University of Insubria
PhD program in Biological and Medical Science

Clinical and Experimental Pharmacology

XXV cycle

Analysis of the interaction between the IGF system and HIF: rationale for cotargeting the two systems in breast cancer.

Director: Prof. Franca Marino

Mentor: Prof. Elena Monti

PhD thesis of:

Monica Mancini

611616

Academic Year 2011/2012

Table of contents

Table of contents

Abstract

The observation that several RTKs can act as promoters of tumor development and growth has brought a revolution in the study of new targets, in the hope of developing more specific anticancer drugs: the Insulin-like Growth Factors system is among the most studied.

The role of this system in the development and progression of a large number of malignancies is well documented. Hence the development of different classes of compounds that can affect this system, in particular directly targeting the type 1 receptor (IGF-1R). Some of these compounds, both monoclonal antibodies (mAbs) against the receptor or small molecules that inhibit the TK activity of IGF-1R, are already undergoing clinical trials, because of their effectiveness in inhibiting the signal. Unfortunately, cancer cells show a high degree of plasticity and redundancy in the proliferative and anti-apoptotic signals, which may contribute to the creation of resistance to specific inhibitors: the identification of these mechanisms, and their overcome, are two key goals in the development of these drugs.

Within this study, we encountered a similar problem: our experiments show that the compound in use, NVP-AEW541 (a specific, low molecular weight inhibitor of IGF-1R catalytic activity), is perfectly capable of inhibiting the signal transduced by the receptor following stimulation with IGF-1, but it appears to lose effectiveness following stimulation with IGF-2. Starting from the observation that IGF-2 seems to be the key factor in this resistance, literature search showed us a growing number of experimental evidences in support of the role of variant A of the insulin receptor (IR-A) in the maintenance of a proliferative signal similar to that classically attributed to IGF-1R: our experiments confirmed the activation of this receptor following exposure to IGF-2, justifying the loss of effectiveness of NVP-AEW541 in regard of the migratory potential of MDA-231 cell line. While the dual inhibition of IGF-1R and IR could overcome this redundancy in the signal, it remains to consider the problems that may occur due to the inhibition of a receptor as essential for cellular metabolism as IR.

A different and probably more practicable strategy would be the inhibition of the key player in the dual activation, IGF-2. The one proposed is a strategy that reflects the physiological mechanism of controlling the levels of IGF-2: in fact normal cells are able to control the levels of circulating IGF-2 by binding the ligand to IGF-2R, followed by its subsequent transport to lysosomes for degradation. During this study we then concentrated also on IGF-2R, observing no significative differences between the cell lines in study.

Following the ligand-based targeting of the IGF system, which effectiveness seems to be proven by an ongoing phase I clinical trial using MEDI-573, actually the only mAb in clinical testing that exerts its effects by neutralizing not only IGF-2 but also IGF-1, we used a neutralizing antibody against IGF-2 (MAB292), making it impossible for it to bind both IGF-1R and IR-A. The use of MAB292 proved to be effective both in combination with NVP-AEW541 and alone: MDA-231 cells seem in fact unable to respond to stimulation with IGF-2 and almost completely lose the ability to migrate.

This strategy could be particularly effective especially considering that, in hypoxic conditions (pO_2 <2.5%), stabilization of HIF-1α leads to an increase in the levels of IGF-2. This increase in fact results

in the resistance of MDA-231 to NVP-AEW541, while MAB292 still retain its effectiveness. We also observed the lack of a direct and defined relationship between HIF-1α and IGF-2R levels: cells seemed not able to compensate to IGF-2 increase by increasing also the receptor levels.

From this observation it's therefore evident that another strategy to overcome the resistance to IGF-1R inhibitors may be the combination with HIF-1α inhibitors. At the present days, the fundamental problem is the lack of specific inhibitors: many chemotherapic drugs, in fact, have a complete or partial inhibitory activity of HIF-1α, that is however accompanied by a number of off-target effects. One of these compounds is Topotecan, a Top-1 poison in use in the treatment of ovarian and small cell lung cancer that inhibits HIF-1α translation by a Top1-dependent but DNA damage-independent mechanism, suggesting that its effect as HIF-1α inhibitor could be mechanistically distinguished from those characterizing its cytotoxic activity. In our case the dual inhibition was obtained with success through the use of Topotecan in combination with NVP-AEW541.

We also introduced the combination between NVP-AEW541 and Luteolin, a compound of natural origin belonging to the flavonoid class that possess anti-proliferative and anti-migratory activity. Despite the more complex comprehension of its effect on HIF-1, which resulted only slightly decreased, the most interesting feature of this compound resulted to be its anti-migratory potential: the use of Luteolin in fact completely inhibited migration in MDA-231.

As a last point in our study, we focused our attention on the metabolic alteration determined by the modulation of insulin/IGF system. Since deregulation of cellular energy metabolism is considered an increasingly important hallmark of cancer, IR and its related metabolic syndromes have become another major focus in the breast cancer research and treatment field. Preliminary analysis of the oxidative and glycolitic profiles of three cell lines showed in first stance an interesting difference at the basal level as well as a modulation of the two processes after treatment with NVP-AEW541 together with MAB292 or with Luteolin. Further experiments would be needed to assess the effective impact of these compounds on cellular metabolism, as well as the metabolic processes and responses in hypoxia.

In conclusion we can say that the dual targeting of IGF-1R and HIF-1 appears to be an interesting therapeutic strategy in breast cancer, especially given the close relationship between these two systems.

Introduction

Structure of the breast

The mammary gland is an organ unique to the class Mammalia, with the specific function in the female to synthesize, secrete, and deliver milk to the newborn upon demand for its optimal nourishment, protection, and development (Medina, 1996). In humans, the life cycle of the female mammary gland is epitomized by drastic changes in composition, architecture, and functionality, mediated by marked changes in gene expression, that characterize its physiological stages of development, all of which are aimed at allowing it to perform its function as a milk-producing organ with the birth of the infant. The key mammary developmental stages include fetal growth, infant (prepubertal) growth, pubertal expansion, pregnancy and lactation-associated remodeling, and post-lactational and post-menopausal involution (Russo and Russo, 2004). A sound knowledge of the development, anatomy, physiology, and regulation of the breast is integral in the understanding of both the normal biology and function of this organ and its benign or malignant pathologies and their successful treatment.

Breast development during life follows a time course of distinct phases. Beginning with the formation of the mammary crest and subsequent primitive mammary buds during embryonic life, it continues with minimal growth during infancy followed by a rapid growth phase at puberty in the female. Unlike most other organs of the body, which develop to a relatively mature state during embryonic life, breast development culminates during the pregnancy and lactation cycle (PLC) when the mammary gland undergoes complete remodeling, maturating into a functional milk-secretory organ. Regression of PLC-induced growth is initiated as weaning is commenced and is completed after involution when the breast regresses to a resting state. Remarkably, the PLC-induced mammary growth and subsequent involution can be repeated at multiple pregnancies during the reproductive life of a female. The cycle is completed with a further phase of involution post-menopause (Hassiotou and Geddes, 2012).

An understanding of the gross anatomy of the breast and its variations has many clinical applications ranging from breastfeeding/lactation support to the detection, diagnosis, and removal of benign and malignant lesions. In this context, the epithelial cells lining the duct walls are at the origin of the majority of breast malignancies (Li et al, 2003), underscoring the importance of a thorough understanding of the anatomy of the breast. Current descriptions of breast anatomy are based on Cooper's dissections of lactating breasts (Cooper, 1840). However, there is renewed interest in investigating breast anatomy, particularly that of the ductal system, with the aim of better understanding the origins of breast cancer and the potential of localized intra-ductal therapies (Going and Mohun, 2006).

The breast is composed of glandular (secretory) and adipose (fatty) tissue supported by a loose framework of fibrous connective tissue called Cooper's ligaments. The secretory tissue is drained by a ductal system that stores and transports milk to the nipple during lactation. The resting breast consists of ductal epithelial tissue embedded within a fibrous stroma. Each duct wall is lined by two

layers of epithelial cells: an inner layer that encapsulates the ductal lumen, and which contains cuboidal epithelial cells, some of which (typically those of the terminal duct) have the potential to further differentiate into milk-secretory cells (lactocytes) during lactation; and a basal/outer layer of contractile myoepithelial cells that tightly surround the luminal layer and have properties of smooth muscle cells. The basal layer lies on the basement membrane (Hassiotou and Geddes, 2012).

Particular anatomical structures of the mammary gland are:

Nipples. The nipple is composed of longitudinal and horizontal smooth muscle fibers relating to the nipple base. These muscles either remain separate or are intermixed with longitudinal fibers often associated with the nipple ducts with horizontally orientated muscles located distally and which provide a sphincter-like function (Tezer et al, 2011).

Breast ductal system. Standard textbook descriptions depict the ductal system as numerous small ductules that drain the alveoli merging to culminate in one main duct that dilates slightly to form a lactiferous sinus. The main duct then narrows before it passes through the nipple and opens onto the nipple surface (Rusby et al, 2007).

Lobes. In women, the glandular tissue is composed of lobes that comprise lobules containing 10–100 alveoli. Each breast lobe is generally considered to exist as a single entity (Love and Barsky, 2004). The arrangement and volume of tissue associated with each lobe within the breast has been confirmed to be highly variable, showing up to 20–30-fold differences in lobe volume (Moffat and Going, 1996).

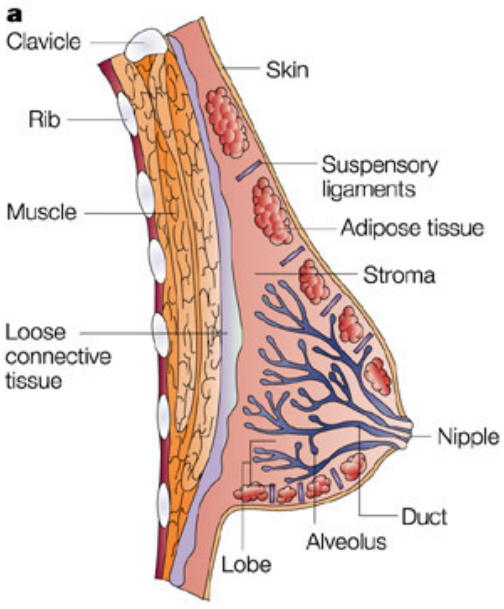

Figure 1 Anatomy of the human mammary gland (*Nature Rev Cancer,* 2002)

Breast cancer

Cancer is increasingly a global problem and breast cancer is not only the most common incident form of cancer in women worldwide, but is the first or second most common in all regions of the world, and responsible for 1.4 million new cases annually (Boyle, 2006). The incidence of breast cancer is increasing almost everywhere throughout the world, although the mortality rate from breast cancer is declining in many high income countries (Boyle, 2012).

Only a decade ago, breast cancer was considered a relatively 'simple' disease in many respects, with focus essentially on quantifying whether a tumour was, or was not, estrogen dependent—a situation that had lasted for a century (Beatson, 1896). It develops following multiple alterations in the molecular signatures, function, and structure of the affected cells and is therefore characterized by various stages. These include cellular immortality, hyperplasia, tumorigenicity, and invasiveness and are structurally evident as an initial epithelial hyperplasia, which develops into cellular atypia and occlusion of the duct, intra-ductal carcinoma, and progression to a locally invasive carcinoma, which can metastasize to various organs, such as the lung, bone, and liver (Medina, 1996; Lu et al, 2009; Oskarsson et al, 2011).

In fact in modern times breast cancer is recognized as a heterogeneous disease encompassing a variety of entities, which are molecularly, morphologically and clinically distinct. Epidemiologic studies employing cDNA microarrays and immunohistochemical markers resulted in breast cancers being classified into five distinct sub-types (Nielsen et al, 2004; Perou et al, 2000):

Luminal A. Estrogen receptor (ER) positive and/or progesterone receptor (PR) positive, human epidermal growth factor receptor 2 (HER2) negative;

Luminal B. ER positive and/or PR positive, HER2 positive;

HER2 overexpressing. ER negative, PR negative, HER2 positive;

Basal-like. ER negative, PR negative, HER2 negative, cytokeratin 5/6 positive and/or epidermal growth factor receptor positive;

Normal breast-like tumours.

However, the most modern definition of breast cancer has evolved, with triple-negative breast cancer (TNBC) defined as ER negative, PR negative and lacking overexpression of HER2; luminal-A cancers are defined as ER positive and histologically low grade; luminal-B cancers are also mostly ER positive but may express low levels of hormone receptor and are often high grade; HER2-positive cancers show amplification and high expression of the HER2 gene (Sotiriou and Pusztai, 2009; Boyle, 2012).

Triple Negative Breast Cancer (TNBC)

The definitional hallmark of TN breast cancer is their negative status for both ER and PR and the lack of over-expression and amplification of the HER2 gene: they represent around 15%-20% of newly diagnosed breast cancer cases. Unfortunately, a consensus on what constitutes a negative hormone receptor status is still lacking: this makes it very difficult to compare data stemming from different studies (Reis-Filho and Tutt, 2008; Viale and Bottiglieri, 2009).

Comparing TN with non-triple negative breast cancers, several clinical differences are noticeable. First of all, TN tumours reportedly affect younger (mean age 47–55 years) women, are more prevalent in the population of women carrying BRCA-1 mutations, their average size at diagnosis is larger than for non-TN tumours, and the prevalence of lymph node metastases is higher. Interestingly, for TN tumours there is no apparent correlation between tumour size and nodal status, and even small tumours with a triple negative phenotype have a higher rate of nodal positivity than non-TN tumours. TN tumours are more common in the African-American population, often manifest clinically as “interval” cancer between consecutive mammograms, and are more likely to be diagnosed by clinical examination, probably due to a more rapid growth rate (Atchley et al, 2008; Viale and Bottiglieri, 2009).

The clinical course of the disease is particularly aggressive, with a tendency to early dissemination to distant organs (particularly in the lungs and brain, less frequently in the bones) irrespective of the axillary lymph node status, and the time between the appearance of distant recurrence to death is much shorter in this type of malignancy than in other types of breast cancer (Diaz et al, 2007). The appearance of distant metastases is rarely preceded by a local recurrence, and if a local relapse occurs it is not predictive of systemic metastases. Moreover, the risk of recurrence is particularly high during the first 3 to 5 years after diagnosis, and it decreases thereafter. Overall survival is shorter than for other breast malignancies, and most deaths occur within the first 5 years after diagnosis. Histologically, TN tumours are more commonly high-grade neoplasms, which lack tubule formation, have prominent nuclear abnormalities and high mitotic count. They have broad pushing tumour borders, and are often characterized by large areas of central or geographic necrosis and fibrosis, often associated with a ribbon-like tumour architecture, and show a prominent lymphocytic infiltrate (Viale and Bottiglieri, 2009). These necrosis and fibrosis have long been identified as a peculiar feature of some high-grade invasive ductal carcinomas with a poor prognosis and early appearance of pulmonary and cerebral metastases. More recently, the biological and clinical implications of the fibrotic foci in breast cancers have been extensively investigated. These foci have been considered as surrogate markers of hypoxia and neoangiogenesis (Van Den Eynden et al, 2007).

The immunohistochemical profile of TN breast cancer has been extensively investigated (Kreike et al, 2007; Reis-Filho and Tutt, 2008; Lerma et al, 2007) and it is characterised by the variable expression of several markers, including cytokeratins 5,14 and 17 (at least one of these cytokeratins is expressed in the vast majority of TN tumours), vimentin (55%), P-cadherin (93%), EGFR (27–37%), PDGFR (31%), IGF-1R (36%), c-kit (11–38%), S-100 protein (22%), p63 (10%), smooth-muscle actin (8%) and by the nuclear accumulation of p53 (50–56%). Cyclin D1 is overexpressed in as many as

51% of the tumours, whereas down-regulation of p27 expression and loss of PTEN has been documented in 56% and 14% of the TN tumours, respectively. The median Ki-67 labelling index is 35% (range 10–90%), and only 20% of the tumours have Ki-67 labelling indexes less than 20% (Lerma et al, 2007).

Approximately 75% of TNBCs express basal markers and, consequently, the triple-negative sub-type is frequently, and erroneously, taken as a surrogate marker for the basal-like sub-type (Atchley et al, 2008). “Basal-like” breast carcinomas have been identified by the hierarchical clustering of the variations in the expression of 496 genes (“intrinsic” gene subset). Basal-like carcinomas show an aggressive clinical course and a poor outcome (Perou et al, 2000). Due to the clinical, biological and histological similarities with TN breast cancers, it has been tempting to consider the triple negative phenotype as the immunohistochemical surrogate for the molecularly-defined basal-like tumours (Kreike et al, 2007). This view, however, is oversimplistic (Viale and Bottiglieri, 2009).

Breast Cancer Therapy

Breast cancer therapy is today based on surgical removal of the primary tumour, postoperative radiation followed by adjuvant endocrine and/or chemotherapy (Lundgren et al, 2007). As already stated, expression of both the ER and PR, as well as the oncogene HER2/neu, are being used to define the most prominent breast cancer subtypes in a clinical context. This is particularly clinically relevant since these three markers represent important predictive markers for systemic therapies to allow for a personalized therapeutic approach. Also, increasing knowledge of the molecular pathology of breast cancer has resulted in the development and validation of a number of therapeutic targets that are currently and increasingly being exploited as part of an individualized targeted therapeutic approach. Targeted agents largely consist in either targeted antibodies or small molecules (Liedtke and Kiesel, 2012). Despite impressive advances in the treatment of patients with breast cancer, there are still patients within each subtype that do not benefit from current therapeutic strategies; therefore, overcoming resistance against these highly efficacious agents is of major importance. Instead, patients with TNBC suffer from a lack of targeted therapies, since endocrine and HER2-targeted agents cannot be used.

Endocrine Therapy

HR-positive breast cancer represents the most common breast cancer subtype and is present in more than 3/4 of patients with breast cancer. Most HR-positive breast cancers are classified as luminal breast cancer when examined using complex gene expression analyses. Luminal breast cancers are divided into luminal A and B breast cancer, the latter comprising a more aggressive

phenotype that may require additional chemotherapy (Liedtke and Kiesel, 2012). The most established targeted therapeutic approach for patients with breast cancer consists in endocrine therapy through the use of agents such as tamoxifen of aromatase inhibitors (Forbes et al, 2008; Goss et al, 2007). More recently, agents targeted against the HER2/neu oncogene (i.e. the humanized antibody Trastuzumab or the small molecule Lapatinib), against EGFR (i.e. the chimeric monoclonal antibody Cetuximab or the small-molecule Tyr-kinase inhibitor Gefitinib) or against VEGF (i.e. the humanized monoclonal antibody Bevacizumab) have been developed and are being used as part of clinical routine in a number of countries worldwide (Conte and Guarnieri, 2012). Nevertheless, there is an urgent need to develop novel (targeted) therapies that overcome (primary and secondary) endocrine resistance: newer biologic agents like Pertuzumab (a humanized monoclonal antibody that blocks HER2 dimerization) and T-DM1 (an antibody-drug conjugate that uses Trastuzumab to specifically deliver a cytotoxic antimicrotubule agent to HER2+ cells), as well as multikinase inhibitors (i.e. Sorafenib, Sunitinib and Dasatinib) and mTOR inhibitors (i.e. Everolimus) are currently undergoing different phases of clinical trial (Liedtke and Kiesel, 2012; Conte and Guarneri, 2012).

TNBC Therapy

TNBC is the most clinically relevant given that patients lacking the predictive markers ER/PR and HER2 cannot benefit from endocrine and HER2-targeted therapy. Patients with TNBC suffer from an adverse prognosis which is in contrast of an increased probability of deriving benefit from chemotherapy (Liedtke et al, 2008). Therefore, in the development of novel and optimization of current therapeutic concepts for patients with TNBC there are several issues that are of particular importance:

(1) Identification of patients with intrinsic resistance against current chemotherapy regimens that should be offered alternative approaches, if available;

(2) Optimization of response to current chemotherapy regimens, for instance through the use of alternative chemotherapy substances or dosages (Isakoff et al, 2011);

(3) Development of novel (targeted) agents.

Possible targets already in study are VEGF (the use of Bevacizumab in TNBC patients is undergoing clinical trial) and PARP (at least in TNBC also BRAC1/2 negative; Iniparib and Olaparib showed promising results in phase II trial, which unfortunately could not be reproduced in phase III). Other therapeutic targets with potential indication for patients with TNBC are MET, mTOR, IGF-1R, SRC, NOTCH, FGFR and ADAM17 (Liedtke and Kiesel, 2012).

Metastatic breast cancer (MBC) therapy

Despite improvements in adjuvant treatment of early breast cancer, about 20% of patients initially diagnosed with regional disease are eventually diagnosed with metastases (Beslija et al, 2009).

Unfortunately, tumors in most patients with metastatic breast cancer (MBC) eventually become unresponsive to systemic therapy. The heterogeneity in the clinical behavior of MBC—as it relates to tumor burden, distribution of metastatic sites, rate of disease progression, and degree of symptoms—indicates the need for flexibility in therapeutic strategies (Alvarez et al, 2010; Guarneri and Conte, 2009). Indeed, the heterogeneous molecular pathways involved in breast cancer provide numerous potential targets for therapeutic intervention. Several targeted agents have been introduced into chemotherapy regimens for MBC in recent years, complementing the existing options (Sánchez-Muñoz et al, 2009; Beslija et al, 2009), and numerous other targeted agents are in preclinical or early clinical stages of development. Treatment of MBC is aimed at stabilizing disease, improving survival, and maintaining quality of life with manageable toxicities; earlier and more aggressive use of traditional chemotherapeutic agents, such as anthracyclines and taxanes, has decreased the number of treatment options in the metastatic setting. However various new approaches to combination therapy are being investigated, including classic and novel cytotoxic agents and targeted therapies (Alvarez et al, 2010). The latter have the potential to improve outcomes in MBC, and their use has increased dramatically over recent years. Overall, single-agent use of targeted therapies has failed to produce dramatic benefit in patients with advanced breast cancer; however significant benefit has been achieved by their use in combination with cytotoxic chemotherapy. One strategy that appears particularly promising is the combination of hormonal therapy with a targeted agent as demonstrated by a significant improvement in PFS of patients with HR_ recurrent advanced breast cancer treated with exemestane in combination with everolimus compared with exemestane alone. Another promising approach is multitarget inhibition as illustrated by the CLEOPATRA trial showing that the addition of pertuzumab to trastuzumab and docetaxel resulted in significantly prolonged PFS as first-line treatment for HER2_ MBC. Despite all these studies, treatment of TNBC remains a major challenge (Conte and Guarneri, 2012).

First-Line Treatment
Anthracyclines: doxorubicin, epirubicin, liposomal doxorubicin, or anthracyline-based combinations
Taxanes: paclitaxel, docetaxel, albumin-bound paclitaxel
Other agents: gemcitabine, capecitabine, vinorelbine, ixabepilone/capecitabine
After Anthracycline Resistance or Failure
Taxane monotherapy (docetaxel every 3 weeks, paclitaxel every 3 weeks or weekly, or nab-paclitaxel every 3 weeks)
Later-Line Treatment
Capecitabine, gemcitabine, liposomal doxorubicin, ixabepilone, eribulin, or vinorelbine as monotherapy or in combination with other cytotoxic agents
HER2-Overexpressing Tumors
Trastuzumab and non–anthracycline-based chemotherapy
Lapatinib in combination with capecitabine as second-line treatment

Table 1 Standard cytotoxic chemotherapy in breast cancer

Resistance to breast cancer therapy

As already stated, despite the advances in the treatment of patients with breast cancer there are still patients within each subtype that do not benefit from current therapeutic strategies. For instance, patients with both luminal (i.e. hormone receptor positive) early breast cancer and those with HER2/neu-positive disease experience resistance to endocrine or HER2/neu-targeted therapy in a significant number of cases (Forbes et al, 2008; Goss, 2007). There is an urgent need to develop novel (targeted) therapies that overcome (primary and secondary) endocrine resistance (Liedtke and Kiesel, 2012).

Intracellular signal transduction is characterized by a network of (often redundant) signaling pathways that mediate mechanisms essential for cellular function and survival. It is well known that there is a close and reciprocal crosslink between growth factor receptor and hormone receptor signaling with regard to mediation of tumor cell stimulation (Schiff et al, 2004). Therefore, it seems plausible that combined use of growth factor receptor inhibitors and endocrine therapy may be used to overcome endocrine resistance. In fact, a number of proof-of-principle studies have been conducted, demonstrating that combination of endocrine and growth-factor targeted therapies may be feasible and effective (Osborne et al, 2011).

Another mechanism of endocrine resistance is mediated through the phosphatidylinositol 3-kinase (PI3K)–Akt– mammalian target of rapamycin (mTOR) signaling pathway (Burstein, 2001; Johnston, 2006). In preclinical models, the use of Everolimus, an mTOR inhibitor, in combination with aromatase inhibitors inhibits proliferation and induces apoptosis The results of these studies demonstrate that dual endocrine and mTOR blockade may result improved endocrine response among patients with HR-positive breast cancer, particularly among those individuals with metastatic disease. Hence, using mTOR inhibition may be a mechanism of overcoming endocrine resistance, particularly among advanced cases. Further substances in this context may be worth exploring (Efeyan and Sabatini, 2010).

One major milestone in the therapy of patients with HER2/neu-positive breast cancer certainly is the development and establishment of the humanized monoclonal antibody Trastuzumab for patients with HER2/neu-positive breast cancer of all disease-stages. In the pivotal registration trial, patients with metastatic HER2/neu-positive breast cancer were randomized to receive either chemotherapy alone or chemotherapy in combination with Trastuzumab. Treatment with Trastuzumab resulted in a significant increase in overall survival, however, even in the combination arm, median OS was still 25.1 months, demonstrating that those patients had eventually experienced either primary or secondary resistance (Slamon et al, 2001).

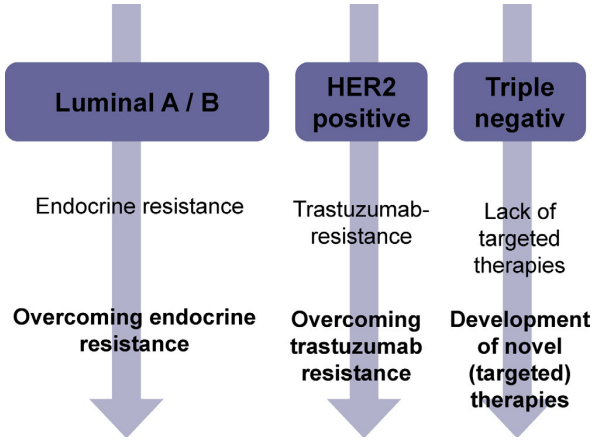

Figure 2 Approaches to optimize therapy among patients with distinct breast cancer subgroups. *(Maturitas, 2012)*

A great number of mechanisms have been suggested to explain the development of trastuzumab resistance: reduced bioavailability; CNS metastases; loss of Trastuzumab binding capacity (Scaltriti et al, 2007); loss of the HER2 extracellular domain (p95); mutation of the Trastuzumab binding domain; downregulation of HER2 expression; activation of downstream signal transduction (Nahta et al, 2009); activating PI3K-mutations; loss of PTEN; activation of alternative signal transduction/growth factor receptors like IGF-R, HER3, MET, etc. (Nahta et al, 2009). The study of these different mechanisms of resistance has lead to the introduction of a number of novel substances to treat patients with Trastuzumab-resistant breast cancer (i.e. Lapatinib, Pertuzumab and T-DM1) (Liedtke and Kiesel, 2012).

The insulin/IGF system

Insulin is conventionally described in medical textbooks as a hormone that plays key roles in the regulation of carbohydrate metabolism, while the insulin-like growth factors are described as important regulators of pre- and post-natal growth. In fact, an insulin-like signal transduction system is present in simple organisms such as *C. elegans*, where it regulates fundamental cellular processes including longevity and energy metabolism (Dong et al, 2007). The emergence of distinct insulin and IGF receptors and the use of these signaling systems to regulate blood glucose concentration is a relatively recent evolutionary development (Pollak, 2008).

The lack of structural variation of insulin and IGFs throughout phylogeny1-3 speaks to their importance in the control of growth and metabolism in multicellular organisms. A key lesson learned from phylogenetic and physiological studies is that metabolism and growth are tightly coupled through

a single signaling receptor in invertebrates, while vertebrates have uncoupled the process of growth and metabolism by separating growth from metabolism into IGF and insulin signaling pathways, respectively (Froesch et al, 1985; Maki, 2010).

As previously stated, the insulin-like growth factor (IGF) system comprises a phylogenetically ancient family of peptides involved in mammalian growth, development and metabolism, as well as in cellular processes such as proliferation, survival, cell migration and differentiation. This family includes three ligands (IGF-1, IGF-2 and insulin), their cell surface receptors [the IGF-1 receptor (IGF-1R), the mannose 6-phosphate/IGF-2 receptor (M6P/IGF-2R), two different isoforms of the insulin receptor (IR-A and IR-B) and the hybrid IR/IGF-1R], six high affinity binding proteins (IGFBP-1 to 6) and their proteases. Moreover, it includes the proteins involved in intracellular signaling distal to IGF-1R, such as the insulin receptor substrate (IRS) family and AKT (Le Roith, 2003; Annunziata et al, 2011).

Ligands

IGF-1 was first identified as a serum factor induced by the pituitary growth hormone (GH) that mediated GH-induced sulfate uptake into the cartilage and was initially referred to as ‘‘sulfation factor’’ (Salmon and Daughaday, 1957). In 1972, the more general term ‘‘somatomedin’’ was proposed to designate a substance in the serum considered to be the intermediary of somatotropin (GH) action on its target tissues. In the meantime, a new factor with insulin-like effects was found in human serum, whose activity could not be suppressed by addition of anti-insulin antibodies: this insulin-like factor was also shown to possess growth-promoting effects. Later, IGF-1 and IGF-2 were purified, and IGF-1 was found to be identical to somatomedin. Both substances were termed ‘‘insulin-like growth factor,’’ for their close homology with insulin and because their effects on cell and tissue growth predominate over those on metabolism (Daughaday et al, 1987). Insulin expression is confined to specialized pancreatic β-cells, and under normal circumstances it is tightly regulated by the level of circulating glucose. In contrast to epidermal growth factor and other tissue growth factors that are relevant to neoplastic disease, insulin functions as a classic hormone, influencing tissues remote from its site of production (Pollak, 2012). IGF-1 has characteristics of both circulating hormone and tissue growth factor. Most IGF-1 found in the circulation is produced by the liver. Regulation of hepatic IGF-1 production is complex, and GH has a dominant role in upregulating IGF-1 gene expression. GH, in turn, is produced by the pituitary gland under the regulation of the hypothalamic factors somatostatin and growth hormone-releasing hormone (GHRH). Initially, it was supposed that virtually all IGF-1 originated in the liver and was transported by endocrine mechanisms to sites of action, but it is now recognized that IGF-1 is also synthesized in other organs where it exerts autocrine or paracrine effects (Annunziata et al, 2011). Similarly to IGF-1, IGF-2 is also expressed both in the liver and in extrahepatic tissues, but is not tightly regulated by GH (Clemmons, 2006).

The human IGF-1 gene is located on the long arm of the chromosome 12. It has a complex structure with multiple promoters and contains six exons, four of which are subjected to alternative

splicing; the alternative transcripts generate different precursors, without altering the structure of the mature peptide (Tricoli et al, 1984; Rotwein et al, 1986). IGF expression is finely regulated at the level of transcription, RNA processing and translation, and the final product is a precursor protein that undergoes proteolytic processing at both ends (Sussenbach et al, 1992). The mature IGF-1 is a single chain 7.5 kDa, 70-amino acid peptide, cross-linked by 3 disulfide bridges.

Since its first characterization, it was evident that IGF-1 displays high degree of homology with insulin, having 48% amino acid identity, identical disulfide bonding and similar tertiary structure. The major structural difference between the IGFs and insulin is that the IGFs retain the C-peptide region that is cleaved from proinsulin, and there is a small D extension to the A chain in the IGF molecules (Blundell et al, 1983). A second important difference is that specific amino acids at positions 3, 4, 15 and 16 within the IGF-1 molecule, which are not present in insulin, confer binding to a family of six high affinity binding proteins (IGFBPs). Therefore, while most insulin circulates in a free form, more than 75% IGF-1 is confined to the vascular compartment as a 150 kDa ternary complex with the acid labile subunit (ALS) and IGFBP-3, the most abundant circulating IGFBP (Firth and Baxter, 2002).

IGF-2 is a 67-aminoacid protein produced by post-translational removal of the COOH-terminal E domain from the precursor molecule, pro-IGF-2 (Duguay et al. 1998); its structure resembles that of IGF-1. Partial cleavage of the E domain results in big IGF-2 (two isoforms; 1-104 or 1-87) which, along with pro-IGF-2, are also found in the circulation. Mature IGF-2 itself can be processed to generate des(37-40) IGF-2 (also known as vesiculin). Little is known about the signaling properties and function of these IGF-2 variants (Marks et al, 2011, Harris and Westwood, 2012). In humans, evidence for the importance of IGF-2 comes from the observation that *Igf2* is maternally imprinted (Giannoukakis et al. 1993). Relaxation of imprinting leads to Beckwith–Wiedemann syndrome (BWS) in which excess IGF-2 is associated with foetal overgrowth. IGF-2 can interact with a number of cell-surface receptors but it binds to the type 1 IGF receptor with highest affinity and therefore it is likely that this receptor mediates much of IGF-2 effect on cellular proliferation, survival, differentiation and migration. However, IGF-1 also binds to IGF-1R and in general, elicits the same effects with greater potency, which has led to some speculation about the specific purpose of IGF-2. In recent years, this has been clarified through the use of receptor inhibitors and a better understanding of the pathways downstream of the type 2 IGF receptor and also, the A isoform of the insulin receptor (IR-A), both of which bind IGF-2 with greater affinity than IGF-2 (Harris and Westwood, 2012).

Receptors

An ancestral insulin-like receptor arose early in evolution and has important roles in *Drosophila melanogaster* and *C. elegans* (Dong et al, 2007; Teleman et al, 2008). The need to regulate cellular uptake of glucose independently of cell survival and proliferation probably led to the evolution of distinct IGFRs and insulin receptors in more complex animals. In humans, IGF-1R and insulin receptor (IR) are widely expressed on normal tissues: both types of receptors are members of the tyrosine kinase class of membrane receptors and have tetrameric structures, characterized by two

'half receptors', each of which in turn comprises a predominately extracellular α-chain that is involved in ligand binding and a predominately intracellular β chain that includes the tyrosine kinase domain (De Meyts, 2004). Cells that co-express the two receptor genes present not only insulin and IGF-1R, but also 'hybrid receptors' formed by an insulin half receptor and an IGF-1 half receptor (Belfiore et al, 2009). These hybrid receptors appear to bind IGF-1 and IGF-2 with high affinity, similarly to IGF-1R. Conversely, insulin is bound with low affinity, which is tenfold lower than that of classical insulin receptors and 20-fold lower than the affinity of hybrids for IGF-1. The biological response elicited by these hybrid receptors can vary, depending on the ligands involved and the specific IR isoforms. However, their physiological role is still unclear (Belfiore et al, 2009).

The biosynthesis and trafficking of the receptors involves the chaperone protein heat shock protein 90 (HSP90), therefore implying that IR and IGF-1R are among the targets of the HSP90-targeting agents that are currently being evaluated for antineoplastic activity (Pollak, 2009).

The insulin receptor exists in two splice variant isoforms, depending on the absence or presence of exon 11 splicing (Belfiore et al, 2009). IR-A (short form) is a fœtally expressed isoform that lacks a region within exon 11. The 'B' isoform only recognizes insulin, but the 'A' isoform, which is the isoform most commonly expressed by tumours, recognizes both insulin and IGF-2 (Pollak, 2012). Insulin is the canonical ligand for IR and most potently activates IR homodimers. However, as already stated, the ability of IGF-2 to activate IR is also well established (Belfiore et al, 2009). Interestingly, affinity of IGF-2 for IR-A is 5-fold tighter than IR-B homodimers. As a result, in addition to its role in metabolic signaling in tissues involved in glucose homeostasis, IR can also promote cell proliferation and survival (Buck et al, 2010).

IGF-1R gene encodes for a single chain 180 kDa 1,367 amino acid precursor. Cleavage of the precursor generates α and β subunits which are glycosylated and proteolytically processed to yield the mature α2β2 receptor (Ullrich, 1986). IGF-1R is closely related to the IR, sharing 70% amino acid identity overall and 84% identity within the catalytic domain (Lawrence et al, 2007) Following ligand binding, the intracellular tyrosine kinase domain autophosphorylates specific tyrosines that act as docking sites for signaling proteins, such as the insulin receptor substrate (IRS) family of proteins (IRS-1 through -4). These proteins then recruit other substrates leading to activation of different signaling cascades, including the phosphatidylinositol 3-kinase (PI3K)–AKT– TOR pathway and the RAF–MAPK pathway, that stimulate cell proliferation and survival (Annunziata et al, 2011).

At the cellular level, signaling downstream of insulin receptors and hybrid receptors is similar but not identical. In each case, the kinase activity of the receptor leads to phosphorylation of members of the insulin receptor substrate (IRS) family of proteins, and this leads to activation of PI3K, AKT and various downstream networks. However, different cell types use this control system to regulate different processes. For example, a major consequence of pathway activation in the liver is the inhibition of gluconeogenesis and the activation of glycogen storage. By contrast, epithelial cells do not express gluconeogenic enzymes and consequences of pathway activation include the stimulation of proliferation and the inhibition of apoptosis (Belfiore et al, 2009).

IGF-2R is structurally unrelated to either IGF-1R or IR as it consists of just a single, primarily extracytoplasmic, polypeptide chain. This receptor binds IGF-2 with greater affinity than IGF-1 and whilst it does not accept insulin as a ligand (El-Shewy and Luttrell, 2009), it does have high affinity for the sugar mannose-6-phosphate (M-6-P), and can therefore bind lysosomal enzymes and other growth factors and cytokines. Cloning of the type 2 IGF receptor cDNA (Morgan et al, 1987) led to the realisation that this receptor was also the cation-independent receptor for M-6-P; given the well-documented role of this receptor in the intracellular transport of lysosomal enzymes, it was suggested that rather than mediating IGF-2 effects, it might be important for clearing IGF-2 from the circulation (Harris and Westwood, 2012). However, although IGF-2R contains neither tyrosine kinase activity nor an autophosphorylation site, it seems to be linked to G-proteins which provides a mechanism for signal transduction (El-Shewy and Luttrell, 2009).

