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Multicellular Tumor Spheroids as a model to study tumor cell adaptations within a hypoxic environment

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Multicellular Tumor Spheroids as a model to study tumor cell adaptations within a hypoxic environment

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ABSTRACT

Hypoxia is a common feature in solid tumors which facilitates the development of therapeutically resistant and aggressive tumor phenotypes. As oxygen levels decrease, cellular metabolism and energy production becomes impaired and the potential for catastrophic DNA damage increases. To prevent cell death, tumor cells undergo several adaptations which prevent depletion of nutrients, minimize DNA damage, and re-acquire blood flow. The mechanisms behind tumor cell survival during hypoxia are poorly understood.

Tumor neovascularization is a critical step in facilitating continued tumor growth. Accordingly anti-angiogenic therapy has shown significant success in preventing progression free survival however increased tumor aggression and alternative neovascularization methods result in decreased overall patient survival. Vasculogenic mimicry is one such proposed method of alternative neovascularization wherein tumor cells transdifferentiate and form de novo vascular structures. Although there are indications that this process is driven by hypoxia and is linked to aggressive tumor phenotypes, there is little known about the signaling pathways inducing such an adaptation.

Evidence from in vitro studies suggest that activation of DNA damage repair proteins in proliferating hypoxic cells is critical for sustained proliferation and cell viability thus implicating repair proteins as potential therapeutic targets. In vivo studies demonstrating a correlation between hypoxia and the formation of γ-H2AX further support this potential. The evaluation of such therapeutic targets is limited by the inability of in vitro monolayer culture models to recreate therapeutically relevant aspects of a 3D in vivo tumor.
Multicellular tumor spheroids (MCTS) represent a 3D *in vitro* culture model in which tumor cell adaptations to the hypoxic tumor microenvironment are recreated. These include the accumulation of metabolic waste, appearance of nutrient gradients, and development of proliferative heterogeneity. MCTS have been used extensively to characterize radiation resistance in a hypoxic environment. We set out to describe the relationship between proliferation, hypoxia, DNA damage repair, and vasculogenic mimicry using the MCTS model. Using MCTS, we demonstrate for the first time the activation of DNA damage repair proteins in hypoxic proliferating cells residing within the MCTS core and further show the ability to study the efficacy of therapeutics targeting this cell population. Although metabolic adaptations and DNA damage repair protein activation are observed in this model, MCTS formed using the liquid overlay method do not experience vasculogenic mimicry. The results presented herein advances the field of tumor biology by validating the utility of MCTS as a model system in which clinically relevant features of a tumor can be recreated and studied. Furthermore our studies identify the Eyes Absent tyrosine phosphatase as a therapeutic target whose activity regulates DNA damage repair under both normoxic and hypoxic conditions and may be involved in vasculogenic mimicry.
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CHAPTER ONE – MODELING TUMOR CELL ADAPTATIONS TO HYPOXIA IN MULTICELLULAR TUMOR SPHEROIDS

Abstract

Under hypoxic conditions, tumor cells undergo a series of adaptations that promote evolution of a more aggressive tumor phenotype including the activation of DNA damage repair proteins, altered metabolism, and decreased proliferation. Together these changes mitigate the negative impact of oxygen deprivation and allow preservation of genomic integrity and proliferative capacity, thus contributing to tumor growth and metastasis. As a result the presence of a hypoxic microenvironment is considered a negative clinical feature of many solid tumors. Hypoxic niches in tumors also represent a therapeutically privileged environment in which chemo- and radiation therapy is less effective. Although the negative impact of tumor hypoxia has been well established, the precise effect of oxygen deprivation on tumor cell behavior, and the molecular signals that allow a tumor cell to survive in vivo are poorly understood. Multicellular tumor spheroids (MCTS) have been used as an in vitro model for the avascular tumor niche, capable of more accurately recreating tumor genomic profiles and predicting therapeutic response. However, relatively few studies have used MCTS to study the molecular mechanisms driving tumor cell adaptations within the hypoxic tumor environment. Here we will review what is known about cell proliferation, DNA damage repair, and metabolic pathways as modeled in MCTS in comparison to observations made in solid tumors. A more precise definition of the cell populations present within 3D tumor models in vitro could better inform our understanding of the heterogeneity within tumors as well as provide a more representative platform for the testing of therapeutic strategies.
Keywords: Hypoxia, Multicellular Tumor Spheroids, Metabolism, DNA Damage Repair,
Proliferation, Cancer

Abbreviations
2D Two dimensional cell culture, monolayer culture
3D Three dimensional
A673 Human Ewing Sarcoma cell line (CRL-1598; ATCC)
ATM Ataxia Telangiectasia
ATR ATM and Rad3-related
BRCA1 Breast Cancer 1
CD31 Platelet Endothelial Cell Adhesion Molecule
CD133 Prominin-1
Chk1/2 Checkpoint kinase 1 and 2
DDR DNA damage repair
ECM Extracellular matrix
GYS1 Glycogen synthase 1
HIF-α Hypoxia Inducible Factor α subunit
H2AX Histone variant 2A.X
γ-H2AX H2AX phosphorylated on serine-139
Kap1 KRAB-associated protein-1
MCTS Multicellular Tumor Spheroids
MLH1 MutL homolog 1
MMP Matrix metallopeptidase
OMS Organotypic Multicellular Sphere
PARP Poly (ADP-ribose) polymerase
PYGL Glycogen phosphorylase L (Liver)
RAD51 RAD51 recombinase
TDTS Tumor Derived Tumor Sphere
TIMP-1 TIMP metallopeptidase inhibitor 1
Background

The majority of solid tumors will develop hypoxia to some degree and tumor hypoxia is a significant prognostic factor that predicts poor patient outcome (Scanlon et al., 2015, Hammond et al., 2014). It is clear from decades of research that hypoxia induces metastasis and invasion, imparts chemo- and radiation resistance, and provides a selective pressure to abrogate pro-apoptotic signaling (Wilson et al., 2011). The clinically relevant nature of hypoxia has prompted investigations into how the tumor microenvironment directs tumor cell biology and function. Although the literature on this topic is extensive (Scanlon et al., 2015; Hammond et al., 2014; Wilson et al., 2011; Bristow et al., 2008; Hanahan et al., 2011; Wang et al., 2017; Hockel et al., 2001) many aspects of tumor cell biology and survival in the context of a 3-dimensional (3D) environment remain poorly understood. For decades the Multicellular Tumor Spheroid (MCTS) model has been used to study clinically relevant aspects of tumor biology, including hypoxia (Leek et al., 2016), protein expression patterns within tumors (Ghosh et al., 2005; Daster et al., 2017; Pacheco Marine et al., 2016) and responses to therapeutics (Ghosh et al., 2005; Daster et al., 2017; Hirschhaeuser et al., 2010; Mehta et al., 2012; Song et al., 2008; Lovitt et al., 2014; Yip et al., 2013; Dufau et al., 2012; Fedrigo et al., 2011; Chan et al., 2008; Kilic et al., 2007; Francia et al., 2004; Wartenberg et al., 2003; Olive et al., 1994). However, relatively few experiments have attempted to use MCTS to further our understanding of tumor cell adaptations within a hypoxic microenvironment. This review aims to describe ways in which MCTS can be used to better simulate solid tumors by detailing key features of MCTS that resemble the in vivo context.
The development of tumor hypoxia

While the term hypoxia is used to describe a wide variety of oxygen concentrations (Hammond et al., 2014; Hockel et al., 2001), it most often refers to the point at which oxygen concentrations have decreased beyond the threshold required for normal cell function. The majority of solid tumors will develop hypoxic regions due to a combination of rapid oxygen depletion, insufficient vascularization, and suboptimal tumor blood flow (Hammond et al., 2014; Hockel et al., 2001). For example, the consumption of oxygen by rapidly proliferating perivascular tumor cells can deplete the limited supply of available oxygen and prevent sufficient oxygenation of subsequent cell layers (Leek et al., 2016, Durand et al., 1977; Groebe et al., 1996; Wagner et al., 2011). While intracellular oxygen is utilized in a variety of reactions, the majority of oxygen consumption is devoted to ATP production through glucose metabolism (Wagner et al., 2011; Herst et al., 2007) where oxygen serves as a terminal electron receptor during oxidative phosphorylation. In addition to consumption through intracellular processes, the physical distance between tumor cells and blood vessels also influences the development of hypoxia. Oxygen diffusion through tissue is limited to approximately 200 μm based on evidence from experimental and mathematical models (Wilson et al., 2011; Grimes et al., 2014). Hypoxia can be further exacerbated by the destruction of angiogenic vessels following cytotoxic or anti-angiogenic therapy (Leek et al., 2016; Al-Hussein et al., 2012; Paez-Ribes et al., 2009; Pinto et al., 2016). Accumulating evidence now suggests that antiangiogenic therapy induces tumor hypoxia which provides a selective pressure for tumors to acquire a more aggressive phenotype leading to therapeutic resistance and tumor progression (Al-Hussein et al., 2012; Paez-Ribes et al., 2009; Pinto et al., 2016). Whether developed as a result of rapid tumor growth or in response to therapeutics, hypoxia is ultimately the result of an imbalance between oxygen availability, consumption, and the physical boundaries to oxygen diffusion inherent to a 3D tissue mass.
Spheroid models for studying hypoxia

The effect of hypoxia on cells has traditionally been studied in monolayer culture. 2D (monolayer) hypoxia experiments are most typically performed by placing tumor cells in a gas-controlled chamber (Hammond et al., 2014). While experimentally straightforward, this method is unable to recreate clinically relevant aspects of tumor biology that can impact on tumor cell behavior and therapeutic response (Mehta et al., 2012; Baker et al., 2012). For example, monolayer cells experience polarized cell adhesion and two dimensional contact with neighboring cells which results in abnormal cell spreading, alterations in the distribution of cell surface receptors, and selection for specific sub-populations of cells best adapted to in vitro growth (Baker et al., 2012). It is also well established that the genomic profiles and therapeutic responses of tumor cells grown in 2D differ from those seen in solid tumors (Ghosh et al., 2005; Daster et al., 2017; Hirschhaeuser et al., 2010; Mehta et al., 2012; Pacheco-Marin et al., 2016; Zhou et al., 2013). Studying hypoxia in vivo is challenging due to the high degree of variation in oxygen tensions within and amongst tumors, and limited ability to definitively identify regions of chronic versus acute hypoxia (Hammond et al., 2014; Dewhirst et al., 2016). For these reasons, there can be a disconnect between in vitro studies and the complex 3D environment of a tumor. MCTS may contribute to bridging this gap.

Several methods are used for generating spherical 3D cultures and are reviewed extensively elsewhere (Leek et al., 2016; Baker et al., 2012; Vinci et al., 2012; Weiswald et al., 2015; Achilli et al., 2014). Common techniques include the liquid overlay technique in which 3D culture is attained by incubating cell suspensions in plates coated with an inert substrate, and the “hanging drop” method wherein cell suspensions are cultured in suspended droplets such that gravity prevents cell attachment and favors cell-cell adhesion (Leek et al., 2016; Weiswald et al., 2015; Timmins et al., 2007). Several factors, including the size of the spheroids, can influence the
behavior of MCTS (Friedrich et al., 2009). Spheroid size and media composition dictate the viability and growth kinetics of 3D cultures due to the development of gradients in oxygen and metabolites (discussed below); therefore 3D culture requires careful design of growth conditions and analytical endpoints (Friedrich et al., 2009). Excellent discussions on the practical considerations needed when designing and interpreting data with 3D culture models have been published elsewhere (Hirschhaeuser et al., 2010; Friedrich et al., 2009).

There are four types of 3D spherical culture models that differ in some important ways (recently reviewed in (Weiswald et al., 2015)). MCTS, Tumor Spheres (TS), Tumor Derived Tumor Spheres (TDTS), and Organotypic Multicellular Spheres (OMS). MCTS are formed from established tumor cell lines and grown in standard culture media. MCTS can be composed of mono- or heterotypic cell populations, the latter being the co-culture of tumor cells with other cell types such as macrophages, endothelial cells, and fibroblasts (discussed later in this review). A variation on MCTS culture is the TS model which has been used as a method for growing tumor stem cells in 3D. These spheres are formed from clonal expansion of single cells suspended in non-adherent conditions supplemented with a specific compliment of growth factors in the culture media (Weiswald et al., 2015). Unlike MCTS derived from established cell lines, TS culture represents a selective population of cells known to be aggressive and likely to contribute to tumor regrowth (Weiswald et al., 2015; Ishiguro et al., 2017). Accordingly, TS may differ in drug response and growth kinetics relative to MCTS owing to the enrichment of the cancer stem cell population (Ishiguro et al., 2017). TDTS are similar to TS however they are formed from partially dissociated tumor tissues (Weiswald et al., 2015; Ishiguro et al., 2017; Ham et al., 2016). In the TDTS model, primary tumor cells are separated from non-tumor cell types and grown in non-adherent conditions. Relative to MCTS and TS, TDTS have been shown to more accurately recreate tumor growth and gene expression profiles. For example, in breast and colon cancer, TDTS mimic
differentiation properties and growth kinetics of the parent tumors more accurately than MCTS from the same tumor background (Weiswald et al., 2015; Theodoraki et al., 2015). TDTS therefore provide a suitable model for studying properties of individual tumors. The OMS model utilizes primary, non-dissociated, tumor tissue comprised of all cell types residing within the tissue at the time of excision which provides additional complexity. The presence of stromal cells can adversely affect tumor therapeutic response; therefore the OMS culture method provides a suitable model in which an individual tumor’s therapeutic response can be studied to predict an in vivo response (Weiswald et al., 2015; Bjerkvig et al., 1990; Christensen et al., 2010).

Each model has distinct advantages and disadvantages (discussed elsewhere (Hirschhaeuser et al., 2010; Lovitt et al., 2014; Baker et al., 2012; Weiswald et al., 2015)) but for maximal control and reproducibility of cell behavior, the MCTS model provides the best coupling of speed with which spheres can be generated, the ability to eliminate influence from non-tumor cell types, and the heterogeneous phenotype of tumor cells incorporated into the sphere. The properties of 3D culture discussed in this review however are applicable to most 3D systems because critical physical properties, such as the development of hypoxia, within the 3D environment are consistent amongst models Hirschhaeuser et al., 2010; Baker et al., 2012; Friedrich et al., 2009; Huh et al., 2015)

**Tumor cell adaptations under hypoxia**

Spheroids are a useful in vitro model of avascular tumor spaces. Spheroids exceeding 400 μm in diameter develop a hypoxic core and activate known survival signaling pathways to maintain cell viability. Tumor cells grown as MCTS, TS, TDTS, and OMS display regional heterogeneity in tumor cell proliferation (Christensen et al., 2010; LaRue, Khalil, & Freyer, 2004; Lawlor, Scheel, Irving, & Sorensen, 2002), metabolic activity (Baker & Chen, 2012; Christensen et al., 2010;
Daster et al., 2017; Favaro et al., 2012; Ghosh et al., 2005; Kumar et al., 2008; Pacheco-Marin et al., 2016), and DNA damage repair (DDR) signaling (Bindra et al., 2005; Bindra & Glazer, 2007; Bindra et al., 2004; Collins, Herve, Keevil, Blaydes, & Webb, 2011; Ghosh et al., 2005). In addition to the influence of the 3D microenvironment, the genetic background of the tumor cells and stromal cell populations also contribute to the specifics of spheroid regionalization (Friedrich, Seidel, Ebner, & Kunz-Schughart, 2009; Kunz-Schughart, Groebe, & Mueller-Klieser, 1996; Stadler et al., 2015; Stock et al., 2016).

Hypoxia in the 3D environment of tumors and MCTS induces the stable expression of hypoxia inducible factors (HIF). In vivo, HIF proteins transcriptionally promote angiogenesis through VEGF-A, glycolysis, and pH control through CA-IX (Loboda, Jozkowicz, & Dulak, 2010). Expression of HIF target genes occurs in tumor regions distal to blood vessels and is commonly used to identify hypoxic conditions (Hammond et al., 2014). Similar patterns have been observed in MCTS wherein HIF target genes (CA-IX, Glut1, VEGF-A) are expressed in the inner, hypoxic cell layers (Leek, Grimes, Harris, & McIntyre, 2016; Menrad et al., 2010; Pires et al., 2010; Pires et al., 2012; Shweiki, Neeman, Itin, & Keshet, 1995). Functionally HIF expression in both MCTS and in tumors has been linked to cell survival through the repression of pro-apoptotic signaling, repression of proliferation, and the regulation of metabolic reprogramming (Menrad et al., 2010; Pescador et al., 2010). In this way, HIF signaling contributes to regionalization of MCTS cell layers and the formation of microenvironments as a function of cell distance from the MCTS surface: limited diffusion of oxygen into MCTS establishes gradients in HIF-α stabilization and subsequently tumor cell behavior. Similarly, rapid proliferation by well oxygenated cell layers and the formation of physical barriers, through tight cell-cell contacts and ECM deposition, generates gradients in glucose, catabolites, and therapeutics (Baker & Chen, 2012; Grantab & Tannock, 2012). The following sections will discuss the ways in which gradient development within the
MCTS model reflects tumor cell adaptations in the avascular tumor space in vivo. These adaptations are schematized in Fig. 1.

**Proliferation gradients within MCTS**

Physiological hypoxia (5% > [O$_2$] > 1%) (Carrera et al., 2014) stimulates cell growth signaling through HIF-α to produce various cytokines and proteins necessary for carrying out cell division (Carrera et al., 2014; Kilic, Kasperekzyk, Fulda, & Debatin, 2007). Maintenance of proliferation of tumor and non-tumor cells requires an adequate supply of biomass (nucleotides, carbon sources, lipids) which is progressively diminished with decreasing oxygen levels resulting in a slowed and eventually halted replication cycle. Severe hypoxia defined as an [O$_2$] < 0.13% (Hammond et al., 2014) will result in replisome disassembly and exit from the cell cycle after approximately 12 to 24 hours (Foskolou et al., 2017; Hammond et al., 2014; Pires et al., 2010). Spheroids larger than 400 μm in diameter develop oxygen gradients representing a range of hypoxic conditions including chronic-severe hypoxia in the spheroid core. Accordingly spheroid proliferation occurs in a regionally specified manner; there is a progressive decrease in the fraction of S-phase cells with the development of hypoxia in the MCTS core (Bjerkvig, Tonnesen, Laerum, & Backlund, 1990; Dufau et al., 2012; Frongia et al., 2009; Khaitan, Chandna, Arya, & Dwarakanath, 2006; LaRue et al., 2004; Laurent et al., 2013; Lobjois, Frongia, Jozan, Truchet, & Valette, 2009; Magnussen et al., 2015; Pires et al., 2010; Pires et al., 2012; Riffle, Pandey, Albert, & Hegde, 2017). In this way, proliferation gradients develop in a size-dependent manner.

In a recent study we found that a population of cycling cells that retain proliferative status in a hypoxic environment can be identified in MCTS larger than 500 μm in diameter formed with A673 (Ewing Sarcoma) or Lewis Lung Carcinoma cells (Riffle et al., 2017). In human tumors, identification of a similar proliferative hypoxic cell population has been negatively correlated to
outcome and it is hypothesized that these cells contribute to tumor recurrence and metastasis (Evans et al., 2007; Evans, Hahn, Magarelli, & Koch, 2001; Evans et al., 2010). The mechanisms underlying tumor cell survival and proliferation within the 3D hypoxic environment is not well studied, however the identification of such a population within the MCTS model indicates that MCTS can be used to advance our understanding of proliferation under hypoxia.

**Hypoxia and the activation of DNA damage repair signaling**

Hypoxia development drives the repression of several DDR proteins which contributes to an increased mutation rate amongst hypoxic cells (Bindra et al., 2005; Bindra & Glazer, 2007; Bindra et al., 2004; Bristow & Hill, 2008; N. Chan et al., 2008; Koshiji et al., 2005; Scanlon & Glazer, 2015). On the other hand, numerous studies have demonstrated hypoxia-dependent activation of DDR proteins leading to stabilization of otherwise damage prone replication forks (Bencokova et al., 2009; Hammond, Denko, Dorie, Abraham, & Giaccia, 2002; Hammond, Dorie, & Giaccia, 2003, 2004; M. M. Olcina et al., 2013; Pires et al., 2010; Pires et al., 2012). Although well described in vitro, there are still significant unknowns regarding hypoxia-induced DDR, including the clinical relevance of such signaling in vivo and the specific proteins involved in this signaling network.

