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Hypoxia-Induced Signaling Promotes Prostate Cancer Progression: Exosomes Role as Messenger of Hypoxic Response in Tumor Microenvironment

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Abstract

Prostate cancer (PCA) is the leading malignancy in men and the second leading cause of cancer-related deaths. Hypoxia (low O_2 condition) is considered an early event in prostate carcinogenesis associated with an aggressive phenotype. In fact, clinically, hypoxia and hypoxia-related biomarkers are associated with treatment failure and disease progression. Hypoxia-inducible factor 1 (HIF-1) is the key factor that is activated under hypoxia, and mediates adaptation of cells to hypoxic conditions through regulating the expression of genes associated with angiogenesis, epithelial-to-mesenchymal transition (EMT), metastasis, survival, proliferation, metabolism, stemness, hormone-refractory progression, and therapeutic resistance. Besides HIF-1, several other signaling pathways including PI3K/Akt/mTOR, NADPH oxidase (NOX), Wnt/β-catenin, and Hedgehog are activated in cancer cells under hypoxic conditions, and also contribute in hypoxia-induced biological effects in HIF-1-dependent and -independent manners. Hypoxic cancer cells cause extensive changes in the tumor microenvironment both local and distant, and recent studies have provided ample evidence supporting the crucial role of nanosized vesicles “exosomes” in mediating hypoxia-induced tumor microenvironment remodeling. Exosomes’ role has been reported in hypoxia-induced angiogenesis, stemness, activation of cancer-associated fibroblasts (CAFs), and EMT. Together, existing literature suggests that hypoxia plays a predominant role in PCA growth and progression, and PCA could be effectively prevented and treated via targeting hypoxia/hypoxia-related signaling pathways.

Keywords

hypoxia; hypoxia-inducible factor 1; prostate cancer; exosomes; biomarkers

I. INTRODUCTION

Prostate cancer (PCA) is the most common non-cutaneous cancer in men, and according to the American Cancer Society reports, 220,800 new cases and 27,540 deaths from PCA are

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estimated in the United States in 2015.[1] To reduce PCA mortality, we need a better understanding of biological events that play a fundamental role in prostate carcinogenesis. In this regard, hypoxia (low oxygen conditions) is considered an early event during prostate carcinogenesis associated with aggressive phenotype, and plays an important role in PCA growth, promotion, metastasis, and hormone-refractory progression.[2–4] Hypoxia induces genetic, metabolic, and proteome-related changes leading to increased glycolysis, angiogenesis, stemness, and selection of resistant clones.[2,5,6] In fact, tumor hypoxia status and hypoxia-related biomarkers are associated with poor prognosis and the major reason for treatment failure and disease relapse.[7–10] Therefore, PCA could be effectively prevented and treated through targeting hypoxia-induced signaling pathways and biological effects.

Under hypoxic conditions, several signaling pathways are activated in cancer cells to adjust to the harsh and inhospitable environment. Although signaling mediated by HIF1 (hypoxia-inducible factor 1) constitutes a potent hypoxic response mechanism, there are several instances of hypoxia responses that are HIF independent, and could be executed by other pathways such as phosphatidylinositol 3-kinases (PI3K)-Akt-mammalian target of rapamycin (mTOR), NADPH oxidase (NOX), Wnt/β-catenin etc.[3,6,11–14] Together, these signaling pathways are instrumental in the adaptation of cancer cells to hypoxic conditions through promoting angiogenesis, anaerobic metabolism, invasiveness, motility, survival, stemness, drug resistance, etc. Besides the genetic/epigenetic changes, hypoxic cancer cells enforce extensive tumor microenvironment remodeling to further improve their survival chances. For example, hypoxic cancer cells promote the growth of endothelial cells in neighboring blood vessels via releasing pro-angiogenic factors. Similarly, hypoxic cancer cells promote the activation of cancer-associated fibroblasts (CAFs) that further supports angiogenesis as well as cancer cells invasiveness, stemness, and immune cells recruitment in the tumor microenvironment. Another interesting feature emerging in the hypoxic tumor environment is a metabolic symbiosis between different cellular components, where hypoxic cells use glucose via glycolysis, generating lactic acid that is consumed by normoxic cells (cancer cells, endothelial cells, fibroblasts, macrophages, etc.) via monocarboxylate transporter 1 (MCT1).[15–18] The use of lactate as primary source of energy by normoxic cells spared the glucose for hypoxic cells in the deeper tissues. Besides, hypoxia in the primary tumor also modulates the environment at distant sites to support metastasis (these sites are known as pre-metastatic niches).[19,20] Recent studies have confirmed that “exosomes” (endosome-derived vesicles of ~30–150 nm in diameter) serve important roles in intercellular communication through transport of bioactive molecules between cells, and that hypoxic cancer cells secrete more and distinct exosomes compared to healthy cells.[21–24] Exosomes are loaded with unique proteins, lipids, nucleic acid (mRNA, miRNA, and DNA, etc.), metabolites, and sugars, and are considered important players in tumor microenvironment remodeling, both local and distant.[19,21,23,24] It is believed that a better molecular understanding of exosomes biogenesis in hypoxic cancer cells as well as their effect on receipt cells could offer new avenues for cancer control.

