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Three Dimensional Engineered Models to Study Hypoxia Biology in Breast Cancer

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Abstract

Breast cancer is the second leading cause of mortality among women worldwide. Despite the available therapeutic regimens, variable treatment response is reported among different breast cancer subtypes. Recently, the effects of the tumor microenvironment on tumor progression as well as treatment responses have been widely recognized. Hypoxia and hypoxia inducible factors in the tumor microenvironment have long been known as major players in tumor progression and survival. However, the majority of our understanding of hypoxia biology has been derived from two dimensional (2D) models. Although many hypoxia-targeted therapies have elicited promising results *in vitro* and *in vivo*, these results have not been successfully translated into clinical trials. These limitations of 2D models underscore the need to develop and integrate three dimensional (3D) models that recapitulate the complex tumor-stroma interactions *in vivo*. This review summarizes role of hypoxia in various hallmarks of cancer progression. We then compare traditional 2D experimental systems with novel 3D tissue-engineered models giving accounts of different bioengineering platforms available to develop 3D models and how these 3D models are being exploited to understand the role of hypoxia in breast cancer progression.

Keywords

Three-dimensional (3D) models; Microtumor models; Breast cancer; Hypoxia; Cancer hallmarks

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⁶.Conflict of interest statement

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1. Introduction

Breast cancer is the second leading cause of mortality among women worldwide.[1] According to the recent GLOBOCAN 2018, cancer incidence and mortality statistics, female breast cancer accounted for almost one in four cases among women with a mortality rate of 11.6% worldwide. [2] Though current conventional chemotherapies and immunotherapies have been successful in management of 70–80% of early stage breast cancer patients, treatment of metastatic breast cancer still remains a challenge. [3] The variable treatment responses are further aggravated by breast cancer heterogeneity at the molecular and cellular level. Breast cancer also exhibits various molecular subtypes namely, luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) positive and triple negative breast cancer (TNBC). [4–6] It is widely accepted that the surrounding tumor microenvironment (TME) that consists of non-cellular factors (oxygen, nutrient, pH gradients, etc.), extracellular matrix (ECM) and cellular factors (tumor cells, stromal cells) plays important role in tumor progression and treatment response.[7–11] Of note, hypoxic changes in surrounding TME are being increasingly highlighted to guide the crosstalk between the hypoxic tumor and stromal cells.[12, 13] The role of hypoxia inducible factors 1 alpha and beta (HIF-1 α and HIF-1 β) in the TME are being extensively studied to understand how hypoxia regulates the hallmarks of tumor progression in breast cancer.[9, 14]

Hypoxia, characterized by low oxygen levels, has been illustrated to activate hypoxia-inducible signaling via HIF-1 α and HIF-1 β , which mediates adaptive changes in tumor cells followed by rapid proliferation of tumor cells and remodeling of tumor vasculature.[15, 16] These hypoxic transformations sequentially guide tumor survival and progression in breast cancer cells via metabolic adaptations, angiogenesis, stromal cell recruitment, migration, tissue invasion, ECM remodeling, extravasation to metastatic sites and drug resistance.[17, 18] The clinical significance of hypoxia in tumor progression has also been studied in breast cancer patients.[19–21] Elevated HIF-1 α levels correlated with poor prognosis and tamoxifen resistance in breast cancer patients.[22] Over-expression of HIF-1 pathway genes (carbonic anhydrase IX (CAIX), vascular endothelial growth factor (VEGF), BCL2 interacting protein 3 (BNIP-3) and ascorbate) correlated with advanced tumor stage and grade, increased necrosis, invasion and poor disease-free survival rates.[23] Similar results were also reported from another study where increased HIF-1 α and CAIX overexpression predicted poor outcome in early-stage triple negative breast cancer patients.[24]

In view of the clinical relevance of hypoxia, different hypoxia inhibitors (Tirapazamine, PR104, TH-302 and Banoxanthane) have been tested in clinical trials; however, hypoxia-targeted therapies combined with current standard-of-care therapies (e.g., chemotherapy or radiotherapy) have exhibited varied outcomes in several clinical trials.[25–28] The failures warrant further investigation into related mechanisms along with the role of TME in influencing therapeutic response. It should be noted that traditional two-dimensional (2D) cell monolayers used to test efficacy of hypoxia-targeted therapies are limited in their translational efficacy as they do not completely emulate the complex *in vivo* tumor cell-cell and cell-ECM interactions of solid tumors. To address the limitations of 2D models, three-

dimensional (3D) models have been recently engineered to effectively recapitulate complex tumor-TME interactions.

In view of these developments, this review first summarizes current knowledge of how complex biological processes and cancer hallmarks are modulated by hypoxia followed by the recent progress in the development of novel 3D models to study cancer biology and finally, how these 3D models have contributed to our understanding of hypoxia biology with particular focus on breast cancer progression.

2. Hypoxia Biology

Hypoxia-inducible factors (HIF-1 α and HIF-1 β) play a major role in tumor survival and progression in breast cancer cells through various cellular processes including metabolic adaptations, promoting tumor vascularization (angiogenesis), stromal cell recruitment, migration, tissue invasion, extracellular matrix modelling, extravasation to metastatic sites and drug resistance.[18, 29] The recent pre-clinical studies in breast cancer patients have also highlighted the promising results of co-administration of HIF inhibitors with chemotherapeutic drugs.[26, 30–35] In the following sub-sections, we summarize the role of hypoxia in promoting various hallmarks of breast cancer progression, which is also comprehensively illustrated in Figure 1.

2.1 Metabolic Stress and Lipolysis

Metabolic reprogramming is rapidly emerging as a major hallmark of cancer; however, the precise metabolic markers still need extensive validation.[36] Breast cancer cells differentially rewire the metabolic adaptations in response to hypoxia in different subtypes of breast cancer. [37] HIF-1 α is reported to mediate coupling of redox regulation and glucose metabolism in breast cancer cells, which modulates stem cell phenotype in breast cancer cells.[38] HIF-1 α also mediates the metabolic responses to hypoxia via a number of mechanisms; increased flux through the glycolytic pathway thereby regulating mitochondrial reactive oxygen species (ROS) production and through changes in the serine synthesis pathway and mitochondrial folate cycle leading to increased production of glutathione and nicotinamide adenine dinucleotide phosphate (NADPH). [17, 38] A recent study reported that the metabolic coupling involving oxidative changes and the Warburg effect in the glycolytic stromal cells promoted mitochondrial metabolism in breast cancer cells. [39] This was driven by the HIF-1 α -mediated activation of nuclear factor kappa B subunit 1 (NF- κ B) and the c-Jun N-terminal kinase (JNK) pathway, which was modulated through ROS production leading to a catabolic phenotype. The expression of caveolin-1 (CAV-1) and monocarboxylate transporter 4 (MCT4) by this catabolic phenotype served as novel stromal cell markers indicative of the Warburg effect in breast cancer cells.[39] Additionally, dysregulation of adipocytes along with that of metabolic substrates (cholesterol, lipids, triglycerides, free fatty acids, hormones, leptin, adiponectin) in the breast TME have also been shown to mediate tumor progression via the induction of hypoxia (recently reviewed in detail [40]). Walsh *et al.* accentuated the critical metabolic plasticity seen in breast cancer stem cells, which allows tumor initiating cells to switch between oxidative phosphorylation and glycolysis guided through metabolic enzymes,

energy and stress sensors, epigenetic chromatin modifications, transcription factors, non-coding RNAs and transcriptional repressors.[41] These mechanisms collectively indicate metabolic adaptations driven by ROS, which further induce catabolic phenotypes (mitophagy, autophagy, and glycolysis) in breast cancer cells and emphasize the importance of deciphering metabolic synergy along with epithelial-stromal interactions in breast cancer cells.

2.2 Apoptosis

Hypoxia mainly activates anti-apoptotic pathways that mediate tumor growth, progression, and survival.[42, 43] In contrast, a few studies have shown activation of growth-inhibitory genes and pro-apoptotic pathways under hypoxic conditions. [44] Hypoxia has been shown to induce cell cycle arrest and apoptosis via different pathways, which are discussed hereafter. HIF-1 α has been shown to interact with p53 in MCF-7 cells transfected with HIF-1 α , which leads to stabilization of p53 and the induction of apoptosis.[45] Synchronous results were reported from another study in which induction of p53 target genes i.e. pleckstrin homology like domain family A member 3 (PHLDA3) and inositol polyphosphate-5-phosphatase (INPP5D) by hypoxia led to apoptosis due to AKT serine/threonine kinase (AKT) inhibition. [46] The interactions and interplay between p53 family members and HIFs have previously been reviewed by Amelio and Melino [47] and Petrova [48]. In another study, expression of BNIP3 (a cell death factor) was upregulated in presence HIF-1 α that was attributed to activation of the BNIP3 promoter containing HIF-1 response elements (HRE). [49] Similar findings were reported from another study where HIF-1 led to induction of BNIP3 and its homologue Nip3 like protein X (NIX) in T47D, MCF-7 and MDA-MB-231 breast tumors cells [50]. Hypoxia has also been shown to induce the TNF-related apoptosis inducing ligand (TRAIL) and the nuclear localization of breast cancer susceptibility gene 1 (BRCA1) in MCF-7 and MDA-MB-468 breast cancer cells, which further enhanced TRAIL-induced apoptosis in breast cancer cells.[51] Hypoxia-induced apoptosis in human breast cancer cells has also been reported to be mediated via signal transducer and activator of transcription 5B (STAT5b) and a caspase-3 dependent pathway. [52]

2.3 Angiogenesis and Vascular Mimicry

Abnormal angiogenesis is a well-established hallmark of tumor progression and survival and is triggered by hypoxic microenvironments. These hypoxic conditions in turn initiates an imbalance between pro- and anti-angiogenic factors. The hypoxic response has been coupled to increased vascularization or angiogenesis via angiogenic sprouting and elevated expression of matrix metalloproteinases (MMPs) in breast cancer cells.[53–55] Additionally, the hypoxic response has also been shown to modulate activation of pro-angiogenic factors i.e. VEGF [56–59], fibroblast growth factor (FGF) [15, 60], platelet-derived growth factor- β (PDGF- β) [61, 62], stromal-derived factor 1 α (SDF-1 α) [63, 64], angiopoietin-1 (Ang-1) [65, 66], angiopoietin-2 (Ang-2) [65, 67] and transforming growth factor beta 1 (TGF- β) [68–70] in breast cancer cells. In totality, elevated expression of these hypoxia-induced pro-angiogenic factors has been reported to promote neo-angiogenesis in breast tumors. In a recent report, elevated high-mobility group box 1 (HMGB1) was reported to increase HIF-1 α expression, which in turn elevated VEGF expression in MCF-7 breast cancer cells

via AKT phosphorylation and the activation of phosphatidylinositol 3-kinase (PI3K) pathway.[71] Further, shRNA mediated knockdown of HMGB1 downregulated HIF-1 α and VEGF, which inhibited vessel formation in MCF-7 cells. [71] Hypoxia induction was also shown to drive expression of ADAM metallopeptidase domain 17 (ADAM17), which resulted in shedding of ectodomain of nectin-4 in the TME, which interacts with integrin- β 4 on endothelial cells. [72] This interaction led to activation of PI3K/AKT, proto-oncogene tyrosine-protein kinase Src (Src) and inducible nitric oxide synthase (iNOS) pathway, which in turn promoted angiogenesis in MCF-10A breast tumor cells. [72]

HIF-1 and hypoxia regulated genes are also being targeted via different drugs to test if they can rewire normal vascularization in breast tumor cells. In one of the recent studies, administration of metformin was shown to inhibit progression in breast cancer cells through decreased hypoxia and induction of vessel normalization via downregulation of PDGF- β . [73] Centchroman (a novel estrogen receptor modulator) was reported to regulate breast cancer angiogenesis through inhibition of HIF-1 α and its downstream target VEGF (i.e. HIF-1 α /VEGFR2 signaling axis) via decreased phosphorylation of extracellular signal regulated kinase (ERK)/AKT in MCF-10A cells. [74] In yet another interesting study, a bromodomain and extra-terminal (BET) inhibitor JQ1 was shown to downregulate HIF regulated genes, carbonic anhydrase (CA9) and vascular endothelial growth factor A (VEGF-A), which inhibited binding of HIF to hypoxia response element in CA9 promoter site in MDA-MB-231 triple negative breast cancer cells. This inhibition of CA9 and VEGF-A in turn inhibited xenograft vascularization in MDA-MB-231 cells. [75]

