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Modeling preclinical cancer studies under physioxia to enhance clinical translation

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Abstract

Oxygen (O_2) plays a key role in cellular homeostasis. O_2 levels are tightly regulated *in vivo* such that each tissue receives an optimal amount to maintain physiologic status. Physiologic O_2 levels in various organs range between 2–9% *in vivo*, with the highest levels of 9% in the kidneys and the lowest of 0.5% in parts of the brain. This physiologic range of O_2 tensions is disrupted in pathologic conditions such as cancer, where it can reach as low as 0.5%. Regardless of the state, O_2 tension *in vivo* is maintained at significantly lower levels than ambient O_2, which is approximately 21%. Yet, routine *in vitro* cellular manipulations are carried out in ambient air, regardless of whether or not they are eventually transferred to hypoxic conditions for subsequent studies. Even brief exposure of hematopoietic stem cells to ambient air can cause detrimental effects through a mechanism termed extraphysiologic oxygen shock/stress (EPHOSS), leading to reduced engraftment capabilities. Here, we provide an overview of the effects of ambient air exposure on stem and non-stem cell subtypes, with a focus on recent findings that reveal the impact of EPHOSS on cancer cells.

Keywords

Physioxia; Breast Cancer; Ovarian Cancer; Cancer Stem Cells; NRF2

INTRODUCTION

The emergence of cyanobacteria symbolizes the transition from anoxic to oxic atmosphere on earth (1). This increasing availability of O_2 is intricately linked to the evolution of major

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life forms on the planet, with more complex multicellular life forms beginning to evolve as O_2 levels began to rise to levels that support cellular bioenergetics and survival (2). The important roles that O_2 plays in the maintenance of life makes it a potentially effective biosignature in the identification of exoplanets that could support life (3, 4).

The present level of atmospheric O_2 is 21% (5), which ironically does not mirror tissue O_2 levels that are typically lower and ranges between 2–9% *in vivo* (6). In addition, distribution of O_2 in various parts within the same organ is heterogenous. Similarly, variations in O_2 distribution are characteristic of pathologic conditions such as tissue hypoperfusion, respiratory impairment, and anemia (7). Solid tumors are also characterized by a heterogenous distribution of blood supply and O_2. In general, the periphery of the tumors has better blood supply and thus, more access to O_2, while the core of the tumors, which is typically poorly perfused can be severely hypoxic (8, 9), a feature that contributes significantly to tumor cell heterogeneity (10–12).

In essence, the physiologic and pathologic O_2 conditions that are characteristic of normal or diseased states play important roles in mediating homeostasis and disease pathogenesis. This calls into question the current approaches to studying mammalian cells *in vitro*. Despite the fact that healthy and cancerous cells reside in significantly lower O_2 tensions, *in vitro* manipulation of many different cell types is carried out under ambient (~19–21%) O_2 conditions (6). The purpose of this review is to provide an overview of findings related to the beneficial effects of studying healthy and cancer cell types under physiologically/ pathologically relevant O_2 conditions. We discuss the impact of ambient O_2 conditions on cancer cell behavior with a focus on findings from our recent work which demonstrated effects of ambient air on signaling pathways, specific tumor cell populations, and response to therapy (13).

OXYGEN CONSIDERATIONS FOR *IN VITRO* STUDIES

The definition of various O_2 levels, especially with regards to how it relates to multicellular organisms is somewhat arbitrary. This is particularly true for the definition of normoxia. While one would imagine that the term “normoxia” describes “normal” physiologic O_2 levels in tissues and organs, it is used to describe ambient O_2 conditions to which tissues and cultured cells are exposed (14). A substantial number of comparative O_2 studies involve analyses of different cell types under normoxic and other O_2 conditions to observe for O_2-dependent differences (15–18). One important O_2 condition to which cells or tissues under normoxia are compared is hypoxia. Hypoxia is the condition whereby tissue O_2 levels are decreased either due to impaired blood supply or decreased blood O_2 levels (19). In the context of *in vitro* and *ex vivo* experimental systems, the hypoxic O_2 level is typically set at conditions <2% O_2 (20) although, some studies have considered O_2 levels ranging between 3% and 7% as hypoxic, depending on experimental design (21–23). Thus, further emphasizing the arbitrary nature of the terms used to describe different O_2 levels.

