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Oxygen-mediated endocytosis in cancer

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Abstract

Solid tumours invariably exhibit regions of hypoxia and up-regulation of receptor tyrosine kinases (RTKs) that trigger multiple signal pathways, including those that govern cell proliferation, survival and motility, ultimately contributing to oncogenesis. Although past studies have shown hypoxia-dependent transcriptional and translational induction of several RTK expression and their respective ligands, recent evidence suggests that hypoxia regulates RTK signalling through endocytosis, a major deactivation pathway of RTKs. Hypoxia-mediated endocytosis is also thought to modulate the activity of a growing list of other membrane-associated proteins such as integrins and Na,K-ATPase. These recent discoveries underscore the emergence of endocytosis as an important hypoxia-mediated regulatory process in cancer.

Introduction

Hypoxia plays a critical role in physiologically normal development such as embryogenesis, in which embryo is in a state of partial hypoxia, which is essential for the control of neovascular and cardiovascular development. Hypoxia also plays pathophysiological roles in human diseases such as ischemia, stroke and cancer [1–3]. For example, tumour cells exhibit molecular adaptation to oxygen deprivation *via* initiation of hypoxia-mediated survival pathways, angiogenesis, erythropoiesis, invasion and metastasis. Ultimately, prolonged hypoxic stress leads to a more aggressive tumour phenotype that is less sensitive to both radiation and chemotherapy [4]. Several lines of independent studies have shown that hypoxia is an indicator of poor prognosis for patients with breast [5–7], cervical [8–10] and non-small cell lung cancers [11], independent of other clinical factors such as tumour size.

Another important feature of tumour growth is the unregulated receptor tyrosine kinase (RTK) activity through constitutive mutational activation, overexpression or defective termination of RTK-mediated signalling [12–17]. Although there have been hypoxia-dependent transcriptional and translational mechanisms described for the enhancement of certain RTK expression, a general unifying mechanism governing hypoxia-induced RTK signalling has been until recently unknown. This review highlights recent discoveries into the causal role of tumour hypoxia in the regulation of RTK and non-RTK endocytosis and the contribution of these hypoxia-adaptive processes to cancer development.

Hypoxia-inducible factors

The hypoxic response is orchestrated by the hypoxia-inducible factor (HIF), a heterodimeric transcription factor consisting of a modular α and a common β subunit, the aryl hydrocarbon receptor nuclear translocator (ARNT) [18–21]. Both α and β subunits belong to the basic helix-loop-helix-Per/Arnt/Sim (βHLH-PAS) superfamily of transcription factors. βHLH region is required for DNA binding while the two N-terminal PAS domains are required

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for dimerization and DNA specificity [18, 22]. The transactivation domain located in the C-terminus recruits transcriptional co-activators required for transcription [23].

Although the constitutively expressed ARNT is an absolute requirement for HIF transcriptional activation [24], the regulation of HIF occurs primarily through oxygen-dependent ubiquitin-mediated degradation *via* the oxygen-dependent degradation (ODD) domain within HIF-α [25, 26]. Under normal oxygen tension or normoxia, HIF-α is hydroxylated at two conserved proline residues in the ODD domain by a family of prolyl hydroxylase domain-containing proteins (PHD). Prolyl-hydroxylation permits the binding between HIF-α and von Hippel-Lindau (VHL) protein, the recognition subunit of an E3 ubiquitin ligase complex called ECV (Elongins BC/Cul2/VHL), which triggers polyubiquitylation and subsequent destruction of HIF-α *via* the 26S proteasome [27, 28]. Under hypoxia, PHD-mediated prolyl-hydroxylation of HIF-α is impaired and consequently escapes ECV recognition. The stabilized HIF-α dimerizes with ARNT, translocates to the nucleus and recruits p300/CBP transcriptional co-activator, thus forming the active HIF complex. HIF binds to the hypoxia-responsive element in the promoter regions of numerous hypoxia-inducible target genes, including *vascular endothelial growth factor*, *glucose transporter 1* and *erythropoietin*, to promote angiogenesis, anaerobic metabolism and erythropoiesis, respectively [28–30].

