CRISPR/Cas9-Editing-Based Modeling of Tumor Hypoxia

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Neha Masarkar, Suman Kumar Ray, Pragati Raghuwanshi, Ashish K. Yadav, and Sukhes Mukherjee

Abstract

One of the critical characteristics of solid tumors is hypoxia, which has been linked to a poor prognosis for cancer patients. Solid tumors frequently exhibit tumor hypoxia, primarily caused by an insufficient and diverse vascular network. Hypoxia, brought on by inadequate blood flow and oxygen supply in tumors, reduces the sensitivity of tumor cells to anticancer therapy. The major cellular stressor, hypoxia, influences various molecular pathways, and as we learn more about the underlying molecular mechanisms, the clinical use of hypoxic modifiers may increase. Hundreds of genes are transcribed by hypoxia-inducible factors (HIFs), which enable cells to adapt to hypoxic settings. HIF-1 upregulation, linked to higher patient mortality in several cancer types, can be brought on by intratumoral hypoxia and genetic changes. On the growth of tumors, HIF-1 activity inhibition has a profound impact. The effectiveness of new HIF-1 inhibitors is being sought after, and their effectiveness as anticancer therapies is being investigated. The HIF-1 transcription factor complex binds to hypoxia-responsive element (HRE) sequences found in the promoters of target genes to mediate the expression of genes that are particular to hypoxia. The use of therapeutic cargo like CRISPR/Cas9 to inhibit HRE-driven gene expression is currently being extensively investigated to develop cancer-specific targeted

Neha Masarkar and Suman Kumar Ray contributed equally with all other contributors.

N. Masarkar · P. Raghuwanshi · A. K. Yadav · S. Mukherjee (✉)
Department of Biochemistry, All India Institute of Medical Sciences, Bhopal, Madhya Pradesh, India
e-mail: sukhes.biochemistry@aiimsbhopal.edu.in

S. K. Ray
Independent Researcher, Bhopal, Madhya Pradesh, India

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therapeutics. The CRISPR/Cas9 technique has provided a window into previously unsolvable issues in our knowledge of cancer genetics, the noncoding genome, and heterogeneity in hypoxic tumors. It also offers new insights into therapeutic vulnerabilities. Combining CRISPR/Cas9-mediated HIF-1 knockdown with other antitumor strategies may make HIF-1 a viable therapeutic knockout target for treating several solid tumor types. A promising approach for altering the tumor microenvironment and preventing the growth and metastasis of hypoxic tumors is the downregulation of HIFs using CRISPR/Cas9, improving the therapeutic benefits of chemotherapy.

Keywords

Anticancer therapy · CRISPR/Cas9 · Gene editing · HIFs · Solid tumors · Tumor hypoxia · Tumor microenvironment

13.1 Introduction

A low oxygen tension level characterizes the pathophysiological characteristic of hypoxia. Once threshold oxygen levels are breached, hypoxia can exist in tissues, including tumors, impairing cellular or organ activities (Walsh et al. 2014). Particularly for malignant solid tumors, tumors' sensitivity to hypoxia is made worse by their abnormal growth. No matter the type of treatment used, the compensatory mechanisms that tumors use due to hypoxia negatively impact the delivery of curative care. Hypoxia sets off a series of actions encouraging tumor growth, strengthening immunity against the tumor, and promoting tumor angiogenesis (Abou Khouzam et al. 2021). The pathological angiogenesis that develops as the tumor grows results in an aberrant tumor vasculature that is randomly formed and leaky. As a result, the abnormally vascularized tumor microenvironment (TME) triggers immune suppression and upholds a persistent hypoxic condition (Lamplugh and Fan 2021). By restoring the vascular integrity of the tumor, the blood flow should improve, hypoxia should be relieved, and antitumor immunity should be reshaped.

Activating the transcription of genes required to avoid hypoxic (low oxygen level) situations is accomplished by hypoxia-inducible factors (HIFs) (Akanji et al. 2019). HIFs are essential for carcinogenesis. Despite clinical studies connecting elevated levels of HIF-1 with aggressive cancer growth and a poor patient prognosis, HIF-1 has been verified as a prospective target for innovative cancer therapies. Furthermore, preventing HIF-1 activity slowed the spread of cancer. As a result, HIF-1 is a promising target for cancer treatment (Mukherjee and Ray 2022). Given that cancer cells are known to be hypoxic, this might be expected. Cancer cells use the HIF-1 protein to activate several metabolic pathways to survive in the hypoxic microenvironment.

Table 13.1 HIF overexpression and its implications on various cancer cell/tumor types

Cancer cell/tumor type	HIF overexpression	Association
Breast LN, positive	HIF-1	Increased mortality
Breast cancer	HIF-1	Apoptosis inhibition
Cervical cancer	HIF-1	Increased mortality
Colorectal cancer	HIF-1 and HIF-2	Increased mortality
	HIF-1	Overexpression of drug efflux proteins
		Autophagy induction
GI stromal tumor of the stomach	HIF-1 and HIF-2	Increased mortality
Gastric	HIF-1	Increased mortality
		Apoptosis inhibition
Renal	HIF-1	Increased mortality
Lung adenocarcinoma	HIF-1	Overexpression of drug efflux proteins
Lung NSCLC	HIF-1	Increased mortality and also decreased mortality
Oropharyngeal SCC	HIF-1	Increased radiation resistance
Ovarian	HIF-1 and HIF-2	Increased mortality with p53
	HIF-1	Overexpression of drug efflux proteins
Esophageal, early stage	HIF-1	Resistance to photodynamic therapy

Furthermore, new anticancer therapy approaches can be developed using the HIF-1 path, as evidenced by cellular and molecular insights. Given the complicated relationships between HIFs, cancer growth, and carcinogenesis, the biological significance of HIFs cannot be overstated. HIF heterodimers, which include vascular endothelial growth factor (VEGF), bind to hypoxia-response elements (HREs) in the promoters of target genes after they are activated (Olenyuk et al. 2004). In the hypoxic microenvironment, there is a unique chance to send these proteins from outside the body to areas of the tumor where the most aggressive and drug-resistant cancer cells tend to live.

The clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein-9 (Cas9), a novel RNA domain-containing endonuclease-based genome engineering technology, has recently been demonstrated to be a powerful technique in the treatment of various cancers due to its multifunctional properties, including high specificity, accuracy, and time-reducing and cost-effective strategies with minimal off-target effects (Ray and Mukherjee 2021). Hypoxia-inducible factor-1 (HIF-1) downregulation using CRISPR/Cas9 is a potential strategy for altering the tumor microenvironment and preventing tumor spread (Li et al. 2019). Table 13.1 displays the effects of HIF overexpression on several cancer cells. The versatile CRISPR/Cas9 system may target and change specific DNA sequences in the genome, including HIFs. The versatility and simplicity of CRISPR have made it possible to quickly make practically any desired modification with more efficiency and at a cheaper cost than previous technologies. However, the CRISPR/Cas9 system's in vivo administration is still tricky. The development of CRISPR/Cas9

genomic editing has revolutionized how we control the genome and make it easier to study the biology of hypoxic tumor cells both in vitro and in vivo (Lino et al. 2018; Wilson and Gilbert 2018). Using the CRISPR/Cas system, we can edit the genome's sequence to downregulate or fix a mutation, dull an overactive gene or both (Fuziwara et al. 2022), and modify the gene expression of HIFs.

13.2 Tumor Hypoxia

Prokaryotic and eukaryotic organisms both require oxygen to survive, so they must be able to detect changes in oxygen levels and react to them. Oxygen-sensing mechanisms have been created to preserve tissue and cell homeostasis as well as to enable adaptation to the persistently low oxygen environments present in situations such as cancer (Maltepe and Saugstad 2009; Tretter et al. 2020). Most malignant tumors exhibit hypoxia, a non-physiological degree of oxygen tension (Ray and Mukherjee 2022). Having progressed but dysfunctional vascularization and acquiring the epithelial-to-mesenchymal transition phenotype, which results in cell motility and metastasis, are all effects of tumor hypoxia (Muz et al. 2015). Through the induction of cell quiescence, hypoxia modifies the metabolism of cancer cells and leads to therapeutic resistance. Hypoxia causes cancer cells to turn on a complex network of cell signaling pathways, such as HIF, PI3K, MAPK, and NF-B. These pathways work together to create positive and negative feedback loops that make the effects of hypoxia stronger or weaker (Jun et al. 2017).

Numerous genes are transcribed by hypoxia-inducible factors, enabling cells to adjust to hypoxic conditions (Fig. 13.1). It is understood that HIF controls the transcription of hundreds of genes (Dengler et al. 2014). HIF is a heterodimer composed of an aryl hydrocarbon nuclear receptor translocator (ARNT), also known as a hypoxia-activated component, and a constitutively expressed subunit (Mandl and Depping 2014; Wood et al. 1996). The subunit comes in three isoforms: HIF-1, HIF-2, and HIF-3. While research on HIF-3 isoforms is not as common, HIF-1 and HIF-2 have received greater attention (Jun et al. 2017; Hashimoto and Shibasaki 2015). Generally speaking, HIF-2 regulates genes comparable to HIF-1, but HIF-3 functions as a negative regulator of these genes. Human malignancies (Wigerup et al. 2016; Moreno Roig et al. 2018) of the bladder, brain, cervix, colon, endometrial, lung, oropharynx, pancreas, skin, and stomach are more deadly when HIF-1 overexpression in tumor biopsies is present. Many HIF target genes provide a mechanistic explanation for the many effects of intratumoral hypoxia on cancer development, as well as the relationship between HIF-1 overexpression and unfavorable outcomes for cancer patients that have been noted (Moreno Roig et al. 2018; Soni and Padwad 2017). HIF-1's potential target genes are beginning to emerge, which may contribute to the development of tumors (Masoud and Li 2015; Weidemann and Johnson 2008). One crucial issue is that different cancer types have different subsets of the HIF-1 target genes that react to hypoxia.

