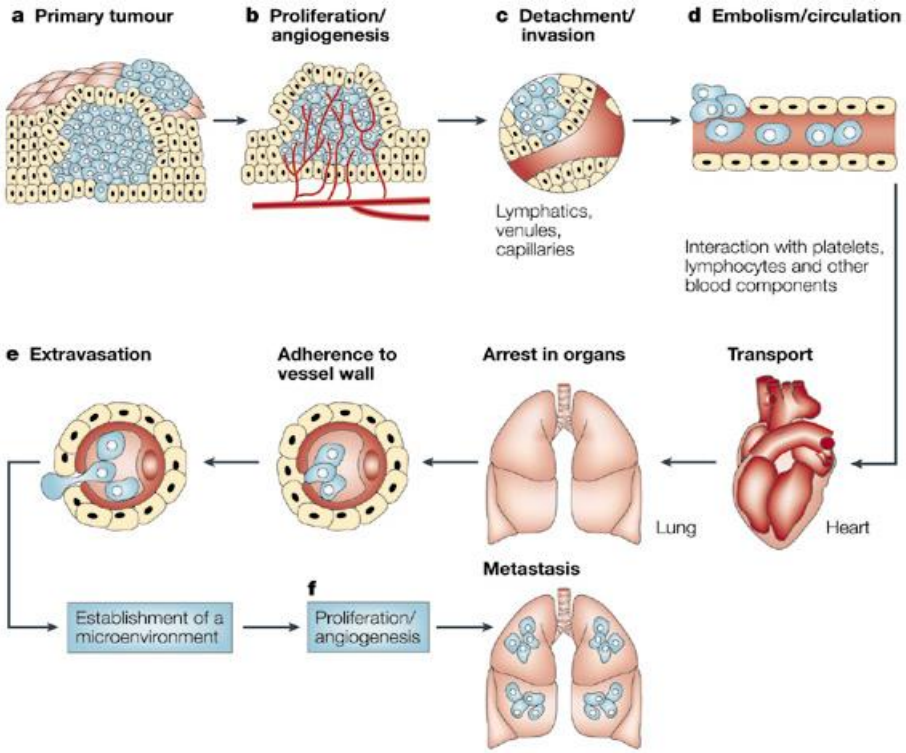

Figure 2: The main steps in the formation of a metastasis.
(a) Cellular transformation and tumor growth. Growth of neoplastic cells is progressive, with nutrients for the expanding tumor mass initially supplied by simple diffusion. (b) Extensive vascularization must occur if a tumor mass is to exceed the limit of oxygen and nutrients' diffusion (1–2 mm in diameter). (c) Local invasion of the host stroma by some tumor cells occurs by several parallel mechanisms. Thin-walled venules, such as lymphatic channels, offer very little resistance to penetration by tumor cells and provide the most common route for tumor-cell entry into the circulation. (d) Detachment and embolization of single tumor cells or aggregates occurs next, but most circulating tumor cells are rapidly destroyed. The tumor cells that survive the circulation become trapped in the capillary beds of distant organs by adhering either to capillary endothelial cells or to subendothelial basement membrane that might be exposed. (e) Extravasation occurs next – probably by mechanisms similar to those that operate during invasion. (f) Proliferation within the organ parenchyma completes the metastatic process. To continue growing, the micrometastasis must develop a vascular network and evade destruction by host defenses. Cancer cells can then invade blood vessels, enter the circulation and produce additional metastases.[14]

1.1.2. The hallmarks of cancer

The hallmarks of cancer consist of eight biological capabilities, acquired throughout carcinogenesis, that help rationalizing the complexity of cancer. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction. Underlying these hallmarks are genome instability, which generates the genetic diversity that expedites their acquisition, and inflammation, which boosts multiple hallmark functions.[10,20] The classical representation of the hallmarks of cancer can be seen in figure 3.

The multistage process of tumor formation is driven by progressive acquisition of activating mutations in dominant growth-enhancing genes (oncogenes) and inactivating mutations in recessive growth-inhibitory genes (tumor suppressor genes). This means that, despite the multitude of genetic and epigenetic alterations found across cancers, a given tumor is mostly driven by a select few changes – those that result in the gain of an oncogene or the loss of a tumor suppressor gene.[1,4,21–23]

Studies by Jain *et al.,*[24] Felsher and Bishop[25] and others show that cancer cells are often addicted to (i.e., physiologically dependent on) the continued activity of specific activated or overexpressed oncogenes for maintenance of their malignant phenotype. Indeed, the inactivation of a single critical oncogene can induce cancer cells to differentiate into cells with a normal phenotype or to undergo apoptosis. This dependency of tumors upon the continued activity of certain oncogenes is called “oncogene addiction”.[1,4,13,21,22]

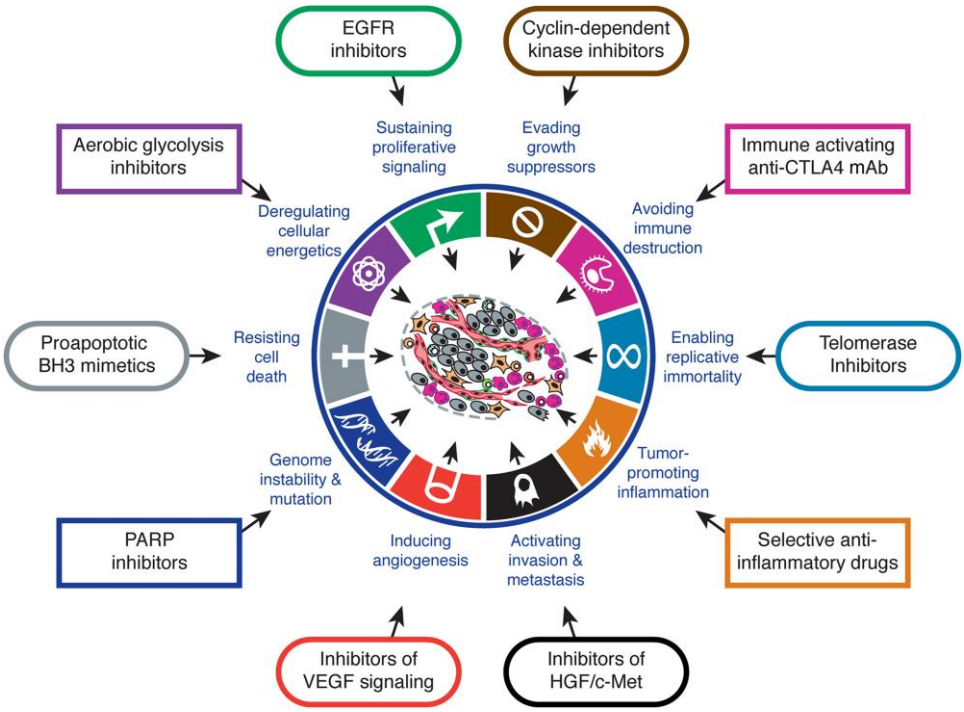

Figure 3: The hallmarks of cancer and their therapeutic targets.
Drugs that interfere with each of the acquired capabilities necessary for tumor growth and progression have been developed and are in clinical trials or, in some cases, approved for clinical use in treating certain forms of human cancer. Additionally, drugs are being developed to target each of the enabling characteristics and emerging hallmarks, which also hold promise as cancer therapeutics. The drugs listed are but illustrative examples; there is a deep pipeline of candidate drugs with different molecular targets and modes of action in development for most of these hallmarks.[20]

1.1.3. Cancer treatment

According to recent studies, around one in every four deaths in the USA is due to cancer.[26] And the burden that cancer represents for patients (even for those who survive the disease) and their families adds even further relevance to these figures. Therefore, it is important to improve current cancer therapies and develop new ones. Currently, the most common cancer treatments are surgery, chemotherapy, radiotherapy, immunotherapy and targeted therapy.[1,27,28]

Surgery consists of the excision of the cancer in its entirety, together with all adjacent tissues into which cancer cells may have spreaded. It is the most effective form of treatment, but it can only be performed early in the disease and before the cancer spreads into tissues that cannot be resected.[1]

Chemotherapy and radiotherapy both consist mostly of inducing DNA damage, which will cause problems in cell division and bring about apoptosis. The main drawbacks of these treatments are that they are not tumor specific, so normal cells that are actively proliferating may also be affected (which explains the severe side effects associated with these strategies) and also that development of resistance to these therapies is a possibility.[1,28]

Immunotherapy is a type of cancer treatment designed to boost the body's natural defenses to fight cancer.[29,30] It has long been recognized that the immune system and malignant cells often coexist in a dynamic equilibrium, and the complex interaction between growing tumors and the immune system may determine the course of disease.[20,29,30] Tumors must develop the ability to evade the immune system in order to proliferate and metastasize.[10,20] The theory of immune surveillance suggests that the immune system is proactively able to eliminate abnormal cells and prevent cancer formation in the body. Studies have shown that patients with compromised or suppressed immune function have an increased risk of developing cancer.[31–35] Clearly, the adaptive immune response is able to control the growth of some tumors, as evidenced by the observation that the presence of tumor-infiltrating lymphocytes (TILs) often is associated with improved overall survival (OS), i.e. the percentage of patients that are alive after being diagnosed and starting treatment is higher for patients with TILs.[36–38] However, the

immune system is rendered ineffective as tumors progress. The goal of cancer immunotherapy is to boost or restore the ability of the immune system to detect and destroy cancer cells by overcoming the mechanisms by which tumors evade and suppress the immune response, in essence, to shift the equilibrium back in favor of immune protection.[34] The traditional approach to immunotherapy has been to increase the frequency of tumor-specific T cells through administration of tumor vaccines, cytokines such as interleukin-2, and adoptive transfer of TILs.[34,39] In the last decade, efforts to improve presentation of tumor antigens to the immune system have focused on antigen-presenting cells such as myeloid dendritic cells. Remarkable achievements in the field have already produced a paradigm shift in melanoma treatment: metastatic melanoma, previously considered incurable, can now be treated with a potentially curative rather than palliative intent.[40] This type of treatment has been gaining more relevance in the cancer research field in recent years and, in 2013, it was considered the breakthrough of the year by *science magazine*.[41]

Targeted therapy refers to a new generation of anti-cancer drugs (small-molecule inhibitors and monoclonal antibodies) designed to interfere with a specific molecular target (typically a protein) that is believed to have a critical role in tumor growth or progression.[20,42–44] The introduction of mechanism-based targeted therapies to treat human cancers has been considered as one of the fruits of decades of remarkable progress of research into the biology of cancer pathogenesis.[20] Targeted therapeutics can be categorized according to their respective effects on one or more hallmark capabilities,[10,20] as illustrated in the examples presented in figure 3. Most of the hallmark-targeting cancer drugs developed to date have been deliberately directed toward specific molecular targets that are involved in one way or another in enabling particular capabilities.[20] Such specificity of action has been considered a virtue, as it presents inhibitory activity against a target while having, in principle, relatively fewer off-target effects and thus less nonspecific toxicity.[20] However, despite continuous breakthroughs in cancer therapy and drug development, targeted therapy is almost inevitably challenged by the occurrence of drug resistance, mainly due to tumor heterogeneity (i.e., the existence of tumor subpopulations harboring distinct mutations – some of which resistant to the drug), therefore allowing

cancer progression.[20,45,46] More recently, with the concept of synthetic lethality (two genes are synthetic lethal if a mutation of either alone is compatible with viability but mutation of both leads to death)[47] and the increasing knowledge about cancer pathways a new era in cancer therapy has emerged. The old concept that one single drug would treat cancer is now outdated; nowadays, most studies focus in finding two (or more) drugs whose combination increases tumor killing and reduces resistance.[48–50] The most common approach to find synthetically lethal interactions are loss of function genetic screens, which have rendered several combinations that were proven to have a major clinical impact.[51,52] One successful example was the discovery that resistance to BRAF inhibition in $BRAF^{V600E}$ mutant colon cancer can be overcomed by co-targeting EGFR (which resulted in three clinical trials).[48] Another appealing example of the concept of synthetic lethality is the discovery of toxicity of Poly (ADP-ribose) polymerase (PARP) 1 and 2 inhibitors in BRCA1 and BRCA2 mutant cells, which has already been clinically validated in breast cancer.[53,54]

