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WHITE PAPER

A TECHNICAL PERSPECTIVE: UNDERSTANDING THE CELLULAR RESPONSE TO HYPOXIA THROUGH *IN VITRO* MODEL SYSTEMS

ABSTRACT

Hypoxia plays a role in carcinogenesis directing a number of molecular pathways that generally promote tumor growth, invasiveness and metastasis. *In vitro* models of hypoxia present various challenges to approximate *in vivo* conditions including adequate regulation of oxygen levels and the duration of hypoxia. To better simulate the natural tumor microenvironment, investigators are increasingly relying on 3D culture models. Monolayer culture conditions tend to induce undesirable molecular and phenotypic cellular changes, whereas sphere models mimic the oxygen, nutrient and growth factor gradients in tumors *in vivo*. Moreover, 3D cultures may contain tumor as well as supporting non-tumor cells or stroma, allowing the study of cellular interactions as may occur *in vivo*. This review presents an overview of various sphere models, highlighting advantages and caveats for the use of current 3D culture systems to evaluate cellular responses to hypoxia in cancer.

INTRODUCTION

Physiological oxygen (physoxia) conditions for human tissues and organs are variable, often ranging between ~5% (e.g., brain) to ~10% (e.g., renal cortex).[1,2] For some tissues, physoxia may normally fall outside of this range, either below at ~1% (e.g., bone marrow and thymus) or above at ~14% (e.g., arterial blood) (**FIGURE 1**).[3-6] However, low oxygenation or hypoxia is often linked to disease and is commonly present in tumors, in which cells may be exposed to a range of low oxygen levels including hypoxia (~0.1-1%) and even anoxia (~0.02%). Hypoxia represents a significant driving force for tumorigenesis, generally promoting aggressive, invasive and metastatic phenotypes by the activation of signaling mechanisms predominantly regulated by hypoxia-inducible factors, HIF-1α and HIF-2α. Cellular adaptations and recent findings on the response to hypoxia in cancer have been recently reviewed: The Cellular Response to Hypoxia.

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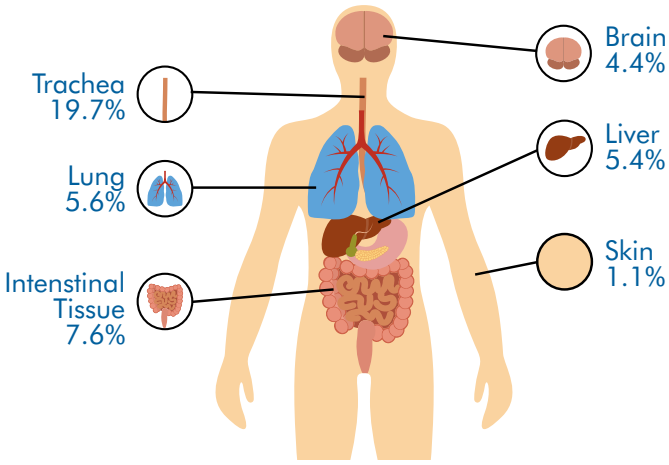

FIGURE 1. Normal median percent oxygen in organs and tissues. Tumors are characterized by the presence of decreased oxygen levels in relation to normal organs and tissues. For example, median percent oxygen associated with tumors in the brain (1.7%), lung (2.2%) and liver (0.8%) may be between 2- to 6-fold below physoxia.[7]

Hypoxia in tumor microenvironments arises from the combined effects of increased cellular proliferation and metabolism, as well as deficiencies in vascular supply and limitations in tissue-oxygen diffusion. Recent findings support that hypoxia-duration (acute vs chronic) critically influences molecular signaling pathways regulating cellular adaptations in tumors.[8-10] Moreover, hypoxia/re-oxygenation cycles are associated with a greater incidence of DNA damage and instability, thought to be induced by reactive oxygen species (ROS).[8-10] Together, these factors result in dynamic changes in oxygen levels in tumor cells which are often difficult to replicate in the laboratory setting.

