Chapter 5
The Functional Interplay Between Pro-oncogenic RUNX2 and Hypoxia-Inducible Factor-1α (HIF-1α) During Hypoxia-Mediated Tumor Progression

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Abstract Solid tumor tissues often have functional and phenotypical heterogeneities, arising at least in part from the local hypoxic tumor microenvironment (generally O_2 concentration is less than 2%). The elevated level of hypoxia is tightly associated with genetic instability, tumor progression, drug resistance, and/or poor clinical outcome after treatment, indicating that hypoxia exerts a strong selection pressure for the survival of cancer stem cells (CSCs) within tumors and also permits their maintenance. Thus, it has become urgent to precisely clarify the molecular basis of how hypoxia could contribute to the acquisition and/or maintenance of the aggressive phenotypes of this deadly disease. Meanwhile, cells keep genomic integrity to avoid genetic instability-mediated tumorigenesis through the proper stress response under normoxia. Upon hypoxia, hypoxia-inducible factor-1α (HIF-1α) which has an O_2-sensing ability accumulates and then facilitates tumor development through an induction of vascular endothelial growth factor (VEGF)-dependent angiogenesis. Therefore, the hypoxic HIF-1α/VEGF regulatory axis plays a vital role during the malignant tumor progression. Intriguingly, pro-oncogenic runt-related transcription factor 2 (RUNX2) has an ability to stimulate HIF-1α-mediated induction of VEGF. Recently, we have found for the first time that RUNX2 contributes to the acquisition of drug-resistant phenotype of malignant tumor cells. In this review, we focus on the functional interplay between HIF-1α/VEGF and RUNX2 within the hypoxic tumor microenvironment. Finally, we would like to discuss the potential therapeutic strategy targeting this tumor hypoxia.

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5.1 Introduction

Accumulating evidence strongly suggests that tumor cell microenvironments including hypoxia play a pivotal role in the acquisition and/or maintenance of malignant phenotypes of advanced tumors. Although tumor cells with a higher proliferation rate consume a large amount of oxygen and nutrients, they are exposed to a serious hypoxic condition. To survive under these severe conditions, hypoxic response takes place within tumors [1, 2].

One of the initial molecular events in response to hypoxia is the stabilization and activation of hypoxia-inducible factor-1α (HIF-1α). HIF-1α, which recognizes and binds to HRE (hypoxia-responsive element) within its target gene promoter/enhancer, is a basic helix-loop-helix family of transcription factor. Under normoxia, the amount of HIF-1α is kept at an extremely low level within cell nucleus through von Hippel-Lindau (VHL) E3 ubiquitin ligase-mediated proteasomal degradation system [3]. Upon hypoxia, VHL is dissociated from HIF-1α, and thereby HIF-1α becomes stable without proteolytic degradation. Stabilized and activated HIF-1α then induces the expression of its numerous target gene products such as pro-angiogenic vascular endothelial growth factor (VEGF), erythropoietin, and enolase [4, 5].

As expected, VEGF plays a central role in hypoxic response to assist the survival of a certain population of tumor cells exposed to serious hypoxic conditions. VEGF promotes the formation of new blood vessels around hypoxic areas, which supply hypoxic tumor cells with the enough amount of oxygen as well as nutrients, and then a certain subset of tumor cells acquires much more malignant phenotypes including the enhanced drug resistance and the increased metastatic potential [6–9]. Indeed, it has been shown that the aberrant overexpression of *VEGF* is closely associated with poor clinical prognosis of the patients with a variety of aggressive tumors [10, 11]. With these in mind, HIF-1α/VEGF regulatory axis in response to hypoxia has been considered to be one of the promising molecular targets for cancer therapy [12].

The evolutionarily conserved small noncoding RNAs, termed microRNAs (miRNAs), regulate their target gene expression primarily through the posttranscription level in a sequence-specific manner [13]. Of interest, increasing evidence strongly indicates that miRNAs are implicated in the regulation of various cellular processes such as hypoxic response [14]. For example, it has been described that miR-622 directly targets *HIF-1*α and then significantly impedes HIF-1α-mediated tumor cell migration as well as invasion in response to hypoxia [15]. On the other hand, miR-630, a HIF-1α-induced miRNA, contributed to the promotion of tumor growth and metastasis [16]. Thus, it is likely that a certain set of miRNAs involved in hypoxic response might serve as critical prognostic indicators and also potential molecular targets for future cancer therapy.