IGFBPs

The bioactivity of IGFs is furthermore modulated by IGFBPs, which have high affinity for both IGF-1 and IGF-2. In general, IGFBPs limit IGFs access to IGF-1R, thereby attenuating the bioactivity of these growth factors (Firth and Baxter, 2002). The tumour suppressor p53, as well as many growth inhibitors including vitamin D, anti-estrogens, retinoids, and transforming growth factor-β (TGFβ), reduce IGF bioactivity by increasing the secretion of IGFBPs. It is of interest that the circulating concentration of insulin (~0.5 nmol per litre) is considerably lower than that of IGF-1 (~20 nmol per litre) or IGF-2 (~90 nmol per litre). This is compatible with the view that insulin has direct access to its receptor, whereas IGF-1 faces binding competition from the IGFBPs, and IGF-2 from both IGFBPs and IGF-2R (Pollak, 2009). However, in certain contexts, overexpression of IGFBPs is associated with increased rather than decreased IGF action, with adverse effects on cancer prognosis and with loss of function of PTEN (So et al, 2008). The mechanisms involved in this aspect of IGFBP physiology remain incompletely described, but are the subject of intense investigation: one hypothesis is that the secretion of these high-affinity IGFBPs increases the concentration of ligands in the tumour microenvironment; whereas these bound ligands are initially in an inactive state, they may be released as continuously bioavailable ligands owing to the action of IGFBP proteases that are secreted by neoplastic cells. The concept that IGFBPs have biological activities that are independent of their IGF binding properties is not new (Firth and Baxter, 2002). A recent study suggests that certain IGFBPs modulate Wnt signalling in a manner that is influenced by the local concentration of IGF ligands. This finding deserves follow-up as it implies the existence of a network linking two important signal transduction pathways. Adding further to the complexity of IGFBP activity is the finding of proteolytic cleavage fragments of IGFBPs in serum; it is unclear if the fragments have biologic activity as well (Maki, 2010).

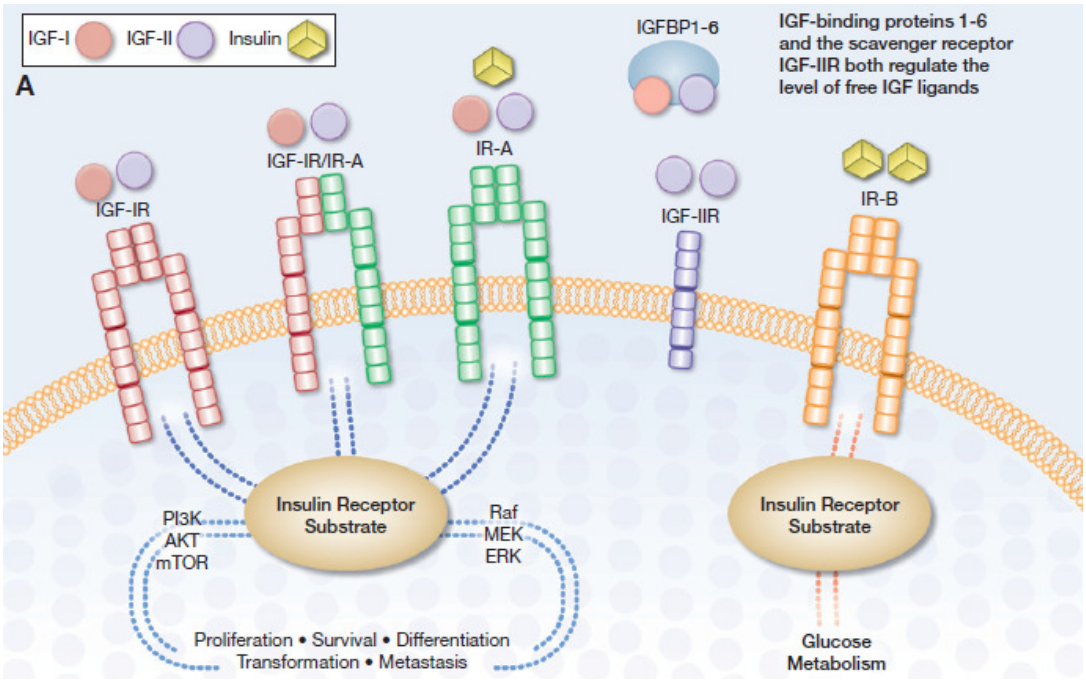

Figure 3 The major components of the IGF axis (the IGF-1R/IR-B hybrid receptors are not represented). *(Cancer Res, 2012)*

Biological effects of IGF-1 and IGF-2

The IGF system is involved in pleiotropic actions, at endocrine, paracrine and autocrine levels. It is activated during fetal development with growth-promoting effects, especially in bone, mammary gland, prostate and muscle, but it is also implicated in postnatal growth and tissue remodeling. For example in the adulthood, IGFs play a key role in skeletal muscle regeneration and hypertrophy and in mammary gland cell proliferation and survival during puberty, pregnancy and lactation. In addition, IGF-1 is a trophic and survival factor for neuronal cells, and it may exert neuroprotective effects in pathologic conditions such as Huntington's disease and Alzheimer. Moreover, the IGF system plays a role in neuronal plasticity and cognitive functions (Annunziata et al, 2011). IGFs also have protective functions in the heart, as assessed by *in vitro* and *in vivo* studies. Indeed, low serum IGF-1 levels are associated with increased risk of ischemic heart disease and stroke (Colao, 2008). IGF-1 is also a mitogen and antiapoptotic factor for vascular cells, including smooth muscle and endothelial cells, and exerts regulatory functions on the immune system. As a net result, IGF-1 seems to have atheroprotective effects. At the cellular level, IGF-1 proliferative effects can be followed by induction of differentiation: it is a renowned mitogen, as expected by its role in promoting somatic growth. Besides its role in somatic growth, IGF-1 exerts metabolic effects. Indeed, it can mediate the anabolic effects of GH by stimulating protein synthesis and preventing proteolysis. Because of its anabolic effects in adults, IGF-1 has been implicated in the treatment of catabolic states associated with illness (Le Roith, 2003). However, IGF-1 and GH have divergent effects on carbohydrate metabolism. While GH has insulin-antagonistic properties and its excess causes insulin resistance, IGF-1 has insulin-like

effects and improves insulin sensitivity. Several evidence indicate that IGF-1 contributes to glucose homeostasis and insulin sensitivity (Clemmons, 2006). Low circulating IGF-1 levels are associated with insulin resistance, as found in individuals with IGF-1 gene deletion. *In vivo* and *in vitro* evidence indicate that IGF-1 can stimulate glucose transport, particularly in skeletal muscle cells, exerting direct glucose-lowering effects via either the IGF-1R or the hybrid receptor. IGF-1 can enhance insulin actions also by inhibiting GH secretion from the pituitary, and therefore preventing its diabetogenic effects, via a negative feedback mechanism (Annunziata et al, 2011).

IGF-2 exhibits proliferative and antiapoptotic actions similar to those of IGF-1: it plays a fundamental role in embryonic and foetal growth, as proven by studies performed on IGF-2 knockout mice, which survive but remain smaller than their wild-type littermates (Maki, 2010). IGF-2 is not expressed in mice after birth, while it is expressed throughout life in humans, where its activity is regulated by genomic imprinting, the gene being inactive on the chromosome inherited from the mother in most normal tissues (Harris and Westwood, 2012). IGF-2 affects foetal growth by influencing placental development and function (through modulation of trophoblast turnover and migration, as well as nutrient transport) and appears to be an important modulator of muscle growth and differentiation, especially in early cardiac development, myogenesis and vasculogenesis.

The insulin/IGF system in cancer

The evolving consensus that insulin and IGFs physiology are relevant to neoplasia arises from converging results from independent lines of investigation. Population studies have provided evidence that relate circulating ligand levels as well as polymorphic variation of relevant genes to cancer risk and prognosis. Laboratory models have provided further evidence that is consistent with the population studies as well as experimental validation of various therapeutic targeting approaches (Pollak, 2009).

Laboratory studies. Experimental investigations of the function of insulin in neoplasia preceded those focusing on the functions of IGFs. Early studies not only showed that insulin at physiologically relevant concentrations stimulates DNA synthesis in breast cancer cells, but also provided evidence that insulin deficiency is associated with less aggressive cancer proliferation in vivo (Osborne et al, 1976). Until the recent resurgence of interest, however, little attention was given to following up on these observations made more than 30 years ago, probably because of the assumption that any attempt to reduce insulin-stimulated signalling in cancers would have severe metabolic consequences for the host. IGF-1R targeting strategies were first proposed over 20 years ago, when IGF-1R (overexpression?) was detected in human cancers (Pollak et al, 1987). Many subsequent *in vitro* and *in vivo* models provide, overall, convincing evidence for a role of IGF1R in neoplasia. Initial *in vitro* experiments demonstrated dose-dependent increases in neoplastic cell proliferation with increasing

IGF-1 concentration. Following studies showed that the transforming action of many oncogenes required, or was facilitated by, IGF signalling. *In vivo* models using naturally occurring mutations associated with low IGF-1 levels, or genetic manipulations that influence ligand levels showed that *in vivo* tumour growth is influenced by the IGF-1 physiology of the host. A translational research approach showed that a pattern of gene expression induced by IGF-1 could predict poor outcome in patients with breast cancer (Creighton et al, 2008). More recently, several drug candidates that target IGF signalling were found to have anti-neoplastic activity *in vivo*, both as single agents and in combination with currently approved drugs, and are currently undergoing clinical trials (Gao et al, 2012). The influence of host hyperinsulinaemia on cancer behaviour has been the subject of recent investigations: in general, these results provide strong (but circumstantial) evidence that hyperinsulinaemia may be a mediator of the adverse effect of obesity on cancer prognosis.

Population studies. Different studies have been undertaken to examine influences of IGF-1 excess and deficiency on cancer in humans. Although they provide limited evidence in favour of a relationship between higher levels of IGF-1 and malignancy, they are not definitive. In both cases, treatment of the endocrine disorder may complicate interpretations, and both conditions are rare enough that assembly of large cohorts is challenging. Epidemiologic research provides direct and circumstantial evidence for the relevance of insulin and IGFs to neoplasia. Examples of circumstantial evidence include observations concerning somatic growth patterns and mammographic density (Pollak, 2009). Given that IGF-1 is known to influence growth patterns, it is of interest that well-controlled studies provide evidence that height and weight at birth (which is related to the concentration of IGF-1 in the umbilical cord) are related to risk of some cancers, and that breast cancer in particular is related to patterns of peripubertal growth (Alhgren et al, 2004). Mammographic breast density, a strong risk factor for breast cancer, has been related both to the level of circulating IGF-1 and to polymorphisms in IGF-related genes. Rigorous prospective studies provided evidence for a relationship between the levels of circulating IGF-1 and the risk of developing prostate, breast, colorectal or other cancers, such that individuals at the high end of the normal range of serum IGF-1 concentration had more than double the risk of a subsequent cancer diagnosis of those at the low end of the normal range (Pollak, 2009). Some of these early reports also described the finding that higher circulating levels of IGFBP3 were associated with reduced cancer risk, which was interpreted as reflecting an influence of IGFBP3 in reducing IGF-1 bioactivity, in keeping with laboratory studies (Giovannucci et al, 2000). However, follow-up studies have failed to confirm these reports, or have revealed more complicated relationships. The basis for these inconsistencies is under investigation by several research groups.

Genetic studies. They provide evidence, methodologically unrelated to serum assays, that implicates IGF-1 physiology in cancer risk. A study suggests that, in some individuals, high levels of IGF-1 are in fact associated with reduced IGF-1R activation owing to subtle variants of IGF-1R that are deficient in signalling activity. In this situation, homeostatic control mechanisms raise the ligand levels in the serum in an attempt to compensate. In such cases, the assumption that higher amounts of ligand in the serum can be used as a surrogate for higher levels of signaling may be false, and this would attenuate any association between IGF-1 serum levels and cancer risk. More work needs to be

done to investigate this issue and to clarify the frequency of thesereceptor variants in different populations (Pollak, 2009).

Aberrant autocrine and paracrine IGF-2 signalling, leading to the enhancement of cell proliferation and resistance to apoptosis, has long been implicated in the initiation and progression of tumour growth (Toretsky and Helman 1996). Epigenetic alterations, such as the loss of DNA imprinting, occur in cancer at least as commonly as genetic mutations. The majority of imprinted genes exist in clusters, and their expression is regulated by the methylation status of CpG-rich cis-elements, known as differently methylated regions (DMRs). The DMRs are differentially methylated on CpG sites by DNA methyltransferases, depending on the parental origin of the allele (Mann et al. 2000). *Igf2* is an example of an imprinted gene; the loss of imprinting (LOI) of the normally silent maternal allele of *Igf2* leads to overexpression of IGF-2 protein and an increased risk of malignancy. Ligation of IR-A by IGF-2 initiates a proliferative response comparable to that of IGF-1R, and aberrant IR-A signalling has been implicated in a number of diseases, including cancer (Belfiore et al, 2009). IGF-2 signalling through IR-A has also been shown to induce differential expression of genes involved in signal transduction, cell cycle, metabolism, angiogenesis and adhesion, when compared with insulin signalling (Harris and Westwood, 2012).

IR-A has long been recognized as the predominant IR isoform expressed by carcinomas of the breast, colon and lung (Frasca et al. 1999). Activation of IR by IGF-2 in human breast cancer cell lines stimulates proliferation, with IGF-2 exhibiting 63% of the potency of insulin. In contrast, IGF-2 signalling through IR in non-malignant human breast cells is less than 1% as potent as insulin (Sciacca et al. 1999).

The tumour suppressor function of the IGF-2R was first demonstrated in 1999, when it was showed that down-regulation of IGF2R expression in JEG-3 choriocarcinoma cells enhanced proliferation *in vitro*, and increased tumour growth rate *in vivo* (O'Gorman et al. 1999). Conversely, IGF-2R overexpression reduced JEG-3 cell proliferation *in vitro*, and decreased tumour growth in nude mice. IGF-2R overexpression did not alter endogenous IGF-2 production, or secretion of the IGF-2R ligands procathepsin D and L, but did promote secretion and activation of latent TGF-β1. Overexpression of a soluble form of the receptor dramatically reduced tumour cell growth in vitro and in vivo, but did not alter the level of TGF-β1 (O'Gorman et al. 2002). These data suggest that increased levels of soluble IGF-2R inhibit cell proliferation. Unlike its murine homologue, human *Igf2r* exhibits biallelic expression, although a few individuals exclusively express the maternal allele. Mutations in *Igf2r*, or loss of heterozygosity at the 6q26–27 locus where *Igf2r* resides, lead to reduced IGF-2R expression and increased circulating concentrations of IGF-2. Loss of biallelic *Igf2r* expression has been reported in cancers of the breast, liver, prostate, lung, adrenal gland, head, neck and endometrium (Martin-Kleiner and Gall 2010). Loss of heterozygosity proximal to the *Igf2r* locus is also predictive of the presence of disseminated tumour cells in the bone marrow of ovarian cancer patients, before and after chemotherapy (Kuhlmann et al. 2011).

The Insulin/IGF system in breast cancer

Numerous evidences have shown that the IGF pathway is strongly implicated in breast cancer.

IGF-1R is over-expressed in about 90% of breast cancer cases and IGF-1R levels are higher in cancer cells than in normal breast tissue or in benign mammary tumors. Furthermore, breast cancer cells over-express both IGF-1R and IR-A and that leads potentially to formation of hybrid IGF-1R–IR-A receptors (HRs) as well (Yang and Yee, 2012). IGF-1R has been associated with the cell's metastasizing potential: more than one decade ago it was demonstrated that inhibition of IGF-1R resulted in suppression of adhesion, invasion and metastasis of breast cancer cells, while that inhibition did not significantly suppress the growth of the primary tumor (Dunn et al, 1999). The IGF/IGF-1R downstream signaling pathways have recently been shown to be directly involved in the metastatic cascade in breast cancer cells and over-expression of IGF-1R has been associated with poor prognosis in patients with early breast cancer (Taunk et al, 2010). Despite data from human breast tumors that demonstrated IGF-1R over-expression and hyper-phosphorylation, the prognostic utility of IGF-1R expression in breast carcinomas is still debated, also due to technical issues (e.g. optimal cut-off point value defining receptor over-expression) (Law et al, 2008). Interestingly enough, it has been shown that IGF-1R expression varies among breast cancer subtypes and is correlated with either good (e.g. luminal cancers) or bad prognosis (e.g. HER2-enriched tumors) (Yerushalmi et al, 2012). More interestingly, IGF-dependent enhanced cellular proliferation has been documented in triple negative breast carcinomas, which can provide new targets for the so far limited therapeutic options (Davidson et al, 2011). Although receptor activation has been observed, neither mutations nor amplification of the gene that encodes IGF-1R have been described. However, it has been shown that certain IGF-1R single nucleotide polymorphisms (SNPs) might be useful predictive factors regarding recurrence of estrogen receptor positive breast cancer patients treated with endocrine agents (Winder et al, 2011).

Several studies indicate a role for IGF-1 in the development of breast cancer. High levels of total IGF-1 in plasma have been reported to be a risk factor for breast cancer in premenopausal women: these findings are supported by a small study reporting that a combination of low plasma IGFBP-3 and high IGF-1 increases the risk of ductal carcinoma *in situ* in pre-menopausal women (Helle, 2004). Significantly higher serum IGF-1 levels have been found in breast cancer patients compared to healthy controls. However, these results are not consistent, and a small recent study did not detect any differences regarding serum IGF-1, while IGBP-3 was decreased in cancer patients. Another report has shown that the IGF-1/IGFBP-3 ratio, which is used as an index of IGF-1 bioavailability, is elevated in pre-menopausal breast cancer patients compared to controls. While plasma IGF-1 levels may be important for the development of breast cancer, the presence of manifest breast cancer disease also influences the IGF system. A number of patients with advanced breast cancer have increased plasma IGFBP-3 protease activity which correlates with clinical stage and alterations in tumour burden due to therapy. Increased IGFBP-3 protease activity is also observed in other cancers and other serious conditions. The occurrence of elevated IGFBP-3 protease activity in a variety of pathological conditions probably reflects a non-specific phenomenon: anyway, elevated IGFBP-3

protease activity may theoretically increase the delivery of IGF-1 to the tumour, but this condition is also associated with lower plasma levels of IGF-1 and -2 (Martin and Baxter, 2011).

To assess the role played by IGF-2 in breast cancer, female transgenic mice were engineered to exhibit enhanced IGF-2 expression in the mammary gland and as a result they displayed an increased incidence of aggressive, metastatic, mammary tumours, implicating chronic IGF-2 signaling as a tumorigenic stimulus (Pravtcheva and Wise 1998). As predicted, when these animals were crossed with transgenic mice overexpressing IGF-2R, their offspring exhibited a significant delay in the onset of mammary tumour formation and reduced tumour burden (Wise and Pravtcheva 2006). Biallelic IGF-2 expression has been observed in human breast cancer samples: one study reported LOI in 67% of benign lesions and 60% of malignant lesions, whereas all control samples displayed normal IGF-2 imprinting (McCann et al. 1996). However, only three benign and five malignant tissue samples were analyzed, so these data must be interpreted with caution. ProIGF-2 has been shown to promote the survival of the MCF7 breast cancer cell line by activating PI3K/Akt signalling and upregulating the expression of the antiapoptotic proteins Bcl-2 and Bcl-XL (Singh et al. 2008). Mature IGF-2 can also promote breast cancer progression by activating ER-α and ER-β in the absence of estrogen. In breast cancer cells, IGF-2 binding to IGF-1R and IR-A was found to induce translocation of ERα and ERβ to the mitochondria, facilitating activation of cell survival pathways (Richardson et al. 2011).

The Insulin/IGF system as prognostic factor

A topic of increasing interest concerns the influence of IGFs and insulin on cancer prognosis, as distinct from cancer risk. Available evidence suggests that hyperinsulinaemia is associated with worse cancer outcome, whereas IGF-1 levels are less important as prognostic factors (Goodwin et al, 2002; Giovannucci et al, 2000). The biological basis for the apparently stronger relationship between insulin levels, as opposed to IGF-1 levels, and cancer is under investigation. One possibility is that the level of IR may be higher than that of IGF-1R in established cancers, whereas the reverse may be the case in at-risk but untransformed epithelial cells. It is also plausible that the levels of circulating IGF-1 or IGF-2 fail to reflect significant local effects of autocrine or paracrine production of these ligands by aggressive cancers (Pollak, 2009). Studies are not consistent regarding this issue. One study reported that expression of IGF-1R is associated with a better relapse-free survival compared to lack of expression. However, others found that expression of IGF-1R was associated with a poor prognosis in the subgroup of ER-negative cancers, or to be without prognostic importance. Increased level of IGF-1R expression in breast cancer specimens was found to inhibit apoptosis, and was associated with an increased risk of relapse after radiation therapy (Helle, 2004). IRS-1 levels, which is phosphorylated after IGF-1R activation, was found to predict worse disease-free survival in small tumours. However, there are also data which indicate that expression of IGF-1R and IRS-1 is decreased in poorly differentiated tumours. Moreover, high levels of immunoreactive IGFBP-3 were found in tumours with poor prognostic features (ER and PR negativity, high S phase, and aneuploidy). Other investigators found that high levels of immunoreactive IGFBP-3 in tumours were associated

with an increased risk of death (Helle, 2004).

The Insulin/IGF system as therapeutic target

The fact that insulin is mitogenic for neoplastic cells in culture was known before the development of the paradigm that targeting peptide growth factor receptors represents a useful strategy for cancer drug development. These provocative results, however, were not pursued with regards to their potential clinical relevance. To understand why, it is necessary to recognize that the general notion of targeting peptide growth factor receptors was not developed at the time. Even if it had existed as a theoretical concept, there were no practical pharmacological strategies available, as there were no examples of drugs that inhibited receptor tyrosine kinase activity, and the use of anti-receptor antibodies as drugs had not been demonstrated yet (Pollak, 2008). However, by the late 1990s, the general interest in these receptors in the context of neoplasia increased, due to increasing epidemiologic evidence for a relationship between circulating levels of IGF-1 and cancer risk, and also because of increasing laboratory evidence for a role for IGF-1 receptors in neoplastic growth. In addition, attention was given to the considerable amount of circumstantial evidence linking insulin and IGF physiology to neoplasia (Toretsky and Helman, 1996; Belfiore et al, 2009). At the same time, the progresses in drug development lead to the introduction of those new classes of compounds (like the mentioned anti-receptor antibodies and RTKi) nowadays used in targeted therapies.

The IGF axis has thus become an increasingly attractive target for cancer research. Progress has been substantial in recent years, and the field has moved to more sophisticated models and clinical trials. Targeted strategies include, on one hand, a reduction of ligand levels or bioactivity, and, on the other hand, inhibition of receptor function using receptor-specific antibodies or small-molecule tyrosine kinase inhibitors. In contrast to the history of early drug development for molecular targets such as ERBB2, many different drug candidates that target the IGF-1R are being evaluated simultaneously in dozens of ongoing clinical trials (Pollak, 2009). Nine mAbs and six small molecules have been tested across approximately 150 phase I to III trials in 16 solid tumor types and certain hematologic malignancies (i.e. multiple myeloma and leukemia). Objective response to IGF-1R–targeting mAb monotherapy is rare, with notable occurrences in approximately 10% of patients with various sarcomas, in particular the Ewing subset. Objective, and in some cases durable, responses have been reported in phase I and phase II studies of mAbs that target IGF-1R (i.e. Figitumumab and Ganitumumab) in adults and children with heavily pretreated sarcomas. Nevertheless, disease stabilization, defined as lack of objective response or progressive disease at 12 weeks, remains the major efficacy signal (Olmos et al, 2011). IGF-targeting agents are generally expected to show their full potential by augmenting the efficacy of endocrine, cytotoxic, or other targeted therapies, possibly by mitigating the development of resistance to otherwise effective interventions. Although combinations of large or small IGF-targeting molecules with various standard-of-care therapies have

generally shown a reasonable toxicity profile, this strategy has yet to translate preclinical promise into therapeutic efficacy. Few of the IGF-1R targeting antibodies under investigation have progressed to or completed phase III testing, facing various problems (Gao et al, 2012). For examples, as recently as 2010, 2 phase III clinical trials were testing Figitumumab (CP-751871) globally, one in conjunction with paclitaxel plus carboplatin and another with Erlotinib. Both of these pivotal trials were discontinued after data showed an increase in serious adverse events (Jassem et al, 2010).

Activators of AMP-activated protein kinase (AMPK), such as metformin, are also being studied not only because they lower the amounts of circulating insulin, but also because there is evidence that they act as anti-proliferative agents by reducing signalling downstream of IR and IGF1R (Pollak, 2009).

Classes of inhibitors

Ligand-targeting approaches

First-generation strategies that included the use of somatostatin analogues to reduce circulating IGF-1 levels were unsuccessful. One of the largest clinical trials using this approach fortunately included a translational science component that showed that the desired suppression of ligand levels was not achieved, so the negative results represent a failure of a particular strategy, rather than evidence that the target is unimportant. Other approaches, such as ligand specific antibodies or growth-hormone antagonists show interesting preclinical potential (Pollak, 2009). MEDI-573 is currently the only mAb in clinical testing that exerts its effects by neutralizing both IGF-1 and IGF-2. Distinct from all the IGF-1R–targeting mAbs under development, MEDI-573 specifically inhibits IGF signaling through IGF-1R and IR-A, as well as through their hybrid receptors. The ongoing phase I clinical trial has shown stabilization of disease in 7 of 16 patients, most of whom had chemoresistant cancers. Importantly, preliminary data strongly suggest that MEDI-573 might achieve this result without inducing hyperglycemia (Menefee et al, 2010). If confirmed, this observation would be consistent with expectations about ligand- versus receptor- based targeting of the IGF axis, specifically with respect to the clinical consequences of sparing IR-B and its various hybrid receptors, each of which has potential to alter glucose metabolism via interactions with insulin (Gao et al, 2012).

Receptor-specific antibodies

Many receptor-specific antibodies have been studied preclinically, and several are being evaluated in clinical trials. To date, the largest clinical experience has been with the Pfizer antibody CP-751871 (Figitumumab). In general, toxicity has been acceptable, and early clinical results have not only revealed activity in terms of pharmacodynamic endpoints, but have also suggested that administration of the antibody together with chemotherapy significantly improves the response rate in patients with non-small-cell lung cancer. The most recent available update showed the largest improvement was in squamous cancers (response rate to chemotherapy alone 41%; with antibody 72%), but squamous lung cancers were noted to express higher levels of IGF-1R than other histological types (Gualberto et al, 2008); ongoing research will reveal whether this early result is confirmed in phase III clinical trial

studies, and if it affects survival endpoints.

Additional IGF-1R specific antibodies have been developed. Those for which early clinical trial data have been reported include AMG-479 (Ganitumumab, Amgen), AVE-1642 (Sanofi-Aventis), IMC-A12 (Cixutumumab, Imclone), MK-0646 (Dalotuzumab, Merck) and R1507 (Roche) (Pollak, 2009). Although these antibodies differ with respect to IgG subclass and serum half-life, they share many similarities. These include a generally favourable toxicity profile without dose-limiting toxicity and disease stabilization or objective response in a minority of patients in phase I single-agent clinical trials. Several of the antibodies have induced objective responses in metastatic, chemotherapy-refractory Ewing sarcoma, although it is clear that not all patients with this disease respond in a similar manner. Initial evaluation of Dalotuzumab included pharmacodynamic studies on neoplastic tissue, which revealed reduction of phospho-Akt and phospho-s6 kinase, both of which function downstream of the receptor, as well as downregulation of receptor levels and reduction in proliferation. Different IGF-1R specific antibodies are now being evaluated in phase II clinical trials for many oncological indications in various combinations with approved agents. As already reported, 2 phase III clinical trials were testing Figitumumab (CP-751871) globally, one in conjunction with paclitaxel plus carboplatin and another with Erlotinib. Both of these pivotal ADVancing IGF-IR in Oncology (ADVIGO) trials were discontinued after data showed an increase in serious adverse events, including mortality, and a low likelihood of meeting the primary endpoint of improved overall survival (Jassem et al, 2010). A compensatory increase in the circulating concentrations of growth hormone and IGF-1 occurs on administration of IGF1R-specific antibodies. This was predicted and is reminiscent of the rise in estrogen levels that results from treatment with estrogen-targeting drugs in premenopausal patients with breast cancer. The hyperglycaemia encountered occasionally with IGF-1R specific antibody treatment probably reflects the insulin resistance that is induced by the high levels of growth hormone (rather than any interaction between the antibody and IR). This possibility is supported by the modest treatment-induced hyperinsulinaemia that has been observed in patients, as well as by correction of hyperglycaemia by the use of metformin. There is no evidence to date that the increase in IGF-1 level can overcome the blocking effect of IGF-1R specific antibodies (Pollak, 2009).

Receptor kinase inhibitors

Several tyrosine kinase inhibitors that inhibit IGF-1R and IR have been developed and found to be active in preclinical models, and some are currently being evaluated in phase I clinical trials: BMS-754807 (Selleckbio), NVP-AEW541 (Novartis) and OSI-906 (Linsitinib, Selleckbio) are promising examples of small molecule inhibitors. Details of relative *in vivo* inhibitory activity for the IR and IGF-1R in different tissues remain unclear. As these compounds were initially expected to also inhibit the function of IR, the possibility of more serious metabolic toxicity than that seen with the IGF-1R specific antibodies required careful investigation. If these small molecules penetrate the blood–brain barrier there is also a theoretical possibility of neurotoxicity (especially with long-term exposure), as IGF-1 signalling has neuroprotective activity in the brain. However, it is possible that these agents will be more potent antineoplastics, if indeed IR present on malignant cells has an important role in neoplastic behaviour. In agreement with this possibility, a model of IR-A mediated resistance to IGF-

1R targeting has been described (Buck et al, 2010). Even if most TKIs show limited selectivity of IGF-1R over IR *in vitro* or *in vivo*, due to the high degree of homology of the intracellular β subunits of the two receptors, new and more selective compounds have been developed and are being tested. Ongoing clinical trials are trying to clarify the relative advantages and disadvantages of receptor-specific antibody and tyrosine kinase inhibitor approaches in terms of both efficacy and adverse effects.

NVP-AEW541

NVP-AEW541 is an optimized IGF-1R kinase inhibitor that selectively distinguishes between the native IGF-1R and the closely related IR (Garcia-Echeverria, 2004). Small molecules belonging to the pyrrolo[2,3-d]pyrimidine class were identified in Novartis in a high-throughput screening of a compound archive as inhibitors of the IGF-1R *in vitro* kinase activity. This compound class was subjected to a medicinal chemistry program aimed at optimizing the potency and selectivity toward the IGF-1R kinase, as well as their drug-like properties. NVP-AEW541 is a representative example of such an optimization process, whose potency and selectivity was first assessed in a series of *in vitro* kinase assays using different recombinant kinase domains or purified kinases and synthetic peptide substrates. The compound was found to inhibit the *in vitro* kinase activity of the recombinant IGF-1R kinase with an IC50 value of 0.15 μM and to be equipotent against the recombinant IR kinase domain. NVP-AEW541 was confirmed to be active toward the IGF-1R kinase (IC 0.086 μM) and to be 27-fold more potent toward the native IGF-1R, as compared to the structurally related native IR. The selectivity observed at the cellular level indicates that, despite the 84% identity of the two receptors kinase domains, there are conformational differences between the native forms of these receptor tyrosine kinases that are not recapitulated by the recombinant kinase domains used in the *in vitro* kinase assay. Thus, at the cellular level, NVP-AEW541 is highly selective against the IGF-1R, as compared to both the IR and other tyrosine kinases. The compound was found to fulfill all the key characteristics expected from an IGF-1R inhibitor. It effectively inhibits the survival function ascribed to IGF signaling and prevents the ability of transformed cells to grow in an anchorage-independent manner, both events occurring at concentrations that are consistent with its capacity to inhibit IGF-1R autophosphorylation. Despite the cellular selectivity achieved toward the IGF-1R, as compared to the IR, there remains a potential risk of such a compound interfering with glucose metabolism. In fact, the two receptors can form heterodimers, a situation where a selective IGF-1R inhibitor would prevent cross-phosphorylation and activation of the associated IR heterodimeric partner. Alternatively, the compound could in principle accumulate at concentrations high enough to also inhibit the IR. To assess this potential risk, plasma glucose and plasma insulin were monitored in mice treated with efficacious doses (50 mg/kg, bid, p.o.) of NVP-AEW541. No significant differences were observed when the plasma glucose and insulin levels of vehicle and compound treated animals were compared after 10 days of treatment. Thus, NVP-AEW541 specifically targets the IGF-1R and represents a novel, potential therapeutic strategy for the treatment of tumor types for which IGF-1R-mediated signaling is a required driving/survival function (Garcia-Echeverria, 2004).

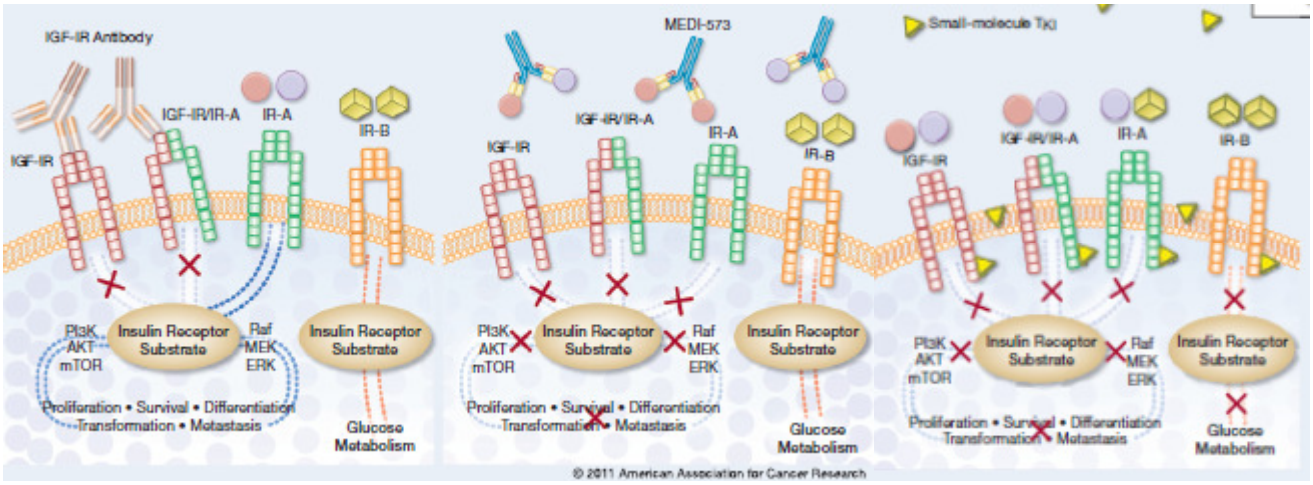

Figure 4 IGF-1R specific-antiboby targeted therapy, IGF ligand-neutralizing antibody targeted therapy and IGF-1R kinase activity inhibition. *(Cancer Res, 2012)*

Metformin and AMPK activators

The biguanide metformin is commonly prescribed in the treatment of type II diabetes because it lowers both glucose and insulin levels. Population studies provided preliminary evidence that it might have anti-neoplastic or chemopreventive activity, thereby motivating further laboratory investigations. Although often referred to as an insulin sensitizer because it lowers insulin levels, recent evidence suggests that the key mechanism of action of metformin is as an activator of the AMPK–LKB1 pathway (Evans et al, 2005). In the liver, this results in inhibition of gluconeogenesis and hepatic glucose output, which in turn reduces circulating glucose level, resulting in a secondary decrease in insulin level. In transformed epithelial cells, metformin, similarly to other AMPK activators, inhibits rather than increases insulin-stimulated proliferation (Zakikhani et al, 2006). Therefore, metformin has two properties of potential oncological relevance: it reduces systemic insulin levels and has direct AMPK–LKB1-dependent growth-inhibitory action. Reduction of systematic insulin levels would be predicted to be of greatest benefit in the important subset of cancer patients who are hyperinsulinaemic and, hence, whose tumours may be growth-stimulated by insulin. The anti-neoplastic actions of metformin (and other AMPK activators) have been modelled in laboratory studies, and found to be more complex than would be expected if they acted only as insulin-lowering agents (Pollak, 2009). Although most models using AMPK activators show anti-proliferative effects, AMPK activation could in certain contexts also enhance cellular survival under stress, a topic which requires further study before large-scale clinical trials can be launched. Further research is required to clarify the extent to which clinically relevant doses of metformin act to activate AMPK in neoplastic tissue as compared with liver tissue, since at the moment there are important knowledge gaps in its pharmacokinetics. However, this remains an important area of investigation, given preliminary evidence regarding metformin from population and clinical studies together with datasets linking hyperinsulinaemia to adverse cancer outcome (Pollak, 2009).

Combinations

Although there have been multiple documented examples of single-agent activity of IGF-1R specific antibodies in Ewing sarcoma and other solid tumours in phase I clinical trial studies, it is commonly assumed, based on the experience with other receptor kinase inhibitors, that combination therapies will have an important role in treatment. This view is consistent with evidence that IGF-1R activation tends to reduce responsiveness to many approved antineoplastic therapies. A few combinations represent obvious priorities (Pollak, 2012). Many targeted therapies are routinely used in combination with other agents, and most trials with agents that target the IGF system have involved combinations selected not on the basis of a specific synergy demonstrated preclinically, but rather on a pragmatic approach involving the addition of a drug candidate to a current standard regiment that has some existing activity but needs to improve its efficacy. Early experience suggests that combining cytotoxics with IGF-1R blockade might be useful. In addition, there is evidence that IR and IGF-1R can have a role in conferring resistance to rapamycin and its analogues; therefore there is interest in combining these with IGF-1R targeting agents. Similarly, there is considerable evidence that IGF-1R mediated signalling confers resistance to therapies that target EGF receptor family members, so simultaneous inhibition of these receptor families is of interest. Combined inhibition of steroid signal transduction and IGF-1R is also proposed for breast and prostate cancer, based on preclinical models. The combinations of a growth-hormone receptor antagonist or metformin with IGF-1R specific antibody would be of interest as this might reduce the GH-induced insulin resistance, hyperglycaemia and hyperinsulinaemia that are associated with IGF-1R targeting, thereby improving tolerability and/or efficacy. Finally, the possibility that IGF-1R inhibition might enhance radiotherapy outcomes is being examined (Pollak, 2009).