This review presents an overview of *in vitro* models and conditions used for the study of cellular adaptations to hypoxia in cancer. The advantages and caveats associated with different models are discussed as well as new advances in the application of 3D cultures that have furthered our understanding of hypoxia in tumorigenesis.

MODELING HYPOXIA *IN VITRO*

EXPERIMENTAL NORMOXIA AND HYPOXIA

Multiple studies support that *in vitro* culture conditions influence hypoxia induced signaling pathways and cellular adaptations.[11, 12, 13] The majority of studies evaluating the effects of hypoxia on cell signaling are based on 2D cell culture approaches. To study cellular adaptations to hypoxia, monolayer cultures are exposed to low oxygen conditions (e.g., 0.02-5%) by incubation in gas controlled chambers or incubators in which a specific mixture of gases is applied (e.g., 95% N_2, 5% CO_2; anoxia).[8,14] Though traditionally considered a model system, monolayer cell cultures present several limitations directly associated with the types of cell-cell and cell-matrix interactions that occur in 2D systems. These interactions promote the development of abnormal morphology and atypical distribution of membrane proteins.[11, 15, 16] Moreover, several factors including nutrients, signaling molecules, and oxygen are distributed differently in monolayer cultures when compared to tumors *in vivo*.[17, 18]

One important challenge for studies focusing on the effects of low oxygenation in tumors is the replication of *in vivo* hypoxic conditions. To induce moderate hypoxia, monolayer cultures of cancer cell lines are frequently exposed to oxygen levels ranging between 0.3 to 4.5%.[7] Exposure to extreme hypoxia or anoxia (<0.1% O_2) has been associated with apoptosis, but is also used in studies to model hypoxic responses.[8]

Another potential caveat of *in vitro* cell culture systems is the oxygen conditions set for controls. In the laboratory setting, the standard oxygen condition (normoxia; ~20% O_2) used for cellular cultures is well above the physiological range, which averages ~6% O_2.[7] Frequently studies use normoxia as a control for comparing and elucidating molecular adaptations to hypoxia, albeit the potential discrepancy that may exist in signaling induced by normoxia vs physoxia.

Finally, acute and chronic hypoxia co-exist in the tumor environment. Significantly, current evidence supports that the duration of hypoxia influences the activation of signaling pathways and tumorigenesis outcome. Acute hypoxia is driven by brief periods of blood flow occlusion, whereas chronic hypoxia is associated with prolonged limitations in oxygen diffusion.[8] Generally, *in vitro* models of acute hypoxia involve incubating cells at <2% O_2 for minutes to hours, although in some studies cells have been exposed to low oxygenation for 72 hours.[8] In contrast for chronic hypoxia, cells are often incubated for several hours to days, and even up to weeks in some studies.[8]

Tumor cells *in vivo* are exposed to variable oxygenation, from normal to anoxic levels. Therefore, to more accurately replicate *in vivo* tumor conditions, a series of 3D sphere culture models have been introduced since the 1970s. Currently, it is well accepted that replication of oxygen gradients mimicking the *in vivo* tumor condition is commensurate with the use of 3D spheroid models.

MODULATING HYPOXIC SIGNALING *IN VITRO*

In vitro hypoxia studies, typically require incubating cells under carefully controlled oxygenation conditions. However studying hypoxia signaling can forgo this gas exchange requirement by using hypoxia-mimetic agents (e.g., $CoCl_2$) or small molecules which directly target the HIF-α dependent pathway.

Two mechanisms have been proposed from *in vitro* studies to explain how $CoCl_2$ may induce a hypoxic-like state. First, cobalt inhibits the hydroxylation of HIF-α by binding to the iron-binding domain of HIF hydroxylase.[19] In the absence of Proline 564 hydroxylation, HIF-1α is stabilized and not targeted for ubiquitination and degradation.[19] The second proposed mechanism suggests that cobalt stabilizes HIF-α by inhibiting its interaction with the von Hippel-Lindau protein (pVHL).[19] Cobalt binds to the oxygen-dependent degradation domain (ODDD) of hydroxylated HIF-α, which prevents its interaction with pVHL and proteasomal degradation.[19]

