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The biological kinship of hypoxia with CSC and EMT and their relationship with deregulated expression of miRNAs and tumor aggressiveness

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Abstract

Hypoxia is one of the fundamental biological phenomena that are intricately associated with the development and aggressiveness of a variety of solid tumors. Hypoxia-inducible factors (HIF) function as a master transcription factor, which regulates hypoxia responsive genes and has been recognized to play critical roles in tumor invasion, metastasis, and chemo-radiation resistance, and contributes to increased cell proliferation, survival, angiogenesis and metastasis. Therefore, tumor hypoxia with deregulated expression of HIF and its biological consequence lead to poor prognosis of patients diagnosed with solid tumors, resulting in higher mortality, suggesting that understanding of the molecular relationship of hypoxia with other cellular features of tumor aggressiveness would be invaluable for developing newer targeted therapy for solid tumors. It has been well recognized that cancer stem cells (CSCs) and epithelial-to-mesenchymal transition (EMT) phenotypic cells are associated with therapeutic resistance and contribute to aggressive tumor growth, invasion, metastasis and believed to be the cause of tumor recurrence. Interestingly, hypoxia and HIF signaling pathway are known to play an important role in the regulation and sustenance of CSCs and EMT phenotype. However, the molecular relationship between HIF signaling pathway with the biology of CSCs and EMT remains unclear although NF- κ B, PI3K/Akt/mTOR, Notch, Wnt/ β -catenin, and Hedgehog signaling pathways have been recognized as important regulators of CSCs and EMT. In this article, we will discuss the state of our knowledge on the role of HIF-hypoxia signaling pathway and its kinship with CSCs and EMT within the tumor microenvironment. We will also discuss the potential role of hypoxia-induced microRNAs (miRNAs) in tumor development and aggressiveness, and finally discuss the potential effects of nutraceuticals on the biology of CSCs and EMT in the context of tumor hypoxia.

Keywords

Hypoxia; HIF; CSC; EMT; miRNAs

1. Introduction

Low concentrations of oxygen in cells or tissues, referred to as hypoxia, are one of the most pervasive microenvironmental stresses and are recognized as the most common features of

solid tumors. Hypoxia has been known to be associated with many aspects of biological processes during tumor development and progression such as cell survival, invasion, angiogenesis, and cellular metabolic alterations. Clinically, hypoxia and its signaling pathway have been shown to be associated with resistance to radiotherapy and chemotherapy, contributing to increased risk of tumor recurrence and metastasis, leading to reduced overall survival rate and increased mortality [1-5]. It is estimated that up to 60% of locally advanced solid tumors exhibit hypoxic (1% O₂ or less, compared to 2–9% O₂ in the adjacent tissues) and/or anoxic (<0.01% O₂, or no detectable oxygen) conditions throughout the whole tumor tissues [6]. Transient hypoxia is related to inadequate blood supply while chronic or prolonged hypoxia is related to increased oxygen diffusion distance due to tumor expansion. Both types of hypoxic conditions are associated with poor outcome of patients diagnosed with solid tumors.

Tumor hypoxia is usually induced by several microenvironmental factors such as inadequate vascularization due to tumor angiogenesis leading to aberrant vessels with altered perfusion; an increase in oxygen diffusion distances due to rapid expansion of tumor cells; and tumor- or therapy-associated anemia leading to the reduced capacity of oxygen transportation [6,7]. Tumor cells usually have a greater capacity to adapt to a hostile, hypoxic environment for survival, compared to normal cells, which contributes to their malignant and aggressive behavior. This adaptation is controlled by many factors, including transcriptional and post-transcriptional changes in gene expression. It has been estimated that up to 1.5% of the human genome are responsive to hypoxia at transcriptional levels [6,7]. Hypoxia inducible factors (HIF) are one of the most critical transcriptional regulators that mediate the adaptation of tumor cells to a hypoxic tumor microenvironment.

In this review, we will discuss the role of HIF signaling pathways in the maintenance of hypoxic cellular response within the tumor microenvironment, and will discuss the role of hypoxia and its signaling pathway in the regulation and sustenance of the phenotypes of cancer stem cells (CSCs) and epithelial-to-mesenchymal transition (EMT). Moreover, we will summarize the molecular interrelation between HIF signaling pathway and other known cellular signaling pathways such as nuclear factor of κ B (NF- κ B), phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B)/mammalian target of rapamycin (mTOR), Notch, Wnt/ β -catenin, and Hedgehog in defining tumor aggressiveness. We will also discuss the state of our knowledge on the potential role of hypoxia-mediated microRNAs (miRNAs) in tumor development and aggressiveness. Finally, we will discuss the state of our knowledge on the role of natural product-derived agents (nutraceuticals) as potential molecular regulators of hypoxia-associated biology of tumor aggressiveness as a potential therapeutics.

2. Hypoxia, HIF, and clinical prognosis in tumor

HIF proteins are the master transcriptional regulators of the cellular response to hypoxic tumor microenvironment [8,9], which are involved in the regulation of many key aspects of tumor development and progression. For example, cell proliferation and survival are mediated through the regulation of the gene expression of HIF downstream targets, cyclin-dependent kinase inhibitor 1A (CDKN1A) and B-cell lymphoma 2 (Bcl2)/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), a cell death inducer during hypoxia; adaptive cellular metabolism mediated through the regulation of glucose transporter 1 (GLUT1), GLUT3, lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1); microenvironmental acidity mediated through the regulation of carbonic anhydrase 9 (CAIX); invasion and metastasis through the regulation of C–X–C chemokine receptor type 4 (CXCR4), mesenchymal–epithelial transition factor (c-MET), matrix metalloproteinases (MMP), and lysyl oxidase (LOX); angiogenesis through vascular endothelial growth factor (VEGF), VEGFR1, and angiopoietin-2 (Ang-2); and stem cell maintenance *via* the

regulation of octamer-binding transcription factor 4 (Oct4) [6,10-13]. The function of these HIF downstream target genes are reviewed elsewhere [14,15], and thus these are not the focus of this article.

A large number of clinical evidence suggest that HIF and its downstream targets are considered as key markers of clinical prognosis of patients diagnosed with solid tumors. Increased expression of HIF-1 has been identified to be associated with poorer prognosis with decreased disease-free survival in several early studies, which has been confirmed by a recent meta-analysis report [3]. Increased expression of VEGF and/or HIF-1 has been shown to be associated with poor prognosis [6,16-19]. The up-regulation of CAIX has also been associated with aggressive features with poor overall and relapse-free survival, consistent with the expression of HIF-1 [20-26]. Both markers are shown to correlate with both primary breast tumor and lymph node metastasis [26,27]. The up-regulation of GLUT1 and lactate dehydrogenase 5 (LDH-5) has been shown to be associated with poor prognosis, consistent with the expression of HIF-1 in many solid tumors [19,26-34]. High expression of BNIP3 in tumors is also reported to be associated with poor prognosis with increased risk of recurrence and decreased disease-free survival [27,35,36], and may be considered as independent prognostic factor for overall survival [27,35-38].

Recently, the expression of HIF-2 or concomitant with the expression of HIF-1 and its downstream targets, VEGF, Oct4, and erythropoietin (EPO), has been shown to be positively associated with poorer prognosis, increased rate of local recurrence, and reduced overall survival rate in various cancers [39-42]. These data clearly suggest that hypoxia and HIF signaling pathway play important roles in tumor development and aggressiveness.

3. Hypoxia, HIF, and treatment resistance in tumor

Hypoxia and HIF pathway have been considered as a negative factor for tumor therapy, and have been identified to be associated with the resistance to radiotherapy and chemotherapy [4,5]. Several clinical studies demonstrated that HIF-1, and its downstream targets, CAIX and VEGF have been associated with resistance to chemotherapy [5,23,43-45], consistent with multiple recent findings [46-50], indicating that hypoxia is associated with chemotherapy resistance.

The relationship between hypoxia and resistance to radiation therapy has also been documented. A recent meta-analysis report in head and neck cancers suggests that hypoxic modification improves tumor control and survival in conjunction with curative intended radiation therapy of head and neck cancers [51]. Another recent meta-analysis report demonstrates that biological markers involved in angiogenesis and hypoxia are associated with poor prognosis of cervical cancer with chemotherapy and radiation therapy [52]. These clinical data suggest that targeting these hypoxia-induced signaling pathways in combination with chemo-radiation therapy may improve survival of patients diagnosed with advanced-stage cervical cancer.

Several experimental studies have confirmed the important role of hypoxia and HIF signaling pathway in the induction of treatment resistance. High expression of HIF-1 enhances the resistance to radiation as assessed by clonogenic survival assay under normoxic and hypoxic conditions [53], whereas inactivation of HIF-1 signaling pathway by HIF-1 inhibitors or siRNA has been shown to increase the sensitivity of tumor to radiation therapy *in vitro* and *in vivo* [13,53,54]. Other experimental studies also revealed that HIF-1 deficiency in various cancer cells, including CSCs, are more susceptible to chemotherapeutics and ionizing radiation therapy than its wild type cells [15,45,55]. These findings are consistent with other findings [56-59], which clearly support that hypoxia-

induced signaling pathway plays an important role in both *de novo* (intrinsic) and acquired (extrinsic) resistance to conventional therapeutics in most solid tumors.

Although the exact molecular mechanism(s) of how hypoxia and HIF pathway lead to therapeutic resistance is not fully understood, several mechanisms have been proposed, such as decreased cytotoxicity due to the lack of oxidation of DNA free radicals by hypoxia, hypoxia-induced cell cycle arrest, compromised drug delivery due to increased distances of tumor cells to aberrant vasculature, impaired DNA repair system, hypoxia-induced extracellular acidification, and altered cellular metabolisms through multiple signaling pathways such as GLUT1 and ATP-binding cassette transporter G2 (ABCG2), a known drug resistant marker, which affects drug efficacy. It has also been considered that hypoxia-induced development and maintenance of CSC and EMT phenotypes would contribute to increased therapeutic resistance. Recently, hypoxia-induced VEGF has been shown to increase treatment resistance, due to the protective role of VEGF against cytotoxic effects of radiation in endothelial cells [60], further suggesting mechanistic role of VEGF in resistance to chemotherapy and radiation therapy [6,61].

4. Hypoxia-inducible signaling pathways in tumor

Emerging evidence suggests that hypoxic-inducible signaling pathways are significantly associated with the development and progression of tumors. As the major transcription regulators responsible for hypoxic stress, HIFs have been demonstrated to play a pivotal role in tumor growth, angiogenesis, invasion, and metastasis (Fig. 1). To date, three HIF family proteins, namely HIF 1, 2, and 3 have been identified in humans. We will discuss the role of these HIF proteins in tumorigenesis as documented in the following paragraphs.

4.1. The role of HIF-1 α signaling in tumor

HIF, a member of per-aryl hydrocarbon receptor nuclear translocator (ARNT)-sim (PAS) family of heterodimeric basic helix–loop–helix (bHLH) transcription factor, are composed of α subunit and β subunit [62-67]. Both α and β subunits belong to bHLH/PAS domain transcriptional factors [66]. To date, there are three subunits of β , namely, 1, 2, and 3 that have been demonstrated. However, HIF-1 β is reportedly the most common heterodimer involved in hypoxic response [10,68-70]. The HIF-1 α subunit (also known as ARNT or ARNT2) is identical to the arylhydrocarbon receptor nuclear translocator, which serves as a heterodimeric partner with the arylhydrocarbon receptor. HIF-1 α is constitutively expressed and present in the nucleus across many cell types, and is regulated in oxygen-independent pattern [71-73]. The *in vivo* experimental studies have revealed that functional loss of HIF-1 α suppresses tumor growth, consistent with decreased expression of VEGF and GLUT3 in hepatoma xenograft models [74,75], suggesting its potential role in tumorigenesis.

