ELSEVIER



# Can hi-jacking hypoxia inhibit extracellular vesicles in cancer?

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**Increasing evidence indicates that extracellular vesicles (EVs) are key players in undesirable cell–cell communication in cancer. However, the release of EVs is not unique to cancer cells; normal cells release EVs to perform physiological roles. Thus, selective inhibition of EV release from cancer cells is desirable. Hypoxia contributes to tumour development and aggressiveness. EV quantities and thus undesirable communications are substantially increased in hypoxia. Targeting hypoxia could selectively inhibit EV release from tumour cells without disturbing physiologically relevant EVs. The unfavourable association between hypoxia and EV release is evident in multiple tumour types; therefore, targeting hypoxia could have a broad therapeutic benefit.**



## Introduction

Although it has long been known that eukaryotic cells release complex vesicular structures into their environment, only in recent years has it been realised that these entities are not merely junk or debris but that they are tailor-made specialised mini-maps of their cell of origin. Over recent years, increasing evidence generated by ourselves and others (for examples see Refs [1–3]) indicates that in fact substantial 'cargos of information' involved in cell–cell communication are transported in the bloodstream and other body fluids in exosomes and ectosomes or microvesicles. These vesicles, collectively termed extracellular vesicles (EVs), are often defined and subgrouped based on size and cellular origin (exosomes ~30–120 nm, endosomal origin; microvesicles or ectosomes >120–1000 nm, from the cell membrane). To help establish standards and best practice, substantial efforts have been invested in developing recommendations for minimal experimental requirements for definition of EVs and their functions [4,5] and in transparent reporting and centralising knowledge in EV research [6]. In reality, however, once outside the cell and released into their environment (for example, the bloodstream), we cannot be certain whether the EVs originated from the endosomal region of cells or directly from the cell membrane. Furthermore, EV size distinctions are not absolute (i.e., there is no known reason why ectosomes budding from the cell membrane cannot be <120 nm). Much of the focus in EV research has been directed at studying EV quantities, their contents, their cells of origin (tumour and/or microenvironment) and the consequences of their release. In conditions such as cancer, inhibiting EV release could have therapeutic benefit.

## EVs in physiological conditions and in cancer

In cancer, accumulating evidence has strongly implicated EVs with having pathophysiological consequences by transferring undesirable information from cell to cell. EVs have been implicated in transmitting drug-resistance characteristics [7–16]; attracting cancer cells to secondary sites, including the brain, as metastases [17–19]; helping cancer cells evade anticancer activities of the immune system [20–23]; stimulating recipient cell proliferation, motility, invasion; and inducing neovascularisation and angiogenesis [24–26]. In parallel, however, as previously reviewed, EVs are released from healthy cells and are associated with a range of physiological functions necessary for good health [27]. For example: EVs have been isolated from milk [28–30] and have been proposed to play a part in the development of the immune system in infants [31]; EVs in amniotic fluid have been suggested to regulate the immune response to maximise foetal survival during pregnancy [32]; and EVs in bile are apparently involved in cholangiocyte regulatory mechanisms [33]. EVs from platelets in the Q2

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bloodstream can have pro-coagulation and anticoagulation properties, balancing coagulation and anticoagulation under healthy circumstances [34]. Thus, when aiming to inhibit EV release from the tumour environment for the benefit of cancer patients, consideration must be given to any potential adverse effects that could be conferred on EVs that are released from normal cells in the body.

## Hypoxia

Hypoxia is a pathophysiological property that is defined as a state of depressed oxygen tension. The presence of hypoxia in solid tumours, first described by Thomlinson and Gray [35], is strongly associated with tumour growth, angiogenesis, malignant progression, metastasis and resistance to therapy, thereby affecting the curability of solid tumours regardless of treatment modality [36]. A key mechanism by which cancer cells and stromal cells of the microenvironment adapt their metabolism to hypoxia is through the transcriptional activity of hypoxia-inducible factors (HIFs). HIFs function as heterodimers that consist of an oxygen-related HIF-1α (or HIF-2α) subunit and a constitutively expressed HIF-1β subunit. Overexpression of HIF-1α in cancer cells has been associated with an unfavourable prognosis in several cancer types [37]. Similarly, the membrane-tethered enzyme carbonic anhydrase isoform IX (CAIX), which is inducible by hypoxia, is selectively associated with cancer. Because CAIX is not expressed at a substantial level in normal tissue (with the exception of the stomach) staining for CAIX expression is now an established marker for tumour hypoxia and a clinical indicator of aggressive cancers with poor prognosis [38].

