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Metabolic Response to Stress Differentiates Heterogeneous Cancer Cells with Varying Metastatic Potential

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Abstract

Intratumoral heterogeneity is ubiquitously present within primary tumors and contributes to intractable behaviors such as metastasis and mutability spatiotemporally. Mounting evidence has shown that heterogeneous cell populations can adversely affect cell metabolism and metastatic potential. The cell's only fluorescent molecules within the electron transport chain, flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NADH), can allow the quantitation of cell metabolism. We demonstrate the use of the optical redox ratio (FAD/(NADH+FAD)) to determine the metabolic behaviors of a heterogeneous panel of cells with varying metastatic programs at normal conditions and following acute hypoxia. At normal conditions, we reveal an attenuation in the optical redox ratio as metastatic potential decreases, not including the non-metastatic cell line. We reveal that reoxygenating the clonogenic cells after hypoxia enabled further differences in the optical redox ratio for the highly metastatic (increased by 43 ± 9%), semi-metastatic (increased by 33 ± 4%), and non-metastatic (decreased by 14 ± 7%) cell lines. This work coalesces two potential strategies for cancer treatment: 1) the optical redox ratio to assess cell metabolic features and therapy-induced changes 2) the method of inducing a "stress" test to identify further differences in heterogeneous cell populations.

1. Introduction

Tumor heterogeneity is a major hallmark of cancer and has hindered the ability to create a ubiquitous form of successful therapy for patients [1-3]. Locoregional tumors possess a variation of heterogeneous cells that arise from subclonal populations, of which only the "fittest" groups are able to contribute to the overall phenotype [4]. The causal role of somatic mutations within the cell groups can lead to adverse behaviors for a particular tumor, which in turn can create mutability spatially and temporally. Additionally, studies have shown that these subpopulations can contribute to metabolic reprogramming [3, 5], metastatic potency [5, 7], treatment resistance [8, 9], and transient dormancy signatures [10], prolonging the duration of treatment for cancer patients. Despite the clinical significance of cancer heterogeneity [11], its effect on the differentiation of malignant and benign carcinomas has gone largely unexplored. Therefore, the causal relationship between heterogeneous cell populations and its genetic, phenotypic and metabolic figures warrants further study.

Deranged cell metabolism is a robust driver of multifaceted cancer growth. Cancer proliferation and function is powered by the inherent production of lactate through anaerobic glycolysis in the presence of oxygen, also known as the Warburg effect [12, 13]. Bypassing mitochondrial respiration (OXPHOS) creates a decreased presence of reactive oxygen species (ROS) and induces glycosylation [14], leading to the deflection of glycolytic intermediates into the pentose phosphate shunt and thus synthesizing lipids, amino acids, and ROS scavengers for increased protection of the cellular antioxidant system [15]. Conversely, recent studies have suggested that the increased presence of OXPHOS [16-18] and ROS [17, 19] is directly proportional to invasive and metastatic potential. Tumor entities with activated oncogenes [20] and a substantial difference in intra/extracellular pH [21], caused by the variation in metabolic routes taken by the cell, can further confer metastatic behaviors. Additionally, the most common sites of metastasis exhibit varied metabolic programs used by metastatic cancer cells [22]. More studies related to these metabolic codependencies can lead the cancer community in a more direct path for investigating prognostic markers that portend a migratory phenotype.

Chance et al. were one of the first of many to assess the redox state of the cell using intrinsic metabolic fluorescence [23]. Within cells, flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NADH), electron transport chain's cardinal acceptor and donor respectively, emit the only forms of autonomous fluorescence with regards to metabolism. With optical imaging, these fluorescent figures can be used to contribute to the optical redox ratio (FAD/(NADH+FAD)), an assessment of cellular metabolic activity [23]. This ratio is a basic indicator of the cell's preferred metabolic pathway; for instance, as glucose is catabolized and used to produce adenosine triphosphate (ATP) within the cell, NADH is predominantly created and lowers the optical redox ratio (ORR). On the other hand, if cellular respiration is chosen for ATP production, then FAD is preferentially formed and hence increases the ORR. Recent reports have rendered the ORR to be substantial because of its sensitivity to dynamic changes in real-time [7, 24]. With the advent of stem cell therapy and regenerative medicine, the ORR can associate metabolic oscillations with the type of stem cell differentiation taken (such as osteogenic, adipogenic, or chondrogenic pathways) [25-27] and allow scientists to exploit the poorly understood mechanisms of these cells, while also correlating the ORR with other confirmed forms of cell analysis [25]. Additionally, reports have shown that the ORR has the ability to detect a metabolic response to radiation or drug therapy [28-30] and differentiate breast cancer types with various receptor expressions [31-33]. Despite the growing number of publications regarding the ORR's ability to differentiate cell type, this study will be the first of its kind to specifically look at the ORR of a heterogeneous breast cancer cell model with metastatic progression.

