

# Hypoxia, partial EMT and collective migration: emerging culprits in metastasis

Running title: Hypoxia and partial EMT during metastasis

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**Abstract:** Epithelial-mesenchymal transition (EMT) is a cellular biological process involved in migration of primary cancer cells to secondary sites facilitating metastasis. Besides, EMT also confers properties such as stemness, drug resistance and immune evasion which can aid a successful colonization at the distant site. EMT is not a binary process; recent evidence suggests that cells in partial EMT or hybrid E/M phenotype(s) can have enhanced stemness and drug resistance as compared to those undergoing a complete EMT. Moreover, partial EMT enables collective migration of cells as clusters of circulating tumor cells or emboli, further endorsing that cells in hybrid E/M phenotypes may be the 'fittest' for metastasis. Here, we review mechanisms and implications of hybrid E/M phenotypes, including their reported association with hypoxia. Hypoxia-driven activation of HIF-1 $\alpha$  can drive EMT. In addition, cyclic hypoxia, as compared to acute or chronic hypoxia, shows the highest levels of active HIF-1 $\alpha$  and can augment cancer aggressiveness to a greater extent, including enriching for a partial EMT phenotype. We also discuss how metastasis is influenced by hypoxia, partial EMT and collective cell migration, and call for a better understanding of interconnections among these mechanisms. We discuss the known regulators of hypoxia, hybrid EMT and collective cell migration and highlight the gaps which needs to be filled for connecting these three axes which will increase our understanding of dynamics of metastasis and help control it more effectively.

**Keywords:** Hypoxia, HIF-1 $\alpha$ , partial EMT, Collective migration, Inflammatory Breast Cancer, E-cadherin, Metastasis.

## Introduction

Metastasis, the leading cause of cancer-related deaths worldwide, is a complicated process involving multiple steps such as cancer cell dissemination, invasion, migration, intravasation, extravasation and colonization [1]. Metastasis has extremely high attrition rates (> 99.8%), because of the limited ability of disseminating cells to overcome the challenging and dynamic microenvironments they face during these steps of the metastatic cascade [2]. Moreover, the co-occurrence of metastasis with dormancy, drug resistance and tumor relapse further adds to the complexity. Metastasis may involve both active (i.e. cells crawl towards blood vessels) and passive processes (i.e. cells are engulfed by leaky vessels) to different extents; why cells metastasize remains an open question which has recently attracted attention on viewing metastasis from an ecological dynamics perspective [3,4]. However, molecular investigation into mediators of metastasis has revealed various hallmarks of metastasis-initiating cells [2]. The presence of hypoxia (or nutrient stress) to varying degrees in the tumor microenvironment and the ability of cancer cells to migrate collectively as a cluster or cohort have emerged as recent culprits in the process [5,6]. Clusters of circulating tumor cells (CTCs) can form a disproportionately higher rate of metastasis as compared to individually migrating CTCs which are typically mesenchymal cells [7–9], suggesting that the disseminating cancer cells follow 'United they stand, divided they fall'.

Signatures of collective migration have been noticed clinically as tumor buds – the clusters of undifferentiated cells mainly in the invasive front of a tumor [10]. These tumor buds in colorectal cancer have been shown to express HIF-1 $\alpha$  [11] – one of the major regulators of cellular hypoxic response, at least for acute and intermittent hypoxia [12]. HIF-1 $\alpha$  has also implicated in driving epithelial-mesenchymal transition (EMT) and its associated traits such as cancer stem cells (CSCs) [13]. EMT is a cell biological process during which epithelial cells lose their traits such as apico-basal polarity and cell-cell adhesion and/or acquire mesenchymal traits such as migration, invasion and adhesion-independent growth [14]. Recent studies have shown that EMT is not a binary process and involves multiple hybrid phenotypes *en route* EMT [15,16]. Cells in these hybrid E/M phenotype(s) tend to display properties of both epithelial and mesenchymal cells, indicating that EMT is being induced but not completed, also termed as partial EMT [17]. Hybrid E/M phenotype has been implicated in collective cell migration of cancer cells [18,19]. The enhanced stemness and drug resistance seen in hybrid E/M cells as compared to fully epithelial or fully mesenchymal cells may offer fitness advantages during various bottlenecks that cancer cells tend to face during the metastatic cascade [20].

Hypoxia is a crucial factor known to be involved in the regulation of various hallmarks of cancer [13,21]. While many normal cells die under hypoxia, cancer cells can adapt to hypoxic condition by reprogramming their gene expression profiles [22]. Hypoxia has been shown to be involved in the induction of EMT, drug resistance and metastasis [23]. Intermittent or cyclic hypoxia – believed to be the most commonly observed scenario in a tumor – has been shown to accelerate tumor growth through cellular adaptation driven by HIF-1 $\alpha$  [24]. Hypoxia can push cells towards a partial EMT [25,26]; however, a better mapping of different extents of hypoxia in terms of duration and/or oxygen concentration with corresponding EMT phenotypes attained remains to be done. In this review, we will briefly discuss mechanisms underlying attaining and stably maintaining the hybrid E/M phenotype(s), its role in mediating stemness

and drug resistance, as well as mediators of varying cellular hypoxic response. We will also connect the dots between hypoxia, partial EMT, and collective cell migration and discuss their cross-regulations and synergistic contribution as drivers of metastasis.

## Defining partial EMT

EMT and its reverse mesenchymal-epithelial transition (MET) are evolutionarily conserved cell biological processes involved in embryonic development, tissue repair and wound healing [27,28]. EMT is believed to facilitate dissemination and migration of primary tumor cells to secondary sites where they may undergo MET to form secondary tumors resulting into cancer metastasis [2]. EMT can be associated with various traits crucial to metastasis such as tumor-initiation, therapy resistance, immune evasion and anchorage-independent growths [15,29].