1.2. Hypoxia, angiogenesis and antiangiogenic therapy

1.2.1. Hypoxia and angiogenesis

Oxygen and nutrients are essential for the life of every cell in the body. Therefore, the establishment of a functional, integrated vascular system is crucial for tissue growth and homeostasis.[55,56] Similarly, tumor development and progression is dependent on angiogenesis, as the recruitment of new blood vessels to the tumor site is required for the delivery of nutrients and oxygen and for the removal of waste products.[57] In fact, for tumors to growth beyond the size of about 1-2 mm^3 they must switch to an angiogenic phenotype.[58,59]

The angiogenic process starts when cancer cells experience hypoxia and mount adaptive responses to deal it.[55] The term hypoxia refers to a condition characterized by a cellular or tissue level of oxygenation lower than normal (in the context of tumors, as having an internal partial pressure of oxygen of less than 10–15mmHg (≈3%)).[60] In most solid tumors, hypoxic areas are frequent events, due to: (a) structural and functional abnormalities of the tumor micro-vessels, (b) an increase in diffusion distances, given the highly proliferative capacity of tumor cells, and (c) tumor-associated anemia leading to a reduced oxygen transport capacity of the blood.[61]

The hypoxia inducible factor (HIF) family of transcription factors has emerged as the master regulator of oxygen tension homeostasis and controls fundamental pathophysiological pathways (figure 4).[62–64] HIF-1 is a heterodimeric protein comprising a constitutively expressed α and β subunits, which is tightly regulated by oxygen availability.[65,66] Under normoxic conditions, HIF-1α is hydroxylated by oxygen-dependent prolyl hydroxylase domain proteins (PHDs), and rapidly targeted for ubiquitination and proteasomal degradation. Furthermore, under normoxic conditions, HIF-1α is hydroxylated at residue Asn803 by factor inhibiting HIF-1 (FIH), which inhibits the recruitment of the Cbp-p300 cofactor and blocks HIF-1-dependent transcriptional activation.[65,66]

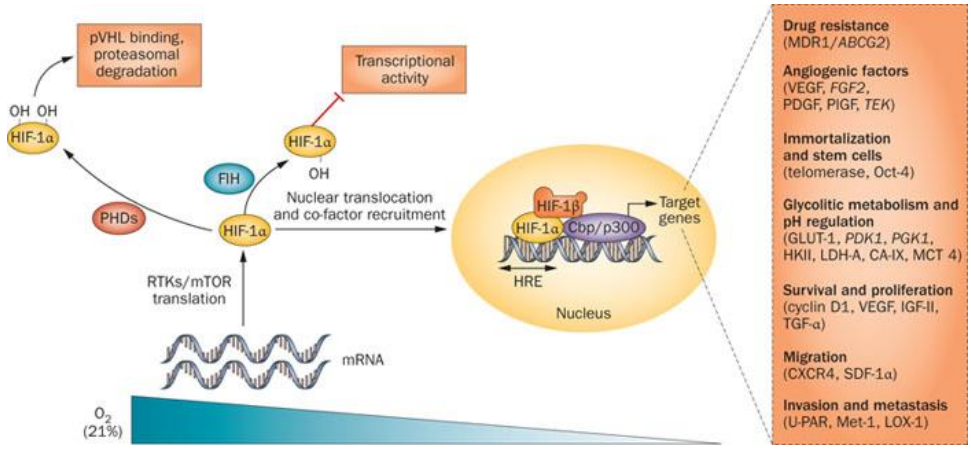

Figure 4: Regulation of HIF-1α and HIF-1α-dependent gene expression.
Under normoxic conditions, HIF-1α is continuously transcribed and translated, but it is rapidly degraded through the pVHL pathway following hydroxylation of Pro402 and Pro564 by PHDs. FIH mediates the hydroxylation of Asn803, which inhibits the recruitment of co-factors for transcription. When the oxygen tension decreases, HIF-1α is stabilized, translocates to the nucleus and dimerises with HIF-1β—enabling transcription of a large number of genes involved in cancer biology. RTKs boost translation of HIF-1α via activation of the mTOR pathway.[64]

Contrarily, in hypoxia, due to the lack of substrate, PHDs become less active and this leads to the HIF-1α stabilization, nuclear translocation and dimerization with HIF-1β.[65,66] By binding to hypoxia responsive elements (HRE) located in the promotor region of several genes, heterodimeric HIF-1 activates the transcription of its numerous targets involved in cellular adaptation to hypoxia, including angiogenesis, metabolism, proliferation, metastasis and differentiation, all together contributing to tumorigenesis.[60,62–64,67]

In hypoxic conditions, HIF gets activated and upregulates the expression of several genes. Some of these are responsible for the production of proangiogenic factors, such as VEGF, which activates endothelial cells (ECs) and promote angiogenesis.[64,67,68] ECs at the leading edge of the vascular sprout extend filopodia and migrate toward angiogenic signals. VEGF activates VEGFR2 to stimulate tip cell migration. Tip cell migration requires basement membrane degradation (in part due to MMP), EC junction loosening (caused by VE-cadherin, ZO-1, and others), and pericyte detachment (regulated by Ang2).[68] VEGF increases the permeability of the vessel, allowing the extravasation of plasma proteins (e.g., fibronectin and fibrinogen) that are deposited as a provisional matrix layer while the preexisting interstitial matrix is remodeled by proteases. Tip cells adhere to the ECM, mediated by integrins, and migrate toward guidance signal molecules (e.g., semaphorins and ephrins). Stalk cells trail behind the tip cell and proliferate to allow sprout elongation

and lumen formation. This system allows vascular migration (by tip cells) and elongation of the shaft (by proliferating stalk cells).[68] When two tip cells meet, they fuse and a connected lumen is formed to allow blood flow through the new vessel. This perfuses the hypoxic tissue, and the resultant oxygen and nutrient delivery leads to decreased levels of angiogenic signals, inactivation of EC oxygen sensors, and increased proquiescent molecules that lead to EC quiescence.[68]

Hypoxia is responsible for many effects in tumor biology: selection of genotypes favoring survival under hypoxia reoxygenation injury (such as TP53 mutations),[69] prosurvival changes in gene expression that suppress apoptosis[70] and support autophagy[71] and the anabolic switch in central metabolism.[72] Hypoxia also enhances receptor tyrosine kinase mediated signaling,[73] tumor angiogenesis,[74] vasculogenesis,[75] the epithelial-to-mesenchymal transition,[76] invasiveness[77] and metastasis,[78] as well as suppressing immune reactivity.[79] In addition, it also contributes to loss of genomic stability through the increased generation of reactive oxygen species (ROS)[80] and the downregulation of DNA repair pathways,[62] as well as contributing to resistance to radiotherapy and chemotherapy.[64]

1.2.2. Antiangiogenic therapy

Antiangiogenic (AA) therapy stems from the fundamental concept that tumor growth, invasion, and metastasis are angiogenesis-dependent.[59,81] The microvascular ECs recruited by tumors (to form new blood vessels) have become an important target in cancer therapy because, unlike cancer cells (the primary target of cytotoxic chemotherapy), that are genetically unstable with unpredictable mutations, the genetic stability of ECs makes them less susceptible to acquired drug resistance.[81] Also, since VEGF is mainly produced by tumor cells promoting angiogenesis, a low side toxicity is expected from this therapy. Therefore, targeting tumor vasculature has arisen as an appealing anti-cancer therapeutic approach.[55,57,82]

To date, ten drugs that target VEGF or its receptors have been approved for the treatment of various malignant diseases[83] – see table 1.

Table 1: Currently antiangiogenic drugs approved for cancer treatment – adapted from Jain K. Rakesh.[83]

Drug	Approved Indication
Bevacizumab	- metastatic colorectal cancer (with chemotherapy) - metastatic nonsquamous NSCLC (with chemotherapy) - metastatic breast cancer (with chemotherapy) - metastatic renal cell carcinoma (RCC) (with IFN-α) - advanced cervical cancer (with chemotherapy)
Sunitinib	- metastatic RCC - gastrointestinal stromal tumor - primitive neuroectodermal tumor
Sorafenib	- metastatic RCC - unresectable hepatocellular carcinoma
Pazopanib	- metastatic RCC - advanced soft tissue sarcoma
Vandetanib	- advanced medullary thyroid cancer
Axitinib	- advanced RCC
Regorafenib	- chemorefractory metastatic colorectal cancer
Aflibercept	- chemorefractory metastatic colorectal cancer
Cabozantinib	- advanced medullary thyroid cancer
Ramucirumab	- metastatic gastric and GEJ cancers - metastatic GEJ cancers (with chemotherapy) - metastatic NSCLC (with chemotherapy)

Antiangiogenic therapies have been widely used in clinical trials, with delayed tumor progression in some patients, leading to improved progression-free survival and overall survival compared with standard therapy. But unfortunately, a significant number of patients either do not respond to AA therapy or rapidly develop resistance to it.[55,64]

The functional consequences of AA therapies on the tumor microenvironment are poorly understood and a matter of debate; at least two contrasting hypotheses have been proposed: (1) AA therapy causes vascular "regression" that results in increased intratumor hypoxia, selection of metastatic clones and resistance to therapy and (2) AA therapy "normalizes" the vasculature, with a consequent decrease in intratumor hypoxia and interstitial pressure, which is associated with improved delivery of chemotherapy.[64,83] These two hypotheses are not necessarily mutually exclusive, but might be cancer and genotype-dependent.[64]

The first hypothesis, is one of the explanations for cancers' (intrinsic or acquired) resistance to AA therapies and the second explains why AA therapies show clinical benefits for a short period of time (in combination with chemotherapy), i.e., AA agents are believed to transiently ''normalize'' the abnormal tumor vasculature, resulting in improved blood perfusion. The latter would decrease hypoxia (known to confer resistance to radio-, chemo- and immune therapies) and increase drug accessibility. Therefore, therapies given during the window of normalization might achieve greater efficacy. The normalized vessels would also resist intravasation of cancer cells from the primary tumor to the blood stream, potentially decreasing metastases.[83–85]

Several mechanisms of resistance to AA therapy have been proposed, however, they all stem from the fundamental concept that vessel regression increases tumor hypoxia.[86,87] The most common escape mechanism is the upregulation of alternative proangiogenic signals (such as FGF, PDGFs and PlGF),[88,89] the use of different modes of vascularization (vessel cooption (by growing around preexisting vessels), vascular mimicry (replacement of ECs by tumor cells), and vasculogenesis (vessel growth from bone marrow–derived progenitor cells))[68,90,91] increasing pericyte coverage of the vasculature[92] and/or activating an invasive phenotype.[93–96] Hypoxic responses have a role in many adaptive mechanisms, as described in the last paragraph of section 1.2.1..