Besides angiogenesis and neo-vascularization, an interesting concept of vascular mimicry (VM) was highlighted in uveal melanomas in the last decade, which has gained impetus over the years in cancer biology. [76] The concept of VM refers to *de novo* formation of microvascular channels by the malignant cell population and has been extensively studied in breast cancer cells.[77–83] This concept of VM has also been linked to hypoxia in breast cancer cells and has been reviewed by Li and colleagues. [84] Though presently we have few studies delineating the mechanism underlying hypoxia induced VM, research is ongoing to explore this dimension of cancer biology. In one of the initial studies, combretastatin A4 phosphate (CA4P), a vascular targeting agent was reported to induce intra-tumoral hypoxia (elevated HIF-1 α) along with a VM network in mice injected with W256 breast cancer cells via activation of the HIF-1 α / erythropoietin producing hepatoma receptor A2 (EphA2)/ PI3K/MMP signaling pathway. [85] Also, treatment of MDA-MB-231 and Hs578T tumor bearing mice with sunitinib, a receptor tyrosine kinase (RTK) inhibitor, was shown to induce VM in TNBC along with upregulation of expression of HIF-1 α , vascular endothelial cadherin (VE-cadherin), matrix metallopeptidase 2 (MMP2) and Twist1, which was not observed in mice injected with non-TNBC cells (MCF-7 and BT474).[86]

Further, VM targeting with 6,6'-bis(2,3-dimethoxybenzoyl)- α , α -D-trehalose (DMBT), an anti-invasive agent, was shown to inhibit hypoxia-induced VM in MDA-MB-231 and MCF-7 breast cancer cells. DMBT treatment decreased expression of HIF-1 α , matrix metallopeptidase 9 (MMP-9), cell division cycle 42 (Cdc42), VE-cadherin, p-AKT, epidermal growth factor receptor (EGFR) and phosphorylated mammalian target of rapamycin (mTOR) via downregulating HIF-1 α /VE-cadherin/MMP signaling pathways.[87]

Tumor protein p53-inducible nuclear protein 1 (TP52INP1) has also been shown to inhibit hypoxia-induced VM by downregulating the ROS/ glycogen synthase kinase 3 beta (GSK-3 β)/Snail signaling axis leading to decreased HIF-1 α , Snail and VE-cadherin expression. [88] In an interesting study, ectopic restoration of miR-204 was illustrated to inhibit hypoxia-induced VM in MDA-MB-231 cells via cooperative downregulation of 13 target proteins involved in VEGF, PI3K/AKT, focal adhesion kinase (FAK)/Src and Raf-1 proto-oncogene, serine/threonine kinase (RAF1)/ mitogen activated protein kinase (MAPK) signaling pathways. [89] In conclusion, the existing scientific literature suggests that HIF-1 α plays essential roles in inducing tumor angiogenesis and VM in breast cancer cells via different signaling cascades.

2.4 Pro-inflammatory Pathways

Tumor hypoxia is known to promote inflammation-mediated metastasis via activation of pro-inflammatory signaling molecules in malignant cells and the adjacent TME. Mechanisms of how hypoxia mediates inflammatory signaling have been discussed in detail in a recent article [90]. The crosstalk between hypoxia and inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and leptin is suggested to deregulate Notch signaling in breast cancer.[91] This deregulated Notch signaling, a critical downstream target of IL-6 induces tumor angiogenesis in breast cancer. It has also been reported that hypoxia-mediated downregulation of estrogen signaling, particularly CCAAT/enhancer binding protein- α (C/EBP α) promoted breast cancer growth via cytokines, IL-1 β /IL-6 and tumor necrosis factor- α (TNF- α) in the microenvironment. [12] HIF-1 α upregulates genes of the purinergic pathway, namely, CD23, CD73, A2A adenosine receptor (A2AAR) and A2B adenosine receptor (A2BAR) of which CD73 plays a major role in tumor progression via downregulating PI3K/AKT and Wnt/ β -catenin pathways. CD73 in the TME has been shown to negatively regulate interferon-gamma (IFN- γ) and TNF- α expression via binding of CD73-derived adenosine to natural killer (NK) cells and macrophages (increased interleukin-4 (IL-4) and interleukin-10 (IL-10) production). Targeting A2 adenosine receptors (A2AR) has been shown to decrease metastasis via elevated expression of granzyme B and perforin.[92] The role of inflammatory mediators namely IL-6, interleukin-8 (IL-8), TGF- β , TNF- α , connective tissue growth factor (CTGF), VEGF, cyclooxygenase-2 (COX-2) and C-C motif chemokine ligand 2 (CCL2) in regulation of hypoxia in different malignancies and their function in tumor progression is discussed.[93] Additionally, the mechanistic inhibition of hypoxia signaling pathways and hypoxia-activated pro-drugs have been shown to modulate the anti-inflammatory response.[29, 94] As hypoxia has been reported to induce these inflammatory mediators, drugs targeting these inflammatory molecules have also been studied to regulate progression in malignancies. [95–99] LY2157299 (Galunisertib, a TGF- β receptor I kinase inhibitor) has been shown to inhibit tumor progression in triple negative breast cancer *in vitro* and has also been shown to alleviate tumor recurrence post paclitaxel treatment in mouse xenograft models. [95] Anti-IL6 treatment has also been shown to inhibit bone metastasis in breast cancer murine model. [99] Vorinostat (8-(hydroxyamino)-8-oxo-N-phenyl-octanamide), a histone deacetylase (HDAC) inhibitor, known to inhibit HIF-1 α translation has been shown to decrease inflammatory mediators (IL-6, interleukin-1 beta (IL-1 β) and TNF- α) in a dose-dependent manner in a mouse model. [98] Human peripheral blood mononuclear cells were reported to

secrete decreased IL-1 β , interleukin-12 (IL-12), TNF- α and IFN- γ upon administration of vorinostat. [96, 97] These studies elicit the role of HDAC inhibitors in modulating HIF-1 α levels along with suppression of inflammatory molecules. Over the recent years, pro-inflammatory molecules modulated via hypoxia have emerged as potential candidates for targeted therapy. However, further studies still need to elucidate the potential of these small molecule inhibitors as specific targeted therapies.

2.5 Migration and metastasis

Epithelial to mesenchymal transition (EMT) and metastasis is intricately regulated by remodeling of the cytoskeleton in breast cancer cells that plays important roles in migration and invasion of breast cancer cells into surrounding TME, intravasation into blood vessels and extravasation and colonization at secondary sites. [100–103] The hypoxic microenvironment is known to be a critical factor in EMT, tumor cell migration and metastasis and was recently reviewed by Gao and colleagues [7], Gilkes [8], Schito and Ray [10] and the Liu group [9]. In addition to activation of well-known hypoxia-mediated signaling pathways including the hepatocyte growth factor (HGF)-c-Met axis, Ras homolog family member A (RhoA)/ Rho associated coiled-coil containing protein kinase 1 (ROCK1) transduction pathway, NOTCH-integrin-linked kinase (ILK) signaling pathways, platelet-derived growth factor B (PDGF) pathway and MAPK/ERK pathway that have been implicated in tumor cell migration, other groups have identified roles for less studied signaling molecules in inducing migration in hypoxic cells. In one of the recent studies, SRY-box-2 (SOX2) was shown to promote hypoxia-induced migration in MDA-MB-231 and MDA-MB-468 breast cancer cells through induction of NEDD9 (neural precursor cell expressed, developmentally down-regulated 9) expression and activation of Rac family small GTPase 1 (Rac1)/HIF-1 α signaling pathway. [104] Also, nuclear factor erythroid-2-related factor 2 (Nrf2) expression was reported to inhibit kelch-like-ECH-associated protein 1 (Keap1) and induce activation of HIF-1 α , glucose-6-phosphate dehydrogenase (G6PD) and Notch 1 (G6PD/HIF-1 α /Notch 1 axis) in MCF-7 and MDA-MB-231 cells. [105] Activation of the Notch signaling pathway modulated downstream Hes family BHLH transcription factor 1 (HES-1) expression, which led to EMT and Nrf-2 driven metastasis in breast cancer cells. Hypoxia was also reported to induce migration in MDA-MB-231 cells via activation of cadherin-22 (a cell-cell adhesion molecule), which is in turn regulated by overexpression of eukaryotic translation initiation factor 4E type 2 (eIF4E2).[106] In another study, epigenetic regulation of zinc finger MYND-type containing 8 (ZMYND8) leading to ZMYND8 acetylation at lysine 1007 and 1034 activated HIF-1 mediated metastasis with recruitment of bromodomain containing 4 (BRD4) and release of RNA polymerase II. [107] These studies highlighted the plausible role of epigenetic mechanism in HIF activation along with HIF-mediated breast cancer migration and metastasis.

2.6 Drug Resistance

It is well-established that hypoxia leads to drug resistance against conventional anti-cancer agents in breast cancer. [108] Different research groups have postulated different mechanisms of inherent or acquired drug resistance, which is a major player in poor treatment response rates and can also predispose patients to early relapse or refractory disease. A recent hypothesis emphasizes the role of cancer stem cells (CSCs), which are

regulated by HIF-2 α , which in turn, modulates the stemness in breast cancer cells while the role of HIF-1 α has been implicated in tumor cell survival. Various mechanisms and signaling cascades responsible for drug resistance involve prominent ABC drug transporters such as P-glycoprotein (P-gp) including breast cancer resistance protein (BCRP), multidrug resistance gene 1 (MDR1), multidrug resistance associated protein (MRP1 and MRP2) responsible for efflux of chemotherapeutic drugs, thereby conferring drug resistance.[109, 110] The regulation of MRP1 and MRP2 in hypoxic microenvironments involves different signaling pathways such as NF- κ B, peroxisome proliferators-activated receptor alpha (PPAR α), constitutive androstane receptor (CAR), pregnane X receptor (PXR), farnesoid X receptor (FXR) and microRNAs (miRNAs) during chemoresistance. [13] The PI3K/AKT signaling pathway has also emerged as a major pathway upregulated in drug resistance wherein hypoxic conditions are known to activate PI3K/AKT coupled to mTOR along with accumulation of HIF-1 α and HIF-2 α . Besides the PI3K/AKT pathway, drug resistance in breast cancer cells is also modulated through MAPK pathway, ERK, MEK (MAPK/ERK), and phospholipase C-gamma (PLC- γ). Drug resistance is also reported to be regulated by the TME including contributions by stromal cells (vascular endothelial cells, immune-inflammatory cells and fibroblasts), extracellular matrix and soluble factors in addition to intrinsic tumor specific pathways involving Ang-2, FGF/FGF receptor (FGFR) and MET proto-oncogene (MET)/HGF pathway. [111, 112] Cancer-associated fibroblasts (CAFs) have been reported to induce CSC phenotypes, which are chemoresistant due to secretion of CCL2.[113] Also, stromal cell-derived factor (SDF-1)/C-X-C motif chemokine 12 (CXCL12) have also been reported to protect small cell lung cancer (SCLC) cells from chemotherapy induced apoptosis.[114] Tumor associated macrophages (TAMs) have also been shown to enhance chemoresistance to paclitaxel in MDA-MB-231 breast cancer cells via cathepsin proteases.[115] Further details into the mechanism of chemoresistance mediated via TME has been discussed by Velaei's group.[112] The detailed mechanisms of drug resistance induced by hypoxia (HIF-1 α and HIF-2 α) in breast cancer cells has also been reviewed by Schoning *et al.* and Mahdi *et al.* [116, 117]

Although the field of hypoxia biology has evolved greatly with deep understanding of downstream signaling pathways, it should be noted that most of these mechanisms were discovered/studied using 2D cultures, which do not recapitulate the *in vivo* TME observed in solid tumors. While 2D cultures can be easily adapted to high throughput assays and drug screening approaches, 2D *in vitro* models have some major limitations for the study of cancer biology. Hence, there are a multitude of efforts to generate physiologically relevant 3D experimental models to mimic the *in vivo* TME of solid tumors. In the following sections, we first assess the limitations of current experimental models followed by general advances in various technologies to generate 3D experimental models recapitulating different aspects of TME, followed by hypoxia-related studies that highlight differences in 2D and 3D cultures. We then discuss 3D experimental models used to investigate the role of hypoxia in promoting various hallmarks discussed above with a focus on breast cancer progression.