IGFs as Predictive Biomarkers

A growing clinical database suggests that efficacy and toxicity of all the compounds targeting the IGF system may be predicted by quantifiable factors such as tumor IGF-1R or circulating IGF ligand levels. Target expression levels such as these, if validated, would serve as relatively straightforward predictive biomarkers for identifying patient subpopulations likely to respond to IGF targeted therapies (Gao et al, 2012). For IGF-1R antibodies, elevated tumor IGF-1R expression and circulating IGF-1 have both been shown to correlate to some degree with responses in early clinical trials. As stated before, data from the phase II study testing the IGF-1R specific mAb Figitumumab in combination with paclitaxel plus carboplatin in NSCLC showed a higher objective response rate in the subset of patients with squamous cell carcinoma histology. Despite the limited sample size, these data are particularly compelling in light of biomarker analysis showing the highest IGF-1R expression in the squamous subtype. In addition, a higher response rate to the combination of paclitaxel plus carboplatin and Figitumumab was observed in epithelial-to-mesenchymal transitional tumors (71%), defined by intermediate E-cadherin and high IRS-1 expression, compared with those in the mesenchymal- like subset (32%), defined by low expression of both E-cadherin and IRS-1. By contrast, sample analysis showed high plasma levels of free IGF-1 and vimentin as predictive of

clinical benefit in the adenocarcinoma subtype. Ancillary data from the NSCLC study of Figitumumab showed improved PFS among patients with high pretreatment-free IGF-1 levels compared with patients with low pretreatment-free IGF-1 levels, pointing to the potential role of this ligand as a predictive marker (Gualberto et al, 2011). These intriguing data are indirectly supported by interim results from a phase I to II study of Figitumumab in 31 patients with relapsed sarcoma, which suggested that pretreatment plasma levels of IGF-1>110 ng/mL conferred a significant treatment advantage compared with lower levels. Overall, these data suggest the value of evaluating circulating IGF-1 as a possible predictive marker in studies of IGF-targeting agents and opportunities for enriching future study populations (Olmos et al, 2011). A growing body of nonclinical and clinical data provide valuable lessons for the development of ligand-based approaches (such as MEDI-573) in terms of patient selection. In particular, they provide a compelling rationale for a priori identification of patients with tumors that overexpress IR-A and IGF-2 in addition to IGF-1R, which may prove a critical determinant of clinical response to monotherapy or combination regimens in molecularly defined subpopulations. This hypothesis has been supported by preclinical studies in which high levels of IR expression and elevated mRNA levels of IR-A compared with IR-B have been found in most of the cancer cell lines tested (Garofalo et al, 2011; Gao et al, 2011). Other molecular markers may also predict tumor response to IGF-targeting compounds. IRS-1 expression levels have been positively correlated with sensitivity of preclinical models to IGF-1R targeting (Kurmasheva et al, 2009). Increased VEGF production by cancer cells following rapamycin treatment has been shown to correlate with synergistic responses to rapamycin and IGF-1R antibody combined therapy in certain sarcoma tumor models . Among the cancers associated with dysregulation of the IGF axis, increased IGF-2/IR-A signaling has been best documented in breast cancer, in which conventional criteria (e.g., ER and/or PR and/or HER2 status, stage, grade, luminal type A vs. type B) together with IR-A signatures may enable identification of and enrichment for subpopulations likely to respond to dual targeting of IGF-1R and IR-A (Belfiore and Frasca, 2008). MEDI-573 has been shown to inhibit IGF-induced proliferation of cells expressing IGF-1R or IR-A, either together or alone, pointing to a distinct theoretical advantage over IGF-1R–targeting antibodies in selected populations. A global clinical trial is currently testing whether MEDI-573 in conjunction with an aromatase inhibitor enhances therapeutic outcomes in patients with advanced ER-positive, HER2-negative breast cancer. Whether these markers achieve the level of validation required to predict for sensitivity to different types of IGF-targeted agents has yet to be confirmed by clinical testing (Gao et al, 2012).

Insulin/IGF system and chemoresistance

The observation that a range of RTKs can function to drive tumorigenesis has revolutionized anticancer drug discovery and development efforts in recent decades. However, tumor cells exhibit a high degree of signaling plasticity, which can contribute to adaptive survival in the presence of RTK inhibitors, and identifying the mechanisms of acquired resistance to these agents is a major goal toward individualizing their use in the clinic. Multiple RTKs can be activated simultaneously within a single cell, and cross talk can exist between them. Cross talk between EGFR and either IGF-1R or

MET can provide adaptive survival for tumor cells when EGFR is targeted individually. Preclinical data highlighting reciprocity for these receptor pairs have spurred the evaluation of combinatorial RTK targeting in the clinic for EGFR inhibitors (Buck et al, 2010). As already reported, there is growing support for IR as a mitogenic driver for tumor cells, and there are several examples in which IGF-1R or IR can compensate for the inhibition of the other in nontransformed cells: indeed, the activity of IGF-2 on IR was first discovered in studying mouse development, where it was found that IR, activated by IGF-2, can compensate for IGF-1R disruption to rescue embryonic growth: other studies have later described enhanced signaling by insulin when IGF-1R is disrupted in tumor cells (Zhang et al, 2007). Of particular significance is the observation that elevated phosphorylation of both IGF-1R and IR has been observed in many human tumor cell lines, thus showing that IGF-1R/IR cross talk is another means exploited by tumor cells to maintain activation of cell survival pathways when IGF-1R is specifically targeted. It is demonstrated that treatment with either insulin or IGF-2 can maintain activation of the AKT pathway when IGF-1R is selectively targeted: insulin concentrations corresponding to mild hyperinsulinemia can promote an increase in phosphorylation of IR and AKT, independent of IGF-1R. Collectively, these data support the approach of cotargeting IGF-1R and IR to deliver enhanced and sustained antitumor activity for cancers that rely on signaling through both of these receptors. Moreover, because resistance to IGF-1R–specific antibodies may emerge via increased IR signaling, dual targeting of IGF-1R and IR by TKIs may be effective following failure of an anti IGF-1R antibody. Identifying biomarkers associated with the activation of IGF-1R and IR will be important for optimally evaluating emerging therapeutic agents (Buck et al, 2010).

Insulin, IGFs and their role in the resistance to breast cancer therapy

Although significant improvement has been achieved and more effective agents are currently available in the treatment arsenal against breast cancer, the vast majority of patients will develop resistance. The IGF pathway has been reported to play an important role in the development of resistance against several DNA-damaging agents, as IGFs protect cancer cells from apoptotic cell death via IGF-1R-mediated activation of the PI-3K/AKT pathway, as well as rescue from drug-induced cytostasis through activation of MAPK pathway signals. The IGF system transduces signals that may also confer a multidrug resistance (MDR) phenotype to cancer cells, by the induction of MDR-related genes such as Mdr-1 (with increased expression of its product, the p-glycoprotein drug efflux pump) and the manganese superoxide dismutase (MnSOD) (Guo et al, 2003). Indeed, IGF-1R signaling has been associated with resistance and increased survival of breast cancer cells treated with 5-fluorouracil, methotrexate, or camptothecin via IGF-1R inhibition of apoptosis (Karamouzis and Papavassiliou, 2012). Inhibition of IGF-dependent signaling with mAbs or TKIs results in enhancement of the cytotoxic effect of several chemotherapeutics, including gemcitabine, irinotecan, etoposide, carboplatin, adriamycin, ifosfamide, navelbine, 5-fluorouracil and vincristine, both *in vitro* and *in vivo* (Tao et al, 2007). Moreover, resistance to hormonal therapy against breast cancer may be partially explained by cross-talk between ER and IGF pathways as well as through the modulation of downstream pathways. One possible mechanism by which breast cancer cells escape tamoxifen-

induced apoptosis may be the activation of the AKT pathway via IGF-mediated signaling, which leads to phosphorylation of ER at Ser-167 and subsequent ligand-independent activation of ER (Campbell, 2001). IRS-1 is the major adaptor protein of IGF-1R/IR but also the mediator of cross-reaction with other membrane associated molecular networks that are responsible for the development of hormone-resistance in breast carcinomas. Additionally, expression levels of IGFBPs were found to be predictive factors for response to hormonal agents. For example, IGFBP-2 mRNA and protein levels have been reported to be over-expressed in cell lines resistant to the anti-estrogens fulvestrant and tamoxifen (Juncker-Jensen, 2006).

The IGF-1R pathway has also been implicated in resistance to radiotherapy (RT). It is believed that it exerts its activity, in part, by modulating ataxia telangiectasia mutated (ATM) function, which controls the response of the cell to DNA damage induced by RT via triggering cell-cycle arrest and apoptosis, as well as DNA repair, and it has been documented that inhibition of IGF-1R signaling enhances sensitivity of breast cancer to RT, thus representing a way to enhance the efficacy of RT in cases of radioresistant tumors (Karamouzis and Papavassiliou, 2012).

Crosstalk and combination therapy in breast cancer

IGF-1R Monoclonal Antibodies and mTOR Inhibitors

mTOR inhibitors affect the S6K1-IRS1 negative feedback loop and result in enhanced PI3K- AKT activation through IGF-1R signaling: if this pathway represents a resistance mechanism for the mTOR inhibitors, then co-targeting IGF-1R and mTOR might result in enhanced clinical benefit over mTOR inhibitor monotherapy. Studies showed that dual inhibition of IGF-1R and mTOR improved antitumor activity in vitro and in breast cancer and other cancer patient tumor samples (Wan et al, 2007). Currently, Merck is determining the benefits of IGF-1R monoclonal antibody (Dalotuzumab) and mTOR inhibitor (Ridaforolimus) combination therapy in breast cancer patients with ER-positive tumors (NCT01220570, NCT01234857). Amgen is evaluating the clinical benefits of combining Ganitumumab with Everolimus in patients having advanced cancers (NCT01061788, NCT01122199). The results of these clinical trials are expected to reveal the benefits of co-targeting IGF-1R and mTOR. It is worth noting that drugs acting as dual inhibitors of PI3K and mTOR, such as NVP-BEZ235, also demonstrated improved antitumor efficacy compared to mTOR inhibitors alone (Yang and Yee, 2012).

Targeting IGF-1R/IR and Estrogen Receptor-α (ERα)

Cross talk between IGF/insulin system and ER signaling pathway is well established. IGF/insulin signaling activates ERα via PI3K/AKT and/or MAPK pathways respectively by phosphorylating ERα Ser167 and/or ERα Ser118. Estrogen increases expression of several key genes in the IGF signaling pathway including IGF-2, IGF-1R, and IRS-1, while decreasing expression of other genes, such as IGFBP-3 and IGF-2R. Thus, the overall effect of estrogen on the IGF/insulin system is to positively regulate signaling. Acquired resistance to anti-estrogen therapies is an important clinical problem. Since ERα may function together with IGF-1R signaling to enhance cell survival, targeting both pathways may have value (Yang and Yee, 2012). More recently, microarray data suggest that a gene

signature (co-regulated by IGF-1 and estrogen) correlated with poor prognosis in human breast cancer, which also implies dual inhibition of IGF-1R and ER pathway may be necessary in certain breast cancer subtypes (Casa et al, 2012). However, the clinical trials using the combination therapy for patients with endocrine-resistant breast cancer have been disappointing. In these trials, most women had already developed resistance to anti-ER therapies. In most of the reported trials, the anti IGF-1R strategies were tested as the second or third line endocrine therapies (Kaufman et al, 2010).

It has been recently shown that tamoxifen-resistant (TamR) cells and tumors lose expression of IGF-1R while maintaining IR expression. These findings suggest IGF-1R is a poor target in TamR tumors and IR might be an alternative option in treating TamR breast cancer (Fagan et al, 2012). Patients with TamR tumors also show loss of IGF-1R at the time of progression on tamoxifen. Thus, endocrine resistant patients might not be the best candidates for anti IGF-1R therapies. However, there are other ways to target IGF-1R and IR with small molecule TKIs, ligand neutralizing antibodies, or even growth hormone receptor antagonists, so the final word about the clinical relevance of these cross talk pathways is not yet settled (Yang and Yee, 2012).

Targeting IGF-1R and Human Epidermal Growth Factor Receptor (EGFR)

About 30 % of the patients with invasive breast cancers have amplification or overexpression of HER2, which is associated with poor prognosis breast cancer. Trastuzumab is a recombinant humanized monoclonal antibody that targets the extracellular domain of HER2: initially it showed outstanding anti-tumor efficacy in patients with HER2 positive breast cancer in combination with cytotoxic chemotherapy. However, not all patients benefit from this regimen and in advanced breast cancer, resistance develops in about one year (Esteva, 2002). IGF-1R and HER2 are reported to form heterodimers in Trastuzumab-resistant breast cells. Furthermore, IGF-1 was shown to activate HER2 signaling in Trastuzumab-resistant breast cancer cells but not parental cells. Inhibition of IGF signaling resulted in restoration of Trastuzumab sensitivity to resistant cells (Browne et al, 2011). These preclinical findings led to several clinical trials aimed at evaluating the benefits of co-targeting IGF-1R and HER2 in Trastuzumab-resistant breast cancer patients (NCT01479179, NCT00788333, NCT00684983, and NCT01111825) (Yang and Yee, 2012).

Targeting IGF/Insulin Signaling and Chemotherapy

Combining either IGF-1R monoclonal antibodies or IGF-1R TKI could enhance doxorubicin drug efficacy (Zeng, 2012). It is demonstrated that giving cytotoxic chemotherapy prior to or concurrently with IGF-1R inhibitors resulted in a better tumor response. In contrast, IGF-1R inhibition prior to cytotoxic chemotherapy did not improve the benefits of doxorubicin and may represent an interference pathway between cytotoxic chemotherapy and IGF-1R inhibitors. These results suggest a combination of IGF-1R blockade and chemotherapy works in a sequence-dependent fashion (Yang and Yee, 2012).

IGF/Insulin System Therapy and Metformin

As noted above, IGF-1R inhibition is predicted and proven to result in compensatory upregulation of circulating IGFs and insulin. These effects may cause hyperinsulinemia and be clinically manifest

as metabolic syndrome or type-2 diabetes. Therefore, combining insulin sensitizing drugs to decrease serum levels of insulin with metformin might be necessary to attenuate the metabolic effects of the anti IGF-1R/IR drugs. The I-SPY2 trial of neoadjuvant breast cancer therapy is testing the therapeutic value of combining Ganitumumab, metformin and paclitaxel. The metformin will help to manage any acquired insulin resistance induced by Ganitumumab (Barker et al, 2009). Metformin also reduces reactive oxygen species in mitochondria, which potentially would be important to inhibit tumorigenesis independent of the effects on glucose metabolism. Thus metformin combined with IGF-1R blockades may not only attenuate the drug-induced hyperglycemia and hyperinsulinemia, but may also exhibit antitumor efficacy (Yang and Yee, 2012).

Hypoxia

Oxygen is essential for eukaryotic life and is inextricably linked to the evolution of multicellular organisms. Proper cellular response to changes in oxygen tension during normal development or pathological processes, such as cardiovascular disease and cancer, is ultimately regulated by the transcription factor, hypoxia-inducible factor (HIF). Over the past decade, unprecedented molecular insight has been gained into the mammalian oxygen-sensing pathway involving the canonical oxygen-dependent prolyl-hydroxylase domain-containing enzyme (PHD)–von Hippel-Lindau tumour suppressor protein (pVHL) axis and its connection to cellular metabolism (Greer et al, 2012).

Hypoxia is a state of reduced oxygen pressure below a critical threshold, which restricts the function of organs, tissues and cells (Hockel M and Vaupel, 2001). Normal oxygen partial pressure (pO_2) levels range from 150 mm Hg in the upper airway to 5 mm Hg in the retina, and a pO_2 <40 mm Hg in arterial blood constitutes hypoxia. Hypoxia can be caused by a reduction in oxygen supply, for example at increased altitude or by localized ischemia caused by the disruption of blood flow to a given area. Many solid tumors also contain hypoxic regions (pO_2 <5 mm Hg), owing to the inability of the local vasculature to supply sufficient oxygen to the rapidly growing tumor because of the rate of growth of the tumour itself and the severe structural abnormality of tumor microvessels (Semenza, 2012). However, hypoxia also has an important and beneficial role in mammalian physiology; indeed, its presence is crucial for proper embryogenesis. At the cellular level, the response to hypoxia includes a switch from aerobic metabolism to anaerobic glycolysis and the expression of a variety of stress proteins regulating cell death or survival. Further adaptations that occur at the tissue level to increase oxygen delivery include the induction of erythropoiesis and angiogenesis (Koh et al, 2008).

Hypoxia Inducible Factors (HIFs)

Human cells require adequate supplies of O_2 on a continuous basis to use as the terminal electron acceptor in the process of mitochondrial respiration that generates ATP, which is used to power most biochemical reactions. Both the delivery and consumption of O_2 are regulated through the activity of hypoxia-inducible factors (HIFs) (Semenza, 2011). As cells proliferate, increased oxygen consumption results in hypoxia, which activates HIFs, leading to transcription of the VEGF gene, which encodes vascular endothelial growth factor, a secreted protein that stimulates angiogenesis and thereby increases oxygen delivery (Semenza, 2012).

Hypoxia-inducible factors are DNA-binding transcription factors that associate with specific nuclear cofactors under hypoxia to transactivate a myriad of genes to trigger various adaptive responses to compromised oxygen tension, mediating the primary transcriptional responses to hypoxic stress in normal and transformed cells (Greer et al, 2012). HIFs are basic helix-loop-helix–PER–ARNT–SIM (bHLH–PAS) proteins that form heterodimeric complexes that are composed of an O_2 -labile α-subunit (HIF-1α, HIF-2α or HIF-3α) and a stable β-subunit (HIF-1β; also known as ARNT). Together, these subunits bind hypoxia-responsive elements (HREs) that contain a conserved 5'-RCGTG-3' core sequence, thereby eliciting transactivation of downstream genes and the adaptive hypoxic response (Keith et al, 2012). Based on genome-wide chromatin immunoprecipitation combined with DNA sequencing or mRNA microarrays (ChIP-seq and ChIP-chip, respectively), the number of direct HIF target genes is currently greater than 800 (i.e. at least one in 30 of all human genes) (Semenza et al, 2012). HIFs also indirectly regulate gene expression by transactivating genes encoding microRNAs and chromatin-modifying enzymes (Schödel et al, 2011). Hypoxic HIF activity is controlled primarily through post-translational modification and stabilization of HIF-1α and HIF-2α subunits, so that HIFα protein levels and overall HIF transcriptional activity increase as cells become more hypoxic (Keith et al, 2012).

HIF-1α was first described by Semenza and colleagues in 1995, when it was shown to have a central role in mediating hypoxia-dependent transcriptional responses (Wang et al, 1995). In 1997, the identification by independent groups of HIF-2α - which was initially called endothelial PAS protein 1 (EPAS1), HIF-related factor (HRF), HIF1α-like factor (HLF) or member of PAS family 2 (MOP2)11 - indicated that HIF regulation was more complex than initially fathomed (Keith et al, 2012). Whereas HIF-1α seems to be expressed in nearly all cell types, RNA in situ hybridization of mouse embryos revealed that the expression of *Epas1* is more restricted and is particularly abundant in blood vessels. This observation led to the hypothesis that the primary role of HIF-2α is to modulate vascular endothelial cell function, an idea that is supported in part by the close correlation of the mRNA expression patterns of EPAS1 and vascular endothelial growth factor A (VEGF-A). A more complex view emerged as HIF-2α protein expression was identified in multiple cell types in hypoxic rat kidney, lung and colonic epithelia, as well as in hepatocytes, macrophages, muscle cells and astrocytes, indicating that both HIF-1α and HIF-2α are co-expressed in many cell types.

Most HIF transcriptional responses have been attributed to HIF-1α and HIF-2α; however, a third HIFα subunit (HIF-3α) has also been described. *HIF3A* mRNA is differentially spliced to produce multiple HIF-3α isoforms that either promote or inhibit the activity of other HIF complexes, although little is currently known about the impact of HIF-3α on tumour progression under hypoxic conditions (Heikkila et al, 2011). Similarly, ARNT2 (HIF-2β) has been identified and shown to regulate neuronal development and to exhibit overlapping activity with HIF-1β; however, its activity in human cancer cells has not been studied in depth (Hirose et al, 1996; Keith et al, 2011). Although it will be important to determine whether (and how) HIF-3α and ARNT2 affect HIF-mediated responses in cancers, the available evidence suggests that HIF-1α and HIF-2α (together referred to as HIFα hereafter) account for the vast majority of HIF-dependent effects on tumour growth and progression that have been described to date (Keith et al, 2012).

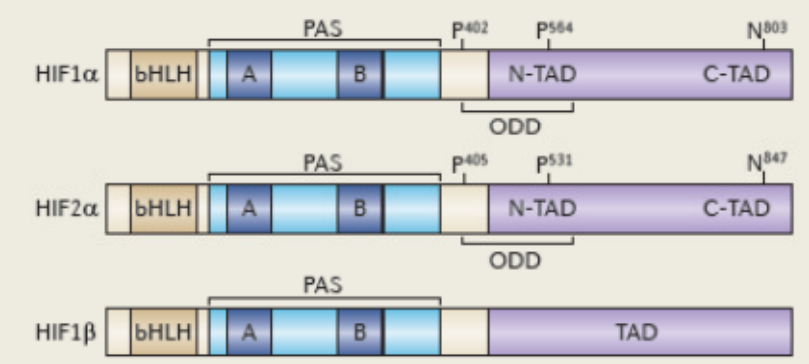

Figure 5 structure of HIF subunits *(Nature, 2012)*

The role of HIFα as transcription factors in the context of oxygen sensing and tumourigenesis has been extensively characterized. While the aforementioned role of HIFα in cell signaling regulation is considered the most important, emerging evidence has implicated a larger role for HIFα in cell biology.

Role of HIF in embryonic development

HIF1α has previously been shown to promote a metabolic switch to glycolysis in response to chronic hypoxia through the transcriptional regulation of key metabolic enzymes such as pyruvate dehydrogenase kinase-1 (PDK1) and lactate dehydrogenase A (LDHA) (Kim et al, 2006). HIF-1α drives a metabolic shift to glycolysis during transition of pluripotent embryonic stem cells (ESCs) to epiblast stem cells (EpiSCs) and maintains pluripotency in ESCs and HSCs (hematopoietic stem cells) through TGFb/Activin/Nodal signalling. These findings suggest that the high expression of HIF-1α and shift to glycolytic metabolism is not just a result of the hypoxic environment of the bone marrow, but is tightly regulated by haematopoietic stem-cell–associated transcription factors. Whether or not unique ESC-associated transcription factors regulate HIFα in ESC remains to be determined (Greer et al, 2012).

Role of HIF in immunity

Inflamed tissues are often hypoxic as a result of decreased perfusion, edema, vascular insult and/or influx of oxygen-consuming immune cells or pathogens (Nizet and Johnson, 2009). Thus, the role of HIFα in host immune responses and inflammation has become of particular interest: one example is its role in promoting differentiation of TH17 cells by several distinct mechanisms.

Role of HIF in Notch-dependent developmental pathways

HIFα can modulate human leukocyte differentiation via canonical and non-canonical pathways. However, HIFα can induce or suppress the differentiation of a multitude of cell types, including but not limited to adipocytes, medulloblastoma precursor cells, neuroblastoma cells, glioblastoma stem cells, and neural crest progenitors. A number of these reports have implicated Notch, a well-established mediator of cellular differentiation processes, as an important effector: an example of the interaction between HIFα and Notch in crystal cell development, which requires direct stabilization of endosomal Notch by HIFα (Greer et al, 2012).

HIF regulation

The induction of HIF-1 activity during hypoxia can be attributed to a variety of factors. HIF-1α is continuously transcribed and translated in hypoxia despite an overall decrease in global protein translation. Additionally, several mechanisms regulate the stability and activity of HIF-1α protein in an oxygen-dependent manner. Recent years have seen great advances in the field of HIF-1α regulation, providing a clearer understanding of established pathways and also introducing new and sometimes controversial findings involving novel regulators and mechanisms controlling HIF-1α levels. The most recent informations involving HIF-1 protein regulation revolve around different important aspects governing the availability of the HIF-1α subunit: its proteasomal degradation, a well-characterized field that has recently seen a wealth of new information, and its transcription as well as translation, two still developing and, at times, controversial fields (Koh et a, 2008).

Degradation

pVHL dependent pathway: ubiquitylation

To enable the rapid response to changes in oxygen levels, cells have evolved a highly sophisticated mechanism for both sensing and adapting to hypoxia. The oxygen-dependent regulation of the HIF-α subunit through its ubiquitin-proteasomal degradation von Hippel Lindau protein (pVHL) pathway has been well studied: under aerobic conditions, HIF-1α is hydroxylated by specific prolyl hydroxylases (PHD1, PHD2 and PHD3) at two conserved proline residues (Pro402 and Pro564) situated within its oxygen-dependent degradation (ODD) domain. This reaction requires oxygen, 2-oxoglutarate and ascorbate as cofactors. Under hypoxic conditions (<5% O_2), PHD activity is inhibited, resulting in HIF-1α stabilization. In addition to the enzymatic inhibition of the PHDs, hypoxia causes perturbations in the mitochondrial electron-transport chain, thus increasing the levels of

cytoplasmic reactive-oxygen species (ROS), which alters the oxidation state of Fe^{2+} (a cofactor for PHD activity) to Fe^{3+}, which cannot be utilized. This alteration inhibits PHD activity and promotes HIF-1α stabilization (Koh et al, 2008). Thus, disruption of mitochondrial function using either pharmacological or genetic inhibition or knockout of the mitochondrial electron-transport chain convincingly prevents HIF-1α stabilization during hypoxia (Simon, 2006). In addition, the essential role of mitochondria in HIF-1α regulation is highlighted by the exclusive enrichment of mitochondrial inhibitors from a library of >600000 diverse compounds by using a HIF-1 reporter assay (Lin et al, 2008). However, the role of ROS in hypoxia and HIF-1 regulation remains controversial, owing to discrepancies in different model systems, a lack of tools for accurate detection of ROS and variability in the severity and length of hypoxia applied. It is believed that future studies will provide a clearer role for the mitochondria in HIF-1α regulation. In addition to mitochondrial-dependent mechanisms, the PHDs are subject to regulation by other factors, including intracellular calcium concentrations and the seven in absentia homologs 1 and 2 (Siah1 and Siah2) E3 ubiquitin ligases (Simon, 2006). HIF-1α hydroxylation facilitates binding of pVHL to the HIF-1α ODD. pVHL forms the substrate-recognition module of an E3 ubiquitin ligase complex comprising elongin C, elongin B, cullin-2 and ring-box 1, which directs HIF-1α poly-ubiquitylation and proteasomal degradation. Recognition of HIF-1α by pVHL is further facilitated by HIF-1α acetylation at Lys532 by arrest-defective-1 (ARD1) N-acetyltransferase, which functions mainly under normoxic conditions. It should be noted, however, that the acetylation of HIF-1α by ARD1 and its importance have been disputed (Koh et al, 2008). The central role of pVHL in HIF-1α regulation is manifest in von Hippel Lindau (VHL) disease, where the inactivation of the VHL gene results in the development of highly vascularized tumors of the kidney, retina and central nervous system (Kaelin, 2007). In addition to pVHL, human double minute 2 (Hdm2), the E3 ligase that binds to and degrades the p53 tumor-suppressor protein, can also induce HIF-1α proteasomal degradation in an oxygen-independent manner via p53–HIF-1α binding (Ravi et al, 2000).

pVHL itself is subject to tight regulation by mechanisms that modulate its stability or its affinity for HIF-1α and other components of the pVHL E3 ubiquitin ligase complex. One such regulator is E2-endemic pemphigus foliaceus (EPF) ubiquitin carrier protein (UCP), a member of the E2 enzyme family: it can specifically ubiquitylate pVHL leading to its degradation. Hence, UCP overexpression causes the proteasomal-dependent degradation of pVHL, resulting in the accumulation of HIF-1α in normoxia (Jung et al, 2006). Other novel regulators of HIF-1α include osteosarcoma-9 (OS-9) and spermidine/spermine-N1-acetyltransferase (SSAT)2, which were both identified as HIF-1α binding partners in a yeast two-hybrid screen. OS-9 overexpression results in the marked reduction of HIF-1α protein levels under both normoxia and hypoxia by promoting pVHL-dependent degradation. OS-9 forms a ternary complex with HIF-1α and PHD2 or PHD3, thereby promoting HIF-1α proline hydroxylation and directing pVHL binding and subsequent proteasomal degradation. SSAT2 simultaneously binds to pVHL and elongin C, thereby stabilizing their interaction and promoting HIF-1α ubiquitylation. Accordingly, SSAT2 overexpression decreases HIF-1α levels, whereas SSAT2 knockdown increases HIF-1α levels under both normoxia and hypoxia. SSAT2 might be a necessary

component of the pVHL E3 ligase complex because it is required for the decrease in HIF-1α levels mediated by overexpression of either PHD2 or pVHL (Koh et al, 2008).

pVHL dependent pathway: de-ubiqitylation

The pVHL-interacting de-ubiquitylating enzyme (VDU2; also called USP20) is the sole HIF-1α de-ubiquitylating enzyme (DUB) identified to date. VDU2 itself can be ubiquitylated and degraded by the pVHL E3 ligase complex. VDU2 binds and de-ubiquitylates HIF-1α in a pVHL-dependent manner, hence salvaging it from proteasomal degradation. The region of pVHL, which is commonly altered in VHL disease, harbors the binding sites for both VDU2 and HIF-1α (Koh et al, 2008).

pVHL dependent pathway: SUMOylation

Hypoxia induces small ubiquitin-like modifier (SUMO)-1 expression and increases HIF-1α SUMOylation, a process that has been shown to lead to its stabilization. However, new evidence indicates that HIF-1a SUMOylation can also lead to HIF-1a degradation. Hypoxia-induced HIF-1α SUMOylation can promote hydroxyproline-independent HIF-1α–pVHL E3 ligase complex binding, thus leading to ubiquitylation and proteasomal degradation. These findings provide evidence for an alternative signal for pVHL binding in the absence of proline hydroxylation. Hence, hypoxia-induced HIF-1α SUMOylation can promote either its stabilization or pVHL-dependent degradation. Further characterization of the type (SUMO 1–3) or nature of HIF-1α SUMO conjugation during hypoxia should shed light on this newly identified mechanism for HIF-1α regulation (Koh et al, 2008).

pVHL independent pathways

Increasing evidence indicates that mechanisms other than pVHL-dependent HIF-1α degradation have an important role in controlling its levels. Compared with pVHL, the regulation of these new pathways seems to be less dependent on oxygen availability and more on specific cellular conditions such as calcium or the presence of growth factors. HIF-1α protein stability is regulated through an oxygen- independent pathway involving the molecular chaperone 90 kDa heat-shock protein (HSP90) and receptor of activated protein kinase (PK)C (RACK1), which compete for binding to HIF-1α. RACK1 homodimerizes and recruits elongin C and other components of the E3 ligase complex to HIF-1α, leading to its ubiquitylation and degradation in a manner mechanistically similar to the pVHL pathway. RACK1–HIF-1α binding is dependent upon the presence of SSAT1, which stabilizes the interaction (Liu et al, 2007.1). It is intriguing that both SSAT1 and SSAT2 bind to and promote HIF-1α ubiquitylation by completely different mechanisms: SSAT2 promotes oxygen and pVHL-dependent HIF-1α degradation, whereas SSAT1 promotes oxygen-independent, RACK1- dependent HIF-1α degradation. The RACK1 pathway can also be regulated by calcium through the activity of calcineurin, a calcium- and calmodulin- dependent and serine/threonine-specific protein phosphatase. Calcineurin A dephosphorylates RACK1 in a calcium-dependent manner, thus blocking RACK1 dimerization and inhibiting RACK1-mediated HIF-1a degradation (Liu et al, 2007.2).

PI3K–Akt signaling activates a variety of signaling cascades with diverse outcomes, including cell survival and death, and is also intricately linked to HIF-1 regulation, not only by inducing HIF-1α

translation in response to growth factors but also through the regulation of HIF-1α protein degradation. However, the modulation of the PI3K–Akt pathway and its role in HIF-1α regulation during hypoxia remains controversial and is highly context dependent. It has been suggested that the PI3K pathway might be activated by short-term hypoxia but inhibited by prolonged hypoxia (Mottet et al, 2003) . Glycogen synthase kinase 3 (GSK3) is phosphorylated and inactivated by Akt: its overexpression results in prolyl-hydroxylation- and pVHL-independent HIF-1α ubiquitylation and proteasomal degradation. Similarly, overexpression of forkhead box (FOX)O4 or of constitutively active FOXO3a, both of which are also negatively regulated by Akt, also represses HIF-1α, the former by inducing pVHL-independent HIF-1α ubiquitylation and degradation and the latter by inhibiting HIF-1α transactivation in a p300-dependent manner. Hence, it is possible that prolonged hypoxia might inhibit the PI3K pathway, resulting in increased GSK3b activity and perhaps also FOXO4 and FOXO3a, which then results in decreased HIF-1α levels and activity (Koh et al, 2008; Mottet et al, 2003).

In summary, the regulation of HIF-1α protein levels through degradation is complex, involving multiple pathways and regulatory factors, some yet to be fully characterized.

Transcription

Surprisingly, relatively little is known about the transcriptional regulation of the *HIF1A* and *EPAS1* genes. Nuclear factor-κB (NF-κB) regulates the transcription of *HIF1A*. Moreover, helper T cell (TH)1 cytokines stimulate this NF-κB–HIF1α pathway to activate a range of HIF1α target genes, whereas the TH2 cytokines interleukin-4 (IL-4) and IL-10 differentially activate EPAS1 expression, although the precise mechanisms involved are not clear. Expression at the *HIF1A* locus, in contrast to the *EPAS1* locus, is also regulated by the SWI/SNF chromatin remodelling protein BRG1-associated factor 57 (BAF57; also known as SMARCE1). Additional investigation into differential *HIF1A* and *EPAS1* transcription is certainly warranted (Keith et al, 2012).

Translation

Under normoxia

A variety of oncoproteins, growth factors and cytokines regulate HIF-1α protein translation in normoxic conditions (Semenza, 2011). In certain contexts, HIF-1α protein induction is dependent on activation of the PI3K/Akt/mTOR and the MAPK pathways (Zhou and Brune, 2006). These pathways phosphorylate the translational repressors (eIF)4E binding proteins (4E-BP1, 4E-BP2 and 4E-BP3) and the ribosomal kinase S6K. eIF4E is an mRNA cap-binding protein that mediates the binding of the eIF4F complex to the 50 cap structures of mRNA. Hypophosphorylated 4E-BP1 binds to eIF4E with high affinity, thereby preventing eIF4F-complex formation and translation initiation. Activation of mTOR and ERK promotes protein synthesis by phosphorylating 4E-BP1 on several sites, hence decreasing its affinity for eIF4E and enabling the formation of the eIF4F complex and subsequent cap-dependent translation. Phosphorylation of S6K by mTOR and ERK controls translation by

phosphorylating components of the translational machinery including the ribosomal protein S6, eIF4B and eEF2K (Sonenberg and Hinnebusch, 2007). It was originally believed that phosphorylation of S6 by S6K stimulates translation by increasing the affinity of ribosomes for the 50-terminal oligopyrimidine tract (50 TOP) motif in certain mRNAs. The putative presence of a 50 TOP motif downstream of nucleotide +32 in the 50 untranslated region (UTR) of HIF-1α that was initially found was proposed to be the mechanism by which mTOR and S6K drive HIF1-α translation (Van den Beucken, 2006). However, it is now believed that the 50 TOP motif is not present in *HIF1A* mRNA, and both S6K and S6 are dispensable for the translational activation (Sonenberg and Hinnebusch, 2007). Hence, the mechanism for S6K-mediated translational regulation of HIF-1α remains unclear.

Under hypoxia

Hypoxia leads to an almost immediate shutdown of general protein translation as a means of decreasing energy consumption during stress. Translation inhibition during hypoxia is regulated by at least two separate pathways. The first pathway, the unfolded protein response (UPR), is activated rapidly (1–2 h) at oxygen concentrations of <1% and stimulates the endoplasmic reticulum (ER) kinase PKR-like ER kinase (PERK), which phosphorylates a crucial regulator of translation initiation, eIF2a. The second pathway, controlled by mTOR, is activated by prolonged hypoxia and inhibits translation by disrupting the eIF4F complex . Thus, the level and length of oxygen deprivation seems to determine the specific mechanism(s) for translation inhibition, each driving distinct gene-expression patterns (Koh et al, 2008). Despite a decrease in global protein translation during hypoxia, a small group of proteins crucial for survival, including HIF-1α, continue to be translated. The evidence supporting the continued translation of HIF-1α was determined using several independent approaches including reporter assays (Young et al, 2008). However, how HIF-1α is selectively translated during periods of global translation inhibition remains incompletely understood. One suggested mechanism is through IRES (internal ribosome entry site) elements. Several studies in the past have reported that the 50 UTR of HIF-1α contains an IRES capable of promoting translation of a downstream reporter in bicistronic reporter assays. However, more recent work convincingly disputes the role of IRES in HIF1-α translation. Moreover, whether an IRES-dependent mechanism contributes substantially to protein translation during hypoxia remains a subject of debate, and observations of protein translation in hypoxia owing to cryptic promoter activity rather than IRES-mediated translation have been reported (Koh et al, 2008). Recent work indicates that, in breast cancer, overexpressed 4E-BP1 and eIF4G function are a hypoxia-activated switch that facilitates cap-independent translation over cap-dependent translation of HIF-1α and other key pro-angiogenic and pro-survival mRNAs (Braunstein et al, 2007). Thus, although translation of HIF-1α and a subset of other stress-survival proteins is maintained or even increased during hypoxia, despite the global decrease in cap-mediated protein translation, the exact mechanism remains unclear. A role for calcium in the regulation of HIF-1α translation during hypoxia is receiving increasing attention. Most cells in fact respond to hypoxia with a sustained increase in cytoplasmic free calcium, but studies addressing the role of calcium in the regulation of HIF-1α levels have been inconsistent with regards to the effect or the mechanism:

this controversy reflects the complexity of calcium-dependent signaling (Koh et al, 2008). Intriguingly, it has been demonstrated that HIF-1α expression in RCC cell lines seems to be regulated by both mTOR-containing kinase complexes mTORC1 and mTORC2, whereas HIF-2α expression is mTORC2-dependent and mTORC1- (Toschi et al, 2008). Other forms of differential translation control have been reported for HIFα proteins. For example, the iron response element (IRE) binding protein 1 (IREBP1; also known as aconitase) was shown to bind a canonical IRE in the 5′ untranslated region (UTR) of EPAS1, thereby inhibiting its translation. This effect seems to be specific for HIF-2α, as IREBP1 fails to bind the *HIF1A* transcript or regulate its translation, despite the presence of a near-consensus IRE in the 5′ UTR of *HIF-1A*. This regulation is also consistent with the identification of HIF-2α as the primary regulator of erythropoiesis and cellular iron metabolism in vivo (Keith et al, 2012).

