HIF-2α: A REGULATOR OF AUTONOMOUS GROWTH IN OVARIAN CARCINOMA

Tahmina Omar

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the
M.Sc. program in Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine
University of Ottawa
Ottawa, Ontario, Canada

September 2012

Statement of Contribution

Gabriel Lachance performed the SKOV3 lentivirus infection and Josianne Payette carried out the OVCA429 transfection. Josianne Payette made the clones for both OVCA429 and SKOV3 controls and shHIF-2α.

James Uniacke did the [^{35}S]-Met experiments regarding global translation.

Joseph Perera provided the hypoxyprobe staining of the spheroid.

Aleksandra Franovic performed the RTK array of the SKOV3 cells.

ABSTRACT

Cancer develops in many organs and tissues in the body through genetic and environmental modifications to acquire the hallmarks of cancer. The hallmarks of cancer allow the cells to become malignant and progress to a tumorigenic state. It has previously been shown in various carcinomas that HIF-2α, a key component in hypoxia adaptation, has a role in autonomous growth, the first hallmark of cancer. Ovarian cancer is the most lethal of the gynecological malignancies and accounts for 3% of new cases in women annually but is the fifth most common cause of death due to cancer. Here, it is shown in two ovarian carcinoma cell lines that HIF-2α is involved in *in vitro* and *in vivo* growth. It is also shown that the effect of HIF-2α is due to its role in autonomous growth and not vascularization with the use of *in vitro* spheroids. From recent findings in the laboratory the oxygen-stimulated translation initiation complex was discovered and HIF-2α is one of its components. In the absence of HIF-2α there is a downregulation in translation in hypoxia in ovarian carcinoma. This is also seen in a HIF-2α translational target, IGF1R and its downstream signaling pathway, which may be involved in autonomous growth as well as other hallmarks of cancer. Taken together, the data in this thesis presents the importance of HIF-2α in autonomous growth and cancer progression in ovarian carcinoma, as well as verifying its role in translation.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

4E-BP	eIF4E-binding proteins
bHLH/PAS	Basic Helix-Loop-Helix/Per-Arnt-Sim
BrdU	5-bromo-2'-deoxyuridine
CA125	cancer antigen 125
CHIP	Carboxyl terminus of HSP70-interaction protein
C-TAD	C-terminal transactivation domain
Cul-2	Cullin-2
DNA	Deoxyribonucleic acid
DNMT3a	DNA methyl transferase 3a
EGFR	Epidermal growth factor receptor
eIFs	eukaryotic initiation factors
EMT	Epithelial-mesenchymal transition
EOC	Epithelial Ovarian Cancer
EPAS1	Endothelial PAS protein 1
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
G418	Neomycin
Glut-1	Glucose transporter-1
HAF	HIF-associated factor
HIFs	Hypoxia inducible factors
H&E	Haematoxylin and Eosin
HRE	Hypoxia response element
HSP70	Heat shock protein 70
IGFR1	Insulin growth factor 1 receptor
IRES	Internal ribosome entry site

ITS	Insulin, transferrin and selenium
m^7-GTP	7-methylguanosine
MEM	Minimum Essential Medium
mRNA	Messenger ribonucleic acids
mTOR	Mammalian target of rapamycin
ODDD	Oxygen dependent degradation domain
PBS	Phosphate buffered saline solution
PDGFR	Platelet derived growth factor receptor
PHDs	Prolyl hydroxylases
PI3K	Phosphatidylinositol 3-kinase
PLC-γ	Phospholipase C-γ
PMSF	Phenylmethylsulfonyl fluoride
PUMA	p-53-upregulated modulator of apoptosis
PVDF	Polyvinylidene difluoride
pVHL	VHL protein
Rbx-1	Ring box protein 1
RCC	Renal Clear Cell Carcinoma
rHRE	RNA hypoxia response element
RT	Reverse transcription
RTKs	Receptor tyrosine kinases
[^{35}S]-Met	Methionine labeled with radioactive sulfur-35
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
shRNA	Short hairpin ribonucleic acid
TGFα	Transforming growth factor-α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

TVS	Transvaginal ultrasonography
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular endothelial growth factor receptor
VHL	von Hipple-Lindau
VHL -/-	Biallelic inactivation VHL mutations

ACKNOWLEDGEMENTS

I would like to thank Dr. Stephen Lee for accepting me into his lab and giving me the opportunity to work on such a wonderful project. It has been a pleasure to be a part of his lab and an extremely rewarding experience. I not only have Dr. Lee to thank but also all the members of his lab. Everyone have been extremely helpful and supportive and I could not asked for a better lab to be a part of. Although my Masters has been somewhat of a bumpy road Dr. Lee has always been understanding, motivating, and assured me that there was light at the end of the tunnel. He has been the driving force encouraging me not to give up and to do the best work I am capable of, and for this I cannot express enough gratitude. I would also like to thank my loving parents and my two sisters because without them I would not have gotten this far. They have not allowed me to give up, been accommodating to me in many ways, and have given me everything and more. I am extremely grateful and privileged to have such a loving family. I want to thank everyone who has contributed in one way or another to my Masters because it has gotten me through to the end. Thank you.

1 INTRODUCTION

1.1 The Biology of Cancer

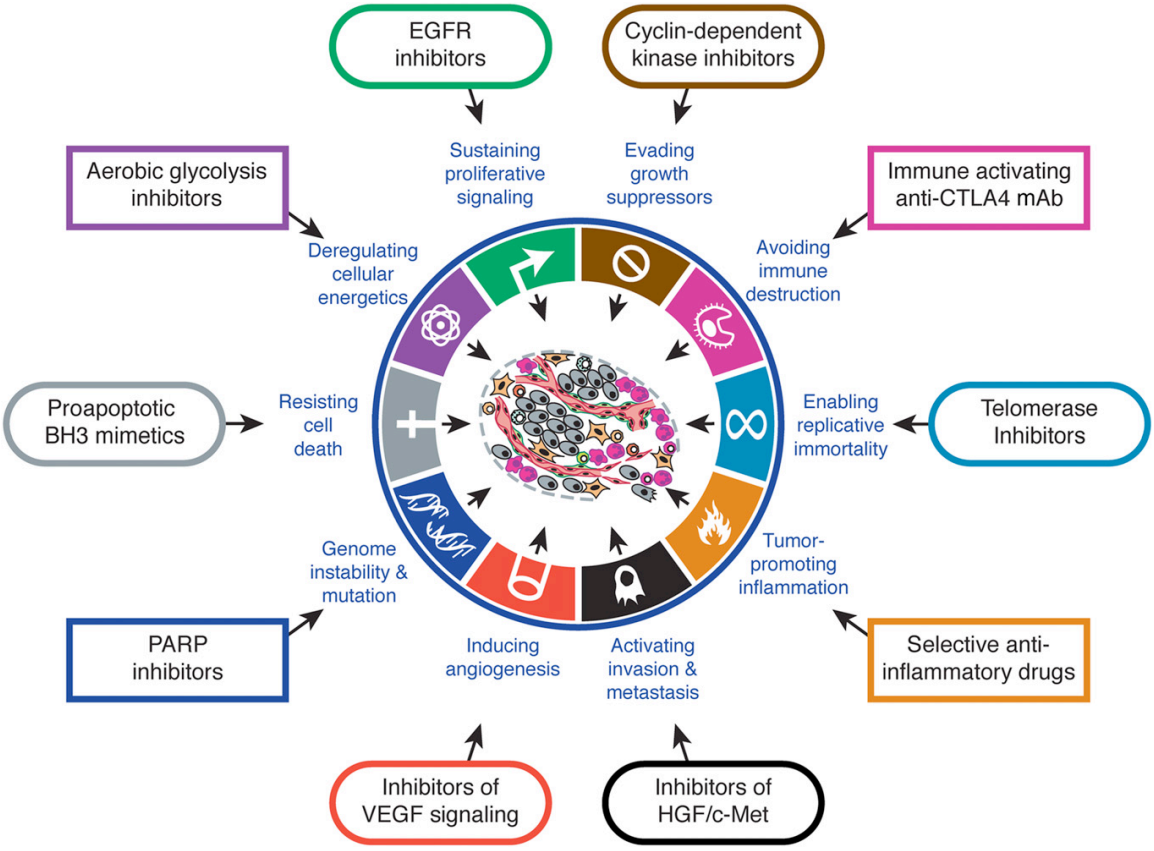
1.1.1 Characteristics of Cancer

Cancer does not define one disease; it is made of more than 200 individual diseases affecting various organs and tissues of the body. The various forms of cancers are named based on the organ or tissue from which they originate (Cancer Care Ontario, 2009). Many factors contribute to the incidence of cancer, including but not limited to, age, sex, race, environment, diet, infection by viruses and genetics (Canadian Cancer Society, 2012). Current statistics indicate 40% of women and 45% of men in Canada will develop cancer during their lifetime, resulting in a mortality rate in which approximately one out of every four Canadians will die from cancer (Canadian Cancer Society, 2011). Cancer arises from changes in the genome to become malignant as well as support from its tumor microenvironment. These mutations can produce either an oncogene with a dominant gain of function or a tumor suppressor with recessive loss of function (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). The transformation from a normal cell into a cancerous cell is a multistep process. A prominent feature of cancer is its ability to rapidly produce abnormal cells that grow into tumors and are able to metastasize to other areas of the body (Rous, 1910; The World Health Organization, 2010).

1.1.2 Hallmarks of Cancer

Cancer cells have defects in the regulatory systems that oversee cellular proliferation and homeostasis (Gatenby and Gillies, 2008). Hanahan and Weinberg have outlined the hallmarks of cancer that govern the alterations of normal cells to evolve progressively into a neoplastic state (Figure 1). The acquisition of all the hallmarks is necessary to allow cells to become malignant and form tumors. All types of human cancer share these particular traits, since mammalian cells contain similar machinery for proliferation, differentiation, and cell death. Therefore, regardless of the array of diversity seen among cancers, they all acquire these hallmark capabilities to become malignant (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). The acquisition of the hallmarks is enabled by genomic instability and mutations, tumor-promoting inflammation as well as the tumor microenvironment (Hanahan and Weinberg, 2011). These hallmarks breach the anticancer defense system to allow chronic proliferation, resistance to cell death, induction of angiogenesis, activation of invasion and metastasis, among others (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). The hallmarks of cancer outline the complexity of the disease with many cancer drug therapies targeted towards the hallmarks since cancer cannot progress without the acquisition of each hallmark (Figure 1) (Hanahan and Weinberg, 2011).

Figure 1. The hallmarks of cancer. Hanahan and Weinberg suggest that cancer cells acquire these 10 essential enabling traits and hallmarks to dictate malignant growth and progression. Many target therapies of cancer are directed towards molecular targets involved in enabling a particular hallmark (Hanahan and Weinberg, 2011).


EGFR inhibitors
Cyclin-dependent kinase inhibitors
Sustaining proliferative signaling
Evading growth suppressors
Aerobic glycolysis inhibitors
Immune activating anti-CTLA4 mAb
Deregulating cellular energetics
Avoiding immune destruction
Proapoptotic BH3 mimetics
Resisting cell death
Enabling replicative immortality
Telomerase Inhibitors
Genome instability & mutation
Tumor-promoting inflammation
PARP inhibitors
Selective anti-inflammatory drugs
Inducing angiogenesis
Activating invasion & metastasis
Inhibitors of VEGF signaling
Inhibitors of HGF/c-Met

1.1.3 Autonomous Growth

The first hallmark of cancer is sustaining proliferative signaling or autonomous growth, a fundamental trait of cancer cells allowing them to sustain proliferation independently (Figure 1) (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). For proliferation to occur growth signaling molecules are produced to instruct entry into cell division in a controlled manner to maintain homeostasis. Normal cells acquire the instructions for fundamental processes such as cell growth and division by paracrine secretion of growth factors from neighboring cells (Rafferty, 1975). Paracrine signaling is conveyed in large part by mesenchymal cells and the growth factors can then bind to cell surface receptors on neighboring cells and enable a signaling cascade (Donjacour and Cunha, 1991; Greil et al., 1989; James and Bradshaw, 1984). Cancer cells deregulate growth factor signaling (Sporn and Roberts, 1985; Sporn and Todaro, 1980). Some cell surface receptors contain an intracellular tyrosine kinase domain and its downstream pathways regulate the progression through the cell cycle and other biological processes, such as cell survival and energy metabolism (reviewed in Blume-Jensen and Hunter, 2001; Ullrich and Schlessinger, 1990). Autonomous growth can be achieved in multiple ways by cancer cells. One method is to produce the growth factor itself that is able to bind to its cognate receptor, resulting in autocrine proliferative stimulation triggering downstream pathways that initiate proliferation (Sporn and Roberts, 1985; reviewed in Blume-Jensen and Hunter, 2001; Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Witsch et al., 2010). Growth factor receptors containing an intracellular tyrosine kinase domain are known as receptor tyrosine kinases (RTKs) and are overexpressed and occasionally mutated in cancer cells (reviewed in Lemmon and Schlessinger, 2010).

1.2 Receptor Tyrosine Kinases

1.2.1 Structure and function of receptor tyrosine kinases (RTKs)

RTKs have important roles in the cell's proliferation and progression to a neoplastic state. They are a family of cell surface receptors discovered over 35 years ago consisting of 58 RTKs that fall into 20 subfamilies (Sporn and Roberts, 1985). RTKs have key roles in cellular processes such as proliferation, differentiation, cell survival, metabolism, migration, and cell-cycle control (reviewed in Blume-Jensen and Hunter, 2001; Lemmon and Schlessinger, 2010; Ullrich and Schlessinger, 1990). The receptors have similar structure consisting of a ligand binding domain in the extracellular region of the cell, a transmembrane helix, a cytoplasmic region that contains the tyrosine kinase domain, the carboxyl C-terminal and juxtamembrane autoinhibitory region (Hanks et al., 1988; Schlessinger, 1988; Williams, 1989; Yarden and Ullrich, 1988). RTK activation occurs by the growth factor binding to the receptor resulting in dimerization of the receptors (Schlessinger, 1988). Some RTKs are already dimerized or even form oligomers before growth factor binding. Regardless if the receptors are already dimerized or not, growth factor binding is still needed to activate the tyrosine kinases (Hammacher et al., 1989; Schlessinger, 1988; Soos and Siddle, 1989). In the dimer/oligomer complex one of the receptors is responsible for phosphorylating one or more tyrosines on the neighboring RTK. The phosphorylated RTK then serves as the site for assembly and activation of its downstream targets recruited by growth factor stimulation (Honegger et al., 1988a; Honegger et al., 1988b). Some of the intracellular signaling pathways activated by RTKs include extracellular signal-regulated kinases (ERK), phosphatidylinositol 3-kinase

(PI3K), and phospholipase C-γ (PLC-γ) as well as transcription factors such as signal transducers and activators of transcription (STAT) proteins (reviewed in Witsch et al., 2010).

1.2.2 Receptor tyrosine kinases in cancer

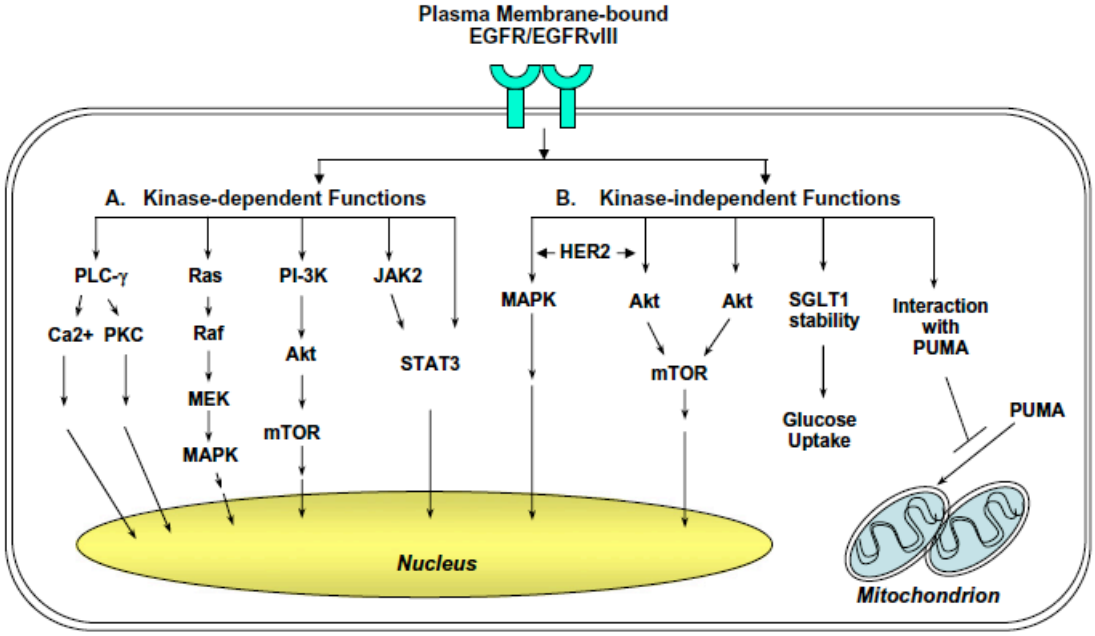
Mutations occurring in RTKs alter the activity, abundance, cellular distribution and regulation in cancer cells enhancing their survival and growth. Deregulated RTK activation in human cancer can occur by autocrine activation, chromosomal translocation, RTK overexpression or gain-of-function mutations (reviewed in Lemmon and Schlessinger, 2010). Cancer cells produce endogenous growth factors that act on its self by the external receptors. Malignant cell growth can be fueled by autocrine secretion of growth factors allowing them not to rely on paracrine secretion as normal cells do. This leads to a constitutive activation of the receptors known as an autocrine proliferation (Sporn and Roberts, 1985; Sporn and Todaro, 1980; reviewed in Blume-Jensen and Hunter, 2001; Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Sporn and Roberts, 1985). A common RTK family that is often altered in cancer is the ErbB family. In fact mutations in ErbB RTKs are present in >20% of solid tumors and in glioblastoma 35% have an overexpression in wild-type and mutated forms of the epidermal growth factor receptor (EGFR), among others (Libermann et al., 1985; Wong et al., 1987; reviewed in Lemmon and Schlessinger, 2010). In breast cancer 20-25% of patients have an increase in ErbB-2/HER2 expression that is associated with poor prognosis (Atlas et al., 2003). Amplification of insulin-like growth factor receptor-1 (IGF1R) is also seen in

breast cancer as well as melanoma leading to the generation of anti-apoptotic signals (Almeida et al., 1994). In the cases where RTKs are overexpressed, it enables the cells to become hyper-responsive to growth factors leading to autocrine activation of the transformed cells (Di Foire et al., 1987; reviewed in Witsch et al., 2010). RTKs are involved in the acquisition of growth autonomy as well as other hallmarks of cancer such as invasive growth, angiogenesis, resistance to apoptosis and evading growth suppression. For example, resistance to apoptosis is acquired by the activation of PI3K/Akt pathway by IGF1R activation inducing potent anti-apoptotic signals (Datta et al., 1997; Kulik et al., 1997; Shelton et al., 2004). The formation of new blood vessels is also important for tumor growth which are formed by vascular endothelial growth factor receptor (VEGFR) signaling (Lynden et al., 2001). These alterations affecting RTKs play a key role in the cell's transformation to a malignant cell with tumorigenic capability (reviewed in Lemmon and Schlessinger, 2010).

1.2.3 Epidermal growth factor receptor (EGFR) in cancer

EGFR is overexpressed in many cancers and is a major focus of targeted therapy (Di Foire et al., 1987; Libermann et al., 1985; Wong et al., 1987; reviewed in Han and Lo, 2012; Lemmon and Schlessinger, 2010 and Witsch et al., 2010). Once EGFR is dimerized it can recruit and activate downstream signaling molecules. Some of its main pathways include PLC-γ protein kinase C (PKC), and PI3K-Akt-mTOR (mTOR-mammalian target of rapamycin) (Figure 2) (reviwed in Han and Lo, 2012). Although this receptor is overexpressed in many cancers, gene amplification is a rare event. Gene

Figure 2. EGFR kinase dependent and independent functions. Kinase dependent function of EGFR/EGFRvIII recruits and phosphorylates downstream signaling molecules affecting growth, differentiation, migration, and inhibition to apoptosis. Kinase independent function of EGFR/EGFRvIII serves to sequester proapoptotic protein p-53-upregulated modulator of apoptosis (PUMA) in the cytoplasm and stabilize sodium-dependent glucose cotransporter-1 (SGLT1) for glucose uptake. Only EGFR and not a mutant form is able to enter the nucleus and act as a transcriptional co-factor for activation of genes involved in tumor proliferation (Han and Lo, 2012).


Plasma Membrane-bound
EGFR/EGFRvIII
A. Kinase-dependent Functions
B. Kinase-independent Functions
PLC-γ
Ca2+
PKC
Ras
Raf
MEK
MAPK
PI-3K
Akt
mTOR
JAK2
STAT3
HER2
MAPK
Akt
Akt
mTOR
SGLT1
stability
Glucose
Uptake
Interaction
with
PUMA
PUMA
Nucleus
Mitochondrion

amplification is found in glioblastoma allowing overexpression of not only the wild-type receptor but also of a mutant, EGFRvIII, lacking a region of its extracellular domain affecting its dimerization arm (Jeuken et al., 2009; Wong et al., 1987). The EGFRvIII mutant is constitutively active independent of ligand stimulation. Mutations are a rare occurrence in EGFR. However, 10% of patients with non-small cell lung cancer are responsive to treatment with EGFR inhibitor due to mutations in the tyrosine kinase domain (Lynch et al., 2004; Sharma at al., 2007). EGFR also has roles independent of its kinase activity affecting malignant tumor biology (Figure 2). EGFR as well as mutant EGFR that have lost its kinase capability are able to survive serum starvation induced cell death (Weihua et al., 2008). It is able to achieve this through interaction with other proteins such as p-53-upregulated modulator of apoptosis (PUMA), which is primarily located in the mitochondria but sequestered by EGFR in the cytoplasm (Figure 2) (Zhu et al., 2010). EGFR can be transported to the nucleus and mitochondria. Nuclear expression of EGFR is found in normal tissue but also in different cancer types such as breast, ovary, lungs, pancreas and glioma. EGFR transports from the cell surface to the nucleus serving as transcription co-factor and upregulating genes such as *cyclin D1* and *c-Myc* that are related to tumor proliferation (Jaganathan et al., 2011; Lin et al., 2001). The role of EGFR translocation to the mitochondria is not well understood but accumulation of mitochondrial EGFR leads to drug resistance to EGFR inhibitors in the treatment of cancers (Han and Lo, 2012). EGFR's presence in tumors is associated with advanced stage tumors, reduced patient survival and resistance to standard therapy (Arteaga, 2002). EGFR has multiple roles and functions aside from its role as a cell surface receptor in a wide range of cancers making it a prominent target for treatment.

1.2.4 Receptor tyrosine kinases as therapeutic targets

RTKs that are overexpressed or containing mutations play key role in the development and progression of cancer as well as many diseases and disorders. Monoclonal antibodies and tyrosine kinase inhibitors have been developed and approved for the treatment of cancers (Reichert and Valge-Archer, 2007; Shawver et al., 2002; reviewed in Lemmon and Schlessinger, 2010). Treatment with tyrosine kinase inhibitors and monoclonal antibodies often have a low to moderate effect on cancer but is enhanced when combined with chemotherapy or radiotherapy (Baselga et al., 2006). Thus far a monoclonal antibody to ErbB-2/HER2 (trastuzumab/Herceptin) has been used to treat breast cancer while a monoclonal antibody to EGFR (cetuximab/Erbitux) as well as tyrosine kinase inhibitors gefitinib (Iressa) and erlotinib (Tarceva) have been used to treat colorectal and head and neck cancer in combination with chemotherapy (Aboud-Pirak et al., 1988; Citri and Yarden, 2006; Reichert and Valge-Archer, 2007; Shawver et al., 2002). To inhibit VEGFR an antibody to its ligand vascular endothelial growth factor (VEGF) (bevacizumab/Avastin) has been manufactured allowing it to inhibit tumor angiogenesis and is used in the treatment of colorectal, lung, and breast cancer (Cobleigh et al., 2003; Johnson et al., 2004: Kabbinavar et al., 2003). Imatinib (Gleevec), a platelet derived growth factor receptor (PDGFR) and KIT inhibitor has been used to treat gastrointestinal-stromal tumors and mast cell leukemia (Shawver et al., 2002). An area of concern in targeted therapy of RTKs is resistance arising in patients treated with these options. Resistance occurs through selective means where new variant of the RTKs arise or overexpression of a different RTK occurs to compensate and continue growth (Engleman

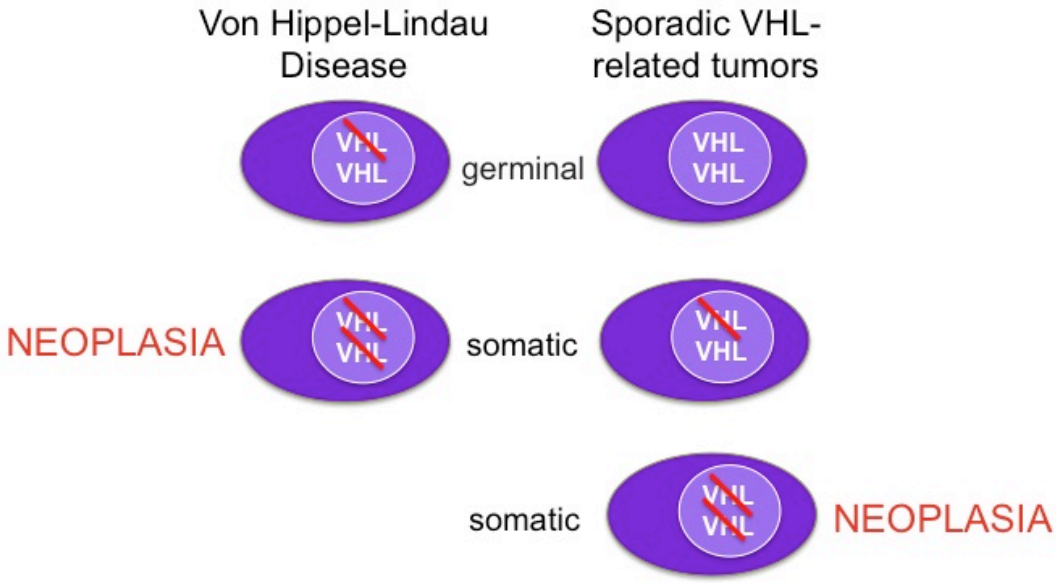
and Setleman, 2008; Sergina and Moasser, 2007). This compensatory mechanism of resistance is seen in breast cancer patients treated with ErbB-2/HER2 antibody who acquire IGF1R signaling to enable growth (Nahta et al., 2005; Sartore-Bianchi et al., 2009). Targeted therapy towards RTKs has been beneficial in the treatment of certain cancers but new therapy options are needed to avoid the issue of acquired resistance.

