International Journal of Research in Medical and Clinical Science
Volume 2, Issue 1, 2024, PP: 52-76
www.journalserapublications.com

Review Article

Induction of Cancer Stem Cells in Tumor Microenvironment and Involvement of HIFs And Carbonic Anhydrase IX in Oncogenesis of Solid Tumors and Hematological Malignancies Including Adult T-Cell Leukemia/Lymphoma (ATL)

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Abstract

Cancer stem cells (CSCs) and leukemia stem cells (LSCs) induced in tumor microenvironment (TME) play important roles in oncogenesis of solid tumors and hematological malignancies. TME contributes to not only induction of CSCs and LSCs but also cancer initiation, proliferation, metastasis and therapy resistance. In addition, hypoxia inducible factors (HIFs) that are induced under hypoxia in TME activate various molecules associated with cancer cell proliferation, survival, metastasis, angiogenesis, immune escape, metabolic regulation and therapy resistance. Carbonic anhydrase IX (CA9) that is one of the gene products induced by HIFs via hypoxia responsive element (HRE) also promotes oncogenesis of solid tumors and hematological malignancies including adult T-cell leukemia/lymphoma (ATL). This review aims to summarize the various mechanisms for induction of CSCs and LSCs in TME and the involvement of HIF and CA9 in oncogenesis of solid tumors and hematological malignancies including ATL. First, we showed induction of CSCs/LSCs by transcription factors, post-transcriptional controls, epigenetic modification, signaling pathways and other extrinsic factors in TME. Then, we indicated the essential roles of HIFs and CA9 in cell proliferation, cell survival, metastasis and therapy resistance. Finally, we shortly discussed the possible therapies to target CSCs, including CA9 inhibitors. From these analyses, it is likely that the HIF-1α/CA9 axis may contribute to finalization of the three-step oncogenesis of ATL

Keywords: Cancer stem cell, hypoxia, tumor microenvironment, HIF, CA9, ATL.

Abbreviations

4E-BP=4E-binding protein, **AML**=acute myeloid leukemia, **AP-1**=activator protein-1, **ARNT**=aryl hydrocarbon receptor nuclear translocator, **AS**=alternative splicing, **ATL**=adult T-cell leukemia/lymphoma, **BC**=blast crisis, **CA9**=carbonic anhydrase IX, **CAF**=cancer-associated fibroblast, **CAR**=chimeric antibody receptor, **CLL-1**=C-type lectin-like molecule-1, **CML**=chronic myeloid leukemia, **CpGIs**=,cytosine-phosphate-guanine islands **CRC**=colorectal cancer, **CSC**=cancer stem cell, **CSL**=C protein binding factor 1/Suppressor of Hairless/Lag-1, **DNMT**=DNA methyltransferase, **Dvl**=Dishevelled, **ECM**=extracellular matrix, **eIF=**eukaryotic translation initiation factor, **EMT**=epithelial-to-mesenchymal transition, **EpCAM**=epithelial cell adhesion molecule, **ERK**=extracellular signal-regulated kinase, **FUSU**=Suppressor of Fused, **Fzd**=Frizzled, **GLI**=glioma-associated oncogene homolog, **GMP**=granulocyte-macrophage progenitor, **GSK3β**=glycogen synthase kinase-3,

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HCC=hepatocellular carcinoma, **HDAC**=histone deacetylase, **Hh**=Hedgehog, **HIF**=hypoxia inducible factors, **HRE**=hypoxia responsive element, **HTLV-1**=human T-cell leukemia virus type 1, **IDH**=isocitrate dehydrogenase, **IGF-1**=insulin-like growth factor-1, **IL**=interleukin, **IL1RAP**=interleukin-1 receptor accessory protein, **IPMN**=intraductal papillary mucinous neoplasm of the pancreas, **JAK**=Janus kinase, **JUN**=c-JUN N-terminal kinase, **KLF4**=Krüppel-like factor 4, **LAC**=lung adenocarcinoma, **LDR=**low density lipoprotein receptor-related protein, **LEF**=lymphoid enhancer-binding factor, **LIC**=leukemia-initiating cell, **lncRNA**=long noncoding RNA, **LSC**=leukemia stem cell, **LSCC**=lung squamous cell carcinoma, **MAML**=mastermind-like, **MAPK**=mitogen-activated protein kinase, **MDS**=myelodysplastic syndrome, **MDSC**=myelo-derived suppressive cell, **MDS/MPN**=yelodysplastic/myeloproliferative neoplasm, **tRNAi**=initiating methionyl tRNA **miRNA**=microRNA, **MMP**=matrix metalloproteinase, **MNK**=MAPK-interacting kinase, **MSI**=Musashi, **mTOR**=mechanistic target of rapamycin, **mTORC1**=mechanistic target of rapamycin complex 1, **NEMO**=NF-κB essential modulator, **NF-κB**=nuclear factor kappa B, **NHL**=non-Hodgkin lymphoma, **NICD**=Notch intracellular domain, **NIK=**NF-κB-inducing kinase, **NSCLC**=non-small cell lung cancer, **NTC=**Notch transcription complex, **Oct4**=octamer-binding transcription factor 4, **PABP**=poly(A)-binding protein, **PBX1**=pre-B-cell leukemia transcription factor 1, **PD-1**=programmed cell death protein 1, **PDAC**=pancreatic ductal adenocarcinom, **PD-L1**=programmed cell death protein 1 ligand 1, **PI3K**=phosphatidylinositol-3-kinase, **PIC=**pre-initiation complex, **PR**=protected region, **PRC2**=polycomb repressive complex 2, **PTEN**=phosphatase and tensin homolog, **RBP**=RNA binding protein, **RBP-J**=recombination signal binding protein for immunoglobulin κ J region, **ROMP**=regulator of phospholipid metabolism, **RSK**=ribosomal S6 kinase, **S6K**=S6 kinase, **SCLC**=small cell lung cancer, **SMAD**=mothers against decapentaplegic homolog, **SMO**=Smootbened, **SOS**=son of sevenless, **SOX2**=sex-determining region Y-box 2, **SP**=specificity protein, **STAT**=signal transducer and activator of transcription, **SUFU**, suppressor of fused protein, **TAM**=tumor-associated macrophage, **TCF**=T cell-specific factor, **TET=**ten eleven translocation, **TGF-β**=T-cell growth factor β, **TIM**3=immunoglobulin and mucin 3, **TGF**=transforming growth factor, **TKI**=tyrosine kinase inhibitor, **TME**=tumor microenvirontment, **TNBC**=triple negative breast cancer, **TNF**=tumor necrosis factor, **Treg**=regulatory T cell, **ZEB**=zinc-finger E-box-binding.

Introduction

Cancer stem cells (CSCs) and leukemia stem cells (LSCs) play important roles in oncogenesis of solid tumors and hematological malignancies. CSCs and LCSs are induced by three factors, i.e., genetic alterations, epigenetic modulations and factors in tumor microenvironments (TME) [1]. Of these, the roles of TME in not only induction of CSCs and LSCs but also cancer initiation, proliferation, metastasis and therapy resistance have attracted many researchers [2-4]. TME consists of tumor cells, stromal cells, epithelial cells and immune cells [5]. Many intrinsic and extrinsic factors in TME cooperatively work in induction of CSCs and LSCs, including epigenetic modulation, transcription factors, post-transcriptional controls, signaling pathways, metabolic controls, hypoxia and others [2,4,6]. In particular, involvement of hypoxia inducible factors (HIFs) in CSC induction under hypoxia in TME have been intensively investigated [7,8]. HIFs also contribute to cancer cell proliferation, survival, metastasis and therapy resistance. In addition, carbonic anhydrase IX (CA9) that is induced by HIF has been suggested to be associated with not only CSC induction [9] but also oncogenesis of numerous solid tumors [10,11] and lymphomas [12,13].