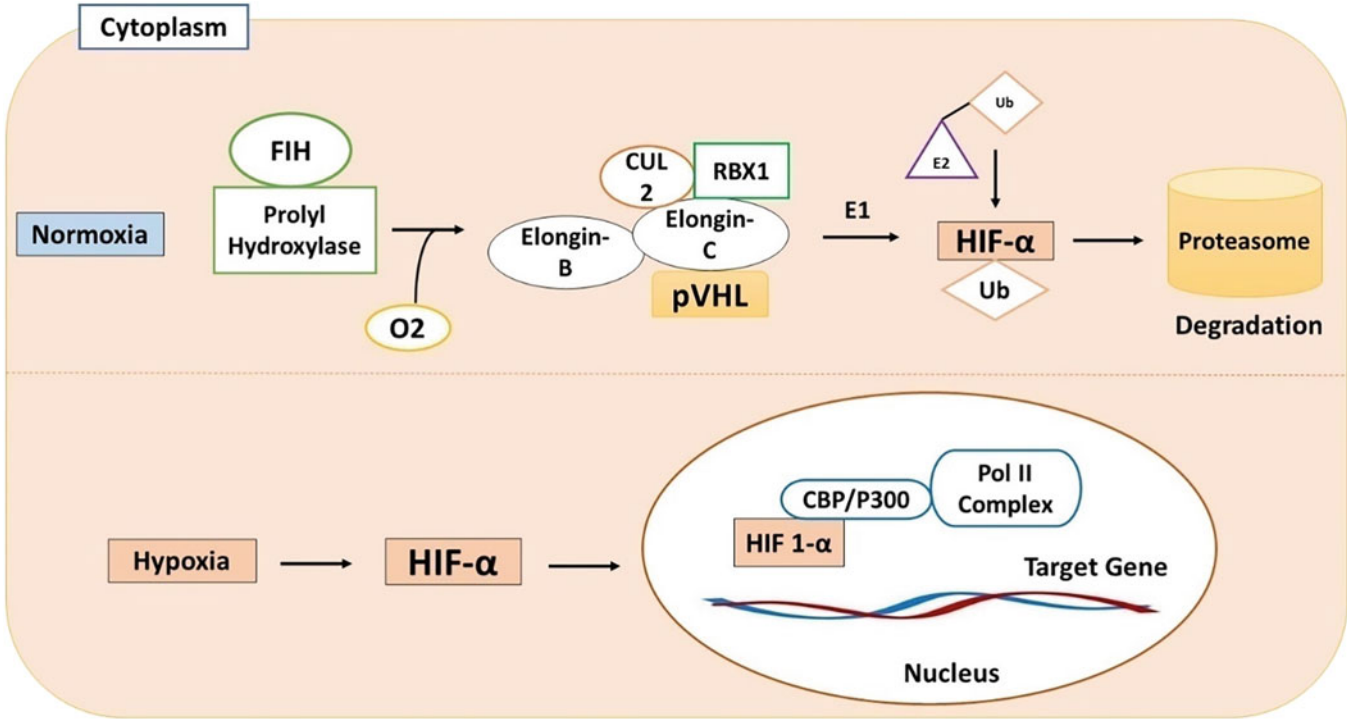

Fig. 13.1 HIF pathway in normoxia and hypoxia: HIF-1 is post-translationally modified by prolyl hydroxylase in the presence of oxygen (O_2) to interact with the von Hippel-Lindau (VHL) complex. Iron chelation prevents prolyl hydroxylase from functioning since it has an iron moiety. VHL is a component of a more extensive complex component consisting of elongin-B, elongin-C, CUL2, RBX1, and an enzyme that conjugates ubiquitin (E2). This complex is involved in the ubiquitylation (Ub) of HIF-1 together with an enzyme that activates ubiquitin (E1). Proteasome inhibitors can prevent the breakdown of HIF-1, which the Ub modification targets. However, HIF-1 cannot change HIF-1 without oxygen. Hence the protein is unaltered. Stabilized HIF-1 is transported to the nucleus, where it interacts with cofactors such as the DNA polymerase II (Pol II) complex, CBP/p300, and aryl hydrocarbon receptor nuclear translocator (ARNT) to bind to hypoxia-responsive elements (HREs) and activate transcription of target genes

13.3 CRISPR/Cas9 Technology: A Gene-Editing Tool

A collection of scientific techniques known as gene editing allows for modifying an organism's DNA. At specific sites in the genome, these technologies enable the addition, removal, or modification of genetic material. There are several methods for genome editing that have been developed. When compared to traditional methods (such as zinc finger nucleases and transcription activator-like effector nucleases), clustered regularly interspaced short palindromic repeats (CRISPR-associated nuclease 9, CRISPR-Cas9) have several advantages, including cost-effectiveness, flexibility, and ease of use (Kato-Inui et al. 2018; Gaj et al. 2016; Akram et al. 2022; Zhang et al. 2020). The most practical technique for gene editing remains CRISPR-Cas9, despite some drawbacks like effective delivery and safety. Furthermore, the CRISPR-Cas9 system has the potential to be used for genome editing, making it a novel therapeutic method for the treatment of diseases brought on by genome alterations, including cancer (Cox et al. 2015; Karimian et al. 2019; Shojaei Baghini et al. 2022).

CRISPR may have had the most influence on cancer research in pooled genomic screens. CRISPR knockout (KO) screens have become the "go-to" tool for examining gene function in cancer due to their simplicity in design, ease of cloning, effectiveness, and continual development of better sgRNA libraries (Shojaei Baghini et al. 2021; Hiranniramol et al. 2020). Positive selection CRISPR screenings continue to hone our understanding of how genes and pathways contribute to cancer in cell lines, organoids, and animals (Xing and Meng 2020; Hazafa et al. 2020). Although CRISPR has made pooled genetic screens possible in more challenging situations, there are countless examples of successful screening investigations in cell lines. For instance, in human colon organoids treated with the TGF-R inhibitor A83-01, Michels and his colleagues (2020) screened a targeted array of tumor suppressors to identify genes that limit tumorigenic expansion (Michels et al. 2020). Researchers have found putative regulators of non-small-cell lung cancer (NSCLC) metastasis using ex vivo transduction of a genome-wide library and subsequent engraftment in recipient mice (Sanghvi et al. 2019; Testa et al. 2018). Although it is difficult to transmit viral or plasmid-based vectors directly to organs in situ, it is conceivable to keep a representation of complicated libraries in vivo. Chow and his colleagues (2018) administered a genome-wide AAV sgRNA library to the brains of inducible Cas9-expressing mice to identify a subset of cancer drivers in the resulting glioblastomas. Table 13.2 is a summary of all the CRISPR/Cas9 clinical trials that are being done to treat different kinds of tumors.

