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The Involvement of the ERK-Hypoxia-Angiogenesis Signaling Axis and HIF-1 in Hepatocellular Carcinoma

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1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and is associated with high resistance to drugs and high mortality. There are multiple factors that influence its molecular pathogenesis but two well established characteristics of malignant transformation in HCC are its hypervascular nature and the upregulation of the Raf/MEK/ERK signaling cascade. MAPK pathway activation can be triggered by important risk factors of hepatocarcinogenesis such as HBV or HCV infection. Furtermore, its deregulation is well documented in human HCC patients and is associated with poor prognosis (Bruix & Llovet, 2009). Constitutive stimulation of both ERK isoforms has been frequently observed in both HCC samples and hepatocarcinoma-derived cell lines and plays a prominent role in the proliferation, invasion and metastasis of HCC cells (Min et al., 2010). These processes are also associated with neoangiogenesis and aberrant vessel formation, which in turn depend on the development of hypoxic regions and VEGF overexpession frequently observed in tumor samples (Rosmorduc & Housset, 2010). The master regulators of the cellular response to oxygen deprivation are the hypoxia inducible transcription factors (HIFs). Their activation results in expression of many genes that contribute to survival and proliferation of malignant cells and, more importantly, resistance to conventional treatments and poor patient outcome (Poon, E. et al., 2009). Interestingly, hypoxia has been observed to lead to ERK activation, which can further stimulate HIF transcriptional activity. This can result in increased expression of HIF target genes that include pro-angiogenic factors and other proteins that facilitate adaptation of tumor cells to their environment (Dimova et al., 2009). In this chapter, we will discuss the cross-talk between these pathways, their contribution to HCC development and progression and their potential as targets of combined therapeutic approaches.

2. Hepatocellular carcinoma

Hepatocellular Carcinoma (HCC) is the fifth most common and third in lethality cancer. It is characterized by intrinsic drug-metabolizing activity that confers resistance to

chemotherapeutic treatment. Mortality is associated with metastasis, recurrence and new tumor development and diagnosis is usually made at intermediate or late stage so that only ~ 20% of cases can undergo surgery that includes resection, ablation or transplantation. Unfortunately, due to the genomic heterogeneity of HCC tumors, the exact molecular pathogenetic and oncogenic alterations that lead to HCC initiation and progression are not completely understood and require further investigation. However, there are several risk factors and pathways known to be associated with the occurrence and development of this type of cancer (Villanueva et al., 2010).

Probably the most important predisposing factor for the development of HCC is liver cirrhosis that usually results from viral infection (HBV or HCV), alcohol abuse or contamination with Aflatoxin B1. Other contributing factors include inflammation and non-alcoholic fatty liver disease (NAFLD). All of these factors can lead to HCC development by triggering cellular events such as proto-oncogene activation, ROS generation and genetic alterations or instability (Frau et al., 2010). Genetic studies of human HCCs resulted in the identification of gene mutations and expression profile alterations. The identified deregulated genes can be associated with important signaling pathways and shed more light in the molecular events that contribute to HCC pathogenesis. According to these analyses, there are three subgroups of human HCC cancers, genetic profile of which corresponds to the deregulation of specific signaling events (Hoshida et al., 2010). One of them is characterized by over-expression of growth factors (EGF, IGF II and HGF being the most prominent) and induction of major signaling pathways such as PI3K/AKT/mTOR and RAS/RAF/MAPK, which affect cell proliferation and survival and, moreover, contribute to the aggressive phenotype of the disease. In another subgroup, the affected genes are related to cell differentiation and liver development. These genes are found downstream of the WNT or the highly similar Hedgehog pathway and their involvement in human hepatocarcinogenesis is still under investigation. Whereas, in the third less-defined group, the early stages of the disease are linked to inflammation-related pathways, with interleukin-6 being a major signaling molecule (Villanueva et al., 2010; Zender et al., 2010). However, all these signaling pathways do not function independently in the context of HCC tumors but they cooperate and influence one another contributing to the progression of the disease.

From these and other studies it has been made clear that ERK pathway activation and neo-angiogenesis are two characteristics of HCCs that greatly facilitate malignant transformation, as they are involved in tumor development, growth and metastasis. Another aspect of HCC, common to many solid tumors, is the creation of hypoxic areas as a result of increased metabolic rate, irregular angiogenesis and tissue inflammation (Rosmorduc & Housset, 2010). The central regulatory elements of cell response to oxygen deprivation are the hypoxia inducible transcription factors (HIFs). After their activation, HIFs induce the expression of their targets, which in their turn facilitate adaption of the cells to the hypoxic environment of the tumor and contribute to survival, proliferation and aggressiveness of cancer cells.

3. ERK pathway and HCC

Extracellular-signal regulated kinases 1 and 2 (ERK 1/2 or else p44/42 MAPK) are serine/threonine protein kinases both homologous and highly similar in their regulatory

mechanisms and functions. They are expressed in all cell types and they integrate extracellular signals such as growth factors and cytokines into cellular responses that promote gene expression, proliferation, survival and migration. ERK activation relies on the binding of ligands to cell membrane receptors and the subsequent activation of the RAS/RAF/MEK signaling cascade resulting in the phosphorylation and activation of ERK1/2 (Pearson et al., 2001). It is well documented that sustained ERK activity is important for the development of many types of tumors including hepatocellular carcinoma. As it has been already mentioned, there is activation of Ras pathway and significant increase of phosphorylated ERK levels in all HCC-derived cell lines. However, Ras mutations that could account for sustained pathway activation are rare in human HCCs (Min et al., 2010). One explanation for constistutive ERK activation in HCCs can be that HBV or HCV viral infection interferes and activates the ERK signaling cascade facilitating thus hepatocarcinogenesis (Chin et al., 2007; Zhao et al., 2005). Another reason may lie with the fact that there is global suppression of negative regulators that control the ERK signaling cascade in HCC cell lines. It has been shown that Ras inhibitors are inactivated in HCC-derived cell lines probably as a result of deregulated methylation and genomic instability (Calvisi et al., 2006). Furthermore, the Raf kinase inhibitor protein (RKIP) has been observed to be downregulated in HCC cells (Lee, H.C. et al., 2006). Significant decrease has also been detected in the phosphatase DUSP1, which restrains ERK activity by dephosphorylation, as a result of ubiquitin-mediated proteolysis triggered by sustained ERK activation in HCC (Calvisi et al., 2008).