There are three mammalian HIF-α: HIF-1α, HIF-2α and HIF-3α [20, 31]. HIF-1α is most ubiquitously expressed whereas the expression of HIF-2α and HIF-3α is predominantly confined to the heart, placenta and lung [32, 33]. The three HIF-α genes share homology in the βHLH and PAS domains but differ significantly in their C-terminal transactivation domains, which may explain overlapping yet distinct transcriptional target genes [20]. HIF-α isoforms have highly conserved ODD domains, which account for their destructive targeting *via* ECV in an oxygen-dependent manner [34, 35]. Although HIF-1α possesses both oncogenic and tumour suppressor properties, HIF-2α overexpression has been invariably implicated in the promotion of transformed phenotype [22, 36]. HIF-3α expression has been observed in several human cancer cell lines, such as colon and prostate cancer cells. However, the significance of the full-length HIF-3α isoform in cancer is unclear [22].

von Hippel-Lindau protein and cancer

Inheritance of a faulty *VHL* gene causes VHL disease that is characterized by the development of tumours in multiple organs, including the brain, spine, retina and kidney. Functional inactivation of VHL is also responsible for the vast majority of sporadic clear-cell renal cell carcinoma, the most common form of kidney cancer and the sixth leading cause of cancer death [37]. Most *VHL* mutations result in the inappropriate accumulation of HIF-α irrespective of oxygen tension due to the inability of mutant VHL to recognize prolyl-hydroxylated HIF-α or to form an active ECV complex [37, 38]. Several lines of evidence support the notion that the deregulation of HIF-α plays a causal role in tumorigenesis and that the negative regulation of HIF-α represents the major tumour suppressor function of VHL.

Endocytosis

Endocytosis is an essential cellular process in which materials from the extracellular space are brought inside the cells by membrane invagination. Endocytosis is an essential cellular homeostatic process in eukaryotic cells that controls an extraordinary array of activities such as signal transduction, neuronal synaptic transmission, nutrient uptake, clearance of apoptotic cells, regulation of intercellular interaction and antigen presentation [39]. Endocytosis can be a constitutive process or triggered by ligand engagement of receptor. Endocytic pathways are categorized into three major types – phagocytosis or pinocytosis, clathrin-dependent endocytosis and clathrin-independent endocytosis.

Phagocytosis or pinocytosis

Phagocytosis is a class of endocytosis that involves internalization of large solid particles (>1.0 μm) such as apoptotic cell debris, viruses and bacteria. Also referred to as cellular 'eating', phagocytosis involves the formation of pseudopods and the generation of large vesicles, mostly restricted to immune leucocytes such as macrophages, neutrophils, monocytes and dendritic cells as a major mechanism for removing pathogens and cell debris [40]. Phagocytosis is an active and highly regulated process that requires actin reorganization regulated by Rho family GTPases. Analyses of ovarian cancer, breast cancer and fibrosarcoma cells suggest that highly metastatic cells also degrade extracellular collagen through a phagocytic pathway [41].

In contrast, pinocytosis is the ingestion of small particles (<0.2 μm) such as extracellular fluid and dissolved molecules [42, 43]. Also referred to as cellular 'drinking', pinocytosis is a process that occurs in all cell types and is mechanistically diverse and highly regulated. Macropinocytosis is a subset of actin-dependent pinocytic process regulated by the Rho family GTPase Rac1. Actin polymerization drives the formation of membrane ruffles and protrusions that collapse back onto and fuse with the plasma membrane, which generates large endocytic vesicles of variable size called macropinosomes that contain large volumes of fluid. Although the mechanics behind this highly regulated process are not well understood, growth factor-induced macropinocytosis may play a role in directed cell migration and cancer metastasis [44].