3. General Overview of current experimental systems used in cancer biology

3.1 Limitations of 2D and *in vivo* models in studying hypoxia biology

Solid tumor microenvironment is composed of tumor and stromal cells (vascular, immune and fibroblast cells) and ECM components where cell-cell interactions, cell-ECM interactions and local gradients of nutrients, growth factors, secreted factors and oxygen regulate cancer cell function and behavior. Traditionally, 2D cell-based *in vitro* tumor models have been extensively used to study hypoxia biology due to the relative ease of their implementation and capacity to provide for high-throughput screening. Traditional 2D cell culture tumor models refer to flat monolayer cell culture on plastic dishes or glass wherein cells are exposed to hypoxia using hypoxic chambers that maintain hypoxic conditions over a defined concentration of pO₂. Cell culture in hypoxic chambers cannot reproduce the spatial oxygen gradients and spatial heterogeneity observed in solid tumors *in vivo*, where only an inner hypoxic core exists. This is because in hypoxic chambers, all cells in 2D are equally exposed to hypoxia. They also do not capture the constantly evolving dynamic TME *in vivo*, and hence, they are considered poor surrogates for evaluating tumor progression as well as drug efficacy (summarized in Table I).

In vivo xenograft models have also been used to study hypoxia biology via gene knock-in/knock-out experiments [118–121]. Xenograft models mainly rely on the manipulation of molecular targets and signaling pathways regulated by hypoxia. Although the xenograft mouse models and patient-derived xenografts (PDXs) better resemble solid tumors than 2D cultures, they do not completely represent the etiology and pathophysiology of tumor development in human.[122, 123] Preclinical animal models rely heavily on mouse tumor models that are expensive and time consuming, thereby limiting the numbers of agents that can be tested and are also limited by ethical considerations. The differences in (for syngeneic immunocompetent models) and/or lack of immune component (immunocompromised mouse xenograft models) and lack of human-specific stromal interactions further limit the relevance of rodent models to human solid tumors.[124, 125] This contributes to poor predictions of drug efficacy and patient responses in clinical trials.

In view of these limitations of 2D and xenograft models, a major advantage of biomimetic tissue-engineered *in vitro* models is that they can be designed to better mimic the cell–cell interactions, cell–matrix interactions, and the heterogeneous TME of human solid tumors observed *in vivo*. Biomimetic *in vitro* 3D models made of human cancer cell lines or human tissues can be exploited to elucidate the role of non-cellular microenvironmental factors (e.g. hypoxia, ECM molecules, etc.) as well as cellular factors (epithelial-stromal cell interactions) in human disease progression within the context of a defined and controlled microenvironment. [126–130] Compared to 2D monolayer cultures, tumor cells cultured in 3D microenvironments experience different cellular cues that modify their responses to various stimuli. For example, tumor cells growing in 3D cell cultures are exposed to dramatically different adhesive, topographical and mechanical forces than cells growing in 2D on treated surfaces.[131, 132] It has been well documented that the 3D microenvironment alters oxygen/nutrient diffusion gradients leading to hypoxia, changes in

pH, etc., which further affect numerous cellular and functional activities including cell morphology, viability, proliferation, differentiation, and migration through altered signal transduction, histone acetylation, gene expression, and protein expression. [132, 133] Additionally, the cell-cell and cell-ECM interactions of cells in solid tumors and multi-layer tumor spheroids constitute a permeability barrier through which therapeutic agents must penetrate leading to altered drug penetration efficacy, drug metabolism, and drug sensitivity. [134, 135] Technological advances in the field of tissue engineering, microfluidics and bioprinting has enabled fabrication of variety of 3D tumor models recapitulating various aspects of the TME. [136]

3.2 Overview of technologies to generate 3D tumor models

Multicellular Tumor Spheroids—The most widely used 3D models to study cancer biology and drug response are multicellular tumor spheroids (MCTS, also called as microtumors, tumoroids, or tumor aggregates) (summarized in Table II). Tumor spheroids can be homotypic (made of single tumor cell types) or heterotypic (aggregates of different tumor cell types or mixture of tumor and stromal cells) and have been widely used to study cell–cell interactions, microenvironmental cues important for tumor growth, and mechanistic investigations of various hallmarks of cancer such as angiogenesis, metastasis, metabolic adaptations, and drug resistance. [137–139] Tumor spheroids are generated using different technologies including spinner flasks, rotatory system, hanging drop, microcarrier beads, bioreactors (Figure 2 A–F), etc. [140–143] However, these techniques are both time-consuming and labor intensive and most importantly, they generate aggregates with a wide range of irregular sizes and shapes, which presents a critical challenge to recreate reproducible physio-chemical microenvironments and makes assay standardization difficult. Indeed, variations in size and shape can play a critical role in the establishment of differential gradients such as hypoxia, pH, nutrients, growth factors, cytokines, waste products, and metabolic stress within the TME, which in turn can affect cancer biology, cell behaviors and cellular responses to the drug treatments.

While tumor spheroids enable studying role of non-cellular microenvironmental factors such as pH, oxygen, and nutrient gradients as well as cell-cell interactions on tumor cell behaviors, they do not recapitulate tumor-stromal cell interactions. This limitation can be overcome by generation of heterotypic spheroids (Figure 2 G–K), which have been used to recreate intra-tumoral heterogeneity, for example by mixing cancer cells of different subtypes. [144, 145] Heterotypic spheroids have also been used to recreate tumor-stromal cell interactions where stromal cells such as immune cells, fibroblasts, endothelial cells, etc. are mixed with tumor cells. [146] [147] For example, McGuigan and colleagues established (TRACER) platform (Tissue Roll for the Analysis of Cellular Environment and Response) to investigate tumor-stroma interactions using co-culture of patient-derived CAFs and squamous carcinoma cells of the hypopharynx (Figure 2L).[147]

3D Scaffolds—3D spheroids can also be grown on prefabricated scaffolds in which cells attach and migrate along the scaffold fibers. Scaffolds are generally seeded with cell lines, sometimes along with growth factors for promoting their growth and providing tumor microenvironment to recapitulate molecular crosstalk and *in vivo* architecture. These

scaffolds can be of natural (gum, gellan, hyaluronic acid, silk, etc.) or synthetic origin (polylactide/glycolide, polycaprolactone or polystyrene) and provide mechanical support to the seeded cells. [148] They can be engineered to emulate the extracellular matrix (ECM) since the ECM is known to promote key pathways that modulate angiogenesis, metastasis, migration, proliferation and cellular organization. [149–152] With the advancements in the field of biomaterial fabrication, scaffolds are being tailored to mimic biophysical and biochemical properties of ECM. The porous poly(lactide-co-glycolide) (PLG) fabricated scaffolds were used to establish *in vivo* microenvironmental cues in a 3D human oral cancer model. In this model, oral squamous cell carcinoma cells (OSCC-3) cultured in PLG scaffolds displayed enhanced malignant potential with increased secretion of VEGF, bFGF and IL-8. [151] Similar results were reported from another study where hypoxic MCF-7 cells seeded on PLG scaffolds showed elevated secretion of angiogenic factors *IL-8* and *VEGF*. [153] Similarly, an open-faced 3D collagen scaffold has been shown to create microenvironment suitable for promoting signaling pathways essential for spheroid formation, differentiation and organization. [154] The major advantages of working with scaffold-based 3D tumor models is the ability to fine tune physico-chemical properties of the scaffold such as scaffold porosity providing more surface area to cells, scaffold surface functionalization to alter tumor cell adhesion properties, and mechanical properties to study role of mechanotransduction in tumor progression. The disadvantages of scaffold-based approaches include scaffold to scaffold variations, non-transparency and thickness creating challenges for imaging, and difficulty in removing cells for protein analysis. [155, 156] One could overcome these shortcomings by improving 3D scaffold material compositions and combining biofabrication techniques (Table II). In addition to the natural and synthetic scaffolds, decellularization of native healthy and diseased tissues is being explored to preserve native ECM structure and composition. [150] Such decellularized matrices are actively being used as bioactive scaffolds for studying tumor progression, EMT as well as a platform for drug testing.[157] The limitations in using decellularized patient-derived matrix to fabricate scaffolds is potentially the availability of patient biopsies and more importantly, the ethical issues associated with obtaining informed content for the same.

Microfluidic devices—Tumor-on-a-chip or microfluidic devices have miniaturized and revolutionized cell culture platforms. Microfluidic devices provide a controlled cellular environment, adaptive to different cellular and metabolic changes and are easy to handle with high reproducibility. [158, 159] A major advantage of microfluidic devices is the feasibility of measuring dynamic cellular changes over time on the same chip rather than a single endpoint analysis. Recently, the application of microfluidic approaches has been extended to investigate cells from liquid biopsies because of their ability to successfully mimic the interactions and flow of different types of cells. Other advantages include feasibility to develop co-culture and cell localization studies involving use of chemical gradients (Table II). However, one of the limitations of tumor-on-a-chip models is its limited scalability for high throughput screening (HTS); we still need to precisely streamline automated loading of micro-physiological components on the model, which is still done manually. This necessitates future developments in tumor-on-a-chip models as both automation and miniaturization need to be well-balanced for microfluidic devices to better respond to HTS requisites of pharmaceutical companies.

Bioreactors—Versatile bioreactors often used in pharmaceutical processes have also been employed to culture tumor cells as spheroids [160] or microcarriers [161]. They help in easy culturing coupled with high throughput production of spheroids (Table II) These bioreactors precisely monitor metabolites, temperature, pH and nutrients under strict control. Perfusion culture systems were later developed, which allowed for monitoring dynamic 3D tumor growth under reduced media conditions to bridge the gap between microfluidic chips and bioreactors. Examples include 3DKube™ [162] and IVtech™ [163]. Perfused bioreactor systems offer the possibility of dynamic visualization due to their transparency and the potential for advancing cancer research by making it feasible to investigate interactions in patient derived 3D tumor cells cultured in a cancer niche. However, bioreactors have their own share of disadvantages like requirement of specialized equipment, less uniformity in cell control and size and unwanted shear force exposure on cells (Table II).

3D Bioprinting—3D bioprinting employs a layer-by-layer deposition technique ideally to capture complexity and spatial heterogeneity of a tissue-like structure by combining tumor/stromal cells and ECM-like biomaterials. [164] The advantage of using these bio-ink hydrogels is the maintenance of cell viability and 3D architecture because they provide suitable mechanical and physicochemical interactions imitating the tumor tissue. An added advantage of bioprinting is minimal manipulation of 3D cell cultures, which enhances its biological replication, consistency and reliability in drug screening assay read-outs. 3D bioprinted models can be used to study the cell–ECM and cell-cell interactions between tumor cells and different stromal cell populations (macrophages, fibroblasts) along with ECM components. [165–167] [168] [169] Although bioprinting is a promising and versatile technique, a major challenge with 3D bioprinting is to simultaneously print material (bio-ink) and the tumor cells without replacing the chemical solvents and maintaining cell viability. Further, standardization of bioprinting protocols will help promote the use of these 3D tumor models in pre-clinical settings.[165]

3.3 Comparative studies between 2D and 3D models

It has been shown that expression of genes and proteins can be differentially regulated in 2D vs. 3D cultures. For instance, hypoxia was reported to differentially regulate estrogen receptor alpha (ER α) expression in 2D and 3D T47D breast cancer cells. In this study, the 3D model consisted of paper-based scaffolds-cells containing a single zone surrounded by a wax-patterned border suspended in collagen I matrix. Surprisingly, ER α mRNA levels were insensitive to hypoxia (1% O₂ via hypoxic chamber) in 3D whereas in 2D, a decrease in ER α transcription activity was reported. [170] In another study, the exposure of MCF-7 breast cancer spheroids and MCF-7 monolayer cells to estradiol showed similar expression profile for estrogen-regulated transcription targets (progesterone receptor (PGR); amphiregulin (AREG) and PDZ containing domain 1 (PDZK1)); however, they showed differentially activated cell-cell interactions pathways [171], which was attributed to differences in cellular differentiation in 2D and 3D cultures. In our own study [172], size-controlled 3D microtumors generated using hydrogel microwells of defined diameter (150 and 600 μ m) showed differential microtumor size-dependent ER α expression. While small non-hypoxic 150 μ m microtumors maintained ER α expression, large hypoxic 600 μ m microtumors displayed loss of ER α expression both at mRNA and protein levels.