There is extensive evidence showing down regulation of numerous proteins involved in homologous recombination, mismatch repair, base excision repair, and nucleotide excision repair under hypoxic conditions (Scanlon & Glazer, 2015). Homologous recombination genes RAD51 and BRCA1, and the mismatch repair protein MLH1 (Collins et al., 2011) were shown to be transcriptionally down regulated in a panel of established cell lines in response to severe hypoxia (<0.5% O₂) (Bindra et al., 2005; Bindra & Glazer, 2007; Bindra et al., 2004). These results were validated in vivo by the finding that both RAD51 and BRCA1 were inversely correlated to markers
of hypoxia in cervical and breast cancer patients (Bindra et al., 2005; Bindra & Glazer, 2007; Bindra et al., 2004; Scanlon & Glazer, 2015). Recent studies using MCTS reported a similar decrease in DDR proteins (Collins et al., 2011; Ghosh et al., 2005). The loss of DDR proteins correlates with a decreased ability to repair double stand breaks under oxygen deprivation (N. Chan & Bristow, 2010; N. Chan et al., 2008; N. Chan et al., 2010; Scanlon & Glazer, 2015). This impaired DDR response is being investigated as a potential therapeutic advantage through contextual synthetic lethality (N. Chan et al., 2010). The concept proposes targeting the remaining DDR factors to induce catastrophic genomic instability. It is assumed that non-malignant cells, with an intact DDR signaling network, will be capable of repairing any therapy-induced damage. Promising results in preclinical experiments suggest that this strategy is effective when targeting PARP proteins in BRCA-deficient tumors (D. A. Chan & Giaccia, 2011; N. Chan & Bristow, 2010; M. Olcina, Lecane, & Hammond, 2010). This success has stimulated interest in the identification of DDR proteins operating under hypoxia as potential therapeutic targets. The ability of MCTS to recapitulate DDR protein loss validates the potential use of this model for future studies into contextual synthetic lethality.

Compelling evidence indicates that hypoxia drives replication stress, which in turn activates the DDR kinases Ataxia Telangiectasia (ATM) and ATM-and-Rad3 related (ATR) (Bencokova et al., 2009; M. M. Olcina, Grand, & Hammond, 2014). These kinases were shown to then signal through phosphorylation of downstream targets including Kap1 (S824), Chk1 (S345), Chk2 (Thr68), and H2AX (Ser139), referred to as γ-H2AX (Bencokova et al., 2009; Hammond et al., 2002; Hammond et al., 2003; Pires et al., 2012). Although ATM is activated, no DNA damage was originally reported in monolayer culture of the cell lines tested (Hammond et al., 2002; Hammond et al., 2003). However, recent studies using MCTS show increased DNA breaks under mild hypoxia using a Ewing Sarcoma cell line (A673) (Riffle et al., 2017). Together, these studies support a
model where ATM and ATR activity leads to stabilization of stalled replication forks and prevention of catastrophic DNA damage, thus allowing continued cell survival and eventual restart of proliferation (Fig. 2). In vivo the consequences of hypoxia induced DDR have been difficult to study. Multiple recent papers reported correlations between γ-H2AX formation and the presence of hypoxia (Banuelos, Banath, Kim, Aquino-Parsons, & Olive, 2009; Nagelkerke & Span, 2016; Olive, Banuelos, Durand, Kim, & Aquino-Parsons, 2010). For example, in carcinomas of the uterine cervix a 1.4 fold enrichment of γ-H2AX within hypoxic tumor regions (identified through carbonic anhydrase immunoreactivity) and a 2.8 fold enrichment in severely hypoxic (pimonidazole positive) regions has been reported (Olive et al., 2010).

Studies exploring DDR in MCTS typically used γ-H2AX staining of trypsin-dissociated spheres to identify the total levels of DNA damage via flow cytometry (LaRue et al., 2004; Olive, Banath, & Sinnott, 2004). More reliable assessment of regional expression can be done through immune labeling of spheroid sections or whole mount imaging. Expression of reporter constructs located downstream of a DDR protein promotor allows for live imaging of DDR response and is of particular interest for the purposes of high throughput screening (Friedrich et al., 2009; Huh, Hamilton, & Ingber, 2011; Lovitt, Shelper, & Avery, 2014; Mondesert et al., 2015). Using these techniques, DNA damage repair has largely been explored in the context of therapeutic response to DNA damaging treatments. We recently used MCTS to describe the spatial correlation between the activation or expression of specific DDR signaling proteins, hypoxia markers, and proliferation (Riffle et al., 2017). Using immunofluorescent labeling of spheroid sections, our findings showed a strong enrichment of γ-H2AX in the hypoxic (HIF-1α, pimonidazole positive), viable cell layers of MCTS. This enrichment and the spatial proximity to necrosis was similar to in vivo studies (Banuelos et al., 2009; Nagelkerke & Span, 2016; Olive et al., 2010). Furthermore we were able to demonstrate for the first time in a 3D context that proliferating hypoxic cells activate DDR
signaling which can be targeted by small molecule inhibitors. Interestingly the response to ATR inhibition differed between spheroids and monolayers, reinforcing the significant differences in cell signaling/response between 2D and 3D environments (Riffle et al., 2017).

Altered metabolic activity in MCTS

Hypoxia within the MCTS model is tightly correlated with glucose deprivation. It has long been known that glucose starvation significantly decreases spheroid growth and viability (Freyer & Sutherland, 1986; Kunz-Schughart et al., 1996; Tannock & Kopelyan, 1986). Studies using radiolabeled glucose and mathematical modeling revealed a critical threshold for glucose and oxygen diffusion into the MCTS core, beyond which chronic starvation results in cell death (Owen, Byrne, & Lewis, 2004; Stock et al., 2016). Recent findings detailing protein expression and metabolic adaptations within MCTS resemble metabolic profiles described in vivo (Bloch et al., 2014; Favaro et al., 2012; Ghosh et al., 2005; Kunz-Schughart et al., 1996; Pacheco-Marin et al., 2016; Witney et al., 2014). The accumulation of glycogen stores under mild hypoxia and subsequent breakdown of these stores into glucose under severe hypoxia represents one such adaptation (Favaro et al., 2012). Our studies using MCTS demonstrated regional increases in glycogen storage in the perinecrotic/hypoxic core of MCTS, correlating hypoxia development with glycogen accumulation (Riffle et al., 2017). These observations correlate well with studies showing enrichment of glycogen in perinecrotic/hypoxic zones of tumor xenografts where hypoxia altered the expression of glycogenic (GYS1) and glycolytic (PYGL) enzymes which are known to regulate MCTS glycogen storage (Favaro et al., 2012). Further glycogen accumulation within hypoxic regions of spheroids and tumor xenografts has been shown in real-time via positron emission tomography (Witney et al., 2014).
In addition to regulation of energy production, metabolic adaptations facilitate the production of reducing equivalents as a means of controlling reactive oxygen species (ROS) (Kim, Kim, & Bae, 2016). It is well established that hypoxia reduces oxidative phosphorylation efficiency which generates increased levels of ROS (Dugan, 1999). These highly reactive species oxidize lipids, proteins, and nucleic acids which disrupts cellular homeostasis and induces potentially catastrophic DNA damage (Cadet, Davies, Medeiros, Di Mascio, & Wagner, 2017; Tafani et al., 2016). To counteract such effects, tumor cells upregulate metabolic pathways that generate NADPH, a powerful reducing equivalent that helps to restore glutathione levels and mitigate the negative impact of ROS (Kim et al., 2016; Tafani et al., 2016). Some such pathways involve glucose metabolism. It was recently shown that detachment of tumor cells from the ECM disrupts glucose metabolism and induces increased ROS species (Schafer et al., 2009). In agreement with this data, the formation and growth of MCTS is dependent on sufficient glucose availability and activation of antioxidant pathways (Favaro et al., 2012; Marin-Hernandez et al., 2017; Schafer et al., 2009). To facilitate growth in MCTS and within hypoxic microenvironments, tumor cells activate signaling through HIF proteins (Loboda et al., 2010). The HIF transcriptional unit is a heterodimeric complex composed of a constitutively expressed HIF-β subunit and a labile HIF-α subunit. In well oxygenated tumor regions, HIF-α proteins are degraded following proline hydroxylation by Prolyl Hydroxylase (PHD) proteins (Loboda et al., 2010). In the absence of oxygen, PHD protein function is diminished resulting in HIF-α stabilization, dimerization with HIF-β, and subsequent transcriptional regulation of numerous proteins. The majority of proteins involved in glycolysis are regulated by the HIF transcriptional complex leading to increased glycolysis under hypoxic conditions (Loboda et al., 2010; Parks, Cormerais, & Pouyssegur, 2017; Pescador et al., 2010). In addition to oxygen deprivation, ROS production impairs PHD function leading to HIF-α stabilization, further contributing to metabolic reprogramming under hypoxic stress (Tafani et al., 2016).
HIF-α stabilization, metabolic reprogramming, and ROS production have all been observed in MCTS with patterns similar to those reported in vivo (Favaro et al., 2012; Jiang et al., 2016; Leek et al., 2016; Riffle et al., 2017; Wartenberg, Fischer, Hescheler, & Sauer, 2000; Wartenberg et al., 2003), reflecting recent reports of increased glycogen storage under hypoxic conditions facilitating continued cell proliferation and survival by increasing NADPH production (Favaro et al., 2012; Pescador et al., 2010). Similarly, metabolic reprogramming under hypoxia includes increased activity from the Isocitrate Dehydrogenase proteins (IDH) which play a significant role in ROS homeostasis and proliferation under hypoxia (Metallo et al., 2011; Wise et al., 2011; Yang, Ye, Guan, & Xiong, 2012). IDH proteins reductively carboxylate α-ketoglutarate to form citrate and NADPH (Wise et al., 2011; Yang et al., 2012). This process has become increasingly appreciated as critical for cell viability under hypoxia by promoting de novo lipogenesis, restoration of cellular glutathione, and production of carbon equivalents for cell signaling (Metallo et al., 2011; Wise et al., 2011; Yang et al., 2012). Not surprisingly, the loss of IDH proteins leads to the suppression of MCTS growth concomitant with increased ROS production (Metallo et al., 2011). IDH appears to be involved in MCTS formation as evidenced by the inability to reproducibly form MCTS from glioma cells containing IDH mutations which alter substrate specificity (Lenting, Verhaak, Ter Laan, Wesseling, & Leenders, 2017). Similar mutations in vivo are associated with increased patient survival due to an impaired HIF response within hypoxic tumor regions (Miroshnikova et al., 2016). These adaptations to neutralize ROS have been schematized in Fig. 3.

The activation of glycolytic pathways is clearly replicated in MCTS as well. Numerous studies have identified increased lactic acid production within hypoxic spheroids which correlates with increased production and activity from glycolytic enzymes (Bloch et al., 2014; Longati et al., 2013; Rodriguez-Enriquez et al., 2008). Increased glycolytic activity is indicative of an adaptive shift
from oxidative phosphorylation to glycolysis as the primary source of ATP, referred to as the Warburg effect (Marin-Hernandez et al., 2017; Weinhouse, 1956; Zu & Guppy, 2004). The ability of MCTS to mimic metabolic adaptations observed in vivo could provide a relevant model system for targeting metabolic pathways in cancer therapy.

Together these studies indicate that hypoxia development within the MCTS model generates metabolic reprogramming leading to increased synthesis of glycogen, IDH reductive carboxylation, and glycolytic activity with similar distributions to that observed in vivo. Comprehensive reviews of metabolic adaptations under hypoxic conditions and the interplay of ROS with this process are available (Eales, Hollinshead, & Tennant, 2016; Kim et al., 2016; Tafani et al., 2016).

**MCTS in co-culture studies**

The MCTS discussed thus far were composed exclusively of tumor cells. However, tumors are diverse in terms of their micro-environmental composition as well as their cellular composition. The adaptation of tumor cells to a hypoxic environment is influenced by the activity of stromal cells including endothelial cells, fibroblasts, adipocytes, macrophages, monocytes, and other cell types (Mao, Keller, Garfield, Shen, & Wang, 2013). As in the case of tumor cells, the hypoxic environment can affect stromal cell behavior (Lau, Ho, & Lee, 2017). Endothelial cells are known to increase proliferation in response to a hypoxic environment and to activate a DDR signaling cascade similar to that described for tumor cells (Economopoulou et al., 2009; Okuno, Nakamura-Ishizu, Otsu, Suda, & Kubota, 2012; Y. Wang et al., 2016). Accumulating evidence suggests that immune cell infiltration into the hypoxic tumor microenvironment increases angiogenesis and tumor metastasis (Guiet et al., 2011; M. Wang et al., 2017). Both adipocytes and fibroblasts contribute significantly to tumor progression through the production of pro-proliferative cytokines and through production of extracellular matrix (ECM) proteins which confer chemo-resistance.
(Correa de Sampaio et al., 2012; Moya, Tran, & George, 2013; Osterholm et al., 2012; Stock et al., 2016; M. Wang et al., 2017; Wen et al., 2017; Yip & Cho, 2013).

Towards accurately recreating the tumor microenvironment in vitro, various co-culture models have been developed wherein MCTS are composed of both tumor and stromal cells. The OMS model is perhaps the most representative form of co-culture system due to the inclusion of all resident stromal cell types within the excised tissue (Oudar, 2000; Weiswald, Bellet, & Dangles-Marie, 2015). OMS have been used as a model for multiple tumor types and accurately reflect tumor growth kinetics (Bjerkvig et al., 1990; Christensen et al., 2010; Weiswald et al., 2015). In glioblastoma for example, the OMS model was found to be superior to MCTS in its ability to recreate the immuno-histochemical profile of in vivo tissues including the expression of many proteins previously correlated to hypoxia-induced aggression (CD31, CD133, P-glycoprotein, and TIMP-1) (Christensen et al., 2010). However, the inclusion of multiple cell types in the OMS model makes it difficult to determine the influence of a specific stromal cell population on the adaptive tumor cell response (Oudar, 2000; Weiswald et al., 2015).

Heterologous spheroid culture in which tumor cells are combined with one or more stromal cell types has provided unique insights into stromal and tumor cell responses to hypoxia (Kunz-Schughart, Heyder, Schroeder, & Knuechel, 2001; Lovitt et al., 2014; Weiswald et al., 2015). The most commonly used methods of heterologous culture can be broadly described as: 1) Spheroid confrontation culture: individual spheroids are formed from stromal cells and separately from tumor cells followed by joint culture in suspension or embedded within an ECM, 2) Spheroid-monolayer culture: pre-formed MCTS are placed atop confluent monolayers, 3) Heterologous spheroid co-culture: tumor and stromal cell suspensions are combined during the sphere forming
process, 4) Spheroid co-cultures in vivo: pre-formed MCTS containing stromal cells are implanted in vivo (Oudar, 2000).

MCTS in co-culture with endothelial cells mimic several processes during hypoxia-induced angiogenesis: oxygen deprivation within the MCTS core stimulates production and accumulation of vascular endothelial growth factors (VEGF) (Shweiki et al., 1995; Sutherland, 1988) which provides a stimulus for endothelial cell recruitment. In vivo, endothelial cell invasion into tumors facilitates metastasis and release of tumor cells from the growth inhibitory effects of a hypoxic environment (M. Wang et al., 2017). Other MCTS co-culture models recreate a similar process and have been used to improve our understanding of the relationship between endothelial cells and tumor cells (Ghosh et al., 2007; Oudar, 2000). For example, placement of tumor spheroids atop confluent endothelial monolayers can result in infiltration by endothelial cells expressing tumor complimentary adhesion molecules or can result in tumor-mediated destruction of endothelial vessels, reminiscent of cell death observed in vivo following vessel cooption (Ghosh et al., 2007; Oudar, 2000). Use of confrontation or heterologous co-culture models can successfully produce vascular networks within spheroids with an increase in tumor cell viability and drug resistance (Wartenberg et al., 2001; Wartenberg et al., 2003). The invasive potential of tumor cells following vascularization is also replicated through the use of heterologous sphere culture systems (Correa de Sampaio et al., 2012; Ehsan, Welch-Reardon, Waterman, Hughes, & George, 2014; Stock et al., 2016). A recent study highlighted this by showing the formation of MCTS with both endothelial and tumor cells could lead to the formation of luminal vessels in which migrating tumor cells can be seen (Ehsan et al., 2014), mimicking the process of tumor intravasation.
Fibroblasts are also a critical stromal cell population that contribute to tumor progression through the release of proliferative and pro-metastatic growth factors and foster the creation of a drug resistant environment by depositing ECM which hinders drug diffusion (Correa de Sampaio et al., 2012; Kunz-Schughart et al., 1996; Oudar, 2000). Each of these characteristics are replicated in heterologous sphere culture (Correa de Sampaio et al., 2012; Kunz-Schughart et al., 2001; Osterholm et al., 2012; Oudar, 2000; Stock et al., 2016; Yip & Cho, 2013). Within a pro-angiogenic environment, fibroblasts take on a mural cell phenotype and augment angiogenesis through the release of VEGF, matrix metalloproteinases (MMP), and other growth factors (Correa de Sampaio et al., 2012; Kunz-Schughart et al., 2001; Osterholm et al., 2012; Oudar, 2000; Stock et al., 2016; Yip & Cho, 2013). In vitro this process can be studied through the “mini-tumor model” in which endothelial cells, fibroblasts, and tumor cells are co-cultured in 3D (Osterholm et al., 2012). This tri-culture model replicated pathological formation of luminal vascular structures bordered by fibroblasts and tumor cells which are both dependent on angiogenic growth factors and responsive to anti-angiogenic therapeutics (Osterholm et al., 2012). This mini-tumor model has significant potential for studying the refractory response of tumor and stromal cells following anti-angiogenic therapy and the specific mechanisms used to circumvent these treatments.

Immune cells are a major regulatory factor during tumor progression (Hanahan & Weinberg, 2011). Within the hypoxic tumor environment, there are indications that immune cells facilitate tumor aggression through increased angiogenesis and immunosuppression (Gottfried, Kunz-Schughart, Andreesen, & Kreutz, 2006; Mao et al., 2013). In vivo immune cells aggregate within hypoxic, peri-necrotic regions (Gottfried, Kunz-Schughart, Andreesen, et al., 2006). These observations have been mirrored in MCTS co-cultures and indicate potential utility for MCTS as an investigational tool to replicate in vivo conditions (Dangles et al., 2002; Stock et al., 2016). In support of this, immune cell influence over angiogenesis has been described by a series of studies.
through the inclusion of monocytes in co-culture with tumor spheroids to show immune-dependent increases in production of VEGF, ECM degrading proteins, and in vivo angiogenesis through MCTS co-culture in skifold window chambers (Bingle, Lewis, Corke, Reed, & Brown, 2006; Dangles et al., 2002; Gottfried, Kunz-Schughart, Andreesen, et al., 2006; Stock et al., 2016). A particular advantage to MCTS co-culture in modelling the hypoxic microenvironment is the accumulation of immunomodulatory metabolites and cytokines.

Levels of lactic acid are commonly high in hypoxic tumor regions due to increased glycolytic activity and HIF mediated production of lactic acid transporters (Perez de Heredia, Wood, & Trayhurn, 2010). In vitro studies show that hypoxia-induced increases in lactic acid and VEGF can decrease dendritic cell differentiation and maturation while increasing monocyte conversion to M2 macrophages, ultimately resulting in immunosuppression (Dikov et al., 2005; Gottfried, Kunz-Schughart, Andreesen, et al., 2006; Gottfried, Kunz-Schughart, Ebner, et al., 2006; Stock et al., 2016; Tang et al., 2012). The MCTS co-culture models are well suited to model a hypoxic microenvironment with high metabolite and growth factor levels. Hypoxic spheroids show higher levels of lactic acid which correlates with decreased immune cell invasion, reduced production of colony stimulating factor (Gottfried, Kunz-Schughart, Andreesen, et al., 2006; Gottfried, Kunz-Schughart, Ebner, et al., 2006) and decreased dendritic cell maturation in hypoxic conditions (Gottfried, Kunz-Schughart, Andreesen, et al., 2006; Selleri et al., 2016).