In this study, we investigated the endogenous fluorescence of a cohort of heterogeneous cells with the ORR. The isogenic cell lines – 4T1, 4T07, 168FARN, and 67NR – originated from the same murine tumor [22, 34-36] and vary by metastatic ability and plasticity [36]. The ORR was analyzed at baseline (normal conditions) and after a short period of hypoxia to reveal a significant difference in metabolism between metastatic cell lines and detect varying responses with respect to metastatic potency. We also confirm the sensitivity of the ORR to variations in oxygen consumption and compare it to a metabolic flux assay *in vitro*. This work coalesces two potential strategies for cancer treatment: 1) the ORR to assess cell metabolic features and therapy-induced changes 2) the method of inducing a "stress" test to identify further differences in heterogeneous cell populations.

2. Methods

2.1 Cell culture and short-term hypoxia

The isogenic mammary cell lines used in this study and listed in Table 1 – 4T1, 4T07, 168FARN, and 67NR – originated from a single tumor [34] and were given to us by Dr. Fred Miller (Karmanos Cancer Institute, Detroit, MI). The receptor statuses for all cell lines are stratified as negative for estrogen (ER), progesterone (PgR), and human epidermal growth factor receptor 2 (HER2), commonly known as triple-negative. Cells were passaged in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 1% nonessential amino acids, and 1% penicillin-streptomycin. All cell work was done within ten passages.

For the acute hypoxia experiments, a gas chamber (Biospherix, Parish, NY) was used to set the incubator atmosphere to .5% oxygen and 5% carbon dioxide and induce hypoxia for a total of 80

minutes (20 minutes to achieve desired atmosphere conditions and 60 minutes of allotted incubation time).

Cell line	Invasion - Metastasis cascade	Metastatic site
67NR	Primary tumor formation only	N/A
168FARN	Primary tumor + local invasion + intravasation	Lymph nodes
4T07	+ Extravasation	Micrometastases in lung, lymph nodes
4T1	+ Metastatic growth	Lung, Liver, Bone

Table 1. Cohort of heterogeneous murine cell lines with varying metastatic potential [7].

2.2 Seahorse metabolic flux analyzer

We have previously detailed the protocol for the Seahorse assay [7]. Essentially, the Seahorse metabolic flux analyzer determines mitochondrial usage by measuring the pH of the cell media before and after assigned drugs are injected sequentially. Cell densities for the panel of cell lines used – 4T1, 4T07, 168FARN, and 67NR – were plated at a concentration of 7500, 10000, 12500, and 15000 cells per well, respectively, on the day prior to experimentation. The same cell densities were plated in a 96-well plate for normalization purposes [36]. The normalized oxygen consumption rate (n-OCR) was established as the oxygen consumption rate (OCR) divided by the proton production rate (PPR).

2.3 Multiphoton imaging

Our multiphoton microscope [7] was used to attain NADH (755 nm) and FAD (860 nm) images. 24 hours prior to imaging, the cell lines were plated at a density of 250000 cells per well in a 6-well plate. Three field of views per plate were taken for three plates for normoxia and one hour following acute hypoxia. Two of these runs were completed.
To assess ORR sensitivity, three images of NADH and FAD (6 total) of the 4T1 cell line were acquired every ten minutes. After three sets of images were taken at baseline, oligomycin, an inhibitor of complex V of the electron transport chain (ETC), was injected and three more sets of images were taken. The same procedure was applied after the subsequent additions of carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP) and a mixture of antimycin A and rotenone, an uncoupler that perturbs the mitochondrial membrane potential and allows oxygen to be optimally consumed and an inhibitor of the ETC's complexes I and III respectively. Figure 4 illustrates the mitochondrial-perturbing drug effect on the ORR in more detail. Following this experiment, a control plate was analyzed at the same time points and exhibited no change in the ORR (data not shown). This was repeated for two more plates.

2.4 Statistics

Statistical differences of the ORR were determined using a nested, two-way analysis of variance test. Hypoxic effects and cell lines were attributed to be fixed effects. Statistical differences of each cell line were evaluated via post-hoc Tukey Honestly Significant Difference tests.