13.4 Hypoxia-Specific Expression of CRISPR-Cas9

The Cas9 protein is a repurposed double-stranded DNA (dsDNA) nuclease that can be programmed to cut any genomic region and is employed for mammalian gene editing (Lino et al. 2018; Davis et al. 2022). By permanently destroying genes necessary for tumor cell viability, CRISPR-Cas9 presents an exceptional potential

Table 13.2 The ongoing CRISPR/Cas9 clinical trials investigations in different tumor types

Target	Study phase	Cancer type	Clinical trial identification
CRISPR/Cas9 inactivates the CISH gene	Phase II	Metastatic gastrointestinal epithelial cancer	NCT03538613
NY-ESO-1-redirected CRISPR edited T-cells (NYCE T-cells)	Phase I	Multiple myeloma melanoma synovial sarcoma myxoid/round cell liposarcoma	NCT03399448
PD-1 knockout	Phase I	Metastatic non-small cell lung cancer	NCT02793856
PD1 knockout	Phase II	Advanced esophageal cancer	NCT03081715
PD-1 and TCR gene knocked out	Phase II	Mesothelin positive multiple solid tumors	NCT03545815
PD-1 knockout	Phase II	Advanced stage Epstein-Barr virus (EBV)-associated malignancies	NCT03044743
CD19 and CD20 or CD22 CAR-T cell immunotherapy	Phase II	Relapsed or refractory leukemia and lymphoma	NCT03398967
CD19 and CAR-T cells (UCART019)	Phase II	Relapsed or refractory CD19+ leukemia and lymphoma	NCT03166878
HPV16-E6/E7 or HPV18 E6/E7 knockout	Phase I	Human papillomavirus-related malignant neoplasm	NCT03057912
TCR and B2M knockout	Phase II	CD19 + leukemia and lymphoma	NCT03166878
Fix NF1 mutation allele	–	Tumors of the central nervous system	NCT03332030
TCR and PD-1 knockout	Phase I	Multiple myeloma	NCT03399448
TCRα, TCRβ, B2M	–	Multiple myeloma	NCT04244656
TCRα, TCRβ	–	Lymphoma-cell lymphoma	NCT04035434
PDCD1	–	Stage IV gastric carcinoma, stage IV nasopharyngeal carcinoma, T-cell lymphoma stage IV, Stage IV adult Hodgkin lymphoma, stage IV diffuse large B-cell lymphoma	NCT03044743
PDCD1	Phase I	Metastatic non-small cell lung cancer	NCT02793856
PDCD1	Phase II	Metastatic renal cell carcinoma	NCT02867332
PDCD1	Phase II	Hormone refractory prostate cancer	NCT02867345
PDCD1	Phase I	Invasive bladder cancer stage IV	NCT02863913
PDCD1	Phase I	Esophageal cancer	NCT03081715
TCRα, TCRβ, PDCD1	–	Solid tumor, adult	NCT03545815
PDCD1	–	B-cell leukemia, B-cell lymphoma	NCT03398967

(continued)

Table 13.2 (continued)

Target	Study phase	Cancer type	Clinical trial identification
TCRα, TCRβ, B2M	Phase II	B-cell leukemia, B-cell lymphoma	NCT03166878
HPK1	Phase I	Leukemia, lymphoma	NCT04037566

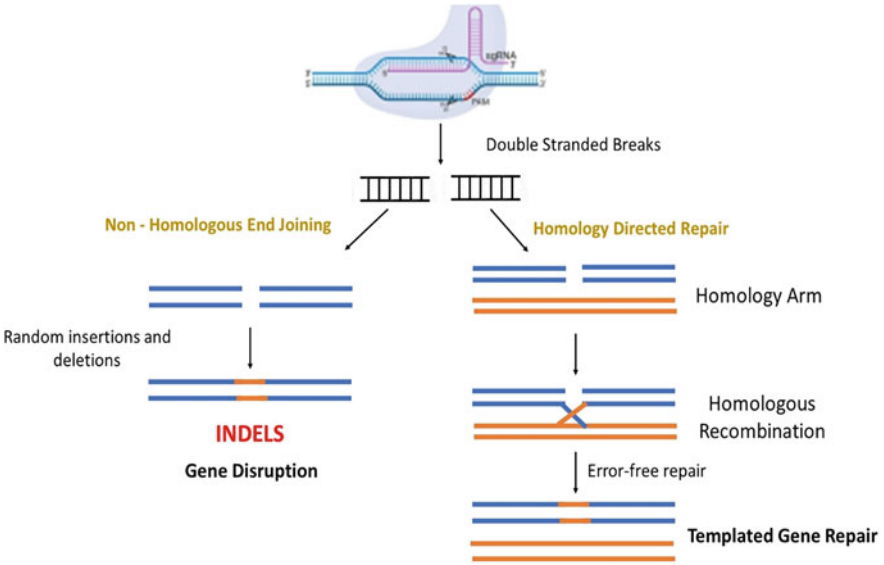

Fig. 13.2 CRISPR/Cas9-mediated gene editing. Either the NHEJ pathway or HDR is used to repair DNA damage. The NHEJ process results in error-prone repair because random insertions and deletions (indels) are inserted on the cut side and ligated. During repair in the HDR pathway, the damaged DNA follows the pattern of the homologous chromosomal DNA. It leads to a restoration that is free of mistakes