At the molecular level, EMT results in transcriptional inhibition and/or loss of membrane localization of epithelial cell-surface marker E-cadherin and gain of mesenchymal markers such as vimentin, N-cadherin,  $\alpha$ -smooth muscle, and fibronectin [30,31]. Many signalling pathways such as Wnt/ $\beta$ -catenin, TGF- $\beta$ , FGF, EGFR, Notch, Hedgehog and BMP signaling and/or alterations in the extra-cellular matrix (ECM) stiffness can induce EMT [17]. EMT progression is often associated with increase in the expression of EMT-inducing transcription factors (EMT-TFs) such as ZEB1/2, SNAI1/2, TWIST, FOXC2 and GSC that can receive signals from the abovementioned signaling pathways [30]. EMT is inhibited and/or reversed by various MET-inducing transcription factors such as GRHL2, OVOL1/2, and ELF3/5, many of which form mutually inhibitory feedback loops with one or more EMT-TFs; for instance, GRHL2 and ZEB1 inhibit each other [32–35], so do OVOL1/2 and ZEB1 [36,37]. ELF3 can repress EMT via inhibiting ZEB1 [38], and ELF5 can inhibit SNAI2 [39]. Such feedback loops have been observed at many instances of cellular differentiation and can often allow for the (co)-existence of two (or more) cell states [40](in this case: epithelial, mesenchymal and hybrid E/M states).

Recent high-throughput studies have emphasized that EMT is not a binary process but can progress through multiple stable hybrid states [41,42] referred often interchangeably as intermediate EMT, intermediate mesenchymal, partial EMT, hybrid epithelial/mesenchymal, incomplete EMT, semi-mesenchymal, EMT-like, metastable [10]. The importance of hybrid E/M state(s) is supported by observations that induction of fully mesenchymal state results into loss of tumor-initiating and/or colonization potential [43,44] while cells in the hybrid E/M state might display maximum tumor-initiation potential when compared with cells in epithelial or mesenchymal states [44–47].

EMT is regulated by complex regulatory networks involving transcription factors, miRNAs, growth factors, non-coding RNAs and epigenetic modifiers [29,30]. Mechanism-based mathematical modelling of these networks has revealed how hybrid E/M states can be attained and stably maintained; for instance, miR-200/ZEB feedback loop can allow three stable states: miR200<sup>high</sup>/ZEB<sup>low</sup> (epithelial), miR200<sup>low</sup>/ZEB<sup>high</sup> (mesenchymal), and miR200<sup>med</sup>/ZEB<sup>med</sup> (hybrid E/M) [48]. Extensions of this model have revealed the existence and signatures of hybrid E/M phenotypes, and identified molecules that may contribute to maintaining these phenotypes such as GRHL2 and OVOL1/2, called as 'phenotypic stability factors' [49–58].

Recent observations using sophisticated techniques such as lineage tracing in patient-derived xenografts, genetically engineered mouse models (GEMM), single-cell RNA-seq, fluorescent

activated cell sorting (FACS), immunohistochemistry (IHC), and microfluidic setups have advanced our understanding of hybrid E/M cells *in vitro* and *in vivo* [15,59]. Single-cell studies have provided evidence of hybrid E/M cells co-expressing markers of both epithelial and mesenchymal cells such as CDH1 (E-cadherin) and VIM (vimentin), thus overcoming the limitations of bulk measurements. For example, experimental studies using FACS analysis have shown breast cancer cells within a population can display phenotypes with varying EMT status, including cells co-expressing both epithelial and mesenchymal markers [45,53]. Similarly, lung cancer cells H1975 have been shown to display hybrid E/M phenotype co-expressing E-cadherin and vimentin over several passages [34]. Multiple single-cell studies have used different epithelial and mesenchymal markers to identify the relative abundance of hybrid E/M cells in cancer cell lines, for example, SNAI/E-cadherin in lung cancer cell lines (A549, LT73, and H460) [60] and in clear cell renal cell carcinoma cells [61], SNAI/E-cadherin/occludin in colorectal cancer [62], epithelial surface marker CD24 and mesenchymal marker CD44 in breast cancer cell line HMLER [45], and vimentin and keratin intermediate filaments in breast cancer cell lines [63]. In addition, hybrid E/M phenotype is also observed in circulating tumor cells (CTCs) [64,65], *in vivo* mouse models [20], lymph node progression of NSCLC patients [66] and in brain metastases and patient samples [67,68]. Moreover, the relative abundance of E-cadherin and vimentin in a cell population is used to classify cell lines into epithelial, mesenchymal and hybrid E/M phenotypes in many cancer types [69–71]. In a recent study, many hybrid E/M states were observed in skin and mammary primary tumors using combinatorial FACS analysis of EPCAM (epithelial cell adhesion molecule) and CD106, CD61, and CD51 – the most heterogeneously expressed ones during EMT [41].

Another widely used method to study heterogeneity in EMT status of cells is through single-cell transcriptomic analysis of tumor cells; head and neck squamous cell tumors showed malignant hybrid E/M cells present at the leading edge [72]. Various transcriptomic signature-based metrics have helped quantify the EMT status of cells along a continuous spectrum; these methods exhibit concordant results among themselves [73]. Characterization of hybrid E/M cells in tumor samples is gaining importance in terms of deciding the course of treatment and thus unravelling the contributions of cells with different EMT phenotypes in metastasis, drug resistance, and disease aggressiveness – an important aspect enabled only by such high-throughput single-cell analysis [74–76].