**MCTS as a model to determine therapeutic efficacy in heterogeneous environments**

Aside from instigating an aggressive phenotype, tumor hypoxia presents several challenges to therapeutic intervention (Minchinton & Tannock, 2006; Wilson & Hay, 2011). Hypoxia has been appreciated for its ability to reduce radiation effectiveness for many decades (Durand & Biaglow, 1977; Hammond & Muschel, 2014; M. Olcina et al., 2010). Since this phenomenon was described,
a significant amount of evidence has amassed demonstrating the ways a hypoxic environment renders tumor cells privileged, or resistant, to multiple therapeutic modalities (Mehta, Hsiao, Ingram, Luker, & Takayama, 2012; Olive & Durand, 1994; Wartenberg et al., 2003; Wilson & Hay, 2011) (Casanovas, Hicklin, Bergers, & Hanahan, 2005; Christensen et al., 2010; Grantab & Tannock, 2012; Harada et al., 2012; Minchinton & Tannock, 2006; Olive et al., 2010). Several studies have detailed the contribution of stromal cells to chemo-resistance in the MCTS model (Correa de Sampaio et al., 2012; Osterholm et al., 2012; Song et al., 2016; Yip & Cho, 2013) and technological advances are allowing more complex co-culture systems to be developed (Huh et al., 2011; Lovitt et al., 2014). The use of MCTS for screening of therapeutics has been extensively reviewed elsewhere (Friedrich et al., 2009).

Conclusions

The negative impact of hypoxia on tumor prognosis warrants a significant effort to better understand and target hypoxic tumor cell adaptations. The MCTS model is an established technique with untapped potential to improve our understanding of subpopulations within a tumor. With growing therapeutic interest in targeting metabolic pathways, DDR proteins, and contextual synthetic lethality, the MCTS model could be used to great effect.

Figure 1 Hypoxia-induced adaptations in a spheroid. Oxygen gradients within tumor spheroids lead to conditions ranging between mild physiological hypoxia to anoxia (represented here by shades of gray). This results in regionalization of tumor cell populations (Freyer & Sutherland, 1986; Sutherland, 1988). Hypoxia develops in the spheroid core due to a combination of oxygen diffusion limitations and rapid consumption from proliferating cells (Durand & Biaglow, 1977; Sutherland, 1988; Zhou et al., 2013). Oxygen deprivation induces glycogen storage to facilitate
subsequent metabolism and continued proliferation under more severe hypoxia (Favaro et al., 2012; Pescador et al., 2010; Riffle et al., 2017). Cycling cells in hypoxic regions experience replication stress (stalled replication forks (Hammond et al., 2002) and DNA damage (Riffle et al., 2017)), inducing activation of DNA damage repair (DDR) signaling. DDR allows temporary tumor cell survival and proliferation upon re-oxygenation, but after prolonged periods (12-24 hours) of severe hypoxia the replisome will disassemble (Foskolou et al., 2017; M. Olcina et al., 2010).

**Figure 2 Hypoxia-induced DNA damage repair signaling.** Cells attempting to proliferate under hypoxic conditions experience slowed replication due to decreased nucleotide pools (Foskolou et al., 2017; M. M. Olcina et al., 2014). In the absence of oxygen, ribonucleotide reductase is impaired in its ability to produce deoxyribonucleotides required for efficient replication (Foskolou et al., 2017). Replication forks stall under these conditions which can lead to DNA damage (Foskolou et al., 2017; Hammond et al., 2002; Hammond et al., 2003; M. M. Olcina et al., 2014; Riffle et al., 2017). Increased DNA damage and replication stress stimulate activation of the DNA damage repair kinases ATM and ATR (Bencokova et al., 2009; Hammond et al., 2002; Hammond et al., 2003). Together these kinases phosphorylate downstream targets resulting in the stabilization of stalled replication forks and preservation of cell viability (Hammond et al., 2003; M. M. Olcina et al., 2014; Riffle et al., 2017).

**Figure 3 Hypoxia induces metabolic adaptations to prevent ROS related damage and maintain proliferation.** Under hypoxic conditions tumor cells experience elevated levels of ROS. To mitigate the negative impact of ROS, tumor cells have been shown to increase glycogen stores which are later broken down into glucose-6-phosphate (Glucose-6-P) (Favaro et al., 2012; Pescador et al., 2010). Hypoxic cells increase flux through the pentose phosphate pathway which produces NADPH and Ribose-5-phosphate (Ribose-5-P). Ribose-5-P serves as a precursor for
DNA nucleotides thus facilitating proliferation (Riganti, Gazzano, Polimeni, Aldieri, & Ghigo, 2012). NADPH provides reducing equivalents that restore glutathione levels and neutralize ROS. NADPH is also generated through reductive carboxylation of α-ketoglutarate by Isocitrate Dehydrogenase proteins (Jiang et al., 2016; Wise et al., 2011). Following oxygen deprivation, glutamine is metabolized to form glutamate and subsequently α-ketoglutarate (Wise et al., 2011). The formation of citrate by IDH proteins has been shown to increase lipogenesis and facilitate proliferation (Metallo et al., 2011).
Figure 1: Hypoxia-induced adaptations in a spheroid

Figure 2: Hypoxia induced DNA damage repair signaling
Figure 3: Hypoxia induces metabolic adaptations to prevent ROS related damage and maintain proliferation.
CHAPTER TWO – Linking hypoxia, DNA damage and proliferation in multicellular tumor spheroids

Introduction

Hypoxia development is a common and clinically relevant feature of solid tumors. Oxygen diffusion through tissue is limited to approximately 200 μm. Beyond this threshold, cellular metabolism and replication become compromised due to insufficient oxygen levels (Hockel & Vaupel, 2001; Wilson & Hay, 2011). As oxygen levels fall, cells initiate a series of adaptations that result in the release of angiogenic growth factors, alterations in metabolic activity, and activation of DNA damage repair pathways to preserve DNA integrity and facilitate continued proliferation (Eales, Hollinshead, & Tennant, 2016; Vizan et al., 2009). Hypoxia inducible factor-1a (HIF-1α) contributes significantly to these adaptive responses through the production of angiogenic growth factors such as Vascular Endothelial Growth Factor A (VEGF-A) and proteins integral to metabolic reprogramming (Fong, 2008; Loboda, Jozkowicz, & Dulak, 2010). Tumor angiogenesis occurs in response to growth factor release however these newly formed vessels are characteristically tortuous, leaky, and inefficient leading to regional cycling of hypoxia/reoxygenation as well as chronic oxygen and nutrient deprivation (Dudley, 2012). Inherent properties of a 3D hypoxic tissue mass foster the formation of a chemo- and radiation resistant microenvironment out of which genetically unstable tumor cells displaying a more aggressive phenotype can grow (Minchinton & Tannock, 2006; Multhoff, Radons, & Vaupel, 2014; Pinto et al., 2016).
Survival of tumor endothelial cells within the hypoxic environment is dependent on DNA damage repair (DDR) signaling (Economopoulou et al., 2009; Okuno et al., 2012). It is well described that the cycling of hypoxia and reperfusion within the tumor vasculature generates bursts of reactive oxygen species (ROS) which induce extensive DNA damage (Dugan LL, 1999; Walshe & D'Amore, 2008). In contrast, chronic exposure to severe hypoxia does not cause DNA damage but results in nucleotide depletion and subsequent replication stress (Foskolou et al., 2017; M. M. Olcina et al., 2014). Hypoxia induced replication stress activates DNA damage repair (DDR) proteins in an effort to stabilize replication forks and to trigger an S-phase block, temporarily preserving genomic integrity and proliferative capacity (Foskolou et al., 2017; M. M. Olcina et al., 2013). Replication stress induces the activation of ATM and ATR kinases which subsequently phosphorylate the downstream targets H2AX ser-139 (γ-H2AX), p53 Ser-15, Chk1 Ser-345, and Chk2 Thr-68 (Hammond et al., 2002; Hammond et al., 2003; M. M. Olcina et al., 2014). These DDR proteins collectively coordinate the formation of a heterochromatic state around the stalled replication fork and prevent new origin firing (M. M. Olcina et al., 2014). This response has primarily been described in vitro through the use of two-dimensional cell culture (2D) with limited evidence of a similar response in vivo. Therefore the relationship between hypoxia, proliferation, and DDR protein activation in the complex three-dimensional (3D) environment of an in vivo tumor is unknown.

Tumor cells grown in 2D culture differ significantly from their in vivo counterparts as assessed by transcriptomic, proteomic, and behavioral profiles (Hirschhaeuser et al., 2010; Longati et al., 2013; Stock et al., 2016; Weiswald et al., 2015). Aspects of 2D cell culture such as cell-cell contact, exposure to environmental factors, and forced polarization can significantly alter tumor cell behavior and response to therapeutics. For these reasons, 3D cell culture through the Multicellular Tumor Spheroid (MCTS) model has been increasingly utilized to screen prospective
therapeutics prior to in vivo studies (Friedrich et al., 2009; Hirschhaeuser et al., 2010; Weiswald et al., 2015). Additionally, MCTS demonstrate regional patterns of proliferation, cell death, and metabolism that resemble in vivo observations (Bloch et al., 2014; Milotti & Chignola, 2010; Olive & Durand, 1994; Sutherland, 1988; Weiswald et al., 2015). Importantly, a 3D hypoxic tumor environment provides an opportunity to determine a physiological relationship between hypoxia, proliferation, and DDR signaling.

In the following work, we set out describe the relationship between hypoxia, metabolic reprograming, DNA damage repair, and proliferation using the MCTS model. MCTS generated from Ewing Sarcoma or Lewis Lung Carcinoma cell lines were used to show that the development of hypoxia temporally and spatially correlates with decreased proliferation, phosphorylation of DDR proteins in Ki67 positive cells, stabilization of HIF-1α, and increased glycogen storage. In characterizing DDR signaling as it relates to hypoxia development, the phosphorylation of ATM and H2AX was observed to regionally correlate with hypoxia markers and to be induced by the hypoxic environment. Pharmacological inhibition of ATM or ATR revealed differences between the contributions of each kinase to DDR signaling and proliferation within the 3D MCTS environment. Collectively our studies present MCTS as a simple and robust model in which DDR associated signaling pathways, and the efficacy of therapeutics targeting these pathways, can be studied.

**Materials and Methods**

*Antibodies and reagents*

Human Ewing Sarcoma A673 (CRL-1598; ATCC, Manassas, VA) and LLC (CRL-1642; ATCC, Manassas, VA); Dulbecco’s Modified Eagles Medium, DMEM, (11,965,092; Thermoscientific, Waltham, MA); Fetal Bovine serum - FBS, (TMS-013-B; Millipore, Billerica, MA); Agar (J637;
Amresco, Solon, OH); DMSO (67–68-5, Sigma, St. Louis, MO); Proox-p110 ProCO₂ Hypoxia Chamber (Biospherix, Parish, NY); Inflatable Glove Bag Model X-37-27 (108D; Glas-Col, Terre Haute, IN); ATM inhibitor KU55933 (S1092; Selleckchem, Houston, TX); ATR inhibitor VE-821 (S8007; Selleckchem, Houston, TX); Click-IT Plus EdU imaging kit (MP10637, Life technologies, Carlsbad, CA).

The following antibodies were used in these studies: mouse anti-γ-H2AX (JBW301; Millipore, Billerica, MA), rabbit anti-Ki-67 (MA5–1452; Thermoscientific, Waltham, MA), rabbit anti-cleaved caspase-3 (5A1E; Cell Signaling Technology, Danvers, MA), mouse anti-HIF-1α (810,958; BD Biosciences, San Jose, CA), mouse anti-phospho-serine 1981 ATM (05–740; Millipore, Billerica, MA), rabbit anti-phospho-serine 428 ATR (2853P; Cell Signaling Technology, Danvers, MA), mouse anti-pimonidazole (HP1; Hypoxyprobe, Burlington, MA), goat anti-mouse IgG (H + L) Alexa Fluor 647 (A-21235; Thermoscientific, Waltham, MA), goat anti-mouse IgG Cyanine 5 (A-10524; Thermoscientific, Waltham, MA), donkey anti-rabbit Alexa Fluor 594 (A-21027; Thermoscientific, Waltham, MA), rabbit anti-phosphoserine 345 Chk1 (2341; Cell Signaling Technology, Danvers, MA), horse radish peroxidase-conjugated goat anti-mouse (A120-11P; Bethyl, Montgomery, TX), horse radish peroxidase-conjugated goat anti-mouse (A120-201P; Bethyl, Montgomery, TX).

Cell Culture

A673 and LLC were maintained in standard culture conditions (37 °C with 5% CO₂) in DMEM with 1% vv⁻¹ penicillin (100 IU ml⁻¹) and streptomycin 100 mg ml⁻¹ supplemented with 10% vv⁻¹ heat inactivated FBS.

MCTS culture
Spheroids were formed through use of the liquid overlay method wherein single cell suspensions (100 μl of A673 or LLC cell suspensions in DMEM supplemented with 2% FBS or 10% FBS were added at a density of $5 \times 10^4$ or $5 \times 10^3$ cells ml$^{-1}$ respectively) were added to 96-well plates previously coated with agar. The agar coating prevents cell adhesion. Plates containing single cell suspensions were incubated stationary in standard cell culture conditions (37 °C, 5% CO$_2$) for 48 h. During this time, tumor cells aggregated forming a single, connected mass of cells that could be moved as a singular unit. After this 48-h formation period, 100 μl of fresh media was added to each well. This makes for a total of 200 μl of total media in each well. After this point, 100 μl of media was removed and replaced with fresh media every 48 h such that total volume within the wells was maintained at 200 μl. In experiments with test compounds or hypoxia treatment, spheroids were individually washed with DMEM and transferred to freshly coated agar plates with 200 μl of fresh medium containing test inhibitors when applicable.

**Hypoxia Treatment**

Hypoxia treatment was performed at 37 °C for 12 h with 1% O$_2$, 5% CO$_2$ in a hypoxia chamber followed by collection in a hypoxic glove bag pre-equilibrated at 1% O$_2$.

**MCTS growth curves**

Growth curves for MCTS were generated using bright-field images captured with a Nikon Eclipse TS100 4× objective (0.1 N.A.). MCTS diameter was determined using ImageJ 1.48v (NIH, Bethesda, MD; [http://imagej.nih.gov/ij](http://imagej.nih.gov/ij)) and converted to μm. Growth curves in Fig. 4 represent the mean diameter ± standard deviation of at least 8 MCTS from 3 independent experiments. LLC growth curves and characterization were performed by Morgan Albert.

**Processing of MCTS for histological assessment**
MCTS were collected in Eppendorf tubes and allowed to settle at the bottom before culture medium was removed, replaced with freshly thawed 4% paraformaldehyde/phosphate-buffered saline (pH 7.4), and incubated at 4 °C for 3 h. When appropriate, spheroids were incubated with EdU (10 μM, 24 h) or Pimonidazole (100 μM, 3 h) prior to fixation. For cryopreservation, fixed MCTS were dehydrated with 30% sucrose and snap-frozen with Optimal Cutting Temperature media (4583; Tissue Tek, Torrance, CA). Cryosections were made at 5 μm thickness and stored at -80 °C. Morphology of MCTS sections was determined with Harris Hematoxylin and Eosin (PolyScience, Warrington, PA) staining. Glycogen storage determined with Periodic Acid (375,810, Sigma, St. Louis, MO) and Schiff’s Base (3,952,016, Sigma, St. Louis, MO) staining, in parallel to sections pre-incubated with 0.5% amylase. No hematoxylin counter stain was used. Morgan Albert aided in the processing of LLC and A673 spheroids.

Immunofluorescent analysis of MCTS sections

Cryo-sections were blocked with antibody specific blocking buffer for 1 h at room temperature in a humidified chamber. Primary antibodies directed against γ-H2AX, Ki-67, cleaved caspase-3, HIF-1α, pATM, and pATR were diluted (1:200) in blocking buffer and incubated on sections for 1 h at room temperature or overnight at 4 °C. After washing with phosphate-buffered saline containing 0.15% Triton X-100, sections were incubated for 1 h at room temperature with fluorescently tagged secondary antibodies diluted (1:200) in blocking buffer, followed by 20-min incubation with Hoechst dye to identify nuclei. Sections were washed and mounted using Fluorgel with DABCO (17985–04; Electron Microscopy Science, Hatfield, PA). Primary antibody dilution for pimonidazole was 1:50. Blocking buffer for γ-H2AX, Ki-67, Hypoxyprobe, and Cleaved Caspase 3 was phosphate-buffered saline supplemented with 10% FBS, 2% BSA, and 0.15% Triton X-100. Blocking for HIF-1α, pATM, pATR was done with phosphate-buffered saline supplemented with 5% donkey serum and 0.15% Triton X-100. EdU was detected following the
manufacturer’s instructions (Life Technologies, Carlsbad, CA); MCTS cryo-sections were permeabilized with 0.5% vv⁻¹ Triton X-100 followed by incubation with Alexa Fluor 647 azide and Hoechst 33,342 nuclear counter stain. Morgan Albert aided in analysis of LLC spheroids.

**ATM and ATR inhibition**

10 μM KU55933 or 2.5 μM VE-821 in DMEM supplemented with 2% FBS and containing 0.025% vv⁻¹DMSO were used. Fresh media containing inhibitors was added to MCTS cultures every 48 h. The vehicle control contained 0.025% vv⁻¹ DMSO in DMEM supplemented with 2% FBS.

**Western blotting**

Cell pellets from exponentially growing cells were lysed by sonication on ice with lysis buffer consisting of 500 mM NaCl, 100 mM HEPES at pH 7.4, 10 mM EDTA, and 0.2% vv⁻¹ NP-40, supplemented with phosphatase inhibitors sodium fluoride (NaF; 50 mM) and sodium orthovanadate (1 mM), proteinase inhibitors phenylmethyl sulfonyl fluoride (PMSF, 1 mM), 1× Proteinase Inhibitor Cocktail (P8340; Sigma, St. Louis, MO), and the reducing agent Dithiothreitol (DTT, 1 mM). Cell debris were removed via centrifugation. Protein concentrations were estimated by Bradford assay, samples were electrophoresed on 10% SDS gels and transferred to PVDF membranes. Membranes were blocked using rapid block buffer (M325, Amresco, Solon OH) for 5 min at room temperature followed by incubation with specific primary and secondary antibodies. Western blots were visualized with enhanced chemi-luminescence (34,078; ThermoScientific, Waltham MA) on X-ray films (53PSF; Worldwide Medical, Bristol PA). All western blotting was performed by Naresh Pandey.
Comet Assay

Ten thousand cells suspended in 1% low melting temperature agarose were diluted in TBE (90 mM Tris, 90 mM Boric Acid, 2 mM EDTA, pH 8.5) and spread atop coverslips previously coated in 1% low melting temperature agarose and allowed to solidify for 10 min at 4 °C. Cells were then lysed for 1 h at 4 °C in 2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1% wv⁻¹ sarkosyl, 1% Triton X-100. Lysed cells were neutralized with TBE. Following alkaline buffer incubation (300 mM NaOH, 1 mM EDTA, pH 12.3) lysates were electrophoresed at 25 V for 40 min at room temperature, dehydrated in 70% ethanol and DNA visualized using propidium iodide (1 μg/ml). Comet assay was performed by Naresh Pandey.

Image acquisition and analysis

Fluorescently stained sections were imaged on a Zeiss confocal microscope at 0.25× magnification across a 1000 μm grid. To determine the number of positive nuclei per spheroid, nuclei were counted as Hoechst-positive cells by using watershed separation and quantification with particle analysis in ImageJ software [12] followed by cell counting to determine the percentage of marker positive nuclei.