Other biochemical based approches rely on the use of small molecules to target different steps in HIF signaling (**TABLE 1**).[14]

TABLE. 1 Modulators of HIF Signaling

	MODULATORS	MECHANISM	POTENCY*
INHIBITORS	TAT-cyclo-CLLFVY (5582)	Selective inhibitor of HIF-1α and HIF-1β dimerization	1.3 μM (IC_{50})
	GN 44028 (5655)	Potent inhibitor of HIF-1α transcriptional activity	14 nM (IC_{50})
	TC-S 7009 (5243)	High affinity and selective HIF-2α inhibitor	81 nM (K_d)
	Echinomycin (5520)	Highly potent and selective HIF-1α inhibitor	29.4 pM (IC_{50})
INDUCERS	DMOG (4408)	Inhibitor of HIF-α prolylhydroxylase; increases HIF-1α levels	
	VH 298 (6156)	Inhibitor of E3 ubiquitin ligase VHL Inhibits interaction between VHL and HIF-α	80-90 nM (K_d)
	ML 228 (4565)	HIF pathway activator Iron chelator	1.23-1.4 μM (EC_{50})

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*Potency of the various inhibitors and inducers may be system and cell type dependent.

One disadvantage of using these chemical modulators is that they only target HIF-dependent signaling. Nevertheless, while their use does not provide evidence about the contribution of other significant pathways involved in hypoxia, which may also influence cellular adaptations, they are instrumental for the understanding of the role of HIFs as master regulators of hypoxia responses.[14]

CANCER CELL CULTURE MODELS AND CURRENT APPLICATIONS

3D cultures enable the study of hypoxia in a context that may more closely replicate the *in vivo* tumor environment. For instance, avascular spheroids are representative of tumor regions distant from blood vessels, and contain microenvironments with diverse oxygenation conditions including oxic, hypoxic (<5%) and anoxic (<0.1%).[11] Similar to hypoxic tumor regions, HIFs are stabilized in spheroids according to graded oxygen levels, reaching maximal expression in cells peripheral to the core.[11] Several types of 3D spherical cultures have been developed as detailed in the next section.

I. MULTICELLULAR TUMOR SPHEROIDS (MCTS)

Multicellular tumor spheroids (MCTS) are formed from single cells, usually cancer cell lines, grown in standard culture media and under conditions that prevent attachment.[11, 15] To maintain non-adherence, cell cultures may be rotated (e.g., with a rotating shaker) and grown using cell culture-ware coated with inert materials such as agar or agarose.[15] Other techniques for producing MCTS include the hanging drop and microparticle based methods.[15] These methods provide specific advantages, for example, in the hanging drop technique, spheroids form in complete absence of substrate interaction, whereas microparticle based approaches allow better control over spheroids' size.[15] MCTS may also be formed by co-culturing tumor cells with supporting cells or stroma such as endothelial and immune cells.

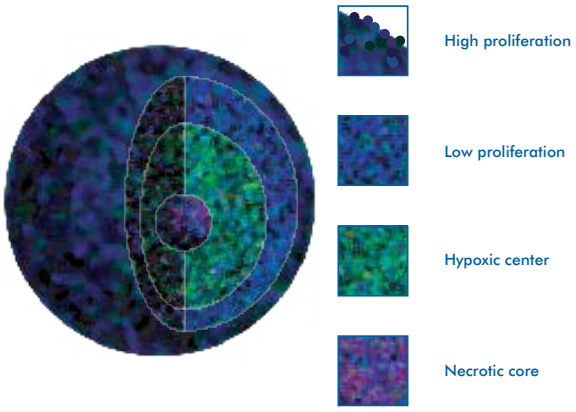

FIGURE 2. Growth patterns in MCTS. Cell proliferation in MCTS depends on nutrients, growth factors, and oxygenation gradients. Various proliferative regions are identifiable in spheroids and correlate with oxygenation levels. Cell proliferation is highest at the outermost layer of the spheroid and decreases towards the hypoxic center. Larger spheroids may contain a necrotic core, in particular for MCTS above 400 µm in diameter.[11,21]