In this review, we describe various signaling pathways that are activated in PCA cells under hypoxic conditions. Even though, we have primarily focused on signaling pathways related to PCA cells, but wherever possible we have also described studies in other cancer cells. We have also discussed the role of exosomes as messenger of hypoxic response in the tumor

microenvironment. In the last, we have discussed the possibility of using hypoxia/hypoxia-related signaling as potential biomarker for disease prognosis as well as drug target in PCA prevention and treatment.

II. HYPOXIA-INDUCED SIGNALING PATHWAYS

Several signaling pathways are activated in cancer cells under hypoxic conditions including HIF, PI3K/Akt/mTOR, NOX, Wnt/β-catenin, and Hedgehog (Fig. 1). These pathways are briefly discussed below in this section.

A. HIF Signaling

HIF belongs to the large family of basic helix-loop-helix proteins and is a heterodimer of a stable HIF-1β subunit (known as the aryl hydrocarbon receptor nuclear translocator), and one of three oxygen-regulated HIF-α subunits (HIF-1α, HIF-2α, and HIF-3α). Depending on the cell type, HIF-1 regulates more than 2% of all human genes controlling energy metabolism, neovascularization, survival, stemness, invasiveness, etc. In fact, HIF is the major mediator of cellular adaptation to hypoxic conditions.[25–27] HIF activation is a multistep process involving HIF-α stabilization, nuclear translocation, hetero-dimerization, transcriptional activation, and interaction with other proteins.[28,29] HIF-1α overexpression is linked with the increased mortality in patients with various malignancies.[7,30–33] This association is primarily based on the HIF-1-mediated regulation of genes that play pivotal roles in the central features of cancer pathogenesis such as angiogenesis, metastasis, invasion, stemness, metabolism, and anti-apoptosis.[27,28,34,35]

Under normoxic conditions, prolyl hydroxylases (PHD1, 2, and 3) hydroxylate the site-specific proline residues of HIF-1α in the presence of oxygen, Fe^{2+}, oxoglutarate (2-OG), and ascorbate. Hydroxylated HIF-1α is recognized by von Hippel-Lindau (VHL), which serves as the targeting subunit of an E3 ubiquitin ligase complex, and tags HIF-1α with polyubiquitin, which is then recognized by proteasome for degradation. Under hypoxic condition, PHDs activity is decreased resulting in lesser HIF-1α prolyl hydroxylation, which in turn leads to decreased rates of HIF-1α polyubiquitination and proteolysis. Therefore, under hypoxia, HIF-1α is stabilized, translocates to nucleus, dimerizes with HIF-1β, and binds to hypoxia response element (HRE) within the promoters of target genes.[4,6,27] HIF-1α also binds with co-activator CBP/p300 and activates the expression of target genes including glucose transporters, glycolytic enzymes, angiogenesis, proliferation, survival, metastasis, stemness, and drug resistance.[2,6,33] HIF-1α and CBP/p300 interaction is also dependent on oxygen level since factor-inhibiting HIF-1α (FIH) is known to hydroxylate HIF-1α at the Asn-803 site in the presence of O_2 preventing the binding of HIF-1α to CBP/p300. HIF-1α can also be regulated through oxygen-independent mechanisms in a cell type-specific manner. For example, HIF-1α can be activated by oncogenic mutations of PTEN (phosphatase and tensin homolog), VHL, succinate dehydrogenase (SDH), and fumarate dehydrogenase (FH).[36] Also, it could also be activated by the increased activity of ERBB2, SRC, RAS/MAPK, and PI3K/Akt/mTOR pathways.[36] Furthermore, HIF-1α is also stabilized by reactive oxygen species (ROS), which block PHD activities and also activate MAPKs.[36]. Similar to HIF-1α, HIF-2α is also regulated by oxygen-dependent

hydroxylation and compared with HIF-1α, it has several overlapping as well as distinct targets. On the contrary, HIF-3α is considered a negative regulator of hypoxia-inducible gene expression.

Several studies have reported that HIF-1α activation regulates several key biological events in hypoxic PCA cells. Ghafar et al. reported that exposure to acute hypoxia even for 24 h increased the aggressive characteristics and survival properties in human PCA LNCaP cells through upregulation of HIF-1α expression.[37] Hypoxia-induced HIF-1α also promoted survival in PCA cells through upregulation of IL8 receptors (CXCR1 and CXCR2).[38] Besides, HIF-1α enhanced the choline kinase expression in hypoxia prostate cancer cell resulting in higher phosphocholine and total choline levels. [39] Horii et al. reported that in PCA cells, HIF-1α interacts with androgen receptor (AR) to activate PSA expression under hypoxic condition.[40] Mitani et al. also reported that under low androgen condition, hypoxia enhances transcriptional activity of AR through HIF-1α.[41] Together, these two studies supported that hypoxia/HIF-1α promotes the advanced hormone-refractory progression of PCA.