This review aims to summarize the various mechanisms for induction of CSCs and LSCs in TME and the involvement of HIF and CA9 in oncogenesis of solid tumors and hematological malignancies including adult T-cell leukemia (ATL). We first show induction of CSCs/LSCs by transcription factors, post-transcriptional controls, epigenetic modification, signaling pathways and other extrinsic factors in TME. Then, we indicate the essential roles of HIFs and CA9 in cell proliferation, survival, metastasis and therapy resistance. Finally, we shortly discuss the possible therapies to target CSCs, including CA9 inhibitors.

Phenotypic Plasticity of Transdifferentiation and Dedifferentiation

EMT

Phenotypic plasticity is a complicated mechanism to escape from cytotoxic chemotherapy, radiotherapy, molecular targeted therapy and immunotherapy. There are

two types of phenotypic plasticity, i.e., transdifferentiation (direct shift from one differentiated cell type to another cell type) and dedifferentiation (reversion from differentiated cell to CSC) [14-18]. Transdifferentiation is represented by epithelial-to-mesenchymal transition (EMT) [18-21] and is found in many solid tumors such as non-small cell lung cancer (NSCLC) [18,22,23], pancreatic ductal adenocarcinoma (PDAC) [24,25], hepatocellular carcinoma (HCC) [26], esophageal cancer [27], gastric cancer [28] and prostate cancer [29]. Several signaling pathways, including T-cell growth factor β (TGF-β), insulin-like growth factor-1 (IGF-1), PI3K (phosphatidylinositol-3-kinase)/AKT/mechanistic target of rapamycin (mTOR), RAS/extracellular signal-regulated kinase (ERK), WNT, Notch, Hedgehog and HIF, are involved in EMT [15,18,23]. In addition, EMT-associated transcription factors (Snail, Twist and ZEB) play important roles in induction of EMT [15,18,23].

CSCs

In turn, CSC is another type of phenotypic plasticity, i.e., dedifferentiation [2,4,6,16]. Existence of LSC is confirmed in hematological malignancies (acute myeloid leukemia [AML] [30] and chronic myeloid leukemia [CML] [31]. However, in lymphoid malignancies such as acute lymphoblastic leukemia (ALL) and non-Hodgkin malignant lymphoma, existence of CSCs has been controversial [32-34]. Instead, existence of leukemia-initiating cells (LICs) has been proposed [33-37]. CSCs and LSCs have potential of self-renewal as well as potential of differentiation [2,4]. Thus, induction of CSCs and LSCs causes serious problems such as therapy resistance as well as cancer initiation, progression and relapse in clinical settings [2,3]. CSCs and LSCs are induced by epigenetic modulation (DNA methylation and histone modification), intrinsic factors (CSC-associated transcription factors and post-transcriptional controls) and extrinsic environmental factors (signaling pathways, metabolic controls, hypoxia and others) in TME [2,4,6]. Important post-transcriptional controls in CSC induction are RNA binding proteins, RNA alternative splicing and noncoding RNAs (microRNAs [miRNAs] and lncRNAs [long noncoding RNAs]), while signaling pathways such as Notch, Hedgehog, WNT, T-cell growth factor β (TGF-β), signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa B (NF-κB) play significant roles in CSC induction. HIF and CA9 under hypoxia in TME are also essential factors.

Surface Markers and Transcription Factors

Surface Markers of CSCs and LSCs

CSC surface markers are useful for diagnosis and prognostic evaluation [3,38-42]. According to Walcher et al. [3], frequent observed CSC markers in each disease are as follows: CD44, CD87, CD90, CD133, CD166 and epithelial cell adhesion molecule (EpCAM) on lung cancer stem cells; CD24, CD29, CD44, CD49, CD61, CD70, CD90, CD133, CXCR4, EpCAM, LGR4 and ProC-R on breast cancer stem cells; CD24, CD44, CD90, CD133, CXCR4, EpCAM, LGR4 and LINGO2 on gastric cancer stem cells; CD24, CD44, CD90, CD133 and EpCAM on liver cancer stem cells; CD24, CD44, CD133, CD166, EpCAM and LGR5 on colorectal cancer (CRC) stem cells; CD33, CD123, C-type lectin-like molecule-1 (CLL-1) and immunoglobulin and mucin 3 (TIM3) on acute myeloid leukemia (AML) stem cells; and CD25, CD26, CD33, CD36, CD117, CD123 and interleukin-1 receptor accessory protein (IL1RAP) on chronic myeloid leukemia (CML) stem cells. Of these, CD44 [42], CD133 [41] and EpCAM [38-40] are the commonly found CSC markers of solid tumors, whereas CD33 [43,44], CD123 [45,46] and CLL-1 [47,48] are the specific LSC markers of hematological malignancies.

CSC-Associated Transcription Factors

Numerous transcription factors such as octamer-binding transcription factor 4 (Oct4), sex-determining region Y-box 2 (SOX2), Krüppel-like factor 4 (KLF4), Nanog, c-Myc and pre-B-cell leukemia transcription factor 1 (PBX1) are involved in induction, maintenance or other functions of CSCs [2-4,49] **(Table 1).** High expression of Oct4 (also known as POU5F1, Oct3 or Oct3/4) is associated with self-renewal, metastasis and therapy resistance [4,50]. In addition, Oct4 activates migration-related gene CXCR4 and invasion-related gene MMP2, MMP9 and TIMP1 [51]. SOX2 also plays important roles in CSC formation, CSC stemness maintenance, tumor initiation, metastasis, EMT induction, resistance to apoptosis and therapy resistance [52]. KLF4, a zinc finger transcription factor, is involved in induction of pluripotent stem cells (iPSCs) and cell cycle arrest [53]. The Myc family consists of c-Myc, L-myc and N-Myc, but c-Myc is relevant to CSC induction. It is indicated that c-Myc maintains CSC stemness and induces CSC-associated metastasis and therapy resistance [54]. Another transcription factor Nanog regulates CSC

stemness, EMT induction, immune evasion and therapy resistance [55]. In addition, PBX1 regulates CSC stemness and induces therapy resistance [56].

Post-transcriptional controls

There are several mechanisms of post-transcriptional control of CSCs/LSCs. However, RNA-binding proteins, (RBPs) RNA alternative splicing(AS) and noncoding RNAs are relevant to the functions of CSCs [2,4] **(Table 1).**

RNA-Binding Proteins

RNA-binding proteins are post-transcriptional regulators of mRNA maturation, turnover, localization and translation [57]. RBPs are also involved in CSC induction. For instance, RBP Musashi (MSI-1 and MSI-2) controls CSC stemness maintenance, proliferation and therapy resistance, and is up-regulated in breast, ovarian and endometrial cancers [58,59]. Another RBP Lin 28 (Lin28a and Lin28b) blocks the synthesis of a tumor suppressor microRNA let-7 and promotes CSC survival and metastasis in ovarian cancer, germ cell tumor and teratoma [60].