Expression of β subunit is regulated in an oxygen-dependent manner and it is induced by cellular hypoxia. Its expression is usually maintained at low levels in most cells with normal oxygen tension [67]. The levels of HIF-1 α are the primary determinant of HIF-1 DNA binding and transcriptional activity [76]. The induction of HIF-1 α is a critical step in the hypoxic response which occurs through the up-regulation of its mRNA expression, protein stabilization, and nuclear localization. Under normoxic condition, post-translational hydroxylation of proline residues at the positions of 402 and 564 (Pro⁴⁰² and Pro⁵⁶⁴) in the oxygen-dependent degradation domain (ODD) of HIF-1 α is induced by oxygen sensor dioxygenase proteins and prolyl hydroxylases (PHD 1, 2, 3), especially PHD2 [77-80]. The hydroxylated HIF-1 α binds to tumor suppressor protein the von Hippel–Lindau (VHL), an E3 recognition component of the ubiquitin ligase complex by these hydroxylated groups of HIF-1 α [81-84]. These interactions lead to rapid degradation of HIF-1 α through a ubiquitin

and proteasome-dependent pathway [83,85-90], leading to the inactivation HIF-1 [88,91]. This hydroxylation of HIF-1 occurs quite rapidly under normoxic conditions, resulting in very rapid HIF-1 turnover, resulting in maintaining the minimal levels of HIF-1 activity [92-94]; however, this regulation is altered under hypoxic condition with the tumor microenvironment as discussed later. In addition to proline hydroxylation, asparagine at the position of 803 (Asn⁸⁰³) in the COOH-terminal transactivation domain (CAD) of HIF-1 can be hydroxylated by the factor-inhibiting HIF (FIH), an oxygen sensor hydroxylase enzyme under normoxic condition [77-80,95]. These modifications inhibit HIF transcriptional activity by preventing the interaction between CAD of HIF-1 and p300/CBP transcriptional co-activator. FIH also can block the interaction between HIF-1 and its co-activators, leading to the inactivation of HIF-1 transcriptional activity. Under hypoxic condition, HIF-1 is stabilized due to hypoxia-induced inhibition of PHD and FIH activities, and then it is translocated into the nucleus where it dimerizes with the constitutively expressed HIF-1 [77,92]. The HIF-1 / heterodimers bind to its cognate transcriptional enhancer element, referred to as hypoxia-response element (HRE) (5'-RCGTG-3', where R is A or G), in the promoter/enhancer regions of a number of HIF target genes in the presence of p300/CBP binding protein (CBP) coactivators [96-98], leading to their transcriptional activation. These HIF responsive gene targets include glucose transporters, glycolytic enzymes, angiogenic growth factors, and several molecules involved in apoptosis and cell proliferation such as GLUT1,3, EPO, transferrin, endothelin-1 (ET-1), inducible nitric synthase (iNOS), heme oxygenase 1, PDK1, lactate dehydrogenase A (LDHA), BNIP3, VEGF, insulin-like growth factor (IGF) and IGF-binding proteins [99,100]. Currently, more than 60 HIF target genes have been identified in human cells, as reviewed elsewhere [13,14,101,102].

VHL plays an important role in the regulation of HIF signaling pathway, and is negatively associated with HIF pathway. Functional loss or mutation of VHL increases the activation of HIF signal pathway. Clinically, mutation in this gene causes VHL disease, congenital polycythemia, and several sporadic tumor types. Several other tumor suppressors such as p53, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), endogenous Akt inhibitor protein, tuberous sclerosis 2 (TSC2) and liver kinase B1 (LKB1) have been shown to regulate the activation of HIF-1. Functional loss or mutation of these genes induces activation of HIF-1 activation even under normoxic conditions [13,103-106].

A number of experimental studies have demonstrated that increased activation of HIF-1 has a role in promoting cell survival, invasion, angiogenesis, and tumor growth [107]. HIF-1 is expressed in a wide range of human cancer cells. High expression of HIF-1 has been identified in many human cancers including brain, breast, colon, lung, pancreatic, prostate and ovarian cancer [10,11,55,100,108,109], and is clinically associated with poorer prognosis of many tumors.

4.2. The role of HIF-2 α signaling in tumor

HIF-2 (also known as endothelial PAS domain protein 1, EPAS1) has been shown to share transcriptional target genes with HIF-1 such as VEGF, tyrosine kinase receptor with immunoglobulin and epidermal growth factor homology 2 (Tie2), Ang-2 and fms-like tyrosine kinase 1 (Flt-1), and also regulated by cellular oxygen tension. HIF-1 and HIF-2 have the homologous DNA-binding sequence binding sites in the promoter/enhancer regions of their target genes [110]. Similar to HIF-1, HIF-2 is stabilized at a wider range of oxygen tensions, ranging from severe hypoxia (<1% oxygen) to more physiologically relevant tension in tumors (2–5% oxygen) [111], and activates downstream transcriptional targets. It has been documented that HIF-2 has a similar role in the induction of angiogenesis *via* upregulation of VEGF production, and may play a dominant role in tumor angiogenesis [112]. Despite their similarities, HIF-2 expression has been shown to be

restricted to endothelial cells of vascular organs and had several unique transcriptional targets such as Oct4 and transforming growth factor- β (TGF- β). These targets are different from the canonical pro-angiogenic hypoxic response, which suggests an important and specific role for HIF-2 in the regulation of other cellular processes such as pluripotency.

The role of HIF-2 in HIF signaling pathway is not fully understood. High expression of HIF-2 has been recently identified in certain cancer cells including CSCs. Emerging evidence from experimental studies suggests that HIF-2 plays an important role in tumorigenesis, for example, inhibition of HIF-2 by its siRNA suppresses the growth of tumor derived from pVHL-defective renal carcinoma cells *in vivo* [113]. One recent experimental study paradoxically showed that HIF-2 over-expression can contribute to the progression of non-small cell lung cancer (NSCLC), whereas HIF-2 deletion promotes Kras-driven lung tumor development [114]. However, multiple recent experimental studies have revealed that HIF-2 is required for maintaining the phenotype and function of various stem cells such as glioblastoma, neuroblastoma, renal cancer, and non-small cell lung cancer [111,115-120]. The clinical data shows that there is a concomitant increased expression of HIF-1 and HIF-2 expression in certain cancers [112,121-123], and that the expression of HIF-2 and/or HIF-1 as well as its downstream targets has been shown to be associated with poorer prognosis of malignant diseases [115,122,124-127]. It appears that there is a crosstalk between HIF-1 and HIF-2 in tumor development and progression. Further molecular and mechanistic investigations are needed in order to fully elucidate the role of HIF proteins in human solid tumors.

4.3. The role of HIF-3 α signaling in tumor

The function and regulation of HIF-3 remain unclear. Limited studies have shown that HIF-3 has several splice variants, and its limited expression pattern has been identified in the eye and in the cerebellum, kidney, heart and lung [128-131]. Some HIF-3 variants have been shown to be a dominant-negative regulator of the HIF-1 and 2 isoforms, and are thought to be direct transcriptional targets of HIF-1 activity under hypoxic conditions [130,131]. It has also been shown *in vivo* that HIF-3 can be induced by insulin-induced hypoglycemia under normoxic conditions in cerebral cortex and kidney [129]. One recent study reveals that the expression of HIF-3 isoforms is cell type specific in vascular cells; however, only one isoform, HIF-3 β , is mediated by hypoxia and regulated by NF- κ B. Overexpression of HIF-3 isoforms decreases hypoxia-induced expression of VEGF and enolase 2, which confirms the notion of a hypoxia-inhibition of HIF-3 as a negative regulator of HIF signaling pathway [132].

The role of HIF-3 and its variants in tumorigenesis has not been optimally exploited. One experimental study has shown that HIF-3 δ , an alternatively spliced variant of human HIF-3 with similar domain structure as the murine inhibitory PAS protein (IPAS), is able to form an abortive transcriptional complex with HIF-2 and prevents the engagement of HIF-2 to the HRE site located in the promoter/enhancer regions of hypoxia-inducible genes. In addition, the re-expression of HIF-3 δ in VHL-null 786-O renal cancer cells *via* adenovirus decreases the endogenous expression of HIF-2 downstream target gene expression and suppresses the growth of 786-O tumor xenografts in severe combined immunodeficiency (SCID) mice. These data suggest that HIF-3 isoforms may have the tumor suppressive function [133].

5. The regulatory role of hypoxia and HIF pathway in CSCs

A number of studies have provided supportive evidence that hypoxia and HIF signaling pathway play pivotal roles in the regulation of the phenotype and function of CSCs, contributing to tumor aggressiveness. The CSCs constitute a sub-population of malignant

cells within a tumor mass and possess the ability to self-renew giving rise to heterogeneous tumor cell populations with a complex set of differentiated tumor cells [134-137]. The theory of CSCs has fundamental clinical implications especially because CSCs have been identified in many malignant tumor tissues and the CSCs are considered to be highly resistant to chemo-radiation therapy [134-138] relative to differentiated daughter cells. This provides an explanation for the clinical observation that tumor regression alone may not correlate with patient survival [138] because of tumor recurrence due to the presence of CSCs. The CSCs have been believed to play critical roles in treatment resistance and cancer metastasis and recurrence after conventional therapy through multiple mechanisms and networks, as reviewed by us recently [137,139,140].

Hypoxia has been recognized as an important factor that regulates the sub-population of normal stem cells and maintains the normal tissues or non-stem cell tissues in a stem cell state during embryonic and adult development [98,141,142]. Emerging experimental evidence indicates that hypoxia also plays an important role in the regulation of the phenotype and function of CSCs by enhancing the self-renewal capacity and maintenance of undifferentiated state of CSCs [143-145], consistent with recent findings in glioma CSCs [146,147]. Hypoxic CSCs robustly retain the undifferentiated phenotype, whereas normoxic CSCs retained differentiated state. The hypoxia-induced phenotype of CSCs is reversible when it re-grows at normoxic conditions [148]. It has been shown that hypoxia is able to maintain the stem-like phenotypes in neuroblastomas and activate signaling pathways that are associated with undifferentiated phenotypes of normal stem cells, including sex determining region Y box 2 (Sox2), Oct4 and Notch-1 signaling [118]. The hypoxia-induced activation of the signature genes of CSCs may be one of the major reasons that hypoxia is associated with increased tumor aggressiveness leading to poorer clinical outcome (Fig. 2).

Emerging evidence suggests that hypoxic areas within a tumor or the areas of necrotic tumor tissues are considered as a niche where CSCs reside [149]. Such hypoxic niche for CSCs may have an important role in the development and progression of tumors. Therefore, it is possible that tumors may evolve and develop from mutation in normal stem cells or from non-stem cell population during hypoxic conditions. The precise mechanism by which hypoxia mediates the phenotype and function of CSCs is not fully understood. It has been documented that hypoxia-mediated phenotype and function of CSCs is regulated by HIF proteins, specifically HIF-1 and HIF-2 mediated signaling pathways, which participates in the transcriptional activation and regulation of HIF targets, such as Oct4 and other CSC markers, as reviewed elsewhere [148,150].