3. Results

3.1 ORR detects changes in oxygen consumption in real-time

We first established the sensitivity of the ORR. The NADH and FAD images of the 4T1 cell line were taken before and after a serial addition of mitochondrial-perturbing drugs (Figure 1A). We have plotted the ORR (Figure 1B) with respect to time and saw that the ORR mimics the changes seen in the n-OCR plot (Figure 1C). We ensured that these variations during the time interval were due to the drugs used by imaging a control plate following the experiment (data not shown). The ORR does detect changes in the oxygen consumption of the cell and can be correlated to an established *in vitro* metabolic flux assay [7, 24].

3.2 Heterogeneous cells exhibit significantly different ORRs based on metastatic potency at baseline and after acute hypoxic conditions

Table 1 exhibits the varying metastatic potential of the cohort of breast cancer cell lines used. 4T1 is able to intravasate, extravasate, and grow overt metastatic nodes within the lung, liver, and bone. 4T07 is not able to create metastatic nodes that grow autonomously. 168FARN does not have any extravasation potential, and 67NR can only form a primary tumor.
Figure 2A exhibits the ORR images of the clonogenic cell lines. We observe an increase of the ORR as metastatic potential increases (not including 67NR) at baseline in Figure 2B and 2C. The highest ORR at normal conditions was shown in the 67NR cells. We further distinguished the cell lines by inducing a hypoxic stress test. 4T1 and 4T07 exhibited a significantly higher ORR after acute hypoxia. There was relatively no change within the 168FARN group, yet within the 67NR cell group, we saw a significant decrease in the ORR after the hypoxic stress test. Our results are concordant with the n-OCR for these cell lines.

3.3 Reoxygenation after hypoxic stress test creates a sentinel indicator of metastatic potential

Figure 3 exhibits the difference in ORR before and after acute hypoxia for each cell group. There was an attrition of the ORR as metastatic potency decreased. Our statistical analysis reveals a significant interaction between the response to hypoxia and specific cell line. This tells us that hypoxia-induced reoxygenation can be potentially used to differentiate heterogeneous cell populations with varying metastatic potential.

4. Discussion

A plethora of studies have focused on aberrant cell metabolism due to its omnipresence within metastatic progression [7, 17, 18, 22, 36]. These reports have shown that the increasing effect of OXPHOS [7, 18, 36] and superoxide production [17, 19, 22] can lead to a pro-migratory phenotype. Our results are concordant with studies that show higher mitochondrial activity as metastatic potential increases [31-33]. Since the ORR can sense the varying changes in oxygen consumption, we can use this optical technique to detect various heterogeneous cell types.
With regards to the sensitivity of the ORR, we see a relatively high standard deviation in Figure 1B. Cell plates are 25 millimeters in diameter and are perfused with 1 milliliter of Seahorse supplemented medium. The drug delivery within the perfused media was most likely not