Physioxia on the other hand describes physiologic O_2 concentrations within tissues and organs and is also referred to as “tissue normoxia” (24). In general, O_2 levels in each tissue or organ are determined by the amount of blood supply to that organ (8). Therefore,

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in highly perfused organs, O_2 levels are typically higher than in less perfused organs and tissues (25). The bone marrow (BM), however, appears to be an exception to this rule, with significant heterogeneity in O_2 distribution. The highly vascular peri-sinusoidal regions have the lowest O_2 levels (1.3%) while the endosteal regions have slightly higher O_2 concentrations (1.8%). The outer periosteal layer is relatively non-hypoxic, with O_2 levels as high as 7%, due to the relatively lower cellularity and reduced metabolic activity, compared to the BM (9). This is in line with the fact that the amount of blood (and O_2) supply to tissues is governed also by their metabolic demands (10). This gradient of O_2 tensions has been described in other organs. In the brain, the mean pO_2 decreases with increasing depth from the dura mater (11, 12). In contrast to the brain tissue, the pO_2 in the skin tissues increases with depth from the epidermis towards the sub-papillary plexus (26). In the kidney, higher cortical O_2 levels, relative to the medulla, has been reported (27, 28). Furthermore, hepatocytes that make up the liver parenchyma are subjected to a gradient of O_2 tensions. Hepatocytes in the periportal zone of the hepatic lobule are exposed to higher amounts of O_2 compared to those located in the perivenous regions. This influences the heterogeneity and plasticity of these cell types with significant impact on differentiation and maturation of hepatocytes and subsequent metabolic zonation (29–32).

Based on these highly regulated variations in the distribution of O_2 among and within tissues/organs, there is a consensus that mammalian cells of diverse types are significantly impacted by exposure to ambient O_2 tension as it exceeds O_2 levels within the *in vivo* microenvironment (33). For this reason, several studies have yielded important data on how culturing and propagating cells under physioxia may be beneficial to understanding cell behavior in the context of their *in vivo* microenvironment. Since physiologic O_2 levels are known to range between 2–9%, experimental physioxic O_2 levels are typically set at 5%, with 2–3% O_2 representing the lower limit (34). Numerous studies have demonstrated the impact of physioxia in preserving important stem cell types such as induced pluripotent, embryonic, and adult stem cells. For example, mesenchymal adipose-derived stem cells (ASCs) grown under hypoxic (2–3% O_2) conditions have been shown to exhibit stemness maintenance, improved proliferative capacity and effective differentiation following osteogenic stimuli (35). Such beneficial effects have also been reported for physioxia on the chondrogenic potential of BM derived mesenchymal stem cells (MSCs) (36–39). Further studies into the differential effects of physioxia on ASCs by Chen and colleagues (40) revealed positive effects of physioxia on their proliferation, migration, angiogenesis, and survival. Data from studies involving four human MSC lines showed beneficial impact of physiologic O_2 tensions on the genetic stability, decreased oxidative stress and resultant survival of mesenchymal stem cells (41). Similar beneficial effects have been demonstrated with central nervous system stem cells (42), neural crest stem cells (43), lacrimal gland derived MSCs (44), nasal olfactory mucosal MSCs (45), umbilical cord MSCs (46), skeletal muscle-derived stem cells (47) and hematopoietic stem cells (48–54). The impact of physioxia on various stem cells has been reviewed extensively (25).

With regards to non-stem cell subtypes, early studies revealed a potential for an increase in the proliferative lifespan of normal diploid cells if they are cultured in O_2 conditions lower than 10% (55). Numerous findings have also been reported with regards to the effects of physioxia on mechanistic processes in non-stem cells (56). Nishikawa and