As stated earlier that the expression of both HIF-1 and HIF-2 are associated with tumor aggressiveness. A number of experimental studies have clearly provided convincing evidence showing that both HIF-1 and HIF-2 plays important roles in the regulation of CSC phenotypes and functions, in a cell type specific manner. For examples, several recent studies have demonstrated that hypoxia-induced HIF-1 promotes the phenotype and function of CSCs, consistent with the up-regulation of HIF-1, and HIF-target genes such as Oct4, Nanog, c-Myc, Notch-1, and CD133, the CSC markers, in various cancer cells including CSCs [144,146,151-154]. Conditional knock-down of HIF-1 attenuates the expansion of hypoxia-induced CD133⁺ CSCs, which are confirmed by other recent findings [155,156]. These results collectively suggest that HIF-1 is required for the maintenance of the phenotype and function of CSCs in certain cancers.

Other recent investigations were focused on the role of HIF-2 in the regulation of CSCs. It has been shown that HIF-2 is highly expressed in CSCs; also known as tumor initiating cells (TIC) of neuroblastoma, glioblastoma, renal cell carcinoma, and breast carcinoma are associated with unfavorable disease outcome [119,120,157]. The expression of HIF-2 can

be induced by hypoxia, and increased expression of CD133 in CSCs [158] and the tumorigenic capacity of glioma stem cells [117]. Conditional loss of HIF-2 increases vessel abnormalities, decreases significantly the proliferation and selfrenewal capacity of CSCs, and suppresses tumor growth and metastasis [159,160]. Forced over-expression of HIF-2 induces stem cell markers Oct4, Nanog, Sox2, and c-Myc, and augments the tumorigenic capacity of the non-stem cell population [159]. It is interesting to note that HIF-2 may participate in the up-regulation of acidity-induced CSC phenotype and function in an oxygen-independent manner [161]. These findings strongly suggest that HIF-2 is required for the phenotype and function of CSCs.

The role of differential expression of HIF-1 and HIF-2 between non-stem cells and CSCs has paid significant attention in recent years, which may depend on the degree and period of hypoxia as well as a specific cell type dependent phenomenon. It has also been shown that HIF-2 is only significantly present in the population of CSCs, and remarkably induced by hypoxia [117,162]. In comparison, HIF-1 is present in both stem and non-stem tumor cells and is only stabilized under more severe hypoxic conditions [117], suggesting the differential roles of these two isoforms in CSCs.

It has been postulated that HIF-1 involves in the adaptation to acute/transient, and HIF-2 to chronic/prolonged hypoxia [115,163]. A recent experimental data has shown that hypoxia associated factor (HAF, also known as SART1), a potent E3 ubiquitin ligase, is highly expressed in various cancer cells and its levels are decreased during acute hypoxia, but increased following prolonged hypoxia [164,165]. HAF can inhibit HIF-1 activity through its direct binding to HIF-1 protein for ubiquitination and degradation in an oxygen- and pVHL-independent mechanism. It is also known that HAF binds to HIF-2, but at a different site than HIF-1, and increases HIF-2 transactivation without causing its degradation. Thus, HAF switches the hypoxic response of the cancer cell from HIF-1 dependent to HIF-2 dependent transcription and activates HIF target genes such as MMP-9, plasminogen activator inhibitor 1 (PAI-1), and Oct-3/4. The switch to HIF-2 dependent gene expression caused by HAF also promotes the expansion of tumor stem cell population, resulting in highly aggressive tumors *in vivo* [164,165]. These results suggest the differential expression and roles of HIF-1 and HIF-2 in the regulation of CSCs, which could indeed be dependent on hypoxic tumor microenvironment. However, the exact molecular mechanism(s) of how HIF-1 and HIF-2 interplay their roles in the regulation of CSC require further in-depth investigation.

6. The regulatory role of hypoxia and HIF pathway in EMT

The acquisition of EMT during tumor development progression is known to play important roles, which is also associated with the formation of the primary mesoderm from upper epiblast epithelium during neural crest cell formation from part of the ectoderm, and in palatal formation during embryonic development [139,166]. The EMT also occurs during adult placenta formation, and in the formation of fibroblasts during inflammation and wound healing after birth [139,166]. EMT is an important biological process by which epithelial-like cells with a cobblestone phenotype acquire mesenchymal characteristics with a spindle-shaped fibroblast-like morphology in human tumors. This process involves a disassembly of cell-cell junctions, such as down-regulation and relocation of E-cadherin and zonula occludens-1 (ZO-1), which are epithelial cell phenotype markers, and down-regulation and translocation of β -catenin from the cellular membrane to the nucleus, re-organization of actin cytoskeleton, and up-regulation of mesenchymal cell phenotype markers (such as vimentin, fibronectin, and N-cadherin). This process confers mesenchymal phenotypic cells to have less cell adhesion capacity, which leads to increased cell migration and invasion, resulting in tumor aggressiveness [167-170].

During the acquisition of EMT phenotype, several transcription factors such as zinc-finger E-box binding homeobox 1 (ZEB1) and ZEB2 (also known as SIP2, septin interacting protein 2), and Snail1 (also known as Snail), Snail2 (also known as Slug), Twist1 (also known as Twist), and E47 (also known as T cell factor, TCF3) have been shown to be critical mediators of EMT phenotype induced by a variety of inducers in different cell lines including cancer cells [170,171]. It has been demonstrated that ZEB1 regulates the expression of genes by binding to ZEB-type E-boxes (CACCTG) within the promoter region of target genes, resulting in chromatin condensation and gene inactivation [170,172]. The expression of E-cadherin, a marker for epithelial cell phenotype is repressed by ZEB1 through its binding to ZEB-type E-boxes in the E-cadherin gene promoter, which is fundamental for the acquisition of EMT phenotype [173]. Although EMT has been originally described in embryonic development, where cell migration and tissue remodeling have a primary role in the regulation of morphogenesis in multi-cellular organisms, recent experimental data and clinical findings have provided supportive evidence showing that EMT plays a pivotal role in tumor aggressiveness mediated *via* induction of cancer cell invasion and migration, and the phenotype and function of CSCs, thereby leading resistance to chemo- and radiation therapy, resulting in tumor recurrence [174].

A large number of data demonstrate that hypoxia induces EMT characteristics in a variety of cells including cancer cells [169,175,176]. Moreover, the hypoxia-induced EMT is tightly mediated by HIF-signaling pathway, which contributes to aggressive tumor growth and invasiveness. For example, several experimental studies have demonstrated that growth under hypoxic condition reprogram epithelial cells to a more mesenchymal phenotype, due to the activation of E-cadherin transcription repressors, leading to the promotion of tumor invasive potential [177-180], consistent with more recent findings [176]. A complex molecular crosstalk between hypoxia-induced pathway and EMT has not yet fully understood. Several aspects of potential molecular mechanisms have been proposed, as reviewed recently [170]. First, the activation of HIF-1 and 2 can induce EMT by up-regulation of EMT-associated transcription factors or repressors such as Twist, Snail, Slug, and SIP1/ZEB2 in many different cancer cells [179,181-183], as supported by more recent findings [184]. Secondly, hypoxia and HIF pathway activate the EMT-associated signaling pathways such as TGF- β , Notch, NF- κ B, Wnt/ β -catenin, and Hedgehog [173,178,185-213]. Next, hypoxia and HIF pathway can induce EMT phenotype or characteristics by the regulation of EMT-associated inflammatory cytokines such as increased expression of hypoxia-induced tumor necrosis factor (TNF- α), interleukin 6 (IL-6), and IL-1 β , which promote the induction of EMT phenotype [214-216]. Finally, hypoxia and HIF pathway can induce EMT by direct or indirect regulation of proteins or enzymes that mediate cell-matrix interactions and facilitate motility and invasion mediated through the regulation of LOX/LOX2, Hey1, Hes1, and urokinase-type plasminogen activator (uPA) expression [217-219]. The data from recent studies suggest that other cellular signaling pathways such as VEGF and epigenetic regulators have been shown to play important roles in the hypoxia-induced EMT in cancer cells [220-223]. For example, hypoxia-induced or HIF-induced histone deacetylase 3 (HDCA3) is reported to be required for hypoxia-induced EMT phenotype [224]. One *in vitro* and *in vivo* study revealed that HIF-2 promotes the expression of several genes associated with EMT phenotype markers such as ZEB1, Snail, SIP1, and vimentin, and increases non-small cell lung tumor growth in xenograft mouse model [116]. Therefore, targeting both HIF pathway and EMT could provide a therapeutic strategy in the future (Fig. 2).

7. The role of VEGF signaling in tumor

Angiogenesis is classically defined as the formation of new blood vessels from the existing vascular bed, which is an essential biological process during development and diseases.

Angiogenesis has been considered as one of the hallmarks of cancer, and plays an essential role in tumor growth, invasion and metastasis. Tumor angiogenesis is induced by hypoxic condition through the regulation of several pro-angiogenic factors including VEGF, one of the major HIF target gene products. The VEGF is a well-known potent endothelial specific mitogen and has a central role in tumor angiogenesis [13,70,102,225]. VEGF and its receptor signaling pathway is one of the major signaling pathways and is best characterized. The increased expression of VEGF has been identified to be associated with a wide range of solid tumors, as stated earlier. Moreover, VEGF has been considered as negative predictor of tumor-related outcome.

In addition to a central role in tumor angiogenesis, emerging evidence suggests that VEGF might have other regulatory roles in tumor aggressiveness by angiogenesis-independent mechanism. Several studies have demonstrated that VEGF or VEGFR-1 is able to inhibit epithelial cell phenotype, and to mediate EMT phenotype in various cells including pancreatic cancer cells through the regulation of EMT-associated transcription factors, Snail, Twist, and Slug [221-223]. Additional studies have confirmed that the activation of VEGF or VEGFR may induce EMT phenotype in cancer cells [220,226,227]. These results suggest that hypoxia-induced VEGF signaling pathway not only acts as a regulator of angiogenesis, but also acts as a potential inducer of EMT, both of which contributes to tumor aggressiveness.

The role of VEGF in the regulation of the phenotype and function of CSCs has not been fully investigated. A recent report has shown that VEGF promotes cardiovascular stem cell migration *via* PI3K/Akt pathway in a concentration-dependent manner under hypoxic conditions [228]. The VEGF receptor inhibitor SU5416 or the PI3K/Akt inhibitor wortmannin suppresses the VEGF-induced cardiac stem cell accumulation and migration [228]. Another study have demonstrated that stem cell-like glioma cells isolated from human glioblastoma samples produce high levels of VEGF, significantly form highly-vascular and hemorrhagic tumor in the brains of immunocompromised mice. Treatment of these CSC-like cells with VEGF inhibitor bevacizumab inhibits their ability to induce endothelial cell migration and tube formation *in vitro*, and suppresses CSC-derived tumor growth [229], which suggests that hypoxia-induced VEGF expression may contribute to tumor initiating capacity through stem cells.

8. The role of NF- κ B in HIF signaling pathway in tumor

HIF signaling pathway is essential for cellular response to hypoxia. However, almost 20 different transcription factors have displayed directly or indirectly hypoxic sensitivity, including NF- κ B. The NF- κ B has been identified to be activated by hypoxia for more than a decade in a wide variety of cells [230,231]. NF- κ B is a family of homo- or heterodimer transcription regulators including RelA (p65), RelB, c-Rel, NF- κ B1 (p105/p50), and NF- κ B2 (p100/p52), which are typically activated following stimulation of cells with pro-inflammatory cytokines such as TNF- α and IL-1, antigens and bacterial liposaccharides (LPS), growth factors, UV light, and reactive oxygen species (ROS) [232,233]. Normally, NF- κ B proteins are sequestered in the cytoplasm of non-stimulated cells by a family of inhibitory proteins of NF- κ B (I κ B- and I κ B-), which bind to NF- κ B and mask its nuclear translocation. Once cells are stimulated, I κ B proteins become phosphorylated by the I κ B kinase complex (IKK- α , β , and γ), and ubiquitinated, and degraded by the proteasomes, eventually leading to nuclear translocation and activation of NF- κ B target gene transcription.