A challenge for the development of MCTS is controlling spheroid size, which influences metabolic pathways and gene expression of constituent cells.[20] MCTS are formed by cellular aggregation and depend on the adhesive properties of constituent cells (**FIGURE 2**).[20] While MCTS may be formed from heterogeneous cell populations, this system does not completely replicate the diversity of cellular interactions occurring *in vivo*. Moreover, as a matrix-free system, MCTS are unable to replicate the cellular-extracellular matrix (ECM) interactions and associated activity of key signaling pathways.[13]

-CORRELATING HYPOXIA AND DNA DAMAGE REPAIR IN MCTS

Recently a MCTS model, derived from Ewing Sarcoma and Lung Carcinoma cells, allowed investigators to correlate hypoxia with an increased incidence of DNA damage and activation of DNA repair pathways.[21] A defined region of hypoxia was identified in MCTS larger than 500 µm in diameter.[11, 21] The green hypoxic region, lying peripheral to a necrotic core, was characterized by the expression of HIF1-α and the presence of proliferating cells. Investigators correlated hypoxia with an increased incidence of DNA breaks and the activation of the DNA damage repair kinase ATM (Ataxia Telangiectasia) (**FIGURE 3**).[21,22] Additionally, activation of ATR (ATM-and-Rad3 related) kinase in this model was shown to play a role in the induction of cellular proliferation within the hypoxic peri-necrotic region. Overall, these findings demonstrated that within MCTS a population of cells undergo adaptations driven by HIF regulated programs, as occurs in tumors *in vivo*. The activation of DNA repair/proliferation pathways provides a plausible mechanism contributing to the survival of cells within hypoxic microenvironments.[21]

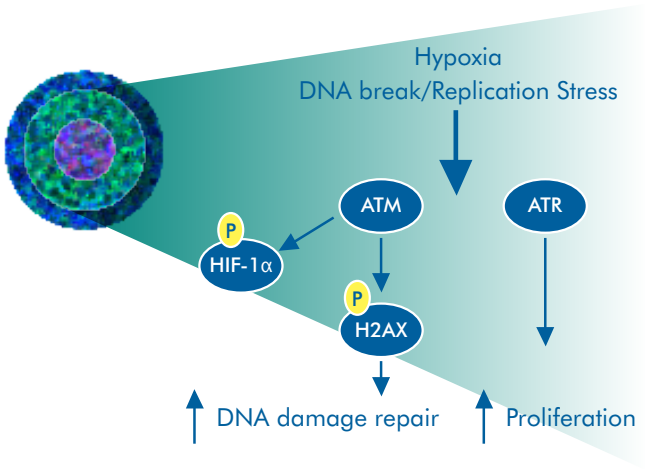

FIGURE 3. Activation of DNA damage repair in a MCTS model. In the hypoxic microenvironment, both ATM and ATR kinases are activated downstream of DNA damage and replication stress. Activated ATM leads to the phosphorylation of H2AX, whereas ATR kinase activity promotes cell proliferation. ATM is a known activator of HIF-1α.

Comparing responses to hypoxia between 3D and 2D cultures demonstrated specific differences in the contribution of ATM/ATR kinases to the phosphorylation of H2AX (Ser-139 phosphorylated or γ-H2AX), a marker for the activation of DNA repair pathways.[21] In the hypoxic region of 3D spheroids, phosphorylation of H2AX was predominantly dependent on ATM, as demonstrated by using specific inhibitors (ATM-KU55933 and ATR-VE-821). In contrast, inhibition of ATM modestly decreased γ-H2AX in 2D cultures, whereas inhibition of ATR induced γ-H2AX.[21] These findings suggest that culture conditions may significantly influence cellular responses to hypoxia leading to differential activation of pathways controlling DNA repair and proliferation.