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colleagues (57) explored the effects of changes in *in vivo* O_2 tensions on osteoclastogenesis. They determined 5% and 2% O_2 levels correspond to physioxia and physiologic hypoxia, respectively, in osteoclasts. O_2 perturbation involving minimal changes within the physiologic range of O_2 tensions significantly impacted osteoclastogenesis via mechanisms presumed to be associated with O_2 sensing roles for ten-eleven translocation (TET) enzymes (57). Other studies have demonstrated the differential effects of ambient and physioxic O_2 levels on cellular redox homeostasis. The underlying hypothesis of these studies being that exposure of cultured cells to ambient air induces development of reactive O_2 species (ROS) that influences cell behavior. Ferguson *et al* (58) evaluated the effects of photodynamic irradiation therapy on A431 human epidermoid carcinoma cells which have either been exposed to ambient O_2 conditions for extended periods or maintained under physioxic conditions. The A431 cells cultured under hyperoxic conditions responded to photodynamic irradiation through increased production of mitochondrial ROS and subsequent upregulation of genes associated with maintenance of redox homeostasis, relative to the cells under physioxia. These differences, however, did not translate into differences in the viability of the treated cells under both O_2 tensions, suggesting that the antioxidant response of the cells treated under hyperoxic O_2 conditions protects the cells from oxidative damage. Ambient air induced increases in cellular ROS may also be associated with the induction of O_2–consuming cellular enzymes including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (Nox4), nitric oxide synthases (NOS) and monoamine oxidase (MAO) (59). This increase in ROS production may cause cells to senesce, die or adapt to these oxidative products. However, cells that adapt to such conditions may experience changes to their phenotypes, which may confound experimental findings (60, 61). A review of the effects of O_2 induced ROS production in *in vitro* cell culture is provided by Jagannathan et al (6).

Therefore, it can be concluded that *in vitro* manipulation of both stem and non-stem cell subtypes should be carried out under O_2 conditions that are optimal for their growth and maintenance of their *in vivo* phenotypes. Indeed, multiple cell types were demonstrated to benefit from O_2 tensions that range between 5–8%, which mirrors physioxic O_2 conditions in tissues and organs *in vivo*. The 5–8% range in O_2 levels was shown to be not too low to cause anoxia and not high enough to induce O_2 induced oxidative injury, but just right – the so called “Goldiloxygen” zone (62).

EXTRAPHYSIOLOGIC OXYGEN SHOCK/STRESS

Hematopoietic stem cells (HSCs) reside in environments with reduced O_2 tension *in vivo* (63, 64). Studies into EPHOSS evaluated the effects of even brief exposure of HSCs (< 1 hour) collected from mouse BM to room O_2, regardless of eventual studies under physioxia or room O_2 (48, 65). The collection and processing of HSCs from BM and cord blood (CB) in ambient air significantly decreased HSC yield due to rapid differentiation into hematopoietic progenitor cells (HPCs). Collection and processing of the BM and CB cells under physioxia (3% O_2) led to increased yield of BM HSCs and BM engraftment capacity as determined via analyses of various phenotypic markers and competitive BM transplantation experiments. The differentiation of HSCs into HPCs occurred through a phenomenon that was referred to as EPHOSS. These studies revealed

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that the mechanisms responsible for EPHOSS may be associated with the increased production of mitochondrial ROS and an associated induction of mitochondrial transition permeability pore (MPTP) opening via regulatory mediation by cyclophilin D. In addition, p53 was implicated in EPHOSS due to positive effects of p53 deletion on HSC yield. Furthermore, hypoxia inducible factor 1 alpha (HIF-1α) and miR210 were linked to EPHOSS although mechanism(s) of this linkage is not well understood (48). Similar effects were observed for the preservation of HSC populations and improved BM engraftment capacity when mobilized mouse peripheral blood was collected at 3% O_2 (53).

In subsequent studies, it was discovered that the collection and processing of aged mouse BM hematopoietic cells at 3% O_2 enhanced the number and engraftment capability of HSCs that were otherwise presumed to be limited in their capacity for long-term repopulation (54). Similarly, despite known deficiency of HSCs and HPCs in the BM of *Fanca* and *Fancc* knockout mice, collection and processing of BM cells from these mice under physioxia led to significant increases in the yield of long-term HSCs (50). Newer mechanistic insights suggest roles for Calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2) and TET2 in HSC to HPC differentiation fate determination and HSC self-renewal respectively (51, 52).