Statistics

Results represent the average of at least 2 separate experiments with a total of at least 8 spheroids per condition ± standard deviation (SD). Statistical analyses were performed using Graphpad PRISM version 5.0 for Mac OSX, http://www.graphpad.com. A t-test was used when two samples/conditions were compared and ANOVA for more than two groups. Significance represents $P < 0.05$. 
Results

**MCTS develop a hypoxic core concurrent with altered glucose metabolism and cell death**

We sought to examine the development of hypoxia in MCTS and its spatial correlation with DDR protein activation, proliferation, necrosis and apoptosis. Typically, spheroid growth is accompanied by the development of an anoxic central core and the formation of two distinct zones: a viable ring of cells on the surface and a central necrotic core (Groebe & Mueller-Klieser, 1996; LaRue et al., 2004; Leek et al., 2016; Milotti & Chignola, 2010; Sutherland, 1988). For this reason, spheroids represent a suitable model for investigating the cellular and biochemical changes that accompany the growth of avascular tumors.

A panel of cell lines representing different malignancies were tested for their ability to rapidly and reproducibly form MCTS. The given cell lines were selected based on their extensive use in the study of tumor microenvironments or based on the ability to perform xenografts in genetically altered mice for future studies. Amongst the cell lines tested, cell lines from Lewis lung carcinoma (LLC) and Ewing sarcoma (A673) reliably formed spheroids and were selected for further characterization. Spheroids were formed in cell specific medium (DMEM + 10% FBS for LLC, and DMEM + 2% FBS for A673). Both cell lines formed loose aggregates within 24 hours followed by the formation of discrete, 3D spheroids approximately 300-420 μm in diameter. Spheroid growth was monitored by light microscopy and plotted in Figure 4a. A673 cells formed the most tightly packed and well-formed spheroids whose growth plateaued at an average diameter of 850 μm 12 days after seeding. LLC spheroids grew to about 1000 μm over 11 – 12 days, with a less tightly packed surface layer of cells than in the case of A673.

In order to assess the temporal and spatial onset of necrosis and hypoxia, representative spheroids were harvested and sectioned when they were <500 μm, 570 – 650 μm and >850 μm
in diameter; time-points were chosen to represent the first formation of a discrete spheroid, an exponential growth phase, and spheroids that had stopped growing. An initial analysis of cell and spheroid morphology was conducted using hematoxylin and eosin (H&E) staining (Fig. 4B). A673 and LLC spheroids consisted of uniformly and densely packed cells with onset of necrosis apparent in the center of spheroids that were 570 – 650 μm in diameter, resembling characteristics described for severely hypoxic tumor regions (Daster et al., 2017; Evans et al., 2001). At their maximum diameter, the majority of A673 and LLC spheroid volumes were composed of necrotic cells (accounting for 50 – 60% of spheroid volume).

Hypoxia development was assessed by immunostaining for the exogenous marker Pimonidazole (Fig. 5B) Pimonidazole, a 2-nitroimidazole, is reductively activated in hypoxic conditions and can then form stable adducts with proteins, peptides and amino acids. Pimonidazole binding to cellular glutathione has been shown to occur at oxygen concentrations below 1.3% and is therefore used as a marker of hypoxia (Chou, Azuma, Varia, & Raleigh, 2004). Pimonidazole retention was first observed in the perinecrotic cell layers of spheroids approximately 570-650 μm in diameter and persisted in all subsequent sizes. To assess the activation of hypoxia activated signaling we then assessed nuclear HIF-1α immunoreactivity (Fig. 5A). Unlike Pimonidazole, HIF-1α was first observed in the core of small spheroids (<500 μm diameter) and was consistently observed in the perinecrotic cell layers of larger spheres. HIF-1α stabilization occurs at higher oxygen concentrations than is required for Pimonidazole retention in cells and thus it is likely that the core of small spheroids (<500 μm in diameter) have developed mild hypoxia not yet severe enough to reduce Pimonidazole (Carrera et al., 2014).

Activation of hypoxia signaling is associated with numerous alterations to cell activity including the kinetics of glucose metabolism and energy production (Eales et al., 2016; Parks, Cormerais, & Pouyssegur, 2017). Among these changes, HIF-1α dependent increases in glycogen storage
has recently been reported to be a mechanism to maintain cell survival and proliferation during hypoxia in multiple cell types (Favaro et al., 2012; Pescador et al., 2010; Riganti, Gazzano, Polimeni, Aldieri, & Ghigo, 2012). With the development of hypoxia in the MCTS core, it is likely that inner spheroid cells activate such survival signaling pathways. The accumulation of glycogen in the hypoxic core of MCTS was determined through Periodic Acid and Schiff's base (PAS) staining (Fig. 5C). In small spheroids HIF-1α (but not Pimonidazole positive) spheroids we observed PAS staining in one of two patterns: sporadically present throughout the small sphere or regionally increased in the spheroid core. In larger spheres, PAS staining was consistently observed in the hypoxic and perinecrotic cell layers. Interestingly the largest spheres (>650 μm) showed PAS staining throughout the viable cell layers indicating a global metabolic shift. PAS staining was confirmed to be representative of glycogen by performing an amylase digestion step prior to PAS staining which resulted in the elimination of positive glycogen staining observed in parallel sections. Taken together, these results confirm the presence of a hypoxic core in spheroids approximately 570 μm in diameter and demonstrate the ability of MCTS to replicate HIF dependent survival mechanisms in a regionally specific manner.

These results show that the relationship(s) between spheroid size and the onset of hypoxia and necrosis are similar in both A673 and LLC spheroids. In the more detailed analyses described below, some studies were restricted to A673, since these cells form more robust and reliable spheroids.

**DDR activation occurs within proliferating cells in the hypoxic MCTS core**

Previous studies have noted the thickness of the outer viable cell layer in MCTS remains relatively constant as the spheroid grows, while the necrotic core increases proportionally with the size of the spheroid (Groebe & Mueller-Klieser, 1996). The viable layer is comprised of an outer proliferative rim that has the best access to nutrients and oxygen while the inner cell layers consist
of quiescent, non-proliferative but viable cells. This viable rim is approximately 100 μm thick in both A673 and LLC spheroids (Fig. 5B).

To identify the distribution of actively proliferating cells, Ki67 immunostaining (present in all phases of the cell cycle except G0) was monitored as a function of spheroid size (Fig. 6A). Cell proliferation was present uniformly throughout small (>500 μm) spheroids, but was more restricted to the cells near the spheroid surface once the diameter exceeded 570 μm. This transition appears to coincide with the onset of severe hypoxia and necrosis in the central core. EdU staining, which marks cells that have successfully proceeded through S-phase of the cell cycle, was concentrated in a narrow band along the spheroid surface (Fig. 6A). Compared with Ki67, EdU was in a much more restricted zone. Such a difference between Ki67 and EdU immunoreactivity is usually indicative of slowed or hindered progression through the cell cycle. Hypoxic conditions have been shown to increase the amount of single stranded DNA due to slowed or stopped replication fork progression (Hammond et al., 2002). This was supported by immunostaining for the single stranded DNA binding protein Replication Protein A subunit 2 (RPA-2) which revealed a strong enrichment in the viable core of spheroids at all sizes (Fig. 6B). Increased expression of RPA-2 has been reported in response to multiple stimuli including DNA damage or ongoing replication; therefore RPA-2 staining, while compelling, is not a conclusive marker of replication stress (Wold, 1997; Yin et al., 2013). Taken together this data strongly implies proliferating hypoxic cells are experiencing replication stress or other such stimuli which lead to failed progression through S-phase. It follows that prolonged exposure to these hypoxic conditions is cytotoxic as indicated by robust staining of the apoptotic marker cleaved caspase 3 in the core of growing spheroids (Fig. 6B). Development of an apoptotic core parallels the development of necrosis (Fig. 3B).
Hereafter, we have narrowed our studies to exclude the largest spheroid size owing to the high degree of cell death, difficulty in maintaining spheroid integrity, and the lack of clearly defined hypoxia response gradients.

**Hypoxia induces DDR signaling in proliferating cells**

The repression of proliferation and enrichment of RPA-2 in the hypoxic core of spheroids is consistent with hypoxia induced replication stress or DNA damage, both of which would be predicted to activate DDR pathways. Immunostaining for nuclear γ-H2AX and 53BP1 was used to monitor activation of DNA damage repair signaling in growing spheroids (Fig. 7). γ-H2AX positive nuclei were present throughout 500 μm spheroids (Fig. 7A,B). It is likely that low levels of replicative stress that occur stochastically during the cell cycle could account for this. Replicative stress leads to stalled replication forks, which trigger a genomic surveillance program that is driven by the ATR kinase (Hammond et al., 2002). Unresolved replication stress can lead to stalled forks and double stranded DNA breaks. In larger spheroids two distinct regions of γ-H2AX staining were seen: one prominent band of γ-H2AX positive cells was present in the central hypoxic area after necrosis was observed, and another smaller population of γ-H2AX positive cells was present on the surface of the spheroids. Immunostaining of parallel sections within a single spheroid demonstrated the regional correlation between severe hypoxia development (HIF-1α and Pimonidazole), decreased EdU incorporation, and formation of γ-H2AX (Fig. 7). Further analysis of DDR activation was done through 53BP1 staining which was observed in a similar pattern to γ-H2AX and notably enriched in the perinecrotic cell layers (Fig. 8). This pattern of γ-H2AX and 53BP1 lead to the conclusion that genotoxic stress, and subsequently activation of DDR proteins is occurring in the hypoxic spheroid core.

It is reasonable to hypothesize that the central area of γ-H2AX staining is a result of hypoxia-induced activation of the ATR/ATM kinases. To this point, extensive phosphorylation of ATM
(pSer1981) was seen in the central, non-necrotic regions of 570 – 650 μm A673 and LLC spheroids, albeit to a lesser extent in LLC (Fig. 8). In contrast, no positive staining was observed for pSer428-ATR. We were further unable to observe positive signal for the indirect marker of ATR activity, pSer345-Chk1. This pattern of ATM activation suggests that ATM is activated in the core of A673 and LLC MCTS in response to hypoxia.

There is compelling evidence that hypoxia by itself does not lead to substantial DNA damage (Comet assay detectable) (M. Olcina et al., 2010; Wilson & Hay, 2011) and that hypoxia-induces a DDR wherein the ATR/ATM kinases become activated in proliferating cells to phosphorylate downstream targets, like γ-H2AX, leading to stabilization of stalled replication forks (Hammond et al., 2014; M. Olcina et al., 2010; M. M. Olcina et al., 2014). Despite severe hypoxia, Ki67 positive cells were clearly visible within the hypoxic cell layers (Fig. 6). These proliferating hypoxic cells are likely experiencing replication stress and would therefore be expected to activate DDR signaling. To establish whether γ-H2AX formation in the hypoxic spheroid core occurred in proliferating cells, spheroids were co-stained for Ki67 and γ-H2AX (Fig. 9). Extensive overlap was observed between the two markers in both A673 and LLC MCTS leading to an important conclusion that MCTS recapitulate a hypoxic environment in which proliferating cells have activated DDR signaling. The survival and proliferation of hypoxic cells in vivo is negatively correlated to patient outcome and the mechanisms of cell survival under these conditions are not fully understood, partially due to the shortcomings of current models (Evans et al., 2001; Evans et al., 2010). Having demonstrated the ability of MCTS to recreate proliferating hypoxic cells which are activating survival signaling, we then sought to further characterize the DDR response and evaluate the therapeutic potential of targeting these pathways.
Hypoxia induces DDR protein activation in MCTS

In addition to hypoxia, the MCTS core has been reported to accumulate metabolic waste, contain a high interstitial fluid pressure, and to experience glucose deprivation (Bloch et al., 2014; Daster et al., 2017; Hirschhaeuser et al., 2010). To specifically determine if hypoxia in the MCTS model is responsible for activation of DDR signaling, small (<500 μm) spheroids not yet containing severe hypoxia where incubated in 1% O₂ for 12 hours followed by immunofluorescent analysis (Fig. 10). In response to hypoxia, proliferation (Ki67) was unaffected however glycogen storage (PAS staining) was prominently shifted to the outer most cell layers confirming that spheroids have engaged in an adaptive response to hypoxia under these conditions similar to what is observed in the core of larger spheres (Fig. 5C). During hypoxic culture, a significant increase in DDR protein (γ-H2AX, pSer1981-ATM, 53BP1, and RPA-2) accumulation was observed in the spheroid core (Fig 10). This increase was observed prominently amongst inner cell layers rather than on surface cells. We have previously shown that spheroids of this size contain an oxygen gradient such that HIF-1α is stabilized in the inner most cell layers (Fig. 5). The regionalization of DDR signaling in hypoxic spheroids implies that 1% O₂ is not sufficient to induce DDR signaling while the inner most, severely hypoxic cell layers have crossed the threshold for such a response.

It would be expected therefore that incubation of monolayer cultures in 1% O₂ would have no effect on DDR signaling.

In contrast, monolayers displayed significant increases in γ-H2AX during hypoxia treatment as determined by immunoblotting and immunofluorescence (Fig. 11). In response to hypoxia γ-H2AX staining appeared in foci which are indicative of DNA strand breaks. Indeed, use of the alkaline comet tail assay confirmed significant increases in DNA strand breaks (Fig. 11). Taken together these results indicate that oxygen deprivation elicits the activation of DDR proteins in both monolayer and 3D culture, however the oxygen threshold for such a response appears to be lower
in 3D cultures. It can be concluded from these studies that the regional increase of DDR proteins in pimonidazole positive spheroids is a direct effect of oxygen deprivation.

**Failure to identify contribution of ROS to MCTS DDR signaling**

Severe hypoxia is known to increase the levels of ROS within cells. To determine if ROS contribute to MCTS DDR activation, spheroids were cultured with the cell permeable compound CM-H$_2$DCFDA. This compound readily diffuses across cell membranes where, following removal of acetate groups by intracellular esterases, the compound CM-H$_2$DCF can bind glutathione species and subsequently be oxidized by ROS species to produce the fluorescent DCF product. Treatment of MCTS with CM-H$_2$DCFDA resulted in prominent staining of the outer most cell layers with little to no staining occurring more than 3 cell layers deep (Fig. 12). Although these results suggest there are no ROS species within the MCTS core, they are not conclusive. There are several possibilities which could explain the lack of fluorescence in the spheroid core including a failure of the dye to penetrate into the inner most cell layers.

In an attempt to address this possibility, spheres were incubated with H$_2$O$_2$ concurrent with CM-H$_2$DCFDA treatment. To determine if H$_2$O$_2$ treatment penetrated into the spheroid core, MCTS were probed for γ-H2AX as an indirect marker of H$_2$O$_2$; oxidative stress following H$_2$O$_2$ treatment increases γ-H2AX signal in similar studies (Dugan LL, 1999). Predictably H$_2$O$_2$ induced a distinct increase in γ-H2AX amongst MCTS cell layers, however no change in DCF fluorescence was observed (Fig. 12). This result could be interpreted as evidence for failed penetration of CM-H$_2$DCFDA into the spheroid core. Alternatively, the concurrent treatment of H$_2$O$_2$ with CM-H$_2$DCFDA could result in the quenching of the available reporter by H$_2$O$_2$ thus limiting the dyes ability to penetrate into the innermost spheroid cell layers. Taken together, these results cannot be used to determine the contribution of ROS in the induction of DDR signaling during hypoxia.
High levels of ROS result in oxidation of the DNA backbone, most commonly at 2-deoxyguansine residues which produces 8-oxo-2-deoxyguanosine (8-Oxo-dG) (Nikitaki, Hellweg, Georgakilas, & Ravanat, 2015). We reasoned that immunohistochemical labeling of 8-Oxo-dG in MCTS sections would label regions of spheroids that are experiencing, or have recently experienced high levels of ROS. As was the case with CM-H$_2$DCFDA, an inability to observe increased signaling with H$_2$O$_2$ treated spheroids prevented our ability to determine the distribution of ROS within MCTS.

To indirectly test the contribution of ROS to MCTS DDR signaling, A673 MCTS were treated with N-acetylcysteine (NAC). NAC has been widely used as an antioxidant due to its ability to restore cellular glutathione levels (Rushworth & Megson, 2014). Treatment of MCTS approximately 500 μm in size with 1 mM and 10 mM NAC resulted in a dose dependent decrease in γ-H2AX levels. While these results can be interpreted as evidence for ROS induced γ-H2AX, it is also possible that NAC reduced γ-H2AX through other means. Decades of research have indicated that NAC itself is a poor antioxidant and primarily neutralizes intracellular ROS through its contribution to glutathione synthesis (Rushworth & Megson, 2014). While NAC has been considered an antioxidant, a series of studies have noted additional effects from NAC treatment that alter cell signaling including the disruption of disulfide bonds present within proteins when used at concentrations as high as 10 mM (De Flora, Izzotti, D’Agostini, & Balansky, 2001; Rushworth & Megson, 2014). For these reasons, it is difficult to conclude that NAC treatment reduces γ-H2AX through the reduction of ROS levels.

In total, we were unable to conclusively determine the distribution of ROS within MCTS due to the lack of a sufficient indicator of ROS production within the MCTS core. Disruption of the spheroid to gain access to the inner cell layers would result in oxidative stress owing to reoxygenation of the hypoxic core and would therefore not provide a reliable assessment of endogenous MCTS
ROS levels. Therefore we conclude that it is not yet clear to what extent ROS contribute to MCTS DDR activation.

**Cell specific ATM and ATR contribution to hypoxia induced DDR signaling**

Having established that hypoxia is inducing in A673 and LLC MCTS, we next set out to determine the primary kinases involved in this response and the effect of small molecule inhibitors targeting specific DDR pathways. Results in Fig. 8 and Fig. 10 imply ATM has a prominent role in the hypoxia induced DDR signaling. The contribution of ATM and ATR kinases to 3D growth and DDR response to hypoxia was assessed using small molecule inhibitors specific for ATM (KU55933) or ATR (VE-821) (Charrier et al., 2011; Hickson et al., 2004). Spheroids were incubated with respective inhibitors for 5 days, spanning the growth period from small (<500 μm) pimonidazole negative (start of treatment) spheres to large (>800 μm) pimonidazole positive spheres. A673 MCTS growth was suppressed by approximately 25% in the presence of either ATM or ATR inhibitors such that vehicle treated spheres grew to a diameter of 860 μm whereas KU55933 or VE-821 treated spheres plateaued in growth (at 660 μm) after 48 hours (Fig. 13E). Assessment of γ-H2AX following 72 hour inhibitor treatment revealed a significant decrease in γ-H2AX throughout all cell layers following ATM, but not ATR inhibition (Fig. 13 A,D). These results are consistent with ATM being the primary kinase upstream of hypoxia induced γ-H2AX and suggest non-redundant contributions of each kinase to spheroid growth. To this point, ATR inhibition significantly decreased the proliferative index (Ki67+ nuclei) which was unaffected in ATM inhibited spheres (Fig. 13 B,D). Both inhibitors increased cleaved caspase 3 staining (Fig. 13 C,D). Together these data indicate that both ATM and ATR kinases contribute to 3D growth and viability of tumor cells in a 3D environment through separate mechanisms such that ATM critically acts upstream of γ-H2AX formation whereas ATR has a more prominent role in regulation of cell cycle progression.
To confirm that ATM kinase phosphorylates H2AX in response to hypoxia, small A673 spheroids (<500 μm) were cultured with the ATM inhibitor KU55933 during 12 hours of hypoxia (1% O₂) exposure. As before, hypoxia induced γ-H2AX in A673 spheroids treated with vehicle and ATM inhibition suppressed γ-H2AX induction (Fig. 14). Monolayer experiments in 2D cultures were subject to PI3KK inhibitor treatments and exposed to mild hypoxia (1% O₂) (Fig. 15). As in spheroids, mild hypoxia induced significant increases in γ-H2AX formation, however upon ATM inhibition, A673 cells had significantly less γ-H2AX induction (Fig. 15).

ATR inhibition during hypoxia treatment was capable of suppressing hypoxia induced γ-H2AX formation as well. This result contrasts with observations in 96 hour treated spheres where ATR inhibition had no effect. Previous reports have demonstrated that ATR activation under hypoxia is decreased after prolonged exposure to hypoxia (M. Olcina et al., 2010). Therefore it is possible that the abrupt onset of severe hypoxia brought on by incubation in 1% O₂ triggers activation of ATR which is absent when spheroids slowly develop severe hypoxia over multiple days of culture. Inhibition of ATR in monolayer cultures revealed a significant increase in γ-H2AX which was further exaggerated under hypoxia. This increase differs from spheroids where ATR inhibition had suppressed hypoxia induced γ-H2AX formation and supports previously reported conclusions that tumor cell drug response is dependent on culture conditions.