The constitutive activation of ERK signaling is essential for cell survival, proliferation and invasion of human HCC cells since Ras pathway inhibition results in growth suppression and cell death. Moreover, siRNA-mediated silencing of ERK2 or elimination of ERK1/2 phosphorylation by silencing of MEK1 resulted in inhibition of cell proliferation and tumor growth suppression in xenograft animal models (Bessard et al., 2008; Gailhouste et al., 2010). Activation of the Raf/MEK/ERK pathway in human HCC biopsy samples can be used as a biomarker of the disease because nuclear phosphorylated ERK levels are higher in tumor samples (Abou-Alfa et al., 2006). Finally, ERK pathway activation is associated with poor patient outcome whereas, inversely, elevated expression levels of pathway inhibitors such as DUSP1 are observed in tumor samples of patients with longer survival time (Calvisi et al., 2008).

The importance of the ERK pathway for HCC occurrence and progression made it an important candidate for targeted therapeutic approaches as shown in case of sorafenib. Sorafenib, a multikinase inhibitor, impedes cell proliferation by targeting the Raf/MEK/ERK signaling cascade at the level of Raf kinase (for which it was originally developed) and also exhibits antiangiogenic properties by targeting the tyrosine kinase activities of the vascular endothelial growth factor receptor-2/-3 (VEGFR-2/-3) and the platelet derived growth factor receptor beta (PDGFR-β) (Liu, L. et al., 2006; Wilhelm et al., 2004). Sorafenib has been recently approved as the first effective systemic drug for treating advanced HCC showing a significant (almost 3 months) increase in patient survival. Also, due to its tolerability in single agent trials, it has been evaluated in combination with other anticancer therapies, including cytotoxic chemotherapy and anti-angiogenic therapy (Llovet et al., 2008). The effectiveness of sorafenib in clinical evaluation highlights the potential of targeting the ERK pathway in the treatment of HCC. Recent experimental studies have

shown that treatment with MEK inhibitors (AZD6244, PD0325901) inhibited cancer cell proliferation and tumor growth in human HCC cell lines and HCC xenografts in animal models (Hennig et al., 2010; Huynh et al., 2007). Moreover, when AZD6244 was tested in combination with sorafenib, it enhanced the anti-tumor activity of sorafenib, suggesting the potential value of ERK pathway inhibition in combinational therapeutic approaches (Huynh et al., 2010).

4. Angiogenesis and HCC

HCC progression requires the development and maintenance of adequate blood supply. This requires vascular endothelial cell proliferation and migration in order to establish a new vascular network. The transformation from a poorly vascular nodule to the hypervascular malignant phenotype of the disease has become a hallmark for diagnosis, treatment and possible future therapeutic approaches (Fernandez et al., 2009). Formation of new vessels with abnormal architecture is linked to fibrinogenesis and cirrhosis, processes that cause extensive cell damage and can lead to malignancy (Rosmorduc & Housset, 2010). The ability to form new vessels is critical not only for the emergence of HCC but also for the growth of metastatic nests and the transition from early to advanced stages of the disease. Indeed, impairing the blood supply that feeds the tumor with transarterial chemoembolization is an effective treatment for patients with advanced HCC and improves their survival (Llovet & Bruix, 2003).

Neoangiogenesis in HCC is a process stimulated by hypoxia, growth factors, oncogenes and nutrient concentration followed by the release of pro-angiogenic growth factors and activation of endothelial cells. The most prominent pro-angiogenic signal, as revealed in a number of studies, is vascular endothelial growth factor (VEGF) (Fernandez et al., 2009; Rosmorduc & Housset, 2010). Its expression can be induced as a result of hypoxia, oncogenic signaling and viral infection, involving MAPK pathway activation and transcriptional regulation by AP1 and HIF-1α (Hassan et al., 2009). Its significant role in HCC is shown by the fact that cells isolated from human tumors are able to produce VEGF by themselves. Hypoxia further enhances VEGF expression both by regulation at the transcriptional level and by stabilization of VEGF mRNA (von Marschall et al., 2001). Moreover, VEGF and its receptors (VEGFR-1 and VEGFR-2) are found over-expressed in patients' samples and their increased levels have been associated with the aggressive phenotype of the disease (Imura et al., 2004). In line with this observation, increased concentration of VEGF in serum samples is directly connected to poor patient outcome after resection or ablation (Poon, R.T. et al., 2004; Poon, R.T. et al., 2007).

Other factors that promote tumor angiogenesis in HCC are FGFs (Fibroblast growth factors), angiopoietins (Ang-2) and platelet-derived growth factor (PDGF). There is evidence that bFGF over-expression is involved in vascular endothelial cell proliferation during tumor angiogenesis in human HCC (Imura et al., 2004). Furthermore, Ang-2 levels as well as its activity through the Tie-2 receptor are increased in human samples and have been correlated with neovascularization and microvessel density (MVD) (Mitsuhashi et al., 2003). Finally, animal studies have shown that the involvement of PDGF-C in HCC development is linked to its role in the initiation of fibrosis (Campbell et al., 2005). Interestingly, it appears that all these factors team up with the VEGF signaling cascade in order to provoke normal or aberrant vessel formation in HCCs.