to treat cancer. Davis and his colleagues (2022) transfected cells with the Cas9 nuclease expressed from either the CBh or the 5HRE promoter to evaluate the viability and effectiveness of a hypoxia-regulated Cas9-expression system (Davis et al. 2022). However, when cells were treated under hypoxic circumstances, and gRNAs were expressed in the presence of the HRE-driven Cas9, a considerable decline in cell viability was seen (Thomas et al. 2021; Schwinn et al. 2018). Researchers have verified that Cas9 cleavage only occurs when the HRE promoter is used to induce the nuclease's expression in hypoxic settings (Lam and Truong 2021). Cancer cells exposed to hypoxia can specifically have their viability reduced by the HRE-promoter-driven Cas9 mechanism. Caki1 clear-cell renal carcinoma cells introduce inactivating mutations into the VHL protein gene using the CRISPR/Cas9 DNA editing system (Fig. 13.2) (Artemov et al. 2018; Wolf et al. 2020).

Because of the mutational change in the reading frame, its functionality has been altered.

13.5 Genome-Wide CRISPR/Cas9 Screening and Progression of Hypoxic Cancer

One of the key hallmarks of solid tumors is hypoxia. Tumor cells can adapt to a hypoxic microenvironment by activating HIFs, especially HIF-1a and HIF-2a (Ziello et al. 2007; Corrado and Fontana 2020). A robust method for evaluating gene function and searching for genes implicated in cancer cell proliferation and metastasis is the genome-scale CRISPR-Cas9 knockout (GeCKO) library (Shalem et al. 2014; Joung et al. 2017). CRISPR screening has advanced hypoxia cancer research significantly in recent years. However, few studies (Hazafa et al. 2020; Afolabi et al. 2021) have employed CRISPR systems to screen essential genes like HIFs that regulate immune evasion or tumor treatment resistance. In a study, Teng and his team (2021) discovered that hypoxia upregulates HIF-1 bound to the CBX8 promoter region and controls the transcriptional level of CBX8 expression. By CRISPR screening, Yang and his team (Yang et al. 2019) have discovered a set of genes linked to the development of pancreatic cancer by CRISPR. According to a combined analysis of clinical samples from patients with pancreatic cancer (Li et al. 2021; Shi et al. 2021), the expression of CBX8 is higher in pancreatic cancer tissue, and increased expression of CBX8 is associated with a poor clinical prognosis (Teng et al. 2021). Under low oxygen conditions, HIF-1 controls the transcription of CBX8, and CBX8 promotes the growth of pancreatic cancer cells by targeting IRS1, which turns on the PI3K/AKT pathway.

13.6 Knockdown of Hypoxia-Inducible Genes by Tumor Target Delivery of CRISPR/Cas9 System

Despite significant advancements in understanding the pathophysiology and spread of various hypoxic malignancies, this issue still poses one of the biggest threats to human health. The primary reasons for a poor prognosis in cancer are invasion and metastasis, which are correlated with epithelial-mesenchymal transition (EMT), which aids in the early-stage dispersion of hypoxic cancer cells (Ribatti et al. 2020; Roche 2018). In light of these difficulties, EMT inhibition might be a workable strategy to lower metastasis and increase the antitumor effects of chemotherapy. Fast tumor growth and limited vascular supply were factors in the development of the hypoxic tumor microenvironment. HIF-1, a component of HIF-1, is essential for tumor cell growth, death, and EMT (Jun et al. 2017; Lv et al. 2016). As a result, HIF-1 has emerged as a promising target for the management of tumor microenvironments and the therapy of cancer. The downstream molecules of HIF-1, such as VEGF, CXCR-4, MMP-9, and TWIST, may have significant

anti-proliferation and anti-metastatic effects on hypoxia-inducible tumor cells by inhibiting the production of HIF-1 (Li et al. 2018; Tam et al. 2020).

Genome editing has shown tremendous promise as a cancer therapeutic over the past few decades and has grown to be an essential tool in genetic engineering. The CRISPR/Cas system, which consists of a short guide RNA (sgRNA) and the Cas9 nuclease, is a hot topic in gene engineering (Xu and Li 2020). The Cas9 nuclease introduces a DNA double-stranded break after sgRNA binds to site-specific DNA sequences. It generates base pairs in the CRISPR/Cas9 system (Alagoz and Kherad 2020). CRISPR/Cas9 can be used for downregulating HIFs thanks to the thoughtful design of sgRNA. There are numerous ways to prepare Cas9 nuclease and sgRNA, including plasmids, proteins, RNAs, or viral vectors. However, because protein and RNA degrade quickly, delivering them to living organisms is still tricky. However, due to the high molecular weight of Cas9-expressing DNA or Cas9 protein and the requirement for nucleus entry, CRISPR/Cas9 also faces more difficulties than RNAi (Lino et al. 2018). The key to the widespread deployment of this cutting-edge technology is effective ex vivo and in vivo administration systems. Studies are focusing more and more on making different materials that will improve the effectiveness of CRISPR/Cas9 systems for systemic delivery (Luther et al. 2018).