In aggregate our data show that hypoxia in both monolayer and spheroid culture induces a survival mechanism wherein ATM is activated and subsequently forms γ-H2AX in proliferating hypoxic cells. The contribution of ATR to this process however is context dependent such that monolayer cultures rely on ATR to prevent accumulation of DNA damage under hypoxia whereas MCTS do not.
Discussion

The maintenance of cell growth and viability within a solid tumor requires sufficient access to oxygen and nutrients. Oxygen deprived tumor micro-regions arise as a result of insufficient tumor perfusion which elicits the production of VEGF-A and other pro-angiogenic growth factors to stimulate neovascularization and tumor cell survival (Chung & Ferrara, 2011; Coleman & Ratcliffe, 2009). Accordingly, antiangiogenic therapies have been developed with promising success in the extension of progression free survival. Contrastingly, their impact on overall survival has fallen short of expectations as a result of tumor cell adaptations under hypoxia which lead to a more aggressive and therapeutically resistant tumor (Al-Husein et al., 2012). The mechanisms allowing tumor cells to survive severe hypoxia and undergo such a transformation are not fully understood, however several studies have suggested that both DNA damage repair (DDR) signaling and altered glycogen metabolism facilitate continued survival and proliferation under hypoxia (Favaro et al., 2012; Pescador et al., 2010; Wilson & Hay, 2011; Witney et al., 2014). While proliferation within the hypoxic environment is associated with a negative patient outcome, no consensus has been reached regarding the relationship between tumor oxygenation and proliferation (Evans et al., 2007; Evans et al., 2001; Evans et al., 2010; Marotta et al., 2011). Such a relationship and its connection to the activation of survival signaling can be studied in the controlled environment of a Multicellular Tumor Spheroid (MCTS). Our studies sought to determine the spatial and temporal relationship between DNA damage repair, glycogen storage, proliferation, hypoxia, and cell death using MCTS.

Typically chronic hypoxia within the tumor microenvironment is accompanied by the development of necrosis and the accumulation of both glycogen and γ-H2AX signal in perinecrotic cells (Banuelos et al., 2009; Evans et al., 2001; Favaro et al., 2012; Nagelkerke & Span, 2016; Olive et al., 2010). A similar relationship was observed in our MCTS where necrosis in the MCTS core...
developed concurrently with the accumulation of both glycogen and γ-H2AX in the perinecrotic, pimonidazole positive cell layers. Such a correlation indicates that hypoxia induces metabolic adaptations and DNA damage repair (DDR) signaling in the core of MCTS. Tumor glycogen storage has been used as a tool in the diagnosis of Ewing Sarcoma and an increase in glycogen storage related to cell survival under hypoxic conditions was previously described in vivo and in MCTS ("Ewing Sarcoma Treatment (PDQ(R)): Health Professional Version," 2002; Favaro et al., 2012; Pescador et al., 2010). Our observations of a spatial correlation between glycogen accumulation and HIF-1α stabilization in the spheroid core indicates the MCTS model is a relevant tool for the study of metabolic adaptations under hypoxia and in the development of therapeutics targeting these pathways.

Previous studies have described a relationship between hypoxia and DDR activation in monolayer cell culture wherein DDR signaling was induced by stalled replication forks under conditions of severe hypoxia (O₂<0.1%) (Hammond et al., 2003; M. M. Olcina et al., 2014). In an attempt to preserve DNA integrity tumor cells initiate a DNA damage response through the activation of ATM and ATR leading to the subsequent formation of γ-H2AX (M. Olcina et al., 2010). ATM and ATR belong to the PI3KK family of proteins known to regulate DDR signaling (Cimprich & Cortez, 2008; Fernandez-Capetillo, Lee, Nussenzweig, & Nussenzweig, 2004; Marechal & Zou, 2013; Rogakou, Pilch, Orr, Ivanova, & Bonner, 1998). Both proteins phosphorylate a number of overlapping substrates however each PI3KK protein is activated in response to specific stress conditions. ATR activity is stimulated by single stranded DNA resulting from replication or single strand breaks while ATM has been shown to be activated by both reactive oxygen species and double strand breaks (Marechal & Zou, 2013). A third PI3KK member (DNA-PK) also phosphorylates H2AX in response to hypertonic conditions or in the presence of apoptotic DNA fragmentation (Podhorecka, Skladanowski, & Bozko, 2010). Under hypoxia, ATR mediated DDR signaling is
likely the result of hypoxia induced single stranded DNA at stalled replication forks. The signal leading to ATM activation under hypoxia is not yet clear however some evidence suggests a combination of replication stress and chromatin modifications leads to ATM autophosphorylation (pATM) and activity (M. M. Olcina et al., 2014).

The activation of DDR signaling by stalled replication forks implies that an attempted proliferation by oxygen deprived cells will elicit this DDR signaling. The distribution of proliferating cells in MCTS has been previously described to occur as a gradient: outer cell layers are highly proliferative whereas perinecrotic cells can be proliferative or quiescent (Folkman & Hochberg, 1973; Freyer & Sutherland, 1986). It follows that attempted proliferation within the hypoxic core of spheroids would be predicted to accumulate γ-H2AX signal. Indeed co-staining with the proliferative marker Ki67 and DNA damage marker γ-H2AX revealed a significant overlap in the hypoxic core of MCTS linking hypoxic cell proliferation and DDR signaling in the 3D hypoxic environment. Ki67 is used clinically to determine the proliferative index of tumors due to its expression throughout the cell cycle and absence from G0 (Whitfield, George, Grant, & Perou, 2006). The observed absence of 5’-ethynl-2’-deoxyuridine (EdU) incorporation within hypoxic spheroid cell layers indicates the overlap of Ki67 and γ-H2AX within these regions may be the result of failed or slowed replication which is supported by observations of increased RPA-2 and 53BP1 signal under hypoxic conditions.

While we were unable to detect ATR activation despite its known role in unstressed replication (Cimprich & Cortez, 2008), the distribution of pATM in growing spheroids correlated to, and was induced by hypoxia development. This pattern of activation implies a potential role for ATM activity in DDR signaling within the hypoxic MCTS core. Consistent with this, pharmacological inhibition of ATM using the small molecule inhibitor KU55933 significantly reduced γ-H2AX signal in A673 cells whereas ATR inhibition had negligible effect on γ-H2AX levels. Both ATR and ATM inhibition
resulted in significant growth reduction of A673 MCTS; similar results have been reported in spheroids formed from glioblastoma cells (Pires et al., 2012). The activation of ATM within the hypoxic core is consistent with the presence of DNA breaks and is supported by monolayer experiments in which hypoxic culture resulted in substantial increases in DNA lesions.

The inhibition of ATR resulted in significant increases in γ-H2AX levels and in pan-nuclear γ-H2AX staining in A673 monolayers. Pan-nuclear γ-H2AX staining is associated with a pre-apoptotic state and has been observed following the loss of ATR activity previously (Bonner et al., 2008; Ester M. Hammond et al., 2004; Pires et al., 2012). This effect is particularly pronounced in cells experiencing replication stress or lacking P53 function (Nieto-Soler et al., 2016). The Ewing Sarcoma cell line used herein (A673) is reported to have high levels of replication stress due to the expression of the EWS/FLI1 transcription factor and is also reportedly P53 deficient (May et al., 2013). The combined presence of increased replication stress and decreased G1 checkpoint signaling may explain why hypoxic stress resulted in observable DNA damage and why these cells were highly sensitive to ATR inhibition. In addition to regulation of DDR, activation of ATR under hypoxia is known to regulate replication through cdc25-CDK2-APC/C^Cdh1 (Martin, Rainey, Santocanale, & Gardner, 2012). The loss of ATR activity during hypoxia may therefore result in unregulated replication origin firing leading to increased DNA damage; a similar trend was reported following ATR inhibition in glioblastoma cells (Pires et al., 2010). The lack of such a response in MCTS treated with the ATR inhibitor indicates that ATR may not be required to maintain DDR signaling in the 3D environment. This is consistent with genomic profiles of MCTS which have shown ATR expression levels to decrease when tumor cells are cultured as spheroids rather than monolayers (Ghosh et al., 2005). The difference in DDR response to ATR inhibition between monolayer cells and MCTS highlights the importance of recreating a 3D tumor environment when validating therapeutic targets.
Conclusions

From these studies we can conclude that MCTS represent a valuable in vitro model which recreates therapeutically relevant features of a hypoxic tumor microenvironment including the accumulation of glycogen and the presence of proliferative hypoxic cells. Using this in vitro model system we show that ATM, but not ATR, is the primary kinase responsible for γ-H2AX formation in the hypoxic core of A673 MCTS, and that targeting either ATM or ATR kinase inhibits spheroid growth, albeit through different mechanisms. We further show that DDR protein activation in response to hypoxia can vary amongst cell lines and that results obtained in MCTS are both complimentary and distinct from monolayer studies. MCTS therefore offer a unique opportunity to study the efficacy of therapeutics targeting cells known to adapt under hypoxic conditions and to subsequently evade conventional therapy.

Figure Legends

**Figure 4.** Growth Characteristics of A673 and LLC MCTS. **a.** Growth curves of A673 (red) and LLC (black) MCTS. The mean diameter of >8 spheroids is plotted with standard deviation to represent MCTS progression from initial formation to final size before collapse. **b.** H&E staining of MCTS cryosections taken from spheres after formation (< 500 μm), during exponential growth phase (550-650 μm) and at maximal size (>750 μm) reveals the development of necrosis in spheres with diameter larger than 500 μm. Shrinkage during processing accounts for smaller diameter during final image analysis.

**Figure 5.** Hypoxia development and glycogen accumulation in growing MCTS. **a.** Immunolabeling of A673 MCTS cryosections for the stable expression of HIF-1α (red), most often associated with decreased oxygen levels, reveals the presence of oxygen gradients in all stages of MCTS growth. **b.** A673 and LLC MCTS cryosections immunolabeled for the hypoxia marker pimonidazole (red).
Retention of this marker occurs in the core of spheroids larger than 500 μm indicating the development of hypoxia with oxygen levels below 1.3%. Immunolabeled sections were counterstained with Hoechst 33342 for nuclear identification. c. Periodic Acid and Schiff’s Base staining (dark pink) demonstrate glycogen storage in A673 and LLC MCTS cryosections.

**Figure 6.** Proliferation and apoptosis in A673 MCTS. a. Cryosections obtained from MCTS spheroids at different sizes were immunostained for the proliferation markers Ki67 (red) and EdU (green) in adjacent sections. b. Representative images of A673 MCTS stained for the single stranded DNA binding protein subunit RPA-2 (red) and the apoptotic marker Cleaved Caspase 3 (green). Hoechst 33342 (blue) counterstain was used in all immunolabeled images for nuclear identification.

**Figure 7.** γ-H2AX formation in A673 and LLC MCTS. a. Representative images of A673 MCTS demonstrates activation of DNA damage repair signaling as determined by nuclear γ-H2AX (red). b. Representative images of LLC MCTS cryosections stained for γ-H2AX (red) reveals a similar pattern of DNA damage repair signaling to that observed in (a). Hoescht 33342 (blue) was used to determine nuclear localization of γ-H2AX signal.

**Figure 8.** ATM activation and accumulation of 53BP1 in MCTS core. a. Representative images showing the detection of ATM activation through auto-phosphorylation of ATM at serine-1981 (pATM, red) in A673 and LLC MCTS approximately 570 μm in diameter. b. Detection of the DNA damage repair protein 53BP1 (red) in an A673 MCTS cryosection obtained from a spheroid approximately 600 μm in diameter. This image is representative of one experiment (5 spheroids). Hoescht 33342 (blue) labels nuclei in both (a) and (b).

**Figure 9.** Coincidence of γ-H2AX, proliferation, and hypoxia in A673 and LLC MCTS. a. γ-H2AX was used as a marker of DNA damage repair signaling. Parallel cryosections from a
representative A673 spheroid approximately 570 μm in diameter demonstrated DNA damage repair signaling within the MCTS hypoxic core through immunostaining adjacent sections of the same spheroid for γ-H2AX (left most red) and the hypoxia markers Pimonidazole (green, Pim) and HIF-1α (right most red). Inner dotted line indicates the necrotic border. Outer dotted line represents the outer most limit of hypoxia signal. b. Representative images of cryosections from A673 and LLC MCTS approximately 570-650 μm in diameter co-labeled for the proliferation marker Ki67 (green) and the DNA damage repair protein γ-H2AX (red). Hoescht 33342 (blue) was used to identify nuclei.

**Figure 10.** DNA damage repair signaling and glycogen storage is promoted by hypoxia. a. The effect of maintaining spheroids (approximately 400-500 μm in diameter) in hypoxic conditions (1% O₂) for 12 hours was monitored. Representative images of A673 MCTS stained for DNA damage repair proteins (in descending order from top to bottom) γ-H2AX, 53BP1, pATM, and RPA-2. Ki67 immunostaining indicated no change in proliferation between hypoxia and normoxic spheroids after 12 hours. Hoescht 33342 staining (blue) was used to identify nuclei. Glycogen storage under hypoxia was assessed using PAS (dark pink) staining. b. The percentage of marker positive nuclei in normoxia (black bar) and hypoxia (striped) is represented. The mean percentage of positive nuclei from >10 spheroids and two independent experiments with standard deviation is plotted. † indicates quantification is representative of 8 spheroids. * indicates p<0.05 using two tailed students T-test. N.S. indicates no significance detected.

**Figure 11.** DNA damage repair signaling is promoted by hypoxia in monolayers a. Hypoxic incubation (1% O₂ for 12 hours) induced DNA damage repair signaling in A673 monolayer cells was assessed using γ-H2AX (red) staining with Hoescht 33342 (blue) nuclear stain. b. Immunobloting of A673 monolayer lysate confirms an increase in γ-H2AX levels under hypoxia (1% O₂). c. Alkaline comet tail assay demonstrated increased tail length during 12 hour incubation
with 1% O$_2$. * indicates $p<0.05$ by two tailed students T-test. N.S. indicates no significance detected. Immunobloting and comet tail assays were performed by Naresh Pandey.

**Figure 12.** Inconclusive results with CM-H$_2$DCFDA staining in A673 MCTS. **a.** Treatment of A673 MCTS (approximately 400-500 μm in diameter) with 500 μM H$_2$O$_2$ for 1.5 hours resulted in significant increases in γ-H2AX signal (red). **b.** Co-treatment of MCTS with the ROS reporter CM-H$_2$DCFDA (green) and H$_2$O$_2$ showed no change in reporter fluorescence localization. **c.** MCTS treated with the ROS scavenger N-Acetyl Cysteine at indicated dosages for 6 hours resulted in a significant decrease in γ-H2AX signal (red) however these results are representative of 1 experiment with approximately 6 spheroids. **d.** Bar graph representing the average percentage of nuclei (blue) in (c.) with γ-H2AX signal (red). * indicates $p<0.05$ using two tailed students T-test. N.S. indicates no significance detected. Significance determined based on one experiment.

**Figure 13.** ATM is linked to γ-H2AX formation and ATR regulates MCTS proliferation with both proteins contributing to cell viability. **a.** Proliferation in A673 MCTS was determined through Ki67 staining (red with blue Hoescht 33342 nuclear counterstain) following 96 hour incubation with vehicle (0.01% DMSO), the ATM inhibitor KU55933 (10 μM) or the ATR inhibitor VE-821 (2.5 μM). Spheroid diameters were less than <500 μm at the time of drug addition and were assessed 96 hours later at an approximate diameter of 700 μm. **b.** The distribution of γ-H2AX (red with blue Hoescht 33342 nuclear counterstain) in MCTS following 96 hour incubation with the ATM inhibitor KU55933 or the ATR inhibitor VE-821. **c.** Cell death in response to 96 hour inhibition of ATM or ATR was monitored with cleaved caspase 3 staining (green with blue Hoescht 33342 counterstain). **d.** Quantification of spheroids represented by parts (a), (b), and (c). Bar graphs represent percentage (mean and standard deviation) of positive cells in at least >15 spheroids and 3 independent experiments. Cleaved caspase 3 integrated density was normalized to spheroid area for comparison. **e.** The effect of ATM (blue) and ATR (red) inhibitors on A673
MCTS growth over time (hours). * indicates p<0.05 using two tailed students T-test. N.S. indicates no significance detected.

**Figure 14.** ATM and ATR regulate hypoxia induced γ-H2AX formation in A673 MCTS. a. The effect of ATM and ATR inhibition in A673 MCTS (<500 μm in diameter) subject to 12 hour incubation with 1% O₂ was determined. ATM and ATR inhibition prevented hypoxia induced γ-H2AX (red with blue Hoescht 33342) formation b. Quantification of γ-H2AX staining in A673 MCTS. Bar graphs represent the percentage (mean and standard deviation) of positive nuclei in at least >8 spheroids and two independent experiments. * indicates p<0.05 by two tailed students T-test. N.S. indicates no significance detected.

**Figure 15.** Hypoxia induces DNA damage in A673 monolayers which correlates with ATM regulated γ-H2AX formation. a. Quantification of results shown in (b.) representing A673 monolayers subjected to 12 hour hypoxia in the presence of ATM inhibitor KU55933 or ATR inhibitor VE-821. Monolayers were immunostained for γ-H2AX (red with blue nuclear counterstain). Bar graphs represent the average percentage of nuclei with 5 or more γ-H2AX foci (with standard deviation) in 3 independent experiments. * indicates p<0.05 by two tailed students T-test. N.S. indicates no significance detected.
Figures

Figure 4. Growth Characteristics of A673 and LLC MCTS
Figure 5. Hypoxia development and glycogen accumulation in growing MCTS

A. <500µm  550-650µm  >750µm

B. A673  100 µm

C. <500µm  550-650µm  >750µm

A673  100 µm

LLC
Figure 6: Proliferation and apoptosis in A673 MCTS

A. <500\mu m

- Ki67

- EdU

100 \mu m

B. 550-650\mu m

- RPA-2

- CC3

>750\mu m
Figure 7: γ-H2AX formation in A673 and LLC MCTS

A. < 500 μm  550-650 μm  > 750 μm
B. < 500 μm  550-650 μm  > 750 μm

Figure 8: ATM activation and accumulation of 53BP1 in MCTS core

A. pATM
B. 53BP1
Figure 9: Coincidence of γ-H2AX, proliferation, and hypoxia in A673 and LLC MCTS

A. γ-H2AX  Pim  HIF-1α

B. A673
   Ki67  γ-H2AX

LLC

100 μm
Figure 10: DNA damage repair signaling and glycogen storage is promoted by hypoxia
Figure 11: DNA damage repair signaling is promoted by hypoxia in monolayer culture

Figure 12: Inconclusive results with CM-H₂DCFDA staining in A673 MCTS
Figure 13: ATM is linked to γ-H2AX formation and ATR regulates MCTS proliferation with both proteins contributing to cell viability

Figure 14: ATM and ATR regulate hypoxia induced γ-H2AX formation in A673 MCTS
Figure 15: Hypoxia induces DNA damage in A673 monolayers which correlates with ATM regulated γ-H2AX formation
CHAPTER 3: TARGETING THE EYA-PTP TO PREVENT HYPOXIC TUMOR CELL DDR AND VASCULOGENIC MIMICRY

The majority of solid tumors develop hypoxia to some extent which can be further exacerbated by therapeutic treatment (Hockel & Vaupel, 2001). Prolonged exposure to this environment is cytotoxic; however, tumor cells have adaptive mechanisms to increase cell survival, retain a proliferative capacity, and decrease hypoxic stress through the stimulation of angiogenesis and DNA damage repair (DDR) pathways (Coleman & Ratcliffe, 2009; M. M. Olcina et al., 2014). The ability of aggressive tumor cells to survive hypoxic conditions is further enhanced by inherent properties of an oxygen deprived environment which affords chemo- and radiation-resistant properties to hypoxic cells (Karakashev & Reginato, 2015; Liu, Lin, & Yun, 2015; Minchinton & Tannock, 2006; Pinto et al., 2016; Wilson & Hay, 2011). For these reasons, tumor hypoxia is a negative prognostic factor and considerable effort has been invested into understanding mechanisms of hypoxic tumor cell survival.