The NF- κ B is a well-known critical transcription factor in a wide range of cancers that regulates the expression of NF- κ B target genes associated with a variety of cellular

processes such as cell survival, proliferation, invasion, migration, angiogenesis and tumor metastasis, leading to the resistance to chemo- and radio-therapy, tumor recurrence or relapse, and increased risk of poor prognosis [234-236]. Moreover, emerging evidence suggests that NF- κ B also plays an important role in EMT and CSCs, contributing to tumor aggressiveness [237-240].

A large number of studies have indicated that hypoxia could activate NF- κ B signaling pathway in a variety of cells including cancer cells. NF- κ B is primarily a regulator of inflammatory and anti-apoptotic gene expression. It is reasonable to believe that NF- κ B activation is required to inhibit apoptosis to enable a hypoxic cell to survive through the period of hypoxic insult. Hypoxic action of NF- κ B signaling pathway also involves promoting hypoxic inflammatory response through the regulation of gene expression of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1, as well as adhesion molecules, enzymes, and pro-inflammatory enzymes [241,242]. It is reasonable to speculate that hypoxia induced NF- κ B activation may be contributing to the maintenance of CSCs and EMT during the development and progression of tumors especially because both HIF and NF- κ B signaling pathways are known to enhance the induction of EMT phenotype and maintenance of stem cell phenotype and function in tumor microenvironment.

HIF-1 has been shown to be required for the activation of NF- κ B [243]. The functional loss or deficiency of HIF-1 decreases NF- κ B activation and p65 expression whereas increased expression of HIF-1 results in the activation of NF- κ B through hyperphosphorylation of I κ B and phosphorylation of p65 at Ser²⁷⁶ in keratinocytes [244]. The HIF-1-mediated activation of NF- κ B has been shown to be consistent with cancer cell survival, invasion, and tumor growth [240,243,245-247]. Therefore, hypoxic activation of HIF pathway plays an important role in the regulation of NF- κ B pathway in tumor microenvironment. The molecular mechanism of how HIF pathway regulates NF- κ B pathway has been partly exploited in several studies, which suggest that HIF pathway activates NF- κ B pathway mainly through the regulation of IKK signaling-mediated regulation of canonical NF- κ B signaling.

Some earlier studies has shown that hypoxia induces NF- κ B activation through the regulation of the activation of IKK signaling, especially IKK- γ [248], which was further confirmed by recent findings [59,247, 249-252]. Hypoxia-induced IKK activation has been considered to be mediated through PHD domain because IKK- γ contains sequence-analogous motifs (LxxLAP motifs), which are known targets for hydroxylation by PHD1 [248]. Pharmacological or genetic inhibition of hydroxylases PHD1 signaling leads to increased NF- κ B signaling [248]. Recently, PHD3 is found to inhibit the phosphorylation of IKK- γ and NF- κ B proteins independent of its hydroxylase activity by blocking the interaction between IKK- γ and heat shock protein 90 (Hsp90) that is required for phosphorylation of IKK- γ in colorectal cancer cells. Knock-down of PHD3 increased IKK signaling and the resistance of these cancer cells to the effects of TNF- α and increased tumorigenesis [253]. Furthermore, two additional new studies have demonstrated that hypoxia-induced IKK kinases such as X-linked inhibitor of apoptosis protein (XIAP) and TGF- β activated kinase 1 (TAK1) could mediate IKK activity *via* IKK- γ , leading to the activation of NF- κ B [245,249]. Conditional deletion of XIAP, a potential E3 ubiquitin ligase, inhibits hypoxia-induced IKK activation. Over-expression of XIAP leads to increased phosphorylation of IKK in both normoxic and hypoxic conditions [249]. These findings suggest that hypoxia causes activation of IKK, specifically *via* IKK- γ , and thus may have a key role in the regulation of NF- κ B pathway under tumor hypoxic microenvironment.

Moreover, FIH is also considered to regulate hypoxic induction of NF- κ B activation through the hydroxylation of NF- κ B components such as p105 and I κ B- because these components

of NF- κ B contains ankyrin repeat domain, which are subject to hydroxylation by FIH [254]. However, the functional consequence of the hydroxylation of I κ B- by FIH is not conclusive [255,256]. Several studies have shown that hypoxic activation of NF- κ B pathway is, at least, in part HIF-1-dependent [243,244,247]. It is speculated that HIF-1 may directly interact with NF- κ B proteins, promoting DNA binding at the promoter region of NF- κ B target genes.

In addition to HIF-mediated activation of NF- κ B, it is known that NF- κ B could regulate HIF pathway in tumorigenesis. A large number of experimental studies have clearly indicated that NF- κ B could directly regulate HIF signaling pathway within the tumor microenvironment because HIF promoter contains active NF- κ B binding site in position -178/-188 [257-259]. The mutation of NF- κ B DNA binding site in the HIF-1 promoter region leads to the loss of hypoxia-induced HIF-1 upregulation [257,259]. Moreover, blocking individual NF- κ B members by its specific siRNAs decreases HIF-1 activity [257,259]. The activation of NF- κ B induced by TNF- α or p50, p65 results in increased levels of HIF-1 mRNA and protein [257,259]. It has also been shown that knockdown of IKK- decreases HIF-1 activity [252], consistent with other recent findings indicating that NF- κ B up-regulates HIF-1 signaling pathway *via* IKK- [260-262]. These results suggest that IKK signaling is required for the activation of HIF-1 activity. Non-hypoxic stimulation such as cytokines especially TNF- α and IL-4, also activate HIF-1 activity through NF- κ B dependent mechanism [254,260,263]. Therefore, NF- κ B regulates HIF-1 signaling pathway to maintain the basal levels of HIF-1 under normoxia condition, and the levels of HIF-1 increases under hypoxia.

Interestingly, one earlier study has shown that NF- κ B induces HIF-2 activation through the interaction with IKK- (also known as NEMO, NF- κ B essential modulator), leading to CBP/p300 recruitment for transcriptional activation of HIF-2 [264]. Moreover, one recent study has shown that NF- κ B directly regulates HIF-1 mRNA and protein in *Drosophila*. NF- κ B-mediated induction of HIF-1 results in the modulation of HIF-2. The over-expression of HIF-1 can rescue HIF-2 protein levels following NF- κ B depletion [265]. Furthermore, NF- κ B has also been found to regulate HIF-3, a negative regulator of HIF signaling pathway [132]. However, the role of NF- κ B in the regulation of HIF-3 in the tumor microenvironment is not fully understood.

One recent clinical study revealed that the expression of HIF-1 is positively associated with the expression of RelA in gastric cancer patients. The *in vitro* experiment showed that over-expression of I κ B- M (suppressor-suppressive mutant form) suppresses tumor growth, angiogenesis, and HIF-1 expression [246], consistent with previous findings indicating that the interaction of HIF and NF- κ B pathways contributes to breast cancer metastasis through the induction of EMT together with migration through p53-lysine acetylation and HDAC-dependent epigenetic mechanism to up-regulate NF- κ B and HIF [266]. Taken together, the interaction between HIF and NF- κ B signaling pathways plays a pivotal role in tumor aggressiveness. However, the precise molecular crosstalk between these pathways in the tumor microenvironment is complex and still requires in-depth investigation. In summary, NF- κ B and HIF pathways appear to have an important role in the tumor microenvironment; however, the precise molecular mechanism of crosstalk between NF- κ B and HIF require further in-depth investigation.

9. The role of PI3K/Akt/mTOR in HIF signaling pathway in tumor

PI3K/Akt/mTOR pathway has been known to play a key role in a wide variety of cellular processes including cell proliferation, adhesion, migration, invasion, metabolism, and survival [267]. Phosphorylation of PI3K by microenvironment stimuli such as growth

factors, cytokines, and hormones results in the activation of Akt, which leads to the phosphorylation of mTOR (an active form). The activation of mTOR complex (mTORC1 and mTORC2) then phosphorylates p70- S6 kinase (S6K1) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4-BP1), *via* mTORC2, finally leading to the up-regulation of gene transcription and translation [268-270].

The tumor suppressor protein, PTEN, a phosphatase opposes the activation of PI3K, thereby down-regulates the level of activated Akt, leading to the reduction of mTOR signaling [268,271,272]. Aberrant activation of PI3K/Akt/mTOR signaling pathway has been identified in many tumors and has been considered to be associated with tumor aggressiveness, in part, mediated through the regulation of multiple cellular signaling pathways involving in the induction of EMT and CSC phenotypes and regulation of their functions [273,274].

A large number of studies have provided supportive evidence showing that hypoxia induces the PI3K/Akt/mTOR signaling pathway, consistent with cell proliferation, maintenance of stem cell function *via* HIF-dependent mechanisms in a wide range of cells including cancer cells [275-281]. The evidence suggests that both HIF and PI3K/Akt/mTOR signaling pathways plays important roles in tumor hypoxic microenvironment, leading to tumor aggressiveness. Thus, there may exist a crosslink between both signaling pathways under hypoxic conditions. Earlier experimental studies have shown that Akt signaling pathway participates in the up-regulation of growth factors-induced HIF-1 activity by increased phosphorylation of eIF4E and p70S6K in various cells including cancer cells [282-284]. A number of recent studies have demonstrated that Akt/mTOR up-regulates HIF-1 expression, and stabilizes HIF-1, and increases HIF-1 synthesis in various cells including cancer cells under normoxic and hypoxic conditions [38,280,281]. It is also known that mTOR stimulates the translation of HIF-1 mRNA into protein [285]. Specific inhibitors for either PI3K, Akt or mTOR decrease the levels of HIF-1, and inhibit hypoxia-induced HIF-1 transactivation and VEGF expression, leading to a decrease in angiogenesis [260,276, 277,279,285-288]. It has also been shown that the loss of PTEN facilitates HIF-1 mediated gene expression, leading to increased tumor growth and tumor vascularity, compared to PTEN expressing prostate cancer cells [289]. These data suggest that PI3K/Akt/mTOR signaling pathway plays important roles in the regulation of HIF signaling pathway during the development and progression of tumors.

The role of HIF signaling in the regulation of PI3K/Akt/mTOR signaling pathway has been exploited in some studies. One earlier study revealed the inhibition of HIF signaling pathway by 2-methoxyestradiol (2ME), an endogenous HIF-1 inhibitor, which significantly enhances the radiation-induced reduction of cell proliferation and survival, consistent with the down-regulation of radiation-induced expression of HIF-1 and Akt/mTOR signaling, and decreased DNA synthesis in breast cancer cells [290]. These results suggest that HIF signaling pathway participates in the regulation of PI3K/Akt/mTOR signaling pathway, thereby promoting cell proliferation and survival, and tumor growth. Taken together, a crosslink between HIF and PI3K/Akt/mTOR signaling pathways within the tumor microenvironment is highly complex, and thus require in-depth molecular studies in the future.