1.3 The VHL Tumor Suppressor Gene

1.3.1 Von Hippel-Lindau (VHL) Disease

Von Hippel-Lindau (VHL) disease is an autosomal dominantly inherited cancer syndrome (Collins, 1894; Lindau, 1927; Von Hippel, 1904). Individuals with this disease are germline heterozygotes, but tumor development occurs when the remaining wild type VHL allele is mutated or lost, following the Knudson two-hit model (Figure 3) (Knudson, 1971; Maher et al., 1990; Tory et al., 1989;). This cancer syndrome is characterized by the increased risk of blood vessel tumors called hemangioblastoma occurring in the central nervous system, retina, as well as in kidney, and adrenal glands. VHL disease also causes renal and pancreatic carcinoma (Binkovitz et al., 1990; Maher et al., 1990; Maher and Kaelin, 1997; Tory et al., 1989). Cancer resulting from VHL somatic mutations or allele loss does not only occur in the syndrome but also in the majority of nonhereditary sporadic renal clear cell carcinoma (RCC) (Figure 3) (Maher et al., 1990, Foster et al., 1994). The VHL gene is located on chromosome 3p25, where many different mutations that cause this disease have been identified (Latif et al, 1993; Stebbins et al., 1999). VHL-associated neoplasms are typically hypervascular with high levels of

Figure 3. Acquisition of cancer by VHL disease and sporadic VHL loss. VHL disease is an inherited cancer syndrome. Individuals with this disease are germline heterozygotes. The development of tumors occurs when the second VHL allele is lost, coinciding with the Knudson two-hit model. VHL loss due to somatic mutations occurs in the majority of nonhereditary sporadic cancers such as renal clear cell carcinoma (RCC).


Von Hippel-Lindau Disease
Sporadic VHL-related tumors
VHL
VHL
germinal
VHL
VHL
NEOPLASIA
VHL
VHL
somatic
VHL
VHL
somatic
VHL
VHL
NEOPLASIA

hypoxia-induced messenger ribonucleic acids (mRNAs) under both hypoxic and normoxic conditions such as VEGF and erythropoietin. VEGF and erythropoietin increase the blood oxygen carrying capacity and delivery accounting for the hypervasculature in VHL-associated cancers (Sato et al. 1994; Vaupel and Harrison, 2004; Wizigmann-Voos et al., 1995).

1.3.2 Function of VHL protein

The VHL gene encodes for a protein, pVHL that is 213 amino acids with a molecular weight of 30 kDa (Duan et al., 1995; Iliopoulos et al., 1995; Latif et al. 1993). pVHL is a tumor suppressor with a gatekeeper function, since restoring the protein in VHL -/- (biallelic inactivation VHL mutations) RCC suppresses the ability of the cells to form tumors in nude mice (Iliopoulos et al., 1995; Kinzler and Vogelsein, 1997; Pause et al., 1998). pVHL forms a complex with elongin B, elongin C, Cullin-2 (Cul-2) and ring box protein 1 (Rbx-1), known as the VBC/Cul-2 complex (Pause et al., 1997). At the C-terminal α domain, pVHL is bound to elongin B and C, which inhibits transcription, while pVHL recruits hypoxia inducible factor (HIF) α-subunits at the N-terminal β domain (Kibel et al., 1995; Ohh et al., 2000; Stebbins et al., 1999). Cul-2 is bound to the elongin C and in turn Rbx-1 is bound to Cul-2 (Kamuna et al., 1999; Lonergan et al., 1998). Mutations are commonly found in the α and β domain of pVHL compromising its function (Stebbins et al., 1999). In the presence of oxygen, the VBC/Cul-2 complex elicits an E3 ubiquitin ligase activity on HIFαs that have been hydroxylated by prolyl hydroxylases (PHDs) at conserved proline residues leading to proteasomal degradation

(Bruick and McKnight, 2001; Cockman et al., 2000; Epstein et al., 2001; Iwai et al., 1999; Ohh et al., 2000). In the VHL disease the cancers have a constitutive expression of HIF-1α and HIF-2α since regulation of these proteins is lost, resulting in the activation of its target mRNAs at normal oxygen conditions (Maxwell et al., 1999).

1.4 Hypoxia Inducible Factors

1.4.1 The role of Hypoxia Inducible Factors (HIFs) in oxygen homeostasis

Cells exposed to a hypoxic environment induce the activation of adaptive responses to meet the demands of metabolic, bioenergetic and redox reactions (Maxwell et al., 2001; reviewed in Majmundar et al., 2010). Hypoxic cells temporarily arrest in cell cycle, reduce energy consumption, and secrete survival and proangiogenic factors. For cells to adapt to acute and chronic hypoxia, the HIF family of transcription factors activate genes involved in glucose uptake and metabolism, extracellular pH control, angiogenesis, erythropoiesis, mitogenesis, and apoptosis (reviewed in Semenza, 1999). Aside from their adaptive function, recent work on HIFs has revealed that they also have roles in physiological and pathological responses (reviewed in Majmundar et al., 2010).

1.4.2 The HIF family of transcription factors

HIFs are basic Helix-Loop-Helix/Per-Arnt-Sim (bHLH/PAS) transcription factors and in mammals there are three genes encoding HIFαs and three genes encoding for HIFβs (ARNT- Aryl hydrocarbon receptor nuclear translocator). HIF-1α and HIF-2α (EPAS1-endothelial PAS protein 1) are most structurally similar and the best

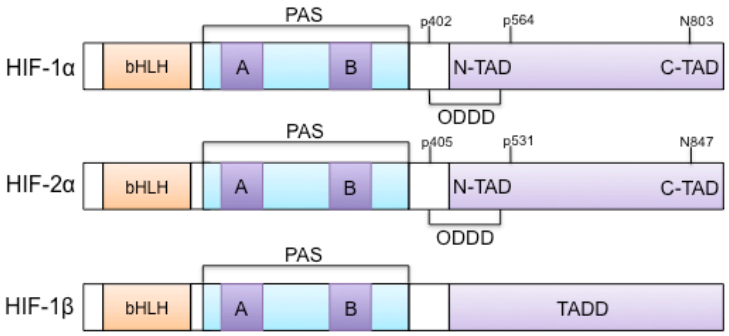
characterized, while the role of HIF-3α (IPAS- Inhibitory PAS domain protein) is less understood (Makino et al., 2001, Makino et al., 2002; Semenza, 2003; Tian et al., 1997; Wang et al., 1995). It is known that HIF-3α exists as multiple splice variants, some of which inhibit HIF-1α and HIF-2α activity (Makino et al., 2001; Makino et al., 2002). HIF-1α and HIF-2α do have some overlapping target genes but their roles are not redundant which is verified by knock out studies in mice. HIF-1α is ubiquitously expressed and null mice die at embryonic day 11 with neural tube defects and cardiovascular malformations (Iyer et al., 1998; Jain et al., 1998; Ryan et al., 1998). HIF-2α is expressed in highly vascularized structures but also in distinct cell populations of the brain, heart, lung, kidney, and liver (Jain et al., 1998; Wiesener et al., 2003). The absence of HIF-2α in mice causes embryonic and perinatal lethality, as well as developmental abnormalities and severe vascular defects (Peng et al., 2000; Tian et al., 1998).

1.4.3 HIF mediated gene activation

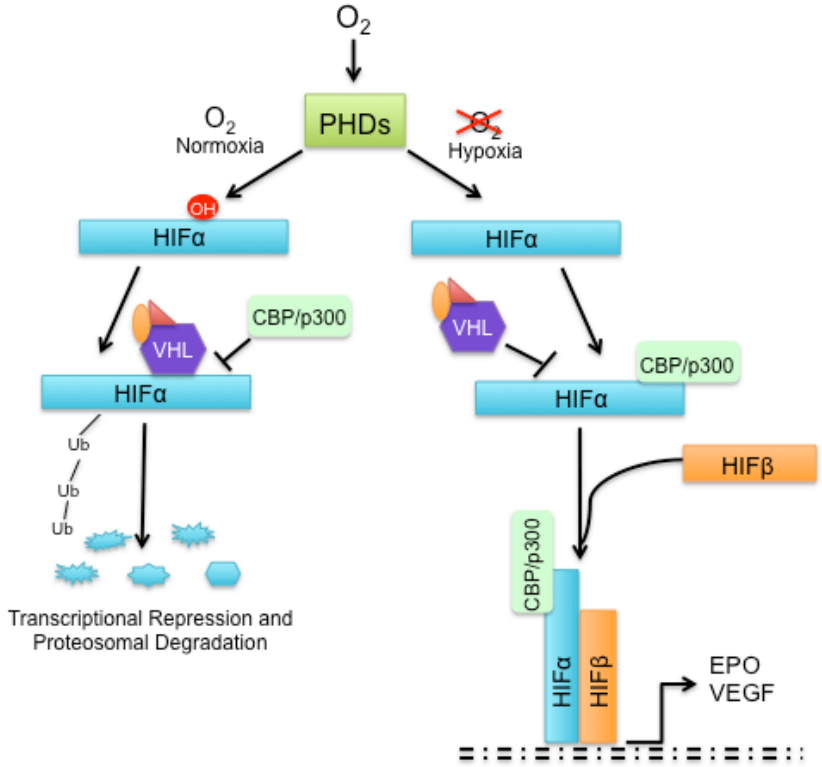
HIFs form heterodimers consisting of an oxygen dependent α subunit and a constitutively expressed nuclear β subunit (Hirose et al., 1996). In normoxia, PHDs hydroxylate and target HIF-1α and HIF-2α for degradation at conserved proline residues in the oxygen dependent degradation domain (ODDD) (Figure 4A and B) (Huang et al., 1998; Pugh et al., 1997). Hydroxylated HIFα is recognized and marked for proteosomal destruction by the VBC/Cul-2 complex (Cockman et al., 2000; Ohh et al., 2000). In hypoxic stress, PHD activity is diminished from the lack of oxygen (Epstein et al., 2001).

Figure 4. Structure of HIF proteins and regulation of the HIFα subunits. A. HIFα proteins are hydroxylated at conserved proline residues in the oxygen dependent degradation domain (ODDD). HIFα proteins heterodimerize with HIF-1β through the bHLH/PAS domains and are able to bind with coactivators through the C-terminal transactivation domain (C-TAD). B. In normal oxygen conditions HIFα is hydroxylated by prolyl-hydroxylases (PHDs). The VBC/Cul-2 complex recognizes HIFαs and marks them for proteasomal degradation. In hypoxia, PHD activity is diminished and HIFα is able to heterodimerize with HIF-1β and activate transcription of target genes.

A


PAS
p402
p564
N803
HIF-1α
bHLH
A
B
N-TAD
C-TAD
ODDD
PAS
p405
p531
N847
HIF-2α
bHLH
A
B
N-TAD
C-TAD
ODDD
PAS
HIF-1β
bHLH
A
B
TADD

B


O2
PHDs
O2
Normoxia
O2
Hypoxia
OH
HIFα
HIFα
VHL
CBP/p300
HIFα
Ub
Ub
Ub
Transcriptional Repression and
Proteosomal Degradation
VHL
CBP/p300
HIFα
HIFβ
CBP/p300
HIFα
HIFβ
EPO
VEGF

Stabilized HIFα proteins can heterodimerize with stable HIF-1β subunits present in the nucleus through their bHLH/PAS domains and subsequently bind with coactivators through their C-terminal transactivation domain (C-TAD) (Figure 4A and B) (Arany et al., 1996; Maxwell et al., 2001). HIF heterodimers recognize and bind to the hypoxia response elements (HREs) in the genome and induce transcription of target genes such as the glucose transporter-1 (Glut-1), erythropoietin, and VEGF (Figure 4B) (Ebert et al., 1995; Maxwell et al., 2001; Minchenko et al., 1994; Semenza and Wang et al., 1992).

1.4.4 Autonomous growth in VHL negative renal clear cell carcinoma

Autocrine proliferative stimulation is seen in RCC that lack the tumor suppressor pVHL (de Paulsen et al., 2001). Bi-allelic inactivation of the VHL tumor suppressor gene causes RCC cells to have a constitutive activation of HIF-1α and HIF-2α (Maxwell et al., 1999). Cancer cells have the capability of engaging in autonomous growth, as reflected by their ability to proliferate in serum-free conditions. VHL -/- RCC cells are able to proliferate in the absence of exogenous growth factors and re-expression of VHL restores HIFα regulation and growth suppression in cultured cells as well as tumor suppression in xenografts (de Paulsen et al., 2001; Gunaratnam et al., 2003; Pause et al., 1998; Smith et al., 2005). Studies with VHL-/- RCC cells suggested that HIF-2α, and not HIF-1α, promotes tumor growth (Kondo et al., 2002; Maranchie et al. 2002; Raval et al., 2005). With the constant presence of HIF-2α, expression of angiogenic factors such as VEGF and mitogenic factors such as transforming growth factor-α (TGFα) are induced (Smith et al., 2005). TGFα is a renal mitogen and its cognate receptor, EGFR, is also

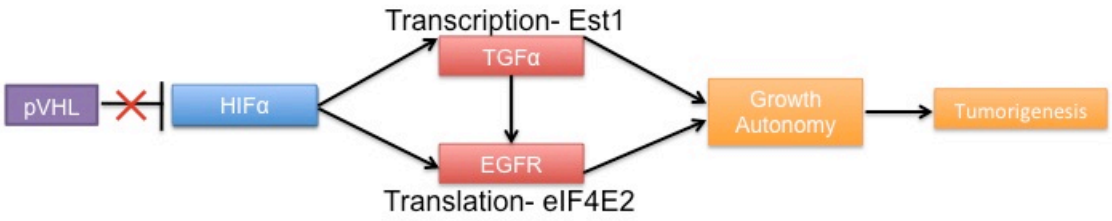
activated in RCC. In the absence of VHL to silence HIF-2α the TGF-α/EFGR pathway is activated (de Paulsen et al., 2001; Gunaratnam et al., 2003; Smith et al., 2005). EGFR activation provides permanent self-sufficiency in growth signaling which drives autonomous growth leading to tumorigenesis of VHL-/-RCC cells (Figure 5) (Smith et al., 2005).

1.5 HIF-2α in cancer

1.5.1 Tumor hypoxia

With solid tumors, the blood supply is often unable to sustain the demand for nutrients and growth (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Vaupel and Harrison, 2004). Tumors tend to be acutely or chronically hypoxic with some areas having a better supply of oxygen than others. This results in the accumulation of HIF-1α and HIF-2α and activation of their targets allowing for adaptation to the environment (Gatenby and Gilles, 2004; Hanahan and Weinberg, 2011, Maxwell, 2001). From tumor hypoxia, HIF-1α and HIF-2α are suggested to be an underlying cause of the Warburg Effect, as they activates genes involved in glucose uptake, metabolism and pH control. The Warburg Effect results from the increase in anaerobic glycolysis, generating an acidic tumor microenvironment (Warburg 1930; Warburg, 1956a; Warburg, 1956b; reviewed in Hanahan and Weinberg, 2011). Growth advantages occur in certain cells that have adapted to the hypoxic microenvironment and expand increasing hypoxia and malignant progression (Vaupel and Harrison, 2004). Another important aspect of the tumor microenvironment is the formation of the tumor-associated stroma. The tumor-

Figure 5. Oncogenic pathway in RCC. VHL is a part of post-translation modification inhibiting HIF-2α at the protein level. In the absence of VHL, HIF-2α is able to transcriptionally activate TGF-α with Est1. HIF-2α is a part of the translation initiation complex that activates EGFR translation with eukaryotic initiation factor 4E2 (eIF4E2), providing permanent self-sufficiency in growth signaling that drives autonomous growth and leads to tumorigenesis of RCC.


Transcription- Est1
TGFα
pVHL
HIFα
Growth Autonomy
Tumorigenesis
EGFR
Translation- eIF4E2

associated stroma is a heterogeneous population of cells undergoing various degrees of differentiation, proliferation, vascularity, inflammation, and invasiveness, contributing to the progression of tumors (Hanahan and Weinberg, 2011). HIFs have been shown to cause tumor growth in nude mice through autonomous growth of the cells as well as non-autonomous effects involving tumor-associated stromal cells engaging in metabolism and angiogenesis (de Paulsen et al., 2001; Kondo et al., 2002). These hypoxic solid tumors have an accumulation in HIF-1α and HIF-2α, which is seen in an array of cancers (Aebersold et al., 2002; Birner et al., 2000; Birner et al., 2001; Birner et al., 2001; Bos et al., 2001; Maxwell et al., 1999; Osada et al., 2007; Schindl et al., 2002; Sivridis et al., 2002; and Talks et al., 2000; reviewed in Bertout et al., 2008).

1.5.2 Tumorigenic capability of HIF-2α

HIF-1α and HIF-2α have been shown to have opposing effects regarding growth and proliferation of cancer cells on c-Myc, p53 and mTOR pathways. HIF-1α tends to have an inhibitory effect towards growth and even induces cell cycle arrest; while on the other hand, HIF-2α promotes growth (An et al., 1998; Gardner et al., 2001; Gordan et al., 2007; Hammond et al., 2002; Roberts et al., 2009; Zhang et al., 2007; reviewed in Keith et al., 2011). In RCC, HIF-2α is able to promote cell growth through the TGFα/EGFR pathway. It was discovered that HIF-2α seemed to be the subunit containing oncogenic properties involved in *in vitro* proliferation and *in vivo* tumorigenesis of VHL-/- RCC cells (Kondo et al., 2002; Raval et al. 2005). EGFR, a translational target of HIF-2α, is stimulated by renal mitogen TGFα, a transcriptional target of HIF-2α (Franovic et al.,

2007; Gunaratnam et al., 2003; Smith et al., 2005; Uniacke et al., 2012). Additional cancers beside RCC have been shown to involve HIF-2α in autonomous growth or in displaying an aggressive phenotype (Table 1). HIF-2α silencing in genetically diverse human cancer cell lines such as glioblastoma (U87MG), colorectal (HCT116), and non-small-cell lung carcinomas (A549), prevented *in vitro* growth and *in vivo* tumorigenesis (Franovic et al., 2009). Similar results were seen in both breast and liver cancer, in which HIF-2α silencing down regulated *in vitro* growth of the cells (Menrad et al., 2010; Stiehl et al., 2011). These studies examined different mutational statuses and tissue origins of cancer cell lines, all indicating HIF-2α is a regulator of autonomous growth (Franovic et al., 2009; Menrad et al., 2010; Stiehl et al., 2011).

1.6 Oxygen-regulated protein synthesis

1.6.1 EGFR is a translational target of HIF-2α

HIF-2α activates the TGFα/EGFR pathway in VHL-/- RCC providing permanent self-sufficient growth (Franovic et al., 2007; Gunaratnam et al., 2003; Smith et al., 2005). Smith et al. identified that TGFα is a specific transcriptional target of HIF-2α promoting growth through the TGFα/EGFR pathway and suppression of EGFR prevented tumorigenesis (Smith et al., 2005). Interestingly, TGFα is also regulated independently and in cooperation with HIF-2α by Ets-1. Ets-1 is a transcription factor that is induced in hypoxia and an example of an oncogene. Ets-1 expression in cancer feeds into the TGFα/EGFR autonomous growth pathway (Holterman et al., 2010). The regulation of TGFα in cancer had been discovered but what about its cognate receptor EGFR?

Table 1. Oncogenic presence HIF-2α in various cancers. In multiple cancer cell lines HIF-2α has shown to be present and have an effect on *in vitro* growth ([a]) or/and *in vivo* tumorigenesis ([b]). HIF-2α has also been associated with an aggressive phenotype and poor prognosis in tumor samples([c]), indicating its oncogenic capability.

Oncogenic presence HIF-2α in various cancers

Cancer Type	Reference
Astrocytoma	Khutua et al., 2003. [c]
Breast Cancer	Stiehl et al., 2011. [a c]
Colorectal carcinoma **Glioblastoma** **Lung cancer**	Franovic et al., 2009. [a b]
Head and neck cancer	Koujourakis et al., 2002. [c]
Hepatocellular carcinoma	Menrad et al., 2010. [a b]
Neuroblastoma	Holmquist-Mengelbie et al., 2006. [a b c]
Non-small-cell lung cancer	Giatromanolaki et al., 2001. [c]
Ovarian cancer	Osada et al., 2007. [c]
Renal clear cell carcinoma	Kondo et al, 2002. [a b] Raval et al., 2005. [a b]

[a] *In vitro* studies, [b] *In vivo* studies, [c] Tumor samples

Franovic et al. discovered that elevated EGFR protein levels were present in a hypoxic/HIF-2α-dependent manner but mRNA levels did not change. This study explained the overexpression of EGFR seen in many cancers where no mutation is detected (Franovic et al., 2007). The next question of interest was by which mechanism is resulting in EGFR protein accumulation in hypoxia? From here the discovery of the oxygen-regulated translation initiation complex was made.

1.6.2 Inhibition of cap-mediated translation

Translation of mRNA into a protein is the final stage of gene expression producing the building blocks of life. During normal oxygen conditions, translation of mRNA into proteins begins with eukaryotic translation initiation factor (eIF) 4E binding to the 7-methylguanosine (m^7-GTP) 5' cap of mRNAs forming a complex with eIF4A and eIF4G known as eIF4F (Edery et al., 1983; Lamphear et al., 1995; Pestova et al., 1996; Sonenberg et al., 1978; reviewed in Gebauer and Hentze, 2004; and Sonenberg and Hinnebusch, 2009). Once eIF4F is assembled at the cap it recruits the preassembled 43S PIC (eIFs 1, 1A, 2, 3 and 5; the Met-tRNA (transfer RNA); and 40S small ribosomal subunit) (Pestova et al., 1996). In certain stresses, such as hypoxia, translation is downregulated due to the inhibition of eIF4E. The interaction between eIF4E and eIF4G is inhibited due to eIF4E-binding proteins (4E-BP) sequestering eIF4E, therefore inhibiting cap-dependent translation (Gingras et al., 1995; Lin et al., 1994; Marcotrigiano et al., 1999; Pause et al., 1994). The kinase, mTOR senses the cell's extracellular environment for amino acid, oxygen and energy availability and phosphorylates 4E-BP

accordingly. In hypoxia, 4E-BP is hypophosphorylated and able to bind eIF4E and inhibit cap-mediated translation. When 4E-BP is hyperphosphorylated by mTOR it releases eIF4E allowing for translation initiation to occur (Khaleghpour et al., 1999; reviewed in Gebauer and Hentze, 2004; Sonenberg and Hinnebusch, 2009). In circumstances when cap-mediated translation is inhibited, an alterative method is internal ribosome entry site (IRES)-mediated translation. Some mRNAs contain an IRES and are able to recruit the 40S with the aid of fewer eIFs (Pestova et al., 2008; Terenin et al., 2008). This mechanism seems to have a minor role in the case of hypoxia (Holcik and Sonenberg, 2005; Young, et al., 2008).

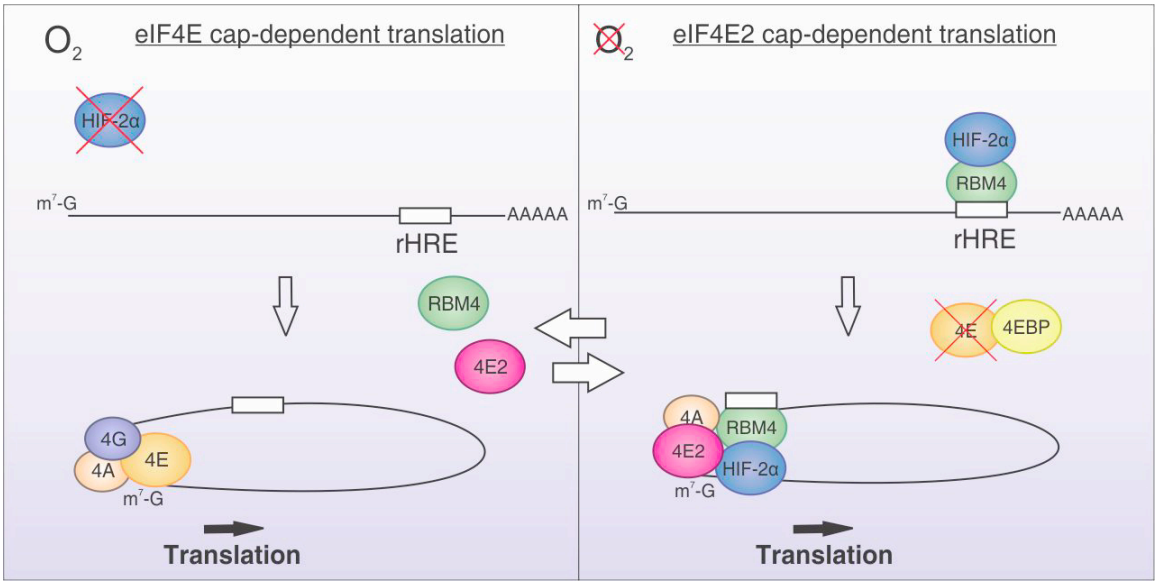
1.6.3 Hypoxia induced translation initiation complex

During cellular starvation and stress, cap-mediated translation is inhibited. In some circumstances such as apoptosis certain mRNAs contain an IRES and translation can occur. In hypoxia, protein synthesis is inhibited for the conservation of energy but mRNAs need to be translated to allow for adaptation to the environment. IRES is a cap-independent method of translation, but it does not seem to be the mechanism used for translation in hypoxia (Young, et al., 2008). In a study by Young et al., hypoxia regulated mRNA containing an IRES such as VEGF and HIF-1α were tested to determine if they used this cap-independent method of translation. Cells were oxygen starved and transfected with synthesized mRNAs and IRES-mediated translation accounted for <1% of translation (Young, et al., 2008). In addition to activating transcription of target genes, HIF-2α also has a role in translation of mRNA into proteins (Uniacke et al., 2012). This

was discovered by the investigation into EGFR overexpression in cancer. It was found that HIF-2α activation increases protein levels of EGFR enhancing its expression and involvement in signaling pathways (Franovic et al., 2007). Cells were treated with transcription inhibitors and an accumulation of EGFR was found in hypoxia in a HIF-2α-dependent manner in glioblastoma and primary cultures of renal epithelial cells. Silencing of HIF-2α prevented *de novo* synthesis of EGFR while the silencing of HIF-1α and HIF-1β had no effect. The silencing of HIF-2α also caused a considerable decrease on global translation while HIF-1α did not (Uniacke et al., 2012).