When cancer drug responses are compared in 2D and 3D tumor models, differential drug sensitivity has been reported to be manifested as either greater resistance or enhanced sensitivity. [132, 138] The drug response to cisplatin and cetuximab was evaluated in head and neck squamous cell carcinoma (HNSCC) cells (LK0858B, LK0902 and LK1122) cultured as monolayers in 2D and as spheroids in ultra-low attachment plates. Counter-intuitively, LK0902 cells were shown to be more sensitive to cetuximab treatment in 3D tumor spheroids in comparison to 2D culture. [173] In another study conducted in colorectal cancer cells, cells cultured in type-I collagen were reported to be sensitive to cetuximab treatment while the 2D cells were non-responsive. [174] In another study, AU565, HCC1569, SKBR3, and BT549 breast cancer cells cultured in matrigel were more sensitive to HER2-targeting agents (lapatinib, pertuzumab and trastuzumab) in 3D compared to cells cultured in 2D.[175] MCF-7 cells cultured in 3D laminin-rich ECM also showed elevated sensitivity to doxorubicin in comparison to 2D monolayer cultures. This was attributed to prevention of activation of p53-DRAM-autophagy.[176]

Based on this evidence, a broad consensus has emerged that *in vitro* 3D tumor models serve better as physiologically relevant preclinical models to evaluate the efficacy of cancer drug leads, and that the application of these models have the potential to improve the success rate of drug candidates that advance into mouse tumor models and clinical trials (Table II). The next section highlights application of 3D models to investigate role of hypoxia in specific hallmarks in breast cancer progression.

4. Three Dimensional Models Used to Study Hypoxia Biology in Breast Cancer

4.1 Metabolic Stress and Lipolysis

Altered cancer cell metabolism under hypoxic conditions exert a selection pressure, which modulates different cellular processes coupled with downregulation of oxidative phosphorylation and inhibition of fatty acids. Altered metabolic adaptations and lipid profile of tumor cells is a well-established hallmark of cancer progression. Currently, lipid profiling and mapping in breast cancer *in vitro* models has been extensively studied using 2D cell culture to investigate the association between lipid production and tumor progression. In view of the technological advancements, 3D models are being used to understand the underlying metabolic changes, which facilitate tumor cell survival and proliferation. Presently, there is only one published study investigating hypoxia-initiated modulation of breast cancer metabolic adaptations. [137] In this study, starvation-induced spatial-temporal metabolic adaptations were investigated in an organotypic microfluidic MCF-10A breast cancer model. To recapitulate the ductal carcinoma in situ (DCIS) microenvironment, the authors developed a microfluidic model, in which MCF-10A cells were grown inside a luminal mammary duct model, embedded in a 3D hydrogel with mammary fibroblasts. [137] A hypoxia sensing dye was added to the collagen hydrogel before polymerization to detect oxygen levels. It was shown that metabolic signaling pathways specific to glycolysis and hypoxia were altered. The study also identified gradients of different metabolic phenotypes across the mammary duct model. Also, administration of the hypoxia-activated prodrug;

Tirapazamine selectively destroyed hypoxic DCIS cells. [137] The results from this study highlights the metabolic adaptations of tumor cells to endure hypoxia.

In addition to the metabolic changes inherent in breast cancer cells undergoing tumor progression and survival, modulation of lipid metabolism is another major source of energy to support tumor cell proliferation. In 2D monolayer models of breast cancer metabolism, aberrant accumulation of lipids along with altered lipid profiles are reported to be associated with malignant progression. [177] This altered lipid metabolism is well-known hallmark of cancer progression. It is also well known that malignant cells store surplus lipids in the form of intracellular droplets or in organelles involved in transport and storage of lipids, which provides the essential energy for tumor proliferation. The recent high-throughput technological advancements in Mass spectrometry (MS) and Raman microscopy has enabled investigations of the lipid profile (lipidomics) and special distribution of lipids in cancer tissues. In one of comprehensive study design, Vidavsky *et al.* [178] mapped and profiled lipid distribution in a 3D breast cancer model of tumor progression. They used liquid chromatography coupled with MS to extensively illustrate the difference in the lipid composition of the MCF-10A breast cancer progression when cultured as 2D monolayers vs. 3D spheroids. Further, lipid staining, and Raman chemical imaging was used to study the spatial distribution of lipids. While the amount of lipids decreased significantly, the ratio of acylglycerols to the membrane lipids increased in 3D cultured cells, suggesting formation of large lipid droplets, which were absent in 2D cultures. In malignant invasive 3D MCF-10A spheroids, lipid accumulation was observed in the hypoxic core of the tumor spheroids with declining gradient towards the periphery. Similarly, large lipid droplets were present in the hypoxic core, which was attributed to activation of the *de novo* sphingolipid synthesis signaling cascade. [178] In another study, Jiang and colleagues investigated the heterogeneous spatial response of tumors to hypoxia using MALDI-MS imaging (MSI) in 3D MDA-MB-231-HRE-tdTomato (hypoxia response element with control of red fluorescent tdTomato protein construct) breast tumor xenografts.[179] The findings from the study highlighted co-localization of lipid species in the hypoxic regions of tumors using principal component analysis-linear discriminant analysis (PCA-LDA) with identification of a total of 34 hypoxia regulated lipid species for which strong positive correlations were observed for phosphatidylcholine (PC) (16:0/18:0; 16:0/18:1; 18:0/18:1 and 18:1/18:1) and sphingomyelin (SM) (d18:1/24:0) suggestive of their high expression in the hypoxic cores. [179]

Tumor metabolic pathways and lipid metabolism have been extensively studied in 2D cultures and still lacks extensive studies using appropriate 3D culture systems, which better recapitulate the *in vivo* malignant tumor progression. In the view of limited scientific literature on the evolution of metabolic stress and lipolysis in hypoxic conditions using 3D models, more well-designed experimental studies are needed to reconnoiter lipid metabolism for therapeutic or targeted modulation.

4.2 Apoptosis

The studies reporting activation of the pro-apoptotic pathway in hypoxic 3D breast cancer models are sparse. In breast cancer cells, the mechanism of action of hypoxia in modulating

pro-apoptotic pathways has not been very well explored in 3D tumor models and presently there has been only one study published. In this study, a 3D scaffold-based MDA-MB-231 breast cancer model recreating a hypoxic environment was used to study its effects on phenotypic features and growth dynamics. [180] A decrease in the percentage of live cells was seen in 3D scaffolds, which was directly proportional to increased number of apoptotic events. Also, the hypoxic niche was shown to induce migration on collagen fibrils eventually leading to cellular senescence in MDA-MB-231 breast cancer cells illustrated by overexpression of senescence associated β -galactosidase marker. MDA-MB-231 breast cancer cells cultured in these scaffolds expressed increased expression of Bcl-2 associated X protein (Bax), caspase 3 and caspase 9 in comparison to monolayer cultures. [180]

4.3 Angiogenesis and Vascular Mimicry

Hypoxia is an established key regulator of tumor angiogenesis and vascular mimicry, which modulates angiogenic events in malignant cells via expression of HIFs. Angiogenesis as a whole has been extensively studied and is shown to have a major correlation with hypoxia. However, along with hypoxia one must take into consideration other potential targets, which might engage in crosstalk with other immune cells and promote tumor angiogenesis.

An interesting biomimetic 3D model was created using macroporous type I collagen scaffolds (average porosity: $85 \pm 6.3\%$) to study how hypoxic microenvironment affects the growth dynamics and phenotypic features in MCF-7 and MDA-MB-231 breast cancer cells. [180] These scaffolds induced hypoxic microenvironment in 3D MCF-7 and MDA-MB-231 breast cancer cells with elevated HIF-1 α protein expression and activation of the glycolytic pathway indicated by high Glut-1 expression. Hypoxia also induced angiogenesis with elevated VEGF levels in scaffold media of MCF7 ($p=0.006$) and MDA-MB-231 cells ($p=0.008$) on day 7 in comparison to monolayer culture cells. [180] In one study [181], microfabricated alginate hydrogels (4% w/v) were used for 3D culture in an oxygen controlled (1% O₂) environment to study how 3D culture influenced hypoxic responses and angiogenesis in MDA-MB-231 breast cancer cells. The findings supported a positive correlation between culture dimensionality and hypoxia response, which further regulated tumor angiogenesis via VEGF. This VEGF-mediated sprouting angiogenesis was reported to be enhanced via the action of IL-8, a pro-inflammatory cytokine. [181] Further, the effect of cyclic hypoxia (exposure to cycles of hypoxia) on angiogenesis and tumor vascularization was also studied in MCF-7 CSCs. [182] MCF-7 cell mammospheres were generated using ultra-low attachment plates and were exposed to hypoxic conditions continuously or intermittently. The secreted media from intermittently hypoxic CSCs showed elevated VEGF levels (1159 ± 90 pg/ml) compared to continuously hypoxic stem cells (934 ± 28 pg/ml) and control normoxic conditioned media (225 ± 24 pg/ml). [182] Similar upregulation of VEGF-A and HIF-1 α expression were reported in another study, which explored the angiogenic potential of MDA-MB-231 bioengineered tumors cultured in collagen I hydrogels exposed to closed vasculature of 150–200 μ m diameter induced hypoxia. [183]

In addition to the role of hypoxia in stimulating angiogenesis in the TME of malignant cells, intra-tumoral hypoxia has also been shown to induce formation of vessel-like tubes from tumor cells (VM). [84] In the context of breast cancer cells, presently there are few published

studies, which have explored VM in 3D matrigel and collagen matrix. In the first study, Walker 256 (W256) rat breast carcinoma cells were cultured in cold matrigel under 2% O₂ in a humidified tri-gas incubator and treated with CA4P, a vascular targeting agent responsible for depolymerizing microtubule. [85] The CA4P treatment elevated HIF-1 α and induced tube formation indicative of VM network in W256 cells. Further, they also established a W256 breast tumor bearing mouse model, which when exposed to CA4P, induced intra-tumoral hypoxia and increased VM network formation *in vivo*. It was proposed that the induction of hypoxia and VM networks by CA4P was due to activation of HIF-1 α /EphA2/PI3K/MMP signaling pathways. [85] In another study, MDA-MB-231 cells cultured in matrigel and transfected with pre-miR-2014 (30nM) were incubated in hypoxic conditions (1% O₂) were observed to induce 3D tunnel formation (matrix-associated VM) in MDA-MB-231 cells. [89] Ectopic restoration of miR-204 was shown to inhibit VM and reduced patterned 3D channels. This miR-204 was reported to impair VM via downregulation of PI3K/AKT, VEGF, FAK/Src and RAF1/MAPK signaling pathways, thereby emphasizing the importance of PI3K/AKT/FAK pathways in VM formation.

Another study explored the influence of topographical organization of high-density collagen matrix *in vivo* on tumor progression, migration and metastasis. [184] The high-density collagen matrix triggered transcriptional response (upregulation of integrin subunit beta 1 (ITGB1), jagged canonical Notch ligand 1 (JAG1) and fibroblast growth factor receptor 1 (FGFR1) leading to motility switch in MDA-MB-231 breast cancer cells independent of matrix stiffness, density or hypoxia in comparison to low-density collagen matrix, which did not show migration. This high-density collagen matrix induced migration post one-week culture *in vitro* along with formation of interconnected network resembling endothelial tubulogenesis/VM. This migration phenotype was identified as collagen-induced network phenotype (CINP), which was shown to be driven by driven by β 1-integrin upregulation and correlated with poor patient survival. [184]

4.4 Pro-Inflammatory Pathways

Recent developments in the field of microfluidic devices have made it possible to recapitulate 3D tumor microenvironments *in vitro* to study the effect of hypoxia on pro-inflammatory pathways. In an initial study, MDA-MB-231 breast cancer cells cultured in collagen I and exposed to 5% O₂ levels were reported to have decreased JNKs, cortactin expression and activated transforming growth factor beta-1 (TGF β 1)/Smad signaling cascade. [14] This dysregulated JNK expression, activated Smad 2/4 and Snail1 further inhibited formation of invadopodia and expression of matrix metalloproteinase 14 (MMP14). [14] Following the initial report, a hypoxic 3D microenvironment was shown to regulate the hypoxic response and angiogenesis via pro-inflammatory pathways. The authors used an alginate-based hypoxic (oxygen-controlled) 3D model and reported elevated IL-8-mediated angiogenic sprouting in a 3D endothelial invasion assay. They further highlighted role of pro-inflammatory molecules and signaling cascade as a major modulators of hypoxic tumor responses in the TME. [181] Similarly, Yoshino and Funamoto demonstrated oxygen-dependent (normoxic: 21% O₂ and hypoxic: 1% O₂) contraction and degradation of ECM when human MDA-MB-231 breast cancer cells were co-cultured with HUVECs in a collagen gel. This was attributed to tumor-endothelial cell interactions coupled with

downregulation of matrix metalloproteinase-7 (MMP-7). [185] Recently, 3D-heterotypic spheroid models (composed of RAW 264.7 macrophages and MDA-MB 231 breast cancer cells) were used to evaluate the role of TAMs in the TME. [186] The effect of hypoxia on this tumor/macrophage heterotypic 3D spheroid model exhibited elevated IL-10 secretion leading to activation of M2 TAMs differentiation pathway in the TME. [186] Further, an *in-silico* agent-based model was used to study hypoxia, chemokine receptor C-C chemokine receptor type 5 (CCR5) and cancer stem cell interactions in MDA-MB-231 cells. The group reported hypoxia-induced tumor progression in MDA-MB-231 cells, further showing that treatment with Maraviroc (a CCR5 inhibitor) decreased tumor cell growth. [187]

Although development of 3D microfluidic platforms made it possible to better replicate the TME *in vitro*, only a limited number of scientific studies have investigated hypoxic microenvironments. Further studies are warranted to understand the precise mechanism of which hypoxia modulates ECM dynamics and the pro-inflammatory pathways in the TME since these may become promising therapeutic targets.