II. TUMORSPHERES (TS)

Tumorspheres (TS) are formed from an expanded population of cancer stem cells (CSCs) grown from single cell suspensions and under conditions that reduce adherence. Single cell suspensions used for TS may be derived from a variety of solid tumors including colon, breast and lung tissues.[15] For maintenance of stemness, the culture media may be supplemented with growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF), hydrocortisone, insulin, progesterone and heparin, although the specific supplementing factors may vary depending on cell- and tissue-type.[15] Markers expressed by CSCs in TS may be influenced by the culture process in a cell line dependent manner.[23]

The hypoxic tumor microenvironment contains CSCs which are thought to play a role in tumor initiation and metastasis.[24] Different from other spheroid models, TS cultures are not meant to replicate the heterogeneous tumor environment, but instead are primarily used to study the properties of CSCs.[15]

-EVALUATING THE ROLE OF HIF-1 IN TUMOR INITIATING CELLS (TICS) IN BREAST CANCER

The ability of mammary tumor epithelial cells (MTECs), with normal or reduced HIF-1α expression, to form tumorspheres was evaluated in a recent study. Schwab's group employed both *in vitro- and in vivo-approaches* to demonstrate that HIF-1α plays a direct role in regulating tumor initiating cells (TICs) in breast cancer.[24] Briefly, MTECs derived from an *in vivo* mouse model, mouse mammary tumor virus polyoma virus middle T (MMTV-PyMT), were transduced *ex vivo* to delete HIF-1α and used in tumorsphere assays to test their potential for *in vivo* tumorigenesis. The tumorsphere assay provides an estimate of cancer stem cell number present in tumor tissue. Sphere formation efficiency was significantly decreased by deletion or reduced expression of HIF-1α.[24] In agreement with the *in vitro* findings, *in vivo* limiting dilution transplantation of MTECs into the mammary pads of mice, demonstrated that HIF-1α expression is required for tumor growth. Also, gene expression analysis of HIF-1α null tumor spheres demonstrated substantial downregulation of specific Notch related genes (e.g., *Notch4, Dll1, Hey1* and *Hey2*). This suggests a role for the Notch pathway in promoting TICs in breast cancer.[24]

Additionally, investigators probed the role of cell culture conditions on HIF-1α stabilization. MTECs grown under chronic hypoxia in 2D vs 3D cultures showed differential regulation of HIF-1α stabilization.[24] For example, MTECs grown in 2D cultures with EGF showed high levels of stabilized HIF-1α, in contrast HIF-1α stabilization in 3D cultures was minimal.[24] These findings suggest that *in vitro* culture conditions may critically influence cellular responses to hypoxia and the regulation of TICs.

III. TUMOR-DERIVED TUMOR SPHERES (TDTS)

Tumor-derived tumor spheres (TDTS) are produced from tumor tissues by partial dissociation, removal of non-tumor cells and grown in culture media, which may be supplemented with growth factors, under non-adherent culture conditions.[11, 15] Adhesion properties typical of some colon cancer cells support sphere formation. Generally this property correlates with malignancy and is absent from normal colon epithelial cells, which fail to adhere and form colospheres.[15] This model is thought to more accurately replicate the tumor environment when compared to MCTS and TS.[11]

-UNDERSTANDING HYPOXIA INDUCED QUIESCENCE IN TDTS

Hypoxia drives survival of cancer cells by triggering various cellular adaptations which often result in treatment resistance. Recently, the pancreatic cancer cell line, AsPC-1, was found to have robust survival properties under chronic hypoxic conditions.[25] AsPC-1 cells survive hypoxia (1% O_2) for several weeks by entering a quiescent or dormant state, a process dependent on AKT inhibition and HIF-1α stabilization.[25] Strikingly, when oxygenation conditions improve, these cells rapidly return to an active state.[25]

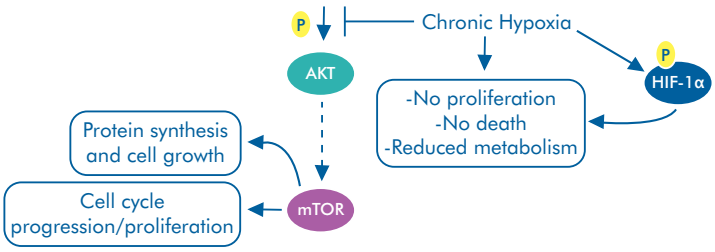

FIGURE 4. Quiescent state in primary colorectal cancer 3D culture (CTOS). Dormancy under conditions of chronic hypoxia is independently regulated by reduced AKT activity and stabilized HIF-1α.