1.3. Colorectal Cancer

1.3.1. Facts & figures

Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer and the second leading cause of cancer death in both men and women in the United States.[97] In Europe, CRC represents the second most common cancer and leading cause of cancer death, in both genders combined.[98] Although there is an increasingly well-described genetic component (accounting for about 5% of disease burden), the dominant causative factors are environmental.[99,100] CRC is therefore a major global health problem that the scientific community must address seriously in order to find new and better solutions.

CRC usually develops slowly, over a period of 10 to 20 years.[101] Most begin as a noncancerous growth, called a polyp, developing on the inner lining of the colon or rectum. The most common kind of polyp is called an adenomatous polyp or adenoma. Although all adenomas have the capacity to become cancerous, less than 10% are estimated to progress to invasive cancer.[102,103] Cancers that develop in glandular cells are called adenocarcinoma, and account for most CRC (approximately 96%).[104] The initial step in CRC tumorigenesis is the adenoma formation, associated with loss of APC. Larger adenomas and early carcinomas then acquire mutations in the small GTPase KRAS (which causes proliferative signaling to be continuously active), followed by loss of chromosome 18q and mutations in TP53.[105]

Given the recognized transition from premalignant adenomas to invasive carcinomas[106] this type of cancer has, in principle, a great potential to be detected in early stages, increasing the likelihood of cure. Yet, because CRC's main detection method (colonoscopy) is quite invasive, most people avoid it until it's too late (i.e., when the cancer has already metastasized). The majority of patients with metastatic CRC (mCRC) cannot be cured and therefore, metastasis remains the major cause of cancer-related death[107]. Yet, over the past years, the outcome of these patients has been improved, mainly due to the introduction of targeted therapies in the clinic.[108,109]

1.3.2. Targeted therapy in CRC

Considerable progress has been made in the treatment of colorectal cancer from the era when 5-fluorouracil (chemotherapeutic drug) was the only effective agent for this disease.[109] In addition to new chemotherapeutic agents, such as oxaliplatin, irinotecan, and capecitabine, the advent of targeted therapies has contributed considerably to the treatment of colon cancer and has improved clinical outcomes. An increased understanding of cancer at the molecular and genetic level has allowed for the development of therapeutics that target the multiple pathways essential to malignant behavior.[45,109]

While currently approved targeted therapies in advanced colorectal cancer (table 2) is limited to monoclonal antibodies against VEGF and EGFR, many more drugs targeting different pathways of oncogenesis are in development.[109]

Sustained angiogenesis is one of the hallmarks of cancer.[10,20] One mechanism responsible for the induction of angiogenesis is altered gene transcription, resulting in overproduction of VEGF at the tissue level.[110] Both the ligand (VEGF) and its receptors (VEGFR) are known to be overexpressed in colon cancer, and their presence indicates a poor prognosis.[111–113] There is also evidence that VEGF inhibition can have direct antitumor effects through a separate mechanism apart from angiogenesis.[114,115] Based on these observations, there is a great rationale for targeting the VEGF pathway in CRC. However, patients either do not respond to the treatment or those who respond eventually develop resistance – the mechanisms have already been described in the last paragraph of section 1.2.2..

Table 2: Currently FDA approved targeted therapies for colorectal cancer – adapted from FDA.[116]

Drug	Target
Bevacizumab	anti-VEGF
Zaltrap	anti-VEGF-A and anti-PlGF
Cetuximab	anti-EFGR
Panitumumab	anti-EGFR
Regorafenib	multi-kinase inhibitor

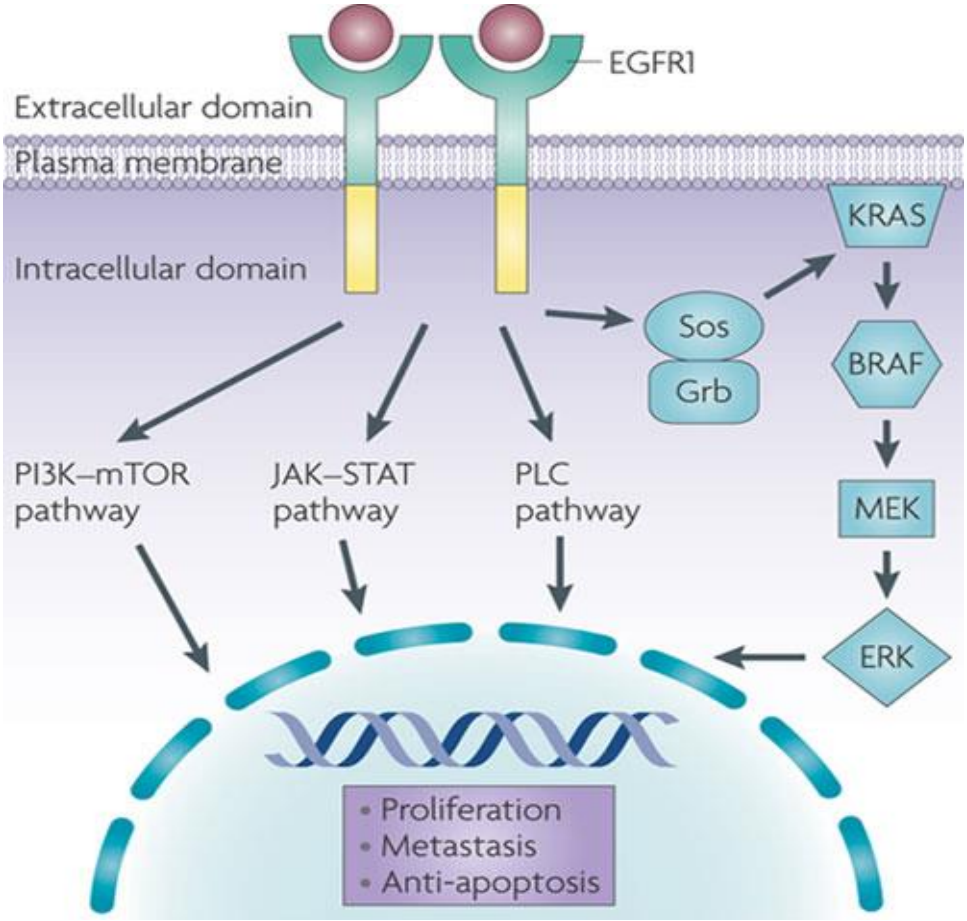

Figure 5: The epidermal growth factor receptor signaling pathway.
Activation of membrane kinases including EGFR by external growth factors initiates receptor dimerization and subsequent activation of the intracellular kinase domain, which in turn leads to the activation of intracellular pathways. Through the small adaptor proteins Sos and Grb, the KRAS signalling cascade is activated, leading to increased proliferation. Part of the KRAS pathway is BRAF, which explains why non-constitutively activated KRAS and BRAF are necessary for EGFR blockade to work.[105]

Unchecked and deregulated growth are also hallmarks of cancer.[10,20] Among the growth factors implicated in the development of colon cancer, EGF and its receptors have been studied the most. EGFR is a member of the ErbB family of receptors, a group that is abnormally activated in many epithelial malignancies, such as CRC.[117] It has been demonstrated that potentially 80% of colon cancers exploit EGFR for their pathogenesis.[118,119] There is also evidence to suggest that EGFR inhibition functions not only through downregulation of growth signals, but also through downregulation of proangiogenic factors including VEGF and IL-8, leading to a decrease in tumor microvessel density.[120] Further supporting the AA effect of EGFR inhibition is the fact that EGFR resistant tumors demonstrate upregulation of VEGF.[121] Based on these observations, there is also a great rationale for targeting the EGFR pathway (figure 5) in CRC.

Investigations into the molecular basis of response to EGFR-blocking antibodies started in 2005 and since then a rapidly accumulating body of knowledge has indicated that resistance to EGFR blockade in mCRC is related to constitutive activation of signaling pathways downstream of EGFR.[100]

Mutations in KRAS were the first to be causally implicated in resistance to EGFR-targeted monoclonal antibodies, leading to the exclusion of patients with chemorefractory mCRC with tumors bearing KRAS mutations from treatment with single-agent cetuximab or panitumumab.[122,123] Also, because not all KRAS wild-type patients benefit from treatment with EGFR-directed therapy, research has flourished to identify additional biomarkers of resistance that could account for the heterogeneity in clinical response.[100]

Several studies based on preclinical models and tumor samples obtained at relapse identified molecular mechanisms that lead to acquired resistance to EGFR blockade in colorectal cancer: (1) mutations in the EGFR extracellular domain[124] (which impairs the binding of the antibody to the receptor); (2) amplification of RTKs[125–127] (such as ERBB2 or MET); (3) mutations in RAS and RAF genes (both point mutations and gene amplification).[128–130]

1.3.3. Combination of anti-EGFR with anti-VEGF therapies in CRC

Multiple cellular pathways influence the growth and metastatic potential of tumors. This creates heterogeneity, redundancy, and the potential for tumors to bypass signaling pathway blockade, resulting in primary or acquired resistance.[131,132] A multifaceted approach, involving targeted inhibition of multiple signaling pathways, may be more effective than inhibition of a single target and may help overcome tumor resistance by blocking potential "escape routes".[131]

Two key elements in the growth and dissemination of tumors are the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor (VEGF). Close relationships between these factors exist: VEGF signaling is up-regulated by EGFR expression and, conversely, VEGF up-regulation (independent of EGFR signaling) seems to contribute to resistance to EGFR inhibition, however, the mechanism behind this is not well known yet.[133–137]

Both EGFR and VEGF are overexpressed in patients with colorectal cancer[112,113,138,139] and several studies have showed that drugs targeting either the EGFR or the VEGF pathways have clinical benefit in several human cancers, either alone or in combination with standard cytotoxic therapies.[131] Therefore, it is expected that the inhibition of both these pathways could improve antitumor efficacy and overcome resistance.

Anti-VEGF treatment used in conjunction with EGFR inhibitors has shown promising results in preclinical and clinical studies. A xenograft study blocking VEGF (with VEGF antisense oligonucleotide) and EGFR (with C225 antibody) demonstrated synergistic antitumor activity,[140] and mice intraperitoneally injected with human colon cancer cells showed improved antitumor activity in response to anti-EGFR antibody (C225) and an anti-VEGFR2 antibody (DC101).[141] Phase I and II clinical studies in mCRC indicate increased efficacy with the combination of anti-VEGF and anti-EGFR therapy, with improved response rate, increased time to progression, and increased overall survival in patients who received cetuximab and bevacizumab[142] versus historical control groups of patients who received cetuximab,[143] bevacizumab monotherapy,[144] or cetuximab plus chemotherapy.[145] This activity of the combination of cetuximab and bevacizumab may be due to the fact that

resistance to EGFR inhibitors is mediated, at least partly, by activating VEGF-dependent signaling.[146,147]

However, two other clinical studies combining chemotherapy, anti-EGFR and anti-VEGF blocking antibodies (CAIRO2 and PACCE) showed disappointing results (figure 6): a significant decrease in progression free survival (i.e., the period of time after the start of treatment of a disease that patients live with stable disease, without it getting worse) and a poorer quality of life, due to the secondary effects of the drug combination.[143,148] The negative results of the CAIRO2 trial might be explained by a negative interaction between cetuximab and bevacizumab. This negative interaction is caused by hypertension, a common side effect of bevacizumab treatment, that was recently shown to correlate with clinical outcome in patients with colorectal cancer.[149] In the study it was observed that hypertension was less frequent in the group treated with chemotherapy plus cetuximab and bevacizumab, in opposition to the group treated with only chemotherapy plus bevacizumab, which suggests a decreased efficacy of bevacizumab when administered in combination with cetuximab.[150] Also, as expected, the KRAS genotype affects the response to anti-EGFR treatment: patients with WT-KRAS tumors have longer progression-free survival than those with mutated-KRAS tumors.[150] However, no clear mechanism explaining the failure of this combination has been found so far, making it an attractive research topic.