Though molecular markers and transcriptomic signatures are extensively used to characterize EMT, cellular motility and morphology traits are another axes to identify phenotypic heterogeneity of cells in a population [77,78]. For example, MDA-MB-468 cells treated with EMT-inducing EGF were segregated into cobble, spindle and circular states based on the cellular morphologies. Through quantitative image analysis and mathematical modelling, the authors showed that these morphological state transitions are reversible and depends upon EGF dose and the level of epidermal growth factor receptor (EGFR) phosphorylation [77]. Similarly, Gaussian mixture model focussing on morphometric features associated with transition from compact to elongated phenotype – such as elongation of subcellular nuclear, vimentin and cytoplasmic features – furnished the EMT status of individual cells in a heterogenous cell population [79]. Quantitative epithelial-mesenchymal scoring method based on cell morphologies using support vector machine learning was developed to classify cells into epithelial and mesenchymal phenotypes. Using this method, breast cancer cells MDA-MB-231 and MCF-7 showed morphological qualities of both epithelial (rounded) and mesenchymal (elongated) cells suggestive of hybrid E/M phenotype [80], indicating that at least a subpopulation of these seemingly ‘pure’ epithelial and ‘pure’ mesenchymal cells [81]

may exhibit hybrid E/M phenotype(s) [82]. Whether these morphological changes correspond with molecular signatures of hybrid E/M remains to be identified; thus, EMT may progress at least semi-independently on these molecular and morphological axes [83]. Thus, a falsifiable definition of partial EMT states at biophysical and/or biochemical states shall be helpful in decoding the unique functional attributes of hybrid E/M phenotypes.

### **Role of partial EMT in collective migration**

Cancer cells detach from the primary tumor and invade surrounding tissues to successfully metastasize to secondary locations. Broadly speaking, two major modes of invasion methods have been reported: single cell (amoeboid- or mesenchymal- like movement) and collective cell migration [84,85]. Cancer cells can switch between these two invasion strategies depending upon environmental stimuli [84,86]. In collective cell migration, cancer cells retain cell-cell adhesions while invading surrounding tissues and can display a variety of migration modalities such as compact cluster/budding of cells, narrow linear strands or broad sheets of cells [87]. A quantitative 3D assessment of tumor budding in pancreatic, breast, colorectal and lung adenocarcinoma tumor buds has shown that collective cell migration is the predominant mechanism of invasion in cancer [88].

Hybrid E/M phenotype(s) have been implicated in collective cell migration. Many traits of hybrid E/M phenotype such as loss of polarity, shift towards spindle shape morphology, partial loss of total and/or membranous E-cadherin and nuclear accumulation of ZEB1 have been shown to be displayed by tumor buds [88]. In *Drosophila melanogaster* model of colorectal cancer, SNAIL homolog Sna was shown to activate partial EMT which facilitated collective cell migration through basement membrane and muscle fibre, leading to polyclonal metastases [89]. Similarly, collective migration in murine model of squamous cell carcinoma and CTC clusters in head and neck cancer patients can be elicited by SNAIL by inducing claudin-11 [90]. A biophysical model that recapitulates the frequency and size distribution of CTC clusters across cancer types has emphasized the importance of cells in multiple hybrid E/M states in forming CTC clusters of varying sizes [91]. CTC clusters can also contain stromal cells such as platelets, immune cells and cancer-associated fibroblasts [92], which protect CTC cluster from shear stress and immune attack while in blood circulation [93] and may secrete cytokines to facilitate colonization [94].

The association of hybrid E/M phenotypes and collective invasion is obfuscated by the variability of molecular mechanisms driving collective invasion. For instance, while primary invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) both display collective invasion, there exist molecular differences: IDC retains E-cadherin and contains adherens junctions and multicellular clusters, ILC lacks E-cadherin and  $\beta$ -catenin and show CD44 at cell-cell contacts [95]. Similarly, collective cell migration in prostate cancer cells lacking  $\alpha$ -catenin was shown to be mediated by N-cadherin junctions [96]. Thus, collective migration does not seem to be exclusively mediated via E-cadherin based cell-cell junctions.

Moreover, the EMT status of cells in collectively invading cohort may vary based on spatial localization. In a recent study investigating collective invasion in cancer cell spheroids using 3D assays, CSC-like cells with a hybrid E/M phenotype were shown to be at the periphery leading the collective invasion [97]. Similarly, in 3D organoid model of invasive primary breast cancer, the leader cells at the invasive boundary showed collective cell migration while staying

attached to the follower cells at the rear end. Leader cells displayed altered polarity and frontal protrusion development, yet maintenance of epithelial markers such as cytokeratin 14 (K14) and p63, pointing towards a hybrid E/M phenotype [98]. Collective dissemination of K14<sup>+</sup> epithelial cell cluster has been shown to cause distant polyclonal metastases in mouse model of breast cancer [8]. Besides K14, plakoglobin, an essential component of desmosomes and adherens junction, has been shown to be important for CTC cluster formation and consequent metastasis. Its knockdown abrogates CTC cluster and suppresses lung metastasis *in vivo*. Consistently, in breast cancer patients, the abundance of CTC clusters and plakoglobin shows adverse outcomes, suggesting a clinical validation of observations in mouse models [99]. Therefore, collective invasion and metastasis can be fuelled by at least a partial retention of epithelial traits [100] such as E-cadherin, which has been shown to be important for survival of invasive ductal carcinomas during invasion, dissemination and colonization [101–103].