Since vascular formation is very important for tumor progression and transition to malignancy, it represents an intriguing therapeutic target for the treatment of HCC. At present, there are several drugs undergone investigation in experimental models or humans and some of them are already in use in various types of cancer. Most of the tested inhibitors target selectively the VEGF pathway and induce arrest of endothelial cell proliferation, regression of the existing vessels or suppress the mobilization of endothelial progenitor cells from bone marrow. These agents range from monoclonal antibodies (mAb) targeting VEGF-A, like bevacizumab (Avastin), to small molecules that inhibit autophosphorylation of VEGF receptors like sorafenib (Nexavar), the single agent that was approved for the treatment of advanced hepatocellular carcinoma in 2007 (Fernandez et al., 2009).

5. Hypoxia and HCC

As many other solid tumors, HCC is characterized by the development of hypoxia. Even in normal liver, emergence of hypoxic conditions can result from its division into areas with different capabilities of oxygen delivery (i.e., 60 to 65 mmHg in the portal area to 30 to 35 mmHg in the perivenous area) and increased cellular metabolic activity, which leads to high oxygen consumption (Rosmorduc & Housset, 2010). Apart from that, the response to trauma or inflammation can cause aberrant vessel formation, irregular blood flow and, finally, poor oxygenation of the cells. Cells respond to oxygen deprivation by activating a number of genes that allow them to adapt and survive. More importantly, hepatocellular carcinoma cells not only survive but also show significant stimulation of their proliferation under hypoxia as opposed to other cancer cell lines (Gwak et al., 2005).

Hypoxia promotes tumor progression through several mechanisms. Under hypoxic conditions, cells shift their metabolism from oxidative phosphorylation to anaerobic glycolysis by the induction of genes that encode glucose transporters, glycolytic enzymes and proteins that remove glycolysis by-products (e.g. lactic acid) from cancer cells. As mentioned above, HCC tumors exhibit epigenetic alterations and signaling pathways activation that stimulate proliferation. These may be supported by tumor hypoxia, which affects the methylation status of HCC cells and, more importantly, activates the ERK pathway (Liu, Q. et al., 2011; Minet et al., 2000). Another very important contribution of hypoxia to HCC development is the stimulation of angiogenesis. Two of the central pro-angiogenic factors in HCC, namely VEGF and angiopoietin 2, are induced in hypoxic conditions and promote the abnormal vessel formation and branching observed in these tumors. Key mediators of these responses and central components of hypoxia signaling within the cell are the hypoxia inducible factors (HIFs).

HIFs are heterodimeric transcriptional complexes that respond to changes of cellular oxygen concentration and activate the expression of hypoxia target genes. These genes encode for proteins involved in processes critical for oncogenesis such as survival, proliferation, invasion and metastasis. Active HIF heterodimers are composed of the constitutively expressed HIF-β subunit (or ARNT; Aryl hydrocarbon Receptor Nuclear Translocator) and the regulated HIF-α subunit, which is over-expressed in many tumors and causes HIF activation and increased transcription of its targets (Semenza, 2010). HCC is not an exception and expression levels of HIF-1α are increased in all stages of the disease and correlate to its progress.

There is plenty of evidence linking HIF-1α to both early and late HCC stages. The genetic instability observed during the premalignant state of HCC can be caused by viral infection. HCV core protein induces HIF-1α and HIF-dependent transcriptional activation of the VEGF gene in HCC-derived cells (Hassan et al., 2009). Liver angiogenesis has been indeed observed in biopsy samples of HCV patients and is possibly an essential step for HCV-related oncogenesis. HIF-1α mRNA and protein levels are also increased in premalignant dysplastic nodules, as observed in both human and animal samples, and cause up-regulation of a number of genes that promote angiogenesis (VEGF), glucose transport (GLUT1) and PI3K/AKT pathway activation (growth factor IGF-II and hepatocyte growth factor receptor c-Met) (Nakamura et al., 2007). Interestingly, it has also been shown that HIF-1α expression at this stage is hypoxia-independent and its levels rise as the disease progresses (Tanaka et al., 2006). These findings indicate the important role of this transcription factor in abnormal gene expression that occurs during early HCC development.

As already mentioned, hypervascularity is a prominent feature of progression to HCC malignancy and HIF-1α over-expression is also directly associated with VEGF expression, microvessel density (MVD) and microvenous invasion in human HCC samples (Huang et al., 2005). Deregulation of the HIF pathway, which comes as a result of HIF-1α over-expression, is often associated with resistance to radiotherapy and chemotherapy, which renders hypoxic tumors highly aggressive and metastatic (Poon, E. et al., 2009). This is also true for primary HCC, in which HIF-1α expression is associated with poor response to radiotherapy, metastasis and low survival rates (Xiang et al., 2011). Therefore, understanding HIF-α regulation may provide valuable information in order to target the HIF pathway as a means for combinational therapeutic strategies against HCC.

6. HIF-α structure and regulation

Three HIF-α isoforms have been so far identified: HIF-1α, HIF-2α and HIF-3α. HIF-1α and -2α have similar structure and are rapidly induced in response to hypoxia. The third family member is not well understood. It exists in several splice variants, one of which acts as a dominant negative regulator of HIF-dependent gene expression as it binds to the HIF-1α subunit to form a nonfunctional complex. Whereas HIF-1α shows broad tissue distribution, HIF-2α is cell-type specific and was shown to have distinct biological roles. However, HIF-1 and HIF-2 can regulate both overlapping and distinct target genes (Poon, E. et al., 2009). More specifically, liver cells express both isoforms, albeit, with different kinetic profiles: HIF-1α responds quickly but returns to basal levels early. In contrast, HIF-2α expression is delayed but prolonged, suggesting a coordinated response of the two subunits to hypoxia (Wiesener et al., 2003).