Although these findings are more directly linked to the hematopoietic system, they provide compelling insights as to how other cell types can be influenced by even short-term exposure to ambient air. Cancer cells have been studied extensively under hypoxia. However, the findings of studies involving HSCs (48) beg the question of whether the initial propagation of cancer cells in air, regardless of eventual transfer to low O_2, influences experimental findings and thus, suggests the need to reevaluate cancer cells in the context of EPHOSS. We explored the impact of EPHOSS on cancer cells utilizing animal models of breast cancer and human cancer cells (13).

EXTRAPHYSIOLOGIC OXYGEN SHOCK/STRESS IN CANCER

Most solid tumors are highly hypoxic (19, 66). However, O_2 concentration in solid tumors is anisotropic (12, 34, 67). Specifically, solid tumors are characterized by regions of physioxia (~8% O_2), hypoxia (~1% O_2) and anoxia (0% O_2) (12). This suggests that cancer cells within solid tumors are not necessarily subjected to a fixed amount of O_2 supply. This notion is supported by reports that solid tumors can undergo cycling hypoxia, which describes spatio-temporal fluctuations in O_2 tension, that leads to alternating levels of hypoxia within the same tumor (68–72). While O_2 concentrations in healthy breast tissues range between 6.8% to 9% or higher, the median O_2 concentration in about 40% of breast tumors is about 3.9% and can be as low as 1.3% (34, 73).

The effects of O_2 levels on tumor cell behavior have been studied extensively, with the vast majority involving comparative studies between ambient O_2 and hypoxia (0.5%–1%), an O_2 concentration required to induce activity of the HIF proteins (74–78). However, majority of these studies were carried out only after the cells have already been exposed to ambient O_2 conditions. Furthermore, majority of preclinical cancer studies, including evaluation of cancer cell response to target drugs were done in room O_2. Interestingly, very few studies

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have explored the toxic effects of ambient O_2 on cancer cells. Early studies showed that FS19 murine sarcoma cells processed from growing tumors and propagated *in vitro* under culture conditions in atmospheric O_2 failed to proliferate, compared to those grown at 5% O_2 (79). More recently, comparative studies involving four patient-derived breast cancer cell lines grown at 5% O_2 and other established, routinely used breast cancer cell lines grown in ambient air revealed increased expression of the HIF-1 target gene, carbonic anhydrase 9 and decreased ROS production at 5% O_2, relative to ambient air (80). Signaling pathway differences and response to targeted drugs were, however, cell line dependent with the conclusion of this study being that breast cancer cells grown at 5% O_2 are mostly similar to those in ambient air. It is, however, important to note that both studies involved the use of cancer cells initially processed in ambient air prior to culture at 5% O_2.

In our recent work (13), we asked whether observations made in HSCs with respect to initial exposure to ambient air also applies to tumor cells. We utilized tumor cells from transgenic mammary tumor mouse models (MMTV-PyMT and MMTV-Her2) and metastatic cells in ascites fluid collected from ovarian cancer patients. Tumor tissues were harvested under physioxia (3% O_2), minced, and split into two parts which were processed into single cells under physioxia and ambient air (21%). The cells were either stained directly for analyses of CSC marker profiles, prepared for proteome analyses, re-transplanted into animals or cultured at 5% O_2 and 21% O_2. To ensure that observed differences were associated only with the differential O_2 tensions and not intertumoral variability, the tumor cells were processed from fractions of the same tumor tissue. The ascites fluid was collected using uniquely designed syringes/needles that had been calibrated to physioxia and ambient air, respectively. The samples were then transferred immediately to the respective O_2 workstations. An important aim of our experimental approach was to ensure that the tumor cells being collected, processed and evaluated under physioxia were never exposed to room O_2 (Fig. 1; A and B). In a combination of flow cytometric, proteomics and sequencing approaches, we demonstrated variations in CSC marker profiles, relevant cell signaling pathways, ROS generation, alternative splicing, and response to targeted drugs due to experimental physioxic or ambient O_2 conditions (Fig. 2) (13).