It is well established that hypoxia/HIF-1α status of the tumor is associated with radio resistance and poor prognosis following radiotherapy. However, exposures to hypoxia even postirradiation activated the HIF-1α and contributed to aggressive phenotype and radio resistance.[42] Ma et al. reported that PCA cells under hypoxia exhibited higher expression of HIF-1α, HIF-2α, and stem cell biomarkers (Oct3/4 and Nanog), as well as increased stemlike properties.[43] Importantly, S100A8 and S100A9 are overexpressed in several neoplasms including PCA and play an important role in proliferation, inflammation, and metastasis. And the expression of these two molecules was increased in hypoxic PCA cells through a direct regulation by HIF-1α.[44] Similarly, CX3CR1 expression was strongly increased in androgen-independent PCA cells following exposure to hypoxia associated with higher motility and invasiveness.[45] Importantly, attenuation of HIF-1α abrogated hypoxia-induced upregulation of CX3CR1, and also prevented their motility and invasiveness under hypoxic environment.[45] Hypoxia also increased the invasiveness of PCA cells through HIF-1α- and TNFα-mediated stabilization of EMT promoter Snail.[46] We have also observed an increase in HIF-1α expression in hypoxic PCA cells associated with activation of several lipogenesis-related molecules and lipid accumulation.[23] Huss et al. have reported that angiogenic switch in TRAMP (transgenic adenocarcinoma mouse prostate transgenic model) mice is driven in part by HIF-1α early in PCA progression.[47] We have also observed that HIF-1α expression increases with the disease progression in TRAMP mice associated with higher levels of several angiogenesis-related biomarkers (VEGF, VEGFR1, VEGFR2, and microvessel density) as well as increased metastasis to distant organs. [48]. Overall, hypoxia-induced HIF-1α signaling plays an important role in the angiogenesis, stemness, lipogenesis, EMT, metastasis, and hormone-refractory progression in PCA cells (Fig. 1).

B. PI3K/Akt/mTOR Signaling

In addition to its role in normal cell, PI3K/Akt/mTOR pathway is also important in the development of several cancers.[49–51] This pathway plays a key role in various cellular functions including proliferation, survival, migration, adhesion, invasion, metabolism, and

angiogenesis.[49–51] PI3K is a family of enzymes that phosphorylates the 3′-OH of the inositol ring of phosphatidyl inositol. PI3K generates an important lipid second messenger called phosphatidylinositol 3,4,5-triphosphate (PIP3), which plays a crucial role in several signaling pathways.[52] Furthermore, PIP3 activates the serine/threonine kinases PDK1 and Akt. On the contrary, the PTEN gene encodes a phosphatase that opposes the action of PI3K, which in turn reduces the level of activated Akt. Akt is known to control protein synthesis and cell growth by leading to the phosphorylation of mTOR. Under hypoxic conditions, the PI3K/Akt/mTOR pathway is activated in cancer cells (including PCA cells), and plays important role in adaptation of cells to low O_2 conditions.[53–57] Several studies have suggested that the PI3K/Akt/mTOR pathway mainly acts through promoting HIF-1α expression under hypoxic conditions.[56–58] In this regard, Wang et al. reported that both Akt and mTOR are activated in multiple myeloma U266 cells and their inhibition by LY294002 and rapamycin, respectively, resulted in inhibition of hypoxia-induced HIF-1α expression.[57] Blancher et al. reported the role of PI3K in regulating the HIF-1α but not HIF-2α expression in breast and renal cancer cells.[54] Chen et al. reported that blocking PI3K/Akt or mTOR activity strongly diminished the hypoxia-induced HIF-1α expression and VEGF secretion in human bladder cancer cells.[59] Interestingly, Manohar et al. identified and tested the efficacy of a novel HIF-1α inhibitor P31555 in PCA cells, and concluded that P3155 also inhibits hypoxia-induced HIF-1α expression through inhibiting the PI3K/Akt/mTOR pathway.[60] Yan et al. reported that Akt and its downstream signaling is activated in hepatocellular carcinoma cells (HCC) and controls the hypoxia-induced EMT in these cells.[55] Importantly, Akt is also activated in PCA cells under chronic hypoxic conditions and plays an important role in the hormone-refractory progression of the disease.[53]

Several studies have aimed at understating the detailed mechanism underlying the PI3K/Akt/mTOR activation and their role in increasing HIF-1α expression in hypoxic cancer cells.[12,61] In an in-depth study, Joshi et al. clearly showed that hypoxia alone might not be sufficient to render HIF-1α resistant to proteasomal cleavage and degradation, and in fact requires PTEN inactivation or activation of PI3K/Akt.[12] This study showed that PI3K inhibitors and PTEN could promote HIF-1α degradation in hypoxic glioma cells through regulating the cytoplasmic localization of MDM2 (murine double mutant 2).[12] Hudson et al. suggested the role of mTOR beyond translational control of HIF-1α, and suggested that mTOR could regulate HIF-1α expression through affecting the oxygen-dependent degradation (ODD) domain in HIF-1α.[13] In another study, Shen et al. reported that under severe hypoxic conditions, gastric cancer cells secrete a higher amount of TGF-β, which activates Akt along with Smad2/3 via autocrine loop.[62] Additionally, ROS role has been suggested in the activation of the PI3K/Akt/mTOR pathway under hypoxic condition, which in turn increases HIF-1α expression.[61] Ader et al. also showed that under hypoxic conditions, sphinogosine kinase 1 (SphK1) is activated in ROS-dependent manner in human PCA PC3 cells and increases the HIF-1α expression dependent on Akt activation and glycogen synthase kinase 3β (GSK3β) inactivation.[63] Therefore, ROS could directly/indirectly activate the PI3K/Akt/mTOR pathway, which could then increase HIF-1α expression (Fig. 1). Overall, it is clear that PI3K/Akt/mTOR activation not only controls HIF-1α expression in normoxic cancer cells but also under hypoxic conditions (Fig. 1). It is also expected that activated PI3K/Akt/mTOR pathway would enhance the expression of their

other target genes involved in cell proliferation, survival, motility, etc., and therefore play an important role in hypoxia-induced biological effects in cancer cells (Fig. 1). However, more studies are warranted to clearly establish the molecular mechanisms involved in the activation of PI3K/Akt/mTOR in hypoxic cancer cell.