Runt-related transcription factor 2 (RUNX2) is one of the RUNX family members composed of RUNX1, RUNX2, and RUNX3. It has been well established that RUNX2 is a master regulator of osteoblast differentiation and bone formation. *RUNX2*-deficient mice displayed a complete loss of bone formation [17, 18]. Consistent with these observations, RUNX2 transactivates a variety of its target genes implicated in osteogenesis such as *type I collagen*, *osteopontin*, and *osteocalcin* [19]. However, this previous point of view has been challenged by the findings showing that, in addition to osteogenesis, RUNX2 has a strong oncogenic potential. Firstly, it has been shown that aberrant overexpression of *RUNX2* is detectable in numerous tumor tissues [20–22]. Secondly, RUNX2 has an ability to transactivate several tumor cell invasion-related genes including *MMP-9* and *MMP-13* [23, 24]. Lastly, we have found that depletion of *RUNX2* significantly improves drug sensitivity of various tumor cells ([25, 26]).

In the present review article, we describe the basic background of hypoxic response of tumor cells and then discuss the potential therapeutic strategies which might overcome hypoxic response-mediated malignant progression of tumor cells based on our current observations.

5.2 Hypoxia-Inducible Factor-1 (HIF-1) and Hypoxic Response of Tumor Cells

To survive, the majority of solid tumor cells exhaust a lot of nutrients and oxygen provided from the surrounding normal vasculature, and thereby tumor tissues become hypoxic (insufficient oxygen levels). Hypoxic tumors appear to become much more aggressive (the reduced cell death, the enhanced drug resistance, the higher resistance to radiotherapy, and the increased metastatic potential) [27–30]. Consistent with these observations, tumor hypoxia is an independent poor prognostic factor for the survival of tumor patients irrespective of treatment modality [31, 32].

To increase their mass under this serious hypoxic condition, tumor cells require the additional nutrients and oxygen. The angiogenesis (the formation of new blood vessels) which plays a pivotal role in malignant tumor progression as well as metastasis is a multistage biological process tightly regulated by a balance between pro-angiogenic and anti-angiogenic signalings [33, 34]. The tumor angiogenesis, which is one of the hallmarks of the hypoxic tumors, is implicated in the accelerated proliferation rate of the vascular endothelial cells [8].

The vascular endothelial growth factor (VEGF) (also known as VEGF-A), which is the most important angiogenic secreted dimeric glycoprotein, promotes these angiogenic processes such as the vascular endothelial cell proliferation and the development of the tumor vessels under the hypoxic condition [35]. As described [36], VEGF family is composed of several structurally and functionally related proteins including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor. Among them, VEGF-A has been the most studied member of VEGF family. Since the secretion of VEGF from the hypoxic tumor cells has

been shown to trigger the advanced tumor development [6, 7, 9], VEGF-mediated formation of tumor vessels is one of the essential hypoxic responses of tumor cells. Therefore, preventing these processes might contribute at least in part to prohibit both aggressive tumor progression and metastasis [10, 11].

Transcriptionally active hypoxia-inducible factor-1 (HIF-1), a basic helix-loop-helix family of transcription factor, is a heterodimer made up of α- and β-subunits. Under normal conditions, HIF-1α is continuously produced and simultaneously degraded through ubiquitin-proteasome breakdown system driven by von Hippel-Lindau (VHL) E3 ubiquitin ligase, whereas HIF-1β is constitutively expressed and kept at constant level within cell nucleus [3]. The immediate molecular response to hypoxia is stabilization of HIF-1α. Upon hypoxia or loss of functional VHL, HIF-1α is stabilized, forms a heterodimeric complex with HIF-1β, and binds to hypoxia-responsive elements (HREs) within its target gene promoters to trigger a concerted transcriptional response [5]. Among HIF-1-target genes, *VEGF* is the extensively studied HIF-1-regulated gene [4]. HIF-1-mediated deregulated expression of VEGF leads to the development of hypoxic tumors through the promotion of the angiogenesis as mentioned above. As expected, the aberrant expression of VEGF has been shown to be associated with the poor prognosis of various types of human tumors [37, 38] (Fig. 5.1). Therefore, prohibition of VEGF-mediated pro-angiogenic pathway improved the efficacy of various anticancer drugs on breast, cervix, stomach, lung, colon, rectum, and ovary carcinomas [39–41].