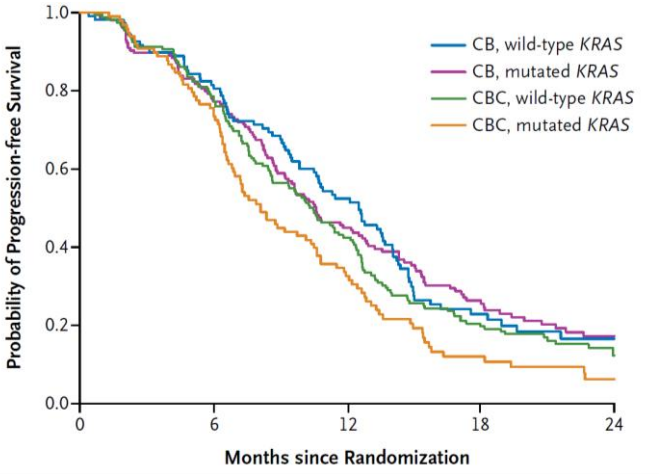

Figure 6: Progression-free survival in the CAIRO II clinical trial.
In this randomized trial in patients with metastatic colorectal cancer, the addition of cetuximab to treatment with capecitabine, oxaliplatin, and bevacizumab resulted in a significant decrease in progression-free survival and a poorer quality of life. The KRAS genotype affects the response to anti-EGFR treatment: patients with wild-type KRAS tumors have longer progression-free survival than those with mutated-KRAS tumors.[150] (CB = chemotherapy + bevacizumab; CBC = chemotherapy + bevacizumab + cetuximab)

1.4. Objectives

Tumor hypoxia, a common feature in most cancers, is further aggravated upon antiangiogenic treatment and, while it can represent a lethal stress to some tumor cells, it can also function as a potent driving force of malignancy and drug resistance for others.[86,87]

A critical step to increase the efficacy of AA therapies and develop new cancer fighting strategies it is to overcome their main cause of resistance – hypoxia. Also, to date, no clear prognostic or predictive markers of response to AA agents have been found, which makes the search for biomarkers to identify patients who may benefit from angiogenesis inhibitors (and also serve as potential drug targets) another critical step to upgrade the efficacy of AA therapies.

This project aims to improve the efficacy of AA agents using an *in vitro* translational approach. In a first part, we intend to find the mechanism(s) underlying the resistance to the combination of bevacizumab and cetuximab showed in the CAIRO2 clinical trial. To that aim, cells were treated with cetuximab, both in normoxia and in hypoxia, to mimic EGFR inhibition in combination with anti-VEGF. Then, using functional (such as colony formation assays) and molecular (e.g. western blotting) assays we tried to uncover the mechanism(s) that confer resistance to the anti-EGFR treatments in hypoxia. In a second part, using the concept of synthetic lethality, we aim to identify novel and powerful synthetic lethal interactions with hypoxia. To do that, we analyzed gene expression of six different CRC cell lines cultured in both normoxia and hypoxia so as to derive a CRC specific hypoxia-signature. We performed a functional genetic screen (using a collection of RNA interference vectors that target the genes of the hypoxia-signature) to identify which of these genes are pivotal for the survival of CRC cells cultivated under hypoxic conditions.

2. Results and Discussion

2.1. Resistance to anti-EGFR treatments in hypoxia in WT4 CRC cells

In the first part of this project, we used an *in vitro* approach to study how CRC cell lines respond to the combination of EGFR and VEGF inhibition. EGFR was inhibited by the monoclonal antibody cetuximab, given its approval for the treatment of CRC patients in the clinic. To mimic the effect of anti-VEGF therapy (which primarily targets tumor ECs of the blood vasculature and therefore cannot be used in an *in* vitro experimental setting) we cultured cells in a hypoxic environment (1% O_2). The efficacy of this treatment was determined in a panel of CRC cell lines using a long term proliferation assay (10-15 days).

Our data shows that despite substantial sensitivity to cetuximab treatment in normoxia (21% O_2), quadruple WT (WT4 – WT for KRAS, NRAS, BRAF and PIK3CA) CRC cell lines (DiFi, Lim 1215, CCK81 and OXCO2) become more resistant to EGFR inhibition when cultured in hypoxia (1% O_2, Figure 7A). We were able to recapitulate this finding using gefitinib and neratinib, two TKIs that inhibit EGFR and EGFR/HER2, respectively (Figure 7B). This hypoxia-dependent phenotype seems to be specific for WT4 CRC cell lines, as proliferation of $KRAS^{MUT}$ (LoVo) or $BRAF^{MUT}$ (HT-29) CRC cell lines was not significantly affected by EGFR inhibition at any of the studied oxygen tensions (Figure 7C). This result was not surprising, especially for the $KRAS^{MUT}$ context, since patients with KRAS mutations respond poorly to cetuximab, which lead to their exclusion from treatment with the EGFR inhibitor by FDA.[151] Of note, all tested cell lines were able to survive for over 15 days at 1% O_2, only displaying a slight impairment in proliferation when compared to 21% O_2 conditions. In addition, these results mirror the clinical trial CAIRO2, where KRAS WT patients did not benefit from the combination of cetuximab and bevacizumab[151]. Therefore this system is of potential interest to study the mechanisms of resistance to EGFR inhibition in hypoxia and, possibly, get some molecular insight to explain and overcome the CAIRO2 disappointing results.

To validate these findings we tested lysates of drug/hypoxia-treated cells with phosphoprotein-specific antibodies that identify the activated state of components of the

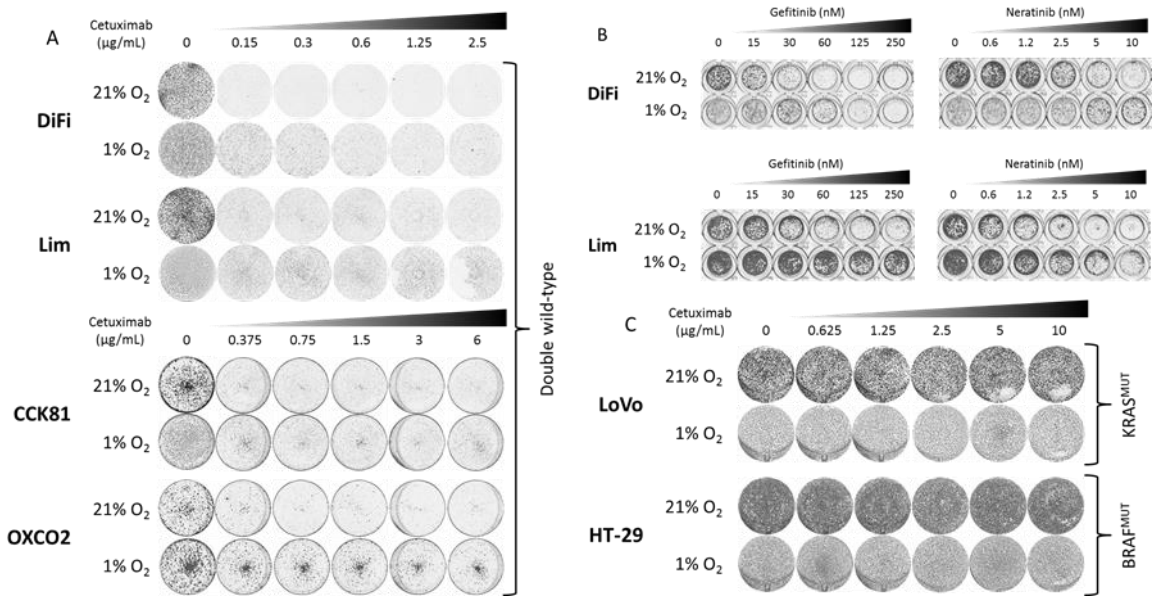

Figure 7: Hypoxia induces resistance to EGFR inhibition in WT4 CRC cell lines.
Long-term colony formation assay of a CRC cell line panel consisting of WT4 (DiFi, Lim, CCK81 and OXCO2), $KRAS^{MUT}$ (LoVo) and $BRAF^{MUT}$ (HT-29) cell lines. Cells were treated with increasing concentrations of cetuximab (A and C) and gefitinib or neratinib (B) and incubated at 21% or 1% O_2 for 10-15 days. For each cell line, all plates were seeded, fixed, stained and scanned at the same time.

survival, proliferation, apoptosis, hypoxia and EGFR signalling pathways (Figure 8). As expected, cetuximab treatment resulted in a decrease in Tyr 1068 phosphorylation of EGFR, which reflects its inhibition. Moreover, CRC cells accumulated the HIF1α transcription factor when cultured in hypoxia, confirming that the hypoxic sensing machinery was active in our experimental setting. Of note, cetuximab decreased HIF1α accumulation in hypoxia, which is concordant with published data, where the use of EGFR inhibitors has been shown to decrease tumor cell expression of HIF-1α and VEGF.[152,153] We also observed that cetuximab-treated cells cultured in hypoxia have more phosphorylated AKT, a known marker of survival,[154] which confirms biochemically the resistance phenotype observed in the long term colony formation assay. In light with these findings, blockage of apoptosis in cetuximab-treated cells in hypoxia is confirmed by the low levels of cleaved PARP (apoptosis marker),[155] indicating that these cells are indeed protected against apoptotic cell death. Phosphorylated ERK is also higher in hypoxia-treated DiFi cells, yet this is not so clear in the other cell lines, which might have to do with the time point when samples were taken for analysis; a time course experiment would elucidate further. Overall, with this experiment we were able to biochemically confirm the long term proliferation assay resistant phenotype.