HIFs in cancer progression

HIFs play key roles in cancer progression, as several specific HIF-regulated genes are involved in many aspects of tumour biology including angiogenesis, stem cell maintenance, metabolic reprogramming, autocrine growth factor signaling, epithelial-mesenchymal transition, invasion, metastasis, and resistance to radiation therapy and chemotherapy. An extensive body of experimental and clinical data has validated HIFs role in cancer: first, in addition to intratumoral hypoxia, loss-of-function for tumor suppressor genes (most notably, VHL), and gain-of-function for oncogenes and viral transforming genes, increase HIF activity; second, levels of HIF-1α or HIF-2α correlate with tumor growth, vascularization, and metastasis both in animal models and in clinical studies. In different cancers, the HIF-dependent expression of these genes may be increased either by genetic alterations or by intratumoral hypoxia (Semenza, 2012).

(a) Increased cell proliferation and survival

Among the first changes that distinguish neoplastic from normal cells are an increased rate of cell proliferation and a decreased rate of cell death as a result of increased expression of secreted growth/survival factors. Often, the same cells express the cognate membrane receptors for these factors, resulting in autocrine signaling. Among the growth/survival factors that are encoded by HIF-regulated genes and participate in autocrine signaling we find: transforming growth factor-α (TGF-α) in clear-cell renal carcinoma; insulin-like growth factor-2 (IGF-2) in colorectal carcinoma; vascular endothelial growth factor (VEGF) in colorectal, gastric, and pancreatic cancer; endothelin 1 (EDN1) in breast, prostate, and ovarian cancer and erythropoietin (EPO) in breast, prostate, and renal cancer and melanoma. Immortalization of cancer stem cells also requires the expression of telomerase (TERT), as well as pluripotency factors and factors that block cellular senescence (Semenza, 2012).

(b) Metabolic reprogramming

The uptake of glucose by metastatic cancer cells is so reliably and markedly increased compared to normal cells that it serves as the basis for the clinical test used to screen cancer patients for occult metastases in which 18F-fluorodeoxyglucose is imaged by positron emission tomography (FDG-PET). HIF-1 mediates the expression of genes encoding glucose transporters (GLUT1, GLUT3) and glycolytic enzymes (ALDOA, ENO1, GAPDH, HK1, HK2, PFKL, PGK1, PKM2, LDHA) that convert glucose to lactate. HIF-1 also actively suppresses mitochondrial oxidative metabolism by increasing the expression of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inactivates pyruvate dehydrogenase, the enzyme that converts pyruvate to acetyl-CoA thereby fuelling the TCA cycle (Semenza, 2011.2). HIF-1 also downregulates oxidative metabolism by activating the expression of the genes encoding BNIP3 and BNIP3L, which mediate mitochondrial-selective autophagy (Bellot et al, 2009). Recent data suggest that in some cell types HIF-1 may also mediate expression of transketolase enzymes (TKT, TKTL2) of the pentose phosphate pathway (PPP), which non-oxidatively catalyze the production of ribose required for nucleic acid synthesis (Zhao, 2010).

(c) Angiogenesis

HIF-1 controls the expression of multiple genes encoding angiogenic growth factors, including vascular endothelial growth factor (VEGF), stromal-derived factor 1 (SDF1), placental growth factor (PlGF), platelet-derived growth factor B (PDGFB), and angiopoietin (ANGPT) 1 and 2 (Rey and Semenza, 2010). In mouse models, inhibition of HIF-1 activity dramatically inhibits tumor vascularization (Liao D and Johnson, 2007).

(d) Epithelial–mesenchymal transition

HIF-1 activates the transcription of genes encoding repressors (i.e. ID2, SNAI1, SNAI2, TCF3) that block the expression of E-cadherin and other proteins that contribute to the rigid cytoskeleton, cell-cell adhesion, and other differentiated characteristics of epithelial cells (Esteban et al, 2006). HIF-1 also mediates expression of genes (TGFA, VIM) that promote flexibility in the cytoskeleton and other characteristics of the mesenchymal phenotype (Gunaratnam et al, 2003).

(e) Invasion and metastasis.

HIF-1 activates transcription of genes encoding proteases that degrade (i.e. CTSC, MMP2, MMP9) or remodel (LOX, LOXL2, LOXL4) the extracellular matrix within the primary tissue and at distant sites of metastasis (Wong et al, 2011) as well as motility (AMF, MET) and permeability factors (VEGF, ANGPT2) that promote the intravasation of cancer cells into blood vessels and cell surface (L1CAM) and secreted (ANGPTL4) proteins that promote extravasation of cancer cells into the parenchyma at metastatic sites such as the lung (Zhang et al, 2012).

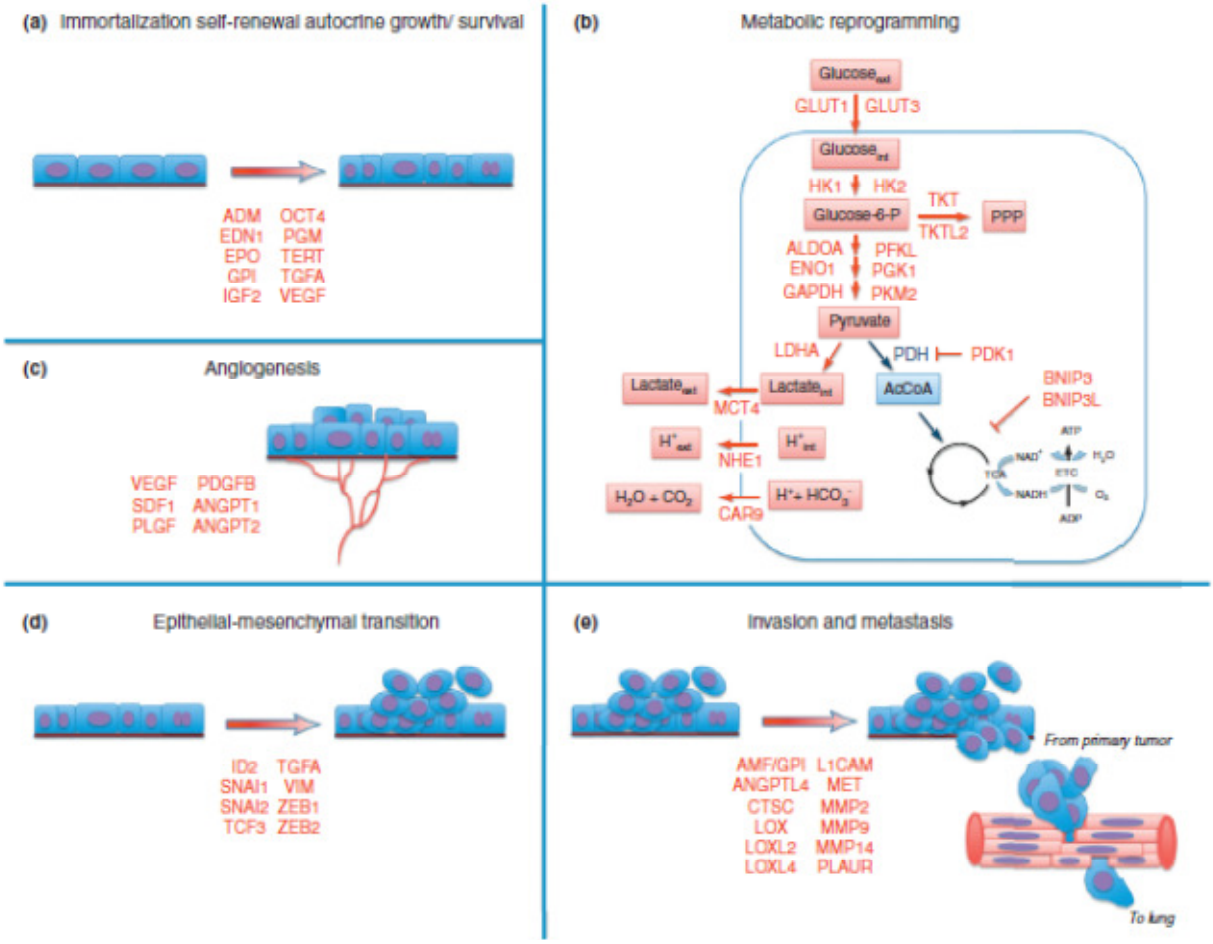

Figure 6 HIF regulation of genes encoding for proteins involved in crucial aspects of cancer progression.
(Trends in Pharmacological Sciences, 2012)

Although HIF-1α and HIF-2α clearly influence tumour progression by directly regulating the expression of unique and shared target genes, recent evidence indicates that these HIFα proteins also affect tumour progression by exerting distinct, often opposing, effects on crucial oncoproteins and tumour suppressors, including MYC, p53 and mTOR. Interestingly, multiple recent studies have also revealed unexpected tumour-suppressive activities of HIF-1α and HIF-2α in specific contexts (Maranchie et al, 2002; Acker et al, 2005). Although initially viewed as having largely overlapping functions, there is now mounting evidence that the two can promote highly divergent, even opposing, outcomes when expressed in the same cell type. It seems that HIF-1α and HIF-2α mediate these disparate responses partly through independent regulation of distinct target genes, but also through direct and indirect interactions with complexes that contain important oncoproteins and tumour suppressors (Keith et al, 2012).

HIFs in breast cancer

As mentioned earlier, hypoxic areas can often be observed in breast cancer samples. Besides the tumours with an obvious hypoxic and necrotic manifestation, some type of hypoxic response is

commonly detected in a large fraction of invasive breast cancer samples. By using HIF-1α as a marker for hypoxia, approximately 25-40% of all invasive breast cancer samples are hypoxic (Vleugel et al, 2005). In general, HIF-1α positivity is linked to large tumour size, high grade, high proliferation, lack of lymph-node metastases and hormone-receptor negativity. It should be noted that it is not obligate with necrotic areas in large tumours and there could accordingly be marked HIF-1α positivity even in small tumours despite the link between hypoxia and tumour size. The large variation of HIF-1α positive tumours in the different studies probably depends on the cutoff used for scoring positive tumours (Lundgren et al, 2007). Although hypoxia is the major factor regulating HIF-1α, it can also be triggered by growth factor stimulation and/or genetic alterations in oncogenes and suppressor genes, and a fraction of the breast cancer samples expressing HIF-1α are probably not hypoxic. It has been shown that out of 44% HIF-1α positive samples, 13.5% expressed HIF-1α in a perinecrotic fashion, whereas 30.5% had a more diffuse HIF-1α staining indicative of an alternative activation of the factor (Vleugel et al, 2005). The HIF-1α downstream target CAIX was further coexpressed with HIF-1α only in the perinecrotic and scattered HIF-1α expressing tumours. The lack of association between HIF-1α and downstream targets in certain high expressing breast cancers also supports differing cellular response depending on the mechanism underlying HIF-1α expression and on the context in which the hypoxia marker appears. On the other hand, when analyzing CAIX in large breast cancer cohorts, there was a strong link to HIF-1α expression as well as to large tumour size, grade, proliferation, and hormone receptor negativity (Brennan et al, 2006). It has been shown also that the presence of hypoxia in a primary breast cancer corresponds to hypoxia markers in lymph-node metastasis, strengthening the hypothesis that besides effects from the microenvironment, the intrinsic angiogenic hypoxia potential of a tumour is maintained during tumour progression. An association between HIF-1α and MET overexpression has also been demonstrated in breast cancer, suggesting that HIF-1α promotes aggressive breast cancer disease by induction of MET. Other reports have shown a strong correlation between HIF-1α and VEGF-C as well as lymphatic microvessel density in human breast cancer. This suggests that HIF-1α could affect tumour-associated lymphangiogenesis, which is a critical parameter in the spreading of breast cancer cells. Hypoxia can further stimulate breast carcinoma cell invasion through general effects on a multitude of invasion/migration-associated gene products, exemplified by matrix metalloproteinase MT1-MMP and MMP2 activation as well as stabilisation of microtubules, and promoting the trafficking of integrins (Lundgren et al, 2007).

Relationship between HIF-1α and ERα

The presence of ERα is an important marker for endocrine treatment response in breast cancer and constitutes a key molecule in targeted treatment of the disease. ERα is also implied in the development and progression of breast cancer and is altogether intimately linked to several important breast cancer features. Interestingly, hypoxia has been shown to decrease ERα protein content in breast cancer cell lines, and several groups have reported specific downregulation of ERα and PR under various hypoxic conditions (1% and 0.1% oxygen). The mechanisms of ERα downregulation during hypoxia are probably dependent on both increased proteasomal degradation and decreased

transcriptional activation (Cho et al, 2005). The transcriptional inhibition of ERα during hypoxia seems to be mediated by the ERK pathway, since downregulation could be inhibited by MEK and ERK inhibitors (Lundgren et al, 2007).

Hypoxia and HIF-1α as prognostic markers in breast cancer

In general there is a rather strong link between the expression of hypoxia markers in breast cancer and aggressive disease, and consequently impaired patient prognosis. Several large studies have observed that HIF-1α protein expression is linked to a worse prognosis, though with some variations in the specific subgroup of breast cancer where HIF-1α was an independent prognostic marker (Lundgren et al, 2007). It should be noted that the majority of the studies analyzing HIF-1α in breast cancer in relation to prognosis have been conducted using immunohistochemistry, thus the evaluation of HIF-1α is in fact troublesome: it is therefore important to include tissues with defined hypoxic areas when analyzing HIF-1α in clinical materials, increasing the validity of the results obtained. The use of CAIX as well as HIF-1α antibodies can serve as positive controls in tissue-based analyses. Studies on HIF-1α in an unselected material of 745 invasive breast cancer samples observed that high HIF-1α expression significantly correlated to poor overall survival and high metastatic risk (Dales et al, 2005). Multivariate statistical analysis further showed that the prognostic value of HIF-1α was independent of other current prognostic markers. The prognostic features of HIF-1α were also significant for the subgroup of lymph node-negative patients. Other studies have shown that HIF-1α is linked to poor survival also in lymph node-positive disease: the true prognostic information of HIF-1α has been assessed also by analyzing a control group of patients who did not receive any adjuvant treatment after surgery. This ensures that the specific link between HIF-1α and tumour aggressiveness was studied without interference of potential associations between HIF-1α and treatment effects. Also, in this study there was a correlation between HIF-1α and prognosis, but specifically in the subgroups of lymph node-positive disease or in grade 1-2 tumours. Rather striking and contrasting results between diffuse and scattered HIF-1α staining have been observed: tumours with scattered staining, indicative of hypoxia-driven HIF-1α expression, were in general more aggressive compared to tumours with diffuse staining (Vleugel et al, 2005). Larger studies of whole sections of breast cancer samples are probably needed in order to fully clarify the different roles for scattered and diffuse HIF-1α in breast cancer. Another problem is the use of slightly different end-points between studies: this should be considered when comparing results from larger studies, and a future aim would be to use the same criteria for tumour aggressiveness.

HIFs as therapeutic targets

HIF inhibitors

The discovery of HIF-1 in the early '90s provided a molecular target associated with intratumor hypoxia that could be used for the development of novel cancer therapeutics. Despite the intrinsic challenges associated with the discovery and development of pharmacological inhibitors of transcription factors, more so in the absence of structural information that could facilitate drug design, many academic groups and pharmaceutical companies have attempted to identify HIF-1 inhibitors. For the most part, efforts have been based on high throughput screening assays aimed at identification of inhibitors of HIF-1 expression and/or transcriptional activity (Melillo, 2007). After several years of attempts and many HIF-1 inhibitors described in the literature, there are several conclusions that can be drawn and considerations that can be made. The common denominator of most, if not all, HIF-1 inhibitors described so far is the lack of specificity, which indicates that they inhibit multiple targets and that HIF-1 inhibition cannot be easily separated from other activities exerted by these agents. This feature of HIF-1 inhibitors may have hampered efforts in validating HIF-1 as a target using pharmacological approaches; nevertheless HIF-1 inhibitors may still have potential applications for therapeutic purposes (Semenza, 2007). A challenge that HIF-1 inhibitors must face to be "validated" as potential therapeutic tools is the evidence that they inhibit the intended target in relevant *in vivo* models and more so in patients with cancer. Indeed, inhibition of HIF-1 expression and/or activity in cell culture is hardly predictive of their potential usefulness as therapeutic agents. However, validation of HIF-1 inhibitors in preclinical models is hindered by the lack of established biomarkers that can be consistently associated with HIF-1 inhibition in tumor tissue. Different endpoints have been measured to assess HIF-1 inhibition in published studies, including but not limited to IHC and/or western blot analysis of HIF-1α protein expression, mRNA expression of HIF-1 target genes and more indirect, surrogate endpoints of HIF inhibition, e.g., angiogenesis and micro-vessels density. Despite these challenges, efforts to validate HIF-1 inhibitors in appropriate *in vivo* models are essential to move these potential therapeutic agents to the clinical setting. This is even more relevant in light of the potential lack of antitumor activity of HIF-1 inhibitors used as single agents. In fact, antitumor activity cannot be and should not be used as a surrogate endpoint for the validation of HIF-1 inhibition, as it is conceptually difficult to envision how HIF-1 inhibition alone may be associated with dramatic tumor shrinkage in xenograft models in which HIF-1 expression in tumor tissue is heterogeneous and focal in nature. Even more challenging is, of course, to generate evidence of HIF-1 inhibition in the clinical setting (Onnis et al, 2009). However, this is a necessary path for the validation of HIF-1 inhibitors in early clinical trials and for the development of this strategy in combination approaches, which appears to be a more promising avenue for the application of HIF-1 inhibitors.

It should be noted that the reports published so far relate for the most part to HIF-1α, although many of these agents may also affect HIF-2α. Both subunits are potential targets of small molecule inhibitors and no clear selectivity, capable of discriminating between inhibition of HIF-1α or HIF-2α,

has been so far convincingly demonstrated (Onnis et al, 2009). According to their putative mechanism of action and although this is an obviously simplified classification, HIF inhibitors could be tentatively divided in agents that modulate:

HIF-1α mRNA expression

Aminoflavone, the active component of the prodrug AFP-464, which is currently in Phase I cancer trials, partially inhibits HIF-1α mRNA expression but almost completely blocks HIF-1α protein expression, suggesting that it decreases both the stability and translation of HIF-1a mRNA (Terzuoli et al, 2010).

HIF-1α synthesis

Drugs that inhibit the translation of HIF-1α mRNA into protein include: mTOR inhibitors, such as rapamycin, temsirolimus (CCI-779), and everolimus (RAD-001); cardiac glycosides, such as digoxin, which have been used for decades to treat heart disease; microtubule targeting agents, such as 2-methoxyestradiol and taxotere; topoisomerase I and II inhibitors, such as topotecan and NSC-644221, respectively; and synthetic oligonucleotides, such as EZN-2968, a locked nucleic acid oligonucleotide that binds to HIF-1α mRNA and blocks its translation, which is currently in Phase I clinical trials. This category also includes anticancer drugs that inhibit receptor tyrosine kinases, such as BCR/ABL, EGFR, and HER2, and thereby indirectly inhibit mTOR activity. YC-1 [3-(50-hydroxy methyl-20-furyl)-1-benzylindazole] may also block HIF-1α protein expression by inhibiting mTOR. PX-478 (S-2- amino-3-[40-N,N,-bis(2-chloroethyl)amino]phenyl propionic acid N-oxide dihydrochloride) inhibits HIF-1α translation by an undetermined mechanism and sensitizes tumor xenografts to radiation therapy: indeed, three different mechanisms have been proposed. Many of the drugs in this category show preferential activity against HIF-1α, seemingly with less effect on HIF-2α (Semenza, 2012).

Topotecan

One of the first agents described to affect HIF-1α protein translation was topotecan (TPT), an FDA approved chemotherapeutic agent currently used as second line therapy for patients with small cell lung cancer or ovarian cancer. Topotecan was originally identified at the National Cancer Institute in a high throughput screen using a cell-based assay of HIF-1 transcriptional activity (Rapisarda et al, 2002). Topotecan is a camptothecin analog that poisons topoisomerase I by inducing the formation of stable Top1-DNA cleavage complexes, which in the presence of DNA replication generate double strand DNA breaks and cytotoxicity. Interestingly, topotecan inhibited HIF-1α translation by a Top1-dependent but DNA damage-independent mechanism, suggesting that cytotoxicity and HIF-1α inhibition could be mechanistically distinguished. Indeed, daily low dose administration of topotecan in a mouse xenograft glioma model caused inhibition of HIF-1α protein expression, angiogenesis and tumor growth (Rapisarda et al, 2004). More recently, it has been shown that administration of daily topotecan in combination with the anti-VEGF antibody bevacizumab exerts synergistic antitumor activity in xenograft models, providing a rationale for clinical development of this combination strategy (Rapisarda et al, 2009). The ability to inhibit HIF-1α protein translation appears to be shared by all the

agents that inhibit Top1. Because topotecan has a short half-life when administered to patients, it is conceivable that other topoisomerase 1 inhibitors with more favorable pharmacokinetics may be more suitable for chronic suppression of the HIF-1 pathway. In this regard an interesting agent is EZN-2208, a PEGylated form of SN38, the active metabolite of CPT-11 (Irinotecan), characterized by improved pharmacokinetics and by remarkable antitumor activity in preclinical models of solid tumors and lymphomas, including CPT-11-resistant tumors (Onnis et al, 2009). One of the criticisms of proposed trials involving compounds such as topotecan is that these are drugs that have already failed to show significant anticancer effects in clinical trials. However, the key distinction that must be made between prior and current trials is that these drugs have previously been administered episodically at maximum tolerated dose as cytotoxic agents, whereas their current utilization as HIF inhibitors involves frequent administration at lower doses in an effort to maintain continuous inhibition of HIF activity. In a recently completed pilot pharmacodynamics study involving 16 patients with advanced cancer and biopsy-proven HIF-1α overexpression, the administration of topotecan orally at 1.6 mg/m2/day _ 5 days/week _ 2 weeks/28-day cycle resulted in decreased tumor blood flow in seven of 10 patients and loss of HIF-1α expression on repeat biopsy in four of seven patients, suggesting that the drug hit the target in vivo (Kummar et al, 2011). During the trial, the topotecan dose was reduced to 1.2 mg/m2/day due to myelosuppression, although it is not known whether this side effect reflected cytotoxicity due to DNA damage or was a direct consequence of HIF inhibition, independent of DNA damage. In either case, it remains to be determined whether two-week-on/two-week-off inhibition of HIF will be an effective anticancer strategy and whether there is a therapeutic window that will allow chronic use of topotecan as a HIF inhibitor (Semenza, 2012).

HIF-1α stability

Drugs that induce the degradation of HIF-1α protein include HSP90 inhibitors, such as 17-allylamino-17-demethoxygeldanamycin, which cause VHL-independent, RACK1-dependent ubiquitylation and proteasomal degradation of HIF-1α; antioxidants, such as ascorbate (vitamin C) and N-acetyl cysteine, which block tumor xenograft growth by promoting HIF-1α degradation through the PHD2–VHL–proteasome pathway; the thioredoxin inhibitor PX-12, which induces HIF-1α degradation that may be due in part to increased expression of SSAT1, a protein that binds to HIF-1α and promotes RACK1-dependent ubiquitylation and proteasomal degradation; Class II histone deacetylase (HDAC) inhibitors, such as LAQ824, which stimulate ubiquitylation of HIF-1α by an undetermined mechanism; Grich oligonucleotides, which represent another nucleic acid-based strategy but in this case the target is HIF-1α protein rather than mRNA; berberine, a natural product that induces HIF-1α degradation and has antiangiogenic effects both in cancer cells and in endothelial cells; Se-methylselenocysteine, which induces HIF-1α degradation and sensitizes hypoxic cancer cells to the effects of the chemotherapy drug irinotecan; and YC-1, a guanylate cyclase activator that induces HIF-1α degradation by an unknown mechanism. YC-1 and deguelin, an HSP90 inhibitor, sensitize tumor xenografts to radiation therapy. Most of the drugs in this category show similar activity against HIF-1α and HIF-2α (Semenza et al, 2012).

HIF heterodimerization

Acriflavine, a drug that was used clinically as an antibacterial agent before the discovery of penicillin, binds directly to the PAS-B subdomain of HIF-1α and HIF-2α and blocks their interaction with HIF-1β, thereby blocking HIF-dependent gene transcription, leading to impaired tumor growth and vascularization (Lee et al, 2009).

HIF DNA-binding activity

Anthracyclines, such as doxorubicin (Adriamycin) and daunorubicin, bind to DNA and block the binding of HIF-1 and HIF-2 in cultured cells and block HIF-1-dependent expression of angiogenic growth factors, leading to impaired tumor growth and vascularization. Echinomycin, another DNA intercalating agent that inhibits HIF-1 activity, has been shown to block lymphoma and acute myelogenous leukemia growth by eradicating cancer stem cells (Semenza, 2012).

HIF-1α-dependent transactivation

The proteasome inhibitor bortezomib, which is approved by the FDA for treatment of mantle cell lymphoma and multiple myeloma, inhibits HIF-1 transcriptional activity by targeting the C-terminal transactivation domain of HIF-1a that interacts with the coactivator p300, although drug treatment does not disrupt the interaction. In prostate cancer cells, bortezomib blocks HIF-1α protein expression by inhibiting phosphatidylinositol-3-kinase/AKT/mTOR and ERK signaling (Molineaux, 2012). Chetomin, a dithiodiketopiperazine metabolite of the fungus *Chaetomium* species, previously characterized as having antimicrobial activity, was also found to be able to block the interaction of HIF-1α and HIF-2α with p300 by disrupting the HIF-interacting domain of p300 (Kung et al, 2004).

Nanobodies

An interesting new class of inhibitors is composed by a new kind of antibodies, llama heavy-chain antibodies, which are also referred to VHH or nanobodies, are stable at high temperatures and can bind antigen in high salt concentrations. The characteristic of small molecular can make nanobidies go through the membrane structure, cripple intracellular viral replication, block enzymatic activity, pass the blood–brain barrier and can be used for various immunological applications like classical antibodies (Hu et al, 2012). Nanobodies against HIF-1 protein are screened based on their activity on essential domain of HIF-1 pathways, e.g. the ODD domain and PAS domain, that are necessary for synthesis, translation, transcription and oxygen-dependent regulation. Nanobodies recognize the domain and specially bind with it, in order to block the synthesis, speed the degradation of HIF-1 protein or affect transcriptional activities of targeted genes. Many researchers have focused their attention on the field. A set of VHH against both human and mouse HIF-1α has been identified (Groot et al, 2006). These VHH were mapped to epitopes within the oxygen-dependent degradation domain (ODDD), and when combined they can also prevent HIF-1α/β dimerization. These anti-HIF-1α VHH were engineered into higher affinity bivalent VHH (Groot et al, 2008). Others nanobodies specific to HIF-1α of pancreatic cancer were isolated from a naive camelidae VHH library. Anti-HIF-1α VHH nanobodies (AHPC) are able to recognize HIF-1α within the Per-Arnt-Sim-B (PAS-B) domain responsible for the dimerization of HIF-1α and HIF-1β and combine with it specifically in order to prevent the dimerization, reduces the level of HIF-1 and blocks tumor proliferation and metastasis (To

et al, 2006). Ablynx (Nanobody®, Ghent, Belgium) is the first company that focused on the discovery and development of nanobodies, for a range of serious human diseases including inflammation, haematology, oncology and pulmonary disease.

Cross talk and combination therapy

In addition to extensive data indicating that HIFs mediate resistance to radiation therapy and chemotherapy (Moeller et al, 2007; Rohwer and Cramer, 2011), there is mounting evidence that HIF-1 activity may contribute to the development of resistance to novel targeted therapies, such as imatinib treatment of chronic myeloid leukemia. HIF-1 appears to mediate resistance to imatinib through metabolic reprogramming, by activating expression of transketolase and thereby increasing glucose flux through the non-oxidative arm of the pentose phosphate pathway (Zhao, 2010). The switch from oxidative to reductive metabolism that is mediated by HIF-1 has the effect of reducing cellular ROS levels, and this may increase resistance to cytotoxic chemotherapy (Santamaria et al, 2006). In the case of VEGF receptor inhibitors, data from several mouse models indicate that treatment, either with the anti-VEGFR2 antibody DC-101 or the small molecule tyrosine kinase inhibitor sunitinib, reduced primary tumor growth and vascularization but increased metastasis, probably because impaired angiogenesis led to increased intratumoral hypoxia and increased HIF activity (Loges et al, 2009). The failure of the anti-VEGF antibody bevacizumab to affect breast cancer progression, which led to revocation of approval by the FDA, may involve HIF-1-dependent expression of other angiogenic growth factors (Tanne, 2011). By contrast, HIF inhibitors dramatically decreased the spontaneous metastasis of human breast cancer cells to the lungs in mouse orthotopic transplantation models by affecting multiple steps in the metastatic process [26,27]. Taken together, these results suggest that combination treatment with HIF inhibitors may improve the efficacy of antiangiogenic agents, a conclusion that is supported by data from mouse models (Wong et al, 2011; Zhang et al, 2011). Traditional chemotherapy may also be more effective when administered with a HIF inhibitor, and many different molecular mechanisms underlie this effect in a cell-type and chemotherapy-specific manner. First, HIFs have been shown to regulate the expression of genes encoding ATP-binding cassette multidrug transporters, including MDR1 (ABCB1) and BCRP (ABCG2), which efflux chemotherapy drugs from cancer cells. Second, HIF-1 inhibits chemotherapy-induced cancer cell senescence. Third, HIF-1 inhibits expression of proapoptotic mitochondrial proteins (BAX, BID) and caspases (CASP3, CASP8, CASP10) and induces expression of antiapoptotic proteins (BCL2, BIRC5). Fourth, HIF-1 prevents chemotherapy-induced DNA damage by inhibiting the expression of topoisomerase IIa protein or the DNA-dependent protein kinase complex. Last but not least, HIF-1-dependent metabolic reprogramming may decrease ROS levels and thereby inhibit chemotherapy-induced cell death (Semenza, 2012).

Flavonoids and cancer

The flavonoids are polyphenolic compounds found as integral components of the human diet. They are universally present as constituents of flowering plants, particularly of food plants. The flavonoids are phenyl substituted chromones (benzopyran derivatives) consisting of a 15-carbon basic skeleton (C6-C3-C6), composed of a chroman (C6-C3) nucleus (the benzo ring A and the heterocyclic ring C), also shared by the tocopherols, with a phenyl (the aromatic ring B) substitution usually at the 2-position. Different substitutions can typically occur in the rings, A and B (Kanadaswami et al, 2005). Several plants and spices containing flavonoid derivatives have found application as disease preventive and therapeutic agents in traditional medicine in Asia for thousands of years: they display a wide range of pharmacologic properties, including anti-inflammatory, anticarcinogenic, and anticancer effects (Ross and Casum, 2002). The much lower risk of colon, prostate and breast cancers in Asians raises the question of whether flavonoid components mediate the protective effects of diets that contain them at high levels by acting as natural chemopreventive and anticancer agents. An impressive body of information exists on the antitumor action of plant flavonoids. *In vitro* research has concentrated on the direct and indirect actions of flavonoids on tumor cells, and has found a variety of anticancer effects, such as cell growth and kinase activity inhibition, apoptosis induction, suppression of the secretion of matrix metalloproteinases and of tumor invasive behavior. Furthermore, some studies have reported the impairment of *in vivo* angiogenesis by dietary flavonoids. Experimental animal studies indicate that certain dietary flavonoids possess antitumor activity. The hydroxylation pattern of the B ring of the flavones and flavonols, such as luteolin and quercetin, seems to critically influence their activities, especially the inhibition of protein kinase activity and antiproliferation. The different mechanisms underlying the potential anticancer action of plant flavonoids await further elucidation. Certain dietary flavonols and flavones targeting cell surface signal transduction enzymes, such as protein tyrosine and focal adhesion kinases, and angiogenesis-inducing processes appear to be promising candidates as anticancer agents. Further *in vivo* studies of these bioactive constituents is deemed necessary in order to develop flavonoid-based anticancer strategies. In view of the increasing interest in the association between dietary flavonoids and cancer initiation and progression, this important field is likely to witness expanded effort and to attract and stimulate further in depth investigations (Kanadaswami et al, 2005).

Luteolin

Luteolin, belonging to the flavone subclass of flavonoids, usually occurs in its glycosylated form in celery, green pepper, perilla leaf, and camomile tea, etc., and as an aglycone in perilla seeds. Recently, a potent anticancer effect of luteolin has been shown in several experiments *in vitro*; however, the bioavailability of luteolin has not yet been fully tested. Only one study on mouse skin cancer development has shown an anticancer effect of the luteolin-containing perilla leaf extract *in vivo* (Ueda et al, 2003), suggesting a potential anticancer effect of any form of luteolin in vivo.

Apoptosis is supposed to be the main mechanism of the anticancer effects of luteolin, although other mechanisms, such as cell cycle inhibition by inactivating cyclin-dependent kinase 2 (CDK2), and antiangiogenesis by inhibiting vascular endothelial growth factor (VEGF)–induced phosphatidylinositol 3V-kinase activity, have been shown (Casagrande and Darbon, 2001; Hasebe et al, 2003). The suggested mechanisms for the luteolin-induced apoptosis include activation of wild-type p53, inactivation of receptor tyrosine kinase, inactivation of topoisomerases, sensitization to tumor necrosis factor-α (TNF-α), imbalance in Bcl-2 family of proteins and inhibition of fatty acid synthase activity (Kanadaswami et al, 2005).

As already stated, Luteolin exerts it activity as antiangiogenic agent by inhibiting VEGF, a HIF-1 target gene: surprisingly, it has been demonstrated that HIF-1α levels are in fact increased rather than decreased after treatment with luteolin (Hasebe et al, 2003). The two results, in apparent contrast, can be explained by the fact that while HIF-1α levels are increased, HIF-1 nuclear activity is in fact inhibited, as we could see also in our laboratory in HCT116, a colon cancer cell line (data not published). Another explanation indicate flavonoids to impair VEGF transcription by an alternative mechanism that did not depend on nuclear HIF levels, for example that flavonoids suppressed hypoxia-induced STAT3 tyrosine phosphorylation and that this activity correlated with their potency as VEGF inhibitors, suggesting that inhibition of STAT3 function may play a role in this process (Ansò et al, 2010). Another study on gastric cancer cells, on the other hand, showed how luteolin significantly enhanced the radioresponse of human tumors transplanted into nude mice: this radiosensitizer effect was accompanied by a a decreased expression levels of both VEGF and HIF-1α (Zhang et al, 2009). The mechanism under luteolin control of HIF-1α remains nowadays unclear.

Cellular metabolism

Metabolism is broadly defined as the sum of biochemical processes in living organisms that either produce or consume energy. It is a dauntingly large sum: more than 8,700 reactions and 16,000 metabolites are now annotated in the Kyoto Encyclopedia of Genes and Genomes. Core metabolism can be simplified to those pathways involving abundant nutrients like carbohydrates, fatty acids, and amino acids, essential for energy homeostasis and macromolecular synthesis in humans. Pathways of core metabolism can then be separated conveniently into three classes: those that synthesize simple molecules or polymerize them into more complex macromolecules (anabolism); those that degrade molecules to release energy (catabolism); and those that help to eliminate the toxic waste produced by the other classes (waste disposal). These pathways are profoundly important. Stated bluntly, they are the sole source of energy that allows life to resist the urge to degrade into entropy. Defining these pathways and understanding their physiological roles have been among the most fruitful pursuits in biological research (De Berardinis and Thompson, 2012). The ‘‘golden age of biochemistry’’ (roughly the 1920s to 1960s) defined most of the metabolic network responsible for

nutrient utilization and energy production in humans and other organisms. These included core activities like glycolysis (Embden, Meyerhof, and Parnas), respiration (Warburg), the tricarboxylic acid (TCA) and urea cycles (Krebs), glycogen catabolism (Cori and Cori), oxidative phosphorylation (Mitchell), and the supremacy of ATP in energy transfer reactions (Lipmann). Biochemistry and the analysis of metabolic pathways dominated basic and medically oriented research during these decades, with some 15 Nobel Prizes in either Physiology/Medicine or Chemistry awarded for work related to energy balance or core metabolic pathways. By the end of this period, it was possible to understand at the level of enzymatic control such complex matters as the temporal and organ-specific regulation of fuel preferences. Research in metabolism has been propelled by the realization that metabolic perturbations accompany common human diseases.