Cells in a partial EMT phenotype exhibiting collective migration as clusters were also seen in a mouse model of pancreatic ductal adenocarcinoma (PDAC). However, these cells lacked the membranous expression of E-cadherin due to its accumulation in recycling endosomes and consequent disrupted trafficking, not due to transcriptional inhibition usually seen in EMT. In this mouse model, a subset of tumors exhibited traditional EMT program with transcriptional repression of epithelial program, including the levels of E-cadherin. In contrast to 'partial EMT' cells, these 'complete EMT' cells migrated as single cells [31] (Fig 1). Thus, the standard analysis of measuring E-cadherin levels at a histopathological or expression level need not be sufficient for evaluating its functional contribution to cell-cell adhesion and collective cell migration [104].

The biochemical and biomechanical interplay of regulation of collective cell migration remains to be better understood. The dynamic and coordinated decision-making in terms of leader and follower cells, as well as their interchange of positions as seen in *in vitro* setups [105–107] may affect the formation and maintenance of CTC clusters and their metastatic propensity. Decoding the mechanisms of non-cell-autonomous plasticity in terms of EMT through juxtacrine and/or paracrine mechanisms [108,109] and its effect on CTC clusters, collective invasion and metastatic propensity are some of the open questions that can help resolve contradictory reports about the dispensability of EMT in metastasis.

### **Partial EMT, stemness and drug resistance**

Successful metastasis of cancer cells from primary tumor to secondary sites requires both the migration of primary cancer cells and their capability to initiate tumor growth at the secondary site. The latter depends upon the tumor initiation potential of the cancer cells, also referred to as 'stemness' [110]. Though still debatable, cancer stem-like cells (CSCs) represent a minor subpopulation of tumor cells capable of tumor initiation (stemness) and clonal expansion through asymmetrical cell division producing both CSCs and non-CSCs [111]. Recent studies have emphasized that cancer cell stemness is a dynamic trait associated with phenotypic plasticity, reflected by switching of CSC to non-CSC and *vice versa* [112]. Initial reports suggested EMT to be coupled with a gain of stemness [113,114]; however, more recent studies have shown that full EMT might reduce tumor initiation potential of cancer cells [41,43–45]. Instead, stemness is most likely maintained by cells in hybrid E/M state rather than cells in pure E or pure M states, as seen across multiple cancer types [20]. A mathematical modelling study coupling regulatory circuits for stemness (LIN28/let-7) and EMT (miR-

200/ZEB) has shown that hybrid E/M cells can gain stemness [115]. This positioning of 'stemness window' on the 'EMT axis' is not fixed; it can be fine-tuned by modulatory factors such as OVOL or Notch signaling [37,116]. Experimentally, breast cancer cells expressing signatures of hybrid E/M phenotype were shown to display greater stemness as compared to pure E or M cells [45] (Fig 1). Similar, hybrid E/M cells isolated from *in vivo* mouse model of prostate cancer showed comparable or enhanced stemness and invasive characteristics as compared to pure E and M cells [43]. Consistent observations were seen in hybrid E/M cells isolated from primary ovarian cancer cultures and ovarian tumors *in situ* [117]. Thus, further understanding of molecular mediators of coupling between partial EMT and stemness of cancer cells will provide important therapeutic vulnerabilities.

An important clinical barrier in the treatment is the development of drug resistance in cancer cells. Cells undergoing EMT and/or CSCs have been shown to be associated with drug resistance as compared to bulk of the tumor and are responsible for tumor regrowth [118]. Accumulating evidence about hybrid E/M promoting stemness proposes the hypothesis that hybrid E/M cells may play a strong role in mediating development of drug resistance. For example, taxanes induced a phenotypic transition to a chemotherapy-tolerant state (CD44+/CD24+) which was previously shown to exhibit a hybrid E/M phenotype [45] in *in vitro* and *in vivo* models of breast cancer subtypes [119]. Similarly, tamoxifen-resistance in MCF-7 breast cancer cells [120], trastuzumab-resistance in HER2-overexpressing breast cancer cells [121] and radiation-resistance in colorectal cancer cells [122] have been shown to be associated with hybrid E/M phenotype. Thus, studying cancer progression by taking a multi-dimensional approach integrating the molecular processes such as EMT, stemness and drug-resistance is crucial for a comprehensive systems-level understanding of the dynamics of cancer metastasis and developing effective therapeutics to curb the same [123].

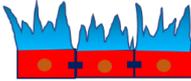
### **Stabilizers of partial EMT phenotype**

Hybrid E/M state has been earlier suggested to be transient and may not represent a stable phenotype [124]. However, many network-based studies have demonstrated the possibility of relatively high stability of hybrid E/M state [17]. Hybrid E/M phenotype can be stably maintained by different cell lines over multiple passages [34,125]. Moreover, cells isolated from *in vivo* tumors contain hybrid E/M cells [41,72,126]. Thus, hybrid E/M state can be a stable phenotype, especially in the presence of phenotypic stability factors (PSFs) such as OVOL, NRF2, GRHL2, NUMB,  $\Delta$ NP63 $\alpha$ , and NFATc that can promote and stabilize hybrid E/M state and increase the mean residence times of cells in hybrid E/M states [34,51,53,55,125,127–129]. Knockdown of these PSFs in H1975 lung cancer cell line impaired a stable hybrid E/M phenotype and collective migration and drove the cells towards a complete EMT [34,128]. Similarly, knockdown of NRF2 in H1975 and RT4 bladder cancer cells inhibited hybrid E/M phenotype and collective cell migration [125]. Many of these PSFs were found to be involved in transitions among multiple hybrid E/M phenotypes *in vivo* in squamous cell carcinoma mouse models [15]. Yet, the role of these PSFs in enabling the formation of CTC clusters remains to be shown.

