Molecular Response to Hypoxia;

from *C. elegans* to cancer

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Cover: Normarski microscopic photograph of a wild-type *Caenorhabditis elegans* specimen in the process of egg laying.

Molecular Response to Hypoxia; from *C. elegans* to cancer

Moleculaire reactie op zuurstofgebrek; van *C. elegans* tot kanker (met een samenvatting in het Nederlands)

Proefschrift

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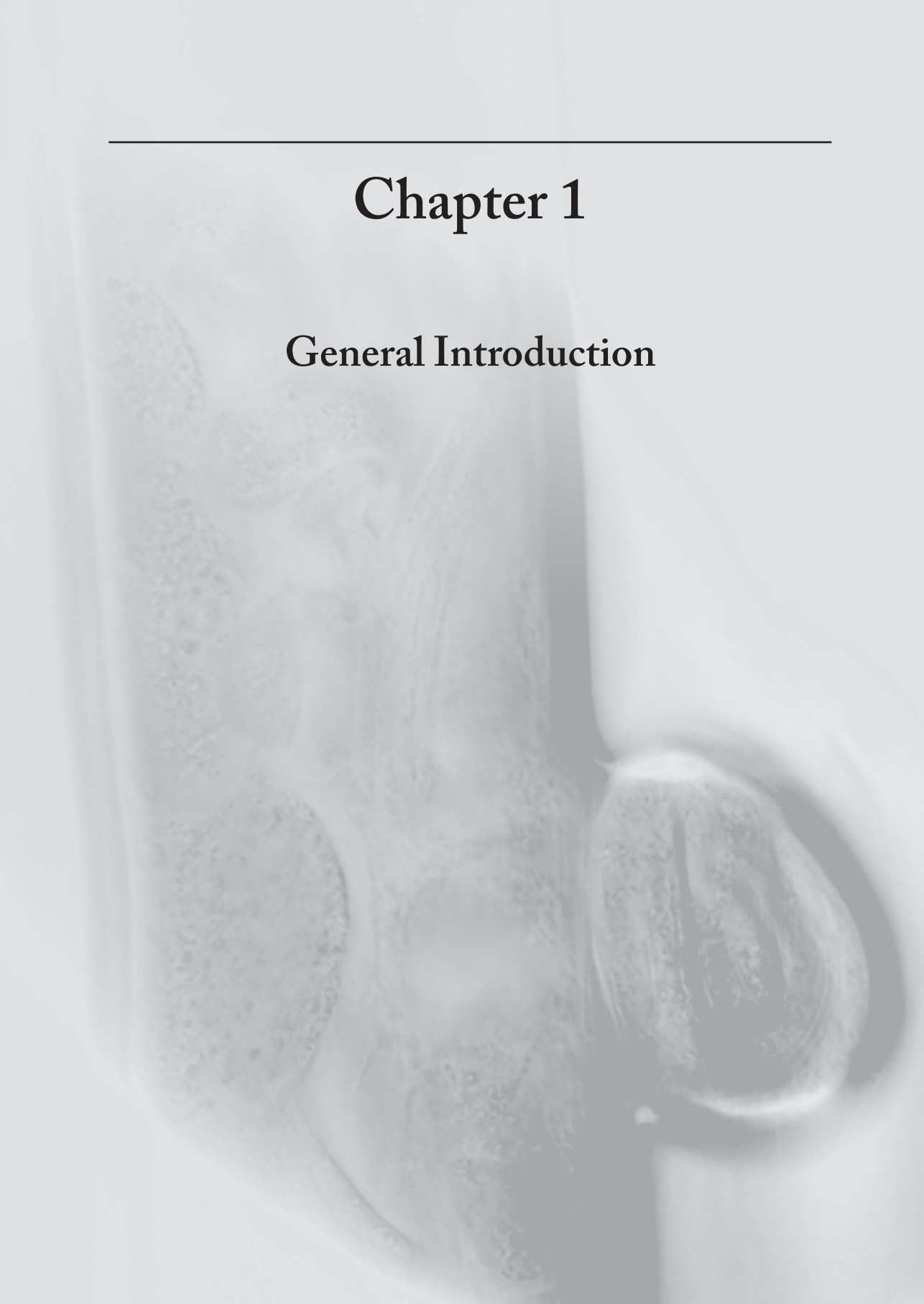
Chapter 1

General Introduction



Hypoxia

Oncogenesis is governed by genetic and epigenetic events that co-opt to malignant progression. The role of the microenvironment in tumorigenesis and maintenance is increasingly appreciated. Oxygen supply is one of the rate limiting microenvironmental factors. As all aerobe organisms rely on oxygen, cancer cells are no exception. Hypoxia is the condition in which the partial oxygen pressure has dropped to levels that are no longer sufficient to sustain normal cellular function. An early event in tumor growth is the emergence of hypoxic areas as intercapillary distances exceed 140 micron (Vaupel et al., 1988). Oxygen tension measurements revealed that average oxygen tension is lower in tumor tissue compared to normal tissue (on average 1-2 % compared to ~7-10%) (Vaupel et al., 1991). Hypoxic tumours are associated with decreased life expectancy (Hockel et al., 1996). This association can be accounted for by a number of factors (Figure 1). First of all, hypoxia confers tumor cells resistant to therapy (Evans & Koch, 2003), which may be due to (1) decreased delivery by restriction of blood supply, (2) decreased cellular uptake, (3) increased efflux by drug transporters, and (4) local acidosis that alters therapeutic efficacy (Comerford et al., 2002; Mahoney et al., 2003). Furthermore, hypoxic cells are often resistant to apoptosis and/or cease cell cycle progression, decreasing sensitivity to agents that target these processes (Carmeliet et al., 1998; Graeber et al., 1996). Radiotherapy is also less effective because of the absence of the "oxygen enhancement effect", which normally generates oxygen radicals, which induce DNA damage within tumour cells. A second explanation for the poor prognosis of hypoxic tumors is the selective pressure applied to tumor cells, when exposed to such a hostile environment lacking oxygen and nutrients. By a Darwinian process, the fittest tumor cells survive and clonally expand. Tumor cells become self-sustaining and resistant to apoptosis, enabling them to migrate and metastasize. Selection might be enhanced by a direct effect of hypoxia on the DNA repair mechanism leading to genomic instability. The hypoxic gene expression profile provides molecular insight into the poor prognosis of hypoxic cancers.

Classical hallmarks of solid cancer include uncontrolled cell growth, inhibition of apoptosis, angiogenesis, invasive and metastatic behavior (Hanahan & Weinberg, 2000). Hypoxia affects most of these hallmarks. Importantly, hypoxia is implicated in a process termed "the angiogenic switch". Judah Folkman in 1971 hypothesized the dependence of tumor growth on neoangiogenesis (Folkman, 1971). The angiogenic switch triggers vessel growth towards low

vessel density tumor areas with low partial oxygen pressure. Hypoxic cells express multiple angiogenic factors, e.g. vascular endothelial growth factor (VEGF) (Carmeliet, 2005). This will be discussed in more detail later. Another important process in cancer progression is the so called "Warburg effect". At lower oxygen concentrations, ATP production through the respiratory chain decreases. Hypoxic cells depend on the (much less efficient) anaerobic glycolysis for their ATP-requirements. Tumor cells in general have an increased glycolytic metabolism, characterized by a increased uptake of glucose, which is converted to lactate, as demonstrated by Otto Warburg (Warburg, 1930). High lactate concentrations and concomitant acidosis are commonly found in metastatic cancer (Schwickert et al., 1995; Walenta et al., 1997; Walenta et al., 2004). Hypoxic and mitogenic signaling in tumor cells are directly responsible for this glycolytic switch in cancer cells (Pasteur effect). Understanding the molecular response to hypoxia will certainly provide further insight into the pathophysiology of progressive cancer.

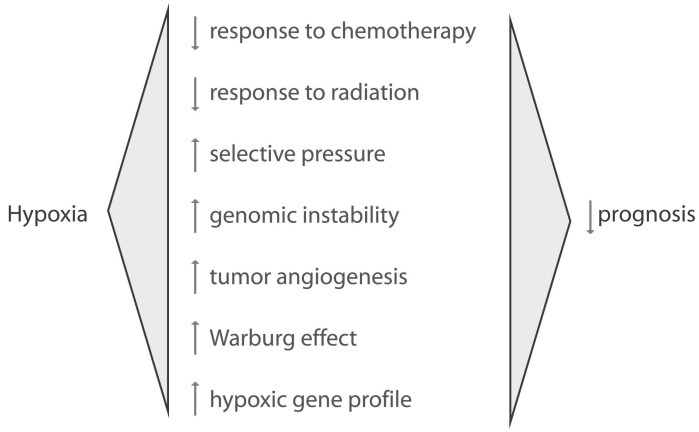


Figure 1.
Schematic drawing of factors influenced by hypoxia that have impact on patient prognosis.

Molecular response to hypoxia

Hypoxic cells alter metabolism and growth rate and produce angiogenic factors, but how is this response regulated? In 1991, Greg Semenza found that the hypoxia-inducible gene erythropoietin (Epo), a growth factor for red blood cells, was regulated by hypoxia-inducible nuclear factors, and later purified the hypoxia-inducible factor-1 alpha (HIF-1α) protein responsible for Epo induction (Semenza et al., 1991; Semenza & Wang, 1992; Wang et al., 1995).

HIF-1α is a member of the basic Helix-Loop-Helix (bHLH)-Per/Arnt/Sim (PAS) transcription factor family of proteins that share an N-terminal bHLH domain followed by a PAS domain. Other family members are HIF-2α (or EPAS1 for endothelial domain PAS protein), HIF-3α (of which a dominant negative splice variant is also expressed called IPAS1 for inhibitory PAS domain protein), HIF-1β (or ARNT1 for aryl hydrocarbon nuclear translocator 1), HIF-2β (ARNT2) and HIF-3β (ARNT3) (Figure 2A) (Semenza, 2000a; Wenger, 2000). The HIF transcription factor is a heterodimer composed of a constitutively expressed beta subunit and a hypoxia-regulated alpha subunit (Wang et al., 1995). At physiological oxygen tensions (normoxia), the alpha subunit is continuously degraded upon synthesis. This is the result of the oxygen-dependent activity of a group of prolyl-hydroxylases (PHD's) that belong to the 2-oxogluterate-dependent-oxygenase family (Bruick & McKnight, 2001; Epstein et al., 2001). In the presence of oxygen, these enzymes hydroxylate the proline residue in the XLAP motifs of the alpha subunits. For HIF-1α these prolines are at position 402 and 564 of the oxygen dependent degradation domain (ODDD), for HIF-2α these are P405 and P531 (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001). Hydroxylation allows binding of a ubiquitin ligase complex, that poly-ubiquitinates HIFα, which directs HIFα for proteasomal degradation. The binding occurs via the Von Hippel Lindau protein (pVHL), an E3 ubiquitin ligase, that can bind only hydroxylated HIFα (Cockman et al., 2000; Maxwell et al., 1999; Ohh et al., 2000). In the absence of oxygen, the PHD activity is reduced, resulting in HIFα protein stability due to reduced prolyl-hydroxylation and pVHL binding. The stable protein translocates to the nucleus and dimerizes to HIFβ, mainly HIF-1β (Wood et al., 1996). The HIF heterodimeric transcription factor subsequently binds to hypoxia response elements (HREs) in the genome, containing the consensus sequence 5'-RCGTG-3', and thereby enhances the expression of target genes (Jiang et al., 1996). In addition to the PHD/pVHL-mediated regulation, HIFα activity is controlled by hydroxylation of an aspargine residue in the C-terminal transactivation domain (N803 for HIF-1α) by the oxygen-dependent hydroxylase factor inhibiting HIF (FIH), which impairs with binding of transcriptional coactivators like p300 and CBP (Hewitson et al., 2002; Lando et al., 2002a; Lando et al., 2002b; Mahon et al., 2001; McNeill et al., 2002). Other regulatory modifications of the HIF protein include S-nitrosilation, sumoylation, acetylation and phosphorylation (Bae et al., 2004; Flugel et al., 2007; Jeong et al., 2002).

Interestingly, PHD and FIH activity is not only regulated by oxygen levels. 2-Oxoglutarate (2-OG), a component of the mitochondrial tricarboxylic acid (TCA) cycle, is also required for PHD/FIH activity (Bruick & McKnight,

2001). Reduced 2-OG levels, e.g. by inactivating mutations in TCA enzymes like fumarate hydratase (FH), result in enhanced HIFα stability and target gene trancription and formation of HIFα-expressing tumors in mice (Isaacs et al., 2005; Pollard et al., 2007). Furthermore, PHD and FIH activity requires ferrous iron [Fe(II)], which explains HIFα stabilization by chelation of iron (Fe(II)) by desferrioxiamine (DFO) or competition by Co(II) ions by treatment with cobalt chloride (Maxwell et al., 1999; Wang & Semenza, 1993b). In addition, reactive oxygen species (ROS) enhance H_2O_2 levels, which results in oxidation of Fe(II) to Fe(III) through the Fenton reaction [H_2O_2+Fe(II)→OH·+OH^- +Fe(III)], reducing PHD/FIH activity (Gerald et al., 2004; Pouyssegur & Mechta-Grigoriou, 2006). Next to metabolites and oxygen, the PHD enzymes and FIH are regulated by proteosomal degradation by the E3 ubiquitin ligase SIAH-1 and -2 (Fukuba et al., 2007; Nakayama et al., 2004). HIF activity is apparently tightly regulated by oxygen- and metabolite-dependent processes (Figure 2B).

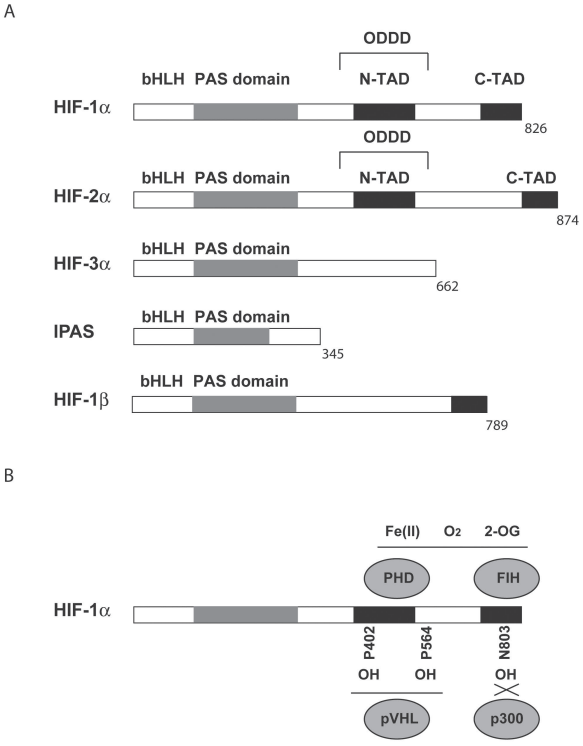


Figure 2.
Schematic drawing of (A) HIF protein domains and (B) main regulatory sites. bHLH; basic helix-loop-helix domain, PAS; Per/Arnt/Sim domain, ODDD; oxygen dependent degradation domain, N-TAD; N-terminal transactivation domain, C-TAD; C-terminal transactivation domain, Fe(II); ferrous iron, 2-OG; 2-oxogluterate.

Also other stress-related pathways regulate HIF. Heat exposure is able to induce HIF-1, which is dependent on the heatshock protein HSP90 (Katschinski et al., 2002). HSP90 interacts with HIF-1α, protecting HIF-1α from VHL-independent degradation by the receptor of activated protein kinase 1 (RACK1) (Liu et al., 2007; Minet et al., 2000). This explains the inhibitory effects of HSP90 inhibitors on HIF-1α activity (Minet et al., 2000). Furthermore, the cupper metabolism gene MURR1 domain (COMMD1) involved in copper homeostasis, was recently found to interact with HIF-1α and HIF-1α stability and transactivation (van de Sluis et al., 2007). In addition, oncogenic signaling affects HIF synthesis, stability and transcriptional activity, as will be discussed below.

Oncogenic regulation of HIF

Tumorigenesis involves inhibition of tumor suppressor genes and activation of oncogenes. The HIF complex is regulated by a classical tumor suppressor; VHL. The Von Hippel Lindau syndrome is a rare autosomal recessive familial cancer syndrome in which a mutated copy of the VHL gene is inherited through the germline. Loss of the remaining wild type allele leads to the development of a spectrum of multiple highly vascularized benign tumors such as hemangioblastioma and others vascular abnormalies such as retinoangiopathy (Friedrich, 1999). This is in agreement with the role of the HIF transcription factors in angiogenesis. Furthermore, patients have a high risk to develop renal cell carcinoma. These tumors are HIF dependent as is shown in elegant mouse models (Kondo et al., 2003; Yan et al., 2007). Somatic VHL mutation in sporadic renal cell carcinoma is also common (Kaelin, 2007). VHL might be the primary regulator for HIF, but its expression is controlled by multiple mechanisms. Many of these involve other known tumor suppressor genes and oncogenes (Bardos & Ashcroft, 2004). As will be further discussed in chapter 2, the phosphoinositol-3-kinase (PI 3-kinase) pathway controls HIF-1α protein expression. The PI 3-kinase pathway is an oncogenic signaling pathway that induces proliferation and inhibits apoptosis (Manning & Cantley, 2007). Receptor tyrosine kinase activation by growth factors like insulin and epidermal growth factor (EGF) activate PI 3-kinase, which in turn phosphorylates inositol lipids to produce phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. This results in phosphorylation of the major PI 3-kinase effector Akt or protein kinase B (PKB) by the phosphatidylinositol-dependent kinase-1

(PDK1) (Burgering & Coffer, 1995). Akt is serine/threonine kinase, which mediates a myriad of signaling cascades. PI 3-kinase activity is counteracted by the phosphatase and tensin homologue (PTEN), a tumor suppressor frequently mutated in human cancer and causative for the multiple hamartoma or Cowden syndrome (Li et al., 1997; Liaw et al., 1997). HIF-1α protein can be induced by EGF, insulin, insulin-like growth factor-2 (IGF-2) and overexpression of the HER-2/*neu* oncoprotein, which is inhibited by PI-3kinase inhibitors or PTEN re-expression (Feldser et al., 1999; Jiang et al., 2001a; Laughner et al., 2001; Zhong et al., 2000; Zundel et al., 2000). This regulation occurs by posttranslational control, presumably via the Akt effector: mammalian target of rapamycin (mTOR) (Brugarolas et al., 2003; Gort et al., 2006; Hudson et al., 2002; Land & Tee, 2007; Treins et al., 2002; Zhong et al., 2000). Furthermore, other Akt effectors have been implicated in HIF regulation, like GSK-3, FOXO4, HMD2, HSP90 and others (Bardos et al., 2004; Flugel et al., 2007; Mottet et al., 2003; Pore et al., 2006; Skinner et al., 2004; Tang & Lasky, 2003; Zhou et al., 2004b). Interestingly, the tumor suppressor proteins TSC1 (hamartin) and TSC2 (tuberin) are also implicated in the regulation of HIF via mTOR (Brugarolas & Kaelin, 2004; Brugarolas et al., 2003). TSC1/2 loss leads to slow growing, highly vascularized benign tumors in the heart, brain, kidneys, eyes and skin of mice. TSC1/2 heterodimers function as a GTPase activating protein for the mTOR interacting Ras homologue enriched in brain (Rheb), which results in inhibition of mTOR activity (Kwiatkowski & Manning, 2005). TSC2-deficiency results in induction of HIF-1α and HIF-2α as a result of reduced mTOR inhibition, although subsequent VEGF induction is probably also regulated by a mTOR independent function of TSC2 (Brugarolas et al., 2003; Liu et al., 2003). Intriguingly, the LKB1 tumor suppressor protein controls the activity of the TSC1/TSC2 tumor suppressor complex. Mutations in LKB1 cause Peutz-Jeghers syndrome (PJS), which is also characterized by the development of hamartomas (Giardiello et al., 1987). LKB1 is normally activated by low ATP/ADP ratio, as seen under hypoxia, and activates AMPK, which in turn phosphorylates and activates TSC2 (Corradetti et al., 2004). AMPK activation inhibits the insulin-induced expression of HIF-1α, via a decrease in mTOR activity (Treins et al., 2006). Although the signaling network is rather complex and emerging, it seems likely that energy and oxygen sensing pathways like the PI 3-kinase/Akt/mTOR pathway, LKB1/AMPK/TSC1/2/mTOR pathway play an important role in HIF regulation and therefore HIF might be a common player in hamartoma syndromes like Cowden, Peutz-Jeghers and tuberous sclerosis complex.

The *TP53* tumor suppressor gene is the most commonly mutated gene in human cancers (Vogelstein & Kinzler, 2004). p53 is a transcription factor that mediates cell cycle arrest or apoptosis in response to DNA damage and other stimuli, including anoxia (Levine et al., 2006). Similarly to HIFα, p53 is normally continuously degraded. HDM2 binds to p53 and recruits an E3 ubiquitin ligase complex that targets p53 for degradation. Upon induction of DNA damage, the protein kinase ATM phosphorylates p53 preventing HDM2 binding. In addition, p53 stabilization and transcriptional activation is enhanced by p300-dependent acetylation. Whereas HIF is induced at oxygen tensions varying from 8 to less then 0.1% oxygen, p53 is induced only by severe hypoxia or anoxia ($pO_2 < 0.2\%$) and its stabilization might be HIF-dependent (An et al., 1998; Hammond & Giaccia, 2005). In addition, microenvironmental changes, like acidosis and nutrient deprivation, are necessary for hypoxic p53 induction (Pan et al., 2004). Hypoxic apoptosis might be dependent on direct interaction of the HIF-1 ODDD to a p53 dimer, but hypoxia might also inhibit cell damage-induced, p53-dependent cell death (Achison & Hupp, 2003; Sanchez-Puig et al., 2005). On the other hand, p53 inhibits HIF-1 activity via binding to the p300-HIF complex (Blagosklonny et al., 1998). Furthermore, p53 induces HIF-1α degradation by promoting HDM2-mediated ubiquitination of HIF-1α (Ravi et al., 2000). Finally, VHL regulates p53 by nuclear translocation, stabilization and activation after genotoxic stress, providing additional evidence for a crosstalk between the HIF and p53 pathways (Roe et al., 2006). This link might be important in clinical management between p53 positive and negative malignancies.

The small GTPase Ras family consists of oncoproteins that cycle from a GDP-bound (inactive) to a GTP-bound (active) state. There are three functional ras genes, H-*ras*, K-*ras* and N-*ras* and in colon and pancreatic cancer activating *ras* mutations are frequent (Bos, 1989). Activated Ras induces the mitogen-activated protein kinase (MAPK) pathway and the PI 3-kinase pathway. H-ras transformed cells display enhanced hypoxic VEGF induction, probably via the PI 3-kinase pathway (Mazure et al., 1997; Mazure et al., 1996). In addition, the Ras effector p42/p44 MAPKs (Erk1 and Erk2) are activated by hypoxia and enhance transcriptional activity of HIF-1α by direct phosphorylation, presumably at S641/643, whereas the other MAPKs (p38 and JNK) do not affect HIF phosphorylation status (Minet et al., 2000; Mylonis et al., 2006; Richard et al., 1999; Sodhi et al., 2001). The MAPKs thereby activate the VEGF promoter via both the Sp1/AP-2 transcription factor and HIF-1 (Mazure et al., 2003). The exact mechanism of MAPK-dependent regulation of HIF however needs further study.

Another oncoprotein implied in HIF signaling is SRC. *C-src* is the proto-oncogene of which the Rous sarcoma virus (RSV) oncogene *v-src* is derived. Inhibition of c-SRC can inhibit hypoxic VEGF induction, whereas overexpression of v-SRC induces HIF-1 signaling via the MAPK route (Jiang et al., 1997; Mukhopadhyay et al., 1995). Effects on HIF signaling are disputed, but the theory is that HIF-1 and STAT3 cooperate downstream of SRC to induce transformation, glycolysis and angiogenesis (Gleadle & Ratcliffe, 1997; Semenza, 2000b). This hypothesis can be readily tested in src-dependent tumours.

The nuclear factor-κB (NF-κB) is a central mediator of inflammatory response, but also plays a role in oncogenesis (Rayet & Gelinas, 1999). Hypoxia induces NFκB stability and activity by tyrosine phosphorylation of the NFκB inhibitor iκB (Koong et al., 1994). Interestingly, inhibition of NFκB blocks hypoxic HIF-1α stabilization and *epo* transcription, whereas microtubule destabilization and TNFα stimulation induce transcription of HIF-1α via a NFκB-dependent mechanism (Figueroa et al., 2002; Jung et al., 2003; Zhou et al., 2003). Cooperation between NFκB and HIF in controlling transcriptional response under hypoxia therefore seems likely.

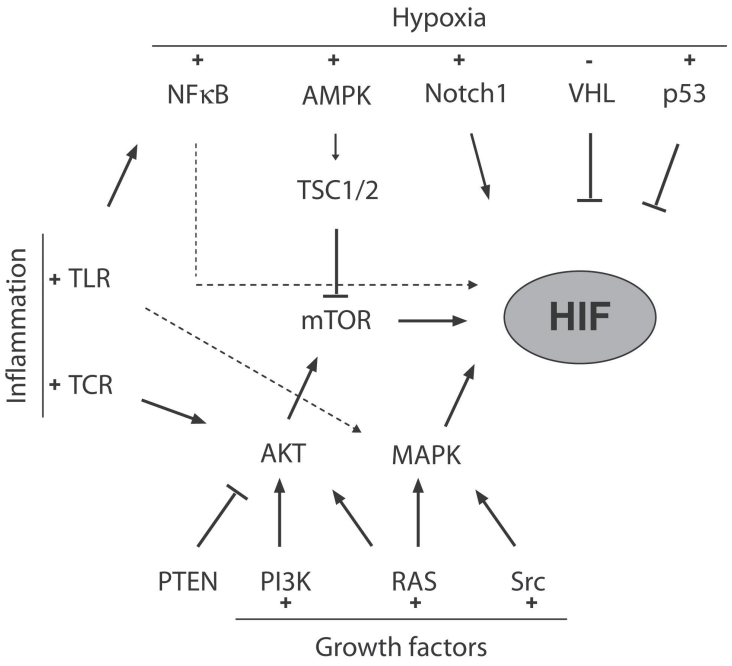


Figure 3.
Schematic drawing of multiple signal transduction pathways affecting HIF signaling.

Recently, a novel interaction was reported of HIF-1α and the cell fate receptor Notch1 (Gustafsson et al., 2005). The Notch proteins are highly conserved membrane proteins that are released upon ligand binding by proteolytic cleavage involving Adam proteases and γ-secretase to constitute transcription factors that regulate cell-fate decision (Mumm & Kopan, 2000; van Es et al., 2005). Hypoxia increases Notch1 signaling to maintain dedifferentiated cell fate (Gustafsson et al., 2005). Under hypoxia HIF-1α associates with the Notch1 intracellular domain and they bind together to Notch responsive promoters, driving Notch1 transcriptional activity. Simultaneously, the ankyrin repeat domains of Notch1 are hydroxylated by FIH and compete with HIF for FIH-dependent hydroxylation, which may result in enhanced HIF activity in the presence of Notch1 (Coleman et al., 2007).

Apparently, HIF is regulated by multiple oncogenic signaling pathways (Figure 3). In addition to oncogenic regulation, HIF is also regulated by inflammatory responses mediated by the toll-like receptor (TLR) and T-cell receptor (TCR) that activate the NFκB, MAPK and PI 3-kinase pathways (Frede et al., 2006; Nakamura et al., 2005). This might result in the inhibition of anti-tumor T-cells within the hypoxic microenvironment (Lukashev et al., 2007). Unraveling the exact background for a regulatory mechanism to kick in will be a great challenge and necessary to determine the functional significance of the many HIF-1 regulating pathways.

Transcriptional regulation by HIF

Functional response to hypoxia is mediated by the HIF transcription factors. Mouse models have shown that HIF-1α is essential for normal development by controlling vascularization and O_2 homeostasis (Iyer et al., 1998; Ryan et al., 1998). The downstream target genes of HIF are diverse and implicated in a broad spectrum of processes. Over 70 of the HIF target genes contain one or more functional HREs within their genomic context. This HRE can be located in the 5' promoter region as well as in 3' regions (in *EPO*), and within intronic regions (in *EGLN3* and *TWIST1*, as demonstrated in this thesis) (Pescador et al., 2005; Semenza et al., 1991). The minimal HRE sequence necessary for interaction with the HIF heterodimer is CGTG with a purine, preferably adenine, at the -1 position (Wenger et al., 2005). Adjacent sequences influence HRE binding and might contain DNA binding sequences for additional transcriptional factors, like SP-1 (in *CAIX*), ATF, CREB-1 (in *LDHA*) and AP-1 (in *VEGF*), that

cooperate with HIF to regulate transcription (Damert et al., 1997; Ebert & Bunn, 1998; Firth et al., 1995; Kaluz et al., 2003). Furthermore, tandem core HREs can constitute a functional HRE (in *Glut-1*, several glycolytic enzymes and *TWIST1* as demonstrated in this thesis) (Wenger et al., 2005). The HIFβ subunit presumably binds the GTG part of the core HRE, whereas HIFα binds the NAC sequence and the dioxine receptor (aryl hydrocarbon receptor or AHR), that can also dimerize to HIFβ, binds TNG containing sequences (Swanson, 2002). Although differential regulation by HIF-1α versus HIF-2α exists, no difference in core HRE composition is found that explains specific HIF-1 or -2 binding (Raval et al., 2005). Recruitment of additional transcription factors and co-factors might explain this, but this is still under debate. Genome wide location analysis of HIF1 and HIF2 binding sequences will be necessary to unravel their distinct biological reponses.

Hypoxic cells need to adjust metabolism as oxygen-dependent ATP generation via oxidative phosphorylation ceases and glycolysis is enhanced, generating the Warburg effect. It is therefore not surprising that many key-enzymes of the glycolysis are under direct control of the HIF transcription complex, namely aldolase A and C, enolase 1 (*ENO1*), glyceraldehyde phosphate dehydrogenase (*GAPDH*), hexokinase-1 and -2 (*HK-1* and *-2*, phosphofructokinase L (*PFK L*), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 and -4 (*PFKFB*-3 and *PFKFB*-4) and phosphoglycerate kinase 1 (*PGK1*) (Graven et al., 1999; Greijer et al., 2005; Lu et al., 2002; Semenza et al., 1996; Semenza et al., 1994). Also enzymes facilitating glycolysis are upregulated, like glucose transporters 1 and 3 (*Glut-1* and *-3*) and lactate dehydrogenase A (*LDHA*) (Ebert et al., 1995; Firth et al., 1995). Anaerobic energy synthesis results in lactate synthesis and thereby acidification of the intracellular milieu. Carbonic anhydrase IX (CAIX) is an enzyme that catalyses the reaction [$H_2O + CO_2 \leftrightarrow H^+ + HCO_3^-$]. CAIX is induced by HIF to neutralize the intracellular pH, resulting in extracellular acidosis (Grabmaier et al., 2004; Pouyssegur & Mechta-Grigoriou, 2006). CAIX expression is often used as a marker for hypoxic areas within tumor tissue and indicates functional HIF-1 (Potter & Harris, 2004).

Regulation of metabolism by HIF is a direct cellular adaptation to oxygen deprivation. In addition, hypoxic tissue regulates mechanisms that influence oxygen supply to the affected tissue areas, including erythropoiesis, vasodilation and neoangiogenesis. HIF-regulated genes involved in erythropoiesis and heme synthesis are erythropoietin (*EPO*), ferrochelatase, the heme binding ATP-binding cassette transporter breast cancer resistance protein (*BCRP* or *ABCG2*),

ceruloplasmin, transferrin and its receptor (Krishnamurthy et al., 2004; Liu et al., 2004; Mukhopadhyay et al., 2000; Rolfs et al., 1997; Tacchini et al., 1999; Wang & Semenza, 1993a). To increase blood supply, vasodilation is induced by HIF-dependent transcription of endothelial NO synthase (*eNOS*), heme oxygenase, endothelin-1, $\alpha_{1\beta}$-adrenergic receptor and adrenomedullin (Coulet et al., 2003; Eckhart et al., 1997; Hu et al., 1998; Lee et al., 1997; Nguyen & Claycomb, 1999). Perhaps the best-studied function of HIF is the induction of angiogenesis. Hypoxic HIF-1 activity is a potent inducer of VEGF (VEGF-A) expression (Forsythe et al., 1996). VEGF-A is a member of the platelet-derived growth factor (PDGF) family of homodimeric glycoproteins. VEGF is a strong angiogenic growth factor, essential for the proliferation and migration of vascular endothelial cells necessary for neoangiogenesis (Ferrara, 2002; Pepper et al., 1991). VEGF is recognized and bound by the receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), which are also both induced by HIF-1 (Gerber et al., 1997). In addition to VEGF, other angiogenic factors are induced by hypoxia, like angiopoietin-1, -2 and -4 (ANGPT1, -2 and -4), placental growth factor (PGF), and platelet-derived growth factor-B (PDGF-B) (Kelly et al., 2003; Yamakawa et al., 2003). The role of HIF in angiogenesis is illustrated by the formation of highly vascularized tumors in VHL disease (Friedrich, 1999). Furthermore, various knockout mouse models have established the importance of HIF for proper vascular development (Carmeliet et al., 1998; Iyer et al., 1998; Ryan et al., 1998).

In addition to oxygen homeostasis and vascularization, regulation of hypoxic survival is a major function of the HIF transcription complex. Both protection from and induction of apoptosis can be under control of the HIF pathway. The best known pro-apoptotic gene induced by HIF-1 is the Bcl-2/adenovirus E1B 19-kDa-protein interacting protein 3 (BNIP3) gene, which can induce apoptosis by sequestering anti-apoptotic Bcl-2 family members (Kothari et al., 2003). Also the pro-apoptotic BH3-only member Noxa is induced by HIF (Kim et al., 2004). Anti-apoptotic genes induced by HIF include Ser/Thr protein phosphatase 5 (*PP5*), myeloid cell factor-1 (*Mcl-1*) and nucleophospmin (Li et al., 2004; Piret et al., 2005; Zhou et al., 2004a). Apoptotic decision making is influenced by the severity of oxygen deprivation and dependent on other signaling pathways as well. For instance, the activation of p53 by severe hypoxia might contribute to pro-apoptotic signaling by HIF (Sanchez-Puig et al., 2005). In addition, HIF-1α phosphorylation status might influence apoptotic potential (Suzuki et al., 2001). Hypoxia-induced G1 arrest on the other hand, is thought to be independent on p53, but dependent on HIF-1α (Goda et al., 2003; Graeber et al., 1994). Hypoxia

induces the cyclin-dependent kinase inhibitors inhibitors (CDKIs) p21 and p27 and hypophosphorylation of the retinoblastoma (Rb) protein, which results in G1 arrest (Gardner et al., 2001; Goda et al., 2003; Green et al., 2001). The exact role of HIF herein has been disputed (Carmeliet et al., 1998; Goda et al., 2003). Cell cycle arrest might play a protective role, ultimately resulting in cell survival under hypoxic conditions.

Tumor hypoxia is associated with metastatic disease (Hockel et al., 1996). Furthermore, experimental mouse models have indicated that HIF functions in metastatic disease (Liao et al., 2007). The hypoxic gene expression profile predicts metastasis (Chi et al., 2006; Nuyten & van de Vijver, 2006). It is plausible that HIF transcriptional activity mediates at least part of this hypoxic profile. Genes regulated by HIF that impact on the ability to metastasize include regulators of epithelial to mesenchymal transduction, enzymes that regulate extracellular matrix (ECM) formation and degradation, adhesion molecules and migratory factors. EMT regulators induced by HIF-1α and/or HIF-2α include SNAIL, SIP1 and TWIST1 (this thesis), which are repressors of E-cadherin expression, a key molecule in maintenance of epithelial cell junctions (Esteban et al., 2006; Evans et al., 2006; Krishnamachary et al., 2006; Peinado et al., 2007). The HIF regulated ECM controlling enzymes include lysyl oxidase (LOX), matrix metalloprotease 2 and 9 (MMP2 & MMP9), and urokinase plasminogen activator receptor (uPAR) (Erler et al., 2006; Graham et al., 1999; Luo et al., 2006; Osinsky et al., 2005). Importantly, the cell surface G-protein-coupled CXC chemokine receptor 4 (CXCR4) and its ligand stromal-derived factor-1 (SDF-1) are regulated by HIF-1α and HIF-2α, facilitating tumor cell invasion and metastatic homing (Liu et al., 2006; Zagzag et al., 2005). Finally, HIF-1α mediates induction of migratory signaling molecules like the hepatocyte growth factor (HGF) receptor c-Met, activation of which is a potent inducer of migration, and autocrine motility factor (AMF) (Funasaka et al., 2005; Niizeki et al., 2002; Pennacchietti et al., 2003).

Last but not least, HIF mediates transcription of the prolyl hydroxylase enzymes that modulate HIF expression itself. PHD2 and PHD3 are both up regulated by HIF (Metzen et al., 2005; Pescador et al., 2005). This results in a negative feedback loop, which facilitates rapid HIFα degradation upon reoxygenation. In addition, HIF regulates transcription of Cited2, a negative regulator of HIF transactivation (Bhattacharya et al., 1999). Taken together, HIF signaling regulates many processes, that sometimes execute conflicting actions (Figure 4) (for a more complete overview see following references) (Semenza, 2003;

Wenger et al., 2005). Understanding differential gene expression by HIF-1 versus HIF-2, the influence of cooperating or antagonizing signaling pathways, and cofactor requirement, will enhance insight in the functional relevance within a specific background.

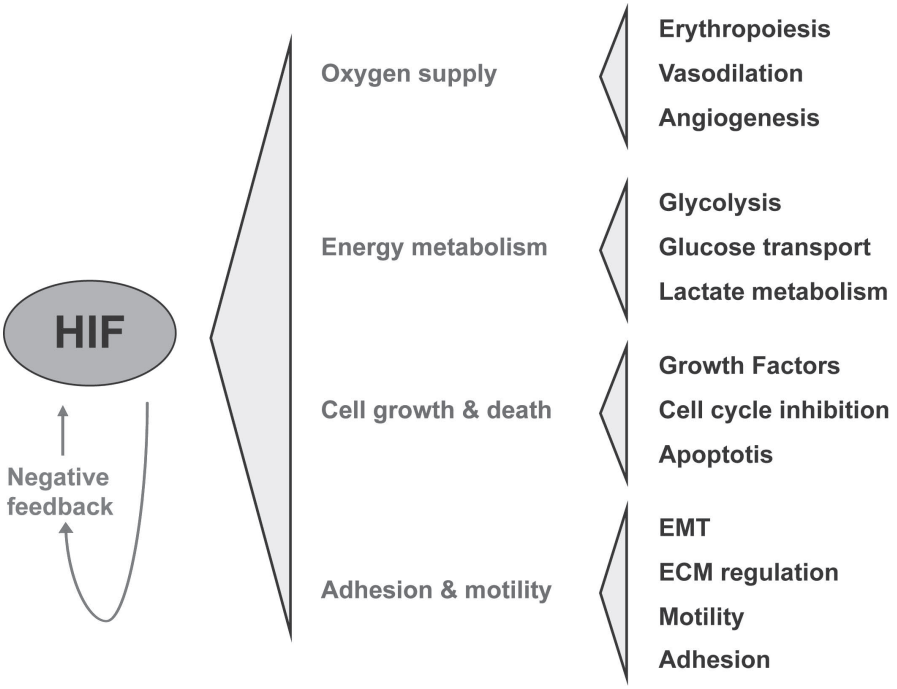


Figure 4.
Schematic representation of biological effects of HIF-mediated transcription. In blue the main biological effects are depicted. These are futher specified in black. In red the negative feedback loop is indicated.

HIF expression in cancer and clinical outcome

Expression of HIF-1α has been extensively studied in multiple cancerous lesions. In breast cancer progression, HIF-1α expression is increasingly detected from ductal carcinoma in situ (DCIS) to invasive ductal breast carcinoma, especially in poorly differentiated lesions (Bos et al., 2001). Overexpression of HIF-1α is associated with human cancer progression and prognosis in epithelial cancers of the head and neck, cervix, colon, breast and lung (Bos et al., 2003; Dales et al., 2005; Giatromanolaki et al., 2001; Gruber et al., 2004; Jokilehto et al., 2006; Kronblad et al., 2006; Kurokawa et al., 2003; Schindl et al., 2002;

Schoppmann et al., 2006; Trastour et al., 2007; Vleugel et al., 2005; Yatabe et al., 2004; Yoshimura et al., 2004). The role of HIF in tumorigenesis is illustrated by the fact that embryonic stem (ES) cells and mouse embryonic fibroblasts (MEFs) lacking HIF-1α have reduced capacity to form teratocarcinomas and fibrosarcomas, respectively (Ryan et al., 1998; Ryan et al., 2000). In addition, HIF-1α overexpression increases tumorigenesis of pancreatic cancer cells, whereas expression of dominant negative HIF-1α decreases this (Akakura et al., 2001; Chen et al., 2003). Furthermore, HIF-1α expression in invasive breast carcinoma, gastric cancer, squamous cell carcinoma of the cervix is correlated with poor prognosis (Bos et al., 2003; Dales et al., 2005; Gruber et al., 2004; Ishikawa et al., 2004; Schindl et al., 2002; Sumiyoshi et al., 2006). In other studies, HIF-1α overexpression in squamous cell carcinoma of the oral floor, renal cell carcinoma and non-small cell lung cancer has been correlated with a good prognosis (Fillies et al., 2005; Lidgren et al., 2005; Volm & Koomagi, 2000). This might be explained by the proapoptotic function of HIF-1α, as HIF-1α depleted ES cell-derived tumors displayed reduced hypoxia-induced apoptosis (Carmeliet et al., 1998). Besides, it has also been suggested that in squamous epithelium, where there is a physiological expression of HIF-1α, HIF-1α expression may be differentiation related. Better differentiated tumors arising in these tissues that inherently have a better prognosis would thereby express more HIF1α, which is thereby an epiphenomenon rather then a carcinogenetic event (Fillies et al., 2005). Hypoxic tumor development has been linked to increased risk for metastasis (Vaupel et al., 2004). HIF overexpression in human cancer might be causative for this, which is supported by the finding that HIF-1α is highly expressed in metastases of breast, prostate, colon and renal cell carcinomas, and overexpression of HIF-1α in primary tumors has been shown to predict presence of bone marrow metastases in breast cancer patients (Evans & Koch, 2003; Woelfle et al., 2003; Zhong et al., 1999). This is in concordance with a mouse model for breast carcinoma, which displayed a decrease in lung metastasis after conditional deletion of HIF-1α (Liao et al., 2007).

The role of HIF-2α in oncogenesis might be more pronounced then HIF-1α, although clinical evidence is minimal at this moment. In VHL-deficient renal cell carcinoma, HIF-2α depletion reverses the malignant phenotype in mouse models and HIF-2α overexpression in VHL-positive background induces tumor growth, underlining the oncogenic activity of HIF-2α (Kondo et al., 2003; Kondo et al., 2002). RCC xenografts expressing HIF-2α show enhanced tumor growth, whereas those expressing HIF-1α do not (Raval et al., 2005).

Knock-in replacement of HIF-1α with HIF-2α in ES cells also resulted in enhanced tumor growth (Covello et al., 2005). HIF-2α expression has been found in several human solid tumor specimen as well as in tumor-associated macrophages (Talks et al., 2000). Furthermore, in colorectal carcinoma, stromal expression of HIF-2α was associated with a decreased survival, indicating that stromal HIF expression contributes to malignant progression (Cleven et al., 2007). This is supported by the observation that implanted tumors can activate the VEGF promoter in recipient stromal cells (Fukumura et al., 1998).

Although the exact role of HIF in tumorigenesis requires further investigation, one can foresee that development of small molecule inhibitors of HIF activity may offer clinical opportunities in treatment of hypoxic cancers. Several drugs have recently been introduced in clinical trials, that display HIF-inhibitory properties, like HSP90 inhibitors (17-AAG), proteasome inhibitors (Bortezomib or Velcade), and inhibitors of signal transduction routes (e.g. mTOR inhibitor rapamycin) (Patiar & Harris, 2006). None of these inhibitors is however specific for HIF, but specific inhibitors are being developed (Groot et al., 2006; Jones & Harris, 2006; Olenyuk et al., 2004; Rapisarda et al., 2002).

