Hypoxia in a 3D in vitro Spheroid Model: the Role on Tumor Progression and Response to Chemotherapy

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November 2015

Abstract
Cancer is a major burden on public health, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012. Better platforms for drug screening are required to improve the success rates of drugs entering clinical trials. Among the features that characterize the tumor microenvironment, hypoxia is present in 90% of solid tumors, and it has been correlated with poor outcome for patients, possibly due to chemoresistance [10]. In this work, a recent 3D in vitro model was used to study the influence of hypoxia on spheroids formed from MDA-MB-231 breast cancer cells. Hypoxia promoted tumor progression, stimulating a significant increase in spheroid growth, showing higher levels of proliferation, and a more persistent and directed invasion mode. HIFs-related genes expression was assessed under hypoxia, being more than three times higher for PLOD2, P4HA1, LDHA and PDK1. Knock down studies were conducted for HIF-1α and HIF-2α, presenting lower invaded distances and proliferative levels under hypoxia, suggesting HIFs-regulation. In addition, IC50 curves were assembled based on proliferation and invaded distance to study chemoresistance against Paclitaxel and Doxorubicin. Both IC50 values were higher under hypoxia for both drugs, showing chemosensitivity. Hypoxic spheroids subjected to low concentrations of Paclitaxel showed more progression than the normoxic control without drug, suggesting chemoresistance. Analogous 2D studies were conducted showing lower IC50 values and thus, the importance of 3D microenvironment for chemoresistance promoted by hypoxia. This work can potentially describe the hypoxic effect, highlighting the need of understanding the influence of the microenvironment in tumor progression.

Keywords: Cancer, Breast cancer, Hypoxia, 3D in vitro model, Spheroid, Tumor Progression, Chemosensitivity, Chemoresistance, Paclitaxel, Doxorubicin, 2D vs 3D, HIFs.

1. Introduction
1.1. Challenge of Anti-cancer Drugs
The widespread and lethal nature of cancer persists, despite billions of dollars of public and private investment. The average success rate for all therapeutic areas is approximately 11%; however, only 5% of agents that have anticancer activity in preclinical development are licensed after demonstrating sufficient efficacy in phase III [1]. Drug discovery is especially difficult in the oncology field, where a lack of profound knowledge of cancer mechanisms is the major obstacle. Moreover, several studies have been suggesting potential explanations for the high attrition rates in cancer drug discovery, such as the compound’s biochemical target, the use of inadequate preclinical models, the incomplete understanding of resistance mechanisms, and the incorrect design and execution of clinical trials [2], [3].

1.2. Cancer Models
Standard models used in cancer drugs research include cultured human tumor cell lines and rodent xenografts, that comprise many of the human lines grown subcutaneously in immunocompromised animals. Among the weaknesses of cancer models, there are important questions to point out. For instance, the artificial nature of tumor cell lines may not represent the native tumor and it is compromised by the high number of cell passages during culture. Moreover, cells in culture lack the architectural and cellular complexity of real tumors, such as the presence of inflammatory cells, vasculature and other stromal components. Despite the fact of xenografts have stroma, it is murine, not human. In addition, the xenograft-host interactions under the skin are potentially different from those in the tissue of tumor origin. Another interesting difference is that normal human tumors develop over years, while mouse xenografts are cultured only during days or weeks. Also, even if a xenograft represents significant aspects of the tumor from which it was derived, it probably captures only a fraction of the total genetic/epigenetic heterogeneity of a given tumor subtype [4]. On the other hand, 3D models include a 3D architecture and allow clinically relevant matrices. Therefore, they can potentially mimic tumor microenvironment and its features such as heterogeneity, tissue architecture, mass transport, invasion, metastasis, cell adhesion and tumor-immune cell interactions. Furthermore, since these models can be turned
into high throughput systems, they can be used as a selection step before the clinical trials on xenografts, decreasing the failure rate, and consequently the investment in the following phases.

1.3. Tumor Microenvironment

The tumor microenvironment is created by the tumor and dominated by tumor-induced interactions. The surrounding tissue of a developing tumor includes proliferating tumor cells, stroma, blood vessels, infiltrating inflammatory cells and a variety of associated tissue cells, that influence gene expression through cell-cell and cell-ECM interactions. The products encoded by these genes control the pathophysiological characteristics of the tumor. For instance, the ECM influences malignant transformation being highly related with the invasion and metastasis abilities of the tumor, and with sensitivity to drug treatment. Moreover, the vasculature of solid tumors is abnormal, being characterized by blood vessels often dilated and convoluted, and branching patterns that feature excessive loops and arteriolar-venous shunts. This abnormal structure gives rise to imbalances between the cellular oxygen consumption and supply rates to the cells causing hypoxia, which is present in 90% of solid tumors.