C. NOX Signaling

The NOX system consists of catalytic subunits [gp91^{phox} (NOX1-5) and p22^{phox}] on the membrane and regulatory subunits (p40^{phox}, p47^{phox}, p67^{phox}, and Rac1/2) toward the cytoplasmic side. The NOX system passes an electron to oxygen and generates superoxide that is rapidly converted to hydrogen peroxide and causes oxidation of redox-sensitive cysteine residues in target molecules. NOX-generated reactive ROS acts as a secondary messenger and is involved in the regulation of several survival and mitogenic signaling pathways.[64–67] Importantly, dysregulated NOX-dependent ROS generation is associated with several chronic diseases including cancer.[68,69] NOX-mediated ROS generation activates several oncogenes (e.g., receptor tyrosine kinases, Src, and Ras), inactivates several tumor suppressor genes (e.g., PTEN, p53 and TSC2),[70] and is considered critical in the transformation as well as maintenance of malignant and resistant phenotype in cancer cells.[64,65,69–73] There are abundant reports suggesting that the NOX system plays an important role in PCA growth and progression.[64–67,73,74] Lim et al. reported that NOX1 is overexpressed in a high percentage of human prostate tumors associated with elevated ROS generation and increased tumorigenicity.[66]

Hypoxia is one of the key elements that promote NOX activity.[75,76] Cycling hypoxic glioblastoma cells showed higher NOX4 expression associated with high ROS levels and radio resistance.[77] Diebold et al. reported that hypoxia rapidly increased NOX4 expression in pulmonary artery smooth-muscle cells (PASMCs).[76] Chromatin immunoprecipitation (CHIP) analysis identified HIF-1α binding to *NOX4* gene regulating its expression, and activated NOX4 maintained the ROS level and supported the proliferation of PASMCs.[76] In response to intermittent hypoxia, HIF-1α increased the NOX2 expression in the central and peripheral nervous system of mice as well as in cultured cells.[75] Contrary to these reports, Block et al. reported that NOX (1 and 4) and p22^{phox} regulate HIF-2α expression in VHL-deficient renal cell carcinoma.[78] Nanduri et al. also reported that HIF-1α activation by intermittent hypoxia requires NOX stimulation by xanthine oxidase in rat pheochromocytoma PC12 cells.[14] J Moon et al. reported that NOX-mediated ROS production plays an important role in HIF-1α activation following hyperthermia treatment in cancer cells.[79] Overall, it seems that in hypoxic cells, HIF-1α, and NOX promote each other's expression in a feed-forward manner and execute hypoxic response (Fig. 1).

D. Wnt/β-Catenin Signaling

The Wnt/β-catenin signaling pathway contributes to the pathogenesis of several diseases including cancer.[80–84] In the absence of Wnts, β-catenin is phosphorylated by the "destruction complex," which includes adenomatous polyposis coli (APC), axin 2, casein kinase 1, and GSK3. The phosphorylated β-catenin is subsequently ubiquitinated and degraded via the proteasomal-mediated pathway. Wnts binding to the Frizzled family of receptors and the LDL-receptor-related proteins (LRP) 5 and 6 corepressors inhibits the

destruction complex allowing β-catenin to accumulate, translocate to the nucleus, and activate Wnt target genes such as cyclin D1, c-myc, etc.[80–82] Importantly, GSK3β could also regulate the stability of HIF-1α as inhibition of GSK3β increased HIF-1α levels, whereas its overexpression reduced HIF-1α levels.[85,86]