Evolutionary Conservation of the HIF-pathway in *C. elegans*

As aerobic organisms depend on oxygen, it is not surprising that the components of the molecular oxygen sensing mechanism are largely conserved throughout evolution. In the nematode *Caenorhabditis elegans* (*C. elegans*) and fruitfly *Drosophila melanogaster*, both highly amendable to genetic manipulation, the HIF pathway is conserved. The genes for HIF-1α and VHL share up to 50% homology to their mammalian homologues. *C. elegans* is a worm-shaped nematode of 10 micron in length, which feeds on bacteria in the soil. The animals are hermaphrodite, which facilitates genetic research, because strains are easily maintained. Genetic crossing is feasible, because a small percentage of animals is male. Furthermore, RNA interference (RNAi) is a powerful research tool in *C. elegans*, as feeding on bacteria expressing double stranded RNA (dsRNA) homologous to a gene, is sufficient to systemically silence the messenger RNA (mRNA) expression of that gene. High through-put screening is therefore feasible because of these properties (Kamath et al., 2003; van Haaften et al., 2004). Classical genetic screening of *C. elegans* for phenotypes has resulted in substantial amount of information. In one of these screens, the lab of Nobel laureate Robert Horvitz identified genes resulting in

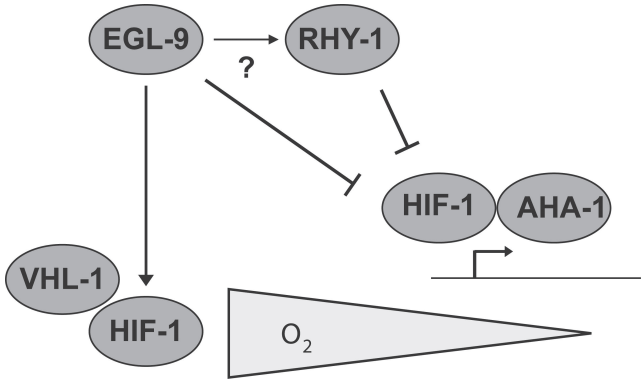


Figure 5.
Schematic drawing of the HIF-1 pathway in *C. elegans*. Under normal oxygen tension EGL-9 function results in VHL-dependent HIF-1 degradation. In addition, EGL-9 and RHY-1 function in a VHL-1 independent pathway to repress HIF-1 activity.

a defect in egg-laying behavior (Trent et al., 1983). These genes were termed *egl*-genes. The exact phenotype varies among mutants, but is characterized by an inability to lay eggs and a subsequent stacking of eggs in the womb. These eggs hatch and rupture the mother, producing viable offspring. The *egl*-genes encode a variety of proteins involved in mesodermal or neuronal development. One of these genes, *egl-9*, encodes the HIF-1 prolyl-hydroxylase homologue (Epstein et al., 2001). The exact etiology of its egg-laying defect is unknown and interestingly, the defect is thermo sensitive. Recently, it was noted that the phenotype is HIF-1 dependent (Bishop et al., 2004). The HIF pathway in *C. elegans* consists of a prolyl-hydroxylase (*egl-9*), which hydroxylates the HIFα subunit (*hif-1*) under normal oxygen tensions (Epstein et al., 2001). This enables binding of *C. elegans* VHL-1 and subsequent HIF-1 degradation. Under hypoxia, EGL-9 is inactive and HIF-1 is stabilized and heterodimerizes to the β-subunit AHA-1, forming the HIF transcription factor (Figure 5) (Jiang et al., 2001b; Powell-Coffman et al., 1998). The HIF target genes in *C. elegans* are mainly involved in metabolism, signal transduction, transport and ECM remodeling (Bishop et al., 2004; Shen et al., 2005). Animals deficient in e*gl-9* have been shown to express high levels of HIF-1 and HIF-1-target genes, as do *vhl-1* deficient animals, albeit to a lesser extent (Bishop et al., 2004; Epstein et al., 2001; Shen et al., 2005; Shen et al., 2006). Therefore, it has been suggested that EGL-9 regulates HIF-1 transcriptional activity in addition to VHL-1-dependent degradation. Recently, a novel regulator of HIF-1, *rhy-1*,

was identified by forward genetic screening (Shen et al., 2006). Presumably RHY-1 and EGL-9 function in VHL-1-independent pathway(s) to repress HIF-1 transcriptional activity.

Wild-type *C. elegans* encounter variable oxygen tensions in the soil and are able to maintain normal metabolic rate and proliferation up to 0.5% oxygen. Animals deficient for *hif-1* are unable to keep homeostasis at lower oxygen tensions (1-0.5 % oxygen) (Jiang et al., 2001b; Padilla et al., 2002; Shen & Powell-Coffman, 2003). This results in severely reduced viability and reproducibility. At lower percentages of oxygen (anoxia), wild type *C. elegans* switches to suspended animation, a process independent of HIF-1 (Padilla et al., 2002). In addition, culturing of wild type *C. elegans* under 25ºC results in enhanced heat endurance, which is dependent on HIF-1 (Treinin et al., 2003). This heat acclimatization also enhances hypoxia and cadmium tolerance.

Because of the high conservation of the HIF pathway, the multiple HIF-dependent phenotypes, and high through-put feasibility, *C. elegans* provides a good model system to study the HIF pathway.

Thesis outline

Understanding the molecular pathways triggered by hypoxia will enhance insight into the pathological mechanism that underlies hypoxia-associated poor prognosis of cancer. This thesis addresses two main questions: i. how is HIF-1α regulated, and ii. how does hypoxia, and particularly HIFα overexpression, result in a poor prognosis?

Chapter 2 reviews the latest insights in HIF-mediated regulation of metastasis. In **chapter 3**, the role of the PI 3-kinase pathway in HIF-1α regulation is studied, providing evidence for a role for this oncogenic pathway in breast cancer. **Chapter 4** shows how p27 is re-expressed in endometroid endometrium carcinoma in a HIF-1α dependent manner and related to cell cycle arrest in perinecrotic tumor areas. In **chapter 5**, *C. elegans* is used as a model system in genetic screens for members of the HIF pathway and candidates are studied in mammalian systems. Here, *TWIST1* is identified as a HIF-2α target gene. **Chapter 6** discusses genetics, epigenetics and expression of the hypoxia-inducible target gene TWIST1 in human breast cancer in more detail. In **chapter 7**, a functional rescue screen for HIF-1α regulators is presented. Finally, **chapter 8** discusses and integrates all chapters.

References

Achison M and Hupp TR. (2003). Hypoxia attenuates the p53 response to cellular damage. *Oncogene*, **22,** 3431-40.

Akakura N, et al. (2001). Constitutive expression of hypoxia-inducible factor-1alpha renders pancreatic cancer cells resistant to apoptosis induced by hypoxia and nutrient deprivation. *Cancer Res*, **61,** 6548-54.

An WG, et al. (1998). Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. *Nature*, **392,** 405-8.

Bae SH, et al. (2004). Sumoylation increases HIF-1alpha stability and its transcriptional activity. *Biochem Biophys Res Commun*, **324,** 394-400.

Bardos JI and Ashcroft M. (2004). Hypoxia-inducible factor-1 and oncogenic signalling. *Bioessays*, **26,** 262-9.

Bardos JI, et al. (2004). Growth factor-mediated induction of HDM2 positively regulates hypoxia-inducible factor 1alpha expression. *Mol Cell Biol*, **24,** 2905-14.

Bhattacharya S, et al. (1999). Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. *Genes Dev*, **13,** 64-75.

Bishop T, et al. (2004). Genetic analysis of pathways regulated by the von hippel-lindau tumor suppressor in Caenorhabditis elegans. *PLoS Biol*, **2,** e289.

Blagosklonny MV, et al. (1998). p53 inhibits hypoxia-inducible factor-stimulated transcription. *J Biol Chem*, **273,** 11995-8.

Bos JL. (1989). ras oncogenes in human cancer: a review. *Cancer Res*, **49,** 4682-9.

Bos R, et al. (2003). Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*, **97,** 1573-81.

Bos R, et al. (2001). Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. *J Natl Cancer Inst*, **93,** 309-14.

Brugarolas J and Kaelin WG, Jr. (2004). Dysregulation of HIF and VEGF is a unifying feature of the familial hamartoma syndromes. *Cancer Cell*, **6,** 7-10.

Brugarolas JB, et al. (2003). TSC2 regulates VEGF through mTOR-dependent and -independent pathways. *Cancer Cell*, **4,** 147-58.

Bruick RK and McKnight SL. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. *Science*, **294,** 1337-40.

Burgering BM and Coffer PJ. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature*, **376,** 599-602.

Carmeliet P. (2005). VEGF as a key mediator of angiogenesis in cancer. *Oncology*, **69 Suppl 3,** 4-10.

Carmeliet P, et al. (1998). Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature*, **394,** 485-90.

Chen J, et al. (2003). Dominant-negative hypoxia-inducible factor-1 alpha reduces tumorigenicity of pancreatic cancer cells through the suppression of glucose metabolism. *Am J Pathol*, **162,** 1283-91.

Chi JT, et al. (2006). Gene expression programs in response to hypoxia: cell type specificity and prognostic significance in human cancers. *PLoS Med*, **3,** e47.

Cleven AH, et al. (2007). Stromal expression of hypoxia regulated proteins is an adverse prognostic factor in colorectal carcinomas. *Cell Oncol*, **29,** 229-40.

Cockman ME, et al. (2000). Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J Biol Chem*, **275,** 25733-41.

Coleman ML, et al. (2007). Asparaginyl hydroxylation of the notch ankyrin repeat domain by factor inhibiting hypoxia inducible factor. *J Biol Chem*.

Comerford KM, et al. (2002). Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. *Cancer Res*, **62,** 3387-94.

Corradetti MN, et al. (2004). Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. *Genes Dev*, **18,** 1533-8.

Coulet F, et al. (2003). Identification of hypoxia-response element in the human endothelial nitric-oxide synthase gene promoter. *J Biol Chem*, **278,** 46230-40.

Covello KL, et al. (2005). Targeted replacement of hypoxia-inducible factor-1alpha by a hypoxia-inducible factor-2alpha knock-in allele promotes tumor growth. *Cancer Res*, **65,** 2277-86.

Dales JP, et al. (2005). Overexpression of hypoxia-inducible factor HIF-1alpha predicts early relapse in breast cancer: retrospective study in a series of 745 patients. *Int J Cancer*, **116,** 734-9.

Damert A, et al. (1997). Activator-protein-1 binding potentiates the hypoxia-induciblefactor-1-mediated hypoxia-induced transcriptional activation of vascular-endothelial growth factor expression in C6 glioma cells. *Biochem J*, **327 (Pt 2),** 419-23.

Ebert BL and Bunn HF. (1998). Regulation of transcription by hypoxia requires a multiprotein complex that includes hypoxia-inducible factor 1, an adjacent transcription factor, and p300/CREB binding protein. *Mol Cell Biol*, **18,** 4089-96.

Ebert BL, et al. (1995). Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences. *J Biol Chem*, **270,** 29083-9.

Eckhart AD, et al. (1997). Characterization of the alpha1B-adrenergic receptor gene promoter region and hypoxia regulatory elements in vascular smooth muscle. *Proc Natl Acad Sci U S A*, **94,** 9487-92.

Epstein AC, 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.

Erler JT, et al. (2006). Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature*, **440,** 1222-6.

Esteban MA, et al. (2006). Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. *Cancer Res*, **66,** 3567-75.

Evans AJ, et al. (2006). VHL Promotes E2 Box-dependent E-cadherin Transcription by HIF-mediated Regulation of SIP1 and Snail. *Mol Cell Biol*.

Evans SM and Koch CJ. (2003). Prognostic significance of tumor oxygenation in humans. *Cancer Lett*, **195,** 1-16.

Feldser D, et al. (1999). Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. *Cancer Res*, **59,** 3915-8.

Ferrara N. (2002). VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer*, **2,** 795-803.

Figueroa YG, et al. (2002). NF-kappaB plays a key role in hypoxia-inducible factor-1-regulated erythropoietin gene expression. *Exp Hematol*, **30,** 1419-27.

Fillies T, et al. (2005). HIF1-alpha overexpression indicates a good prognosis in early stage squamous cell carcinomas of the oral floor. *BMC Cancer*, **5,** 84.

Firth JD, et al. (1995). Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements. *J Biol Chem*, **270,** 21021-7.

Flugel D, et al. (2007). Glycogen synthase kinase 3 phosphorylates hypoxia-inducible factor 1alpha and mediates its destabilization in a VHL-independent manner. *Mol Cell Biol*, **27,** 3253-65.

Folkman J. (1971). Tumor angiogenesis: therapeutic implications. *N Engl J Med*, **285,** 1182-6.

Forsythe JA, et al. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*, **16,** 4604-13.

Frede S, et al. (2006). Bacterial lipopolysaccharide induces HIF-1 activation in human monocytes via p44/42 MAPK and NF-kappaB. *Biochem J*, **396,** 517-27.

Friedrich CA. (1999). Von Hippel-Lindau syndrome. A pleomorphic condition. *Cancer*, **86,** 2478-82.

Fukuba H, et al. (2007). Siah-1 facilitates ubiquitination and degradation of factor inhibiting HIF-1alpha (FIH). *Biochem Biophys Res Commun*, **353,** 324-9.

Fukumura D, et al. (1998). Tumor induction of VEGF promoter activity in stromal cells. *Cell*, **94,** 715-25.

Funasaka T, et al. (2005). Regulation of phosphoglucose isomerase/autocrine motility factor expression by hypoxia. *Faseb J*, **19,** 1422-30.

Gardner LB, et al. (2001). Hypoxia inhibits G1/S transition through regulation of p27 expression. *J Biol Chem*, **276,** 7919-26.

Gerald D, et al. (2004). JunD reduces tumor angiogenesis by protecting cells from oxidative stress. *Cell*, **118,** 781-94.

Gerber HP, et al. (1997). Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J Biol Chem*, **272,** 23659-67.

Giardiello FM, et al. (1987). Increased risk of cancer in the Peutz-Jeghers syndrome. *N Engl J Med*, **316,** 1511-4.

Giatromanolaki A, et al. (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,** 881-90.

Gleadle JM and Ratcliffe PJ. (1997). Induction of hypoxia-inducible factor-1, erythropoietin, vascular endothelial growth factor, and glucose transporter-1 by hypoxia: evidence against a regulatory role for Src kinase. *Blood*, **89,** 503-9.

Goda N, et al. (2003). Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. *Mol Cell Biol*, **23,** 359-69.

Gort EH, et al. (2006). Hypoxia-inducible factor-1alpha expression requires PI 3-kinase activity and correlates with Akt1 phosphorylation in invasive breast carcinomas. *Oncogene*, **25,** 6123-7.

Grabmaier K, et al. (2004). Strict regulation of CAIX(G250/MN) by HIF-1alpha in clear cell renal cell carcinoma. *Oncogene*, **23,** 5624-31.

Graeber TG, et al. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature*, **379,** 88-91.

Graeber TG, et al. (1994). Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. *Mol Cell Biol*, **14,** 6264-77.

Graham CH, et al. (1999). Hypoxia-mediated stimulation of carcinoma cell invasiveness via upregulation of urokinase receptor expression. *Int J Cancer*, **80,** 617-23.

Graven KK, et al. (1999). Identification of an oxygen responsive enhancer element in the glyceraldehyde-3-phosphate dehydrogenase gene. *Biochim Biophys Acta*, **1447,** 208-18.

Green SL, et al. (2001). p21(Cip1) and p27(Kip1) regulate cell cycle reentry after hypoxic stress but are not necessary for hypoxia-induced arrest. *Mol Cell Biol*, **21,** 1196-206.

Greijer AE, et al. (2005). Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). *J Pathol*, **206,** 291-304.

Groot AJ, et al. (2006). Identification by phage display of single-domain antibody fragments specific for the ODD domain in hypoxia-inducible factor 1alpha. *Lab Invest*, **86,** 345-56.

Gruber G, et al. (2004). Hypoxia-inducible factor 1 alpha in high-risk breast cancer: an independent prognostic parameter? *Breast Cancer Res*, **6,** R191-8.

Gustafsson MV, et al. (2005). Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Dev Cell*, **9,** 617-28.

Hammond EM and Giaccia AJ. (2005). The role of p53 in hypoxia-induced apoptosis. *Biochem Biophys Res Commun*, **331,** 718-25.

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

Hewitson KS, et al. (2002). Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J Biol Chem*, **277,** 26351-5.

Hockel M, et al. (1996). Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res*, **56,** 4509-15.

Hu J, et al. (1998). Hypoxia regulates expression of the endothelin-1 gene through a proximal hypoxia-inducible factor-1 binding site on the antisense strand. *Biochem Biophys Res Commun*, **245,** 894-9.

Hudson CC, et al. (2002). Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin. *Mol Cell Biol*, **22,** 7004-14.

Isaacs JS, et al. (2005). HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. *Cancer Cell*, **8,** 143-53.

Ishikawa H, et al. (2004). Expression of hypoxic-inducible factor 1alpha predicts metastasis-free survival after radiation therapy alone in stage IIIB cervical squamous cell carcinoma. *Int J Radiat Oncol Biol Phys*, **60,** 513-21.

Ivan M, et al. (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science*, **292,** 464-8.

Iyer NV, et al. (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev*, **12,** 149-62.

Jaakkola P, et al. (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science*, **292,** 468-72.

Jeong JW, et al. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. *Cell*, **111,** 709-20.

Jiang BH, et al. (1997). V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. *Cancer Res*, **57,** 5328-35.

Jiang BH, et al. (2001a). Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. *Cell Growth Differ*, **12,** 363-9.

Jiang BH, et al. (1996). Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J Biol Chem*, **271,** 17771-8.

Jiang H, et al. (2001b). The Caenorhabditis elegans hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. *Proc Natl Acad Sci U S A*, **98,** 7916-21.

Jokilehto T, et al. (2006). Overexpression and nuclear translocation of hypoxia-inducible factor prolyl hydroxylase PHD2 in head and neck squamous cell carcinoma is associated with tumor aggressiveness. *Clin Cancer Res*, **12,** 1080-7.

Jones DT and Harris AL. (2006). Identification of novel small-molecule inhibitors of hypoxia-inducible factor-1 transactivation and DNA binding. *Mol Cancer Ther*, **5,** 2193-202.

Jung YJ, et al. (2003). Microtubule disruption utilizes an NFkappa B-dependent pathway to stabilize HIF-1alpha protein. *J Biol Chem*, **278,** 7445-52.

Kaelin WG, Jr. (2007). The von Hippel-Lindau tumor suppressor protein and clear cell renal carcinoma. *Clin Cancer Res*, **13,** 680s-684s.

Kaluz S, et al. (2003). Expression of the hypoxia marker carbonic anhydrase IX is critically dependent on SP1 activity. Identification of a novel type of hypoxia-responsive enhancer. *Cancer Res*, **63,** 917-22.

Kamath RS, et al. (2003). Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. *Nature*, **421,** 231-7.

Katschinski DM, et al. (2002). Heat induction of the unphosphorylated form of hypoxia-inducible factor-1alpha is dependent on heat shock protein-90 activity. *J Biol Chem*, **277,** 9262-7.

Kelly BD, et al. (2003). Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. *Circ Res*, **93,** 1074-81.

Kim JY, et al. (2004). BH3-only protein Noxa is a mediator of hypoxic cell death induced by hypoxia-inducible factor 1alpha. *J Exp Med*, **199,** 113-24.

Kondo K, et al. (2003). Inhibition of HIF2alpha is sufficient to suppress pVHL-defective tumor growth. *PLoS Biol*, **1,** E83.

Kondo K, et al. (2002). Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. *Cancer Cell*, **1,** 237-46.

Koong AC, et al. (1994). Hypoxia causes the activation of nuclear factor kappa B through the phosphorylation of I kappa B alpha on tyrosine residues. *Cancer Res*, **54,** 1425-30.

Kothari S, et al. (2003). BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. *Oncogene*, **22,** 4734-44.

Krishnamachary B, et al. (2006). Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. *Cancer Res*, **66,** 2725-31.

Krishnamurthy P, et al. (2004). The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. *J Biol Chem*, **279,** 24218-25.

Kronblad A, et al. (2006). Hypoxia inducible factor-1alpha is a prognostic marker in premenopausal patients with intermediate to highly differentiated breast cancer but not a predictive marker for tamoxifen response. *Int J Cancer*, **118,** 2609-16.

Kurokawa T, et al. (2003). Overexpression of hypoxia-inducible-factor 1alpha(HIF-1alpha) in oesophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. *Br J Cancer*, **89,** 1042-7.

Kwiatkowski DJ and Manning BD. (2005). Tuberous sclerosis: a GAP at the crossroads of multiple signaling pathways. *Hum Mol Genet*, **14 Spec No. 2,** R251-8.

Land SC and Tee AR. (2007). Hypoxia inducible factor 1alpha is regulated by the mammalian target of rapamycin (mTOR) via an mTOR-signalling motif. *J Biol Chem*.

Lando D, et al. (2002a). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev*, **16,** 1466-71.

Lando D, et al. (2002b). Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science*, **295,** 858-61.

Laughner E, et al. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol Cell Biol*, **21,** 3995-4004.

Lee PJ, et al. (1997). Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. *J Biol Chem*, **272,** 5375-81.

Levine AJ, et al. (2006). The P53 pathway: what questions remain to be explored? *Cell Death Differ*, **13,** 1027-36.

Li J, et al. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science*, **275,** 1943-7.

Li J, et al. (2004). Hypoxia-induced nucleophosmin protects cell death through inhibition of p53. *J Biol Chem*, **279,** 41275-9.

Liao D, et al. (2007). Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. *Cancer Res*, **67,** 563-72.

Liaw D, et al. (1997). Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat Genet*, **16,** 64-7.

Lidgren A, et al. (2005). The expression of hypoxia-inducible factor 1alpha is a favorable independent prognostic factor in renal cell carcinoma. *Clin Cancer Res*, **11,** 1129-35.

Liu MY, et al. (2003). Up-regulation of hypoxia-inducible factor 2alpha in renal cell carcinoma associated with loss of Tsc-2 tumor suppressor gene. *Cancer Res*, **63,** 2675-80.

Liu YL, et al. (2004). Regulation of ferrochelatase gene expression by hypoxia. *Life Sci*, **75,** 2035-43.

Liu YL, et al. (2006). Regulation of the Chemokine Receptor CXCR4 and Metastasis by Hypoxia-inducible Factor in Non Small Cell Lung Cancer Cell Lines. *Cancer Biol Ther*, **5**.

Liu YV, et al. (2007). RACK1 competes with HSP90 for binding to HIF-1alpha and is required for O(2)-independent and HSP90 inhibitor-induced degradation of HIF-1alpha. *Mol Cell*, **25,** 207-17.

Lu S, et al. (2002). Identification of an additional hypoxia responsive element in the glyceraldehyde-3-phosphate dehydrogenase gene promoter. *Biochim Biophys Acta*, **1574,** 152-6.

Lukashev D, et al. (2007). Hypoxia-dependent anti-inflammatory pathways in protection of cancerous tissues. *Cancer Metastasis Rev*, **26,** 273-9.

Luo Y, et al. (2006). Over-expression of hypoxia-inducible factor-1alpha increases the invasive potency of LNCaP cells in vitro. *BJU Int*.

Mahon PC, et al. (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev*, **15,** 2675-86.

Mahoney BP, et al. (2003). Tumor acidity, ion trapping and chemotherapeutics. I. Acid pH affects the distribution of chemotherapeutic agents in vitro. *Biochem Pharmacol*, **66,** 1207-18.

Manning BD and Cantley LC. (2007). AKT/PKB Signaling: Navigating Downstream. *Cell*, **129,** 1261-74.

Masson N, et al. (2001). Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *Embo J*, **20,** 5197-206.

Maxwell PH, et al. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature*, **399,** 271-5.

Mazure NM, et al. (2003). Protein kinases and the hypoxia-inducible factor-1, two switches in angiogenesis. *Curr Pharm Des*, **9,** 531-41.

Mazure NM, et al. (1997). Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. *Blood*, **90,** 3322-31.

Mazure NM, et al. (1996). Oncogenic transformation and hypoxia synergistically act to modulate vascular endothelial growth factor expression. *Cancer Res*, **56,** 3436-40.

McNeill LA, et al. (2002). Hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) catalyses hydroxylation at the beta-carbon of asparagine-803. *Biochem J*, **367,** 571-5.

Metzen E, et al. (2005). Regulation of the prolyl hydroxylase domain protein 2 (phd2/egln-1) gene: identification of a functional hypoxia-responsive element. *Biochem J*, **387,** 711-7.

Minet E, et al. (2000). ERK activation upon hypoxia: involvement in HIF-1 activation. *FEBS Lett*, **468,** 53-8.

Mottet D, et al. (2003). Regulation of hypoxia-inducible factor-1alpha protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3beta pathway in HepG2 cells. *J Biol Chem*, **278,** 31277-85.

Mukhopadhyay CK, et al. (2000). Role of hypoxia-inducible factor-1 in transcriptional activation of ceruloplasmin by iron deficiency. *J Biol Chem*, **275,** 21048-54.

Mukhopadhyay D, et al. (1995). Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. *Nature*, **375,** 577-81.

Mumm JS and Kopan R. (2000). Notch signaling: from the outside in. *Dev Biol*, **228,** 151-65.

Mylonis I, et al. (2006). Identification of MAPK phosphorylation sites and their role in the localization and activity of hypoxia-inducible factor-1alpha. *J Biol Chem*, **281,** 33095-106.

Nakamura H, et al. (2005). TCR engagement increases hypoxia-inducible factor-1 alpha protein synthesis via rapamycin-sensitive pathway under hypoxic conditions in human peripheral T cells. *J Immunol*, **174,** 7592-9.

Nakayama K, et al. (2004). Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia. *Cell*, **117,** 941-52.

Nguyen SV and Claycomb WC. (1999). Hypoxia regulates the expression of the adrenomedullin and HIF-1 genes in cultured HL-1 cardiomyocytes. *Biochem Biophys Res Commun*, **265,** 382-6.

Niizeki H, et al. (2002). Hypoxia enhances the expression of autocrine motility factor and the motility of human pancreatic cancer cells. *Br J Cancer*, **86,** 1914-9.

Nuyten DS and van de Vijver MJ. (2006). Gene expression signatures to predict the development of metastasis in breast cancer. *Breast Dis*, **26,** 149-56.

Ohh M, et al. (2000). Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat Cell Biol*, **2,** 423-7.

Olenyuk BZ, et al. (2004). Inhibition of vascular endothelial growth factor with a sequence-specific hypoxia response element antagonist. *Proc Natl Acad Sci U S A*, **101,** 16768-73.

Osinsky SP, et al. (2005). Hypoxia level and matrix metalloproteinases-2 and -9 activity in Lewis lung carcinoma: correlation with metastasis. *Exp Oncol*, **27,** 202-5.

Padilla PA, et al. (2002). Dephosphorylation of cell cycle-regulated proteins correlates with anoxia-induced suspended animation in Caenorhabditis elegans. *Mol Biol Cell*, **13,** 1473-83.

Pan Y, et al. (2004). p53 cannot be induced by hypoxia alone but responds to the hypoxic microenvironment. *Oncogene*, **23,** 4975-83.

Patiar S and Harris AL. (2006). Role of hypoxia-inducible factor-1alpha as a cancer therapy target. *Endocr Relat Cancer*, **13 Suppl 1,** S61-75.

Peinado H, et al. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer*, **7,** 415-28.

Pennacchietti S, et al. (2003). Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell*, **3,** 347-61.

Pepper MS, et al. (1991). Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. *Biochem Biophys Res Commun*, **181,** 902-6.

Pescador N, et al. (2005). Identification of a functional hypoxia-responsive element that regulates the expression of the egl nine homologue 3 (egln3/phd3) gene. *Biochem J*, **390,** 189-97.

Piret JP, et al. (2005). Hypoxia-inducible factor-1-dependent overexpression of myeloid cell factor-1 protects hypoxic cells against tert-butyl hydroperoxide-induced apoptosis. *J Biol Chem*, **280,** 9336-44.

Pollard PJ, et al. (2007). Targeted inactivation of fh1 causes proliferative renal cyst development and activation of the hypoxia pathway. *Cancer Cell*, **11,** 311-9.

Pore N, et al. (2006). Akt1 activation can augment hypoxia-inducible factor-1alpha expression by increasing protein translation through a mammalian target of rapamycin-independent pathway. *Mol Cancer Res*, **4,** 471-9.

Potter C and Harris AL. (2004). Hypoxia inducible carbonic anhydrase IX, marker of tumour hypoxia, survival pathway and therapy target. *Cell Cycle*, **3,** 164-7.

Pouyssegur J and Mechta-Grigoriou F. (2006). Redox regulation of the hypoxia-inducible factor. *Biol Chem*, **387,** 1337-46.

Powell-Coffman JA, et al. (1998). Caenorhabditis elegans orthologs of the aryl hydrocarbon receptor and its heterodimerization partner the aryl hydrocarbon receptor nuclear translocator. *Proc Natl Acad Sci U S A*, **95,** 2844-9.

Rapisarda A, et al. (2002). Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. *Cancer Res*, **62,** 4316-24.

Raval RR, et al. (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,** 5675-86.

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

Rayet B and Gelinas C. (1999). Aberrant rel/nfkb genes and activity in human cancer. *Oncogene*, **18,** 6938-47.

Richard DE, et al. (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,** 32631-7.

Roe JS, et al. (2006). p53 stabilization and transactivation by a von Hippel-Lindau protein. *Mol Cell*, **22,** 395-405.

Rolfs A, et al. (1997). Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. *J Biol Chem*, **272,** 20055-62.

Ryan HE, et al. (1998). HIF-1 alpha is required for solid tumor formation and embryonic vascularization. *Embo J*, **17,** 3005-15.

Ryan HE, et al. (2000). Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. *Cancer Res*, **60,** 4010-5.

Sanchez-Puig N, et al. (2005). Binding of natively unfolded HIF-1alpha ODD domain to p53. *Mol Cell*, **17,** 11-21.

Schindl M, et al. (2002). Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer. *Clin Cancer Res*, **8,** 1831-7.

Schoppmann SF, et al. (2006). Hypoxia inducible factor-1alpha correlates with VEGF-C expression and lymphangiogenesis in breast cancer. *Breast Cancer Res Treat*, **99,** 135-41.

Schwickert G, et al. (1995). Correlation of high lactate levels in human cervical cancer with incidence of metastasis. *Cancer Res*, **55,** 4757-9.

Semenza GL. (2000a). HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol*, **88,** 1474-80.

Semenza GL. (2000b). Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. *Crit Rev Biochem Mol Biol*, **35,** 71-103.

Semenza GL. (2003). Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*, **3,** 721-32.

Semenza GL, et al. (1996). Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem*, **271,** 32529-37.

Semenza GL, et al. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. *Proc Natl Acad Sci U S A*, **88,** 5680-4.

Semenza GL, et al. (1994). Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem*, **269,** 23757-63.

Semenza GL and Wang GL. (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,** 5447-54.

Shen C, et al. (2005). Roles of the HIF-1 Hypoxia-inducible Factor during Hypoxia Response in Caenorhabditis elegans. *J Biol Chem*, **280,** 20580-8.

Shen C and Powell-Coffman JA. (2003). Genetic analysis of hypoxia signaling and response in C elegans. *Ann N Y Acad Sci*, **995,** 191-9.

Shen C, et al. (2006). The Caenorhabditis elegans rhy-1 Gene Inhibits HIF-1 Hypoxia-Inducible Factor Activity in a Negative Feedback Loop That Does Not Include vhl-1. *Genetics*, **174,** 1205-14.

Skinner HD, et al. (2004). Vascular endothelial growth factor transcriptional activation is mediated by hypoxia-inducible factor 1alpha, HDM2, and p70S6K1 in response to phosphatidylinositol 3-kinase/AKT signaling. *J Biol Chem*, **279,** 45643-51.

Sodhi A, et al. (2001). MAPK and Akt act cooperatively but independently on hypoxia inducible factor-1alpha in rasV12 upregulation of VEGF. *Biochem Biophys Res Commun*, **287,** 292-300.

Sumiyoshi Y, et al. (2006). Overexpression of hypoxia-inducible factor 1alpha and p53 is a marker for an unfavorable prognosis in gastric cancer. *Clin Cancer Res*, **12,** 5112-7.

Suzuki H, et al. (2001). Dephosphorylated hypoxia-inducible factor 1alpha as a mediator of p53-dependent apoptosis during hypoxia. *Oncogene*, **20,** 5779-88.

Swanson HI. (2002). DNA binding and protein interactions of the AHR/ARNT heterodimer that facilitate gene activation. *Chem Biol Interact*, **141,** 63-76.

Tacchini L, et al. (1999). Transferrin receptor induction by hypoxia. HIF-1-mediated transcriptional activation and cell-specific post-transcriptional regulation. *J Biol Chem*, **274,** 24142-6.

Talks KL, et al. (2000). The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. *Am J Pathol*, **157,** 411-21.

Tang TT and Lasky LA. (2003). The forkhead transcription factor FOXO4 induces the down-regulation of hypoxia-inducible factor 1 alpha by a von Hippel-Lindau protein-independent mechanism. *J Biol Chem*, **278,** 30125-35.

Trastour C, et al. (2007). HIF-1alpha and CA IX staining in invasive breast carcinomas: prognosis and treatment outcome. *Int J Cancer*, **120,** 1451-8.

Treinin M, et al. (2003). HIF-1 is required for heat acclimation in the nematode Caenorhabditis elegans. *Physiol Genomics*, **14,** 17-24.

Treins C, et al. (2002). Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. *J Biol Chem*, **277,** 27975-81.

Treins C, et al. (2006). AMPK activation inhibits the expression of HIF-1alpha induced by insulin and IGF-1. *Biochem Biophys Res Commun*, **342,** 1197-202.

Trent C, et al. (1983). Egg-laying defective mutants of the nematode Caenorhabditis elegans. *Genetics*, **104,** 619-47.

van de Sluis B, et al. (2007). Increased activity of hypoxia-inducible factor 1 is associated with early embryonic lethality in Commd1 null mice. *Mol Cell Biol*, **27,** 4142-56.

van Es JH, et al. (2005). Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature*, **435,** 959-63.

van Haaften G, et al. (2004). Gene interactions in the DNA damage-response pathway identified by genome-wide RNA-interference analysis of synthetic lethality. *Proc Natl Acad Sci U S A*, **101,** 12992-6.

Vaupel P, et al. (1988). Evaluation of oxygen diffusion distances in human breast cancer using cell line specific in vivo data: role of various pathogenetic mechanisms in the development of tumor hypoxia. *Adv Exp Med Biol*, **222,** 719-26.

Vaupel P, et al. (2004). Tumor hypoxia and malignant progression. *Methods Enzymol*, **381,** 335-54.

Vaupel P, et al. (1991). Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O2 tension measurements. *Cancer Res*, **51,** 3316-22.

Vleugel MM, et al. (2005). Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in invasive breast cancer. *J Clin Pathol*, **58,** 172-7.

Vogelstein B and Kinzler KW. (2004). Cancer genes and the pathways they control. *Nat Med*, **10,** 789-99.

Volm M and Koomagi R. (2000). Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer. *Anticancer Res*, **20,** 1527-33.

Walenta S, et al. (1997). Correlation of high lactate levels in head and neck tumors with incidence of metastasis. *Am J Pathol*, **150,** 409-15.

Walenta S, et al. (2004). Lactate in solid malignant tumors: potential basis of a metabolic classification in clinical oncology. *Curr Med Chem*, **11,** 2195-204.

Wang GL, et al. (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,** 5510-4.

Wang GL and Semenza GL. (1993a). Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. *J Biol Chem*, **268,** 21513-8.

Wang GL and Semenza GL. (1993b). Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood*, **82,** 3610-5.

Warburg O. (1930). Constable and Co., Ltd: London.

Wenger RH. (2000). Mammalian oxygen sensing, signalling and gene regulation. *J Exp Biol*, **203,** 1253-63.

Wenger RH, et al. (2005). Integration of oxygen signaling at the consensus HRE. *Sci STKE*, **2005,** re12.

Woelfle U, et al. (2003). Molecular signature associated with bone marrow micrometastasis in human breast cancer. *Cancer Res*, **63,** 5679-84.

Wood SM, et al. (1996). The role of the aryl hydrocarbon receptor nuclear translocator (ARNT) in hypoxic induction of gene expression. Studies in ARNT-deficient cells. *J Biol Chem*, **271,** 15117-23.

Yamakawa M, et al. (2003). Hypoxia-inducible factor-1 mediates activation of cultured vascular endothelial cells by inducing multiple angiogenic factors. *Circ Res*, **93,** 664-73.

Yan Q, et al. (2007). The hypoxia-inducible factor 2alpha N-terminal and C-terminal transactivation domains cooperate to promote renal tumorigenesis in vivo. *Mol Cell Biol*, **27,** 2092-102.

Yatabe N, et al. (2004). HIF-1-mediated activation of telomerase in cervical cancer cells. *Oncogene*, **23,** 3708-15.

Yoshimura H, et al. (2004). Prognostic impact of hypoxia-inducible factors 1alpha and 2alpha in colorectal cancer patients: correlation with tumor angiogenesis and cyclooxygenase-2 expression. *Clin Cancer Res*, **10,** 8554-60.

Yu F, et al. (2001). HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. *Proc Natl Acad Sci U S A*, **98,** 9630-5.

Zagzag D, et al. (2005). Stromal cell-derived factor-1alpha and CXCR4 expression in hemangioblastoma and clear cell-renal cell carcinoma: von Hippel-Lindau loss-of-function induces expression of a ligand and its receptor. *Cancer Res*, **65,** 6178-88.

Zhong H, et al. (2000). Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res*, **60,** 1541-5.

Zhong H, et al. (1999). Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res*, **59,** 5830-5.

Zhou G, et al. (2004a). Ser/Thr protein phosphatase 5 inactivates hypoxia-induced activation of an apoptosis signal-regulating kinase 1/MKK-4/JNK signaling cascade. *J Biol Chem*, **279,** 46595-605.

Zhou J, et al. (2003). Tumor necrosis factor-alpha causes accumulation of a ubiquitinated form of hypoxia inducible factor-1alpha through a nuclear factor-kappaB-dependent pathway. *Mol Biol Cell*, **14,** 2216-25.

Zhou J, et al. (2004b). PI3K/Akt is required for heat shock proteins to protect hypoxia-inducible factor 1alpha from pVHL-independent degradation. *J Biol Chem*, **279,** 13506-13.

Zundel W, et al. (2000). Loss of PTEN facilitates HIF-1-mediated gene expression. *Genes Dev*, **14,** 391-6.

Chapter 2

Review: Hypoxic Regulation of Metastasis via Hypoxia Inducible Factors

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Abstract

Metastases formation is a major factor in disease progression and accounts for the majority of cancer deaths. The molecular mechanisms controlling invasion, dissemination to blood or lymphatic systems and spread of tumor cells to distant organs are still poorly understood. Recent observations indicate that the metastatic phenotype may already be present during the angiogenic switch of tumors. Intratumoral hypoxia correlates with poor prognosis and enhanced metastases formation. The Hypoxia Inducible Factors (HIFs) are key molecules in the hypoxic response and play critical roles during tumor cell expansion by regulating energy metabolism and the induction of angiogenesis. Increasing evidence implicates HIF function in metastatic cell characteristics, like epithelial to mesenchymal transition and cell detachment, invasion and seeding. Here, we review the link between tumor cell hypoxia and the acquisition of metastatic behavior. We hypothesize that polyclonal tumor selection by hypoxia enhances metastatic capacity by transcriptional control of key regulators of metastasis. This polyclonal hypoxic gene profile potentially develops into a metastatic profile, positioning hypoxia as a central phenomenon in metastasis formation.

Introduction

Outgrowing metastases are the major cause of human cancer mortality. The acquisition of full metastatic capacity of cancer cells requires several sequential rate-limiting steps: i. cancer cells must detach from the primary tumor, ii. invade into the surrounding stroma, iii. intravasate into the blood or lymphatic vessels, iv. spread into the capillary bed of distant organs, and v. extravasate and colonize into the recipient organs (Gupta & Massague, 2006). It is well established that tumor cell specific alterations (cell autonomous) are not sufficient to drive the metastatic cascade but that continuous interaction with the microenvironment (non-cell autonomous) is needed to permit tumor cell survival. One of crucial rate-limiting events during human tumor growth is encountered when tumors outgrow the pre-existing vasculature leading to hypoxia. Intratumoral hypoxia is a hallmark of solid cancer. Hypoxic tumors have a higher tendency to metastasize and are clinically characterized by therapy resistance and a poor prognosis (Bos et al., 2003; Dales et al., 2005). Tumor cell hypoxia affects various key steps during the metastatic cascade and selects for metastatic variants (Vaupel et al., 2004). Central components in the cellular hypoxia response are the Hypoxia Inducible Factors (HIFs). Here, we review how hypoxia directly affects gene-expression programs driving metastatic spread. We summarize the increasing evidence implicating HIFs in several key steps in the metastatic cancer cascade. Finally, we formulate a concept of how the early hypoxic microenvironment drives the selection of polyclonal metastatic cells contributing to more aggressive tumor behavior.

Hypoxia and HIF

In mammalian cells, the HIF transcriptional complex is a key regulator of the local and systemic responses to hypoxia that occur during normal development and (patho-) physiological processes (Semenza, 2003). Intratumoral hypoxia, defined roughly when intercapillary distances exceed 140 micron, correlates with poor prognosis and has become an important clinical parameter (Hockel et al., 1999; Movsas et al., 2000). Cancer cells adapt to hypoxic environments by converting to glycolytic energy metabolism, induction of angiogenesis and cellular survival programs, and this acute cellular response to hypoxia is mediated by the HIF basic helix-loop-helix (bHLH) transcription factor family (Pouyssegur et al., 2006). HIF is a heterodimeric protein complex composed of a constitutively expressed bèta subunit, HIF-1β or aryl hydrocarbon receptor

nuclear translocator (ARNT), and an oxygen-regulated alpha subunit both of which belong to the bHLH-PAS (Per, ARNT, SIM) protein family (Wang et al., 1995). Mammalian cells have three HIFα isoforms. HIF-1α and HIF-2α have been studied best and will be considered here. HIFα stability is tightly regulated at the posttranslational level by oxygen concentration. Under normal oxygen tension (normoxia), HIFα is constitutively hydroxylated and acetylated at multiple proline and lysine residues in the Oxygen Dependent Degradation Domain (ODDD) and on asparagine residues in the c-terminal transactivation domain (N832). These modifications enhance binding of HIFα to the Von Hippel-Lindau (VHL) protein, the recognition component of an E3 ubiquitin ligase complex and regulate assembly of HIFα/-β heterodimers with transcriptional co-activators. Prolyl hydroxylation marks HIFα for ubiquitination and proteosomal degradation (for a review see (Maxwell et al., 2001)). Thus, under normoxia HIFα protein is continuously degraded. Under hypoxic conditions the activity of prolyl hydroxylases is attenuated since they require ferrous-ions and O_2 for their function, which leads to HIFα stabilization. After nuclear translocation, HIFα/ARNT heterodimers regulate transcription by binding hypoxia responsive elements (HRE) (Pugh et al., 1991).

Oncogenic regulation of HIF

HIF protein stability and expression can also be increased by tumor specific genetic alterations in oncogenes or tumor suppressor genes. For example, in the Von Hippel Lindau hereditary cancer syndrome the loss of VHL leads to attenuated proteosomal degradation of HIFα and predisposes to a variety of highly vascularized malignant and benign neoplasms (Kaelin, 2002; Maher, 2004). Also in sporadic cancers deregulation of oncogenes and tumor suppressor genes affects HIF signaling. For instance, the phosphatidylinositol-3-kinase (PI 3-kinase)/Akt kinase pathway directly impacts on HIF stability and HER-2/*neu* overexpression or the tuberous sclerosis complex gene TSC-2 (Tuberin) loss induce HIF-1α stabilization via a mammalian target of rapamycin (mTOR)-dependent pathway (Semenza, 2003). Furthermore, HIF-1α stabilization depends on the TP53 tumor suppressor protein (Ravi et al., 2000). Other pathways implicated in HIF regulations are the mitogen-activated protein kinase (MAPK), nuclear factor-kappaB (NF-kB) and Notch signaling pathways (Gustafsson et al., 2005; Mazure et al., 2003; Zhou et al., 2003).