Besides intracellular PSFs, another stabilizer of hybrid E/M state can be combinations of EMT-inducing and MET-inducing signals. For example, MCF-10A and DLD1 cells when treated simultaneously with TGF- $\beta$  and vascular endothelial growth factor A (VEGF-A), demonstrated a hybrid E/M phenotype [129]. Similarly, combinatorial treatment of oral squamous cell

carcinoma (OSCC) cells with TGF- $\beta$  and retinoic acid (known inducer of epithelial differentiation and opposes TGF- $\beta$  signaling) resulted into enrichment of post-EMT CSC cell population with CD44<sup>high</sup>EpCAM<sup>low/-</sup> CD24<sup>+</sup> cell surface markers exhibiting enhanced phenotypic plasticity and therapeutic resistance [130], suggesting enrichment of hybrid E/M state [45]. Other signaling pathways such as SRC proteins, SRC2 and SRC3 [131], colony-stimulating factor 1 (CSF-1) and its receptor CSF-1R [132] have been shown to be involved in maintaining hybrid E/M state.

Another layer of regulation of EMT and hybrid E/M states is epigenetic modifications [133]. Epigenetic changes can control the propensity and frequency of reversible or irreversible EMT or MET [134]. Prolonged exposure of epithelial cells to EMT inducing signal promotes complete EMT and inhibits recovery of these cells back to the epithelial state possibly because of stabilization of long-term transcriptional activity as a result of epigenetic feedback on the miR200/ZEB loop [134–136], thus offering a possible mechanism of different degrees of perturbation required to induce MET [137]. The chromatin modifier protein HMGA2 has been shown to regulate epithelial-mesenchymal plasticity and is upregulated in hybrid E/M and mesenchymal cells of mouse prostate cancer [138]. Different histone modifications and/or CpG methylation are involved in regulation of epithelial genes in the hybrid E/M states [139], including the regulation by GRHL2, a MET-TF which can also facilitate the accessibility of epithelial enhancers [140]. Besides, the Mbd3/NuRD complex, in combination with activities of HDACs, and Tet2 hydroxylase, are important regulators of epithelial-mesenchymal plasticity in EMT models of murine breast cancer [141]. Future work decoding the integrated regulatory network for hybrid E/M phenotypes at transcriptional, post-transcriptional and epigenetic levels will be important in decoding their dynamics and functional attributes.

Hybrid epithelial/mesenchymal phenotype in metastasis 	'Completely' mesenchymal phenotype in metastasis 
Collective cell migration; can associate with tumor/stromal cell to form tumor emboli	Single cell migration
Enhanced stemness with high tumor initiation capacity	Reduced stemness/tumor-initiation capacity
Higher survival in bloodstream and more efficient in metastasis	Less efficient in metastasis

**Fig 1: Attributes of partial EMT.** Comparison of functional attributes of cells displaying hybrid E/M phenotype with those displaying completely mesenchymal cells, during metastasis

## Hypoxia

Hypoxia, i.e. reduced oxygen levels, is a crucial factor in tumor microenvironment (TME) that can regulate multiple hallmarks of cancer such as angiogenesis, invasion, EMT, stemness and immune evasion [13,142]. Similar to EMT and/or CSC phenotypes in a tumor, intratumoral hypoxia can also be heterogenous spatially and/or temporally such that certain regions of a tumor are hypoxic ( $O_2$  concentration between 1-2% or below) while surrounding regions are

well oxygenated (normal physiological O<sub>2</sub> concentration ranges between 4.6-9.4%) [143–145]. Based on the dynamical profile, tumor hypoxia can be classified into acute, chronic and cyclic hypoxia. Acute hypoxia, also called as perfusion-limited hypoxia, arises due to the temporary blockage of capillaries because of the pressure exerted by the growing tumor mass or irregular erythrocyte flow and lasts for shorter timescales (from few minutes to few hours) as the capillaries may regain blood circulation [146,147]. On the other hand, chronic hypoxia, also called as diffusion-limited hypoxia, arises due to the increased distance between the tumor cells and the nearest blood capillaries as a result of increased cellular density due to over-proliferation of cancer cells. It can last for >24 hours and these regions can show necrosis because of complete oxygen deprivation [6,146]. The third category, cyclic hypoxia, also called intermittent hypoxia (IH), can arise due to a transient shut down of inefficient tumor vasculature resulting into periodic cycles of hypoxia and reoxygenation in tumor cells. The timescale, duration and frequency of intermittent hypoxia-reoxygenation cycles may vary from minutes to days [12,148–150] and may cause reoxygenation injury due to increased oxidative stress and free radical formation leading to tissue damage [151].

Hypoxia inducible factors (HIFs) are known to be the master regulators of gene expression under oxygen-deprived conditions. Transcriptional regulation by HIF proteins is mediated by a heterodimeric HIF complex consisting of HIF $\alpha$  and HIF $\beta$  subunits, which bind to HIF responsive element (HRE) present in the promoter region of target genes [152]. While stability of HIF $\alpha$  is oxygen-dependent, HIF $\beta$  is expressed constitutively. Members of prolyl hydroxylase domain (PHD) family function as oxygen-level sensor, which, under normoxia, hydroxylate two proline residues present at the oxygen-dependent degradation domain of HIF $\alpha$ . These hydroxylated proline residues are recognized by the von-Hippel Lindau (VHL) protein, which acts as substrate recognition protein of an E3 ubiquitin ligase complex that targets HIF $\alpha$  for ubiquitination and proteasomal degradation. Hypoxia inhibits degradation of HIF $\alpha$  by inhibiting PHD resulting in accumulation of HIF $\alpha$  [146]. Three HIF $\alpha$  proteins have been identified in metazoans – HIF-1 $\alpha$ , -2 $\alpha$  and -3 $\alpha$ . HIF-1 $\alpha$  and HIF-2 $\alpha$  share 48% amino acid sequence and have similar structures; however, they are differentially expressed and target different genes [153,154]. While HIF-1 $\alpha$  is ubiquitously expressed, HIF-2 $\alpha$  show tissue specific expression [146]. Similar to HIF-1 $\alpha$  and HIF-2 $\alpha$ , HIF-3 $\alpha$  contains bHLH and PAS domains but lacks C-terminal transactivation domain and has been shown to display HIF-1 $\alpha$  regulatory function [155,156].