In the absence of angiogenesis, rapid tumor growth generates hypoxic tumor regions which suppresses tumor cell proliferation and ultimately leads to cell death if oxygenation is not restored (Hammond et al., 2014; Wilson & Hay, 2011). Oxygen deprived cells rely on DDR signaling to maintain viability (Scanlon & Glazer, 2015). Recent studies point to the activation of a DDR signaling cascade under hypoxia whereby ATM and ATR become activated to phosphorylate H2AX (forming γ-H2AX) at stalled replication forks leading to prolonged survival and proliferative viability (M. Olcina et al., 2010; M. M. Olcina et al., 2014). Identifying proteins involved in this response and the value of targeting such proteins is a topic of ongoing research. Tumor cells have been shown to survive hypoxia through multiple additional adaptations including the stimulation of neovascularization via canonical (angiogenesis) and non-canonical (vasculogenic
mimicry, VM) methods (Hockel & Vaupel, 2001; Maniotis et al., 1999). During VM hypoxia triggers a transendothelial phenotypic switch wherein tumor cells begin to express a combination of tumor, developmental, and endothelial markers (Seftor et al., 2012). These de-differentiated cells are then able to invade the ECM and form tubular structures capable of conducting blood and plasma, thereby perfusing an otherwise hypoxic region. Accumulating evidence suggests VM is one method by which tumor cells circumvent anti-angiogenic therapy and has primarily been observed in highly aggressive tumor types. These associations have encouraged research into VM as a potential therapeutic target in aggressive cancers (Schnegg, Yang, Ghosh, & Hsu, 2015).

The Eyes Absent (EYA) family of proteins has recently been identified as a therapeutically relevant target in several malignancies (Blevins, Towers, Patrick, Zhao, & Ford, 2015). The EYA tyrosine phosphatase (EYA-PTP) is unique in that EYA proteins utilize an aspartate nucleophile to catalyze tyrosine dephosphorylation (J. P. Rayapureddi, Kattamuri, Chan, & Hegde, 2005; Jayanagendra P. Rayapureddi et al., 2003). This atypical mechanism has allowed the development of small molecules targeted against the EYA-PTP activity (Pandey et al., 2013; Tadjuidje et al., 2012). Salient to tumor hypoxia, the EYA-PTP is known to be involved in tumor cell invasion and to regulate cell survival during developmental hypoxia through unknown substrates (Cook et al., 2009; Pandey et al., 2010; Tadjuidje & Hegde, 2013). Preliminary data indicate the EYA-PTP may be involved in both VM and tumor cell DDR in vivo. Based on these findings, we set out to determine if EYA proteins were involved in two tumor cell adaptations under hypoxia: DDR signaling and VM. Using two dimensional (2D) monolayer cultures, we found EYA-PTP inhibition prevented 2D VM. We then assessed the presence of VM in the three dimensional (3D) MCTS model but concluded that no VM was occurring. DDR within MCTS was disrupted following EYA-PTP inhibition such that γ-H2AX could be observed in all cell layers which contrasted with control spheroids where γ-H2AX was localized to the central core. Collectively
these studies validate EYA-PTP as a unique target capable of inhibiting multiple tumor cell adaptive responses under hypoxic conditions.

**Materials and Methods**

**Cell lines**

Human Ewing Sarcoma A673 (CRL-1598; ATCC, Manassas, VA); Mouse Lewis Lung Carcinoma LLC (CRL-1642; ATCC, Manassas, VA); Mouse B16-F10 (CRL-6475; ATCC, Manassas, VA); Human 1205Lu\(^1\) (RRID:CVCL_5239, Coriell Institute, Camden, New Jersey ); Human WM1366 (RRID:CVCL_6789, Coriell Institute, Camden, New Jersey).

**Antibodies and reagents**

Dulbecco’s Modified Eagles Medium (DMEM) (11,965,092; Thermoscientific, Waltham, MA); MCDB-153 Medium (M7403, Sigma-Aldrich, St. Louis, MO), L-15 Medium Leibovitz (L4386, Sigma-Aldrich, St. Louis, MO); Fetal Bovine serum - FBS, (TMS-013-B; Millipore, Billerica, MA); Agar (J637; Amresco, Solon, OH); DMSO (67–68-5, Sigma, St. Louis, MO); Matrigel (354230, Corning, Bedford, MA) ; Benzaron was obtained from Sigma Aldrich (St. Louis, MO) and stored as a 100mM stock in DMSO (Sigma)

The following antibodies were used in these studies: mouse anti-γ-H2AX (JBW301; Millipore, Billerica, MA), rabbit anti-Ki-67 (MA5–1452; Thermoscientific, Waltham, MA), rabbit anti-cleaved caspase-3 (5A1E; Cell Signaling Technology, Danvers, MA), goat anti-mouse IgG (H + L) Alexa Fluor 647 (A-21235; Thermoscientific, Waltham, MA), donkey anti-rabbit Alexa Fluor 594 (A-21027; Thermoscientific, Waltham, MA), rat anti-CD31 (553369, BD Bioscience, San Jose, CA),

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\(^1\) This cell line has since been discontinued by the distributor due to mouse cell line contamination
anti-Laminin (MA1-21194, Thermoscientific, Waltham, MA), mouse anti-VE-Cadherin (SC52751, Santa Cruz, Santa Cruz, CA).

**Cell Culture**

A673, B16, and LLC were maintained in standard culture conditions (37 °C with 5% CO₂) in DMEM with 1% v/v penicillin (100 IU ml⁻¹) and streptomycin 100 mg ml⁻¹ supplemented with 10% v/v heat inactivated FBS. 1205LU and WM1366 were maintained in MCDB-153 Medium with 25% (v/v) Leibovitz medium, 14 mM Sodium Bicarbonate, 1% v/v penicillin (100 IU ml⁻¹) and streptomycin 100 mg ml⁻¹ supplemented with 10% v/v heat inactivated FBS to a final pH of 7.4.

**MCTS culture**

Spheroids were formed through use of the liquid overlay method as described in Chapter 2. In experiments with Benzarone, spheroids were individually washed with DMEM and transferred to freshly coated agar plates with 200 μl of fresh medium containing test inhibitors when applicable.

**MCTS growth curves**

Growth curves for MCTS were generated as described in Chapter 2.

**Processing of MCTS for histological assessment**

Processing was performed as described in Chapter 2.

**Immunofluorescence analysis of MCTS sections**

Cryo-sections were blocked with antibody specific blocking buffer for 1 h at room temperature in a humidified chamber. Primary antibodies directed against γ-H2AX, Ki-67, CD31, Neuropilin-2, and VE-Cadherin were diluted between (1:50 and 1:200) in blocking buffer and incubated on sections for 1 h at room temperature or overnight at 4 °C. After washing with phosphate-buffered
saline containing 0.15% Triton X-100, sections were incubated for 1 h at room temperature with fluorescently tagged secondary antibodies diluted (1:200) in blocking buffer, followed by 20-min incubation with Hoechst dye to identify nuclei. Sections were washed and mounted using Fluorgel with DABCO (17985–04; Electron Microscopy Science, Hatfield, PA). Primary antibody dilution for Laminin was 1:50. Blocking buffer for γ-H2AX, Ki-67, and CD31 was phosphate-buffered saline supplemented with 10% FBS, 2% BSA, and 0.15% Triton X-100. Blocking for Neuropilin-2, VE-Cadherin, and Laminin was done with phosphate-buffered saline supplemented with 5% donkey serum and 0.15% Triton X-100.

**Tube formation assay**

Single cell suspensions of B16, LLC, or 1205LU were diluted in DMEM supplemented with 2% FBS. A673 cells were diluted in Endothelial Growth media supplemented with 2% FBS, bFGF (4ng/ml), VEGF (2ng/ml) and EGF (10ng/ml). Matrigel was thawed on ice for 3 hours prior to use. 40 μl of matrigel was added to each well of a 96 well plate and incubated for a half hour at 37 degrees Celsius. 100 μl of cell suspension was added to matrigel coated wells and incubated for the indicated times. Tube formation assays were performed by Naresh Pandey and Stephen Riffle.

**EYA inhibition**

Indicated concentrations of Benzaronine in DMEM supplemented with 2% FBS and containing 0.025% vv⁻¹DMSO were used. Fresh media containing inhibitors was added to MCTS cultures every 48 h. The vehicle control contained 0.025% vv⁻¹ DMSO in DMEM supplemented with 2% FBS.

**Image acquisition and analysis**
Fluorescently stained sections were imaged on a Zeiss confocal microscope at 0.25× magnification across a 1000 μm grid. To determine the number of positive nuclei per spheroid, nuclei were counted as Hoechst-positive cells by using watershed separation and quantification with particle analysis in ImageJ software [https://imagej.nih.gov/ij/] followed by cell counting to determine the percentage of marker positive nuclei. Cords in tube formation assay were quantified by NeuronJ analysis in ImageJ software [https://imagej.nih.gov/ij/]

Statistics
Results represent the average of at least 2 separate experiments with a total of at least 3 independent experiments and 8 spheroids per condition (where appropriate) ± standard deviation (SD). Statistical analyses were performed using a two tailed student’s t-test. Significance represents \( P < 0.05 \).

EYA-PTP inhibition prevents 2D vasculogenic mimicry

Given that EYA is known to regulate tumor cell invasion and survival under stress (Pandey et al., 2010; Robin et al., 2012), we hypothesized that inhibition of EYA proteins would impair the vasculogenic potential of aggressive tumor cells. To determine if EYA proteins are involved in VM, we monitored the ability of tumor cells to form tubes atop matrigel with or without the EYA-PTP inhibitor Benzarone (BZ). The human tumor cell lines A673, WM1366, and 1205LU were selected to represent different malignant backgrounds (Ewing sarcoma, nonmetastatic melanoma, and metastatic melanoma respectively). The mouse cell lines LLC and B16-F10 were also tested to allow for future comparisons to be done in genetically modified mouse models. Of the cell lines used, only the human melanoma cell line WM1366 was unable to form tubes. The failure to form tubes may reflect the non-aggressive background of this cell line which is derived from a primary melanoma reported as non-aggressive. Contrastingly, human cell lines from
melanoma (1205LU)\(^2\) and Ewing Sarcoma (A673) as well as murine cell lines from Lewis Lung carcinoma (LLC) and melanoma (B16-F10) were able to form robust tubes within 18 hours of seeding. A673 cells have been reported to be poorly-vasculogenic and previous studies reported that A673 cells did not form tubes atop matrigel (Pardali et al., 2011; van der Schaft et al., 2005). This may be reflective of growth factor availability which is supported by the observation that A673 cells did not form tubes in standard culture media (as used in previous experiments) but rather required endothelial growth media supplemented with bFGF (4ng/ml), VEGF (2ng/ml) and EGF (10ng/ml).

To ask if the EYA-PTP contributed to in vitro tube formation, cells were incubated with the EYA-PTP small molecule inhibitor BZ during the tube formation process. Incubation with BZ abrogated tube formation and resulted in focal aggregation of all cell lines tested (Fig. 16). The inability of tumor cells to form tubular structures in the presence of BZ suggests a role for EYA in the tube formation process. Given that EYA has previously been shown to regulate tumor cell invasion, it is likely that impairment of VM is due in part to decreased invasion. In support of this, it was observed that BZ inhibited cells took longer amounts of time to form tube like networks when compared to vehicle treated controls. (Fig. 17). Previous results have reported similar effects on tumor and endothelial cell lines (Pandey et al., 2010; Tadjuidje et al., 2012). Together these observations indicate that EYA proteins may contribute to VM. More detailed studies are required to determine the mechanism through which the EYA-PTP contributes to VM.

**VM is not observed in free floating MCTS**

A previous study reported the presence of laminin networks within melanoma spheroids which suggested that MCTS could be used to model VM (Larson et al., 2014). We previously

\(^2\) This cell line has since been discontinued by the distributor due to mouse cell line contamination
characterized the MCTS model as one in which a severely hypoxic core develops resembling in vivo avascular regions (Riffle et al., 2017). Such environments are also known to promote VM and have been described in Ewing Sarcoma among many other malignancies (Cao et al., 2013; van der Schaft et al., 2005). We attempted to identify VM in A673 and LLC MCTS that were approximately 550-650 μm in size due to the clearly defined presence of a hypoxic core. We failed to observe positive staining for markers of VM including CD31 and VE-Cadherin (Fig. 18). Laminin staining could be seen within these MCTS however there were no clearly discernable networks (Fig. 18). PAS staining has been used extensively to identify VM within patient samples (Cao et al., 2013). We previously reported the presence of PAS positive cell layers within the inner layers of spheroids which correlated with the development of hypoxia (see Chapter 2, Fig. 5) (Riffle et al., 2017). Incubation of spheroid sections with amylase enzyme significantly reduced PAS staining suggesting that the PAS stain was marking glycogen stores rather than extracellular matrix. Furthermore, MCTS PAS staining appeared to be ubiquitous throughout defined spheroid regions which contrast with VM in which looping networks of PAS channels is a defining characteristic (Maniotis et al., 1999). Finally, at no point could a lumen be identified within these PAS positive regions. Taken together, we conclude that no VM occurs in MCTS under the conditions tested.

It is possible that extracellular matrix (ECM) is required for VM and is not supplied in sufficient quantity through MCTS culture in the liquid overlay technique. Several ECM components are known to promote VM (Hendrix et al., 2002; Hess, Seftor, Seftor, & Hendrix, 2003; Seftor, Seftor, Kirschmann, & Hendrix, 2002; Seftor et al., 2001). Within melanoma spheroids in which VM was described, laminin networks could be seen in spheroids grown atop matrigel (Larson et al., 2014). Networks of laminin formed in a nestin dependent process which appeared to be continuous with the matrigel, suggesting that components within the matrigel may prompt the vasculogenic
process (Larson et al., 2014). To test this possibility, we formed B16 mouse melanoma spheroids atop matrigel. B16 MCTS contained similar PAS and laminin positive networks (Fig. 19). Both PAS and laminin appeared continuous with matrigel as in other studies (Larson et al., 2014). Laminin is a significant component in matrigel, therefore it’s possible that laminin within the MCTS is derived from matrigel (Hughes, Postovit, & Lajoie, 2010). H&E staining of similar spheroids was done to determine if luminal vessels were present (Fig. 19). Although rare, H&E staining revealed the presence of lumen like structures in these spheroids however parallel sections did not show a continuous lumen suggesting that these structures are not representative of a vascular network. This data is consistent with ECM networks existing within MCTS grown atop matrigel.

Similar studies were performed using LLC MCTS grown in the liquid overlay method and then transferred to matrigel once the spheroids had developed a hypoxic core. LLC MCTS were observed to invade the surrounding matrigel and form tube like, looping structures (Fig. 17). These structures contained filopodia at the leading tip and were PAS positive (Fig. 17). This tube forming ability of MCTS was eliminated in BZ treated cultures however it could not be determined whether this was due to the inhibition of migration or proliferation due to the length of time in which spheroids were exposed to BZ. Future studies would benefit from time course analysis to determine when tubes form followed by a limited treatment period during the time in which neovascularization takes place.

Collectively, our data indicate that VM like qualities in MCTS such as the formation of ECM networks and tube like structures is promoted by matrigel and that free floating spheroid culture is not sufficient to observe these phenomena.
EYA-PTP loss or inhibition in MCTS results in an impaired DDR

Previous work in our lab identified a role for the EYA-PTP in endothelial cell survival through the regulation of hypoxia induced DDR (Y. Wang et al., 2016). These studies showed that targeting the EYA-PTP was a successful method for preventing pathological angiogenesis in vivo which raised the possibility of using BZ as an antiangiogenic tumor chemotherapeutic. Tumors are known to experience severe hypoxia in response to antiangiogenic drug treatment which induces development of an aggressive tumor phenotype (Pinto et al., 2016). Furthermore the loss of vasculature hinders our ability to deliver therapeutic compounds to hypoxic tumor regions (Minchinton & Tannock, 2006). Loss of EYA-PTP activity has previously been shown to decrease tumor cell migration, proliferation, and survival (Pandey et al., 2010). Taken together, small molecule inhibitors of the EYA-PTP have the ability to inhibit tumor angiogenesis and to potentially decrease cell survival and aggression in the subsequent hypoxia.

To specifically determine the effect of EYA-PTP inhibition on tumor cells in the avascular niche, we utilized the MCTS model previously described wherein LLC or A673 MCTS develop oxygen gradients that culminate in a severely hypoxic, necrotic core bordered by growth impaired cells with active DDR signaling (Riffle et al., 2017). Severe hypoxia is absent from spheres 500 μm in diameter but develops before reaching 700 μm. CRISPR technology was used to knockout the Eya3 homologue from LLC cells (Fig. 20). This cell line predominantly expresses the Eya3 homologue and substantially less of the homologue Eya1, all of which have been correlated to tumor progression (Blevins et al., 2015). We reasoned the loss of Eya3 would result in impaired DNA damage repair based on the known role of EYA in regulating cell γ-H2AX formation following DNA damage (Cook et al., 2009). Knockdown of Eya3 was confirmed through immunoblotting.

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*This work was performed by Naresh Pandey of the Hegde Lab*
LLC Eya3 knockdown cells (hereafter abbreviated as LLC-CKO) were capable of forming MCTS whose growth paralleled that of wild type cells (Fig. 20B). Analysis of DDR signaling in LLC-CKO MCTS revealed a significant decrease in γ-H2AX positive nuclei (Fig. 20C, D). Together these results indicate that Eya3 regulates the formation of γ-H2AX within hypoxic and normoxic environments and contributes to tumor cell growth in 3D culture. The decreased formation of γ-H2AX following loss of Eya3 is consistent with the current model wherein EYA tyrosine phosphatase activity is required for the dephosphorylation of Y142 on H2AX. Failure to do so prevents recruitment of homologous recombination proteins and impairs the DNA damage response (Cook et al., 2009).

Based on this model, we predict that use of the small molecule inhibitor BZ to suppress EYA-PTP activity will disrupt DDR and result in the accumulation of DNA breaks. To determine the effect of EYA inhibition during the development of severe hypoxia, A673 and LLC spheroids (500 μm in diameter) were cultured with varying concentrations of BZ for 72-96 hours, the time frame in which hypoxia develops and DDR signaling is concentrated in the MCTS core. Treating the spheroids prior to hypoxia development is similar to BZ treatment of in vivo tumors which have been treated at approximately 200 mm³ volume, an early stage where there is not believed to be significant hypoxia. BZ treatment inhibited MCTS growth in a dose dependent manner (Fig. 21) which correlated with decreased Ki67 (Fig. 22). The significant loss of Ki67 signal indicates a role for the EYA-PTP in tumor cell proliferation. Total levels of γ-H2AX within BZ treated LLC spheroids were not significantly decreased; however, the distribution pattern of γ-H2AX appeared to expand to all cell layers abolishing the regionalization observed in vehicle treated spheres (Fig. 22). The redistribution of γ-H2AX staining indicates an impaired DDR signaling in outer cell layers leading to the accumulation of DNA damage in these layers and provides evidence that the EYA tyrosine phosphatase is a critical component in the repair of DNA strand breaks. These results are similar
to trends observed in monolayer cultures\textsuperscript{4} and in tumor xenografts where EYA-PTP inhibition stimulated phosphorylation of H2AX and a loss of regionalization in tumor xenografts\textsuperscript{5,6}. This similarity to \textit{in vivo} studies indicates that LLC MCTS can be used to study and predict the effect of EYA-PTP inhibitors \textit{in vivo}.

**Discussion**

The preliminary data presented in this chapter addresses whether the EYA-PTP contributes to tumor cell adaptations under hypoxic conditions. Previously published works indicated the EYA-PTP influences cell survival under genotoxic conditions through the regulation of DDR signaling (Cook et al., 2009; Robin et al., 2012; Y. Wang et al., 2016). Several lines of evidence indicate a role for EYA proteins in pathological angiogenesis and tumor cell aggression (Blevins et al., 2015; Y. Wang et al., 2016). Taken together, these findings support the hypothesis that the EYA-PTP is involved in hypoxic tumor cell survival and represents a viable therapeutic target within aggressive hypoxic tumor regions.