Due to limited vascular supply, a typical hallmark of pancreatic cancer that is detrimental to the survival of tumor cells is the hypoxic microenvironment. The expression of specific genes and the metabolism of tumor cells are altered by factors like HIF-1, which increase the likelihood of metastasis (Huang et al. 2017). EMT may result from the activation of E-cadherin transcriptional repressors in hypoxic circumstances. HIF-1 overexpression controls several downstream molecules, including VEGF and MMP-9, which are connected to angiogenesis, EMT, and the proliferation of tumor cells (Wigerup et al. 2016; Petrova et al. 2018). It is also associated with abnormal p53 accumulation in cancer. Clinical samples have further demonstrated the relationship between HIF-1 and the VEGF and MMP-9 that are produced downstream. It has been suggested that stopping HIF-1 makes gemcitabine work better against cancer.

13.7 CRISPR/Cas9-Mediated Hypoxia-Inducible Factor-1α Knockout Enhances the Antitumor Effect

Although gene therapy and immunotherapy have made tremendous strides in treating solid tumors, their application in patients is currently limited due to their high-cost and labor-intensive nature. Therefore, adopting cutting-edge technology like CRISPR/Cas9 to treat hypoxia cancer may be a practical and inexpensive option. Translational medicine may use precise gene knockout to alter HIFs, and the CRISPR/Cas9 system is one of the best tools for this. With the help of a small guide RNA (sgRNA), which is made up of a target complementary CRISPR RNA (crRNA) and an additional trans-activating crRNA (tracrRNA) (Ran et al. 2013; Karvelis et al. 2013), the system can successfully target desired genomic locations. Technologically, tailored gRNAs and CRISPR/Cas9 can be expressed in a particular

cell by a delivery vector like a lentiviral vector. Base pairing between the crRNA sequence and the target DNA sequence allows the CRISPR/Cas9 endonuclease to target a particular genomic location, resulting in a double-stranded DNA break and a targeted gene alteration that disrupts the target gene. The CRISPR/Cas9 system seems like an excellent way to treat hypoxic cancer, both in the lab and in people, when there are genetic abnormalities and an unusually high expression of essential oncogenic proteins.

Hypoxia-induced oxygen-dependent transcription factor HIF1 is crucial for the maintenance of cancer stem cells as well as the aggressiveness, enhanced angiogenesis, and chemotherapy resistance of hypoxic tumors (Zhang et al. 2021). By boosting the expression of matrix metalloproteinase 2 (MMP2) and MMP9, HIF-1 precisely activates the hypoxia pathways, causing the overexpression of VEGF, activating the epithelial-mesenchymal transition (EMT), tumor invasiveness, and metastasis (Barillari 2020; Hapke and Haake 2020). According to research, HIF-1 is elevated in hepatocellular carcinoma and linked to portal vein metastases and hepatic capsular invasiveness. Furthermore, HIF1 regulates the production of the multidrug resistance protein (MDR), and its protein product P-glycoprotein (P-gp) can transport chemotherapeutic medicines out of cells (Badowska-Kozakiewicz et al. 2017). Therefore, inhibiting HIF1 may be a therapeutic strategy for treating many hypoxia-related cancers, including hepatocellular carcinoma. Liu and his team (2018) investigated the role of the human HIF-1 gene in the human liver cancer cell line SMMC-7721 using a lentivirus-mediated CRISPR/Cas9 system (Liu et al. 2018). Under low oxygen levels caused by CoCl2, the HIF1 disruption caused by the lentivirus decreased cell growth, migration, and invasion and induced apoptosis.

13.8 HIF-1α-Knockout via CRISPR/Cas9 Suppresses HIF-1α Expression and Impairs Cell Invasion and Migration

Cellular invasion is significantly influenced by hypoxia and HIF-1, which serve as its primary regulators. According to mounting data, a reservoir of cells resembling those of healthy stem cells may persist within tumors and be capable of promoting carcinogenesis. Numerous hypoxic tumors have been reported to contain these cells, also known as cancer stem cells (CSCs) or tumor-initiating cells. The ability of hypoxia to keep stem cells in their undifferentiated condition has been demonstrated. Méndez and his colleagues (Méndez et al. 2010) have shut down the expression of HIF-1 and assessed the migration and invasion capacity of these glioma cells to understand better the role that HIF-1 plays in glioma cell migration in vitro and in vivo. A gene expression profile study was conducted to find potential genetic pathways responsible for the decreased migration in vitro, decreased invasiveness in vivo, and decreased capacity to form tumor spheres in cells knocked down for HIF-1.

To identify sgRNAs specifically targeting the gene loci of HIF-1α, researchers have designed sgRNAs to target the exon of HIF-1α (Naeem et al. 2020). The efficiency of HIF-1αknockout is well established in the experimental mice models.

Immunohistochemical examination by Ding and his colleagues (2006) revealed that HIF-1α is highly expressed in the hypoxic hepatocellular carcinoma and CRISPR/Cas9 efficiently disrupted the expression levels of the HIF-1αgene and its targets, including VEGF, in liver cancer cells and xenograft tumor tissues (Ding et al. 2006). In vitro study can be possible using hypoxia mimetic cells by infecting the experimental cells with the lentiviruses and $CoCl_2$ (He et al. 2015). The disruption of HIF 1α with the CRISPR/Cas9 system inhibits hypoxic cancer cell migration and invasion, particularly under the hypoxic microenvironment.