RNA Alternative Splicing

RNA alternative splicing is another mechanism to induce CSCs [2]. For instance, exon-8,9 deleted mis-spliced glycogen synthase kinase 3β (m-GSK3β) induces blast crisis (BC) of CML and LSC generation [61]. In chronic phase (CP), granulocyte-macrophage progenitors (GMPs)

Table 1. *Involvement of transcription factors and post-transcriptional control in induction, maintenance and other functions of CSCs.*

Molecules	Mechanisms	CSC-associated functions	References
Oct4	Transcription factors	Self-renewal, metastasis, therapy resistance	[50]
SOX2	Transcription factors	CSC formation, metastasis, therapy resistance	[52]
KLF4	Transcription factors	Induction of iPSCs, cell cycle arrest	[53]
c-Myc	Transcription factors	Stemness maintenance, metastasis, therapy resistance	[54]
Nanog	Transcription factors	Stemness maintenance, immune evasion, therapy resistance	[55]
PBX1	Transcription factors	Stemness maintenance, therapy resistance	[56]
MSI	Post-transcriptional control (RBP)	Stemness maintenance, proliferation, therapy resistance	[58,59]
LIN28	Post-transcriptional control (RBP)	Survival, metastasis	[60]
exon-8,9-deleted GSK3β	Post-transcriptional control (RNA AS)	Increased expression of β-catenin, LSC generation of CML	[61]
mis-spliced BCL-XL, SHP-1, PYK2	Post-transcriptional control (RNA AS)	LSC generation of AML	[63]
miRNA21, miRNA221	Post-transcriptional control (miRNA)	Stemness maintenance, metastasis, therapy resistance	[64]
ROMP	Post-transcriptional control (lncRNA)	Stemness maintenance, relapse, therapy resistance	[66]

Abbreviations. *CSC=cancer stem cell, LSC=leukemic stem cell, Oct4=octamer-binding transcription factor 4, SOX2=sex determining region Y-Box-2, KLF4=Krüppel-like factor 4, PBX1=pre-B-cell leukemia transcription factor 1, MSI=Musashi, iPSC=pluripotent stem cell, RBP=RNA-binding protein, GSK3β=glycogen synthase kinase 3β, AS=alternative splicing, CML=chronic myeloid leukemia, AML=acute myeloid leukemia, miRNA=microRNA, lncRNA=long noncoding RNA, ROMP=regulator of phospholipid metabolism.*

of CML express high level of *Bcr-Abl fusion* gene products, while high expression of β-catenin in GMP reflects the progression from CP to BC CML [62]. Full length of GSK3β destructs β-catenin, but m-GSK3β does not reduce the expression of β-catenin. In BC GMP, functionally defected m-GSK3β is highly expressed and aberrant high expression of β-catenin is induced, resulting in self-renewing LCS generation of CML [61]. In addition, mis-spliced *BCL2L1* (BCL-XL), *PTPN6* (also known as SHP-1) and *PTK2B* (PYK2) are associated with LSC generation of AML [63].

Noncoding RNAs

In addition, there are many noncoding RNAs, but microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) are important for CSC regulation in cancer. Of numerous miRNAs, miRNA21 and miRNA221 are relevant to CSC stemness maintenance, CSC-associated metastasis and CSC-associated therapy resistance in various cancers [64]. Many lncRNAs also play roles in CSC-associated tumorigenesis and CSC-associated metastasis [65]. For instance, a lncRNA ROMP (regulator of phospholipid metabolism) in breast cancer up-regulates a phospholipid metabolism-associated PLA2G16 by stabilizing PLA2G16 mRNA and activates PI3K/AKT, WNT/β-catenin, and Hippo/YAP signaling pathways, resulting in CSC stemness maintenance, relapse and therapy resistance [66].

Epigenetics

Epigenetic modification is one of the post-transcriptional control in CSC initiation and cancer promotion. Epigenetics is defined as "an epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence." [67] Since the term 'epigenetics' was proposed by Conrad Waddington in 1942 [68], various roles of epigenetic modulation in cancer initiation, progression, metastasis and therapy resistance have been clarified [69,70]. Recently, involvement of epigenetic alterations in tumor oncogenesis and progression via CSC has been intensively investigated [71,72] (**Table 2**). Many epigenetic modifications are independent of genetic alterations. However, at the initial stage, epigenetic alterations in DNA methylation and chromatin remodeling can be caused by genetic mutations [71]. In epigenetic modification of CSC induction, DNA modulation (DNA methylation and demethylation) and chromatin modification (remodeling) are critical mechanisms.

DNA Methylation

DNA methylation of cytosine-phosphate-guanine islands (CpGIs) that are located in the promoter regions of genes affects activation or repression of associated genes,

Table 2. *Epigenetic alterations in CSCs and LSCs, caused by gene mutations.*

Mutations	Epigenetic types	Mechanisms	Diseases	References
DNMT3A(R882H)	DNA methylation	Activation of stemness genes (*Meis1, Mn1* and *Hoxa*)	AML	[75]
DNMT3A(R882H)	DNA methylation	Suppression of differentiation-associated genes (*Cebpa*, *Cebpe* and *PU.1*) via recruitment of PRC1	AML	[76]
DNMT3A and TET2 mutations	DNA methylation	Enhanced self-renewal of HSCs and pre-LSCs with inhibition of differentiation	AML	[77]
IDH1(R132H)	DNA methylation	Induction of cell proliferation and CD24-positive stem cell feature	Glioma	[78]
H3F3A(K27M)	Histone modification	PRC2 activity inhibition by H3.3(K27M), reduction of H3K27me3, and induction of stemness properties	DIPG	[80]
EZH2 and TET2 mutations	Histone modification	Induction of HSC proliferation and MDS phenotype with reduction of H3K27me3	MDS, MDS/MPN	[81]

Abbreviations. *CSC=cancer stem cell, LSC=leukemic stem cell, HSC=hematopoietic stem cell, DNMT=DNA methyltransferase, IDH=isocitrate dehydrogenase, PRC2=polycomb repressive complex 2, AML=acute myeloid leukemia, TET=ten eleven translocation, DIPG=diffuse intrinsic pontine glioma, EZH2=enhancer of zeste homolog 2, MDS=myelodysplastic syndrome, MDS/MPN=myelodysplastic/myeloproliferative neoplasm*.

inducing activation of self-renewal capacity of CSCs and LSCs. DNA methyltransferases (DMTF1, DMTF3A and DMTF3B) transfer a methyl group to C5 of cytosine in CpG islands, converting 5-methyl cytosine (5mC). On the contrary, ten eleven translocation dioxygenases (TET1, TET2 and TET3) start the demethylation cycle by hydroxylation of 5mC into 5-hydroxymethyl cytosine (5hmC) and by further oxidation into 5-formyl cytosine (5fC) and 5-carboxyl cytosine (5caC). Then, thymine DNA glycosylase (TDG) converts 5fC and 5caC into unmethylated cytosine, resulting in termination of the demethylation cycle [73]. In addition, isocitrate dehydrogenase (IDH) that converts isocitrate to α-ketoglutarate is a cofactor for TET [2]. Hypermethylation of CpG islands in promoter regions induces silencing of corresponding genes. However, since the gene encoding DNA methyltransferase 3A DNMT3A (*DNMT3A*) is frequently mutated (20~30% in AML and 10~20% in other hematological malignancies) [74] and the loss-of-function of mutated DNMT3A inhibits its enzymatic function, cell expansion of pre-LSCs is induced [71]. For instance, DNMT3A(R882H) mutant directly binds to *cis*-regulatory enhancer sites, inducing focal hypomethylation of CpGIs, and activates stemness genes (*Meis1-Mn1-Hoxa* gene cluster), which contributes to expansion of LSCs in AML [75]. Furthermore, in DNA-methylation independent manner, DNMT3A(R882H) mutant recruits polycomb repressive complex 1 (PRC1), and PRC1 suppresses the expression of differentiation-associated genes (*Cebpa*, *Cebpe* and *PU.1*), resulting in block of differentiation of LSCs in AML [76]. A combination of *DNMT3A* and *TET2* also induces enhancement of self-renewal of hematopoietic stem cells (HSC) and pre-LSCs and inhibits their differentiation [77]. In addition, frequent *IDH1(R132H)* mutation is observed in low grade gliomas. With hypermethylation of CpGIs, IDH(R132H) mutant induces proliferation of astrocytes and a CD24-positive stem cell feature [78].