Clinical Significance of HIF

Direct oxygen measurements into tumours has revealed that areas of necrosis are surrounded by poor oxygenated areas (Vaupel et al., 1991). In those areas hypoxia-induced nuclear HIF-1α protein can be readily detected by immunohistochemistry (Figure 1). Overexpression of HIF-1α is associated with human cancer progression in epithelial cancers of the head and neck, cervix, colon, breast and lung (for a review see (Vaupel, 2004)). In breast cancer, HIF-1α expression is an independent predictor of poor clinical outcome, with a greater predictive value then other hypoxic markers like carbonic anhydrase IX (CAIX) (Bos et al., 2003; Trastour et al., 2007). HIF-1α expression correlates with a therapy resistance and primary tumor HIF-1α overexpression has been shown to predict presence of bone marrow metastases in breast cancer patients (Trastour et al., 2007; Woelfle et al., 2003). Moreover, HIF-1α is not only expressed in primary tumors, but also in metastatic lesions and HIF-1α expression predicts early relapse (Dales et al., 2005; Zhong et al., 1999). Gene expression profiling has revealed a unique set of transcriptional targets in hypoxic breast cancers (Chi et al., 2006). These clinical studies suggest an important role for HIF in disease progression. We will now discuss the molecular evidence supporting this hypothesis.

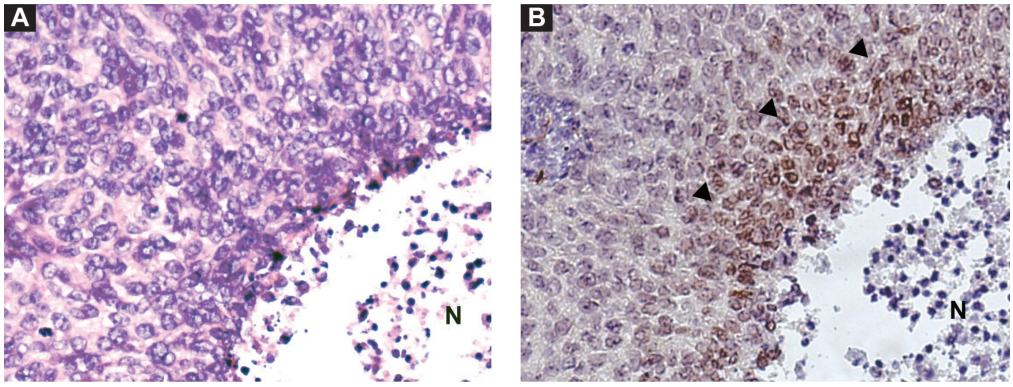

Figure 1.
Perinecrotic staining of HIF-1α. Photograph show a 40x magnification of an endometriod endometrial carcinoma with the necrotic area indicated by N. A) shows a haematoxilin-eosin staining. B) shows immunohistochemical staining for HIF-1α, indicated by black arrows. Note the perinecrotic nature of the staining.

Cancer Cell Detachment and EMT

Cancer cell detachment is the initial event in metastases formation of carcinomas and is marked by the loss of cell adhesion molecules and the acquisition of mesenchymal phenotypes (Christofori, 2006). This process is termed epithelial to mesenchymal transition (EMT) although this process is never complete and some epithelial characteristics remain (Christiansen & Rajasekaran, 2006). A hallmark of EMT is the loss of E-Cadherin, an adhesion molecule frequently lost in human cancer and causal in tumor formation and metastasis (Derksen et al., 2006; Perl et al., 1998). Loss of E-cadherin enhances cancer cell motility and resistance to anoikis (Derksen et al., 2006; Vleminckx et al., 1991). EMT is a crucial process during normal development of multicellular organisms (Huber et al., 2005). EMT is controlled by AKT-, Wnt-, Notch-, and Hedgehog-signaling pathways and can be induced by a number of growth factors, like transforming growth factor β (TGF-β), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and fibroblast growth factors (FGF) (Huber et al., 2005). Several transcription factors critical for mesoderm formation, like SNAI1 (SNAIL), ZEB and certain bHLH factors, are known to induce EMT through repression of E-Cadherin and induction of mesenchymal gene expression (Peinado et al., 2007). Recent findings implicate the HIF pathway in EMT induction, acting through these mesodermal fate genes. In VHL-deficient renal cell carcinomas, both HIF-1α and HIF-2α are involved in the downregulation of E-Cadherin expression (Esteban et al., 2006; Evans et al., 2006; Krishnamachary et al., 2006). One of the EMT regulating factors regulated by HIF signaling is SNAI1, a zinc-finger transcription factor indispensable for mesoderm development (Esteban et al., 2006; Evans et al., 2006; Imai et al., 2003). In addition to SNAI1, other known repressors of E-Cadherin, like TCF3 (E12/E47), ZEB1 (yEF1/ ZFHX1A), and ZEB2 (SIP1/ ZFHX1B), are upregulated in VHL-deficient renal cell carcinomas (Esteban et al., 2006; Krishnamachary et al., 2006). Using genetic screens in *C. elegans*, we recently identified ceTWIST (Helix-loop-helix-8 or HLH-8) as a critical downstream target in hypoxia sensing (Gort et al., 2007). *TWIST1* is directly regulated by HIF-2α under hypoxia. *TWIST1*, the Saethre-Chotzen syndrome susceptibility gene, is a master regulator of mesodermal fate and a bHLH repressor that like SNAI1, binds E-box containing promoters. In invasive lobular breast cancer, TWIST1 contributes to metastasis by promoting EMT through down regulation of E-cadherin and high levels of TWIST1 correlates with high disease recurrence in breast and ovarian cancer (Hosono et al., 2007; Martin et al., 2005; Yang et al., 2004). Whereas SNAI1-mediated repression

of E-cadherin indirectly leads to activation of mesenchymal markers such as Vimentin (Batlle et al., 2000; Cano et al., 2000), TWIST1 can directly activate N-Cadherin expression, a marker for EMT progression (Alexander et al., 2006). Thus both SNAI1 and TWIST1 appear to orchestrate the Cadherin switch during EMT. A central role for hypoxia and HIF-dependent regulation EMT provides an attractive model in which hypoxia in early tumorigenesis triggers cancer cell detachment and invasion.

Extracellular matrix modulation and invasion

Strong evidence for a role of hypoxia and HIF as regulators of ECM degradation and tumor cell migration comes from the analysis of Erler and collegues who identified the ECM crosslinking enzyme lysyl oxidase (LOX) as a direct HIF-1α target (Erler et al., 2006). LOX is a copper-dependent amine oxidase that plays a critical role in the biogenesis of connective tissue matrices by crosslinking elastin and collagen and has been shown to enhance tumor cell migration (Payne et al., 2005). LOX expression in hypoxic cancers correlates with poor patient survival and inhibition of LOX activity in a mouse model of metastatic breast cancer significantly reduced liver/bone metastases. (Erler et al., 2006). Although the mechanism whereby LOX increases metastatic behavior is unresolved it may directly affect motility by regulating FAK-dependent adhesion to collagen. These data warrant the development of LOX inhibitors as anti-metastatic therapeutics. Interestingly, LOX homologues LOXL2 and LOXL3 interact and cooperate with SNAI1 in the downregulation of E-cadherin in carcinoma progression (Peinado et al., 2005). Regulation of matrix metalloproteinases (MMPs), urokinase type plasminogen activator (uPAR) and Cathepsin D by hypoxia may provide additional means for hypoxic ECM modulation (Semenza, 2003).

Cell motility and invasion

Hypoxic tumor cells adopt a multitude of strategies in addition to neoangiogenesis to overcome oxygen deprivation by inducing cell motility to colonize oxygen proficient areas. This process is thought to occur independent of the angiogenic switch and has been termed the “invasive switch” (Pennacchietti et al., 2003). Scatter factor or hepatocyte growth factor (HGF) is a pleiotropic cytokine which stimulates proliferation and invasion through its receptor, the

tyrosine kinase proto-oncogene c-Met (Furge et al., 2000). c-Met is frequently overexpressed or mutated in human cancers (Birchmeier et al., 2003). Hypoxia in a HIF-dependent manner directly promotes invasion by inducing c-Met transcription and sensitizing cells to HGF stimulation. c-Met is required and sufficient for hypoxia-induced invasive growth (Eckerich et al., 2007; Hara et al., 2006; Pennacchietti et al., 2003). Thus, hypoxia unleashes a positive feedback loop between HIF and c-Met that fuels invasive growth. c-Met-induced motility is also enhanced by hypoxic modulation of cell surface integrin signaling. Integrins are adhesion receptors with bidirectional signaling between plasma membrane and ECM, that play essential roles in motility and migration (Danen & Yamada, 2001). MG-63 human osteosarcoma cells exposed to the hypoxia-mimicking agent $CoCl_2$ upregulate both integrin α5 (the fibronectin receptor) and integrin α2 (the alpha chain of the collagen receptor), resulting in increased adhesion (Indovina et al., 2006). Upregulation of the integrin α5β1 dimer via HIF-1α might explain HER-2/*neu*-dependent fibronectin ligation, resulting in increased survival of mammary adenocarcinoma cells, since HER-2/*neu* can regulate HIF expression under normoxic conditions (Laughner et al., 2001; Spangenberg et al., 2006). Conversely, integrin-dependent signaling affects HIF activity itself via integrin-linked kinase (ILK)-mediated AKT activation (Tan et al., 2004). Fibroblasts lacking HIF-1α fail to upregulate the GTPase *RhoA*, a core transducer of integrin signaling critical in stress fiber formation, during hypoxia (Greijer et al., 2005). Thus hypoxia and HIF enhance motility by acting at multiple levels to facilitate migration towards nutrient and oxygen rich areas within oxygen-deprived tissues.

Acidosis and invasion

Cancer cells starved for oxygen and nutrients switch to anaerobic glycolysis (the Pasteur effect) as an alternative energy source in a process that is predominantly HIF dependent (Gatenby & Gillies, 2004). Anaerobic respiration produces lactic acid and adenosine triphosphate (ATP) from glucose, which results in acidosis. Acidosis is a general feature of human tumors and has been shown to promote tumor cell invasion by destruction of adjacent non-cancerous tissue and ECM degradation (Gatenby & Gillies, 2004). Importantly, tumor-induced acidosis can negate the effect of anti-cancer therapeutics that only function under physiological pH (Mahoney et al., 2003). To prevent intracellular acidosis, HIF induces the transcription of carbonic anhydrase XI (CAIX) that leads to increased HCO_3^- uptake, thereby neutralizing the intracellular

pH albeit at the expense of extracellular acidosis, which is further increased by the H^+ ions expelled out of the cell by proton pumps (Pouyssegur et al., 2006). In addition, HIF-1α induces the expression of the glycolytic enzyme phosphoglucose isomerase (PGI or autocrine motility factor (AMF)), a major cell motility stimulating factor (Funasaka et al., 2005; Niizeki et al., 2002). Hypoxia-induced cell motility can be inhibited by PGI inhibition. Clearly, the numerous metabolic changes induced by hypoxia, in part dependent on HIF, can promote metastatic behavior. This has led to the concept that combination therapies targeting both angiogenesis as well as intracellular (pH) metabolism/ regulation may enhance therapeutic efficiency in cancer (Pouyssegur et al., 2006).

Angiogenesis and intravasation

The production of pro-angiogenic factors by hypoxic (cancer) cells that induce endothelial cell proliferation and vessel sprouting is perhaps the best studied function of HIF. The"angiogenic switch" is a hallmark of human solid cancer progression (Carmeliet et al., 1998; Ferrara, 2002). In 1971, it was already proposed that the capacity of tumor cells to induce angiogenesis promotes tumor cell progression and that anti-angiogenic therapy may improve clinical outcome (Folkman, 1971). Key players in this process are vascular endothelial growth factors (VEGF), and VEGF receptors 1 (VEGFR1 or Flt-1), which are directly regulated by HIF (Forsythe et al., 1996), (Gerber et al., 1997). Significantly, VEGF-A activation was found to be crucial for tumor angiogenesis in a mouse model for multistage pancreatic cancer (Inoue et al., 2002). The VEGF gradient secreted by tumor cells induces sprouting of endothelial cells, which express VEGFR1 and VEGFR2 (Carmeliet, 2005). Loss of HIF-1α in endothelial cells (EC) impairs hypoxia-induced angiogenesis via a VEGF-VEGFR autocrine loop, showing that hypoxia also affects EC function directly, perhaps by impaired VEGFR expression (Tang et al., 2004). In addition to VEGF, other angiogenic factors are regulated by hypoxia, like angiopoietin-1, -2 and -4 (ANGPT1, -2 and -4), placental growth factor, and platelet-derived growth factor-B (Kelly et al., 2003; Yamakawa et al., 2003). Altogether, hypoxia-induced angiogenesis is emerging as an important mechanism controlling metastatic capacity by recruitment of blood vessels and lymphatic circulation. This has already resulted in therapeutic strategies, using anti-angiogenic agents like VEGF receptor blocking agents (e.g. Bevacizumab or Avastin®)(Gordon et al., 2001). At face value, anti-angiogenic treatment may also have tumor promoting effects

by inducing hypoxia and selection of metastatic variants that use alternative strategies (i.e migration) to overcome nutrient deficiency. Paradoxically pro-angiogenic treatment of tumors by activating the Notch-Delta signaling pathway may also provide therapeutic benefit (Thurston et al., 2007). Delta-like 4 Notch ligand (DLL4) signaling through Notch1 and Notch4 receptors is critical for embryonic vascular development (Gale et al., 2004; Krebs et al., 2004). VEGF produced by both hypoxic tumor cells and endothelial cells upregulates DLL4 and Notch signaling in normal and tumor endothelial cells (Diez et al., 2007; Hellstrom et al., 2007). Blocking DLL4/Notch signaling using neutralizing antibodies stimulates non-productive angiogenic sprouting and branching in tumors induced by VEGF and leads to increased tumor hypoxia and decreased tumor growth (Noguera-Troise et al., 2006; Ridgway et al., 2006). Thus, a delicate balance between VEGF and DLL4/Notch signaling is required for proper (tumor) angiogenesis. Disruption of this signaling cascade results in a decrease of functional angiogenesis (VEGF) or increase in non-functional angiogenesis (DLL4). Interestingly, anti-DLL4 blocking strategies also proved effective in tumors resistant to anti-VEGF treatment, providing rationale for new combination therapies targeting both DLL4 and VEGF. The recently discovered link between HIF and Notch signaling might play an important role in controlling this balance (Gustafsson et al., 2005).

Survival

Cancer cells continuously enter the circulation yet only few seed and produce deadly metastatic tumors. Generally, loss of interaction with the environment triggers apoptosis (anoikis). Cancer cells can escape anoikis by repression of apoptosis. For instance, overexpression of the neurotrophin receptor TrkB protects from cell death induced by cell-matrix dissociation (Douma et al., 2004). TrkB expression can be up regulated directly by HIF-1, but not HIF-2, under hypoxic conditions (Martens et al., 2007). Furthermore, TrkB can regulate VEGF expression via HIF-1 (Nakamura et al., 2006). In addition, c-Met signaling confers resistance to anoikis and supports anchorage independence (Longati et al., 1996). Furthermore, SNAI1/2 and TWIST1 act on anti-apoptotic and prosurvival pathways by antagonizing p53- and c-Myc-induced apoptosis, respectively (Maestro et al., 1999; Wu et al., 2005). Direct evidence for a role of hypoxia in resistance to anoikis is lacking, but hypoxia does increase metastatic potential by promoting cell survival in metastatic colonies (Greijer & van der Wall, 2004; Zhang & Hill, 2007).

Seeding

Secondary tumor formation follows arrest and seeding. Cells arrest in narrow capillary beds of the organ vasculature which is an active process of cell-cell/cell-matrix interaction, involving among others, the G-protein-coupled CXC chemokine receptor 4 (CXCR4) and the CXCR4 ligand stromal cell-derived factor-1α (SDF-1α or CXCL12), and integrin-fibronectin interaction (Gupta & Massague, 2006). Expression of CXCR4 in lung-, breast-, ovarian-, renal-, and prostate cancer has been implicated in increasing metastatic potential and expression of SDF-1α is found in target tissue of metastases (Muller et al., 2001). Staller et al. showed for the first time that CXCR4 expression is regulated by hypoxia in a HIF-2α dependent manner (Staller et al., 2003). In addition, SDF-1 is also upregulated in VHL deficient cells, suggesting an autocrine pathway (Zagzag et al., 2005). In non-small cell lung cancer cells, activation of the epidermal growth factor receptor also results in up regulation of CXCR4, which was dependent on PI 3kinase/AKT/mTOR-mediated activation of HIF-1α (Liu et al., 2006; Phillips et al., 2005). In addition, HIF-mediated induction of SDF-1α in endothelial cells functions to recruit circulating CXCR4-positive tumor cells or endothelial progenitor cells (EPC) to ischemic tissue (Ceradini et al., 2004; Zagzag et al., 2005). This provides three strategies by which the CXCR4-SDF-1 interaction influences cancer cell seeding; i. hypoxic tumor cells express CXCR4 and seed after SDF-1 recognition in distant non-hypoxic tissue, ii. hypoxic tumor cells express SDF-1 which might recruit CXCR4 expressing cells by chemotaxis, and iii. hypoxic cancer cells expressing both CXCR4 and SDF-1 establish an autocrine signaling pathway, which results cancer cell autonomy. These are interesting findings and indicate that disruption of the CXCR4-SDF axis may be exploited to target hypoxic tumor seeding, using blocking antibodies (Gonzalo et al., 2000).

Polyclonal selection of metastasis by hypoxia

Apart from the effects of oxygen deprivation on genomic stability and environmental selection, resulting in progression of more aggressive malignancy, hypoxia induces a gene expression program, which facilitates metastatic spread. Molecules involved in angiogenesis, cell detachment, cell motility, invasiveness and chemotaxis are under direct control of the hypoxia-regulated HIF pathway (Figure 2).

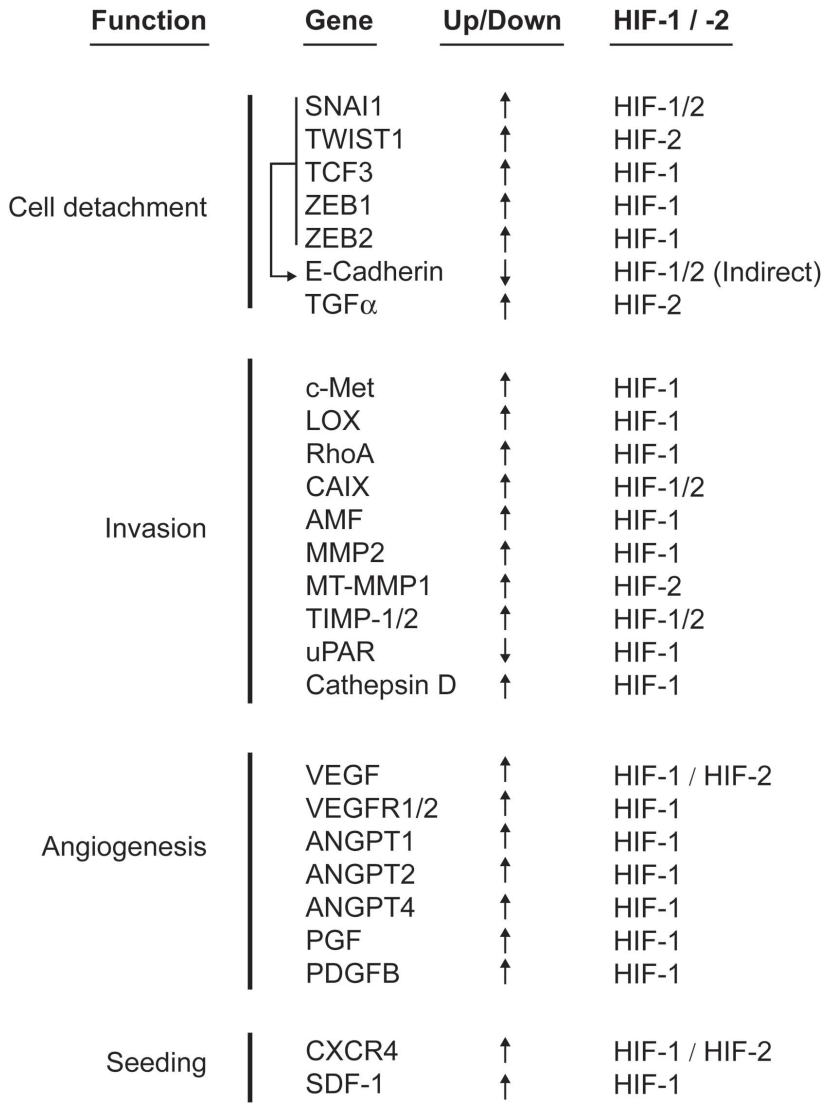

Figure 2.
Hypoxic regulation of genes involved in metastasis. Genes are divided by functional implication in metastasis. Furthermore, up or down regulation under hypoxia and involvement of HIF transcription factors is indicated.

The long held paradigm and working model that continuous selection of rare spontaneous variants within cancers emerge and drive tumor formation and progression is under scrutiny (Nowell, 1971). Fidler and colleagues were among the first to demonstrate that B16 melanoma cells have an intrinsic cellular heterogeneity with respect to subpopulations of cancer cells with different metastatic capacity (Fidler & Kripke, 1977; Poste et al., 1981). This has recently

been validated by Minn et al. who observed that pre-existing subpopulations of MDA-MB-231 breast cancer cells determine bone or lung metastasis (Minn et al., 2005). Importantly, this polyclonal heterogeneity stabilizes the metastatic capacity of clonal subpopulations within tumors suggesting that some form of cooperative interaction exists between subclones that determines the frequency of metastases (Fidler & Kripke, 1977). Support for this has been obtained from the discovery that primary breast cancers already harbor the metastasis (poor-prognosis) gene expression signature, suggesting that most primary tumor cells have the capacity to metastasize rather than evolve through the emergence of rare metastatic variants (van 't Veer et al., 2002; van de Vijver et al., 2002). Remarkably, this signature seems applicable to epithelial tumors in general, suggesting this is in part an intrinsic biological property of cancer cell populations (Ramaswamy et al., 2003). In retrospect it seems obvious to propose that this polyclonality and heterogeneity is driven in part by the presence of cancer stem cells. Importantly, a significant fraction of signature genes are stromal-derived suggesting a critical role for tumor-host microenvironment in driving tumor progression and metastasis (Liotta & Kohn, 2001). This is underscored by the analysis that tumor-derived stromal expression signatures might provide equally strong clinical classifiers as tumor cell signatures (Roepman et al., 2006).

We would like to extend the polyclonal selection working model by proposing that intratumoral hypoxia is a driving force behind the polyclonal expansion of metastatic subpopulations within primary tumors (Figure 3). This can explain both the clonality of secondary tumors, as well as early on-set whole tumor population malignancy, because gene signatures of multiple clones add up to constitute the poor-prognosis gene-signature. Intratumoral hypoxia is not only restricted to necrotic areas but occurs throughout tumors and, therefore affects the tumor population as a whole. We have screened both breast and endometrial carcinomas that display high HIF levels for activating HIF mutations but have found none to date (Horree et al., submitted) (Vleugel et al., 2005b). This is consistent with a model for polyclonal adaptation rather than monoclonal selection of metastatic clones. Indeed, deletion of HIF-1α in a mouse model for breast cancer demonstrates that HIF is critical for tumor progression and metastasis but not for tumor initiation (Liao et al., 2007). Further, breast cancers with bone marrow metastatic cells had higher HIF-1α expression on the RNA and protein level than those without metastatic cells in the bone marrow (Woelfle et al., 2003). Hypoxia (through HIF) regulates a myriad of metastagenic genes. The hypoxia-response signature predicts prognosis independently and contributes to the poor-prognosis gene signature in breast cancer (Bos et al., 2003; Chi et al., 2006; Nuyten & van de Vijver, 2006; Vleugel et al., 2005a). Identification of intratumoral hypoxia might serve as a prognostic factor in clinical decision making.

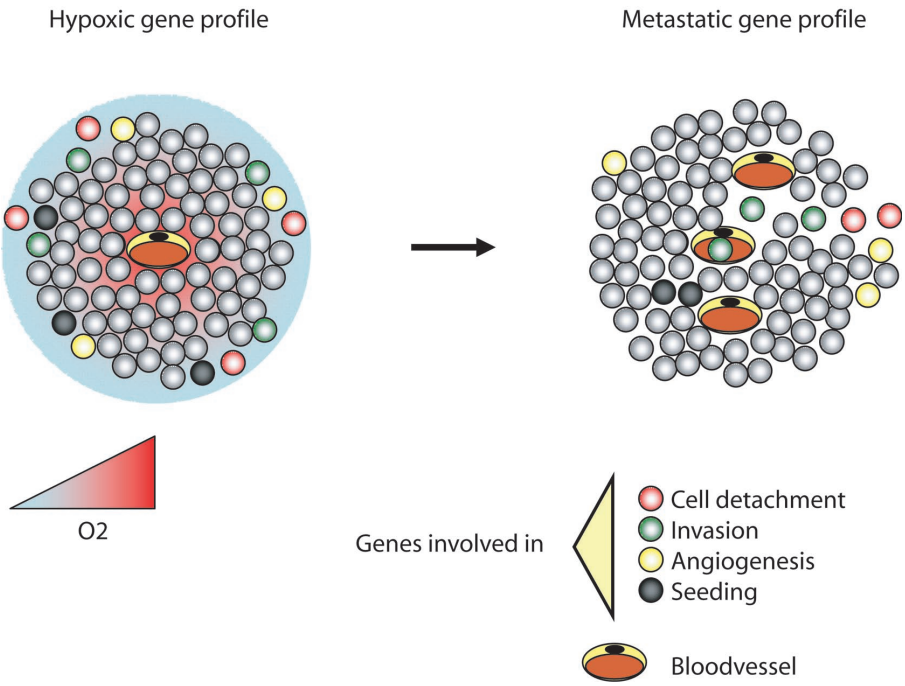

Figure 3.
The hypoxic gene profile versus the metastatic gene profile. On the left, the hypoxic gene profile is illustrated. The oxygen gradient is indicated from red (normal oxygen levels) to blue (hypoxia). Cells that lie in the periphery of the blood supply become hypoxic and start expressing genes involved in metastasis as indicated by different colors. On the right, a metastatic tumor is depicted, containing cells that express genes involved in each sequential step in metastasis formation, thereby constituting the metastatic gene profile. More bloodvessels are present here because of the stimulatory effects of hypoxia on angiogenesis. Cells expressing gene products functionally involved in the different steps of metastasis formation are depicted in concordance with these function; i.e. angiogenesis, cell detachment, invasion and seeding. The hypoxic gene profile might develop into a functional metastatic one, and therefore provide a predictive value.

Conclusion

In conclusion, the direct effect of hypoxia on cell adhesion molecules and matrix degrading enzymes provides a framework for testable hypotheses in mouse models and cell systems. From a clinical point of view, identification of the molecular networks regulating enhanced metastatic behavior provide important anti-cancer targets that may aid in the diagnosis and management of metastatic disease. Further elucidation of the hypoxia/HIF-mediated transcriptional networks will provide exciting insights into cancer biology and highlight diagnostic and therapeutic avenues to be explored in the near future.

References

Alexander NR, et al. (2006). N-cadherin gene expression in prostate carcinoma is modulated by integrin-dependent nuclear translocation of Twist1. *Cancer Res*, **66,** 3365-9.

Batlle E, et al. (2000). The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol*, **2,** 84-9.

Birchmeier C, et al. (2003). Met, metastasis, motility and more. *Nat Rev Mol Cell Biol*, **4,** 915-25.

Bos R, et al. (2003). Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*, **97,** 1573-81.

Cano A, et al. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol*, **2,** 76-83.

Carmeliet P. (2005). VEGF as a key mediator of angiogenesis in cancer. *Oncology*, **69 Suppl 3,** 4-10.

Carmeliet P, et al. (1998). Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature*, **394,** 485-90.

Ceradini DJ, et al. (2004). Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med*, **10,** 858-64.

Chi JT, et al. (2006). Gene expression programs in response to hypoxia: cell type specificity and prognostic significance in human cancers. *PLoS Med*, **3,** e47.

Christiansen JJ and Rajasekaran AK. (2006). Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer Res*, **66,** 8319-26.

Christofori G. (2006). New signals from the invasive front. *Nature*, **441,** 444-50.

Dales JP, et al. (2005). Overexpression of hypoxia-inducible factor HIF-1alpha predicts early relapse in breast cancer: retrospective study in a series of 745 patients. *Int J Cancer*, **116,** 734-9.

Danen EH and Yamada KM. (2001). Fibronectin, integrins, and growth control. *J Cell Physiol*, **189,** 1-13.

Derksen PW, et al. (2006). Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell*, **10,** 437-49.

Diez H, et al. (2007). Hypoxia-mediated activation of Dll4-Notch-Hey2 signaling in endothelial progenitor cells and adoption of arterial cell fate. *Exp Cell Res*, **313,** 1-9.

Douma S, et al. (2004). Suppression of anoikis and induction of metastasis by the neurotrophic receptor TrkB. *Nature*, **430,** 1034-9.

Eckerich C, et al. (2007). Hypoxia can induce c-Met expression in glioma cells and enhance SF/HGF-induced cell migration. *Int J Cancer*, **121,** 276-83.

Erler JT, et al. (2006). Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature*, **440,** 1222-6.

Esteban MA, et al. (2006). Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. *Cancer Res*, **66,** 3567-75.

Evans AJ, et al. (2006). VHL Promotes E2 Box-dependent E-cadherin Transcription by HIF-mediated Regulation of SIP1 and Snail. *Mol Cell Biol*.

Ferrara N. (2002). VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer*, **2,** 795-803.

Fidler IJ and Kripke ML. (1977). Metastasis results from preexisting variant cells within a malignant tumor. *Science*, **197,** 893-5.

Folkman J. (1971). Tumor angiogenesis: therapeutic implications. *N Engl J Med*, **285,** 1182-6.

Forsythe JA, et al. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*, **16,** 4604-13.

Funasaka T, et al. (2005). Regulation of phosphoglucose isomerase/autocrine motility factor expression by hypoxia. *Faseb J*, **19,** 1422-30.

Furge KA, et al. (2000). Met receptor tyrosine kinase: enhanced signaling through adapter proteins. *Oncogene*, **19,** 5582-9.

Gale NW, et al. (2004). Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc Natl Acad Sci U S A*, **101,** 15949-54.

Gatenby RA and Gillies RJ. (2004). Why do cancers have high aerobic glycolysis? *Nat Rev Cancer*, **4,** 891-9.

Gerber HP, et al. (1997). Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J Biol Chem*, **272,** 23659-67.

Gonzalo JA, et al. (2000). Critical involvement of the chemotactic axis CXCR4/stromal cell-derived factor-1 alpha in the inflammatory component of allergic airway disease. *J Immunol*, **165,** 499-508.

Gordon MS, et al. (2001). Phase I safety and pharmacokinetic study of recombinant human anti-vascular endothelial growth factor in patients with advanced cancer. *J Clin Oncol*, **19,** 843-50.

Gort EH, et al. (2007). The TWIST1 oncogene is a direct target of hypoxia-inducible factor-2alpha. *Oncogene*, doi:10.1038/sj.onc.1210795.

Greijer AE, et al. (2005). Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). *J Pathol*, **206,** 291-304.
Greijer AE and van der Wall E. (2004). The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J Clin Pathol*, **57,** 1009-14.
Gupta GP and Massague J. (2006). Cancer metastasis: building a framework. *Cell*, **127,** 679-95.
Gustafsson MV, et al. (2005). Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Dev Cell*, **9,** 617-28.
Hara S, et al. (2006). Hypoxia enhances c-Met/HGF receptor expression and signaling by activating HIF-1alpha in human salivary gland cancer cells. *Oral Oncol*.
Hellstrom M, et al. (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature*, **445,** 776-80.
Hockel M, et al. (1999). Hypoxic cervical cancers with low apoptotic index are highly aggressive. *Cancer Res*, **59,** 4525-8.
Hosono S, et al. (2007). Expression of Twist increases the risk for recurrence and for poor survival in epithelial ovarian carcinoma patients. *Br J Cancer*, **96,** 314-20.
Huber MA, et al. (2005). Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol*, **17,** 548-58.
Imai T, et al. (2003). Hypoxia attenuates the expression of E-cadherin via up-regulation of SNAIL in ovarian carcinoma cells. *Am J Pathol*, **163,** 1437-47.
Indovina P, et al. (2006). Hypoxia and ionizing radiation: changes in adhesive properties and cell adhesion molecule expression in MG-63 three-dimensional tumor spheroids. *Cell Commun Adhes*, **13,** 185-98.
Inoue M, et al. (2002). VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. *Cancer Cell*, **1,** 193-202.
Kaelin WG, Jr. (2002). Molecular basis of the VHL hereditary cancer syndrome. *Nat Rev Cancer*, **2,** 673-82.
Kelly BD, et al. (2003). Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. *Circ Res*, **93,** 1074-81.
Krebs LT, et al. (2004). Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes Dev*, **18,** 2469-73.
Krishnamachary B, et al. (2006). Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. *Cancer Res*, **66,** 2725-31.
Laughner E, et al. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol Cell Biol*, **21,** 3995-4004.
Liao D, et al. (2007). Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. *Cancer Res*, **67,** 563-72.
Liotta LA and Kohn EC. (2001). The microenvironment of the tumour-host interface. *Nature*, **411,** 375-9.
Liu YL, et al. (2006). Regulation of the Chemokine Receptor CXCR4 and Metastasis by Hypoxia-inducible Factor in Non Small Cell Lung Cancer Cell Lines. *Cancer Biol Ther*, **5,** 1320-6.
Longati P, et al. (1996). Hepatocyte growth factor is a pleiotropic factor protecting epithelial cells from apoptosis. *Cell Death Differ*, **3,** 23-8.
Maestro R, et al. (1999). Twist is a potential oncogene that inhibits apoptosis. *Genes Dev*, **13,** 2207-17.
Maher ER. (2004). Von Hippel-Lindau disease. *Curr Mol Med*, **4,** 833-42.
Mahoney BP, et al. (2003). Tumor acidity, ion trapping and chemotherapeutics. I. Acid pH affects the distribution of chemotherapeutic agents in vitro. *Biochem Pharmacol*, **66,** 1207-18.
Martens LK, et al. (2007). Hypoxia inducible factor-1 (HIF-1) is a transcriptional activator of the TrkB neurotrophin receptor gene. *J Biol Chem*., **282**, 14379-88.
Martin TA, et al. (2005). Expression of the transcription factors snail, slug, and twist and their clinical significance in human breast cancer. *Ann Surg Oncol*, **12,** 488-96.
Maxwell PH, et al. (2001). The pVHL-hIF-1 system. A key mediator of oxygen homeostasis. *Adv Exp Med Biol*, **502,** 365-76.
Mazure NM, et al. (2003). Protein kinases and the hypoxia-inducible factor-1, two switches in angiogenesis. *Curr Pharm Des*, **9,** 531-41.
Minn AJ, et al. (2005). Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. *J Clin Invest*, **115,** 44-55.
Movsas B, et al. (2000). Increasing levels of hypoxia in prostate carcinoma correlate significantly with increasing clinical stage and patient age: an Eppendorf pO(2) study. *Cancer*, **89,** 2018-24.
Muller A, et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature*, **410,** 50-6.

Nakamura K, et al. (2006). Brain-derived neurotrophic factor activation of TrkB induces vascular endothelial growth factor expression via hypoxia-inducible factor-1alpha in neuroblastoma cells. *Cancer Res*, **66,** 4249-55.
Niizeki H, et al. (2002). Hypoxia enhances the expression of autocrine motility factor and the motility of human pancreatic cancer cells. *Br J Cancer*, **86,** 1914-9.
Noguera-Troise I, et al. (2006). Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature*, **444,** 1032-7.
Nowell PC. (1971). Genetic changes in cancer: cause or effect? *Hum Pathol*, **2,** 347-8.
Nuyten DS and van de Vijver MJ. (2006). Gene expression signatures to predict the development of metastasis in breast cancer. *Breast Dis*, **26,** 149-56.
Payne SL, et al. (2005). Lysyl oxidase regulates breast cancer cell migration and adhesion through a hydrogen peroxide-mediated mechanism. *Cancer Res*, **65,** 11429-36.
Peinado H, et al. (2005). A molecular role for lysyl oxidase-like 2 enzyme in snail regulation and tumor progression. *Embo J*, **24,** 3446-58.
Peinado H, et al. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer*, **7,** 415-28.
Pennacchietti S, et al. (2003). Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell*, **3,** 347-61.
Perl AK, et al. (1998). A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature*, **392,** 190-3.
Phillips RJ, et al. (2005). Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha. *J Biol Chem*, **280,** 22473-81.
Poste G, et al. (1981). Interactions among clonal subpopulations affect stability of the metastatic phenotype in polyclonal populations of B16 melanoma cells. *Proc Natl Acad Sci U S A*, **78,** 6226-30.
Pouyssegur J, et al. (2006). Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature*, **441,** 437-43.
Pugh CW, et al. (1991). Functional analysis of an oxygen-regulated transcriptional enhancer lying 3' to the mouse erythropoietin gene. *Proc Natl Acad Sci U S A*, **88,** 10553-7.
Ramaswamy S, et al. (2003). A molecular signature of metastasis in primary solid tumors. *Nat Genet*, **33,** 49-54.
Ravi R, et al. (2000). Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. *Genes Dev*, **14,** 34-44.
Ridgway J, et al. (2006). Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature*, **444,** 1083-7.
Roepman P, et al. (2006). Dissection of a metastatic gene expression signature into distinct components. *Genome Biol*, **7,** R117.
Semenza GL. (2003). Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*, **3,** 721-32.
Spangenberg C, et al. (2006). ERBB2-mediated transcriptional up-regulation of the alpha5beta1 integrin fibronectin receptor promotes tumor cell survival under adverse conditions. *Cancer Res*, **66,** 3715-25.
Staller P, et al. (2003). Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. *Nature*, **425,** 307-11.
Tan C, et al. (2004). Regulation of tumor angiogenesis by integrin-linked kinase (ILK). *Cancer Cell*, **5,** 79-90.
Tang N, et al. (2004). Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. *Cancer Cell*, **6,** 485-95.
Thurston G, et al. (2007). The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth. *Nat Rev Cancer*, **7,** 327-31.
Trastour C, et al. (2007). HIF-1alpha and CA IX staining in invasive breast carcinomas: prognosis and treatment outcome. *Int J Cancer*, **120,** 1451-8.
van 't Veer LJ, et al. (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature*, **415,** 530-6.
van de Vijver MJ, et al. (2002). A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med*, **347,** 1999-2009.
Vaupel P. (2004). The role of hypoxia-induced factors in tumor progression. *Oncologist*, **9 Suppl 5,** 10-7.
Vaupel P, et al. (2004). Tumor hypoxia and malignant progression. *Methods Enzymol*, **381,** 335-54.
Vaupel P, et al. (1991). Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O2 tension measurements. *Cancer Res*, **51,** 3316-22.
Vleminckx K, et al. (1991). Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell*, **66,** 107-19.
Vleugel MM, et al. (2005a). Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in invasive breast cancer. *J Clin Pathol*, **58,** 172-7.
Vleugel MM, et al. (2005b). Mutation analysis of the HIF-1alpha oxygen-dependent degradation domain in invasive breast cancer. *Cancer Genet Cytogenet*, **163,** 168-72.

Wang GL, et al. (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,** 5510-4.

Woelfle U, et al. (2003). Molecular signature associated with bone marrow micrometastasis in human breast cancer. *Cancer Res*, **63,** 5679-84.

Wu WS, et al. (2005). Slug antagonizes p53-mediated apoptosis of hematopoietic progenitors by repressing puma. *Cell*, **123,** 641-53.

Yamakawa M, et al. (2003). Hypoxia-inducible factor-1 mediates activation of cultured vascular endothelial cells by inducing multiple angiogenic factors. *Circ Res*, **93,** 664-73.

Yang J, et al. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*, **117,** 927-39.

Zagzag D, et al. (2005). Stromal cell-derived factor-1alpha and CXCR4 expression in hemangioblastoma and clear cell-renal cell carcinoma: von Hippel-Lindau loss-of-function induces expression of a ligand and its receptor. *Cancer Res*, **65,** 6178-88.

Zhang L and Hill RP. (2007). Hypoxia Enhances Metastatic Efficiency in HT1080 Fibrosarcoma Cells by Increasing Cell Survival in Lungs, Not Cell Adhesion and Invasion. *Cancer Res*, **67,** 7789-97.

Zhong H, et al. (1999). Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res*, **59,** 5830-5.

Zhou J, et al. (2003). Tumor necrosis factor-alpha causes accumulation of a ubiquitinated form of hypoxia inducible factor-1alpha through a nuclear factor-kappaB-dependent pathway. *Mol Biol Cell*, **14,** 2216-25.

Chapter 3

Hypoxia Inducible Factor-1α expression requires PI 3-kinase activity and correlates with Akt1 phosphorylation in invasive breast carcinomas

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Abstract

Hypoxia Inducible Factor-1 alpha (HIF-1α) is the regulatory subunit of the heterodimeric transcription factor HIF-1 and the key factor in cellular response to low oxygen tension. Expression of HIF-1α protein is associated with poor patient survival and therapy resistance in many types of solid tumors. Insight into HIF-1α regulation in solid tumors is important for therapeutic strategies. In this study, we determined the pathophysiological relevance of HIF-1α regulation by the oncogenic PI 3-kinase/Akt signaling pathway. We modeled the physiology of hypoxic tumor regions by culturing carcinoma cells under low oxygen tension in the absence of serum. We observed that hypoxic induction of HIF-1α protein was decreased by serum deprivation. Overexpression of dominant active Akt1 restored HIF-1α expression, whereas inhibition of PI 3-kinase activity reduced hypoxic HIF-1α protein levels to a similar extent as serum deprivation. Immunohistochemical analysis of 95 human breast cancers revealed that lack of Akt1 phosphorylation correlates with low HIF-1α levels. To our knowledge this is the first reported comparison between HIF-1α expression and Akt phosphorylation in human carcinomas. We conclude that Akt activity is physiologically relevant for HIF-1α expression in breast cancer. This implies that HIF-1α function might be therapeutically targeted by inhibition of the PI 3-kinase/Akt pathway.

The hypoxia inducible factor-1 (HIF-1) is the main regulator of cellular adaptation to oxygen deprivation (Semenza, 2003; Semenza, 2004). HIF-1 is a heterodimeric transcription factor composed of a constitutively expressed subunit (HIF-1β or ARNT), and a regulatory subunit (HIF-1α), which is continuously degraded under normoxia (Jiang et al., 1996; Wang et al., 1995). Expression of HIF-1α protein in solid tumors has been associated with tumor aggressiveness and poor clinical outcome (Birner et al., 2000; Bos et al., 2003; Bos et al., 2001; Dales et al., 2005; Vleugel et al., 2005). Therefore, insight into HIF-1α regulation in solid tumors is important for therapeutic strategies. The mechanism of oxygen-dependent regulation of HIF-1α has been studied extensively. Under normoxia HIF-1α is hydroxylated by specific prolyl hydroxylases, which allows binding of the E3 ubiquitin ligase Von Hippel-Lindau protein (pVHL) and subsequent HIF-1α degradation by the proteasome (Bruick & McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001; Maxwell et al., 1999). When oxygen tension is severely reduced, HIF-1α is no longer hydroxylated and becomes stabilized. Stabilization of HIF-1α results in the transcription of HIF-1 target genes, such as vascular endothelial growth factor (*VEGF*) (Forsythe et al., 1996; Levy et al., 1995; Semenza, 2003). In addition to VHL-dependent regulation, oncogenic signaling pathways regulate HIF-1α. For instance, the PI 3-kinase/Akt or Protein Kinase B (PKB) signal transduction cascade has been implicated in the regulation of HIF-1α either under hypoxic conditions or in response to stimulation of tyrosine kinase receptors (Fukuda et al., 2002; Jiang et al., 2001). The exact contribution of the PI 3-kinase pathway to HIF-1α regulation is still subject of discussion (Alvarez-Tejado et al., 2002; Arsham et al., 2002).