5.3 Maintenance and Propagation of Cancer Stem Cells (CSCs) Under Hypoxia

It has been well known that phenotypic and functional heterogeneity is a common property of a variety of solid tumors, arising at least in part from tumor cell microenvironments. According to cancer stem cell (CSC) theory, which might recapitulate the primary tumors, only a small subset of tumor cell population has a strong pro-oncogenic potential. A subset of tumor cells which expresses cell surface normal stem cell markers such as CD24 and CD44 has been shown to be much more tumorigenic [42]. In support of these findings, it has been described that malignant glioma and colon cancer include CSCs [43, 44]. Growing evidence indicates that CSCs cause the acquisition of malignant phenotypes of advanced tumors including drug resistance and recurrence after therapy [45].

Since hypoxia is one of the critical properties of tumor cell microenvironments, it is possible that hypoxia-induced HIF-1α might contribute to the maintenance and/or survival of CSCs. Bos et al. demonstrated that the expression level of HIF-1α protein in breast cancer tissues is larger in poorly differentiated than well-differentiated lesions, indicating that HIF-1α plays a role in the maintenance of the undifferentiated state of aggressive tumors [46]. Soeda et al. found that hypoxia promotes cell survival of undifferentiated CD133-positive glioma-initiating CSCs through the activation of HIF-1α, whereas CSC differentiation is markedly

prohibited under hypoxia [47]. CD133 has been considered to be one of the molecular markers of CSC population [48]. Based on their results, depletion of HIF-1α led to a massive reduction in the sphere-forming ability of glioma CSCs. Collectively, CSCs are obviously dependent on HIF-1α for their survival, self-renewal, and tumor growth.

Moreover, Heddleston et al. found that hypoxia increases number of CSC population and enhances the stem-like phenotype of the established tumor cell lines [49]. Similarly, Dong et al. described that hypoxia potentiates CSC sphere formation and causes an increase in number of CD44-positive colon cancer CSC subpopulations [50]. From their observations, the expression level of inhibitor of DNA-binding protein 2 (Id2) in colon cancer CSCs was significantly elevated in response to hypoxia through the activation of pro-oncogenic Wnt/β-catenin signaling pathway. Consistently, silencing of *Id2* markedly attenuated hypoxia-mediated CSC sphere