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is the best-characterized endocytic pathway. CME is triggered by the binding of ligand to its cognate receptor and occurs in two stages divided into multiple

steps [45, 46]: (1) Internalization, which covers the steps from the targeting of receptors in clathrin-coated pits to the formation of clathrin-coated vesicles and (2) intracellular receptor trafficking, which covers the steps from early endosomes to sorting endosomes, late endosomes and lysosomes. The major coat protein is clathrin, composed of three heavy chains and three light chains that form a three-legged structure called triskelion. The adaptor protein 2 is required to initiate coated pit assembly on the membrane through binding to the cytoplasmic tails of receptor molecules. Once a clathrin-coated pit has formed, dynamin, a recently identified GTPase, self-assembles into helical rings and stacks to form a collar at the neck of a clathrin-coated invagination and pinches off the vesicle from the plasma membrane [47]. CME is also a highly selective process and at least two types of internalization signal have been described: the tyrosine-based motif (YXXΦ, in which Φ is a hydrophobic residue) and the dileucine-based motif (LL) [48–51].

Once a clathrin-coated vesicle is severed from the membrane, these vesicles lose their clathrin coat, merge with early endosomes and sorted, either to be recycled to the plasma membrane or to be targeted to the lysosome [52]. For example, following its rapid internalization to the early sorting endosomes, the transferrin receptor, a well-studied clathrin cargo, is returned to the plasma membrane [53]. Other receptors, such as the epidermal growth factor receptor (EGFR), are primarily destined for late endosomes and ultimately lysosomes where the receptors and ligands are degraded in the acidic milieu containing digestive enzymes (acid hydrolases) [51, 54].

Intracellular trafficking of receptors involves a series of membrane budding and fusion events [55]. These are regulated by specific cytosolic and membrane-associated protein factors, including a group of Ras-like small GTPases called Rabs [56]. The current view is that Rabs are involved in specifying the correctness of membrane–membrane interactions at either the docking or fusion level or both [57]. For example, Rab4 and Rab11 control the function and formation of endosomes involved in recycling while Rab7 regulates membrane transport from the early endosomal stage through to the late endosomal and lysosomal stages. Rab5 controls membrane trafficking in the early endocytic pathway, thereby dictating the sorting function in endocytosis [57]. Rabaptin-5 is one of the most well-characterized Rab5 effectors having two Rab5 binding sites, which suggests that rabaptin-5 plays a role in the tethering of early endosomes [58]. Rabaptin-5 also has an N-terminal Rab4 binding domain, which suggests that it has a role in endosome recycling [59]. Early endosomal antigen 1 (EEA1) is another Rab5 effector [60]. EEA1 is also involved in the tethering/docking and fusion of early endosomes and contains two Rab5 binding sites, as well as a C-terminal FYVE domain that specifically binds to phosphatidylinositol 3-phosphate (PI3P) [61].

Clathrin-independent endocytosis

Endocytosis of membrane and fluid also occurs through a clathrin-independent pathway. Clathrin-independent endocytosis can be distinguished from CME by its slow kinetic characteristics, as opposed to the rapid internalization that occurs *via* CME. Clathrin-independent internalization may be responsible for the uptake of molecules that do not use coated pits, such as GPI-anchored proteins, lipids and pathogens. Among the various types of clathrin-independent endocytosis, caveolin-mediated endocytosis is one of the more extensively studied pathways.

Although caveolae were first identified over 50 years ago [62], our understanding of the function of these unique organelles is just beginning to emerge. Caveolae are uncoated, highly abundant, omega (Ω) shaped (50–100 nm in diameter) invaginations on the plasma membrane that are present in most cell types [63]. They demarcate lipid rafts, or domains in the plasma membrane that are enriched in cholesterol and sphingolipids [64]. Caveolins, a group of oligomeric cholesterol-binding proteins that insert into the membrane as a hairpin loop, are the major structural proteins that are essential for the formation of caveolae [65–67]. The biological role for caveolae continues to be debated. Caveolae have been implicated in the regulation of signal transduction, endocytosis and the maintenance of cholesterol homeostasis [63, 68]. Caveolin phosphorylation, dynamin and Rab5 activity and actin polymerization are required for caveolin-mediated endocytosis. The caveolar cargoes are diverse, ranging from lipids, proteins and lipid-anchored proteins to pathogens. Caveolin-mediated endocytosis is a major pathway of entry for some viral pathogens, such as SV40 and some adenoviruses [69, 70].