β-catenin expression is reported to be higher in PCA compared with normal prostate tissue and associated with disease progression.[11,87] Mitani et al. reported the role of β-catenin along with HIF-1α in AR activation under low androgen conditions leading to hormone-refractory PCA progression.[11] This study showed that under hypoxic conditions, HIF-1α binds to β-catenin and promotes its nuclear translocation, and together they enhance AR transactivation by accelerating N-terminal and C-terminal interaction.[11] HIF-1α also modulates Wnt/βcatenin signaling in hypoxic embryonic stem cells by enhancing βcatenin activation and the expression of the downstream effectors LEF-1 and TCF-1.[88] Wnt/β-catenin pathway also plays an important role in HIF-1α-mediated EMT in human prostate carcinoma cells.[83] To et al. reported that hypoxia activated β-catenin levels independent of classical APC and p53 pathways but stimulated by PI3K/Akt signaling in a Nurr77-dependent manner in colon cancer cells.[89] Furthermore, the β-catenin-Nurr77 feed-forward loop promoted the migration, invasion, and EMT in these cells.[89] Liu et al. also reported that hypoxia-induced β-catenin activation increases the invasiveness and metastasis of hepatocellular carcinoma cells (HCCs) through promoting EMT and MMP2 (matrix metalloproteinase 2) expression.[84] More importantly, positive expression of β-catenin in HCC tissue microarray was associated with HIF-1α expression, and co-expression of β-catenin and HIF-1α was correlated with shorter overall patient survival and time to disease recurrence. Zhang et al. further confirmed that Wnt/βcatenin signaling enhances hypoxia-induced EMT and survival in HCC by increasing HIF-1α activity.[90] Similarly, Liu et al. reported that β-catenin activation under hypoxic conditions plays an important role in regulating the invasiveness of gastric cancer cells through activating the urokinase plasminogen activator (uPA) and MMP7 expression.[91] Interestingly, this study found that β-catenin could regulate HIF-1α expression in hypoxic cancer cells. In another study, Scholten II et al. reported that Wnt/βcatenin signaling is downregulated by hypoxia in osteosarcoma cells (OS), which appears to rely on both HIF-independent and -dependent mechanisms.[81] This study suggested that an inverse association between Wnt/βcatenin and HIF, but interestingly further downregulation of Wnt/βcatenin, mitigated the hypoxia-induced chemoresistance in these cells.[81] Santoyo-Ramos et al. suggested even complicated interaction between Wnt/βcatenin and HIF-1.[82] PIn hypoxic colon cancer cells, HIF-1α promoted Wnt/βcatenin signaling, EMT, and stemness, while HIF-2α seems to negatively regulate Wnt/βcatenin signaling.[82] Furthermore, Lim et al. reported that HIF1α could obstruct Wnt/βcatenin signaling pathway by inhibiting the hARD1-mediated activation of βcatenin.[92] Verras et al. also reported that tumor hypoxia could block Wnt/βcatenin signaling through the induction of endoplasmic stress in cancer cells; however, tumor cells harboring common βcatenin pathway mutations were insensitive to this hypoxic effect.[93] These outcomes were considered as hypoxia-induced selective pressure for the growth of cancer cells with mutations in the βcatenin pathway.[93] Overall, Wnt/βcatenin signaling seems to have significant cross talk with the HIF-1 pathway and plays an important role in

inducing the growth, EMT, metastasis, and androgen-independent growth in hypoxic cancer cells (Fig. 1).

E. Hedgehog Signaling

The Hedgehog (Hh) signaling pathway plays a critical role in numerous processes during embryonic development including cell growth, differentiation patterning, and organogenesis. In normal adult tissue, the Hh pathway regulates stem cell population maintenance, tissue repair, and regeneration. Aberrant activation of Hh signaling has been reported in several malignancies including PCA.[94–97] Gli1 is the transcriptional target of the Hh pathway and an activator of target genes including cyclin D1/D2, N-myc, bcl2, VEGF, MMP9, oct4, sox2, etc.[96] In the absence of Hh ligands, patched 1 (PTCH1) holds Smoothened (SMO), a seven-transmembrane spanning protein, in an inactive state and further downstream signaling is inhibited. On binding of Hh ligands to PTCH1, SMO dissociates from PTCH1, signaling is transduced, and Gli1 is activated, which then activates the expression of target genes. Onishi et al. reported that hypoxia activates the Hh signaling pathway in pancreatic ductal adenocarcinoma cells (PDACs) by increasing the transcription of SMO in a ligand-independent manner and increases the invasiveness of PDAC.[97] Importantly, this study showed that silencing of HIF1α did not affect the transcription of Hh-related genes under hypoxia as well as silencing of SMO or Gli1 did not affect HIF1α expression under hypoxia, suggesting that Hh and HIF1α pathways act independent of each other.[97] Lei et al. also reported that Hh signaling plays an important role in hypoxia-induced EMT and invasiveness of pancreatic cancer cells, but surprisingly this study suggested that activation of Hh signaling under hypoxic conditions is dependent on HIF-1α activation.[98] However, these two studies employed different pancreatic cancer cell lines, and that could be the possible reason for HIF1α dependent or independent activation of Hh signaling. Lei et al. reported a similar role of Gli1 in hypoxia-induced EMT and invasiveness of breast cancer cells.[99] In another study, Zhou et al. reported that SHH-Gli1 activities are increased under hypoxia in renal cell carcinoma (RCC) cells mediated by HIF2α.[100] Interestingly, this study also showed that SHH-Gli1 signaling positively regulates HIF2α expression in normoxic RCC cells.[100] There is increasing evidence suggesting the active role of Hh signaling in the development and progression of PCA[94–96] but to our knowledge role of Hh signaling has not been investigated in hypoxic PCA cells.

III. EXOSOMES AS MESSENGER OF HYPOXIC RESPONSE

Exosomes are small vesicles originating from late endosomes and secreted into the extracellular milieu after the fusion of multivesicular bodies (MVBs) with the plasma membrane. Exosomes have been implicated in the pathogenesis of several diseases including cancer.[21,101] Recent literature has established a critical role for these nanosized vesicles in intercellular communication, primary tumor growth, angiogenesis, pre-metastatic niches preparation, metastasis, drug resistance, immunosuppression, and disease relapse.[21,102–105] Importantly, hypoxic cancer cells secrete a higher amount of exosomes loaded with unique cargo proteins, miRs and lipids, and promote tumor growth and progression.[22,104] King et al. showed that breast cancer cells secrete a higher amount of exosomes under hypoxic condition in an HIF-1α-dependent manner.[22] This study also found increased level of