1.4. Hypoxia

Hypoxia can influence tumor, either by acting as a stress factor that decreases growth by slowing proliferation, or causes cell death due to apoptosis or necrosis events, or on the other hand, by serving as a promoter of malignant progression and increased resistance to cancer treatments. In this last scenario, as cells proliferate, the oxygen consumption increases leading to hypoxia, which activates HIFs, promoting the transcription of the VEGF gene, that encodes vascular endothelial growth factor, leading to angiogenesis, and thereby increases oxygen delivery. The abnormal blood vessels formed within solid tumors cause severe hypoxia, and to adapt to this microenvironment, cancer cells activate physiological HIFs-mediated responses. The resulting lethal phenotype comprises several mechanisms, such as proliferation increase, angiogenesis, metabolic reprogramming, epithelial-mesenchymal transition, invasion and metastasis, multidrug resistance gene (MDR1) upregulation, apoptosis, among others.

1.5. Tumor Microenvironment and Drug Response

Resistance of human tumors to anticancer drugs is frequently associated to gene mutations, however the limited ability of drugs to penetrate tumor tissue and to reach all tumor cells in a potentially lethal concentration is also a cause for chemoresistance. Moreover, the microenvironment heterogeneity leads to proliferative gradients and to regions of hypoxia and acidity, that consequently influence the sensitivity of tumor cells to drug treatment. For instance, tumor cell proliferation is decreased by the increasing distance from tumor blood vessels, and since most chemotherapeutic drugs target cell proliferation, they get less effective. In addition, hypoxia has been associated to HIFs-activation of genes that promote angiogenesis and cell survival. This mechanisms may result in the expansion of populations of cells with altered biochemical pathways with a potential drug-resistant phenotype, for instance with loss of sensitivity to p53-mediated apoptosis or a deficiency in DNA mismatch repair. Tumor pH can also influence the cytotoxicity of anticancer drugs, since molecules diffuse passively across the cell membrane most efficiently in the uncharged form, and with a low extracellular pH in tumors, weakly basic drugs are protonated and display decreased cellular uptake.

1.6. Spheroid Model for Drug Screening

Generally, 3D tumor models allow a better parallel of the tumor. However, only a small number of these systems are sufficiently well characterized to resemble the tumor in vivo, specifically spheroid models. Cells located in the spheroid periphery reflect the in situ situation of actively cycling tumor cells near the capillaries, while inner cells become quiescent and eventually die via apoptosis or necrosis. Overall, the pathophysiological gradients and the cellular arrangement are comparable to the situation in avascular tumor nodules, micrometastases or inter-capillary microregions of solid in vivo tumors. In this work, the spheroid model used was based in a previously described system. Besides the clinically relevant matrix and the larger dimensions that these spheroids can take, this system incorporates a second controllable parameter, thought the optimization of a proliferative assay, the possibility for RNA extraction, and the high throughput potential. The hypoxic condition was provided from the beginning by using an adequate chamber with a 1% oxygen atmosphere.

1.7. A Selection Phase in Drug Discovery

Cancer research is currently focused on chemotherapy treatments and the initial experiments in drug development utilize 2D cultures of cancer cell lines, followed by animal models. 3D models are being introduced to better mimic the in vivo situation, and therefore, decrease the number of drugs that are unsuccessfully tested in animal models. The tumor cell spheroid model, that due to its fully 3D architecture and cell organization, gives origin to similar chemical gradients that eventually lead to events that occur naturally in real tumors, is particularly important. Among the tumor microenvironment features, hypoxia is the leading cause of tumor aggressiveness, mainly due to the activation of hypoxia-inducible factors that are related to several mechanisms of adaptation. Therefore, it is critical to increase the resemblance with the in vivo context, so these models can be used as a selection phase before starting clinical trials, contributing to the reduction of investment in inefficient anticancer drugs. In this work, a spheroid model is used to understand how hypoxia can affect tumor progression and response against drug treatments. Since this is a high throughput system, and it encompasses hypoxia-adaptation mechanism, it is a potential model to be used as a selective step in drug discovery.
2. Materials & Methods

2.1. Cell Culture

MDA-MB-231 and MCF-7 cells (offered by Dr. Danielle Gilkes, Wirtz Lab) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen). HT1080 cells (offered by Dr. Angela Jiminez, Wirtz Lab) were cultured similarly, but supplemented with 0.1% Gentamicin (Sigma). Cells were maintained in a humidified environment at 37°C and 5% CO₂ during culture and live cell imaging. HIF Knock Downs were also offered for Dr. Daniele Gilkes, and obtained as published before [25]. MDA-MB-231 GFP-labelled were offered by Dr. Meng Heng Lee and obtained as previously published [26].

2.2. Spheroid Formation

Cell spheroids were formed in non-adhesive round-bottom 96-well plates following a protocol previously defined [24]. Briefly, monolayers of cells were trypsinized and resuspended in spheroid formation media – (3:1) DMEM and Methocult H4100 (Stemcell Technologies), with a density of 1 x 10^5 cells/ml. This cell solution was seeded, resulting in 10 kCells into each well of the 96-well plate. Cells were then spun down in the well-plate at 1,200 RPM for 7 minutes, for both sides to guarantee roundness. Plates were incubated under standard conditions during 2 days for HT1080 cells, and for 3 days in case of MDA-MB-231 and MCF-7.