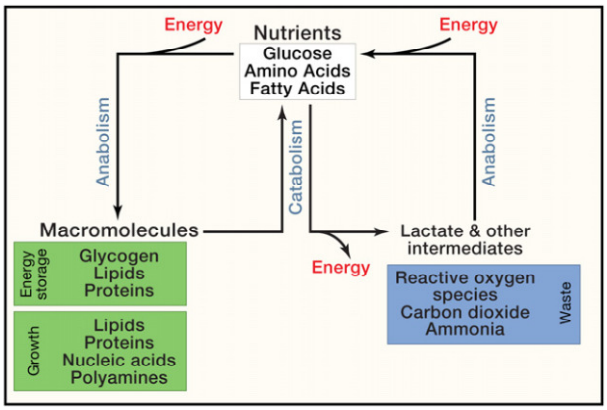

Figure 7 An simplified overview of core metabolism, focusing on the use of major nutrients (glucose, amino acids, and fatty acids) to produce or store energy and to grow. *(Cell, 2012)*

Ongoing exploration of cell biology and disease has recently stimulated a renaissance of interest in small-molecule metabolism (McKnight, 2010). The last 10 years have revealed a host of functions for metabolites and metabolic pathways that could not have been predicted from a conventional understanding of biochemistry. As a result, it is no longer possible to view metabolism merely as a self-regulating network operating independently of other biological systems. Rather, metabolism impacts or is impacted by virtually every other cellular process. Recent work has identified numerous regulatory mechanisms that either link cell signaling to the orchestration of metabolic pathways or enable cells to sense fuel availability and transmit the information through signaling networks. The integration of biochemical pathways in the cellular response to growth factors is a good example. In most mammalian cells, growth occurs only when promoted by extracellular ligands. These growth factors stimulate signal transduction pathways such as the PI3K/Akt/mTOR pathway. Activation of this and other pathways alter the phosphorylation states of numerous targets, which together coordinate the cellular activities that culminate in cell division. But a successful transition from a resting state to growth can only occur if metabolism is reprogrammed to meet the rising demands of proliferation. Growth factor-induced signaling coordinates these functions, including maintaining a bioenergetic state permissive for growth (Lum et al, 2005). In particular, the PI3K/Akt/mTOR pathway stimulates both a rapid increase in essential nutrient uptake and the proper allocation of these nutrients into catabolic and anabolic pathways to produce energy and macromolecules, respectively (Gibbons et al,

2009). Disruption of any of these metabolic effects renders the growth factor ineffective. Dynamic mechanisms also sense cellular energy status and regulate the balance between anabolism and catabolism. Whereas the PI3K/Akt/mTOR pathway promotes anabolism and suppresses catabolism, AMP-activated protein kinase (AMPK) does the reverse. This serine-threonine kinase is a ‘‘fuel sensor’’ activated during compromised bioenergetic states such as acute nutrient deprivation and hypoxia (Hardie, 2011). By phosphorylating a number of key targets, AMPK inactivates energy-consuming, growth-promoting pathways like protein and lipid synthesis and activates catabolism of fatty acids and other fuels. This enables the cell to reset the balance between energy supply and demand. Interestingly, AMPK also regulates a p53- dependent cell-cycle checkpoint activated by glucose deprivation in cultured cells, thereby limiting growth in energetically unfavorable states (Jones et al, 2005).

Metabolism and cancer

Cancer is a prime example of a common human disease with genetically defined, pathological metabolic perturbations. Altered cellular metabolism is a hallmark of cancer, contributing to malignant transformation and to the initiation, growth, and maintenance of tumors (Hanahan and Weinberg, 2011). Although the recent renaissance in metabolism research, in particular work in basic regulation of core metabolic pathways, owes much to cancer cell biology (Bensaad et al, 2006; Gao et al, 2009; Vander Heiden et al, 2010), the principle of perturbed metabolism in tumors is very old, dating almost to the era of early work in chemical carcinogens and viruses as cancer-promoting agents. Otto Warburg performed the first rigorous work in cancer metabolism in the early 1920s: in 1924 he found that carcinoma slices from rats and humans consumed much more glucose and secreted much more lactate than normal tissue, even when presented with enough oxygen to metabolize glucose completely to CO_2 (De Berardinis and Thompson, 2012). As already stated, in most mammalian cells glycolysis is inhibited by the presence of oxygen, which allows mitochondria to oxidize pyruvate to CO_2 and H_2O. This inhibition is termed the ‘Pasteur effect’, after Louis Pasteur, who first demonstrated that glucose flux was reduced by the presence of oxygen (Racker, 1974). This metabolic versatility of mammalian cells is essential for maintenance of energy production throughout a range of oxygen concentrations. The shift towards glycolisys was interpreted as a fundamental change in the way glucose metabolism is regulated in cancer cells and was termed the ‘‘Warburg effect’’ (Warburg, 1956): still it is a critical feature of solid tumors and the basis of diagnostic cancer imaging techniques, such as fluorodeoxyglucose positron emission tomography (FDG-PET) (Shanmugam et at, 2009). After Warburg, generations of cancer biologists and biochemists refined his hypothesis and attempted to provide mechanistic explanations for it. Basically, these studies have confirmed the central observation that many tumors can outcompete surrounding tissue for glucose, but the molecular basis of the Warburg effect and rationale behind cancer cells undergoing aerobic glycolysis have not been completely elucidated (Mucaj et al, 2012).

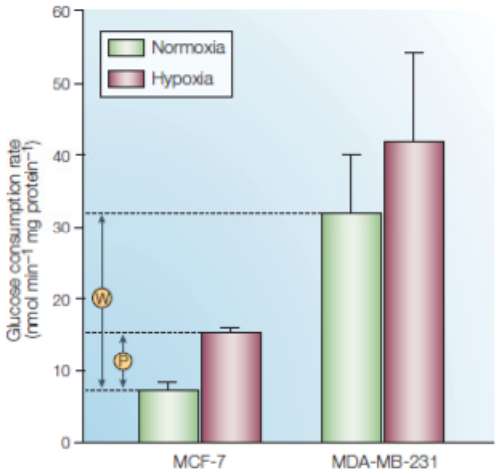

Figure 8 Pasteur and Warburg effects in non-invasive (MCF-7) and metastatic (MDA-MB-231) breast cancer cell lines. *(Nature, 2004)*

In contrast to Warburg's original hypothesis, damaged mitochondria are not at the root of the aerobic glycolysis exhibited by most tumor cells. Most tumor mitochondria are not defective in their ability to carry out oxidative phosphorylation. Instead, in proliferating cells, mitochondrial metabolism is reprogrammed to meet the challenges of macromolecular synthesis. This possibility was never considered by Warburg and his contemporaries. Advances in cancer metabolism research over the last decade have enhanced our understanding of how aerobic glycolysis and other metabolic alterations observed in cancer cells support the anabolic requirements associated with cell growth and proliferation. It has become clear that anabolic metabolism is under complex regulatory control directed by growth-factor signal transduction in nontransformed cells (Ward and Thompson, 2012). Yet despite these advances, the repeated refrain from traditional biochemists is that altered metabolism is merely an indirect phenomenon in cancer, a secondary effect that pales in importance to the activation of primary proliferation and survival signals (Hanahan and Weinberg, 2011). One common hypothesis to unify these metabolic pathways is that aggressive tumor growth requires a restructuring of metabolism to meet the bioenergetic and biosynthetic demands of rapid cell growth and to protect the cells against stresses induced by a harsh microenvironment (Semenza, 2010). Metabolic flux studies in cancer cells have validated this model, emphasizing the integration of these three core pathways (DeBerardinis et al. 2007). Thus, cancer is a paradigm for how perturbed metabolism at the cellular level contributes to disease. What drives metabolic reprogramming in tumour cells is, as in IEMs (Inborn Errors of Metabolism diseases), the fact that metabolic idiosyncrasies of are genetically defined, resulting from the same mutations that promote malignancy. However, in IEMs, germline mutations elicit wholesale changes in metabolism that tend to reduce overall fitness. Cancer mutations are, in general, acquired somatically and are associated with metabolic effects that appear to increase fitness and growth at the cellular level. Two different classes of mutations can reprogram metabolism in tumours. First, many human oncogenes and tumor suppressors regulate glucose metabolism: tumour-promoting mutations in these genes tend to converge on a metabolic phenotype of enhanced glycolysis and energy production, thereby contributing to self sufficiency of growth and evasion of growth-suppressive signals. Second,

metabolic reprogramming can occur as the direct effect of enzyme mutations: this was first observed in a subset of patients with IEMs who displayed an increased risk of cancer (DeBerardinis and Thompson, 2012). Recently, it has become apparent that cancer is also associated with metabolic mutations confined to the tumor. In these diseases, the metabolic enzymes behave genetically as oncogenes or tumour suppressors (Frezza et al, 2011; Thompson, 2009). These forms of cancer are a unique opportunity to determine the cell-intrinsic consequences of metabolic perturbations. The first such examples were mutations in TCAcycle enzymes in familial cancer syndromes. Mutations in succinate dehydrogenase (SDH), an oxidoreductase complex that functions in both the ETC and TCA cycle, were identified in dominantly inherited familial paraganglioma. Mutations in the TCA-cycle enzyme fumarate hydratase (FH) have been identified in familial syndromes characterized by susceptibility to renal cell cancer and leiomyomatosis (smooth muscle tumors of the uterus and skin). In families with SDH- or FH-deficient tumors, affected individuals inherit one mutation, and their tumors display loss of the wild-type allele. Thus, both SDH and FH are tumor suppressors. Other enzyme mutations may function as oncogenes. Genomic sequencing of gliomas and acute myelogenous leukemia identified mutations in two isoforms of NADP+-dependent isocitrate dehydrogenase (IDH1 and IDH2). These enzymes normally oxidize isocitrate to α-ketoglutarate, with NADP+ reduced to NADPH in the process. IDH1 and IDH2 mutations in cancer are somatically acquired, present on only one allele, and confined to the enzyme's active site. Thus, unlike FH and SDH, it was difficult to make a case for IDH1 and IDH2 as tumor suppressors. The surprising involvement of IDH1, IDH2, SDH, and FH in cancer has prompted efforts to identify other metabolic genes whose alteration at the genomic level causes or facilitates tumorigenesis. This work recently uncovered phosphoglycerate dehydrogenase (PHGDH), an enzyme required to produce the amino acids serine and glycine from glucose. Serine and glycine are crucial intermediates in a variety of biosynthetic processes, including the production of nucleotides, proteins, glutathione, creatine, and methylated DNA. High levels of PHGDH protein expression relative to normal tissue correlate with disease severity in breast cancer patients (DeBerardinis and Thompson, 2012).

HIFs and cancer metabolism

Cancer cells are characterized by rapid proliferation and require adaptive metabolic responses to allow continued biosynthesis and cell growth in the setting of decreased oxygen (O_2) and nutrient availability. The hypoxia-inducible factors (HIFs) are a common link between adaptation to low O_2, changes in cancer metabolism, and malignant progression. The HIF-α subunits differentially regulate metabolic enzymes and other key factors involved in glycolysis, changes in redox status, and oxidative phosphorylation. Importantly, metabolic changes can, in turn, regulate HIF activity. Finally, changes in metabolism under hypoxia lead to important crosstalk between cancer cells and the stromal compartment of the microenvironment (Mucaj et al, 2012). The placement of HIF-1 both upstream and downstream of cancer metabolism results in a feed-forward mechanism that may play a major role in the development of the invasive, metastatic, and lethal cancer phenotype (Semenza, 2010.2).

Glycolysis allows for continued ATP production without the need for O_2-dependent oxidative phosphorylation, and is thus an important pathway under hypoxic conditions. Metabolic intermediates from the glycolytic pathway can also be funneled into macromolecular biosynthesis for continued growth. HIF-1α activates transcription of genes encoding glucose transporters and other glycolytic enzymes such as LDHA, PGK-1, and HK1 and thus plays an important role in the glycolytic switch. The HIFs prevent entry into the TCA cycle in part by preventing acetyl coenzyme A (acetyl-CoA) production from pyruvate, and thus redirect glucose away from the oxidative phosphorylation pathway. Pyruvate dehydrogenase (PDH), the enzyme responsible for conversion of pyruvate to acetyl-CoA, is indirectly regulated by HIF activity through the modifying enzyme pyruvate dehydrogenase kinase (PDK). PDK1 is upregulated by HIF-1α expression and in turn deactivates PDH. Hypoxia and HIF increase glucose/carbon flux through the glycolytic pathway while minimizing input into the TCA cycle and oxidative phosphorylation (Semenza, 2010.2). Hypoxia and HIFs have been implicated in promoting reverse flux through the TCA cycle and lipogenesis through activity of IDH1 (Metallo et al, 2011): in fact renal cell lines lacking VHL exhibit constitutive reductive carboxylation of glutamine to α-ketoglutarate even at normal O_2 tensions. An obvious role exists for HIF-mediated control of mammalian cell metabolism and acetyl-CoA production for lipogenesis under hypoxia. Cells may adjust to low O_2 tensions through changes in metabolism and methods of ATP production, but are also known to adaptively alter strategies for macromolecular biosynthesis, using metabolic intermediates to fuel nucleotide and lipid synthesis. Hypoxic cells have unique metabolic needs and respond through HIF-regulated changes in metabolism and protein synthesis.

A recent study has established a novel connection between PKM2 and HIF-1α that is independent of its enzymatic properties. PKM2, which has been previously established to be a transcriptional target of HIF-1α, has also been demonstrated to physically interact with HIF-1α in the nucleus, positively modulating HIF-1α transcriptional activity (Luo et al, 2011). Taken together, these findings suggest that PKM2 plays an important role in malignant metabolic reprogramming under low O_2 conditions, by enhancing the pro-glycolytic signals controlled by HIF-1α.

In addition to HIF-1α, the PHD2-VHL pathway also regulates HIF-2α, which can dimerize with HIF-1β and activates target gene expression: increased levels of both HIF-1α and HIF-2α are observed in many human cancers and associated with increased patient mortality (Semenza, 2010.2). Studies in mice suggest that HIF-2α may regulate SOD2 and other genes encoding antioxidant proteins. Thus, whereas HIF-1α inhibits oxidant generation, HIF-2α promotes antioxidant generation. It is therefore of interest that the NAD+-dependent deacetylase sirtuin 1 (SIRT1) was found to bind to, deacetylate, and increase transcriptional activation by HIF-2α but not HIF-1α. Another NAD+-dependent enzyme is poly(ADPribose) polymerase 1 (PARP1), which was recently shown to bind to HIF-1α and promote transactivation through a mechanism that required the enzymatic activity of PARP1. Thus, transactivation mediated by both HIF-1α and HIF-2α can be modulated according to NAD+ levels (Semenza et al, 2010.2). Further studies are required to understand the significance of these novel findings in the context of cancer cell metabolism.

Materials and Methods

Cell lines

During this study a panel of 4 human breast cancer cell lines was analyzed: T47D and MCF-7 ($ER\alpha^+$, PR^+, $HER2^-$), MDA-231 ($ER\alpha^-$, PR^-, $HER2^-$) and SK-BR3 ($ER\alpha^-$, PR^-, HER2+) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) through the Tumour Microenvironment Laboratory (TML, SAIC-NCI Frederick, MD). Cells were routinely cultured as monolayer at 37°C and 5% CO_2 in RPMI medium supplemented with 5% fetal bovine serum (FBS, Euroclone) and 1% antibiotics (penicillin/streptomycin, Sigma Aldrich) for T47D, MCF-7 and MDA-231 or in McCoy's medium supplemented with 10% FBS and 1% antibiotics for SK-BR3.

Drugs

NVP-AEW541 was kindly provided by Novartis Pharma (Basel, Switzerland): stock solution of this drug was prepared in DMSO and stored at -20°C. MAB292, rhIGF-1 and rhIGF-2 were purchased from R&D Systems: stock solutions were prepared following the manufacturer instructions and stored at -20°C. Topotecan (NSC 609699) was kindly provided by the TML (SAIC-NCI Frederick, MD). Luteolin was purchased from Sigma Aldrich: stock solution was prepared in EtOH and stored at -20°C. Working dilutions of all drugs were prepared in culture media immediately before use.

RT- and real time-PCR

To analyze the constitutive levels of the various components of the IGF and HIF systems, as well as the effect of the different compounds on cellular transcription levels, cells were seeded in dishes and let to grow for 24 hours before being put in different conditions of oxygenation (pO_2 21% or 1%) and/or treated with the different compounds (NVP-AEW541 1 µM, Topotecan 250 nM, Luteolin 25 µM, MAB292 5 µg/mL) for 24 or 48 hours. Cells treated with rhIGF-1 or rhIGF-2 (50 ng/mL for 15 minutes) were starved for 4 hour previous the exposure. Total RNA was then extracted following the manufacturer instructions (RNeasy kit, Quiagen) and quantitated (ND-1000, NanoDrop, ThermoScientific): 250 ng (in 10 µL) mRNA were added to 15 µL of RT solution (High Capacity cDNA Reverse Transcription Kit, Applied Biosystem) and retrotranscribed following the optimized protocol: 25°C for 10', 37°C for 120', 85°C for 5' and 4°C ∞ . The cDNA thus obtained was used in a real time-PCR reaction: 2.5 ng (3.75 µL) of cDNA were added with 21.25 µL of a solution containing 2X Master Mix (TaqMan or SYBR, Applied Biosystems), 3 µM reverse and forward primers (final concentration 300 nM, custom made, IDT), 20X eukaryotic 18S rRNA endogenous control (VIC/TAMRA probe, Applied Biosystems) and H_2O. The run was performed in a thermocycler following the protocol: 95°C for 10', 95° for 15'' and 60°C for 60'' (the last two steps were repeated 45 times) and the results analyzed by the ABI7300 software (Applied Biosystems). Reverse and forward primer sequences are reported in table 2.

Gene	Forward primer	Reverse primer
IGF-1 (NM_000618)	5'-TCTCTTCTACCTGGCGCTGT-3'	5'-AAGCAGCACTCATCCACGAT-3'
IGF-2 (NM_000612)	5'-CTTCCAGACACCAATGGGAAT-3'	5'-GTCCCCACAGACGAACTGGA-3'
IGF-1R (NM_000875)	5'-CTAAACCCGGGGAACTACACAG-3'	5'-TTCACAGAGGCATACAGCAC-3'
IGF-2R (NM_000876.2)	5'-TACAACTTCCGGTGGTACACC-3'	5'-CATGGCATACCAGTTTCCTCCA-3'
IR-A (NM_001079817)	5'-TGAGGATTACCTGCACAACG-3'	5'-ACCGTCACATTCCCAACATC-3'
IR-B (NM_000208)	5'-CGTCCCCAGAAAAACCTCTTC-3'	5'-GGACCTGCGTTTCCGAGAT-3'
IGFBP-3 (NM_000598.4)	5'-CAGAGACTCGAGCACAGCAC-3'	5'-GATGACCGGGGTTTAAAGGT-3'
HIF-1α (NM_001243084.1)	5'-TTTGAGGACTTGCGCTTTCA-3'	5'-TTTGAGGACTTGCGCTTTCA-3'
HIF-2α (NM_001430.4)	5'-AGCAGATGGACAACTTGTACCTGA-3'	5'- TGTCGCCATCTTGGGTCAC-3'
VEGF-A (NM_001025366.2)	5'-GGAGGCGCAGCGGTTAG-3'	5'-AACCCGGATCAATGAATATCAAA-3'
PDK-1 (NM_002610.3)	5'-CAAGAATGCAATGAGAGCCACTA-3'	5'-CCAGCGTGACATGAACTTGAA-3'
Actin (NM_001100.3)	5'-CATTCGCGACAGGATGCA-3'	5'-CTCAGGAGGAGCAATGATCTTGA-3'
B2M (NM_004048.2)	5'-GACTTGTCTTTCAGCAAGGA-3'	5'-ACAAAGTCACATGGTTCACA-3'

Table 2 Reverse and forward primer sequences used for real time-PCR

SDS page e Western Blot

Constitutive levels of IGF-1R, IGF-2R, IR and HIF-1α as well as activation of IGF-1R and IR were evaluated on 3 of the 4 cell lines in our panel (T47D, MCF-7 and MDA-231). To analyze the effects of NVP-AEW541 compound on IR/IGF-1R signaling pathway starved cells were treated for 24 hours with NVP-AEW541 (1μM) and then exposed to rhIGF-1 or rhIGF-2 (50 ng/mL, 15 minutes). In a second set of experiments, we followed MAB-292 (5 μg/mL) inhibitory effects on IR/IGF-1R-related signaling pathways by exposing the three cell lines to the compound for 24 hours in standard medium and after exposure to rhIGF-2. Finally, after having assessed the effects of hypoxia on the insulin/IGF system, we evaluated the effect of the combination of the two compounds on both IGF-1R and IR phosphorylation, as well as the effect of the combination NVP-AEW541/Topotecan (1 μM/250 nM) and NVP-AEW541/Luteolin (1 μM/25 μM) in hypoxic conditions. Cell lysates were prepared in RIPA buffer containing protease inhibitor cocktail (Sigma Aldrich). To determine phosphorylation status the lysis buffer was added with phenylmethylsulfonylfluoride (PMSF, 1 mmol/L) and sodium orthovanadate (Na_3VO_4, 1 mmol/L). Protein concentration was determined by Bradford Method (BioRad) or BCA Method (Pierce) and equivalent amounts of total cell lysate (100μg) were added with

sample buffer 2X (Laemmli, Sigma Aldrich), separated by 7% or 10% acrylamide SDSpage under denaturating conditions and transferred onto PVDF membrane. Membranes were incubated overnight with primary antibodies: anti-phospho-IGF-1Rβ(Tyr1316), dilution 1:1000 (Cell Signaling); anti-phospho-IRβ (Tyr1361), dilution 1:1000 (Cell Signaling); anti-IGF-1R, dilution 1:1000 (Cell Signaling); anti-IR, dilution 1:1000 (Cell Signaling); anti-IGF-2R, dilution 1:1000 (Acam), and anti HIF-1α, dilution 1:300 (Novus Biological). As a control of the correct loading membranes were also incubated with anti-actin antibody, dilution 1:5000 (Sigma Aldrich). Then they were incubated with secondary anti-rabbit or anti-mouse antibody conjugated to horseradish peroxidase, dilution 1:10000 (Amersham). Membranes were revealed by enhanced chemiluminescence Western blotting detection reagents (Amersham and Pierce) and detected on films (Hyperfilm ECL, Amersham).

ELISA assay

Constitutive levels of IGF-2 and IGFBP-3 were evaluated in 3 of the 4 cell lines in our panel. Cells were seeded in 6 well plates and let to grow for 48 hours before being starved and put in different conditions of oxygenation (pO_2 21% or 1%). 24 hours later supernatants were collected and stored at -80°C or immediately quantitated through ELISA kit specific for IGF-2 or IGFBP-3 (Insight Genomics and R&D Systems) following the manufacturer protocols.

Flow Cytometry

Constitutive levels of IGF-1R, IGF-2R and IR were evaluated on all 4 cell lines in our panel. Cells were seeded in 6 well plates and let to grow for 48 hours before being put in different conditions of oxygenation (pO_2 21% or 1%). 24 hours later cells were collected, counted and incubated for 1 hour at 4°C with specific conjugated antibodies (IGF-1R/PE, IGF-2R/FSC and IR/PE, R&D Systems) as well as IgG isotype control antibodies (R&D Systems). Red and green fluorescence was then read at a flow cytometer (Guava easyCyte, Millipore). Percentage of fluorescent cells present in IgG isotype controls was subtracted to the corresponding samples during analysis.

SRB

To evaluate the IC50 of luteolin in all cell lines we performed SRB assay. Cells were seeded in 96 well/plates, let to grow for 24 hour and then treated with crescent concentration of luteolin (0.5-50 μM). Then they were fixed with TCA 50% for 1 hour at 4°C at different times (T0, 6h, 24h and 48h). They were stained with SRB for 15 minutes, rinsed in Acetic acid 0.1%, air-dried and the staining was dissolved in Tris. The absorbance was then read at a spectrophotometer (490 nm) and the values thus obtained used to draw proliferation curves and calculate the IC50. The IC50 of NVP-AEW541 was already known from previous experiments in our laboratory. The IC50 of Topotecan was kindly provided from the TML.

Scratch Wound Healing Assay

To evaluate the effect of the different compounds on the migration potential of the most aggressive cell line among our panel, MDA-231, cells were seeded at high density in specific supports (µ-dish Culture Inserts, Idibi) and let to grow for 24 hour. Cells were then exposed to the different compounds, put in different conditions of oxygenation (pO_2 21% or 1%) and the inserts were removed: pictures of the wound scratch were taken at the time of removal (T0) and at regular times for a total of 48 hours. The experiment has been repeated two times; all the treatments and condition were tested at the same time.

Boyden Chamber assay

To further asses the migration potential of MDA-231, the effects of the different compounds in study were determined also by Boyden Chamber Assay. Boyden chambers (CellBiolabs Inc.) were pre-incubated for 30 minutes at 37°C in 24 well plates containing on the bottom 600 µL of medium and FBS (1:1): cells were then added on top of the chambers and let to grow and migrate for 24 or 48 hours. At the end-points, the chambers were removed from the plate, fixed and stained in two steps (Eosin + AzurA/AzurB, 2 minutes each) to colour the cell that migrated in the chamber and finally photographed. Analysis didn't involve counting the number of migrating cells but only the visible differences between the treatments.

Phospho arrays (Kinase and RTK)

To investigate the overall effects of the treatment with Luteolin at the protein level, we performed two phospho arrays on MDA-231 cells, the first one investigating the activation of the different protein-kinases and the second one on a panel of tyr-kinase receptors. Cells were seeded in dishes and let to grow for 24 hours before being put in different conditions of oxygenation (pO_2 21% or 1%) and/or treated with Luteolin 25 µM for 24 hours. Cells lysates were prepared using the specific lysis buffers included in the two different kits and used to perform the array following the manufacturer protocol (R&D Systems).

Seahorse XF24

The Seahorse XF24 (Seahorse Bioscience) instrument measures the rate of change of analytes (currently dissolved oxygen and pH) in the media immediately surrounding living cells cultured in a microplate (24 well/plate). Changes in the extracellular media are caused by the consumption or production of analytes by the cells. Therefore, a sensitive measurement of the media flux can be used to determine rates of cellular metabolism with great precision and in a totally non-invasive, label-free manner. A unique feature of the XF technology is its ability to make accurate and repeatable measurements in as little as five minutes. This is accomplished by isolating an extremely small volume (less than 10 µL) of media above the cell monolayer. Cellular metabolism causes rapid, easily

measured changes to the “microenvironment” in this small volume. Typically, a measurement cycle is performed for 2-5 minutes. During this time, the media is gently mixed and is followed by a short temperature recovery period. The analyte levels are then measured until the oxygen concentration drops approximately 20-30% and media pH declines approximately 0.1-0.2 pH units. The measurement is performed using 24 optical fluorescent biosensors embedded in a sterile disposable cartridge that is placed into the Seahorse 24 well tissue culture microplate. Baseline metabolic rates are typically measured 3-4 times, and are reported in pmol/min for OCR (and PPR) and in mpH/min for ECAR. Compound is then added to the media and mixed for 5 minutes, and then the post-treatment OCR and ECAR measurements are made and repeated. As cells shift metabolic pathways, the relationship between OCR and ECAR (PPR) changes. Because XF measurements are non-destructive, cells can be profiled over a period of minutes, hours or days. Shifts in substrate utilization and metabolism, characteristic of cancer cells, can be detected conveniently and simultaneously with this instrument through detection of the oxygen consumption rate (OCR), to quantify mitochondrial respiration, and the extracellular acidification rate (ECAR), an indicator of glycolysis (together with the proton production rate or PPR). Measuring the ECAR (or PPR) provides a method for detection and quantitation of glycolytic flux in cancer cells in response to genetic changes, or to drugs. The XF Analyzer simultaneously measures OCR and ECAR, providing a more comprehensive assessment of cellular bioenergetics, and analysis of the dynamic interplay between the two major energy-yielding pathways in cancer cells and other cell types. These parameters are determined by measuring OCR after the sequential injection of oligomycin to inhibit the ATP synthase and preventing phopshorylating respiration, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) to uncouple the mitochondrial inner membrane and allow for maximum electron flux through the ETC, therefore causing a rapid energy consumption without ATP generation (this causes an increase in OCR due to the uncoupling activity but also an increase in ECAR due to the attempt to create ATP using glycolysis), and rotenone (to inhibit complex I and block mitochondrial function) or antimycin (to inhibit complex III). To better evaluate the glycolisis process it is also possible to perform a run that contemplates the sequential injection of glucose, oligomycin and 2-DG (2-deoxyglucose).

The XF24 system include a bench top analyzer and touch screen controller, disposable measurement/compound delivery cartridges, microplates for cell culture, calibration media and XF24 software, as well as a plate preparation station (PS) for exchanging media in the tissue culture plate. Cells are seeded 24 hour prior the run in the specific microplate and let to grow overnight: at the same time the sensors are put in the PS to hydrate in 1 mL of a specific solution. The following day the medium is removed from the cells and a specific DMEM (without glucose, it can be added with the desired concentration) is added: the plate is then placed in the PS (at 37°C without CO_2) for about 60 minutes. In the meanwhile working solutions of the different compounds are prepared and put in the different ports of the cartridge. The plate with the cells is placed in the instrument, let to calibrate and then the cartridge is added for the run.

Data analysis

The XF Extracellular Flux Analyzer uses the OCR measurements to determine six main parameters that describe key aspects of mitochondrial function in a cellular context: basal respiration, "ATPlinked" production, proton leak, maximal respiration, spare respiratory capacity and non-mitochondrial respiration (Figure 9a). The difference between the basal OCR and the oligomycin-insensitive OCR yields the amount of oxygen consumption that is ATP-linked. The balance of the basal OCR comprises O_2 consumption due to proton leak and non-mitochondrial sources. The injection of FCCP allows for uninhibited movement of protons across the mitochondrial inner membrane and effectively depletes the mitochondrial membrane potential resulting in measurement of the maximal oxygen consumption without dependence on ATP/ADP transport. The difference between the FCCP-stimulated rate and the basal OCR yields an estimate of the spare capacity (the amount of oxygen consumption that is available for cells to use in times of increased ATP demand). The injection of rotenone prevents any O_2 from being consumed and thus yields the rate of non-mitochondrial O_2 consumption.

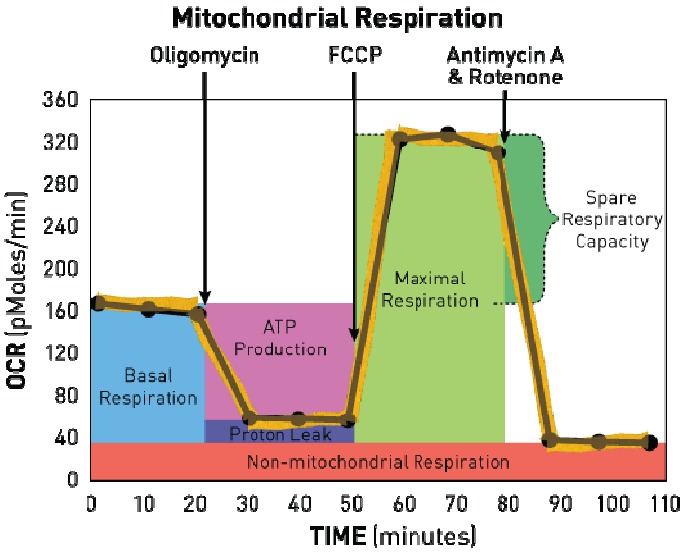

Figure 9a Oxidative profile measured through OCR detection after sequential injection of oligomycin, FCCP and roteneone. *(www.seahorsebio.com, 2012)*

The Analyzer can also use the ECAR measurements after injection of glucose, oligomycin and 2-DG to determine four parameters: glycolisis, glycolitic capacity, glycolitic reserve and non-glycolitic acidification (Figure 9b). Unfortunately the analysis of the glycolitic profile could not be assessed extensively due to lack of time.

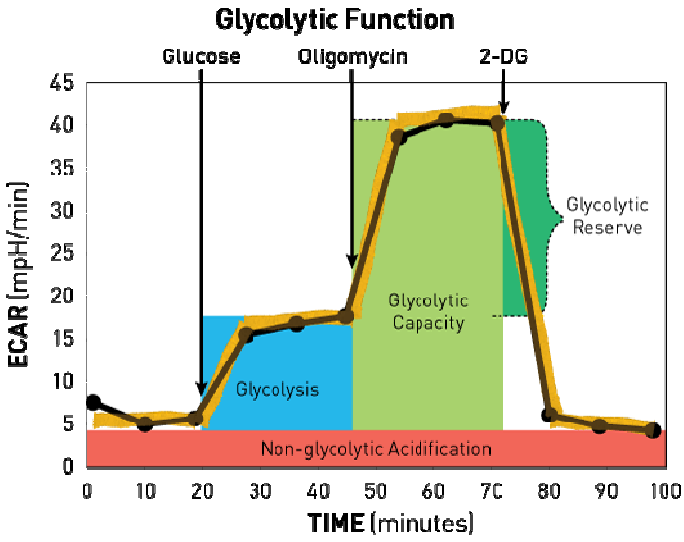

Figure 9b Glycolitic profile measured through ECAR detecttion after sequential injection of glucose, oligomycin, and 2-DG. (www.seahorsebio.com, 2012)

Results

Analysis of the constitutive levels of insulin/IGF and HIF systems components

mRNA expression

The first step in our study contemplated the characterization of the panel of breast cancer cell lines regarding the insulin/IGF and HIF systems: we started with the analysis of the constitutive levels of expression of the different genes of the two systems, preformed by real time-PCR.

Concerning the IGF system, first of all it is interesting to notice the difference between the two ligands: while IGF-1 mRNA levels result undetectable in all cell lines in both conditions (pO_2 21% and pO_2 1%), IGF-2 is on the contrary detectable in all of them, with a noticeable level of variability among cell lines. As expected, in three of the four cell lines we observed an increase in the mRNA expression in hypoxia, in agreement with the fact that IGF-2 is one of HIF-target geneg: interestingly, we could not detect any increase in the MCF-7 cell line, that we used as a term of comparison to investigate the differences in basal expression of all the components of the systems (figure 10). IGF-1R and IGF-2R are both detectable: type-1 receptor shows higher levels of expression compared with type-2 receptor. IGF-1R results decreased in hypoxia in all cell lines, with MDA-231 showing the smallest decrease (70% of mRNA left compared to normoxia), while IGF-2R results slightly decreased in hypoxia in all cell lines, with MDA-231 showing the biggest decrease (70% of mRNA left compared to normoxia). When comparing the four cell lines, contrary to our expectations, MDA-231 were observed to posses the lowest levels of IGF-1R and the highest of IGF-2R: this profile of expression would be more expected in a non-aggressive cell line, like for example MCF-7, which in fact showed basically the opposite profile (figure 11). Anyway, these data cannot be discussed without the analysis of the levels in proteins effectively present at the cellular level: proteins analysis was performed and will be presented later in this chapter. Other components of the IGF system that were investigated were IGFBP-3 and IR. IGFBP-3, the most abundant regulator of IGF tissue delivery was expressed at low levels in all cell lines with the exception of MDA-231, and its levels were increased in hypoxia especially in MDA-231 but again with the exception of MCF-7, where we observed the opposite behavior. Analysis of IR contemplated the use of two different primers specific for the two variants, IR-A and IR-B: as reported in literature, all cell lines showed the presence on high levels of the variant A, predominant on variant B (figure 12). MDA-231 in particular possessed the highest levels of IR-A which remained unchanged in hypoxia (even after starvation).

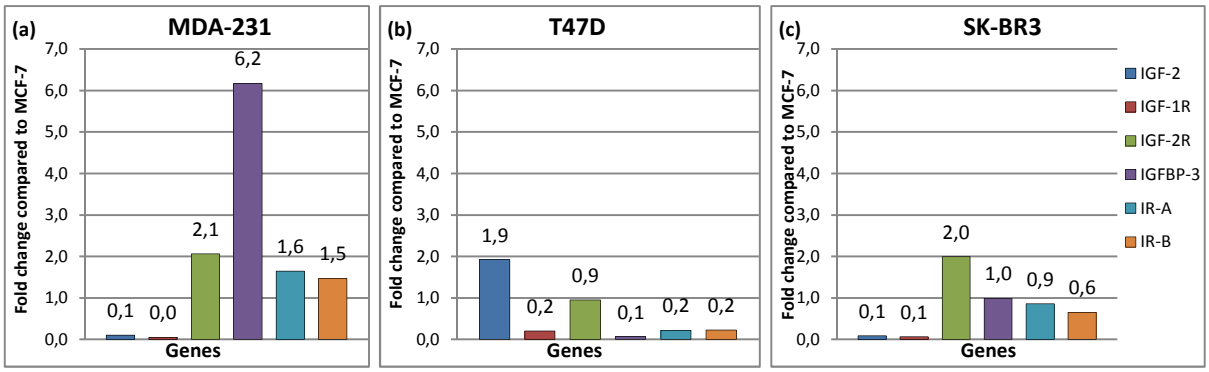

Figure 10 Fold change in the expression of the insulin/IGF system genes in MDA-231 (a), T47D (b) and SK-BR3 (c) compared to MCF-7, used as a term of comparison for the other three cell lines.

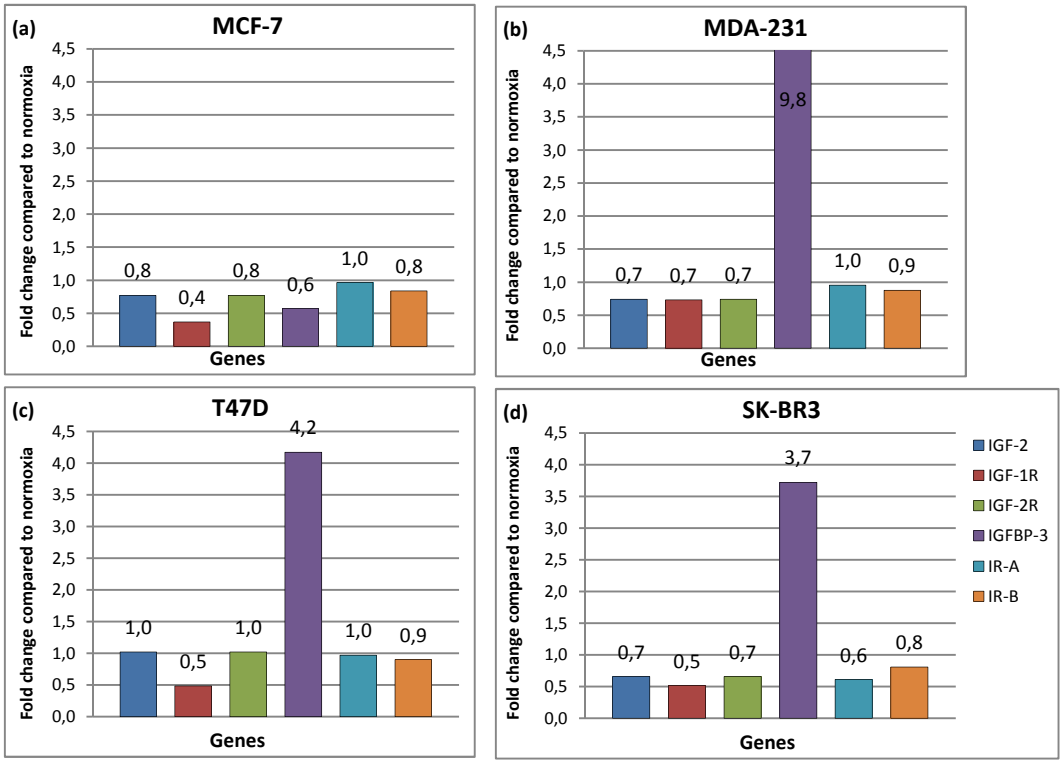

Figure 11 Fold change in the expression of the insulin/IGF system genes in MCF-7 (a), MDA-231 (b), T47D (c) and SK-BR3 (d) in hypoxic condition (pO_2 1%) compared to normoxic expression (pO_2 21%).