homogenized properly during experimentation. On the contrary, the Seahorse instrument is optimized to avoid these kinds of variation. Without the exogenous drug injections, however the variability of the ORR is as accurate as the Seahorse metabolic assay (Figure 2B).
Though others have seen a lower ORR (hence a higher glycolytic phenotype) for non-metastatic cells, our results exhibited a significantly higher ORR for the non-metastatic 67NR cells compared to the other clonogenic cell lines. One report reveals that the metabolomics for the syngeneic panel of metastatic cells (4T1, 4T07, and 168FARN) includes Krebs cycle and glycolytic metabolites whereas the non-metastatic form of the panel (67NR) only had the latter [16], which is consistent with another set of cells with metastatic progression [37]. Hence, the 67NR cells should have a much lower ORR than the one seen in this study. Simões et al. have recently shown that succinate dehydrogenase (SDH) is much lower in the 67NR than in the 4T1 cells [36]. SDH is a major factor of OXPHOS and is the only enzyme that takes part in the Krebs cycle and ETC. However, the limited presence of SDH lessens the amount of NADH being produced by succinate in the Krebs cycle [38], increasing the ORR in the 67NR cells. We have shown that n-OCR (OCR/PPR) measurements have concordant trends with the ORR. Looking at the constituents of the n-OCR, there was a 3x increase in the 4T1's OCR and PPR measurements compared to the 67NR's, creating similar n-OCR measurements at baseline. Our Seahorse measurements underpins the results of others [22, 36]; The n-OCR and ORR of these cell lines further demonstrates the rationale for inducing a hypoxic stress test to further differentiate heterogeneous cell lines.
To create a biomimetic atmosphere, we have chosen to incubate the cell lines in .5% oxygen for sixty minutes [39]. Tumor cells have been shown to experience short-term hypoxia (within minutes to days at a time) as the orientation of the tumor vasculature is being restructured due to neoplastic growth [40, 41]. The hypoxia-mediated response of pancreatic cells with metastatic progression following .5 or 2 hours of hypoxia has been shown to augment the ability to migrate, invade, and survive whereas longer durations of hypoxia would decrease the ability to reproduce and increase the amount of cell death and motility [42]. A higher presence of metastasis-driven cells *in vivo* [43, 44] and cancer stem cells [45] are prevailing with more reoccurring hypoxia periods. Additionally, cyclic hypoxia has been shown to present higher prognostic value than the conventional use of gene signatures in the clinic [46].
Altogether, our results established metabolic differences based on varying metastatic potential in the ORR for a set of heterogeneous breast cancer cell populations. A hypoxic stress test further differentiated metastatic from non-metastatic cells and can prove indicative of a migratory phenotype. For the future, we would like to assess the hypoxia-mediated responses for breast cancer subtypes such as PgR + and ER + cells to exploit any paradigm differences seen, similarly to what we have done with these triple-negative cells. Additionally, we would like to gestate a metastatic model of 67NR through repeated cycles of hypoxia to fully understand the putative factors of tumor hypoxia on metastasis.

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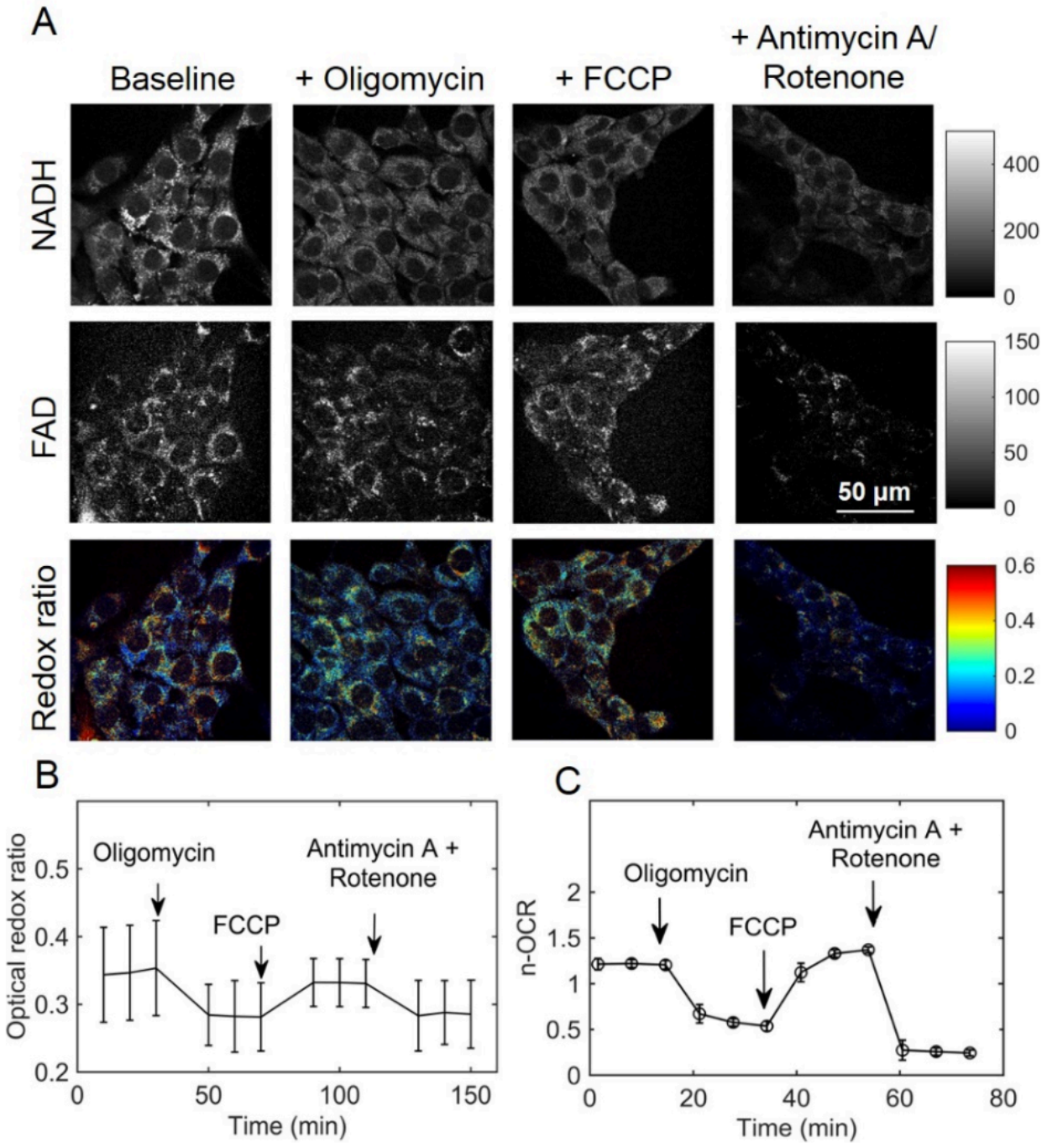