10. The role of Notch in HIF signaling pathway in tumor

Notch signaling pathway plays an important role in the regulation of a wide range of fundamental cellular processes such as proliferation, stem cell maintenance, differentiation during embryonic and adult development and homeostasis of adult self-renewing organs [291-295]. The proteins encoded by Notch genes (Notch-1, 2, 3, 4) can be activated by

interacting with a family of its ligands. The extracellular domain of Notch consists of many EGF-like repeats, which participate in receptor-ligand binding. Notch signaling is initially activated through the receptor-ligand interaction between two neighboring cells. Upon activation, Notch is cleaved, releasing the intracellular domain of the Notch (NICD) through a cascade of proteolytic cleavages by the metalloprotease TNF- α -converting enzyme (TACE) and a γ -secretase complex. The released NICD is then translocated into the nucleus for transcriptional activation of Notch target genes such as Hes1, Hey1, and cyclin D1 [294-296]. The γ -secretase inhibitors (GSI) can suppresses the Notch signaling pathway by binding to γ -secretase complex, leading to the inactivation of this enzyme activity [294,297]. A large number of evidence suggest that Notch signaling pathway plays a pivotal role in tumor aggressiveness by regulation of cell proliferation, apoptosis, invasion, as well as the induction of EMT and CSC functions [291,298-300]. There are some limited reports showing that Notch proteins could exert tumor suppressive effects in few cancers such as lung and skin cancers. However, a large number of studies have demonstrated that Notch proteins have oncogenic in wide range of cancers. Oncogenic or tumor suppressive activities of Notch proteins depend on the cellular context [299].

Several earlier studies have demonstrated that hypoxia-induced HIF-1 α binds, stabilizes and activates Notch signaling, leading to cell proliferation and invasion, and maintenance of stem cells in an undifferentiated cell state in various cells including cancer cells as well as stem cells and precursor cells [144,301-303], which have been confirmed by recent findings [304]. Over-expression of Notch-1 can rescue the γ -secretase inhibitor-induced apoptosis of lung cancer cells under hypoxic conditions [301]. It is also known that hypoxia increases Notch-1 mRNA and protein as well as Notch-1 signaling pathway through HIF-1 dependent mechanism [178,301,305,306], supported by recent findings [158,307]. Hypoxic mediated activation of Notch signaling pathway has been shown to involve cell survival, Notch-induced EMT, maintenance of stem cell self-renewal capacity, and tumor cell invasion and migration [178,217,308,309]. It has also been shown that hypoxia induces Notch signaling pathway by binding HIF-1 α to Hes-1 promoter regions, leading to the induction of EMT and increased cell invasion and migration in breast cancer [217]. Recently, HIF-2 α was found to induce Notch signaling pathway in stem cells [119,120]. Moreover, it has also been documented that the accumulation of both HIF-1 α and 2 proteins involves the hypoxic activation of Notch signaling pathway [217]; however, the exact mechanism of how HIF regulates Notch signaling pathway within the tumor microenvironment is not fully understood.

It has been shown that hypoxia induces HIF-mediated Notch-1 signaling *via* Akt pathway in melanoma cells [305] and that both Notch and HIF-1 α are involve in the promotion of hypoxia-induced VEGF expression in mouse embryonic stem cells [310]. Two studies have provided evidence showing that this complex crosstalk may be mediated by FIH. Specifically, Notch proteins contain ankyrin repeat domains which can be hydroxylated by FIH, leading to the reduced Notch signaling pathway [311-313]. It has been speculated that FIH has more binding affinity than HIF-1 α , which leads to NICD sequestration of FIH away from HIF-1 α , relieving HIF-1 α from repression of FIH. Hydroxylation on HIF-1 α has also been shown to be decreased in the presence of NICD [311], and therefore, the binding of HIF-1 α to HRE promoter sites is more efficient [312]. However, the in-depth investigation is required to elucidate the role and the crosstalk between these two signaling pathways in tumor hypoxic microenvironment.

11. The role of Wnt/ β -catenin in HIF signaling pathway in tumor

Wnt/ β -catenin signal pathway modulates cell growth by increasing β -catenin levels, β -catenin nuclear localization, and binding to the lymphoid-enhancing factor (LEF)/TCF

family of transcription factors, leading to the expression of its target genes in controlling cell growth and proliferation [314-317]. A number of experimental studies have provided evidence for a direct role of Wnt signaling pathway in the embryonic and stem cell development through the regulation of phenotype and self-renewal capacity of stem cells. Moreover, deregulation of Wnt/ β -catenin signaling pathway has been observed in many cancers [318,319]. The activation of the Wnt signaling pathway in epidermal stem cells of transgenic mice can result in epithelial cancers [320], and induce EMT phenotype [320-322]. It has also been shown that Wnt/ β -catenin signaling pathway is involved in the induction of EMT [174,323]. Therefore, Wnt/ β -catenin signaling pathway plays an important role in the regulation of CSCs and EMT [167,174,322,324-329].

Emerging evidence suggests that Wnt/ β -catenin pathway plays a role in tumor microenvironment through a crosslink with HIF pathway. The exact mechanism of the crosstalk between these signaling pathways is not fully understood. Two early studies have demonstrated that hypoxia inhibits β -catenin-TCF4 complex formation and transcriptional activity in colon cancer SW480 cell by HIF-1 β -catenin competition with TCF4 binding directly to β -catenin. However, β -catenin can enhance HIF-mediated transcription, thereby promoting cell survival and adaptation to hypoxia [330,331]. Other multiple studies have shown that hypoxia induces the activation of Wnt/ β -catenin pathway by HIF-dependent mechanisms [286,324,332-334]. Furthermore, over-expression of HIF-1 β -catenin pathway in prostate cancer cells, consistent with cell invasion and the induction of EMT. Conditional knock-down of β -catenin attenuated the EMT characteristics induced by over-expression of HIF-1 [334]. HIF-2 was also shown to enhance the Wnt/ β -catenin/TCF signaling pathway by interacting with β -catenin, leading to cell growth and proliferation in renal cancer cells [286]. These data suggest that hypoxic activation of HIF-mediated Wnt/ β -catenin pathway, at least in part, contributes to hypoxia-induced EMT in the development and progression of tumor. More interestingly, hypoxia can induce stem cell growth self-renewal, consistent with increased expression of HIF-1 β -catenin and increased activation of Wnt/ β -catenin [335]. Conditional knock-down of HIF-1 inhibits the induction of Wnt effectors, LEF1 and TCF1, potentially by interrupting the HIF-1 binding to the promoters of LEF1 and TCF1 in mouse stem cells, which is in direct agreement with an interesting report [336], suggesting that HIF-mediated Wnt/ β -catenin pathway promote the maintenance of stem cell function.

12. The role of Hedgehog in HIF signaling pathway in tumor

Another important signaling pathway potentially associated with the regulation of HIF pathway is the Hedgehog (Hh) signaling pathway. The Hedgehog signaling pathway is a major regulator of cell differentiation, tissue polarity and cell proliferation [337,338]. The Hh family consists of three secreted proteins including sonic Hedgehog (Shh), desert Hedgehog (Dhh), and Indian Hedgehog (Ihh). The Hh proteins are activated by multiple processes such as cleavage, and lipid modification. Binding of lipid-modified Hh to its receptors Patched 1 or Patched 2 (PTCH1 or PTCH2), an inhibitor of Smoothened (Smo) leads to the loss of PTCH activity, and consequent activation of Smo, which transduces the Hh signal to the cytoplasm. Subsequently, the activated Smo causes the activation of Gli (glioma-associated oncogene family zinc finger protein) family of transcriptional effectors through complex interactions with Costal2 (Cos2), Fused (Fu) and Suppressor of fu (Sufu), leading to the up-regulation of PTCH, Wnt, and bone morphogenetic protein (BMP). Therefore, the Hh ligands, Shh, Dhh and Ihh stimulate Gli transcription factors which constitute the final effectors of the Hh signaling pathway.

Emerging evidence clearly suggests the activation of Hh signaling in various human cancers, including basal cell carcinomas, medulloblastomas, leukemia, renal, gastrointestinal, lung,

cervical, ovarian, breast and prostate cancers [339,340]. Furthermore, because Hh plays a central role in the control of cell proliferation and differentiation of both embryonic stem cells and adult stem cells, the aberrant activation of Hh signaling could lead to the generation of CSCs and the development of tumor [329]. Recent studies have shown that activation of Hh signaling is critically related to the features of CSCs and EMT in many types of cancers including colon, gastric, esophagus, hepatic, and other cancers [337,338,341,342], suggesting that Hh signaling pathway may play a key role in the regulation of CSCs and EMT within the tumor microenvironment.

Emerging evidence also suggests that Hh signal pathway may be involved in the regulation of HIF pathway under hypoxic conditions. Earlier studies have shown that Hh pathway has a key role in the regulation of angiogenesis [343]. Specifically, hypervascularization of the neuroectoderm has been observed following transgenic overexpression of Shh in the dorsal neural tube of Zebra fish [343]. These observations clearly suggest the role of Hh signal pathway in angiogenesis mediated through the regulation in the expression of pro-angiogenic factors, VEGF, Ang-1 and -2 [344-346]. Thus, there may be a crosstalk between HIF and Hh pathways during cell development and differentiation.

One experimental study reveals that hypoxia induces not only the increased expression of IGF-2 and VEGF but also the activation of their upstream regulatory genes, HIF-1 and Shh in embryonic rat heart. Inactivation of expression of both HIF-1 and Shh pathways by its inhibitors, respectively, resulted in the suppression of each other [347], consistent with recent findings [348].

However, the complex crosstalk between these pathways within the tumor microenvironment remains unclear. Recently, it has been shown that hypoxia activates Hh signaling pathway in a ligand-independent manner by up-regulation of the gene expression of Smo, Gli1, and MMP9 in pancreatic cancer cells. Conditional knockdown of Smo decreases the transcription of Hh downstream targets, Gli1 and MMP9, and suppress tumor invasiveness under hypoxic conditions [349]. One recent clinical study showed that the elevation of Shh expression and low Smo expression are associated with a clinical tumor hypoxic indicator, HP5 (the percentage of oxygen pressure reading in each tumor less than 5 mmHg) in cervical cancer [339]. These data suggest that hypoxia-mediated Hh signaling pathway may play an important role within the tumor microenvironment.

13. The regulatory role of hypoxia-mediated miRNAs in tumor

The microRNAs (miRNAs) are small non-protein-coding RNAs (around 19–24 nucleotides) that function as post-transcriptional gene regulators by its specific binding to the 3' untranslated region (3'-UTR) of target mRNAs to control protein synthesis or degradation of the mRNAs [350]. miRNAs are currently recognized as regulators of the expression of most genes, and consequently play critical roles in a wide array of biological processes, including cell differentiation, proliferation, death, metabolism and energy homeostasis [314,315]. Sufficient evidence has suggested that miRNAs might have an important role in development and progression of tumors. The altered expression of miRNAs has been associated with clinical prognosis of tumor, resistance to chemo-radiation therapy, tumor recurrence and/or relapse. Tumor hypoxia is a major event during the development and progression of tumor. A large number of miRNAs have been reported to be responsive to hypoxia in a wide range of cells or tissues. Moreover, a number of miRNAs have been identified to be associated with hypoxia and HIF signaling pathways in various cancer cells [351-354]. Thus hypoxia-mediated miRNAs may play an important role in tumor aggressiveness through the regulation of cellular signaling pathways including HIF pathway. Targeting these hypoxia-mediated miRNAs might provide a novel therapeutical strategy for

the prevention and/or treatment of cancer. The following paragraphs will provide evidence in support of several miRNAs that are associated with many solid tumors and their regulation under normoxic and hypoxic conditions driving tumor aggressiveness.

13.1. The up-regulation of hypoxia-mediated miRNAs in tumor

13.1.1. The role of miR-21—miR-21 has been considered as a pro-oncogenic molecule. The clinical studies has shown that increased expression of miR-21 is associated with poor clinical prognosis in a wide variety of cancers such as pancreatic cancer, prostate cancer, breast cancer and brain cancer [355,356]. It has been reported that the increased expression of miR-21 results in the decreased expression of PTEN, a known tumor suppressor in cancers [357,358]. The miR-21 has also been reported to show anti-apoptotic, proliferative, invasive and angiogenic properties in cancer cells [356,359,360].