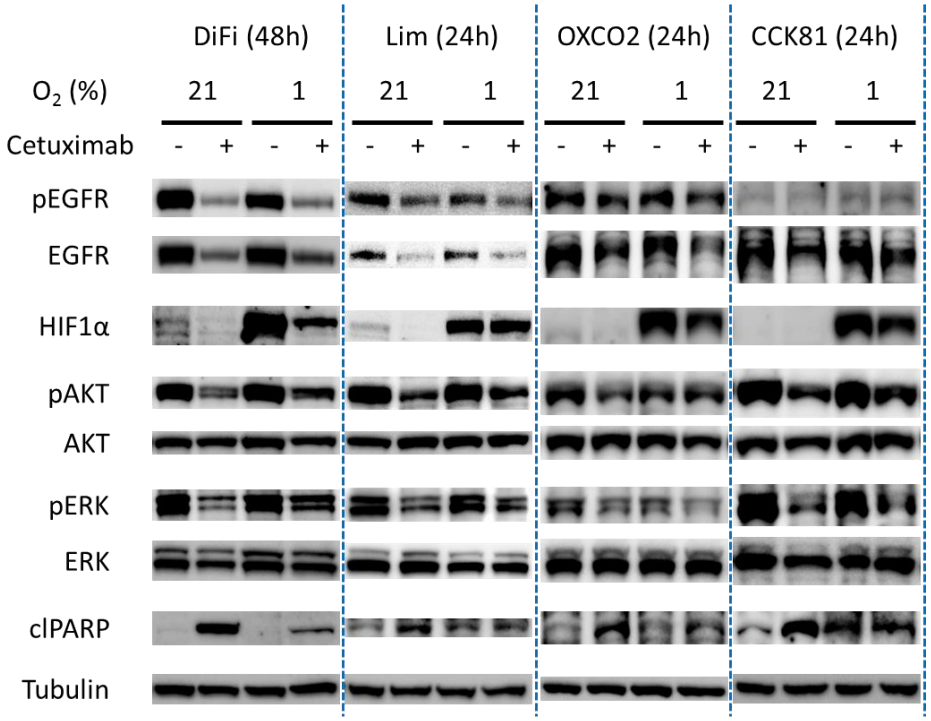

Figure 8: Hypoxia induced resistance to cetuximab in WT4 CRC cell lines confirmed by Western blot. Biochemical responses of DiFi, Lim 1215, OXCO2 and CCK81 cells to cetuximab, hypoxia, or their combination, were documented by western blot analysis. Cells were harvested at 24 hours (Lim 1215, OXCO2 and CCK81) or 48 hours (DiFi) after cetuximab treatment (DiFi cells were treated with 1 μg/mL of cetuximab and Lim 1215, OXCO2 and CCK81 cells were treated with 5 μg/mL of cetuximab). Treatment with cetuximab resulted in a decrease of EGFR (and consequently pEGFR) as expected; in hypoxia, cells treated with cetuximab exhibit a higher phosphorylation of AKT and ERK as compared with normoxia-treated cells and as a consequence, normoxia-treated cells express more cleaved PARP than hypoxia-treated cells. HIF1α served as a control for the hypoxia treatment and tubulin served as a loading control.

HIF is the master regulator of the cellular adaptations to hypoxia. Given that the resistance to EGFR inhibition in the cell lines used in our study only occurs in hypoxia we investigated whether HIF was mediating the resistance phenotype. We addressed this question by performing gain and loss of function experiments, where we treated cells with gefitinib or neratinib in combination with DMOG (HIF stabilizer) or digoxin (HIF inhibitor). We observed that treatment with DMOG (dimethyloxalylglycine, a HIF stabilizer agent by inhibition of PHDs) did not confer resistance to EGFR inhibition in normoxia (Figure 9A) and that treatment with digoxin did not sensitize cells to EGFR inhibition in hypoxia (Figure 9B). These results indicate that HIF is not mediating the resistance to EGFR inhibition in hypoxia. These findings are concordant to our previous biochemical analysis where upon cetuximab treatment in hypoxia HIF accumulation was reduced comparing to the untreated cells.

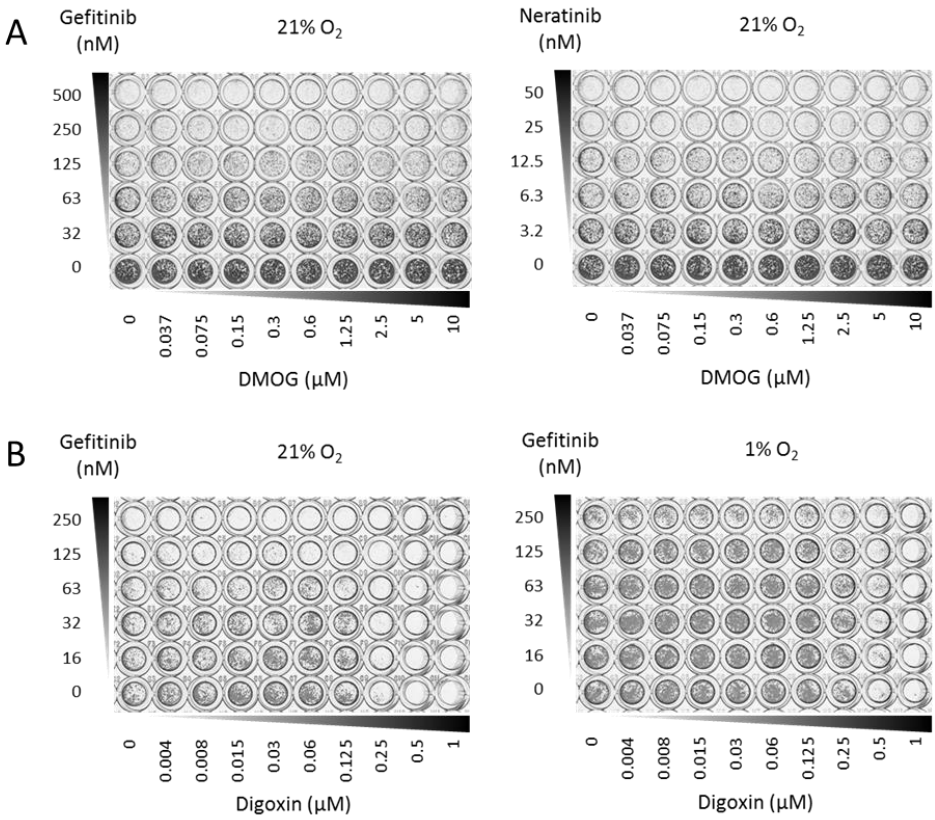

Figure 9: HIF is not mediating the resistance to EGFR inhibition in hypoxia.
A, Response of Lim cells to the combination of EGFR inhibitors gefitinib or neratinib and DMOG. Lim cells were cultured with increasing concentrations of EGFR inhibitor gefitinib or neratinib alone, DMOG alone, and their combination. Cells were incubated at 21% for 10 days. B, Response of Lim cells to the combination of gefitinib and digoxin. Lim cells were cultured with increasing concentrations of gefitinib alone, digoxin alone, and their combination. Cells were incubated at 21% or 1% O_2 for 10 days. All plates were seeded, fixed, stained and scanned at the same time.

This result increases the novelty potential of this project, given that most described resistance mechanisms in hypoxia are HIF-dependent.[156,157]

Amplification of the MET receptor is a known driver of resistance to anti-EGFR therapies in CRC[158] and although it has also been reported as a hypoxic (HIF1) target gene,[159] both features were never assessed together in the context of CRC. To test if MET was implicated in the resistance to EGFR inhibition in hypoxia we treated Lim 1215 cells with EGFR inhibitors (gefitinib or neratinib) in a combinatory matrix with increased concentrations of the MET inhibitor crizotinib in both normoxia and hypoxia (Figure 10A).

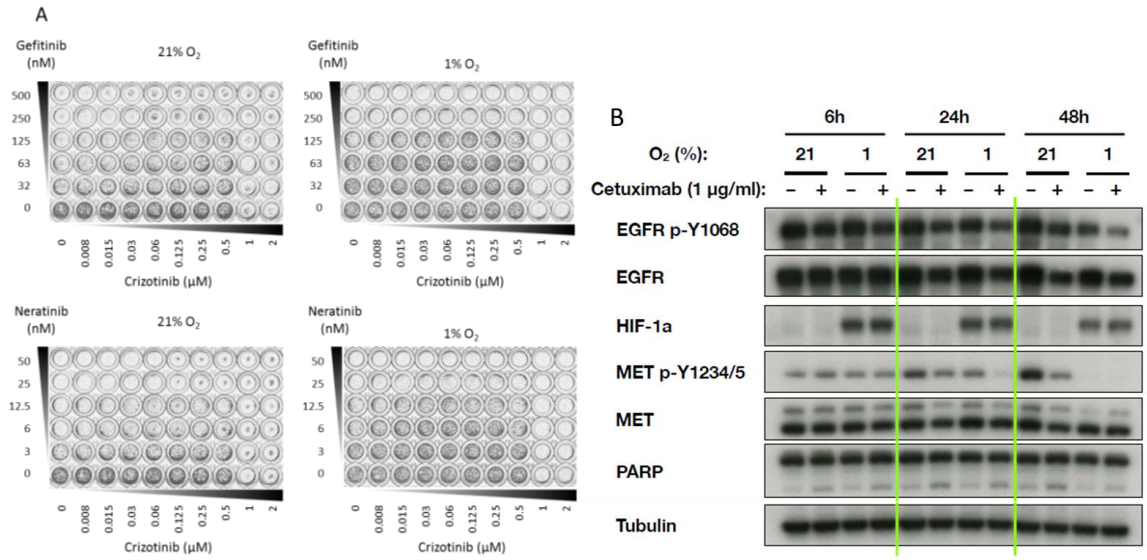

Figure 10: MET is not implicated in the resistance to EGFR inhibition in hypoxia.
A, MET inhibition does not sensitize Lim 1215 cells to EGFR inhibition in hypoxia. Lim 1215 cells were cultured with increasing concentrations of EGFR inhibitor gefitinib alone, MET inhibitor crizotinib alone, and their combination. Cells were incubated at 21% or 1% O_2 for 10 days. All plates were seeded, fixed, stained and scanned at the same time. B, Hypoxia and cetuximab downregulate MET phosphorylation. Biochemical responses of DiFi cells to cetuximab, hypoxia, or their combination, were documented by western blot analysis. Cells were harvested at 6, 24 and 48 hours after cetuximab treatment (1 μg/mL). Treatment with cetuximab resulted in downregulation of pMET. Hypoxia also results in pMET downregulation.

Cells were relatively insensitive to crizotinib and no synergistic activity was observed between EGFR and MET inhibition, with cells still exhibiting resistance to EGFR inhibition in hypoxia compared to normoxia. This result was confirmed by western blot where we observed that combination of hypoxia and cetuximab actually downregulated MET phosphorylation (Figure 10B), and thus excludes a role of MET in the resistance to EGFR inhibition in hypoxia.

Upregulation of RTKs is one of the most common mechanisms of acquired resistance to targeted cancer therapies,[160] therefore to address if any RTK upregulation was causing resistance to EGFR inhibition in WT4 CRC cell lines in hypoxia we performed a Phospho-RTK array in Lim 1215 cells (Figure 11). In this experiment, cetuximab treatment efficiently reduced phosphorylated EGFR levels. We observed an upregulation of phosphorylated HER3, INSR and IGF1R in cetuximab-treated cells, although INSR and IGF1R phosphorylation was higher in normoxia. Unexpectedly there was not any upregulated RTK exclusively in hypoxia-treated cells, therefore excluding RTK upregulation (at least those RTKs present in this assay) as the cause of resistance to EGFR inhibition in WT4 CRC cell lines in hypoxia. HER3 is a member of the Erbb family and upon

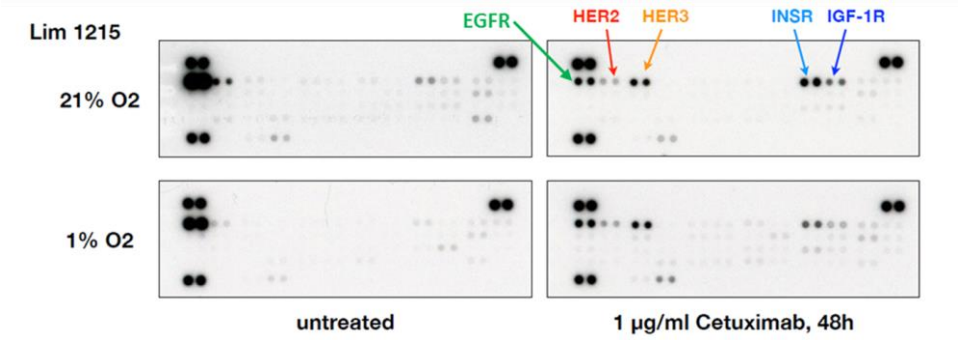

Figure 11: RTK upregulation excluded as the cause of resistance to EGFR inhibition in WT4 CRC cell lines in hypoxia.
RTK phosphorilation in Lim 1215 cells was analysed using a phospho-RTK array. Cells were harvested 48 hours after cetuximab treatment. Treatment with cetuximab resulted in upregulation of HER3, INSR and IGF1R in Lim 1215 cels.