4.5 Migration

Several 3D breast tumor models are being developed to understand the underlying mechanisms of tumor migration. The initial attempt to decipher the migration profile of breast cancer cells was carried out by Funamoto and colleagues [188] who designed a novel microfluidic platform to study the effect of controlled hypoxic microenvironment on MDAMB-231 cells. The microfluidic platform designed by the group consisted of a central 3D gel which acted as an extracellular matrix. This central region was flanked by media channels and peripheral gas channels that maintained the controlled hypoxic microenvironment (0% O₂). [188] This microfluidic platform was used for imaging the migratory phenotypes of MDA-MB-231 cells every 10 min for >8h and enhanced migration was reported under hypoxia via activation of hypoxia associated signaling pathways in comparison to normoxic conditions. A rotatory culture method was used to generate 3D SUM159 and SUM149 breast spheroids, which were exposed to 1% O₂ in a modular incubator chamber and evaluated for selectivity of hypoxia to induce integrin receptor expression, migration, and metastasis. The results highlighted the role of integrin alpha 5 (ITGA5) in inducing migration and metastasis to lymph nodes and lungs under hypoxic conditions (induced via elevated HIF-1 α expression). [189] An innovative 3D breast tumor platform was developed consisting of photo-crosslinked hydrogel encapsulated with 21PT and 21MT-2 breast cancer cells derived from patient samples, which were exposed to 5% O₂ levels in a tri-gas incubator. [190] This study showed decrease in tumor size and density, increase in EMT transition and migration, that was coupled to upregulation of lysyl oxidase (LOX). The model was also used to generate a breast cancer and lung cells contact model by stacking 3D hydrogel constructs with breast cancer cells onto lung mesenchymal cell (LMC) laden hydrogels. This unique model showed migration of breast cancer cells towards LMC cells under hypoxic conditions. In an interesting study carried out on MCF-7 and T47D spheroids cultured using matrigel and exposed to variable oxygen gradients in a hypoxic chamber (0.2–1% oxygen), hypoxia was shown to induce miR-191 in a time-dependent manner, which promoted migration in breast spheroids via upregulation of TGF- β 2 in the

hypoxic TME along with upregulation of its downstream target genes VEGFA, CTGF, SMAD family member 3 (SMAD3) and bone morphogenetic protein 4 (BMP4). [191]

3D models have also been exploited to determine the efficacy of inhibitors targeting hypoxia signaling pathway. Interestingly, the efficacy of five novel ureido-substituted CAIX inhibitors (FC11409B, FC9398A, S4, FC9403, and FC9396A) were assessed under normoxic (21%) and hypoxic (0.5% O₂) in 3D breast spheroids cultured in spinner flasks, an *ex-vivo* explant model, and a MDA-MB-231 xenograft model. [192] Post-CAIX treatment, 3D breast cancer cells were shown to have decreased cell migration, extravasation and invasion potential. S4, FC9403A and FC9398A inhibited invasion into collagen while FC9403A was also reported to reverse established invasion. FC9398A was shown to reduce xenograft tumor growth. [192] Similar results were reported from another study where CAIX inhibitor (CAI017) inhibited migration in a dose-dependent manner in MDA-MB-231 cells cultured in matrigel and hypoxic (1% O₂) conditions and orthotopic mice bearing breast tumors.[193] CAI017 inhibited migratory phenotype via modulation of downstream regulation of the mTORC1 axis. [193] In addition to CAIX inhibitors, LOX inhibitors have also been shown to inhibit migration and EMT. [190]

Currently, there are no preclinical *in vitro* models that show tumor progression in real time in the same tumor model using the same cell line without any genetic manipulations or any artificial culture conditions. Our lab has previously engineered an array of uniform size hydrogel microwells using microfabricated PDMS micro-posts of defined diameter and height (*Figure 3A*)[172, 194–197] The hydrogel microwells are made of polyethylene glycol dimethacrylate (PEGDMA), a non-adhesive polymer that does not allow cell-hydrogel interaction, and thus, maximizes cell-cell interaction forming hundreds of uniform size cell aggregates (referred henceforth as ‘microtumors’). The size of microtumor is determined by the diameter of the microwells on the array, the compaction ability of the cells used and the cell seeding density.[194, 195] For example, T47D breast cancer cells seeded on a device containing 150 μm diameter hydrogel microwells result in microtumors of average diameter of $128 \pm 16 \mu\text{m}$ on day 6 (referred as ‘small’ microtumors) while that containing 600 μm diameter hydrogel microwells result in microtumors of average diameter of $550 \pm 57 \mu\text{m}$ (referred as ‘large’ microtumors) by day 6.[195] Each $1 \times 1 \text{ cm}^2$ device of 150 μm size microwells generates 350–450 small microtumors while each $1 \times 1 \text{ cm}^2$ device of 600 μm size microwells generates 60–80 large microtumors.[195] Thus, the hydrogel microwell platform can generate hundreds of defined yet uniform size microtumors from the same parent cells. We have shown that precise control over microtumor size naturally creates spatial oxygen/nutrient diffusion gradients leading to controlled yet reproducible hypoxic environments.[195] Hypoxia, ROS, and propidium iodide (PI)+ cells were seen in the large microtumor cores, along with increased HIF-1 α and metabolic stress-induced Glut-1 expression, but not in non-hypoxic small microtumors (*Figure 3B*). Interestingly, without any additional stimulus, large T47D microtumors exhibit a migratory phenotype as early as day 3–4 while small microtumors generated from the same parent cells never migrate (*Figure 3C*).[172, 195, 196] That is, the migratory phenotype emerged spontaneously based on differential microtumor size only. These results are consistent with the clinical findings that critical genetic mutations pre-exist prior to the onset of tumor migration/invasion, and that microenvironmental factors, like tumor size-induced hypoxia and metabolic stress, drive

transition from a non-migratory to a migratory phenotype.[141, 198–202] In addition, we observed the emergence of heterogeneous cellular populations inside each large microtumor ('intra-tumoral' heterogeneity) based on the expression of the epithelial marker, E-Cadherin (E-Cad) and mesenchymal marker, Vimentin (VIM) along with tumor-to-tumor variation in the migration kinetics ('inter-tumoral' variation).[195, 196] Thus, by manipulating microtumor size alone and without any genetic manipulations or exogenous stimuli, we reproducibly generated two distinct phenotypes from the same noninvasive parent breast cancer cells: small non-hypoxic microtumors represent a non-migratory phenotype whereas large hypoxic microtumors exhibit a migratory phenotype. These microtumor models recapitulate three key hallmarks of pre-invasive to invasive disease progression that is observed *in vivo*: 1) Increasing tumor size drives hypoxia and metabolic stress; 2) Heterogeneous tumor cells spontaneously emerge; and 3) Cells begin to migrate from the parent tumor.

Another interesting feature of our microtumor model is that the large microtumors exhibit partial or hybrid EMT phenotype (Figure 3D) without loss of E-cad along with upregulation of mesenchymal markers, Snail, Slug and Vim. [195] Our large T47D microtumors recapitulate the collective migration observed in clinical samples of carcinoma.[203, 204] Additionally, the large microtumors were shown to retain their migratory phenotype even after removal of hypoxia suggesting that the migratory phenotype is irreversible.[195] To further delineate the mechanisms underlying this irreversible migratory phenotype, we generated non-hypoxic, yet migratory microtumor models by exposure of small non-hypoxic microtumors to the conditioned media of large microtumors. [196] This study showed that the hypoxic secretome, specifically, soluble E-cad, MMP9 and fibronectin in the conditioned media of large migratory microtumors induced collective migration in the non-hypoxic, non-migratory small microtumors. We proposed a novel two-stage tumor progression mechanism, where hypoxia is important in the early 'initiation stage' of tumor progression, and the positive feedback loop among the secretome factors works synergistically to maintain the migratory phenotype ('maintenance stage'). Computational and experimental studies in 3D microtumor models (using cell lines and patient derived cancer cells) showed that inhibition of tumor-secreted factors effectively halts microtumor migration despite tumor-to-tumor variation in migration kinetics, while inhibition of hypoxia is effective only within a time window and is compromised by tumor-to-tumor variation, supporting our notion that hypoxia initiates migratory phenotypes but does not sustain it.

In summary, 3D models have advanced our understanding of tumor-intrinsic, cell-cell and cell-ECM mediated mechanisms related to hypoxia, EMT and migration.

4.6 Drug Resistance

Hypoxia is widely known to promote drug resistance to conventional chemotherapeutic drugs and radiation therapy. However, the precise mechanism as to how hypoxic cells acquire drug resistance still remains unknown. It is postulated that reduced metabolism, genetic instability, loss of p53-mediated sensitivity to apoptosis or activation of genes regulating multiple drug resistance (e.g. MDR1 encoding P-gp) may be key modulators in acquisition of drug resistance. Recently, numerous studies have also highlighted the role of

CSCs with attributes of self-renewal, drug resistance and tumor relapse along with regulation of miRNAs as major contributors for failure of cancer treatments. In view of the limitations of 2D experimental systems, 3D models have become the choice of experimental models due to their ability to replicate tumor biology *in vivo* and investigate responses to chemotherapeutic agents. 3D spheroids or scaffold-based models are also important for the study of tumor drug resistance. Using the conventional ultralow attachment plate for spheroid formation, different groups have tried to explore the drug resistance biology in breast cancer cells. In one of the initial studies, 3% O₂ in a humidified hypoxic chamber was identified as sufficient to induce HIF-1 α activation in MCF-7 spheroids.[205] In this study, MCF-7 spheroids were resistant to doxorubicin (3 μ mol/L), which was associated with elevated P-gp expression via activation of HIF-1 α . [205] In another study, [206] MCF-10A cells exposed to humidified hypoxic chamber (1% O₂) induced HIF-1 α activation, which in turn, induced lapatinib resistance via activation of the downstream Erb-B2 receptor tyrosine kinase 2 (ERBB2) signaling cascade, ERK/AKT. The study also elucidated that administration of trametinib (a MEK inhibitor) or over-expression of dual-specificity phosphatase 2 (DUSP2) reversed hypoxia-induced lapatinib resistance.[206] Recent technology advancements, have provided new 3D models to study the effect of hypoxia on drug resistance, which are summarized below.