Increased expression of miR-21 has been reported in cancer cells such as breast cells under hypoxic conditions [351,352]. Over-expression of miR-21 in DU145 cells increases the expression of HIF-1 and VEGF and increases tumor angiogenesis. Over-expression of miR-21 target gene, PTEN, inhibits tumor angiogenesis through the down-regulation of HIF-1 and VEGF in cancer cells [361]. Thus, HIF-1 is a key downstream target of miR-21 in the regulation of tumor angiogenesis. It has also been shown that over-expression of miR-21 could promote the survival in bone marrow mesenchymal stem cells under hypoxic condition. Down-regulation of miR-21 increase apoptosis of mesenchymal stem cells [362]. Our unpublished data shows that hypoxia leads to increased expression of miR-21 in pancreatic and prostate cancer cells, consistent with the expression of VEGF, and self-renewal capacity of CSC-like cells. The knock-down of miR-21 expression by its siRNA inhibitor decreased the expression of VEGF and self-renewal capacity in pancreatic and prostate cancer sphere-forming cells under hypoxic condition, which suggests that hypoxia-induced miR-21 expression might play a critical role within the tumor microenvironment, contributing to tumor aggressiveness.

13.1.2. The role of miR-107—miR-107 has been found to be up-regulated in breast cancer and colon cancer cells under hypoxic condition [351]. A recent study from human colon cancer specimens revealed that miR-107 is a p53 target molecule and p53 directly increases the expression of miR-107 via its 5'-UTR [363]. The over-expression of miR-107 decreases the transactivation of an endogenous HIF-1 3'-UTR-driven luciferase reporter gene and HIF-1 protein level. Mutation of the miR-107 target sites attenuates this regulation. Moreover, the over-expression of miR-107 decreases the induction of VEGF expression by chemical-induced hypoxic conditions [363]. These data suggest that miR-107 might inhibit VEGF mediated angiogenesis through the down-regulation of HIF-1 mediated signaling pathway. However, further investigation is required to elucidate the role of miR-107 in tumor aggressiveness and its role within the tumor hypoxic microenvironment.

13.1.3. The contribution of miR-181b—The expression of miR-181b has been reported to be associated with clinical prognosis [364-366]. Altered expression of miR-181b has been found in various malignancies. However, the results are not consistent [364-369]. Several experimental studies have shown that miR-181b increases tumor growth, invasion, metastasis, and multidrug resistance by targeting Bcl-2 and MMP signaling in human cancer cell lines [370-372]. One report indicates that miR-181b function as a tumor suppressor in glioma cells [373]. These data suggest that oncogenic or tumor suppressive activities of miR-181b may depend on the cellular context.

It has been documented that hypoxia induces the expression of miR-181b in various cells including cancer cells [365,374]. The conditional inhibition of miR-181b suppresses proliferation of retinoblastoma cells [375]. The increased expression of miR-181b has been observed in malignant patients with progressive stage, not with stable stage [365]. However, the exact function of hypoxia-mediated miR-181b in tumor microenvironment is still not clear.

13.1.4. The role of miR-210—miR-210 has been identified as hypoxia-induced miRNA in large number of experimental studies. It has been documented that hypoxia induces the increased expression of miR-210 in all the cells tested including cancer cells [351,374,376-383]. Clinically, the increased expression of miR-210 has been associated with poor prognosis in breast cancer and other cancers. The high serum level of miR-210 has also been found in lymphoma patients [384,385]. It has also been shown that the hypoxia-induced miR-210 increases the expression of VEGF and CAIX in pancreatic cancer cells by a HIF-1 -dependent mechanism [378,383], suggesting a regulatory role in tumor angiogenesis [351,353,354,386-388]. Hypoxia-induced miR-210 has also been shown to modulate DNA repair pathway. Forced over-expression of miR-210 was found to suppress the levels of radiation sensitive 52 (RAD52), a key factor in homology-dependent repair (HDR), leading to impaired DNA repair and genetic instability [389]. These data suggest that hypoxia-induced miR-210 plays an important role in the tumor microenvironment, suggesting that targeting miR-210 would provide a novel therapeutic strategy for the treatment of human malignancies.

13.1.5. Contribution of miR-373—miR-373, a hypoxia-mediated miRNA, has been shown to be a mediator of DNA repair systems in various cells including cancer cells *via* a HRE binding site in the miR-373 promoter [389,390]. It has been shown that miR-373 is up-regulated in a HIF-1 -dependent manner in hypoxic cells [374,389,390]. Moreover, forced over-expression of miR-373 resulted in decreased levels of the nucleotide excision repair proteins, RAD23B and RAD52, both of which are found to be down-regulated in hypoxia, which provides new mechanistic insight into the effect of hypoxia-mediated miRNAs on DNA repair and genetic instability in tumor.

13.2. Down-regulation of hypoxia-mediated miRNAs in tumor

13.2.1. The role of Let-7—A large number of studies have documented that let-7 family has a key regulatory role in the development and progression of tumor by targeting multiple signaling pathways. The expression of let-7 has been shown to be negatively associated with clinical outcomes. Several let-7 family members, for example, let-7b and c have been identified as negative regulators of EMT and CSCs through the regulation of PTEN, CSC marker Lin28b in pancreatic and prostate cancer cells [391-395]. Recently, most of let-7 family members such as let- 7a, b, c, e, f, and g have been reported to be responsive to hypoxic condition in human cancer cells [387]. The results show that hypoxia can lead to the down-regulation of let-7a, b, c, d, e, f, and g in human nasopharyngeal carcinoma cells. One of the hypoxia-mediated let-7 member, let-7b has been validated to be a putative VEGF target miRNA [387], suggesting that let-7 may act a regulatory role in the tumor microenvironment by targeting angiogenesis; however, the exact molecular mechanism of hypoxia-mediated let-7 expression and loss of expression within the tumor hypoxic microenvironment is not clear.

13.2.2. The significance of miR-20—miR-20a and b have been found as hypoxia-mediated miRNAs. Hypoxia results in decreased expression of miR-20a and b in human nasopharyngeal carcinoma cells. The miR-20a and b have been considered as a putative VEGF targeting miRNA [387]. A recent *in vitro* study has shown that by using pre-miR-20b

and anti-miR-20b transfection, miR-20b can inhibit the expression of VEGF in HIF-dependent mechanism under chemically-induced hypoxic condition in breast cancer cells [396], which suggest that miR-20 may be a negative regulator of VEGF activity.

13.2.3. The role of miR-22—Emerging evidence suggests that miR-22 plays an important role in tumorigenesis in a cell type specific manner. It has been shown that miR-22 is up-regulated in human senescent fibroblasts and epithelial cells, but down-regulated in various cancer cell lines such as colon cancer, liver cancer, ovarian cancer, and ER⁺ breast cancer cells [397-402]. The down-regulation of miR-22 is reported to be associated with poor prognosis of liver cancer [400]. A number of experimental studies have demonstrated that re-expression of miR-22 decreases cell growth, invasion, and metastasis in various cancer cells by targeting PTEN, p21, and p53 [397-400]. These findings suggest that miR-22 may act as a tumor suppressor. It has also been shown that hypoxia causes decreased expression of miR-22 in colon cancer cells. Re-expression of miR-22 results in the inhibition of HIF-1 expression, consistent with repressing the expression of VEGF under hypoxic condition. Knock-down of endogenous miR-22 enhances hypoxia-induced expression of HIF-1 and VEGF in cancer cells, suggesting that hypoxia-mediated miR-22 expression may have an anti-angiogenic effect through the down-regulation of HIF-1 and VEGF expression in the development and progression of tumor [403].

13.2.4. The significance of miR-101—miR-101 has been reported to act as anti-oncogenic molecule. A number of experimental studies have demonstrated that miR-101 plays a protective role in tumor aggressiveness through inhibition of the phenotype and function of CSCs mediated by inactivation of enhancer of zeste homolog 2 (EZH2), an epigenetic regulator of cell survival, proliferation, and CSC function, in various cancer cells [358,404]. The knock-down of EZH2 by its siRNA suppresses cell proliferation, invasion, and the self-renewal capacity of CSCs in human pancreatic cancer MiaPaCa-2 cells, consistent with the inhibition of gene expression of CSCs. Re-expression of miR-101 results in the inhibition of EZH2 expression consistent with the suppression of the self-renewal capacity of CSCs in MiaPaCa-2 cells and its CSC-like sphere cells [358]. It has been reported that hypoxia decreases the expression of miR-101 by HIF-dependent mechanism in prostate cancer cells [405]. Moreover, increased expression of HIF-1 / by chemically-induced hypoxic condition leads to the down-regulation of miR-101 expression whereas re-expression of miR-101 decreases cancer cell invasion and migration, consistent with decreased expression of EZH2 [405], suggesting hypoxia-mediated expression of miR-101 may have a key role within the tumor microenvironment *via* inhibition of CSC function. However, the precise role and the crosstalk between miR-101, HIF and CSC function in tumor aggressiveness require for further in-depth investigation.

The expression of other hypoxia-mediated miRNA, such as 23a,b and miR-26a,b have been reported in several studies [374,387] although the results are not consistent. Altered expression of these miRNAs has also been observed in certain malignancy [406-410]; however, the exact functions of these hypoxia-mediated miRNAs in the development and progression of tumor is required to be defined in the future. It is important to note that targeted inactivation or reexpression of lost miRNAs in the tumor could be a novel therapeutic strategy. Although up-regulation or down-regulation of miRNAs is experimentally possible through gene transfer technology, their application in humans is very limited, suggesting that alternate approach must be devised to regulate miRNAs as discussed in the following paragraphs.

14. Natural product-based HIF inhibitors

In the following paragraphs, we are discussing the role of several natural agents collectively called nutraceutical that may be important regulator of HIF and targeted miRNAs.

14.1. Soy isoflavone

Isoflavones are found primarily in the members of the Leguminosae family. Foods such as soy, lentil, bean, and chickpea are the most common sources of isoflavones; however, soybean is the food that contains abundant amounts of isoflavones. Genistein, daidzein, and glycitein are three major components of isoflavones found in soybeans and soy protein-rich products. Several epidemiological and clinical studies have shown that isoflavone-rich soy products could have protective effects against various cancers such as prostate cancer [411-414].

A large number of experimental studies from our laboratory and others have shown that isoflavones, particularly genistein and daidzein, elicit anti-tumor effects by targeting NF- κ B, Wnt, Notch-1, and Akt pathways in many cancers [207,415-420]. Due to its anti-tumor properties, isoflavones have been proposed as potential HIF-1 inhibitor. Our recent studies have shown that soy isoflavone causes sensitization of radiation-induced cell death in prostate cancer cells associated with decreased levels of apurinic/apyrimidinic endonuclease (APE)/redox factor 1 (Ref1), a redox activator of transcription factors, including NF- κ B and HIF-1. These observations suggest that soy isoflavone may show an antitumor effect by targeting HIF-1 pathway. Furthermore, we have demonstrated that pre-treatment with soy isoflavone genistein or daidzein inhibits sarcoma (Src)/signal transducer and activator of transcription 3 (STAT3)/HIF-1 activity induced by radiation and decreased nuclear translocation of HIF-1 [421,422]. These findings suggest that soy isoflavones may have inhibitory effects on HIF-signaling pathway in tumor microenvironment, which could also be due to deregulation of miRNAs. Our recent studies have demonstrated that soy isoflavone treatment increases the expression of let-7b, c, d, and e, and decreases the expression of miR-21 consistent with its anti-tumor activity in human pancreatic cancer cells [394,423,424]. These data suggest that soy isoflavone has an anti-tumor activity, which is in part by targeting HIF pathway and hypoxia-mediated miRNAs in the development and progression of tumor.