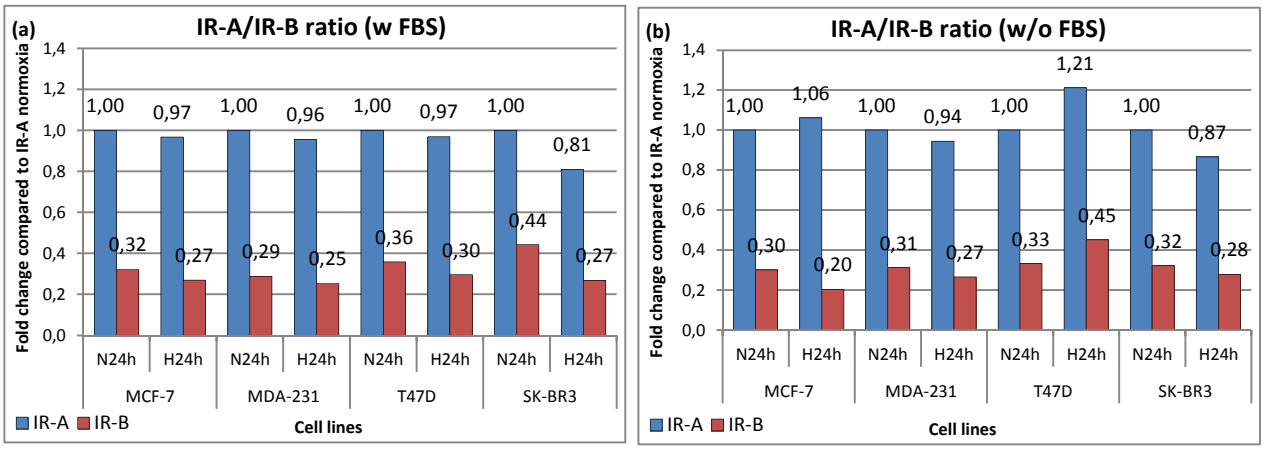

Figure 12 Comparison between IR-A and IR-B expression in all cell lines, in the presence (a) or absence (b) of FBS in the culture media.

Concerning the HIF system, we analyzed the expression of the α subunit, both 1 and 2. HIF-1α mRNA levels were comparable in the four cell lines in our panel, with MDA-231 showing the highest levels, and a slight decrease in hypoxia, due to the general cellular shut down in stress conditions. Considering HIF-2α, its levels were very high in SK-BR3 and MDA-231, high in T47D and very low in MCF-7 (figure 13): these levels were maintained in hypoxia in all cell lines. We analyzed also two HIF-target genes, VEGF-A and PDK-1. VEGF-A was expressed in all cell lines: T47D exhibited the lowest expression, while MDA-231 showed the highest. In all cell lines we could observe an increment in mRNA in hypoxia. PDK-1 mRNA followed a similar pattern, with MCF-7 expressing the lowest levels but also the largest increase in hypoxia (figure 14).

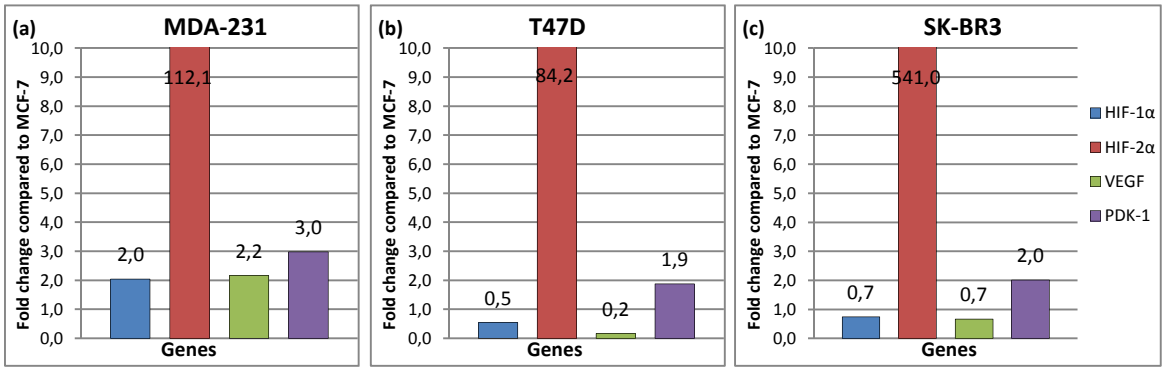

Figure 13 Fold change in the expression of the HIF system genes in MDA-231 (a), T47D (b) and SK-BR3 (c) compared to MCF-7, used as a term of comparison for the other three cell lines.

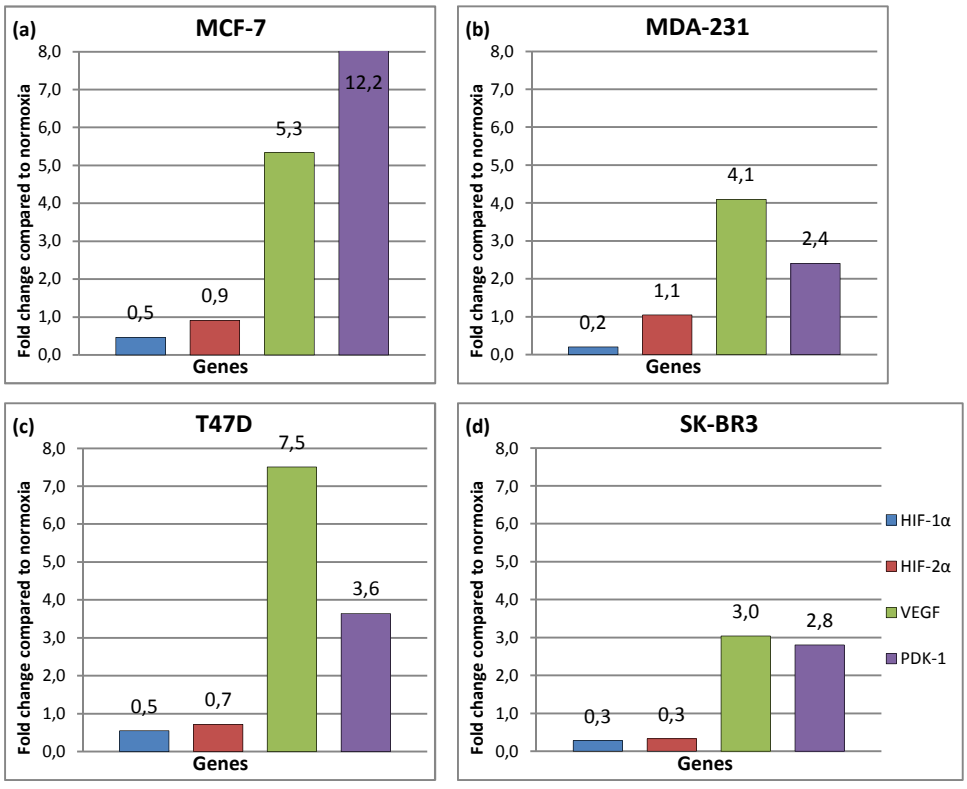

Figure 14 Fold change in the expression of the HIF system genes in MCF-7 (a), MDA-231 (b), T47D (c) and SK-BR3 (d) in hypoxic condition (pO_2 1%) compared to normoxic expression (pO_2 21%).

Protein expression

Together with mRNA expression we also investigated the constitutive levels of proteins actually present in the cells. We performed ELISA assays to verify the concentration of IGF-2 and IGFBP-3 secreted in the medium (we decided not to perform ELISA of IGF-1 considering the absence of the transcript of said protein) and we could observe an interesting pattern which includes both proteins. In fact while the levels of both resulted comparable in the three cell lines analyzed (MCF-7, MDA-231 and T47D) in normoxic condition, when subjected to hypoxic conditions the three cell lines behaved in two opposite ways. MCF-7 and T47D showed a substantial decrease in IGF-2 protein levels and stable levels of IGFBP-3: on the contrary, MDA-231 displayed a remarkable raise in both proteins (figure 15).

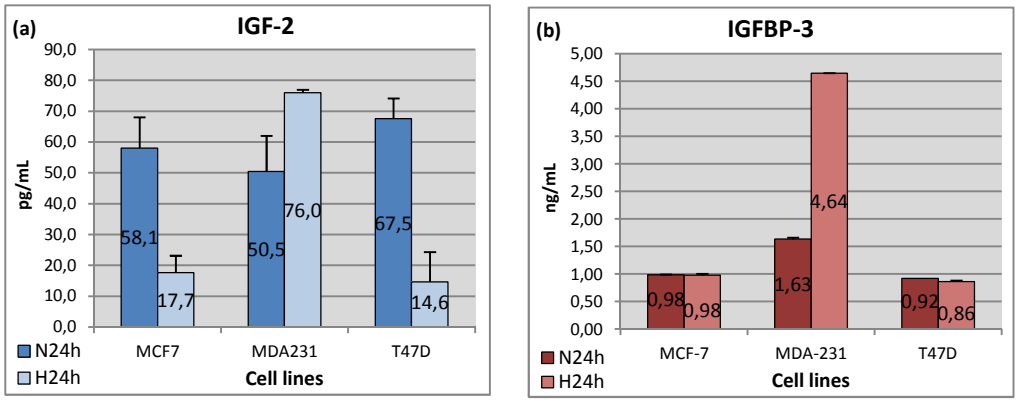

Figure 15 Quantification of the concentration in supernatant of IGF-2 (a) and IGFBP-3 (b) through ELISA assay in normoxic and hypoxic conditions.

At the same time we also performed flow cytometric evaluation of the four cell lines to assess the presence of the receptors (IGF-1R, IGF-2R and IR) in cell membranes. Flow analysis confirmed the PCR data about IGF-1R, showing the highest presence in MCF-7 and T47D; IGF-2R expression was comparable in all cell lines and showed no significant differences between normoxia and hypoxia (similarly to IGF-1R). The same situation was observed also in IR levels with the exception of MCF-7, where a decrease was detected under hypoxic conditions (figure 16).

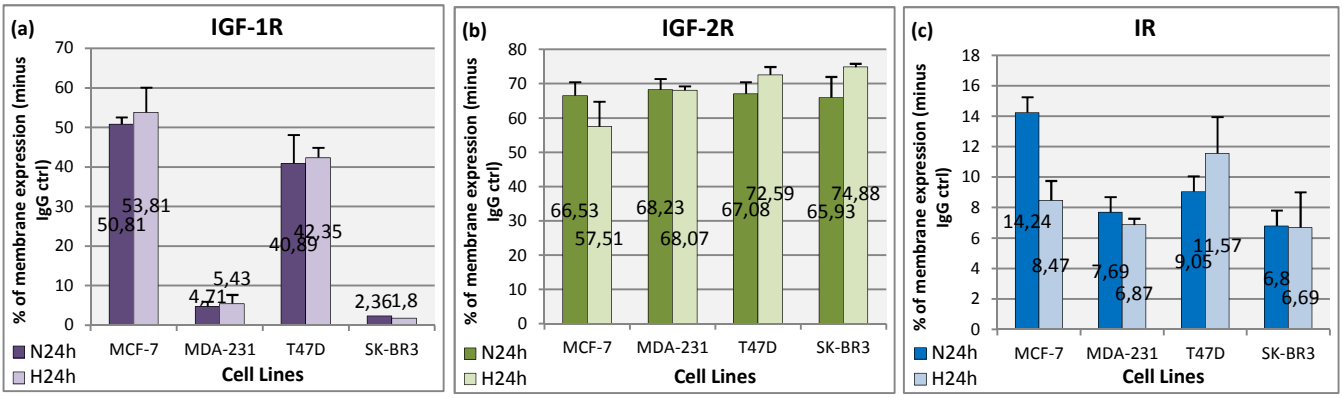

Figure 16 Quantification of the membrane expression of IGF-1R (a), IGF-2R (b) and IR (c) through flow cytometry in normoxic and hypoxic conditions.

NVP-AEW541 ability to inhibit IGF-1R-dependent signal transduction

To determine the efficacy of NVP-AEW541 to inhibit the signal activated by the IGFs through IGF-1R the different cell lines were treated with the drug alone or in combination with rhIGF-1 or rhIGF-2. The ability of NVP-AEW541 to abrogate receptor phosphorylation was assessed: figure 17 shows how indeed IGF-1R phosphorylation was lost in the presence of NVP-AEW541 after exposure to either IGFs in the three cell lines tested.

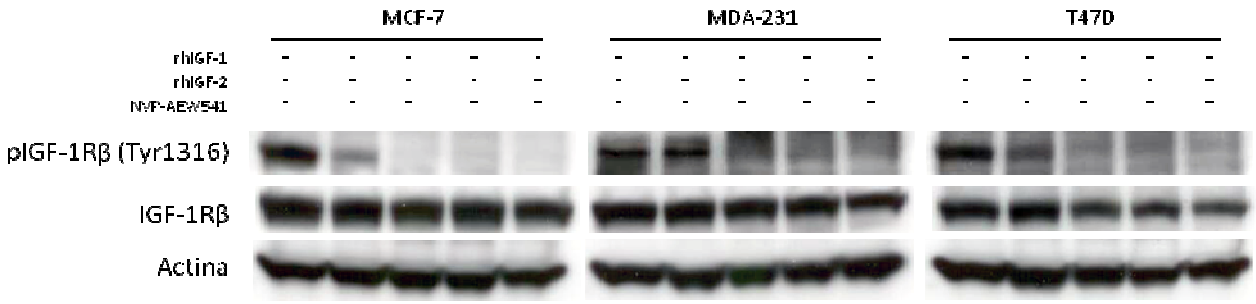

Figure 17 Levels of IGF-1R and its phosphorylation in normoxia following the treatment with NVP-AEW541 (alone and with rhIGF-1 or -2) through Western Blot. As a control of the correct loading actin levels were evaluated.

At the same time we also studied the ability of NVP-AEW541 to inhibit the migration of MDA-231 cells using two different assays (figure 18): with both the Scratch Wound Healing assay (a) and the Boyden chamber assay (b) we could observe how NVP-AEW541 reduced the migration potential of the cell line in the presence of rhIGF-1 while the administration of rhIGF-2 resulted in a loss of effectiveness of the IGF-1R inhibitor.

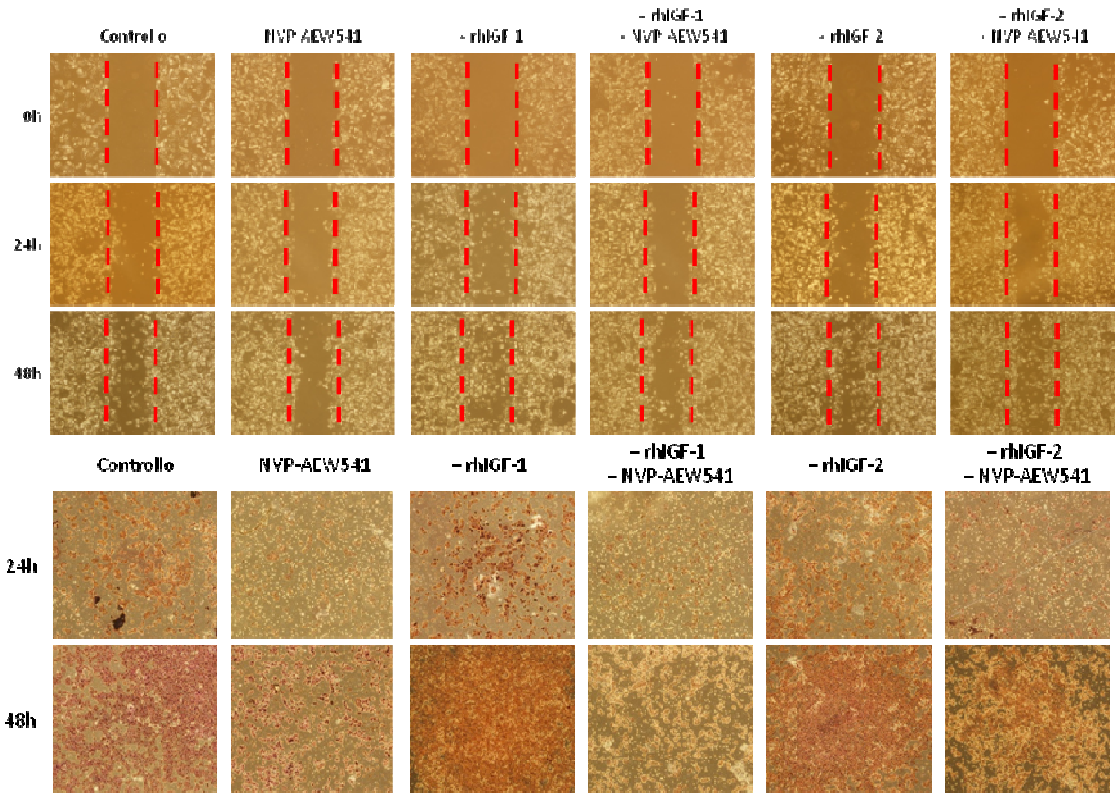

Figure 18 Analysis of the ability to migrate of MDA-231 cell line in normoxia in the presence of NVP-AEW541 treatment (alone and with rhIGF-1 and -2) through Scratch Wound Healing Assay (a) and Boyden Chamber Assay (b).

Analysis of the mechanism of resistance to the anti-migratory effect of NVP-AEW541 following stimulation with rhIGF-2

After observing the inability of NVP-AEW541 to inhibit cell migration as a result of rhIGF-2 administration, we decided to investigate the mechanism of resistance behind this inability. Starting from literature data we analyzed the presence in the cell lines under investigation of the variant A of the IR, the one capable of binding IGF-2: as reported above, real time PCR experiments confirmed that all cell lines possessed an abundance of IR-A transcript. We then evaluated the ability of rhIGF-2 to phosphorylate IR, thus activating a downstream pathway of survival in a manner comparable to IGF-1R: this phosphorylation was actually detectable after administration of exogenous IGF-2, especially in MDA-231 (the cell line showing also the more consistent expression of the protein), although not at high levels (figure 19). In MDA-231 we also investigated the activation of Akt following treatment with NVP-AEW541 and exposure to rhIGF-2, detecting a partial phosphorylation of the protein (not shown in figure).

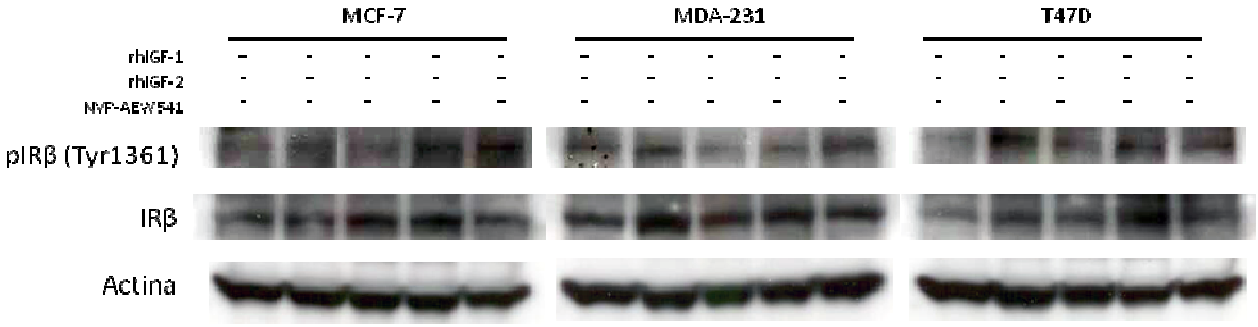

Figure 19 Levels of IR and its phosphorylation in normoxia following the treatment with NVP-AEW541 (alone and with rhIGF-1 or -2) through Western Blot. As a control of the correct loading actin levels were evaluated.

Overcoming the resistance to NVP-AEW541 (1)

Combined inhibition of IGF-1R and IGF-2: concomitant use of NVP-AEW541 and MAB292

Given the role played by IGF-2 in the development of resistance to IGF-1R inhibition, the next step in our study was the inhibition of the same IGF-2 through the use of a neutralizing antibody (MAB292), alone or in combination with NVP-AEW541. Figure 20 shows how, following treatment with MAB292, IR resulted in fact not phosphorylated even after exposure to IGF-2: the difference is especially noticeable in MDA-231.

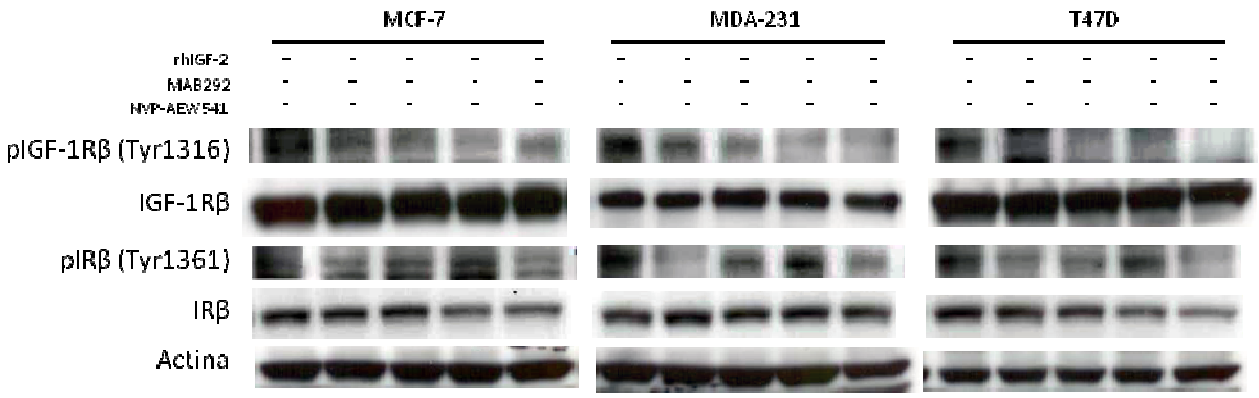

Figure 20 Levels of IGF-1R and IR expression as well as phosphorylation in normoxia following the treatment with MAB292 (alone and with rhIGF-1 or -2) through Western Blot. As a control of the correct loading actin levels were evaluated.

We also observed that IGF-2-dependent migration was effectively inhibited in both assays (figure 21) and that the combination of NVP-AEW541 and MAB292 lead to an almost complete abrogation of the migratory capacity of MDA-231.

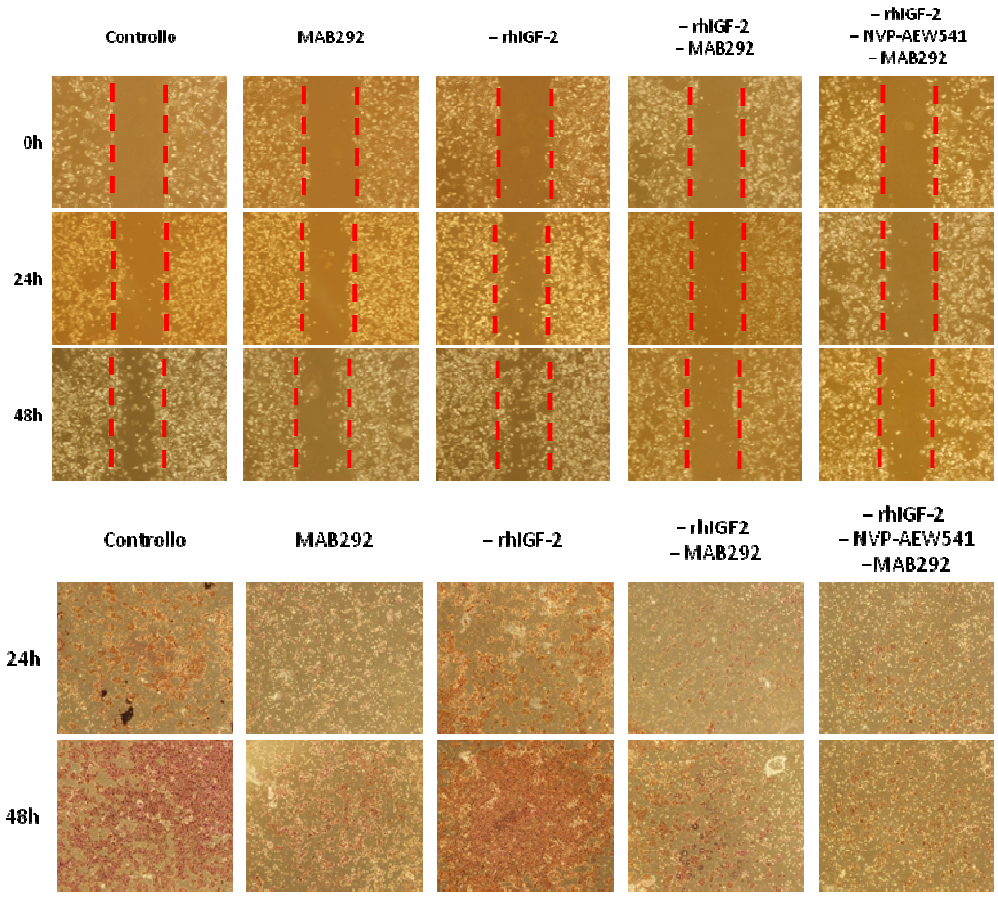

Figure 21 Analysis of the ability to migrate of MDA-231 cell line in normoxia in the presence of MAB292 treatment (alone and in combination with rhIGF-2 and/or NVP-AEW541) through Scratch Wound Healing Assay (a) and Boyden Chamber Assay (b).

Effect of HIF-1α stabilization on the efficacy of NVP-AEW541

Starting from literature data, the next step was to consider the interaction between the insulin/IGF and HIF systems, especially the direct effect of HIF-1 activation on IGF-2 levels. As reported above, through real time PCR we were able to observe an increase in IGF-2 mRNA in all cell lines, with the exception of MCF-7, under hypoxic conditions; an actual increase in the levels of circulating protein was demonstrated only in MDA-231, that was in fact the one cell line were we the decrease of the anti-migratory action of NVP-AEW541 under hypoxic conditions was most evident. The role of endogenous IGF-2 release in the resistance to NVP-AEW541 is further supported by the observation that MAB292 inhibited migration in both assays, similarly to the results obtained following exposure to exogenous IGF-2 (figure 22).

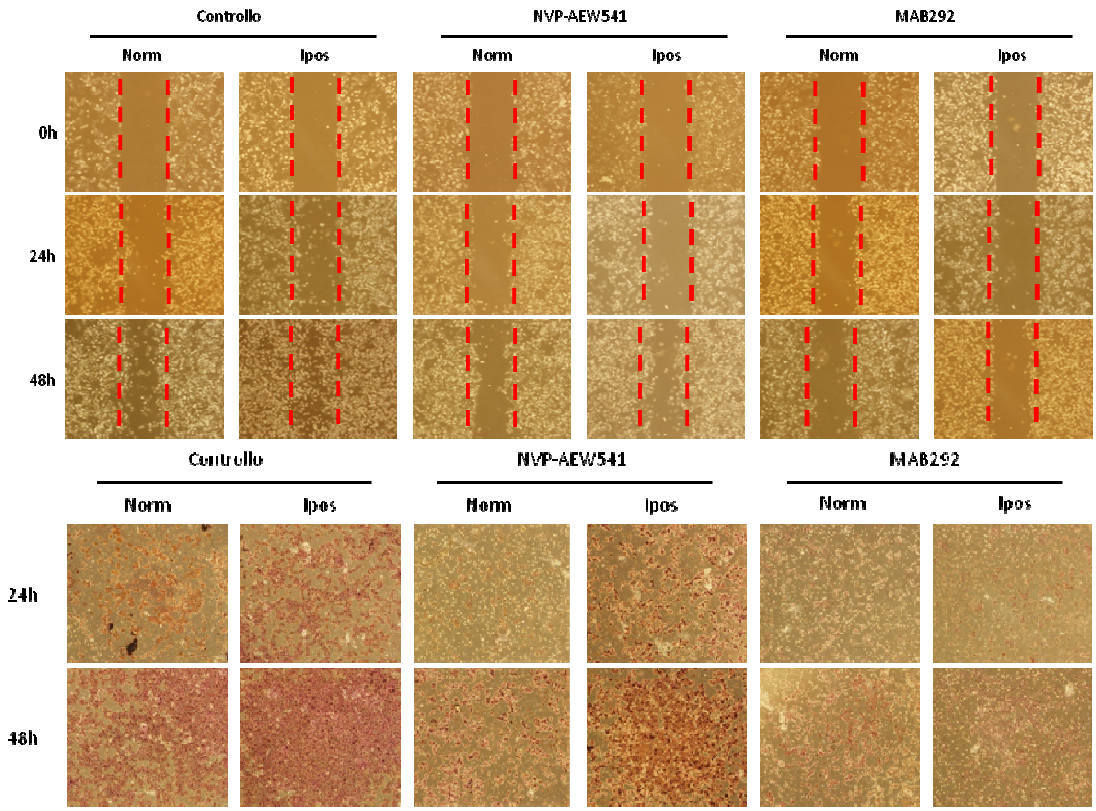

Figure 22 Analysis of the ability to migrate of MDA-231 cell line in hypoxia in the presence of NVP-AEW541 or MAB292 treatment through Scratch Wound Healing Assay (a) and Boyden Chamber Assay (b).

Western Blot analysis confirmed IR to be partially phosphorylated in hypoxia (in contrast to what was observed in normoxia) following exposure to NVP-AEW541 alone, while the use of MAB292, alone or in combination with NVP-AEW541, lead to the loss of such phosphorylation (figure 23).

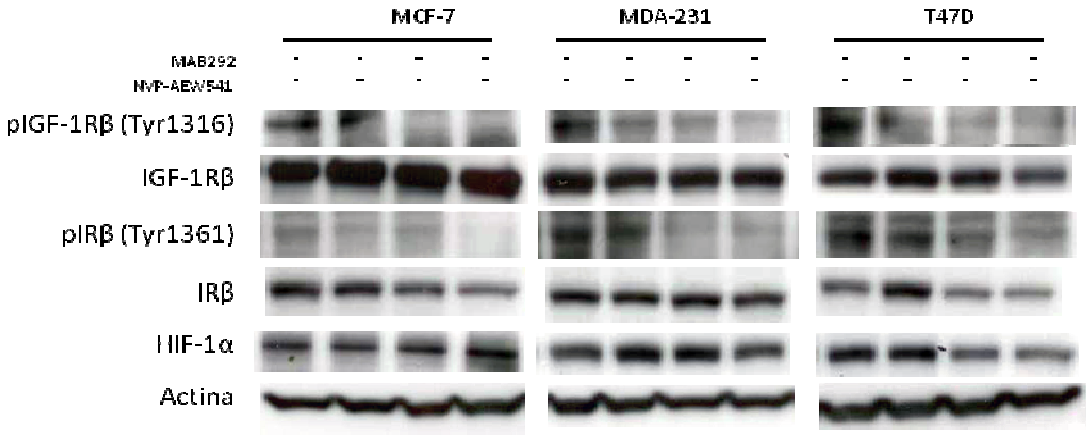

Figure 23 Levels of IGF-1R and IR expression as well as phosphorylation in hypoxia following the treatment with MAB292 and/or NVP-AEW541 through Western Blot. As a control of the correct loading actin levels were evaluated.

Overcoming the resistance to NVP-AEW541 (2)

Combined inhibition of IGF-1R and IGF-2: use of NVP-AEW541 together with HIF inhibitors

Given the ability of hypoxia to determine resistance to selective IGF-1R inhibition through increased production of IGF-2, we finally evaluated the efficacy of the combination of NVP-AEW541 with HIF-1 inhibitors.

To achieve HIF-1 inhibition, we initially used Topotecan, a topoisomerase I poison that inhibits HIF-1α mRNA translation (Onnis et al, 2010). As a first step, we performed real time PCR as well as Western Blot analysis to confirm its efficacy as a HIF-1α inhibitor: as expected, HIF-1α mRNA and protein levels were almost decreased to zero, and the level of HIF-related gene mRNAs (like IGF-2, VEGF-A and PDK-1) was restored to normoxic levels or (figure 24a). We proceeded then to study its effect on insulin/IGF system and it was then possible to notice how the combination with NVP lead to a decrease in IR phosphorylation in hypoxia (figure 25), following the reduction in transcript levels of IGF-2; the effect of the combined treatment was also visible on migratory capacity in both assays (figure additional 26).

Finally, the inhibition of HIF-1 has also been obtained through the use of Luteolin, a compound of natural origin with anti-proliferative and anti-migratory abilities. As reported in literature, we observed a decrease in the transcript levels of HIF-relate genes (IGF-2, VEGF-A and PDK-1, figure 24b) even in the absence of a marked inhibition on HIF-1α protein levels, that resulted slightly decreased in MDA-231, but not comparable to what we observed with Topotecan (figure 25): it is necessary anyway to point out the fact that after treatment with Luteolin we observed a general shut down of the transcription processes of all genes, including that of housekeeping genes like actin and B2M (not shown in figure). After treatment with Luteolin, alone or in combination with NVP-AEW541, we could observe both IGF-1R and IR inhibition: the effectiveness of Luteolin was obvious especially on

proliferation (investigated through SRB assay) and migration with both assays, in combination or not with NVP (figure 26).

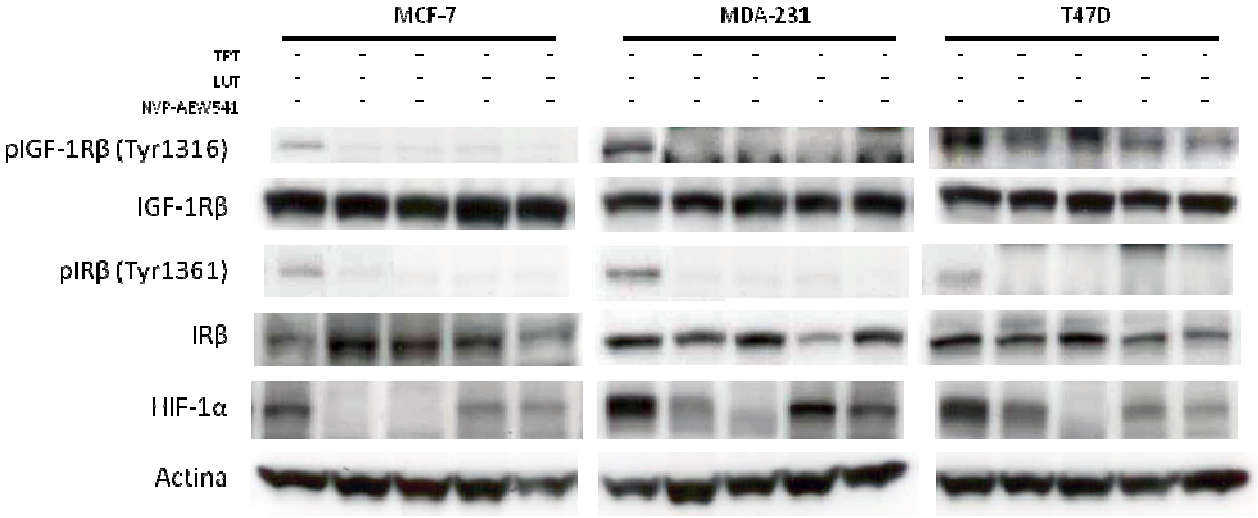

Figure 25 Levels of IGF-1R and IR expression as well as phosphorylation in hypoxia following the treatment with topotecan and luteolin, alone or in combination with NVP-AEW541, through Western Blot. As a control of the correct loading actin levels were evaluated.