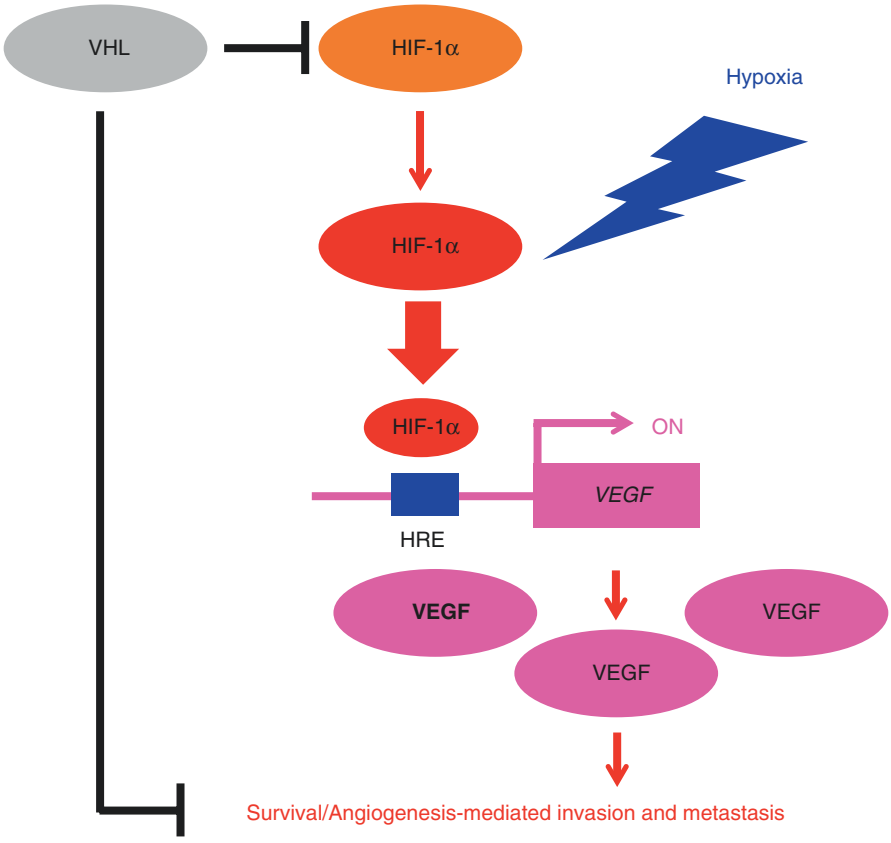

Fig. 5.1 HIF-1α is a critical determinant for hypoxic response. Upon hypoxia, HIF-1α becomes stabilized and transactivates its target genes including *VEGF*. VEGF-mediated angiogenesis contributes to the acquisition and/or maintenance of malignant phenotypes of aggressive tumors

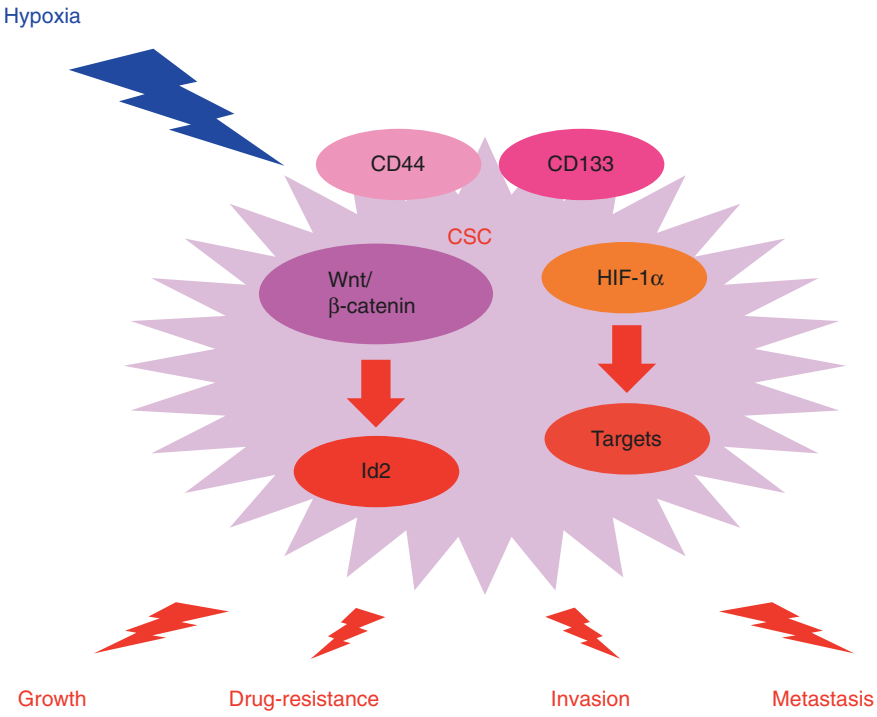

Fig. 5.2 Molecular basis of hypoxic response of cancer stem cells (CSCs). When CD44-/CD133-positive cancer stem cells (CSCs) within tumors are exposed to hypoxia, in addition to HIF-1α, Wnt/β-catenin pro-oncogenic pathway becomes activated and promotes Id2-mediated tumorigenesis

formation and also tumor metastasis in vivo. Intriguingly, highly aggressive pancreatic and colon cancer cells expressed a large amount of Id2 [51, 52].