In addition to caveolin-mediated endocytosis, caveolin-independent pathways represent a rapidly evolving field of study. The molecular details of how vesicles are formed and how these pathways are regulated are not well understood [71]. Advances in our understanding of these pathways come primarily from the identification of cargo molecules internalized through non-clathrin pathways and the identification of possible regulators [72]. Using regulators as a basis for categorization, clathrin- and caveolin-independent pathways can be grouped into RhoA-dependent [73], Cdc42-dependent [74] and Arf6-dependent types [75]. However, this approach has not been entirely satisfactory. There is still disagreement about how many of these clathrin-independent pathways exist and confusion about how they may overlap mechanistically and functionally. Adding to the confusion or complexity is the fact that many cargos can be internalized through multiple pathways. This is perhaps best illustrated by differences in the endocytosis of EGFR. Although EGFR is internalized through CME at low physiological concentrations of EGF, EGFR may be internalized by caveolin-mediated endocytosis at higher ligand concentrations [76, 77].

Hypoxia-mediated regulation of RTKs

Both tumour hypoxia and dysfunction of RTKs can contribute to cancer development and resistance to conventional cancer therapy. These two factors are intertwined, and previous work has shown that tumour hypoxia can up-regulate signalling through EGFR and other RTKs. EGFR is one of the most studied RTKs. It is

overexpressed or highly activated in more than 80% of all solid tumours and is associated with increased metastasis, therapeutic resistance and poor prognosis [78]. Franovic *et al.* provided evidence that EGFR expression can be induced by the hypoxic microenvironment, while the activation of HIF-2α in the core of solid tumours results in increased EGFR mRNA translation [79]. Moreover, short hairpin RNA mediated inhibition of EGFR is sufficient to abolish HIF-dependent tumorigenesis in multiple VHL$^{-/-}$ RCC cell lines [80]. EGFR regulates HIF-α expression through the PI3K-Akt signalling axis in non-small cell lung cancer [81–83]. The crosstalk between EGFR and HIF signalling pathways has been reported to increase resistance to apoptosis under normoxic conditions in human breast cancer cells [84].

Other studies have shown that tumour hypoxia can up-regulate signalling *via* the hepatocyte growth factor (HGF)/c-Met pathway as well [85]. HGF normally stimulates growth, migration and epithelial-to-mesenchymal transition in a range of cell types, including epithelial, blood, neural and skin cells as well as hepatocytes [86]. Pennacchietti *et al.* revealed that under hypoxic cell culture conditions, c-Met, the receptor for HGF, is increased at both the transcription and protein levels, thus making it more available for ligation with HGF [87]. Under hypoxia, increased c-Met expression and HGF sensitization promote tumour cell invasiveness [87, 88]. Moreover, hypoxia has also been shown to induce tyrosine phosphorylation of the platelet-derived growth factor receptor, activate PI3K/Akt cascade that leads to glycogen synthase kinase-3 inactivation, and enhance vascular endothelial growth factor receptor expression [89].

Hypoxia-mediated RTK endocytosis

Hsu *et al.* provided evidence that VHL$^{-/-}$ RCC cells exhibit increased surface abundance of fibroblast growth factor receptor 1 upon ligand stimulation and downstream target activation, such as ERK1/2, compared to VHL-reconstituted RCC counterpart [90]. VHL was shown to interact with the metastasis suppressor Nm23, a protein known to regulate dynamin-dependent endocytosis at the level of internalization, suggesting that VHL promotes internalization process of fibroblast growth factor receptor 1 [90].

Whether hypoxia influenced RTK turnover upon ligand engagement was, until recently, unknown. Yi *et al.* asked whether the common observation of tumour hypoxia and RTK overexpression in solid tumours were causally linked, and revealed that hypoxia or loss of VHL prolonged EGFR half-life through HIF-mediated delay of endosome formation and the eventual degradation of EGFR cargo in lysosomes [91]. The activation of signalling components downstream of EGFR was correspondingly prolonged, resulting in enhanced cell proliferation and survival. The deceleration in endocytosis was due to the attenuation of Rab5-mediated early endosome fusion *via* HIF-dependent down-regulation of rabaptin-5, a critical Rab5 effector, at the transcriptional level. Primary kidney and breast tumours with strong hypoxic signatures consequently exhibited significantly lower expression of rabaptin-5 mRNA and protein [91]. These findings suggest an unprecedented role for the general oxygen-sensing pathway in classical endocytosis that explains, at least in part, why and how RTK signalling is accentuated under hypoxia, and provide an oncogenic model in which tumour hypoxia or hyperactivation of HIF prolongs RTK-mediated signalling by delaying endocytosis-mediated deactivation of receptors.