Hypoxic condition in TME show variability in terms of duration of hypoxia and levels of oxygen during hypoxia. HIF-1 $\alpha$  and HIF-2 $\alpha$  show differential stability based on hypoxic condition. For example, HIF-2 $\alpha$  but not HIF-1 $\alpha$ , has been shown to regulate the hypoxic response at higher oxygen level (5% O<sub>2</sub>) in neuroblastoma cells [157,158]. Similarly, both HIF-1 $\alpha$  and HIF-2 $\alpha$  are stabilized at 1% O<sub>2</sub> but HIF-1 $\alpha$  stayed active only for small duration, while HIF-2 $\alpha$  was stabilized for longer duration, suggesting a crucial role of HIF-1 $\alpha$  in mediating acute responses and switching to HIF-2 $\alpha$  mediated response during prolonged hypoxia. HIF-1 $\alpha$  protein show highest peak at 4-8 hours of hypoxia and then gradually decreases while HIF-2 $\alpha$  stabilizes later and remain stabilized for longer hours (24-72 hours) [157,158].

On the other hand, dynamics of HIF-1 $\alpha$  and HIF-2 $\alpha$  stabilization during cyclic hypoxia show completely a different trend. Studies have shown that cyclic hypoxia response is largely, if not entirely, driven by HIF-1 $\alpha$ . In different combinations of durations and frequencies of hypoxia-reoxygenation (H-R) cycles in cancer and endothelial cells, HIF-1 $\alpha$  protein levels have been observed to be high during the hypoxic phase of H-R cycles but lost during reoxygenation

phase [159,160]. Moreover, levels of some of the HIF-1 $\alpha$  regulated genes were found to be upregulated during reoxygenation phase in a stress-granule dependent manner where the sequestered HIF-1 $\alpha$  transcripts were released upon reoxygenation [161]. Multiple regulatory mechanisms have been shown to govern the enhanced stability of HIF-1 $\alpha$  during cyclic hypoxia such as activation of protein kinase A and ROS mediated HIF-1 $\alpha$  stabilization [160,162–164].

With the realization that intermittent/cyclic hypoxia may be a more common phenomenon in TME as compared to chronic hypoxia, recent focus has been moving to identify similarities and differences in response of cells to cyclic hypoxia vs. those in chronic hypoxia. For example, under IH, cancer cells have shown to display greater metastasis in mouse models of human cancers, as compared to chronic hypoxia [165–167]. IH has been shown to increase angiogenesis [168–171] and the survival of endothelial cells under proapoptotic stimuli with enhanced migration and tubulogenesis in a HIF-1 $\alpha$ -dependent manner [168,169]. Similarly, IH has been shown to increase stem-like properties in breast cancer and neuroblastoma cells [172,173]. In addition, as compared to chronic hypoxia, IH resulted into greater selection of gastric cancer cells with higher self-renewal and survival properties [174]. Increased HIF-1 $\alpha$  under IH has been shown to upregulate mesenchymal markers such as vimentin, SNAIL, N-cadherin in different cancer cells [175–177]. Moreover, in tumor regions experiencing cyclic hypoxia in glioblastoma xenografts, HIF-1 $\alpha$  has been shown to induce chemoresistance against doxorubicin and BCNU by upregulating the expression of ABCB1 efflux transporter [178]. Similarly, higher radioresistance in cancer cells under cyclic hypoxia in a HIF-1 $\alpha$ -dependent manner has been observed [161,162,169]. Together, these studies suggest that cyclic hypoxia aggravates cancerous properties of tumor cells to a greater extent and the effects could be mediated by HIF-1 $\alpha$  as evidenced by some studies.

### **HIF-1 $\alpha$ mediated regulation of partial EMT and collective migration**

Cells in a hybrid E/M state show large phenotypic plasticity [82,138], thus potentially enabling cells to switch between different cell states such as CSC and non-CSC, drug-tolerant and drug-sensitive. One common molecular player which might be playing a crucial role in increasing the aggressiveness of cancer cell during IH and partial EMT is HIF-1 $\alpha$ . In this section, we will discuss how HIF-1 $\alpha$  is involved in regulating factors associated with EMT and try to connect HIF-1 $\alpha$  with partial EMT. Pancreatic cancer cells grown under hypoxic condition were shown to display partial EMT in HIF-1 $\alpha$  dependent manner with reduced E-cadherin and increased vimentin as compared to cells grown in normoxic or hyperoxic conditions [26].

Hypoxia induced EMT is shown to be mediated by HIF-1 $\alpha$  which has been shown to regulate many molecules involved in EMT [13]. For example, HIF-1 $\alpha$  and TGF $\beta$  has been shown to facilitate the activation of one another in hepatocyte, endothelial and cancer cells [179–182]. In addition, HIF-1 $\alpha$  regulates TGF $\beta$ 1/SMAD3 signaling to promote breast cancer progression [183]. Similarly, induction of EMT by Wnt/ $\beta$ -catenin has been shown to be driven by HIF-1 $\alpha$  in prostate and hepatocellular carcinoma [184,185].