The aim of our study was to gain insight into the pathophysiological relevance of HIF-1α regulation by the PI 3-kinase/Akt pathway. To do so, we considered a model to study HIF-1α regulation in the hypoxic regions of a tumor. Lack of oxygen supply from the blood vessels coincides with decreased availability of nutrients and growth factors. This unfavorable environment results in a strong selective pressure for the most adaptive and malignant tumor cells (Vaupel & Mayer, 2005). Culturing cells under hypoxia and low serum conditions reflects the physiology of these hypoxic tumor regions. To study HIF-1α in this model cervical carcinoma-derived HeLa cells and breast carcinoma-derived MCF-7 cells were cultured in the presence or absence of fetal calf serum (FCS) for 24 hours followed by incubation in a hypoxic environment (1% O_2) for 4 hours. In the presence of serum HIF-1α protein levels were induced by hypoxia in both cell lines (Figure 1a). However, in serum-deprived HeLa and MCF-7

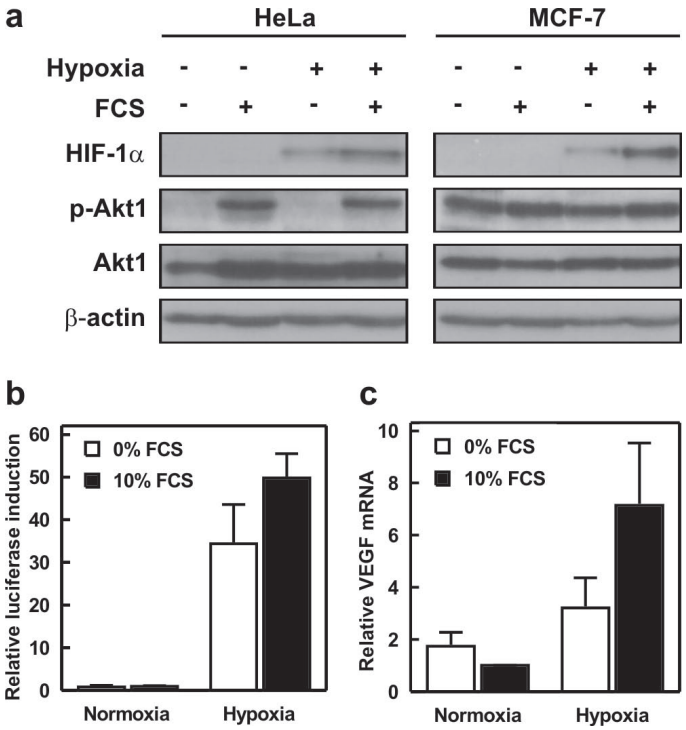


Figure 1.
Induction of HIF-1α protein and function by hypoxia is impaired after serum deprivation. **a)** Western analysis of serum- and oxygen-deprived cells. Cells were cultured in Dulbecco's Modified Eagle Medium (Gibco BRL, UK) supplied with 10% Fetal Calf Serum (FCS) (Gibco BRL, UK). Cells were serum-maintained or -deprived for 24 hours and incubated for 4 hours under hypoxia (1% O_2) in an Invivo$_2$ 1000 Workstation (Biotrace, UK). Cells were lysed in Laemmli sample buffer (Laemmli, 1970). Twenty microgram protein/lane was subjected to SDS-PAGE followed by Western blot analysis. Blots were incubated overnight with anti-HIF-1α (1:250, BD Transduction Laboratories, CA, USA), anti-Akt (phospho S473) (1:1000, Abcam, UK), anti-Akt (1:1000, Abcam, UK) or anti-β-Actin (1:5000, US Biological, MA, USA) at 4ºC. Enhanced Chemo Luminescence (ECL, Amersham Biosciences, UK) was used for signal detection according to manufacturers' protocol. **b)** HIF-1 reporter analysis in serum- and oxygen-deprived cells. HeLa cells were cotransfected with TK Renilla (Promega Corporation, WI, USA) and either empty pGL3 (Promega Corporation, WI, USA) or pGL3 vector containing five hypoxia responsive elements (5xHRE) (Shibata et al., 1998) 24 hours prior to treatment using Lipofectamin 2000© (Invitrogen, CA, USA) according to manufacturers' protocol. Cells were serum-maintained or -deprived for 24 hours and subsequently incubated under hypoxia (1% O_2) for 16 hours or left untreated. Cells were lysed in 1x Passive lysis buffer (Promega Corporation, WI, USA) according to manufacturers' protocol. Experiments were performed in triplicate. Samples were assayed with a dual luciferase kit (Promega Corporation, WI, USA) and measured using a Veritas™ 96-well plate luminometer (Turner Biosystems, CA, USA). All values were normalized using TK Renilla expression. Data represent the 5xHRE value divided by pGL3 value. **c)** Quantitative vascular endothelial growth factor (VEGF) mRNA analysis in serum- and oxygen-deprived cells. HeLa cells were serum-maintained or -deprived for 24 hours and subsequently incubated under hypoxia (1% O_2) for 24 hours or left untreated. Total RNA was isolated from cell lysates using Trizol followed by chloroform/phenol extraction. cDNA was made using a Reverse Transcriptase kit (Roche, Germany) and oligo (dT) primers (Invitrogen, CA, USA). Quantitative PCR was performed with AssayOnDemand kits for VEGF and hydroxymethylbilane synthase (HMBS) (Applied Biosystems, CA, USA) using an ABI7900 analyzer. Data were analyzed using the SDS2.2.1 program (Applied Biosystems, CA, USA). VEGF expression was normalized using expression levels of HMBS. cDNA from HeLa cells incubated with serum under normoxia served as a reference.

cells only little HIF-1α protein was detectable after incubation under hypoxic conditions. To study presumable influences of FCS on the activity of the PI 3-kinase pathway, phosphorylation of Akt1 on serine 473 was determined (Alessi et al., 1996). In serum deprived HeLa and MCF-7 cells Akt1 phosphorylation was decreased compared to serum maintained cells, whereas total Akt1 levels remained unchanged. Interestingly, the effect of serum deprivation on levels of HIF-1α induction coincides with the effect on Akt1 phosphorylation. VEGF transcription is induced under hypoxia in a HIF-1 dependent manner (Levy et al., 1995). We studied VEGF regulation under hypoxia to determine whether or not the inhibition of HIF-1α protein induction by serum deprivation resulted in reduced HIF-1 transcriptional activity. HeLa cells were transiently transfected with a luciferase reporter construct under control of five Hypoxia Responsive Elements (HRE) derived from the VEGF promotor (Shibata et al., 1998). Transcriptional activity of HIF-1 was detected when cells were cultured under hypoxic conditions in the presence of serum (Figure 1b). In the absence of serum, activation by hypoxia was distinguishably attenuated. In addition, VEGF mRNA expression in HeLa cells was analyzed by quantitative PCR to confirm effects of serum deprivation on endogenous gene regulation by HIF-1α. Expression of VEGF mRNA was induced in HeLa cells after incubation in a hypoxic environment in the presence of serum (Figure 1c). In the absence of serum, VEGF mRNA expression was significantly less induced. In conclusion, these data show that serum deprivation impairs induction of HIF-1α protein and subsequent gene regulation by HIF-1 in response to hypoxia, which corresponds with a decrease in Akt1 phosphorylation.
To test whether the observed reduction in hypoxia-induced HIF-1α protein expression in HeLa after serum deprivation was caused by the reduced PI 3-kinase activity, serum-deprived HeLa cells were transfected with constitutively active Akt1 (Burgering & Coffer, 1995). This resulted in substantial induction of HIF-1α protein levels by hypoxia (Figure 2a).

Overexpression of constitutively active Akt1 did not enhance hypoxic HIF-1α expression in the presence of serum, which suggests that the effects of serum maintenance and Akt1 overexpression are not additive. Under normoxic condition, HIF-1α protein expression was not induced by constitutively active Akt1, whereas overexpressed HIF-1α could be detected, showing that Akt1 activity alone is not sufficient for HIF-1α induction (Figure 2b). To study whether the PI 3-kinase/Akt pathway is necessary for HIF-1α protein expression, HeLa cells were cultured in the presence or absence of serum and treated with the PI 3-kinase inhibitor LY294002. Inhibition of PI 3-kinase resulted in reduction in

hypoxia-induced HIF-1α protein levels to a similar extent as serum deprivation (Figure 2c). Expression of HIF-1α protein was not completely abolished. This might be explained by basal translation of HIF-1α, which is enhanced by PI 3-kinase activity (Hudson et al., 2002). The mammalian target of rapamycin (mTOR) is an effector in the PI-3 kinase pathway involved in translational regulation by phosphorylating 4EBP and p70S6K (Hay & Sonenberg, 2004).

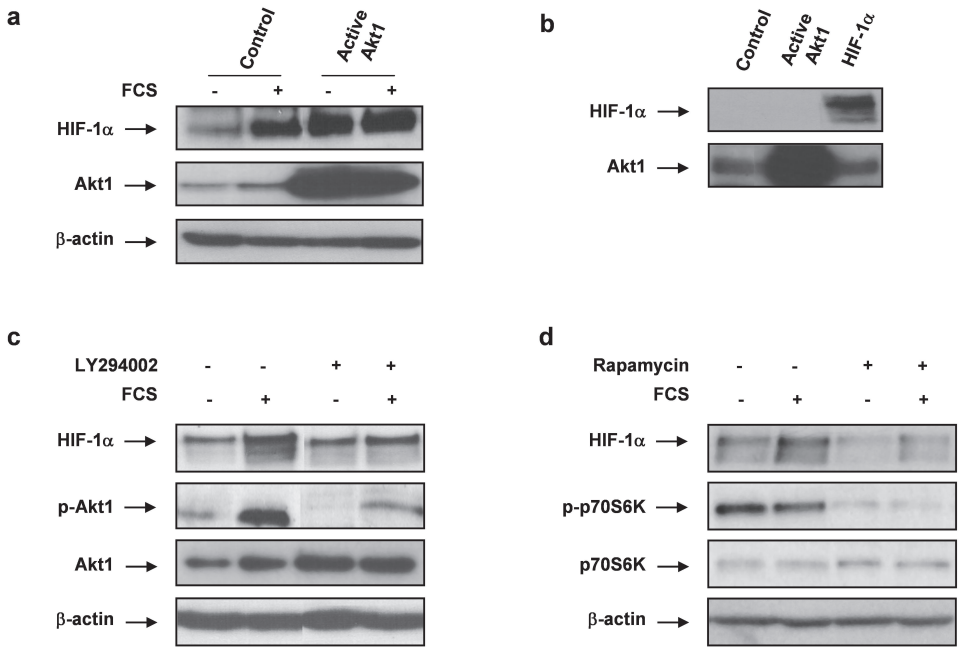
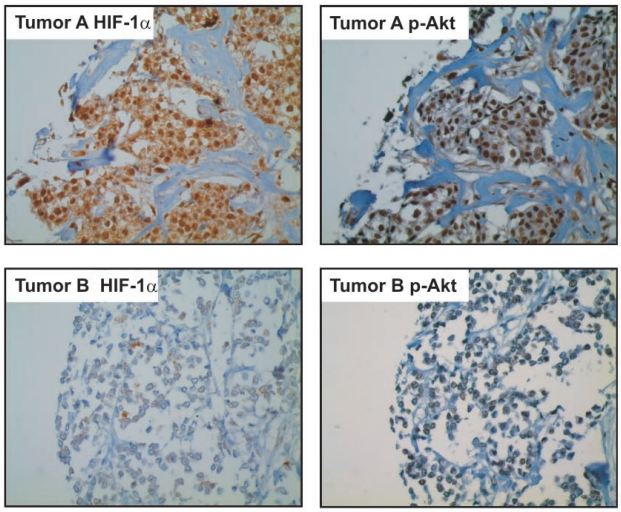


Figure 2.
PI 3-kinase activity is required for hypoxic induction of HIF-1α **a)** Western analysis of hypoxic and **b)** normoxic HeLa cells overexpressing active Akt1. HeLa cells were transfected with pSG5-gag- Akt1 (Burgering & Coffer, 1995) or with pCEP4-HIF-1α (Jiang et al., 1996), or an empty pcDNA3 vector (Invitrogen, CA, USA) 24 hours prior to treatments using lipofectamin 2000© according to manufacturers' protocol. When indicated cells were serum-maintained or -deprived for 24 hours prior to incubation for 4 hours under hypoxia (1% O_2). Western blot analysis was performed as described in Figure 1a legend. **c)** Western analysis of hypoxic HeLa cells treated with a PI 3-kinase inhibitor. HeLa cells were treated with 10 μM of the PI 3-kinase inhibitor LY294002 (Sigma-Aldrich Co., MO, USA) for 30 minutes prior to incubation for 4 hours under hypoxia (1% O_2). When indicated cells were serum-maintained or -deprived for 24 hours prior to hypoxia. Western blot analysis was performed as described in Figure 1a legend. d) Western analysis of hypoxic HeLa cells treated with Rapamycin. HeLa cells were treated with 12.5 μM of the mTOR inhibitor Rapamycin (Sigma-Aldrich Co., MO, USA) 24 hours prior to incubation for 4 hours under hypoxia (1% O_2). When indicated cells were serum-maintained or -deprived for 24 hours prior to hypoxia. Western blot analysis was performed as described in Figure 1a legend.

To address whether the specific mTOR inhibitor rapamycin had an effect on HIF-1α levels, HeLa cells were cultured in the absence or presence of serum and treated with rapamycin. Hypoxic induction of HIF-1α was reduced by rapamycin in the presence of serum (Figure 4d). Further, serum deprivation only resulted in minor additional impairment of HIF-1α induction. These data indicate that lack of PI 3-kinase or mTOR activity severely limits HIF-1α protein expression. Based on these results in our *in vitro* model, one would expect that HIF-1α expression in solid tumors largely depends on Akt activity. Akt1 phosphorylation on serine 473 has been suggested to be associated with aggressiveness in carcinomas (Kirkegaard et al., 2005; Kreisberg et al., 2004; Schmitz et al., 2004). However, no association studies with HIF-1α have been reported yet. We therefore examined the expression and correlation of HIF-1α protein and phosphorylated Akt1 in 129 cases of invasive breast cancer. Hematoxylin-eosin stained sections were used to identify representative areas of tumor tissue in tissue blocks, from which a tissue microarray was constructed. The average age of the patients was 65.5 years, 40 percent were lymph-node positive and the average tumor size was 2.26 cm^2. The tissue microarray was analyzed by immunohistochemistry for HIF-1α protein and for phosphorylated Akt1. Staining could be analyzed for both proteins in 95 cases. Phosphorylated Akt1 was observed in the majority of the breast carcinomas, but not in normal breast tissue. An Akt1-score was assigned to each case based on product of the percentage of positive tumor nuclei and semi-quantitatively assessed staining intensity (0-3), taking the mean Akt1-score as cut-off to discriminate between high and low Akt1 phosphorylation. Low Akt1 phosphorylation was observed in 38 patients. From these patients only 6 patients (16%) had HIF-1α expression in more than 5% of the tumor cells and none had HIF-1α expression in more than 50% of the tumor cells (Figure 3a & 3b).

In patients with high Akt1 phosphorylation the HIF-1α expression levels were not necessarily high or low. Therefore, we conclude that a low expression of phosphorylated Akt1 correlates with low expression of HIF-1α in breast cancer ($p=0.026$ for HIF-1α expression less then 5%). This is in agreement with our observations that reduced PI 3-kinase/Akt activity in serum-deprived cells limits HIF-1α expression and that a high level of active Akt alone is not sufficient for HIF-1α accumulation. Most likely HIF-1α protein expression requires both inhibition of pVHL-dependent HIF-1α degradation by hypoxia and Akt-induced HIF-1α translation. Therefore, lack of hypoxia in some of the tumor samples is a plausible explanation for this observation. Furthermore, we can not exclude the involvement of other signaling pathways.

a



b

Association of Akt-1 phosphorylation and HIF-1α protein expression

		Akt-1 phosphorylation		
HIF-1α levels	*All (95)*	*Low (38)*	*High (57)*	*P value*
<5%	68	32 (33.68%)	36 (37.89%)	
≥5%	27	6 (6.32%)	21 (22.11%)	0.026
<50%	84	38 (40.00%)	46 (48.42%)	
≥50%	11	0 (0.00%)	11 (11.58%)	0.004

Figure 3.
HIF-1α protein expression correlates with Akt1 phosphorylation. **a)** Immunohistochemical analysis of invasive breast carcinoma tissue. Tumor A represents breast carcinoma tissue with a high HIF-1α expression and a high Akt1 phosphorylation score. Tumor B represents breast carcinoma tissue with low HIF-1α expression and a low Akt1 phosphorylation score. The tissue array was constructed by taking 4-5mm cores from neutral buffered formaldehyde-fixed paraffin blocks of breast cancer tissues obtained from the archives of the Department of Pathology of the VU University Medical Center, Amsterdam, using a dedicated instrument (Beecher, WI, USA). The use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients (van Diest, 2002). Immunohistochemistry for HIF-1α was performed as described previously (Bos et al., 2003). Antigen retrieval for phosphorylated Akt1 was done by boiling sections in citrate buffered solution for 15 minutes. Staining was performed with anti-Akt (phospho S473) (1:100, Abcam, UK) followed by incubation with PowerVision Poly-Hrp-anti Ms/Rb/Rt IgG (Biotin-free, Ready to use, Immuno Vision Technologies, CA, USA). **b)** Association of Akt1 phosphorylation and HIF-1α protein expression. Data was analyzed using SPSS for Windows, version 12.0.1 (SPSS Inc., IL, USA). The nonparametric chi-square test was used to evaluate correlations between HIF-1αexpression and Akt1 phosphorylation. Two-sided *P* values < 0.05 were considered significant.

Even though oxygen-dependent hydroxylation of HIF-1α remains the main regulatory mechanism, the PI 3-kinase/Akt pathway takes an important place in HIF-1 regulation. It remains to be determined which effectors of the PI 3-kinase/Akt pathway are responsible for the observations made in this study. Many downstream targets of Akt have been implicated in regulating HIF-1α protein expression and function, like TSC-2, mTOR, GSK-3β, FOXO4, HDM2 and heat shock proteins (Bardos et al., 2004; Brugarolas et al., 2003; Hudson et al., 2002; Mottet et al., 2003; Skinner et al., 2004; Tang & Lasky, 2003; Treins et al., 2002; Zhong et al., 2000; Zhou et al., 2004). Some of these Akt effectors have been indicated to regulate translation of HIF-1α, whereas others might regulate HIF-1α degradation (Brugarolas et al., 2003; Hudson et al., 2002; Zhou et al., 2004). The effects of serum deprivation on HIF-1α could still be observed after inhibition of degradation of HIF-1α (data not shown). Furthermore, treatment with rapamycin reduced hypoxia-induced HIF-1α expression. This might implicate that Akt mainly influences HIF-1α synthesis, partially via a mTOR dependent mechanism.

In breast cancer Akt activity predicts clinical outcome (Kirkegaard et al., 2005; Schmitz et al., 2004). We are the first to report a link between Akt phosphorylation status and HIF-1α expression in invasive breast carcinomas. Our data show that active Akt is necessary for high HIF-1α expression. Therefore, it might be possible to target HIF-1α-mediated tumor aggressiveness through inhibition of Akt activity. Clinical trials using Akt or PI 3-kinase inhibitors might give more insight into the mechanism by which Akt-dependent regulation of HIF-1α affects tumor aggressiveness of hypoxic tumors and patient survival.

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References

Alessi DR, et al. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. Embo J, 15, 6541-51.

Alvarez-Tejado M, et al. (2002). Lack of evidence for the involvement of the phosphoinositide 3-kinase/Akt pathway in the activation of hypoxia-inducible factors by low oxygen tension. J Biol Chem, 277, 13508-17.

Arsham AM, et al. (2002). Phosphatidylinositol 3-kinase/Akt signaling is neither required for hypoxic stabilization of HIF-1 alpha nor sufficient for HIF-1-dependent target gene transcription. J Biol Chem, 277, 15162-70.

Bardos JI, et al. (2004). Growth factor-mediated induction of HDM2 positively regulates hypoxia-inducible factor 1alpha expression. Mol Cell Biol, 24, 2905-14.

Birner P, et al. (2000). Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res, 60, 4693-6.

Bos R, et al. (2003). Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. Cancer, 97, 1573-81.

Bos R, et al. (2001). Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst, 93, 309-14.

Brugarolas JB, et al. (2003). TSC2 regulates VEGF through mTOR-dependent and -independent pathways. Cancer Cell, 4, 147-58.

Bruick RK and McKnight SL. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. Science, 294, 1337-40.

Burgering BM and Coffer PJ. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature, 376, 599-602.

Dales JP, et al. (2005). Overexpression of hypoxia-inducible factor HIF-1alpha predicts early relapse in breast cancer: retrospective study in a series of 745 patients. Int J Cancer, 116, 734-9.

Epstein AC, 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.

Forsythe JA, et al. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol, 16, 4604-13.

Fukuda R, et al. (2002). Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. J Biol Chem, 277, 38205-11.

Hay N and Sonenberg N. (2004). Upstream and downstream of mTOR. Genes Dev, 18, 1926-45.

Hudson CC, et al. (2002). Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin. Mol Cell Biol, 22, 7004-14.

Ivan M, et al. (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science, 292, 464-8.

Jaakkola P, et al. (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science, 292, 468-72.

Jiang BH, et al. (2001). Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. Cell Growth Differ, 12, 363-9.

Jiang BH, et al. (1996). Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem, 271, 17771-8.

Kirkegaard T, et al. (2005). AKT activation predicts outcome in breast cancer patients treated with tamoxifen. J Pathol, 207, 139-46.

Kreisberg JI, et al. (2004). Phosphorylation of Akt (Ser473) is an excellent predictor of poor clinical outcome in prostate cancer. Cancer Res, 64, 5232-6.

Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-5.

Levy AP, et al. (1995). Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. J Biol Chem, 270, 13333-40.

Maxwell PH, et al. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature, 399, 271-5.

Mottet D, et al. (2003). Regulation of hypoxia-inducible factor-1alpha protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3beta pathway in HepG2 cells. J Biol Chem, 278, 31277-85.

Schmitz KJ, et al. (2004). Prognostic relevance of activated Akt kinase in node-negative breast cancer: a clinicopathological study of 99 cases. Mod Pathol, 17, 15-21.

Semenza GL. (2003). Targeting HIF-1 for cancer therapy. Nat Rev Cancer, 3, 721-32.

Semenza GL. (2004). Hydroxylation of HIF-1: oxygen sensing at the molecular level. Physiology (Bethesda), 19, 176-82.

Shibata T, et al. (1998). Enhancement of gene expression under hypoxic conditions using fragments of the human vascular endothelial growth factor and the erythropoietin genes. Int J Radiat Oncol Biol Phys, 42, 913-6.

Skinner HD, et al. (2004). Vascular endothelial growth factor transcriptional activation is mediated by hypoxia-inducible factor 1alpha, HDM2, and p70S6K1 in response to phosphatidylinositol 3-kinase/AKT signaling. J Biol Chem, 279, 45643-51.

Tang TT and Lasky LA. (2003). The forkhead transcription factor FOXO4 induces the down-regulation of hypoxia-inducible factor 1 alpha by a von Hippel-Lindau protein-independent mechanism. J Biol Chem, 278, 30125-35.

Treins C, et al. (2002). Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. J Biol Chem, 277, 27975-81.

van Diest PJ. (2002). No consent should be needed for using leftover body material for scientific purposes. For. BMJ, 325, 648-51.

Vaupel P and Mayer A. (2005). Hypoxia and anemia: effects on tumor biology and treatment resistance. Transfus Clin Biol, 12, 5-10.

Vleugel MM, et al. (2005). Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in invasive breast cancer. J Clin Pathol, 58, 172-7.

Wang GL, et al. (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, 5510-4.

Zhong H, et al. (2000). Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res, 60, 1541-5.

Zhou J, et al. (2004). PI3K/Akt is required for heat shock proteins to protect hypoxia-inducible factor 1alpha from pVHL-independent degradation. J Biol Chem, 279, 13506-13.

Chapter 4

Hypoxia Inducible Factor-1α is essential for hypoxic p27 induction in endometrioid endometrial carcinoma

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Abstract

Hypoxia-inducible factor 1α (HIF-1α) plays an essential role in the adaptive response of cells to hypoxia. The cyclin-dependent kinase inhibitor p27(Kip1) is highly expressed in the normal endometrium but is lost during endometrial carcinogenesis. However, in high grade cancers p27 re-expression is observed. We analysed the role of HIF-1α in hypoxia-induced expression of p27 *in vitro* and *in vivo* in endometrial cancer. Paraffin-embedded specimens from endometrioid endometrial carcinoma (n=39) were stained immunohistochemically for HIF-1α, p27 and Ki67. HEC-1B, an endometrial carcinoma cell line, was cultured under normoxic or hypoxic conditions in the presence or absence of transiently expressed short hairpin RNAs targeting HIF-1α. Protein expression of p27 and HIF-1α was assessed by Western blotting. Immunohistochemical staining revealed perinecrotic HIF-1α expression in 67% of the cases and p27 staining central in the tumor islands, mostly around necrosis in 46% of the cases. In 50% of the tumors with perinecrotic HIF-1α expression p27 and HIF-1α perinecrotic/central co-localisation was observed. In these tumor sections hypoxia-associated p27 expression showed less proliferation around necrosis. Analysis of cultured endometrial carcinoma cells demonstrated that p27 protein expression is induced by hypoxia. This induction was abrogated by transient knockdown of HIF-1α using RNAi. Furthermore, hypoxia induced cell cycle arrest in HEC-1B cells. We conclude that in endometrioid endometrial carcinoma, p27 re-expression by hypoxia is HIF-1α dependent and leads to cell cycle arrest. This may contribute to survival of cancer cells in hypoxic parts of the tumor.

Introduction

Lack of oxygen supply to tumor tissue results in hypoxic areas. This is important in carcinogenesis because it results in a more aggressive phenotype with increased invasiveness and proliferation, formation of metastases and poorer survival (Hockel et al., 1996; Vaupel & Mayer, 2005). Moreover, hypoxic tumors are more resistant to radiotherapy and chemotherapy (Vaupel & Mayer, 2005). In reaction to hypoxia, cells alter their metabolism and activate survival genes. Hypoxia-inducible factor 1 (HIF-1) plays an essential role in the adaptive cellular response to hypoxia. HIF-1 is a transcription factor and consists of an alpha and a beta subunit. Both subunits are basic helix loop helix proteins that contain a PAS (PER-Aryl hydrocarbon nuclear translocator-SIM) domain (Wang et al., 1995). Under normoxia, the HIF-1α protein has a very short half-life due to oxygen-dependent polyubiquination by the Von Hippel-Lindau (VHL) tumor suppressor protein, which targets HIF-1α for proteasomal degradation (Cockman et al., 2000). Lack of oxygen abrogates this process, which results in stable HIF-1α protein. HIF-1β is constitutively expressed, and therefore HIF-1α protein levels determine the amount of HIF-1 formed. Target genes of HIF-1 control glucose transporters, glycolytic enzymes, gluconeogenesis, high-energy phosphate metabolism, growth factors, apoptosis, erythropoiesis, haem metabolism, iron transport, vasomotor regulation and nitric oxide synthesis (Lee et al., 2004).

During carcinogenesis, the cell cycle is progressively deregulated (Horree et al., 2007b; Hui et al., 1998; Pucci & Giordano, 1999). Overexpression of cell cycle stimulating factors such as the cyclins and cyclin dependent kinases (CDKs), and reduced activity of cyclin dependent kinase inhibitors (CDKIs) are frequently found in tumours (Alfsen et al., 2003; Gamboa-Dominguez et al., 2007; Horree et al., 2007b; Hutter et al., 2006; Vrekoussis et al., 2005). Cell cycle deregulation is correlated with a more malignant subtype, a higher proliferation rate, recurrence and a worse survival in different tumors (Alfsen et al., 2003; Gamboa-Dominguez et al., 2007; Hutter et al., 2006; Vrekoussis et al., 2005).

Hypoxia leads to cell cycle arrest, but the role of HIF-1 is not fully understood. Hypoxia reduces proliferation and increases apoptosis in wild-type embryonic stem (ES) cells, but not in HIF-1α knock-out ES cells (Carmeliet et al., 1998). Furthermore, some genes involved in cell cycle control are regulated by hypoxia either in a HIF-1α-dependent (p53, p21/WAF/Cip1, Bcl-2) or HIF-

1α-independent (GADD153) manner, suggesting that there are at least two different adaptive responses to oxygen deprivation (Carmeliet et al., 1998). The CDKI p27(Kip1) inhibits the kinase activity of cyclin-cdk complexes, particularly cyclinE-cdk2 and cyclinD-cdk4 activity, resulting in cell cycle arrest (Massague, 2004). Regulation of p27 by mitogenic stimuli involves p27 transcription, translation, stability and localization, and results in release of the cyclin-cdk complexes. CyclinD-cdk4 then phosphorylates the retinoblastoma protein pRB, which attenuates repression of the mitogenic E2F transcription factor, resulting in G1 to S progression (Sherr & Roberts, 1999). It is a matter of debate whether the induction of p27 by hypoxia is HIF-1α dependent (Carmeliet et al., 1998; Gardner et al., 2001; Goda et al., 2003; Mack et al., 2005).
Endometrial cancer is the most common malignant tumor of the female genital tract. Estimated incidence of cancer in the uterine corpus in the US is 39,080 for 2007 (6% of all cancers) (Jemal et al., 2007). HIF-1α is overexpressed in many cancers, including endometrial cancer and is associated with poor prognosis in stage 1 endometrial cancer (Horree et al., 2007a; Sivridis et al., 2002; Zhong et al., 1999).

In a recent study on cell cycle regulation during endometrial carcinogenesis, we observed a decrease in p27 expression in endometrial cancer compared to normal tissue and precursor lesions (Horree et al., 2007b). This has also been described before (Ozkara & Corakci, 2004). However, we observed a high re-expression p27 protein in perinecrotic (i.e. hypoxic) areas of endometrial carcinomas. Here, we analyzed the involvement of p27 in the hypoxic response *in vitro* and *in vivo* in endometrial cancer.

Our results demonstrate a critical role for p27 in the HIF-1 mediated hypoxic response of endometrial cancer cells *in vitro* and suggest a similar mechanism for cell survival in perinecrotic endometrial cancers.

Material and Methods

Patients and Tissues

Paraffin-embedded clinical specimens from endometrioid adenocarcinoma (EC, n=39) were selected from the archives of the Department of Pathology of the University Medical Center, Utrecht, The Netherlands. These tissues were derived from patients operated between 1991 and 2004. None of the carcinoma patients received preoperative radio- or chemotherapy. Table 1 gives an overview of the patient demographics and main pathological features.

Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital (van Diest, 2002).

Table 1. Patient characteristics and demographics

Clinicopathologic features of patients with endometrioid endometrial carcinoma		N=39	
		n	(%)
Age (yrs)	Mean	62.5	
	Minimum	40	
	Maximum	85	
Grade	1	11	(28.2%)
	2	17	(43.6%)
	3	11	(28.2%)
Myometrial Invasion	< 50%	17	(43.6%)
	≥ 50%	22	(56.4%)
Stage	1	13	(33.3%)
	2	10	(25.6%)
	3	11	(28.2%)
	4	5	(12.8%)

Immunohistochemistry

HIF-1α, p27 and Ki67 were immunohistochemically stained on 5 μm thick paraffin slides (Horree et al., 2007a; Horree et al., 2007b). Slides were deparaffinized with xylene and serial ethanol dilutions. For HIF-1α, target retrieval solution (Dako, Glostrup, Denmark) was used for antigen retrieval with all slides placed in a water bath for 45 minutes at 97°C. A cooling off period of 20 minutes preceded the incubation of the HIF-1α mouse monoclonal (BD Biosciences, Pharmingen, Lexington, USA), at a dilution of 1:500. The catalysed signal amplification system (Dako) was used according to the manufacturer's instructions to detect HIF-1α. For p27 and Ki67 staining, endogenous peroxidase activity was blocked for 30 minutes with a buffer solution containing peroxide, followed by antigen retrieval (citrate buffer, pH 6.0, 20 minutes). Next, for p27, slides were incubated with the primary antibody (anti-kip1/p27, BD Transduction Laboratories, USA; dilution 1:500, overnight 4°C), followed by the secondary antibody (Powervision (Poly-HRP-anti Ms/Rb/RtIgG biotin free), ImmunoVision Technologies, Brisbane, CA, USA; ready to use). For Ki67, slides were further processed in an automatic staining device. Incubation with the primary antibody (anti-Ki67, Immunotech, Beckman Coulter, Fullerton, CA, USA; dilution 1:100, 60 minutes, room temperature) was followed by the secondary antibody (biotinylated horse-anti-

mouse, diluted 1:500, Vector BA-2000, Vector Laboratories, Burlingame, CA, USA), followed by streptavidin-biotin complex, diluted 1:1000, Immunotech). All slides were developed with diaminobenzidine followed by haematoxylin counterstaining. Before the slides were mounted all sections were dehydrated in alcohol and xylene Appropriate positive and negative controls were used throughout.

Evaluation of Staining
Two authors (PvD, NH) scored all slides blinded to clinicopathologic data and results of the other stainings. The percentage of dark, homogenously stained nuclei was estimated as before (Bos et al., 2003; Horree et al., 2007a; Vleugel et al., 2005), ignoring cytoplasmic staining. The pattern of HIF-1α staining was described as perinecrotic, diffuse, or mixed. For p27, the pattern of staining was noted as perinecrotic, centrally in tumor islands, diffuse, or mixed. A perinecrotic staining is a type of staining in which cells only stain in the close vicinity of a necrotic field. As central and perinecrotic patterns were seen in combination and both likely represent staining in hypoxic areas, these were further grouped. The pattern of Ki67 staining was classified as diffuse (no association with necrosis) or anti-perinecrotic (less staining around necrosis).

HIF-1α/p27 double staining
To show colocalization of HIF-1α and p27, immunohistochemical double staining was performed. Slides were deparaffinized and dehydrated with xylene and serial ethanol dilutions. Endogenous peroxidase was blocked with a buffer containing peroxide for 15 minutes. Then, antigen retrieval was performed with EDTA buffer pH 9.0 for 20 minutes. After cooling down, slides were incubated with anti-HIF-1α antibody (mouse monoclonal, BD Transduction, dilution 1:50, overnight 4°C), followed by incubation with the secondary antibody Powervision (Poly-HRP-anti Ms/Rb/RtIgG biotin free, ImmunoLogic, ImmunoVision Technologies, Brisbane, CA, USA, ready to use) for 30 minutes. All slides were developed with diaminobenzidine followed by incubation with anti-p27 monoclonal antibody (Kip1, Transduction, dilution 1:500, overnight 4°C). Afterwards, slides were incubated with Fast Red substrate (Dako). Before the slides were mounted all sections were air-dried and put through xylene.

Cell culturing
Endometrial carcinoma cells, HEC-1B, obtained from American Type Culture Collection (LGC Promochem, Middlesex, UK) were cultured in minimal eagles medium (ATCC, LGC Promochem, Middlesex, UK) containing penicillin-streptomycin (Gibco BRL, UK) and 10% fetal calf serum (Gibco BRL, UK). For hypoxic treatment cells were incubated at 1% 0_2 in a hypoxia working station (Ruskin Technology, Leeds, United Kingdom).

Plasmids and transfection
Cells were transfected using Lipofectamin 2000© (Invitrogen, CA, USA) according to manufacturer's protocol. The HIF-1α knockdown plasmid was generated by ligation of annealed oligo sequences 5'-GATCCCCGGACAAG TCACCACAGGACTTCAAGAGA GTCCTGTGGTGACTTGTCCTTTTTG GAAA-3' and 5'-AGCTTTTCCAAAAAGGACA AGTCACCACAGGACT CTCTTGAAGTCCTGTGGTGACTTGTCCGGG-3' into Bgl II and Hind III digested pSuper-puro (kindly provided by Dr. Reuven Agami, Dutch Cancer Institute, Amsterdam).

Western blotting
Per lane, 20 μg protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blot analysis. The following antibodies were used; anti-HIF-1α (1:250) and p27 (1:1.000) (BD Transduction Laboratories, CA, USA), anti-β-actin (1:5.000) (US Biological, MA, USA), and goat anti-mouse IgG + IgM HRP conjungate (1:10.000) (Biosource, CA, USA). Enhanced Chemo Luminescence (ECL, Amersham Biosciences, UK) was used for signal detection according to the manufacturer's protocol.

FACS analysis
Cells were labelled with BrdU (Sigma-Aldrich Co., MO, USA) for two hours prior to trypsinisation and collection in 1ml of medium. Subsequently, cells were fixated by gently adding 4.5ml of 100% ethanol and left overnight. Cells were permeabilized and DNA was denatured by adding 500μl of a solution containing 5M HCl and 0.5% TritonX-100 for 20 minutes and were stained with anti-BrdU-FITC (Roche, IN, USA) (1:100) in PBS containing 1% BSA and 0.1% Tween-20 for 30 minutes. After washing the cells in PBS, they were stained with Propidium Iodide (Sigma-Aldrich Co., MO, USA) for 20 minutes and analyzed using a FACS Calibur system (BD Transduction Laboratories, CA, USA).

Statistical Analysis
To assess correlations between p27 expression and Ki67 patterns and clinicopathological data the Fisher's exact test was used. Two sided p-values <0.05 were considered significant. All statistical analyses were performed by using SPSS for Windows version 12.0.1., 2003 (SPSS Inc., Chicago, IL).

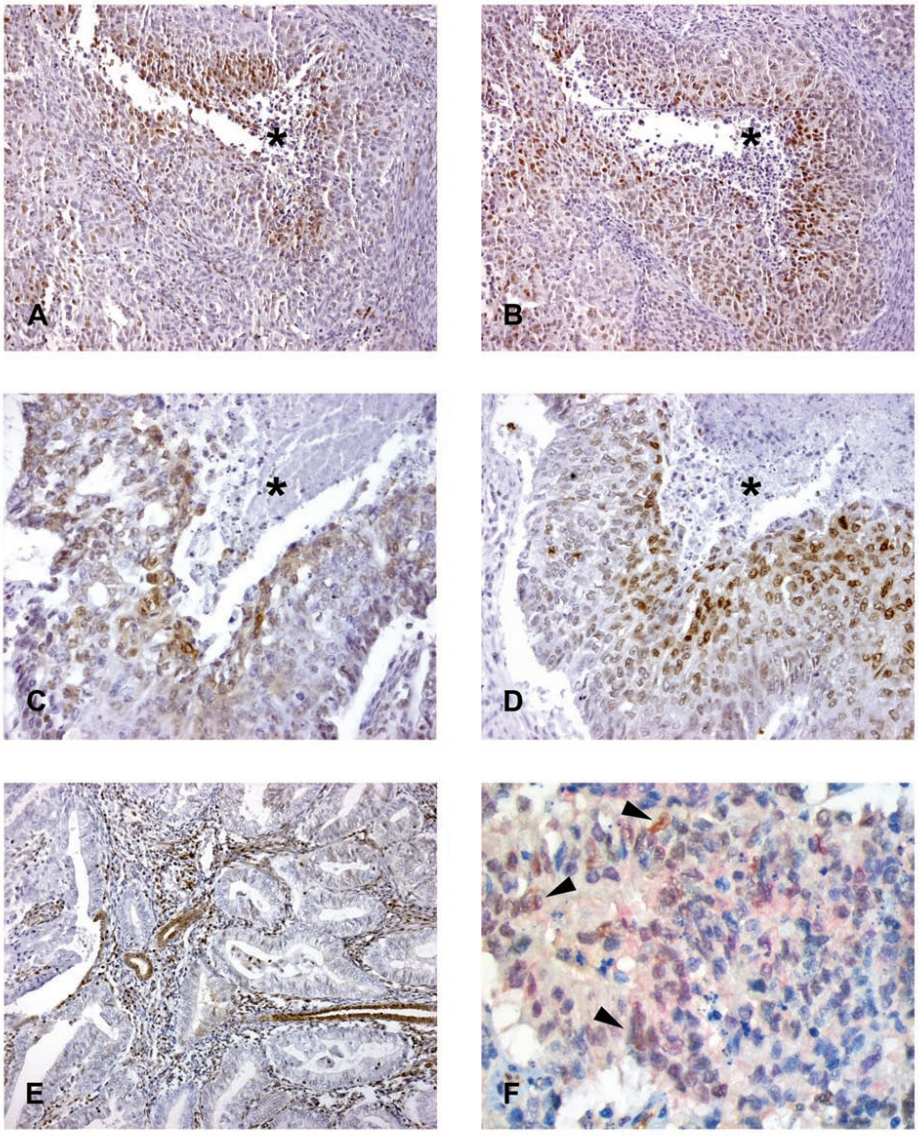

Figure 1. Immunohistochemical staining of p27 and HIF-1α in endometrial carcinoma.
Typical patterns are shown: A and B same tumor and consecutive slide with perinecrotic staining of p27 (A), perinecrotic staining of HIF-1α (B); C and D same tumor and consecutive slide with perinecrotic staining of p27 (C), perinecrotic staining of HIF-1α (D); tumor slide with 3 normal glands (E) the normal glands and the stroma around the glands express p27 and the tumor glands show loss of p27 staining; double staining p27 and HIF-1α (F) with examples of double positive cells indicated by arrows. Asterisk implies necrosis in the tumor.

Results

HIF-1α and p27 are perinecrotically co-expressed in endometrial cancers

To study the relationship between HIF-1α and p27 in endometrial carcinoma, we analyzed 39 tumor tissue sections for HIF-1α and p27 protein expression. We observed three patterns of HIF-1α immunostaining: exclusively perinecrotic (representing areas with low oxygen levels) (6/39, 15.4%), diffuse (11/39, 28.2%) or mixed (20/39, 51.3%), the remaining 2 (5.1%) were HIF-1α negative [21]. Expression of p27 showed differential types of staining patterns: a central/perinecrotic pattern (14/39, 35.9%), a diffuse pattern (17/39, 43.6%), or a mixed pattern (4/39, 10.3%), the remaining 4 (10.3%) were p27 negative. In 50% of the tumors (13/26) with perinecrotic HIF-1α expression, we observed central/perinecrotic p27 staining. In 9 of these, p27 expression was confined to these perinecrotic areas, suggesting hypoxia-induced p27 re-expression (Figure 1A-E). Co-expression of p27 and HIF-1α in perinecrotic areas was confirmed by HIF-1α/p27 double immunohistochemistry as shown in figure 1F.

Clinicopathological correlations

Staining pattern of p27 did not correlate with the clinicopathological survival parameters, age, stage or myometrial invasion. Central/perinecrotic p27 pattern was more often seen in higher grade endometrial cancers ($p = 0.039$).

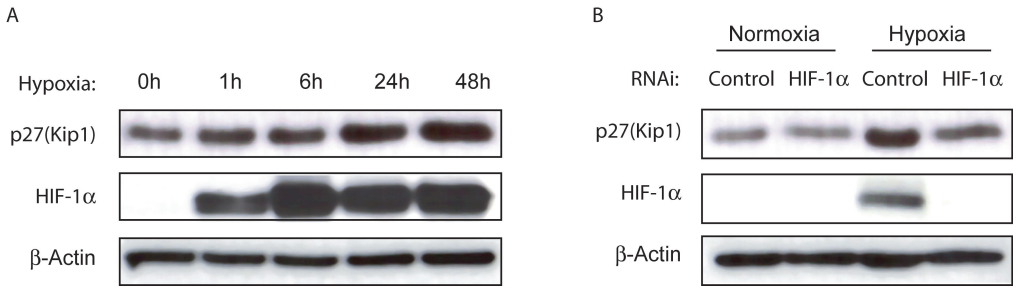

Figure 2. Hypoxia induces p27 expression in a HIF-1 dependent manner.
A. Western blot analysis of HIF-1α, p27 and β-actin in HEC-1B cells cultured under hypoxic conditions for indicated periods of time.
B. Western blot analysis of HIF-1α, p27 and β-actin in HEC-1B cells transiently transfected with (lanes 2 and 4) and without (lanes 1 and 3) a knockdown vector targeting HIF-1α, under normoxic (lanes 1 and 2) and hypoxic conditions (lanes 3 and 4). Control cells were transfected with an empty vector. When indicated cells were treated for 24 hours under hypoxic conditions. These blots are representative of at least two independent experiments.

p27 expression under hypoxia is HIF-1α dependent

Since we observed a co-expression of p27 and HIF-1α in a significant fraction of endometrial cancer, we addressed whether p27 is induced by hypoxia. Therefore, we analyzed p27 expression in HEC1B human endometrial carcinoma cells cultured for different time periods under hypoxic conditions. Normoxic HEC1B cells did not express HIF-1α and had modest p27 levels. Induction of HIF-1α levels was seen after 1 hour of hypoxia, which peaked at 6 hours and persisted up to 48 hours of hypoxia (Figure 2A). The expression of p27 was increased after 24 hours of hypoxia and after 48 hours of hypoxia. To study whether this induction is dependent on HIF-1α, we used RNA interference (RNAi) to knock down HIF-1α in HEC1B cells. RNAi against HIF1α markedly reduced hypoxia-induced HIF-1α levels (Figure 2B). This reduced HIF-1α completely blocked the induction of p27 by hypoxia, indicating that hypoxia-induced p27 expression is HIF-1α dependent.