## Assessing the influence of hypoxia on EV quantities, contents and activities

When simulating hypoxia *in vitro*, typically 0.1–1.0% oxygen (sometimes described as severe and moderate hypoxia, respectively) [39] is used acutely for 4–72 h and compared to 21% oxygen to represent normoxia. Of note, 21% oxygen as normoxia represents atmospheric $O_2$ levels and is typically used for *in vitro* studies in tissue culture incubators. However, the physiological levels of $O_2$ are reported to be between 3% and 8% in tissues [40]. Twenty-one percent oxygen is likely to be hyperoxic, not truly representing $O_2$ levels in tissues *in vivo*. For EV isolation for analysis, although an increasing number of methods and variations of methods have been used, those involving ultracentrifugation are still the most favoured [41].

Considering the emerging research data available, one of the first such studies associating EVs with hypoxia involved cultured A431 skin squamous cells that, when exposed to hypoxic conditions, were found to have disrupted the extracellular matrix, increasing cell–cell adhesion, invasiveness and production of a secretion containing many EV-associated proteins [42]. This suggested that the proteins and EVs secreted from the cells facilitated metastasis and angiogenesis.

## EV quantities

Some studies have specifically reported increased quantities of EVs to be released under hypoxic compared with normoxic conditions. For example, in breast cancer cell lines (MCF-7, SKBR3, MDA-MB-231) acute hypoxia versus normoxia significantly increased the quantities of EVs released, when collected using centrifugation steps of up to 10 000 × *g* [43]. Furthermore, as detailed further below, Umezu *et al*. reported that, under chronic hypoxic conditions for 6–7 months, the quantities of EVs released from multiple myeloma cell lines (RPMI8226, KMS-11, U266) were approximately twice those released following acute hypoxia [44]. Of note, for EV isolation this study used ExoQuick™ which has been described as one of the precipitation solution-based techniques that does not succeed in extracting all exosomal particles but does co-precipitate nonexosomal impurities and thus is not considered to be EV-specific [45]. The direct cause and mechanism of increased EV biogenesis during hypoxia is still unknown.

## EV contents, tubule formation and angiogenesis, migration, invasion and metastasis

Focusing on glioblastoma multiforme (GBM), a malignant tumour type that is characterised by hypoxia, Svensson *et al*. reported conditioned medium from U87-MG GBM cells exposed to hypoxia to have increased levels of tissue factor (TF) — the vast majority of which was associated with EVs with sizes and protein markers indicative of exosomes [46]. TF and VIIa (which together form the extrinsic pathway of coagulation), carried in EVs of U87-MG cancer cells that were isolated using ultracentrifugation steps of up to 100 000 × *g*, were shown to have potential in activating protease-activated receptor 2 (PAR-2; coagulation factor II) signalling in healthy human umbilical vein endothelial cells (HUVECs). This indicates that GBM cell-derived EVs could constitute an important signalling mechanism in hypoxia-driven modulation of nonmalignant cells in the tumour microenvironment. The same group subsequently showed that EVs from plasma procured from GBM patients before surgery, when compared with plasma EVs from age- and gender-matched individuals ($n$ = 12 each), were enriched in hypoxia-regulated mRNAs and proteins [including matrix metalloproteinases (MMPs), interleukin (IL)-8, platelet-derived growth factors (PDGFs), caveolin 1 and lysyl oxidase], closely reflecting the oxygenation status of cultured GBM cells and patient tumours. Furthermore, EVs from hypoxic versus normoxia GBM cells significantly accelerated growth and angiogenesis of a GBM xenograft model *in vivo* when co-injected with U87-MG cells [47].

Treatment of the triple-negative breast cancer MDA-MB-231 cells with dimethyloxaloylglycine (DMOG) to induce HIF response or, alternatively, reduce HIF expression (with HIF siRNA) supported HIF being causally involved in increased EV release. Additionally, cellular miR-210 (associated with poor survival in breast cancer) was found to be significantly increased in hypoxic breast cancer cells (MCF-7) and thus their corresponding EVs, although it should be noted that throughout this study they typically used ExoQuick™ for EV isolation [39]. Building on this work, Wang *et al*. reported that exposure of breast cancer cells (MDA-MB-231) to hypoxia augmented the release of EVs collected by relatively low-speed centrifugation (i.e., 500 × *g* for 10 min, 2000 × *g* for 20 min and 10 000 × *g* for 70 min) that was mediated by HIF-dependent expression of RAB22A — a member of the RAB family of small GTPases that, when GTP-bound, interacts with early-endosomal antigen 1 and could be involved in the trafficking of and interaction between endosomal compartments [43].

Furthermore, pre-culture of MDA-MB-231 cells with EVs from hypoxic conditions compared to normoxic conditions showed *in*





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