Histone Modification

There are many histone modifications such as methylation, acetylation, phosphorylation, ubiquitylation, sumoylation or ADP ribosylation [79]. Of these, histone methylations at lysine (K) and arginine (R) residues are relevant to CSC control [4], and histone lysine methylation is carried out at three levels, i.e., mono-, di- and tri-methylation. Gene activation is induced by histone H3 lysine 4 (H3K4), histone H3 lysine 36 (H3K36) and histone H3 lysine 79 (H3K79), while gene repression is associated with histone H3 lysine 9 (H3K9), histone H3 lysine 27 (H3K27) and histone H4 lysine 20 (H4K20) [72]. *H3F3A* encodes the H3 variant H3.3. Over 70% of diffuse intrinsic pontine gliomas (DIPGs) harbor *H3F3A* mutation, and the most common alteration is H3.3(K27M), i.e., lysine K27 is replaced by methionine M. The H3.3(K27M) inhibits polycomb repressive complex 2 (PRC2)-mediated methyltransferase activity via binding to the catalytic site of the PRC2, resulting in reduced expression of H3K27me3 [80]. Finally reduction of H3K27me3 induces stemness properties of neural precursor cells. Enhancer of zeste homolog 2 (EZH2) is a core component of PRC2. According to Muto et al. [81], *EZH2* mutations and *TET2* mutations are found in 11.8% (14/119) and 19.3% (23/119) of the total 119 patients with myelodysplastic syndrome (MDS) and myelodysplastic/myeloproliferative neoplasm (MDS/MPN), respectively, and the concurrent *EZH2* and *TET2* mutations are found in 6.7% (8/119) of these patients. Concurrent depletion of *EZH2* and *TET2* induces proliferation of HSC and generates an MDS phenotype in mice with reduction of H3K27me3 [81].

Signaling pathways

In addition to the intrinsic factors above mentioned, various extrinsic factors (signaling pathways and metabolic controls including hypoxia) in TME are involved in CSC induction [4]. Signaling pathways (Notch, Hedgehog, WNT, TGF-β, signal transducer and activator of transcription 3 [STAT3] and nuclear factor κ B [NF-κB]) are relevant to CSC induction [2,4,72] (**Table 3**).

Notch

Notch is a cell-contact dependent signaling and Notch receptor is a transmembrane receptor. There are four Notch receptors (Notch1-4) and six ligands (DLL1-4 and JAG1/2). When a ligand binds to the Notch receptor, Notch intracellular domain (NICD) is released from the receptor via successive cleavages by furin-like protease, ADAM protease and γ-secretase. Then, NICD translocates to the nucleus and forms a Notch transcription complex (NTC) that consists of NICD, the DNA binding factor C-protein binding factor/Suppressor of Hairless/Lag-1 (CSL, also known as recombination signal binding protein for immunoglobulin κ J region [RBP-J]) and coactivators of the mastermind-like (MAML) families. The NTC binds to Notch regulatory elements (NREs) and recruits transcriptional coregulators, leading to initiation of transcription of Notch

target genes [82]. In esophageal adenocarcinoma cell lines, Notch induces CSC subpopulation (induction of CSC surface markers LDH1, CD133, CD25, LGR5 and MSI2) and CSC maintenance (CSC-associated transcription factors Oct4, MSI and SOX2) [83]. CSC formation induced by Notch signaling is also found in glioblastoma, ovarian cancer and breast cancer [82].

Hedgehog

There are three Hedgehog ligands (Sonic Hedgehog [Shh], Indian Hedgehog and Desert Hedgehog). When the Shh ligand binds to the 12-domain transmembrane receptor Patched (PTCH1 and PTCH2), the inhibition of another 7-domain transmembrane receptor Smoothered (SMO) by PTCH is derepressed. Then, activated SMO inhibits Suppressor of Fused (FUSU), inducing the dissociation of glioma-associated oncogene homolog (GLI1, GLI2 and GLI3) from SUFU. Finally, full-length GLI (or GLI activator [GLIA]) translocates into the nucleus and activates transcription of target genes [83a]. This canonical Shh signaling pathway is supposed to induce CSC via activation of CSC-associated transcription factors (Nanog, c-Myc and Oct-4) in pancreatic cancer [84]. Induction of CSC or LSC by the Shh signaling is also observed in breast cancer, prostate cancer, glioblastoma, multiple myeloma and CML [84a]. Crosstalk between canonical Shh signaling and NF-κB signaling is important for malignant progression of breast cancer [85].

WNT

The canonical WNT signaling pathway involves the transcription cofactor β-catenin in transcription regulation of target genes. When the WNT ligand binds to the Frizzled (Fzd) receptor and the coreceptor low density lipoprotein receptor-related protein 5/6 (LDR5/6), the Dishevelled (Dvl) protein is recruited. Then, the Dvl relieves the inactivation of β-catenin by the degradation complex consisting of Adenomatous polyposis coli (APC), Axin, GSK-3β and casein kinase 1 (CK1), leading to the stabilization of β-catenin and its translocation into the nucleus. The β-catenin forms the transactivation complex with T-cell specific factor (TCF)/lymphoid enhancer-binding factor (LEF). This β-catenin-TCF/LEF complex activates expression of target genes [86]. In CRC, the CSC surface markers Lgr5, CD44, CD133 and CD166 are up-regulated by the canonical WNT/β-catenin signaling [87]. CSC induction by the canonical WNT/β-catenin signaling is found in breast cancer, gastric cancer, pancreatic cancer and melanoma [4].

Table 3. *Signaling pathways that induce CSCs.*

Signaling pathways	Mediated by	Target molecules	Diseases	References
Notch	NTC (= NICD/CSL/MAML complex)	CSC surface markers (LDH1, CD133, CD25, LGR5, MSI2), CSC-associated transcription factors (Oct4, MSI, SOX2)	Esophageal cancer	[83]
Canonical Hedgehog	GLI	CSC-associated transcription factors (Nanog, c-Myc, Oct-4)	PDAC	[84]
Canonical WNT/β-catenin	β-catenin-TCF/LEF complex	CSC surface markers (Lgr5, CD44, CD133, CD166)	CRC	[87]
TGF-β	SMAD2/SMAD3/SMAD4 complex	CSC surface marker (CD166), CSC-associated transcription factor (SOX2)	Breast cancer	[89]
Canonical IL-6/JAK/STAT3	STAT3 dimer	CSC surface marker (CD133)	HCC	[92]
Canonical NF-κB	p50/RelA heterodimer	CSC surface marker (CD44), CSC-associated transcription factors (Nanog, SOX2, Oct4)	Ovarian cancer	[95]