Hypoxia induces a G1 and G2/M arrest

Expression of p27 induces cell cycle arrest by blocking the G1 to S-phase transition [14]. As hypoxia induces p27, we wondered whether hypoxia can induce G1 arrest in endometrial carcinoma cells. Therefore we compared the cell cycle profile of HEC1B cells cultured for 24 hours under hypoxic or normoxic conditions by FACS analysis. Hypoxia resulted in a decrease in

p27 re-expression leads to diminished proliferation around necrosis

Previous results suggest that one of the roles of p27 re-expression after oxygen deprivation in endometrial carcinomas is the induction of cell cycle arrest. To support this hypothesis we investigated the expression of cell division marker Ki67 in the perinecrotic tumor areas. 39 tumor sections were immunohistochemically stained for Ki67 and scored for patterns of staining. In 5 tumors Ki67 count was too low to be assessed for staining pattern and therefore not considered in this analysis. Table 2 shows staining results. An anti-perinecrotic staining was observed in 12 tumors, a diffuse staining in 22 cases. Perinecrotic p27 expression colocalized significantly with anti-perinecrotic staining of Ki67 in 9/14 cases (64.3%, p=0.012, Fischer's exact test) (Figures 3C, D and E). These data indicate that hypoxic p27 re-expression functions to cease cell growth in perinecrotic tumor fields.

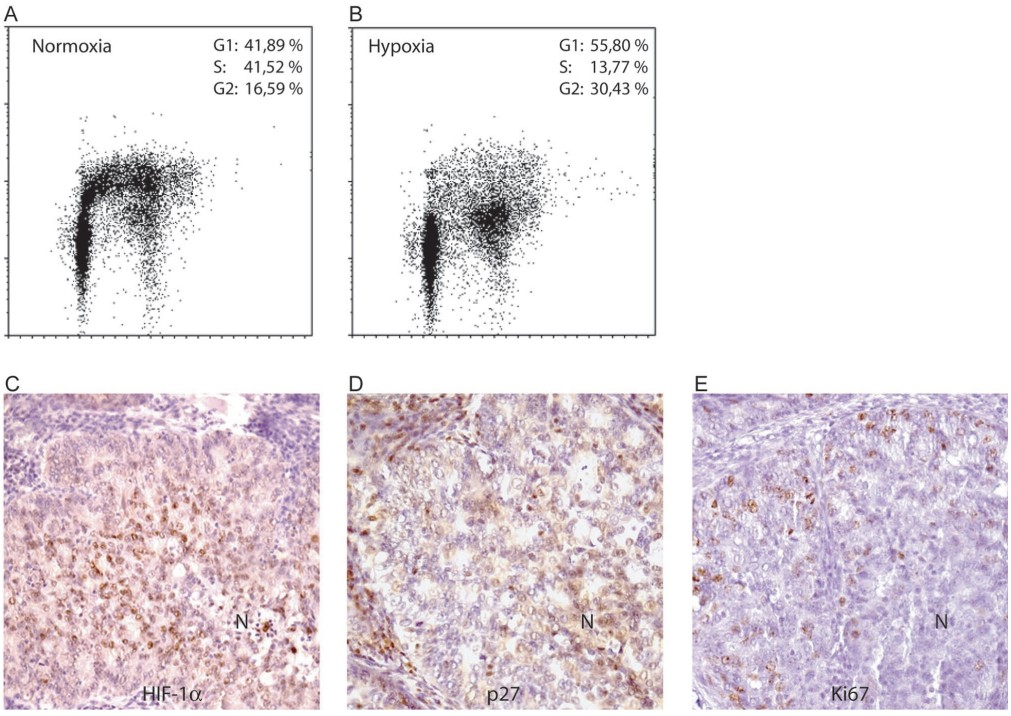

Figure 3. FACS analysis and Ki67 staining.
(A-B) HEC1B cells were cultured for 24 hours under normoxic or hypoxic conditions, labeled with BrdU (y-axis) and Propidium Iodide (x-axis) and subjected to FACS. Per experiment 10.000 cells were counted. G1, G2 and S-phase content were calculated by manual gating. The same gates were used for both the normoxia (A) and hypoxia (B) experiments. Results were independently reproduced three times and a representative experiment is shown. (C-E) Consecutive slides of the same tumor showing perinecrotic HIF-1α staining (C), central/perinecrotic p27 staining (D) and anti-perinecrotic Ki67 staining (E). N = Necrosis. amount of cells in the S-phase from 41,52% to 13,77%, whereas both G1 and G2/M phase were enriched from 41,89% to 55,80 and from 16,59 to 30,43% respectively (Figures 3A and B). This demonstrates that hypoxic endometrial carcinoma cells undergo a block in G1 and G2/M in response to hypoxia.

Table 2. Cross table of patterns of immunohistochemical p27 and Ki67 staining of endometrial cancer.

		Ki67		Total
		Diffuse	Anti-perinecrotic	
p27	Negative	3	0	3
	Diffuse and mixed	14	3	17
	Central and perinecrotic	5	9	14
Total		22	12	34

Discussion

HIF-1α is a key regulator for survival of hypoxic tumor cells. However, the function of HIF-1α in hypoxia-induced cell cycle arrest is less well understood. We investigated associations between HIF-1α and p27 in endometrioid endometrial carcinoma, the most frequent histological subtype of endometrial cancer. Often p27 expression was seen around necrotic, hypoxic areas in otherwise p27 negative cancers. As p27 is highly expressed in the normal endometrium but gets down regulated during carcinogenesis, this points to hypoxia induced perinecrotic re-expression of p27 (Horree et al., 2007b). Perinecrotic re-expression of p27 was more often seen in higher grade endometrial cancers. We propose that the central expression of p27 that was sometimes observed in solid tumor groups in tumors with perinecrotic p27 expression may be an early indicator of hypoxia. This perinecrotic expression of p27 has not been described before in endometrial carcinomas but has been noted in melanomas and in gliomas, although these studies did not compare p27 to HIF-1α expression (Murphy et al., 2004; Zagzag et al., 2003).

Hypoxia is known to upregulate p27 but there are some conflicting data on the role of HIF-1 in this process and implications for G1/S cell cycle arrest (Box & Demetrick, 2004; Carmeliet et al., 1998; Gardner et al., 2001; Goda et al., 2003; Green et al., 2001). Hypoxic regulation of p27 has been shown to be regulated via a HIF-1 independent region of the proximal p27 promoter in mouse embryonic fibroblasts (MEFs), although it has also been shown that gene silencing of HIF-1α by small interfering RNA reduces p27 protein and mRNA levels (Gardner et al., 2001; Mack et al., 2005). Furthermore, in HIF-1α wild-type splenic B lymphocytes induction of p27 was seen after hypoxia, which was absent in HIF-1α double knock-out cells. However, HIF-1α knockout ES cells still upregulate p27 after hypoxia (Carmeliet et al., 1998). Green et al showed that p21/p27 double knock-out mice are still able to initiate a G1/S arrest, however Mack et al showed that in VHL knock-out MEFs increase of p27 was associated with diminished proliferation (Green et al., 2001; Mack et al., 2005). Thus, these studies do not uniformely show whether p27 is regulated by HIF-1α.

Differences between other studies and our results might be explained by different experimental cell systems used, which is endorsed by data from Box et al., who showed that different cell lines differed in their ability to upregulate p27 and to undergo G1/S cell cycle arrest in response to hypoxia within one study with equal experimental environmental conditions (Box & Demetrick, 2004).

However, our results provide evidence for *in vivo* dynamics apart from *in vitro* results. We show that the induction of p27 during hypoxia is HIF-1α-dependent in the endometrial carcinoma cell line HEC1B. Our study provides further insight into the mechanism governing the re-expression of p27 in perinecrotic areas of endometrial cancer.

Recently, p27 was shown to be regulated by the energy sensing LKB1-AMPK pathway, via AMPK-dependent phosphorylation, which increased stability of p27 (Liang et al., 2007). The stable form of p27 was sufficient to induce autophagy, an evolutionarily conserved process wherein catabolism of intracellular organelles generates energy. Hypoxia can also induce LKB-AMPK activity because of failure to generate sufficient ATP required for cellular functions (Laderoute et al., 2006; Lee et al., 2003; Marsin et al., 2000). This process turned out to be independent of HIF-1α in MEFs (Laderoute et al., 2006). In DU145 cells (prostate cancer) however, it has been shown that AMPK activity itself is critical for the HIF-1 transcriptional activity and its target expression, which implicates that HIF-1 is situated downstream in this pathway (Lee et al., 2003). It might be of significance to search for the role of AMPK in hypoxic, HIF-1α dependent induction of p27 in (endometrial) cancer development.

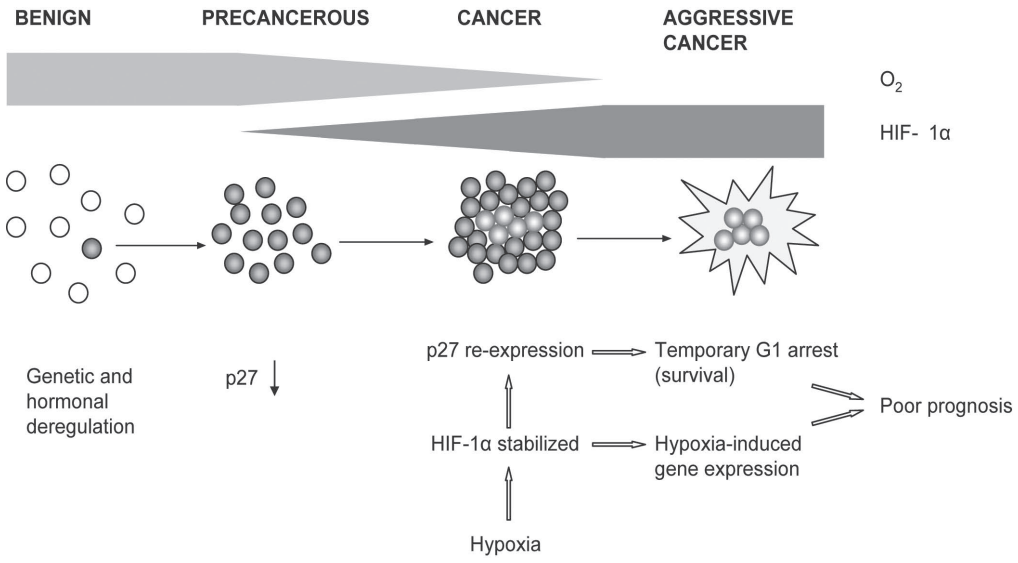

Figure 4. Schematic illustration of the HIF-1α-p27 pathway in endometrial carcinogenesis.

In addition to p27, earlier research has focused on the interaction between HIF-1α and other cell cycle regulators and apoptosis. The role of HIF-1 in hypoxia to induce apoptosis is nicely reviewed by Greijer and van der Wall (Greijer & van der Wall, 2004). P53 is an important regulator of the cell cycle and apoptosis. Severe hypoxia (less then 0.2%O_2) induces p53 in a HIF-1α dependent manner (An et al., 1998; Carmeliet et al., 1998). Hypoxic apoptosis might involve interaction between HIF-1α and p53, as well as induction of Bax, downstream effector of p53 (Hansson et al., 2002; Sanchez-Puig et al., 2005; Wincewicz et al., 2007). P53 and HIF-1α are competitors for the cofactor p300 and MDM2-mediated degradation (Blagosklonny et al., 1998; Chen et al., 2003; Ravi et al., 2000). Thus, a loss of wild-type p53 might be associated with increased tumor growth during hypoxia because of diminished apoptosis and augmented HIF-1α induced transcriptional activation of downstream factors for survival of tumor cells. This might include perinecrotic p27 expression.
We conclude that in endometrioid endometrial carcinoma, p27 up regulation by hypoxia is HIF-1α dependent. Furthermore, hypoxic HEC1B cells undergo a partial G1 arrest, corroborated by lack of staining for the proliferation marker Ki67 in perinecrotic p27/HIF-1α expressing cells in tumor sections. This cell cycle arrest may contribute to survival of cancer cells in hypoxic tumor areas. We propose a model for endometrioid carcinogenesis, which involves early loss of p27, which facilitates proliferation, and late HIF-1-mediated re-expression of p27, that results in dormancy of these hypoxic cells (Figure 4). Combined with the hypoxia induced expression of genes regulating supply of energy, growth factors and other survival factors, this may promote survival and adaptation of sub clones within the tumor that may contribute to metastatic disease and poor clinical outcome.

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References

Alfsen GC, et al. (2003). The prognostic impact of cyclin dependent kinase inhibitors p21WAF1, p27Kip1, and p16INK4/MTS1 in adenocarcinomas of the uterine cervix: an immunohistochemical evaluation of expression patterns in population-based material from 142 patients with international federation of gynecology and obstetrics stage I and II adenocarcinoma. *Cancer*, **98,** 1880-9.

An WG, et al. (1998). Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. *Nature*, **392,** 405-8.

Blagosklonny MV, et al. (1998). p53 inhibits hypoxia-inducible factor-stimulated transcription. *J Biol Chem*, **273,** 11995-8.

Bos R, et al. (2003). Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*, **97,** 1573-81.

Box AH and Demetrick DJ. (2004). Cell cycle kinase inhibitor expression and hypoxia-induced cell cycle arrest in human cancer cell lines. *Carcinogenesis*, **25,** 2325-35.

Carmeliet P, et al. (1998). Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature*, **394,** 485-90.

Chen D, et al. (2003). Direct interactions between HIF-1 alpha and Mdm2 modulate p53 function. *J Biol Chem*, **278,** 13595-8.

Cockman ME, et al. (2000). Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J Biol Chem*, **275,** 25733-41.

Gamboa-Dominguez A, et al. (2007). Prognostic significance of p21WAF1/CIP1, p27Kip1, p53 and E-cadherin expression in gastric cancer. *J Clin Pathol*, **60,** 756-61.

Gardner LB, et al. (2001). Hypoxia inhibits G1/S transition through regulation of p27 expression. *J Biol Chem*, **276,** 7919-26.

Goda N, et al. (2003). Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. *Mol Cell Biol*, **23,** 359-69.

Green SL, et al. (2001). p21(Cip1) and p27(Kip1) regulate cell cycle reentry after hypoxic stress but are not necessary for hypoxia-induced arrest. *Mol Cell Biol*, **21,** 1196-206.

Greijer AE and van der Wall E. (2004). The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J Clin Pathol*, **57,** 1009-14.

Hansson LO, et al. (2002). Two sequence motifs from HIF-1alpha bind to the DNA-binding site of p53. *Proc Natl Acad Sci U S A*, **99,** 10305-9.

Hockel M, et al. (1996). Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res*, **56,** 4509-15.

Horree N, et al. (2007a). Hypoxia and angiogenesis in endometrioid endometrial carcinogenesis. *Cell Oncol*, **29,** 219-27.

Horree N, et al. (2007b). Progressive derailment of cell cycle regulators in endometrial carcinogenesis. *J Clin Pathol*.

Hui AM, et al. (1998). Cell cycle regulators and human hepatocarcinogenesis. *Hepatogastroenterology*, **45,** 1635-42.

Hutter G, et al. (2006). Differential effect of epigenetic alterations and genomic deletions of CDK inhibitors [p16(INK4a), p15(INK4b), p14(ARF)] in mantle cell lymphoma. *Genes Chromosomes Cancer*, **45,** 203-10.

Jemal A, et al. (2007). Cancer statistics, 2007. *CA Cancer J Clin*, **57,** 43-66.

Laderoute KR, et al. (2006). 5'-AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments. *Mol Cell Biol*, **26,** 5336-47.

Lee JW, et al. (2004). Hypoxia-inducible factor (HIF-1)alpha: its protein stability and biological functions. *Exp Mol Med*, **36,** 1-12.

Lee M, et al. (2003). AMP-activated protein kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells. *J Biol Chem*, **278,** 39653-61.

Liang J, et al. (2007). The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat Cell Biol*, **9,** 218-24.

Mack FA, et al. (2005). Decreased growth of Vhl-/- fibrosarcomas is associated with elevated levels of cyclin kinase inhibitors p21 and p27. *Mol Cell Biol*, **25,** 4565-78.

Marsin AS, et al. (2000). Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Curr Biol*, **10,** 1247-55.

Massague J. (2004). G1 cell-cycle control and cancer. *Nature*, **432,** 298-306.

Murphy M, et al. (2004). Hypoxia regulation of the cell cycle in malignant melanoma: putative role for the cyclin-dependent kinase inhibitor p27. *J Cutan Pathol*, **31,** 477-82.

Ozkara SK and Corakci A. (2004). Significantly decreased P27 expression in endometrial carcinoma compared to complex hyperplasia with atypia (correlation with p53 expression). *Pathol Oncol Res*, **10,** 89-97.

Pucci B and Giordano A. (1999). Cell cycle and cancer. *Clin Ter*, **150,** 135-41.

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

Sanchez-Puig N, et al. (2005). Binding of natively unfolded HIF-1alpha ODD domain to p53. *Mol Cell*, **17,** 11-21.

Sherr CJ and Roberts JM. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev*, **13,** 1501-12.

Sivridis E, et al. (2002). Association of hypoxia-inducible factors 1alpha and 2alpha with activated angiogenic pathways and prognosis in patients with endometrial carcinoma. *Cancer*, **95,** 1055-63.

van Diest PJ. (2002). No consent should be needed for using leftover body material for scientific purposes. For. *BMJ*, **325,** 648-51.

Vaupel P and Mayer A. (2005). Hypoxia and anemia: effects on tumor biology and treatment resistance. *Transfus Clin Biol*, **12,** 5-10.

Vleugel MM, et al. (2005). Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in invasive breast cancer. *J Clin Pathol*, **58,** 172-7.

Vrekoussis T, et al. (2005). Endothelial p21(WAF1/CiP1) cell cycle inhibitor is down-regulated in breast cancer. *Anticancer Res*, **25,** 2743-8.

Wang GL, et al. (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,** 5510-4.

Wincewicz A, et al. (2007). Cumulative expression of HIF-1-alpha, Bax, Bcl-xL and P53 in human colorectal cancer. *Pathology*, **39,** 334-8.

Zagzag D, et al. (2003). Expression of p27KIP1 in human gliomas: relationship between tumor grade, proliferation index, and patient survival. *Hum Pathol*, **34,** 48-53.

Zhong H, et al. (1999). Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res*, **59,** 5830-5.

Chapter 5

The *TWIST1* oncogene is a direct target of Hypoxia Inducible Factor-2α

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Abstract

Hypoxia-inducible Factors (HIFs) are highly conserved transcription factors that play a crucial role in oxygen homeostasis. Intratumoral hypoxia and genetic alterations lead to HIF activity, which is a hallmark of solid cancer and is associated with poor clinical outcome. HIF activity is regulated by an evolutionary conserved mechanism involving oxygen-dependent HIFα protein degradation. To identify novel components of the HIF pathway we performed a genome wide RNA interference screen in *C. elegans*, to suppress HIF-dependent phenotypes, like egg-laying defects and hypoxia survival. In addition to *hif-1* (HIFα) and *aha-1* (HIFβ), we identified *hlh-8*, *gska-3* and *spe-8*. The *hlh-8* gene is homologous to the human oncogene *TWIST1*. We show that *TWIST1* expression in human cancer cells is enhanced by hypoxia in a HIF-2α-dependent manner. Furthermore, intronic hypoxia response elements of *TWIST1* are regulated by HIF-2α, but not HIF-1α. These results identify *TWIST1* as a direct target gene of HIF-2α, which may provide insight into the acquired metastatic capacity of hypoxic tumors.

Introduction

One of the hallmarks of solid cancer development is reduced tissue oxygen tension (hypoxia) within the tumor, encountered when tumors outgrow the surrounding blood supply (Vaupel et al., 2001). Hypoxia is correlated with therapy resistance and enhanced metastatic potential, resulting in poor prognosis in many cancer types (Vaupel & Mayer, 2005). The Hypoxia Inducible Factor (HIF) transcription factor plays a central role in the molecular response to hypoxia. In human cells the HIF complex consists of a constitutively expressed β-subunit and an α-subunit, which is regulated by oxygen tension (Semenza, 2004). In the presence of oxygen, HIFα is hydroxylated on proline residues by prolyl hydroxylases (PHDs). Hydroxylated HIF proteins are substrates for the VHL tumor suppressor protein that acts as an E3-ubiquitin ligase targeting hydroxylated-HIFα for proteasomal degradation. Under hypoxia, HIFα is not hydroxylated and becomes stabilized, dimerizes with HIFβ and translocates to the nucleus where it mediates target gene activation. Among HIF target genes are glycolytic genes [e.g. Phosphoglycerate Kinase 1 (*PGK1*)] and genes involved in angiogenesis [e.g. Vascular Endothelial Growth Factor (*VEGF*)], which are key players in the hypoxic response (Greijer et al., 2005). The human genome encodes three homologous HIFα proteins, respectively HIF-1, -2, and -3α (Ema et al., 1997; Gu et al., 1998; Wang et al., 1995) and three prolyl hydroxylases (Epstein et al., 2001; Semenza, 2001). The mechanism of oxygen sensing via the HIF pathway is conserved throughout evolution. The nematode Caenorhabditis elegans (C. elegans) has a single homologue for each component (HIF-1 (HIFα), AHA-1 (HIFβ), VHL-1 (VHL), and the dioxygenase EGL-9 that regulates HIF prolyl hydroxylation) (Shen & Powell-Coffman, 2003). This reduced complexity of the HIF-pathway facilitates genetic analysis. In its natural habitat *C. elegans* is exposed to variable oxygen tension in the soil. Wild type animals are capable of maintaining metabolism and reproduction in conditions up to 0.5% oxygen. *C. elegans* carrying deletions in *hif-1* are apparently healthy under standard culture conditions, but exhibit high levels of lethality in 0.5% or 1% oxygen (Jiang et al., 2001; Padilla et al., 2002). Furthermore, *egl-9* mutants have constitutive HIF activity, due to loss of HIF-1 hydroxylation (Epstein et al., 2001). These mutants are egg-laying defective (Trent et al., 1983). Eggs are maintained in the gonads where they eventually hatch, resulting in laceration of the vulva, killing the mother, while producing viable offspring. Mutation of both *hif-1* and *egl-9*, suppresses the egg-laying defect, demonstrating that this phenotype is HIF-1 dependent (Bishop et al., 2004). Because of the ability to conduct genome-wide loss of function analysis

using RNAi feeding in *C. elegans*, we chose to exploit the *egl-9* mutant phenotype to uncover novel HIF pathway components.

Using this approach, we identified novel genes required in the EGL-9/HIF-1 pathway and for survival under hypoxia. One of these is *hlh-8*, a basic helix loop helix transcription factor involved in *C. elegans* vulval development (Harfe et al., 1998). The *hlh-8* gene is homologous to the human oncogene *TWIST1*. We show that *TWIST1* is regulated in a HIF-dependent manner under hypoxia in mammalian cells. These findings provide novel insight into how intratumoral hypoxia can promote cancer metastasis (Woelfle et al., 2003).

Material and Methods

Strains and nematode culturing

Standard *C. elegans* culturing methods were used. The following *C. elegans* strains were used: wild-type Bristol N2, ZG31 *hif-1(ia4)*, JT307 *egl-9(sa307)*, MT1201 *egl-9(n571)* and RB1034 *gska-3(ok970)* (source Caenorhabditis Genetics Center, and *C. elegans* knock-out consortium (RB1034)). Strains were maintained at 20˚C. RNA interference (RNAi) was performed by feeding worms Escherichia coli strain HT115 (DE3) expressing double stranded (ds) RNA. Plasmids for dsRNA production were derivatives of the L4440 vector and were obtained from J. Ahringer (Cambridge, United Kingdom).

RNAi screen in Liquid 96-Well Culture

Inoculation and induction of bacteria and high throughput screening in liquid culture was done as previously described (Kamath & Ahringer, 2003; van Haaften et al., 2004). Worm cultures were synchronized by bleaching and hatched in M9 at 20°C overnight. For RNAi liquid cultures 20-30 L1 larvae were resuspended in 50 µl of M9 per well in flat-bottom 96-well tissue-culture plates and 75 µl of bacteria were added. The egg-laying defective phenotype is thermosensitive and therefore, animals were cultured at 25˚C (Trent et al., 1983). Per RNAi, twenty-five synchronized L1 larvae from one of both *egl-9* defective strains (sa307 and n571) were cultured. Occurrence of F1 (eggs and larvae) was determined between 48 and 72 hours of growing under permissive conditions of 25˚C. Addresses displaying progeny after 48 hours in one or both strains and/or after 72 hours in both strains were marked positive and verified by sequencing.

Hypoxia sensitivity Assay

RNAi bacteria from an overnight culture in Luria broth medium containing 50 μg·ml-1 ampicillin were induced with 0.25 mg·ml-1 isopropylthiogalactoside at 37°C for 4 h and then seeded on 4-cm nematode growth-medium RNAi plates containing 50 μg·ml-1 ampicillin and 200 μg·ml-1 isopropylthiogalactoside. Roughly 30 synchronized L1 larvae (P0) were seeded on fresh RNAi plates and incubated for 24 hours at 25°C under 20% oxygen. Subsequently, the plates were exposed to hypoxia (1% O2) in an Invivo 1000 hypoxia workstation (Ruskin Technology, Leeds, United Kingdom) for 120 hours at 25°C. The amount of viable offspring (F1) was determined.

Cell culturing, transfection and luciferase assay

HeLa cells, PC3 cells, HCT116 cells and HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (Gibco BRL, UK) supplied with 10% Fetal Calf Serum (FCS) and antibiotics (Gibco BRL, UK). When indicated, cells were exposed to hypoxia (1% O2) using the hypoxic workstation. HeLa cells were transfected using Lipofectamine 2000© (Invitrogen, CA, USA) according to the manufacturers protocol. HEK293T were transfected using polyethylenimine (Polysciences, PA, USA). Stable cell lines were generated by infection of HeLa cells expressing an ecotropic receptor with supernatant from Phoenix cells transfected with pRetroSuper containing hairpin sequences directed against either HIF-1α or HIF-2α. Polyclonal lines were cultured continuously under 2 μg/ml puromycin.

For luciferase reporter assays, cells were lysed in passive lysis buffer (Promega Corporation, WI, USA) according to the manufacturers protocol. Experiments were performed in triplicate. Samples were assayed with a dual luciferase kit (Promega Corporation, WI, USA) and measured using a VeritasTM 96-well plate luminometer (Turner Biosystems, CA, USA). All values were normalized using TK-Renilla (Promega) expression.

Cloning, plasmids and RNAi

The 5' intronic region of *TWIST1* was cloned by PCR on genomic DNA using forward primer (1); 5'-GCGCCATTGCTGCTGTCAC-3', and reverse primer (2); 5'-GGACGGACGGGAGGGACC-3'. The 3' intronic region of TWIST1 was cloned by PCR on genomic DNA using forward primer (3); 5'-cagtccacctcgatttcctc-3', and reverse primer (4); 5'-gtgaccctgggtgtctctgt-3'. Products were subcloned from pCR2.1 (Invitrogen, CA, USA) into pGL3-prom (Promega Corporation, WI, USA) using KpnI and XhoI (Roche, Switzerland). Base pair substitutions were constructed by PCR on plasmid DNA using (A)

forward primer (3) and reverse primer (5); 5'-cctgtcacgcacactcaaacgcac-3', or reverse primer (6); 5'cctgtcaaacacactcacgcgcac-3', and (B) forward primer (7); 5'-gtgcgtttgagtgtgcgtgacagg-3', or forward primer (8); 5'-gtgcgcgtgagtgtgtttgacagg-3', and reverse primer (4). The fragment obtained after PCR on PCR products A and B using forward primer (3) and reverse primer (4) was subcloned as described above. All constructs were verified by sequencing. The expression plasmids pCEP4-HIF-1α and pcDNA3-HIF-2α and the 5xHRE/hCMVmp-luc reporter construct were previously described (Forsythe et al., 1996; Shibata et al., 1998; Tian et al., 1997). Full-length HIF-2α-Flag was constructed by digestion of pcDNA3-HIF-2α using BamHI and cloning into the p3XFLAG-CMV™-10 expression vector (Sigma, MO, USA). This plasmid was then *EcoRI* digested and the removed fragment was replaced by a PCR-derived fragment from pcDNA3-HIF-2α using primers forward 5`-cgcgaattcATGACAGCTGACAAGGAGAAGAAAAGGAG-3` and reverse 5`-tggtagaattcataggctgagcggccaagcagctc-3`. Next, the plasmid was HindIII digested and overhangs were filled in with Klenow and backligated, resulting in HIF-2α-Flag. HIF-2α-FlagΔ1-278 was generated by digestion of the HIF-2α-Flag construct using *EcoRI* and subsequent backligation, losing the *EcoRI* fragment. The HIF-2α DNA binding domain construct (HIF-2α-Δ281-870) contains amino acid 1 to 280, encompassing the bHLH domain and part of the PAS domain. Short interfering RNA oligos were obtained from Ambion (CA, USA); siRNA ID #114178 (HIF-1β), #114177(HIF-1β), #147201(HIF-1β) and Silencer®negative control #1 siRNA. Knockdown plasmids were generated by ligation of annealed oligo sequences 5'-GATCC CCGGACAAGTCACCACAGGACttcaagagaGTCCTGTGGTGACTTGTC CTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAGGACAAGTCACCAC AGGACtctcttgaaGT CCTGTGGTGACTTGTCCGGG-3' for HIF-1α and 5'-GATCCCCGGAGACGGAG GTGTTCTATTTCAAGAGAATAGAACACCT CCGTCTCCTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAGGAGACG GAGGTGTTCTATTCTCTTGAAATAGAACACCTCCGTCTCCGGG-3' for HIF-2α into *BglII* and *HindIII* digested pRetroSuper-puro (kindly provided by Dr. Reuven Agami, Netherlands Cancer Institute, Amsterdam).

RNA analysis

RNA from total worm lysate and total mammalian cell lysates was prepared by homogenation in Trizol reagent (Invitrogen, CA, USA) followed by chloroform/phenol extraction. cDNA was prepared from 1 μg of RNA using Reverse Transcriptase (Roche, Germany) and oligo-(dT) primers (Invitrogen, CA, USA). Quantitative PCR for C. elegans genes was performed using SYBR green

(Applied Biosystems, CA, USA). Specific primer sequences for F22B5.4 and *inf-1* were kindly provided by dr. Powell-Coffman (Shen et al., 2006). Q-PCR for human genes was performed using commercially available TaqMan assays for PGK1 (Hs99999906_m1), TWIST1 (Hs00361186), and hydroxymethylbilane synthase (HMBS) (Hs00609297_m1) (Applied Biosystems, CA, USA) using an ABI7900 analyzer (Applied Biosystems, CA, USA). Data were analyzed using the SDS2.2.1 program (Applied Biosystems, CA, USA). HMBS was used for normalization.

Western blotting

20 μg protein/lane was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blot analysis. The following antibodies were used; anti-HIF-1α and anti-HIF-1β (BD Transduction Laboratories, CA, USA), anti-HIF-2α (NOVUS Biologicals, CO, USA), anti-Flag M2 (Sigma, MO, USA), anti-β-actin (US Biological, MA, USA), goat anti-mouse IgG + IgM HRP conjungate (Biosource, CA, USA) and goat anti-rabbit IgG HRP conjungate (Biorad, CA, USA). Enhanced Chemo Luminescence (ECL, Amersham Biosciences, UK) was used for signal detection according to the manufacturers protocol.

Results

Genome-wide screening allows identification of HIF-pathway members

C. elegans egl-9 mutants display an egg-laying defect, visible by an accumulation of eggs in the animal and no or few laid eggs (Trent et al., 1983). The *egl-9* phenotype is suppressed in *hif-1* and *egl-9* double mutants, suggesting that HIF-1 pathway members downstream of *egl-9* are able to modulate the *egl-9* egg-laying defect (Bishop et al., 2004). We reasoned that we could exploit this epistatic relationship to uncover novel genes in the hypoxic response pathway. First, we tested whether knocking down *hif-1* via RNA interference would produce a similar suppression as genetic ablation. Indeed *hif-1* RNAi suppressed the egg-laying phenotype of both *egl-9* mutants tested (*sa307* and *n571*) visible by the presence of eggs and larvae in the medium (Figure 1A).

Subsequently, we performed a genome wide RNAi screen in liquid culture using a RNAi library targeting 86% of *C. elegans* genes (Kamath et al., 2003; van Haaften et al., 2004). We identified 137 independent RNAi's that suppressed the *egl-9* phenotype. (Figure 1B, supplementary data). These included both

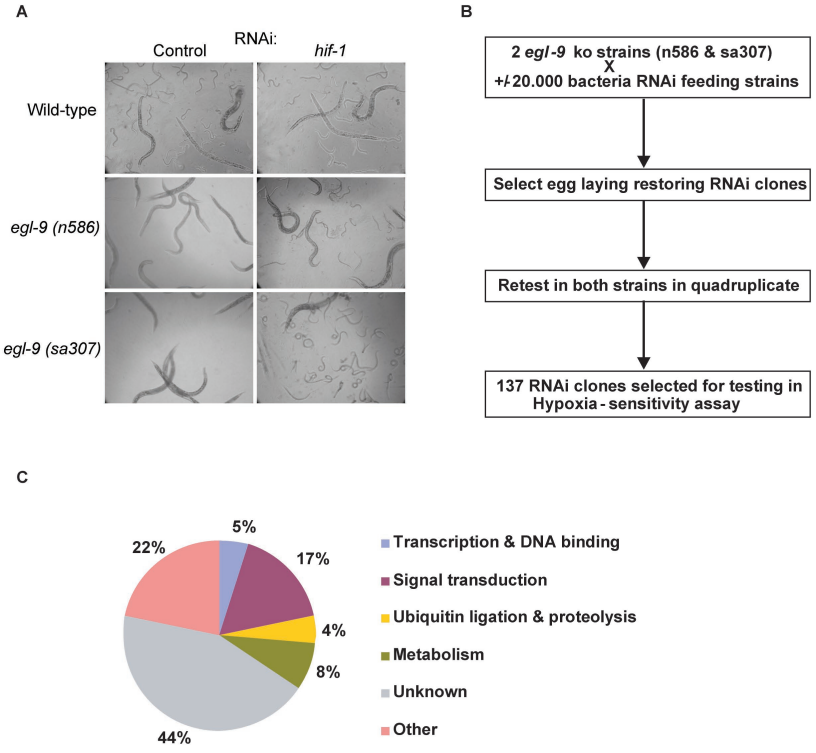

Figure 1.
Genome-wide screening for *egl-9* phenotype suppression to identify HIF pathway members. (a) Photographs of liquid cultures of *C. elegans* strains carrying wild type, n586 or sa307 alleles for *egl-9*. Animals were treated with *hif-1* RNAi (right panels) or empty vector control (left panels). (b) Schematic drawing of screening set-up and selection procedures. (c) Representation of 137 genes identified in the egg-laying defect screen organized by gene ontology.

RNAi's directed against *hif-1* and *aha-1*, validating our screen. Furthermore, several genes involved in ubiquitin ligation, like *ubc-20* and *ubc-21*, and genes involved in MAPK and AKT signaling pathways were identified (Figure 1C, supplementary data).

HIF-1, AHA-1, SPE-8, GSKA-3 and HLH-8 are necessary for hypoxic survival
Suppressors of the *egl-9* phenotype do not necessarily function in the hypoxia response. Animals with a loss of function mutation in *hif-1* are known to adapt poorly to oxygen levels between 0.5 and 2% O2 (Jiang et al., 2001). To enrich for genes that function in the hypoxia pathway, we rescreened candidate RNAi's for enhanced sensitivity to hypoxia. Wild type animals were grown under either normoxic or hypoxic (1% O2) conditions and the amount of viable offspring was

determined. Under these conditions ~25% of the wild type offspring survived prolonged exposure to hypoxia (figure 2A). Of the 137 RNAi clones examined, five resulted in a dramatic decrease in viability to less than 1% under hypoxia compared to normoxia (Figure 2A). As expected, these RNAi clones included *hif-1* and *aha-1*. In addition, RNAi foods directed against three additional genes (*spe-8*, *gska-3* and *hlh-8*) were identified. To confirm this, we tested hypoxia sensitivity of available mutant strains *hif-1* and *gska-3*. Both knockout strains phenocopied hypoxia sensitivity similar to genetic knockdown (Figure 2B). Hypoxia sensitivity might be explained by defects in HIF-1 function, caused either by reduced protein levels (*hif-1* RNAi) or reduced activity (*aha-1* RNAi). Next we determined the effect of RNAi on the HIF-1 target gene F22B5.4 (Bishop et al., 2004; Shen et al., 2005). In control animals, F22B5.4 was induced approximately 200 fold by hypoxia compared to normoxia (Figure 2C). RNAi against *hif-1* and *aha-1* almost completely abolished F22B5.4 induction, consistent with their role in HIF-1 mediated transcription. RNAi directed against *spe-8*, *gska-3* or *hlh-8* however did not reduce F22B5.4 induction, suggesting that these genes do not regulate F22B5.4 expression.

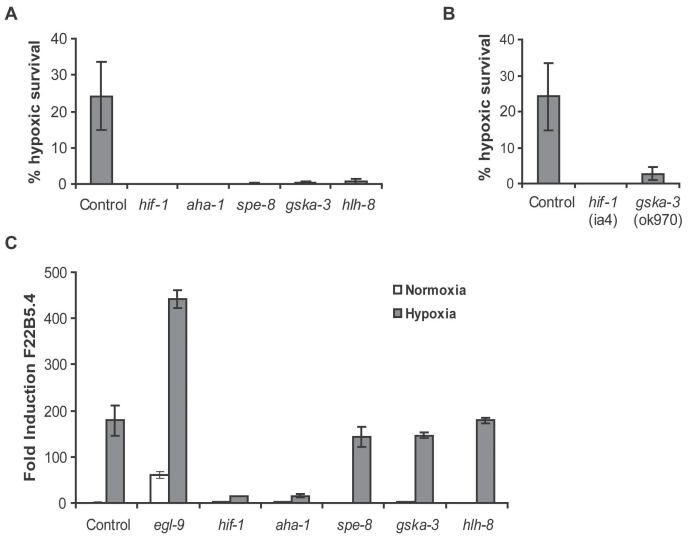

Figure 2.
Analysis of hypoxic survival and HIF-1 transcriptional activity. (a) Hypoxic survival assay of N2 wild-type and RNAi treated animals at 1% O2.. Error bars represent standard deviations of five experiments. (b) Hypoxic survival assay *hif-1* and *gska-3* deficient strains grown at 1% O2. Error bars represent standard deviations of five experiments. (c) Quantitative RT-PCR analysis of the hypoxia responsive gene F22B5.4 on N2 wild-type and RNAi treated animals under normoxia and hypoxia (1%O2). Expression was normalized to the housekeeping gene *inf-1*. Error bars represent standard deviations of technical duplicates. In each experiment percentage of viability is corrected to the corresponding viability under normoxia.

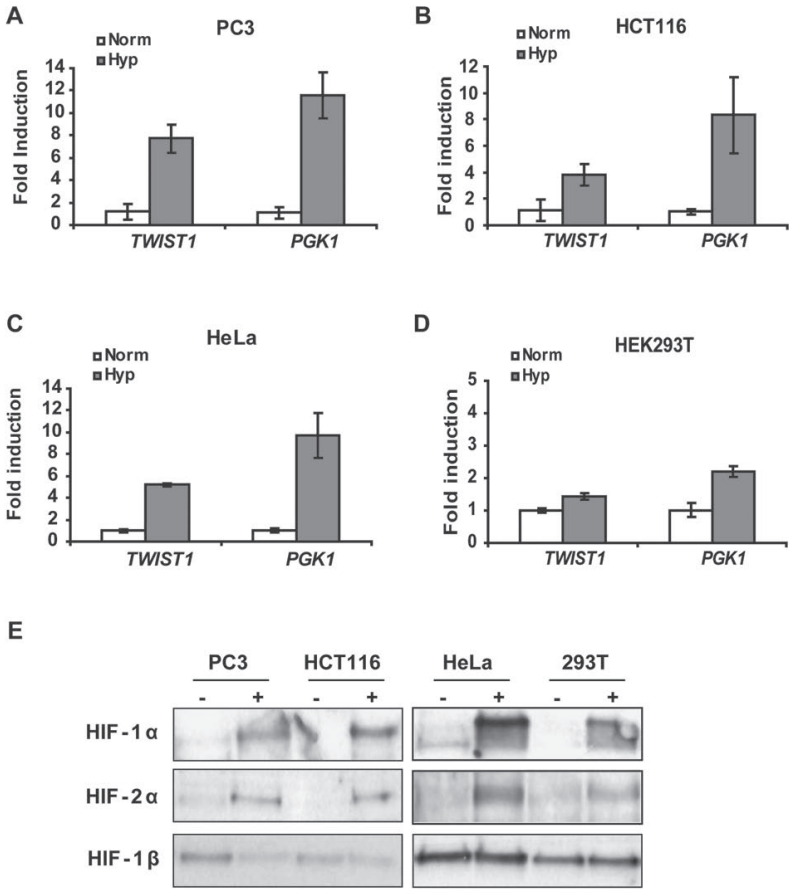

Figure 3.
TWIST1 mRNA is induced by hypoxia. Quantitative RT-PCR analysis for TWIST1 and PGK1 in (a) PC3, (b) HCT116 , (c) HeLa and (d) HEK293T cells incubated at 1% O2 or normal oxygen tension (21%) for 16 hours. Error bars represent standard deviations from triplicate experiments. (e) Western blot analysis for HIF-1α, HIF-2α and HIF-1β of indicated cell lines incubated under hypoxia for 16 hours or not. Note stabilization of HIF-1α and HIF-2α under hypoxia. HIF-1β remain unaltered and are used as loading control.

Human hlh-8 *homologue* TWIST1 *is induced by hypoxia*

Human *TWIST1* is homologous to *C. elegans hlh-8*, as demonstrated by sequence comparison, which shows that the HLH-domain has 59%-63% sequence identity to Twist-family members in other species (Corsi et al., 2002; Harfe et al., 1998). Expression of *TWIST1* has been shown to induce metastatic capacity and anti-apoptotic activity of human cancer cells (Maestro et al., 1999; Yang et al., 2004). Hypoxia has been implicated in similar processes (Hockel & Vaupel, 2001). To study the relationship between *TWIST1* and hypoxia, we

investigated the expression of *TWIST1* mRNA in human cell lines after exposure to hypoxia using a *TWIST1*-specific TaqMan assay. In cervix carcinoma cells (HeLa), prostate cancer cells (PC3), and colon cancer cells (HCT116), *TWIST1* expression was induced four to eight fold after twenty-four hours of hypoxia (Figure 3A-C) concurrent with *PGK1* induction, a validated HIF target (Kress et al., 1998). In contrast, we could not detect significant *TWIST1* induction in human embryonic kidney cells (HEK293T) (Figure 3D). *TWIST1* induction correlated with HIF-2α that was expressed and stabilized under hypoxia in all cell types studied, except for HEK293T cells, which showed no *TWIST1* induction (Figure 3E).