Together, in addition to HIF-1α, Wnt/β-catenin-dependent augmentation of Id2 might be involved in the maintenance and/or survival of CSCs in response to hypoxia (Fig. 5.2).

5.4 Regulatory Role of MicroRNAs in Response to Hypoxia

MicroRNAs (miRNAs) are evolutionarily conserved small noncoding regulatory RNAs of around 22 nucleotides in length, which recognize and bind to the 3′-untranslated regions (3′-UTR) of their target mRNAs, repressing their translation [13]. Bioinformatic analysis indicates that more than 30% of protein-coding genes might be regulated by miRNAs [53]. A growing body of evidence strongly suggests that numerous siRNAs act as tumor suppressors or oncogene products. For example, Cheng et al. demonstrated that miR-622 has an ability to downregulate HIF-1α

and then prohibits metastatic spread of lung cancer in mouse xenograft model [15]. Xue et al. found that hypoxia-mediated repression of miR-15-16, which targets angiogenic *FGF2*, promotes tumor angiogenesis and metastasis [54].

In contrast, Rupaimoole et al. found that miR-630 reduces the expression level of Dicer, which is involved in miRNA biogenesis, and stimulates tumor growth as well as metastasis [16]. Ge et al. described that miR-421 targets *E-cadherin* as well as *caspase-3* and then stimulates metastasis [55]. According to their results, the expression level of miR-421 was significantly higher in advanced gastric cancer tissues than localized ones. Similarly, Devlin et al. revealed that miR-210 is upregulated in response to hypoxia and contributes to tumor metastasis [56]. In addition, miR-382 has been shown to be angiogenic miRNA targeting tumor suppressor PTEN (phosphatase and tensin homolog) [57]. Since the expression of miR-630, miR-421, miR-210, and miR-382 has been shown to be regulated by HIF-1α, it is worth noting that a certain subset of miRNAs responsible for hypoxia-induced tumor invasion/metastasis participates in HIF-1α-mediated oncogenic pathway. In this connection, several miRNAs implicated in hypoxic response might provide potential therapeutic clues to overcome malignant phenotypes of advanced tumors.

5.5 Pro-oncogenic Property of Runt-Related Transcription Factor 2 (RUNX2)

Runt-related transcription factor 2 (RUNX2) is a nuclear sequence-specific transcription factor responsible for the induction of bone formation and osteoblast differentiation. Indeed, *RUNX2*-deficient mice died just after birth and exhibited a complete loss of bone formation [17, 18]. In support of these observations, RUNX2 transactivates a number of osteogenic indicators such as type I collagen, osteopontin, and osteocalcin through RUNX2-responsive elements within their promoter regions [19].

Meanwhile, accumulating evidence demonstrated that *RUNX2* is highly expressed in a variety of tumor tissues as compared to their corresponding normal ones. For example, Pratap et al. described that *RUNX2* is aberrantly overexpressed in breast and prostate cancers [21]. Kayed et al. demonstrated that pancreatic ductal adenocarcinoma tissues highly express *RUNX2* as compared to the normal pancreas [20]. In addition, Wang et al. revealed that the expression level of *RUNX2* is higher in ovarian cancer tissues than normal ovarian ones [22]. Lastly, Boregowda et al. found that RUNX2 is highly expressed in melanoma tissues relative to melanocytes [58]. Accordingly, it has been shown that the expression level of *RUNX2* is employed as a prognostic indicator of non-small cell lung carcinoma patients [59]. These observations imply that, in addition to osteoblast differentiation and bone formation, RUNX2 has a strong pro-oncogenic potential in vivo.

Recently, we have found for the first time that RUNX2 is tightly linked to the drug-resistant phenotype of malignant tumor-derived cells such as osteosarcoma U2OS cells and pancreatic cancer AsPC-1 cells ([25], [26]). The drug resistance has been considered to be one of the hallmarks of advanced tumors [30]. From our observations, siRNA-mediated knockdown of *RUNX2* remarkably improved adriamycin (ADR) and gemcitabine (GEM) sensitivity of U2OS and AsPC-1 cells, respectively.