14.2. Curcumin

Curcumin (diferuloylmethane) is a bioactive compound, which is derived from the plant *Curcuma longa* (Linn) grown in tropical Southeast Asia [425-427]. Curcumin has been received considerable attention due to its pronounced anti-inflammatory, anti-oxidative, immunomodulating, anti-atherogenic, and anti-carcinogenic activities. Curcumin has been shown to inhibit the growth of a variety of tumor cells. A large number of experimental and clinical studies have shown that curcumin can induce cell apoptosis, inhibit cell proliferation, cell invasion, and tumor growth, consistent with its regulation of multiple cellular signaling pathways such as NF- κ B, Notch, Wnt, and Hh [416,417,420,428-430]. The inhibitory role of curcumin in the regulation of HIF pathway has also been exploited in a number of experimental studies. It has been shown that curcumin has the binding affinity to HIF-1 with the low energy [431], and to decrease the protein levels of HIF-1, HIF-2, and HIF-1, and VEGF in various cancer cells such hepatoma and breast carcinoma cells under hypoxic conditions, consistent with the inhibition of angiogenesis *in vitro* [432,433]. The xenograft animal study also reveals that curcumin treatment suppresses human liver tumor growth by inhibition of HIF signaling pathway [434].

Recently, we have developed a novel synthetic analog of curcumin, 3,4-difluoro-benzo-curcumin, referred to as difluorinated-curcumin (CDF) [429,435], which shows greater bioavailability in multiple tissues, and also inhibits cell growth, DNA-binding activity of NF- κ B, Akt, cyclooxygenase 2 (COX2), and the production of PGE₂ and VEGF [357,358]. Moreover, CDF suppresses the tumor growth of human pancreatic cancer in xenograft mouse models, consistent with the inactivation of NF- κ B, EZH2, COX2, VEGF, and CSC function [358]. Our unpublished data shows that CDF decreases miR-21 expression, the production of VEGF, and CSC self-renewal capacity, wound healing capacity in pancreatic and prostate cancer cells under hypoxic conditions, consistent with the inhibition of the gene expression of VEGF and HIF-1 α , suggesting that CDF could function as a potential inhibitor of HIF signaling pathway in cancer cells. Several recent studies have shown that curcumin or its analog increases the expression of let-7b, c, d, e, and i and miR-22, and decreases the expression of miR-21, consistent with its anti-tumor activity in various cancer cells [357,436-438], suggesting a significant role of curcumin and its analog in anti-tumor activity, which appears to be in part by targeting HIF and hypoxia-mediated miRNAs.

14.3. Tea polyphenols

Consumption of green tea has been associated with human health including the prevention of cancers and heart disease. Several epidemiological studies have shown lower incidence of certain cancers such as prostate cancer among Asian men with a high dietary intake of green tea, which suggests that green tea could be a chemopreventive agent against cancers [439]. One report from Japan's Public Health Center-based Prospective Study revealed that the high consumption of green tea is associated with decreased risk of advanced prostate cancer [440]. Green tea and its constituents have been investigated both *in vitro* and *in vivo*. Green tea contains several catechins including epicatechin (EC), epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCG). However, EGCG has been shown to be the most potent for the inhibition of tumorigenesis and the reduction of oxidative stress among these catechins [441]. A number of experimental studies have demonstrated that EGCG exerts an anti-tumor activity *in vitro* and *in vivo*, potentially associated with its inhibition of NF- κ B, Wnt, and Hh pathways in various cancers, such as lung, colon, prostate, colon, and breast cancers [185,442-448]. The regulatory effect of EGCG on HIF-1 α pathway has been reported in several *in vitro* and *in vivo* studies although the results are not consistent. Some experimental studies showed the inhibitory effect of EGCG on HIF-1 α and VEGF activities in various cancer cells [449-452], consistent with increased expression of let-7 in hepatocellular carcinoma cells [453]. However, other reports have shown some opposite effect of EGCG on HIF through the deregulation of miR-210 and PHD [454-457]. Due to the differences of doses, treatment duration, and cell types, these inconsistent findings require further in-depth investigation to elucidate the role of EGCG in the regulation of HIF pathway and hypoxia-mediated miRNAs in the tumor microenvironment.

14.4. Resveratrol

Resveratrol (trans-3,5,4'-trihydroxystilbene) is a polyphenol compound found in the skin of red grapes, berries and peanuts [458]. Increased evidence from experimental studies has shown that resveratrol could suppress many types of cancers by regulation of cell proliferation and apoptosis mediated through multiple cellular signaling pathways such as Akt, Wnt, Hh, and NF- κ B [458-464]. The inhibitory role of resveratrol in the regulation of HIF-pathway has been exploited as documented by increased number of experimental studies. It has been shown that resveratrol exerts a protective effect against the hypoxia-induced lesion in various normal cells by inhibition of HIF-1 α activity [465-467]. It has also been shown that resveratrol inhibits the activation of HIF-1 α and the expression of VEGF and MMP9, leading to the suppression of hypoxia-mediated cell invasion and migration in various cancer cells [468-470]. These findings suggest that resveratrol may act as an inhibitor

of HIF signal pathway within the tumor microenvironment. There are some limited reports showing that resveratrol treatment could decrease miR-21 expression and increase the expression of miR-22 in lung and colon cancer cells by microarray assay [471,472]. More studies are required to elucidate the significance of resveratrol in the regulation of HIF pathway and hypoxia-mediated miRNAs in tumorigenesis.

14.5. Indole-3-carbinol and 3,3'-diindolylmethane

3,3'-Diindolylmethane (DIM) is one of the dimeric product of indole-3-carbinol (I3C) which is produced from naturally occurring glucosinolates contained in a wide variety of plants including members of the family Cruciferae such as broccoli. Under the acidic conditions of the stomach, I3C undergoes extensive and rapid self-condensation reactions to form several derivatives and DIM is one of the major derivatives. Epidemiological studies have indicated that human exposure to indoles through consumption of cruciferous vegetable could decrease cancer risk [473]. DIM has been shown to reduce oxidative stress and stimulate the expression of anti-oxidant response element-driven gene, suggesting the anti-oxidant function of indole compounds [474,475]. Several experimental studies have shown that DIM inhibits tumorigenesis and cancer cell growth, and induces apoptosis in cancer cells *in vitro* and *in vivo*. These findings suggest that DIM could serve as a potent agent for the prevention of tumor progression and/or treatment of cancers, potentially associated with its inhibition of multiple cellular signaling pathways such as NF- κ B, Akt, and Wnt as documented by recent studies from our laboratory and others [473-478].

Moreover, one *in vitro* study has demonstrated that DIM reduces the level of HIF-1 α , its transcriptional activity, as well as the gene expression of key hypoxia responsive factors, VEGF, furin, enolase-1, GLUT1, and phosphofructokinase. Its inhibitory effect on HIF activity is consistent with increased rate of the prolyl-hydroxylase- and proteasome-mediated degradation of HIF-1 α , thereby leading to decreased rate of HIF-1 α transcription [479]. Furthermore, we have recently demonstrated that nutritional grade BR-DIM (absorption-enhanced form) inhibited the radiation-induced activation of NF- κ B and HIF-1 DNA activities in prostate cancer cells, thereby leading to enhanced radiation-induced cell killing and tumor growth inhibition [480]. These data clearly suggest that DIM may act as an inhibitory mediator of HIF signaling pathway. Our recent experimental data showed that DIM treatment increases the expression of let-7b, c, d, and e and decreases the expression of miR-21 and miR-22b in human pancreatic cancer cells [394]. Another report showed increased expression of miR-21 by DIM [481]. Therefore, the regulatory role of DIM in tumorigenesis is in part mediated *via* targeting HIF pathway and hypoxia-mediated miRNAs; however, the role of DIM still requires further investigation.

14.6. Lycopene

Lycopene is one of the major deep-red pigments responsible for the color of tomatoes and its products. Tomatoes and its products including ketchup, tomato juice, and pizza sauce, are the richest sources of lycopene in the daily diets of people in the USA. Lycopene is a potent anti-oxidant agent. It has been known that lycopene is a biologically active carotenoid displaying high physical quenching rate constant with singlet oxygen, which suggests its high activity as an anti-oxidant agent. One early epidemiological study has demonstrated that frequent consumption of tomato products is associated with a decreased risk of prostate cancer [482]. The inverse associations between plasma lycopene and prostate cancer have also been reported in several human studies [483,484]. Experimental studies also showed that lycopene inhibited cell growth in various cancers through the regulation of cell-cycle-related genes. One *in vivo* animal study revealed that lycopene has anti-tumor effects that could be potentiated by vitamin E, an anti-oxidant that is also present in tomatoes [485], confirming the anti-tumor activity of lycopene. Our previous phase II clinical trial has

shown that lycopene supplements reduce tumor size and prostate-specific antigen (PSA) level in localized prostate cancer [486,487], suggesting its promising effects on prostate cancer prevention and/or treatment. Several experimental studies have shown that its anti-tumor effects are clearly associated with its inhibition of IGF/Akt, Wnt, Wnt/ β -catenin, NF- κ B, and androgen receptor (AR) signaling [482,484,486-488].

The regulatory effect of lycopene on HIF activity has been exploited recently. One docking analysis study revealed that lycopene has its binding affinity to HIF-1 α protein with low energy, suggesting its potential inhibitory activity of HIF-1 α [431]. However, in one animal model for cerebral ischemia-reperfusion injury established by middle cerebral artery occlusion (MCAO), the rats pre-treated with the high dose of lycopene (20 mg/kg) for 15 days showed increased expression of HIF-1 α mRNA in the cerebral cortex after 24 h of cerebral ischemia-reperfusion injury [489]. To our knowledge, there is no report linking anti-tumor activity of lycopene and hypoxia-mediated miRNAs in the development and progression of tumor. The detailed mechanistic role of lycopene on HIF activity and hypoxia-mediated miRNAs requires further in-depth investigation.

14.7. Vitamin D

Vitamin D is one of the essential nutrients for health in humans. There are two major forms of vitamin D, namely vitamins D₂ and D₃ in the diets. The active form of vitamin D in the body is 1,25-dihydroxyvitamin D (1,25-(OH)₂D), which can be made from either vitamin D₂ or vitamin D₃. Several epidemiologic and experimental studies have suggested that higher intakes of vitamin D from food and/or supplements lead to higher blood levels of vitamin D that are associated with reduced risks of cancer [490,491]. It has been reported that reduced levels of active vitamin D in the body resulted in a higher incidence and mortality of prostate cancer [490,491]. These findings suggest that vitamin D could be useful as a preventive agent for cancers. A large number of *in vitro* and *in vivo* studies have shown that vitamin D inhibits cell growth, invasion, and angiogenesis in tumors through the regulation of multiple signaling pathways, including Wnt, VEGF, and NF- κ B [492,493]. Moreover, one experimental study showed that 1,25-(OH)₂D₃ (0.1–1 μ M) inhibits cell proliferation and protein expression of HIF-1 α and VEGF in various human cancer cells under hypoxic conditions. 1,25-(OH)₂D₃ also inhibited HIF-1 transcriptional activity as well as HIF-1 target genes, VEGF, ET-1, and GLUT1. These inhibitory effects of vitamin D on cancer cells are known to be a HIF-1 α -dependent mechanism [492]. However, another *in vitro* study shows that 1,25-(OH)₂D (10 nM) increased both mRNA and protein levels of HIF-1 α in H-ras-transfected MCF10A breast epithelial cells and its parental cells under normoxic conditions [494]. Moreover, two recent experimental studies have shown that vitamin D treatment up-regulates the expression of miR-21 and miR-22 and down-regulates the expression of miR-20a and b, and miR-181b in malignant cells [495,496]. However, the precise role of vitamin D on HIF-signaling and hypoxia-mediated miRNAs is not yet clear. Further investigation is required to clarify the role of vitamin D in the regulation of HIF signaling pathway and hypoxia-mediated miRNAs within the tumor microenvironment.