TWIST1 *induction is HIF-2α dependent*

To investigate whether *TWIST1* induction is mediated by HIF activity, cells were treated with the iron chelator, desferroxiamine (DFO), that inhibits prolyl hydroxylase activity leading to HIFα stabilization (Epstein et al., 2001). DFO induced both *TWIST1* and *PGK1* expression in HeLa cells (Figure 4A). HIF-1β is the constitutively expressed common component of the HIF transcription complex, and is essential for its activity (Wood et al., 1996). To address whether hypoxic induction of *TWIST1* required HIF activity, we used RNAi to knockdown HIF-1β expression (Figure 4B). RNAi against HIF-1β severely reduced hypoxic induction of both *PGK1* and *TWIST1* expression in HeLa cells (Figure 4C and D). To study if HIFα was necessary for hypoxic *TWIST1* induction, we generated HeLa cell-lines with stable knockdown of HIF-1α or HIF-2α (Figure 4E). Whereas in HIF-1α knockdown cells *PGK1* and *TWIST1* was up regulated in a hypoxia-dependent manner similar to the parental line, HIF-2α knockdown cells up regulated *PGK1* but failed to induce *TWIST1* mRNA after hypoxia (Figure 4F and G).

HIF-2α regulates TWIST1 *intronic hypoxia response elements*

To determine whether HIF induces *TWIST1* directly, we screened the genomic *TWIST1* sequence for consensus HIF binding elements or HREs (Wang et al., 1995). We identified two potential HREs 1(CGCGTG) and 2(TGCGTG) within 6 bp proximity within the only intron of *TWIST1*, located immediately downstream of the coding region that terminates in exon 1 (Figure 5A). To test whether HIF was sufficient to induce expression from these sequences we generated pGL3-based luciferase reporter constructs containing genomic fragments upstream and downstream from the *TWIST1* open reading frame (Figure 5A). A 600 bp sequence 5' of the *TWIST1* transcript containing the TATA box was not activated by either HIF-1α or HIF-2α (Figure 5B, D and E). In contrast, reporter constructs containing the potential HREs in the 3'- intronic sequence were induced in a dose-dependent manner by HIF-2α, but not by HIF-1α, whereas

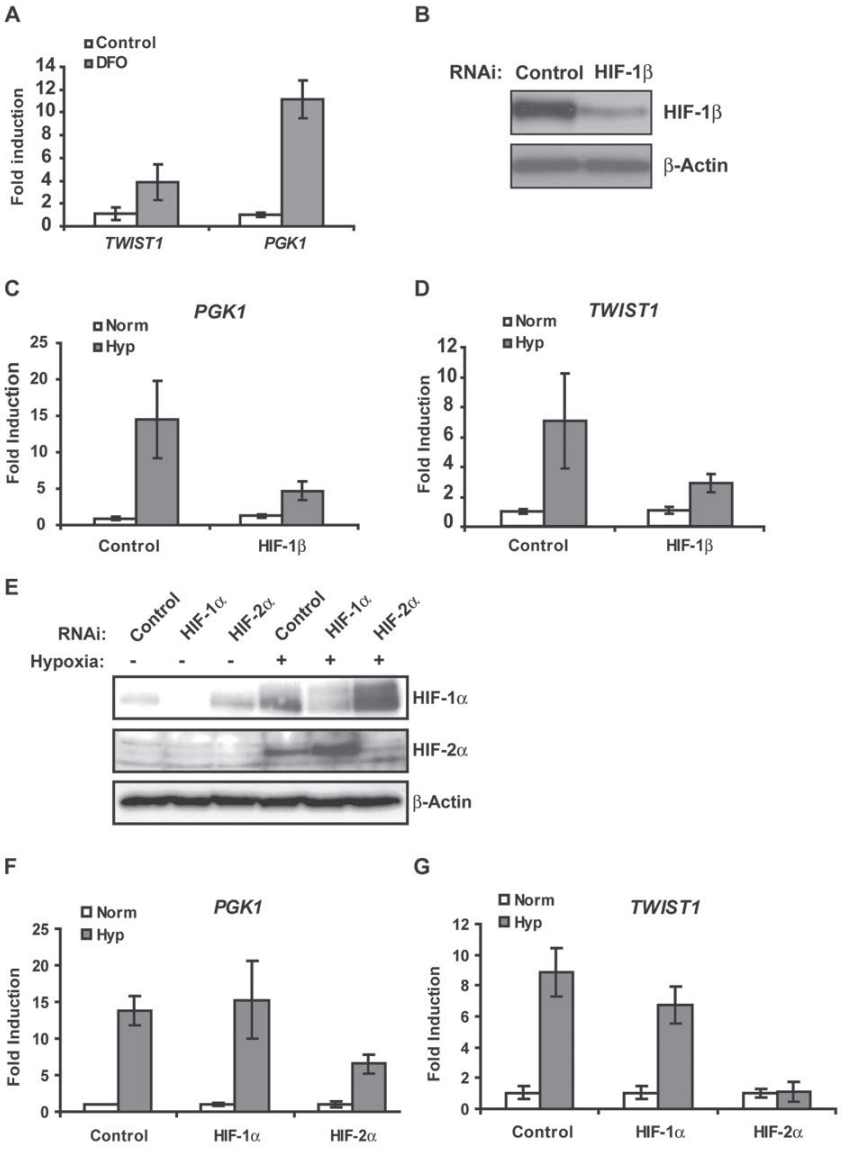

Figure 4.
Induction of *TWIST1* is HIF-dependent. (a) Quantitative RT-PCR analysis for *TWIST1* and *PGK1* in HeLa cells treated with DFO for 16h.. (b) Western blot analysis for HIF-1β in HeLa cells treated with HIF-1β or control siRNA, Note efficient HIF-1β knockdown. β-Actin was used as loading control (d) Quantitative PCR analysis for *PGK1* (c) and *TWIST1* (d) in HeLa cells treated with HIF-1β or control siRNA . (e) Western blot analysis of HeLa cells with stable HIF-1α or HIF-2α RNAi using pSuper-ShRNA indicating efficient knockdown of hypoxia-induced HIF-1α or HIF-2α (f) and (g) quantitative RT-PCR analysis for *PGK1* and *TWIST1* in HIF-1α and HIF-2α knockdown HeLa cells. Error bars represent standard deviations from triplicate experiments.

a VEGF-derived HRE reporter was induced by both HIF-1α and HIF-2α (Figure 5C, D and E). Importantly, mutating either HRE 1 or 2 completely abolished *TWIST1*-reporter activity, demonstrating that both HREs are essential for *TWIST1* regulation by HIF-2α.

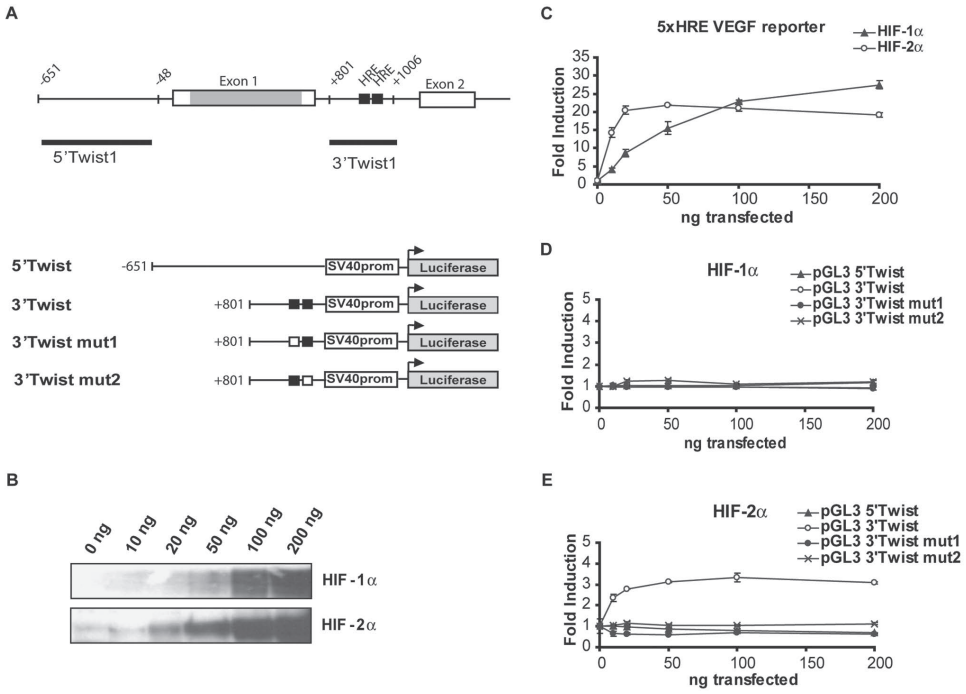

Figure 5.
HIF-2α induces transcription via intronic *TWIST1* HREs. (a) Schematic drawing of *TWIST1* genomic region showing both exons (white boxes), the coding sequence (grey box) and both HRE elements (black boxes). Luciferase based reporters were generated containing an SV40 basal promoter fused to *TWIST1*genomic sequences. Substituted elements are represented by white box. (b) Western blot analysis for HIF-1α and HIF-2α of HEK293T cells transfected with increasing amounts of HIF-1α or HIF-2α used in reporter assays. (c) Transcription assays showing induction of 5xHRE-VEGF reporter activity by HIF-1α or HIF-2α under normoxia and the effect of HIF-1α (d) or HIF-2α (e) on wild type and HRE mutated *TWIST1* reporters. HIF-2α but not HIF-1α induces 3'*TWIST* reporter. Fold induction was corrected using co-transfected TK-Renilla expression. Error bars represent a triplicate experiment.

TWIST1 *regulation requires HIF-2α DNA-binding domain*

To further investigate the transcriptional regulation of *TWIST1* by HIF-2α, we expressed a HIF-2α mutant lacking the basic DNA binding domain (HIF-2α-Δ1-278, Figure 6A and B) (Erbel et al., 2003). Wild type HIF-2α induced both 3'*TWIST1* and *VEGF* reporter activity. In contrast, overexpression of HIF-2α-Δ1-278 mutant did not result in 3'*TWIST1* and *VEGF* reporter activity (Figure 6C and D). In addition, overexpression of the HIF-2α DNA binding domain alone (HIF-2α-Δ281-870) did not induce the 3'*TWIST1* reporter activity (data not shown). These results demonstrate that HIF-2α DNA binding is necessary for TWIST1 induction, appointing to a direct interaction of HIF-2α with the 3'TWIST1 HRE.

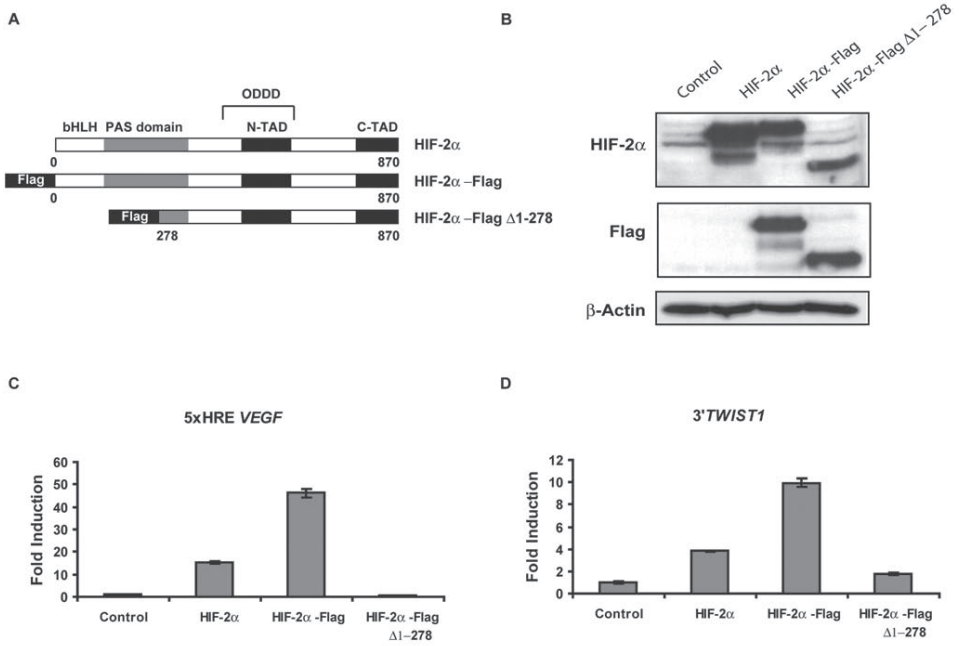

Figure 6.

(a) Schematic representation of HIF-2α expression constructs with or without DNA binding domain. (b) Western blot analysis of HEK293T cells transfected with HIF-2α expression constructs. (c) and (d) Luciferase assay of HEK293T cells transfected with indicated reporter and expression constructs.

Discussion

Tumor hypoxia is a common event in solid cancer progression and leads to aggressive tumors that are highly angiogenic and are associated with treatment failure and poor clinical prognosis (Semenza, 2003). Because hypoxia is encountered early during cancer cell expansion, tumor hypoxia is a relevant therapeutic target. The HIF-VHL axis is essential in the hypoxic response of tumor cells and highly conserved throughout evolution (Dekanty et al., 2005; Jiang et al., 2001). Over expression of both HIF-1α and HIF-2α is seen in many human cancers (Semenza, 2003). Although the HIF-1α and HIF-2α proteins are very similar and are both induced upon hypoxia, they elicit different transcriptional responses and have different roles during embryonic development (Raval et al., 2005; Ravi et al., 2000; Wang et al., 2005; Wiesener et al., 2003).

In this study, we performed a genome wide loss of function screen to identify novel components of the HIF pathway in *C. elegans* and validated their role in hypoxia response in mammalian (cancer) cells. We uncovered a total of 137 *C. elegans* genes that in one or both *egl-9* deficient strains suppressed the egg-laying defect. These genes could be categorized according to gene ontology in a small number of groups including transcription and DNA binding, ubiquitin ligation and proteolysis, metabolism, signal transduction and a group of proteins with unknown function (Figure 1 C, Supplementary data). In addition to *hif-1* and *aha-1*, we found *akt-2*, a homologue of the human AKT oncoprotein, which is involved in regulation of HIF-1α protein expression (Bardos & Ashcroft, 2004; Dekanty et al., 2005; Gort et al., 2006). Furthermore, an unknown protein (Y39A3CR.6, Supplementary Data) with significant homology to mammalian HIF-1β-like protein was found (Ikeda & Nomura, 1997). Although the *egl-9* mutant phenotype is HIF-1 dependent, the etiology is more complicated, since *vhl-1* deficient worms have comparable HIF-1 levels, but are not egg laying defective (Shen et al., 2006). This points to a VHL-independent regulation of HIF activity by EGL-9. Indeed, in addition to its role in regulating HIF protein stability through hydroxylation, the human EGL-9 homologue EGLN1 also suppresses HIF transcription in the nucleus (To & Huang, 2005). This may in part explain the higher transcriptional activity of HIF target genes and the more severe phenotype of *egl-9* deficient animals (Shen et al., 2006). Furthermore, EGL-9 and the recently identified HIF-1 regulator RHY-1 function in a VHL-1 independent pathway to repress HIF-1 activity, which can explain a VHL-1 independent function of EGL-9 (Shen et al., 2006).

To enrich for HIF-1 dependent genes from the primary candidates we performed an additional selection using a hypoxia survival assay. Using this assay we recovered both *hif-1* and *aha-1*, demonstrating that AHA-1 is necessary for HIF-1-mediated transcription and HIF-1-dependent phenotypes (Jiang et al., 2001). We identified three novel genes previously unknown to play a role in the C. elegans HIF pathway. Two of these, *spe-8* and *gska-3*, are predicted homologues of the human tyrosine kinase FER and the serine/threonine glycogen synthase kinase GSK-3 respectively, which have previously been reported to play a role in HIF regulation in mammalian cells (Mottet et al., 2003; Salem et al., 2005). Using *gska-3* deficient animals, we confirmed increased hypoxia sensitivity observed with *gska-3* knockdown. We were unable to demonstrate reduced HIF-1 target gene expression, suggesting these genes function downstream or parallel to *hif-1*, or are regulated in a spatially or temporally restricted manner that impeded robust detection. In addition, we identified *hlh-8*, the *C. elegans* homologue of human oncogene *TWIST1* (Harfe et al., 1998). HLH-8 is involved in *C. elegans* vulval development, which might explain its role in egg-laying defects (Harfe et al., 1998).

In mammalian cells, we show that *TWIST1* expression is induced by hypoxia, which requires HIF function. Furthermore, we show that HIF-2α, but not HIF-1α, regulates *TWIST1* expression through intronic HREs 3' of the *TWIST1* coding sequence. Regulation of 3' sequences by the HIF transcription complex are not unusual. Indeed, one of the best characterized HIF target genes, erythropoietin, is regulated via 3' HRE sequences (Pugh et al., 1991; Wang & Semenza, 1993). Nevertheless, we cannot exclude additional regulation of *TWIST1* via other unidentified HREs.

TWIST1 belongs to a family of bHLH transcription factors that regulates mesodermal cell fate in *Drosophila* (Szymanski & Levine, 1995). Loss of *TWIST1* expression is associated with the hereditary Saethre-Chotzen syndrome that is characterized by inappropriate fibroblast growth factor receptor 2 (FGFR2) expression (Wilkie & Morriss-Kay, 2001). TWIST1 acts as an inhibitor of N-MYC induced apoptosis, establishing *TWIST1* as an oncogene (Maestro et al., 1999). In human breast cancers, high TWIST1 levels correlate with invasive lobular carcinoma, a highly infiltrating tumor type associated with loss of E-cadherin expression and required for breast cancer metastases in a murine model for breast cancer (Yang et al., 2004). TWIST1 represses E-cadherin, which results in the activation of mesenchymal markers and cell motility suggesting that TWIST1 promotes epithelial-to-mesenchymal transition. TWIST1 may also

regulate the expression of the bHLH repressor SNAIL that acts as a boundary repressor by down regulating the expression of ectodermal genes within the mesoderm (Ip et al., 1992). The HIF pathway can also regulate E-Cadherin repression, presumably via SNAIL (Esteban et al., 2006). Our data suggest that this might be in concert with TWIST1.

Hypoxia-induced *TWIST1* expression illustrates how cells can acquire phenotypic characteristics like increased motility, scattering and invasiveness associated with aggressive tumor behavior (Hanahan & Weinberg, 2000). Interestingly, HIF-2α has been shown to enhance aggressive tumor behavior, (Holmquist-Mengelbier et al., 2006; Kondo et al., 2003). This is consistent with HIF-2α-dependent regulation of *TWIST1* as shown here. Possibly, HIF-2α controls gene expression in (hypoxia-induced) metastatic disease, whereas HIF-1α plays a more prominent role during normal oxygen homeostasis. This is supported by the finding that HIF-1α does not induce metastasis formation in a transgenic mouse model, but does facilitate this process (Liao et al., 2007). The regulation of *TWIST1* by the HIF pathway in mammalian cells provides an attractive explanation for the poor survival seen in patients with HIF overexpression (Bos et al., 2003). It will be interesting to study *TWIST1* expression in hypoxic tumor models and relate this to metastatic propensity.

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Supplementary data concerning the egg laying screen in *C. elegans* is available at http://www.nature.com/onc/journal/vaop/ncurrent/suppinfo/1210795s1.html and in thesis page 100-102.

References

Bardos JI and Ashcroft M. (2004). Hypoxia-inducible factor-1 and oncogenic signalling. *Bioessays*, **26,** 262-9.

Bishop T, et al. (2004). Genetic analysis of pathways regulated by the von hippel-lindau tumor suppressor in Caenorhabditis elegans. *PLoS Biol*, **2,** e289.

Bos R, et al. (2003). Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*, **97,** 1573-81.

Corsi AK, et al. (2002). Characterization of a dominant negative C. elegans Twist mutant protein with implications for human Saethre-Chotzen syndrome. *Development*, **129,** 2761-72.

Dekanty A, et al. (2005). The insulin-PI3K/TOR pathway induces a HIF-dependent transcriptional response in Drosophila by promoting nuclear localization of HIF-alpha/Sima. *J Cell Sci*, **118,** 5431-41.

Ema M, et al. (1997). A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. *Proc Natl Acad Sci U S A*, **94,** 4273-8.

Epstein AC, 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.

Erbel PJ, et al. (2003). Structural basis for PAS domain heterodimerization in the basic helix--loop--helix-PAS transcription factor hypoxia-inducible factor. *Proc Natl Acad Sci U S A*, **100,** 15504-9.

Esteban MA, et al. (2006). Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. *Cancer Res*, **66,** 3567-75.

Forsythe JA, et al. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*, **16,** 4604-13.

Gort EH, et al. (2006). Hypoxia-inducible factor-1alpha expression requires PI 3-kinase activity and correlates with Akt1 phosphorylation in invasive breast carcinomas. *Oncogene*, **25,** 6123-7.

Greijer AE, et al. (2005). Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). *J Pathol*, **206,** 291-304.

Gu YZ, et al. (1998). Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. *Gene Expr*, **7,** 205-13.

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

Harfe BD, et al. (1998). Analysis of a Caenorhabditis elegans Twist homolog identifies conserved and divergent aspects of mesodermal patterning. *Genes Dev*, **12,** 2623-35.

Hockel M and Vaupel P. (2001). Biological consequences of tumor hypoxia. *Semin Oncol*, **28,** 36-41.

Holmquist-Mengelbier L, et al. (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.

Ikeda M and Nomura M. (1997). cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS protein (BMAL1) and identification of alternatively spliced variants with alternative translation initiation site usage. *Biochem Biophys Res Commun*, **233,** 258-64.

Ip YT, et al. (1992). The dorsal gradient morphogen regulates stripes of rhomboid expression in the presumptive neuroectoderm of the Drosophila embryo. *Genes Dev*, **6,** 1728-39.

Jiang H, et al. (2001). The Caenorhabditis elegans hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. *Proc Natl Acad Sci U S A*, **98,** 7916-21.

Kamath RS and Ahringer J. (2003). Genome-wide RNAi screening in Caenorhabditis elegans. *Methods*, **30,** 313-21.

Kamath RS, et al. (2003). Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. *Nature*, **421,** 231-7.

Kondo K, et al. (2003). Inhibition of HIF2alpha is sufficient to suppress pVHL-defective tumor growth. *PLoS Biol*, **1,** E83.

Kress S, et al. (1998). Expression of hypoxia-inducible genes in tumor cells. *J Cancer Res Clin Oncol*, **124,** 315-20.

Liao D, et al. (2007). Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. *Cancer Res*, **67,** 563-72.

Maestro R, et al. (1999). Twist is a potential oncogene that inhibits apoptosis. *Genes Dev*, **13,** 2207-17.

Mottet D, et al. (2003). Regulation of hypoxia-inducible factor-1alpha protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3beta pathway in HepG2 cells. *J Biol Chem*, **278,** 31277-85.

Padilla PA, et al. (2002). Dephosphorylation of cell cycle-regulated proteins correlates with anoxia-induced suspended animation in Caenorhabditis elegans. *Mol Biol Cell*, **13,** 1473-83.
Pugh CW, et al. (1991). Functional analysis of an oxygen-regulated transcriptional enhancer lying 3' to the mouse erythropoietin gene. *Proc Natl Acad Sci U S A*, **88,** 10553-7.
Raval RR, et al. (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,** 5675-86.
Ravi R, et al. (2000). Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. *Genes Dev*, **14,** 34-44.
Salem Y, et al. (2005). Fer kinase sustains the activation level of ERK1/2 and increases the production of VEGF in hypoxic cells. *Cell Signal*, **17,** 341-53.
Semenza GL. (2001). HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus. *Cell*, **107,** 1-3.
Semenza GL. (2003). Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*, **3,** 721-32.
Semenza GL. (2004). Hydroxylation of HIF-1: oxygen sensing at the molecular level. *Physiology (Bethesda)*, **19,** 176-82.
Shen C, et al. (2005). Roles of the HIF-1 Hypoxia-inducible Factor during Hypoxia Response in Caenorhabditis elegans. *J Biol Chem*, **280,** 20580-8.
Shen C and Powell-Coffman JA. (2003). Genetic analysis of hypoxia signaling and response in C elegans. *Ann N Y Acad Sci*, **995,** 191-9.
Shen C, et al. (2006). The Caenorhabditis elegans rhy-1 Gene Inhibits HIF-1 Hypoxia-Inducible Factor Activity in a Negative Feedback Loop That Does Not Include vhl-1. *Genetics*, **174,** 1205-14.
Shibata T, et al. (1998). Enhancement of gene expression under hypoxic conditions using fragments of the human vascular endothelial growth factor and the erythropoietin genes. *Int J Radiat Oncol Biol Phys*, **42,** 913-6.
Szymanski P and Levine M. (1995). Multiple modes of dorsal-bHLH transcriptional synergy in the Drosophila embryo. *Embo J*, **14,** 2229-38.
Tian H, et al. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev*, **11,** 72-82.
To KK and Huang LE. (2005). Suppression of hypoxia-inducible factor 1alpha (HIF-1alpha) transcriptional activity by the HIF prolyl hydroxylase EGLN1. *J Biol Chem*, **280,** 38102-7.
Trent C, et al. (1983). Egg-laying defective mutants of the nematode Caenorhabditis elegans. *Genetics*, **104,** 619-47.
van Haaften G, et al. (2004). Gene interactions in the DNA damage-response pathway identified by genome-wide RNA-interference analysis of synthetic lethality. *Proc Natl Acad Sci U S A*, **101,** 12992-6.
Vaupel P, et al. (2001). Oxygen status of malignant tumors: pathogenesis of hypoxia and significance for tumor therapy. *Semin Oncol*, **28,** 29-35.
Vaupel P and Mayer A. (2005). Hypoxia and anemia: effects on tumor biology and treatment resistance. *Transfus Clin Biol*, **12,** 5-10.
Wang GL, et al. (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,** 5510-4.
Wang GL and Semenza GL. (1993). General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci U S A*, **90,** 4304-8.
Wang V, et al. (2005). Differential gene up-regulation by hypoxia-inducible factor-1alpha and hypoxia-inducible factor-2alpha in HEK293T cells. *Cancer Res*, **65,** 3299-306.
Wiesener MS, et al. (2003). Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. *Faseb J*, **17,** 271-3.
Wilkie AO and Morriss-Kay GM. (2001). Genetics of craniofacial development and malformation. *Nat Rev Genet*, **2,** 458-68.
Woelfle U, et al. (2003). Molecular signature associated with bone marrow micrometastasis in human breast cancer. *Cancer Res*, **63,** 5679-84.
Wood SM, et al. (1996). The role of the aryl hydrocarbon receptor nuclear translocator (ARNT) in hypoxic induction of gene expression. Studies in ARNT-deficient cells. *J Biol Chem*, **271,** 15117-23.
Yang J, et al. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*, **117,** 927-39.

Supplementary data

clone	function	gene or description	sa307	n586
Transcription				
F38A6.3	transcription	*hif-1*	3	3
C25A1.11	transcription	*aha-1*	3	3
Y39A3CR.6	transcription	Protein of unknown function	2	2
C41G7.5	transcription	*ahr-1*	2	0
C02B8.4	transcription	*hlh-8*	1	0
ZC155.2	DNA binding	Protein of unknown function	1	0
Y67A6A.2	transcription	*nhr-62*	1	0
Signal Transduction				
C36B1.10	serine/threonine kinase	*gska-3*	2	2
F28H7.1	G-coupled protein receptor	*srj-22*	2	2
R151.4	tyrosine kinase	Putative tyrosine protein kinase	1	2
C09H6.2	phospho tyrosine binding	lin-10	1	2
C38C3.4	serine/threonine kinase	Serine/threonine protein kinase	1	2
F46A9.3	potassium channel	*twk-29*	1	2
M02H5.11	G-coupled protein receptor	*srh-35*	2	0
F28H6.1	serine/threonine kinase	*akt-2*	0	2
F34D6.3	potassium channel	*sup-9*	2	0
F53G12.6	tyrosine kinase	*spe-8*	2	0
R05D8.6	transmembrane receptor	*srg-51*	1	0
F13E6.2	kinase	Member of the adenylate kinase family	0	1
F53B6.7	kinase/unknown	Protein of unknown function	0	1
F20E11.2	G protein-coupled receptor	*srsx-2*	0	1
K07D4.7	Rho-GEF	*tag-218*	1	0
ZK721.1	unknown	*tag-130*	1	0
F21F3.5	acetylcholine receptor	*unc-38*	0	1
F52F12.3	tyrosine kinase	*mom-4*	0	1
C49C3.10	serine/threonine kinase	Serine/threonine protein kinase	0	1
T22C1.8	tyrosine phosphatase	Putative tyrosine protein phosphatase	0	1
K03D3.1	unknown	*srz-74*	1	0
H24O09.1	unknown	*srbc-40*	0	1
C18B10.7	G protein-coupled receptor	*str-190*	1	0
Ubiquitin ligation and proteolysis				
F40G9.3	Ubiquitin-protein ligase	*ubc-20*	1	2
F08G12.4	Ubiquitin-protein ligase	*vhl-1*	0	2
C44E12.1	metallopeptidase	Protein of unknown function	1	0
F44F1.1	unknown	Member of the calpain protease protein family	1	0
C06E2.3	Ubiquitin-protein ligase	*ubc-21*	0	1
F53A9.1	metallopeptidase	Protein of unknown function	1	0
Metabolism				
W01H2.2	unknown	Protein of unknown function	2	2
F41D3.11	unknown	Protein of unknown function	2	2
C37E2.1	oxidoreductase	Member of isocitrate dehydrogenase family	0	1
Y56A3A.12A	amidase	Protein of unknown function	1	0
DY3.5	unknown	*pqn-26*	0	1
F42A9.4	electron transport	*cyp-33E3*	0	1
C25A8.4	hydrolase	Protein of unknown function	1	0
C26H9A.1	oxidoreductase	*vha-7*	1	0
F10F2.2	PRFG synthase	Phosphoribosylformylglycinamidine synthetase	1	0
F56H11.4	fatty acid metabolism	*elo-1*	1	0
Y38C9B.1	electron transport	*cyp-29A3*	1	0
DNA repair				
C23H4.6	DNA repair	Putative coiled-coil protein	0	2
T28A8.7	DNA repair	*mlh-1*	1	0
Y47G6A.11	DNA repair	*msh-6*	0	1
Y62E10A.5	DNA dealkylation	*agt-1*	0	1

Supplementary data continued

clone	function	gene or description	sa307	n586
Structure				
B0511.5	unknown	Protein of unknown function	2	2
C43H8.3	unknown	Protein with putative laminin-G domain	0	1
F38A6.2	structure	echinoderm microtubule-associated-like protein	0	1
Y38C1BA.a	structure	*col-109*	1	0
Transport				
K11G9.5	transport	inorganic phosphate cotransporter	2	2
B0280.12	transport	glr-2	1	2
B0361.3	unknown	organic cation transporter	1	0
F40F4.2	transport	*lbp-1*	1	0
C05E11.5	transport	*amt-4*	1	0
T23H4.1	transport	*inx-20*	1	0
Y53G8AR.7	transport	Predicted transporter	1	0
Other				
F56A6.4	embryonic development		2	2
H31G24.1	unknown	Formin homology family member	2	2
ZK849.5	unknown	Member of the membrane protein family	2	2
C55F2.2	reproduction	Protein of unknown function	2	1
Y9C9A.18	unknown	*str-159*	1	2
Y40D12A.2	serine protease	Putative carboxypeptidase	1	2
K07A12.4	elongation factor	member of translation elongation factor family	2	0
F46F2.3	embryonic development	Protein of unknown function	1	0
C18B10.9	unknown	*str-29*	1	0
ZK688.2	unknown	Member of the putative membrane family	1	0
C50F2.6	protein folding	*fkb-5*	1	0
F54D7.2	larval development	CDP-alcohol phosphotransferases	0	1
T05G5.4	embryonic development	Protein of unknown function	0	1
M88.5	nucleic acid binding	Protein containing putative KH domains	1	0
T06G6.9	protein folding	*vbp-1*	1	0
Unknown				
C41D7.1	unknown	Protein of unknown function	2	2
F21C10.9	unknown	G protein-coupled receptor	2	2
W02H3.1	unknown	Protein of unknown function	1	2
F47F2.2	unknown	Protein of unknown function	1	2
C36F7.5	unknown	Protein of unknown function	1	2
R13H8.2	unknown	Protein of unknown function	1	2
C37A5.10	unknown	Protein of unknown function	1	2
T10B11.6	unknown	Member of an uncharacterized protein family	1	2
Y73F8A.10	unknown	Protein of unknown function	1	2
F46C8.3	unknown	Protein of unknown function	1	2
C48E7.1	unknown	Protein of unknown function	1	2
T20F10.2	unknown	Protein contains a putative coiled-coil domain	1	2
C06E7.2	unknown	Protein of unknown function	1	2
F01F1.2	unknown	Protein of unknown function	1	2
Y39E4A.1	unknown	Protein of unknown function	1	2
ZK20.4	unknown	Protein of unknown function	1	2
R148.1	unknown	*xbx-7*	0	2
C46A5.6	unknown	Protein of unknown function	2	0
F13D2.4	unknown	Protein of unknown function	0	2
Y53G8AM.7	unknown	Protein of unknown function	0	2
Y73F8A.13	unknown	Protein of unknown function	2	0
C06A6.1	unknown	Protein of unknown function	2	0
W01C8.4	unknown	Protein of unknown function	0	2
C39E9.8	unknown	Protein of unknown function	2	0
F20D1.7	unknown	Leucine-rich repeat (LRR) protein	0	2
T28C6.2	unknown	Protein of unknown function	0	1
R04D3.2	unknown	Member of an uncharacterized protein family	1	0
F49B2.3	unknown	Protein of unknown function	1	0

Supplementary data continued

clone	function	gene or description	sa307	n586
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C45G9.11	unknown	Protein of unknown function	0	1
T08H10.3	unknown	Protein of unknown function	1	0
ZC190.5	unknown	Protein of unknown function	0	1
F56C3.3	unknown	Protein of unknown function	1	0
C27F2.5	unknown	Protein of unknown function	1	0
C16C8.7	unknown	Protein of unknown function	0	1
W07B3.1	unknown	Protein of unknown function	0	1
C05E4.8	unknown	Protein of unknown function	1	0
T05A10.4	unknown	Protein of unknown function	0	1
T23B3.5	unknown	Protein of unknown function	0	1
Y106G6H.1	unknown	Protein of unknown function	0	1
F28D9.4	unknown	Protein with similarity to human PIGL	0	1
ZK1151.3	unknown	Protein with weak similarity to PLEC1	1	0
F41D3.9	unknown	Member of an uncharacterized protein family	0	1
T16H12.9	unknown	Protein of unknown function	0	1
M03F4.1	unknown	Protein of unknown function	0	1
B0272.5	unknown	Protein of unknown function	0	1
C18B12.1	unknown	Protein of unknown function	0	1
Y37E11B.10	unknown	Protein of unknown function	1	0
F15E6.5	unknown	Protein of unknown function	1	0
C46C11.1	unknown	Protein of unknown function	1	0
C46H11.7	unknown	Member of an uncharacterized protein family	0	1
Y71F9B.10	unknown	*sop-3*	1	0
F56H6.9	unknown	Protein of unknown function	1	0
C49C8.2	unknown	Protein of unknown function	0	1
ZC190.9	unknown	Protein of unknown function	1	0
F09E10.7	unknown	Protein of unknown function	1	0
H02F09.4	unknown	Member of reverse transcriptase family	1	0
C12D12.4	unknown	Protein of unknown function	0	1
F47C8.1	unknown	Protein of unknown function	0	1

Legend to supplementary data.

Table shows cosmid number, name, function and extent of egg-laying defect for 137 genes identified in the egg-laying defect screen. Gene ontology classes are derived from Wormbase (http://www.wormbase.org). A score of 1 represents mild suppression, 2 represents medium suppression and 3 represents major suppression.

Supplementary table is available at oncogenes website (http://www.nature.com/onc/journal/vaop/ncurrent/suppinfo/1210795s1.html).

Chapter 6

TWIST1 expression and methylation in breast cancer

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Abstract

TWIST1 is an anti-apoptotic and pro-metastatic transcription factor involved in regulation of epithelial to mesenchymal transition (EMT). It is expressed in a number of epithelial cancers. In breast cancer, TWIST1 expression has been found to relate to metastases formation. Interestingly, methylation of the *TWIST1* promoter that generally silences gene expression frequently occurs in breast cancer. This does not fit with the paradigm of an oncogene. We therefore studied *TWIST1* methylation in relation to its expression in normal breast tissue and invasive breast cancers. We found that *TWIST1* promoter methylation is significantly enhanced in malignant compared to healthy breast tissue. Furthermore, TWIST1 protein expression was significantly enhanced in breast malignancy compared to matched healthy tissue from the same patients. There was no correlation between *TWIST1* promoter methylation and protein or RNA expression. This might point to a biphasic role of TWIST1 in breast cancer development.

Introduction

TWIST1 belongs to the basic-helix-loop-helix (bHLH) family of transcription factors and is implicated in lineage-specific cellular differentiation and survival (Lee et al., 1999; Maestro et al., 1999). *TWIST1* function in vertebrates governs early mesodermal patterning and osteogenesis (Bialek et al., 2004). Individuals with germ-line haploinsufficiency of the *TWIST1* gene suffer from the hereditary disorder Saethre-Chotzen syndrome (SCS, acrocephakosyndactyly type III), characterized by premature craniosynostosis and limb, head and face anomalies (Wilkie & Morriss-Kay, 2001). In cancer development, *TWIST1* functions as a prometastatic oncogene. Expression of TWIST1 protein counteracts the pro-apoptotic effects of N-MYC by repression of *ARF* and thereby hampers p53 function (Maestro et al., 1999). In a metastatic breast cancer mouse model, *TWIST1* was necessary for onset of metastasis (Yang et al., 2004). *TWIST1* induces epithelial to mesenchymal transition (EMT). EMT is a developmental program that enables epithelial cells to undergo a mesenchymal cell fate (Thiery, 2002). This is characterized by differential regulation of several epithelial and mesenchymal marker genes, such as E-Cadherin, N-Cadherin, Vimentin, α-SMA and β-Catenin. Loss of E-Cadherin, a common phenomenon in breast cancer, is an important hallmark of EMT and a predictor of poor prognosis in various cancers (Thiery, 2002). EMT is orchestrated by trancriptional activition and repression by a group of transcription factors, including SNAI1, SNAI2, ZEB1A, ZEB1B and TWIST1 (Peinado et al., 2007). TWIST1 regulates EMT by repression of E-Cadherin and induction of N-Cadherin (Alexander et al., 2006; Yang et al., 2004). In addition to its anti-apoptotic and pro-metastatic function, *TWIST1* overexpression induces angiogenesis and chromosomal instability (Mironchik et al., 2005).

At the DNA level, cancer progression is characterized by genetic and epigenetic events. Methylation of CpG islands in promoter regions of tumor suppressor genes is such an epigenetic event (Baylin & Herman, 2000). Methylation abrogates proper TATA Binding Protein (TBP) binding to the promoter, ultimately resulting in reduced expression of genes that play important roles in the cell cycle, cell adherence, cell signaling, DNA repair and apoptosis (Widschwendter et al., 2001; Widschwendter & Jones, 2002). Human breast carcinomas exhibit *TWIST1* promoter hypermethylation at high frequency, ranging from 16 to 77% (Bae et al., 2004; Evron et al., 2001; Fackler et al., 2003; Fackler et al., 2004; Martin et al., 2005; Mehrotra et al., 2004) (Suijkerbuijk et al, submitted). Moreover, *TWIST1* methylation is a good predictor of human

breast cancer presence (Fackler et al., 2004). These findings postulate *TWIST1* methylation as an interesting cancer biomarker, but its functional meaning remains a puzzling one. A role of *TWIST1* as an oncogene seems plausible, although this is contradictory to its frequent methylation in breast cancer, which would suggest that TWIST1 expression protects from carcinogenesis.

To further elucidate the putative role of TWIST1 in breast carcinogenesis, we studied the relation between *TWIST1* promoter methylation and expression in normal and malignant breast tissue specimens.

Material and Methods

Patients

Seventy-six invasive breast carcinomas were obtained from the Pathology department of the University Medical Center Utrecht. Use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients in our hospital (van Diest, 2002). H&E-stained slides of the paraffin blocks were reviewed by a pathologist (PvD) to confirm the presence of malignancy in tumor samples. Histologic type was assessed according to the WHO. Grade was assessed according to the Nottingham system, and the estrogen (ER), progesterone (PR) and HER-2/*neu* receptors were assessed by standard immunohistochemistry (van Diest et al., 1992). We selected 34 patients with invasive ductal breast cancer supplemented with a set of 42 patients with invasive lobular cancer as these show loss of E-Cadherin expression. Table 1 shows baseline clinicopathologic features of 76 breast cancers studied for *TWIST1* promoter methylation and TWIST1 expression (missing cases not listed).

Cell culturing, western analysis and immunofluorescence

HeLa cells and MCF-7 cells were cultured in Dulbecco's modified Eagles medium (DMEM) (Gibco BRL, London, UK) supplemented with 10% fetal calf serum (FCS) (Gibco) and penicillin-streptomycin (Gibco). Western analysis was performed as described previously (Gort et al., 2007). A previously described antibody against TWIST1 was used (Mironchik et al., 2005). For immunofluorescence cells were seeded on glass slides, which were subsequently fixed using 4% paraformaldehyde. After permeabilisation in 0.1% Triton-X100 for 20 minutes and blocking in 5% bovine serum albumin and 0.1% goat serum (DAKO, Glostrup, Denmark) for 2 hours, slides were incubated with the anti-TWIST1 antibody (1:50) for 1 hour. After washing, slides were incubated with a secondary FITC-conjungated anti-rabbit antibody (DAKO) for 1 hour and subsequently mounted in Vectashield (Vector Laboratories Inc, Burlinghame, CA).

Table 1. Cohort characteristics.

Clinicopathologic features of ductal breast carcinoma patients		N = 76 N	(%)
Type	Ductal	34	(44.7%)
	Lobular	42	(55.3%)
Grade	I	6	(7.9%)
	II	14	(18.4%)
	III	20	(26.3%)
Lymph node status	Negative	36	(47.4%)
	Positive	33	(43.4%)
ER	Negative	11	(14.5%)
	Positive	58	(76.3%)
PR	Negative	28	(36.8%)
	Positive	40	(52.6%)
HER-2/*neu*	Negative	27	(35.5%)
	Positive	8	(10.5%)
Age (years)	Mean	61.1	
	Minimal	37.0	
	Maximum	88.0	
Mitotic index	Mean	19.9	
Tumor size (cm)	Mean	8.1	

DNA isolation, RNA isolation and quantitative RT-PCR

For isolation of genomic DNA from parafine embedded specimen, a 10 µm unstained section was deparaffinized by treatment of two times five minutes xylene, and the relevant tissue was scraped from the slide. Fifty µl of TNES (10mM Tris/150 mM NaCl/2 mM EDTA/0.5% SDS) extraction buffer containing 250 ng salmon sperm DNA (Invitrogen Corp., Carlsbad, CA) and 100 µg proteinase K (Invitrogen Corp., Carlsbad, CA), was added to the tissue. After 4h rotation at 52°C, samples were heat inactivated for 5 min at 99ºC and stored at 4ºC.

RNA and genomic DNA were isolated from frozen material by dissolving three 10 μm thick sections in 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA) followed by chloroform/phenol extraction. The aqueous phase was used to precipitate RNA. The interphase was used to precipitate DNA. cDNA was prepared from 1 μg of RNA using Reverse Transcriptase (Roche) and a 1:1 oligo-(dT) : hexamer primermix (Invitrogen). Q-PCR was performed using commercially available TaqMan assays for *TWIST1* (Hs00361186), and the housekeeping gene hydroxymethylbilane synthase (*HMBS*) (Hs00609297_ m1) (Applied Biosystems, Foster City, CA, USA) using an ABI7900 analyzer (Applied Biosystems). Data were analyzed using the SDS2.2.1 program (Applied Biosystems), using HMBS for normalization.

Quantitative multiplex methylation-specific PCR
QM-MSP was performed as previously described (13, 14). Briefly, 13.5μl of isolated DNA was heated at 99ºC for 10 min, quick chilled on ice after which 1.5 μl freshly prepared 2 M NaOH was added. Thirty-five μl of 4.5 M sodium bisulfite (Sigma, St Louis, MO, USA) containing 1 mM hydroquinone (Sigma; both freshly prepared; mixed just before adding) was added to the sample, after which it was kept at 55ºC in the dark under oil for 4h.