2.3. 3D Type I Collagen Matrices

Collagen matrices were prepared as previously described by mixing culture medium and 10X Reconstitution Buffer 1:1 (v/v), with soluble Rat Tail type I Collagen in Acetic Acid (BD Biosciences) [27]. The final collagen concentration was 2 mg/ml. 1M NaOH solution was added to normalize the pH to 7, according to the manufacturer’s indication. All reagents were kept chilled in an ice bath, and precaution was taken to avoid bubble formation.

2.4. Spheroid Embedment

As described previously [24], 800 µl of (1:1) Agarose and Media solution was added in the bottom of 8mm ID square cells (Vitrocom) – referred as cuvette – in order to facilitate the image processing in this system. After sterilization and pre-warming, 600 µl of collagen solution were added in each cuvette, which were then exposed for 25 seconds to 37°C through a water bath, in order to have a preliminary collagen crosslinking. Spheroids were slowly aspirated with a 1 ml pipette from the 96-well plate, and placed on the lid of a 10 mm petri-dish. The formation media was discarded and the spheroid was aspirated into a 200 µl pipette tip with 3µl of collagen solution. Once in the pipette tip, the spheroid was placed in the middle of the collagen solution to guarantee a 3D microenvironment. The cuvettes were then incubated under standard conditions for 30 minutes before adding 200 µl of warm culture medium on the top of the gel. The spheroids were maintained for 7 days in normoxia (oxygen at 20%) or hypoxia (oxygen at 1%). To increase high throughput potential, this system was transferred to 48-well plates (Corning). 500 µl of collagen solution were added to each well. The spheroid embedment was performed similarly as in the cuvettes.

2.5. 2D Cultures

2D studies were conducted similarly to the 3D spheroid trials to allow a relevant comparison. Therefore, these cultures were maintained in 6-well plates and started with an initial seeding of 10 kCells per well. These cultures were kept for 5 or 7 days in normoxic and hypoxic conditions.

2.6. Hypoxia

Hypoxic conditions were obtained by using a modular incubator chamber (Billups-Rothenberg) flushed with a gas mixture containing 1% O₂, 5% CO₂, and 94% N₂, that was maintained inside the regular incubator at 37°C.

2.7. Image Collection and Handling

Using a swept field microscope – Nikon Eclipse Ti – the mid-plane of spheroids was imaged using an adequate grid size. Afterward, the stitching process was completed using Image J and NIS-Elements software, considering an overlap of 10%. The cell spheroid area was obtained using NIS-Elements by manually tracing the cell spheroid periphery.

2.8. Viability Assay with Presto Blue

Presto Blue reagent (Invitrogen) is a resazurin-based solution that functions as a cell viability indicator by using the reducing power of living cells to quantitatively measure the proliferation of cells. This reagent contains a cell-permeant compound that is blue in color and virtually nonfluorescent. When added to cells, this reagent is modified by the reducing environment of the viable cell and turns red, becoming highly fluorescent. This reagent was added to the media (1:10) and it is not harmful for the cells. After a exposure time of 20 hours for spheroid culture, and 4 hours for 2D cultures, a sample of 100 µl from each well was transferred to a 96-well assay plate (Corning) and the fluorescence was read using a plate reader Spectra Max Gemini XPS (Molecular Devices). This process was performed in the first and last days of culture in order to obtain a ΔFluorescence.

2.9. Gel Degradation for Cell Extraction

After spheroid growth, collagen gels were incubated for 2 hours with Collagenase A version 19 (Roche Diagnostics) solution for a final concentration of 1 mg/ml. After some mechanical disruption using a pipette tip, the collagen gels were incubated for 10 minutes with 3µl of Trypsin. Afterward, a second round of mechanical disruption was completed and the cell suspension was transferred to an Eppendorf tube.

2.10. Cell Counting

The resulting cell suspension was spun down for 5 minutes at 1500 rpm. The supernatant was discarded and the cells were resuspended with 100 µl of fresh media. The cell counting was performed using a Countess Automated Cell...
Centrations after 20 hours for consistency. After 5 or 7 days takes 4 hours, drugs were added in the same working conditions and provided consistent results in different trials. Concerning 2D cultures, although the viability assay only uses viabilities to keep the viability assay protocol and allow cell's adaptation to the new environment, the drug treatments started at the first day of experiment (20 hours after spheroid embedment). Therefore, after this period, the medium was replaced with Presto Blue containing media and the required drug concentration.