Abbreviations. *NTC=Notch transcription complex, NICD=Notch intracellular domain, CSL=C-protein binding factor/Suppressor of Hairless/Lag-1, MAML=mastermind-like, GLI= glioma-associated oncogene homolog, PDAC=pancreatic ductal adenocarcinoma, TCF=T-cell specific factor, LEF=ymphoid enhancer-binding factor, CRC=colorectal cancer, TGF-β=transforming growth factor-β, SMAD=mothers against decapentaplegic homolog, IL-6=interleukin 6, JAK= Janus kinase, STAT3=signal transducer and activator of transcription 3;HCC=hepatocellular carcinoma, NF-κB= nuclear factor κ B.*

TGF-β

TGF-β is a family of ligands to the TGF receptor type 1 (TβR1) family and the TβR2 family [88]. When a TGF-β binds to the TβR2, the TβR1 is recruited, and the complex composing TGF-β, TβR1 and TβR2 is formed. Then, the activated TGF-β/TβR1/TβR2 phosphorylates mothers against decapentaplegic homolog 2 (SMAD2) and SMAD3, and the complex of phosphorylated SMAD2/SMAD3 that is combined with SMAD4 translocates into the nucleus. The complex SMAD2/SMAD3/SMAD4 binds to the promoter regions of target genes, inducing activation of these genes. In breast cancer, the TGF-β/SMAD signaling activates expression of the CSC surface marker CD166 and the CSC-associated transcription factor SOX2 [89]. CSC formation is induced by the TGF-β/SMAD signaling also in gastric cancer, HCC, lung cancer and pancreatic cancer [3].

STAT3

STAT3 signaling is activated by various signaling pathways [90]. However, the STAT3 signaling pathway via interleukin 6 (IL-6) and Janus kinase (JAK), i.e., the IL-6/JAK/STAT3 signaling pathway, is essentially important for its contribution to CSC induction [90]. In TME, IL-6 is highly expressed, and this elevated IL-6 directly binds to IL-6 receptor (IL-6R, or IL-6Rα) on the cell surface, and the IL-6/IL-6R forms a trimer with glycoprotein 130 (gp130, also known as IL-6Rβ or CD130), i.e., the trimer complex IL-6/IL-6R/gp130. The pair of this activated trimer forms a heterohexamer complex consisting of two IL-6, two IL-6R (= IL-6Rα) and two gp130 (= IL-6Rβ). Then, this heterohexamer complex IL-6/IL-6R/gp130 recruits and phosphorylates JAKs (JAK1, JAK2, JAK3 and tyrosine protein kinase 2 [TYK2]), and the activated JAKs phosphorylate further STAT3 at the Tyr702 residue, resulting in dimerization of STAT3 and translocation of the STAT3 dimer into the nucleus [91]. Finally, the STAT3 dimer binds to the consensus response elements in the promoter of target dimer genes, inducing activated transcription of numerous target genes encoding regulators of various cellular functions including CSC generation. This canonical IL-6/JAK/STAT3 signaling induces CSCs that is confirmed by activated expression of CSC surface marker CD133 in HCC [92]. CSC generation by the IL-6/JAK/STAT3 signaling is also confirmed in breast cancer, colon cancer and prostate cancer [2]. Interaction between the IL-6/JAK/STAT3 signaling and HIF-1α is indicated [92].

NF-κB

The NF-κB family of transcription factors consists of five members, i.e., p65 (RelA), RelB, c-Rel, p50 (its precursor is p105) and p52 (its precursor is p100). NF-κB signaling pathways are classified into canonical and noncanonical ones. Canonical NF-κB signaling is activated by various cell membrane receptors, including tumor necrosis factor receptor (TNFR), T-cell receptor (TCR), IL-1R, Toll-like receptor (TLR) and others. The NF-κB heterodimer p52/RelA is sequestered in the cytoplasm by inhibitor of NF-κB (IκB) proteins (IκBα, IκBβ, IκBε, p105 [p50 precursor], p100 [p52 precursor], Bcl3, IκBζ and IkBNS) in the form of IκB/p52/RelA complex. When canonical NF-κB signaling is activated, IκB is phosphorylated by the inhibitor of NF-κB kinase (IKK) complex, which consists of two catalytic subunits IKKα and IKKβ and a regulatory subunit IKKγ (also known as NF-κB essential modulator [NEMO]). Then, the NF-κB heterodimer p52/RelA is released from IκB, and the released p52/RelA translocates into the nucleus and activates transcription of target genes [93,94]. Noncanonical NF-κB signaling is activated by tumor necrosis factor receptor (TNFR) superfamily members. Another NF-κB heterodimer p100/RelB is in the cytoplasm. When noncanonical NF-κB signaling is activated, NF-κB-inducing kinase (NIK) is activated. Then, NIK activates IKKα, and IKKα in turn phosphorylates p100 and induces ubiquitination of p100 to generate p52, leading to translocation of the NF-κB heterodimer p52/RelB into the nucleus for transactivation of target genes [93,94]. In ovarian cancer, activation of the canonical NF-κB signaling by TLR2 enhances CSC self-renewal, increased expression of a CSC surface marker (CD44), induction of CSC-association transcription factors (Nanog, SOX2 and Oct4), and activation of IL-6 [95]. CSC generation by NF-κB signaling is also observed in breast cancer, prostate cancer, pancreatic cancer and glioblastoma [2]. Crosstalk of canonical NF-κB signaling with STAT3, WNT/β-catenin and Notch signaling is indicated [2]. In addition, crosstalk of noncanonical NF-κB signaling with AKT is suggested [93].

HIF UNDER HYPOXIA IN TME

TME

TME consists of tumor cells, mesenchymal stem cells (MSCs), immune cells (regulatory T cell [Treg], cytotoxic T cell, helper T cell [Th], B cells, natural killer cells [NK-cells], neutrophils, tumor-associated macrophages [TAMs],

and myeloid-derived suppressive cells [MDSCs]), stromal cells (cancer-associated fibroblasts [CAFs], vascular endothelial cells, adipocytes, and stellate cells), dendritic cells, signaling molecules, cytokines, blood vessels, and the extracellular matrix (ECM) [6,96]. In addition, there are metabolic controls such as glycolysis, glutaminolysis, lipogenesis and hypoxia in TME [2,4,6]. These cellular and non-cellular components in TME induce CSCs, metastasis of cancer cells and resistance to chemotherapy and radiotherapy [2-4,8,49,97]. In TME, a hypoxia stress causes serious conditions for maintenance and proliferation of cancer cells. To compensate its negative outcomes, hypoxia inducible factors, HIF-1α, HIF-2α and HIF-3α, are induced [13,98,99]. Certainly MSCs [100,101], TAMs [101a], MDSCs [102] and CAFs [103] play supportive roles in cancer promotion, but HIFs are the most crucial to driving cancer initiation, progression, metastasis and survival under hypoxia [5,7,13,16,99,104,105]. In addition, HIFs activate CA9, and this HIF/CA9 axis promotes oncogenesis of various solid tumors [10,11,106] and lymphomas [13,107,108] including ATL [12,16,17]. Thus, we focus on HIF and CA9.