Both HIF-1α and HIF-2α contain basic helix loop helix (bHLH) and PER-ARNT-SIM (PAS) domains in their NH_2-terminal regions that mediate heterodimerization and binding to specific DNA regulatory sequences called hypoxia response elements (HREs) (Fig. 1). The PAS domain contains two conserved repeats termed PAS-A and PAS-B. In their C-terminal regions, both contain transactivation domains (TAD) that mediate the transcription of their targets. Oxygen sensitivity and regulation relies in a structural feature called oxygen depended degradation (ODD) domain that lies inside the central region of HIF-α (Semenza, 2003).

In terms of its oxygen-dependent or -independent regulation, HIF-1α is more extensively investigated. HIF-2α, on the other hand, is regulated in a similar fashion by oxygen but many elements of its oxygen-independent regulation still remain unclear. Under normal oxygen conditions, HIF-1α is constantly produced and destroyed in a process that involves von Hippel-Lindau (VHL)-mediated polyubiquitination and subsequent proteasomal degradation (Schofield & Ratcliffe, 2005). Interaction of HIF-1α with VHL, a tumor suppressor protein and a subunit of an E3 ubiquitin ligase complex, requires the hydroxylation of two proline residues within the ODD domain of HIF-1α. This hydroxylation is mediated by three conserved prolyl hydroxylases (termed PHDs or HPHs), the activity of which depends on the availability of oxygen, iron and 2-oxoglutarate (Semenza, 2001). When oxygen becomes sparse, hydroxylation is impaired, HIF-1α is stabilized and is imported into the nucleus, where it dimerizes with ARNT and activates the expression of its target genes. However, oxygen dependent-regulation does not only rely on HIF-1α destruction alone but also on the control of its activity. Another oxygen-sensitive hydroxylase called FIH (Factor Inhibiting HIF-1) modifies HIF-1α in one asparagine residue situated inside its C-terminal TAD region (Asn803) and interferes with the association of HIF-1α with the transcriptional co-activator CBP/p300 (Lancaster et al., 2004a).

Apart from oxygen tension, HIF-1α expression and activity are additionally stimulated by oxygen-independent mechanisms that respond to oncogenic activation, growth factors, cytokines and variations of the cellular environment (e.g. pH). There are multiple levels at which cells can control HIF-1α activity, ranging from transcriptional and translational regulation to post-translational modifications. Its transcriptional activation responds to inflammatory stimuli as IKKβ activation causes increased HIF-1α gene expression in a NF-κB-dependent fashion in the liver of hypoxic mice (Rius et al., 2008). The signal transducer and activator of transcription 3 (Stat3) is also involved in the regulation of HIF-1α mRNA synthesis (Niu et al., 2008) and mediates the transcriptional suppression of HIF-1α by the eIF2α kinase PKR (Papadakis et al., 2010). Activation of the phosphatidylinositol 3-kinase/AKT pathway leads to elevated translation of HIF-1α mRNA and increased HIF-1α protein levels (Bardos & Ashcroft, 2005). Post-translationally, HIF-1α is regulated either through its association with other proteins or its modification by number of different enzymes. Protein interactions like binding to the molecular chaperone HSP90 can increase HIF-1α stability by inhibiting VHL-independent degradation (Isaacs et al., 2002; Katschinski et al., 2004). On the other hand, the protein RACK1 competes with HSP90 for binding to the HIF-1α N-terminal PAS-A domain. The RACK1-HIF-1α interaction, which is stabilized by the protein SSAT1 and inhibited by Sept9-v1, promotes increased ubiquitination and degradation of HIF-1α irrespective of oxygen levels (Amir et al., 2009; Baek et al., 2007; Liu, Y.V. et al., 2007). MgcRacGAP (male germ cell RacGTPase Activating Protein), identified using the yeast two-hybrid system, is another protein that interacts with and inhibits HIF-1α. MgcRacGAP over-expression inhibits HIF-1α transcriptional activity, without lowering HIF-1α protein levels or altering its subcellular localization (Lyberopoulou et al., 2007).

Apart from hydroxylation, other posttranslational modifications of HIF-1α include SUMOylation, acetylation, S-nitrosylation and phosphorylation by a number of different kinases. HIF-1α can be SUMOylated but the role of this modification remains controversial; certain reports claim that SUMO conjugate stabilizes HIF-1α while others suggest that deSUMOylation of HIF-1α is necessary for its stability and activity (Bae et al., 2004; Berta et al., 2007; Carbia-Nagashima et al., 2007; Cheng et al., 2007). In contrast,

acetylation of HIF-1α by the acetyltransferase ARD1 has a negative impact by facilitating its interaction with VHL (Jeong et al., 2002), although its effect on HIF-1α stability has also been later disputed (Wei & Yu, 2007). Nitrogen oxide (NO) can interfere with PHD function as well as cause HIF-1α S-nitrosylation, which can stabilize HIF-1α in tumor-associated macrophages (Li et al., 2007; Wei & Yu, 2007).