2.11. RNA Extraction
After spheroid growth, gels were removed from the well and washed with PBS. Then, they were transferred to Eppendorf tubes (2 per each). TRIzol (Life Technologies) was added to collagen gels for 10 minutes at room temperature. Mechanical disruption was promoted by an initial step using a pipette tip, followed by 4 syringe passages intercalated with vortex shaking. The solution was spun down for 5 minutes at 5000 rpm and the supernatant was collected. For 2D cell cultures, the RNA extraction with TRIzol addition in each well was followed by a manual scrubbing step for 1 minute. RNA purification was conducted according to manufacturer's protocol (Direct-zol RNA MiniPrep 200 Preps, Zymo).

2.12. qRT-PCR
cDNA was synthesized with using iScript cDNA synthesis kit (Bio-Rad). qPCR was performed with SYBR Green qPCR master mix (Fermentas) using a Bio-Rad iCycler. The expression of each target mRNA relative to 18S rRNA was calculated based on the threshold cycle (Ct), as 2−ΔΔCt, where ΔCt = CtTarget−Ct18S and ΔΔCt = ΔCt_control−ΔCt_test. Forward and Reverse Primer sequences are as follows: PLOD2, GAGGCGGCCGACAAATG-GAG and CTTGGTGGTTGCCCTTTGGAA; P4HA1, CCTGAGACTGAGAAATTGAACACACAG and GGGTTCAT-ACGTCTCCCACTC; VEGF, CTGGCTCGTCTGGCTC and TGCTTGGAAAGATGACTCG; LDHA, ATCTTGACC-TACGTGGCTTGGA and CCATACAGGCACACTGGAATCTC; PKM2, ACCAGGACAGCCAATACAG and CCGAGGATCCCATCTTC; GLUT1, CCGGGCCAAGGTTGGCTTAA and TGACGATCCGGCAGCAGAT; P53, GGTCTGCTG-ATCTCCAGAAAG and CAGATGCTCTCCGGATGATG; and 18S, GAGGATGAGGGTTGAACTGTT and AAGATGACCAGCC-CCCTCA.

2.13. Drug Treatments
The chemotherapeutic agents tested in this system were Paclitaxel or Taxol (Life Technologies), that was diluted in DMSO, according to supplier’s recommendation. Regarding spheroids, to keep the viability assay protocol and allow cell’s adaptation to the new environment, the drug treatments started at the first day of experiment (20 hours after spheroid embedment). Therefore, after this period, the medium was replaced by fresh media with adequate drug volume to have the desired final concentration. The working concentrations were selected in order to have an IC50 curve for both conditions. The dilutions were made successively and provided consistent results in different trials. Concerning 2D cultures, although the viability assay only takes 4 hours, drugs were added in the same working concentrations after 20 hours for consistency. After 5 or 7 days of exposure, the medium was replaced with Presto Blue containing media and the required drug concentration.

2.14. Oxygen Measurement
As described before, diffused oxygen was measured noninvasively using a commercially available sensor dish reader (SDR; PreSens) capable of reading diffused oxygen levels from an immobilized fluorescent patch. All measurements were performed in a controlled environment under standard conditions, and were taken every 15 seconds.

2.15. Live-cell Tracking of Individual Cells within Spheroids
The mid–plane of multicellular spheroids containing 10% of fluorescently labelled GFP-MDA-MB-231 cells was imaged at low magnification (10X) every 14 minutes, for 16 hours, using a Nikon Confocal Microscope A1. Single cell measurements were performed by tracking single cells using Metamorph image recognition software (Molecular Devices).

2.16. Data and Statistical Analysis
The mean squared displacement (MSD) was calculated as previously described. Collected data was organized for analysis in Excel (Microsoft). Mean values, Standard Error of the Mean (MEAN) and statistical analysis were calculated and plotted using GraphPad Prism (4.02, GraphPad Software). Where it was adequate, statistical analysis were conducted to compare means, namely two-tailed unpaired t-tests, and one-way and two-way ANOVA with Bonferroni post-test. In all presented data, *, **, *** and ns, indicate a p value <0.0001, <0.001, <0.01, <0.05, and >0.05, respectively. α = 0.05 was used for all significance. For IC50 curves fitting, after plotting log(drug concentration) and response values – as invaded distance and fluorescence reading – GraphPad Prism was used to fit the curves to nonlinear regression, specifically among ‘Dose-response curves – Inhibition’ panel.

3. Results
3.1. Hypoxia Promotes Tumor Progression
MDA-MB-231 cells (human breast adenocarcinoma) are metastatic and sensitive to hypoxia, upregulating more than 26 genes under this condition. Several studies have been conducted to understand hypoxia influence in this cell line.