Regulation of HIFs

Hypoxia in TME up-regulates first HIF-1α, then HIF-2α and finally HIF-3α. HIF-1α and HIF-2α are transcription activators in context-dependent manners, while HIF-3α may play a inhibitory role in HIF-dependent transcription. Under hypoxia, stable HIF-1α accumulates in the cytoplasm. Then, HIF-1α translocates into the nucleus and forms a heterodimer with constitutively expressed HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator [ARNT]), and the HIF-1α/HIF-1β heterodimer complex with recruitment of CBP/p300 transcriptional coactivator directly binds to the hypoxia responsive element (HRE) on the promoter region of target genes to activate transcription of mRNAs [5]. Similarly, HIF-2α forms a heterodimer complex HIF-2α/HIF-1β. Transcriptional activation by HIF-1 and HIF-2 under hypoxia stimulates broad target genes [5,109].

Noncanonical Activation of HIFs by Other Signaling Pathways

In addition to the induction of HIF-1α under hypoxia (known as canonical regulation of HIF), expression of mRNA and protein of HIF-1α is increased by PI3K/AKT/mTOR and/or RAS/MER/ERK signaling by activation of HIF-1α mRNA translation (noncanonical regulation of HIF) [109-112] (**Table 4**). Eukaryotic mRNA translation is composed of four successive phases, i.e., 1. initiation, 2. elongation, 3. termination, and 4. ribosome recycling [113-115]. The mRNA has two characteristic structures, i.e., 5′ 7-methylguanosine (m7G) cap [113,116] and 3′ poly(A) tail [117]. The translation initiation of mRNA is processed in eight phases [113,118,119]: 1. Formation of the ternary complex (eIF2 [eukaryotic translation initiation factor 2], GTP and initiating methionyl tRNA [Met-tRNAi]; 2. Formation of the 43S pre-initiation complex (PIC) that consists of the ternary complex (eIF2/GTP/Met-tRNAi), the 40S ribosomal subunit, eIF1, eIF1A, eIF3 and eIF5 [120]; 3. Activation of mRNA by the eIF4F complex, which consists of eIF4E, eIF4G and eIF4A [114], with assistance

Table 4. *Noncanonical activation of HIFs by various signaling pathways.*

Signaling pathways	Mechanisms	Effects	References
PI3K/AKT/mTOR	Activation of 4E-BPs by mTORC1	Activation of HIF-1α mRNA translation	[122,123]
PI3K/AKT/mTOR	Activation of S6Ks by mTORC1	Activation of HIF-1α mRNA translation	[123-124]
PI3K/AKT/mTOR	Loss of *PTEN*	Activation of HIF-1α mRNA translation	[125,126]
RAS/ERK	Activation of RSKs by ERK1/2	Activation of HIF-1α mRNA translation	[119,127]
RAS/ERK	Activation of MNKs by ERK1/2	Activation of HIF-1α mRNA translation	[119,128]
p38 MAPK	Activation of MNKs by p38 MAPK	Activation of HIF-1α mRNA translation	[119,128]
RAS/ERK	Activation of CBP/p300 by ERK1/2	Activation of HIF-1α transcription activity	[129]
NF-κB	Binding of p50/RelA to the *HIF-1α* promoter region	Activation of HIF-1α mRNA transcription	[130-132]

Abbreviations. *HIF=hypoxia inducible factor, PI3K=phosphatidylinositol-3-kinase, mTOR=mechanistic target of rapamycin, 4E-BP=4E-binding protein, mTORC1=mechanistic target of rapamycin complex 1, S6K=S6 kinase, PTEN=phosphatase and tensin homolog, ERK=extracellular signal-regulated kinase, RSK=ribosomal S6 kinase, MAPK=mitogen-activated protein kinase, MNK=MAPK-interacting kinase*

of eIF4B and poly(A)-binding protein (PABP) [121]; 4. Attachment of the 43S PIC to the activated mRNA; 5. Scanning of the 5´ UTR of mRNA in 5´ to 3´ direction by 43S PIC; 6. Recognition of start codon (AUG) and formation of the 48S initiation complex; 7. Joining of 60S ribosomal subunit to the 48S initiation complex and concomitant release of eIF2-GDP and other factors, including eIF1, eIF3, eIF4B, eIF4F and eIF5; 8. Hydrolysis of eIF5B-bound GTP and release of eIF1A and GDP-bound eIF5B from the 80S initiation complex of ribosome. Then elongation phase starts.

When PI3K is activated by ligand binding, activated PI3K activates AKT and then mTOR complex 1 (mTORC1). mTORC1 is involved in translation activation of mRNA via 4E-binding proteins (4E-BPs: 4E-BP1, -BP2 and -BP3) and S6 kinases (S6Ks: S6K1 and S6K2) [109]. On the one hand, mTORC1 phosphorylates 4E-BPs [110,114,115]. Phosphorylation of 4E-BPs induces their release from eIF4E, leading to the association of eIF4E with eIF4G and, finally, the assembly of the mRNA-cap binding eIF4F complex (eIF4E, eIF4G and eIF4A). The activated eIF4F complex induces activation of mRNA and stimulates translation of HIF-1α mRNA [122-124]. On the other hand, mTORC1 activates S6Ks [100,114,115]. Then activated S6Ks phosphorylate multiple components of translation, and finally activate eIF4B, leading to the activation of HIF-1α mRNA translation [122-124]. In addition, phosphatase and tensin homolog (PTEN) is a negative regulator of PI3K. Loss of *PTEN* in turn activates the translation of HIF-1α mRNA [125,126]. Similarly, RAS/ERK signaling is also involved in activation of HIF-1α mRNA translation via activation of 90 kDa ribosomal S6 kinases (RSKs) and MAPK-interacting kinases (MNKs) [119]. On the one hand, ERK1/2 activate RSKs. Then activated RSKs activate eIF4B [127], leading to the activated translation of HIF-1α mRNA [119]. On the other hand, ERK1/2 activate MNKs. Then activated MNKs phosphorylate eIF4E [128], resulting in activated translation of HIF-1α mRNA [119]. Furthermore, p38 mitogen-activated protein kinase (MAPK) also activates MNKs [128], leading to the activated translation of HIF-1α mRNA [119]. In addition, ERK1/2 phosphorylate CBP/p300, resulting in activation of HIF-1α transcription activity [129]. Transcription of the HIF-1α mRNA is also increased by direct binding of NF-κB p50/RelA to the HIF-1α promoter region -197 to -188 [130-132].

Induction of CSCs and LSCs by HIFs

HIF-1α induces CSCs and LSCs by activating various signaling pathways [8] (**Table 5**). For instance, HIF-1α induces CSCs that are confirmed by the CSC surface markers (ABCG2 and CD133) and the CSC-associated transcription factors (SOX2, Oct4 and ALDH1) via activation of miR-1275, WNT/β-catenin and Notch signaling in lung adenocarcinoma (LAC) [133]. HIF-1α also activates Notch signaling, resulting in induction of CSCs with a surface marker CD44 in breast cancer [134]. Furthermore, HIF-1α up-regulates expression of transcription factors ALDH1A1, Nanog and SOX2 through activation of WNT/β-catenin signaling in esophageal squamous cell carcinoma [135]. In addition, HIF-2α induces CSCs (confirmed by CSC-associated transcription facrtors c-Myc, Oct4 and Nanog) via activation of WNT and Notch signaling in breast cancer [136]. HIF-2α also protects LSCs (with a surface marker CD34) from apoptosis induced by reactive oxigen species (ROS) in AML [137]. CSC/LCS induction by HIF-1α and HIF-2α is also confirmed in gastric cancer, pancreatic cancer, prostate cancer or bladder cancer [8].