CSCs are important for cancer progression (81–84). To evaluate the impact of ambient air exposure to CSC marker profiles, we selected LGR5 and TSPAN8 as markers of mammary tumor stem cells. LGR5 is a biomarker of CSCs (85) which contributes to breast cancer progression via the Wnt/beta-catenin signaling axis (86). TSPAN8 is upregulated in breast CSCs and maintains stemness via expression of genes such as NANOG and OCT4 (87). Flow cytometric analyses revealed an enrichment of $LGR5^+$ cells in both PyMT and Her2/Neu tumor models under physioxia, relative to ambient air. However, no significant changes were observed in the number of TSPAN8 positive cells in both models. Importantly, we demonstrated that the observed increase in number of $LGR5^+$ cells is not transient, as similar observations were made following re-transplantation of the collected tumor cells into syngeneic female FVB/N mice. 2D culture conditions were however found to deplete $LGR5^+$ cell populations under both physioxia and ambient air. We circumvented this effect via mammosphere culture conditions, which showed an enrichment of $LGR5^+$/$TSPAN8^+$ tumor cells. Other stem cell markers, including CD61, CD49f and EpCAM also exhibited O_2 dependent changes, but observations varied based on tumor model, culture or transplant

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conditions. To further confirm enrichment of CSCs under physioxia, we demonstrated an increase in the expression of stemness associated genes, including Bmp6, Zeb1 and Gli2 compared to ambient air.

ROS are produced as by-products of O_2 metabolism in a regulated fashion to maintain cellular homeostasis (88). The oxido-reductive processes that regulate cellular ROS production are regulated in a spatial and temporal fashion (89–91). We evaluated the impact of the differential O_2 tensions on ROS production in various cellular compartments using membrane-permeant redox-sensitive fluorescent probes that were analyzed via flow cytometry. We observed increased levels of nuclear ROS production in the tumor cells under physioxia. However, cytoplasmic and mitochondrial ROS production showed no significant difference. We then proceeded to ask whether there is a correlation between LGR5 and/or TSPAN8 positivity and nuclear ROS production. $LGR5^+$ cells were enriched within the nuclear ROS producing cell population under physioxia and ambient air. This enrichment was, however, more significant under physioxia. Thus, $LGR5^+$ cells likely generate ROS, which probably influences their activity. Surprisingly, the increase in nuclear ROS in tumor cells under physioxia did not translate into activation of NRF2 in these cells. NRF2 is associated with oxidative stress and mediates activation of antioxidant genes (92–94). The collection/processing of tumor cells in ambient O_2 led to a stabilization of NRF2. On the other hand, KEAP1, which is a negative regulator of NRF2 (95, 96) was differentially expressed under physioxia. This differential activity of NRF2 in ambient air likely occurred due to stresses induced by exposure of the cells to room O_2. We speculate that the increased expression of NRF2 in ambient air may have contributed to the observed decrease in nuclear ROS. The adaptation of routinely used tumor cell lines to continuous propagation in ambient O_2 is likely due to sustained development of an antioxidant response to O_2 exposure and subsequent protection from oxidative stress. In support of this notion, FS19 murine sarcoma cells already adapted to growth in ambient air became more sensitive to O_2 following inhibition of glutathione (79). Indeed, glutathione has been reported to be elevated in multiple forms of cancer (97) and novel therapies targeted against glutathione are being explored as potential anticancer agents (98, 99). It would be interesting to determine how much of the elevated levels of glutathione and antioxidant enzymes such as superoxide dismutase (100) in various cancers is influenced by exposure of tissues/cell to ambient air. It is likely that some of the observed anticancer effects of glutathione inhibitors (101) are due at least in part to the loss of antioxidant protection from O_2 associated oxidative stress. In addition, immortalization and transformation of certain cell lines for cancer studies may protect the cells from the detrimental effects of excessive O_2, possibly due to a decreased potential for replicative senescence (6). Furthermore, mechanisms associated with enhanced glycolytic processes in immortalized and cancer cells may confer adaptative properties that allow them to grow in high O_2 levels for extended periods (6, 102–104). While the role of NRF2 in the mediation of oxidative processes in cancer cells has been studied extensively (105–107), it may be necessary to reevaluate some of its functions under physioxia, while concurrently eliminating ambient air as a confounding factor.