3.1.1. Hypoxia promotes proliferation and invasion
As expected, MDA-MB-231 spheroids presented a larger occupied area under hypoxia, in particular after day 3 (Figure A,B). The invaded distance on day 7 is significantly different between normoxic and hypoxia, respectively 567.6±218 (SEM) µm and 736.2±213 (SEM) µm (Figure C). Despite the fact that hypoxia effect increases with time, day 5 was selected as the last point for drug testing to optimize time between experiments. There are several different mechanisms of defense potentially inherent to hypoxic tumor cells. In this work, tumor progression of MDA-MB-231 is explored under two mechanisms: proliferation and invasion.
Figure 1: Hypoxia promotes tumor progression through a response that combines proliferative and invasive mechanisms. A) MDA–MB–231 spheroids progression over 7 days under normoxia and hypoxia. The hypoxic spheroids show a larger occupied area and more branching. B) Variation of the spheroid radius over the 7 days. C) Invaded distances at day 7. D) Fluorescence readings from the viability assay based on Presto Blue at day 7. E) Number of live cells resulting from direct counting after 7 days of spheroid growth. F) Motility descriptors determined by plotting the logarithmic form of MSD simplified formula. G) Comparison of motility descriptor values. H) Directedness of single cells within the spheroid. I) Single cell trajectories within the spheroid, during 16 hours. In each image, the detail highlights the representative trajectories of each condition. J) Total travelled distance during 16 hours of exposure. K) NET distance travelled during the 16 hours of exposure. L) Magnitude of instantaneous velocity during 16 hours.

The viability assay based on Presto Blue reagent (Figure 1.D) presented fluorescence values of 1624 ± 32.3 (SEM) AU and 2055 ± 85.5 (SEM) AU for normoxia and hypoxia, respectively. These data were corroborated with direct cell counting, which showed an equivalent difference, with values of 53.3 ± 1.8 (SEM) kCells for normoxia, and 70.7 ± 3.4 (SEM) kCells under hypoxia (Figure 1.E). These data suggest that proliferation is an active mechanism of tumor progression under hypoxia, which was shown on previous studies [35], [36].

Spheroid invasion was analyzed as previously demonstrated using the MSD method [24], [29]. In MDA-MB-231 spheroids, hypoxia led to a significantly higher motility descriptor, 0.97 ± 0.06 (SEM), when compared to normoxic conditions, 0.76 ± 0.06 (SEM) (Figure 1.F,G). This difference indicates that hypoxia promotes a more persistent invasion mode, suggesting the existence of an invasive mechanism activation. In previous work, it was demonstrated that hypoxia increases tumor invasiveness and metastatic potential by the induction of genes encoding proteins involved in several stages of the invasiveness process, acting by different mechanisms [10], [37], [38], [39], [40]. Together, these data suggest that tumor progression in MDA–MB–231 spheroids is promoted by hypoxia, leading to a response combining both proliferative and invasive mechanisms.

3.1.2 Hypoxia promotes directedness in cell movement

To further understand the invasive mechanism in this system as a response to hypoxia exposure, MDA–MB–231 spheroids with 15% of GFP-labelled cells were monitored for 16 hours, allowing the tracking of single cells. Directedness, defined as the total displacement divided by the total travelled distance, was significantly higher under hypoxia, with a value of 0.29 ± 0.04 (SEM), and 0.20 ± 0.02 (SEM) for normoxia (Figure 1.H). Analyzing cell trajectories, as a function of time and radius, cells under hypoxia showed more elongated paths, while under normoxia, they tend to move in circulatory and random movements (Figure 1.I). These data suggest that hypoxia promotes a more directed trajectory towards the periphery of the spheroid. The total distance travelled by the cells is similar under both conditions (Figure 1.J), which indicates that hypoxia does not promote more single cell movement. Looking to the NET distance, which can be translated by the travelled distance in each time-lapse in the radial direction, hypoxia showed a value of 1127 ± 18.4 (SEM) µm, while normoxia presented 771 ± 8.5 (SEM) µm. These data are in agreement with the directedness hypothesis, since it shows that in each time-lapse, hypoxic cells move more in radial direction towards the spheroid periphery (Figure 1.K). The single cell velocity is similarly irregular under both conditions (Figure 1.L), which suggests that hypoxia does not promote an effect on it. Together, these data demonstrate that hypoxia increases the invasive potential of MDA–MB–231 spheroids by stimulating directed trajectories in a radial direction, which corroborates the previous presented data, where hypoxia promoted a higher persistent invasion mode (Figure 1.F,G). These observations can potentially be explained by the fact that hypoxia...
promotes ECM remodelling to facilitate metastasis. Briefly, cancer cells and associated stromal cells that have been exposed to hypoxia are transcriptionally reprogrammed to produce matrix metalloproteinases (MMPs), which degrade the basement membrane surrounding a tumor, facilitating cell movement. Consequently, collagen fibers within the interstitial matrix get aligned, which function as a highway for local invasion, intravasation and metastasis [8].

Therefore, if the spheroid growth is only due to proliferation and invasion mechanisms, it is possible to quantify each contribution based on fluorescence readings and single cell directness, respectively. Considering the percentages of hypoxic effect over the normal condition in each mechanism, and comparing them with the same percentage difference in spheroid grow, a rough estimative can be made, showing that spheroid progression involves 62% of invasion and 38% of proliferation.