5.6 Functional Implication of RUNX2 in the Regulation of the Hypoxic Response

Since RUNX2 has a pro-oncogenic function, it is conceivable that RUNX2 might be closely implicated in the regulation of hypoxic response. Of interest, Pratap et al. described that RUNX2 transactivates tumor cell invasion-related *MMP-9* gene in bone metastatic tumor cells [24]. Similarly, Mendoza-Villanueva et al. found that RUNX2 directly regulates the expression of *MMP-13* and promotes the malignant invasion of breast cancer cells [23]. Forced expression of RUNX2 in prostate cancer cells stimulated their invasiveness [60]. In accordance with these observations, depletion of *RUNX2* resulted in a significant reduction of migration and invasion rate of colon cancer cells [61]. Furthermore, it has been described that pro-oncogenic PI3K/AKT pathway directly or indirectly augments the expression and the transcriptional activity of RUNX2, while RUNX2 also stimulates PI3K/AKT pathway, indicating that this positive feedback loop regulatory mechanism is one of the major driving forces during the malignant tumor cell progression [62]. Thus, it is possible that RUNX2 participates in the acquisition of the malignant phenotypes of the aggressive tumors such as invasion and metastasis.

Consistent with the above-mentioned findings, it has been also shown that RUNX2 has an ability to enhance *VEGF* transcription [63]. Of note, Lee et al. revealed that RUNX2 stabilizes HIF-1α through the inhibition of VHL-mediated ubiquitination of HIF-1α and stimulates the transcriptional activity of HIF-1α [64]. Kwon et al. reported that RUNX2 forms a complex with HIF-1α in cell nucleus and is then efficiently recruited onto *VEGF* promoter region [65]. Additionally, it has been described that hypoxia-mediated induction of RUNX2 drives the malignant progression of numerous tumors through the direct upregulation of anti-apoptotic Bcl-2 [66]. Together, it is likely that RUNX2 potentiates HIF-1α and thus promotes angiogenesis-mediated tumor cell invasion and/or metastasis in response to hypoxia (Fig. 5.3).