Direct HIF-1α phosphorylation can be an efficient way to rapidly and reversibly regulate HIF-1 activity in response to different stimuli. HIF-1α phosphorylations can be activating, such as the one at Thr^{796} in the C-TAD domain of HIF-1α that impairs his interaction with FIH-1 (Lancaster et al., 2004b). On the contrary, phosphorylation by glycogen synthase kinase 3 (GSK3) at three residues (Ser^{551}/Thr^{555}/Ser^{589}) within the HIF-1α N-TAD drives HIF-1α to VHL-independent proteasomal degradation and down-regulates its activity (Flugel et al., 2007). We have recently described HIF-1α phosphorylation by casein kinse 1 (CK1) which also negatively affects HIF-1 activity (Kalousi et al., 2010). CK1δ targets Ser^{247} in the PAS-B domain of HIF-1α and does not affect its stability or localization but interferes with the ability of HIF-1α to form an active complex with ARNT under hypoxia. Over-expression of CK1δ inhibited, whereas, inhibition or silencing of CK1δ stimulated the activity of HIF-1 in several different cell lines, including hepatoma-derived cancer cells. Furthermore, inhibition of CK1 activity in HCC- cells (Huh7) resulted in significantly higher proliferation rates under hypoxia, highlighting the importance of HIF-1 activity for hypoxic adaptation and suggesting an anti-proliferative role for CK1δ (Kalousi et al., 2010). Others, recent but less characterized modifications of HIF-1α include phosphorylation of Ser^{696} by ATM kinase and Ser^{576}/Ser^{657} by Plk3, both of which activate HIF-1α by stabilizing its protein levels (Cam et al., 2010; Xu et al., 2010). Finally, the longer known, best studied and probably most relevant to HCC direct phosphorylation of HIF-1α is the one mediated by ERK (p44/42 MAPK), which will discussed extensively in the next section.

Transcriptional activity of HIF-1α ultimately depends on its nuclear accumulation. In order to enter the nucleus HIF-1α uses more than one import pathways. The first one involves the presence of a classical bipartite-type nuclear localization signal (NLS) in the C-terminal part of HIF-1α and interaction with importin α to mediate its translocation to the nucleus (Depping et al., 2008; Kallio et al., 1998; Luo & Shibuya, 2001). Moreover, recent work from our lab has shown that HIF-1α active transport through the nuclear pore complex can be mediated by multiple import receptors that, apart from importin α family members, also include importins 4 and 7 (Chachami et al., 2009) . Interaction with importins 4 and 7 involves the NH_2-terminal part of HIF-1α (amino acids 1-251), which also contains the bHLH and PAS-A domains, but the exact nature of the NLS that mediates their association is still unclear. The operation of more than one different pathways may ensure the fast and efficient translocation of HIF-1α inside the nucleus as part of an effective cellular response to hypoxic stimuli (Fig. 1). However, the time that HIF-1α spends inside the nucleus and, ultimately, its activity depends also on its nuclear export rate, which is mediated by the major mammalian exportin CRM1 and regulated by the Raf/MEK/ERK pathway (see below).

7. ERK pathway and HIF-1α

The MAPK pathway is one of the two best known major signal transduction pathways regulating HIF-1α activity (the other is PI3K/AKT). Apart from being induced by growth

factors, cytokines and oncogenes, the MAPK pathway is also activated by hypoxia in several different cell lines including hepatoma HepG2 cells (Liu, C. et al., 2005; Minet et al., 2000; Mottet et al., 2002) and was suggested to affect both HIF-1α mRNA translation and HIF-1 transcriptional activity (Fukuda et al., 2002; Richard et al., 1999). This was shown by the use of MEK1 inhibitor PD98059 which decreased HIF-1-dependent gene expression in a number of cell lines including the hepatoma-derived Hep3B (Minet et al., 2000; Salceda et al., 1997). Also, studies from our own and other groups using Gal4-HIF-1α fusion proteins have shown that the ERK pathway is involved in hypoxia-dependent HIF-1α transactivation domain function in different cell lines (Lee, E. et al., 2002; Mylonis et al., 2006). Finally, HIF-1α can be directly phosphorylated by ERK (p44/p42 MAPK) both in vitro and in vivo but is not a direct target of the other MAPK family members (p38 or c-JNK) (Dimova et al., 2009).

Although there had been efforts to identify the exact HIF-1α sites phosphorylated by ERK, these remained unknown until quite recently. The issue was clarified by our recent work using in vitro phosphorylation assays, mass spectrometry and site directed mutagenesis, which led to the indentification of two conserved serine residues (Ser^{641} and Ser^{643}) as the major ERK phosphorylation sites on HIF-1α (Mylonis et al., 2006). Furthermore, inhibition of HIF-1α phosphorylation by mutagenesis of the ERK target sites (conversion of both Ser^{641}/Ser^{643} into Ala) or treatment with the ERK pathway inhibitor PD98059 impaired nuclear accumulation of HIF-1α and, consequently, decreased its transcriptional activity. However, when cells expressing the phosphorylation-deficient mutant of HIF-1α were treated with Leptomycin B (a specific inhibitor of CRM1-dependent nuclear export) nuclear localization of the mutant HIF-1α was restored and its activity was partially recovered, suggesting that lack of ERK-depended phosphorylation reduces nuclear concentration of HIF-1α by excessive nuclear export into the cytoplasm (Mylonis et al., 2006).

These first results indicated that the mechanism by which ERK phosphorylation regulates HIF-1α activity lies downstream of its synthesis and stabilization steps and involves regulated nucleocytoplasmic shuttling. However, this kind of regulation requires that HIF-1α possesses a nuclear export signal (NES) in addition to its NLS. We were, indeed, able to show the presence of such a signal in the form of an atypical hydrophobic NES (^{632}**M**ED**I**K**ILI**^{639}), situated in close proximity to the serine residues 641/643 modified by ERK (Mylonis et al., 2008). This NES interacts strongly with CRM1 but only when ERK-dependent phosphorylation of HIF-1α is impaired. These data also support the idea that regulation of HIF-1α nuclear shuttling is the major – if not exclusive – mechanism through which ERK-mediated phosphorylation controls HIF-1 activity since phospho-mimetic mutation of Ser^{641} into Glu or mutation of the NES renders the mutant form of HIF-1α largely resistant to MAPK-pathway inhibition. Furthermore, the NES mutation "suppressed" the Ser^{641}/Ser^{643}→Ala double mutation and HIF-1α lacking both NES and ERK-sites regained wild-type properties in terms of localization and activity (Mylonis et al., 2008). Taken together, our data, which were also confirmed in a hepatoma-derived cell line (Huh7), support the following model, also shown in Fig. 1. After stabilization, HIF-1α interacts with multiple nuclear import receptors (importins α/β, 4 and 7) and is transported into the nucleus through the nuclear pore compexes (NPCs). Once in the nucleoplasm and with the HIF-1α NES exposed, there are two possible scenarios. If the MAPK/ERK pathway is inactive, as in a quiescent cell, CRM1 will bind to the NES and return HIF-1α to the