Signaling Pathways and Target Genes via HRE activated by HIFs

In turn, the HIF-1α/HIF-1β complex activates various signaling pathways (**Table 6**), including Notch [133,134], WNT [133,135], STAT3 [138], TNF-β [139], Hedgehog [140], NF-κB [141], PI3K/AKT/mTOR [142] or p38 MAPK [143] signaling pathways [104,144]. As already noted, the HIF-2α/HIF-1β activates Notch and WNT signaling pathways [136].

In addition, HIFs activates various target genes via HRE. Taken together, HIFs are involved in a plethora of functions, including angiogenesis (vascular endothelial growth factor [VEGF], TGFs [transforming growth factors]), erythropoiesis (erythropoietin [EPO]), proliferation (insulin-like growth factors [ILGs], TGF-α, EPO), apoptosis inhibition (Bcl2, BAX), immune escape (MDSCs, programmed cell death protein 1 [PD-1], programmed cell death protein 1 lignad 1 [PD-L1]), energy metabolism (glucose transporter 1 [GLUT1], GLUT3, hexokinase 1 [HK1], HK2, monocarboxylate transporter 4 [MCT4]), acidosis/pH regulation (CA9), metastasis (matrix metalloproteinase 2 [MMP2], MMP9), and therapy resistance (EMT induction [Snail, Twist, ZEB], CSC induction [SOX2, Oct4, ALDH1, Nanog]) [5,99,145,146].

Table 5. *Induction of CSCs/LSCs by HIF and CA9 under hypoxia.*

Signaling	Mediated by	Targeted molecules	Diseases	References
HIF-1α/ HIF-1β	miR-1275, WNT, Notch	CSC surface markers (ABCG2, CD133), CSC-associated transcription factors (SOX2, Oct4, ALDH1)	LAC	[133]
HIF-1α/ HIF-1β	Notch	CSC surface marker (CD44)	Breast cancer	[134]
HIF-1α/ HIF-1β	WNT/β-catenin	CSC-associated transcription factors (ALDH1A1, Nanog, SOX2)	Esophageal SSC	[135]
HIF-2α/ HIF-1β	WNT, Notch	CSC-associated transcription factors (c-Myc, Oct4, Nanog)	Breast cancer	[136]
HIF-2α/ HIF-1β	Suppression of ROS	LSC surface marker (CD34)	AML	[137]
CA9	mTORC1, Notch	CSC surface marker (CD44, CD133)	Breast cancer	[164]
CA9	WNT/β-catenin	CSC-associated transcription factors (Nanog, SOX2)	Breast cancer	[9]

Abbreviations. *CSC=cancer stem cell, LCS=leukemia stem cell, HIF=hypoxia inducible factor, LAC=lung adenocarcinoma, SSC=squamous cell carcinoma, ROS=reactive oxigen species, AML=acute myeloid leukemia, CA9= carbonic anhydrase IX, mTORC1=mechanistic target of rapamycin complex 1.*

Table 6. *Signaling pathways activated by HIF-1α and HIF-2α.*

HIFs	Activated signaling pathways	References
HIF-1α/HIF-1β	Notch	[133,134]
HIF-1α/HIF-1β	WNT	[133,135]
HIF-1α/HIF-1β	STAT3	[138]
HIF-1α/HIF-1β	TNF-β	[139]
HIF-1α/HIF-1β	Hedgehog	[140]
HIF-1α/HIF-1β	NF-κB	[141]
HIF-1α/HIF-1β	PI3K/AKT/mTOR	[142]
HIF-1α/HIF-1β	p38 MAPK	[143]
HIF-2α/HIF-1β	Notch	[136]
HIF-2α/HIF-1β	WNT	[136]

Abbreviations. *HIF=hypoxia inducible factor, STAT=signal transducer and activator of transcription, TNF=tumor necrosis factor, NF-κB=nuclear factor kappa B, PI3K=phosphatidylinositol-3-kinase, mTOR=mechanistic target of rapamycin, MAPR=mitogen-activated protein kinase.*

CA9 UNDER HYPOXIA IN TME

Regulation of CA9

Under hypoxia in TME, CA9 is induced by direct binding of HIF-1α/HIF-1β with recruitment of the CBP/p300 transcriptional coactivator [147] to the HRE [148] in the *CA9* promoter region [99,149-152]. *CA9* is activated exclusively by the HIF-1α/HIF-1β, but by neither HIF-2α/HIF-1β nor HIF-3α/HIF-1β [153,154]. In the *CA9* promoter region (−173; +31) [155], there are 6 *cis*-acting elements, i.e., five with a positive promoter function (HRE, protected region [PR]1, PR2, PR3 and PR5) and one with a negative repressor function (PR4) [150]. Activator protein-1 (AP-1) binds to the PR2 [155,156] and enhances the transcription of *CA9* [150]. AP-1 is a transcription factor of leucine zipper family and forms a homodimer jun/jun (c-jun, junB, junD) or a hererodimer jun/fos (s-fos, fosB, fra-1, fra-2, ATF-2, CREB) [157]. For activation of AP-1, Jun is phosphorylated by c-JUN N-terminal kinase (JNK) [157,158,159,160], while fos is phosphrylated by p38 MAPK [159,162] in the ERK/MAPK signaling pathway. In addition, specificity protein-1 (SP-1) and SP-3 bind to PR3 and/or PR5 [156,163] to enhance transcription of *CA9*.

Induction of CSCs by CA9

CA9 induces CSCs by activating several signaling pathways (**Table 5**). Once activated by the HIF-1α/HIF-1β with recruitment of CBP/p300, CA9 activates mTORC1 and then Notch singling, resulting in induction of CSCs identified by the CSC surface markers CD44 and CD133 in breast cancer [164]. In addition, CA9 induces CSC-associated transcription factors Nalog and SOX-2 via the activation of WNT/β-catenin in breast cancer [9].

Multiple Functions of CA9 in Cancer Initiation, Proliferation, Survival And Metastasis

Involvement of CA9 in cancer initiation, promotion and metastasis has been intensively investigated. Thus, it is indicated that CA9 plays multiple roles in oncogenesis (**Table 7**). First, CA9 corrects intracellular pH to counterbalance the acidosis caused by excessed glycolysis in cancer cells, allowing cancer cell proliferation [165-167]. In fact, high expression of CA9 is a marker of poor prognosis and is observed in various solid tumors [10,11], including NSCLC [168-170], CRC [171,172,173], gastric cancer [174], HCC [175,176], PDAC [177,178], breast cancer [164,179,180,181], renal cell carcinoma [182,183], brain tumor [184] and cervical cancer [185] as well as in hematological malignancies such as classical Hodgkin lymphoma [186,187], B-cell non-Hodgkin lymphoma (NHL) [13,107,108], T-cell acute lymphoblastic leukemia/lymphoma [188] and ATL [12]. Second, CA9 induces inhibition of apoptosis, leading to cancer cell survival [185,189,190]. Third, CA9 is also involved in cell adhesion and invasion, promoting cancer cell metastasis [191-193]. Fourth, as already noted, CA9 induces CSCs in breast cancer [9,164]. This means that both cancer inititation and therapy resistance are caused by CA9. Fifth, CA9 also induces EMT in prostate cancer [194], resulting in therapy resistance. Sixth, high expression of CA9 in ATL cell lines is correlated with ability of tumorigenicity [12]. This CA9 functions also can promote lymphoma cell expansion and relapse.

