14-3-3σ Expression Effects G2/M Response to Oxygen and Correlates with Ovarian Cancer Metastasis

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Abstract

Background: In vitro cell culture experiments with primary cells have reported that cell proliferation is retarded in the presence of ambient compared to physiological O2 levels. Cancer is primarily a disease of aberrant cell proliferation, therefore, studying cancer cells grown under ambient O2 may be undesirable. To understand better the impact of O2 on the propagation of cancer cells in vitro, we compared the growth potential of a panel of ovarian cancer cell lines under ambient (21%) or physiological (3%) O2.

Principal Findings: Our observations demonstrate that similar to primary cells, many cancer cells maintain an inherent sensitivity to O2, but some display insensitivity to changes in O2 concentration. Further analysis revealed an association between defective G2/M cell cycle transition regulation and O2 insensitivity resultant from overexpression of 14-3-3σ. Targeting 14-3-3σ overexpression with RNAi restored O2 sensitivity in these cell lines. Additionally, we found that metastatic ovarian tumors frequently overexpress 14-3-3σ, which in conjunction with phosphorylated RB, results in poor prognosis.

Conclusions: Cancer cells show differential proliferative sensitivity to changes in O2 concentration. Although a direct link between O2 insensitivity and metastasis was not determined, this investigation showed that an O2 insensitive phenotype in cancer cells to correlate with metastatic tumor progression.

Introduction

Cell lines derived from cancer patients provide an experimentally manipulable model system that facilitates investigations into cancer biology and its therapy. The unlimited proliferation potential of cancer cells is a major hallmark of malignancy, however the use of standard tissue culture protocols often restricts potential of cancer cells to correlate with metastatic tumor progression.

Oxygen, in addition to nutrients and growth factors, is vital for proper cell growth and its availability has a direct impact on cellular metabolism, signaling pathways, proliferation, differentiation and survival [3,11,12,13]. Many in vitro investigations have demonstrated the advantages of physiological O2 for tissue culture. For example, the biological behavior of primary cell cultures with a physiological concentration of O2 (2.7–5.3%) is far superior compared to the standard practice of growing cells under atmospheric or “ambient” O2 concentration (21% O2) [4]. In fact, these two growth conditions are known to result in distinct metabolic and molecular characteristics [13].

The importance of considering O2 tension in cancer biology is well established. For example, the fact that many cancers exist in a ‘hypoxic’ state has led to the development of hypoxia-targeted therapy [14,15]. In general the hypoxic concentration of O2 is...
<1% for most solid tumors, however the hypoxic concentration could vary based on the cell types and the normal perfusion status [16] and additionally, hypoxia tends to inhibit cell proliferation [17]. Physiological O₂ tension varies from 2.7–3.3% in the interstitial space [18], where many primary tumors reside, to 14.7% in the arterial circulation and lungs, where migrating and potentially metastatic cancer cells are often found. Therefore, cancer studies that are only conducted in ambient (21%) O₂ may miss pertinent biological observations. This may be particularly important when attempting to study the progression of cancer to metastatic disease, which is a significant event in cancer etiology and is associated with poor prognosis [19]. Considering the differences in O₂ tension in different compartments of the body, an understanding of the effect of O₂ concentration on cancer cell proliferation could provide useful insights into the mechanisms involved in the pathological progression of cancer.