13.9 CRISPR/Cas9-Based HIF-1α Disruption Suppresses Cell Proliferation and Induces Cell Apoptosis

Hypoxia can trigger apoptosis (Greijer and van der Wall 2004). Cells either undergo apoptosis (cells that die) or adapt to hypoxia and survive, depending on how severe the hypoxia is. A cell cannot undergo energy-dependent apoptosis in a hypoxic environment devoid of nutrients and instead becomes necrotic. The balance of apoptosis regulatory proteins is delicate. The occurrence of hypoxia in solid tumors is frequent. After repeated episodes of hypoxia, cells become accustomed to this environmental stress, leading to selection for resistance to hypoxia-induced apoptosis. These resistant tumors may be less responsive to therapy and have a more aggressive phenotype. Stopping growth is a typical cellular response to hypoxia (Muz et al. 2015; Vaupel 2008).

The activation of the HIF-1 transcription factor is a crucial aspect of the hypoxic response. Molecular oxygen is now used by mammalian cells to produce energy. Different oxygen concentrations can cause changes in a cell's metabolic state and growth rate, affecting how the cell reacts to the environment. Recent research suggests that hypoxia can affect cell proliferation in two ways: through programmed cell death and growth arrest (Hubbi and Semenza 2015; Pucci et al. 2000). In addition, hypoxia can cause apoptosis in transformed cells by activating the p53 pathway (Leszczynska et al. 2015). It can be a powerful mechanism for selecting p53 mutants in hypoxic tumor cell populations.

On the other hand, non-transformed hypoxic cells can experience cell cycle arrest at the G1/S interface without suffering any long-term viability changes (Goda et al. 2003; Chen 2016). Further research found through in vitro experiments cell cycle progression under hypoxia can be evaluated in vitro. Researchers have discovered that as the cell cycle advances, there is a simultaneous decrease in the proportion of cells in the G0/G1 phase and an increase in the G2/M phase (Bertoli et al. 2013). In addition, the CRISPR/Cas9 system's disruption of HIF 1 prevents hypoxic tumor cells from proliferating and triggers cell apoptosis. This problem may be caused by how CRISPR/Cas9 changes the cell cycle, so it would be helpful to know the cell cycle progression profile of any HIFs of hypoxic cancer caused by CRISPR/Cas9 treatment (Hazafa et al. 2020; Geisinger and Stearns 2021).

13.10 Hypoxia-Responsive Gene Editing to Reduce Tumor Thermal Tolerance for Mild Photothermal Therapy

CRISPR-Cas9, an emerging biological technology, is crucial in treating diseases such as hypoxia cancers and gene editing. The CRISPR-Cas9 system has been delivered using various techniques, such as electroporation, microinjection, viral vectors, and other nonviral vectors. Viral delivery methods among in vivo delivery systems likely result in unintended immunogenicity and carcinogenesis. Despite the enormous nonviral solutions developed to address biosafety concerns, it is still challenging to implement the on-demand release of CRISPR-Cas9 that is activated explicitly by the hypoxic tumor microenvironment (Leszczynska et al. 2015). Rapid cell division and microvessel destruction at the tumor site diminish oxygen delivery due to increased oxygen consumption and restricted blood flow. In most solid tumor microenvironments, hypoxia is a common symptom of an imbalance between inadequate oxygen supply and rising metabolic demand (Abou Khouzam et al. 2021; Eales et al. 2016). The trait encourages the development of more robust platforms for effective cancer treatment methods.

On the one hand, photothermal therapy (PTT) and hyperthermia, which uses light to produce localized heat, have attracted growing interest in tumor therapy due to their noninvasive and spatiotemporally programmable methods modes (Ferroni et al. 2019). On the other hand, a customized CRISPR/Cas9 system delivery technique responds to hypoxia to further realize mild hyperthermia. Using azobenzene-4, 4′-dicarboxylic acid, a hypoxia-responsive azobenzene linker, the CRISPR/Cas9 system is covalently cross-linked on Au nanorods at normal oxygen partial pressure (p-AZO). As a result, the N-N double bond of p-AZO can be reduced, and aniline derivative is produced due to the imbalance of cellular redox states in the hypoxic microenvironment of tumor cells (Chun et al. 2021; Hielscher and Gerecht 2015). To precisely knock down the HSP90 gene, the Cas9/sgRNA ribonucleic protein complex is released from AuNRs, which significantly lowers the heat tolerance of tumor cells (Dong et al. 2016). To further investigate the controllable release of Cas9, Briolay and his team (2021) used liver microsomes in a hypoxic environment. They showed that hypoxia caused Cas9 protein to be released, a good thing for gene editing targeting tumors and precision medicine (Briolay et al. 2021).

13.11 Genome-Wide CRISPR/Cas9 Deletion Screen for Tumor Cell Viability in Hypoxia

Hypoxia and other tumor-related microenvironmental circumstances can significantly impact mitochondrial function, triggering metabolic adaptations that support tumor cell survival and spread. For example, mitochondrial activity is crucial for maintaining tumor cell proliferation by producing ATP via OXPHOS and synthesizing precursors for biomass accumulation, such as amino acids, lipids, and nucleotides; mitochondrial activity is crucial for maintaining tumor cell proliferation (Vander Heiden et al. 2009; Liberti and Locasale 2016). Because of this,

mitochondria are essential for ensuring that cells and tissues get enough oxygen, and it has been found that they control when intracellular hypoxia starts to happen.