From this recent work, an oxygen-regulated translation initiation complex that mediates selective cap-dependent protein synthesis in the absence of eIF4E was discovered. This complex includes HIF-2α, RNA binding protein RBM4, RNA helicase eIF4A, and a cap-binding protein eIF4E2, which is an eIF4E homolog (Uniacke et al., 2012). eIF4E2 is a cap binding protein like eIF4E but has a weaker affinity for the cap than eIF4E (Zuberek et at., 2007). When eIF4E is sequestered by 4E-BP, eIF4E2 has the opportunity to initiate translation. A consensus RNA hypoxia response element (rHRE) recruits the complex to a number of mRNAs. Once the complex is assembled at the rHRE, it captures the m^7-GTP cap and directs the mRNAs to polysomes for translation (Figure 6). Over 4000 targets were identified to contain and rHRE that could potentially use this complex in translation initation in hypoxic conditions. Some of the target mRNAs include other RTKs besides EGFR such as IGF1R and PDGFRα, which have roles in autonomous growth and the progression of cancer (Uniacke et al., 2012).

Figure 6. Hypoxia stimulated translation initiation complex. In normal oxygen condition, eukaryotic translation initiation factor 4E (eIF4E) binds to the m^7-GTP 5' cap of mRNAs. Hypoxia causes eIF4E sequestration and stimulates the formation of a complex consisting of HIF-2α, the RNA binding protein RBM4, a RNA helicase eIF4A, and a cap-binding protein eIF4E2 (an eIF4E homolog). This complex is recruited to the RNA hypoxia response element (rHRE) of mRNAs and targets them to the polysome for translation (Uniacke et al., 2012).


O2
eIF4E cap-dependent translation
HIF-2α
m7-G
AAAAA
rHRE
RBM4
4E2
4G
4A
4E
m7-G
Translation
O2
eIF4E2 cap-dependent translation
HIF-2α
RBM4
m7-G
AAAAA
rHRE
4E
4EBP
4A
RBM4
4E2
HIF-2α
m7-G
Translation

1.6.4 HIF-2α as a therapeutic target

With the recent finding of HIF-2α's involvement in hypoxic cap-mediated translation it makes it an excellent target for cancer therapy. Tumors have a variance of hypoxia due to improper vascularization leading to the stabilization of HIF-1α and HIF-2α allowing the cells to adapt to these conditions (Hanahan and Weinberg, 2011; Vaupel and Harrison, 2004). Cancer cells seem to utilize the HIF-2α-RBM4-eIF4E2 complex to activate translation of a proteome vital for its presence and persistence. This hypoxic translation initiation complex was discovered by taking an in depth look at the overexpression of EGFR, a common theme in a variety cancers (Di Foire et al., 1987; Libermann et al., 1985; Wong et al., 1987; reviewed in Han and Lo, 2012; Lemmon and Schlessinger, 2010; and Witsch et al., 2010). EGFR is a target mRNA of this complex as well as the RTKs, PDGFRα and IGF1R, which have roles in the adaptive response to hypoxia and the proliferation of cancerous cells (Bos et al., 2005; Franovic et al., 2007; Nishi et al., 2002; Singleton et al., 1996). By targeting the interaction between HIF-2α and eIF4E2 in cancer therapy it will hopefully inhibit translation in hypoxic cells without affecting eIF4E translation, therefore only targeting the cancer cells.

1.7 Ovarian Cancer

1.7.1 Ovarian Carcinoma

Ovarian cancer accounts for 3% of new cases of cancer in women annually and is the most lethal of all gynecological cancers (American Cancer Society, 2012). In women, it is the fifth most common cause of death due to cancer, after lung, breast, colorectal

and pancreatic cancers (American Cancer Society, 2012). Ovarian cancer forms from three tissues; 85-95% from epithelial cells, 5-8% from stromal cells, and 3-5% from germ cells. Epithelial ovarian cancer (EOC) or ovarian carcinoma is the most lethal and the most common form of ovarian cancer (Young et al., 1994). Ovarian cancer can arise from malignant transformation of ovarian surface epithelium as well as from the fallopian tubes, deposits of endometriosis or from the surface of the peritoneal cavity since they are histologically similar (Bettochi et al., 1982; Hovadhanakul et al., 1976; Lamping and Blythe. 1977; Takashina et al., 1988; Yanai-Inbar et al 1995). Ovarian carcinoma has five histological subtypes consisting of serous, mucinous, clear cell, endometrioid, and transitional cell/Brenner tumors, with serous ovarian carcinoma being the most common. Tumors that do not fall under these histological subtypes are known as undifferentiated surface epithelial adenocarcinomas (Shaw et al., 2004).

1.7.2 Risk factors of ovarian cancer

A key risk factor of ovarian cancer is a strong family history in either ovarian or breast cancer and hereditary BRCA1 and BRCA2 germline mutations which are only identifiable in approximately 10-15% of patients. Repair of genomic damage diminishes in women with defective BRCA1 and BRCA2 function, leading to increased risk of the disease (Chen et al., 2006; Risch et al., 2006). Genomic mutations do have a key role in the development of many cancers. In ovarian carcinoma somatic mutations are present in *PT53 (*60-80%), *PTEN* (3-8%), *KRAS* (>20%), as well as *PI3KCA*, and *AKT* (Campbell et al., 2004; Carpten et al., 2007; Hennessy and Mills, 2006; Shaw et al., 1999). Additional

risk factors include not bearing children, early menarche, late menopause and increasing age. Factors that reduce the risk of ovarian carcinoma are oral contraceptive use, pregnancy, lactation, and tubal ligation (Collaborative Group on Epidemiologocal Studies of Ovarian Cancer, 2008; Hankinston et al., 1993). Ovarian cancer spreads locally to the opposite ovary, the uterus, and then finally throughout the peritoneal cavity. Distant metastases are rare but they can reach the liver, lungs, pleura, adrenal glands, and spleen (Young et al., 1994). Tumor stage is the most important prognostic parameter. Over 70% of all cases are diagnosed at advanced stages III or IV with poor prognosis according to the International Federation of Gynecology and Obstetrics (FIGO) (Table 2) (Pecorelli et al., 1999). Long-term survival in patients with stage I or II of the disease is 60-90%, while patients with advanced stage III or IV have a much lower long-termsurvival of 10% or less (McGuire et al., 1996; Pecorelli et al., 1999; Young et al., 1990).

1.7.3 Symptoms, diagnosis, and treatment

Diagnosis of ovarian cancer is difficult because of the nonspecific symptoms. While there is no single cause of ovarian cancer, personal or family history of cancer may place a woman at risk (Canadian Cancer Society, 2012). Some of the symptoms include: stomach discomfort or pain, swelling or bloating, back pain, lump in the abdomen, abnormal vaginal bleeding, constipation, diarrhea and nausea (Health Canada, 2010). These symptoms can also be caused by other health conditions and often lead to

Table 2. **The International Federation of Gynecology and Obstetrics (FIGO) stages of ovarian cancer.** Stages of ovarian cancer associated with the location and spread of the disease. Survival rate is indicated at each stage, which decreases greatly with the spread of the cancer. Prognosis is strongly associated with the stage at diagnosis as well as the histologic grade (Pecorelli et al., 1999; Roett and Evans, 2009).

*Stage**	*Spread*	*Five-year survival rate*
I	Limited to ovaries	90 percent
II	Pelvic extension	60 to 80 percent
III	Peritoneal implants and/or retroperitoneal or inguinal lymph nodes	20 percent
IV	Distant metastases	Less than 10 percent

**—Stage classification also includes more specific delineations (e.g., stages IA to IC). Tumor-node-metastasis staging by the American Joint Committee on Cancer is also applicable.*

abdominal imaging rather than pelvic imaging and cancer antigen (CA) 125 testing, which allows the cancer to remain undetected (Canadian Cancer Society, 2012; Smith, L.H. et al., 2005). Serum CA125 concentration does not have the sensitivity or specificity to function alone in screening. A combination of CA125 measurement and transvaginal ultrasonography (TVS) is used. If ovarian carcinoma is suspected on the basis of physical examination, CA125 screening and TVS imaging is done. If ovarian carcinoma is detected, exploratory laparotomy is conducted to determine the histological grade, which allows for tumor removal (Cannistra, 2004; Cheng et al., 2004). Standard therapy involves surgery along with platinum and taxane based adjuvant chemotherapy to prevent recurrence and improve survival (Bell et al., 2006; McGuire et al, 1996; Piccart et al, 2000; Ozols et al., 2003; Vasey et al., 2004; reviewed in Reibenwein and Krainer, 2008). Tumor stage, age, general health and post-operative residual tumor are all significant parameters in the survival and prognosis of patients (Burges and Schmalfeldt, 2011). Patients with stage I or II ovarian cancer will have improved survival if 3-6 cycles of cisplatin or carboplatin-based adjuvant therapy is prescribed after surgical staging (Advanced Ovarian Cancer Trialists Group, 1998; Bell et al., 2004; Elit et al., 2004; Trimbo et al., 2003; reviewed in Burges and Schmalfeldt, 2011; Hennessy et al., 2009; Reibenwein and Krainer, 2008). 20-40% of these patients do not respond to the initial platinum-based chemotherapy and those that have advanced stages are at high risk of disease recurrence (reviewed in Hennessy et al., 2009). Therefore, a new treatment option is needed for ovarian cancer patients.

1.7.4 RTKs expression and therapy in ovarian cancer

Standard therapy for ovarian cancer is surgery to remove the tumor but relapse is high in patients without optimal surgery and most develop resistance to current therapy (Younge et al., 1990). Platinum based adjuvant chemotherapy is considered to be the best strategy to prevent its recurrence and improve survival (Trimbos, et al., 2003). Currently, efforts are being made to optimize adjuvant chemotherapy to improve patient outcome with early and late stages of ovarian cancer, relapse and resistance to current therapy. Targeting the signaling pathways involving RTKs is a potential target since they have been identified to play a role in ovarian cancer carcinogenesis and many therapies have already been approved for use in various cancers (reviewed in Reibenwein and Krainer, 2008). Overexpression of RTKs such as ErbB-2/HER2 (25-30%), EGFR (55-98%), and KIT (12-26%) in ovarian cancer have led to Phase II clinical trials of monoclonal antibodies and tyrosine kinase inhibitors already in use for treatment of other cancers (Schmandt et al., 2003; See et al., 2003; Serrano-Olvera et al., 2006; reviewed in Reibenwein and Krainer, 2008). Clinical trials were done on patients who were resistant to standard therapy or have stage III or IV of the disease. Treatment with a monoclonal antibody or tyrosine kinase inhibitor resulted in a minimal percent of patients achieving remission (Schmandt et al., 2003; See et al., 2003; Serrano-Olvera et al., 2006; reviewed in Reibenwein and Krainer, 2008). An important note of targeting RTKs in ovarian cancer as in others cancers is first determining if it is overexpressed. Ovarian cancer seems to vary case by case in which RTKs are overexpressed, therefore each patient has to be assessed and a treatment plan made accordingly.

1.7.5 HIFs in ovarian cancer

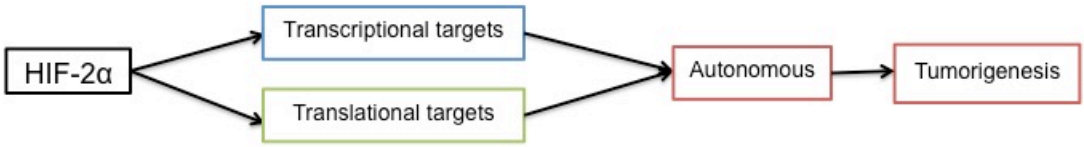
In ovarian carcinoma, HIF-1α and HIF-2α have been shown to be present in human tumor samples due to tumor hypoxia (Osada et al., 2007; Talks et al., 2000). In a paper by Osada et al., HIF-1α and HIF-2α as well as VHL were examined in ovarian carcinoma tumors. HIF-1α staining was observed mostly in the cytoplasm, but with sporadic nuclear staining. No significant difference was seen in cytoplasmic staining in benign, borderline and malignant tumors. A greater percent of cells containing HIF-1α nuclear staining (33%) was seen in malignant tumors and was higher in stages III and IV than in stages I and II. HIF-2α staining (75%) was mainly cytoplasmic with infrequent nuclear expression. Benign tumors were negative of HIF-2α cytoplasmic staining with the majority of HIF-2α present in malignant tumors, especially those in stages III and IV (Osada et al., 2007). Recent work has shown that HIF-2α is a component of the translation initiation complex in hypoxia, which may explain its presence in the cytoplasm (Uniacke et al., 2012). Nuclear staining was seen in malignant tumors regardless of their stage. In patients where HIF-2α positive borderline tumor samples were taken, poorer survival rates were seen, while HIF-1α expression was determined to be an independent prognosistic factor (Osada et al., 2007). HIF-1α when silenced in ovarian carcinoma cell line indicated an increase in proliferation *in vitro* and xenografts were equivalent in volume to control cells. This indicates that HIF-1α is not responsible for the autonomous growth and tumorigenesis seen in ovarian carcinoma (Favaro et al., 2008). VHL expression was cytoplasmic and reduced expression was seen in ovarian carcinoma in comparison to benign or borderline tumors. Loss of heterozygosity was

also detected, although no association was observed in correlation with HIFα expression. This demonstrates that HIF-1α and HIF-2α have distinct roles in ovarian carcinoma and that a greater look into HIF-2α's role may be valuable (Osada et al., 2007).

1.8 Rationale

HIF-2α is upregulated in ovarian carcinomas compared to adjacent tissue and benign tumor samples, as well as RTKs, which adversely affects the outcome of patients (Osada et al., 2007; Schmandt et al., 2003; See et al., 2003; Serrano-Olvera et al., 2006; Talks et al. 2000; reviewed in Reibenwein and Krainer, 2008). It has been shown in genetically diverse human cancer cell lines that HIF-2α silencing decreased *in vitro* growth and tumorigenesis (Franovic et al., 2009). Carcinomas develop from tissues of similar embryonic origins that have a number of different functions. Epithelial cells use a universal mechanism to exit the quiescent state by the regulated production of exogenous growth factors activating RTKs (Coller et al., 2006). Cancer cells have a continual activation of RTK pathways since they have acquired the capability to produce endogenous growth factors and their cognate receptors resulting in proliferation, which is seen in various epithelial cancers (Blume-Jensen and Hunter, 2001; Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Sporn and Roberts, 1985; Witsch et al., 2010). The cause of ovarian cancer remains unknown and based on its epithelial origin and the presence of HIF-2α in this cancer, HIF-2α may be responsible for its autonomous growth leading to tumorigenesis (Figure 7) (Osada et al., 2007; Talks et al., 2000). HIF-2α plays an important role in translation in hypoxia and some of its target mRNAs

Figure 7. Hypothesized model of autonomous growth in ovarian carcinoma. HIF-2α is active in ovarian carcinoma due to tumor hypoxia. We propose that HIF-2α, a component of the oxygen-regulated translation initiation complex, activates transcriptional and translational targets leading to autonomous growth and tumorigenesis of ovarian carcinoma.


HIF-2α
Transcriptional targets
Translational targets
Autonomous
Tumorigenesis

include RTKs, which are upregulated in ovarian carcinoma and bestow growth autonomy (Schmandt et al., 2003; See et al., 2003; Serrano-Olvera et al., 2006; Uniacke et al., 2012; reviewed in Reibenwein and Krainer, 2008). Further examination of HIF-2α in ovarian carcinoma may prove HIF-2α should be investigated as a therapeutic target.

1.9 Statement of hypothesis and objectives

In ovarian carcinoma, we hypothesize that HIF-2α is a regulator of autonomous growth and tumorigenesis. We propose to test this hypothesis with the following objectives:

Objective #1. Determine the presence of HIF-2α in OVCA429 and SKOV3 ovarian carcinoma cell lines and screen shHIF-2α clones from both cell lines for HIF-2α silencing.

The presence of HIF-2α will be determined in OVCA429 and SKOV3 cell lines and shHIF-2α (short hairpin RNA) clones will be screened for HIF-2α silencing.

Objective #2. Determine the effect of HIF-2α silencing on autonomous growth of ovarian carcinoma.

By culturing the shHIF-2α clones in serum-free medium supplemented with insulin-transferrin-selenium (ITS), the ability of cells to proliferate autonomously can be determined by 5-bromo-2'-deoxyuridine (BrdU) incorporation.

Objective #3. Identify the effect of HIF-2α on total translation and RTKs that may be involved in autonomous growth of ovarian carcinoma.

Using [S^{35}]-methionine ([S^{35}]-Met) incorporation, global translation can be monitored and RTKs will be screened to see if they are affected by HIF-2α silencing.

Objective #4. Determine HIF-2α silencing effects on the growth of *in vitro* avascular spheroids.

HIF-2α induces transcriptional and translational activation of an array of genes and mRNAs allowing the cells to adapt to the hypoxic environment. To determine if the effects seen in silencing HIF-2α are due to impairments in autonomous growth and not to angiogenesis, experiments with avascular spheroids will be performed.

Objective #5. Perform a xenograft assay with shHIF-2α clones to determine the effect of HIF-2α on tumorigenesis in ovarian carcinoma.

In nude mice, shHIF-2α cells will be injected along with parental or control cells to establish that HIF-2α is necessary for ovarian carcinoma tumorigenesis.

2 MATERIALS AND METHOD

2.1 Cell lines and Culture

The two cell lines, OVCA429 and SKOV3 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). OVCA429 cells were maintained in Minimum Essential Medium (MEM) Alpha Modification Medium (HyClone/Thermo Fisher Scientific Inc., Logan, UT) supplemented with 0.1% MEM nonessential amino acids (NEAA) (Invitrogen, Burlington, ON). SKOV3 cells were maintained in McCoy's 5A Modified Medium (HyClone/Thermo Fisher Scientific Inc., Logan, UT) and both mediums were supplemented with 7.5% fetal bovine serum (FBS) (HyClone/Thermo Fisher Scientific Inc., Logan, UT), 100 U/mL penicillin and 100 μg/mL streptomycin (HyClone/Thermo Fisher Scientific Inc., Logan, UT). In normoxia cells were kept at 37°C in 21% O_2 and 5% CO_2 environment. Cells incubated in the hypoxia chamber were at 37°C in 1% O_2, 5% CO_2 and N_2-balanced atmosphere for the indicated time periods. In cases where serum free medium was used, medium was supplemented with 1% (v/v) ITS (Invitrogen, Burlington, ON).

2.2 RNA interference

OVCA429 was transfected with two different shRNA targeting HIF-2α mRNA with the following sequences (5'-3'): HIF2A (GACAAGGUCUGCAAAGGGUUU) and (CAGGAUGUAAUUGAACGAUUU) (Franovic et al., 2009). Two complimentary single stranded DNA (deoxyribonucleic acid) oligonucleotides were designed with

overhangs encoding *BamHI/HindIII* restriction enzyme sites and were annealed by an incubation in 1x DNA annealing solution for 3 min at 90°C followed by 1 hour at 50°C (Ambion). The annealed inserts were ligated into p*Silencer* 3.1-H1 neo vector (Ambion). A p*Silencer* 3.1-H1 neo vector encoding scrambled shRNA served as the control. Cells containing HIF2α silencing were then selected for and maintained using neomycin (G418). For transfections Effectene transfection reagent (Qiagen, Valencia, CA) was used to generate the stable cell line. The cells were plated in a 10 cm plate so the following day they would be 70% confluent. The cells were rinsed and supplemented with fresh media and transfected with 2 μg of the plasmid DNA. The following day the plate was split into two 15 cm plates and incubated in G418 containing medium. Within a few days colonies forming from a single cell began to grow. These colonies where transferred to a 24-well plate and when confluent expanded to larger plates remaining in the G418 medium. Immunoblot analysis was then used to determine HIF-2α silencing in the clones.

2.3 Lentiviruses

SKOV3 was transfected with two different shHIF-2α sequences as well using lentiviral infection and were selected for with puromycin to achieve cell populations with a knock down in HIF-2α. Lentiviral vectors expressing human HIF-2α shRNAs (3804 and 3805) were purchased from Sigma-Aldrich (Oakville, ON). Scrambled shRNA vector was obtained from Addgene Inc. (Cambridge, MA). Viruses were produced by transfection of HEK 293T with shRNA, pLP1, pLP2, and pLP/VSV-G plasmids

(Invitrogen, Burlington, ON). Virus particles were collected by spinning 72 h post-transfection and stored at -80°C. SKOV3 cells were infected with supernatant from transfected HEK 293T and 4ng/ml Polybrene (Sigma, Oakville, ON) 24H after plating. Medium was replaced the following day with medium containing puromycin. With the infected cells monoclonal colonies were made in the same fashion as described in section 2.2. Briefly, the 10 cm plate was split into two 15 cm plates allowing for colonies to form from a single cell. These colonies were moved to a 24-well plate and expanded to a 10 cm plate. To determine silencing of HIF-2α had occurred immunoblot analysis was done to screen the clones.

2.4 Immunoblot Analysis

For immunoblot analysis cells were harvested using two different lysis buffers. For HIF-1α and HIF-2α, cells were harvested and lysed in 4% sodium dodecyl sulfate (SDS) in phosphate buffered saline solution (PSB) and denatured for 5 min at 95°C. For RTKs and phosphoproteins analysis, cells were lysed for 1 hour at 4°C in 1 mM Tris-HCl pH 7.4, 1% Triton X-100, 0.2% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin and 1 μg/ml aprotinin. Lysates were then centrifuged (12 000 × *g* for 10 min at 4°C) and the supernatants were collected. Protein concentrations were quantified using a BCA protein assay kit (Pierce, Rockford, IL). 40-50 μg of protein was used for HIF-1α and HIF-2α, while 10-20 μg of protein was used for RTKs and phosphoproteins immunoblot analysis.

For immunoblotting, equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Mississauga, ON). Membranes were stained with Ponceau S to ensure the transfer and equal loading of proteins had occurred. Membranes were washed in PBS with 0.1% Tween-20 and then blocked for 1 hour at room temperature in PBS with 0.1% Tween-20 containing 5% skim milk powder before being place in the primary antibody for overnight incubation at 4°C. All immunoblots were repeated three time (n=3) and immunoblots shown are representive of all three trials. Monoclonal antibodies were used to detect EGFR (Labvision, Fremont, CA) Met (Cell Signaling, Beverly, MA), and HIF-1α (Novus, Littleton, CO). Polyclonal antibodies used to detect HIF-2α (Novus, Littleton, CO); IGF1R, PDGFRα, Akt, Phospho-Akt (S473), Phospho-ERK (T20/Y204) (Cell Signaling, Beverly, MA), ERK1/2 (Promega, Madison, WI) and Actin (Sigma, Oakville, ON).

2.5 Real-Time PCR Analysis

RNA extraction was achieved using TriPure Isolation Reagent (Roche, Indianapolis, IN). Reverse transcription (RT) was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) using random hexamer primers. RT samples were then used to measure HIF-1α and HIF-2α mRNA by real-time PCR with IQ SYBR Green Supermix (Bio-Rad, Mississauga, ON) and data was recorded and analyzed using MX3000P thermocycler (Stratagene, Cedar Creek, TX). The cycling conditions used were 95°C (5 min); 35 cycles of 94°C (30 sec), 60°C (30 sec) and 72°C

(30 sec); and 95°C (1 min), 55°C (30 sec) and 95°C (30 sec). The relative abundance of target mRNA was calculated using the delta-delta CT method with all samples normalized to housekeeping gene *36B4* mRNA and relative to parental cells in normoxia (Gevry et al., 2009; Livak and Schmittgen, 2001). For both cell lines RT-PCR was performed three times and for each RT-PCR product real-time PCR was done (n=3). Error bars represent the standard error of mean (SEM) and Student t-test was used to determine if the silencing was significant compared to control samples. The primers used were HIF-1α: forward 5'-CAGCCGCTGGAGACACAATC-3', reverse 5'-GAAAGTTCCAGTGACTCTGG -3'; HIF-2α: forward 5'-GTCTGCAAAGGGTTTTGGGG -3', reverse 5'- TGTGAGGTGCTGCCACCAG -3', and housekeeping gene 36B4: forward 5'- CGACCTGGAAGTCCAACTAC -3', reverse 5'- ATCTGCTGCATCTGCTTG.

2.6 Autonomous Growth Assay

Cell proliferation assay was performed as previously described (de Paulsen et al., 2001). Briefly, cells were plated in FBS-containing medium so adhesion could occur. Medium was then change to serum free conditions supplemented with ITS and incubated for 48 hour in normoxia or hypoxia. After 48 hours, BrdU label (1:1000) was then added to the medium for a 2 hour incubation so incorporation could occur. The cells were kept at the same condition for the 2 hour BrdU labeling period as they were in for the 48 hour incubation. Cells were then rinsed with PBS, fixed, and stained by indirect immunofluoresence using the BrdU Labeling and Detection kit I (Roche, Indianapolis,

IN) and nuclei were stained using Hoechst (Hoechst 33342 1:2000: Invitrogen, Burlington, ON). Cells were mounted onto slides using Fluoromount G (Electron Microscopy Sciences, Hatfield, PA). The ratio of BrdU-labeled to Hoechst-stained cells was determined by counting five representative fields acquired by fluorescence microscopy (Zeiss Axiovert S100TV microscope, Thornwood, NY) and ImageJ software. The autonomous growth was repeated three times (n=3) in normoxia and two times (n=2) in hypoxia for OVCA429 and SKOV3. Error bars represent the SEM and Student t-test was used to calculate significant difference.