To address this hypothesis, we utilized the MCTS model. Our previous studies have shown that MCTS recreate a hypoxic microenvironment in which DDR signaling occurs within clinically relevant cell populations. In the present study, we found EYA-PTP activity to be a critical regulator of DDR signaling in the MCTS model which when targeted with BZ, led to the loss of regionalization in $\gamma$-H2AX signal indicating an accumulation of DNA damage in cell layers residing within all microenvironments of the spheroid. This finding is consistent with similar work in which tumor xenografts treated with BZ showed a significant increase in $\gamma$-H2AX which correlated with a similar loss of regionalization in $\gamma$-H2AX positive nuclei\textsuperscript{6}.

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\textsuperscript{4} This work was performed by Naresh Pandey of the Hegde Lab
\textsuperscript{5} This work was performed by Dr. Yuhua Wang and Dr. Hemabindu Chintala of the Hegde Lab
\textsuperscript{6} Unpublished data generated by Dr. Yuhua Wang, Dr. Hemabindu Chintala, and Naresh Pandey of the Hegde lab.
It is interesting that the loss of Eya3 results in significant decreases in γ-H2AX while the inhibition of the Eya-PTP had no effect on total γ-H2AX levels in LLC spheroids. There are several potential reasons for this discrepancy including the ability of BZ to inhibit all EYA homologues. The EYA-PTP catalytic pocket is highly conserved amongst homologues and observations during the initial characterization of Benzbromarone (BBR), the parent compound to BZ, reported similar IC50 values when incubated with the EYA3 or EYA2 enzymatic domain (Tadjuidje et al., 2012). Therefore the discrepancy in γ-H2AX levels between Eya3 knockout cells and BZ treated cells reflects the difference between total loss (Eya1 and 3) and partial loss (Eya3) of EYA-PTP activity. From these results it appears that total loss of EYA-PTP activity leads to the accumulation of DNA damage whereas partial loss does not. Knockout of all Eya homologues (Eya1 3) within these cells is one method to address this potential. An alternative explanation is that BZ treatment induces DNA damage, through unintended targets. Recent studies using the parent compound to BZ, Benzbromarone (BBR) show the potential for uncoupling of mitochondrial oxidative phosphorylation in the presence of BBR (Felser et al., 2014; M. Shirakawa, Sekine, Tanaka, Horie, & Ito, 2015). It is possible therefore that Benzolone similarly uncouples oxidative phosphorylation leading to increased reactive oxygen species. Future studies should seek to knockout all Eya homologues to confirm the phenotype observed with BZ is the result of Eya inhibition.

In addition to regulation of DDR signaling in hypoxic tumor cells, we presented evidence that EYA-PTP activity is involved in tube formation by tumor cells, a feature used to measure VM in vitro (Hendrix, Seftor, Hess, & Seftor, 2003). While we were unable to conclusively determine if VM occurred in the MCTS model, multiple tumor cell lines formed tubular structures atop matrigel in an EYA-PTP dependent manner. The signaling pathways through which EYA is acting to enable VM are not clear, however the EYA-PTP has a documented role in tumor cell migration and
invasion (Pandey et al., 2010) therefore our results likely reflect an invasion defect. Consistent with this interpretation, time course imaging shows reduced aggregation of tumor cells into cord like structures when B16 melanoma cells were treated with the EYA-PTP inhibitor BZ. Additionally, the loss of tube like structures and a decreased migration of cells from LLC MCTS cultured atop matrigel are consistent with an impaired ability for tumor cells to invade the ECM. Our experiments also demonstrate MCTS culture atop agar (liquid overlay method) is not sufficient to induce VM like qualities however culture atop matrigel induces formation of ECM networks and tube like structures.

Our studies demonstrate the MCTS model can be used to predict the effect of EYA-PTP inhibition on tumor growth and proliferation in vivo and provides preliminary evidence that the EYA-PTP may be involved in hypoxia induced adaptations such as VM. This is summarized in figure 23. The demonstration of decreased tube formation in the presence of EYA-PTP inhibitors provides the first evidence that EYA proteins may be involved in VM. Based on the results of this work, future studies regarding VM in MCTS should incorporate ECM components to facilitate VM like activities. Taken together these studies indicate that targeting EYA proteins could prevent tumor progression through the inhibition of proliferation, DNA damage repair, and vasculogenic mimicry. Our findings enable future studies to utilize the MCTS model for the study of hypoxic tumor cell drug response simultaneous to the study of tumor cells within a normoxic environment.
Figure Legends for EYA-PTP studies

Figure 16. *In vitro* tube formation of multiple tumor cell lines is inhibited by Benzarone treatment. 

a. Brightfield images 16-18 hours after treatment of the Ewing Sarcoma cell line A673, the murine Melanoma cell line B16-F10, and the human Melanoma cell line 1205LU with DMSO (Veh) or Benzarone (BZ). Treatment of cells occurred at the time of seeding with 7.5 μM BZ in A673, 10 μM BZ in B16-F10, and 5 μM BZ in 1205LU. 

b. Bar graphs representing the quantification of total tube cord length (arbitrary units) in at least 3 independent experiments. * indicates p<0.05 by two tailed students T-test. N.S. indicates no significance detected.

Figure 17. EYA inhibition decreases the rate of B16 tube formation and formation of tube like structures protruding from LLC MCTS. 

a. B16-F10 cells 3 hours, (b) 6 hours and (c) 18 hours after seeding single cell suspensions atop matrigel with DMSO (Veh) or 5 μM Benzarone (BZ). This experiment was performed only one time, therefore there are no quantifications. 

d. Lewis Lung Carcinoma MCTS approximately 500 μm in diameter formed tubes several days after being embedded in matrigel. Treatment with 5 μM BZ when spheroids are embedded in matrigel results in decreased expansion of cells from MCTS and a notable absence of tube like structures. 

e. 63x images of PAS stained tubes extending from an LLC spheroid seeded atop matrigel. Left panel shows the column of tightly packed cells which ends in a tip cell displaying filopodial like structures (right panel). These results are representative of 2 independent experiments and have not been quantified.

Figure 18. A673 MCTS do not contain VM markers. A673 MCTS approximately 550-650 μm in diameter were immunostained for VM markers (red) CD31 (a.), VE-cadherin (b.), and Laminin (c.) with Hoescht 33342 blue counter stain.
**Figure 19.** B16 MCTS formation atop matrigel results in ECM incorporation.  

- **a.** H&E staining of B16 MCTS formed atop matrigel.  
- **b.** B16 MCTS formed atop matrigel were collected and stained with PAS (left, *pink*) and immunostained for laminin (right, *red* with *blue* Hoescht 33342) in serial sections. This experiment was performed one time with a total of approximately 5 spheroids.

**Figure 20.** Eya3 knockout reduces DNA damage repair signaling, but not growth in LLC MCTS.  

- **a.** CRISPR-Cas9 guide RNA (gdRNA) sequences and target sites within the mouse Eya3 genomic transcript. 3 gdRNA plasmids were used.  
- **b.** Growth of wild type (*black*) and CRISPERE knockout (*green*) MCTS over time in hours (hr).  
- **c.** LLC wild type and Eya3 knockout MCTS at approximately 700 μm in diameter immunostained for γ-H2AX (*red*) with (*blue*) nuclear counterstain.  
- **d.** Bar graph representing the average percentage of γ-H2AX positive nuclei (with standard deviation) in 3 independent experiments and at least >8 spheroids per condition. * indicates p<0.05 by two tailed students T-test.

**Figure 21.** Benzaron shows dose dependent inhibition of A673 and LLC MCTS growth.  

- **a.** MCTS diameter was monitored over time (hours) following treatment of A673 and MCTS spheroids with indicated concentrations of the EYA small molecule inhibitor Benzaron (BZ). Spheroids were treated at approximately 400-500 μm in diameter. The average diameter with standard deviation of at least >20 spheroids is plotted.

**Figure 22.** EYA tyrosine phosphatase regulates proliferation and formation of γ-H2AX in LLC MCTS.  

- **a.** LLC MCTS proliferation (Ki67, *yellow*) and **b.** DNA damage repair signaling (γ-H2AX, *red*) was monitored after 72 hour incubation with the EYA tyrosine phosphatase inhibitor Benzaron (BZ) compared to 0.01% DMSO vehicle (Veh) treated spheroids. Spheroid diameters were less than <500 μm at the time of drug addition and were assessed 72 hours later at an approximate diameter of 650 μm. Quantification of spheroids represented is shown to the right
with the mean and standard deviation of at least >14 spheroids and two independent experiments. Black bar represents vehicle treated spheroids, blue bar represents 2.5 μM BZ, red bar represents 5 μM BZ. * indicates p<0.05 by two tailed students T-test. N.S. indicates no significance detected.

**Figures**

**Figure 16:** *In vitro* tube formation of multiple tumor cell lines is inhibited by Benzaron treatment
Figure 17: EYA inhibition decreases the rate of B16 tube formation and formation of tube-like structures protruding from LLC MCTS.
Figure 18: A673 MCTS do not contain VM markers

A. CD31

B. VE-Cad

C. Laminin
Figure 19: B16 MCTS formation atop matrigel results in ECM incorporation

A. [Images showing MCTS formation atop matrigel, with annotations indicating ECM incorporation.]

B. [Images showing PAS and Laminin staining, indicating ECM incorporation.]
Figure 20: Eya3 knockout reduces DNA damage repair signaling, but not growth in LLC MCTS.

A. | gdRNA # | gdRNA sequence | Target sequence | Exon# |
--- | --- | --- | --- | --- |
1 | GGTGCAGGCACCATAGCACT | AGTGCTATGGTCCTGACC | 10 |
2 | TTGCAGATGAGTTGGGTAC | GATACCCACTTCATCTGCAA | 9 |
3 | CAGTACCTGTTTGAGATG | CATCTCAAACAGGTACTG | 9 |

B. [Graph showing growth over time]

C. [Images showing Wild Type and Eya3 CKO with γ-H2AX staining]

D. [Bar graph showing % nuclei with γ-H2AX: Wild Type vs. Eya3 CKO]
Figure 21: Benzarone shows dose dependent inhibition of A673 and LLC MCTS growth

A. A673

LLC

Diameter (µm)

Time (hr)

Vehicle  2-2.5 µM BZ  5 µM BZ  10 µM BZ
Figure 22: EYA tyrosine phosphatase regulates proliferation and γ-H2AX formation in LLC MCTS

A. Veh 2 μM BZ 5 μM BZ

B. Veh 2 μM BZ 5 μM BZ

% Ki67+ Nuclei

% γ-H2AX+ Nuclei

N.S.

*
CHAPTER 4: DISCUSSION AND SIGNIFICANCE

The studies presented in this thesis work advance our understanding (summarized in Fig. 23) of hypoxic tumor cell biology and the Eyes Absent proteins (EYA 1-4) as viable targets in the prevention of tumor cell survival under hypoxia. In the following sections we will discuss our current understanding of cell survival in the hypoxic tumor microenvironment and how our data contributes to this field as a whole.

The extent to which DNA damage repair (DDR) signaling occurred in response to hypoxia development within 3D tumor tissue is poorly understood. Previous studies using 2D culture have indicated that DDR signaling occurs in proliferating tumor cells under hypoxic conditions, facilitating their continued survival and proliferation (M. Olcina et al., 2010). In vivo, a similar response has been reported during the process of pathological angiogenesis in which hyper-proliferative endothelial cells experience replication stress and DNA damage within a hypoxic environment (Economopoulou et al., 2009). In both cases, genotoxic stress leads to the formation of γ-H2AX and the activation of the DDR kinases ATR and ATM (Wilson & Hay, 2011). In vivo, tumor cells likely activate a similar hypoxic DDR response. Correlations between tumor hypoxia and γ-H2AX have been reported further supporting a link between the two (Banuelos et al., 2009; Nagelkerke & Span, 2016; Olive et al., 2004; Olive et al., 2010). Separately, proliferation within hypoxic tumor regions has been correlated with poor patient survival outcomes (Evans et al., 2007; Evans et al., 2010; Evans et al., 2004). Although in vitro studies indicate that hypoxia induces phosphorylation of DDR proteins within proliferating tumor cells (Hammond et al., 2014), there has been no evidence to show that this occurs in complex, three-dimensional tumor environments. There is reason to believe that the tumor cell response to hypoxia in 2D in vitro studies is different than that observed within the 3D hypoxic microenvironment that develops In
Several studies have demonstrated that monolayer culture induces gene expression profiles that are substantially different compared to their in vivo equivalents which correlate with altered drug response and tumor cell behavior (proliferation, migration, survival) (Daster et al., 2017; Francia et al., 2004; Ghosh et al., 2005; Kunz-Schughart et al., 1996; Kunz-Schughart et al., 2001; Pacheco-Marín et al., 2016). For example, ATR kinase expression decreases during 3D culture relative to 2D culture settings (Ghosh et al., 2005). Similar alterations in DDR proteins have been described in vivo (Scanlon & Glazer, 2015). Therefore the contribution of DDR signaling to hypoxic cell survival, and how this relates to proliferation within the hypoxic tumor environment, remains unknown. Our studies provide evidence that tumor cells attempting to proliferate within a 3D hypoxic environment experience a proliferation block which is accompanied by phosphorylation of DDR proteins (Fig. 6-9).

The time course for tumor cells to survive hypoxia and proceed onto continued growth is limited to 12-24 hours {Bencokova, 2009; Foskolou, 2017; Hammond, 2002; Hammond, 2004; Olcina, 2014; Scanlon, 2015} following the initiation of replication stress. Past 12 hours the replisome is disassembled and replication restart is less likely to occur. In vitro studies indicate that DNA damage accumulates beyond 24 hours of severe hypoxia and cell death ensues. This data indicates that there is a small window of time between when tumor cells begin to proliferate under hypoxia, and when they succumb to cell death due to the accumulation of damage. In the MCTS model, this is reflected in the central apoptotic core in which chronic hypoxia and glycoxia leads to apoptosis. The cells in the perinecrotic cell range displaying γ-H2AX and Ki67 reflect cells that are currently in the window of time where DDR proteins have been activated, and the cell is attempting to survive. Apoptotic/necrotic cells represent those cells who were subject to extreme conditions for too long of time.
Our work sets the stage for further exploration of DNA repair proteins and their contribution to tumor cell survival under hypoxia. Such studies hold potential to identify therapeutic targets involved in tumor progression. Using the MCTS model, therapeutic efficacy within normoxic and hypoxic cells can be simultaneously measured. In this regard, we provide compelling evidence that inhibition of critical DDR proteins, such as ATM, leads to decreased DDR and increased tumor cell death (Fig. 13-15, Fig. 21,22). Oxygen-deprived tumor regions present a significant challenge to therapeutics due to limitations in drug penetration, negative pressure gradients owing to interstitial fluid buildup, and decreased penetration of weakly acidic drugs amidst accumulating acidic metabolites (Minchinton & Tannock, 2006). The ability of inhibitors to penetrate into the MCTS core and alter DDR signaling despite the presence of a severely hypoxic center provides support for further development of these and similar compounds for targeting hypoxic cells in vivo.

**EYA and DDR under hypoxia**

EYA proteins have a highly conserved C-terminal domain, known as the EYA domain (ED), in which protein-protein interactions take place and a tyrosine phosphatase catalytic pocket resides (Rebay, 2015; Tadjuidje & Hegde, 2013). A non-classical tyrosine phosphatase, the EYA proteins utilize a nucleophilic aspartate residue to catalyze phosphate removal which contrasts with classical, cysteine-based phosphatases (J. P. Rayapureddi et al., 2005; Jayanagendra P. Rayapureddi et al., 2003). The EYA tyrosine phosphatase, hereafter referred to as the EYA-PTP, has been implicated in several signaling pathways that contribute to tumor progression (Blevins et al., 2015). While many substrates have been proposed, to date the most experimentally validated EYA-PTP substrate is the minor histone variant H2AX which contains a constitutively phosphorylated c-terminal Tyrosine (Tyr142) (Cook et al., 2009; A. Xiao et al., 2009). Following DNA strand breaks, the EYA-PTP dephosphorylates Y142 which allows the recruitment of the Mediator of Damage Checkpoint 1 (MDC1) and subsequently the anchoring of repair proteins
including the MRN complex and ATM (Cook et al., 2009). Recruitment of these proteins directs the damaged cell towards DNA repair whereas failure to remove Y142 phosphorylation results in apoptotic signaling through JNK recruitment to phospho-H2AX (Cook et al., 2009). The influence of the EYA-PTP on DDR and cell survival has been demonstrated in developmental and pathological processes (Tadjuidje & Hegde, 2013). Notably, the loss of EYA proteins from Ewing Sarcoma cells was recently shown to increase apoptosis and the accumulation of DNA damage thus implicating the EYA proteins in tumor cell survival and DDR (Robin et al., 2012). In keeping with this, the same study demonstrated a sensitization of tumor cells to DNA damaging agents following loss of EYA proteins. Similar results were reported in endothelial cells during pathological angiogenesis (Y. Wang et al., 2016). Here it was shown that EYA-PTP activity was required for endothelial cell survival during hypoxia-driven retinal neovascularization. These studies show that pharmacological or genetic loss of EYA activity results in increased endothelial cell death and decreased formation of DDR complexes. Extrapolating from these results, it is likely that the EYA-PTP is involved in tumor cell survival under hypoxia through mediation of DDR signaling in both hypoxic tumor cells and within pathological neovessels; and unpublished data from our lab support this.

The inhibition of EYA-PTP activity using BZ resulted in significant reduction in MCTS and tumor xenograft proliferation (Ki67 staining). In contrast, loss of EYA3 from LLC cells in the LLC-CKO cell line had no effect on MCTS diameter. This may reflect that both the EYA3 and EYA1, or just EYA1 homologues are involved in proliferation such that loss of EYA3 is not sufficient to reduce cell proliferation. With BZ treatment however, all homologues are inhibited and thus proliferation decreases.

Our observation that inhibition of the EYA-PTP resulted in a loss of regionalization in γ-H2AX formation in both LLC MCTS and tumor xenografts suggests that the MCTS model can be used
to predict the effect of EYA inhibitors across spatially defined populations in vivo. Unpublished results from our lab have shown a similar response by tumor cells to BZ treatment in vivo. The similarity in response between MCTS and tumor xenografts led us to conclude that MCTS can be used to predict the efficacy of EYA inhibitors in vivo. However, this effect may be cell line or species specific based on the response of A673 cells to 2.5 µM BZ. There are currently 3 classes of EYA-PTP inhibitors in development.

The first family of compounds identified as EYA-PTP inhibitors were derived from the uricosuric agent Benzbromarone (BBR) and its dehalogenated derivative, Benzaron (BZ) (Pandey et al., 2013; Tadjuidje et al., 2012). Initial identification of these compounds was achieved using a high-throughput screen which was then validated through phosphatase assays using a 10 amino acid phospho-peptide representing the C-terminal phospho-tyrosine 142 region of γ-H2AX. Molecular docking models showed that BBR and BZ bind within the catalytic pocket in an uncompetitive fashion thus abrogating phosphatase activity (Tadjuidje et al., 2012). Structure-activity relationship studies have gone further to show that BBR, BZ, and a well-studied metabolic product of BBR, 6-OH-BBR, are all capable of potent EYA phosphatase inhibition (Pandey et al., 2013). Since its discovery, these compounds have been shown to decrease endothelial cell proliferation, migration, tubulogenesis, angiogenic sprouting, and pathological angiogenic growth through a combination of in vitro, ex vivo, and in vivo models (Pandey et al., 2013; Tadjuidje et al., 2012; Y. Wang et al., 2016). Additionally, BZ and BBR were shown to be effective inhibitors of tumor cell invasion and growth in vitro and in vivo (Pandey et al., 2013).

In addition to BBR and BZ, other compounds have been identified as EYA tyrosine phosphatase inhibitors. MLS000544466 is a compound containing N-arylidenebenzohydrazide identified in a high-throughput screen as an allosteric inhibitor of the EYA2 phosphatase (Krueger et al., 2014). Initial characterization of this compound family used purified ED domains from both EYA3 and
EYA2 to demonstrate allosteric inhibition of the EYA2, but not EYA3, phosphatase in OMFP phosphatase assays. It was further shown that MLS000544466 binds with magnesium in lipophilic conditions, however this binding did not appear to be the mechanism for inhibition (Krueger et al., 2014). While the mechanisms of MLS000544466 inhibition and specificity is yet unknown, the excessive migration induced by EYA2 overexpression in MCF10a cells was inhibited by this compound. Virtual screening methods have identified a series of compounds that are predicted to inhibit EYA proteins through chelation of the coordinating magnesium ion in the EYA catalytic pocket, however these compounds have yet to be tested in cell based systems (Park et al., 2011; Park, Ryu, & Kim, 2012).