3.1.3 Role of hypoxia-inducible factors

To adapt to the hypoxic microenvironment, cancer cells activate physiological responses to hypoxia that are mediated by Hypoxia-inducible Factors (HIFs), such as tumor growth, vascularization, metabolic reprogramming, invasion, metastasis, and resistance to radiation therapy and chemotherapy [13]. To look for the HIFs-regulation in this system, the expression of six hypoxia-related genes was analyzed, namely VEGF, PKM2, LDHA, PDK1, P4HA1 and PLOD2 [41].

MDA-MB-231 spheroids expressed significantly more under hypoxia five of the six tested genes, which suggests that the hypoxic tumor progression demonstrated before is related with the activation of HIFs (Figure 2A). The expression of LDHA, PDK1 and PKM2 present a metabolic adaptation. Interestingly, PLOD2 and P4HA1 present high expression, which is usually a hallmark for collagen alignment mechanisms [40]. These data are in agreement with the previous suggestion, that invasion as a response to hypoxia is mainly driven by ECM remodelling.

The activation of HIFs was also studied by knockdown of HIF1-α and HIF2-α in MDA-MB-231 spheroids. Both knockdowns promoted a reduction in spheroid size under hypoxia (Figure 2B), suggesting that both HIFs play an important role in MDA-MB-231 progression. Significant decreases were observed regarding invaded distance on both knock downs (Figure 2C), while only a slight reduction was observed in respect to proliferation (Figure 2D), which might be explained by the small size of spheroids. Since PLOD2 and P4HA1 are HIF-1-related genes [40], [41], these data demonstrate that tumor progression under hypoxia in this system is highly related to invasion, and it is regulated by both hypoxia-inducible factors.

3.2 Hypoxia promotes Chemoresistance

The presence of hypoxia decreases the effectiveness of chemotherapeutic drugs which require oxygen as electron acceptor to induce cell death. Moreover, tumor microenvironment, particularly hypoxia, induces cellular adaptations which contribute to tumor cell resistance against chemotherapeutic agents [10], [11]. HIF-1α overexpression identified by immunohistochemistry of tumor biopsies is associated with increased mortality in multiple studies involving thousands of patients with breast cancer [43]. In vitro studies started to be conducted to understand how hypoxia can promote drug resistance by HIFs activation [44], [45], [46], [47].

3.2.1 Hypoxia promotes resistance to Paclitaxel

MDA-MB-231 spheroids were treated with Paclitaxel (Taxol), a commonly used chemotherapeutic agent against breast cancer that interferes with the normal breakdown of microtubules. After five days of treatment, hypoxia promoted less chemo sensitivity, showing a significant difference in spheroid branching (Figure 3A). The invaded distance of spheroids treated under hypoxia is significantly higher than the ones exposed to standard conditions (Figure 3B). For
instance, comparing no drug treatment to 100 pM of Paclitaxel, normoxic spheroids invaded around 50% less, while under hypoxia they invaded around 30% less. Regarding proliferation, the viability assay fluorescence readings showed a significant advantage for spheroids under hypoxia (Figure 3.C). As an example, comparing again no drug treatment to 100 pM of Paclitaxel, spheroids under standard conditions showed around 12% less viability, while under hypoxia they showed around 7% less. Moreover, comparing the responsiveness of a spheroid cultured under standard conditions without drug treatment, with one exposed to low concentrations of Paclitaxel – from 1 to 100 pM – under hypoxia, it is remarkable to observe that even in Paclitaxel presence, hypoxia still promotes higher invaded distance and proliferation.

The difference between hypoxic and normoxic responses in each Paclitaxel concentration, allows the conclusion that for higher concentrations, namely 1 nM and 10 nM, hypoxia does not show resistance with regards to invaded distance, and the same conclusion can be taken for 10 nM of Paclitaxel, concerning proliferation data (Figure 3.D).

IC50 curves were assembled for both conditions, based on the two different studied responses. In respect to invaded distance, hypoxic spheroids presented an IC50 of 137.6 ± 63.1 (SE) pM, while under standard conditions the IC50 was 62.2 ± 58.6 (SE) pM (Figure 3.E). Regarding the proliferation data, spheroids under hypoxia showed an IC50 of 532.8 ± 74.4 (SE) pM, while when exposed to normoxic conditions presented 242.9 ± 82.8 (SE) pM (Figure 3.F). Since proliferation-based IC50 values are higher than the ones obtained based in invaded distance, it is reasonable to consider that Paclitaxel has a stronger effect on invasive response. This observation can be explained by the fact that invasive cells are the ones on spheroid surface, which make them the first target of the treatment. This comparison suggests that Paclitaxel, which is currently used with the aim of stopping cell division, could instead be used in lower concentrations to prevent metastasis.

### 3.2.2 Hypoxia decreases sensitivity to Doxorubicin

MDA-MB-231 spheroids were treated with Doxorubicin, a drug that works by intercalating DNA, often used in combination chemotherapy against breast cancer.