EGFR inhibition its phosphorylation is increased; HER3 phosphorylation acts as a compensatory mechanism to EGFR phospho-downregulation.[161] We hypothesize that INSR and IGF-1R, two important membrane receptors involved in glucose metabolism, are upregulated by cells as an attempt to cope with the toxicity of the EGFR inhibition in normoxia. It has been shown that CRC cells with hyper-activated IGF-1R pathway can escape anti-EGFR mediated cell death through continued activation of the PI3K pathway,[162] however, in the tested cell lines this was not sufficient and cells eventually die. In hypoxia, as cells are able to cope better with EGFR inhibition, INSR and IGF1R rescue mechanism seems to be less required.

Development of mutations in the RAS/RAF signaling pathway is a mechanism of resistance to anti-EGFR therapies in CRC. Mutations in the RAS/RAF oncogenes have been found in CRC patients who become resistant to cetuximab treatment, however, it is not completely clear if these mutations developed during cetuximab treatment or if they were already preexistent, but in a very low number to be detected.[122] To test if our cells developed mutations in the oncogenes KRAS, BRAF or NRAS after treatment with EGFR inhibitors in hypoxia, we sequenced DiFi and Lim 1215 cells after two weeks of treatment (with and without cetuximab at 21% or 1% O_2), when the resistance phenotype was noted. No KRAS, BRAF or NRAS hot spot mutations were found in these cells lines in the tested conditions, which also excluded this hypothesis as the cause of resistance.

Another possible mechanism to explain the resistance to EGFR inhibition in WT4 CRC cells is the activation of RAS by hypoxia, since it has been shown that $KRAS^{WT}$ CRC cells cultured in hypoxia have higher levels of activated RAS than in normoxia.[163] To test this hypothesis, we measured the amount of active RAS by performing a GTP-RAS pull-down followed by western blot analysis (Figure 12). We observed that, indeed, DiFi cells have higher active RAS levels when cultured in hypoxia. Strikingly, upon cetuximab treatment, cells cultured in normoxia barely have active Ras while hypoxia cells maintain higher levels of active Ras. This feature explains why WT4 CRC cells display higher levels of pAKT and pERK and reduced levels of cleaved PARP when treated with cetuximab in hypoxia.

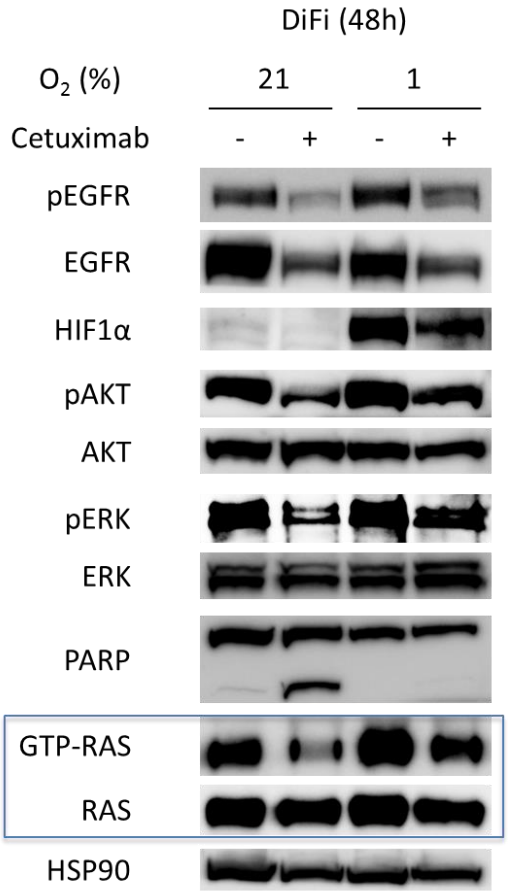

Figure 12: Hypoxia activates RAS, which promotes cell proliferation and protects cells from apoptosis. Biochemical responses of DiFi cells to cetuximab, hypoxia, or their combination, were documented by western blot analysis. DiFi cells were harvested at 48 hours after cetuximab treatment (1 µg/mL). A GTP-RAS pull-down was performed with part of the lysates. Treatment with cetuximab resulted in a decrease of EGFR (and consequently pEGFR) as expected; GTP-RAS is higher in hypoxia, both in the cetuximab treated and untreated cells. In hypoxia, cells treated with cetuximab exhibit a higher phosphorylation of AKT and ERK as compared with normoxia-treated cells. Treated cells express more cleaved PARP in normoxia than in hypoxia. HIF1α served as a control for the hypoxia treatment and HSP90 served as a loading control.

To test if WT4 CRC cells are dependent on the PI3K pathway for their survival upon EGFR inhibition in hypoxia, we blocked EGFR together with PI3K pathway. To do that we performed a loss of function experiment, where we blocked the PI3K pathway using the AKT inhibitor MK2066 (Figure 13A). Lim 1215 cells treated with MK2066 still exhibited resistance to EGFR inhibition in hypoxia, however, since the activity of the drug was not confirmed by western blot, and given the lack of cell killing by this inhibitor in the range of concentrations used no definitive conclusions can be made at this point. Therefore, we performed the same loss of function experiment but using a PI3K inhibitor (GDC0441) (Figure 13B). Cells treated with GDC0441 also still exhibited resistance to EGFR inhibition in hypoxia, indicating that the PI3K/AKT signaling pathway is not essential for the survival of these cells to EGFR inhibition in hypoxia, as the resistance phenotype was not reversed with the combination. The blockage of PI3K-AKT signaling with GDC0441 in this experiment seems more efficient than with MK2066, since it killed cells at higher concentrations in both oxygen tensions.

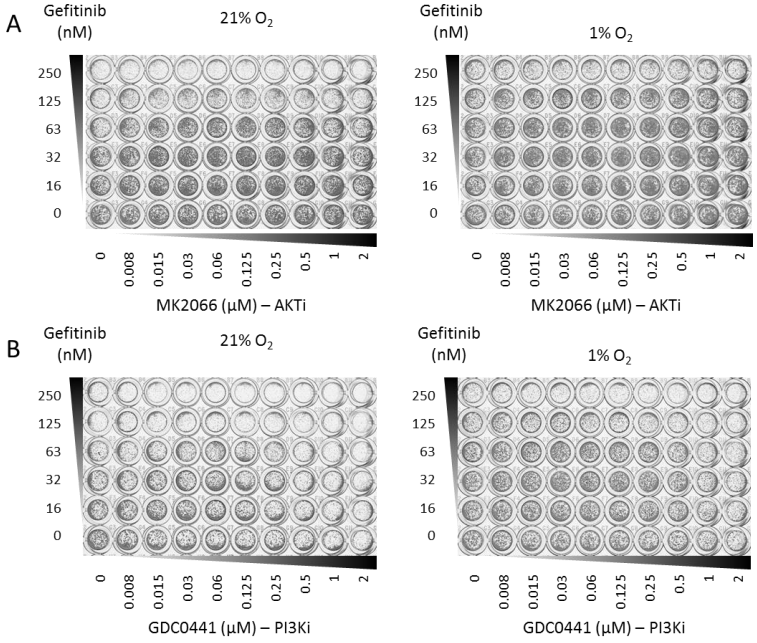

Figure 13: EGFR and PI3K inhibition does not intercept resistance to EGFR inhibition in hypoxia in WT4 CRC. Lim 1215 cells were cultured with increasing concentrations of EGFR inhibitor gefitinib alone, PI3K inhibitor GDC0441 or AKT inhibitor MK2066 alone, and their combinations. Cells were incubated at 21% or 1% O2 for 10 days. All plates were seeded, fixed, stained and scanned at the same time.

We also tested the dependency of our cells on the MAPK pathway for their survival upon EGFR inhibition in hypoxia. It has been previously shown that the combination of EGFR and MEK inhibition can intercept the acquisition of resistance to anti-EGFR therapies in CRC,[129] yet never in the context of hypoxia or in combination with AA agents. We tested this hypothesis by performing a loss of function experiment, where we blocked the MAPK pathway using MEK inhibitors Selumetinib or Trametinib in Lim 1215 cells (Figure 14). We observed that MEK inhibition is highly effective in killing Lim 1215 cells in normoxia and that the combination with EGFR inhibitor further increases cell killing. On the other hand, in hypoxia, Lim 1215 cells were quite resistant to MEK inhibition; however, at higher concentrations, it was possible to see some synergy between the combination of EGFR and MEK inhibition. This experiment suggests that Lim 1215 cells are more dependent on the MAPK pathway for their survival to EGFR inhibition in hypoxia, as the hypoxic-resistance phenotype can be reversed with this combination.

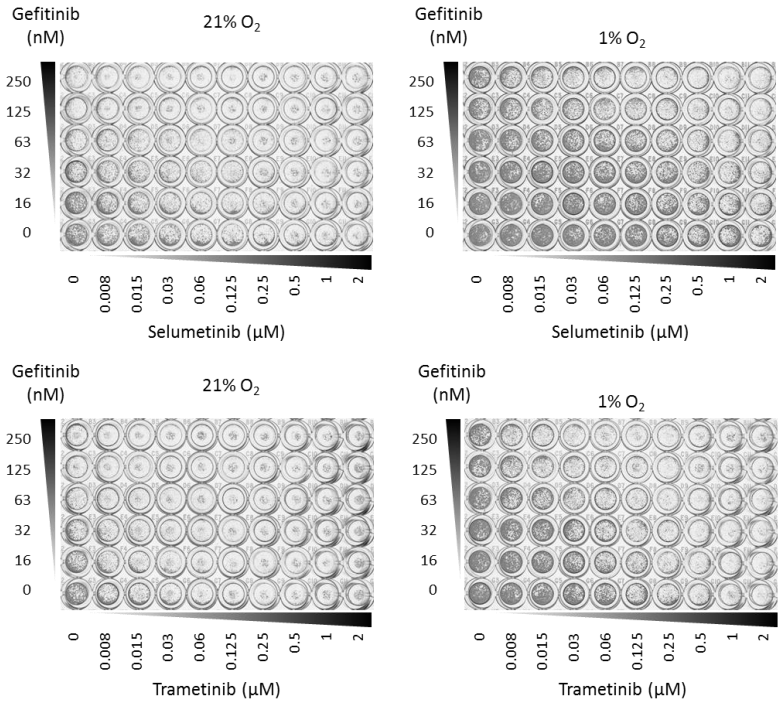

Figure 14: EGFR and MEK inhibition intercept resistance to EGFR inhibition in hypoxia in WT4 CRC.
Response of Lim 1215 cells to the combination of EGFR and MEK inhibitors. Lim cells were cultured with increasing concentrations of EGFR inhibitor gefitinib alone, MEk inhibitor Selumetinib or Trametinib alone, and their combination. Cells were incubated at 21% or 1% O2 for 10 days. All plates were seeded, fixed, stained and scanned at the same time.

Our findings explain why cells are able to resist EGFR inhibition in hypoxia, shed some light on how to reverse the resistance and have ruled out HIF, MET, RTK upregulation or acquisition of mutations in RAS and BRAF oncogenes as the cause for the resistance. However, the mechanism that leads to the activation of Ras in hypoxia still remains unknown.