In addition to NRF2 signaling, maintenance of tumor tissues/cells under physioxia impacted several other signaling pathways including YAP signaling, Cyclin D1/cell proliferation, alternative splicing machinery, and response to targeted therapies. For example, cells under

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physioxia expressed lower levels of cyclin D1, which correlated with lower proliferation rate compared to cells maintained in ambient air. This is in line with previous observations that spheroids derived from HCT116 colon adenocarcinoma cells under physioxia exhibited significantly decreased growth rates, compared to those grown in ambient air (108). Additionally, cells under physioxia were less sensitive to targeted therapies. This is consistent with the notion that cancer cells with stem cell properties are slow proliferating and slow proliferating cells are less sensitive to therapies (109–112). These findings raise the question of ideal experimental conditions for cancer tissue collection, processing, and culturing for drug screening studies. Furthermore, since the epigenome significantly affects sensitivity to targeted therapies, our published report on the effects of O_2 tension on the levels of the epigenetic regulator BRD4 (13) and other studies showing O_2 sensing roles for epigenetic regulators TET2, KDM5A and KDM6B (51, 113, 114) suggests that observed differences in drug sensitivity under physioxia and ambient O_2 may be partially influenced by differences in epigenome. However, most other studies on the influence of O_2 tension on epigenome compared the epigenome under hypoxia with ambient air without considerations for initial exposure of the cells in hypoxia to ambient air. Studies involving fate-mapping of hypoxic cells revealed that tumor cells that experienced intratumoral hypoxia exhibited gene expression patterns distinct from those that were exposed to hypoxia *in vitro* (115). Although many different factors may have contributed to this finding, there is a likelihood that *in vivo* hypoxic conditions diminish ambient air induced cellular changes.

CONCLUSION AND PERSPECTIVES

Numerous studies have evaluated the impact of physiologically and pathologically relevant O_2 on different cell types, including stem, adult and cancer cells of diverse types. However, these prior studies were done using tissues and cells that were initially collected, processed and propagated under ambient air before eventual transfer to workstations with low O_2 levels. We demonstrated the impact of ambient air on stemness phenotype, intracellular signaling and response to therapy. Our studies examined the effect of exposing tumor tissues to ambient O_2 for one hour compared to maintenance throughout at physiologic O_2. Rationale for considering one hour exposure to ambient air is that, in our experience, it typically takes one hour for tumor tissues to arrive to research labs after surgery if the process of collection and distribution is well coordinated. These studies are of importance, considering the limited translatability of preclinical findings into clinical findings in cancer research. The observed differences in the response of the studied tumor cells to therapy based on differences in O_2 levels suggests a need for the consideration of O_2 in preclinical cancer studies. More importantly, the differential impact of ambient air and physioxia on relevant signaling pathways and cellular biomarkers provide a compelling basis for the evaluation of cancer cells in the context of EPHOSS. For example, the development of effective therapies against NRF2, based on its presumed importance for cancer progression has been met with inconsistencies (116–118). Data from our recent study adds another layer of complexity to this inconsistency, considering the sensitivity of NRF2 signaling to differential O_2 tensions.

Cancer cell quiescence is associated with resistance to therapy and a quiescent state is characteristic of cancer cells with stemness properties (111, 112). We demonstrated the

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impact of the differential O_2 tensions on the expression of stemness associated biomarkers. In line with these findings, signaling pathways associated with cell cycle progression were found to be less active under physioxia, with the tumor cells under physioxia being less proliferative than those in ambient air. We speculate that the combined differences in stemness marker profile and signaling pathway differences culminate in the development of a quiescent state that contributes to the development of resistance to therapy under physioxia. The perturbation of this intricate network perhaps contributes in part to the efficacy of hyperbaric cancer treatment in sensitizing cancer cells to some chemotherapeutic drugs (119–121), considering the impact of high dose O_2 treatment on cancer cell metabolism and their redox state (121).

Metastasis is the primary cause of cancer death (122, 123). There has been limited progress in developing metastasis-targeted therapies. Paired genome analysis of primary tumors and metastasis have revealed differences in metastasis compared to primary tumors but none as new therapeutic targets (124, 125). Other studies showed very minimal differences in the genome of primary and metastatic tumors (126, 127). A recent study that analyzed treatment-naïve, primary and metastatic breast cancer identified potentially druggable alterations in genes associated with cancer epigenetics, stemness and drug resistance in metastasis although the bulk of the genomic changes observed were similar in both the primary and metastatic tumors (128). It is possible that epigenome/transcriptome of metastatic cells undergo metastasis organ-site specific changes based on tissue O_2 levels, which genomic sequencing cannot identify. Therefore, studies that compare signaling in metastasis with signaling in primary tumor may need to consider O_2 levels in organ site of metastasis while modeling their studies. This is especially relevant for studies on brain metastasis where O_2 levels are quite low compared to other organs.