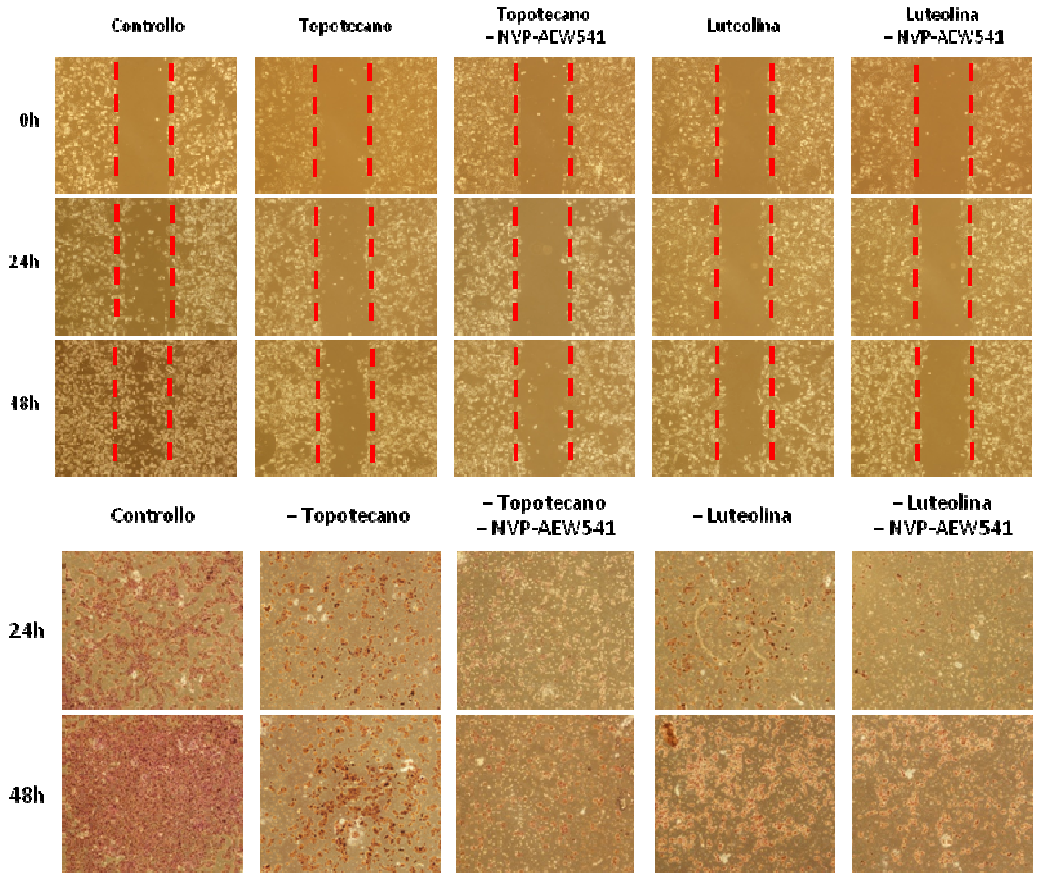

Figure 26 Analysis of the ability to migrate of MDA-231 cell line in hypoxia in the presence treatment with Topotecan or Luteolin, alone or in combination with NVP-AEW541, through Scratch Wound Healing Assay (a) and Boyden Chamber Assay (b).

Analysis of Lutein effects on the different cell lines

Based on the activity profile described in the literature for luteolin, we also performed a screening of the biological activity of this compound, including cytotoxicity assays and phosphoarray analysis under normoxic or hypoxic conditions.

Cytotoxicity

The survival curves obtained for the four h cell lines indicate that the antiproliferative effect of Luteolin was not significantly affected underhypoxic conditions (figure 27). When comparing its efficacy in the different cell lines, MCF-7 cells appeared to be more sensitive, than the other three cell lines, displaying a similar behavior. Based on the the IC50 values estimated from the survival curves,: for the subsequent experiments we decided to use the subtoxic concentration of 25 µM.

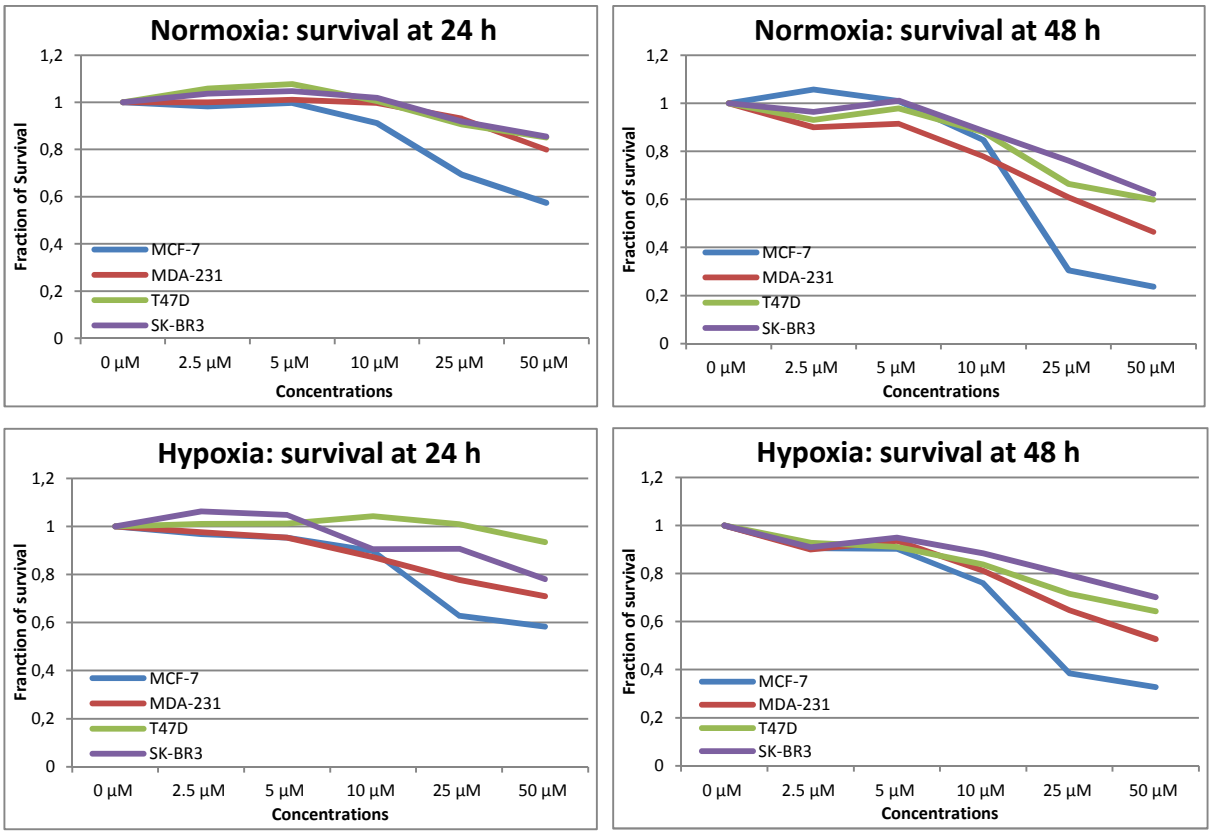

Figure 27 Fraction of survival of the four cell lines after treatment with different concentration of Luteolin, in both normoxic and hypoxic conditions.

Analysis of the profile of activation of PK and RTK

To investigate the overall effects of the treatment with Luteolin at the protein level, we performed two phospho arrays on MDA-231 cells, the first one investigating the activation of the different protein-kinases and the second one on a panel of receptors tyr-kinase. We opted to evaluate the modulation of the different proteins after treatment in both normoxic and hypoxic conditions.

The two maps in figure 28 represent variations in the activation of the two class of proteins: the resulting profile is complex, showing both increase and decrease in the phosphorylation in different sets of PK and RTK. We mainly focused on VEGF receptors, particularly R1, as well as on the STAT (Signal Transducer and Activator of Transcription) proteins, and Akt, whose phosphorylation was significantly, if not altogether completely inhibited after treatment. Other proteins of interest that warranting further investigation were: Paxillin, a protein involved in cell anchorage and consequently in the migration process, that was inhibited; Chk-2 (activated); AMPKα -1 and -2 (both inhibited); and Axl (strongly activated). Activation of EGFR and ERK was also observed, but was less clear-cut than the results obtained for the other genes. As a last consideration, IR activation also seemed increased by phosphoarray analysis, but this result is contradicted by the total inhbition observed by western blot analysis.

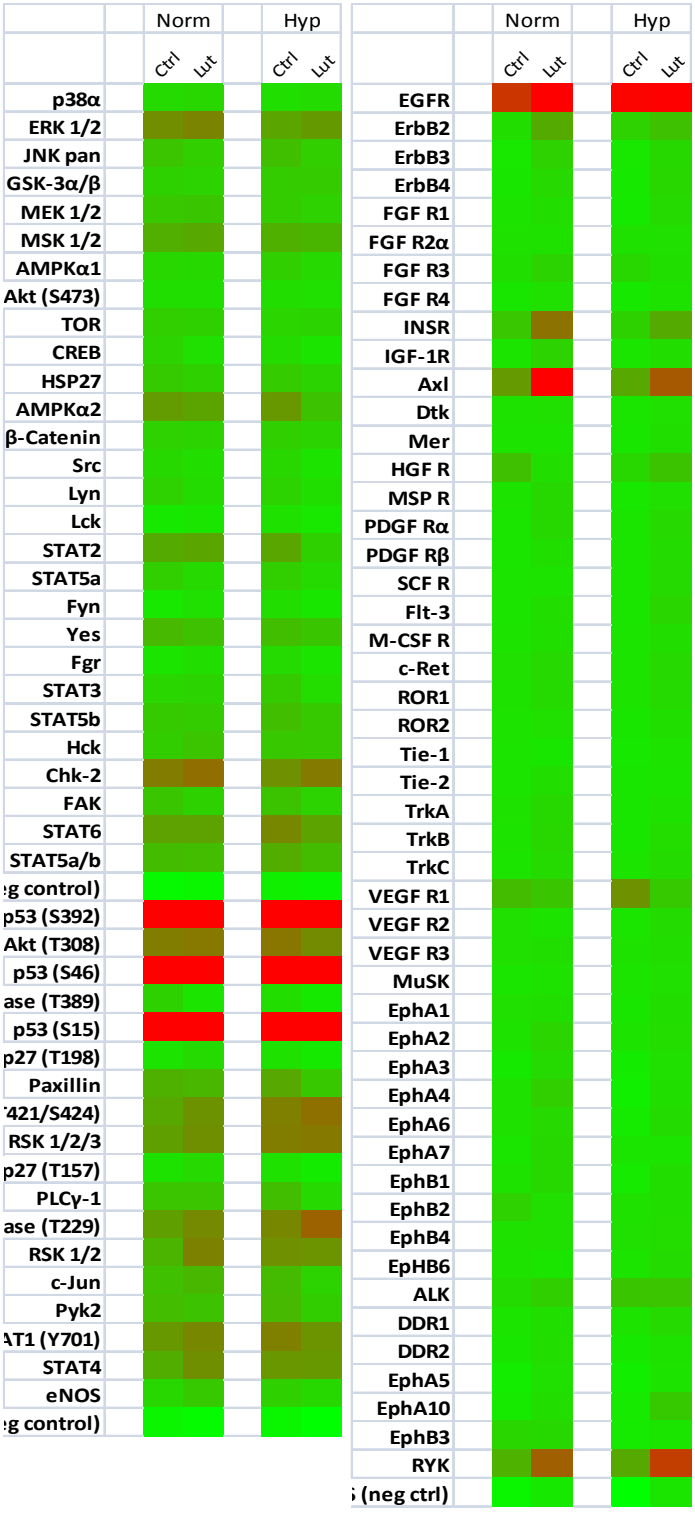

Figure 28 Profile of activation of PK and RTK of MDA-231 cell line in normoxic and hypoxic condition, with and without treatment with Luteolin 25 µM.

Analysis of the metabolic profile (Seahorse XF24)

Constitutive levels

Given the implication of the insulin receptor in the present study, and its role in the metabolic activity of the cells, we finally assessed the metabolic profile of three of the cell lines in our panel. This part of the project was made possible by the collaboration with the TML in Frederick. Figure 29 shows the efficiency of oxidative respiration and glycolysis under basal conditions The three cell lines possess almost overlapping oxidative profiles (a), the glycolytic capacity under normoxic conditions was significantly lower for MCF7 cells than for the other two cell lines (b); in this regard, the metabolic profile of MCF7 cells more closely resembles that observed in normal cells rather than that of tumor cells, notoriously characterized by an increase in aerobic glycolysis (the so-called "Warburg effect").

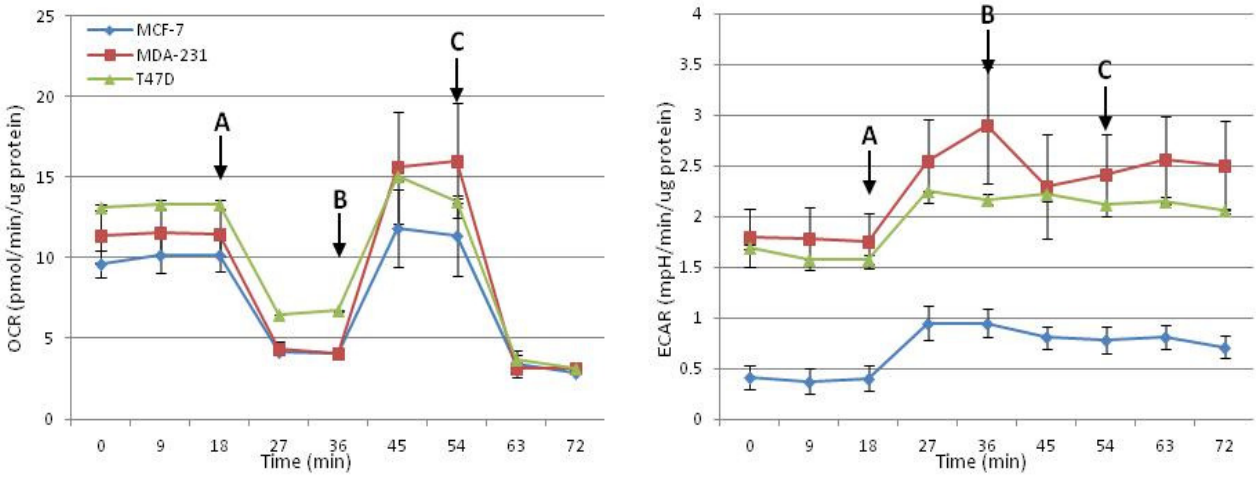

Figure 29 Metabolic profile of the three cell lines under consideration displayed by parameters such as oxygen consumption (OCR, an indicator of oxidative phosphorylation, a) and acidification of the culture medium (ECAR, an indicator of glycolysis, b) detected after sequential injections of oligomycin (1 µM, A), FCCP (0.5 or 0.125 µM, B) and rotenone (0.1 µM, C).

Modification on the metabolic profile following inhibition of the IGF system or treatment with Luteolin

Further preliminary experiments were performed following combined exposure to NVP-AEW541 and MAB292 or Luteolin (Figure 30): interestingly, inhibition of the IGF system, as well as exposure to Luteolin) seems to affect the efficiency of both metabolic processes, albeit with different behavior in the different cell lines.

Further experiments are required to assess the full impact of these compounds on cellular metabolism, as well as the metabolic processes and responses in hypoxia: this last part was not practicable at the moment due to the absence of an hypoxic chamber in which to put the Seahorse XF24 and perform the experiments. The TML is actually still waiting for approval of said hypoxic chamber.

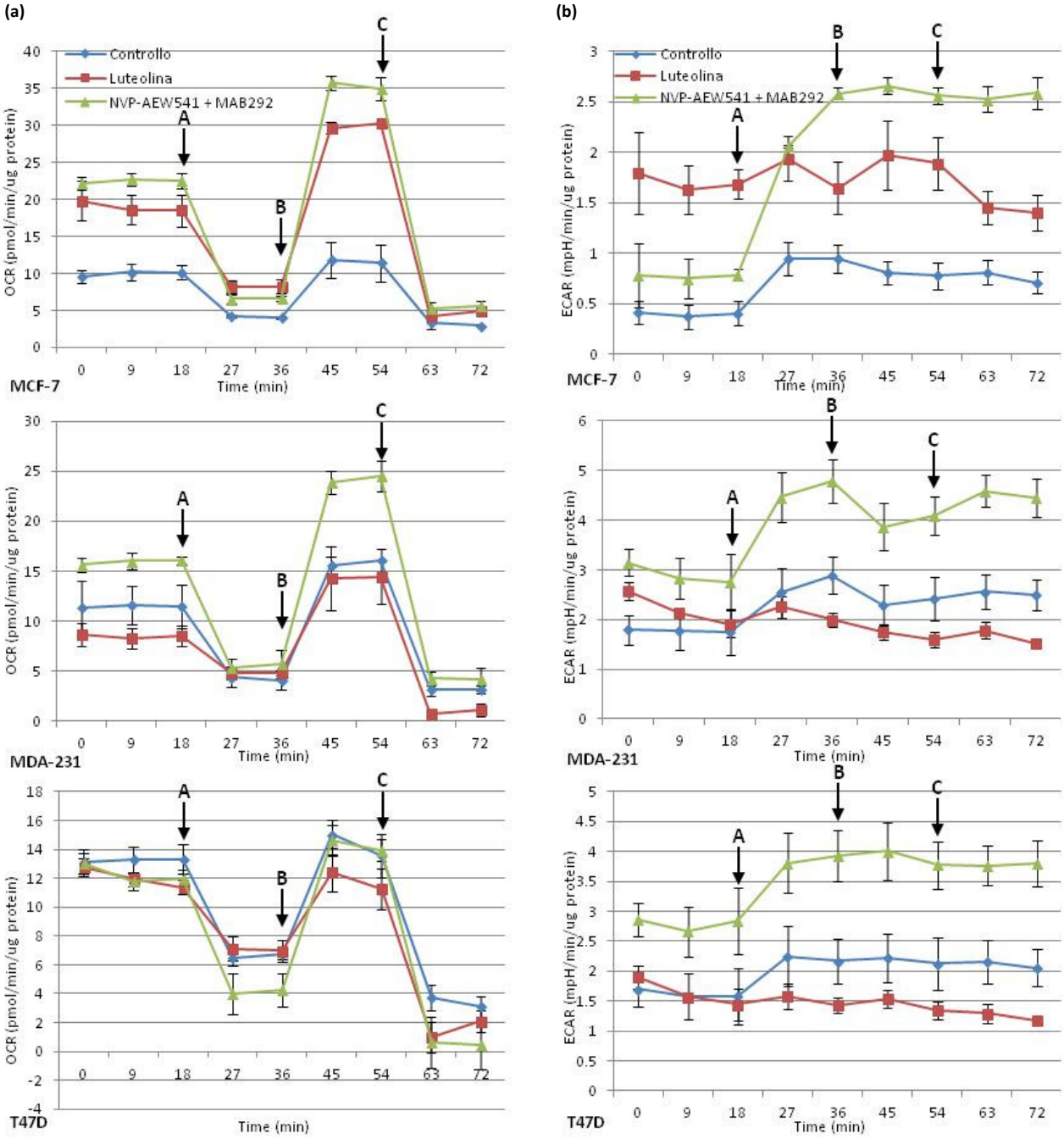

Figure 30 Changes in the metabolic profile of the three cell lines after treatment with Luteolin or NVP-AEW541 + MAB292, viewed through parameters such as oxygen consumption (OCR indicator oxidative phosphorylation, a) and acidification of the culture medium (ECAR, an indicator of glycolysis, b) detected after sequential injections of oligomycin (1 µM, A), FCCP (0.5 or 0.125 µM, B) and rotenone (0.1 µM, C).

Discussion

The observation in the last decade that several receptor tyrosine kinase can act as promoters of tumour development and growth has led to a revolution in the study of new pharmacological targets, in the hope of being able to develop more specific anti-cancer drugs: examples of pathways targeted with success are the HER2/neu family (i.e. the humanized antibody Trastuzumab or the small molecule Lapatinib) or EGFR (i.e. the chimeric monoclonal antibody Cetuximab or the small-molecule Tyr-kinase inhibitor Gefitinib). These compounds have been developed in the last fifteen years and are now being used as part of clinical routine in a number of countries worldwide (Conte and Guarnieri, 2012).

One of the most studied systems in this field appears to be that of the *Insulin-like Growth Factors* (IGF): in this case, however, the development of targeted agents is still in clinical trials of different phases. In fact, many different

The insulin/IGF pathway holds crucial role in metabolism, cell growth, differentiation and proliferation. Aberrant regulation of the IGF system has been attributed to the pathogenesis of different tumour types, in particular in breast cancer, where it has been shown to contribute to various stages of breast carcinogenesis. Therefore, the IGF axis has emerged as a meaningful therapeutic target for oncology drug development that is strongly supported by preclinical studies and drug candidates that target IGF-1R are being evaluated simultaneously in dozens of ongoing clinical trials (Pollak, 2009). However, this complex system presents distinct developmental challenges, and demonstration of meaningful clinical benefit remains elusive (Karamouzis and Papavassiliou, 2012).

While reduction of IGF levels can be achieved through several different approaches, including inhibition of the GH-releasing hormone (GHRH)–GH axis or the neutralization of the ligands, IGF-1R inhibition is by far the most extensively explored. Two different strategies have been developed for the inhibition of IGF-1R, using either monoclonal antibodies (mAbs) against the receptor (i.e. Figitumumab, Ganitumumab, Cixutumumab) or small molecules that inhibit the TK activity of IGF-1R (i.e. Linsitinib, NVP-AEW541, BMS-754807). IGF-targeting agents are generally expected to show their full potential by augmenting the efficacy of endocrine, cytotoxic, or other targeted therapies, possibly by mitigating the development of resistance to otherwise effective interventions. Although combinations of large or small IGF-targeting molecules with various standard-of-care therapies have generally shown a reasonable toxicity profile, this strategy has yet to translate preclinical promise into therapeutic efficacy.

Unfortunately, indeed, tumour cells show a high degree of plasticity and redundancy in proliferative and anti-apoptotic pathways, which may contribute to the development of resistance to specific inhibitors: the identification of these mechanisms of resistance, and their overcoming, are two fundamental goals in the development of these targeted drugs, to identify their effectiveness and their possible actual use in the clinic. An example of signal redundancy is given by EGFR, whose cross-talk with IGF-1R or MET may determine an adaptive response following inhibition of EGFR itself. Another example is the cross-talk between IGF-1R and ERα in breast cancer. In cases like these, the most effective solution lies in the combined inhibition of the different players in the maintaining of the signal.

Within this study we encountered a similar problem: the experiments conducted have in fact shown how the drug in use, NVP-AEW541, a specific, low molecular weight inhibitor of the catalytic activity of IGF-1R (Garcia-Echeverria, 2004), is perfectly able to inhibit the signal transduced by the receptor following stimulation with IGF-1, but in fact seems to lose effectiveness following stimulation with IGF-2, which was especially evident for one of the cell lines under study (MDA-231, featuring a TNBC phenotype and the most aggressive characteristics) to migrate even in the presence of the drug, even though Western blot analysis showed how the compound exerted its inhibitory function on IGF-1R regardless of the type of ligand used for its stimulation.

Starting from the hypotesis that IGF-2 acts as a key factor in the observed resistance, a search of the literature has yielded an increasing number of experimental evidence in support of the role of the insulin receptor (IR), in particular of its splicing variant A upon binding IGF-2, in the maintenance of a proliferative signal similar to that classically attributed to activation of IGF-1R (Buck et al, 2010). For this reason early inhibitors that had been abandoned because of their lack of selectivity for IGF-1R versus IR could be considered again of interest.

Indeed the results of our study are in line with those reported in literature concerning the presence of the variant A of the insulin receptor in breast carcinomas (Frasca et al, 1999; Pollack, 2012). In contrast with IR-B, this variant is capable of binding IGF-2 with higher affinity over insulin itself, and to maintain a proliferative signal even after inhibition of IGF-1R. our results show that IR was especially activated after stimulation with rhIGF-2, but even rhIGF-1 elicited some degree of activation, despite the lower affinity of the receptor for this ligand, probably due to the particularly high concentration of exogenous IGF-1. This activation could (at least partially) justify the dramatic loss of effectiveness of NVP-AEW541 following stimulation with rhIGF-2 in regard of migration of MDA-231 cells. Further evidence confirming that IGF-1R inhibition can be bypassed in this cell line comes from partial activation of Akt despite NVP-AEW541 treatment.

While dual IGF-1R/IR inhibition could prevail over this signal redundancy, the role played by IR in cellular metabolism raises serious concerns regarding the safety of this approach. Actually, variant A of the receptor differs from the classical variant B only in the absence of a single exon (exon 11, coding for a fragment of 12 aa in the C-terminal portion of the α subunit of the receptor) and this makes it particularly difficult to create compounds able to discern between the two variants. Evidence from a number of clinical trials of both antibodies directed against the IGF-1R and inhibitors of tyrosine kinase activity indicates that administration of currently available compounds able to inhibit one or both receptors lead to a transient hyperglycemia (Pollak, 2008; Gao et al, 2012; Karamouzis and Papavassiliou, 2012). In this regard, we performed preliminary study of the metabolic profile of the cell lines in basal conditions and after inhibition of the IGF system (through the use of different strategies that do not involve the direct targeting of IR): the results obtained confirmed that this modulation actually affects the levels of cellular metabolism (data will be further discussed later in this section).

A different and probably more feasible strategy would be to hit IGF-2, i.e. the ligand responsible of the combined IGF-1R/IR activation. The *Igf2* gene is an example of imprinted gene and loss of

imprinting (LOI) in the maternal gene (normally silenced) leads to increased protein expression and a consequent increase in the risk of developing cancers (Harris and Westwood, 2012). The strategy proposed in the present study closely mimics the physiological mechanism controlling IGF-2 levels: in fact normal cells control the levels of circulating IGF-2 by binding the ligand to IGF-2R, followed by internalization and lysosomal degradation. The tumour suppressor function of IGF-2R was first demonstrated 15 years ago, and later it was shown that overexpression of a soluble form of the receptor dramatically reduced tumour cell growth *in vitro* and *in vivo*, suggesting the ability of IGF-2R to inhibit cell proliferation. Mutations in *Igf2r*, or loss of heterozygosity (LOH) at the 6q26–27 locus where *Igf2r* resides, lead to reduced IGF-2R expression and increased circulating concentrations of IGF-2. Loss of biallelic *Igf2r* expression has been reported in cancers of the breast, liver, prostate, lung, adrenal gland, head, neck and endometrium (Martin-Kleiner and Gall 2010). The different cell lines used in the present study exhibited similar levels of IGF2R expression, as assessed by PCR, western blot and flow cytometry.

MEDI-573 is the only mAb currently in clinical testing that exerts its effects by neutralizing not only IGF-2 but also IGF-1. Distinct from all the IGF-1R–targeting compounds under development, MEDI-573 can thus inhibit IGF signaling through both IGF-1R and IR-A, as well as through their hybrid receptors. The ongoing phase I clinical trial has shown promising results and strongly suggested that MEDI-573 might achieve this result without inducing hyperglycemia (Menefee et al, 2010). If confirmed, this observation would be consistent with expectations about ligand- versus receptor-based targeting of the IGF axis, specifically with respect to the clinical consequences of sparing IR-B and its various hybrid receptors, each of which has the potential to alter glucose metabolism via interactions with insulin (Gao et al, 2012).

To confirm the validity of this strategy in breast cancer, we used a neutralizing antibody specific for IGF-2 (MAB292) thus making impossible for the ligand to bind and activate both IGF-1R and IR-A. MAB292 was effective as single agent and even more in combination with NVP-AEW541: following exposure to this antibody, IR-A was predominantly unphosphorylated, in contrast to the effect of NVP-AEW541 alone ; particularly, MDA-231 cells were totally unable to respond to stimulation with rhIGF-2 and, more interestingly, they totally lost the ability to migrate.

IGF2 neutralization could be a particularly winning strategy, especially considering the fact that, at the low oxygen levels that are frequently encountered within the tumor mass (pO2 1%), the activation of pathways as a result of HIF-1α stabilization of and subsequent dimerization with HIF-1β leads to increased IGF2 levels of the same IGF-2, as reported in the literature and observed in our experiments both at transcript and protein levels of especially in the case of MDA-231 cells (Semenza, 2012). This increase is possibly implicated in hypoxia-dependent resistance of MDA-231 cells to the administration of NVP-AEW541, similarly to what has been observed following administration of exogenous IGF-2, although obviously to a lesser extent, considering the different (lower) concentrations of IGF-2 present in hypoxic conditions compared to those administered. As confirmation of the key role of IGF-2 in the resistance to NVP-AEW541 in the presence of low oxygen concentrations, treatment with MAB292 under hypoxic conditions was able to inhibit the migration of

MDA-231 with the same efficacy shown in normoxia following exposure to rhIGF-2, and combined treatment NVP-AEW541/MAB292 again determined the total loss of the migratory capacity, seemingly as a result of the loss of IR phosphorilation. Considering the close relationship between the IGF and HIF system, we decided to further investigate IGF-2R presence in the cell lines, and in particular its regulation under hypoxic conditions: the modulation of this receptor in the presence of low pO2 in fact has not been extensively studied. Actually our experiments could not detect a direct and defined relationship between HIF-1α and IGF-2R levels: we observed a reduction in mRNA levels in hypoxia in all cell lines, but this decrease could not be detected at the protein level (either in membrane expression, evaluated by flow cytometry, or in total protein levels assessed by western blot). Further and more detailed studies are needed to assess the possible correlation between the two proteins. However, it should be emphasized that the use of soluble forms of the receptor, in theory a possible strategy to lower IGF-2 concentration, is actually of difficult practicability. Analysis of IGF-2R levels is probably more interesting and useful as a marker of tumour aggressiveness and/or worse prognosis.

Based on this observation it is therefore evident that a further strategy to overcome the resistance to NVP-AEW541 (and to IGF-1R inhibitors in general) given by IGF-2 overexpression may be the simultaneous inhibition of HIF-1α. The discovery of HIF-1 (and later HIF-2) and its role in activating mechanisms of proliferation and survival in conditions of oxygen concentrations lower than the physiological (pO_2 <2.5%) has led to a great interest in the development of compounds capable of inhibiting the major actors, mainly HIF-1α and HIF-2α. At present, the major problem in targeting HIFs is the lack of specific inhibitors: many chemotherapic drugs, in fact, have a complete or partial inhibitory activity on HIF-1α, but this is often accompanied by a number of off-target effects. Information published so far relates for the most part to HIF-1α, although many of these agents may also affect HIF-2α. Specific inhibitors of HIF-1α and/or HIF-2α are currently under validation (Semenza, 2007; Onnis et al, 2010).

In the present study, HIF-1α inhibition was obtained through the use of Topotecan, a chemotherapeutic in use in the management of ovarian and small cell lung cancer. Topotecan it is a Top-1 poison that inhibits HIF-1α translation by a Top1-dependent but DNA damage-independent mechanism, suggesting that its effect as HIF-1α inhibitor could be mechanistically distinguished from those characterizing its cytotoxic activity. One of the criticisms involving compounds such as Topotecan is that these are drugs that have already failed, as single citotoxic agents, to show significant anticancer effects in clinical trials: however, the key distinction that must be made is that their current utilization as HIF inhibitors involves frequent administration at lower doses in an effort to maintain continuous inhibition of HIF activity (Semenza, 2012). As evidenced by our experiments, the presence of Topotecan (administrated at subtoxic concentrations, so that it could exert its role as HIF-1 inhibitor to a greater extent compared to its activity as a cytotoxic drug) determined a restore in the effectiveness of NVP-AEW541 in hypoxia following the reduction in the levels of IGF-2 (both mRNA and protein) as a consequence of HIF-1α inhibition, demonstrated not only in the levels of HIF-1α protein but also in the levels of HIF-1 target genes (the already mentioned IGF-2 but also VEGF-A and PDK1). IR, as well as IGF-1R, were un-phosphorylated after treatment and the efficacy on

migration was especially noticeable.

A similar effect on migration, if not even more prominent, was also observed following the use of Luteolin, a natural compound belonging to the flavonoid class that can be found as integral components of the human diet and possess anti-proliferative and anti-migratory activity: in this case, despite the inhibition of HIF-1α investigated by Western Blot resulted not significant at the cellular level, we could observe a reduction in the levels of IGF-2 (both at mRNA and protein level) and a synergistic effect with NVP-AEW541.

The case of Luteolin is more complex in regard to its effect on HIF-1. Literature data report how this compound seems in fact to be able to inhibit HIF-1 nuclear activity more rather than affect α subunit levels, that were reported to be increased or decreased in different studies (Hasebe et al, 2003; Zhang et al, 2009). Anyway, starting from our data the most interesting feature of this compound, more than its activity on HIFs, resulted to be its anti-migratory potential: the use of Luteolin, as already stated, completely inhibited migration in MDA-231. However, the use of Luteolin in chemotherapy is far from being near: apart from the need to test its effective bioavailability, the need to use high concentration of the compound even in in vitro experiments and the observation after treatment of a general inhibition in mRNA transcription may be incompatible with its use in therapy.

As a last point in our study, we focused our attention on the metabolic alteration determined by the modulation of insulin/IGF system. Since deregulation of cellular energy metabolism is considered an increasingly important hallmark of cancer, IR and its related metabolic syndromes have become another major focus in the breast cancer research and treatment field. Both obesity and type 2 diabetes mellitus could lead to hyperinsulinemia, which has been reported to activate insulin receptors in normal breast epithelial cells and in neoplastic tissues, increase the risk of developing breast cancer in patients with metabolic syndromes, promote metastatic progression, and associate with poor prognosis in breast cancer patients (Yang and Yee, 2012). Moreover, as already reported above, the pharmacological modulation of a system as fundamental for metabolism as it is the one comprising insulin and IGFs rises the necessity to analyze in depth the effects of this modulation.

Preliminary analysis of the oxidative and glycolitic profiles of three cell lines showed in first stance an interesting difference at the basal level. MCF-7, in fact, notoriously the less aggressive of the three, resulted to have a glycolitic profile closer to normal cells than MDA-231 or T47D, which showed an increase in glycolysis even in presence of oxygen, the so called Warburg effect nowadays considered a hallmark in cancer. This increase in glycolysis is accompanied by a reduction in the oxidative processes, in agreement with literature data. What is even more interesting is the fact that when treated with NVP-AEW541 combined with MAB292 all cell lines showed an increase in the efficiency of the oxidative phosphorilation. The same result was obtained after treatment with Luteolin. Further experiments would be needed to assess the effective impact of these compounds on cellular metabolism, as well as the metabolic processes and responses in hypoxia, which could not be analyzed in this study. A more specific and in depth analysis also of the glycolitic process is necessary to assess the effects of IGF inhibition on metabolism: our preliminary study indeed showed

modifications also in the glycolitic efficiency that need to be further investigated.

Overall, we can conclude that the combined inhibition of IGF-1R and HIF-1 appears to result in a synergistic effect on the migratory capacity of breast cancer cells in this study and it may therefore be a really interesting therapeutic strategy, especially given the close relationship shared by the two systems and the consideration that the use of this combined therapy could improve to outcome in patients diagnosed with metastases that eventually become unresponsive to systemic therapy. In addition to their use in overcoming the resistance to classical therapies, the inhibition of IGF and HIF system could be used at the first stages of breast cancer to prevent the progression to MBC.

Thus, in the present study we have been able to observe that the activation of HIF-1 leads to a variation in the levels of transcription of genes of the IGF system involved in proliferation and migration (in particular an increase in IGF-2) resulting in the creation of a mechanism of resistance to IGF-1R specific inhibition. On the other hand, it is also interesting to observe as a last consideration how the activation of IGF-1R as a result of exposure to both ligands lead to an increase in the transcription of genes HIF-1 dependent (such as VEGF and PDK1, in addition to the already mentioned IGF-2) suggesting the existence of an autocrine loop of survival in the cell lines under examination (at least in MDA-231) and thus making it even more interesting to explore the possibility to combine the inhibition of the two systems.

References

Acker T et al. **Genetic evidence for a tumor suppressor role of HIF-2α.** *Cancer Cell* 2005, 8:131–141.

Ahlgren M et al. **Growth patterns and the risk of breast cancer in women.** *N Engl J Med* 2004, 351:1619–1626.

Alvarez RH et al. **Emerging targeted therapies for breast cancer.** *J Clin Oncol* 2010, 28:3366-3379.

Annunziata M et al. **The IGF system.** *Acta Diabetol* 2011, 48:1–9.

Ansò E et al. **Flavonoids inhibit hypoxia-induced vascular endothelial growth factor expression by a HIF-1 independent mechanism.** *Biochem Pharmacol* 2010, 79(11):1600-1609.

Atchley DP et al. **Clinical and pathologic characteristics of patients with BRCA-positive and BRCA-negative breast cancer.** *J Clin Oncol* 2008, 26:4282–4288.

Autier P et al. **Disparities in breast cancer mortality trends between 30 European countries: retrospective trend analysis of WHO mortality database.** *Br Med J* 2010, 341:c3620.

Barker AD et al. **I-SPY 2: an adaptive breast cancer trial design in the setting of neoadjuvant chemotherapy**. *Clin Pharmacol Ther* 2009, 86(1):97–100.

Beatson GT. **On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases.** *Lancet* 1896, 148:b104–b107.

Belfiore A and Frasca F. **IGF and insulin receptor signaling in breast cancer.** *J Mammary Gland Biol Neoplasia* 2008, 13:381–406.

Belfiore A et al. **Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease.** *Endocr Rev* 2009, 30(6):586–623.

Bellot G et al. **Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains.** *Mol Cell Biol* 2009, 29:2570–2581.

Bensaad K et al. **TIGAR, a p53-inducible regulator of glycolysis and apoptosis.** *Cell* 2006, 126:107–120.

Beslija S et al. **Third consensus on medical treatment of metastatic breast cancer.** *Ann Oncol* 2009, 20:1771-1785.

Blundell TL et al. **Tertiary structures, receptor binding, and antigenicity of insulin like growth factors.** *Fed Proc* 1983, 42(9):2592–2597.

Boyle P. **The globalisation of cancer.** *Lancet* 2006, 368:629–630.

Boyle P. **Triple-negative breast cancer: epidemiological considerations and recommendations.** *Annals of Oncology* 2012, 23(Suppl6):vi7–vi12.

Braunstein S et al. **A hypoxia-controlled cap-dependent to cap-independent translation switch in breast cancer.** *Mol Cell* 2007, 28:501–512.

Browne BC et al. **Inhibition of IGF1R activity enhances response to trastuzumab in HER-2-positive breast cancer cells.** *Ann Oncol* 2011, 22(1):68–73.

Buck E et al. **Compensatory Insulin Receptor (IR) Activation on Inhibition of Insulin-Like Growth Factor-1 Receptor (IGF-1R): Rationale for Cotargeting IGF-1R and IR in Cancer.** *Mol Cancer Ther* 2010, 9:2652-2664.

Burstein HJ. **Novel agents and future directions for refractory breast cancer.** *Seminars in Oncology* 2011, 38(Suppl2):S17–24.

Campbell RA et al. **Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance.** *J Biol Chem* 2001, 276:9817–9824.

Casa AJ et al. **Estrogen and insulin-like growth factor-I (IGF-I) independently down-regulate critical repressors of breast cancer growth.** *Breast Canc Res Treat* 2012, 132(1):61–73.

Casagrande F and Darbon JM. **Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cycle-dependent kinases CDK2 and CDK1.** *Biochem Pharmacol*

References

2001, 61:1205–1215.

Cho J et al. **Cobalt chloride induced estrogen receptor alpha down-regulation involves hypoxia-inducible factor-1alpha in MCF-7 human breast cancer cells.** *Mol Endocrinol* 2005, 19:1191–1199.