One possible explanation for the activation of RAS in hypoxia is by the increased secretion of cytokines in hypoxia,[164,165] which could act on an autocrine manner and be activating a receptor, which in turn would lead to the activation of RAS. To test this hypothesis we performed a cytokine array in DiFi cells (Figure 15). We observed that several cytokines are upregulated in hypoxia and that this upregulation is maintained upon cetuximab treatment, which indicates that this hypothesis can be right. Further validation of this concept can be done using conditioned medium (from cells grown in hypoxia) in cells that will be cultured in normoxia to verify if the later become resistant to cetuximab due to soluble factors released to the media. Transcription analysis, as well as ELISA, of each of these soluble cytokines should also be performed in the experimental setting. This would validate our hypothesis but we would still need to find which are the most important cytokines for the resistance mechanism and how they are activating Ras. To do that we can, for example, knock-down/inhibit each of the cytokines that are upregulated in hypoxia and check if these cells are still more resistant to cetuximab in hypoxia or if the phenotype is reversed. Interestingly, some of these proteins —IL-8, CXCL1, VEGF— are known to be expressed on a NF-kB dependent manner,[165–167] making this transcription factor an interesting target for future investigation. Given that VEGF is secreted by WT4 CRC cells in hypoxia, the use of anti-VEGF(R) drugs should also be considered *in vitro*.

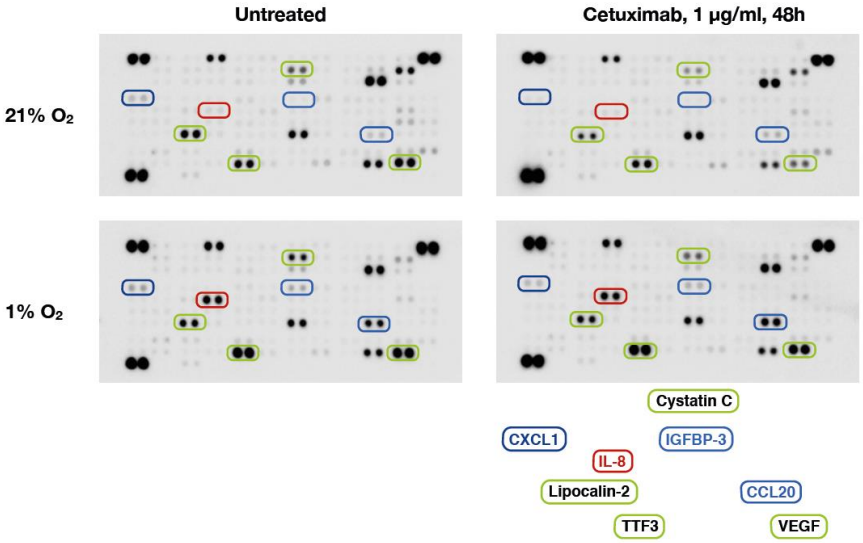

Figure 15: Hypoxia increases the production of cytokines.
Cytokine production in DiFi cells was analyzed using a cytokine array. The supernatant medium was collected 48 hours after treatment with 1µg/mL of cetuximab. Hypoxic cells overproduce Cystatin C, CXCL1, IGFBP-3, IL-8, Lipocalin-2, CCL20, TTF3 and VEGF and this overproduction is maintained upon treatment.

2.2. Loss of function genetic screen in Lim 1215 cells

In the second part of this project we used a more unbiased approach to find synthetic lethal interactions with hypoxia and also to find what can be driving resistance to EGFR inhibition in hypoxia. To do that, we analyzed the gene expression profile of six different WT4 CRC cell lines (Lim 1215, DiFi, OXCO2, CCK 81, C99 and HCA 46) cultured in hypoxia (and compared with normoxia condition). These results were crossed with data from WT4 CRC patient samples to make sure our in vitro signature had significance in a clinical context. We then derived a specific WT4 CRC hypoxia-signature, containing only the genes that overlapped in both data sets. We used this signature to build a shRNA library containing all the shRNAs that target these genes and used this library to perform a loss of function genetic screen in Lim 1215 cells.

If any of the genes present in our library are pivotal for the survival of WT4 CRC cells cultured under hypoxic conditions, cells that are knockdown (KD) for these genes should appear dropped in the whole cell population, when compared to the population of cells cultured in normoxia. After the analysis of the screen we identified 4 genes statistically significant as being synthetic lethal with hypoxia. We proceeded with the validation of this hits by KD these genes with individual shRNAs. By the end of this internship this validations was not yet completed.

Similarly, if any of the genes present in this hypoxia library are pivotal for the survival of WT4 CRC cells cultured with cetuximab and under hypoxic conditions, cells that are KD for these genes should appear dropped in the cell population, when compared to the population of cells cultured without cetuximab under hypoxic conditions. After the analysis of the screen no hits were found. This can be explained by the fact that our library is relatively small (comprising only 107 genes) and also because it was designed mainly to find synthetic lethal interactions with hypoxia. To increase the chances of finding synthetic lethal hits with cetuximab/hypoxia, we should repeat the screen using a bigger library, for example, containing shRNAs that target all human kinases genes.

3. Conclusions and Future Perspectives

In the first part of this work we showed that WT4 CRC cell lines are resistant to EGFR inhibition when cultured in hypoxia. These results are in concordance with two clinical trials (CAIRO2 and PACCE) which concluded that the combination of chemotherapy with anti-EGFR and anti-VEGF drugs is not recommended for the treatment of mCRC in clinical practice.[148,150] These trials' results were unexpected given that both EGFR and VEGF are critical pathways in CRC pathogenesis,[112,113,138,139] therefore providing a strong rationale for targeting these two pathways. Understanding why this combination is not being effective was the main goal of this first part of our work. Several EGFR resistance mechanisms have been already described in the literature; we tested all of them, but none validated in hypoxia, which brings novelty to this work and calls for more investigation. We have shown that the resistance phenotype is caused by the activation of Ras in hypoxia, however the mechanism behind this activation is still not known. Our last experiments point to the involvement of cytokines in this activation but more work is necessary to further elucidate on this.

Our laboratory was a pioneer in the development of the technology to perform synthetic lethality genetic screens. We took advantage of this technology to try to answer our first question —what is causing the resistance to EGFR inhibition in hypoxia— using a more unbiased approach and also to look for novel synthetic lethal interactions with hypoxia. We performed a loss of function genetic screen in Lim 1215 cells. We identified four genes as potential candidates to be synthetic lethal with hypoxia. By the end of the internship the validation of these hits was still ongoing, so no results are presented in this thesis. We didn't identify any genes as potential candidates to explain the resistance phenotype.

Discovering the mechanism underlying the resistance to EGFR inhibition is of high biological significance and for this reason the project will be continued. The results of a future screen using a larger library should provide essential information to help unveiling the resistance mechanism of EGFR inhibition in hypoxia. Some biochemical experiments will follow to validate all colony formation assays (confirm the activity of the used drugs). To increase clinical significance of the project the resistance phenotype will be validated *in*

vivo. Moreover, RNAseq experiments will be conducted to elucidate about genes/cellular pathways whose overexpression/activation under hypoxia are maintained upon EGFR inhibition and thus can elucidate on the resistance mechanism.

Finally, the ongoing validation of the hits found in the screen to be synthetic lethal with hypoxia should provide novel targets to exploit in combination with antiangiogenic therapies to increase therapeutic responses and reduce resistance. And in case these genes overlap with genes found on patients who respond to AA therapies (and don't overlap with patients who don't respond to AA therapies) they might serve as biomarkers for the selection of patients who may benefit from AA therapies. This is of great clinical significance, as so far no biomarkers of response to AA therapies have been found.

4. Materials and Methods

4.1. Cell culture

During this research project we used nine different cell lines; six are derived from WT4 CRC patients (Lim 1215, DiFi, OXCO2, CCK 81, C99 and HCA 46), two are derived from $KRAS^{MUT}$ (LoVo) and $BRAF^{MUT}$ (HT-29) patients, and the other one is derived from human embryonic kidney cells (HEK-293T). Lim 1215 cells were used in all the assays performed during our project; DiFi, OXCO2, CCK 81, LoVo and HT-29 were used only in validation assays; and HEK-293T cells were used for lentivirus production. These cell lines were selected based on their genotype, culturing characteristics and, in the case of the CRC cells, also the ability to proliferate for at least 2 weeks in hypoxic conditions (1% O_2).

All cells were cultured at 37°C and with 5% CO_2; the oxygen percentage in normoxia was 21% and in hypoxia 1%. The eight CRC cell lines were cultured in RPMI 1640 medium (Gibco) and HEK-293T cells were cultured in DMEM medium (Gibco). Both mediums were supplemented with: 10% Fetal Bovine Serum (Thermo Scientific), 1% Penicillin/Streptomycin (Gibco) and 1% L-Glutamine (Gibco).

The list of drugs used throughout our project can be found in table 3.

Table 3: List of drugs used during the project and their targets.

Drug Name	Target
Cetuximab	EGFR
Gefitinib	EGFR
Neratinib	EGFR and HER2
MK2066	AKT
GDC0441	PI3K
Selumetinib	ERK
Trametinib	ERK
Crizotinib	MET
Digoxin	HIF1α
DMOG	PHDs

4.2. Functional assays

4.2.1. Long-term proliferation (colony formation) assays

Cells were seeded (according to table 4) and placed in 21% or 1% O_2 incubators overnight, to allow cell-attachment to the plates. Culture media (and drugs) were refreshed every 2-3 days. When control wells (wells not subjected to drugs) were confluent, cells were fixed using a solution of 2% formaldehyde in phosphate-buffered saline (PBS) for 2 hours, after which they were stained, using a solution of 0.1% crystal violet in water for up to 10 minutes. The staining solution was removed, plates were washed with water and left to dry for 1 or 2 days, after which plates were scanned and stored. All experiments were repeated at least three times.

Table 4: Cell seeding information

Cell Line	Cells seeded in 6-well plates	Cells seeded in 12-well plates	Cells seeded in 96-well plates
Lim	20000	10000	1500
DiFi	40000	20000	2000
LoVo	20000	10000	1500
HT-29	20000	10000	1500
CCK81	40000	20000	2000
OXCO2	40000	20000	2000

4.2.2. Western Blot

Cells were washed with chilled PBS on ice, lysed with RIPA buffer (25mM Tris - HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Complete (Roche) and phosphatase inhibitor cocktails II and III (Sigma)) and proteins were extracted by incubating the samples for 30 min on ice, vortexing every 10 minutes. Samples were then centrifuged for 10 minutes at 14.000 rpm at 4°C.

Protein concentration was quantified by Bicinchoninic Acid (BCA) assay (Pierce BCA, Thermo Scientific). 50 μg of protein (denatured with DTT followed by 5 minutes heating at

95°C) was then loaded in a 4-12% polyacrylamide gel, being separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for approximately 90 minutes at 165 volts. Proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane, using 330 mA for 90 minutes. Membranes were then placed in blocking solution (5% bovine serum albumin (BSA) in PBS with 0.1% Tween-20 (PBS-T). Subsequently, membranes were probed with primary antibody in blocking solution and left incubating overnight at 4 °C with constant shaking. Membranes were washed 3 times for 10 minutes with PBS-T, followed by one hour incubation at room temperature with the second antibody (HRP conjugated, 1:10000) in blocking solution. Membranes were washed 3 times for 10 minutes in PBS-T. Finally, the Western Blot was developed by adding a chemiluminescence substrate (ECL, Thermo Scientific) to the membranes, which was then detected using a ChemiDoc (Bio-Rad). The antibodies used in this project are listed in Table 5.