The findings of our study provide new insights into the impact of initial and prolonged exposure of tumor cells to ambient air. Surprisingly, we detected no increases in HIF signaling in tumor cells under physioxia, suggesting that some of the signaling differences observed under physioxia may be independent of HIF activation. This is, however, in contrast to other reports that both normal and cancer cells cultured at 5% O_2 exhibited an increase in the levels of transcriptionally active HIF-1α, with resulting impact on proliferative capacity (129, 130), perhaps, indicating cell line to cell line differences. Most studies that examined HIF-independent hypoxia signaling compared signaling under hypoxic state with ambient air. Few of these HIF-independent signaling under hypoxia may be active under physioxia as well. In this regard, multiple differentially abundant proteins were found to overlap in three B-cell lymphoma cell lines under physioxia and hypoxia, suggesting that distinct mechanistic processes may govern tumor cell response to physioxic and hypoxic conditions (131). Therefore, it may be necessary to reevaluate few of these signaling pathways in a gradient of O_2 levels instead of the fixed 1% to 21% O_2 comparison.

Ambient air induced changes in stem cell marker profiles, transcriptome and signaling networks suggest the need for preclinical cancer studies to be carried out in the context of EPHOSS. An additional factor to consider in such studies would be creating an *in vitro* system that more closely mimics the tumor microenvironment *in vivo*. This is especially important for studying tumor cells in the context of their interactions with other cells that are

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present within a microenvironment that is characterized by decreased O_2 levels (132–134). Considering the fact that 2D culture systems are limited by factors such as a lack of a tumor microenvironment, perturbation of gene expression and changes in tumor cell phenotype (135, 136), 3D co-culture systems that allow for cancer cell interaction with other relevant cell types (137–140) in O_2 controlled experimental settings may aid better understanding of cancer cell behavior. While O_2 considerations alone by no means address all the challenges associated with ensuring translatability of cancer research, we propose that it should be considered one more step forward in ongoing efforts to improve the status quo.

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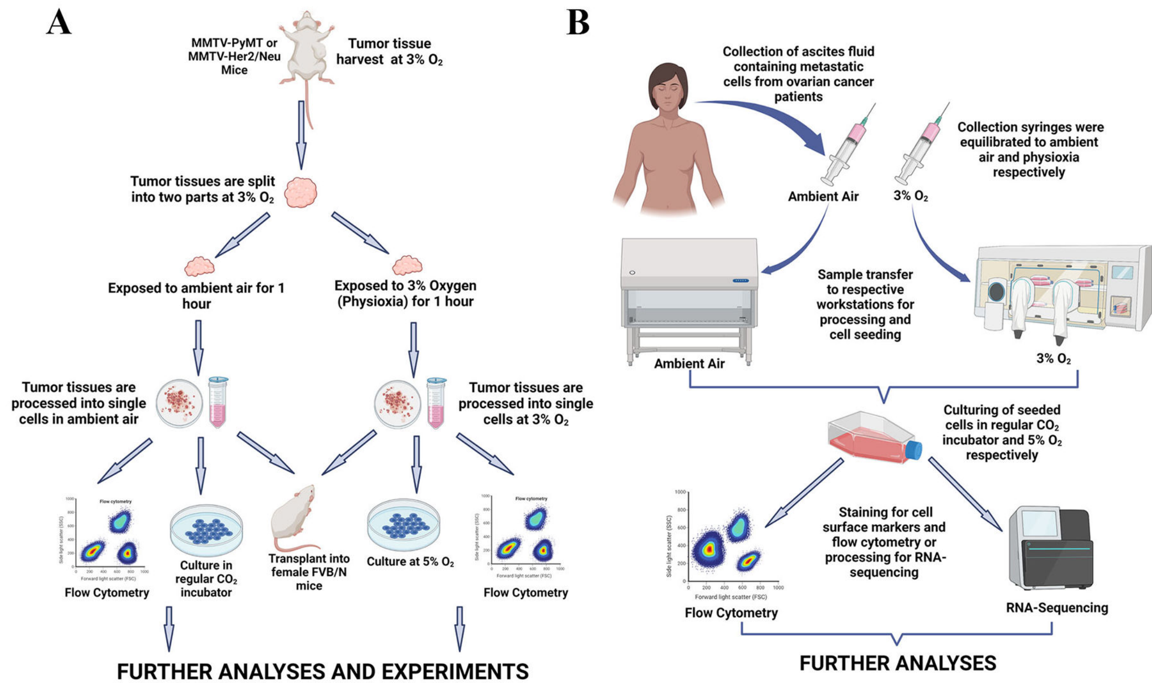