Figure 1. ORR detects changes in oxygen consumption in real-time. (A) Representative NADH, FAD, and ORR images for the 4T1 cells are shown at 20, 60, 100, and 140 minutes following the injection of mitochondrial-perturbing drugs. (B) Longitudinal ORR plot is shown following the subsequent addition of drugs. (C) Seahorse metabolic flux analyzer's n-OCR plot is shown following the serial addition of the same drugs. All error bars denote the standard deviation of the mean plate value [7].

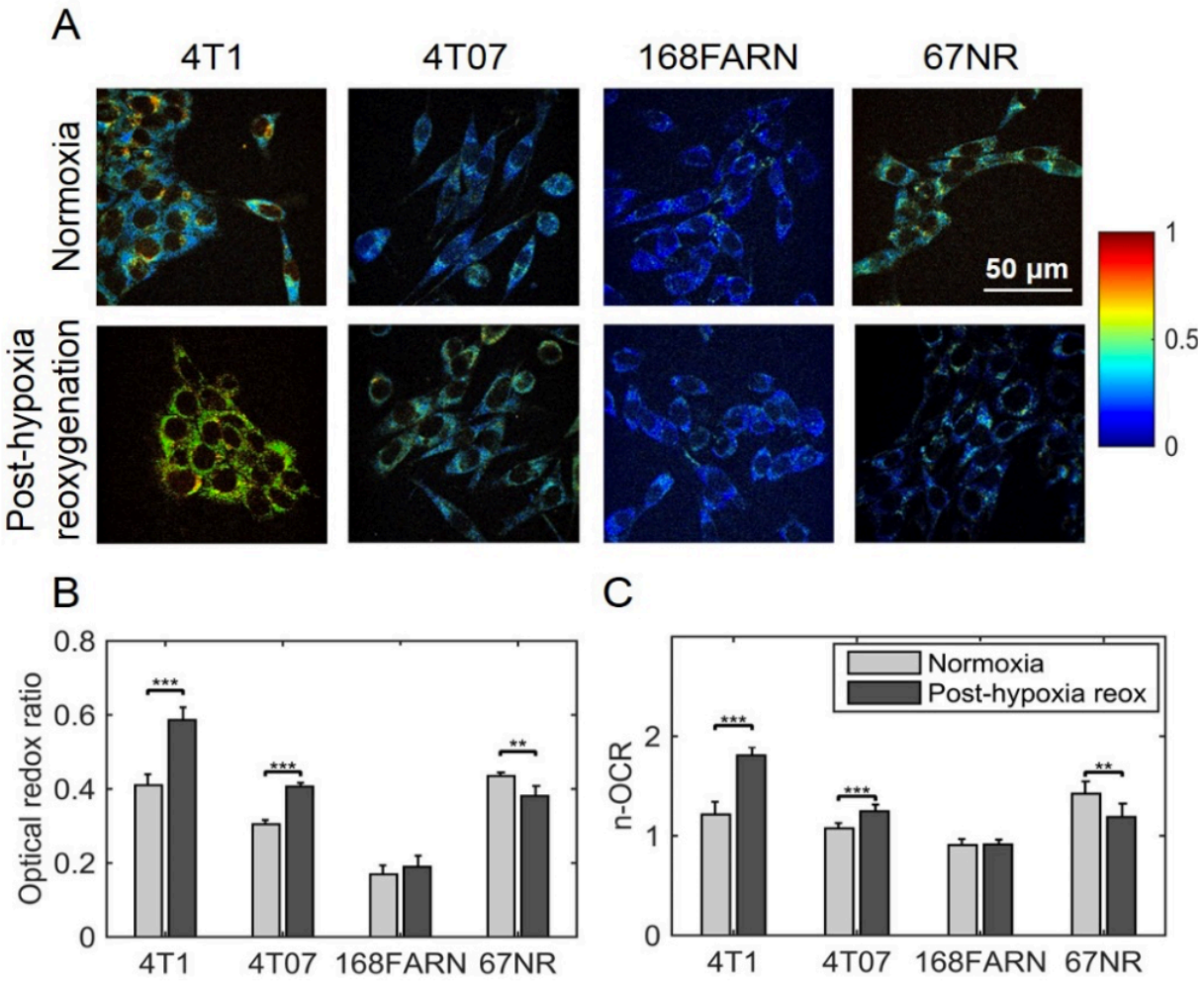

Figure 2. Heterogeneous cells exhibit significantly different ORRs based on metastatic potency at baseline and after acute hypoxic conditions. (A) ORR images are shown for the panel of breast cancer cells used at baseline and following our hypoxic stress test. (B) ORR is shown for each cell line at baseline and following acute hypoxia. (C) n-OCR is shown for each cell line at baseline and following acute hypoxia. ** represents $p < .01$ and *** represents $p < .0001$. All error bars denote the standard deviation of the mean plate value [7].