Multiple EMT-TFs are induced by HIF-1 $\alpha$  under hypoxic condition. HIF-1 $\alpha$  directly binds to HREs present in the promoter of TWIST, an inducer of EMT and activates it in hypopharyngeal and breast cancer cells [186]. Regulation of SNAIL by HIF-1 $\alpha$  has been shown through direct binding to HRE in mouse C166 cells [187] and through transcription activation mediated by

HDAC3 in cancer cells [188]. Additionally, HIF-1 $\alpha$  mediated activation of SNAIL was found in hepatocellular and lung carcinoma [189,190]. SLUG is also suggested to be regulated by direct binding of HIF-1 $\alpha$  to HREs present in its promoter [191], and ZEB2 activation along with SNAIL activation and E-cadherin repression is shown to be mediated by HIF-1 $\alpha$  in renal cancer cells [192]. HIF-1 $\alpha$  mediated direct regulation of ZEB1 is shown in colorectal cancer [193], bladder cancer [194], glioblastoma [195] and pancreatic cancer cells [196].

HIF-1 $\alpha$  mediated activation of cancer-associated fibroblasts (CAFs) in the presence of COX-2 and NF- $\kappa$ B have been shown to induce EMT in prostate cancer cells in a ROS dependent manner [197], suggesting non-cell autonomous EMT induction by proinflammatory signals. Another example of non-cell autonomous signaling leading to induction of EMT is through MMP-2 and MMP-9. ROS stabilized HIF-1 $\alpha$  has shown to induce expression of CAIX in CAFs, which is required for activation of stromal cell delivered MMP-2 and MMP-9 proteins in prostate cancer cells [198]. Pro-inflammatory cytokines such as TNF $\alpha$ , IL-1 $\beta$  and IL-6 have been shown to induce HIF-1 $\alpha$  expression, which drives EMT in prostate and renal cancer cells [199,200]. In addition, TNF $\alpha$  stabilizes SNAIL by activating NF- $\kappa$ B pathway in cancer cell lines [201,202] and NF- $\kappa$ B activity induces HIF-1 $\alpha$  transcription and protein accumulation [203].

Under hypoxia, HIF-1 $\alpha$  driven cellular response activates Notch signaling, which has been shown to activate Snail/Slug, Twist and Zeb1/2 as well [204,205]. In ovarian cancer cells, Notch signaling can potentiate HIF-1 $\alpha$  expression which stabilized SNAIL in LOX-dependent manner [206]. Thus, multiple feedback loops may regulate the interconnection between hypoxia and EMT. Intermittent hypoxia has shown to increase expression of HIF-1 $\alpha$  and NRF2, a key regulator of oxidative stress [163]. NRF2 and HIF-1 $\alpha$  show a complex crosstalk where NRF2-TRX1 induction enhanced HIF-1 $\alpha$  expression in IH [207]. Moreover, NRF2 has been implicated in stabilization of the hybrid E/M state in lung and bladder cancer cells [125], suggesting a regulatory function of HIF-1 $\alpha$  in hybrid E/M phenotype. Exposure of MDA-MB-468 and T47D breast cancer cell line to hypoxia-induced Snail expression without affecting E-Cadherin levels consolidates the proposed connection between partial EMT and hypoxia [208]. Finally, the periodic stabilization and degradation of HIF-1 $\alpha$  during hypoxia and reoxygenation of cyclic hypoxia, respectively might result into periodic TGF $\beta$  signaling through BAMBI, a glycoprotein related to type I TGF $\beta$  receptor, which has been shown to be regulated by HIF-1 $\alpha$  under hypoxic condition [209]. This, in turn, may promote hybrid E/M state, because long-term EMT inducing signal has shown to inhibit the recovery of cells back to epithelial state [134].

Hybrid E/M cells are considered to be poised for collective cell migration; however, the current connection between hypoxia and collective cell migration remains unclear in cancer. Tumor buds – clusters of undifferentiated cells seen in invasive edge of colorectal cancer – have been shown to express HIF-1 $\alpha$  [11]. Invasive edge has been reported to contain HIF-1 $\alpha$ -positive cells [210], and is enriched in more mesenchymal phenotypes [211–213] in other cancers too, but the presence of hypoxia at invasive edge is not exclusive – it has been reported for internal tumor core as well [214]. Therefore, the connection between hypoxic response and a partial or complete EMT remains ambiguous. HIF-1 $\alpha$  has been shown to induce varying degrees of EMT by inhibiting E-cadherin [215–217] and/or upregulating SNAIL and ZEB1 [193,215]. However, HIF-1 $\alpha$  has been shown to favour ameboid migration in epithelial cancers [218,219] by inducing collective-to-ameboid transition (CAT) in breast and head and neck cancer spheroids also, as observed in 3D culture experiments. Based on TWIST-dependent EMT induction, ameboid cells were classified as EMT-like and EMT-