2.7 Protein synthesis by [^{35}S]-Met incorporation

For [^{35}S]-Met (methionine labeled with radioactive sulfur-35) incorporation, 500 000 cells were grown in 10 cm plates for 24 hours in normoxia and then an additional 24 hours in normoxia or hypoxia. An hour prior to the 48 hour end point, media was changed to glutamine-, methionine- and cysteine-free Dulbecco Modified Eagle Medium (DMEM) fro 30 min. [^{35}S]-Met [33 μCi/mL] was then added to the cells for 30 min and lysed in 1 mL of modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 1% Igepal, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4 and 1 mM NaF, 1 μg/mL aprotinin, leupeptin and pepstatin) for 1 hour at 4 ^{o}C. Samples were run on a 6% SDS-PAGE gel, dried at 80 ^{o}C for 90 min and exposed to X-ray film (Kodack BioMax MS Film) overnight at -80 ^{o}C. [^{35}S]-Met labeling was performed once (n=1). [^{35}S]-Met incorporation for global translation was determined by subtracting background pixel intensity from the band pixel intensity using Adobe

Photoshop CS5.1 software and is represented as a percentage.

2.8 In vitro Tumor Spheroids

Spheroid formation was achieved by the liquid overlay technique and has been previously described (Kunz-Schughart et al., 1998; Lieubeau-Teillet et al., 1998; Smith et al., 2005; Sutherland, 1988). Briefly, 10^5 cells were plated in 24-well plates coated with 1% of preheated Seaplaque agarose in 1mL of medium per well (Bio Basic Canada Inc, Markham, ON). To encourage cell-cell adhesion the plates were swirled in a circular motion for 30 min after plating and then placed in the incubator for 30 min. This was repeated until the cells came together and formed a spheroid. The spheroids were grown in FBS containing medium that was changed every 3 days by removing 500 µl of medium and supplementing it with a fresh 500 µl of medium. The spheroids were grown for 5-7 days and were then frozen for histological and immunofluorescence analysis.

2.9 Histology and Immunofluorescence

Spheroids were frozen in O.C.T. embedding medium (Miles Inc., Elkhart, IN) by submerging it into cold isopentane (2-methylbutane) (Acros Organics, New Jersey). The frozen tissue was then sectioned (10-12 µm) onto slides for histological and immunofluorescence analysis. For Haematoxylin and Eosin (H&E) staining slides were fixed in 10% formalin (BDH Chemicals, Toronto, ON) for 20 min and then immersed in Haematoxylin (Fisher Scientific, Dair Lawn, NJ) stain for 1 min. Slides were rinsed in water and then in 1% acid alcohol (concentrated HCl in 70% ethanol) to wash away

excess stain. The slides where then dipped in in 0.5% lithium carbonate, an alkaline solution to intensify the blue stain of Haematoxylin. The tissue was then counterstained in Eosin for 45 sec and dehydrated in two washes of 95% ethanol, two washes of absolute ethanol and lastly in two washes of toluene (Fisher Scientific, Fair Lawn, NJ). Slides were mounted with a glass cover slip using peramount (Fisher Scientific, Fair Lawn, NJ). Tissue sections were viewed with the Zeiss Axiophot microscope with a 40X objective.

For immunofluorescence analysis the spheroid tissue samples were fixed in 4% paraformaldehyde (BDH Chemicals, Toronto, ON) in PBS. Sections were washed in 3 changes of PBS for 5 min each change and blocked for 30 min in blocking buffer (1:20 of FBS in washing buffer: 0.8% bovine serum albumin (BSA: Wisent Inc., St. Bruno, PQ) in PBS). Sections are then washed in washing buffer for 5 min and incubated in Ki67 monoclonal antibody (Dako, Burlington, ON) (1:100 dilution in incubation buffer (washing buffer with 1:100 of FBS)) for 1 hour. Slides were then washed in 3 changes of washing buffer for 10 min and incubated for 30 min in Alexa Flour 488 goat anti-mouse secondary antibody (Invitrogen, Burlington, ON) (1:200 dilution in incubation buffer). Nuclei were stained using Hoechst 33342 (Invitrogen, Burlington, ON) (1:5000 dilution in incubation buffer) for 10 min. The slides were then washed in 3 changes of washing buffer for 15 min each and 3 changes of PBS for 5 min each before being mounted with glass coverslips using Fluormount G. Ki67 immunofluoresence was viewed with Zeiss Axio Observer D1 with the 60X objective and 4 representative periphery fields and 4 representative core fields of Ki67 expressing cells to Hoechst-stained cells were counted. Ki67 immunofluorescence was performed once (n=1) and error bars represent the stardard deviation. A 20X was used to show a larger area of the spheroid.

2.10 Nude Mouse Xenograft Assay

Nude mouse xenograft assay were performed as previously described (Iliopoulos, 1995; Smith et al., 2005). Female CD-1 nude mice (Charles River, Wilmington, MA) were injected subcutaneously into their flank with 10^7 cells diluted in 200 µl of sterile PBS. Parental and control cells were injected into the left side of the mouse with the shHIF2α clones on the right. On a weekly basis tumor dimensions were measured and recorded. Mice were sacrificed 8-12 weeks post-injection according to ACVS protocol at the University of Ottawa facility. Final tumor volumes were measured at the time of kill and the error bars represent the standard deviation of the final tumor volumes..

3 RESULTS

3.1 Identification of HIF-1α and HIF-2α expression in ovarian carcinoma cells lines

3.1.1 Time course of HIF-1α and HIF-2α expression

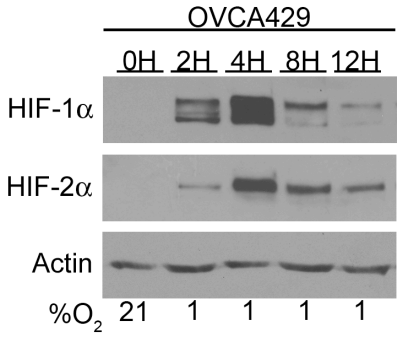
To identify the role of HIF-2α in ovarian carcinoma, OVCA429 and SKOV3 clear cell ovarian carcinoma cell lines were used. SKOV3 is cisplatin resistant with an inactivating mutation in *TP53* and a mutation in adenomatous polyposis coli (*APC*) gene downregulating the protein (ATCC, 2011; Jarrett et al., 2001; Yaginuma and Westphal, 1992). For OVCA429 no somatic mutations have been identified in *TP53* or *KRAS* but an activating mutation has been identified in *PIK3CA* (Kuo et al., 2010). HIF-2α silencing was accomplished by RNA interference using two different shRNA sequences. Only silencing of HIF-2α was performed and not of HIF-1α, since it has been previously shown in various epithelial cancers as well as ovarian carcinoma to have no affect or even increase autonomous growth *in vitro* and tumorigenesis *in vivo* (Favaro et al., 2008; Franovic et al., 2009). In ovarian carcinoma, silencing of HIF-1α pointed towards an increase in proliferation of cells *in vitro* in comparison to control cells and tumor size and weight was comparable to control tumors (Favaro et al., 2008).

To begin the screening of these cell lines for clones containing HIF-2α silencing, a time course was done to determine the time points at which HIF-1α and HIF-2α expression are detectable at in hypoxia. Detecting HIF-1α is important as it indicates that

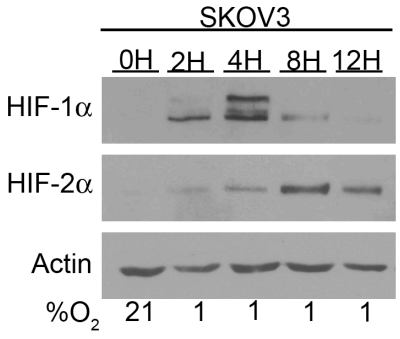
the silencing in these cell lines is specific to HIF-2α and it is a requirement of the screening process of the clones. Parental cells were incubated in the hypoxia chamber over a 12 hour period and lysate was collected at various time-points. For OVCA429, HIF-1α has an increase in expression at the earlier time points and decreases at 8 and 12 hours. HIF-2α has highest expression at 4 hours that decreased at 8 and 12 hours. For both while present at all time-points, greatest expression is seen at 4 hours (Figure 8A). Therefore, after a 4 hour hypoxic incubation lysate for the shHIF-2α clones was collected to be screened by immunoblot analysis. For SKOV3, HIF-1α expression is elevated at the 4 hour time-point and decreased at the later time points. HIF-2α required a longer hypoxic incubation to be strongly induced, which is seen at 8 hours (Figure 8B). At two time points, 4 hours for HIF-1α and 8 hours for HIF-2α lysate was collected for SKOV3 so both HIFα proteins could be detected. In both cell lines HIF-1α displays two bands due to post-translational modifications such as hydroxylation, phosphorylation, and acetylation among others (Jeong et al., 2003; Masson and Ratcliffe, 2003; Richard et al., 1999; reviewed in Brahimi-Horn et al., 2005). In OVCA429 the upper band is present at all time points where in SKOV3 the lower band is present at all time points (Figure 8). It is unknown whether the different bands indicate the various forms of post-translational modifications of the HIFα proteins.

Figure 8. Time course of HIF-1α and HIF-2α expression in hypoxia. Parental cells were incubated in the hypoxia chamber over a 12 hour period and lysate was collected at various time points. A. OVCA429 has the greatest level of expression of HIF-1α and HIF-2α at 4 hours but both proteins are present at all time points. B. SKOV3 has a peak of HIF-1α expression at 4 hours, where HIF-2α has the highest expression at 8 hours. Actin served as a loading control and experiments were repeated three times (n=3).

A


OVCA429
0H 2H 4H 8H 12H
HIF-1α
HIF-2α
Actin
%O2 21 1 1 1 1

B


SKOV3
0H 2H 4H 8H 12H
HIF-1α
HIF-2α
Actin
%O2 21 1 1 1 1

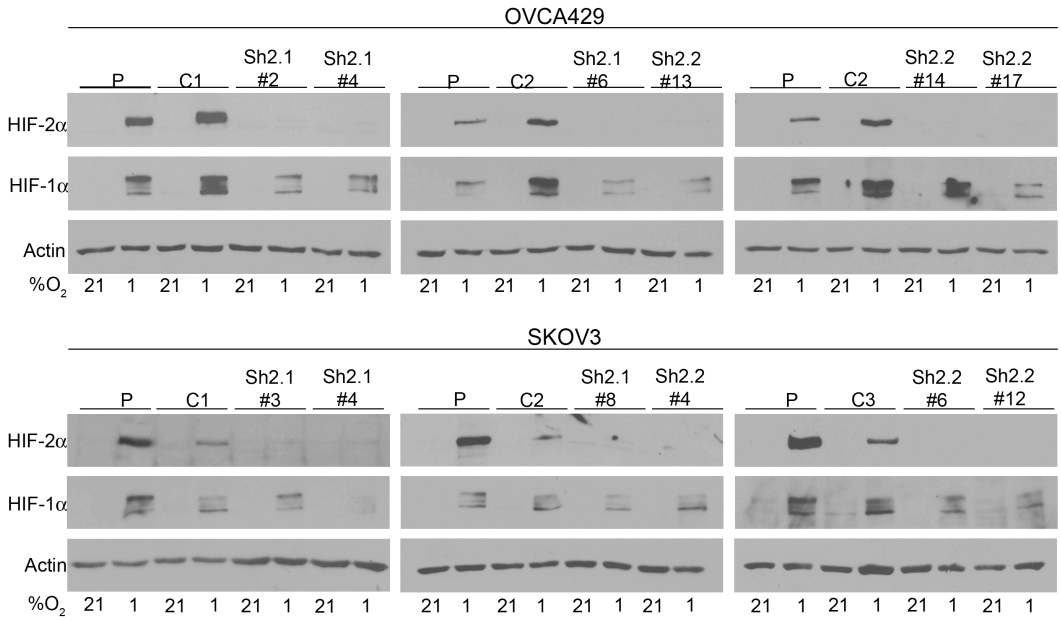
3.1.2 Immunoblot and real-time PCR analysis of shHIF-2α ovarian carcinoma cells

For screening of OVCAR429 shHIF-2α clones, the cells were incubated in the hypoxia chamber for 4 hours. At this time-point, lysate was collected for immunoblot analysis. shHIF-2α clones were screened and three Sh2.1 clones and three Sh2.2 clones have a decrease in or no detectable level of HIF-2α when compared to parental and control cells (Figure 9A top). For Sh2.1 #4 and Sh2.2#13 a slight expression of HIF-2α is seen, while Sh2.1 #2, Sh2.1 #6, Sh2.2 #14 and Sh2.2 #17 have no detectable expression compared to parental and control cells. Sh2.2#14 has comparable HIF-1α expression to control #2, while the rest of the shHIF-2α clones expressed HIF-1α comparable to parental but are lower than control #1 and #2. Control #1 and #2 have a higher level of expression of HIF-1α and HIF-2α than the parental cells. Controls are a scrambled non-targeting shRNA sequence. The scrambled shRNA control seems to be targeting RNA that is increasing HIFα levels. HIF-1α is expressed in all the shHIF-2α clones and although the levels are not comparable to the controls except for Sh2.2#14, levels are comparable to parental cells indicating that silencing is specific to HIF-2α.

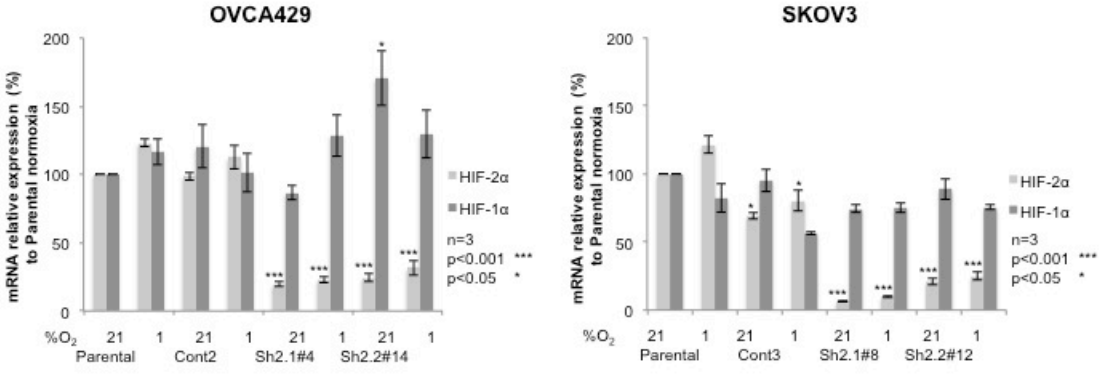
SKOV3 shHIF-2α clones had lysate collected after an 8 hour hypoxic incubation for HIF-2α detection and 4 hours for HIF-1α. The Sh2.1 #3 and #4 clones have almost no HIF-2α expression in comparison to the parental, control #1 and #2. Sh2.2 #4 has a very slight expression in HIF-2α where Sh2.1 #8, Sh2.2 #6 and #12 have no HIF-2α expression when compared to parental and controls #2 and #3. Control cells have a

Figure 9. Screening of shHIF-2α clones by immunoblot and real-time PCR analysis. A. OVCA429 (top) Sh2.1#4 and Sh2.2#13 clones have a slight expression of HIF-2α expression, while the other clones have no detectable expression. All OVCA429 clones express HIF-1α comparable to parental cells but were lower than the control cells except for Sh2.2#14 which is equivalent to control #2. Actin acted as a loading control. SKOV3 (bottom) Sh2.1 #3 and #4 and Sh2.2 #4 have a decrease in HIF-2α expression where Sh2.1 #8 and Sh2.2 #6 and #12 have no expression of HIF-2α detectable. All clones express HIF-1α in comparison to parental and control cells except Sh2.1 #4 where its expression is lower. For SKOV3 immunoblots actin shown is a loading control for HIF-2α and HIF-1α ponceau S stain is shown in Appendix A (Figure 1). All immunoblots were repeated three times and shown here are representative blots. B. Real-time PCR of OVCA429 (left) and SKOV3 (right) parental, control and shHIF-2α clones. mRNA levels are relative to parental cells in nomoxia and normalized to housekeeping gene *36B4*. Statistically significant decrease in HIF-2α is seen in the shHIF-2α clones compared to controls in normoxia indicated by the asterisks for both cell lines. OVCA429 Sh2.2#14 had a statistically significant increase in HIF-1α in normoxia compared to control #2 in normoxia. SKOV3 control #3 has a statistically significant decrease in HIF-2α compared to parental cells. Average of the three trials (n=3) is shown here, error bars represent the SEM and statistical significance was calculated using the Student t-test.

A


OVCA429
P
C1
Sh2.1 #2
Sh2.1 #4
P
C2
Sh2.1 #6
Sh2.2 #13
P
C2
Sh2.2 #14
Sh2.2 #17
HIF-2α
HIF-1α
Actin
%O2 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1
SKOV3
P
C1
Sh2.1 #3
Sh2.1 #4
P
C2
Sh2.1 #8
Sh2.2 #4
P
C3
Sh2.2 #6
Sh2.2 #12
HIF-2α
HIF-1α
Actin
%O2 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1 21 1

B


OVCA429
mRNA relative expression (%) to Parental normoxia
200
150
100
50
0
HIF-2α
HIF-1α
n=3
p<0.001 ***
p<0.05 *
%O2 21 1 21 1 21 1 21 1 21 1
Parental Cont2 Sh2.1#4 Sh2.2#14
SKOV3
mRNA relative expression (%) to Parental normoxia
200
150
100
50
0
HIF-2α
HIF-1α
n=3
p<0.001 ***
p<0.05 *
%O2 21 1 21 1 21 1 21 1 21 1
Parental Cont3 Sh2.1#8 Sh2.2#12

lower expression of HIF-2α compared to SKOV3 parental cells. The scrambled non-targeting shRNA seem to be negatively affecting HIF-2α levels. HIF-1α was detected in all clones similar to parental and controls cell except for Sh2.1 #4, where HIF-1α is barely detectable (Figure 9A bottom) (HIF-1α loading control is in Appendix A, Figure 1).

To determine if the scrambled shRNA controls affected HIFα at the mRNA or protein level real-time PCR was carried out. Relative expression of the mRNA was compared to parental cells in normoxia. OVCA429 cells were incubated in the hypoxia chamber for 4 hours and then RNA was extracted along side its normoxic counterpart. Parental and control cells have similar levels of HIF-2α mRNA with a slight induction in hypoxia compared to its normoxic counterparts (Figure 9B right panel). The shHIF-2α clones have a 3-5 fold statistically significant decrease of HIF-2α in normoxia and hypoxia compared to control cells in normoxia indicated by the asterisks (Figure 9B left panel). For HIF-1α, fairly similar levels of mRNA were seen in all cells and conditions, except for Sh2.2#14 in normoxia has a statistically significant increase in of HIF-1α mRNA expression compared to control #2 cells in normoxia (Figure 9B right panel). Control #2 has similar levels of mRNA compared to parental cells for both HIF-1α and HIF-2α, indicating that the increase in expression seen at the protein level in scrambled shRNA controls is not due to the mRNA levels.

SKOV3 cells were incubated in the hypoxia chamber for 8 hours and RNA was extracted along side its normoxic counterpart. Sh2.1#8 has approximately a 10-15 fold

decrease in HIF-2α and Sh2.2#12 has a 4-5 fold decrease. Both shHIF-2α clone has a statistically significant decrease in HIF-2α mRNA levels compared to control #3 cells in normoxia. Control #3 has a statistically significant decrease of HIF-2α mRNA expression compared to parental cells in normoxia (Figure 9B right panel). HIF-1α mRNA levels are similar in parental, control #3 and shHIF-2α clones (Figure 9B right panel). SKOV3 control #3 has a statistically significant decrease in mRNA levels of HIF-2α, which may account for the decrease seen at the protein level.

3.2 Silencing of HIF-2α prevents autonomous growth of ovarian carcinoma cells

An autonomous growth assay was performed to determine if there was an inhibition in proliferation due to the absence of HIF-2α expression. This was done by BrdU incorporation, a synthetic nucleoside and an analogue of thymidine. It is incorporated into the DNA of replicating cells, substituting for thymidine during synthesis. A specific antibody to BrdU is then used to detect its incorporation, indicating the cells that were actively replicating their DNA. The shHIF-2α clones were set up alongside parental and control cells for the autonomous growth assay. By removing the FBS from the medium, the exogenous growth factors are removed and in the absence of HIF-2α, the shHIF-2α clones should have a decrease in proliferation if the hypothesis is correct. HIF-2α activates growth factors and RTKs that are involved in autonomous growth of cancer cells (Franovic et al., 2007; Franovic et al., 2009; Smith et al., 2005; Uniacke et al., 2012). Serum free medium is supplemented with 1% ITS, which is needed for in vitro

growth and survival of mammalian cells. Cells grown in the presence of serum act as the control to illustrate that in regular *in vitro* culture conditions with the aid of exogenous growth factors proliferation is not affected.

In normoxia, OVCA429 parental and control cells grown in ITS have a 20-25% of BrdU incorporation. Sh2.1 #4, #6, and Sh2.2 #14 have under a 10% BrdU incorporation whereas Sh2.1 #2, Sh2.2 #13 and #17 are just over 10%. All the shHIF-2α clones have a statistically significant inhibition in proliferation in comparison to control #2 indicated by the asterisks. The shHIF-2α clones having a statistically significant decrease in proliferation are worthy candidates for the xenograft assay. OVCA429 parental, controls and shHIF-2α cells grown in the presence of FBS have approximately a 40% BrdU incorporation indicating that with the aid of exogenous growth factors proliferation is not affected by HIF-2α silencing (Figure 10A).

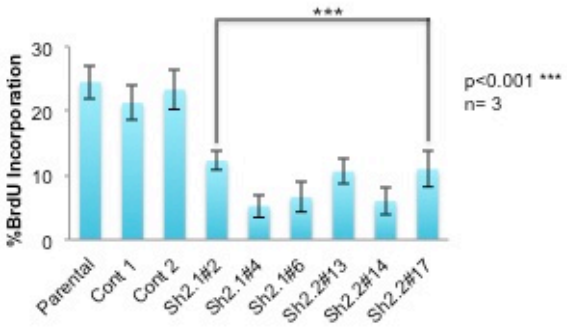
The autonomous growth assay was then repeated in hypoxia since it provides similar conditions to a tumor microenvironment. This was done with shHIF-2α clones Sh2.1#4 and Sh2.2#14 alongside parental and control #2 cells. Equivalent results are seen when compared to cell cultured in normoxia (Figure 10B). All cells grown in medium containing FBS have a 32-37% BrdU incorporation, whereas parental and control #2 cells grown in ITS have approximately 25% BrdU incorporation. shHIF-2α grown in ITS have a 5% or less BrdU incorporation in comparison to control #2 cells and have a statistically significant decrease in proliferation designated by the asterisks (Figure 10B).

Figure 10. Silencing of HIF-2α prevents autonomous growth of OVCA429 *in vitro*. A. In normoxia, OVCA429 parental and control cells grown in ITS have approximately a 20-25% BrdU incorporation where the shHIF-2α clones are approximately 10% or lower. All the clones have a statistically significant inhibition in proliferation in comparison to control #2 cells indicated by the asterisks. OVCA429 cells grown in serum conditions (FBS) have approximately a 40% BrdU incorporation. Experiments were repeated three times (n=3) and shown here is the mean result of the three trials. Error bars represent the SEM and Student t-test was done to determine the significant differences. B. Similar results are seen with the cells grown in hypoxia. Parental and control #2 have a 25% BrdU incorporation and the shHIF-2α clones have 5% or less being statistically significant inhibition in proliferation in comparison to control #2 cells when grown in ITS, indicated by the asterisks. Cells grown in serum conditions have a 30%-40% BrdU incorporation. Experiments were repeated twice in hypoxia (n=2) with the mean of the experiments shown here. Error bars indicate the SEM and significant difference was determined by Student t-test.

A

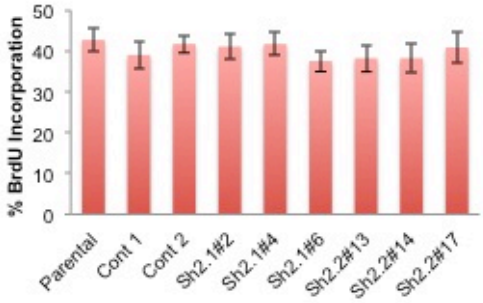
NORMOXIA

OVCA429-ITS



p<0.001 ***
n= 3

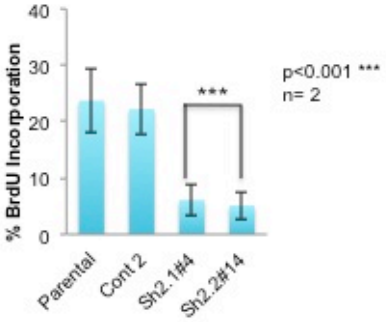
OVCA429-FBS



B

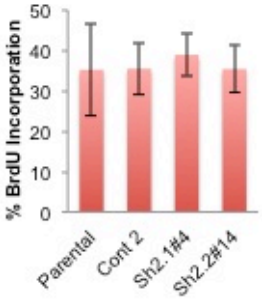
HYPOXIA

OVCA429-ITS



p<0.001 ***
n= 2

OVCA429-FBS



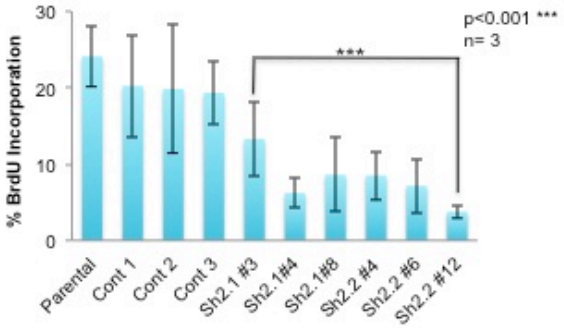
SKOV3 autonomous growth assay was performed in the same fashion as OVCA429. In normoxia, parental cells grown in ITS have a 25% BrdU incorporation and the control cells have approximately 20% incorporation. All shHIF-2α clones have a BrdU incorporation of 10% or less, therefore displaying a statistically significant decrease in proliferation in comparison to control #1 cells. All cells grown in FBS have a BrdU incorporation between 30-40% (Figure 11A). The autonomous growth assay was then repeated in hypoxia to illustrate a condition similar to a tumor microenvironment. In ITS supplemented medium, parental and control #1 cells have a 20% incorporation where the shHIF-2α clones have less than 5% BrdU incorporation and are statistically significant in comparison to control #1 cells (Figure 11B). The autonomous growth assay indicates that HIF-2α has a role in autonomous growth of ovarian carcinoma cell lines. Silencing HIF-2α has a negative effect on proliferation in normoxia and hypoxia when the cells are cultured in ITS supplemented medium. By immunoblot analysis when the membrane is exposed for a longer period of time HIF-2α is present in both normoxic and hypoxic conditions (Appendix A, Figure 2). The autonomous growth assay indicates that basal level of HIF-2α is functional in normoxia as well as when it is induced in hypoxia shown in parental and control cells (Uchida et al., 2004; Wiesener et al., 1998). In the absence of HIF-2α in the shHIF-2α clones there is a decrease in proliferation when cells are in serum free ITS supplemented medium (Figures 10 and 11).