Hypoxia, which contributes to the course of the disease and is linked to treatment resistance and a poor prognosis in patients with solid malignancies, is a defining hallmark of the tumor microenvironment. Metazoan cells can change to keep cellular homeostasis and stay alive. They have developed several pathways, like the HIF pathway, that can sense and respond to changes in oxygen levels. With the help of CRISPR/Cas9 gene-editing technology, scientists can now study how cells respond to stimuli across the whole genome. Researchers used CRISPR/Cas9 deletion screening in different environments (normoxia-glucose, hypoxia-glucose, and normoxia-galactose) to determine how tumor cells depend on nuclear-encoded mitochondrial genes and non-mitochondrial genes for survival when oxygen or glucose is plentiful or scarce (Thomas et al. 2021).

The fundamental response of tumor cells to hypoxia is the loss of mitochondrial genes, including OXPHOS genes like succinate dehydrogenase subunit C (SDHC), which enhances the proliferation of U2OS cells as well as HeLa and MCF7 cells and downregulates the expression of OXPHOS proteins (Pustylnikov et al. 2018; Luo et al. 2020). In addition, respiration in renal cancer cells has been slowed down because of how the HIF pathway affects the activities of MYC and PGC1, which control the creation of new mitochondria (Li et al. 2020). Aside from the genes involved in OXPHOS, it is interesting that the CRISPR/Cas9 deletion screen is more common in hypoxia-glucose than in normoxia-glucose.

13.12 CRISPR/Cas9-Mediated Altered Expression of HIF-1α Enhances the Antitumor Effect

A therapeutic knockout target for the characteristics of hepatocellular cancer, HIF1, has the potential to be effective. In an orthotopic hepatocellular carcinoma model, CRISPR/Cas9-mediated genome editing demonstrated an anticancer effect in addition to extending survival (Shojaei Baghini et al. 2022). Furthermore, it is well-known that angiogenesis plays a crucial role in the initiation, development, advancement, metastasis, and recurrence of hepatocellular carcinoma. HIF-1 is the main factor that controls angiogenesis in low-oxygen environments. It is also good to tell how well someone with hepatocellular carcinoma will do. The CRISPR/Cas9 protein (Choi et al. 2016) and an HIF1-specific sgRNA were delivered by the lentiviral vector (LV-H721) and may be helpful for highly effective HIF1 modification in experimental liver tumor cells. HIF1 deletion decreases cell proliferation in hypoxic environments and is associated with enhanced cell death in hypoxic cancer, showing that the CRISPR/Cas9 technology effectively targets HIF1. Highly effective tumor genome engineering was accomplished by merely combining the expression of CRISPR/Cas9 and sgRNAs (Fuziwara et al. 2022). However, the absence of effective delivery mechanisms and the toxicity of the Cas9 nuclease restrict the use of the lentiviral CRISPR/Cas9 system in clinical situations. Notably, lentivirus infection and the CRISPR/Cas9 mechanism that mediates the deletion of an

inevitable gene result in off-target events and irreversible insertional inactivation of genes. Therefore, it would be ideal for creating a delivery vector and a CRISPR/Cas9 system that primarily targets the genomes of cancer cells.

13.13 Future Outlook

Although the qualitative and quantitative variations in the hypoxia response by both cell types are unknown, one of the main physiological differences between tumor cells and normal cells is their capacity to survive under hypoxic conditions. Hypoxia stimulates a complex transcriptome, including signaling pathways downstream of HIF-1 and other signaling pathways, in both tumor and normal cells. Most solid tumors exhibit hypoxia due to changes in the local vascular blood flow that cause oxygen deprivation. This event offers a rare chance to attack only the hypoxic tumor microenvironment while sparing the surrounding normoxic tissue. Additionally, it has been demonstrated that selectively altering tumor cells that express HIF1 can increase survival and slow the spread of metastatic disease, supporting the idea that targeting these cells is a promising therapeutic approach. Additionally, other genes and pathways have been discovered that merit additional research into their potential as therapeutic targets for tumor cells that are both normoxic and hypoxic.

The CRISPR-Cas9-based technologies discussed here have great promise for eradicating some of the most aggressive and resistant therapy cells found in hypoxic tumors. They merit additional investigation for improved tumor selectivity and delivery. The combination of CRISPR-based technologies with single-cell multi-omics methodologies opens up a vast array of potential applications for examining HIF gene function and tumor heterogeneity. The combination of spatial transcriptomics with pooled CRISPR libraries will be a powerful method for studying the effects of gene disruption on the relationships between hypoxic tumor microenvironments. The HIF pathway facilitates the mechanism for cellular oxygen homeostasis and its response to a low oxygen situation.

A crucial factor in cancer metastasis is the regulation, or dysregulation, of the HIF pathway, which is associated with a poor prognosis for cancer. HIF is a desirable target for chemotherapy against malignant cells because of its role in cancer development. Perhaps using HIF inhibitors in conjunction with conventional therapy will be therapeutically beneficial. Using a tumor-targeted lipid-based CRISPR/Cas9 delivery method may allow HIF-1, a crucial regulator of the hypoxic tumor microenvironment, to be suppressed. In vivo downregulation of HIF-1 by the CRISPR/Cas9 system in combination with chemotherapy could be used to treat diseases that have spread to other body parts.

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