cytoplasm, keeping, thus, its nuclear concentration and subsequent activity low. However, if ERK is active (in response to hypoxia or other oncogenic stimuli), it will phosphorylate HIF-1α and mask its NES, thereby trapping HIF-1α inside the nucleus, promoting its accumulation and maximizing its activity. Subsequent interaction with ARNT will form an active HIF-1 heterodimer, which can bind to DNA and stimulate transcription. This model does not exclude the possibility that ERK-mediated phosphorylation has additional, albeit minor, effects on HIF-1α regulation such as promoting interaction of phosphorylated HIF-1α with another nuclear factor or stimulating the activity of HIF-1α partners such as CBP/p300 (Sang et al., 2003).

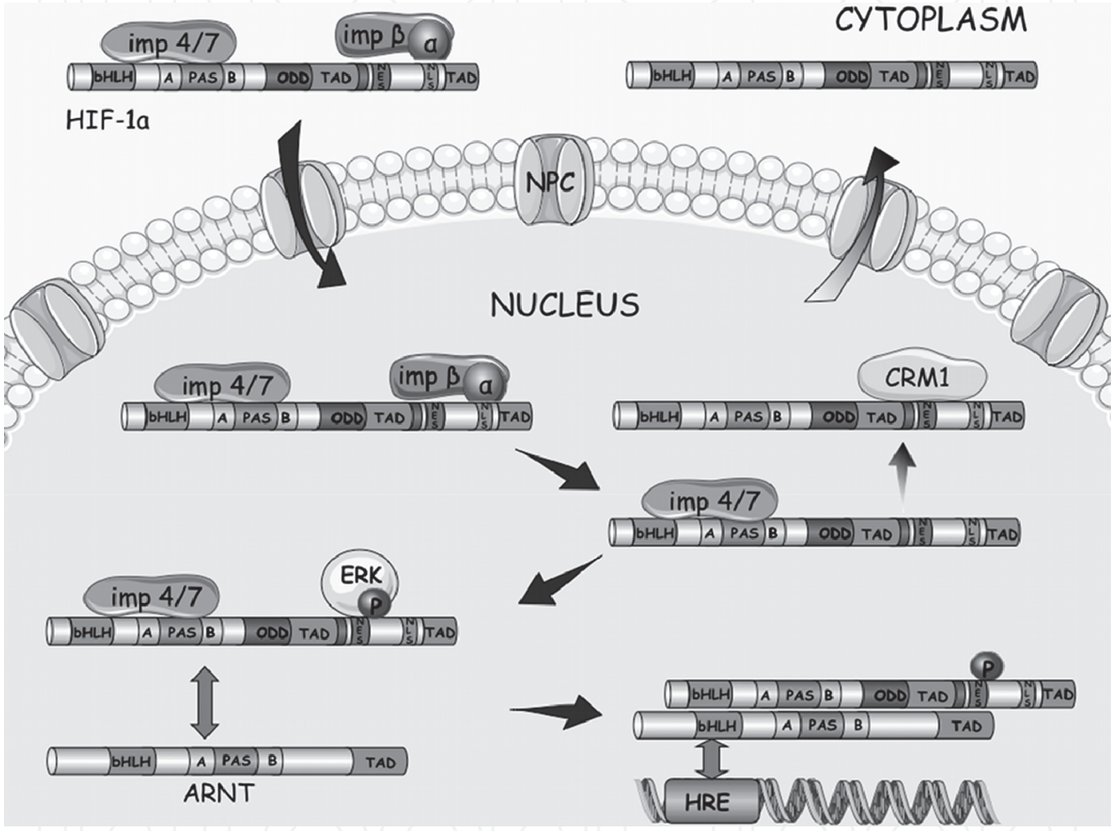

Fig. 1. HIF-1α regulation by ERK-mediated phosphorylation.

This schematic model shows how nucleocytoplasmic shuttling and activity of HIF-1α is controlled by ERK. HIF-1α is transported into the nucleus by nuclear import receptors (importins α/β, 4 and 7) but also contains an NES. In the absence of modification by ERK, binding of CRM1 to the NES returns HIF-1α to the cytoplasm and limits its activity by shortening its intranuclear resident time. When ERK phosphorylates HIF-1α, the NES is masked, interaction with CRM1 is inhibited and HIF-1α is allowed to accumulate inside the nucleus and attain maximal activity by forming an active HIF-1 heterodimer with ARNT and binding to DNA to stimulate transcription (see text for relevant references).

The link between ERK and HIF-1 appears not to be unidirectional. Many reports have shown that hypoxia induces the expression of several members of the DUSP (dual