When MDA-MB-231 spheroids encapsulated in collagen matrix were placed in a hypoxic chamber (1% O₂), it was found that hypoxia stimulated proliferation of spheroids in cells treated with paclitaxel (IC₅₀ 532.8 \pm 74.4) and doxorubicin (IC₅₀ 21.0 \pm 28.5 nM) *vs.* the untreated tumor spheroids emphasizing the role of hypoxia induced chemoresistance.[207] Similarly, hypoxic MDA-MB-231 spheroids cultured in 0.6% agar showed activated cleaved Notch 1 levels and AMP-activated protein kinase (AMPK). [208] In another study, a semisolid culture system was established for MCF-7 spheroids and exposed to hypoxic chamber (0.1% pO₂ and 5% CO₂) to study effect of hypoxia on stemness.[209] The group reported that the hypoxic MCF-7 cells were resistant to paclitaxel, gemcitabine and adriamycin and overexpressed miR-210 in breast cancer stem cells, which suppressed E-cad expression by targeting open reading frame the (ORF) of E-cad. [209] In another study, the hanging drop method was used to generate BT-474 spheroids, which were then exposed to hypoxia using two different methods *i.e.* 1% O₂ in hypoxic chamber and CoCl₂ method.[210] An increase in breast cancer stem cell population was observed in hypoxic HER2+ BT-474 spheroids (CD44+/CD24^{low}) with acquired trastuzumab resistance. [210] In a study carried out in MDA-MB-157 triple negative breast cancer cells, tumor spheroids were generated using polymeric aqueous two-phase system (ATPS) technology, which generated uniform yet controlled size spheroids (100 μ m and 200 μ m) depending on cell densities (1.5*10⁴ cells for 100 μ m and 1.0*10⁵ for 200 μ m spheroids). [211] The larger MDA-MB-157 spheroids treated with doxorubicin (100*10⁻⁹M) showed ten-fold higher drug resistance (IC₅₀ of 481*10⁻⁹M) than smaller spheroids. Further, the group co-treated larger spheroids with doxorubicin (100*10⁻⁹M) and TH-302 (a hypoxia activated prodrug, 10*10⁻⁶M) and the combined treatment was seen to decrease cell viability by 30% and had a combination index (CI) of 0.02 (measure of high synergistic effect of two drugs). The larger MDA-MB-157 spheroids were also seen to express the cancer stem cell markers, namely, CD24, CD133 and Nanog. These results were supported by the hypothesis of hypoxia leading to transcriptional

activation of genes encoding stem cells. [211] In a recent study from another group [212], hypoxia induced a switch via SNAT2/Solute carrier family 38 member 2 (SLC38A2) signaling regulation, which was responsible for generation of endocrine resistance in hypoxic (0.1% O₂) MCF-7 spheroids. Additionally, the 3D-heterotypic spheroid model (tumor cell (MDA-MB 231) and macrophage (RAW 264.7)) discussed above has also been used to evaluate the effect of paclitaxel treatment. [186] The first heterotypic spheroid model, mimicked the TAMs infiltrated into tumor mass and showed resistance to paclitaxel while in the second model, macrophages diffusely seeded around the spheroids showed the same metabolic profiles irrespective of the presence of tumor spheroids.[186] The findings from this study highlight the synergistic growth pattern of TAMs and tumor cells in a heterospheroid model mimicked *in vivo* conditions which play a role in paclitaxel drug resistance. In yet another interesting study, Alhawarat and colleagues illustrated higher chemoresistance in prolonged hypoxia treated CSC spheroids.[182] The 3D-MCF-7 CD44⁺/CD24⁻ CSCs under normoxic conditions exhibited high drug resistance to doxorubicin (4.08 fold) in comparison to parent 2D cultures, which increased further (3.5 fold) when hypoxic MCF-7 CSCs were exposed to repetitive long-term continuous/intermittent cycles of hypoxia for four months.[182]

We have shown differential drug response in our non-migratory small (150 μm) vs. migratory large microtumor models (600 μm) highlighting a significant role for the TME in dictating molecular mechanisms involved in tumor progression and consequently, tumor response to molecular therapies.[172] Although small and large microtumors were generated from the same ER⁺ parental T47D cell line, size-dependent microenvironmental changes such as hypoxia, ROS, and pro-angiogenic factors induced downstream signaling such as loss of ER-α and upregulation of VEGF in large microtumors. Such differential molecular changes between 150 and 600 μm microtumors further led to differential response to ER-targeted anti-estrogen, and anti-VEGF antibodies without much difference in EGFR-targeted tyrosine kinase inhibitor (TKI), gefitinib response (Figure 3E). Non-migratory small microtumors retaining ERα expression were more sensitive to anti-estrogen 4-hydroxytamoxifen similar to early stage breast cancer patients while migratory large microtumors with loss of ERα showed endocrine resistance; however, upregulated VEGF levels rendered them more sensitive to anti-VEGF therapy similar to the advanced stage breast cancer patients. With similar expression levels of signaling molecules involved in the EGFR pathway, both small and large microtumors responded in a size-independent manner to EGFR-targeted TKI gefitinib. We confirmed that differential drug response observed in 150 and 600 μm microtumors was not due to microtumor size-mediated drug diffusion limitations but indeed due to the differential microenvironment-mediated signaling changes. This study underscores the importance of TME in regulation of molecular mechanisms involved in tumor progression and hence, it warrants development of physiologically relevant 3D models that recapitulate underlying molecular mechanisms of disease progression. These efforts will be instrumental for effective screening of targeted therapies. These studies collectively provide the scientific evidence of hypoxia induced chemoresistance (Table III). This also leads the way for therapeutic evaluation of key pathway molecules modulating hypoxia induced chemo-resistance and their inhibitors, which is important to develop better treatment modalities.

5. Future Prospects and Conclusion

This review highlights the role of hypoxia in modulating cellular signaling pathways in directing oncogenic transformations breast cancer progression at different phases of tumor progression. The hypoxic transformations in the tumor cells mediated via expression of hypoxia-inducible factors promote tumor survival and progression in breast cancer cells via metabolic adaptations, angiogenesis, stromal cell recruitment, migration, tissue invasion, extracellular matrix modelling, extravasation to metastatic sites and drug resistance. It is important to note that the current understanding of molecular mechanisms in cancer biology in general and hypoxia biology specifically is heavily derived from 2D cultures that do not recapitulate important components of TME: tumor cells, stromal cells, and non-cellular factors (hypoxia, pH, metabolic stress, ECM, etc.). Hence, there is significant need to validate the currently known mechanisms using physiologically relevant 3D models. We have highlighted several studies that have used 3D *in vitro* culture systems to mimic invasive behavior of cancer cells, epithelial and mesenchymal transition, and cell-cell/cell-ECM interactions. We have also highlighted studies with effort to understand the effect of therapeutic agents on signaling pathways in a 3D microenvironment. 3D tumor spheroids still remain the most widely used model for studying tumor biology. Although the field is recognizing the importance of 3D models in cancer biology and is moving in the right direction, most studies discussed here have utilized hypoxic chambers to create hypoxic microenvironments in 3D.

Recent technological advances in tissue engineering and materials science have launched newer methods for generating 3D models like bioreactors, bioprinters and advanced complex microfluidics. These methods are being fine-tuned to offer greater control over culture complexity in terms of incorporating multiple components of TME as well as culture conditions such as oxygen, nutrient supply etc. Technologies are also evolving to monitor/ image dynamic changes in cellular behaviors in 3D cultures over a period of time. Similarly, there are efforts to characterize and validate the changes in 3D cultures using genomic and transcriptomic profiles. With the advantages of 3D models, these insights will provide us the understanding of the evolution of the TME in 3D and its relevance to clinical samples.

Although there are significant technological advancements in generating variety of 3D tumor models and a huge repertoire of available models to choose from, translation of these models to study hypoxia biology in particular is a long way ahead. This is supported by the limited studies, which have exploited the strengths of 3D models to understand the fundamental role of hypoxia biology in regulating breast cancer progression. It should also be borne in mind that each 3D model may not be able to recapitulate all aspects of *in vivo* TME and disease pathology and that the context, the hypothesis will play significant role in selecting the appropriate model. Hence, it is important to determine the essential components of the 3D models to successfully test the hypothesis. It is equally important to consider validation process for the 3D models. The validation steps could be related to the validation of the known molecular mechanisms or the clinical response to the known drugs. The validation could also include use of patient-specific samples to support the findings observed in these *in vitro* 3D models. In order to advance our understanding of genetic and epigenetic mechanisms involved in hypoxia mediated tumor progression and to unravel novel

therapeutic targets for benefit of the patients, it is instrumental that we promote crosstalk among multiple disciplines including cancer biology, pharmacology, materials science, bioengineering and bioinformatics.

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Abbreviations

2D	Two-dimensional
3-D	Three-dimensional
TME	Tumor microenvironment
ECM	Extracellular matrix
TNBC	Triple negative breast cancer
HIF-1	Hypoxia inducible factor 1
HIF-1α	Hypoxia inducible factor 1 alpha
HIF-1β	Hypoxia inducible factor 1 beta
SNAT2	Sodium-coupled neutral amino acid transporter 2
CAIX	Carbonic anhydrase IX
VEGF	Vascular endothelial growth factor
BNIP-3	BCL2 interacting protein 3
ROS	Reactive oxygen species
NADPH	Nicotinamide adenine dinucleotide phosphate
JNK	c-Jun N-terminal kinase
NF-κB	Nuclear factor kappa B subunit
CAV-1	Caveolin-1
MCT4	Monocarboxylate transporter 4
PHLDA3	Pleckstrin homology like domain family A member 3
INPP5D	Inositol polyphosphate-5-phosphatase
HRE	HIF-1 response element
NIX	Nip3 like protein X
TRAIL	TNF-related apoptosis inducing ligand

BRCA1	Breast cancer susceptibility gene 1
STAT5b	Signal transducer and activator of transcription 5B
MMPs	Matrix metalloproteinases
FGF	Fibroblast growth factor
PDGF-β	Platelet-derived growth factor- β
SDF-1α	Stromal-derived factor 1 α
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
TGF-β	Transforming growth factor beta 1
PPARα	Peroxisome proliferators-activated receptors
mTOR	Mammalian target of rapamycin
MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal-regulated kinase
MAPK/ERK	MEK
TAMs	Tumor associated macrophages
P-gp	P-glycoprotein
HMGB1	High-mobility group box 1
AKT	AKT serine/threonine kinase
PI3K	Phosphatidylinositol-3-kinase
iNOS	Inducible nitric oxide synthase
Src	proto-oncogene tyrosine-protein kinase Src
ADAM17	ADAM metalloproteinase domain 17
BET	Bromodomain and extra-terminal
CA9	Carbonic anhydrase
VEGF-A	Vascular endothelial growth factor A
VM	Vascular mimicry
CA4P	Combretastatin A4 phosphate
RTK	Receptor tyrosine kinase
VE-cadherin	Vascular endothelial cadherin

DMBT	6,6'-bis(2,3-dimethoxybenzoyl)- α , α -D-trehalose
EGFR	Epidermal growth factor receptor
EphA2	Erythropoietin producing hepatoma receptor A2
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
Cdc42	Cell division cycle 42
TP53INP1	Tumor protein p53-inducible nuclear protein 1
GSK-3β	Glycogen synthase kinase 3 beta
FAK	Focal adhesion kinase
RAF1	Raf-1 proto-oncogene, serine/threonine kinase
IL-1	Interleukin-1
IL-6	Interleukin-6
C/EBPα	CCAAT/enhancer binding protein- α
TNF-α	Tumor necrosis factor- α
A2AAR	A2A adenosine receptor
A2BAR	A2B adenosine receptor
NK	Natural killer
IL-4	Interleukin-4
IL-10	Interleukin-10
A2AR	A2 adenosine receptors
CTGF	Connective tissue growth factor
CCL2	C-C motif chemokine ligand 2
COX-2	Cyclooxygenase-2
HDAC	Histone deacetylase
IL-1β	Interleukin-1 beta
IL-12	Interleukin-12
EMT	Epithelial to mesenchymal transition
IL-8	Interleukin-8
HGF	Hepatocyte growth factor

RhoA	Ras homolog family member A
ROCK1	Rho associated coiled-coil containing protein kinase 1
ILK	Integrin-linked kinase
PDGF	Platelet-derived growth factor B
SOX2	SRY-box-2
NEDD9	Neural precursor cell expressed, developmentally down-regulated 9
Nrf2	Nuclear factor erythroid-2-related factor 2
Keap1	Kelch-like-ECH-associated protein 1
G6PD	Glucose-6-phosphate dehydrogenase
HES-1	Hes family BHLH transcription factor 1
eIF4E2	Eukaryotic translation initiation factor 4E type 2
ZMYND8	Zinc finger MYND-type containing 8
BRD4	Bromodomain containing 4
CSCs	Cancer stem cells
BCRP	Breast cancer resistance protein
MDR1	Multidrug resistance gene 1
MRP1/2	Multidrug resistance associated protein 1/2
PPARα	Peroxisome proliferators-activated receptor alpha
CAR	Constitutive androstane receptor
PXR	pregnane X receptor
FXR	Farnesoid X receptor
FGF	Fibroblast growth factor
FGFR	FGF receptor
CAFs	Cancer-associated fibroblasts
SDF-1	Stromal cell-derived factor
CXCL12	C-X-C motif chemokine 12
SCLC	Small cell lung cancer
PDXs	Patient-derived xenografts

MCTS	Multicellular tumor spheroids
DMD	Digital micromirror device
TRACER	Tissue roll for the analysis of cellular environment and response
PDMS	Polydimethylsiloxane
hBM_MSCs	Human bone marrow-derived mesenchymal stem cells
HUVECs	Human umbilical vein endothelial cells
OD	Osteo-differentiated
HTS	High-throughput screening
GEM	Global Eukaryotic Microcarriers
3D-PREDICT	3D-prediction of patient-specific response
ERα	Estrogen receptor alpha
PGR	Progesterone receptor
AREG	Amphiregulin
PDZK1	PDZ containing domain 1
HNSCC	Head and neck squamous cell carcinoma
DCIS	Ductal carcinoma in situ
MS	Mass spectrometry
MSI	MALDI-MS imaging
PCA-LDA	Principal component analysis-linear discriminant analysis
PC	Phosphatidylcholine
SM	Sphingomyelin
Bax	Bcl-2 associated X protein
MMP14	Matrix metalloproteinase 14
MMP-7	Matrix metalloproteinase-7
CCR5	C-C chemokine receptor type 5
ITGA5	Integrin alpha 5
LOX	Lysyl oxidase
LMC	Lung mesenchymal cells

SMAD3	SMAD family member 3
PEGDMA	Polyethylene glycol dimethacrylate
E-cad	E-Cadherin
VIM	Vimentin
miRNAs	MicroRNAs
DUSP2	Dual-Specificity Phosphatase 2
ERBB2	Erb-B2 receptor tyrosine kinase 2
AMPK	AMP-activated protein kinase
ORF	Open reading frame
ATPS	Aqueous two-phase system
CI	Combination index
SLC38A2	Solute carrier family 38 member 2
FGFR1	Fibroblast growth factor receptor 1
JAG1	Jagged canonical Notch ligand 1
ITGB1	Integrin subunit beta 1

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Highlights

- Role of hypoxia in directing oncogenic transformations i.e. metabolic adaptations, angiogenesis, stromal cell recruitment, migration, tissue invasion, extracellular matrix modeling, extravasation to metastatic sites and drug resistance via modulation of cellular signaling pathways.
- Techniques to develop 3D *in vitro* models replicating tumor microenvironments and hallmarks of cancer progression such as metabolic stress, angiogenesis, epithelial and mesenchymal transition, migration, invasion, and drug resistance.
- Opportunities and challenges for the translation of 3D models to understand the fundamental role of hypoxia biology in regulating breast cancer progression.