14.8. Chaetocin

Chaetocin, a class of thiodioxopiperazine, is the naturally occurring agent produced by *Chaetomium* species fungi and has a wide range of biologic activities, including antimicrobial, anti-inflammatory and anticancer effects [497]. It has been shown that chaetocin has HIF inhibitor activity in cancer cells through the inhibition of the interaction between HIF-1 α and p300, leading to the down-regulation of VEGF expression [498], consistent with recent findings which indicate that chaetocin inhibits the expression of HIF-1 α and VEGF in various hepatoma cells, and suppress the growth of HIF-1 α ^{+/+} fibrosarcoma in a xenograft mouse model [499]. These findings suggest that chaetocin may

act as a potential HIF inhibitor. To our knowledge, there is no report linking chaetocin, HIF pathway, and hypoxia-mediated miRNAs in the tumor microenvironment.

14.9. Manassantins

Manassantins and dineolignans are classes of lignoid-containing naturally occurring compounds which are isolated from the roots of the plant *Saururus chinensis* and have been known to have various biological activities, such as neuroleptic, anti-inflammatory and acyl-CoA:cholesterol acyl-transferase (ACAT) inhibitory activities and these have been used as supplement [500-502]. The anti-inflammatory activity of manassantins is mediated by its inhibition of NF- κ B transactivation activity [503]. Manassantin A and B have been considered as potent HIF inhibitors because manassantin A and B exert their inhibitory activity against the hypoxia-induced HIF-1 and VEGF expression at the concentration range of 1 nM to 1 μ M, and a complete inhibition was observed at 100 nM in T47D human breast tumor cells [504,505]. However, the precise molecular mechanism has not been fully elucidated, suggesting further in-depth investigation especially in models with tumor hypoxic microenvironment.

15. Zinc

Zinc is an essential trace mineral element required for human health, and it is known to participate in the activation of approximately 300 enzymes, and it is also involved in the regulation of over 2000 zinc-dependent transcription factors, which are involved in DNA and protein synthesis, cell division, and other metabolisms. Zinc deficiency can cause growth retardation, delayed sexual maturation, depressed immune response, and cause abnormal cognitive functions. Zinc has been considered as an anti-inflammatory and antioxidant agent [506,507]. A large body of epidemiological and clinical studies have demonstrated that zinc deficiency is associated with increased risk of certain cancers, such as prostate, esophageal and oral cancers [508-512]. The data from *in vitro* and *in vivo* studies clearly suggest that zinc may have anti-tumor activity mediated through multiple cellular signaling mechanisms including NF- κ B [513-516]. One recent study has shown that zinc inhibits hypoxia-induced increase in HIF-1 DNA-binding activity and thereby regulates HIF-1-dependent mRNA expression of EPO, and decreases the stability of HIF-1 in the hypoxic astrocyte cells, consistent with reduction in the nuclear translocation of hypoxia-induced assembly of HIF-1/HIF-1 complex. These data suggest that zinc may have an important role in the regulation of HIF signaling pathway within the tumor microenvironment [517]; however further pre-clinical and clinical investigation is warranted.

16. Conclusions and perspectives

We attempted to summarize the “state-of-our-knowledge” on the role of hypoxia and HIF signaling pathway in tumor aggressiveness as succinctly as possible and during such attempt we could not cite all the published results, and thus we sincerely apologize to those authors whose work could not be cited. In summary, the evidence has clearly provided a strong support showing that HIF signaling pathway plays an important role in the induction and maintaining of CSC and EMT phenotypes and regulates their functions mediated through the regulation of multiple complex signaling molecules within the tumor microenvironment. Moreover, emerging evidence also suggests that HIF signaling pathway plays an important role in the regulation of NF- κ B, PI3K/Akt/mTOR, Notch, Wnt/ β -catenin, and Hh signaling not only during normal but also plays important roles during the development and progression of tumors. Furthermore, a number of hypoxia-mediated miRNAs have been shown to mediate cancer cell proliferation, invasion, migration, angiogenesis, and the self-renewal capacity of CSCs through HIF-dependent mechanisms. Therefore, the complexity

of the kinship of hypoxia and the regulation of HIF signaling pathway, the biological functions of CSCs, EMT, and their regulation through deregulated expression of miRNAs are important biological processes (Fig. 3), which must be investigated both in pre-clinical and clinical settings. Understanding of precise molecular event regulated by hypoxia would allow in designing targeted approach for the development of newer therapeutics, which would be useful for improving the overall survival of patients diagnosed with malignancies especially solid tumors. To that end, it is our perspective that nutraceutical could serve as promising novel agents to fulfill that requirement, which of course need further preclinical and clinical investigations.

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List of abbreviations

ABCG2	ATP-binding cassette transporter G2
ACAT	acyl-CoA cholesterol acyl-transferase
Akt	protein kinase B
Ang-2	angiopoietin 2
APE	apurinic/apurimidinic endonuclease
AR	androgen receptor
Bcl2	B-cell lymphoma 2
bHLH	basic helix–loop–helix
bHLH-PAS	Per-Arnt/AhR-Sim basic helix–loop–helix
BMP	bone morphogenetic protein
BNIP3	Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3
BR-DIM	absorption-enhanced form of DIM
CAD	COOH-terminal transactivation domain
CAIX	carbonic anhydrase 9

CBP	CREB binding protein
CD44	the cluster of differentiation 44
CDF	difluorinated-curcumin
CDKN1A	cyclin-dependent kinase inhibitor 1A
c-MET	mesenchymal–epithelial transition factor
Cos2	Costal2
COX2	cyclooxygenase 2
CSC/CSCs	cancer stem cell/cancer stem cells
CXCR4	C–X–C chemokine receptor type 4
Dhh	desert Hedgehog
DIM	3,3 -diindolylmethane
EC	epicatechin
EGCG	epigallocatechin-3-gallate
eIF4E	eukaryotic initiation factor 4E
EMT	epithelial-to-mesenchymal transition
EPO	erythropoietin
ET-1	endothelin 1
EZH2	enhancer of zeste homolog 2
FIH	the factor-inhibiting HIF
Flt-1	fms-like tyrosine kinase 1
Fu	fused
Gli	glioma-associated oncogene family zinc finger protein
GLUT	glucose transporters
GSI	-secretase inhibitors
HAF	hypoxia associated factor
Hey1	hairy/enhancer-of-split related with YRPW motif 1
HDCA3	histone deacetylase 3
HDR	homology-dependent repair
Hh	Hedgehog
HRE	hypoxia-response element
Hsp90	heat shock protein 90
I3C	indole-3-carbinol
IGF-1	insulin-like growth factor 1
Ihh	Indian Hedgehog
IL-6	interleukin 6
I B	inhibitory proteins of NF- B

IKK	I B kinase
iNOS	inducible nitric oxide synthase
IPAS	inhibitory PAS protein
LDH5	lactate dehydrogenase 5
LEF	lymphoid-enhancing factor
LEF	lymphoid-enhancing factor
Lin-28	a conserved regulator of cell fate succession in animals
LKB1	liver kinase B1
LOX	lysyl oxidase
LPS	liposaccharides
miRNAs	microRNAs
2ME	2-methoxyestradiol
MMP	matrix metalloproteinases
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
NF- B	nuclear factor of B
NICD	intracellular domain of the Notch
Notch1	Notch homolog 1
NSCLC	non-small cell lung cancer
Oct4	octamer-binding transcription factor 4
ODD	oxygen-dependent degradation domain
P70S6K1	p70-S6 kinase 1
PAS	per-aryl hydrocarbon receptor nuclear translocator (ARNT)-sim
PKD1	pyruvate dehydrogenase kinase 1
PHD	prolyl hydroxylase
PSA	prostate-specific antigen
PTEN	phosphatase and tensin homolog deleted on chromosome 10
RAD52	radiation sensitive 52, a DNA damage repair protein
Ref1	redox factor 1
ROS	reactive oxygen species
S6K1	ribosomal protein S6 kinase
SCID	severe combined immunodeficiency
Shh	sonic Hedgehog
SIP2	septin interacting protein 2
Smo	smoothened
Sox2	sex determining region Y box 2

STAT	signal transducer and activator of transcription
Sufu	suppressor of fu
TACE	TNF- α -converting enzyme
TGF-	transforming growth factor-
TAK1	TGF- β activated kinase 1
TCF	T cell factors
TIC	tumor initiating cells
Tie2	tyrosine kinase receptor with immunoglobulin and epidermal growth factor homology 2
TNF-	tumor necrosis factor
TSC	tuberous sclerosis
uPA	urokinase-type plasminogen activator
3'-UTR	3'-untranslated region
VEGF	vascular endothelial growth factor
VEGFR1	VEGF receptor 1
VHL	von Hippel–Lindau
XIAP	X-linked inhibitor of apoptosis protein
ZEB1	zinc-finger E-box binding homeobox 1
ZO-1	zonula occludens-1

Fig. 1. Hypoxia driven tumor angiogenesis. Hypoxic tumor microenvironment enhanced through desmoplastic stroma results in activation of HIF proteins that influences aberrant miRNA expression dependent angiogenic signaling resulting in tumor angiogenesis.

Fig. 2. Hypoxia as a driver of aggressive tumor phenotype. HIF (HIF-1 and HIF-2) activation drives two critical tumor promoting pathways (1) increase in CSC markers (Oct4, Nanog, Sox2 and Snail) and (2) EMT promoting markers (ZEB2, Snail, Twist, Wnt, Slug, Notch and TGF- β). These HIF driven pathways interact with one another leading to enrichment/enhancement in both CSC and EMT cell populations that in turn is a driver of aggressive tumor type.

Fig. 3.

Potential pathways linking hypoxia to CSCs and EMT in tumor aggressiveness. Hypoxic stress increases the stability and expression of the HIFs, especially HIF-1 and 2 in the cells under tumor microenvironment. The activation of HIF signaling pathways up-regulate (1) tumor angiogenesis *via* the regulation of VEGF, VEGFR1, and Ang-2; (2) the maintenance of CSC phenotype and function *via* the regulation of Oct4, Nanog, and Sox2; (3) the induction of EMT characteristics *via* regulation of ZEB2, Snail, Twist, Slug, TGF- β , Wnt, and Notch; and (4) the metabolic adaptation of hypoxic stress *via* regulation of Glut 1,2, CAIX, LDH-5, IGF-2, and BNIP3. These regulations result in tumor growth, invasion, metastasis, treatment resistance, and recurrence. Complex interlinking of HIF pathway to NF- κ B, Akt/mTOR, Notch, Wnt/ β -catenin, and Hedgehog signaling pathways increases the contribution of HIF signaling pathway to tumor aggressiveness by the regulation of angiogenesis, CSCs, EMT, and metabolic shifts. Hypoxia also induces the altered expression of miRNAs, for examples, the up-regulation of miR-21, miR-210, and miR-373; and the down-regulation of let-7, miR-20, miR-22, and miR-101 in the tumor cells. The deregulation of the hypoxia-associated miRNAs may enhance tumor angiogenesis, CSC and EMT characteristics through the modulation of multiple signaling pathways. Natural agents such as isoflavones, curcumin, DIM, lycopene, resveratrol, and EGCG have been demonstrated to modulation of these signaling pathways, potentially by the differential regulation of hypoxia-mediated miRNAs, leading to the inhibition/attenuation of tumor aggressiveness.