specificity phosphatase also called MAPK phosphatase, MKP) family, which inactivate the MAPKs, including ERK, by dephosphorylation (Bermudez et al., 2011; Bernaudin et al., 2002; Laderoute et al., 1999; Liu, C. et al., 2005; Seta et al., 2001). Expression of DUSP1 in HepG2 cells may be the reason for the loss of ERK activation after prolonged exposure to hypoxia (Liu, C. et al., 2005), suggesting that hypoxia-inducible DUSPs may play a critical role in the spatio-temporal regulation of MAPK signaling. Furthermore, HIF-1 is itself required for the induction of DUSP6 (Bermudez et al., 2011), suggesting the operation of the negative feedback loop shown in Fig. 2. Since excessive and prolonged activation of ERK or HIF-1 may lead to apoptosis (Cagnol et al., 2006; Carmeliet et al., 1998), this negative feedback may act to limit ERK and, consequently, HIF-1α activity to a threshold compatible with cell survival and proliferation under hypoxia. On the other hand, HIF-1 has been recently shown to inhibit DUSP2 transcription causing DUSP2 suppression and prolonged phosphorylation of ERK, which increased chemoresistance and malignancy in human cancer cells under hypoxia (Lin et al., 2011). This suggests the additional operation of a feed-forward (or positive feedback) loop (also shown in Fig. 2) that can explain the down-regulation of DUSP2 and parallel elevation of ERK and HIF-1 activity in hypoxic cancer cells. It is, however, ambiguous the fact that hypoxic DUSP2 suppression was not observed in hepatoma cell lines and clinical samples of cancerous liver had similar levels of DUSP2 mRNA as those derived from normal tissue (Lin et al., 2011) suggesting limited and tissue-specific relevance of this mechanism.

This tight connection between ERK activity and HIF-1α can be targeted for controlling HIF-1 activity (Fig. 2). Indeed, nuclear accumulation and activity of HIF-1α was impaired when cancer cells were treated with natural occurring compounds such as flavonoids that inhibit the MAPK pathway and ERK-dependent HIF-1α phosphorylation (Triantafyllou et al., 2008). Moreover, the transient expression of a 43 amino acid HIF-1α peptide that contains the ERK modification sites and can be itself an ERK substrate (termed MTD: MAPK target domain) caused nuclear exclusion and loss of activity of endogenous HIF-1α (Mylonis et al., 2008). Finally, we have recently shown that the flavonoid kaempferol could act as a potent inhibitor of hepatoma cancer (Huh7) cell viability by inhibiting ERK activation and causing cytoplasmic mislocalization and inactivation of HIF-1α (Mylonis et al., 2010). Kaempferol could play a dual role in impairing cancer cell growth. It exhibited a mild effect on Huh7 cell survival under normal oxygen concentration, most likely due to inhibition of the MAPK pathway, which is critical for cell proliferation. However, kaempferol exerted a much stronger negative effect under hypoxia, apparently by additionally blocking HIF-1 activity required for cell viability at 0.1 - 1% O_2, conditions that are physiologically more relevant to those inside a tumor growing in vivo. This provided proof-of-principle for the potential use of kaempferol or other HIF-1α phosphorylation inhibitors as anti-HCC agents, since they could selectively target cancer cells normally exposed to hypoxia. The potential of kaempferol to inhibit both ERK and HIF-1 was at low μM concentration (IC_{50} close to 5 μM), which falls within the plasma flavonoid concentration range achievable by dietary intake alone (Gates et al., 2007; Manach et al., 2005). As a naturally occurring dietary substance without known side effects, kaempferol could be a good candidate for further evaluation, as chemopreventive or therapeutic compound, in controlled prospective studies of HCC patients along or in combination with other established conservative and interventional therapies.

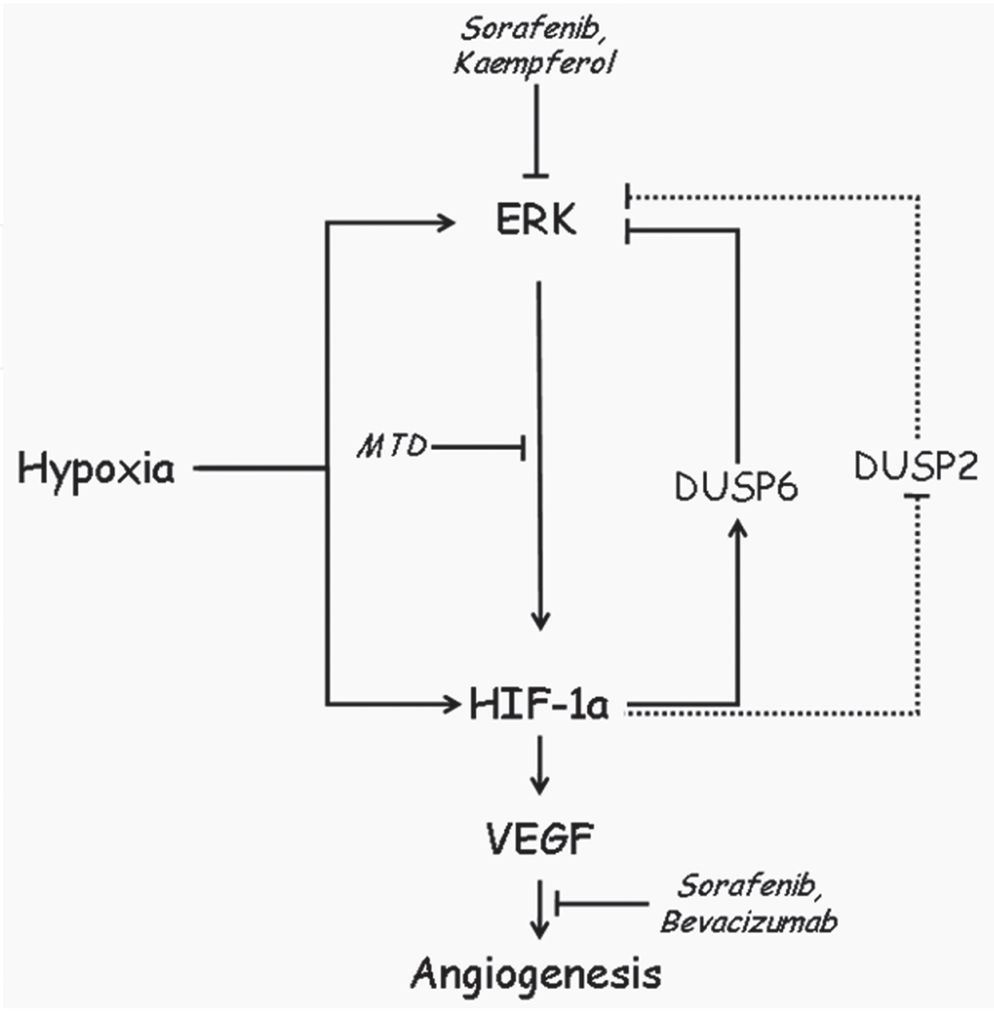

Fig. 2. The hypoxia-ERK-HIF-angiogenesis axis, feedback regulations and possible sites of targeted intervention.