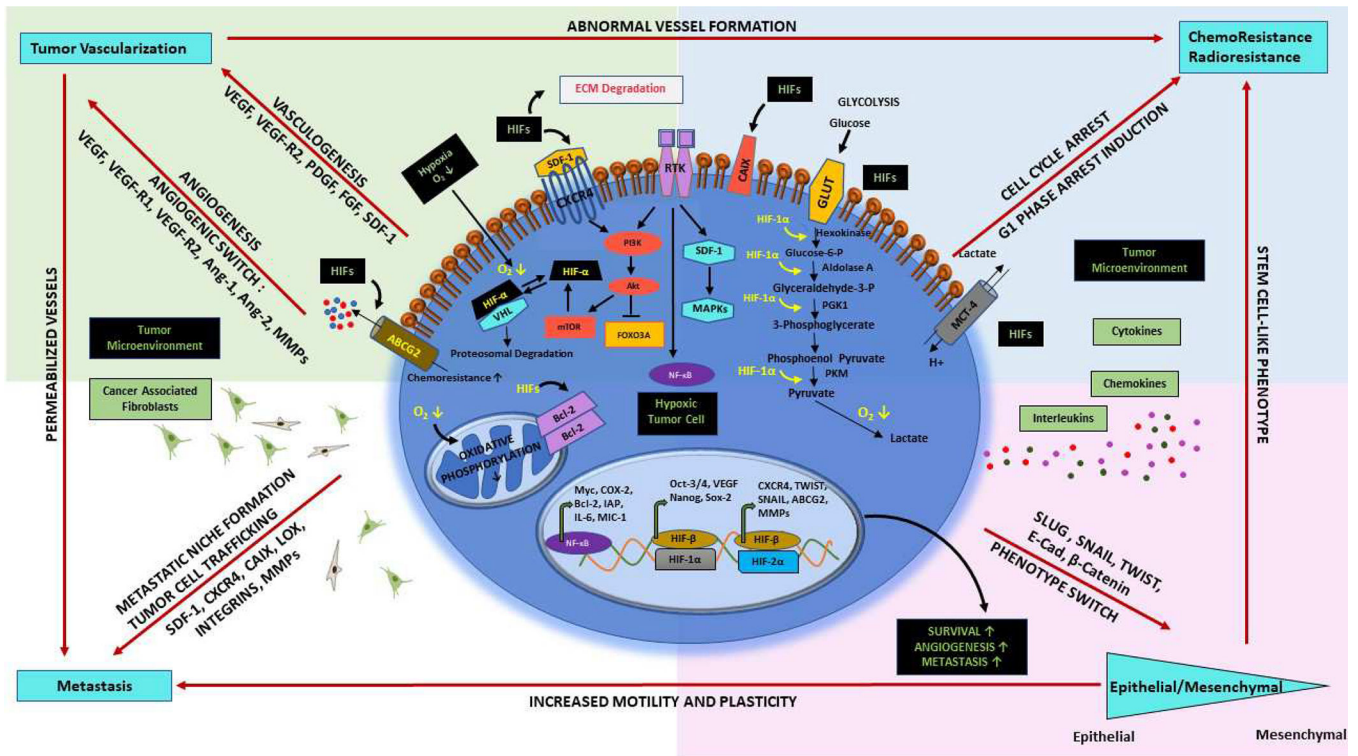


Figure 1: A schematic representation of hypoxia signaling cascades modulated by hypoxia inducible factors (HIFs) in tumor cells.

HIFs promote metabolic adaptations, oxidative stresses, angiogenesis, stromal cell recruitment, extracellular matrix modelling, extravasation, metastasis, migration and drug resistance through different signaling pathways. ATP-binding cassette subfamily G member 2 (ABCG2), AKT serine/threonine protein kinase B (AKT), Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), B-cell lymphoma 2 (Bcl-2), Carbonic anhydrase IX (CAIX), Cyclooxygenase-2 (COX-2), C-X-C motif chemokine 4 (CXCR4), E-cadherin (E-cad), Forkhead box O3 (FOXO3A), Fibroblast growth factor (FGF), Glucose transporter 1 (GLUT-1), Lysyl oxidase (LOX), Inhibitor of apoptosis (IAP), Interleukin-6 (IL-6), Mitogen activated protein kinase (MAPK), Phospholipase C-gamma (PLC- γ), Monocarboxylate transporter 4 (MCT-4), Macrophage inhibitory cytokine-1 (MIC-1), Matrix metalloproteinases (MMPs), Mammalian target of rapamycin (mTOR), Nuclear factor kappa light chain enhancer of activated B cells (NF- κ B), Platelet derived growth factor (PDGF), Phosphoglycerate kinase 1 (PGK1), Phosphatidylinositol 3-kinase (PI3K), Pyruvate kinase (PKM), Receptor tyrosine kinase (RTK), Stromal cell-derived factor 1 (SDF-1), Vascular endothelial growth factor (VEGF), Vascular endothelial growth factor receptor 1 (VEGFR1), Vascular endothelial growth factor receptor 2 (VEGFR2), von Hippel-Lindau tumor suppressor (VHL).

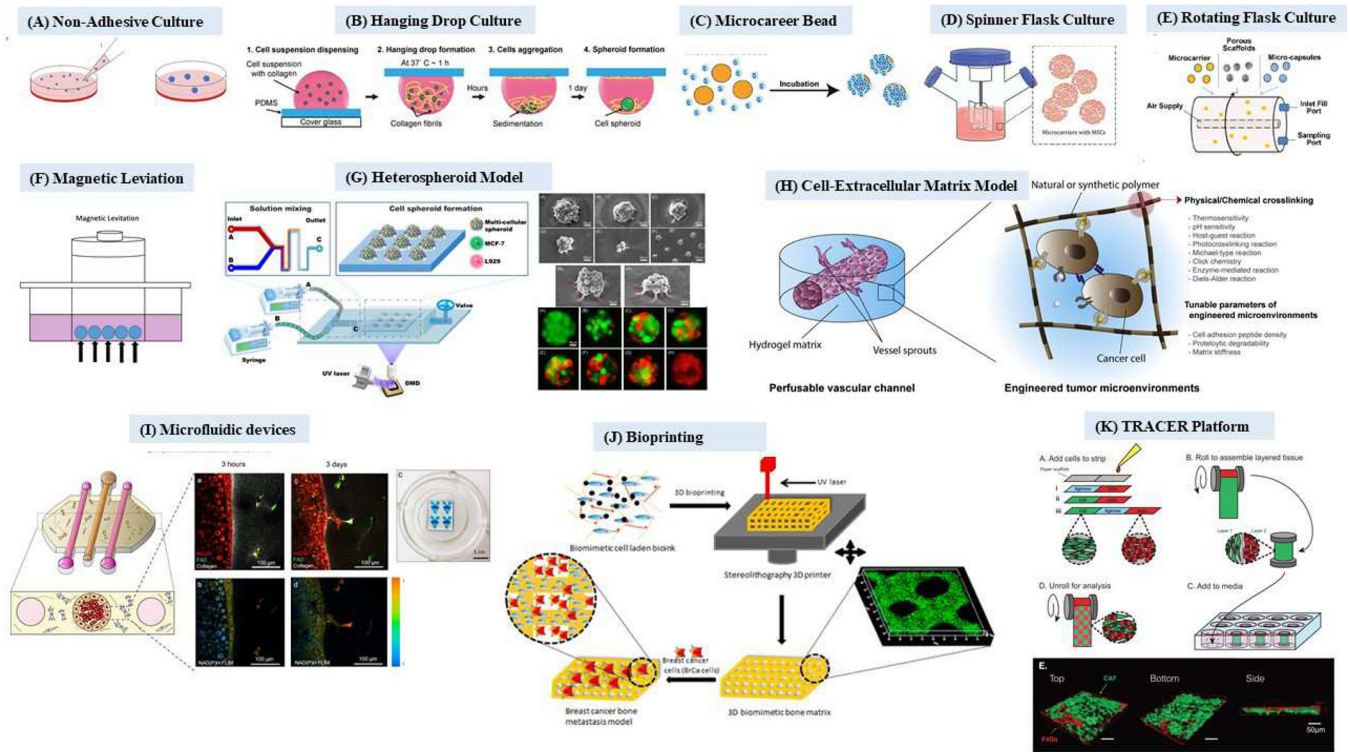


Figure 2: A schematic representation of three-dimensional (3D) models. (A) Non-adhesive culture; Adapted with permission [213], **(B) Hanging drop culture;** Adapted with permission [214], **(C) Microcarrier beads;** Adapted with permission [215], **(D) Spinner flask culture;** Adapted with permission [216], **(E) Rotating flask culture;** Adapted with permission [217] **(F) Magnetic levitation, (G) Heterospheroid model;** Adapted with permission [146], **(H) Cell-extracellular matrix model;** Adapted with permission [218], **(I) Microfluidic device;** Adapted with permission [137], **(J) Bioprinting;** Adapted with permission [219], **(K) TRACER platform;** Adapted with permission [147].