miR-210 in exosomes released by hypoxic breast cancer cells. Umezu et al. reported that hypoxia-resistant multiple myeloma (HR-MM) cells produced more exosomes than the parental cells under normoxia or acute hypoxia conditions with significantly higher miR-135 expression.[102] Moreover, exosomal miR-135 from HR-MM cells directly suppressed the FIH-1 and enhanced the tube formation in endothelial cells. Kucharzewska et al. reported that exosomes mediate hypoxia-dependent intercellular signaling in highly malignant brain tumor glioblastoma multiforme (GBM) cells.[101] The molecular profile of hypoxic exosomes reflected the hypoxic response of GBM donor cells and GBM patient's tumors and were enriched in MMP9, pentraxin 3, IL8, platelet-derived growth factor (PDGF), and plasminogen activator inhibitor 1 (PAI1).[101] This study also showed that exosomes derived from hypoxic GBM cells strongly induced angiogenesis *in vitro*, *ex vivo*, and *in vivo* through phenotypic modulation of endothelial cells. Tadokoro et al. also reported that exosomes derived from hypoxic leukemia cells enhanced tube formation in human umbilical vein endothelial cells via miR-210.[103] Parolini et al. demonstrated that exosome release and uptake is increased in an acidic pH, suggesting that acidic microenvironment in hypoxic tumors could contribute to the increased rate of exosomal release and uptake by cancer cells.[106] In an earlier study, Park et al. also showed that under hypoxic conditions tumor cells secrete proteins and exosomes responsible for increased angiogenesis and metastatic potential.[107] Aga et al. reported the presence of transcriptionally active HIF-1α in the exosomes of nasopharyngeal carcinoma cells that played a role in the induction of receipt cells.[108] Under hypoxic conditions, adipocyte-released exosomes promoted lipogenesis in 3T3-L1 recipient cells.[109] Besides exosomes, hypoxia significantly enhanced the microvesicles (formed by direct budding from the membranes; ~ size 100–≥1000 nm) biogenesis in a HIF1α- and RAB22A GTPase-dependent manner, and promoted the formation of focal adhesions, invasiveness, and metastasis in naïve breast cancer cells.[104]

We have recently reported that exosomes secreted by PCA cells under hypoxic conditions induce EMT, invasiveness, and stemness in naïve PCA cells as well as promote CAF phenotype in naïve normal prostate fibroblasts.[24] We have also reported that hypoxic PCA exosomes are loaded with unique proteins with higher expression of canonical markers, MMP2/9, and signaling molecules compared to normoxic PCA exosomes.[24] In another study, we reported that hypoxic PCA exosomes are loaded with a significantly higher amount of triglycerides, and hypoxic PCA exosomes-induced invasiveness could be inhibited by celecoxib.[23] Furthermore, this study suggested the important role of lipids in the biogenesis of exosomes under both normoxic and hypoxic conditions.[23] In a recent review, Sceneay et al. summarized the role of exosomes in the preparation of pre-metastatic niches and metastasis, suggesting that exosomes secreted by hypoxic cancer cells could have systemic effects and could promote cancer metastasis.[19] It is also suggested that by using the advanced mass spectrometry based proteomics analysis of tumor-derived exosomes, it is possible to estimate the oxygenation status of the patient's tumors.[110] Therefore, exosomes could be a useful biomarker in making appropriate treatment decisions in the clinic. Overall, there is ample evidence suggesting that exosomes could mediate the biological effects of hypoxic cancer cells in the tumor microenvironment (Fig. 2).

IV. HYPOXIA/HYPOXIA-INDUCED SIGNALING MOLECULES AS BIOMARKER FOR PCA DIAGNOSIS AND PROGNOSIS: TRANSLATIONAL RELEVANCE

There are several reports supporting that hypoxia in prostate tumor is an early event and also an independent risk factor for disease progression and treatment failure.[7–10,111] Hypoxia in tumor is measured by analyzing tissues for hypoxia-related biomarkers (mostly HIF-1α and HIF-1α regulated genes) or by using pimonidazole dye (a 2-nitro-imidazole compound that forms a covalent bond with cellular macromolecules at oxygen levels below 1.3%) or pO_2 is directly measured using Eppendorf microelectrodes. There are several studies suggesting that HIF-1α could be a good biomarker to predict PCA progression as well as treatment outcomes.[7,112] HIF-1α is overexpressed in primary prostate cancers as compared with normal prostate epithelium.[113] Du et al. confirmed the high levels of HIF-1α in prostate cancer as compared with benign prostate hyperplasia (BPH) and normal tissue.[114] Pipinikas et al. suggested that HIF-1α mRNA levels in blood could be useful in improving the diagnosis of early stages of PCA.[115] Therefore, HIF-1α overexpression appears to be an early event in PCA pathogenesis.