HIF-1α, induced by hypoxia and activated by ERK-mediated phosphorylation, stimulates the production of VEGF that promotes angiogenesis. Induction of DUSP6 (and possibly other DUSPs, not shown) by HIF-1 deactivates ERK as a negative feedback mechanism, while suppression of DUSP2 can be part of an opposing feed-forward mechanism (not occurring in liver cells). This circuitry can be interrupted by agents that inhibit ERK activation (such as sorafenib and kaempferol), block HIF-1α phosphorylation (such as MTD) or impair VEGF function (such as sorafenib and bevacizumab). For further details and relevant reference see text.

8. Targeting the HIF pathway in HCC

Induction of the HIF- pathway may not come as a result of the cancer alone, but it can also be attributed to conventional HCC treatment such as chemoembolization which has been widely used in unresectable cases of hepatocellular carcinoma (Llovet & Bruix, 2003). However, restriction of the blood flow in the treated tissue generates hypoxic conditions

and activation of the HIF pathway, which may lead to a phenotypic change that renders cancer cells more invasive and metastatic, with subsequent failure of treatment as shown in animal primary liver cancers (Patsenker et al., 2009). Therefore, suppression of HIF-1 activity can be especially beneficial when combined with conventional treatments or future therapeutic agents that target other pathways related to HCC. This is already evident in experimental models, in which inhibition of HIF-1α expression enhanced the efficacy of doxorubicin in suppressing HCC cell growth through stimulation of apoptosis and down-regulation of VEGF (Liu, F. et al., 2008).

Cell based methods have been used to screen for HIF-1 inhibitors and many small molecules have been identified that impair cancer cell growth by reducing HIF-1α protein levels – through blocking its expression or enhancing its degradation – or by impairing its transcriptional activity (Semenza, 2010; Wilson & Hay, 2011). Another novel method to identify HIF-1 inhibitors is screening, recognition and isolation of new bioactive compounds from natural sources with molecularly imprinted polymers (MIPs), which have been developed using as templates known compounds that interfere with HIF-1 activity (Lakka et al., 2011). Of course, the potential application of these agents in cancer therapy relies on the outcome of clinical trials. However, the search for HIF-1 inhibitors revealed that several already tested and established anti-cancer drugs such as topotecan, a topoisomerase inhibitor, geldanamycin, an HSP90 inhibitor, and trichostatin A, a histone deacetylase inhibitor, can also block HIF-1 activity (Ibrahim et al., 2005; Poon, E. et al., 2009; Rapisarda et al., 2004). Given the ERK-HIF connection, extensively discussed above, another way to suppress HIF-1 would be to inhibit the Raf/MEK/ERK signaling pathway. The approval of sorafenib, a Raf inhibitor and anti-angiogenic agent, as single agent against HCC probably demonstrates this principle. Although, the effect of sorafenib on HIF-1 activity has not been directly studied in HCC, sorafenib has been shown to interfere with the HIF pathway in models of melanoma and neuroblastoma (Kumar et al., 2007; Nilsson et al., 2010). It is possible that combination of sorafenib with other agents that also target ERK (such as kaempferol or other naturally occurring substances with similar properties), HIF-1α (such as MTD) or VEGF (such as bevacizumab or inhibitors of the VEGF receptor) could be more efficient in treating HCC and preventing acquirement of resistance (Fig. 2).

9. Conclusion

Aberrant angiogenenesis, MAPK pathway activation and hypoxia contribute to the aggressiveness of hepatocellular carcinoma. This property is further enhanced by the fact that these processes positively influence one another in a way that adds up to the severity of the disease. Furthermore, they all involve as key component HIF-1α (Fig. 2), so its targeting provides an attractive strategy to treat hypoxic and highly angiogenic tumours like HCC. Combination of HIF-1α inhibitors with existing treatments or new targeted therapies like sorafenib may prove to be beneficial for the treatment of the disease.

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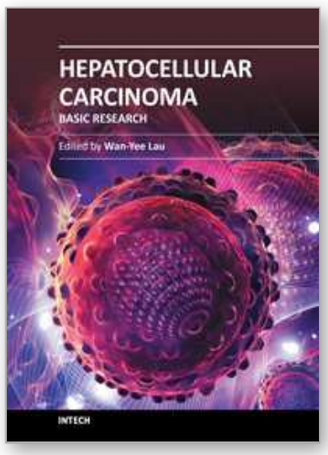

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Hepatocellular Carcinoma represents a leading cause of cancer death and a major health problem in developing countries where hepatitis B infection is prevalent. It has also become increasingly important with the increase in hepatitis C infection in developed countries. Knowledge of hepatocellular carcinoma has progressed rapidly. This book is a compendium of papers written by experts to present the most up-to-date knowledge on hepatocellular carcinoma. This book deals mainly with the basic research aspect of hepatocellular carcinoma. The book is divided into three sections: (I) Biomarkers / Therapeutic Target; (II) Carcinogenesis / Invasion / Metastasis; and (III) Detection / Prevention / Prevalence. There are 18 chapters in this book. This book is an important contribution to the basic research of hepatocellular carcinoma. The intended readers of this book are scientists and clinicians who are interested in research on hepatocellular carcinoma. Epidemiologists, pathologists, hospital administrators and drug manufacturers will also find this book useful.

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