Hypoxia and Rab11-mediated recycling of integrin

Integrins belong to a family of transmembrane receptor proteins composed of heterodimeric complexes of α and β chains. Integrins are involved in mediating cell–cell and cell–extracellular matrix (ECM) adhesion, and transduce signals from the ECM to the cell interior or 'outside-in' signalling, as well as coordinate signals to the extracellular space from inside the cell or 'inside-out' signalling [92]. Integrin dysfunction is a common event in cancer development, especially in metastasis and cancer invasion. Integrins are internalized through both CME and clathrin-independent endocytosis [93].

Yoon *et al.* recently demonstrated that hypoxia stimulates Rab11-mediated recycling of integrin $\alpha_6\beta_4$-containing vesicles to the plasma membrane [94]. This function is thought to be important in cell invasion since overexpression of a dominant-negative form of Rab11 blocks hypoxia-induced invasion [94]. Hypoxia-stimulated Rab11c (also called Rab25) was shown to directly associate with integrin $\alpha_5\beta_1$, which enables a pool of the recycling integrins to be retained at the cell front, promoting invasion into fibroblast-derived ECM [95]. Clinically relevant is that Rab11c has been found to be overexpressed in more than 50% of ovarian and breast cancers and is associated with shorter survival [96]. These findings suggest that hypoxia-mediated recycling of integrins by Rab11 plays an important agonistic role in tumour progression and invasion.

Hypoxia-mediated Na,K-ATPase endocytosis

Down-regulation of Na,K-ATPase, an ATP-dependent ion pumping system, *via* endocytosis is associated with the metastatic behaviour of several cancers [97]. In alveolar epithelial cells, acute hypoxia promotes Na,K-ATPase endocytosis, resulting in the inhibition of Na,K-ATPase activity [98–100]. Prolonged hypoxia leads to RhoA-dependent degradation of plasma membrane Na,K-ATPase [99] and intriguingly, occurs only in the presence of VHL [100]. The overexpression of Na,K-ATPase has been associated with the development of prostate cancer [101] and colorectal cancer [102]. These findings support the notion that hypoxia-mediated Na,K-ATPase endocytosis is involved in cancer development.

p38 MAPK in hypoxia-mediated endocytosis

p38 MAPK is well known stress-activated MAPK and an important regulator of cancer progression [103]. Several lines of evidence suggest a role of p38 MAPK in endocytosis. For example, p38 MAPK controls the endocytic trafficking of various growth-related