After five days of treatment, hypoxia promoted less chemosensitivity showing differences in spheroid branching (Figure 4.A). Moreover, the invaded distance of spheroids treated under hypoxia is always higher than the ones cultured under normoxia (Figure 4.B). For instance, comparing no drug treatment to 10 nM of Doxorubicin, normoxic spheroids invaded around 58% less, while under hypoxia they invaded around 49% less. In respect to proliferation, despite both conditions being closer than in terms of invaded distance, the developed viability assay also showed higher values for spheroids exposed to hypoxia (Figure 4.C). As an example, comparing no drug to 10 nM of Doxorubicin, spheroids under standard conditions showed around 14% less proliferative levels, while under hypoxia they showed 13% less. The difference between hypoxic and normoxic responses in each Doxorubicin concentration is approximately constant regarding invaded distance. The same conclusion can be taken based on proliferation data, with a slight decrease for 1 µM, which can indicate that from this value on, hypoxia may not promote an effect in chemosensitivity (Figure 4.D).

Regarding invaded distance-based curve, hypoxic spheroids presented an IC50 of 0.54 ± 26 (SE) nM, while
Figure 4: Hypoxia affects responsiveness to Doxorubicin treatment. A) MDA-MB-231 spheroids on day 5 of exposure to Doxorubicin treatment, under hypoxic and normoxic conditions. B) Invaded distance under Doxorubicin exposure in normoxia and hypoxia on day 5. C) Fluorescence readings from viability assay performed in spheroids exposed to Doxorubicin treatment under hypoxia and normoxia for 5 days. D) Difference between hypoxic and normoxic conditions on the day 5 of exposure to Doxorubicin treatment, regarding invaded distance (right) and fluorescence readings from viability assay (left). E) IC50 curves under normoxia and hypoxia based on invaded distance measurements for Doxorubicin treatment. F) IC50 curves for normoxic and hypoxic conditions based on fluorescence measurements for Doxorubicin treatment.

under standard conditions the IC50 was $0.13 \pm 0.74$ (SE) nM (Figure 4.F). Regarding the proliferation data, spheroids under hypoxia showed an IC50 of $21.0 \pm 28.5$ (SE) nM (Figure 4.G). Similarly with Paclitaxel treatment, proliferation-based IC50 values are higher, which suggests that invasive cells on spheroid surface are the first ones to be affected. Both analytical methods demonstrate that hypoxia promotes less chemosensitivity to Doxorubicin treatment due to the higher responsiveness in drug presence when compared with the trial under standard conditions. It is important to highlight that the difference in drug response under hypoxia and normoxia is much more significant in Paclitaxel treatment than with Doxorubicin. As before, these results are also in agreement with other studies [53], [54].

3.3. 3D microenvironment reduces drug response

It is becoming clear that screening of anti-cancer drugs on a 2D platform with cell lines is not suitable to precisely select clinically active drugs. The 3D models have potential for better simulating the in vivo tumor, and several studies have been conducted to understand the fundamental differences between the two systems in terms of drug response [55], [56]. Steps for the establishment of 3D tumor spheroid-based functional assays are starting to be taken, and showed key differential sensitivities to targeted agents between 2D and 3D cultures, suggesting enhanced potency of some agents against cell migration/invasion compared with proliferation [57], [58], [59].

In this work, MDA-MB-231 cells were exposed to Paclitaxel and Doxorubicin, as 3D spheroids and 2D cultures. In both drug treatments, the viability decrease was higher in 2D cultures, which means that in 2D, cells lose more viability comparatively to the control – almost 70% when exposed to increasing Paclitaxel doses (Figure 5.A), and approximately 80% regarding Doxorubicin (Figure 5.B) – while 3D spheroids kept the viability drop around 40% in the highest tested concentrations. These observations suggest that the 3D environment reduces chemosensitivity.

IC50 curves were also assembled for 2D cultures based on fluorescence readings resulting from the viability assay. With respect to Paclitaxel, MDA-MB-231 spheroids under hypoxia showed an IC50 of $532.8 \pm 74.4$ (SE) pM, while when exposed to normoxic conditions presented $242.9 \pm 82.8$ (SE) pM. On the other hand, MDA-MB-231 cells cultured in 2D showed $265.2 \pm 13.1$ (SE) pM under hypoxia and $255.2 \pm 19.4$ (SE) pM for normoxic conditions (Figure 5.C). Concerning Doxorubicin, spheroids under hypoxic conditions showed an IC50 of $21.0 \pm 28.5$ (SE) nM, while when exposed to normoxia presented $18.1 \pm 87.0$ (SE) nM. In 2D cultures, MDA-MB-231 cells presented $18 \pm 33.3$ (SE) nM and $1.6 \pm 48.3$ (SE) nM for hypoxic and normoxic conditions, respectively (Figure 5.D). It is important to highlight the fact that even in 2D cultures, hypoxia can play a role in chemosensitivity, showing slightly higher proliferative values. Even with a higher viability decrease in 2D, the Paclitaxel IC50 for both models under normoxic conditions are quite similar, suggesting that the 3D microenvironment by itself does not influence Paclitaxel-sensitivity. Regarding Doxorubicin, 2D cultures seem to be more responsive. Under hypoxia, the IC50 value in the 3D model is approximately double of the one obtained in 2D culture for Paclitaxel treatment, and more than 10 times for Doxorubicin trial. These data, particularly with regards to Paclitaxel, demonstrate the importance of 3D environment in drug re-
response promoted by hypoxia, allowing higher chemoresis-
tance. Therefore, it is interesting to highlight the impor-
tance of a 3D collagen matrix in tumor surroundings under
hypoxia regarding chemosensitivity enhancement.