Cancer cells that have acquired mutations in either oncogenes or tumor suppressor genes display a characteristic uncontrolled proliferation phenotype [20]. For example, tumor suppressors such as p53 or RB act as “molecular gatekeepers” known to affect cell cycle progression. Mutation of such factors facilitates unlimited proliferation in cancer cells [20]. Cell cycle progression involves a sequential series of events catalyzed by cyclins and cyclin-dependent kinases (CDKs) [21], and in normal cells is a tightly regulated process. The tumor suppressor p53 is a master regulator of G1/S and G2/M phase transition in the cell cycle [22] and is known to have an important role in responding to oxygen concentration, particularly hypoxia (<1% O₂) [23] or hyperoxia (95% O₂) [24]. Although examining the effect of extreme O₂ conditions is both important and revealing, it must be noted that these previous studies did not investigate the response of p53 at physiological (3%) O₂ and ambient (21%) O₂. p21 and 14-3-3 generally resulted in a significant decrease in cell proliferation for four of the ovarian cancer cell lines (A2780, OVCAR3, OVCAR8 and HOC8) compared to 3% O₂ (Figure 2). The only exception observed was with HOC8 cells in the presence of the highest concentration of serum (10% v/v), where an insignificant O₂-dependent growth effect was observed (Figure 2). Presumably the lack of response in HOC8 results from a dominant influence of serum, which was not observed with A2780, OVCAR5 and OVCAR8. In contrast, there was no significant effect on the growth of SKOV3 and HeyA8 cell lines by increasing the O₂ concentration to 21%, irrespective of serum concentrations (Figure 2). The observed exception was HeyA8 cultured under 2% serum, which showed decreased cell proliferation at 21% O₂ compared to 3% O₂ (p<0.001). In contrast to the effect of O₂ levels, increasing the concentration of serum resulted in a proportional growth increase in the ovarian cancer cell lines A2780, OVCAR3 and OVCAR8 (p<10⁻⁵; Figure 2). The concentration of serum had a moderate influence on growth in SKOV3 and HeyA8 (Figure 2); a serum concentration between 2 and 10% had a significant effect (p<10⁻⁵) in SKOV3, while HeyA8 serum concentration between 2 and 10% serum had the greatest effect at 3% O₂ (p<10⁻⁵) (Figure 2). Increasing serum concentration from 6% to 10% had little effect on growth of HeyA8, SKOV3 and HOC8 (Figure 2). Together, it appears that both oxygen levels and serum concentration affect the growth of these ovarian cancer cell lines, but in an independent fashion. As expected from work by others with primary cells [4], we observed that the majority of the ovarian cancer cells displayed decreased cell proliferation at ambient O₂ concentration compared to physiological O₂ concentration. However, two cell lines did not appear to have inhibited cell proliferation at the highest (ambient) O₂ levels. We therefore categorized the ovarian cancer cell lines based on these differences, being either O₂ sensitive (A2780, OVCAR3, OVCAR8 and HOC8) or insensitive (SKOV3 and HeyA8) (Figure 2). Overall, these differences suggest heterogeneity in growth regulation responses to physiological cues of O₂ levels in these cultured cell lines.

It is possible that the apparent O₂ insensitivity and differences in proliferation resulted from differences in the doubling time of each cell line. For example, if SKOV3 and HeyA8 (the O₂ insensitive cell lines) proliferate more slowly, O₂ dependent proliferation changes may be too trivial to measure. Therefore, we measured the cell doubling time for all ovarian cancer cell lines. Our results showed that under standard tissue culture conditions (10% serum and 21% O₂) the doubling time for all ovarian cancer cell lines were somewhat similar (<24 hours) except for HOC8, which had an extended doubling time of about 45.5 ± 4.9 hours (Table S1, and see Methods S1). Therefore, most of the ovarian cancer cell lines were dividing at an approximately equal rate, and gross difference in doubling time is unlikely to be a factor in the observed proliferation differences between cell lines under different conditions.

Oxygen sensitivity correlates with dynamic changes in the S and G2 phases of the cell cycle

Considering the differences in proliferation observed for ovarian cancer cell lines grown under either 3% or 21% O₂, we examined the effect of O₂ concentration on the growth potential of six ovarian cancer cell lines using physiological (3% O₂) and ambient (21% O₂) oxygen concentrations. Since the serum present in cell culture medium can also have a dominant influence on growth, we also tested the effect of various concentrations of serum. Regardless of the amount of serum present in the growth medium, culturing in 21% O₂ generally resulted in a significant decrease in cell proliferation for four of the ovarian cancer cell lines (A2780, OVCAR3, OVCAR8 and HOC8) compared to 3% O₂ (Figure 2).
whether O2 concentration alters the cell cycle profile of each cell line. Irrespective of serum concentration, comparing 3% O2 to 21% O2 resulted in a significant decrease in the percentage of cells that were in the G1 phase of the cell cycle and a significant increase in the percentage of cells in S phase (Table 1), which was expected based on previous observations made with primary cells [27]. Furthermore, in three of the O2 sensitive cell lines (A2780, OVCAR5 and OVCAR8) the percentage of the cell population in the G2 phase was increased significantly in 21% O2. However a significant increase in G2 was not observed in the fourth O2