Our studies provide a unique model for the screening of these new compounds and allows for researchers to determine the direct effect of EYA inhibition in tumor cells, separate from secondary effects that result from decreased angiogenesis and effects in other cell types.

**ROS in MCTS**

Hypoxia-induced oxidative stress has been described within multiple pathologies (Hammond et al., 2003; Okuno et al., 2012; Ranchoux et al., 2016; W. Xiao et al., 2015). Recent studies identify oxidative stress as a critical feature during hypoxia-induced pathological neovascularization which induces activation of DDR proteins (Okuno et al., 2012). As oxygen levels decrease, reactive oxygen species (ROS) increases in response to mitochondrial oxidative phosphorylation impairment (Dugan LL, 1999). Increased ROS leads to the oxidation of nucleotide bases and subsequent DNA damage (Nikitaki et al., 2015). We asked if ROS species were enriched in the MCTS core and if these radicals coincided with DNA damage repair signaling, however we were unable to conclusively determine the presence of ROS within MCTS. We utilized CM-H2DCFDA to detect ROS species. This compound is cell-permeable until cellular esterases remove the diacetate groups (Chen, Zhong, Xu, Chen, & Wang, 2010). In the presence of ROS DCF will
become oxidized and fluoresce. A series of publications have reported use of CM-H₂DCFDA to study ROS in MCTS (Wartenberg, Diedershagen, Hescheler, & Sauer, 1999; Wartenberg, Fischer, Hescheler, & Sauer, 2000; Wartenberg, Ling, et al., 2003; Wartenberg, Ling, et al., 2001; Wartenberg, Schallenberg, Hescheler, & Sauer, 2003). These investigations reported the presence of ROS in MCTS approximately 500 μm in diameter; however the spatial distribution of ROS within these MCTS and the effect of ROS on DDR was not explored. In our studies, we observed intense DCF staining in the outer spheroid layers which suggests that ROS production is limited to the outer, non-hypoxic cell layers. However, we could not conclusively determine if CM-H₂DCFDA was capable of penetrating in the spheroid despite use of H₂O₂ (primary radical species believed to oxidize CM-H₂DCFDA) as a positive control (Chen et al., 2010). Treatment of spheroids with the ROS scavenger N-acetylcysteine demonstrated a significant reduction in γ-H₂AX indicating that ROS could be inducing γ-H₂AX within small A673 spheroids, however there have been recent studies suggesting that NAC may influence cellular protein function through the disruption of protein disulfide bonds (Rushworth & Megson, 2014). Furthermore, NAC has been shown to influence a number of cell signaling pathways which may account for changes in γ-H₂AX levels following NAC treatment (De Flora et al., 2001). For these reasons, we cannot determine to what extent ROS are contributing to DNA damage within MCTS.

**Regionally defined glycogen storage within MCTS**

In addition to DDR signaling, tumor cells are known to undergo metabolic adaptations to survive hypoxia. Recently, it was shown that mild hypoxia induces a “pre-conditioning” phenotype within tumor cells where HIF-1α stabilization drives the expression of Glycogen Synthase 1 and the subsequent accumulation of glycogen stores (Favaro et al., 2012; Pescador et al., 2010). This phenomenon has been observed in the perinecrotic portions of tumors as well as in vitro (Favaro et al., 2012). We demonstrate the first in vitro example of increased glycogen storage occurring
in a discrete, regionally defined population of cells bordering severely hypoxic cell layers. The clearly defined regionalization of glycogen storage within MCTS was demonstrated to be the result of hypoxia development. Important to note is that not all hypoxic cell layers were PAS positive; rather, glycogen storage appeared to be limited to the outer most hypoxic cell layers. This limited occurrence is in line with pre-conditioning of hypoxic cells where increasing glycogen stores occur at the onset of hypoxia; with severe hypoxia development however, cells can breakdown glycogen for metabolic and anti-oxidant purposes (Favaro et al., 2012; Pescador et al., 2010). The recreation of this process in the MCTS model will allow future studies to examine how glycogen storage contributes to tumor cell survival within the 3D environment and the efficacy of targeting such a process.

**EYA-PTP activity in tumor cell proliferation**

EYA proteins contribute to cellular proliferation under pathological and developmental contexts (Tadjuidje & Hegde, 2013; Y. Wang et al., 2016). Our lab has previously shown EYA-PTP activity promotes endothelial and tumor cell proliferation. Our demonstration that EYA-PTP inhibition results in significant growth reduction and decreased proliferation within MCTS extends these findings to the 3D context and parallels *in vivo* results. It is unknown through which substrate the EYA-PTP regulates proliferation. Candidate substrates include the Estrogen Receptor beta (ER-β) which was recently shown to be dephosphorylated at tyrosine 42 by EYA2 in breast cancer cells (Yuan et al., 2014). This dephosphorylation was said to prevent ER-β from repressing replication, thereby facilitating tumor cell proliferation. More recently, it was suggested that the EYA-PTP was not strictly a tyrosine phosphatase but rather acted as a dual specific tyrosine-threonine phosphatase capable of dephosphorylating a threonine residue of c-myc (Thr58) (J. Li et al., 2017). Phosphorylation of Thr58 on c-myc was shown to increase ubiquitination and degradation of c-myc. It was proposed that by dephosphorylating this residue, EYA contributes to
increased c-myc stabilization and increased proliferation of breast cancer cells (J. Li et al., 2017). Whether the EYA-PTP contributes to tumor progression through regulation of transcriptional output is not clear. In favor of the EYA-PTP activity exerting influence over transcription, it was shown using breast cancer cell lines expressing a mutant EYA1 (D327A) which lacks phosphatase function that EYA1-mediated the transcription of cyclin D1 which was dependent on the tyrosine phosphatase activity (Wu et al., 2013). Furthermore, in Medulloblastoma it was recently shown that Gli1 mediated transcription required tyrosine phosphatase activity and ultimately increased tumor growth (Eisner et al., 2015). In contrast, we reported previously that the overexpression of EYA proteins increased breast cancer cell proliferation independent of the EYA-PTP activity (Pandey et al., 2010). Consistent with this, mounting evidence indicates that drosophila eya-mediated transcription is unaffected by loss of eya tyrosine phosphatase activity (Jin, Jusiak, Bai, & Mardon, 2013). Therefore it appears that the EYA-PTP contributes to proliferation in a context specific manner. Our findings using the MCTS model indicate the EYA-PTP activity is required for 3D tumor cell growth. Use of LLC cell lines in which Eya3 was knocked out demonstrated that Eya3 loss is not sufficient to affect proliferation. This could reflect redundancy among the Eya homologues present in LLC. Previous results using BZ demonstrated a decrease in proliferation which, combined with knockout data, may support this conclusion. An alternative reading of these results could indicate non-specific effects from BZ treatment which result in growth inhibition. Such a conclusion is not supported by preliminary data produced by other members of the Hegde lab in which Eya3 knockout LLC cells do not show additional growth loss with BZ treatment. Taken together, this information suggests that loss of a single Eya homologue is not sufficient to elicit growth defects; however, the role of EYA proteins in proliferation appears to be complex and future studies should aim to explore this topic.
Modeling Vasculogenic mimicry

Vasculogenic Mimicry (VM) has been reported in a broad range of malignancies encompassing Melanoma, Ewing’s sarcoma, Non-small Cell Lung cancer, Mesothelioma, Breast cancer, and multiple others (Cao et al., 2013; Kirschmann, Seftor, Hardy, Seftor, & Hendrix, 2012). Retrospective analysis of patient samples has shown that a minor subset (23%) of tumors are designated as VM positive however such a designation is strongly correlated to aggressive disease and is not dependent on tumor size (Cao et al., 2013). Importantly, several recent studies have identified increased VM in tumors subject to antiangiogenic treatment raising the possibility that VM is stimulated by hypoxia and contributes to therapeutic resistance (Du et al., 2014; Hendrix et al., 2003; Hendrix et al., 2002; S. Li, Meng, Guan, Guo, & Han, 2016; Pinto et al., 2016; E. A. Seftor et al., 2002). The association of hypoxia, VM, and a negative patient outcome suggests that VM, or the development of VM associated markers, is likely an adaptation to the hypoxic environment that may facilitate tumor survival and dissemination. Despite such correlations, the relevance of VM to overall tumor progression remains unclear.

Functional studies of VM in vivo have suggested that VM channels are capable of directly connecting to endothelial-lined vessels and directing blood flow into the VM network, thus perfusing an otherwise hypoxic region (Hendrix et al., 2016). It has also been suggested that these channels can scavenge and circulate blood leaked into the hypoxic region from nearby vessels or blood lakes (van der Schaft et al., 2005). Evidence for such a role is derived from observations of blood cells in extravascular portions of tumors which, in some instances, were noted to be arranged in single file along PAS positive channels (Hendrix et al., 2003; Maniotis et al., 1999; Sood et al., 2002; van der Schaft et al., 2005). More direct evidence of this perfusion was provided through Doppler ultrasound imaging of nude mice harboring aggressive human cutaneous melanoma xenografts (Seftor et al., 2012). Angiography of patient-derived xenografts
from inflammatory breast cancers show blood flow into portions of a tumor that were later histologically identified as VM positive through H&E staining and the absence of mouse CD31 staining (Frenkel et al., 2008). Although several studies have discussed VM, to date there is no conclusive marker for definitive identification of VM in vivo.

The study of VM relies heavily on two models: in vitro tube formation and analysis of tumor sections (Seftor et al., 2012). VM has been observed in tumor xenografts and patient samples (Pinto et al., 2016). Initial descriptions of VM used Periodic Acid and Schiff’s base staining (PAS) as a method for histological identification of glycoprotein rich matrices such as that of the vascular basil lamina (Maniotis et al., 1999). Building on previous observations (Mueller et al., 1998), Maniotis et al noted PAS positive tracts within uveal melanoma samples that appeared to be connected in a looping pattern. Transmission electron microscopy identified the presence of erythrocytes within acellular, PAS positive tumor micro regions that were lined by cells lacking endothelial markers (Factor VIII related antigen, CD34, KDR). Angiograms of uveal melanoma patients revealed a similar looping network of perfused vessels (Maniotis et al., 1999). The lack of endothelial cell staining, the looping network similarities between PAS positive staining and angiographic results, and the presence of erythrocytes led to the conclusion that these networks represented tumor cell-lined vascular channels, otherwise known as vasculogenic mimicry. Studies of VM in subsequent years would utilize a wide range of markers to identify VM, however the primary method is similar to that employed by Maniotis et al. – PAS staining to identify networks that are negative for endothelial cell markers (Kirschmann et al., 2012). Examples of such combinations include parallel staining with PAS, VE-cadherin (CD144), CD31, or VWF (Kirschmann et al., 2012; Seftor et al., 2012). This method of identifying VM can be complicated by tumor cell expression of endothelial markers during VM including VE-cadherin (Hess et al., 2006). There is significant need for a reproducible model in which VM occurs in the context of a
3D hypoxic microenvironment. Such a model would enable the better understanding of processes that contribute to VM and the context in which they occur.

*In vitro* VM studies consist of plating tumor cells atop, or within an extracellular matrix that allows aggressive cells with VM potential to form a series of focal aggregates, or “nests”, interconnected by cells arranged in a tubular network, or “cords”. Variations of this model interchange the matrix used with the most common being either type I collagen or matrigel. Initial descriptions of VM utilized this method and further showed the formation of a lumen through the use of indocyanine green (Maniotis et al., 1999). Much of what has been reported in regards to molecular signaling during VM is derived from this model.

We hypothesized that MCTS could be used to better model VM in the 3D hypoxic environment. Several lines of evidence indicated that MCTS would develop VM. One such indication is the demonstration that MCTS develop a hypoxic core which is bordered by PAS positive cells undergoing survival signaling (Fig. 5). Additionally, previous studies have used spheroids that were formed from melanoma cells atop matrigel to show the presence of laminin positive networks which correlated with a phenotypic change towards a more aggressive cell type (Larson et al., 2014). In spheroids formed from human Ewing Sarcoma cells and murine melanoma or lung carcinoma cells, we were unable to demonstrate VM (Fig. 17). As discussed, there are several ways to interpret this data. Given that CD31 was not detected in the absence of endothelial cells, the lack of clearly identifiable luminal structures and the absence of laminin networks in free-floating spheroids suggest that VM is not occurring. There is extensive literature indicating a critical role for ECM during the process of VM (Hendrix et al., 2002; R. E. Seftor et al., 2002; Seftor et al., 2001), therefore free-floating spheroids may not produce the necessary ECM components to enable VM. When cultured atop matrigel, MCTS contained extensive laminin staining; however, it was unclear whether this staining was indicative of luminal vessels or the
remodeling of matrigel components (Fig. 18). In addition to laminin networks, Lewis Lung Carcinoma spheroids invaded into the surrounding matrigel and were seen to form vascular-like protrusions (Fig. 16); however we were not able to examine these to determine if a lumen was present. These findings indicate that ECM is a necessary component when modeling VM and also provides preliminary evidence that hypoxic tumor cells may not be the source of this vasculogenic ECM. These results raise the question of where VM-inducing ECM originates in patient samples. It is possible that ECM is derived from stromal cells such as fibroblasts, or that an additional environmental component is necessary to encourage ECM production by tumor cells which was not present in our model. Based on the lack of VM within our free-floating spheroids, future studies can determine the necessary ECM components required to form VM in the hypoxic 3D environment through inclusion or exclusion of specific ECM proteins (collagen IV, Laminin, etc). Additionally, the contribution of stromal cells can be determined through co-culture of fibroblasts with tumor cells in the MCTS model to determine if stromal cells promote this process.

Although there is still much to be uncovered regarding the process of vasculogenic mimicry, it is clear that some of these findings have significant clinical impact. For example, the presence of PAS-positive networks within a tumor sample has a clear correlation with aggressive tumor subtypes and a negative patient outcome (Cao et al., 2013). The process leading to these PAS patterns, whether through VM or other means, does appear to be correlated with hypoxia development. Therefore, an investigation into the origin of this PAS reactive material would hold potential therapeutic benefit. Our own findings indicate that ECM components are critical when modeling VM. The significance, origin, and contribution of PAS channels to overall tumor progression is not yet clear and warrants further study.
Eyes Absent proteins as a component in Vascularogenic Mimicry

Detailed analyses of the pathways contributing to VM have shown that highly aggressive tumor cells begin to de-differentiate and take on a multi-lineage phenotype. Markers associated with embryonic development (Nodal, Twist, and Notch4), endothelial cell lineage (Epha2, VE-cadherin, VEGFR1), and hypoxia (HIF-1α) have all been identified as active, and sometimes necessary components in the formation of VM (Hendrix et al., 2016; Kirschmann et al., 2012; Strizzi, Hardy, Kirsammer, Gerami, & Hendrix, 2011). The evidence thus far has led to the proposed model wherein hypoxia induces a series of changes leading to the production of VEGF-A, VEGF-R1, and MMP’s (Kirschmann et al., 2012). Secretion of MMP proteins allows cleavage of a key ECM component: Laminin-5y2 chain (Seftor et al., 2001). The resulting cleavage products are Laminin-5y2' and Laminin-5y2x, which increase cell migration and can induce VM in non-aggressive tumor cells. Additionally, the proteolysis of ECM components excises embedded growth factors which can further induce phenotypic changes (Hess et al., 2003). VEGF-A signaling for example is known to drive VM through autocrine binding with VEGF-R and subsequent stimulation of VE-cadherin, EphA2, MMP-2 and -9, and the production of developmental proteins (J. Y. Wang et al., 2008).

The initial cue to undergo a VM-like transformation is unclear however compelling evidence has been used to indicate that hypoxia is a primary driving factor (Du et al., 2014; S. Li et al., 2016). For example, transplantation of aggressive melanoma cells into the ischemic hind limb of mice results in formation of chimeric blood vessels comprised of both melanoma and mouse endothelial cells, whereas in the absence of ischemia tumor cells aggregated into tumors (Hendrix et al., 2002). In agreement with the strong correlation between VM and hypoxia in patient samples, HIF-1α stabilization has been shown to be a central component contributing to VM through the increased production of the VM promoting/stabilizing proteins VEGF-A, VEGF-R1, EphA2, Twist,
Nodal, Osteopontin, and Cox-2 in aggressive melanoma cells (Cao et al., 2013; Du et al., 2014; S. Li et al., 2016). Furthermore, hypoxia was shown to indirectly stimulate production of VE-cadherin, Tissue Factor, and PEDF. Our studies using MCTS indicate that hypoxia alone is not sufficient to induce VM and that VM-like features are induced by ECM components. Our works contribute to the culmination of studies thus far which have described a complex signaling network involving developmental and angiogenic signaling cascades regulated by the hypoxic microenvironment.

While EYA proteins have been shown to be critically involved in these processes, substrates through which EYA is acting to regulate these cellular activities is unclear (Eisner et al., 2015; Pandey et al., 2010; Wu et al., 2013; Yuan et al., 2014). Relevant studies by our lab indicate that EYA-PTP activity contributes to invasion and motility through an unknown cytoplasmic substrate. In these studies, the overexpression of EYA3 containing a nuclear localization signal failed to increase tumor cell motility whereas overexpression of wild-type protein significantly increased motility (Pandey et al., 2010). Pharmacological and genetic inhibition of the EYA-PTP resulted in a significant decrease in endothelial cell invasion in vitro and in vivo with similar results in tumor cell lines (Pandey et al., 2013; Tadjuidje et al., 2012; Y. Wang et al., 2016). Collectively, this supports EYA-PTP activity as a mediator of cell motility and invasion through an unknown cytoplasmic substrate, the activity of which enables processes associated with tumor hypoxia such as angiogenesis, tumor cell invasion, and potentially vasculogenic mimicry.

Ultimately, these studies establish the MCTS model as a valuable tool which can be used and manipulated to better understand the hypoxic microenvironment. By describing the presence of active DDR signaling in proliferative hypoxic cells located in the core of MCTS, we enable future studies to explore how DDR signaling under hypoxia contributes to tumor progression and how to best target these cells.
**Future Directions**

An important question that arises from these studies pertains to the proliferating hypoxic cells and the relevance of DDR signaling to overall tumor progression. Our results, in combination with previous monolayer studies (M. M. Olcina et al., 2014), indicate that active proliferation in the hypoxic tumor environment is accompanied by DDR signaling. Whether this happens *in vivo*, and if this DDR signaling facilitates tumor regrowth, is currently unknown. Building on the results of our work, the MCTS model could be used to answer this question through fate mapping of proliferating hypoxic cells. Similar studies have utilized Cre recombinase constructs containing an oxygen-dependent degradation domain to ensure Cre activity would only occur during hypoxia (Harada et al., 2012; Kimura et al., 2015). In combination with a cell cycle Cre promoter and a Cre dependent reporter (flox-stop-flox GFP), the fate of proliferating hypoxic cells could be tracked following therapeutic treatment or reoxygenation.

Additional avenues for future study include the determination of DDR protein contribution to VM, the role of the EYA tyrosine phosphatase in VM, and determination of how stromal cells affect tumor cell adaptations to hypoxia including their impact on DDR signaling, glycogen storage, and VM.
Figure 23: Summary of findings and conclusions

MCTS > 500 μm in diameter

| Hypoxia development | • Necrotic/apoptotic    |
|                     | • Pimonidazol & HIF-1α positive |
| Metabolic adaptation| • Increased glycogen storage |
| Regionalization of proliferation | • Ki67 positive |
| | • EdU negative |
| Activation of DNA damage repair proteins | • γ-H2AX enriched in Ki67 positive cells |
| | • ATM activation |
| MCTS can be used to predict the effects of therapeutics on spatially defined populations | • Hypoxia drives glycogen storage, activation of DNA damage repair proteins, and DNA damage |
| | • Targeting ATM & ATR suppresses γ-H2AX, proliferation, and viability |
| | • MCTS resemble spatial effects of EYA-PTP inhibition |
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