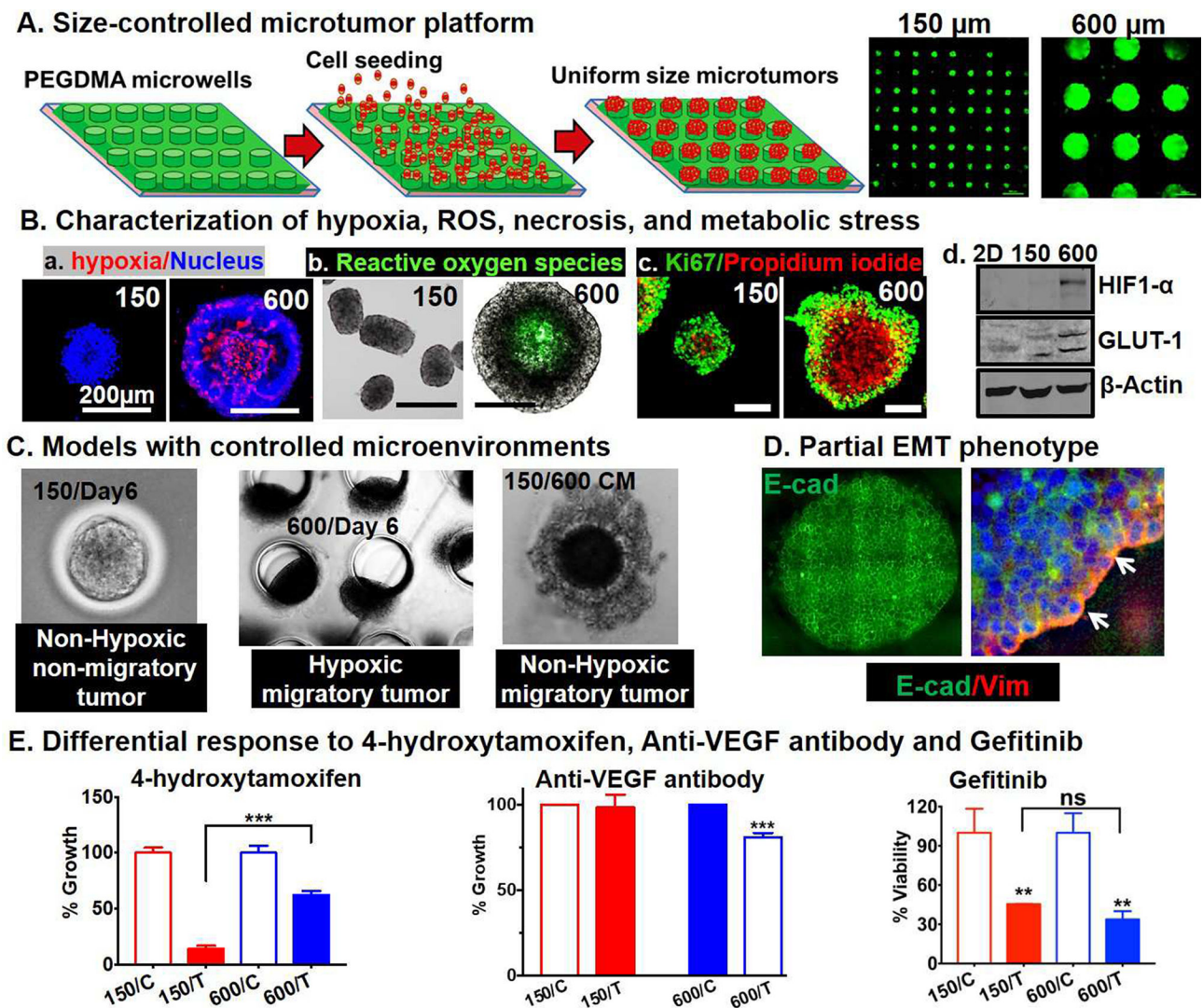


Figure 3: Size controlled 3D uniform micro-tumors recapitulate tumor microenvironment.

(A) Fabrication of 150 μ m and 600 μ m micro-well devices on polyethylene glycol dimethacrylate 1000 (PEGDMA) hydrogel microarrays to develop 3D micro-tumors. (B) (a-c) Large microtumors, 600 μ m show high hypoxia, reactive oxygen species (ROS) in the core of tumor spheroids and high Ki67 staining in the periphery. (d) 600 μ m microtumors express high hypoxia inducible factor 1 (HIF-1) and glucose transporter 1 (GLUT-1) (C) Microscopic images showing non-migratory and non-hypoxic - 150 μ m micro-tumors, hypoxic and migratory - 600 μ m micro-tumors and non-hypoxic, migratory 150 μ m micro-tumors incubated with 600 μ m media. (D) Migratory front in 600 μ m micro-tumors show tumor heterogeneity and both E-cadherin (E-cad) and Vimentin (VIM) expression representative of the partial- epithelial to mesenchymal transition (EMT) phenotype. (E) Differential response of 150 μ m and 600 μ m micro-tumors treated with 4-hydroxytamoxifen (4-OHT), anti-vascular endothelial growth factor (VEGF) and gefitinib.

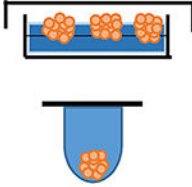
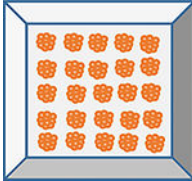
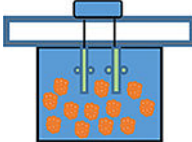
Table I:

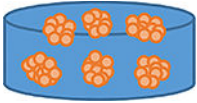
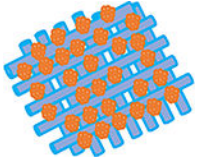
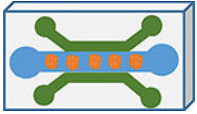
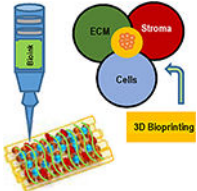
Two-dimensional (2D) vs. three-dimensional (3D) cell culture models.

Culture Techniques	Two-dimensional (2D)	Three dimensional (3D)
<i>In vivo</i> like	Do not mimic <i>in vivo</i> tissue and interactions	Mimics <i>in vivo</i> tissues and interactions
Culture formation	Few minutes to hours	Few hours to few days
Culture Quality	<ul style="list-style-type: none"> • High performance • High reproducibility • Easy to interpret • Long-term cultures 	<ul style="list-style-type: none"> • Low performance • Low reproducibility • Difficult to interpret • Short-term cultures • Difficult to maintain
Architecture	Cells interact partially	<ul style="list-style-type: none"> • Physiological interactions • Cell-Cell, Cell-extracellular matrix, Cell-Growth factors interactions
Assess to essential compounds	Equally exposure to nutrients, oxygen, metabolites and signaling molecules in growth media	Variable access- Nutrients, Oxygen, Metabolites and signaling molecules access is modulated on surface and core
Cell Proliferation	High proliferation rate in monolayer	Replicate <i>in vivo</i> cell proliferation
Cell Polarity	Partial polarization	Accurately replicate cell polarization
Cell cycle stage	Cells in same stage due to equal exposure to media	Heterogeneous cells in different cell cycle stages in spheroids (proliferating, hypoxic, quiescent, necrotic)
Cell Morphology	Flat, sheet-like monolayer cells	Aggregate/spheroid structures
Stiffness	High stiffness ($\sim 3 \times 10^9$ Pa)	Low stiffness (> 4000 Pa)
Cell interactions	<ul style="list-style-type: none"> • Limited cell-cell interaction • Limited cell-extracellular matrix interaction • No 'Niches' 	<ul style="list-style-type: none"> • Replicate <i>in vivo</i> cell-cell interaction • Replicate <i>in vivo</i> cell-extracellular matrix interaction • Environmental 'Niches' present
Drug response	Lack of correlation between human tumors undergoing drug screening in comparison to 2D cells	3D tumor spheroids show similar drug resistance pattern as in patients
Maintenance Cost	<ul style="list-style-type: none"> • Cost-effective • Commercially available media 	<ul style="list-style-type: none"> • Expensive • Time-consuming

Table II:

Advantages and challenges of three-dimensional (3D) tumor models.

Techniques	Graphical Representation	Advantages	Challenges	Research Stage	Ref
Liquid Overlay Culture and Hanging Drop		<ul style="list-style-type: none"> • Simple to use • Uniform spheroid formation • Co-culture feasibility • Control cell size • Transparent • Inexpensive • High reproducibility • Ease of harvesting • Long term culture 	<ul style="list-style-type: none"> • Limited flexibility • Lack reproducibility • Lack cell-matrix interaction • Heterogeneous cell lineage spheroids • Low throughput • Spheroid handling and transfer critical 	<ul style="list-style-type: none"> • Basic Research • Drug Discovery • Personalized Medicine 	[210, 220, 221]
Hydrogel		<ul style="list-style-type: none"> • Inexpensive • Uniform sized microtumors • Co-culture feasibility • High reproducibility • Customizable microtumor size • Cell-ECM interactions 	<ul style="list-style-type: none"> • Gel Composition • Gel-to-gel variations and structural changes • Non-Transparent • Gelling Mechanism • Handling microtumors • Isolation of specific microtumors • Low throughput 	<ul style="list-style-type: none"> • Basic Research • Drug Discovery 	[222–226]
Bioreactors		<ul style="list-style-type: none"> • Simple to culture • High-throughput production • Easily accessible spheroids • Motion of culture assist nutrient transport 	<ul style="list-style-type: none"> • Specialized equipment required • Cell Number/size cannot be controlled • Cells exposed to shear force 	<ul style="list-style-type: none"> • Basic Research • Tissue Engineering • Cell Expansion 	[161–163, 227–233]

Techniques	Graphical Representation	Advantages	Challenges	Research Stage	Ref
Scaffolds		<ul style="list-style-type: none"> • Medium cost • Co-culture feasibility • Customizable 	<ul style="list-style-type: none"> • Scaffold-to scaffold variation • Not transparent • Difficulty in cell removal 	<ul style="list-style-type: none"> • Basic Research • Drug Screening • Cell Expansion • Drug Discovery 	[157, 234–237]
Microtitre plate		<ul style="list-style-type: none"> • Controlled spheroid size • Cell growth and assays in same well • HTS/HCS compatible 	<ul style="list-style-type: none"> • Limited work on ECMs 	<ul style="list-style-type: none"> • Basic Research • Tissue Engineering • Anticancer Drug Screening 	[132]
Microfluidics		<ul style="list-style-type: none"> • Chemical gradients • Cell localization studies • Transparent • Co-culture feasibility 	<ul style="list-style-type: none"> • Fluid control • Environment control • Require expertise • High microfabrication cost 	<ul style="list-style-type: none"> • Basic Research • Tissue Engineering 	[238–240]
3D Bioprinting		<ul style="list-style-type: none"> • Chemical and physical gradients • Co-culture feasibility • Customized architecture • High-throughput production 	<ul style="list-style-type: none"> • Expensive 3D bioprinting machine required • Challenges of cell/material used 	<ul style="list-style-type: none"> • Cancer Pathology • Anticancer Drug Screening • Cancer Treatment • Tissue Engineering 	[165, 168, 169, 241]

Extracellular matrix (ECM), High-throughput setting (HTS), High content screening (HCS).

Table III:*In vitro* studies of drug resistance in 3D-breast cancer tumor models

Cell line model	Drugs Tested	Conclusion	Ref
MCF-7	Doxorubicin (3 μ M, 18 h) Verapamil (Pgp inhibitor, 100 μ M) YC-1 (5 μ M, 24h)	MCF-7 3D-spheroids resistant to doxorubicin associated with elevated Pgp expression via activation of HIF-1 α	[205]
MDA-MB-231	Paclitaxel (1–10nM) Doxorubicin (0–10nM)	Hypoxic spheroids resistant to paclitaxel and doxorubicin show progression	[207]
BT-549			
BT-474			
T-47D			
MCF-7	Paclitaxel Doxorubicin	Dense spheroid formation simulates hypoxia, anti-apoptotic features and drug resistance	[138]
DA-MB-231			
HCC-1954			
MCF10A-ERBB2 MTEC-Neu SK-BR3	Lapatinib (1 μ M) Trametinib (MEK inhibitor)	HIF-1 blocks lapatinib mediated effects in ERBB2 cells and maintain downstream ERK signaling via inhibition of DUSP2. Trametinib treatment reverses hypoxia- mediated lapatinib resistance	[206]
MDA-MB-157 3D	Doxorubicin (100*10 ⁻⁹ M) 5-Fluorouracil TH-302 (Hypoxia-activated prodrug)	Combination treatment (TH-302 and doxorubicin) targets drug resistant spheroids	[211]
MDA-MB-231 RAW 264.7	Paclitaxel (10ng/mL) Tyrphostin AG 1478(EGFR inhibitor)	Macrophages in heterotypic spheroids increase resistance to paclitaxel	[186]
BT-474		HER2+ BT-474 spheroids resistant to trastuzumab with increased breast cancer cells	[210]
MCF-7	Doxorubicin	Hypoxic cancer stem cells illicit high drug resistance to doxorubicin compared to normoxic cancer stem cells	[182]
MCF-7 MDA-MB-231	Paclitaxel Gemcitabine Adriamycin	Survival rates of breast cancer stem cells high in MCF-7 spheroid cells with drug treatment	[209]
MDA-MB-231	Doxycycline (5 μ g/ μ L) Doxorubicin (1 μ M)	AMPK mediates hypoxia induced drug resistance in MDA-MB-231 cells via Notch1 signaling	[208]
MCF-7	Tamoxifen Fulvestrant	Hypoxia induces switch in SNAT2/SLC38A2 regulation which leads to resistance to anti-hormone therapy	[212]

P-glycoprotein (Pgp), Hypoxia inducible factor 1 alpha (HIF-1 α), Erb-B2 receptor tyrosine kinase 2 (ERBB2), Extracellular signal regulated kinase (ERK), Dual specificity phosphatase 2 (DUSP2), Mitogen activated protein kinase (MEK), Epidermal growth factor receptor (EGFR), Human epidermal growth factor receptor 2 (HER2), 5'-Adenosine monophosphate activated protein kinase (AMPK), Sodium-coupled neutral amino acid transporter 2 (SNAT2), Solute carrier family 38 member 2 (SLC38A2).