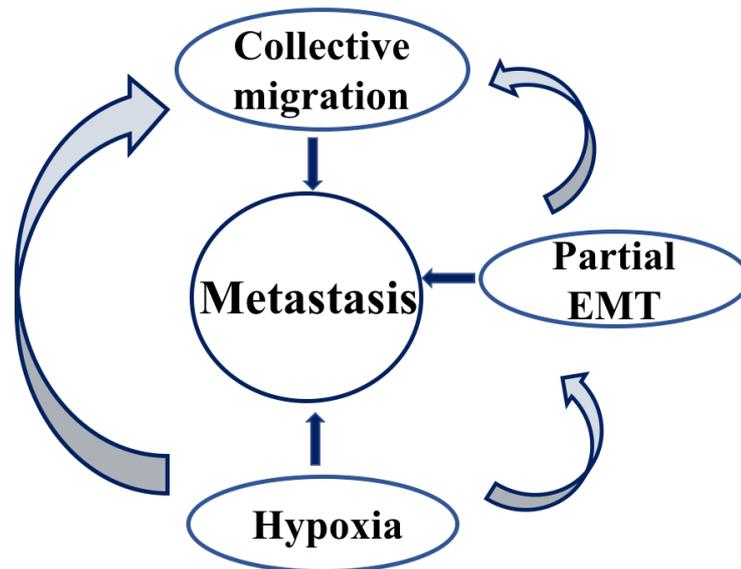
independent cells. HIF-1 $\alpha$  was shown to facilitate ameboid dissemination from collective invasion strands with reduced E-cadherin levels [218]. Enhanced migration of endothelial cells under hypoxia as compared to normoxia in a microfluidic experimental setup has shown to be associated with loosening of intercellular junction resulting from reduced expression of VE-cadherin [220], consistent with reports of hypoxia involved in directed collective migration of border cells in developmental scenarios [221]. In prostate cancer cells, hypoxia induces the expression of SLUG – often associated with a partial EMT [17] – but does not lead to a complete loss of E-cadherin [222], thus possibly endorsing the hypoxia-partial EMT connection. In brain tumor, cells under hypoxia did not exhibit faster migration as compared to normoxia in either scratch or spheroid based experiment [223]. Thus, further analysis is required to understand how hypoxia/ HIF-1 $\alpha$  can drive partial and/or complete EMT and the corresponding functional consequences for metastasis, bearing in mind the intrinsic plasticity of cancer cell migration modes [224,225].

### **IBC: a hallmark of pEMT, hypoxia and aggressiveness**

Inflammatory breast cancer (IBC) is a rare type of breast cancer accounting for 2-4% of all breast cancer patients, but accounts for 7-10% of breast cancer related mortality [226]. IBC is characterized by extensive skin invasion and the formation of tumor cell emboli (clusters) within the breast and dermal lymphatic system. Characteristics of emboli include cell-cell adhesions mediated by E-cadherin [227]. While accumulating studies suggest that hypoxia-induced EMT is required for metastasis in many cancer types [228], the necessity of complete EMT has been debated over recent times and at least revisited in IBC model systems [226]. Compelling evidences from both *in vitro*, and *in vivo* as well as cell line modeling studies suggest that IBC cells exploit the cell cluster-based metastasis through partial retention of epithelial traits [226]. On the other hand, recent studies highlighted the pro-oncogenic role of E-Cadherin wherein E-cadherin promotes metastasis in multiple models of breast cancer [101,229]. Several mechanisms have been attributed for the pro-tumorigenic functions of E-Cadherin. For instance, E-cadherin promotes *in vivo* growth of SUM149 tumor cells through its role in stabilizing HIF-1 $\alpha$  by unknown mechanism(s) leading to regulation of energy metabolism in the aggressive tumor cells [230]. Other mechanisms include, but not limited to, the role of E-cadherin in downregulating TGF- $\beta$  signaling [101]. Notably, TGF $\beta$ -induced single cell motility involves the regulation of EMT [231]. However, under scenarios of deficient TGF $\beta$  signaling, cells are restricted to collective movement and lymphatic spread after partial EMT [232]. Recent studies showed that SMAD3 nuclear expression, a surrogate marker of TGF $\beta$  signaling, is downregulated in cancer cells from IBC patients as compared to non-IBC [233]. In the absence of SMAD3 expression in IBC tumor emboli, collectively invading cancer cells with higher expression of E-Cadherin enables partial EMT that can lead to higher metastatic potential and the tendency for lymphatic dissemination over the hematogenous route [233].

Additionally, under the conditions rich in extracellular matrix, the colony-stimulating factor-1 (CSF-1)/CSF-1R axis plays a key role in driving EMT to E/M phenotype in IBC cells such as SUM149 and SUM190 [132]. Interestingly, a quantitative proteomics study revealed that exosomes (membrane bound extracellular vesicles) produced by hypoxic tumor cells are highly enriched in immunomodulatory proteins and chemokines including CSF-1 that can modify the metabolic shift in infiltrating monocyte-macrophages that enables better evasion of the host immunity and enhance tumor progression [234]. Altogether, these studies suggest

that hypoxia plays an important role in regulating factors and signaling mechanisms enabling partial EMT culminating in aggressiveness of the tumor growth (Fig 2).



**Fig 2: Model describing the interconnection among hypoxia, partial EMT and collective cell migration in metastasis.** Hypoxia induces hybrid E/M phenotype, which can promote collective migration. Collective migration, hypoxia, and hybrid E/M phenotypes all contribute to metastasis in a potentially synergistic manner. A better molecular characterization about the effects of hypoxia on collective cell migration remains to be done.

## Conclusion

Hypoxia has been shown to increase metastasis by augmenting EMT and other hallmarks of cancer. Hybrid E/M phenotype has been implicated in conferring stemness and drug resistance, and shown to facilitate collective cell migration, thus increasing metastasis and tumor initiation capability of cancer cells at the secondary sites. Thus, in multi-dimensional regulation of metastasis, a detailed mechanistic and dynamic understanding of how hypoxia, hybrid E/M phenotypes and collective cell migration are interconnected with each other will help to understand molecular details of cancer metastasis and development of novel therapeutics.

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