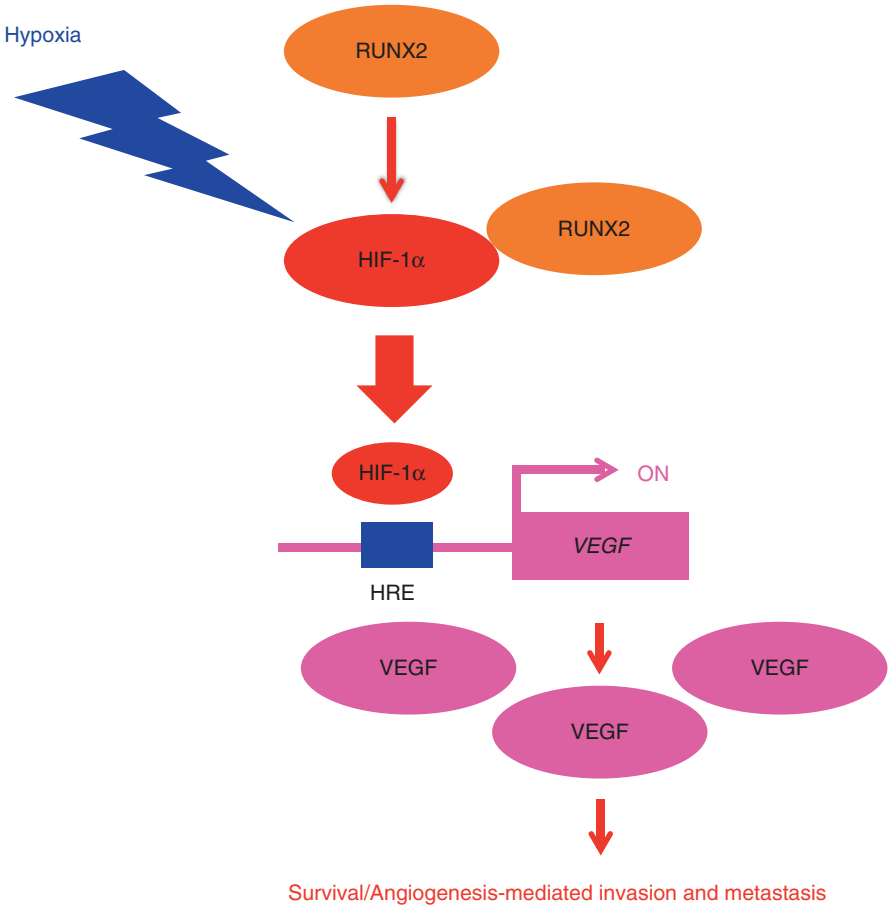

Fig. 5.3 RUNX2 augments HIF-1α-dependent hypoxic response. RUNX2 directly interacts with HIF-1α, enhances its sequence-specific transactivation ability, and thereby augments HIF-1α-dependent hypoxic response

5.7 Future Therapeutic Strategy Targeting Hypoxic Response

As described above, hypoxia-dependent tumor angiogenesis plays a critical role in the promotion of invasion and/or metastasis. These findings prompted us to develop anti-tumor drugs which block HIF-1α-/VEGF-induced angiogenesis. To our

knowledge, a number of VEGF-targeting drugs have been produced and approved for clinical treatment. For example, bevacizumab, a monoclonal antibody against VEGF-A, has been approved for the treatment of the patients with renal cancer [67]. In addition to VEGF, blocking VEGF receptor (VEGFR)-mediated signaling might be a promising approach to develop anti-tumor drugs. Recently, Li et al. described that a small chemical compound termed DW10075 selectively prohibits kinase activity of VEGFR [68]. However, chemotherapeutic drugs targeting HIF-1α/VEGF/VEGFR have limited efficacy against malignant tumors and sometimes cause adverse effects [69].

Given that RUNX2 further stimulates HIF-1α-mediated induction of VEGF [63], it is highly possible that depletion of *RUNX2* suppresses an aggressive progression of malignant tumors through the downregulation of VEGF. Intriguingly, several lines of evidence imply that certain miRNAs might regulate the expression of *RUNX2*. For example, the diminished expression of miR-135 or miR-203 led to the enhancement of *RUNX2* expression in metastatic breast cancer cells [70]. According to their results, miR-135 and miR-203 were highly expressed in normal breast epithelial cells, whereas *RUNX2* was undetectable. In contrast, metastatic breast cancer tissues expressed *RUNX2* but not miR-135 or miR-203.

Similarly, exogenous expression of miR-34c in osteosarcoma cells decreased the expression level of *RUNX2* [71]. Thus, it is likely that miRNA-mediated downregulation of *RUNX2* causes the efficient suppression of malignant phenotypes of the aggressive tumors.

Alternatively, we have found that siRNA-mediated silencing of *RUNX2* improves the efficacy of anti-tumor drugs [25, 26]. Unfortunately, it has been well known that siRNA is extremely unstable and thus its effect is transient. To overcome this weakness, Zorde Khvalevsky et al. developed a local prolonged siRNA delivery system (termed LODER) [72]. According to their results, LODER system blocked the degradation of siRNA and released intact siRNA slowly into tumor cells over a few months. Collectively, it is suggestive that LODER system overcomes the present siRNA delivery obstacles, and thus siRNA-mediated knockdown of *RUNX2* by employing LODER as a delivery system is an attractive therapeutic strategy for the treatment of the patients with malignant tumors.

5.8 Conclusion

A growing body of evidence implies that tumor microenvironments composed of several cell populations including tumor cells play vital roles in the regulation of the malignant tumor progression under severe hypoxic condition. Hypoxia-dependent stabilization and nuclear access of HIF-1α stimulate the expression of VEGF, which contributes to tumor cell survival, invasion, and metastasis through the formation of new blood vessels. Meanwhile, RUNX2 is associated with HIF-1α and further potentiates its sequence-specific transactivation ability. Thus, RUNX2/HIF-1α

regulatory axis is essential for VEGF-mediated malignant tumor progression in response to hypoxia, and RUNX2 might be one of the attractive molecular targets for cancer therapy.

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Conflicts of Interest The authors have no conflict of interest.

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