Ranasinghe et al. measured HIF-1α expression by immunohistochemistry (IHC) in 100 human prostate tumors, which were collected following radical prostectomy or transurethral resection of the prostate (TURP).[7] They found that HIF-1α expression in tumors is an independent risk factor for disease progression associated with significantly reduced metastasis-free survival and CRPC (castrate-resistant prostate cancer)-free survival.[7] More importantly, this study also found that prostate tumors lacking HIF-1α expression did not metastasize or developed CRPC.[7] Yasuda et al. also characterized the role of HIF-1α (by IHC) in predicting the success of neo-adjuvant hormone therapy in patients with adenocarcinoma.[112] This study also found that strong HIF-1α expression after neoadjuvant hormone therapy suggests a relatively high risk of disease recurrence and treatment failure. Stewart et al. showed that the transcript levels of hypoxia-associated genes LOX (lysyl oxidase) and GLUT-1 (glucose transporter-1) were significantly higher in PCA compared to BPH tissue and correlated with Gleason score.[116] In another study, Vergis et al. concluded that for PCA patients treated with radiotherapy, higher HIF-1α and VEGF (one of the HIF-1α regulated gene) expression is significant predictor of a shorter time to biochemical failure independent of clinical tumor stage, Gleason Score, serum PSA concentration, and dose of radiotherapy.[10] Similarly, for patients treated with surgery, the higher expression of HIF-1α, VEGF, and osteopontin (also regulated by HIF-1α) was associated with a shorter time to biochemical failure independent of pathological tumor stage, Gleason score, serum PSA concertation, and margin status.[10] This study clearly suggested that HIF-1α expression or expression of HIF-1α regulated genes could be a predictor of the biochemical failure independent of treatment modality.

Similar findings, as above for HIF-1α, were reported when hypoxia was measured through pimonidazole or microelectrodes. Ragnum et al. characterized the hypoxia area in primary tumors by using pimonidazole.[8] PCA patients were injected pimonidazole (IV) the day before robot-assisted laparoscopic radical prostatectomy (RALP). This study found that pimonidazole immunoscore is significantly higher for tumors at a high clinical stage and

with lymph node metastasis.[8] Pimonidazole gene signature also reflected an aggressive hypoxic PCA phenotype characterized by upregulation of proliferation, DNA repair, and hypoxia-response gene.[8] Carnell et al. also reported that increased pimonidazole staining was observed in tumors with high Gleason score.[117] Milosevic et al. conducted one of the largest clinical studies of PCA hypoxia in 247 patients, with localized PCA before radiotherapy with or without hormonal therapy, with direct measurement of tumor oxygen levels by using ultrasound-guided transrectal needle-electrode.[9] This study found that tumor hypoxia is associated with early biochemical relapse after radiotherapy and predicts local recurrence.[9] Earlier, Turaka et al. reported that hypoxia in prostate cancer (low mean hypoxic prostate/muscle pO_2 ratio) significantly predicts for poor long-term biochemical outcome.[118]

Therefore, it is clear that hypoxia and/or the activation of hypoxia-related signaling in prostate tumors could determine treatment success as well as disease progression. These results also suggest that PCA growth and progression could be effectively prevented via targeting hypoxia/hypoxia-related signaling. This rationale is strongly supported by recent studies suggesting the efficacy of HIF-1α inhibitors to prevent the disease progression and treatment failure.[4,34,119,120] Platz et al. identified that nonspecific HIF-1α inhibitor digoxin, a cardiac glycoside derived from foxglove used to treat congestive heart failure and arrhythmia, has strong efficacy to inhibit the proliferation of androgen-dependent and -independent PCA cells in cell culture.[119] Furthermore, this study based on the clinical data from the Health Professional Follow-Up study (47,884 men followed up from 1986 through 2006) concluded that compared with nonusers, men who regularly used digoxin had a 25% lower risk of PCA, including disease that was potentially lethal.[119] This study strongly suggests the potential usefulness of digoxin in reducing the risk of developing PCA or to treat undetected PCA. In a retrospective review of prospectively collected medical records, Ranasinghe et al. concluded that nonspecific HIF-1α inhibitors (digoxin, metformin, and angiotensin-2 receptor blocker) increase the progression-free survival and decrease the risk of developing CRPC and metastasis in PCA patients on continuous androgen deprivation therapy.[120] Extensive efforts are now being put into targeting HIF-1 and associated pathways by small molecules inhibitors (e.g., YC-1 and PX-478), HIF-1 DNA activity (e.g., polyamides and echinomycin), and HIF-1 transcriptional activity (e.g., chetomin, proteasome inhibitors, and amphotericin B).[4,121]

V. CONCLUSIONS

Hypoxia is one of the hallmarks of solid tumors, endowing them with malignant, aggressive, and treatment-refractory characteristics. HIF-1α is the major signaling pathway that is activated under hypoxic conditions and promotes angiogenesis, survival, EMT, metastasis, etc. However, several other signaling pathways such as PI3K/Akt/mTOR, NOX, Wnt/catenin, and Hedgehog are also activated under hypoxic conditions, and play a vital role in hypoxia-induced biological effects. It is also clear now that exosomes play an important role in transferring message from hypoxic cancer cells to normoxic cells in the tumor microenvironment, thereby contributing toward cancer growth, progression, and metastasis. Clinical studies clearly suggest the potential of hypoxia/hypoxia signaling biomarkers in the prognosis of the disease as well as in making key treatment decisions. However, we need to

further develop reliable noninvasive methods to longitudinally measure hypoxia status in the tumors. In this regard, exosomes could be potentially useful in determining the hypoxia status of the tumors, but we need to establish biomarkers (proteins, miRNA, and lipids) that are distinctly present in the exosomes released by hypoxic cancer cells. Overall, further research in this area could be potentially beneficial in the effective management and control of cancer.