Microspin ion exchange columns (Amersham Biosciences, Piscataway, NJ, USA) were used for purification according to the manufacturer's directions. After 5 min incubation, a mixture of 212μl of H_2O, 130 μl 10 M NH_4OAc, 3μl glycogen and 1 ml absolute ethanol was used for precipitation (at -20ºC overnight). The next day, after 30 min centrifuge 13.000 RPM at 4ºC, drainage and washing with 75% ethanol, the pellet was dissolved in 5μl H_2O. Five μl dissolved DNA was multiplexed in a 50 μl PCR reaction using MSP buffer (16.6 mM NH_4SO_4, 67 mM Tris pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1% DMSO), 0.0625 mM dNTP, 0.1 μM $MgCl_2$, 10 U Platinum Taq (Invitrogen), and 100 ng of each reverse and forward primer. The external (non-CpG dependent) primers for *TWIST1* were: TWIST Ext F gagatgagatattatttattgtg; TWIST Ext R cctcccaaaccattcaaaaac. PCR conditions were as follows: 95ºC for 5 min, 36 cycles of 95ºC for 30 sec, 56ºC for 30 sec and 72ºC for 45 sec, followed by an extension cycle of 72ºC for 5 min. Human sperm DNA (HSD) was used as a negative control, *Sss*I treated MDA-MB-231 cells as a positive control. The PCR products were diluted 1:5 with water and stored at -20°C.

Two μl of 1:5 diluted multiplexed DNA were used for real time PCR in a final volume of 25 μl, using 2.5 μl 10x MSP buffer (as above), 200 μM dNTP, 1.25 U Ramp Taq (Denville Scientific Inc., South Plainfield, NJ, USA), 1X

ROX (Invitrogen). A gene-specific primer and probe set containing 600 nM each of two primers (forward and reverse) and 200 nM labeled probe (Applied Biosystems) was added to the reaction mix. The internal primers and probes for *TWIST1* were: TWIST RT-FM gttagggttcgggggcgttgtt; TWIST RT-RM ccgtcgccttcctccgacgaa; TWIST RT-FUM ggtttgggggtgttgtttgtatg; TWIST RT-RUM cccacctcctaaccaccctcc; TWIST M probe aaacgatttccttccccgccgaaa; TWIST UM probe aaacaatttccttccccaccaaaaca. PCR conditions were 95°C for 7 min, followed by 40 cycles of 95°C for 15 s and 65°C for 1 min. A standard curve (dilutions 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}), and a 80K copy number control were included for extrapolating percent methylation from the U and M curves as described previously (14). Percentage methylation was calculated as: 100x [amount of methylated DNA/ (amount of methylated DNA + unmethylated DNA)].

Immunohistochemistry

TWIST1 was immunohistochemically stained on 5 μm thick paraffin slides. Slides were deparaffinized with xylene and serial ethanol dilutions. Endogenous peroxidase activity was blocked for 30 minutes with a buffer solution containing peroxide, followed by antigen retrieval (boiling in citrate buffer, pH 6.0, 20 minutes). Slides were incubated overnight with the anti-TWIST1 antibody (1:100), or E-Cadherin antibody (extracellular portion, Zymed, San Francisco, CA, USA) followed by the secondary antibody (Powervision (Poly-HRP-anti Ms/Rb/Rt IgG biotin free), ImmunoVision Technologies, Brisbane, CA, USA; ready to use). All slides were developed with diaminobenzidine followed by haematoxylin counterstaining. Before the slides were mounted all sections were dehydrated in alcohol and xylene. Appropriate positive and negative controls were used throughout. Percentage of positively stained nuclei was estimated by an experienced pathologist (PJvD) in all cancers. In addition, in those cases where normal breast tissue was present, the nuclear staining intensity of cancer cells was scored in comparison with normal breast cells as higher, similar or lower. E-Cadherin membrane staining was scored as absent, weak, moderate or strong

Statistical analysis

Mann-Whitney test was used for comparing medians between groups. The Chi-square test was used for comparing frequency distributions. Pearson correlations were used for assessing the association between continuous variables. Wilcoxon and Sign tests were used to compare paired data. SPSS 12.0.1 for Windows was used for statistical analysis. Statistical tests were considered statistically significant at two-sided $P < 0.05$.

Results

TWIST1 *promoter methylation*

Mean percentage *TWIST1* methylation in paraffin embedded specimen of the 76 invasive cancers was 33.7% (median 16.7%). There was no difference in mean percentage of *TWIST1* methylation between ductal (33.9%, n=34) and lobular cancers (33.4%, n=42). *TWIST1* promoter methylation was not associated with age, mitotic index, tumor size, grade, or with lymph node, HER2/*neu*, ER, or PR status. Mean methylation in 12 available frozen normal breast specimens (5.92%) was significantly lower (p=0.004, Wilcoxon test) compared to that of matched frozen malignant tissue (42.5%) (Figure 1). Methylation in paraffin-embedded malignant specimen was 51.64%, which was not significantly different from frozen specimen (p=0.594, Wilcoxon test).

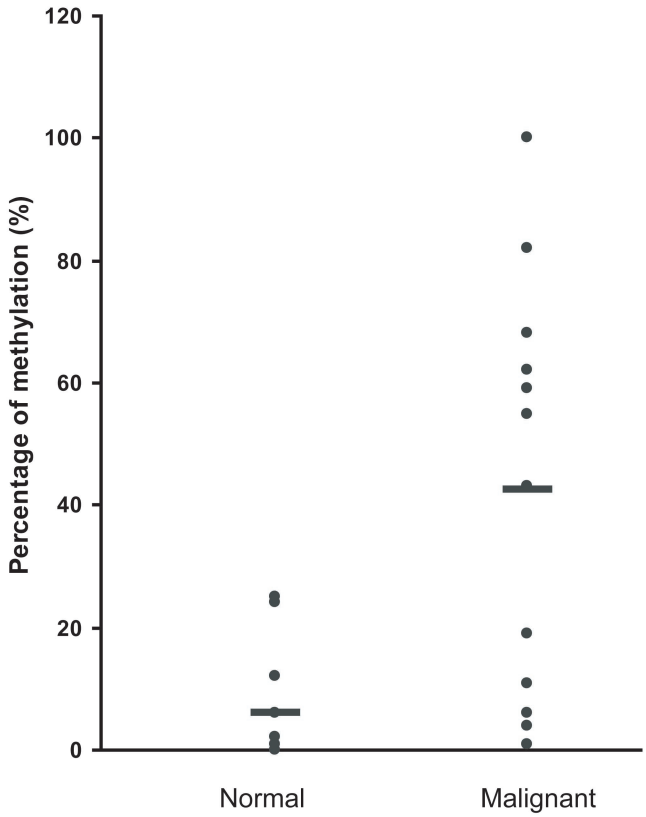

Figure 1.
TWIST1 promoter methylation in breast cancer and normal breast tissue. Each sample is indicated. Mean value is indicated by a line.

TWIST1 protein expression

To study expression of TWIST1 in breast cancer specimen a previously described antibody was used (Mironchik et al., 2005). This antibody was validated by western analysis of MCF-7 cells overexpressing exogenous TWIST1 and immunofluorescence analysis of endogenous TWIST1 in HeLa cancer cells, displaying nuclear staining (Figure 2 A and B). Staining of breast cancers using immunohistochemistry revealed a nuclear pattern in most malignant cells (Figure 2 C-F). In addition, normal breast regularly showed nuclear staining. Mean percentage of stained malignant nuclei was 50.73% (median 50.00%, n=71). There was no difference in TWIST1 expression between ductal and lobular cancers (p=0.972, Mann-Whitney test). No associations between TWIST1 expression and clinicopathological data were found. Of the 65 patients with normal and malignant cells in the same slide, TWIST1 expression was unchanged in 44 cases, enhanced in malignant cells in 17 patients and decreased in the tumor in 4 cases (p=0.007, Sign test).

E-Cadherin protein expression

Specimen were stained for E-cadherin. Table 2 shows the correlation of E-Cadherin expression with clinicopathological features and TWIST1. As expected, lobular carcinoma expressed significantly less E-Cadherin ($p<0.000$, Chi-square test). No associations were found with other clinicopathologic parameters. Pearson's correlation did not reveal any correlation between TWIST1 and E-Cadherin protein expression.

Table 2. E-Cadherin associations.

Feature		E-Cadherin low	E-Cadherin high	p-value
Type (n=70)	Ductal	7 (10.0%)	22 (31.4%)	< 0.000
	Lobular	39 (55.7%)	2 (2.9%)	
Lymph node status (n=63)	Negative	19 (30.2%)	14 (22.2%)	0.458
	Positive	20 (31.7%)	10 (15.9%)	
TWIST1 (n=70)	≤50%	28 (40.0%)	11 (15.7%)	0.229
	>50%	18 (25.7%)	13 (18.6%)	

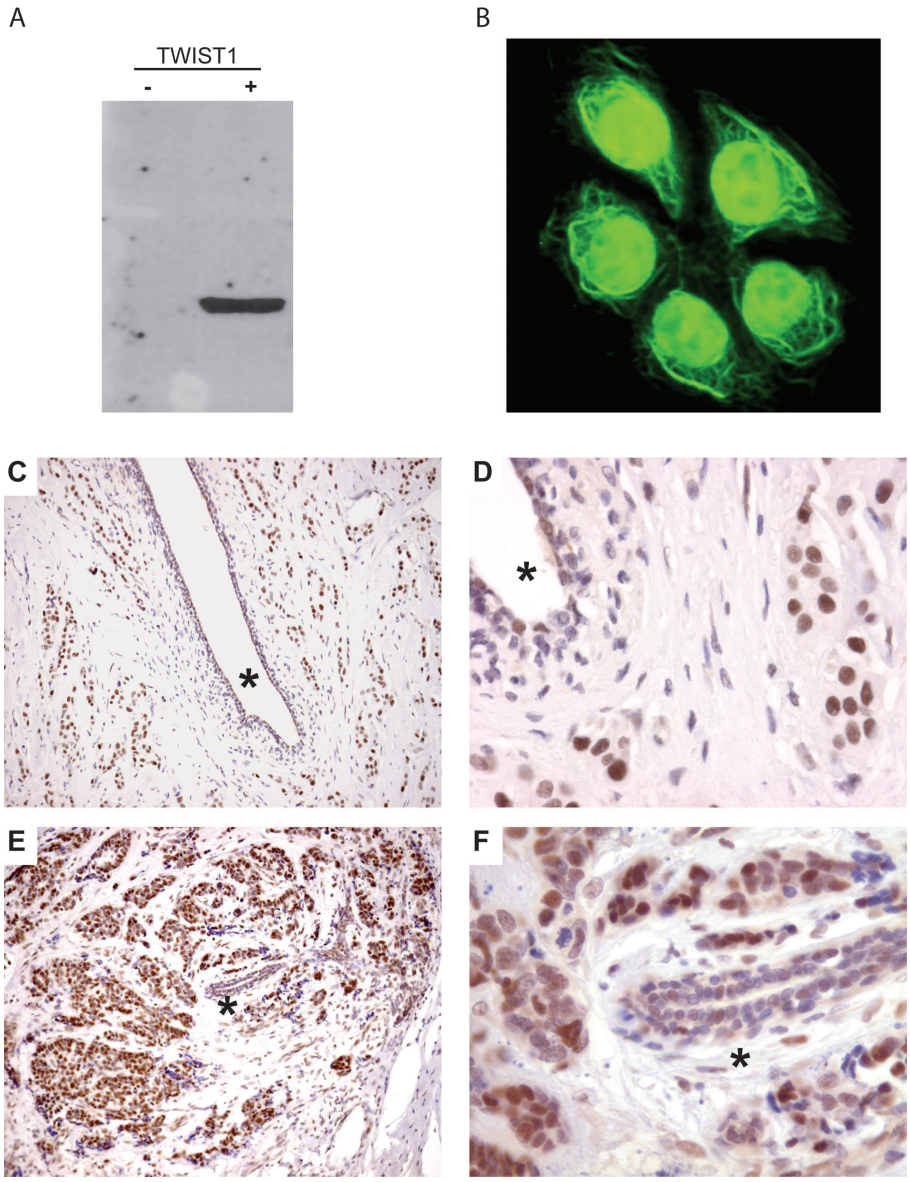

Figure 2.
TWIST1 protein analysis. A) Western blot analysis for TWIST1 of MCF-7 cells either overexpressing TWIST1 or control. B) Immunofluorescence analysis of HeLa cells endogenously expressing TWIST1. Clear nuclear staining is observed together with some cytoskeletal staining. C-F) Immunohistochemistry of TWIST1 in breast carcinoma. Normal breast duct are present (marked by asterisk) with moderate TWIST1 expression in the nuclei surrounded by invasive cancer cells strongly positive for TWIST1. C and D are the same specimen and E and F are the same specimen. C and E show 10x magnification and D and F show 63x magnification.

Relation between TWIST1 promoter methylation status and expression
TWIST1 protein expression did not correlate to *TWIST1* promoter methylation (Pearson correlation coefficient -0.032, p=0.792). To analyze the relation between *TWIST1* promoter methylation and *TWIST1* expression in more detail, we analyzed *TWIST1* mRNA of seven healthy tissue samples and matched malignant tissue. Results were normalized to *TWIST1* mRNA expression levels in HeLa cell lysates. Mean relative RNA expression in normal tissue was 0.95 (median 0.65) and the mean relative RNA expression in malignant tissue was 1.40 (median 0.82), which was not a significant difference (p=1.00, Wilcoxon). RNA analysis of 30 available frozen malignant specimen revealed a mean relative *TWIST1* mRNA expression of 0.95 (median 0.72), which was also not significantly different compared to normal tissue (p=0.578, Mann-Whitney test). Furthermore, no correlation between *TWIST1* methylation status and mRNA expression could be demonstrated in malignant specimens (Pearson correlation coefficient -0.081, p=0.693).

Discussion

In this study, *TWIST1* expression and promoter methylation was studied in breast carcinoma specimens and adjacent normal breast tissue. *TWIST1* expression has been correlated to invasive lobular carcinoma in association with E-Cadherin loss (Yang et al., 2004). *TWIST1* promoter methylation has been suggested to occur less frequently in lobular compared to ductal breast carcinomas (Fackler et al., 2003; Martin et al., 2005). However, in another study, Bae et al described a higher frequency of *TWIST1* promoter hypermethylation in lobular compared to ductal malignancies (Bae et al., 2004). Our study does not support differential *TWIST1* promoter methylation and protein expression between ductal and lobular breast carcinomas. *TWIST1* promoter methylation has been found to be enhanced in breast cancer specimen compared to normal breast tissue from reduction mammoplasty and prophylactic mastectomy samples (Suijkerbuijk et al., submitted). The present study shows that healthy tissue is less frequently methylated for *TWIST1* compared to surrounding malignant tissue from the same patient. This implies that methylation is a progressive event in breast carcinogenesis. Nevertheless, mean methylation percentage in healthy tissue adjacent to a breast tumor in our study group was still higher than the percentages described in completely normal breast tissue in our previous study (Suijkerbuijk et al., submitted). This might point to an early contribution of *TWIST1* promoter methylation and perhaps a role in field cancerization.

We found frequent expression of TWIST1 in invasive breast cancers, in accordance with previous breast cancer studies (Martin et al., 2005; Mironchik et al., 2005; Watanabe et al., 2004; Yang et al., 2004). TWIST1 protein expression was also observed in ductal carcinoma *in situ* in the present study (data not shown), which together suggests that TWIST1 overexpression is involved in breast cancer development, maybe even in an early stage. TWIST1 levels were similar in ductal and lobular cancers, even though lobular cancers with their loss of E-Cadherin expression are thought to have a higher degree of EMT. We did not find any correlation between E-Cadherin loss and TWIST1 expression, however. Interestingly, TWIST1 nuclear expression was also observed in normal ducts in some patients, implying that TWIST1 may have a physiological role in normal breast tissue. However, when comparing paired normal and malignant tissues, TWIST1 was significantly more expressed in malignant nuclei, fitting with the paradigm that *TWIST1* is an oncogene.

The fact that high frequency and degree of promoter methylation of *TWIST1* was in the present study paralleled by increased expression remains puzzling. Furthermore, besides the apparent lack of inverse correlation between promoter methylation and protein expression, mRNA levels between normal and malignant tissue were not clearly different. This does not reflect the difference in protein levels and therefore post-transcriptional regulation of TWIST1 is likely. It remains possible that the methylation observed in the proximal part of the *TWIST1* promoter does not relate to expression of TWIST1, but of genes in genomic proximity. Alternatively, it is possible that *TWIST1* promoter methylation is an early event, which precedes compensatory TWIST1 overexpression. Indeed, *TWIST1* haploinsufficient patients display a higher breast cancer incidence (Sahlin et al., 2007). In case of promoter methylation, compensatory mechanisms might regulate *TWIST1* via unmethylated sequences in the distal promoter or in 3' enhancer sequences. Indeed *TWIST1* is regulated by hypoxia, a common feature in solid cancer, via 3' enhancer sequences (Gort et al., 2007). To further elucidate these mechanisms, the relation between *TWIST1* methylation and expression should be addressed *in vitro* models. Previous studies have shown that increased TWIST1 expression is enhanced in lymph node metastases and predicts survival in breast and other cancers (Kyo et al., 2006; Lee et al., 2006; Martin et al., 2005; Mironchik et al., 2005; Song et al., 2006; Yuen et al., 2007). Although TWIST1 expression did not correlate to lymph node status, it will be interesting to further study TWIST1 in relation to clinical outcome.

We conclude that *TWIST1* promoter methylation is not related to TWIST protein expression in the normal and malignant breast. *TWIST1* promoter methylation is however a strong marker of breast cancer. The observed increased TWIST1 protein levels in breast cancer cells fits with the proposed oncogenic role of *TWIST1* in breast carcinogenesis.

References

Alexander NR, et al. (2006). N-cadherin gene expression in prostate carcinoma is modulated by integrin-dependent nuclear translocation of Twist1. *Cancer Res*, **66,** 3365-9.

Bae YK, et al. (2004). Hypermethylation in histologically distinct classes of breast cancer. *Clin Cancer Res*, **10,** 5998-6005.

Baylin SB and Herman JG. (2000). DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet*, **16,** 168-74.

Bialek P, et al. (2004). A twist code determines the onset of osteoblast differentiation. *Dev Cell*, **6,** 423-35.

Evron E, et al. (2001). Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. *Lancet*, **357,** 1335-6.

Fackler MJ, et al. (2003). DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. *Int J Cancer*, **107,** 970-5.

Fackler MJ, et al. (2004). Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. *Cancer Res*, **64,** 4442-52.

Gort EH, et al. (2007). The TWIST1 oncogene is a direct target of hypoxia-inducible factor-2alpha. *Oncogene*.

Kyo S, et al. (2006). High Twist expression is involved in infiltrative endometrial cancer and affects patient survival. *Hum Pathol*, **37,** 431-8.

Lee MS, et al. (1999). TWIST, a basic helix-loop-helix transcription factor, can regulate the human osteogenic lineage. *J Cell Biochem*, **75,** 566-77.

Lee TK, et al. (2006). Twist overexpression correlates with hepatocellular carcinoma metastasis through induction of epithelial-mesenchymal transition. *Clin Cancer Res*, **12,** 5369-76.

Maestro R, et al. (1999). Twist is a potential oncogene that inhibits apoptosis. *Genes Dev*, **13,** 2207-17.

Martin TA, et al. (2005). Expression of the transcription factors snail, slug, and twist and their clinical significance in human breast cancer. *Ann Surg Oncol*, **12,** 488-96.

Mehrotra J, et al. (2004). Very high frequency of hypermethylated genes in breast cancer metastasis to the bone, brain, and lung. *Clin Cancer Res*, **10,** 3104-9.

Mironchik Y, et al. (2005). Twist overexpression induces in vivo angiogenesis and correlates with chromosomal instability in breast cancer. *Cancer Res*, **65,** 10801-9.

Peinado H, et al. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer*, **7,** 415-28.

Sahlin P, et al. (2007). Women with Saethre-Chotzen syndrome are at increased risk of breast cancer. *Genes Chromosomes Cancer*, **46,** 656-60.

Song LB, et al. (2006). The clinical significance of twist expression in nasopharyngeal carcinoma. *Cancer Lett*, **242,** 258-65.

Thiery JP. (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*, **2,** 442-54.

van Diest PJ. (2002). No consent should be needed for using leftover body material for scientific purposes. For. *BMJ*, **325,** 648-51.

van Diest PJ, et al. (1992). Reproducibility of mitosis counting in 2,469 breast cancer specimens: results from the Multicenter Morphometric Mammary Carcinoma Project. *Hum Pathol*, **23,** 603-7.

Watanabe O, et al. (2004). Expression of twist and wnt in human breast cancer. *Anticancer Res*, **24,** 3851-6.

Widschwendter M, et al. (2001). Epigenetic downregulation of the retinoic acid receptor-beta2 gene in breast cancer. *J Mammary Gland Biol Neoplasia*, **6,** 193-201.

Widschwendter M and Jones PA. (2002). DNA methylation and breast carcinogenesis. *Oncogene*, **21,** 5462-82.

Wilkie AO and Morriss-Kay GM. (2001). Genetics of craniofacial development and malformation. *Nat Rev Genet*, **2,** 458-68.

Yang J, et al. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*, **117,** 927-39.

Yuen HF, et al. (2007). Significance of TWIST and E-cadherin expression in the metastatic progression of prostatic cancer. *Histopathology*, **50,** 648-58.

Chapter 7

A functional screen for regulators of Hypoxia Inducible Factor-1α

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Unpublished data

Abstract

Nutrient and oxygen supply from the microenvironment plays an important role in tumor progression. Oxygen limitation (hypoxia) invariably occurs when tumors outgrow the capacity of the supporting vasculature. Intratumoral hypoxia is one of the major hallmarks of solid tumor growth. Cancer cells undergo genetic and adaptive changes that allow them to survive and proliferate under hypoxic conditions. These changes contribute to aggressive tumor behavior and poor clinical response to treatment. Central in this response is the transcription factor Hypoxia Inducible Factor (HIF), which regulates expression of genes important in response to hypoxia by regulation of transcription from hypoxia responsible elements (HREs). Tumors expressing the hypoxia-dependent subunit HIF-1α display a worse prognosis then those not expressing this protein. Understanding the regulatory pathways controlling HIF-1α expression is therefore of clinical importance. Here, we present a functional rescue screen for HIF-1α regulation based on HRE controled puromycin resistance screening using cDNA overexpression libraries. Genes identified by this approach might function in regulation of HIF-1α expression.

Introduction

Expression of Hypoxia Inducible Factor-1α (HIF-1α) is under control of oxygen-dependent regulatory mechanisms, but also oncogenic pathways play a role (Semenza, 2003). Overexpression of HIF-1a has been correlated with poor prognosis in multiple solid cancer types (Bos et al., 2003). In human breast cancer, perinecrotic expression of HIF-1α represents hypoxia-induced HIF-1α, but additionally diffuse HIF-1α expression remains unexplained (Vleugel et al., 2005). This might reflect micro-hypoxic areas, as well as normoxic HIF-1α expression as a result of genetic changes in HIF-1α regulatory mechanisms. Because HIF-1α expression is of prognostic relevance, the mechanisms that result in normoxic HIF-1α expression are potentially clinically relevant. Here, we present a functional rescue screen to identify novel genes that can induce HIF-1α under hypoxia. Unfortunately, this screen has not been validated. Therefore the data presented here should be considered carefully. However, because the data is not published previously, this appendix might provide information for further research.

Material and Methods

Cell culturing

HeLa cells, Phoenix and 4C12 cells were cultured in Dulbecco's Modified Eagle Medium (Gibco BRL, UK) supplied with 10% Fetal Calf Serum (FCS) and antibiotics (Gibco BRL, UK). The 4C12 cell line was derived from NIH3T3 cells cotransfected with a HRE-puromycin resistance gene together with GFP and a neomycin resistance marker and selected using G418 (Sigma, MO, USA). When indicated, cells were exposed to hypoxia (1% O2) using the hypoxic workstation. For proteasomal inhibition MG132 (Sigma, MO, USA) was used at indicated concentrations. Rescue experiments were stained using Coomassie Blue (Bradford, CA, USA) after fixation in methanol.

Infection, transfection and luciferase assay

Phoenix packaging cells were used to generate ecotropic retroviruses as described (Serrano et al., 1997). 4C12 cells were infected in three consecutive rounds with viral supernatant supplemented with 4 μg/mL polybrene. HeLa cells were transfected using Lipofectamine 2000© (Invitrogen, CA, USA) according to the manufacturers protocol. For luciferase reporter assays, cells were lysed in passive lysis buffer (Promega Corporation, WI, USA) according to the manufacturers protocol. Experiments were performed in triplicate. Samples were assayed with a dual luciferase kit (Promega Corporation, WI, USA) and measured using a VeritasTM 96-well plate luminometer (Turner Biosystems, CA, USA). All values were normalized using TK-Renilla (Promega) expression.

PCR, cloning and plasmids

The HRE-puromycin resistance construct was generated by replacing the luciferase gene from p11WT, encompassing the HIF-1-binding site of VEGF promoter at position –985 and –939 (generous gift from Dr. Gregg Semenza) with the puromycin resistance gene from pBabePuro (Forsythe et al., 1996). For identification of retroviral cDNAs, long term PCR was used to amplificate large products using an expand PCR kit (Roche, Switzerland) according to manufacturers protocol and using primers pMX-F 5'-TCTCCAAGCTCACTTACAGGC-3' and pMX-R 5'-TTCGAAGTCGATGACGGCAG-3'. A 3.5 kb product derived from PCR on clone A31.5 was ligated into pCR2.1 (Invitrogen, CA, USA). PMX-eIF3C and pCDNA3-eIF3C were constructed by restriction of pCR2.1-eIF3 using *EcoR*I and subsequent ligation of the 3.5 kb fragment into *EcoR*I digested pMX, respectively pCDNA3.

Western blotting

20 μg protein/lane was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blot analysis. The following antibodies were used; anti-HIF-1α and anti-HIF-1β (BD Transduction Laboratories, CA, USA), anti-eIF3C (Santa Cruz, CA, USA), anti-COX-2 (Cell Signaling Technology, MA, USA), anti-Glut-1 (A3536, Dako, CA, USA), anti-β-actin (US Biological, MA, USA), goat anti-mouse IgG + IgM HRP conjungate (Biosource, CA, USA) and goat anti-rabbit IgG HRP conjungate (Biorad, CA, USA). Enhanced Chemo Luminescence (ECL, Amersham Biosciences, UK) was used for signal detection according to the manufacturers protocol.

Results

HIF-1 functions as a transcription factor and binds to hypoxia response elements (HREs) in the DNA (Wenger et al., 2005). To design a functional screen, a puromycin resistance gene was cloned to be expressed under control of a HRE consensus sequence (5'-CAGCTG-3') plus flanking sequences, derived from the human VEGF promoter (Forsythe et al., 1996; Shibata et al., 1998). NIH3T3 cells were stably transfected to express this construct. Stable clones that were resistant to puromycin-induced cell-death under hypoxic conditions (1% oxygen), but not under hypoxia were selected (Figure 1).

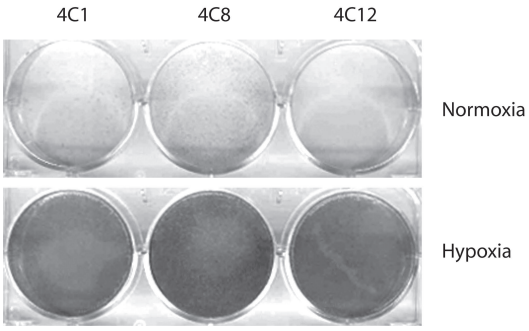


Figure 1.
NIH3T3 clones stably expressing the HRE-puromycin resistance construct were cultured under normoxia or hypoxia in the prescence of puromycin for 3 days. Coomassie staining was used to visualize survival.

The stable clone 4C12 was expanded. Subsequently, 4C12 cells were infected using a high complexity cDNA library generated from peripheral blood lymphocytes of a patient suffering from polycythaemia vera, a myeloproliferative disorder (Shvarts et al., 2002). Cells were exposed to puromycin under normoxia and resistant colonies were isolated and expanded. Using Moloney superinfection, integrated proviruses were recovered and a second round screen was performed by infecting fresh 4C12 cells (Jacobs et al., 2000). Second round colonies were isolated and expanded (Figure 2). Genomic DNA from second round colonies was isolated and retrovirally expressed cDNAs were recovered by PCR using retrovirus specific primers (Figure 2A). Common PCR products from secondary clones derived from one primary clone were sequenced. A list of genes present within recovered cDNA sequences is shown (Table 1).

Among these is eukaryotic Initiation Factor 3 C (eIF3C). This encodes a protein involved in the protein translation complex eIF3. The eIF3 complex is the largest among the initiation factors and consists of 11 non-identical subunits (Asano et al., 1997a; Asano et al., 1997c). It is involved in processes during protein translation like ternary complex stabilization, 40S ribosome binding, facilitation of mRNA binding to the 40S ribosome, promotion of the dissociation of 40S and 60S ribosomal subunits and internal ribosomal entry, which enables CAP-independent translation of mRNAs (Asano et al., 1997a; Lopez de Quinto et al., 2001). The eIF3C cDNA was cloned back into the original viral vector and 4C12 cells infected with eIF3C were resistant to puromycin, demonstrating that eIF3C was the cDNA responsible for the original colony (Figure 3).

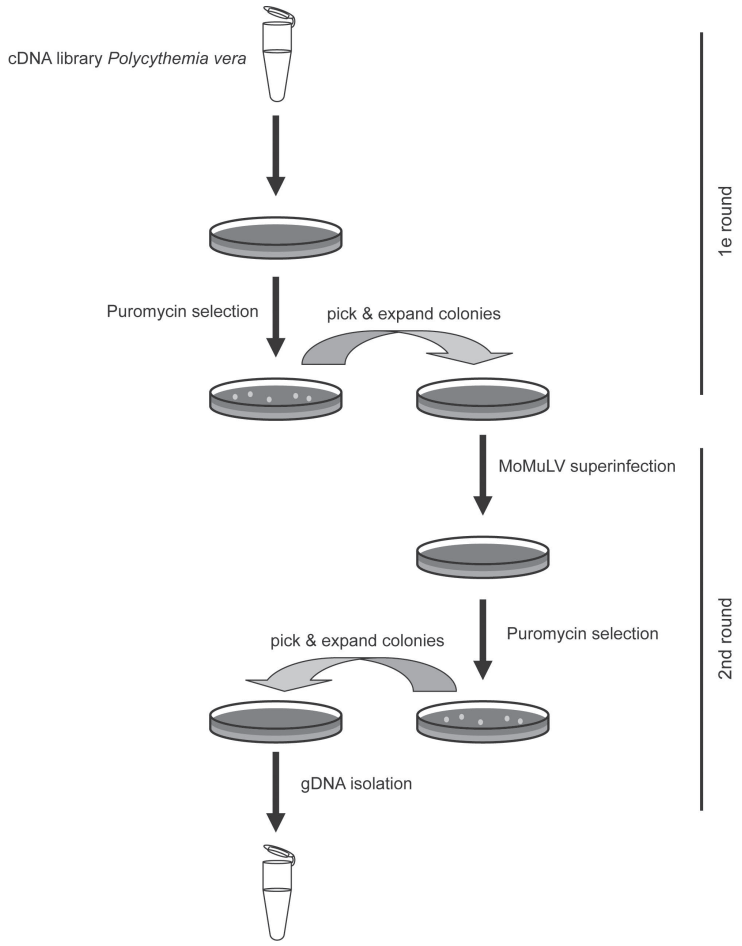


Figure 2.
Schematic model of screening approach.

Table 1. Identified genes with clone number and independent rescue data.

Clone	Insert	Rescue
A1	Integrin α subunit 5	N.A.
A2	Nucleotide Oligomerisation Domain 27 (NOD27)	Yes
A31	elongation Initiation Factor 3 110 kD subunit (eIF3C)	Yes
B5	NEDD9 interacting protein with Calponin homology (NICAL)	No
B14a	Rab1B	No
B14b	Folic Acid Receptor γ	No
B23	Myosin 1F	N.A.
B30	RPL19 (ribosomal protein L19)	N.A.

(N.A. is not available.)

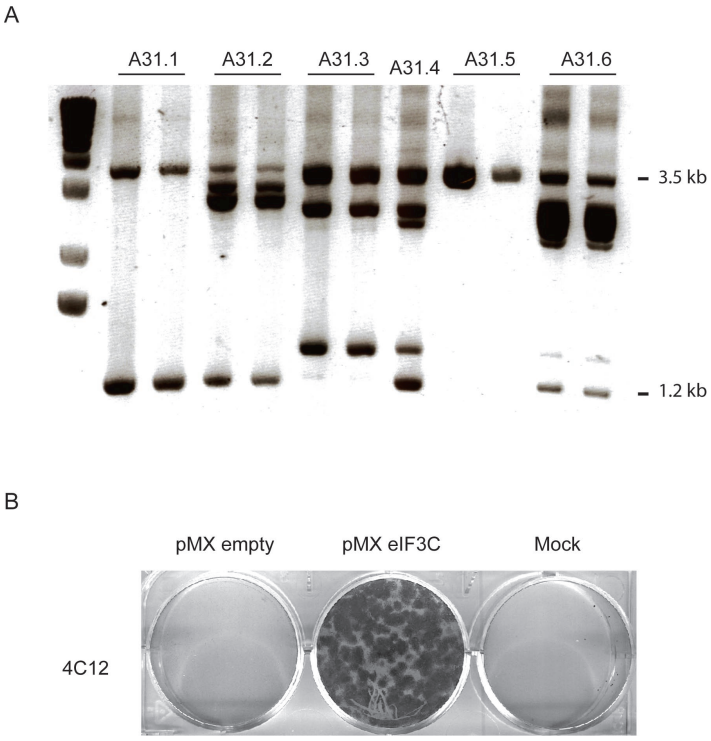


Figure 3.
A) PCR products of secondary clones of A31, which was found to contain the eIF3C sequence (3.5kb band). B) Independent rescue experiment after infection using pMX subcloned eIF3C sequence. Coomassie staining was used to visualize survival.

To study the effect of eIF3C on HIF-1α, HeLa cells were transfected with eIF3C and cultured under normoxia or under hypoxia. No changes in HIF-1α expression was detected under normoxia, whereas HIF-1α expression was induced by hypoxia and this was enhanced by overexpression of eIF3C (Figure 4A). Treatment of HeLa cells with the proteasome inhibitor MG132 under normoxic conditions resulted in stable HIF-1α expression (Figure 4B).

This was not further enhanced by eIF3C overexpression, suggesting that eIF3C does not influence HIF-1α synthesis, at least under normoxia. Analysis of HIF-1α transcriptional activity by reporter gene analysis and target gene expression by protein analysis, revealed no further enhancement of hypoxic HIF-1α activity by eIF3C overexpression.

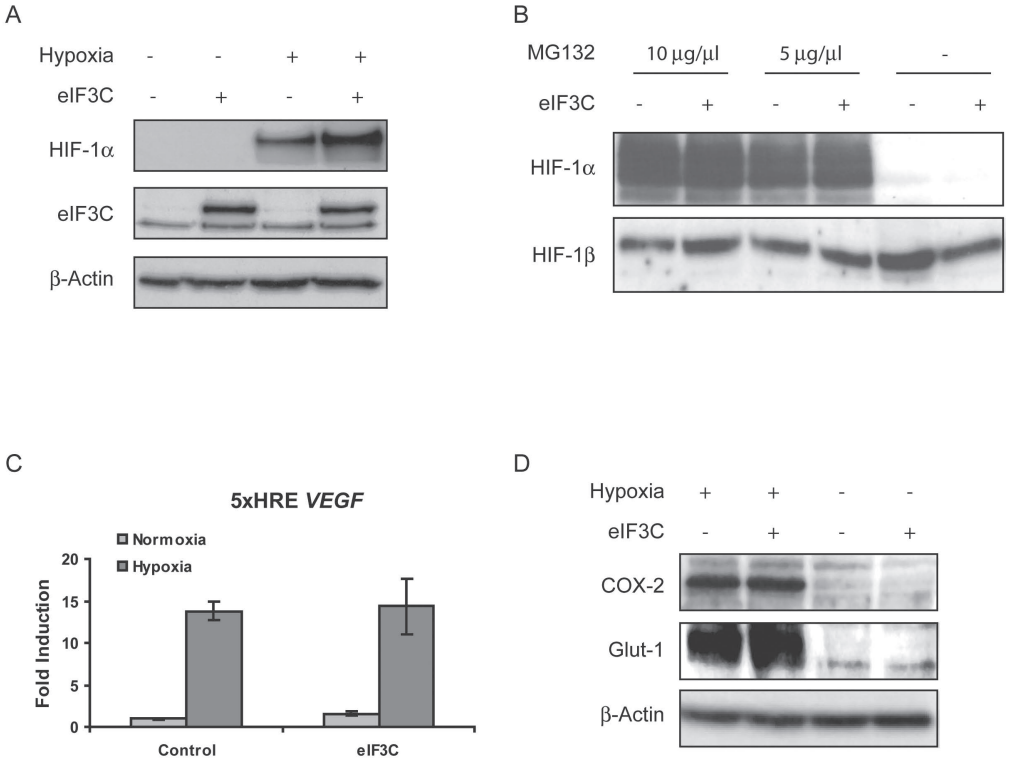


Figure 4.
A) Western blot analysis of indicated proteins. HeLa cells were transfected with eIF3C or control vector and were 48 hours later cultured for 6 hours under normoxia or hypoxia (1% O_2). B) Western blot analysis of indicated proteins. HeLa cells were transfected with eIF3C or control vector and were 48 hours later cultured in the presence of indicated amount of MG132 for 2 hours. C) Luciferase assay using 5xHRE reporter. HeLa cells were transfected with eIF3C or control vector and were 48 hours later cultured for 16 hours under normoxia or hypoxia (1% O_2). Western blot analysis of indicated proteins. HeLa cells were transfected with eIF3C or control vector and were 48 hours later cultured for 16 hours under normoxia or hypoxia (1% O_2).

Discussion

The puromycin resistance screen provides a novel method to identify HIF-1α regulators, but major drawbacks compromise its use in further research. First of all, we were unable to biochemically proof that any of the identified cDNAs can induce normoxic HIF-1α activity. The eIF3C overexpression might enhance hypoxic HIF-1α expression, but not its trancriptional activity. Technical difficulties hampered proper analysis of HIF-1α expression in the first and second round colonies, including difficulties in reproducible HIF-1α detection in murine cells. Novel antibodies have been developped to overcome this problem (Groot et al., 2006). By analysis of identified cDNAs in human cells, it is possible that initial effects are lost, however unlikely, as the cDNA library is of human origin. Second drawback is that the original cell line 4C12 used to perform the screen has in some experiments lost its capacity to confer puromycin resistance under hypoxia. This puts question marks to whether the original screen is reproducible and hampers validation of newly identified cDNAs. Third drawback is the high amount of false positives. Although the original screen contained a negative control that did not show any resistance, later experiments did find spontaneously resistant colonies. Other strategies to pinpoint primary positives to those that regulate HIF-1α using stable cell lines expressing a HRE-reporter gene were unsuccesful, therefore this remains an obstacle.

Nevertheless, the eIF3C overexpression did appear to influence HIF-1α protein expression under hypoxia. Further research concerning eIF3C did include analysis of CAP versus IRES dependent translation of HIF-1α, because IRES-mediated translation of HIF-1α has been reported, but no effects were observed (data not shown) (Lang et al., 2002). Recently, it was reported that another member of the eIF3 complex, the tumor suppressor eIF3E (Int6 or p48), is able to bind HIF-2α and thereby targets it for proteasomal degradation (Asano et al., 1997b; Chen et al., 2007). It might be interesting to further investigate the role of eIF3 in HIF-2α regulation, but this will most probably not explain our screening results, since transcriptional activity of HIF was not altered by eIF3C.

In conclusion, screening for HIF-1α regulators is a difficult process that requires watertight screening conditions and high sensitivity. A similar system using human cells might facilitate validation, but alternative systems should be considered.

References

Asano K, et al. (1997a). Conservation and diversity of eukaryotic translation initiation factor eIF3. *J Biol Chem*, **272,** 1101-9.

Asano K, et al. (1997b). The translation initiation factor eIF3-p48 subunit is encoded by int-6, a site of frequent integration by the mouse mammary tumor virus genome. *J Biol Chem*, **272,** 23477-80.

Asano K, et al. (1997c). Structure of cDNAs encoding human eukaryotic initiation factor 3 subunits. Possible roles in RNA binding and macromolecular assembly. *J Biol Chem*, **272,** 27042-52.

Bos R, et al. (2003). Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*, **97,** 1573-81.

Chen L, et al. (2007). Mammalian tumor suppressor Int6 specifically targets hypoxia inducible factor 2 alpha for degradation by hypoxia- and pVHL-independent regulation. *J Biol Chem*, **282,** 12707-16.

Forsythe JA, et al. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*, **16,** 4604-13.

Groot AJ, et al. (2006). Identification by phage display of single-domain antibody fragments specific for the ODD domain in hypoxia-inducible factor 1alpha. *Lab Invest*, **86,** 345-56.

Jacobs JJ, et al. (2000). Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers. *Nat Genet*, **26,** 291-9.

Lang KJ, et al. (2002). Hypoxia-inducible factor-1alpha mRNA contains an internal ribosome entry site that allows efficient translation during normoxia and hypoxia. *Mol Biol Cell*, **13,** 1792-801.

Lopez de Quinto S, et al. (2001). IRES interaction with translation initiation factors: functional characterization of novel RNA contacts with eIF3, eIF4B, and eIF4GII. *Rna*, **7,** 1213-26.

Semenza GL. (2003). Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*, **3,** 721-32.

Serrano M, et al. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*, **88,** 593-602.

Shibata T, et al. (1998). Enhancement of gene expression under hypoxic conditions using fragments of the human vascular endothelial growth factor and the erythropoietin genes. *Int J Radiat Oncol Biol Phys*, **42,** 913-6.

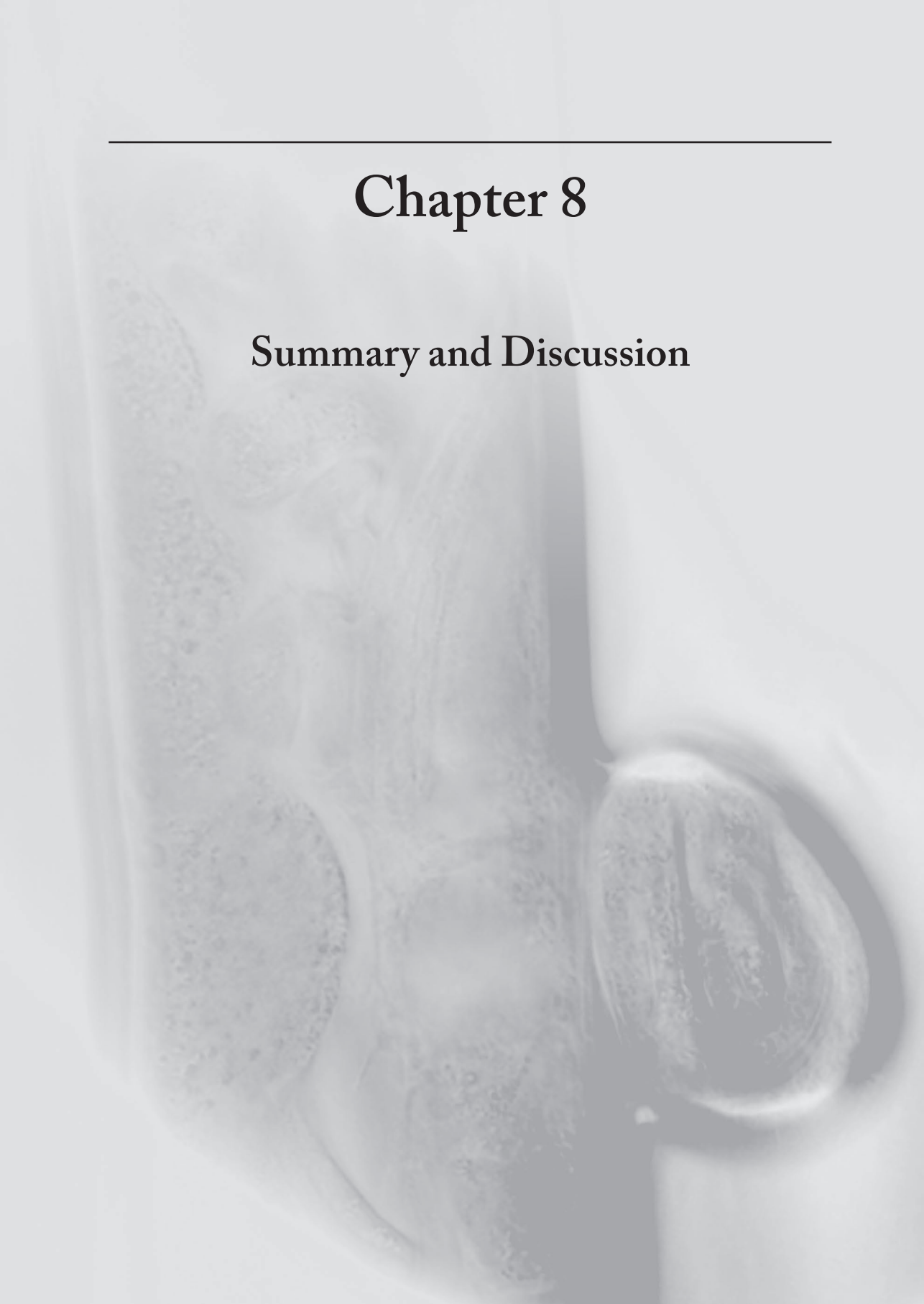
Shvarts A, et al. (2002). A senescence rescue screen identifies BCL6 as an inhibitor of anti-proliferative p19(ARF)-p53 signaling. *Genes Dev*, **16,** 681-6.