Most cancer cell lines are unable to survive chronic hypoxic conditions for more than several days. Therefore, a model system based on the use of primary tumor tissue may be more amenable to these conditions and may facilitate the study of cancer cell adaptations to chronic hypoxia. The colorectal cancer tissue originated spheroid culture system (CTOS), has allowed investigators to probe cellular responses to chronic hypoxia (1% O_2 for 14 days) and nutrient deprivation.[25,26] Under these conditions, investigators found that activation of AKT was also reduced in colon cancer cells and lead to a quiescent state (**FIGURE 4**).[25] Importantly, dormancy of colorectal cancer cells in this model was reversible as previously demonstrated for the AsPC-1 cell line.[25] To determine if dormancy is a cellular adaptation associated with therapy resistance, the chemo sensitivity of cells in CTOS was evaluated under conditions of chronic hypoxia and nutrient deprivation.[25] Dormant cells were able to tolerate treatment with 5FU or SN38 and showed re-establishment of growth once normal culture conditions were restored. Therefore, by using this 3D *in vitro* culture system, investigators demonstrated that dormancy of colon cancer cells is a cellular adaptation to hypoxia involved in treatment resistance.[25]

IV. ORGANOTYPIC MULTICELLULAR SPHEROIDS (OMS)

Organotypic multicellular spheroids (OMS) are derived from tumors by simple dissection and without further processing. Similar to other sphere cultures, OMS are grown under non-adherent culture conditions in standard culture media. Because OMS retain the complex cellular constituents of the parent tumor they may serve as perfect models for personalized therapy, providing a more accurate assessment of a tumor's response to treatment.[11]

-CORRELATING CANCER STEM CELL DISTRIBUTION AND HYPOXIA IN OMS

Organoids may be derived from different tumor-types, -sites and even from metastases of primary tumors. One of the main advantages of organotypic cultures is the preservation of cellular interactions including the cross-talk between stem and non-stem cells and between cells exposed to different oxygenation levels.[27] A recent study used the OMS culture system to explore the distribution of CSCs in relation to oxygenation in glioblastomas.[27]

Organoids derived from patient's glioblastomas conserved CSCs with invasive potential.[27] CSCs within these spheroids were identified by the expression of several pluripotency markers including SOX2, OLIG2 and TLX.[27] CSCs differentially expressed these markers, demonstrating heterogeneity in stem cell populations within glioblastomas.[27] Interestingly, SOX2/OLIG2 positive CSCs were more abundantly distributed towards the peripheral and better oxygenated organoid regions. This was evidenced by the inverse distribution of SOX2/OLIG2 CSCs in relation to carbonic anhydrase positive cells (CA-IX) (**FIGURE 5**).[27] In contrast, expression of these markers within the hypoxic core was more heterogeneous, suggesting that hypoxia may drive a specific distribution of CSCs.[27] Cellular heterogeneity represents one key challenge for the effective treatment of glioblastomas. Therefore, models that replicate the *in vivo* tumor environment may facilitate the identification of molecular factors underscoring glioblastoma heterogeneity.[27]

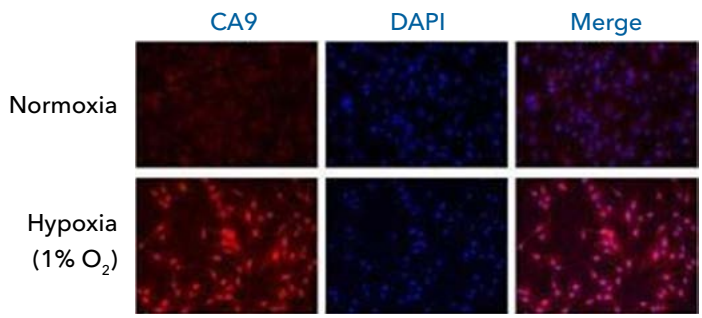

FIGURE 5. Carbonic anhydrase IX is expressed in solid tumors and serves as a marker of hypoxia.[28] Immunocytochemistry/Immunofluorescence: Carbonic Anhydrase IX/CA9 Antibody [NB100-417] - Analysis using the DyLight 488 conjugate of NB100-417. Staining of Carbonic Anhydrase IX (red) in human glioma U87 cells. DAPI counterstains nuclei (blue).