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**Figure 1. Cancer cell proliferation is markedly suppressed by the standard cell culture conditions used for in vitro experiments.**

Equal numbers of A2780 ovarian cancer cells were seeded in a 10 cm petri dish and were routinely maintained under 3% O2 (physiological) or 21% O2 (ambient). The increase in cell numbers was determined by counting manually once in three days, and the total cell numbers were estimated and plotted using linear scale (in Graph A) and log scale (in Graph B).

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**Figure 2. Ovarian cancer cells grown under physiological and ambient O2 show differential proliferation response.** Ovarian cancer cell lines were cultured under 3% or 21% O2 and the extent of proliferation was determined following 3 days of growth (see Materials and Methods section). For each cell line, the percent of cell proliferation at 3% O2 (light shaded bars) and at different concentrations of serum was compared with proliferation under standard tissue culture conditions consisting of 21% (ambient) O2 (dark shaded bars) and 10% FBS. The error bars represent the standard deviations of mean and statistical significant (by student T Test) differences in proliferation between 3% and 21% O2 for each concentration of serum is indicated by an asterisk ([*] p<0.05, [**] p<0.001 and [***] p<0.0001).

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sensitive cell line, HOC8 (Table 1). Similar to HOC8, the O2 insensitive cell lines, SKOV3 and HeyA8, did not display a significant alteration in the proportion of cells in the G2 phase of the cell cycle when grown under 3% O2 or 21% O2 (Table 1). Considering that the O2 sensitive cell lines proliferated more slowly at 21% O2 compared to 3% O2 despite having smaller proportions of their cell population in G1 and a increased proportions in S and G2, we conclude that these cells must be progressing more slowly through the cell cycle. However, for the O2 insensitive cell lines and HOC8 (with the significantly extended doubling time), we did not observe a significant increase in the percentage of cells in G2 when the O2 levels were increased. These results suggest that although the G1 and S phases of the cell cycle are responding similarly to changes in O2 concentration in both O2 sensitive and insensitive cell lines, it is the G2 phase of the cell cycle that is not responsive to O2 concentration in the O2 insensitive cell lines. Therefore, the difference in cell cycle response observed with these ovarian cancer cell lines might be at the level of regulation during the cell cycle progression from G2 to M phase. It is also possible that the changes observed with G2 and O2 sensitivity in these cancer cell lines is reflected in the mitotic component of the cell cycle. Our observation of the mitotic cells present in the O2 sensitive and insensitive cell lines grown under 3% and 21% O2 supports this conclusion; the O2 sensitive cell lines show a proportionate decrease in the mitotic cell population observed at 21% O2 compared to 3% O2 (Figure 3), corresponding to an accumulation of cells at G2 at 21% O2 (Table 1). Similarly, in the O2 insensitive cell lines (HeyA8 and SKOV3) the proportion of mitotic cells remained unaltered regardless of O2 concentrations (Figure 3). This is expected because, as noted previously (Table 1), the proportion of cells at G2 in the O2 insensitive cell lines were also unaffected by O2 concentration. We conclude that most cancer cells retain an ability to regulate cell cycle in response to changes in O2 concentration comparable to wild type cells [27]. However, some cancer cells may lose O2 concentration dependent control of cell cycle (as in the O2 insensitive cancer cell lines), resulting in a distinct phenotype.