Figure 11. HIF-2α silencing impairs autonomous growth of SKOV3. A. In normoxia, SKOV3 parental and control cells grown in medium supplemented with ITS have a BrdU incorporation of approximately 20-25%. The shHIF-2α clones have approximately 10% or lower BrdU incorporation displaying a statistically significant decrease in proliferation in comparison to control #1 cells indicated by the asterisks. All SKOV3 cells grown in medium containing serum have about a 30%-40% BrdU incorporation. Autonomous growth assay in normoxia was performed three time (n=3) and the mean results are shown here. B. In hypoxia, shHIF-2α cells grown in ITS have a statistically significant inhibition in proliferation in comparison to control #1 cells at 5% or less indicated by the asterisks. All cells grown in serum containing medium have just over a 30% BrdU incorporation. The autonomous growth assay performed in hypoxia was repeated twice (n=2). Statistical analysis for both normoxic and hypoxic autonomous growth assay indicates the significant decrease in proliferation determined by Student t-test and error bars represent the SEM.

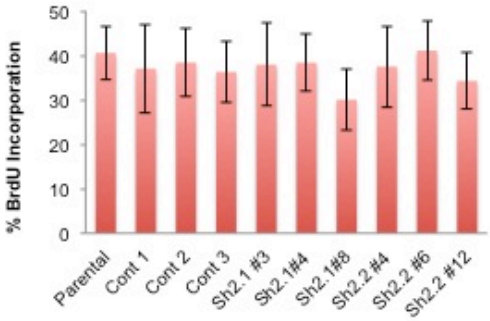
A

NORMOXIA

SKOV3-ITS



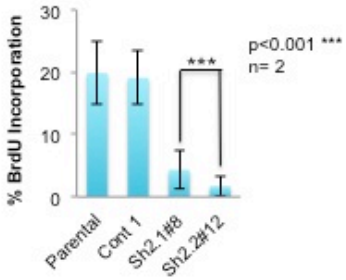
SKOV3-FBS



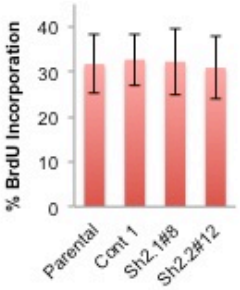
B

HYPOXIA

SKOV3-ITS



SKOV3-FBS



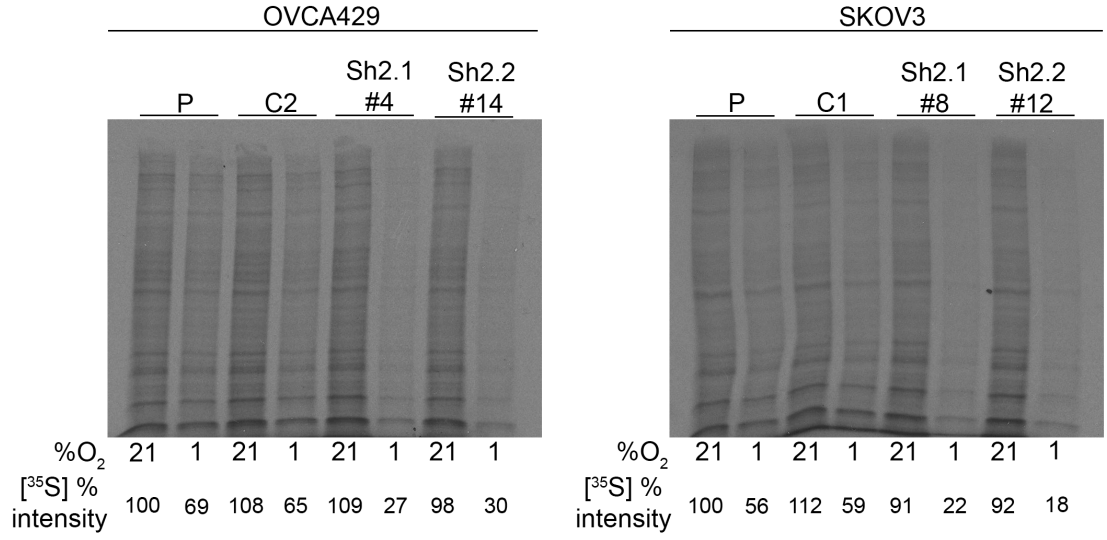
3.3 HIF-2α silencing downregulates translation in ovarian carcinoma

In recent work by our lab, HIF-2α was identified as a component of the translation-initiation complex during hypoxia (Uniacke et al., 2012). The silencing of HIF-2α and the affect it has on global translation can be determined by [^{35}S]-Met labeling. A 30 min pulse of [^{35}S]-Met was given to the cells and lysate was collected. This was done for both OVCA429 and SKOV3 cells and their lysates were run on SDS-PAGE gels. In normoxia, global translation is similar for all the cells indicated by the band intensity as a percentage relative to parental normoxic cells. In hypoxia, parental and controls cells have in between a 30-50% decrease in translation compared to its normoxic counterpart (Figure 12A). The shHIF-2α clones in hypoxia have a 70-80% decrease in translation compared to their normoxic counterparts and approximately a 40% decrease compared to parental and control cells in hypoxia (Figure 12A). There is a decrease of translation in all the cells in hypoxia but there is a further downregulation in the shHIF-2α clones.

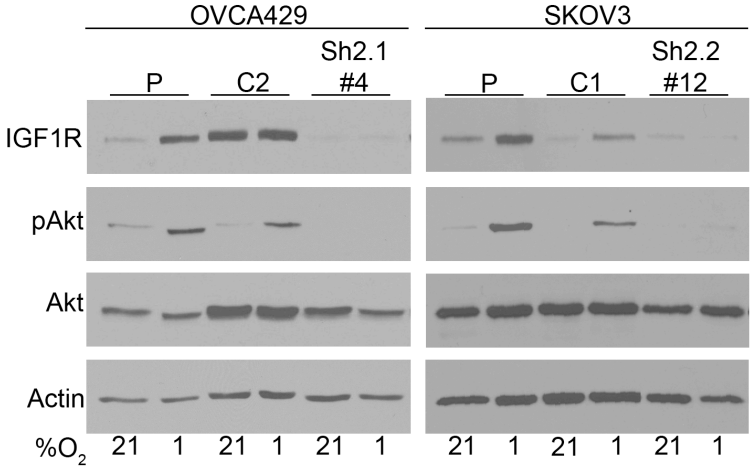
RTKs were found to be translational targets of the hypoxic translation initiation complex (Uniacke et al., 2012). Previously, in our lab a RTK array had been done for SKOV3 cells with HIF-2α silencing (Apendix A, Figure 3). RTKs having decreased expression in the HIF-2α silenced cells compared to control cells were then screened again by immunoblot analysis for both cell lines. The RTKs that were screened are EGFR, IGF1R, HER3, and Met. PDGFRα was also screened since it was shown to be a target mRNA of the HIF-2α−RMB4−eIF4E2 complex (Uniacke et al., 2012). No difference in expression was seen with HER3 and PDGFRα. EGFR and Met did shown a

Figure 12. Silencing of HIF-2α downregulates global translation and IGF1R expression. A. [^{35}S]-Met labeled OVCA429 and SKOV3 shHIF2α clones in hypoxia have a downregulation in global translation by 40% compared to parental and control cells in hypoxia indicated by the band intensity. [^{35}S]-Met labeling was performed once (n=1). B. IGF1R has an induction seen in hypoxia for OVCA429 parental cells and SKOV3 parental and control #1 cells while in the shHIF2α clones have almost no expression. OVCA429 control #2 has high levels of expression in normoxia and hypoxia. Phosphorylated Akt (pAkt) has an induction in parental and control cells in hypoxia with barely any expression in the shHIF2α clones. Immunoblot analysis of IGF1R and Akt was repeated three times (n=3) with representative blots shown here. Actin served as the loading control.

A


OVCA429
SKOV3
P
C2
Sh2.1 #4
Sh2.2 #14
P
C1
Sh2.1 #8
Sh2.2 #12
%O2 21 1 21 1 21 1 21 1
[35S] % intensity 100 69 108 65 109 27 98 30
%O2 21 1 21 1 21 1 21 1
[35S] % intensity 100 56 112 59 91 22 92 18

B


OVCA429
SKOV3
P
C2
Sh2.1 #4
P
C1
Sh2.2 #12
IGF1R
pAkt
Akt
Actin
%O2 21 1 21 1 21 1 21 1 21 1 21 1

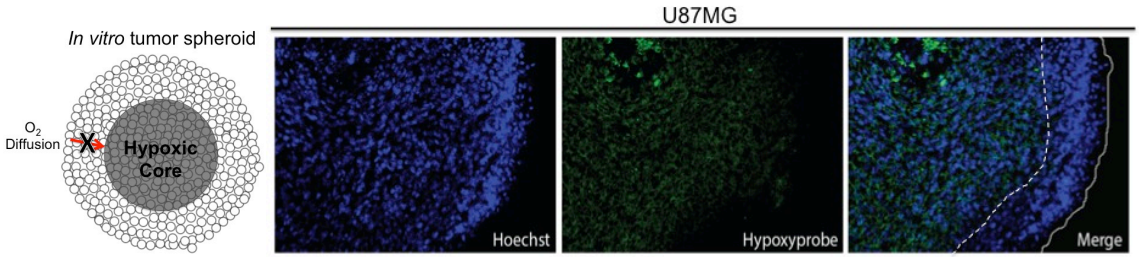
decrease in expression in the shHIF-2α clones but results were not consistant. In both OVCA429 and SKOV3 a decrease in expression of IGF1R was seen in the shHIF-2α clones (Figure 12B). OVCA429 and SKOV3 parental cells have an induction of IGF1R in hypoxia, which is also seen in SKOV3 control #1. OVCA429 control #2 has high levels of IGF1R in both normoxia and hypoxia. Phosphorylated Akt (pAkt), a downstream target of IGF1R also has an induction in parental and control cells in hypoxia with almost no expression in the shHIF-2α clones (Figure 12B). ERK1/2 is also a downstream target of IGF1R but results were not consistent for this signaling molecule.

3.4 *In vitro* tumor spheroid proliferation is prevented by HIF-2α silencing

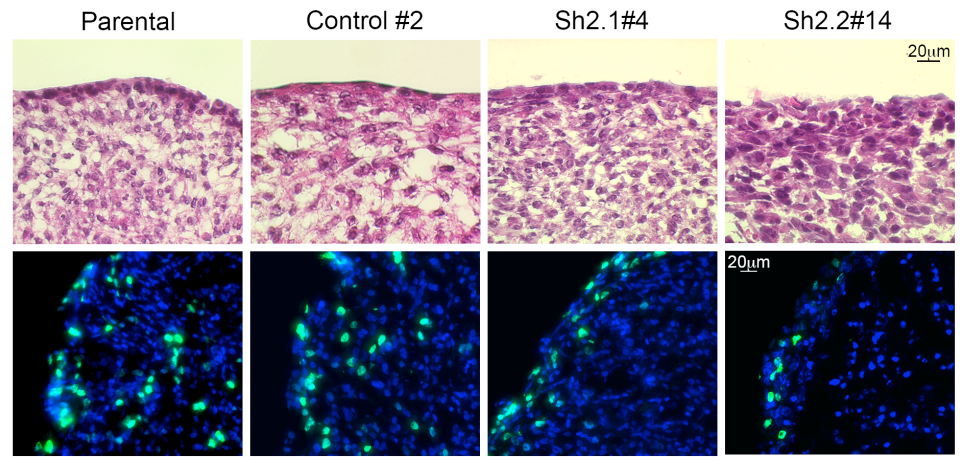
HIFα proteins have a number of transcriptional and translational targets. They have many angiogenic targets that are also upregulated in a number of cancers such as VEGF. During the development of the tumors, angiogenic factors are activated and remain on, resulting in new vessel development to maintain the demands for tumor growth (Hanahan and Folkman, 1996; Shweiki et al., 1992). *In vitro* tumor spheroid formation provides a 3-dimensional avascluar system to determine that the effect of silencing HIF-2α results in defects in the autonomous growth system and not from defects in vascularization. On the day of plating, spheroids had a loose appearance and after an overnight incubation, the spheroids became more compact, with a dense core. *In vitro* spheroids have a hypoxic core due to the fact that oxygen is unable to diffuse the distance through the layers of cells and therefore an upregulation of HIFα proteins is seen (Figure 13A) (Franovic et al.,

Figure 13. Avascular OVCA429 tumor spheroids have a decrease in proliferation in the absence of HIF-2α. A. Hypoxyprobe staining of U87MG spheroids delineating the hypoxic core. B. H&E staining illustrate the compaction of the spheroids with Sh2.1#14 showing the greatest level of compaction compared to parental and control #2, while Sh2.2#14 is the least compact (top panel) (40X). Parental and control #2 spheroids have Ki67 expression with more at the periphery than the core. The shHIF2α clones also have Ki67 expression at the periphery and some in the core but a decrease was seen in comparison to parental and control #2 (bottom panel pictures with 20X). C. Parental spheroid has 25% Ki67 staining at the periphery and 20% in the core. Ki67 expression for control #2 spheroid is 30% at the periphery and 25% at the core. A decrease of Ki67 expression is seen in the shHIF2α spheroids with around 10% of expression at the periphery and 3-5% in the core. 4 representative areas of periphery and 4 of the core (60X) were counted to obtain a percentage of Ki67 expression to Hoechst positive cells. Experiment was performed once (n=1) and error bars represent the standard deviation (representative pictures in Appendix, A Figure 4A).

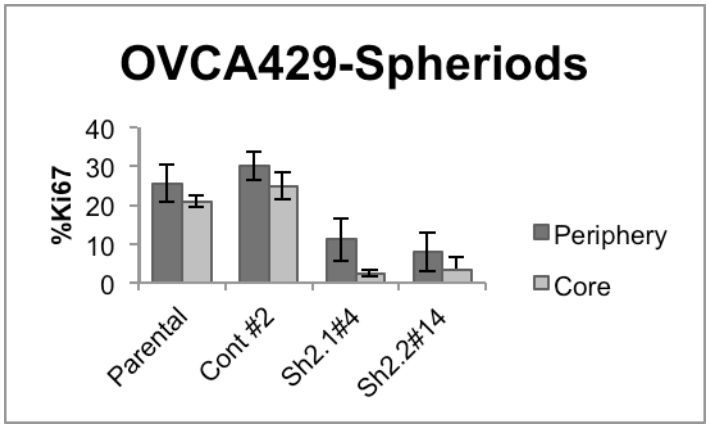
A


U87MG
In vitro tumor spheroid
O2 Diffusion
Hypoxic Core
Hoechst
Hypoxyprobe
Merge

B


OVCA429
Parental
Control #2
Sh2.1#4
Sh2.2#14
20μm
20μm

C


OVCA429-Spheriods
%Ki67
40
30
20
10
0
Parental
Cont #2
Sh2.1#4
Sh2.2#14
Periphery
Core

2007; Grote et al., 1977; MacDougall and McCabe, 1967). The hypoxic core of spheroids is indicated by hypoxyprobe staining. Hypoxyprobe is added to the cell monolayer before they are plated for spheroid formation. The hypoxyprobe is incorporated by the cells and when the spheroid becomes hypoxic the hypoxyprobe is activated and can be detect using a specific antibody shown in U87MG glioblastoma spheroid (Figure 13A). The spheroids were grown for 5-7 days and then frozen for immunofluorescence analysis using an antibody to Ki67. Ki67 is a proliferation marker and is present during all active phases of the cell cycle but absent in G_0 (Scholzen and Gerdes, 2000).

OVCA429, Sh2.1#4 has a great level of compaction compared to parental, control #2 with Sh2.2#14 having the least amount of compaction (Figure 13B top panel). Ki67 is seen strongly on the outer surface of parental and control #2 spheroids and is also present in the core . The shHIF-2α clones have a decrease in Ki67 expression seen mostly at the periphery (Figure 13B bottom panel). Ki67 expression is 25% at the periphery and 20% in the core for parental spheroids, where control #2 has 30% at the periphery and 25% in the core. Periphery staining in the shHIF-2α spheroids is approximately 10%, where core staining is around 3-5% (Figure 13C) (Appendix A, Figure 4A). A decrease in proliferation in comparison to parental and control #2 is seen in the shHIF-2α spheroids. The periphery of the spheroids have a higher Ki67 expression than the core since oxygen is available to the outer layers in parental and control #2 spheroids. The cores of the spheroids are hypoxic and with HIF-2α growth is able to occur but in the absence of HIF-2α a decrease in proliferation is present in the shHIF-2α clones (Figure 13C).

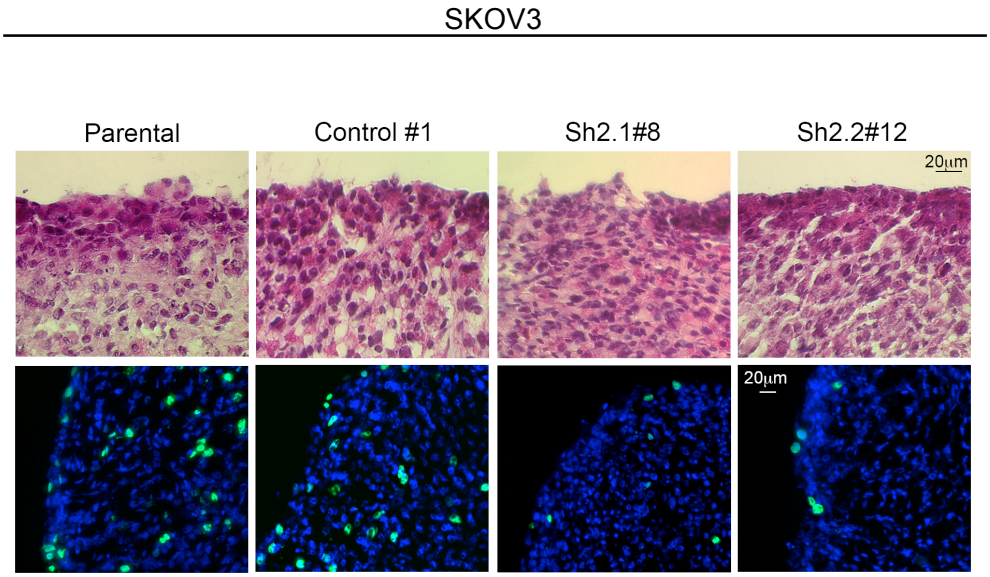
Similar results are seen in SKOV3 spheroids for Ki67 expression. SKOV3 spheroids are very compact, with Sh2.1#8 more so than the others shown by H&E staining (Figure 14A top panel). In parental and control #1 cells, Ki67 expression is seen in the outer surface as well as in the hypoxic core, while the shHIF-2α clones have a decrease in Ki67 expression at the periphery and hypoxic core (Figure 14A bottom panel). Approximately 25% of cells in parental and control #1 spheroids express Ki67, at the periphery and approximately 20% in the core. Periphery Ki67 expression is approximately 5% in the shHIF-2α spheroids with 2% in the core (Figure 14B) (Appendix A, Figure 4B). *In vitro* spheroid formation indicates that the effects of HIF-2α silencing are not from insufficient vascularization, but due to defects in autonomous growth.

3.5 HIF-2α is necessary for in vivo tumor proliferation

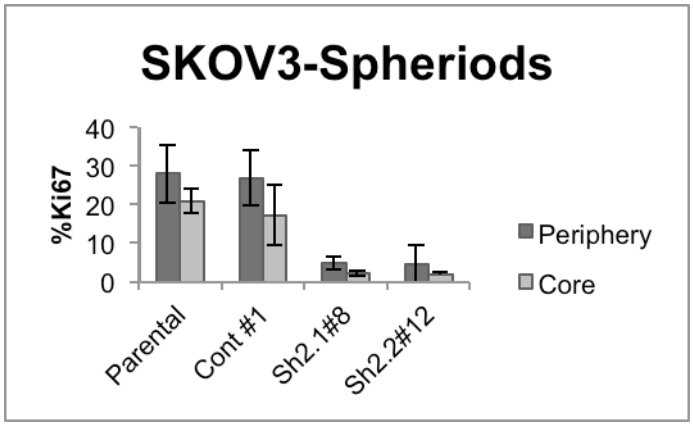
It has previously been shown that HIF-2α is necessary for *in vivo* tumorigenesis in various carcinomas (Franovic et al., 2009; Holmquist-Mengelbie et al., 2006; Kondo et al, 2002; Menrad et al., 2010; Raval et al., 2005). Thus far HIF-2α has downregulated proliferation *in vitro* in 2-dimensional cultures (serum free) and tumor spheroids. There is also a decrease in global translation and expression of IGF1R in hypoxia. Xenografts have previously been done with OVCA429 and SKOV3 parental cells and took approximately two months for tumors to form when injected into the peritoneal cavity (Shaw et al., 2004). SKOV3 cells when injected subcutaneously formed tumors with two months of injection (Nielsen et al., 2000). Knowing that the cells contain the

Figure 14. Growth of SKOV3 in vitro avascular spheroids is downregulated due to HIF2α silencing. A. SKOV3 spheroids are compact with Sh2.1#8 more so than the others (top panel). Parental and control #1 have Ki67 expression in the hypoxic core but more so at the outer edge. The shHIF2α spheroids have a decrease of Ki67 expression at the periphery and core. B. Parental and control #1 spheroids have approximately 25% Ki67 expression at the periphery and 20% in the core. Periphery expression of Ki67 is approximately 5% with the core being approximately 2% in the shHIF2α spheroids. Representative areas of the periphery and core (4 each at 60X) were counted to obtain a percentage of Ki67 expression to Hoechst positive cells. Experiment was performed once (n=1) and error bars represent the standard deviation (representative pictures in Appendix A, Figure 4B).

A


SKOV3
Parental
Control #1
Sh2.1#8
Sh2.2#12
20μm
20μm

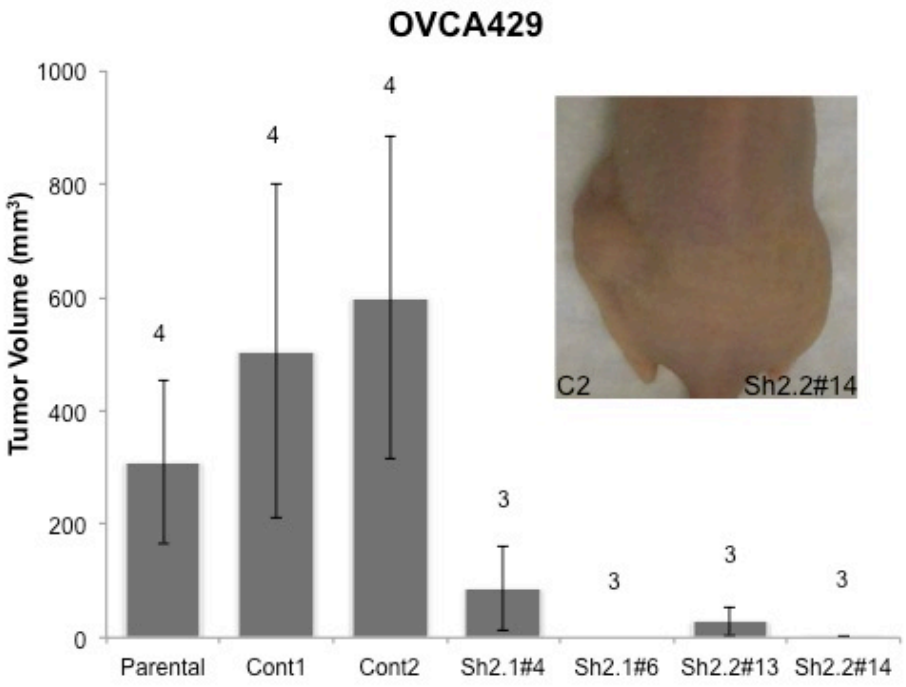
B


SKOV3-Spheriods
%Ki67
40
30
20
10
0
Parental
Cont #1
Sh2.1#8
Sh2.2#12
Periphery
Core

capacity to form xenografts, 10^7 cells were injected subcutaneously into the flank of female CD1 nude mice. All mice were sacrificed at three months except if the tumors became to large then the mouse was sacrificed before the three month endpoint. These mice lack their thymus and are therefore unable to produce T-cells, resulting in immunodeficiency (Charles River, 2011). For OVCA429 each shHIF-2α clone was injected into three mice alongside parental or control cells. Parental, control #1 and control #2 xenografts were in between 300-600 mm^3 in volume, while no xenografts formed for Sh2.1#6. Average tumor volume was less than 100 mm^3 for Sh2.1#4, Sh2.2#13 and Sh2.2#14.

SKOV3 shHIF-2α clones were also tested to see if tumor formation would occur. Clones Sh2.1#3, Sh2.1#8 and Sh2.2#12 were each injected in three mice on the right with either parental, control #1 or control #3 on the left side. Tumor formation did not occur in shHIF-2α clone while parental cells formed tumors after two months of being injected. No tumor formation occurred in the control cells. The xenograft assay with SKOV3 does need to be repeated with new controls and shHIF-2α. From the xenograft assay with OVCA429 it shows that parental and control cells were capable of tumor formation while silencing HIF-2α disabled or impaired tumor formation in the shHIF-2α clones.

Figure 15. HIF-2α silencing decreases the tumorigenic capability of OVCA429. Endpoint tumor volumes of xenografts for OVCA429 parental, control and shHIF-2α clones. In OVCA429 parental, control #1 and control #2 xenografts formed tumors approximately 300-600 mm^3 in volume. No tumor formation occurred for Sh2.1#6, while Sh2.1#4, Sh2.2#13 and Sh2.2#14 have small tumor formation with average tumor volumes less than 100 mm^3. The number of mice injected for parental, control and shHIF-2α clones is indicated above the average volume bar. Error bars indicate the standard deviation and the xenograft assay was performed once (n=1).