3D CANCER CELL CULTURE MODELS: HOW DO THEY COMPARE?

Generally, the value of sphere cultures as model systems lies in the ability to replicate a variety of tumor properties including cellular composition and specific chemical gradients present *in vivo*. However, different 3D models have unique properties that make them more or less suitable for certain applications (TABLE 2).

TABLE 2. Comparison of 3D Tumor Cell Culture Models[15]

3D TUMOR SPHERICAL CULTURES	ADVANTAGES	DISADVANTAGES
MCTS Derived from single cancer cell lines or a combination of tumor and stroma cells	• Spheres may be formed within ~1 week • Several methods to generate spheres have been developed • Amenable to high-throughput applications	• Variable spheroid size may impact assay reproducibility[17] • Optimal cell concentration for sphere needs to be experimentally determined[20] • Heterogeneous spheres do not fully replicate tumor conditions[20]
TS Derived from clonally expanded cancer stem cells (CSCs)	• May be derived from cell lines or from tumor dissociated CSCs within ~1 week • CSC markers facilitate enrichment of specific CSC populations (e.g., flow cytometry) • Allows evaluation of self-renewal properties	• A combination of several cellular markers is needed for purification of CSCs[15] • Fusion and aggregation may lead to spheres being formed from more than one CSC clone[15] • Spheres may contain differentiated tumor cells[15]
TDTS Derived from partially dissociated tumor tissue	• Spheres formed quickly (e.g., 1 day for colospheres) • May be maintained for prolonged periods (e.g., 2 weeks for colospheres) • Cryopreservation of spheres is possible • Better model, compared to MCTS, for replication of tumor conditions and response to therapy	• Not amenable to transfection[15] • Not ideal for high-throughput approaches due to heterogeneity between spheres or spheroids[15, 29]
OMS Derived from tumors without dissociation	• Spheres may form quickly within 1 week (varies depending on tissue) • Best model for replication of the in vivo tumor environment • Best model for the evaluation of individualized responses to therapy	• Not amenable to transfection[15] • Not ideal for high-throughput approaches due to heterogeneity between spheres or spheroids[15, 29]

SUMMARY

Hypoxia in tumors is a factor that drives therapy resistance. Understanding the signaling mechanisms activated by low oxygenation and their outcome for tumorigenesis has required the development of *in vitro* systems that more accurately replicate the tumor environment. Several 3D sphere cultures models have been developed to date, providing investigators biologically meaningful systems in which to probe the effects of hypoxia in tumors.

Studies with spheroid models, such as MCTS, have demonstrated that various oxygen microenvironments develop in 3D cultures, from oxic to anoxic, similar to *in vivo* tumors. Moreover, within hypoxic microenvironments in MCTS, cellular proliferation and DNA damage co-exist, providing the opportunity to uncover mechanisms for DNA repair that may be recalled under stress conditions. Understanding the specific signaling pathways activated that enable tumor cell survival under hypoxia, is key for target identification and for the development of effective therapies. Among the different sphere models available, the MCTS may be most amenable to high-throughput drug screening.

Cancer stem cells play a critical role in the propagation of tumors to distant sites. The TS model, consisting of tumor derived stem cells, provides a platform to investigate the inherent properties that allow these cells to bypass treatment and metastasize. Finally, both the TDTS and OMS models allow investigators to study responses to hypoxia in a setting that more accurately matches the cellular environment of an individual's tumor. By virtue of their preserved heterogeneity, these models may provide tools for a more effective and in some cases a more tailored approach to cancer treatment

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