Figure 1.

Schematic representation of experimental workflow. **A.** Mouse mammary tumor tissues were harvested under physioxia, split into two parts and processed into single cells at respective oxygen tensions. Processed cells were either stained for flow cytometry, cultured for 5 days or transplanted into female FVB/N mice for further studies. **B.** Ascites fluid was collected from ovarian cancer patients with uniquely designed syringes previously equilibrated to ambient air and physioxia. Collected samples were transferred immediately to respective workstations, processed and cultured for 5 days prior to flow cytometric and sequencing analyses. Figures were created with BioRender.com.

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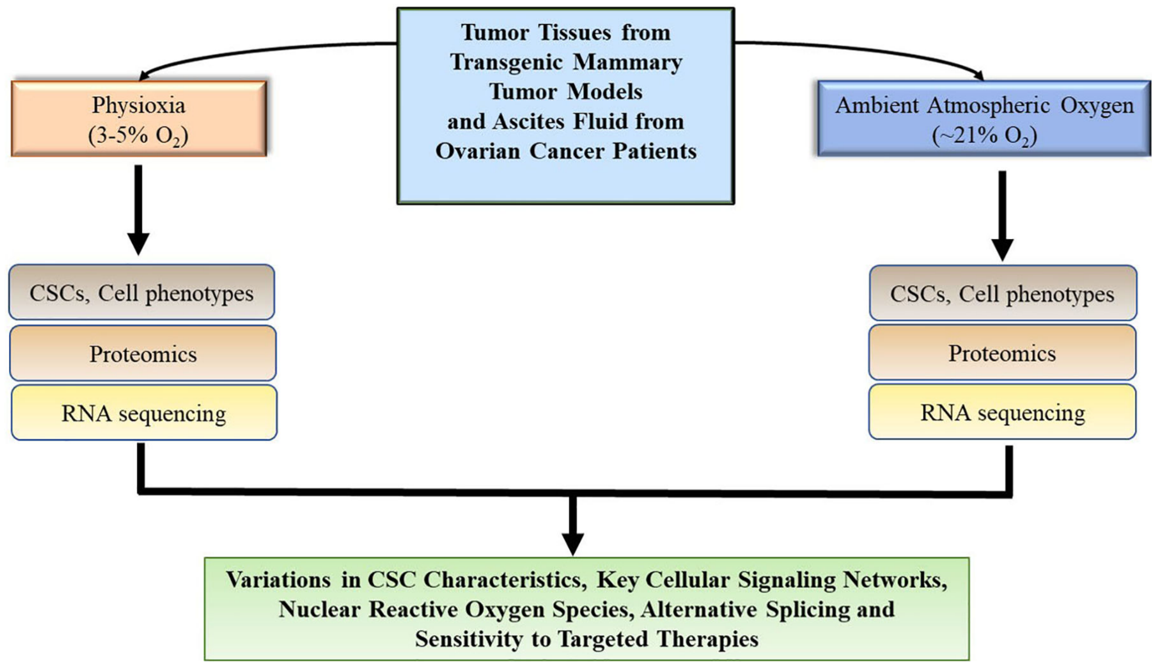

Figure 2.
Summary of experimental findings. Flow cytometric analyses of cell phenotypes, proteomics and sequencing analyses of tumor cells in ambient air and physioxia revealed differences in relevant signaling pathways, generation of reactive oxygen species, alternative splicing and response to targeted drugs.