OVCA429
Tumor Volume (mm3)
1000
800
600
400
200
0
4
4
4
3
3
3
3
C2
Sh2.2#14
Parental
Cont1
Cont2
Sh2.1#4
Sh2.1#6
Sh2.2#13
Sh2.2#14

4 DISCUSSION

4.1 Summary of major evidence

The investigation of the role of HIF-2α in ovarian carcinoma began by determining at what hypoxic time points the HIFα proteins were present to allow for screening of the shHIF-2α clones. In OVCA429, HIF-1α and HIF-2α are both present at 4 hours while in SKOV3 HIF-1α peaked at 4 hours while HIF-2α peaked at 8 hours (Figure 8). HIF-2α is strongly induced and continuously expressed in hypoxia, where HIF-1α is known to have transient expression, expressed acutely at very low oxygen levels (O_2 0-1%), and have a shortened half-life the longer it is exposed to a hypoxic environment (Berra et al., 2001; Holmquist-Mengelbier et al, 2006; Marxsen et al., 2004). HIF-1α is more sensitive to PHD2 and PHD3 degradation. It is also degraded by specific E3 ubiquitin ligases such as HIF-associated factor (HAF) and heat shock protein 70 (HSP70) and carboxyl terminus of HSP70-interaction protein (CHIP) (Holmquist-Mengelbier et al, 2006; Koh et al., 2008, Koh et al., 2011; Luo et al., 2010; Marxsen et al., 2004). HIF2α is not affected by either of these two E3 ubiquitin ligases that act independently of VHL (Koh et al., 2008, Koh et al., 2011; Luo et al., 2010).

After screening clones and identifying ones with a knock down in HIF-2α the autonomous growth assay was done. OVCA429 and SKOV3 cells with HIF-2α silencing have a significant decrease in proliferation in comparison to control cells *in vitro* when cultured in serum free medium in normoxia and hypoxia (Figure 10 and 11). By immunoblot analysis HIF-2α is detectable when the membrane is exposed for a longer

period of time and mRNA levels of HIF-2α was similar in parental and control cells in normoxia and hypoxia (Figure 9B) (Appendix A, Figure 2). Post-translational regulation of HIFαs decreases their protein levels but the basal level of HIF-2α has a potent effect. This is seen in the autonoumous growth assay where a decrease in proliferation is not only present in hypoxia but also in normoxia in the shHIF-2α clones (Figure 10 and 11) (Uchida et al., 2004; Wiesener et al., 1998). To determine that the effect of silencing HIF-2α was affecting autonomous growth and not angiogenesis, 3-dimensional *in vitro* avascular spheroids were used. Spheroids made with the shHIF-2α cells have a decrease in the proliferation marker Ki67, therefore confirming that silencing of HIF-2α was affecting autonomous growth in the ovarian carcinoma cell lines (Figure 13 and 14).

From the autonomous growth assay it was shown that in the absence of exogenous growth factors, shHIF-2α clones have impeded proliferation (Figure 10 and 11). Silencing HIF-2α halts the production of RTKs as well as other proteins, inactivating their downstream signaling and preventing proliferation. [^{35}S]-Met labeling was used to determine the effect of silencing HIF-2α on global translation in ovarian carcinoma. In the absence of HIF-2α there is a 40% decrease in global translation in hypoxia compared to parental and control cells (Figure 12A). Certain RTKs have been found to be translational targets of the hypoxic translation initiation complex, where HIF-2α is one of the components. IGF1R in parental and control cells have an induction in hypoxia except for OVCA429 control #2, where in the shHIF-2α clones a downregulation is seen in hypoxia (Figure 12B). Akt is a downstream target of IGF1R and it followed the same protein expression profiles as IGF1R. An induction of pAkt is present in hypoxia in

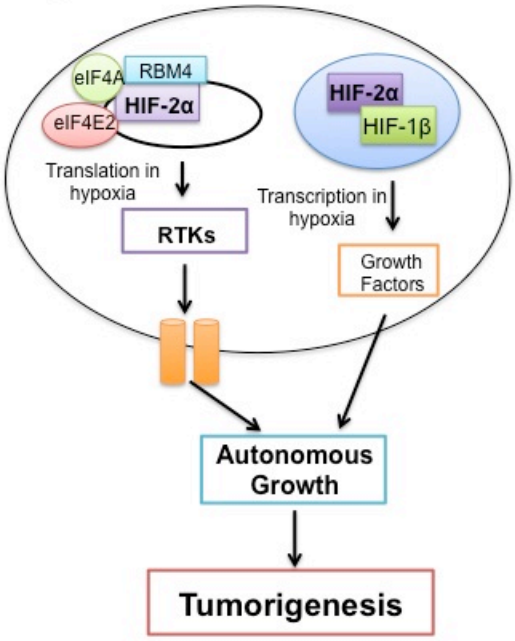
parental and control cells, where the shHIF-2α clones have barely any expression. The Akt pathway has roles in protection from apoptosis, glucose metabolism, proliferation, transcription and migration (Kuemmerle, 2003; Shelton et al. 2004; Torres Aleman, 2005). OVCA429 and SKOV3 were then used for the nude mice xenograft assay to assess the tumorigenic effect of silencing HIF-2α. For OVCA429 small or no tumor formation occurred from the shHIF-2α where parental and control cells formed tumors (Figure 15). SKOV3 parental cells formed tumors, where no tumor formation occurred for the shHIF-2α clones. The SKOV3 controls also did not form tumors. New SKOV3 controls and shHIF-2α clones will have to be made to test the hypothesis. Taken together, the data suggests that HIF-2α activates autonomous proliferation through the activation of its transcriptional and translational targets (growth factors and RTKs) leading to tumorigenesis in ovarian carcinoma (Figure 16).

4.2 HIF-2α the oncogenic protein

Cancer is diverse in many ways since it occurs in all different cell types of the body. Mutations occurring in genes can produce oncogenes or tumor suppressors that deregulate signaling pathways promoting a cancer phenotype. These changes allow the acquisition of the hallmarks of cancer, such as autonomous growth. These oncogenes, tumor suppressors and deregulated signaling pathways are seen in all cancers but not every cancer employs the same factors to be able to proliferate and form tumors (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). There are many different contributing factors to tumorigenesis in cancer cells but we believe that HIF-2α is a

Figure 16. Autonomous growth and tumorigenesis in ovarian carcinoma. The activation of HIF-2α in ovarian carcinoma due to tumor hypoxia activates its transcriptional and translational targets such as growth factors and RTKs, respectively. The elevated activation of RTK and their downstream signaling pathways leads to autonomous growth resulting in tumorigenesis of ovarian carcinoma.

Autonmous growth and tumorigenesis in ovarian carcinoma



recurring theme. As tumor formation and expansion occurs hypoxia is developed from inadequate vascularization. Due to tumor hypoxia HIF-1α and HIF-2α are active to aid in the adaption to the tumor microenvironment. HIF-1α and HIF-2α activation is seen in an array of cancers and thought to be involved in the progression of cancer (Aebersold et al., 2002; Birner et al., 2000; Birner et al., 2001; Birner et al., 2001; Bos et al., 2001; Maxwell et al., 1999; Osada et al., 2007; Schindl et al., 2002; Sivridis et al., 2002; and Talks et al., 2000; reviewed in Bertout et al., 2008; Keith et al., 2011; Vaupel and Harrison, 2004). Although much of the focus of studies has been on HIF-1α, we have focused on HIF-2α. These two HIFα isoforms are highly conserved at the protein level but they have more than just overlapping roles in the response to hypoxia. HIF-2α seems to be the oncogenic protein of the two having an effect on growth and tumor formation in cancer.

4.3 HIF-2α and autonomous growth

HIF-2α has shown to be involved in sustaining proliferative signaling or autonomous growth (Franovic et al. 2009; Menrad et al., 2010; Stiehl et al., 2011). HIF-2α is present in ovarian carcinoma samples at all stages of the disease but an investigation of the role it plays in the development of ovarian carcinoma had not been completed (Osada et al., 2007; Talks et al., 2000). We as well as other laboratories have previously shown in various epithelial cancers regardless of tissue type and genetic position that HIF-2α activation is required for autonomous growth *in vitro* and for tumorigenesis *in vivo* (Franovic et al. 2009; Menrad et al., 2010; Stiehl et al., 2011).

By the autonomous growth assay, proliferation is decreased in ovarian carcinoma cells when HIF-2α is silenced and cultured in serum free medium (Figure 10 and 11). By culturing the cells in serum free medium supplemented with ITS, the exogenous growth factors are removed that aid in proliferation. Translation of RTKs containing an rHRE is halted in the absence of HIF-2α affecting proliferation of the cells (Figure 12B). Although RTKs are translational targets of HIF-2α, some growth factors are transcriptional targets. TGFα is a transcription target of HIF-2α and a renal mitogen in RCC. The TGF-α/EFGR pathway is activated in RCC due to HIF-2α activity resulting in autonomous growth (de Paulsen et al., 2001; Gunaratnam et al., 2003; Smith et al., 2005). HIF-2α is also known to activate other growth factors transcriptionally that may play a role in ovarian carcinoma autonomous growth such as insulin growth factor-1 (IGF1), since its receptor, IGF1R, is affected by HIF-2α silencing (Figure 12B) (Akeno et al., 2002; Scharf et al., 2005).

4.4 HIF-2α and translation

In hypoxia the mechanism used by cells for translation initiation was unknown. IRES-mediated initiation is an alternative method but has shown not to be the replacement mechanism. In a study by Young et al., hypoxia regulated mRNA containing an IRES such as VEGF and HIF-1α were tested to determine if they used this cap-independent method of translation. Cells were oxygen starved and transfected with synthesized mRNA and IRES-mediated translation accounted for <1% of translation (Young, et al., 2008).

HIF-1α and HIF-2α are activated to aid in the adaptation to the hypoxic environment and function to activate transcription of target genes. These transcribed genes need to be translated as well to carry out the response to hypoxia. The oxygen-regulated translation initiation complex HIF-2α−RMB4−eIF4E2 seems to be the main mechanism used for translation in hypoxia. Without HIF-2α or/and RMB4 there is a approximately a 40% decrease in global translation in hypoxia compared to control cells (Uniacke et al., 2012). Experiments for the discovery of this complex was done in U87MG glioblastoma cells and in renal proximal tubular epithelial cells. Here, it is shown that in the absence of HIF-2α in ovarian carcinoma cells there is also a 40% decrease in global translation as well as in IGF1R and its downstream target pAkt (Figure 12). The Akt pathway is one of the main pathways activated by IGF1R, leading to protection from apoptosis. IGF1R is a transmembrane protein and upon ligand binding, the tyrosine protein kinase is activated and phosphorylation of tyrosine residues occurs (Shelton et al. 2004; Torres Aleman, 2005). The tyrosine kinase activity of the IGF1R increases, leading to phosphorylation of associated substrate proteins such as PI3K leading to activation of Akt (Kuemmerle, 2003). Activation of Akt inactivates several proteins that are involved in apoptosis. Akt also has roles in other cellular processes such as glucose metabolism, cell proliferation, transcription and cell migration (Kuemmerle, 2003; Kulik et al., 1997; Shelton et al. 2004; Torres Aleman, 2005).

IGF1R overexpression has been seen in cisplatin resistant cell lines (Eckstein et al., 2009; Reibenwein and Krainer, 2008). SKOV3 is a cisplatin resistant cell line and an induction of IGF1R is seen in hypoxia and is also present in OVCA429 (ATTC, 2011).

IGF1R monoclonal antibodies and inhibitors are currently in clinical trials but have not yet been approved for use in cancer therapy (Brokaw et al., 2007; Mabuchi et al., 2007; Marone et al., 2008; Spentzos et al., 2007; Weroha et al., 2008). Instead of targeting individual RTKs for cancer therapy that often acquire resistance, targeting HIF-2α would affect multiple proteins associated with tumor progression.

4.5 HIF-2α and the Hallmarks of Cancer

The hallmarks of cancer and their enabling characteristics come together allowing tumor formation and metastasis. The role of HIF-2α in autonomous growth of cancer has already been demonstrated and it is also involved in multiple hallmarks. The PI3K/Akt pathway is activated by IGF1R kinase activity, a HIF-2α translational target, inducing potent anti-apoptotic effects and therefore resisting cell death (Datta et al., 1997; Kulik et al., 1997; Shelton et al. 2004; Uniacke et al., 2012). VEGF, a HIF-2α transcriptional target is upregulated in cancer (Carmeliet, 2005). It is involved in inducing angiogenesis since tumors require nutrients and oxygen (Lynden et al., 2001). Tumor-associated neovasculature is formed by angiogenesis. The process of angiogenesis is rather quiescent in normal adult cells except in certain circumstances were it is turned on for a short period of time such as during wound healing (Hanahan and Folkman, 1996). In tumors the angiogenic switch is an early event in the invasiveness of cancers and almost always remains on since HIFαs are present due to tumor hypoxia (Hanahan and Folkman, 1996; Raica et al., 2009).

Carcinomas progress to a higher pathological grade of malignancy shown by their invasive ability and metastasis. E-cadherin, a cell-to-cell adhesion molecule forms adherens junctions between adjacent epithelial cells building epithelial sheets. A downregulation of E-cadherin alters the cell's physiology and attachment to the extracellular matrix leading to invasion and metastasis (Mareel et al., 1994; Oka et al., 1994). In VHL defective RCC cell lines HIF activation is necessary to suppress E-cadherin. Cancer cells express a hypoxic proteome and with the presence of HIF-2α, E-cadherin's function is perturbed. A combined effort from both HIF-1α and HIF-2α are needed for the suppression of E-cadherin. The epithelial-mesenchymal transition (EMT) program is also involved in invasion and metastasis, an acquired attribute usually present in development (Chrisriansen and Rajasekaran, 2006; Waerner et al., 2006; Xu et al., 2006). The activation of the EMT program in mice with lung carcinoma expressing HIF-2α and containing a mutant *KRAS* have increased tumor burden and invasiveness with shortened survival (Kim et al., 2009).

An emerging hallmark is reprogramming energy metabolism. Cancer cell undergo an energy adjustment to use anaerobic glycolysis. Anaerobic glycolysis is used to produce energy rather than pyruvate continuing onto oxidative phosphorylation in the mitochondria due to the decrease in oxygen availability. This observation was first made by Otto Warburg who found even in the presence of oxygen cancer cells still utilize anaerobic glycolysis leading to an acidic tumor environment known as the Warburg effect (Warburg, 1930; Warburg 1956a; Warburg 1956b; reviewed in Gatenby and Gilles, 2004; Hanahan and Weinberg, 2011). Cancer cells compensate for the lower efficiency

of energy production by upregulating glucose transporters, especially Glut-1 a transcriptional target of both HIF-1α and HIF-2α to upregulate glycolysis. HIF-2α has a number of roles in the acquisition of multiple hallmarks of cancer.

4.6 Therapeutic targeting of HIF-2α

With the new discovery of the translational role of HIF-2α, the focus of target therapy should be towards this protein. Cancer cells are hypoxic and due to the HIF-2α−RBM4−eIF4E2 complex they display a hypoxic proteome enabling the cancerous cells to expand and form tumors (Uniacke et al., 2012; Vaupel and Harrison, 2004). By targeting cells utilizing the hypoxic translational machinery, therefore the cancerous cells, it would not interfere with regular eIF4E translation in a patient. Bacteriostatic antibiotics inhibit the protein synthesis in the bacteria causing them not to be able to proliferate (Pankey and Sabath, 2004). Inhibiting HIF-2α's role in translation would work in a similar fashion as a bacteriostatic antibiotic by inhibiting translation resulting in no proliferation and eventually the cancerous cells would not be able to survive without the building blocks of life.

Inhibitors and monoclonal antibodies to GFs and RTKs have been successful in the treatment of certain cancers but eventually resistance occurs (Engleman an Settleman, 2008; Sergina and Moasser, 2007). By targeting HIF-2α, multiple RTKs and proteins involved in tumor formation and progression would be targeted. The HIF-2α−eIF4E2 contact would be the ideal interaction to inhibit in the oxygen-regulated complex. RBM4 is involved in alternative splicing of pre-mRNA, translation and RNA silencing. It is

ubiquitously expressed in cells with a stronger expression in certain tissues and cell types (Markus and Morris, 2009). By inhibiting HIF-2α−RBM4 it may affect the binding of co-factor proteins to RBM4 during regular eIF4E translation as well as inhibit its other functions in noncancerous cells. There are currently many compounds that inhibit HIFα activity that could be screened for an inhibitory effect on the translational activity of HIF-2α allowing multiple hallmarks to be targeted at once.

4.7 Future directions

In ovarian carcinoma cell lines with a lack of HIF-2α, translation is decreased, autonomous growth is impeded and tumor formation is reduced. HIF-2α has been silenced in a number of carcinomas and decreased *in vitro* proliferation and *in vivo* tumorigenesis (Franovic et al. 2009; Menrad et al., 2010; Stiehl et al., 2011). The next step would involve producing stable cell lines with eIF4E2 silencing to observe its effects on tumorigenesis. In our most recent paper, silencing of eIF4E2 was performed in U87MG hindering its association with HIF-2α−RBM4 and downregulating global hypoxic translation. It would be beneficial to expand the role eIF4E2 beyond the U87MG cell line. This is a current project in our lab and stable cell lines with eIF4E2 silencing are being produced.

Stable cell lines with eIF4E2 silencing have been generated in U87MG glioblastoma, HCT116 colorectal and 7860 RCC. With the cell lines containing the eIF4E2 silencing, a nude mice xenograft assay will be completed. In the xenograft assay with sheIF4E2 we

expect to see similar results to that of the shHIF-2α clones. Within the first to second week post injection with the cells an initial mass forms. This mass is likely hypoxic since we have previously shown in shHIF-2α initial masses there was a decrease of Ki67. Therefore, no tumor formation should occur with sheIF4E2 cells. Once it is determined that no tumor formation occurs from eIF4E2 silencing a search should begin to find compounds that are able to inhibit the HIF-2α−eIF4E2 association.

This could be done with compounds currently known to inhibit HIF-2α to see if oxygen-regulated translation is inhibited. These compounds could be added to the various cell lines (U87MG, 7860, HCT116, OVCA429 and SKOV3) to find a compound that inhibits the HIF-2α−eIF4E2 association. The screen would involve culturing the cells in hypoxia in the presence of the compounds. Lysate could then be collected and immunoblot analysis can be done to see if there is a decrease in translational targets such as EGFR and IGF1R. Cell proliferation and cell survival could also be tested by the autonomous growth and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, respectively. Compounds that have an inhibitory effect could be moved to the next level of experiments and be tested in mice. Mice would be injected subcutaneously with the cancer cell lines. Once tumors are formed the compounds could be fed to the mice to see if they are capable of inhibiting tumor growth. Tumors could be excised and stained with Ki67 for proliferation and TUNEL for apoptosis to determine if the compounds have an inhibitory effect on hypoxic translation. The targeting of HIF-2α in the oxygen-mediated translational complex seems to be a promising cancer therapy targeting many tumorigenic proteins. With the compounds having an inhibitory effect on

tumor growth, clinical trials could be set up as the next stage of testing.

Another current project in the lab is looking into how there is a constitutive expression of HIF-2α in cancer. We have found that there is a loss of DNA methyl transferase 3a (DNMT3a) that is responsible for methylating and silencing EPAS1, which may be an initial stage in cancer cells. In RCC low grade tumor samples and overt carcinomas have a loss of DNMT3a compared to its adjacent renal tissue leading to HIF-2α activation and autonomous growth. The introduction of DNMT3a in RCC cell lines establishes the same effect seen when HIF-2α is silenced. In renal epithelial cells silencing of DNMT3a was able to upregulate HIF-2α and activate the TGF-α/EFGR pathway. This discovery is now in the process of being expanded to see if DNMT3a silencing is an initial step in multiple carcinomas and then to rescue tumor formation in xenografts by reintroducing DNMT3a.

4.8 Conclusion

In OVA429 and SKOV3 ovarian carcinoma cell lines, here it has been shown that the silencing of HIF-2α decreases *in vitro* proliferation and *in vivo* tumorigenesis. In the absence of HIF-2α global translation is diminished as well as the RTK, IGF1R, which is involved in cell proliferation and survival. HIF-2α is a component in the oxygen-regulated translational initiation machinery and without it translation in hypoxia is downregulated. Portions of tumors are hypoxic due to its vasculature not being able to supply it with an efficient amount of oxygen. Ovarian carcinoma displays a hypoxic

proteome since HIF-2α has a role in activating transcription and translation leading to autonomous proliferation and tumorigenesis (Figure 16). HIF-2α has proven to be a major player in the oncogenic progression of ovarian carcinoma and should be a focus for targeted cancer therapy.

5 REFERENCES

Aboud-Pirak, E., Sergent, T., Otte-Slachmuylder, C., Abarca, J., Trouet, A., and Schneider, Y.J. (1988). Binding and endocytosis of a monoclonal antibody to a high molecular weight human milk fat globule membrane-associated antigen by cultured MCF-7 breast carcinoma cells. Cancer Res *48*, 3188-3196.

Advanced Ovarian Cancer Trialists Group. (1998). Chemotherapy in advanced ovarian cancer: four systematic meta-analyses of individual patient data from 37 randomized trials. Advanced Ovarian Cancer Trialists' Group. Br J Cancer *78*,1479–87.

Aebersold, D.M., Burri, P., Beer, K.T., Laissue, J., Djonov, V., Greiner, R.H., and Semenza GL. (2001). Expression of hypoxia-inducible factor-1alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. Cancer Res *61*, 2911–2916.

Akeno, N., Robins, J., Zhang, M., Czyzyk-Krzeska, M.F., and Clemens, T.L. (2002). Induction of vascular endothelial growth factor by IGF-I in osteoblast-like cells is mediated by the PI3K signaling pathway through the hypoxia-inducible factor-2alpha. Endocrinology *143*, 420-425.

Almeida, A., Muleris, M., Dutrillaux, B., and Malfoy, B. (1994). The insulin-like growth factor I receptor gene is the target for the 15q26 amplicon in breast cancer. Genes Chromosomes Cancer *11*, 63-65.

American Cancer Society. (2012). Cancer Facts & Figures 2012. Atlanta, GA.

An, W.G., Kanekal, M., Simon, M.C., Maltepe, E., Blagosklonny, M.V., and Neckers, L.M. (1998). Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature *392*, 405-8.

Arany, Z., Huang, L.E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M.A., Bunn, H.F., and Livingston, D.M. (1996). An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci U S A *93*, 12969-12973.

Arteaga, C.L. (2007). HER3 and mutant EGFR meet MET. Nature Medicine *13*, 675-677.

Atlas, E., Cardillo, M., Mehmi, I., Zahedkargaran, H., Tang, C., and Lupu, R. (2003). Heregulin is sufficient for the promotion of tumorigenicity and metastasis of breast cancer cells in vivo. Mol Cancer Res *1*, 165-175.

Baselga, J., Perez, E.A., Pienkowski, T., and Bell, R. (2006). Adjuvant trastuzumab: a milestone in the treatment of HER-2-positive early breast cancer. The oncologist *11 Suppl 1*, 4-12.

Bell, J., Brady, M.F., Young, R.C., Lage, J., Walker, J.L., Look, K.Y., Rose, G.S., Spirtos, N.M., and Gynecologic Oncology, G. (2006). Randomized phase III trial of three versus six cycles of adjuvant carboplatin and paclitaxel in early stage epithelial ovarian carcinoma: a Gynecologic Oncology Group study. Gynecol Oncol *102*, 432-439.

Berra, E., Richard, D.E., Gothie, E., and Pouyssegur, J. (2001). HIF-1-dependent transcriptional activity is required for oxygen-mediated HIF-1alpha degradation. FEBS letters *491*, 85-90.

Bertout, J.A., Patel, S.A., and Simon, M.C. (2008). The impact of O2 availability on human cancer. Nat Rev Cancer *8*, 967-975.

Bettochi, S., Restaino, A., Lucisano, F., Orlando, E., Ferreri, R., Ierardi, G.M., and Selvaggi, L. (1982). Epidemiological factors and prophylaxis of ovarian tumors. Eur J Gynaecol Oncol *3*, 192-205.

Binkovitz, L.A., Johnson, C.D., and Stephens, D.H. (1990). Islet cell tumors in von Hippel-Lindau disease: increased prevalence and relationship to the multiple endocrine neoplasias. AJR Am J Roentgenol *155*, 501-505.

Birner, P., Schindl, M., Obermair, A., Plank, C., Breitenecker, G., and Oberhuber, G. (2000). Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res *60*, 4693–4696.

Birner, P., Gatterbauer, B., Oberhuber, G., Schindl, M., Rössler, K., Prodinger, A., Budka, H., and Hainfellner, J.A. (2001). Expression of hypoxia-inducible factor-1 alpha in oligodendrogliomas: its impact on prognosis and on neoangiogenesis. Cancer *92*, 165–171.

Birner, P., Schindl, M., Obermair, A., Breitenecker, G., and Oberhuber, G. (2001). Expression of hypoxia-inducible factor 1alpha in epithelial ovarian tumors: its impact on prognosis and on response to chemotherapy. Clin Cancer Res *7*, 1661–1668.

Blume-Jensen, P., and Hunter, T. (2001). Oncogenic kinase signalling. Nature *411*, 355-365.

Bos, R., Zhong, H., Hanrahan, C.F., Mommers, E.C., Semenza, G.L., Pinedo, H.M., Abeloff, M.D., Simons, J.W, van Diest, P.J., and van der Wall, E. (2001). Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst *93*, 309–314.

Bos, R., van Diest, P.J., de Jong, J.S., van der Groep, P., van der Valk, P., and van der Wall, E. (2005). Hypoxia-inducible factor-1alpha is associated with angiogenesis, and expression of bFGF, PDGF-BB, and EGFR in invasive breast cancer. Histopathology *46*, 31-36.

Brahimi-Horn, C., Mazure, N., and Pouysségur, J. (2005). Signalling via the hypoxia-inducible factor-1alpha requires multiple posttranslational modifications. Cell Signal *17*, 1-9.

Brokaw, J., Katsaros, D., Wiley, A., Lu, L., Su, D., Sochirca, O., de la Longrais, I.A., Mayne, S., Risch, H., and Yu, H. (2007). IGF-I in epithelial ovarian cancer and its role in disease progression. Growth Factors *25*, 346–54.

Bruick, R.K., and McKnight, S.L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. Science *294*, 1337-1340.

Burges, A., and Schmalfeldt, B. (2011). Ovarian cancer: diagnosis and treatment. Dtsch Arztebl Int *108*, 635-641.

Campbell, I.G., Russell, S.E., Choong, D.Y., Montgomery, K.G., Ciavarella, M.L., Hooi, C.S., Cristiano, B.E., Pearson, R.B., and Phillips, W.A. (2004). Mutation of the PIK3CA gene in ovarian and breast cancer. Cancer Res *64*, 7678-7681.