Oxygen insensitivity correlates with altered G2/M components

Thus far we have demonstrated that O2 sensitive cell cycle response at the G2/M transition is lacking in the O2 insensitive cell lines. We therefore went on to characterize this observation further by determining what component of G2/M regulation is deficient in the O2-insensitive cancer cells. The major effector of G2/M transition is CDC2 [22,28]. CDC2 forms a complex with cyclin B [29,30], which phosphorylates various structural proteins resulting in the collapse of the nuclear envelope, condensation and segregation of chromosomes [30,31] and inactivation of other cell cycle regulatory proteins such as WEE1, RB and CDC25C [30,32]. In normal cells, the overall levels of CDC2 protein are kept constant throughout the cell cycle [33] and are regulated by post-translational modification [33] and cellular localization [30,31]. Once the Tyr15 residue on CDC2 is dephosphorylated by CDC25C, activated CDC2 forms a complex with cyclin B, accumulates in the nucleus, and promotes the G2/M transition [30,33,34]. This occurs in a stepwise fashion through increasing amounts of nuclear CDC2 protein [30]. Our examination of total CDC2 protein and phosphorylated CDC2 protein revealed that both are considerably lower in the O2-insensitive cell lines (HeyA8 and SKOV3) compared to the O2-sensitive cell lines (Figure 4A). Although the levels of CDC2 were relatively high in the O2-sensitive cell lines (A2780, OVCAR5 and OVCAR8) (Figure 2), we observed a decrease in Tyr15 phosphorylation status regardless of O2 concentration for A2780, OVCAR5 and OVCAR8 with increasing serum levels (Figure 4A). This correlates with the observation that increasing serum concentration causes increased cellular proliferation and results in a concomitant reduction in the proportion of cells in G2/M (compare with Table 1). However, no overt O2-dependent alteration in either total or phosphorylated cyclin B or CDC25C was observed in the O2 sensitive cell lines (A2780, OVCAR5, OVCAR8 and HOC8) compared to O2 insensitive cell lines (HeyA8 and SKOV3) (Figure 4A). Therefore, it appears that the observed decrease in the cell population in G2 in 21% O2 might not be dependent on phosphorylation-mediated inactivation of CDC2. It should be noted that these experiments were performed in asynchronously growing cells, and therefore it is possible that transient differences in CDC2 status were missed. Interestingly, the levels of CDC2, Cyclin B and CDC25c (the negative regulator of CDC2) were considerably lower in O2 insensitive cell lines (HeyA8 and SKOV3) compared to O2 sensitive cell lines (A2780, OVCAR5, OVCAR8 and HOC8) (Figure 4A). These observations suggest an inherent deficiency in the core components involved in the G2/M progression in the O2 insensitive cell lines.

p53, p21 and 14-3-3 σ are factors which have the ability negatively to influence CDC2 activity and G2/M transition [22]. Current understanding is that p53 and p21 influence cell cycle in hypoxic and hypoxic conditions [23,24,35,36]. Considering the reduced levels of CDC2 and the apparently defective G2/M checkpoint in the O2 insensitive cell lines (HeyA8 and SKOV3), we explored the possibility that impairment was due to a defect in any of these molecular regulators. Western blot analysis found p53 and p21 to be overexpressed in one O2-insensitive cell line (HeyA8). However, both were absent in the other O2-insensitive cell line (SKOV3), and the expression pattern for these proteins remained unaltered regardless of changes in O2 or serum concentration (Figure S1), suggesting that neither p53 nor p21 is relevant to CDC2’s function in O2 sensitivity. Interestingly, we observed a considerable elevation in the expression of 14-3-3 σ (Figure 4A) in the O2 insensitive cell lines (HeyA8 and SKOV3) compared to the O2-sensitive cell lines. Although, the level of 14-3-3 σ expression was considerably lower in all O2-sensitive cell lines compared to HeyA8 and SKOV3, we did observe an increase in the expression of 14-3-3 σ at 21% O2 with A2780 (Figure 4A). Although
concentrations than O2 levels. The other target for CDC2-mediated inactivation by phosphorylation is WEE1, which can also reciprocally inhibit CDC2 function by phosphorylation [37]. We observed increased phosphorylation of WEE1 in the O2-sensitve cell lines (A2780, OVCAR5 and OVCAR8), but not in HeyA8 (Figure 4B). This pattern was largely recapitulated for total WEE1 protein levels (Figure 4B). Therefore, the absence of phospho-WEE1 in the O2-insensitive cell lines does not indicate an absence of CDC2 activity, but rather an absence of the WEE1 substrate. From these results we concluded that despite the reduced amounts of CDC2 in the O2-insensitive cell lines, CDC2 is functionally active and uninhibited by the increased levels of 14-3-3 \(\sigma\). It should be noted that RB and CDC2 act upon each other to regulate each others function [38], and phosphorylation status of RB [26] or CDC2 [39] could influence E2F mediated expression of cell cycle components required for a dynamic proliferative progression.