Table 5: List of antibodies used for western blot during the project.

Target protein	Molecular weight	Isotype	Brand
EGFR	170	Mouse	BD Biosciences
EGFR p-Y1068	175	Rabbit	Abcam
HIF1α	120	Rabbit	Cayman
AKT	60	Rabbit	Cell Signalling
AKT p-S473	60	Rabbit	Cell Signaling
ERK 1/2	42, 44	Rabbit	Santa Cruz
ERK p-Y204	42, 44	Mouse	Santa Cruz
MET	140	Rabbit	Cell Signalling
MET p-Y1234/5	145	Rabbit	Cell Signaling
Cleaved PARP	89	Rabbit	Cell Signalling
Tubulin	55	Mouse	Sigma

4.2.3. GTP-RAS pull down

Cells were washed with chilled PBS on ice, lysed, incubated and centrifuged (see first paragraph of western blot). Protein concentration was quantified by BCA. 600 μg of protein was used to specifically pull down active Ras using an active Ras pull-down and detection kit (Thermo Scientific). After GTP-Ras pull down, samples were reduced and normal western blot protocol was followed.

4.2.4. Phospho RTK array

Cell lysates were diluted and incubated with the Human Phospho-RTK Array (R&D Systems), according to the kit's instructions. After binding the extracellular domain of both phosphorylated and unphosphorylated RTKs, unbound material was washed away. A pan anti-phospho-tyrosine antibody conjugated to horseradish peroxidase (HRP) was used to detect phosphorylated tyrosines on activated receptors by chemiluminescence.

4.2.5. Cytokine array

Cell culture supernatant was diluted and incubated overnight with the Proteome Profiler Human XL Cytokine Array (R&D Systems), according to the kit's instructions. The membranes were washed to remove unbound material followed by incubation with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were then applied, and a signal was produced at each capture spot corresponding to the amount of protein bound.

4.3. WT4 CRC hypoxia-specific library

Six different WT4 CRC cell lines (Lim 1215, DiFi, OXCO2, CCK 81, C99 and HCA 46) were cultured for 3 days, in both normoxia and hypoxia, after which cells were collected in Trizol and handed in for RNA sequencing. Data was analyzed with the help of Andreas Schlicker (a bioinformatician from our laboratory) and a WT4 CRC hypoxia-specific signature was derived, containing the genes that were significantly overexpressed only in hypoxia (based in the RNAseq data) and that also overlapped with genes expressed in WT4 CRC patients. The signature consists of 107 genes (Table 6).

Table 6: Genes present in the WT4 CRC hypoxia-signature.

#	Gene Name	Ensembl Gene ID	#	Gene Name	Ensembl Gene ID	#	Gene Name	Ensembl Gene ID
1	ADRA2A	ENSG00000150594	37	TNS1	ENSG00000079308	73	HIF3A	ENSG00000124440
2	ALOX5	ENSG00000275565	38	VLDLR	ENSG00000147852	74	PDZD3	ENSG00000172367
3	ALOX5	ENSG00000012779	39	PPFIA4	ENSG00000143847	75	SCIN	ENSG00000006747
4	AOAH	ENSG00000136250	40	CYP4F2	ENSG00000186115	76	SYT8	ENSG00000149043
5	ARNT	ENSG00000143437	41	STC2	ENSG00000113739	77	AHNAK2	ENSG00000185567
6	BNIP3	ENSG00000176171	42	BAIAP3	ENSG00000007516	78	ELFN2	ENSG00000166897
7	C4BPB	ENSG00000123843	43	CLIC3	ENSG00000169583	79	PRAP1	ENSG00000165828
8	CA9	ENSG00000107159	44	SLC16A4	ENSG00000168679	80	LGI4	ENSG00000153902
9	CACNA1C	ENSG00000151067	45	GCNT3	ENSG00000140297	81	PSORS1C1	ENSG00000206458
10	CALB1	ENSG00000104327	46	AKAP12	ENSG00000131016	82	LDHD	ENSG00000166816
11	CDA	ENSG00000158825	47	AATK	ENSG00000181409	83	SLC5A9	ENSG00000117834
12	CKB	ENSG00000166165	48	PLCH2	ENSG00000276429	84	CREG2	ENSG00000175874
13	CNGA1	ENSG00000198515	49	KIAA0319	ENSG00000137261	85	UNC13D	ENSG00000092929
14	COL1A1	ENSG00000108821	50	MUC12	ENSG00000205277	86	GPR115	ENSG00000153294
15	COL17A1	ENSG00000065618	51	MSLN	ENSG00000102854	87	SLC29A4	ENSG00000164638
16	CP	ENSG00000047457	52	B3GALT5	ENSG00000183778	88	C11orf86	ENSG00000173237
17	CYP1A1	ENSG00000140465	53	PNMA2	ENSG00000240694	89	GPR110	ENSG00000153292
18	ARID3A	ENSG00000116017	54	BTNL3	ENSG00000168903	90	SLC39A5	ENSG00000139540
19	EDN1	ENSG00000078401	55	PADI2	ENSG00000117115	91	TMEM150B	ENSG00000180061
20	EDN2	ENSG00000127129	56	OBSL1	ENSG00000124006	92	ENTPD8	ENSG00000188833
21	EFNA2	ENSG00000099617	57	CADM1	ENSG00000182985	93	KPNA7	ENSG00000185467
22	EGR1	ENSG00000120738	58	FBXO2	ENSG00000116661	94	GABRE	ENSG00000102287
23	FABP1	ENSG00000163586	59	SRPX2	ENSG00000102359	95	CAPN14	ENSG00000214711
24	FN1	ENSG00000115414	60	PADI1	ENSG00000142623	96	GABRE	ENSG00000102287
25	FOS	ENSG00000170345	61	ANGPTL4	ENSG00000167772	97	PPP1R3G	ENSG00000219607
26	GABRE	ENSG00000102287	62	PLAC8	ENSG00000145287	98	PDE4C	ENSG00000105650
27	HAS3	ENSG00000103044	63	KRT20	ENSG00000171431	99	FER1L4	ENSG00000088340
28	IL6	ENSG00000136244	64	RHOF	ENSG00000139725	100	PTPRR	ENSG00000153233
29	MGAT3	ENSG00000128268	65	RNF186	ENSG00000178828	101	REN	ENSG00000143839
30	MST1	ENSG00000173531	66	LEPREL1	ENSG00000090530	102	SPOCK1	ENSG00000152377
31	MUC1	ENSG00000185499	67	TNFRSF19	ENSG00000127863	103	SULT2B1	ENSG00000088002
32	CEACAM6	ENSG00000086548	68	PMEPA1	ENSG00000124225	104	TFF1	ENSG00000160182
33	SERPINE1	ENSG00000106366	69	ALPK3	ENSG00000136383	105	TFF2	ENSG00000160181
34	PCK1	ENSG00000124253	70	RRAGD	ENSG00000025039	106	TFF3	ENSG00000160180
35	PDE4C	ENSG00000105650	71	ACE2	ENSG00000130234	107	TGFB3	ENSG00000119699
36	PPP1R3C	ENSG00000119938	72	PRSS22	ENSG00000005001			

Based on this signature, we built a library containing all the shRNAs that target the genes present in this signature. The shRNA vectors were purchased from the "The RNAi Consortium" (TRC).

4.3.1. Library picking

To build the library we picked *E.coli* bacteria (from the whole genome TRC library - stored at -80 °C in glycerol stocks), which contain a pLKO vector with an insert of the desired shRNA and also an antibiotic resistance marker. Bacteria were cultured overnight at 37 °C in 2X LB medium (20g of Bacto-Tryptone, 10g of yeast extract, 10g of NaCl in 1L of water) with carbenicillin. Plasmid DNA was isolated from the bacteria using a standard DNA isolation protocol (from Roche), after which the DNA concentration was measured (using a Nanodrop ND1000 system).

4.3.2. Lentivirus preparation

The lentivirus was prepared by transfecting HEK-293T cells with plasmid DNA from the shRNA library. To do that, we mixed 1 µg of plasmid DNA of our library, 1 µg of lentivirus packaging mix (pMD2.G envelope plasmid and pMDLg/pRRE packaging plasmid) and 6 µL of polyethylenimine (PEI) in 100 µL DMEM medium (quantities used in a 6-well plate setting). The mix was vortexed briefly, incubated for 15 minutes at room temperature and added to one million HEK-293T cells seeded the day before in 2 mL of DMEM medium. Cells were incubated overnight; the next day the medium was refreshed; after 48 hours of incubation, the medium containing the lentivirus was filtered (by 20-µm filters), collected and stored at -80°C.

4.3.3. Loss of function pooled shRNA screen

Lim 1215 cells were seeded and infected with the lentivirus containing library, using a complexity of 2000-fold (which means that each shRNA from the library has to be present in at least 2000 cells) and a multiplicity of infection (MOI) of approximately 0.3 (to make sure cells are infected with only one lentivirus, delivering only one shRNA; this is very

important because multiple integrations would lead to confounding results due to passenger effect). 24 hours after the infection we started the selection of the cells that got infected by adding puromycin (1 µL/mL) to the medium. When the selection was finished (to know this, when we start puromycin selection we also add puromycin to cells that didn't receive any lentivirus and therefore aren't resistant to antibiotic – when these cells are all dead it means the selection is finished), cells were trypsinized and reseeded according to the layout of the screen – figure 16.

Cells were seeded in three technical replicates for each of the five arms: time-point zero (cells at the day of seeding, to allow selecting for straight lethal hits), 21% O_2 treated and untreated and 1% O_2 treated and untreated. Media was refreshed every 2-3 days. After 1 and 2 weeks the untreated and treated arms were collected, respectively. Genomic DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen), according to manufacturer's protocol and viral DNA recovered by PCR.

PCRs were performed, to add barcodes to the different replicates in order to identify them after sequencing. Samples were purified using High Pure PCR Product Purification Kit (Roche), according to manufacturer's protocol. A 1% agarose gel in 0.5x TBE was run (with 4 µl of PCR product) to confirm PCR amplification. Purified PCR products were analyzed by deep sequencing to identify the number of shRNA inserts present in the cell population.

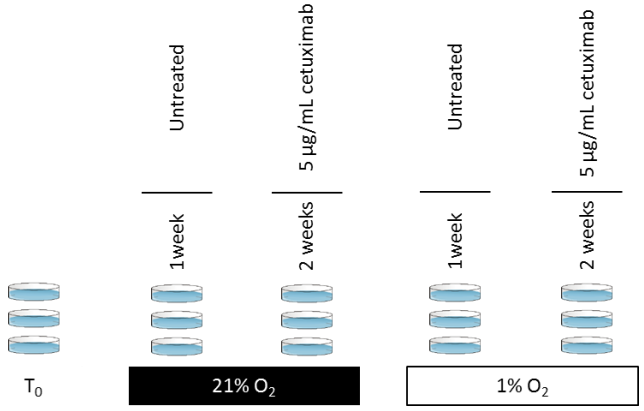

Figure 16: Synthetic lethality screen layout.
In this screen cells are seeded in three technical replicates in each arm, to increase the statistical relevance of the results. Also, a time point zero (T_0) is included, as a reference of comparison. To find synthetic lethal interactions with hypoxia, cells are cultured for one week at 21% and 1% O_2. To find out genes involved in the resistance to cetuximab, cells are cultured for two weeks with cetuximab (5µg/mL) at 21% and 1% O_2.

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