Canadian Cancer Society, Statistics Canada. (2011). Canadian Cancer Statistics 2011: Featuring Colorectal Cancer.

Cannistra, S.A. (2004). Cancer of the ovary. N Engl J Med *351*, 2519-2529.

Carmeliet, P. (2005). VEGF as a key mediator of angiogenesis in cancer. Oncology *69*, 4–10.

Carpten, J.D., Faber, A.L., Horn, C., Donoho, G.P., Briggs, S.L., Robbins, C.M., Hostetter, G., Boguslawski, S., Moses, T.Y., Savage, S.*, et al.* (2007). A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature *448*, 439-444.

Chen, S., Iversen, E.S., Friebel, T., Finkelstein, D., Weber, B.L., Eisen, A., Peterson, L.E., Schildkraut, J.M., Isaacs, C., Peshkin, B.N.*, et al.* (2006). Characterization of BRCA1 and BRCA2 mutations in a large United States sample. J Clin Oncol *24*, 863-871.

Cheng, K.W., Lahad, J.P., Kuo, W.L., Lapuk, A., Yamada, K., Auersperg, N., Liu, J., Smith-McCune, K., Lu, K.H., Fishman, D.*, et al.* (2004). The RAB25 small GTPase determines aggressiveness of ovarian and breast cancers. Nat Med *10*, 1251-1256.

Christiansen, J.J., and Rajasekaran, A.K. (2006). Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. Cancer Res *66*, 8319-8326.

Citri, A., and Yarden, Y. (2006). EGF-ERBB signalling: towards the systems level. Nature reviews *7*, 505-516.

Cobleigh, M.A., Langmuir, V.K., Sledge, G.W., Miller, K.D., Haney, L., Novotny, W.F., Reimann, J.D., and Vassel, A. (2003). A phase I/II dose-escalation trial of bevacizumab in previously treated metastatic breast cancer. Semin Oncol *30*, 117-24.

Cockman, M.E., Masson, N., Mole, D.R., Jaakkola, P., Chang, G.W., Clifford, S.C., Maher, E.R., Pugh, C.W., Ratcliffe, P.J., and Maxwell, P.H. (2000). Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem *275*, 25733-25741.

Collaborative Group on Epidemiological Studies of Ovarian Cancer. (2008). Ovarian cancer and oral contraceptives: collaborative reanalysis of data from 45 epidemiological studies including 23 257 women with ovarian cancer and 87 303 controls. Lancet *371*, 303–314.

Coller, H.A., Sang, L., and Roberts, J.M. (2006). A new description of cellular quiescence. PLoS Biol *4*, 83.

Collins, J.V. (1894). Plea for Teaching the History of Mathematics. Science *23*, 44.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.

de Paulsen, N., Brychzy, A., Fournier, M.C., Klausner, R.D., Gnarra, J.R., Pause, A., and Lee, S. (2001). Role of transforming growth factor-alpha in von Hippel--Lindau (VHL)(-/-) clear cell renal carcinoma cell proliferation: a possible mechanism coupling VHL tumor suppressor inactivation and tumorigenesis. Proc Natl Acad Sci U S A *98*, 1387-1392.

Di Fiore, P.P., Pierce, J.H., Kraus, M.H., Segatto, O., King, C.R., and Aaronson, S.A. (1987). erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science *237*, 178-182.

Donjacour, A.A., and Cunha, G.R. (1991). Stromal regulation of epithelial function. Cancer Treat Res *53*, 335-364.

Duan, D.R., Humphrey, J.S., Chen, D.Y., Weng, Y., Sukegawa, J., Lee, S., Gnarra, J.R., Linehan, W.M., and Klausner, R.D. (1995). Characterization of the VHL tumor suppressor gene product: localization, complex formation, and the effect of natural inactivating mutations. Proc Natl Acad Sci U S A *92*, 6459-6463.

Ebert, B.L., Firth, J.D., and Ratcliffe, P.J. (1995). Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences. J Biol Chem *270*, 29083-29089.

Eckstein, N., Servan, K., Hildebrandt, B., Politz, A., von Jonquieres, G., Wolf-Kummeth, S., Napierski, I., Hamacher, A., Kassack, M.U., Budczies, J., *et al.* (2009). Hyperactivation of the insulin-like growth factor receptor I signaling pathway is an essential event for cisplatin resistance of ovarian cancer cells. Cancer Res *69*, 2996-3003.

Edery, I., Humbelin, M., Darveau, A., Lee, K.A.W., Milburn, S., Hershey, J.W.B., Trachsel, H., and Sonenberg, N. (1983). Involvement of eukaryotic initiation factor 4A in the cap recognition process. J Biol Chem *258*, 11398-11403.

Elit, L., Chambers, A., Fyles, A., Covens, A., Carey, M., and Fung, M.F. (2004). Systematic review of adjuvant care for women with Stage I ovarian carcinoma. Cancer *101*, 1926-1935.

Engelman, J.A., and Settleman, J. (2008). Acquired resistance to tyrosine kinase inhibitors during cancer therapy. Current opinion in genetics & development *18*, 73-79.

Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., *et al.* (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell *107*, 43-54.

Favaro, E., Nardo, G., Persano, L., Masiero, M., Moserle, L., Zamarchi, R., Rossi, E., Esposito, G., Plebani, M., Sattler, U., *et al.* (2008). Hypoxia inducible factor-1alpha inactivation unveils a link between tumor cell metabolism and hypoxia-induced cell death. American journal of pathology *173*, 1186-1201.

Foster, K., Crossey, P.A., Cairns, P., Hetherington, J.W., Richards, F.M., Jones, M.H., Bentley, E., Affara, N.A., Ferguson-Smith, M.A., and Maher, E.R. (1994). Molecular genetic investigation of sporadic renal cell carcinoma: analysis of allele loss on chromosomes 3p, 5q, 11p, 17 and 22. Br J Cancer *69*, 230-234.

Franovic, A., Gunaratnam, L., Smith, K., Robert, I., Patten, D., and Lee, S. (2007). Translational up-regulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer. Proc Natl Acad Sci U S A *104*, 13092-13097.

Franovic, A., Holterman, C.E., Payette, J., and Lee, S. (2009). Human cancers converge at the HIF-2alpha oncogenic axis. Proc Natl Acad Sci U S A *106*, 21306-21311.

Gardner, L.B., Li, Q., Park, M.S., Flanagan, W.M., Semenza, G.L., and Dang, C.V. (2001). Hypoxia inhibits G1/S transition through regulation of p27 expression. J Biol Chem *276*, 7919-7926.

Gatenby, R.A., and Gillies, R.J. (2004). Why do cancers have high aerobic glycolysis? Nat Rev Cancer *4*, 891-899.

Gebauer, F., and Hentze, M.W. (2004). Molecular mechanisms of translational control. Nature reviews *5*, 827-835.

Gevry, N., Hardy, S., Jacques, P.E., Laflamme, L., Svotelis, A., Robert, F., and Gaudreau, L. (2009). Histone H2A.Z is essential for estrogen receptor signaling. Genes & development *23*, 1522-1533.

Giatromanolaki, A., Koukourakis, M.I., Sivridis, E., Turley, H., Talks, K., Pezzella, F., Gatter, K.C., and Harris, A.L. (2001). Relation of hypoxia inducible factor 1 alpha and 2 alpha in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. Br J Cancer *85*, 881-890.

Gingras, A.C., and Sonenberg, N. (1997). Adenovirus Infection Inactivates the Translational Inhibitors 4E-BP1 and 4E-BP2. Virology *237* 182–186.

Gordan, J.D., Bertout, J.A., Hu, C.J., Diehl, J.A., and Simon, M.C. (2007). HIF-2α promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. Cancer Cell *11*, 335–347.

Greil, W., Rafferzeder, M., Bechtner, G., and Gartner, R. (1989). Release of an Endothelial Cell Growth Factor from Cultured Porcine Thyroid Follicles. Mol Endocrinol *5*, 858-867.

Grote, J., Susskind, R., and Vaupel, P. (1977). Oxygen diffusion constants D and K of tumor tissue (DS-carcinosarcoma) and their temperature dependence. Adv Exp Med Biol *94*, 361-365.

Gunaratnam, L., Morley, M., Franovic, A., de Paulsen, N., Mekhail, K., Parolin, D.A., Nakamura, E., Lorimer, I.A., and Lee, S. (2003). Hypoxia inducible factor activates the transforming growth factor-alpha/epidermal growth factor receptor growth stimulatory pathway in VHL(-/-) renal cell carcinoma cells. The Journal of biological chemistry *278*, 44966-44974.

Hammacher, A., Mellstrom, K., Heldin, C.H., and Westermark, B. (1989). Isoform-specific induction of actm reorganization by plateletderrved growth factor suggests that the functionally active receptor is a dimer. EMBO J *8*, 2489-2495,

Hammond, E.M., Denko, N.C., Dorie, M.J., Abraham, R.T., and Giaccia, A.J. (2002). Hypoxia links ATR and p53 through replication arrest. Mol Cell Biol *22*, 1834-43.

Han, W., and Lo, H.W. (2012). Landscape of EGFR signaling network in human cancers: biology and therapeutic response in relation to receptor subcellular locations. Cancer letters *318*, 124-134.

Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell *86*, 353-364.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hankinson, S.E., Hunter, D.J., Colditz, G.A., Willett, W.C., Stampfer, M.J., Rosner, B., Hennekens, C.H., and Speizer, F.E. (1993). Tubal ligation, hysterectomy, and risk of ovarian cancer. A prospective study. J Am Med Assoc *270*, 2813-2818.

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 247, 42-52.

Hennessy, B.T., Coleman, R.L., and Markman, M. (2009). Ovarian cancer. Lancet *374*, 1371-1382.

Hennessy, B.T., and Mills, G.B. (2006). Ovarian cancer: homeobox genes, autocrine/paracrine growth, and kinase signaling. Int J Biochem Cell Biol *38*, 1450-1456.

Hirose, K., Morita, M., Ema, M., Mimura, J., Hamada, H., Fujii, H., Saijo, Y., Gotoh, O., Sogawa, K., and Fujii-Kuriyama, Y. (1996). 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 *16*, 1706-1713.

Holcik, M., and Sonenberg, N. (2005). Translational control in stress and apoptosis. Nature reviews *6*, 318-327.

Holmquist-Mengelbier, L., Fredlund, E., Lofstedt, T., Noguera, R., Navarro, S., Nilsson, H., Pietras, A., Vallon-Christersson, J., Borg, A., Gradin, K., Poellinger, L., and Pahlman, S. (2006). Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer Cell *10*, 413-23.

Holterman, C.E., Franovic, A., Payette, J., and Lee, S. (2010). ETS-1 oncogenic activity mediated by transforming growth factor alpha. Cancer Res *70*, 730-740.

Honegger, A., Dull, T. J., Bellot, F.. Van Obberghen. E., Szapary. D., Schmidt, A., Ullrich, A., and Schlessinger, J. (1988a). Biological acbvitres of EGF-receptor mutants with individually altered autophosphorylation sites. EMBO J *7*, 3045-3052.

Honegger, A., Dull, T. J., Szapary, D., Komoriya. A., Kris. R. Ullrich, A. and Schlessmger, J. (1988b). Kmetic parameters of the protein tyrosine kinase activity of EGF-receptor mutants with indivtdually altered autophosphorylation sites. EMBO J *7*, 3053-3060.

Hovadhanakul, P., Nuerenberger, S.P., Ritter, P.J., Taylor, H.B., and Cavanagh, D. (1976). Primary transitional cell carcinoma of the fallopian tube associated with primary carcinomas of the ovary and endometrium. Gynecol Oncol 1976 *4*, 138-43.

Huang, L.E., Gu, J., Schau, M., and Bunn, H.F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A *95*, 7987-7992.

Iliopoulos, O., Kibel, A., Gray, S., and Kaelin, W.G., Jr. (1995). Tumour suppression by the human von Hippel-Lindau gene product. Nature medicine *1*, 822-826.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y., and Semenza, G.L. (1998). Cellular and developmental control of O2 homeostasis by hypoxiainducible factor 1a. Genes Dev *12,* 149–162.

Iwai, K., Yamanaka, K., Kamura, T., Minato, N., Conaway, R.C., Conaway, J.W., Klausner, R.D., and Pause, A. (1999). Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. Proc Natl Acad Sci U S A *96*, 12436-12441.

Jaganathan, S., Yue, P., Paladino, D.C., Bogdanovic, J., Huo, Q., and Turkson, J. (2011). A functional nuclear epidermal growth factor receptor, SRC and Stat3 heteromeric complex in pancreatic cancer cells. PloS one *6*, e19605.

Jain, S., Maltepe, E., Lu, M.M., Simon, C., and Bradfield, C.A. (1998). Expression of ARNT, ARNT2, HIF1a, HIF2a and Ah receptor mRNAs in the developing mouse. Mech Dev *73,* 117–123.

James, R., and Bradshaw, R.A. (1984). Polypeptide growth factors. Annu Rev Biochem *53*, 259-292.

Jarrett, C.R., Blancato, J., Cao, T., Bressette, D.S., Cepeda, M., Young, P.E., King, C.R., and Byers, S.W. (2001). Human APC2 localization and allelic imbalance. Cancer Res *61*, 7978-7984.

Jeong, J.W., Bae, M.K., Ahn, M.Y., Kim, S.H., Sohn, T.K., Bae, M.H., Yoo, M.A., Song, E.J., Lee, K.J., and Kim, K.W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell *111*, 709-20.

Jeuken, J., Sijben, A., Alenda, C., Rijntjes, J., Dekkers, M., Boots-Sprenger, S., McLendon, R., and Wesseling, P. (2009). Robust detection of EGFR copy number changes and EGFR variant III: technical aspects and relevance for glioma diagnostics. Brain pathology *19*, 661-671.

Johnson, D.H., Fehrenbacher, L., Novotny. W.F., Herbst, R.S, Nemunaitis, J.J., Jablons, D.M., Langer, C.J., DeVore, Gaudreault, J., Damico, L.A., Holmgren, E., and Kabbinavar F. (2004). Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol *22*, 2184-91.

Kabbinavar, F., Hurwitz, H.I., Fehrenbacher, L., Meropol, N.J., Novotny, W.F., Lieberman, G., Griffing, S., and Bergsland, E. (2003). Phase II, randomized trial comparing Bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol *21*, 60–65.

Kamura, T., Koepp, D.M., Conrad, M.N., Skowyra, D., Moreland, R.J., Iliopoulos, O., Lane, W.S., Kaelin, W.G., Jr., Elledge, S.J., Conaway, R.C.*, et al.* (1999). Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science *284*, 657-661.

Keith, B., Johnson, R.S., and Simon, M.C. (2011). HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. Nat Rev Cancer *12*, 9-22.

Khaleghpour, K., Pyronnet, S., Gingras, A.C., and Sonenberg, N. (1999). Translational homeostasis: eukaryotic translation initiation factor 4E control of 4E-binding protein 1 and p70 S6 kinase activities. Mol Cell Bio *19*, 4302-10.

Kibel, A., Iliopoulos, O., DeCaprio, J.A., and Kaelin, W.G., Jr. (1995). Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C. Science *269*, 1444-1446.

Kim, W.Y., Perera, S., Zhou, B., Carretero, J., Yeh, J.J., Heathcote, S.A., Jackson, A.L., Nikolinakos, P., Ospina, B., Naumov, G.*, et al.* (2009). HIF2alpha cooperates with RAS to promote lung tumorigenesis in mice. The Journal of clinical investigation *119*, 2160-2170.

Kinzler, K.W., and Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. Nature *386*, 761, 763.

Knudson, A.G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A *68*, 820-823.

Koh, M.Y., Darnay, B.G., and Powis, G. (2008). Hypoxia-associated factor, a novel E3-ubiquitin ligase, binds and ubiquitinates hypoxia-inducible factor 1alpha, leading to its oxygen-independent degradation. Mol Cell Biol *28*, 7081-7095.

Koh, M.Y., Lemos, R., Jr., Liu, X., and Powis, G. The hypoxia-associated factor switches cells from HIF-1alpha- to HIF-2alpha-dependent signaling promoting stem cell characteristics, aggressive tumor growth and invasion. Cancer Res *71*, 4015-4027.

Kondo, K., Klco, J., Nakamura, E., Lechpammer, M., and Kaelin, W.G., Jr. (2002). Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer cell *1*, 237-246.

Kuemmerle, J.F. (2003). IGF-I elicits growth of human intestinal smooth muscle cells by activation of PI3K, PDK-1, and p70S6 kinase. American journal of physiology *284*, G411-422.

Kulik, G., Klippel, A., and Weber, M.J. (1997). Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol Cell Biol *17*, 1595-1606.

Kunz-Schughart, L.A., Kreutz, M., and Knuechel, R. (1998). Multicellular spheroids: a three-dimensional in vitro culture system to study tumour biology. Int J Exp Pathol *79*, 1-23.

Kuo, K.T., Mao, T.L., Chen, X., Feng, Y., Nakayama, K., Wang, Y., Glas, R., Ma, M.J., Kurman, R.J., Shih, I.M., and Wang, T.L. (2010). DNA copy numbers profiles in affinity-purified ovarian clear cell carcinoma. Clin Cancer Res *16*, 1997-2008.

Lamphear, B.J., Kirchweger, R., Skern, T., and Rhoads, R.E. (1995). Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. J Biol Chem *270*, 21975-2183.

Lamping, J.D., and Blythe, J.G. (1977). Bilateral Brenner tumors: a case report and review of the literature. Hum Pathol *8*, 583-585.

Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F.M., Orcutt, M.L., Stackhouse, T., Kuzmin, I., Modi, W., Geil, L.*, et al.* (1993). Identification of the von Hippel-Lindau disease tumor suppressor gene. Science *260*, 1317-1320.

Lemmon, M.A., and Schlessinger, J. (2010). Cell Signaling by receptor tyrosine kinases. Cell *141*, 1117-1134.

Libermann, T.A., Nusbaum, H.R., Razon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M.D., Ullrich, A., and Schlessinger, J. (1985). Amplification and overexpression of the EGF receptor gene in primary human glioblastomas. Journal of cell science *3*, 161-172.

Lieubeau-Teillet, B., Rak, J., Jothy, S., Iliopoulos, O., Kaelin, W., and Kerbel, R.S. (1998). von Hippel-Lindau gene-mediated growth suppression and induction of differentiation in renal cell carcinoma cells grown as multicellular tumor spheroids. Cancer Res *58*, 4957-4962.

Lin, T.A., Kong, X., Haystead, T.A., Pause, A., Belsham, G., Sonenberg, N., and Lawrence, J.C.,Jr. (1994). PHAS-I as a link between mitogen-activated protein kinase and translation initiation. Science *266*, 653–656.

Lin, S.Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K.Y., Bourguignon, L., and Hung, M.C. (2001). Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nature cell biology *3*, 802-808.

Lindau, A. (1927). Zur Frage der Angiomatosis Retinae und Ihrer Hirncomplikation. Acta Ophthal *4*, 193–226.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif *25*, 402-408.

Lonergan, K.M., Iliopoulos, O., Ohh, M., Kamura, T., Conaway, R.C., Conaway, J.W., and Kaelin, W.G., Jr. (1998). Regulation of hypoxia-inducible mRNAs by the von Hippel-Lindau tumor suppressor protein requires binding to complexes containing elongins B/C and Cul2. Mol Cell Biol *18*, 732-741.

Luo, W., Zhong, J., Chang, R., Hu, H., Pandey, A., and Semenza, G.L. (2010). Hsp70 and CHIP selectively mediate ubiquitination and degradation of hypoxia-inducible factor (HIF)-1alpha but Not HIF-2alpha. The Journal of biological chemistry *285*, 3651-3663.

Lyden, D., Hattori, K., Dias, S., Costa, C., Blaikie, P., Butros, L., Chadburn, A., Heissig, B., Marks, W., Witte, L.*, et al.* (2001). Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med *7*, 1194-1201.

Mabuchi, S., Altomare, D.A., Cheung, M., Zhang, L., Poulikakos, P.I., Hensley, H.H., Schilder, R.J., Ozols, R.F., and Testa, J.R. (2007). RAD001 inhibits human ovarian cancer cell proliferation, enhances cisplatin-induced apoptosis, and prolongs survival in an ovarian cancer model. Clin Cancer Res *13*, 4261–4270.

MacDougall, J.D.B., and McCabe, M. (1967). Diffusion Coefficient of Oxygen through Tissues. Nature *215*, 1173-1174.

Maher, E.R., and Kaelin, W.G., Jr. (1997). von Hippel-Lindau disease. Medicine (Baltimore) *76*, 381-391.

Maher, E.R., Yates, J.R., and Ferguson-Smith, M.A. (1990). Statistical analysis of the two stage mutation model in von Hippel-Lindau disease, and in sporadic cerebellar haemangioblastoma and renal cell carcinoma. J Med Genet *27*, 311-314.

Majmundar, A.J., Wong, W.J., and Simon, M.C. (2010). Hypoxia-inducible factors and the response to hypoxic stress. Molecular cell *40*, 294-309.

Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H., Cao, Y., Berkenstam, A., and Poellinger, L. (2001). Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature *414*, 550-554.

Makino, Y., Kanopka, A., Wilson, W.J., Tanaka, H., and Poellinger, L. (2002). Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3alpha locus. J Biol Chem *277*, 32405-32408.

Maranchie, J.K., Vasselli, J.R., Riss, J., Bonifacino, J.S., Linehan, W.M., and Klausner, R.D. (2002). The contribution of VHL substrate binding and HIF1-alpha to the phenotype of VHL loss in renal cell carcinoma. Cancer cell *1*, 247-255.

Marcotrigiano, J., Gingras, A.C., Sonenberg, N., and Burley, S.K. (1999). Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G. Mol Cell *3*, 707–716.

Mareel, M., Vleminckx, K., Vermeulen, S., Yan, G., Bracke, M., and van Roy F. (1994). Downregulation in vivo of the invasion-suppressor molecule E-cadherin in experimental and clinical cancer. Princess Takamatsu Symp *24*, 63-80.

Markus, M.A., and Morris, B.J. (2009). RBM4: a multifunctional RNA-binding protein. Intern J Biochem Cell Biol *41*, 740-743.

Marone, R., Cmiljanovic, V., Giese, B., and Wymann, M.P. (2008). Targeting phosphoinositide 3-kinase - moving towards therapy. Biochim Biophys Acta *1784*, 159–185.

Marxsen, J.H., Stengel, P., Doege, K., Heikkinen, P., Jokilehto, T., Wagner, T., Jelkmann, W., Jaakkola, P., and Metzen, E. (2004). Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF-alpha-prolyl-4-hydroxylases. The Biochemical journal *381*, 761-767.

Masson, N., and Ratcliffe, P.J. (2003). HIF prolyl and asparaginyl hydroxylases in the biological response to intracellular O(2) levels. J Cell Sci *116*, 3041-9.

Maxwell, P.H., Pugh, C.W., and Ratcliffe, P.J. (2001). Activation of the HIF pathway in cancer. Curr Opin Gen Dev *11*, 293-299.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271-275.

McGuire, W.P., Hoskins, W.J., Brady, M.F., Kucera, P.R., Partridge, E.E., Look, K.Y., Clarke-Pearson, D.L., and Davidson, M. (1996). Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. N Engl J Med *334*, 1-6.

Menrad, H., Werno, C., Schmid, T., Copanaki, E., Deller, T., Dehne, N., and Brune, B. (2010). Roles of hypoxia-inducible factor-1alpha (HIF-1alpha) versus HIF-2alpha in the survival of hepatocellular tumor spheroids. Hepatology *51*, 2183-2192.

Minchenko, A., Salceda, S., Bauer, T., and Caro, J. (1994). Hypoxia regulatory elements of the human vascular endothelial growth factor gene. Cell Mol Biol Res *40*, 35-39.

Nahta, R., Yuan, L.X., Zhang, B., Kobayashi, R., and Esteva, F.J. (2005). Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. Cancer Res *65*, 11118-11128.

Nielsen, U.B., Adams, G.P., Weiner, L.M., and Marks, J.D. (2000). Targeting of bivalent anti-ErbB2 diabody antibody fragments to tumor cells is independent of the intrinsic antibody affinity. Cancer Res *60*, 6434-6440.

Nishi, H., Nishi, K.H., and Johnson, A.C. (2002). Early Growth Response-1 gene mediates up-regulation of epidermal growth factor receptor expression during hypoxia. Cancer Res *62*, 827-834.

Ohh, M., Park, C.W., Ivan, M., Hoffman, M.A., Kim, T.Y., Huang, L.E., Pavletich, N., Chau, V., and Kaelin, W.G. (2000). Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat Cell Biol *2*, 423-427.

Oka, H., Shiozaki, H., Kobayashi, K., Inoue, M., Tahara, H., Kobayashi, T., Takatsuka, Y., Matsuyoshi, N., Hirano, S., Takeichi, M., *et al.* (1993). Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. Cancer Res *53*, 1696-701.

Osada, R., Horiuchi, A., Kikuchi, N., Yoshida, J., Hayashi, A., Ota, M., Katsuyama, Y., Melillo, G., and Konishi, I. (2007). Expression of hypoxia-inducible factor 1alpha, hypoxia-inducible factor 2alpha, and von Hippel-Lindau protein in epithelial ovarian neoplasms and allelic loss of von Hippel-Lindau gene: nuclear expression of hypoxia-inducible factor 1alpha is an independent prognostic factor in ovarian carcinoma. Human Pathology *38*, 1310-1320.

Ozols, R.F., Bundy, B.N., Greer, B.E., Fowler, J.M., Clarke-Pearson, D., Burger, R.A., Mannel, R.S., DeGeest, K., Hartenbach, E.M., Baergen, R.; Gynecologic Oncology Group. (2004). Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study. J Clin Oncol *21*, 3194-3200.

Pankey, G.A., and Sabath, L.D. (2004). Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. Clin Infect Dis *38*, 864-870.

Pause A., Belsham G.J., Gingras A.C., Donzé O., Lin T.A., Lawrence J.C.,Jr., and Sonenberg N. (1994). Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. Nature *371*, 762–767.

Pause, A., Lee, S., Worrell, R.A., Chen, D.Y., Burgess, W.H., Linehan, W.M., and Klausner, R.D. (1997). The von Hippel-Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. Proc Natl Acad Sci USA *94*, 2156-2161.

Pause, A., Lee, S., Lonergan, K.M., and Klausner, R.D. (1998). The von Hippel-Lindau tumor suppressor gene is required for cell cycle exit upon serum withdrawal. Proc Natl Acad Sci USA *95*, 993-998.