Clemmons DR. **Involvement of insulin-like growth factor-i in the control of glucose homeostasis.** *Curr Opin Pharmacol* 2006, 6(6):620–625.

Colao A. **The gh-igf-i axis and the cardiovascular system: clinical implications.** *Clin Endocrinol (Oxf)* 2008, 69(3):347–358.

Conte PF and Guarneri V. **The Next Generation of Biologic Agents: Therapeutic Role in Relation to Existing Therapies in Metastatic Breast Cancer.** *Clinical Breast Cancer* 2012, 12(3):157-166.

Cooper AP. **The Anatomy of the Breast.** London: Longman, Orme, Green, Brown and Longmans, 1840.

Creighton CJ et al. **Insulin-like growth factor-I activates gene transcription programs strongly associated with poor breast cancer prognosis.** *J Clin Oncol* 2008, 26:4078–4085.

Dales JP et al. **Overexpression of hypoxia-inducible factor HIF-1alpha predicts early relapse in breast cancer: retrospective study in a series of 745 patients.** *Int J Cancer* 2005, 116:734–739.

Daughaday WH et al. **On the nomenclature of the somatomedins and insulin-like growth factors.** *J Clin Endocrinol Metab* 1987, 65(5):1075–1076.

Davison Z et al. **Insulin-like growth factor-dependent proliferation and survival of triple negative breast cancer cells: implications for therapy.** *Neoplasia* 2011, 13:504–515.

De Meyts P. **Insulin and its receptor: structure, function and evolution.** *Bioessays* 2004, 26:1351–1362.

DeBerardinis RJ et al. **Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis.** *Proc Natl Acad Sci USA* 2007, 104:19345–19350.

De Berardinis RJ and Thompson CB. **Cellular Metabolism and Disease: What Do Metabolic Outliers Teach Us?** *Cell* 2012, 148:1132-1144.

Diaz LK et al. **Triple negative breast carcinoma and the basal phenotype: from expression profiling to clinical practice.** *Adv Anat Pathol* 2007, 14:419–430.

Dong MQ et al. **Quantitative mass spectrometry identifies insulin signaling targets in *C. elegans*.** *Science* 2007, 317:660-663.

Duguay SJ et al. **Post-translational processing of the insulin-like growth factor-2 precursor: Analysis of O-glycosylation and endoproteolysis.** *J Biol Chem* 1998, 273:18443–18451.

Dunn SE et al. **A dominant negative mutant of the insulin-like growth factor-I receptor inhibits the adhesion, invasion, and metastasis of breast cancer.** *Cancer Res* 1998, 58:3353–61.

Efeyan A and Sabatini DM. **mTOR and cancer: many loops in one pathway.** *Current Opinion in Cell Biology* 2010, 22:169–76.

El-Shewy HM and Luttrell LM. **Insulin-like growth factor-2/mannose-6 phosphate receptors.** *Vitam Horm* 2009, 80:667–697.

Esteban MA et al. **Regulation of E-cadherin expression by VHL and hypoxia-inducible factor.** *Cancer Res* 2006, 66:3567–3575.

Esteva FJ et al. **Phase II study of weekly docetaxel and trastuzumab for patients with HER-2-overexpressing metastatic breast cancer.** *J Clin Oncol* 2002, 20(7):1800–1808.

Evans JM et al. **Metformin and reduced risk of cancer in diabetic patients.** *BMJ* 2005, 330:1304–1305.

Fagan DH et al. **Acquired resistance to tamoxifen is associated with loss of the type I insulin-like growth factor receptor (IGF1R): implications for breast cancer treatment.** *Cancer Res* 2012, Epub ahead of print.

Firth SM and Baxter RC. **Cellular actions of the insulin-like growth factor binding proteins.** *Endocr Rev*

2002, 23(6):824–854.

Forbes JF et al. **Effect of anastrozole and tamoxifen as adju-vant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial.** *Lancet Oncology* 2008, 9:45–53.

Frasca F et al. **Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells.** *Mol Cell Biol* 1999, 19:3278–3288.

Frezza C et al. **Inborn and acquired metabolic defects in cancer.** *J Mol Med* 2011, 89:213–220.

Froesch ER et al. **Actions of insulin-like growth factors.** *Annu Rev Physiol* 1985, 47:443-467.

Gao J et al. **Targeting the Insulin-like Growth Factor Axis for the Development of Novel Therapeutics in Oncology.** *Cancer Res* 2012, 72(1):3–12.

Gao J et al. **Dual IGF-I/II-neutralizing antibody MEDI- 573 potently inhibits IGF signaling and tumor growth.** *Cancer Res* 2011, 71:1029–1040.

Gao P et al. **c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism.** *Nature* 2009, 458:762–765.

Garcia-Echeverria C et al. **In vivo antitumor activity of NVP-AEW541—A novel, potent, and selective inhibitor of the IGF-IR kinase.** *Cancer Cell* 2004, 5:231-239.

Garofalo C et al. **Efficacy of and resistance to anti-IGF-1R therapies in Ewing's sarcoma is dependent on insulin receptor signaling**. *Oncogene* 2011, 30:2730–40.

Giannoukakis N et al. **Parental genomic imprinting of the human IGF2 gene.** *Nat Genet* 1993, 4:98–101.

Gibbons JJ et al. **Mammalian target of rapamycin: discovery of rapamycin reveals a signaling pathway important for normal and cancer cell growth.** *Semin Oncol* 2009, 36(Suppl3):S3–S17.

Giovannucci E et al. **A prospective study of plasma insulin-like growth factor-1 and binding protein-3 and risk of colorectal neoplasia in women.** *Cancer Epidemiol Biomarkers* Prev 2000, 9:345–349.

Going JJ and Mohun TJ. **Human breast duct anatomy, the 'sick lobe' hypothesis and intraductal approaches to breast cancer.** *Breast Cancer Res Treat* 2006, 97:285–291.

Goodwin PJ et al. **Fasting insulin and outcome in early-stage breast cancer: results of a prospective cohort study.** *J Clin Oncol* 2002, 20:42–51.

Goss PE et al. **Efficacy of letrozole extended adjuvant therapy according to estrogen receptor and progesterone receptor status of the primary tumor: National Cancer Institute of Canada Clinical Trials Group MA.17.** *Journal of Clinical Oncology* 2007, 25:2006–2011.

Greer SN et al. **The updated biology of hypoxia-inducible factor.** *The EMBO Journal* 2012, 31:2448–2460.

Groot AJ et al. **Identification by phage display of single-domain antibody fragments specific for the ODD domain in hypoxia-inducible factor1alpha.** *Lab Invest* 2006, 86:345-356.

Groot AJ et al. **Conditional inactivation of HIF-1α using intrabodies.** *Cell Oncol* 2008, 30(5):397-409.

Gualberto A et al. **Characterization of NSCLC patients responding to anti-IGF-IR therapy.** ASCO Annual Meeting *on J Clin Oncol* 2008, 26 (Suppl): abstr 8000.

Gualberto A et al. **Pre-treatment levels of circulating free IGF-1 identify NSCLC patients who derive clinical benefit from figitumumab.** *Br J Cancer* 2011, 104:68–74.

Guarneri V and Conte P. **Metastatic breast cancer: therapeutic options according to molecular subtypes and prior adjuvant therapy.** *Oncologist* 2009, 14:645-656.

Guo G et al. **Manganese superoxide dismutase-mediated gene expression in radiation-induced adaptive responses.** *Moll Cell Biol* 2003, 23:2362-2378.

Hanahan D and Weinberg RA. **Hallmarks of cancer: the next generation.** *Cell* 2011, 144:646–674.

Hardie DG. **AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function.**

References

Genes Dev 2011, 25:1895–1908.

Harris LK and Westwood M. **Biology and significance of signalling pathways activated by IGF-II.** *Growth Factors* 2012, 30(1):1–12.

Hasebe Y et al. **Specific Inhibition of Hypoxia-inducible Factor (HIF)-1a Activation and of Vascular Endothelial Growth Factor (VEGF) Production by Flavonoids.** *Biol Pharm Bull* 2003, 26(10):1379-1383.

Hassiotou F and Geddes DT. **Anatomy of the Human Mammary Gland: Current Status of Knowledge.** *Clinical Anatomy* 2012, 26(1):29–48.

Heikkila M et al. **Roles of the human hypoxia-inducible factor (HIF)-3α variants in the hypoxia response.** *Cell Mol Life Sci* 2011, 68:3885–3901.

Helle SI. **The insulin-like growth factor system in advanced breast cancer.** *Best Practice & Research Clinical Endocrinology & Metabolism* 2004, 18(1):67–79.

Hirose K et al. **cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS factor (Arnt2) with close sequence similarity to the Aryl hydrocarbon receptor nuclear translocator (Arnt).** Mol. Cell. Biol. 1996, 16:1706–1713.

Hockel M and Vaupel P. **Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects.** *J Natl Cancer Inst* 2001, 93:266–276.

Hu Y et al. **Recent agents targeting HIF-1α for cancer therapy.** *J Cell Biochem* 2012, Epub ahead of print.

Isakoff SJ et al. **TBCRC009: a multicenter phase II study of cisplatin or carboplatin for metastatic triple-negative breast cancer and evaluation of p63/p73 as a biomarker of response.** ASCO Annual Meeting on *J Clin Oncol* 2011, 29(Suppl): abstr 1025.

Jassem J et al. **Randomized, open label, phase III trial of figitumumab in combination with paclitaxel and carboplatin versus paclitaxel and carboplatin in patients with non-small cell lung cancer (NSCLC).** ASCO Annual Meeting on *J Clin Oncol* 2010, 28(Suppl): abstr 7500, 15s.

Johnston SR. **Clinical efforts to combine endocrine agents with targeted therapies against epidermal growth factor receptor/human epidermal growth factor receptor 2 and mammalian target of rapamycin in breast cancer.** *Clin Cancer Res* 2006, 12:1061s–8s.

Jones RG et al. **AMP-activated protein kinase induces a p53-dependent metabolic checkpoint.** *Mol Cell* 2005, 18:283–293.

Juncker-Jensen A et al. **Insulin-like growth factor binding protein 2 is a marker for antiestrogen resistant human breast cancer cell lines but is not a major growth regulator**. *Growth Horm IGF Res* 2006, 16:224–239.

Jung CR et al. **E2-EPF UCP targets pVHL for degradation and associates with tumor growth and metastasis.** *Nat Med* 2006, 12:809-816.

Kaelin WG. **Von hippel-lindau disease**. *Annu Rev Pathol* 2007, 2:145–173.

Kanadaswami C et al. **The Antitumor Activities of Flavonoids.** *In vivo* 2005, 19:895-910.

Karamouzis MV and Papavassiliou AG. **Targeting insulin-like growth factor in breast cancer therapeutics.** *Crit Rev Oncol Hematol* 2012, 84:8–17.

Kaufman PA et al. A **Randomized, Double-Blind, Placebo-Controlled, Phase 2 Study of AMG 479 With Exemestane (E) or Fulvestrant (F) in Postmenopausal Women With Hormone-Receptor Positive (HR+) Metastatic (M) or Locally Advanced (LA) Breast Cancer (BC).** *Cancer Res* 2010, 70(76s).

Keith B et al. **Targeted mutation of the murine arylhydrocarbon receptor nuclear translocator 2 (Arnt2) gene reveals partial redundancy with Arnt.** *Proc Natl Acad Sci USA* 2001, 98:6692–6697.

Keith B et al. **HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression.** *Nat Rev Cancer* 2012, 12:9-22.

Kim JW et al. **HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia.** *Cell Metab* 2006, 3:177–185.

Koh MY et al. **HIF-1 regulation: not so easy come, easy go.** *Trends Biochem Sci* 2008, 33 (11):526-534.

Kreike B et al. **Gene expression profiling and histopathological characterization of triple-negative/basallike breast carcinomas.** *Breast Cancer Res* 2007, 9:R65.

Kuhlmann JD et al. **Loss of heterozygosity proximal to the M6P/IGF2R locus is predictive for the presence of disseminated tumor cells in the bone marrow of ovarian cancer patients before and after chemotherapy.** *Genes Chromosomes Cancer* 2011, 50(8):598–605.

Kummar S et al. **Multihistology, target-driven pilot trial of oral topotecan as an inhibitor of hypoxia-inducible factor-1a in advanced solid tumors.** *Clin Cancer Res* 2011, 17:5123–5131.

Kung et al. **Small molecule blockade of transcriptional coactivation of the hypoxia-inducible factor pathway**. *Cancer Cell* 2004, 6:33-43.

Kurmasheva RT et al. **The insulin-like growth factor-1 receptor-targeting antibody, CP-751,871, suppresses tumor-derived VEGF and synergizes with rapamycin in models of childhood sarcoma.** *Cancer Res* 2009, 69:7662–7671.

Law JH et al. **Phosphorylated insulin-like growth factor-i/insulin receptor is present in all breast cancer subtypes and is related to poor survival.** *Cancer Res* 2008, 68:10238-10246.

Lawrence MC et al. **Insulin receptor structure and its implications for the IGF-1 receptor.** *Curr Opin Struct Biol* 2007, 17: 699–705.

Le Roith D. **The insulin-like growth factor system.** *Exp Diabesity Res* 2003, 4(4):205–212.

Lee K et al. **Acriflavine inhibits HIF-1 dimerization, tumor growth, and vascularization.** *Proc Natl Acad Sci USA* 2009, 106:17910–17915.

Lerma E et al. **Immunohistochemical heterogeneity of breast carcinomas negative for estrogen receptors, progesterone receptors and HER2/neu (basal-like breast carcinomas).** *Mod Pathol* 2007, 20:1200–1207.

Li CI et al. **Risk of mortality by histologic type of breast cancer among women aged 50 to 79 years.** *Arch Intern Med* 2003, 163:2149–2153.

Liao D and Johnson RS. **Hypoxia: a key regulator of angiogenesis in cancer.** *Cancer Metastasis Rev* 2007, 26:281–290.

Liedtke C and Kiesel L. **Breast cancer molecular subtypes – Modern therapeutic concepts for targeted therapy of a heterogeneous entity.** *Maturitas* 2012, Dec;73(4):288-294.

Liedtke C et al. **Response to neoadjuvant therapy and longterm survival in patients with triple-negative breast cancer.** *J Clin Oncol* 2008, 26:1275–1281.

Lin X et al. **A chemical genomics screen highlights the essential role of mitochondria in HIF-1 regulation.** *Proc Natl Acad Sci* USA 2008, 105:174–179.

Liu YV et al. **RACK1 competes with HSP90 for binding to HIF-1a and is required for O2-independent and HSP90 inhibitor-induced degradation of HIF-1α.** *Mol Cell* 2007.1, 25:207–217.

Liu YV et al. **Calcineurin promotes hypoxia-inducible factor 1α expression by dephosphorylating RACK1 and blocking RACK1 dimerization.** *J Biol Chem* 2007.2, 282:37064–37073.

Loges S et al. **Silencing or fueling metastasis with VEGF inhibitors: antiangiogenesis revisited.** *Cancer Cell* 2009, 15:167–170.

Love SM and Barsky SH. **Anatomy of the nipple and breast ducts revisited.** *Cancer* 2004, 101:1947–1957.

Lu X et al. **ADAMTS1 and MMP1 proteolytically engage EGF-like ligands in an osteolytic signaling cascade for bone metastasis.** *Genes Dev* 2009, 23:1882–1894.

Lum JJ et al. **Growth factor regulation of autophagy and cell survival in the absence of apoptosis.** *Cell* 2005, 120:237–248.

Lundgren K et al. **Hypoxia and breast cancer: prognostic and therapeutic Implications.** *Cell Mol Life Sci*

References

2007, 64:3233–3247.

Luo W et al. **Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1.** *Cell* 2011, 145(5):732–744.

Maki RG. **Small Is Beautiful: Insulin-Like Growth Factors and Their Role in Growth, Development, and Cancer.** *J Clin Oncol* 2010, 28:4985-4995.

Mann JR et al. **Methylated DNA sequences in genomic imprinting.** *Crit Rev Eukaryot Gene Expr* 2000, 10:241–257.

Maranchie JK et al. **The contribution of VHL substrate binding and HIF1-α to the phenotype of VHL loss in renal cell carcinoma.** *Cancer Cell* 2002, 1:247–255.

Marks AG et al. **Plasma distribution and signaling activities of IGF-II precursors.** *Endocrinology* 2011, 152:922–930.

Martin JL and Baxter RC. **Signalling pathways of insulin-like growth factors (IGFs) and IGF binding protein-3.** *Growth Factors* 2011, 29(6):235–244.

Martin-Kleiner I and Gall TK. **Mannose-6-phosphate/insulinlike growth factor 2 receptor (M6P/IGF2R) in carcinogenesis.** *Cancer Lett* 2010, 289:11–22.

McCann AH et al. **Biallelic expression of the IGF2 gene in human breast disease.** *Hum Mol Genet* 1996, 5:1123–1127.

McKnight SL. **On getting there from here.** *Science* 2010, 330:1338–1339.

Medina D. **The mammary gland: A unique organ for the study of development and tumorigenesis.** *J Mammary Gland Biol Neopasia* 1996, 1:5–19.

Melillo G. **Hypoxia-inducible factor 1 inhibitors.** *Methods Enzymol* 2007, 435:385–402.

Menefee M et al. **MEDI-573, a dual IGF-1/-2 neutralizing antibody, blocks IGF-1R and IR-A signaling and maintains glucose homeostasis in a Phase 1 study for advanced solid tumors.** 22nd EORTC NCI-AACR Symposium 2010, (Berlin and Philadelphia: AACR Abstr 3LB).

Metallo CM et al. **Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia.** *Nature* 2011, 481(7381):380–384.

Moffat DF and Going JJ. **Three dimensional anatomy of complete duct systems in human breast: Pathological and developmental implications.** *J Clin Pathol* 1996, 49:48–52.

Moeller BJ et al. **Hypoxia and radiotherapy: opportunities for improved outcomes in cancer treatment.** *Cancer Metastasis Rev* 2007, 26:241–248.

Molineaux SM. **Molecular pathways: targeting proteasomal protein degradation in cancer.** *Clin Cancer Res* 2012, 18:15–20.

Morgan DO et al. **Insulin-like growth factor II receptor as a multifunctional binding protein.** *Nature* 1987, 329:301–307.

Mottet D et al. **Regulation of hypoxia-inducible factor-1α protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3b pathway in HepG2 cells.** *J Biol Chem* 2003, 278:31277–31285.

Mucaj V et al. **Effects of hypoxia and HIFs on cancer metabolism.** *Int J Hematol* 2012, 95:464–470.

Nielsen TO et al. **Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma.** *Clin Cancer Res* 2004, 10:5367–5374.

Nizet V and Johnson RS. **Interdependence of hypoxic and innate immune responses.** *Nat Rev Immunol* 2009, 9:609–617.

O'Gorman DB et al. **Decreased insulin-like growth factor-II/mannose 6-phosphate receptor expression enhances tumorigenicity in JEG-3 cells.** *Cancer Res* 1999, 59:5692–5694.

O'Gorman DB et al. **Insulin-like growth factor-II/mannose 6-phosphate receptor overexpression reduces growth of choriocarcinoma cells in vitro and in vivo.** *Endocrinology* 2002, 143:4287–4294.

Olmos D et al. **Targeting the insulin-like growth factor 1 receptor in Ewing's sarcoma: reality and expectations.** *Sarcoma* 2011, e pub 402508.

Onnis B et al. **Development of HIF-1 Inhibitors for Cancer Therapy.** *J Cell Mol Med* 2009, 13(9A):2780–2786.

Osborne CK et al. **Hormone responsive human breast cancer in long-term tissue culture: effect of insulin.** *Proc Natl Acad Sci USA* 1976, 73:4536–4540.

Osborne CK et al. **Gefitinib or placebo in combination with tamoxifen in patients with hormone receptor positive metastatic breast cancer: a randomized phase II study.** *Clin Cancer Res* 2011, 17(5):1147-1159.

Oskarsson T et al. **Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs.** *Nat Med* 2011, 17:867–874.

Perou CM et al. **Molecular portraits of human breast tumours.** *Nature* 2000, 406:747–752.

Pollak M. **Targeting insulin and insulin-like growth factor signalling in oncology**. *Curr Opin Pharmacol* 2008, 8:384–392.

Pollak M. **Insulin and insulin-like growth factor signalling in neoplasia.** *Nat Rev Cancer* 2008, 8(12):915-28. Erratum in: *Nat Rev Cancer* 2009, 9(3):224.

Pollak M. **The insulin and insulin-like growth factor receptor family in neoplasia: an update.** *Nat Rev Cancer* 2012, Feb 16;12(3):159-169.

Pollak M et al. **Presence of somatomedin receptors on primary human breast and colon carcinomas.** *Cancer Lett* 1987, 38:223–230.

Pravtcheva DD and Wise TL. **Metastasizing mammary carcinomas in H19 enhancers-Igf2 transgenic mice.** *J Exp Zool* 1998, 281:43–57.

Racker E. **History of the Pasteur effect and its pathobiology.** *Mol Cell Biochem* 1974, 5:17–23.

Rapisarda A et al. **Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway.** *Cancer Res* 2002, 62:4316–4324.

Rapisarda A et al. **Schedule-dependent inhibition of hypoxia-inducible factor-1alpha protein accumulation, angiogenesis, and tumor growth by topotecan in U251-HRE glioblastoma xenografts.** *Cancer Res* 2004, 64:6845–6848.

Rapisarda A et al. **Increased antitumor activity of bevacizumab in combination with hypoxia inducible factor-1 inhibition.** *Mol Cancer Ther* 2009, 8:1867–1877.

Ravi R et al. **Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α.** *Genes Dev* 2000, 14:34–44.

Reis-Filho JS and Tutt AN. **Triple negative tumours: a critical review.** *Histopathology* 2008, 52:108–118.

Rey S and Semenza GL. **Hypoxia-inducible factor-1- dependent mechanisms of vascularization and vascular remodelling.** *Cardiovasc Res* 2010, 86:236–242.

Richardson AE et al. **Insulin-like growth factor-2 (IGF-2) activates estrogen receptoralpha and -beta via the IGF-1 and the insulin receptors in breast cancer cells.** *Growth Factors* 2011, 29:82–93.

Rohwer N and Cramer T. Hypoxia-mediated drug resistance: novel insights on the functional interaction of HIFs and cell death pathways. Drug Resist Updat 2011, 14:191–201.

Ross JA and Kasum CM. **Dietary flavonoids: bioavailability, metabolic effects, and safety.** *Annu Rev Nutr* 2002, 22:19–34.

Rotwein P et al. **Organization and sequence of the human insulin-like growth factor I gene. Alternative RNA processing produces two insulin-like growth factor-I precursor peptides.** *J Biol Chem* 1986, 261(11):4828–4832.

References

Rusby JE et al. **Breast duct anatomy in the human nipple: Three-dimensional patterns and clinical implications.** *Breast Cancer Res Treat* 2007, 106:171–179.

Russo J and Russo IR, **Development of the human breast.** *Maturitas* 2004, 49:2–15.

Salmon WD Jr and Daughaday WH. **A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro.** *J Lab Clin Med* 1957, 49(6):825–836.

Sánchez-Muñoz A et al. **Targeted therapy of metastatic breast cancer.** *Clin Transl Oncol* 2009, 11:643-650.

Santamaria, G et al. **Efficient execution of cell death in nonglycolytic cells requires the generation of ROS controlled by the activity of mitochondrial H+-ATP synthase.** *Carcinogenesis* 2006, 27:925–935.

Schiff R et al. **Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance.** *Clinical Cancer Research* 2004, 10:331S–336S.

Schödel J et al. **High-resolution genome-wide mapping of HIF binding sites by ChIP-seq.** *Blood* 2011, 117:e207–e217.

Sciacca L et al. **Insulin receptor activation by IGF-II in breast cancers: Evidence for a new autocrine/paracrine mechanism.** *Oncogene* 1999, 18:2471–2479.

Semenza GL. **Evaluation of HIF-1 inhibitors as anticancer agents.** *Drug DiscovToday* 2007, 12:853–859.

Semenza GL. **Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics.** *Oncogene* 2010, 29:625–634.

Semenza GL. **HIF-1: upstream and downstream of cancer metabolism.** *Current Opinion in Genetics & Development* 2010.2, 20:51–56.

Semenza GL. **Oxygen sensing, homeostasis, and disease.** *N Engl J Med* 2011, 365:537–547.

Semenza GL. **Regulation of metabolism by hypoxia-inducible factor 1.** *Cold Spring Harb Symp Quant Biol* 2011.2, 76:347-53.

Semenza GL. **Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy.** *Trends in Pharmacological Sciences* 2012, 33(4):207-214.

Shanmugam M et al. **Targeting the Warburg effect in hematological malignancies: from PET to therapy.** *Curr Opin Oncol* 2009, 21(6):531–536.

Simon MC. **Mitochondrial reactive oxygen species are required for hypoxic HIFa stabilization.** *Adv Exp Med Biol* 2006, 588:165–170.

Singh SK et al. **Precursor IGF-II (proIGF-II) and mature IGF-II (mIGF-II) induce Bcl-2 And Bcl-X L expression through different signaling pathways in breast cancer cells.** *Growth Factors* 2008, 26:92–103.

Slamon DJ et al. **Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2.** *N Engl J Med* 2001, 344:783–792.

So A et al. **Insulin-like growth factor binding protein-2 is a novel therapeutic target associated with breast cancer.** *Clin Cancer Res* 2008, 14:6944–6954.

Sonenberg N and Hinnebusch AG. **New modes of translational control in development, behavior, and disease.** *Mol Cell* 2007, 28:721–729

Sotiriou C and Pusztai L. **Gene-expression signatures in breast cancer**. *N Engl J Med* 2009, 360:790–800.

Sussenbach JS et al. **Structure and expression of the human insulin-like growth factor genes.** *Growth Regul* 1992, 2(1):1–9

Tao Y et al. **Mechanisms of disease: signaling of the insulin-like growth factor 1 receptor pathway – therapeutic perspectives in cancer.** *Nat Clin Pract Oncol* 2007, 4:591–602.

Tanne JH. **FDA cancels approval for bevacizumab in advanced breast cancer.** *BMJ* 2011, 343:d7684.

Taunk NK et al. **Prognostic significance of IGF-1R expression in patients treated with breast-conserving**

surgery and radiation therapy. *Radiother Oncol* 2010, 96:204–208.

Teleman AA et al. **Nutritional control of protein biosynthetic capacity by insulin via Myc in *Drosophila.*** *Cell Metab* 2008, 7:21–32.

Terzuoli E et al. **Aminoflavone, a ligand of the aryl hydrocarbon receptor, inhibits HIF-1a expression in an AhR-independent fashion.** *Cancer Res* 2010, 70:6837–6848.

Tezer M et al. **Smooth muscle morphology in the nipple-areola complex.** *J Morphol Sci* 2011, 28:171–175.

Thompson CB. **Metabolic enzymes as oncogenes or tumor suppressors.** *N Engl J Med* 2009, 360:813–815.

To KK et al. **The phosphorylation status of PAS-B distinguishes HIF-1alpha from HIF-2alpha in NBS1 repression.** *EMBO J* 2006, 25:4784-4794.

Toretsky JA and Helman LJ. **Involvement of IGF-II in human cancer.** *J Endocrinol* 1996, 149:367–372.

Toschi A et al. **Differential dependence of hypoxia-inducible factors 1α and 2α on mTORC1 and mTORC2.** *J Biol Chem* 2008, 283:34495–34499.

Tricoli JV et al. **Localization of insulin-like growth factor genes to human chromosomes 11 and 12.** *Nature* 1984, 310(5980):784–786.

Ueda H et al. **Inhibitory effect of Perilla leaf extract and luteolin on mouse skin tumor promotion.** *Biol Pharm Bull* 2003, 26:560–563.

Ullrich A et al. **Insulin-like growth factor i receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity.** *EMBO J* 1986, 5(10):2503–2512.

Van den Beucken T et al. **Translational control of gene expression during hypoxia.** *Cancer Biol Ther* 2006, 5:749–755.

Van den Eynden GG et al. **A fibrotic focus is a prognostic factor and a surrogate marker for hypoxia and (lymph)angiogenesis in breast cancer: review of the literature and proposal on the criteria of evaluation.** *Histopathology* 2007, 51:440–451.

Vander Heiden MG et al. **Evidence for an alternative glycolytic pathway in rapidly proliferating cells.** *Science* 2010, 329:1492–1499.

Viale G and Bottiglieri L. **Pathological definition of triple negative breast cancer.** *Eur J Cancer* 2009, 45 (Suppl 1):5-10.

Vleugel MM et al. **Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in invasive breast cancer.** *J Clin Pathol* 2005, 58:172–177.

Wan X et al. **Rapamycin induces feedback activation of Akt signaling through an IGF-1Rdependent mechanism.** *Oncogene* 2007, 26(13):1932–1940.

Wang GL et al. **Hypoxia-inducible factor 1 is a basic-helix–loop– helix–PAS heterodimer regulated by cellular O2 tension.** *Proc Natl Acad Sci USA* 1995, 92:5510–5514.

Warburg O. **On the origin of cancer cells.** *Science* 1956, 123:309–314.

Ward PS and Thompson CB. **Metabolic Reprogramming: A Cancer Hallmark Even Warburg Did Not Anticipate.** *Cancer Cell* 2012, 21:297-308.

Winder T et al. **Use of insulin-like growth factor (IGF) pathway polymorphism IGFIR rs2016347 to predict tumor recurrence in estrogen receptor-positive breast cancer patients.** ASCO Annual Meeting on *J Clin Oncol* 2011, 29(Suppl): abstr538.

Wise TL and Pravtcheva DD. **Delayed onset of Igf2-induced mammary tumors in Igf2r transgenic mice.** *Cancer Res* 2006, 66:1327–1336.

Wong CC et al. **Hypoxia-inducible factor 1 is a master regulator of breast cancer metastatic niche formation.** *Proc Natl Acad Sci USA* 2011, 108:16369–16374.

Yang Y and Yee D. **Targeting Insulin and Insulin-Like Growth Factor Signaling in Breast Cancer.** *J*

References

Mammary Gland Biol Neoplasia 2012, Epub ahead of print.

Yerushalmi R et al**. Insulin-like growth factor (IGF-1R) in breast cancer subtypes.** *Breast Cancer Res Treat* 2012, 132:131–142.

Young RM et al. **Hypoxia-mediated selective mRNA translation by an internal ribosome entry site-independent mechanism.** *J Biol Chem* 2008, 283:16309–16319.

Zakikhani M et al. **Metformin is an AMP kinasedependent growth inhibitor for breast cancer cells.** *Cancer Res* 2006, 66:10269–10273.

Zeng X et al. **Enhancement of doxorubicin cytotoxicity of human cancer cells by tyrosine kinase inhibition of insulin receptor and type I IGF receptor.** *Breast Canc Res Treat* 2012, 133(1):117–126.

Zhang H et al. **Down-regulation of type I insulin-like growth factor receptor increases sensitivity of breast cancer cells to insulin.** *Cancer Res* 2007, 67:391–397.

Zhang H et al. **HIF-1-dependent expression of angiopoietin-like 4 and L1CAM mediates vascular metastasis of hypoxic breast cancer cells to the lungs.** *Oncogene* 2012, 31(14):1757-1770.

Zhao F. **Imatinib resistance associated with BCR–ABL upregulation is dependent on HIF-1a-induced metabolic reprogramming.** *Oncogene* 2010, 29:2962–2972.

Zhang Q et al. **Radiosensitization effect of luteolin on human gastric cancer SGC-7901 cells.** *J Biol Regul Homeost Agents* 2009, 23(2):71-78.

Zhou J and Brune B. **Cytokines and hormones in the regulation of hypoxia inducible factor-1a (HIF-1a).** *Cardiovasc Hematol Agents Med Chem* 2006, 4:189–197.

Acknowledgements

And here I am, again at the end of a “trip”. Sorry for the ones that are not really fond of English, but since all this work was done in English, I think I should finish in the same language…

This time I will be quicker in my acknowledgements than last time, but that doesn’t mean they will be less sincere; it’s just a sign of the time passing, and my wisdom -aka age- increasing!

The first mention goes without any doubt to the lab where I spent these three years: to Elena, the one that gave me the chance to do the job I would really like to go on doing after this experience, not only here but also on the other side of the Ocean, and gave me all the advice I needed; to Marzia, always there when I had questions, not only job-related; to all the students that crossed paths with mine for their stage. There were lots of them, but a special mention goes of course to the ones I spent more time with, that now are not just “the people of the lab” anymore, but Friends: Elisa, my little “Arciduchessa” and that’s saying all, because otherwise I’ll have to write a paper on you my dear; Chiara, my little “Pistonbrilla”, and I stop here with you also; SuperI; Gracile Gloria; Simon Binding; ENrika…

Sorry if I’m not writing down all the things I would like to say, but it would take really too long, so I’ll just tell you a very heartfelt THANK YOU for everything. And my only advice for the future is… don’t let our “comfort cabinet” become as empty as I found it coming back from the USA!

Talking about USA, a special mention is absolutely needed for the LONG list of people working with me in the TML (I know both of you remember it very well!!!): another heartfelt THANK YOU to Annamaria and Nikki, for everything you’ve done for me when I was there, on the other side of the Ocean, in the lab and outside. I’ll see you soon, you won’t easily free yourselves of me…eventually, I’m going to have some babysitting to do! And a big thank you also to Erik (don’t worry, I’m not forgetting of you either).

Coming back to this side of the Ocean, it’s decidedly time to mention the Friends I have here: you know who you are. Yes, all of you, that stayed with me through happy and sad times (and there had been lots of both of them). To the ones I know since I was a little bit older than a toddler (Anto, Sabry, Dany and then Sara and Laura, now with little Sofia!), the ones that came later though my life (Sarah, Luisa, Silvia, Angelo, Matteo, Andrea ect…), the ones that came with university and similar (Miky, Roby etc…) and the last ones (but not really in order of affection) appeared to stay in my life (Vale, Ste etc…). To all of you, that have never forgot about me even when I was far away even for so long, THANK YOU. You cannot even imagine what you mean to me; let’s just say A LOT.

And then, of course, the biggest THANK YOU goes to my family; mum, dad, Marco and granny. Because, as I already said in other occasions, you’ve always let me take my decisions and you supported all of them, always. And that’s the most comfort a person can find in her family. THANK YOU again.

At last, only a line to you, that will not be here, but will in fact BE WITH me. Thank you.

Acknowledgements

Okay, okay, here's the translation…

Ed eccomi qui, di nuovo alla fine di un "viaggio". Mi dispiace per quelli che non sono realmente appassionati di inglese, ma dato che tutto questo lavoro è stato fatto in inglese, penso dovrei finire nella stessa lingua ...

Questa volta sarò più veloce nei miei ringraziamenti dell'ultima volta, ma questo non significa che saranno meno sinceri, è solo un segno del tempo che passa, e della mia saggezza –o meglio età– che aumenta sempre più!

La prima menzione va senza alcun dubbio al laboratorio dove ho trascorso questi tre anni: a Elena, colei che mi ha dato la possibilità di fare il lavoro che vorrei davvero poter continuare a fare anche dopo questa esperienza, e non solo qui, ma anche dall'altra parte dell'Oceano, oltre ad avermi dato tutti i consigli di cui avevo bisogno; a Marzia, sempre presente quando avevo domande, e non solo di lavoro; a tutti gli studenti che hanno incrociato il proprio percorso con il mio per il loro stage. Sono stati tanti, ma una menzione speciale va ovviamente a quelli con cui ho trascorso più tempo, che ora non sono solo più "quelli del lab", ma Amici: Elisa, la mia piccola "Arciduchessa", e questo è dire tutto, perché altrimenti dovrei scrivere un paper su di te, mia cara; Chiara, la mia piccola "Pistonbrilla", e mi fermo qui anche con te; SuperI; Gracile Gloria; Simon Binding; Enrika ...

Scusate se non scrivo tutte le cose che vorrei dire, ma ci vorrebbe davvero troppo tempo, quindi mi limiterò a lasciarvi un molto sentito GRAZIE per tutto. E il mio unico consiglio per il futuro è ... non lasciate che il nostro "comfort cabinet" diventi vuoti come l'ho trovato di ritorno dagli Stati Uniti!

A proposito di USA, una menzione speciale è assolutamente necessaria per la LUNGA lista di persone che hanno lavorato con me in TML (so che voi due ve la ricordate molto bene!): un altro sentito GRAZIE ad Annamaria e Nikki, per tutto quello che avete fatto per me quando ero lì, dall'altra parte dell'Oceano, in laboratorio e fuori. Ci vediamo presto, non sarà facile liberarvi di me ... alla fine, mi aspetta un pò di lavoro da baby-sitter! E un grande grazie anche a Erik (non preoccuparti, non mi dimentico nemmeno di te).

Tornando al di qua dell'Oceano, è decisamente il momento di parlare degli amici che ho qui: sapete bene a chi mi riferisco. Sì, voi tutti, che siete rimasti con me nei momenti felici e tristi (e ce ne sono stati un sacco di entrambi). Per quelli che conosco da quando ero un pò più grande di un cucciolo (Anto, Sabry, Dany e Sara e Laura, ora con la piccola Sofia!), Quelli che sono arrivati in seguito nella la mia vita (Sarah, Luisa, Silvia, Angelo, Matteo, Andrea ecc ...), quelli che son arrivati con università e simili (Miky, Roby, ecc ...) e gli ultimi (ma decisamente non in ordine di affetto) apparsi per rimanere nella mia vita (Vale, Ste, ecc ...). A tutti voi, che non vi siete mai dimenticati di me, anche quando ero lontano, anche per così tanto tempo, GRAZIE. Non potete nemmeno immaginare cosa significhiate per me; diciamo solo TANTO.

E poi, naturalmente, il più grande GRAZIE va alla mia famiglia: mamma, papà, Marco e nonna. Perché, come ho già detto in altre occasioni, mi hanno sempre permeso di prendere le mie decisioni e sempre sostenuto, sempre. E questo è il massimo appoggio una persona possa trovare nella sua famiglia. GRAZIE di nuovo.

Infine, solo una linea per te, che non sarai qui, ma che in realtà SARAI CON me. Grazie.