Vleugel MM, et al. (2005). Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in invasive breast cancer. *J Clin Pathol*, **58,** 172-7.

Wenger RH, et al. (2005). Integration of oxygen signaling at the consensus HRE. *Sci STKE*, **2005,** re12.

Chapter 8

Summary and Discussion



The molecular response to hypoxia is diverse and complicated. In this thesis, several aspects of this response are described and multiple model systems are used to define the molecular pathways involved. In Chapter 1, the regulation of HIF-1α, the major transcription factor that plays a crucial role in the cellular adaptation to oxygen is reviewed. HIF transcription factors directly regulate angiogenesis, glycolysis and cell survival (Semenza, 2003). Furthermore, hyperactivity of the HIF pathway is often associated with poor prognosis in cancer patients (Bos et al., 2003). Therefore, understanding the HIF pathway is of major importance in the development of treatment strategies that target tumor survival and other malignant cancer properties influenced by hypoxia. In Chapter 2, a review is presented which dissects the plethora of signaling pathways regulated by HIF proteins that may explain the aggressive nature of hypoxic tumors and poor clinical outcome. The major regulatory mechanisms controlling HIF expression and activity are known and involve oxygen sensors that modify the HIF protein to alter its properties in terms of stability and interactions (Semenza, 2003). Additional regulation of HIF stability and activity is mediated by multiple signal transduction cascades. One of these is the oncogenic PI 3-kinase-Akt pathway (this thesis) (Bardos & Ashcroft, 2004). Understanding oncogenic HIF regulation is important to define the interplay between hypoxia and malignant progression.

In chapter 3 a model is presented to study the molecular behavior of cells in hypoxic tumor areas. As these cells are generally restricted from blood supply, they lack oxygen, nutrients and ectopic growth factors. To study mechanisms that alter HIF-1α function under these pathophysiological circumstances, cells grown in the absence of serum and oxygen were studied and found to have a defect in HIF-1α stabilization compared to hypoxic cells in serum-maintained culture. We found that activation of the PI 3-kinase/Akt pathway overcomes this reduction in HIF-1α levels. It seems likely that robust HIF-1α expression required the PI 3-kinase/Akt pathway. This may have direct clinical relevance since breast cancers that lack Akt-1 pathway activity, almost invariably lack HIF-1α expression. In breast cancer, expression of Her-2/neu is a strong predictor of prognosis and a powerful target for anti-cancer therapy (Hynes & Lane, 2005). Since Her-2/neu expression activates the PI 3-kinase pathway and induces HIF-1α, Her-2/neu positive breast cancer might have enhanced hypoxic HIF-1α expression (Laughner et al., 2001). Because inhibition of mTOR by Rapamycin reduces HIF-1α levels comparable to inhibition of PI 3-kinase, it seems likely that mTOR signaling is involved. Therapeutic exploitation of Rapamycin is in development and part of its effect is inhibition of angiogenesis; a HIF-driven

phenotype (Phung et al., 2006). Control of HIF-mediated VEGF regulation by mTOR can partly explain Rapamycins therapeutic value. It will be interesting to await the results from ongoing clinical trials studying Trastuzumab (Herceptin) and Sirolimus (Rapamune, Rapamycin) combinatory therapy and to evaluate HIF expression in these tumor specimens (for more information see http://clinicaltrials.gov/ct/show/NCT00411788).

Recently, expression of HIF-1α in endometroid endometrium carcinoma progression has been described (Horree et al., 2007). In chapter 4, we observed re-expression of the cell cycle regulator p27 (Kip1, CDKN1B) in perinecrotic tumor areas, where HIF-1α is expressed. p27 is a CDK inhibitor that binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4/6, leading to cell cycle inhibition at the G1-checkpoint (Toyoshima & Hunter, 1994). We show in endometrium carcinoma cell lines that hypoxic p27 induction is HIF-1α dependent. It seems likely that hypoxic p27 regulation is dependent on more than HIF alone. PTEN inactivation is often seen in endometrial carcinoma, which results in enhanced PI-3kinase-Akt signaling. While on one side this might promote HIF signaling and induce p27 expression, Akt-mediated repression of the forkhead transcription factor FOXO4 (AFX) activity should result in repression of p27 (Medema et al., 2000). HEC1B cells that lack PTEN expression, upregulated p27 under hypoxia, but HEC1A cells that retain PTEN activity did only very mildly, which was not altered when PI 3-kinase signaling was induced by insulin (Figure 1a). PTEN status did not correlate with hypoxic re-expression of p27 in endometrial tumor specimen (data not shown). Therefore, the role of PTEN in hypoxic p27 expression remains undefined. Another pathway involved might be the energy sensing LKB1-AMPK pathway. In hypoxic cells the ATP/ADP ratio decreases, resulting in LKB1-AMPK activation. LKB1-AMPK function in p27 regulation is also potentially bivalent. Activation of the pathway decreases HIF signaling by mTOR inhibition, which would attenuate hypoxic p27 expression. On the other hand, LKB1-AMPK can phosphorylate p27, which results in an increased stability. Therefore, analysis of p27 phosphorylation status in tumor specimens might elucidate involvement of LKB1-AMPK in endometrial cancer. The essential role for HIF-1α in hypoxic p27 induction does not need to be direct, and a HIF-1 independent region of the proximal p27 promoter is supposed to mediate this (Gardner et al., 2001). Interaction of HIF-1α with the c-Myc oncogene might relieve c-Myc-mediated repression of the p27 promoter (Koshiji et al., 2004). Whether this interaction occurs in endometrial carcinoma remains to be determined.

Expression of p27 is generally lost during endometrial cancer progression, which results in cell proliferation. We hypothesize that hypoxia induced re-expression of p27 in perinecrotic tumor areas functions to halt tumor cell proliferation. This mechanism leads to survival of hypoxic tumour cells and enables a HIF driven change in gene expression profile. The growth of arrest of these hypoxic tumour cells may explain resistance to chemotherapy, which is often directed to target dividing cells. Eventually, these cells may constitute a metastatic cell pool, considering the hypoxic gene profile present in these cells. Interestingly, hypoxic cells decide between death and survival, which is illustrated by intratumoral necrosis surrounded by perinecrotic quiescent cells. In addition to p27 induction, hypoxia reduces expression of cyclins, like Cyclin B1, D1 and E, most likely in a HIF-1α independent fashion (Figure 1b). HIF-mediated regulation of the cell cycle inhibitor p27 points to a protective role, which can be overruled by p53-dependent apoptosis as conditions become almost anoxic (<0.1% O_2) (An et al., 1998). Less severe hypoxia (~1% O_2) does not induce p53 (Figure 1b), therefore perinecrotic HIF expression in endometrium carcinoma probably acts to promotes tumor cell survival. Oxygen sensing is important for all aerobic organisms and it is therefore not surprising that the oxygen sensing pathways are essentially conserved across species. In chapter 5 we exploited this and analysed the HIF-1 pathway in the nematode *Caenorhabditis elegans*. We made use of the recent finding that *C. elegans* lacking the HIF-1 prolyl hydroxylase EGL-9 that are defective in normal egg-laying, are rescued by concomitant *hif-1* knock down. After screening the whole genome for egg laying restoring genes, we found 137 genes capable of reverting the phenotype. False positives and negatives are inevitable when conducting such a large screen.

For example, temperature and oxygen distribution is critical and 96-well format screening does influence the addresses scored positive and therefore rescreening using differential gridding is necessary. We rescreened the positives from this first screen for other HIF-related phenotypes, like hypoxia survival. We identified five genes with severe defects in hypoxic adaptation. As a proof of principle the HIFα and HIFβ homologues were also recovered from this candidate gene based screen. In addition, *gska-3*, *spe-8* and *hlh-8* were identified. Unfortunately, we were unable to reveal the exact mechanism by which these genes interfere with hypoxic survival. Spatial and temporal restricted expression patterns impaired robust validation of these targets using quantitative PCR on whole worm preparations. Nevertheless, we were able to provide functional insight into the mammalian homologue of one of these genes, *hlh-8* (encoding HLH-8 or ceTWIST).

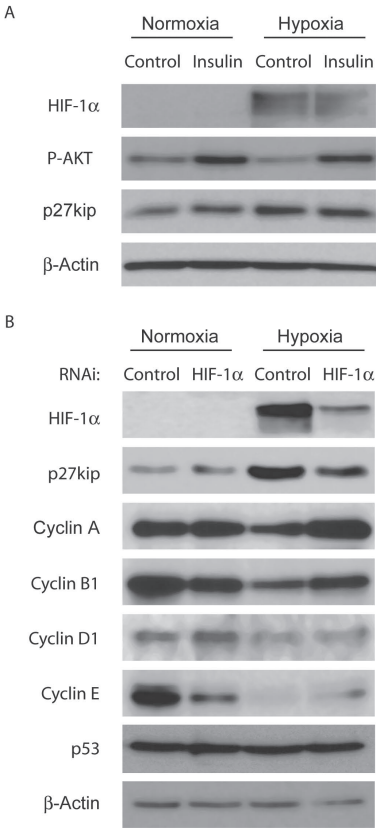


Figure 1.
Western blot analysis of indicated proteins. A) HEC1A cells were cultured in the absence or presence of 1μM insulin (Sigma St Louis, MO, USA) for 24 hours and were simultaneously cultured under normoxic or hypoxic conditions. B) HEC1B cells were transfected with a knock-down plasmid against HIF-1α or a control and 24 hours after transfection cultured under normoxic or hypoxic conditions for 24 hours.

We found that human *TWIST1* is induced by hypoxia in a HIF-2α dependent fashion and that HIF-2α regulates *TWIST1* via two HREs located in an intronic sequence, 3' of the TWIST1 coding region. Recently, *TWIST1* related EMT-inducing genes, like *SIP1* and *SNAI1*, were also found to be regulated by hypoxia (Esteban et al., 2006; Krishnamachary et al., 2006). These are thought to function as repressors E-Cadherin. E-Cadherin loss is one of the hallmarks of metastatic breast cancer. However, we were unable to show E-cadherin repression in hypoxic cell cultures that induced *TWIST1*. Therefore, the molecular pathways downstream of hypoxic TWIST1 remain speculative. TWIST1 has been shown to inhibit oncogene-induced apoptosis by inhibiting the ARF-MDM2-p53 pathway (Maestro et al., 1999). The effects of TWIST1 on oncogene-induced apoptosis are interesting in view of the refractory nature of hypoxic tumors to respond to chemo or radiotherapy. Indeed tumor cells lacking TWIST1 are sensitized to chemotherapy providing a rationale for clinical intervention (Kwok et al., 2005; Wang et al., 2004). Under hypoxic conditions, enhanced TWIST1 expression might also function anti-apoptotic, because

hypoxic HeLa cells transfected with TWIST1 siRNAs display more apoptotic nuclei than controls (unpublished data). Thus TWIST-1 in hypoxic cells may act to suppress p53 dependent apoptosis and enable a p27 mediated growth arrest. The role of TWIST1 in hypoxia-induced EMT is interesting, but so far only supported by the observation that hypoxic NMuMG cells, mouse mammary cells, display similar phenotype under hypoxia as after stimulation with TGFβ, which is known to induce TWIST1 expression (Thuault et al., 2006). It will be important to further investigate the relation between hypoxia and metastasis in mouse models in the presence or absence of TWIST1. Furthermore, the striking difference in capacity of HIF-1 and HIF-2 to regulate *TWIST1* warrents further investigation. In VHL-deficient renal cell carcinoma HIF-2, not HIF-1 is responsible for the tumorigenic phenotype. Stromal expression of HIF-2 has been correlated with poor outcome. Therefore, the role of HIF-2 in cancer development is only emerging and likely to be at least is as important as that of HIF-1. Altogether, establishing *TWIST1* as a downstream target of HIF-2α is likely important, because hypoxia and metastasis are associated.

TWIST1 expression has been causally linked to metastasis in mammary carcinoma mouse models (Yang et al., 2004). Furthermore, *TWIST1* promoter methylation has been shown to predict malignant disease in breast tissue (Suijkerbuijk et al, unpublished) (Fackler et al., 2003). In chapter 6, we examine *TWIST1* expression and methylation status in mammary carcinoma specimens and relate this to malignant progression. Whereas in healthy control tissue we found low *TWIST1* promoter methylation, in tumors TWIST-1 methylation was elevated.. Promoter methylation is generally considered to repress gene expression, and therefore this finding was somewhat surprising. The lack of correlation between *TWIST1* mRNA or protein expression and methylation status in these tumours suggests that *TWIST1* promoter methylation does not affect mRNA expression. Recently, patients haploinsufficient for *TWIST1* were found to have an increased risk for the development of breast cancer (Sahlin et al., 2007). An altenative hypothesis worth testing is that loss of *TWIST1* is an early initiating event, either by mutation or methylation. In time, a subset of cells might start re-expressing TWIST1, via methylation independent regulatory mechanisms. To address this we sequenced the helix-loop-helix domain of TWIST1, known to contain mutations in patients with the Saethre-Chotzen syndrome, in a small cohort (n=40), but did not find any mutations (Kress et al., 2006). Possibly, tumorigenesis selects for high TWIST-1 expressing cells (driven by hypoxia or mutations) that leads to subclones with metastatic potential. It will therefore be interesting to correlate methylation and expression between healthy, primary

and metastatic tumours. The HIF-2 responsive elements we found that drive hypoxic *TWIST1* expression are in the first *TWIST1* exon, 2 kb downstream of the methylated CpG islands within the *TWIST1* promoter. Albeit very speculative, one can imagine a role for hypoxia-induced *TWIST1* expression despite a methylated 5' promoter CpG island. This model could explain early *TWIST1* promoter methylation which is correlated with maligancy, but retains expression, which ultimately might cause metastatic disease. This is supported by the observation that TWIST1 is sometimes found perinecrotically expressed in breast cancers (Figure 2). This observation is however preliminary and other TWIST1 regulating factors are most certainly involved. It will therefore be important to relate TWIST1 expression to patient survival.

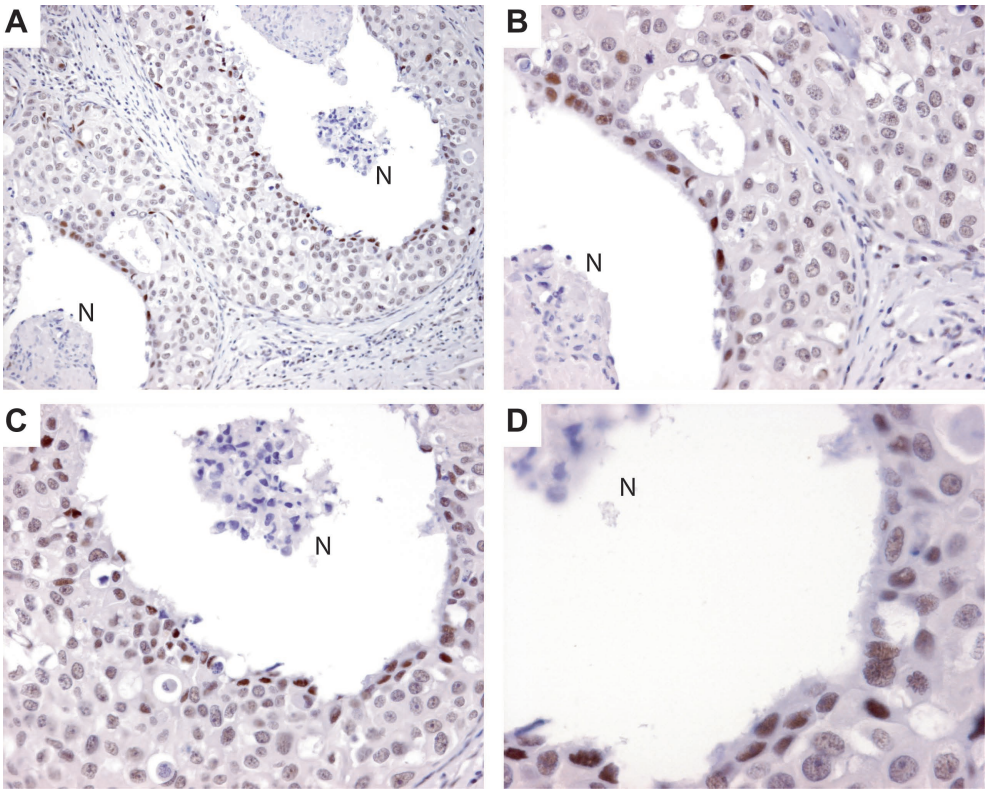


Figure 2.
Immunohistochemical analysis of TWIST1 expression in breast cancer showing perinecrotic expression. Necrosis is indicated by N. A is a 10x magnification, B and C are 20x magnification and D is a 40x magnification.

As mentioned earlier, insight into HIF-1α regulation is of clinically importance. In chapter 7 a functional rescue screen for HIF-1α regulators is discussed. This screen identified eIF3C as a potential regulator of HIF-1α, but we were unable to confirm this in independent experiments. Recently, eIF3E (Int6) was found to be involved in HIF-2α regulation (Chen et al., 2007). Therefore, unraveling the role of eIF3C in HIF signaling might still be interesting.

This thesis provides further insight into hypoxic signaling, upstream and downstream of HIF-1 and HIF-2 (Figure 3). By integrating genetics with cell biology and biochemisty we have uncovered several novel aspects of HIF signaling relevant to the normal hypoxia response as well that in cancer cells. The main conclusions are 1) the PI-3kinase pathway plays an important role in HIF-1α regulation in breast carcinoma, 2) p27 is re-expressed in endometrial carcinoma in a HIF-1α dependent manner, 3) *TWIST1* is induced by HIF-2α, 4) screening genetic model systems like *C. elegans* allows novel insight into mammalian hypoxic signaling pathways, and 5) the relation between *TWIST1* methylation and expression in breast cancer is more complex then assumed.

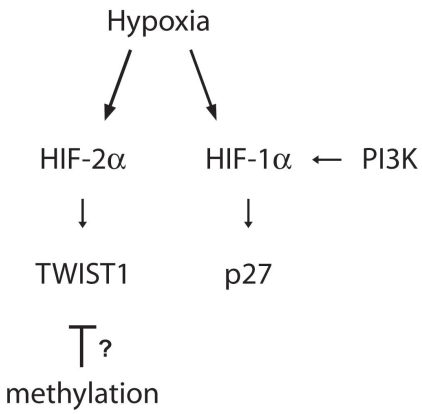


Figure 3.
Schematic representation of novel aspects of hypoxic signaling.

References

An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV and Neckers LM. (1998). Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. *Nature*, **392,** 405-8.

Bardos JI and Ashcroft M. (2004). Hypoxia-inducible factor-1 and oncogenic signalling. *Bioessays*, **26,** 262-9.

Bos R, van der Groep P, Greijer AE, Shvarts A, Meijer S, Pinedo HM, et al. (2003). Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*, **97,** 1573-81.

Chen L, Uchida K, Endler A and Shibasaki F. (2007). Mammalian tumor suppressor Int6 specifically targets hypoxia inducible factor 2 alpha for degradation by hypoxia- and pVHL-independent regulation. *J Biol Chem*, **282,** 12707-16.

Esteban MA, Tran MG, Harten SK, Hill P, Castellanos MC, Chandra A, et al. (2006). Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. *Cancer Res*, **66,** 3567-75.

Fackler MJ, McVeigh M, Evron E, Garrett E, Mehrotra J, Polyak K, et al. (2003). DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. *Int J Cancer*, **107,** 970-5.

Gardner LB, Li Q, Park MS, Flanagan WM, Semenza GL and Dang CV. (2001). Hypoxia inhibits G1/S transition through regulation of p27 expression. *J Biol Chem*, **276,** 7919-26.

Horree N, van Diest PJ, van der Groep P, Sie-Go DM and Heintz AP. (2007). Hypoxia and angiogenesis in endometrioid endometrial carcinogenesis. *Cell Oncol*, **29,** 219-27.

Hynes NE and Lane HA. (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer*, **5,** 341-54.

Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC and Huang LE. (2004). HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. *Embo J*, **23,** 1949-56.

Kress W, Schropp C, Lieb G, Petersen B, Busse-Ratzka M, Kunz J, et al. (2006). Saethre-Chotzen syndrome caused by TWIST 1 gene mutations: functional differentiation from Muenke coronal synostosis syndrome. *Eur J Hum Genet*, **14,** 39-48.

Krishnamachary B, Zagzag D, Nagasawa H, Rainey K, Okuyama H, Baek JH, et al. (2006). Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. *Cancer Res*, **66,** 2725-31.

Kwok WK, Ling MT, Lee TW, Lau TC, Zhou C, Zhang X, et al. (2005). Up-regulation of TWIST in prostate cancer and its implication as a therapeutic target. *Cancer Res*, **65,** 5153-62.

Laughner E, Taghavi P, Chiles K, Mahon PC and Semenza GL. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol Cell Biol*, **21,** 3995-4004.

Maestro R, Dei Tos AP, Hamamori Y, Krasnokutsky S, Sartorelli V, Kedes L, et al. (1999). Twist is a potential oncogene that inhibits apoptosis. *Genes Dev*, **13,** 2207-17.

Medema RH, Kops GJ, Bos JL and Burgering BM. (2000). AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature*, **404,** 782-7.

Phung TL, Ziv K, Dabydeen D, Eyiah-Mensah G, Riveros M, Perruzzi C, et al. (2006). Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. *Cancer Cell*, **10,** 159-70.

Sahlin P, Windh P, Lauritzen C, Emanuelsson M, Gronberg H and Stenman G. (2007). Women with Saethre-Chotzen syndrome are at increased risk of breast cancer. *Genes Chromosomes Cancer*, **46,** 656-60.

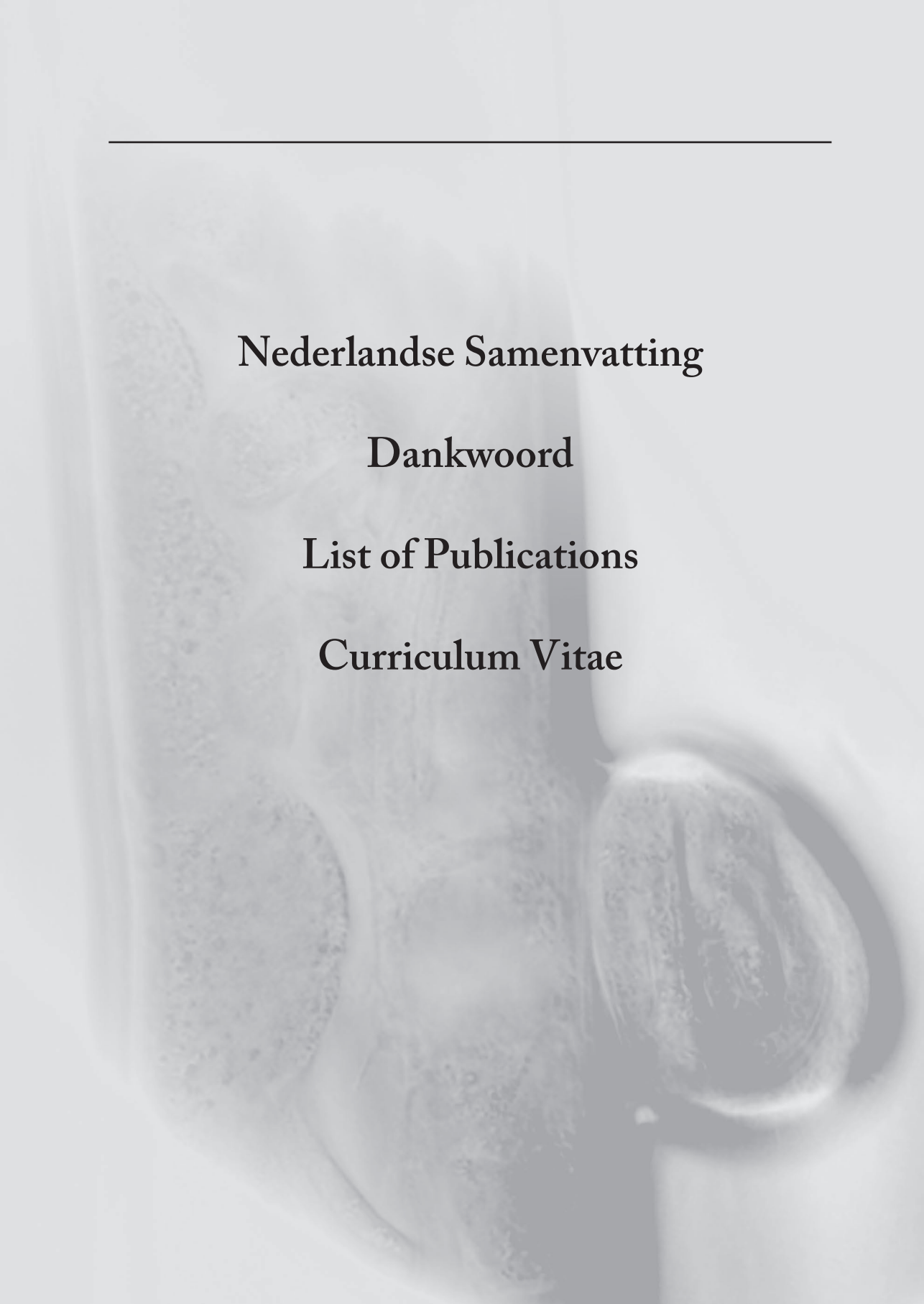
Semenza GL. (2003). Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*, **3,** 721-32.

Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin CH and Moustakas A. (2006). Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition. *J Cell Biol*, **174,** 175-83.

Toyoshima H and Hunter T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell*, **78,** 67-74.

Wang X, Ling MT, Guan XY, Tsao SW, Cheung HW, Lee DT, et al. (2004). Identification of a novel function of TWIST, a bHLH protein, in the development of acquired taxol resistance in human cancer cells. *Oncogene*, **23,** 474-82.

Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*, **117,** 927-39.



Nederlandse Samenvatting

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Nederlandse Samenvatting

Moleculaire reactie op zuurstofgebrek; van *C. elegans* tot kanker

Bij het ontstaan van kanker spelen, naast genetische en epigenetische processen, factoren uit de micro-omgeving van de kankercellen een rol. Een van die factoren is het zuurstofgehalte. Net als gezonde cellen hebben kankercellen zuurstof nodig. Die zuurstof wordt gebruikt om suiker te verbranden waardoor energie gegenereerd wordt. De energie komt vrij in de vorm van adenosinetrifosfaat (ATP), een stof die bij bijna alle moleculaire processen in het lichaam nodig is en daarom ook wel de munteenheid van de cel genoemd wordt. Minder zuurstof betekent dus minder ATP en daardoor onstaan problemen in de functie van de cel.

Zuurstof wordt in ons lichaam opgenomen in de longen en via het bloed vervoerd naar de cellen in de overige organen. Normaal gesproken liggen deze cellen dicht genoeg in de buurt van de kleine bloedvaten in de organen om hieruit zuurstof te ontvangen. Door de ongeremde groei van een kankergezwel ontstaan er in de tumor gebieden die niet goed doorbloed worden en daardoor minder zuurstof krijgen. Wanneer het zuurstofgehalte in de cel te laag is om een normale celfunctie te behouden, spreken we van zuurstofgebrek, ofwel hypoxia. Het is bekend dat het frequent voorkomen van hypoxische gebieden in tumoren gepaard gaat met een slechte prognose voor de patient. Hoe dat precies veroorzaakt wordt is niet goed bekend. Wel zijn er verschillende hypothesen die dit zouden kunnen verklaren. Zo zijn hypoxische cellen minder gevoelig voor chemotherapie en bestraling. Daarnaast veranderen cellen hun gedrag onder hypoxische omstandigheden.

Cellen die in zuurstofnood komen zullen daarop reageren door een aantal processen op gang te zetten, zoals de aanmaak van nieuwe bloedvaten (angiogenese) en veranderde energiehuishouding. Dit is een poging van deze cellen om te overleven. Om deze processen op gang te zetten treden er moleculaire veranderingen in de cellen op. Het belangrijkste molecuul hierbij is de hypoxia induceerbare factor (HIF). Deze factor bestaat uit twee verschillende eiwitten, die de HIFalfa en de HIFbeta subunit genoemd worden. De HIFbeta subunit is altijd in de cel aanwezig, maar de HIFalfa subunit wordt normaliter continu afgebroken na aanmaak. Dit gebeurt doordat zuurstofgevoelige enzymen een hydroxielgroep aan de HIFalfa subunit zetten (hydroxylatie). Deze chemische modificatie zorgt ervoor dat het tumorsuppressor Von Hippel Lindau eiwit

aan HIFalfa kan binden en signaalmoleculen aan HIFalfa zet (ubiquitinering). Hierdoor wordt HIFalfa afgebroken. Voor dit hele proces is zuurstof nodig. Zonder zuurstof zal HIFalfa niet afgebroken worden en samen met HIFbeta tot HIF vormen. Er bestaan drie verschillende HIFalfa eiwitten, HIF-1, -2, en -3alfa, welke ieder kunnen binden aan de HIFbeta subunit. De alfa subunit bepaalt dus of we te maken hebben met een HIF-1, -2, of -3 complex. HIF-1 en HIF-2 zijn het best bestudeerd. Het HIF complex is een transcriptiefactor. Dat wil zeggen dat HIF het DNA kan binden en bepaalde genen laat aflezen. HIF reguleert meer dan honderd genen die betrokken zijn bij de reactie op zuurstofgebrek. Een belangrijk gen hierin is het vasculaire endotheliale groei factor (VEGF) gen, welke het VEGF eiwit produceert, dat een rol speelt in het maken van nieuwe bloedvaten. Andere belangrijke HIF-gereguleerde genen zijn die, die enzymen uit de glycolyse produceren, de enige route waarmee uit suiker ATP gemaakt kan worden zonder zuurstof.

Al deze veranderingen hebben tot doel de hypoxische cel (tijdelijk) te laten overleven zonder zuurstof en zo snel mogelijk uit deze zuurstofarme situatie te komen. Men kan zich voorstellen dat dit in het geval van een hypoxische kankercel niet wenselijk is. Het aanwezig zijn van het zuurstof-gereguleerde HIFalfa eiwit in de cellen van solide tumoren, onder andere hoofd/hals-, baarmoederhals-, darm-, long-, en borstkanker, voorspelt progressief ziekteverloop en een slechte prognose voor de patient. Kennis van de moleculaire processen die een rol spelen bij zuurstofgebrek is belangrijk voor het begrip van de relatie tussen zuurstofgebrek en een slechte prognose. Dit proefschrift bestudeert zowel de regulatie van de HIF eiwitten, als de regulatie van genen door de HIF eiwitten.

In **hoofdstuk 1** wordt een introductie gegeven over hypoxia en de HIF eiwitten. **Hoofdstuk 2** is een review artikel over de relatie tussen de HIF eiwitten en het uitzaaien van kanker (metastasering). De laatste paar jaren is steeds meer duidelijk geworden dat de HIF transcriptiefactoren genen reguleren die betrokken zijn bij verschillende processen tijdens het uitzaaien. Veel genen die door HIF gereguleerd worden zijn ook aanwezig in het zogenaamde metastatische genprofiel. In dit hoofdstuk wordt de hypothese besproken dat hypoxia de drijvende kracht is bij het ontwikkelen van polyklonale tumorcelpopulaties binnen de primaire tumor, die ieder bijdragen aan het uitzaaiingsproces.

In **hoofdstuk 3** wordt een celkweekmodel voor tumorcellen in hypoxische gebieden beschreven, waarbij borstkankercellen in het lab onder zuurstof- en serumgedepriveerde condities worden gekweekt. Zonder serum en de

daarin aanwezige voedingstoffen en signaalmoleculen, is er minder HIF-1alfa eiwit aanwezig onder hypoxische omstandigheden. Door een moleculaire signaleringsroute aan te zetten, namelijk de PI 3-kinase route, herstelt de oorspronkelijke situatie zich. Wanneer tumormateriaal van borstkankerpatienten wordt bestudeerd, wordt inderdaad gevonden dat in de afwezigheid van een actieve PI 3-kinase-route het HIF-1alfa eiwit ook minder aanwezig is. De PI 3-kinase-route is een kankerveroorzakende (oncogene) moleculaire route. Er zijn reeds medicijnen die kanker proberen te remmen door deze route te blokkeren. Het is aannemelijk dat deze medicijnen ook een effect op de HIF-functie zullen hebben.

In **hoofdstuk 4** wordt de relatie tussen HIF-1alfa eiwitexpressie en de celcyclusremmer p27 bestudeerd in kanker van het baarmoederslijmvlies (endometrium). In tumormateriaal van patienten met endometriumkanker blijkt p27 vaak aanwezig te zijn in gebieden rond stervende cellen (perinecrotisch). Deze gebieden zijn meestal hypoxisch en HIF-1alfa is daar dan ook vaak aanwezig. In een celkweekmodel blijkt p27 door zuurstofgebrek te worden geinduceerd en dit is afhankelijk van HIF-1alfa expressie. Tumorcellen hebben normaliter een ongeremde celcyclus. Daarom is een mogelijke functie van p27 het tijdelijk remmen van de celcyclus in de perinecrotische gebieden, om overleving van de tumorcellen mogelijk te maken. Dit draagt waarschijnlijk bij aan een slechte prognose voor de patient.

Hoofdstuk 5 beschrijft het gebruik van het modelsysteem *Caenorhabditis elegans* in onderzoek naar het HIF pathway. *C. elegans* is een nematode, een worm-vormig diertje van ongeveer een milimeter lang. De genen betrokken bij het HIF-pathway, HIFalfa, HIFbeta, VHL, en de hydroxylerende enzymen (prolylhydroxylases ofwel PHD), zijn in de evolutie relatief onveranderd gebleven tussen *C. elegans* en de mens. Bepaalde *C. elegans*-stammen, die de PHD homoloog genaamd *egl-9* missen, kunnen geen eieren leggen, maar sparen deze op in de baarmoeder tot de eieren uitkomen en de moeder uitkruipen. In deze stammen wordt HIF-1 niet gehydroxyleerd en is dat dus ook zonder zuurstofgebrek stabiel. Het *egl-9* phenotype kan opgeheven worden wanneer het *hif-1* gen ook uitgeschakelt wordt. Door alle genen in het *C. elegans* genoom een voor een uit te schakelen in de *egl-9* deficiente stammen en te kijken naar het leggen van eieren, werden nieuwe genen gevonden die betrokken zijn in het HIF-pathway. Een hiervan is het *hlh-8* gen. De humane homoloog hiervan is het metastase geassocieerde gen *TWIST1*. In humane kankercellen hebben we aangetoond dat het *TWIST1* gen door zuurstofgebrek wordt opgereguleerd.

Dit blijkt afhankelijk te zijn van het HIF-2 complex. Mogelijk draagt *TWIST1* expressie onder zuurstofgebrek bij aan de slechte prognose van hypoxische tumoren.

Omdat expressie van het TWIST1 eiwit in borstkanker door eerdere onderzoeken essentieel is bevonden voor het uitzaaien, wordt TWIST1 expressie in borstkanker verder bestudeerd in **hoofdstuk 6**. Het epigenetische proces van methylering van een genpromotorsequentie zorgt doorgaans voor verminderde expressie van dat gen. Verrassend genoeg is methylering van de *TWIST1*-promotor gecorrelleerd met het ontstaan van onder andere borstkanker. Ook in dit hoofdstuk wordt die relatie gevonden. Daarnaast blijkt TWIST1 eiwit expressie ook progressief in borstkanker gevonden te worden en is er geen relatie tussen methylering en expressie. De exacte rede hiervoor blijft onduidelijk. Wel kan geconcludeerd worden dat *TWIST1* methylering mogelijk bruikbaar is als kankermarker.

In **hoofdstuk 7** wordt een functionele screeningsmethode gebruikt om nieuwe genen te identificeren die HIFalfa reguleren. Hiertoe wordt gebruik gemaakt van een celkweekmodel met cellen die genetisch zo gemodificeerd zijn, dat ze resistent worden tegen het antibioticum puromycine wanneer HIF actief is. Door in deze cellen met behulp van virussen genen tot expressie te brengen en vervolgens ze met puromycine te behandelen, kunnen genen gevonden worden die HIF-functie reguleren. Een aantal genen werd geidentificeerd, maar helaas is een rol in HIF-regulatie voor geen van deze genen bevestigd op andere manieren.

De resultaten uit dit proefschrift hebben een belangrijke basis gelegd voor verder onderzoek op onze afdeling naar de relatie tussen zuurstofgebrek en moleculaire veranderingen die tot uitzaaiing kunnen leiden. Het begrip van de moleculaire processen rondom het HIF-pathway zal op termijn bijdragen aan de ontwikkeling van therapieën voor de groep hypoxische tumoren en de geassocieerde slechte prognose.

Dankwoord

Als ik in enige mate de indruk gewekt heb dat ik alles in mijn eentje gedaan heb, dan wordt het tijd dit uit de wereld te helpen. Ik ben erg veel mensen dankbaar voor hulp en ondersteuning de afgelopen vierenhalf jaar. Hier volgt een uitgebreide selectie.

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Eelke

List of Publications

Gort EH, Groot AJ, van Diest PJ, Vooijs M;
Review; Hypoxic Regulation of Metastasis via Hypoxia-inducible Factors.
Current Molecular Medicine (In press)

Gort EH, Horrée N, van der Groep P, Heintz APM, Vooijs M, van Diest PJ;
Hypoxia-inducible factor-1 alpha is essential for hypoxic induction of p27 in endometrioid endometrial carcinoma.
Journal of Pathology. 2008 Jan;214(1):38-45.

Gort EH, van Haaften G, Verlaan I, Plasterk RHA, Shvarts A, van der Wall E, van Diest PJ, Tijsterman M, Vooijs M;
The basic helix-loop-helix transcription factor *TWIST1* is a direct target of Hypoxia-inducible factor-2alpha.
Oncogene, 2007 Sep 17; Epub ahead of print, doi:10.1038/sj.onc.1210795.

Cuppen E, Gort EH, Hazendonk E, Mudde J, Nijman IJ, Guryev V, Plasterk RHA;
Efficient target-selected mutagenesis in *Caenorhabditis elegans*: towards a knockout for every gene.
Genome Res. 2007 May;17(5):649-58. Epub 2007 Apr 6.

Gort EH, Groot AJ, Derks van de Ven TLP, van der Groep P, Verlaan I, van Laar T, van Diest PJ, van der Wall E, Shvarts A;
Hypoxia-inducible factor-1alpha expression requires PI 3-kinase activity and correlates with Akt1 phosphorylation in invasive breast carcinomas.
Oncogene. 2006 Oct 5;25(45):6123-7. Epub 2006 May 8.

Groot AJ, Verheesen P, Westerlaken EJ, Gort EH, van der Groep P, Bovenschen N, van der Wall E, van Diest PJ, Shvarts A;
Identification by phage display of single-domain antibody fragments specific for the ODD domain in hypoxia-inducible factor 1alpha.
Lab Invest. 2006 Apr;86(4):345-56.

Curriculum Vitae

Eelke Hiddo Gort werd geboren op 24 oktober 1978 te Utrecht. Hij doorliep de basisschool in Berlare (België) en het Stedelijk Gymnasium in Breda. Hierna studeerde hij Medische Biologie te Utrecht. Zijn eerste stage deed hij bij de afdeling Fysiologische Chemie en Centrum voor Biomedische Genetica in het Universitair Medisch Centrum te Utrecht onder begeleiding van dr. N. de Ruiter en prof. dr. J.L. Bos, aan het kleine GTP-ase Ral. Zijn tweede stage deed hij bij de Functional Genomics groep van het Hubrecht Laboratorium voor Ontwikkelingsbiologie onder begeleiding van dr. E. Cuppen, dr. M. Tijsterman en prof. dr. R.H.A. Plasterk, waarbij hij werkte aan het maken van een *C. elegans* reverse genetics database. Hij ontving zijn doctoraaldiploma op 23 juni 2003. Vervolgens startte hij zijn promotieonderzoek naar de hypoxisch induceerbare HIF eiwitten bij de afdeling Pathologie aan de Vrije Universiteit te Amsterdam onder leiding van prof. dr. P.J. van Diest en dr. A. Shvarts, en prof. dr. E. van der Wall van de afdeling Medische Oncologie. Vanaf 1 januari 2004 werd dit onderzoek voortgezet aan de afdeling Pathologie in het Universitair Medisch Centrum te Utrecht en vanaf januari 2006 werd dit onderzoek gesuperviseerd door dr. M. Vooijs. Gedurende een periode van anderhalve maand in 2006 voerde Eelke onderzoek uit aan het Johns Hopkins Medical Center te Baltimore, USA, bij de afdeling Radiologie onder leiding van dr. V. Raman in het kader van een International exchange program van het UMC Utrecht. In januari 2006 ontving hij een Keystone Symposia Scholarship Award tijdens de Hypoxia Meeting 2006 te Breckenridge (Colorado, USA). Inmiddels is hij gestart met de masteropleiding Geneeskunde "Selective Utrecht Medical Masters (SUMMA)".

Eelke Hiddo Gort was born 24th of Oktober 1978 in Utrecht. He went to primary school in Berlare (Belgium) and attended high school at the Stedelijk Gymnasium in Breda. He studied Medical Biology in Utrecht. His first internship was at the dept. of Fysiological Chemistry and Center for Biomedical Genetics at the University Medical Center in Utrecht, supervised by dr. N. de Ruiter and prof. dr. J.L. Bos, studying the small GTP-ase Ral. The second internship was at the Functional Genomics group in the Hubrecht Laboratory for Developmental Biology in Utrecht, supervised by dr. E. Cuppen, dr. M. Tijsterman en prof. dr. R.H.A. Plasterk, contributing to a *C. elegans* reverse genetics database. He received his Master of Science degree 23th of June 2003. Subsequently, he initiated his PhD research on the hypoxia inducible HIF proteins at the dept. of Pathology at the Free University in Amsterdam supervised by prof. dr. P.J. van Diest and dr. A. Shvarts, and prof. dr. E. van der Wall of the dept. of Medical Oncology. As from Januari 1th 2004, this research was continued at the dept. of Pathology at the University Medical Center in Utrecht and as from Januari 2006 it was supervised by dr. M. Vooijs. During a one-and-a-half month period, Eelke conducted his research at the Johns Hopkins Medical Center in Baltimore, USA, at the dept. of Radiology under supervision of dr. V. Raman as part of an International exchange program of the UMC Utrecht. In Januari 2006, he received a Keystone Symposia Scholarship Award during the Hypoxia Meeting 2006 in Breckenridge (Colorado, USA). Recently, he started the "Selective Utrecht Medical Masters (SUMMA)" to become a medical doctor.