In summary, the O2-sensitive cell lines (A2780, OVCAR5 and OVCAR8) showed increased expression of CDC2 and cyclin B combined with low level of 14-3-3 \(\sigma\) expression. This suggests that the cell cycle components required for a dynamic proliferative response to differences in the O2 concentration is present in these cell lines. However in the O2-insensitive cell lines that express high levels of 14-3-3 \(\sigma\) and low levels of CDC2 and CDC25C such a dynamic cell cycle response to changes in O2 concentration could be impaired. We therefore pursued the possibility that this inverse correlation between 14-3-3 \(\sigma\) and CDC2 might be important for the O2-sensitive regulation of G2/M transition.

### 14-3-3 \(\sigma\) and mitotic progression in oxygen sensitivity

Our previous observations suggest an association between elevated level of 14-3-3 \(\sigma\) and O2-insensitivity that needs to be confirmed. Therefore, we wanted to confirm that 14-3-3 \(\sigma\) does indeed affect O2-dependent proliferation. For this part of the study, we restricted our analysis to two cell lines with wild type p53: the O2-sensitive A2780 [40], and O2-insensitive HeyA8 cell lines [41].
So far we have used Western blot analysis to monitor the overall expression levels of 14-3-3s and CDC2 (Figure 4A). However, since the functional responses of these proteins are dependent on their cellular localization, we used immunofluorescence to determine their cellular location under 3% O2 and 21% O2. In the O2-sensitive A2780 cell line, the localization of 14-3-3s was restricted to the cytoplasm under 3% O2 (Figure 5A), but was found in both the nucleus and cytoplasm at 21% O2 (Figure 5A). CDC2 was distributed throughout the cell and its localization was unaffected by O2 concentration. It therefore appears that nuclear exclusion of 14-3-3s correlates with a decreased fraction of cells in the G2/M phase and an uninhibited cell cycle progression when A2780 is grown at 3% O2, as noted before (Table 1). In contrast, the O2-insensitive HeyA8 cell line showed high levels of 14-3-3s and low levels of CDC2 (Figure 4A), with a considerable amount of 14-3-3s in the cytoplasm (Figure 5A). Further, 14-3-3s remained excluded from the nucleus even at 21% O2 in the HeyA8 cells (Figure 5A). CDC2 was distributed throughout the cell and its localization was unaffected by O2 concentration. These observations were further verified by Western blot analysis of nuclear and cytosolic cell fractions obtained from these cells (Figure 5B). Finally, to confirm the effect on G2/M transition, we determined the proportion of those cells in M phase for different O2 concentrations using the mitosis specific marker phospho-histone H3. In the O2-sensitive A2780 cells, under 21% O2, we observed a decrease in the mitotic index (P<0.001), compared to 3% O2 (Figure 5C). No such O2-dependent change in mitotic index was observed for the O2-insensitive HeyA8 cells (Figure 5C). These results support our initial conclusion, that the O2-insensitive cells lines have a deficiency in regulating cell cycle progression at G2/M in response to increased O2 levels (Figure 2).