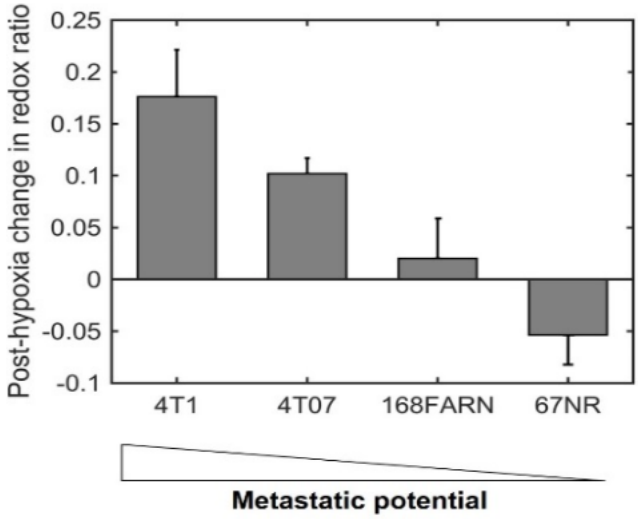

Figure 3. Reoxygenation after hypoxic stress test creates a sentinel indicator of metastatic potential. ORR differences between baseline and after acute hypoxia for all cell groups are shown. All error bars denote the standard deviation and were found by using this equation: $\sqrt{(sd_1)^2 + (sd_2)^2}$, sd_1 and sd_2 denote the standard deviations of the cell groups at baseline and following hypoxia, respectively [7].

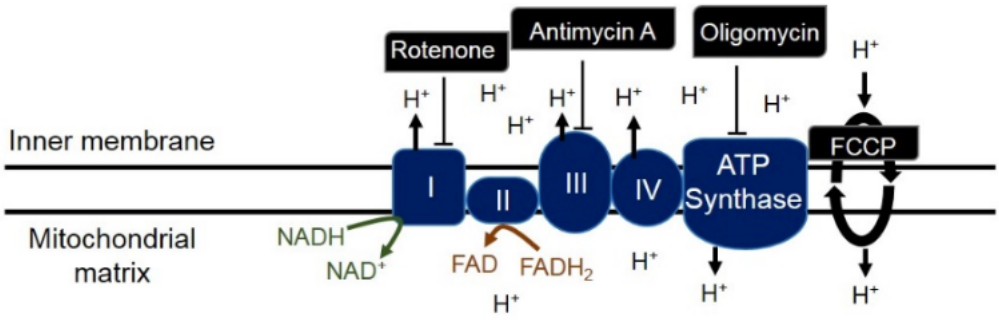

Figure 4. The mitochondrial-perturbing drug effect on the ORR. Oligomycin inhibits complex V of the ETC and decreases OXPHOS, enabling the decrease in ORR. FCCP perturbs the mitochondrial membrane potential and allows oxygen to be optimally consumed, leading to an increase in ORR. The mixture of rotenone and antimycin A inhibit complexes I and II of the ETC and essentially eliminates OXPHOS, decreasing the ORR [7].

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