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ABBREVIATIONS

APC	adenomatous polyposis coli
AR	androgen receptor
BPH	benign prostate hyperplasia
CAFs	cancer-associated fibroblasts
CXCR	CXC chemokine receptor
EMT	epithelial-to-mesenchymal transition
HIF-1	hypoxia-inducible factor 1
FH	fumarate dehydrogenase
FIH	factor-inhibiting HIF-1α
Hh	Hedgehog
IL8	interleukin 8
MAPK	mitogen-activated protein kinase
MDM2	murine double mutant 2
mTOR	mammalian target of rapamycin
PAI1	plasminogen activator inhibitor 1
PCA	prostate cancer
PDGF	platelet-derived growth factor
PHD	prolyl hydroxylase
PI3K	phosphatidylinositol 3-kinases
SDH	succinate dehydrogenase
VEGF	vascular endothelial growth factor

VEGFR vascular endothelial growth factor receptor

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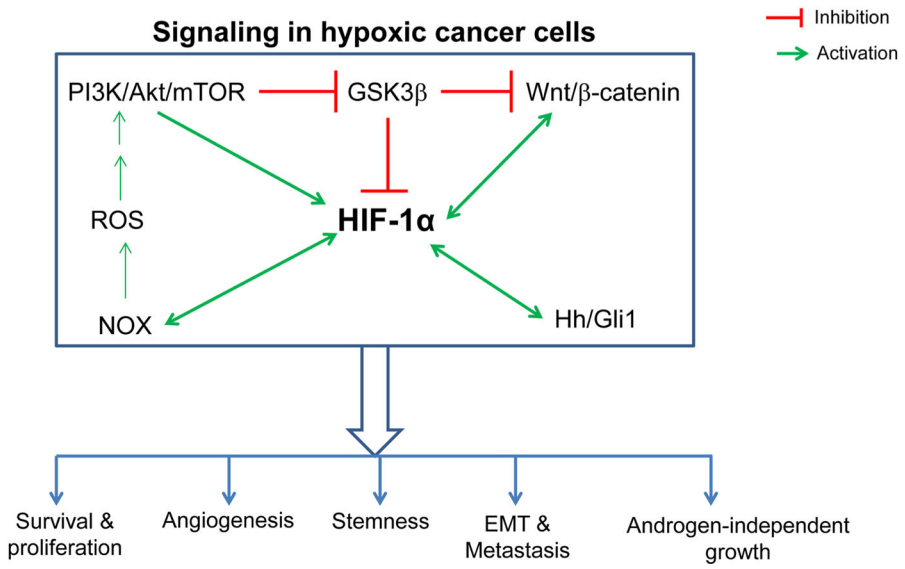

FIG. 1.

Activation of signaling pathways in hypoxic cancer cells. In hypoxic cancer cells, several signaling pathways are activated including HIF-1α, PI3K/Akt/mTOR, Wnt/β-catenin, NOX, and Hedgehog. These signaling pathways have significant cross talk, and together they contribute to hypoxia-induced cancer cells survival, proliferation, angiogenesis, stemness, EMT, metastasis, and androgen-independent growth.

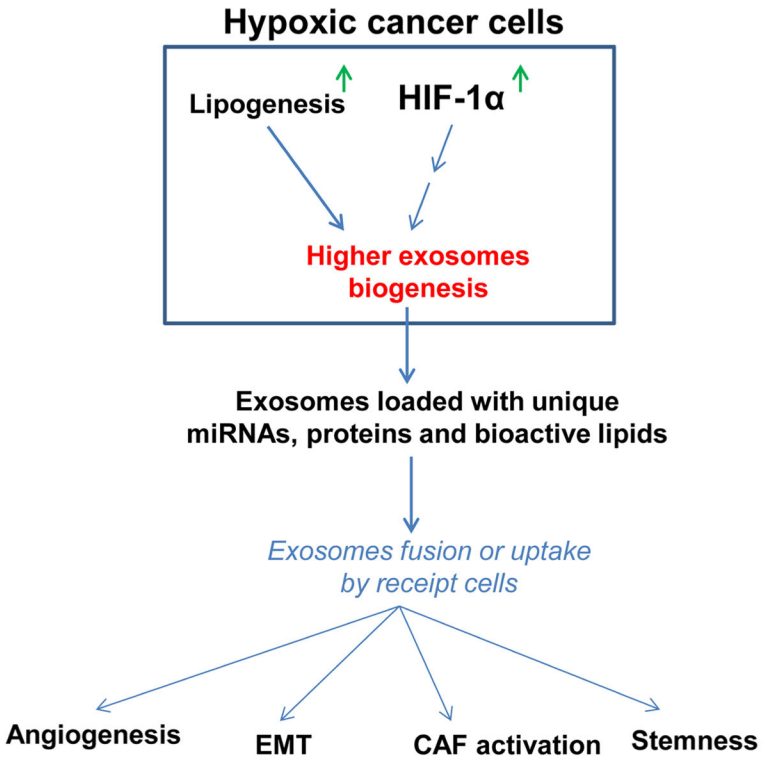

FIG 2.

Exosomes act as messenger of hypoxic response in cancer cells. Biogenesis of exosomes is increased in hypoxic cancer cells dependent on HIF-1α and cellular lipid level, and in turn hypoxic cancer cells secrete higher amounts of exosomes loaded with unique cargo (miRNAs, proteins, and bioactive lipids). Hypoxic cancer cells exosomes uptake by receipt cells in the tumor microenvironment results in higher angiogenesis, EMT, CAF activation, and increased stemness.