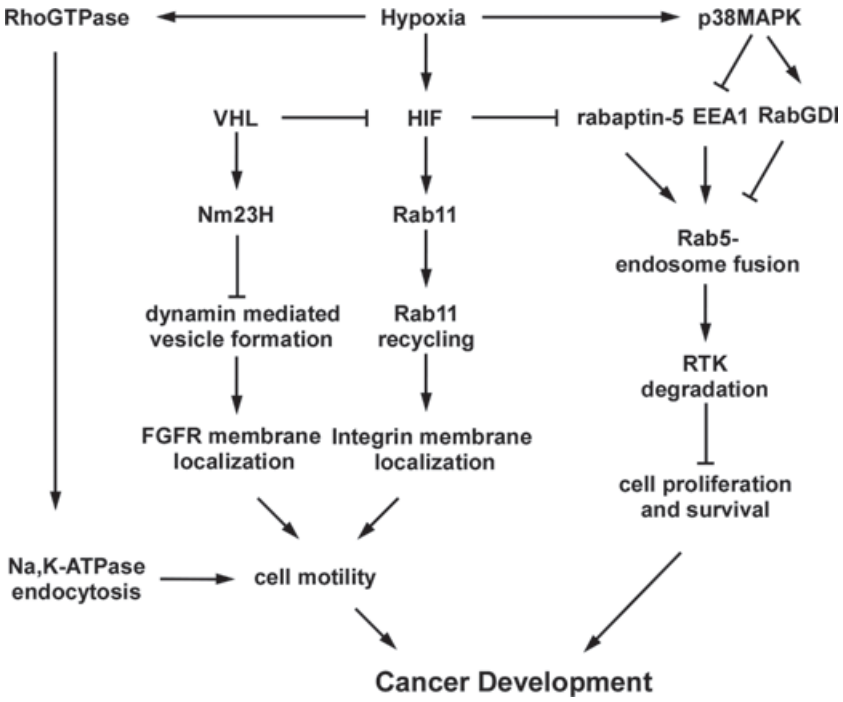

Fig. 1 Hypoxia-mediated endocytosis in cancer development. See text for details.

cell surface receptors and transporters through its ability to phosphorylate EEA1 at Thr1392, thus blocking EEA1-mediated homotypic fusion and subsequent accumulation of early endosomes [104, 105]. p38 MAPK is also required for phagolysosome biogenesis and the endocytosis of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor [104, 106]. p38 MAPK phosphorylates and activates the Rab GDP dissociation inhibitor (RabGDI), which enhances its inhibitory activity toward Rab5, attenuating or terminating the endocytic process [104]. Given that p38 MAPK responds to a wide range of stimuli, including hypoxia [107], it is reasonable to predict that p38 MAPK modulates hypoxia-mediated endocytosis.

Concluding remarks

The cellular sorting machinery, which displays many genetic and post-translational alterations in tumours, is regulated by oxygen tension. The current and aforementioned view of hypoxia-regulated endocytosis is summarized in Fig. 1. Although it is becoming increasingly clear that oxygen influences the activity of several proteins along the endocytic pathway, perhaps most remarkable to-date is rabaptin-5, which regulates both early endosome fusion and endosome recycling. A loss or attenuated expression of rabaptin-5 may contribute to oncogenesis by prolonging the retention and activity of RTKs, such as EGFR and platelet-derived growth factor receptor, as well as potentially non-RTKs in early endosomes. Rab11, which regulates cargo recycling, has been shown to be overexpressed in ovarian and breast cancers, and to increase integrin recycling in response to hypoxia. Increased membrane localization of integrins is an important and common process in tumour invasion and metastasis. In addition, p38 MAPK inhibits Rab5-dependent endosome tethering and fusion by blocking EEA1 and activating RabGDI by phosphorylation. Moreover, Rho family small GTPases, such as RhoA, Rac1 and Cdc42, which are involved in both CME and clathrin-independent endocytosis [108], have been shown to be regulated by hypoxia [109–111]. The precise molecular mechanism(s) by which oxygen affects the role of Rho GTPase in endocytosis remains to be an outstanding question. The observation that hypoxia-mediated endocytosis and degradation of Na,K-ATPase is RhoA dependent [99] offers a promising avenue for deciphering the role of hypoxia in Rho GTPase-mediated endocytosis. Another question of interest is whether HIF modulates other proteins than those involved in CME. One such candidate is caveolin, which is deregulated in several cancers [68]. In addition, caveolin-deficient mice show that caveolae and caveolins play a prominent role in various pathological conditions, especially cancer [68]. Interestingly, caveolin-1 has both tumour suppressor and oncogenic properties [112]. However, the role of hypoxia in caveolin-mediated endocytosis is unclear.

Current studies indicate that hypoxia regulates endocytosis in several ways and that this regulation is important for tumorigenesis. The lessons that emerge from these and continuing studies examining the links between hypoxia and endocytosis will undoubtedly provide better insight into the fundamental role of

hypoxia-mediated endocytosis in cancer development and optimistically provide a novel foundation for alternative cancer treatments. There are and will be many more important answers to be solved in this new area of oxygen-mediated endocytosis research, but perhaps the most interesting answer will be to the question that has yet to be realized.

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