Table 7. *Multiple functions of CA9 in cancer initiation, proliferation, survival and metastasis.*

Functions	Effects	References
Correction of intracellular pH	Proliferation	[165-167]
Apoptosis inhibition	Survival	[185,189,190]
Cell adhesion, invasion	Metastasis	[191-193]
CSC induction	Cancer initiation, therapy resistance	[9,164]
EMT induction	Therapy resistance	[194]
Tumorigenicity	Expansion, relapse	[12]

Abbreviations. *CA9=carbonic anhydrase IX, CSC=cancer stem cell, EMT=epithelial-to-mesenchymal transition.*

The HIF/CA9 axis and finalization of the ATL three-step oncogenesis

ATL is a refractory hematological malignancy [16,17,94,195-202], caused by human T-cell leukemia virus type 1 (HTLV-1) [196,203]. Since the early studies on ATL [204-208], its oncogenic steps have been for a long time in enigma [209-213], because the HTLV-1-derived oncogenes, i.e., Tax [214,215] and HTLV-1 basic leucine zipper (HBZ) [216-219], may not be the decisive factors to finalize its long-term multistep oncogenesis [16,17,94,220-224] (**Table 8**). On the contrary, additional events in host cells are suspected to be crucial to completion of its final step oncogenesis [220-224]. In this regard, the activated expression of NF-κB [221,225,226], AP-1 [227,228], AKT [229,230], HIFs [230] and CA9 [12] are the essentially important clues to clarification of the final step of the ATL three-step oncogenesis [223]. In fact, CA9 expression is confirmed on primary ATL cells in lymph nodes [12]. From these data, it is likely that the HIF-1α/CA9 axis may contribute to finalization of the three-step oncogenesis of ATL [16,94,223,224].

Therapies targeting CSCs

Induction of CSCs itself is a mechanism to escape from anti-cancer therapies such as cytotoxic chemotherapy, radiotherapy, molecular targeted therapy and immunotherapy. Thus, development of novel therapies targeting CSCs is a quite challenging issue.

Various Inhibitors

To overcome this dilemma, various therapeutic preclinical and clinical trials have been pursued, including surface

Table 8. *Three-step oncogenesis of ATL.*

Steps	Clonality	Responsible molecules	References
I. Early	Polyclonal	Tax	[214,215]
II. Intermediate	Oligoclonal	HBZ	[216-219]
III. Final	Monoclonal	Additional events in host cells	[16,17,94,220-224]

Abbreviations. *ATL=adult T-cell leukemia/lymphoma, HBZ=HTLV-1 basic leucine zipper factor.*

marker inhibitors, transcription factor inhibitors, metabolism inhibitors (including HIF-1α inhibitors), signaling inhibitors (Notch signaling inhibitors, Hedgehog signaling inhibitors, WNT/β-catenin signaling inhibitors, TGF-β signaling inhibitors, and JAK/STAT3 signaling inhibitors), targeting epigenetic modifications (DNMT inhibitors and histone deacetylase [HDAC] inhibitors), and immunotherapy (including CAR-T cell therapy) [2-4,6]. However, we have not yet obtained the sufficiently effective drugs targeting CSCs.

HIF-1α Inhibitors

Many clinical trials have been carried out by the direct HIF-1α inhibitor HZN-2986 (anti-sense HIF-1α oligonucleotide) and other indirect HIF-1α inhibitors including camptothecin (downregulating HIF-1α via HIF-1α-targeting miRNAs), rapamycin analog (suppressing the expression of HIF-1α protein via inhibition of mTORC1), digitoxin (suppressing the translation of HIF-1α mRNA), 2-methoxiestradiol (suppressing the translation of HIF-1α mRNA), HDAC inhibitor romidepsin (destabilizing HIF-1α), and proteasome inhibitors (suppressing HIF-1α at multiple levels). However, these trials cannot prove the sufficient efficacy against CSCs of various cancers (lymphomas, metastatic liver cancer, hepatocellular carcinoma, cervical cancer, colorectal cancer, prostate cancer, breast cancer, CML or gastric cancer) [7]. Toxicity is another problem in these clinical trials using these direct and/or indirect HIF-1α inhibitors.

Anti-CA9 Monoclonal Antibodies and CA9 inhibitors

Investigation into targeting CA9 with both monoclonal antibodies and small molecule inhibitors has been accelerated for clinical applications [231,232]. Girentuximab is a chimeric monoclonal antibody binding to the cell surface CA9 molecule [233]. The phase 2 clinical trial using girentuximab for patients with high-risk clear cell renal cell carcinoma after nephrectomy failed to prove the efficacy [234]. Now experimental trials by means of other novel monoclonal antibodies against CA9 [235-237], anti-CA9 antibody-drug conjugates (ADC) [238] and CA9-targeted chimeric antibody receptor (CAR)-T therapy [239,240,241] are under investigation. In contrast, preclinical trials by means of small molecule inhibitors of CA9 (SLC-0111 and RC44) have proved their efficacy [9,164,231,232]. The phase 1 study using the inhibitor SLC-0111 succeeded in proving its safety and tolerability [242]. Certainly efficacy of the single regimen of CA9 inhibitors is moderate, but the combination of the CA9 small molecule inhibitors with conventional cytotoxic chemotherapy [243], immunotherapy [244] or radiation therapy [245] are realistic therapies to target CSCs [232]. Furthermore, the multitargeting approaches by the combination of CA9 inhibitors with hybrid drugs against a variety of disorders have been developed [246]. Taken together, these novel combination therapies using CA9 inhibitors and other therapies can pave the way toward promising therapeutic strategies against CSCs.

Conclusion

CSCs/LSCs have self-renewal ability and cause initiation, progression, relapse and therapy resistance in various solid tumors and hematological malignancies. We showed that CSCs/LSCs are induced by the various intrinsic factors (epigenetic modulation [DNA methylation and histone modification], CSC-associated transcription factors and post-transcriptional control such as RBPs, RNA AS and miRNA/lncRNA) and the extrinsic factors (signaling pathways [Notch, Hedgehog, WNT, TGF-β, STAT3 and NF-κB signaling pathways] and metabolic factors (represenred by hypoxia) in TME. In particular, we clarified the essential roles of HIF and CA9 in not only CSC induction under hypoxia in TME but also promotion of cancer cell proliferation, survival, metastasis, and therapy resistance. Because of the therapy resistance caused by CSC itself, development of the therapies targeting CSCs is quite difficult. In fact, most of preclinical and clinical trials using inhibitors against the intrinsic and extrinsic factors that induce CSCs in TME have not yet proved the efficacy.

However, the preclinical trials by means of CA9 small molecular inhibitors have proved the efficacy and a clinical trial proved its safety and tolerance. The combination therapies of CA9 inhibitors with cytotoxic chemotherapy, immunotherapy and radiation therapy can offer promising therapeutic strategies against CSCs. From these analyses, it is likely that the HIF-1α/CA9 axis may contribute to finalization of the three-step oncogenesis of ATL. Further research is required for its detailed clarification.

Author Disclosure Statement

The author declares that there is no conflict of interests regarding this paper.

Acknowledgement

I cordially acknowledge Professor Emeritus Kazuo Sugamura, MD, PhD, at Tohoku University for his intensive instruction, encouragement and discussion about the ATL research on activation of AKT and CA9.

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Cite this article: Mitsuru Sakitani. *Induction of Cancer Stem Cells in Tumor Microenvironment and Involvement of HIFs And Carbonic Anhydrase IX in Oncogenesis of Solid Tumors and Hematological Malignancies Including Adult T-Cell Leukemia/Lymphoma (ATL). International Journal of Research in Medical and Clinical Sciences. 2024;2(1): 52-76.*