4. Conclusions

Cancer is the leading cause of morbidity and mortality
worldwide [60]. Despite heavy investment in cancer Re-
search & Development, it was shown that 95% of potential
anticancer drugs entering clinical development fail [61], [1].
The use of inadequate preclinical models is one of the rea-
sons for drug failure [3]. In order to improve preclinical
platforms it is necessary to implement the features that
characterize the tumor microenvironment. Among them,
hypoxia is present in 90% of solid tumors, and it has been
correlated with poor outcome for patients, possibly due to
chemoresistance mechanisms [10].

In this work, MDA-MB-231 spheroids were used to study
hypoxia influence in two distinct responses: tumor progres-
sion and chemosensitivity. Regarding proliferation, both
used methods showed a significant increase under hypoxia,
while invasion showed a significantly more persistent inva-
sion mode. Tracking single cells revealed that directedness
of cell movement was significantly higher, and cell trajec-
tories revealed more elongated paths under hypoxia. NET
displacement was also higher in hypoxic cells. Therefore,
hypoxia promotes aligned cell trajectories towards the pe-
riphery of the spheroid, consistent with the persistent in-
vasion mode, probably due to ECM remodelling by meta-
loproteinases activation [8]. Quantifying the contributions
of both mechanisms showed that tumor progression occurs
based on 62% of invasion and 38% of proliferation.

To check for HIFs regulation in this system, six hypoxia-
related genes were analyzed, showing three times more ex-
pression in five of these genes. PLOD2 and P4HA1 ex-
pression are usually related with collagen alignment mech-
nisms [8]. By knocking down HIF1-α and HIF2-α, MDA-
MB-231 spheroids showed a significant reduction in size
and a decrease in proliferation levels under hypoxia. To-
gether, these data demonstrate that tumor progression under
hypoxia is regulated by both hypoxia-inducible factors, with
particular highlight for HIF1-α as a potential main mediator
of the invasive response.

Hypoxia effect in drug response was also studied, using
two compounds currently employed against breast can-
cer. After five days of treatment, hypoxia promoted less
chemosensitivity, showing higher invaded distance and pro-
liferation under both tested drugs. In terms of IC50, both
assembled curves – based on invaded distance and prolifera-
tions readings – showed higher values for hypoxia under
the treatment of both drugs, suggesting once more that hypoxia
promotes chemosensitivity. IC50 values based in invaded
distance were lower than the ones based on proliferation,
suggesting for instance that lower doses of Paclitaxel could
be used to reduce invasion in combination with other therapies for tumor size reduction, decreasing its harmful potential. The progression of a spheroid cultured under standard conditions without drug treatment was lower than the one correspondent to a hypoxic spheroid exposed to low concentrations of Paclitaxel – below or 100 pM, which suggests a chemoresistance effect against this drug, in agreement with similar studies.[48][49][50][51][52]

3D spheroids and 2D cultures were compared in terms of drug response. In both drug treatments, the viability decrease was higher in 2D cultures, suggesting that the 3D environment promotes less chemosensitivity. Regarding Paclitaxel treatment, IC50 values are very similar for both models under normoxia, suggesting that the 3D microenvironment by itself may not influence Paclitaxel-sensitivity. Overall, hypoxic spheroids showed higher IC50 values for both tested drugs, when compared to 2D cultures, showing higher resistance in 3D, in terms of proliferation. Therefore, the 3D collagen matrix in tumor surroundings allows the existence of physical barriers to drugs and other metabolites diffusion, leading to a response more related with the in vivo situation. Moreover, under hypoxia, the HIF-s regulation is potentially more relevant since these 3D models can encompass matrix remodelling to facilitate invasion.

The implementation of improved drug platforms is then critical to decrease the rates of failure of cancer drugs in clinical trials, as well as it will be helpful to better understand drug effect in more complex tumor microenvironment.

Acknowledgements

The author would like to acknowledge the advisers of the project, Dr. Denis Wirtz and Dr. Daniele Gilkes, for frequent guidance and trust, and Dr. Nuno Bernardes, for all the support. The author also wants to thank all members of Wirtz Lab, in particular Dr. Angela Jiminez for the introduction to this model, and Josh DiGiacomo, for the contributions in data analysis and assistance during lab experiments.

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