Pecorelli, S., Benedet, J.L., Creasman, W.T., and Shepherd J.H. (1999). FIGO staging of gynecologic cancer. 1994-1997 FIGO Committee on Gynecologic Oncology. International Federation of Gynecology and Obstetrics. Int J Gynaecol Obstet *64*, 5-10.

Peng, J., Zhang, L., Drysdale, L., and Fong, G.H. (2000). The transcription factor EPAS-1/hypoxia-inducible factor 2a plays an important role in vascular remodeling. Proc Natl Acad Sci USA *97*, 8386–8391.

Pestova, T.V., Shatsky, I.N., and Hellen, C.U. (1996). Functional dissection of eukaryotic initiation factor 4F: the 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. Mol Cell Biol *16*, 6870-6878.

Pestova, T.V., de Breyne, S., Pisarev, A.V., Abaeva, I.S., and Hellen, C.U. (2008). eIF2-dependent and eIF2-independent modes of initiation on the CSFV IRES: a common role of domain II. EMBO J *27*, 1060-1072.

Piccart, M.J., Bertelsen, K., James, K., Cassidy, J., Mangioni, C., Simonsen, E., Stuart, G., Kaye, S., Vergote, I., Blom, R., *et al*. (2000). Randomized intergroup trial of cisplatin-paclitaxel versus cisplatin-cyclophosphamide in women with advanced epithelial ovarian cancer: three-year results. J Natl Cancer Inst. 2000 *92*, 699-708.

Pugh, C.W., O'Rourke, J.F., Nagao, M., Gleadle, J.M., and Ratcliffe, P.J. (1997). Activation of hypoxia-inducible factor-1; definition of regulatory domains within the alpha subunit. J Biol Chem *272*, 11205-11214.

Rafferty, K.A. Jr. (1975). Epithelial cells: growth in culture of normal and neoplastic forms. Adv Cancer Res *21*, 249-72.

Raica, M., Cimpean, A.M., and Ribatti, D. (2009). Angiogenesis in pre-malignant conditions. Eur J Cancer *45*, 1924–1934.

Raval, R.R., Lau, K.W., Tran, M.G., Sowter, H.M., Mandriota, S.J., Li, J.L., Pugh, C.W., Maxwell, P.H., Harris, A.L., and Ratcliffe, P.J. (2005). Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Mol Cell Biol *25*, 5675-5686.

Reibenwein, J., and Krainer, M. (2008). Targeting signaling pathways in ovarian cancer. Expert opinion on therapeutic targets *12*, 353-365.

Reichert, J.M., and Valge-Archer, V.E. (2007). Development trends for monoclonal antibody cancer therapeutics. Nat Rev Drug Discov *6*, 349-356.

Richard, D.E., Berra, E., Gothié, E., Roux, D., and Pouysségur, J. (1999). p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. J Biol Chem *274*, 32631-3267.

Risch, H.A., McLaughlin, J.R., Cole, D.E., Rosen, B., Bradley, L., Fan, I., Tang, J., Li, S., Zhang, S., Shaw, P.A*., et al.* (2006). Population BRCA1 and BRCA2 mutation frequencies and cancer penetrances: a kin-cohort study in Ontario, Canada. J Natl Cancer Inst *98*, 1694-1706.

Roberts, A.M., Watson, I.R., Evans. A.J., Foster, D.A., Irwin, M.S., and Ohh, M. (2009). Suppression of hypoxia-inducible factor 2alpha restores p53 activity via Hdm2 and reverses chemoresistance of renal carcinoma cells. Cancer Res *69*, 9056-9064.

Roett, M.A., and Evans, P. (2009). Ovarian cancer: an overview. American family physician *80*, 609-616.

Rous, P. (1910). A transmissible avain neoplasm (sarcoma of the common fowl). Journal of Experimental Medicine *12,* 696-705.

Ryan, H.E., Lo, J., and Johnson, R.S. (1998). HIF-1a is required for solid tumor formation and embryonic vascularization. EMBO J *17,* 3005–3015.

Sartore-Bianchi, A., Martini, M., Molinari, F., Veronese, S., Nichelatti, M., Artale, S., Di Nicolantonio, F., Saletti, P., De Dosso, S., Mazzucchelli, L*., et al.* (2009). PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. Cancer Res *69*, 1851-1857.

Sato, K., Terada, K., Sugiyama, T., Takahashi, S., Saito, M., Moriyama, M., Kakinuma, H., Suzuki, Y., Kato, M., and Kato, T. (1994). Frequent overexpression of vascular endothelial growth factor gene in human renal cell carcinoma. Tohoku J Exp Med *173*, 355-360.

Scharf, J.G., Unterman, T.G., and Kietzmann, T. (2005). Oxygen-dependent modulation of insulin-like growth factor binding protein biosynthesis in primary cultures of rat hepatocytes. Endocrinology *146*, 5433-5443.

Schindl, M., Schoppmann, S.F., Samonigg, H., Hausmaninger, H., Kwasny, W., Gnant, M., Jakesz, R., Kubista, E., Birner, P., and Oberhuber, G. (2002). Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer. Clin Cancer Res *8*, 1831–1837.

Schlessingec, J. (1988). Signal transduction by allosteric receptor olrgomenzation. Trends Biochem Sci *13*, 443-447.

Schmandt, R.E., Broaddus, R., Lu, K.H., Shvartsman, H., Thornton, A., Malpica, A., Sun, C., Bodurka, D.C., and Gershenson, D.M. (2003). Expression of c-ABL, c-KIT, and platelet-derived growth factor receptor-beta in ovarian serous carcinoma and normal ovarian surface epithelium. Cancer *98*, 758-764.

Scholzen, T., and Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. J Cell Physiol *182*, 311-322.

See, H.T., Kavanagh, J.J., Hu, W., and Bast, R.C. (2003). Targeted therapy for epithelial ovarian cancer: current status and future prospects. Int J Gynecol Cancer *13*, 701-734.

Semenza, G.L. (1999). Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol *15*, 551-578.

Semenza, G.L. (2003). Targeting HIF-1 for cancer therapy. Nat Rev Cancer *3*, 721-732.

Semenza, G.L., and Wang, G.L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol *12*, 5447-5454.

Serrano-Olvera, A., Duenas-Gonzalez, A., Gallardo-Rincon, D., Candelaria, M., and De la Garza-Salazar, J. (2006). Prognostic, predictive and therapeutic implications of HER2 in invasive epithelial ovarian cancer. Cancer Treat Rev *32*, 180-190.

Sergina, N.V., and Moasser, M.M. (2007). The HER family and cancer: emerging molecular mechanisms and therapeutic targets. Trends in molecular medicine *13*, 527-534.

Sharma, S.V., Bell, D.W., Settleman, J., and Haber, D.A. (2007). Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer *7*, 169-181.

Shaw, T.J., Senterman, M.K., Dawson, K., Crane, C.A., and Vanderhyden, B.C. (2004). Characterization of intraperitoneal, orthotopic, and metastatic xenograft models of human ovarian cancer. Mol Ther *10*, 1032-1042.

Shaw, P. A., Zweemer, R. P., McLaughlin, J. Narod, S. A., Risch, H., Jacobs, IJ. (1999). Characteristics of genetically determined ovarian cancer. Mod Pathol *79*, 124.

Shawver, L.K., Slamon, D., and Ullrich, A. (2002). Smart drugs: tyrosine kinase inhibitors in cancer therapy. Cancer Cell *1*, 117-123.

Shelton, J.G., Steelman, L.S., White, E.R., and McCubrey, J.A. (2004). Synergy between PI3K/Akt and Raf/MEK/ERK pathways in IGF-1R mediated cell cycle progression and prevention of apoptosis in hematopoietic cells. Cell cycle *3*, 372-379.

Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature *359*, 843-845.

Singleton, J.R., Randolph, A.E., and Feldman, E.L. (1996). Insulin-like growth factor I receptor prevents apoptosis and enhances neuroblastoma tumorigenesis. Cancer Res *56*, 4522-4529.

Sivridis, E., Giatromanolaki, A., Gatter, K.C., Harris, A.L., and Koukourakis, M.I. (2002). Association of hypoxia inducible factors 1alpha and 2alpha with activated angiogenic pathways and prognosis in patients with endometrial carcinoma. Cancer *95*, 1055–1063.

Smith, K., Gunaratnam, L., Morley, M., Franovic, A., Mekhail, K., and Lee, S. (2005). Silencing of epidermal growth factor receptor suppresses hypoxia-inducible factor-2-driven VHL-/- renal cancer. Cancer Res *65*, 5221-5230.

Smith, L.H., Morris, C.R., Yasmeen, S., Parikh-Patel, A., Cress, R.D., and Romano, P.S. (2005). Ovarian cancer: can we make the clinical diagnosis earlier? Cancer *104*, 1398-1407.

Soos, M. A., and Saddle, K. (1989). lmmunologrcal relationshrps betweenreceptors for insulin and insulrn-like growth factor I. Biochem J *263*, 553-563.

Sonenberg, N., Morgan, M.A., Merrick, W.C., and Shatkin, A.J. (1978). A polypeptide in eukaryotic initiation factors that crosslinks specifically to the 5'-terminal cap in mRNA. Proc Natl Acad Sci USA *75*, 4843-4847.

Sonenberg, N., and Hinnebusch, A.G. (2009). Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell *136*, 731-745.

Spentzos, D., Cannistra, S.A., Grall, F., Levine, D.A., Pillay, K., Libermann, T.A., and Mantzoros, C.S. (2007). IGF axis gene expression patterns are prognostic of survival in epithelial ovarian cancer. Endocr Relat Cancer *14*, 781–90.

Sporn, M.B., and Todaro, G.J. (1980). Autocrine secretion and malignant transformation of cells. N Engl J Med *303*, 878–880.

Sporn, M.B., and Roberts, A.B. (1985). Autocrine growth factors and cancer. Nature *313*, 745-747.

Stebbins, C.E., Kaelin, W.G., Jr., and Pavletich, N.P. (1999). Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. Science *284*, 455-461.

Stiehl, D.P., Bordoli, M.R., Abreu-Rodriguez, I., Wollenick, K., Schraml, P., Gradin, K., Poellinger, L., Kristiansen, G., and Wenger, R.H. (2011). Non-canonical HIF-2alpha function drives autonomous breast cancer cell growth via an AREG-EGFR/ErbB4 autocrine loop. Oncogene *31*, 2283-2297.

Sutherland RM. (1988). Cell and environment interactions in tumor microregions: the multicell spheroid model. Science *240*, 177-184.

Talks, K.L., Turley, H., Gatter, K.C., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J., and Harris, A.L. (2000). The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. The American journal of pathology *157*, 411-421.

Takashina, T., Ono, M., Kanda, Y., Sagae, S., Hayakawa, O., and Ito, E. (1988). Cervicovaginal and endometrial cytology in ovarian cancer. Acta Cytol *32*, 159-62.

Terenin, I.M., Dmitriev, S.E., Andreev, D.E., and Shatsky, I.N. (2008). Eukaryotic translation initiation machinery can operate in a bacterial-like mode without eIF2. Nat Struct Mol Biol *15*, 836-841.

Tian, H., Hammer, R.E., Matsumoto, A.M., Russell, D.W., and McKnight, S.L. (1998). The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev *12,* 3320–3324.

Tian, H., McKnight, S.L., and Russell, D.W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev *11*, 72-82.

Torres Aleman, I. (2005). Role of insulin-like growth factors in neuronal plasticity and neuroprotection. Advances in experimental medicine and biology *567*, 243-258.

Tory, K., Brauch, H., Linehan, M., Barba, D., Oldfield, E., Filling-Katz, M., Seizinger, B., Nakamura, Y., White, R., Marshall, F.F.*, et al.* (1989). Specific genetic change in tumors associated with von Hippel-Lindau disease. J Natl Cancer Inst *81*, 1097-1101.

Trimbos, J.B., Vergote, I., Bolis, G., Vermorken, J.B., Mangioni, C., Madronal, C., Franchi, M., Tateo, S., Zanetta, G., Scarfone, G*., et al.* (2003). Impact of adjuvant chemotherapy and surgical staging in early-stage ovarian carcinoma: European Organisation for Research and Treatment of Cancer-Adjuvant ChemoTherapy in Ovarian Neoplasm trial. J Natl Cancer Inst *95*, 113-125.

Uchida, T., Rossignol, F., Matthay, M.A., Mounier, R., Couette, S., Clottes, E., and Clerici, C. (2004). Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha. J Biol Chem *279*, 14871-1478.

Ullrich, A., and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell *61*, 203-212.

Uniacke, J., Holterman, CE., Lachance G., Franovic, A., Jacob, MD., Fabian, MR., Payette, J., Holcik, M., Pause, A., and Lee, S. (2012). An oxygen-regulated switch in the protein synthesis machinery. Nature *486*, 126-129.

Vasey, P.A., Jayson, G.C., Gordon, A., Gabra, H., Coleman, R., Atkinson, R., Parkin, D., Paul, J., Hay, A., and Kaye, S.B. (2004). Phase III randomized trial of docetaxel-carboplatin versus paclitaxel-carboplatin as first-line chemotherapy for ovarian carcinoma. J Natl Cancer Inst *96*, 1682-1691.

Vaupel, P., and Harrison, L. (2004). Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response. The oncologist *9 Suppl 5*, 4-9.

Von Hippel, E. (1904). Ueber eine sehr seltene Erkrankung der Netzhaut. Albrecht von Graefes Arch Ophthal *59*, 83–106.

Waerner, T., Alacakaptan, M., Tamir, I., Oberauer, R., Gal, A., Brabletz, T., Schreiber, M., Jechlinger, M., and Beug, H. (2006). ILEI: a cytokine essential for EMT, tumor formation, and late events in metastasis in epithelial cells. Cancer Cell *10*, 227-239.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A *92*, 5510-5514.

Warburg O. H. (1930). The Metabolism of Tumours (translated from the German edition by F. Dickens). Constable & Co. Ltd. London, 11-25.

Warburg, O. (1956a). On the origin of cancer cells. Science *123*, 309-314.

Warburg, O. (1956b). On respiratory impairment in cancer cells. Science *124*, 269-270.

Weihua, Z., Tsan, R., Huang, W.C., Wu, Q., Chiu, C.H., Fidler, I.J., and Hung, M.C. (2008). Survival of cancer cells is maintained by EGFR independent of its kinase activity. Cancer cell *13*, 385-393.

Weroha, S.J., and Haluska, P. (2008). IGF-1 receptor inhibitors in clinical trials - early lessons. J Mammary Gland Biol Neoplasia *13*, 471–483.

Wiesener, M.S., Turley, H., Allen, W.E., Willam, C., Eckardt, K.U., Talks, K.L., Wood, S.M., Gatter, K.C., Harris, A.L., Pugh, C.W., Ratcliffe, P.J., and Maxwell, P.H. (1998). Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. Blood *92*, 2260-2268.

Wiesener, M.S., Jurgensen, J.S., Rosenberger, C., Scholze, C.K., Horstrup, J.H., Warnecke, C., Mandriota, S., Bechmann, I., Frei, U.A., Pugh, C.W.*, et al.* (2003). Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB J *17*, 271-273.

Williams, L. T. (1989). Srgnal transduction by the platelet-dewedgrowth factor receptor. Science *243*, 1564-1570.

Witsch, E., Sela, M., and Yarden, Y. (2010). Roles for growth factors in cancer progression. Physiology *25*, 85-101.

Wizigmann-Voos, S., Breier, G., Risau, W., and Plate, K.H. (1995). Up-regulation of vascular endothelial growth factor and its receptors in von Hippel-Lindau disease-associated and sporadic hemangioblastomas. Cancer Res *55*, 1358-1364.

Wong, A.J., Bigner, S.H., Bigner, D.D., Kinzler, K.W., Hamilton, S.R., and Vogelstein, B. (1987). Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification. Proc Natl Acad Sci USA *84*, 6899-6903.

Xu, J., Wang, R., Xie, Z.H., Odero-Marah, V., Pathak, S., Multani, A., Chung, L.W., and Zhau, H.E. (2006). Prostate cancer metastasis: role of the host microenvironment in promoting epithelial to mesenchymal transition and increased bone and adrenal gland metastasis. Prostate *66*, 1664-73.

Yaginuma, Y., and Westphal, H. (1992). Abnormal structure and expression of the p53 gene in human ovarian carcinoma cell lines. Cancer Res *52*, 4196-4199.

Yanai-Inbar, I., Siriaunkgul, S., and Silverberg, S.G. (1995). Mucosal epithelial proliferation of the fallopian tube: a particular association with ovarian serous tumor of low malignant potential? Int J Gynecol Pathol *14*, 107-13.

Yarden, Y., and Ullrich, A. (1988). Growth factor receptor tyrosine kinases. Annu Rev Biochem *57*, 443-478.

Young, R.C., Walton, L.A., Ellenberg, S.S., Homesley, H.D., Wilbanks, G.D., Decker, D.G., Miller, A., Park, R., and Major, F., Jr. (1990). Adjuvant therapy in stage I and stage II epithelial ovarian cancer. Results of two prospective randomized trials. N Engl J Med *322*, 1021-1027.

Young, R. H., Clement, P. B., Scully, R. E. The ovary. In Sternberg, S. S., Antonioli, D. A., Mills, S. E., Carter, D., Oberman, H. A., eds. Diagnostic Surgical Pathology. 2nd ed. New York, NY: Raven Press; 1994.

Young, R.M., Wang, S.J., Gordan, J.D., Ji, X., Liebhaber, S.A., and Simon, M.C. (2008). Hypoxia-mediated selective mRNA translation by an internal ribosome entry site-independent mechanism. The Journal of biological chemistry *283*, 16309-16319.

Zhang, H., Gao, P., Fukuda, R., Kumar, G., Krishnamachary, B., Zeller, K.I., Dang, C.V., and Semenza, G.L. (2007). HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. Cancer Cell *11*, 407-420.

Zhu, H., Cao, X., Ali-Osman, F., Keir, S., and Lo, H.W. (2010). EGFR and EGFRvIII interact with PUMA to inhibit mitochondrial translocalization of PUMA and PUMA-mediated apoptosis independent of EGFR kinase activity. Cancer letters *294*, 101-110.

Zuberek, J., Kubacka, D., Jablonowska, A., Jemielity, J., Stepinski, J., Sonenberg, N., and Darzynkiewicz, E. (2007). Weak binding affinity of human 4EHP for mRNA cap analogs. RNA *13*, 691–697.

Websites

American Type Cell Collection (ATCC). Product Description. "Cell Biology: SKOV3 ". (2011). Retrieved May 18th, 2012).
<http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=HTB-77&Template=cellBiology>

Cancer Care Ontario. "Cancer System Overview". (2009). Retrieved January 21st, 2012.
<https://www.cancercare.on.ca/ocs/csoverview/>

Canadian Cancer Society. "Environmental risk factors for cancer". (2012) Retrieved April 27th, 2012.
<http://www.cancer.ca/Canadawide/Prevention/Environment%20and%20you/Risk%20factors%20for%20cancer.aspx?sc_lang=en>

Charles River. "Reseach animal models" (2011). Retrieved November 12, 2011.
<http://www.criver.com/ENUS/PRODSERV/BYTYPE/RESMODOVER/RESMOD/Pages/CD-1NudeMouse.aspx>

Health Canada. "Ovarian Cancer Awareness Month". (2010). Retrieved October 2nd, 2011.
<http://www.hc-sc.gc.ca/ahc-asc/minist/messages/_2010/2010_09_10-eng.php>

The World Health Organization. “Cancer”. (2010). Retrieved January 7th, 2012.
<http://www.who.int/mediacentre/factsheets/fs297/en/index.html>

6 Appendix A

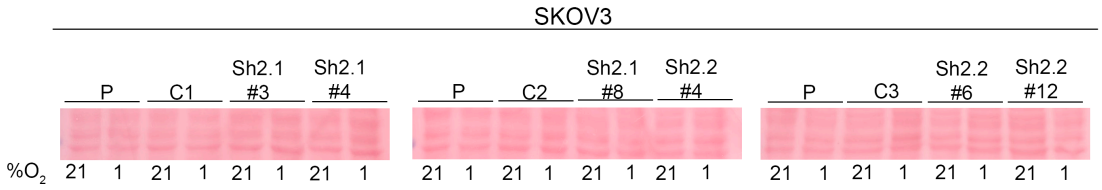


Figure 1. Ponceau S staining of SKOV3 immunoblots for HIF-1α. For HIF-1α detection, SKOV3 cells where incubated in the hypoxia chamber for 4 hours and then lysate was collected. Loading of the 4 hour lysate used to detect HIF-1α is shown by Ponceau S staining.

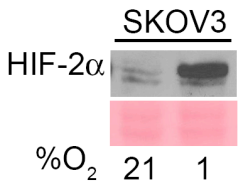


Figure 2. The presence of HIF-2α in normoxia in SKOV3 cells. HIF-2α is detectable in normoxia by immunoblot analysis when the membrane is exposed for a longer period of time shown here in parental SKOV3 cells.

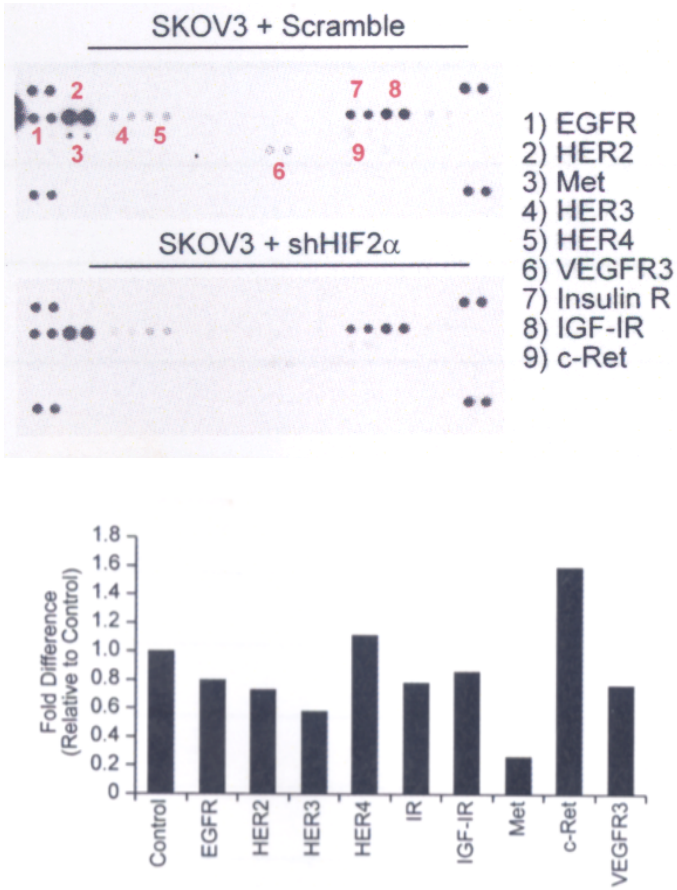
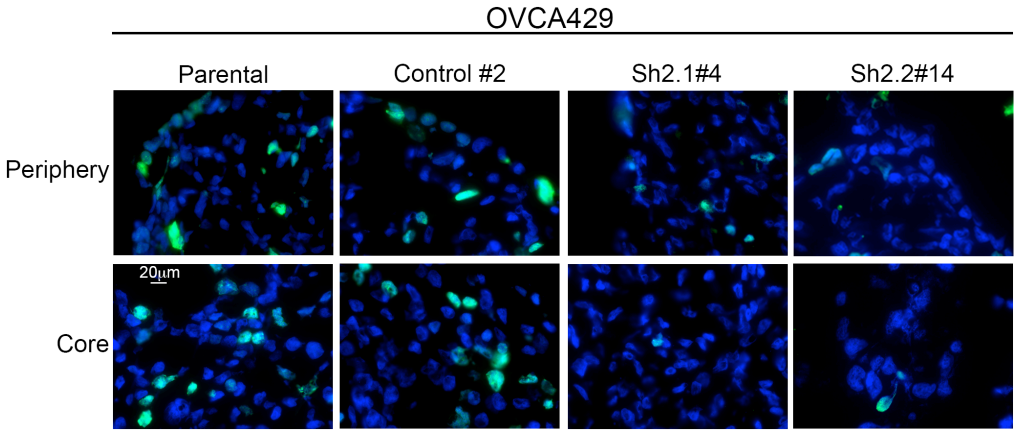


Figure 3. RTK array of SKOV3 cells. RTKs expression of shHIF-2α cells compared to control cells. EGFR, HER2, HER3, insulin receptor (IR), IGF1R, Met and VEGFR3 all have a decrease in expression in the shHIF-2α cells compared to control cells. Individual RTKs where then chose to be screened by immunoblot analysis in both OVCA429 and SKOV3.

A



B

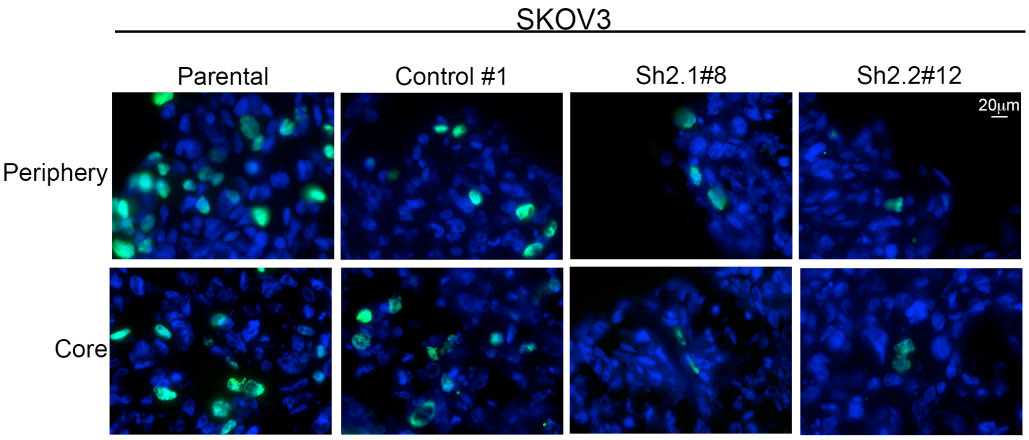


Figure 4. Ki67 expression in OVCA429 and SKOV3 spheroids. A. and B. Representive pictures of OVCA429 and SKOV3 at 60X. These pictures where counted to determine the ratio of Ki67 positive cells to Hoechst positive cells.