The levels and cellular localization of 14-3-3s correlate with O2-sensitive proliferation. To demonstrate a direct relationship, we examined whether over-expression of 14-3-3s could render O2-sensitive A2780 cells insensitive to O2 and conversely whether reducing the levels of 14-3-3s in O2-insensitive HeyA8 cells could restore O2-sensitivity. Transient over-expression of 14-3-3s in A2780 cells reduced cell proliferation (Figure 5D) and resulted in loss of O2-sensitivity. Therefore, merely increasing 14-3-3s expression results in its inability to regulate G2/M in the absence of any further genetic alterations. Conversely, RNAi-mediated silencing of 14-3-3s expression in HeyA8 cells (Figure 5E - Western blot) resulted in a substantial increase in proliferation under 3% O2 (Figure 5E - Bar graph). Interestingly, when the cells from the same siRNA transfection were placed at 21% oxygen, 14-3-3s protein expression was induced, reducing the knockdown effect of the siRNA. This observation also suggests an O2-dependent transcriptional response by 14-3-3s. Despite this transcriptional response, we still observed a muted growth phenotype at 21% O2 under these conditions. Together these experiments demonstrate that 14-3-3s is a critical factor for controlling ovarian cancer cell proliferation in response to O2 concentration.

14-3-3s is frequently highly expressed in ovarian cancer and its ineffectiveness in controlling CDC2 is relevant to ovarian tumor pathology

Considering that increased expression of 14-3-3s provides some indication of impaired G2/M control, it is possible that cancer cell lines that express high levels of 14-3-3s are O2-insensitive. The O2-insensitive ovarian cancer cell lines we have
thus far characterized have high 14-3-3 σ (Figure 5A) and low CDC2 protein levels. It is conceivable that the same phenotypic defect might result from cells with unchecked CDC2 activity, irrespective of 14-3-3 σ levels. To determine the frequency of commonly available cancer cell lines that have the hallmarks of O2-insensitivity, we used a reverse phase protein array (RPPA) and screened 57 different ovarian cancer cell lines for the levels of 14-3-3 σ and CDC2, as well as phospho-RB as an indicator of CDC2 activity. Cell lines with the same name but from different labs or different passages were considered to be different. We therefore set the analysis criteria on the RPPA array to detect high phospho-RB (P-RB) and either high 14-3-3 σ or high CDC2. In the context of high levels of P-RB, this criteria should indicate that either 14-3-3 σ is dysfunctional or that active CDC2 is uninhibited.
Perhaps due to methylated 14-3-3 σ or inhibition of CDC2 degradation [42]. We observed that of the 57 ovarian cancer cell lines represented in the RPPA, 29 cell lines (49%) showed high levels of 14-3-3 σ (Figure 6A) of which 16 cell lines (28%) also had increased P-RB, corresponding to the O2 insensitivity pattern we have described. Amongst these 16 cell lines, 6 also have increased levels of CDC2 while the remainder had decreased levels of CDC2. This suggests that this protein profile is not exclusive to the cell lines we originally identified and might be representative of a relatively common phenomenon. We therefore determined whether this O2-insensitive associated 14-3-3 σ/CDC2/P-RB protein profile is also observed in ovarian tumor samples. Using the same criteria as with the cell line RPPA, we examined 205 ovarian tumor specimens using RPPA. This analysis revealed that 27% of ovarian tumors (56) had elevated levels of both 14-3-3 σ and P-RB, and amongst these, 34 also had elevated levels of CDC2 expression (Figure 6B). These results are very comparable with the RPPA analysis of the ovarian cancer cell lines (Figure 6A).

Ovarian cancer has a poor survival rate and this is often associated with metastatic progression [43]. The O2-insensitive associated 14-3-3 σ/CDC2/P-RB protein profile suggests an unrestricted G2/M control in response to changes in O2 levels, such as a migrating or metastatic cancer would encounter. Therefore, it is possible that this protein profile is associated with poor prognosis. Using the O2-insensitive associated protein profile (high P-RB with either high 14-3-3 σ or high CDC2) we identified 47 of 158 tumors with associated clinical data. A Kaplan-Meier survival estimate shows that patients with the O2-insensitive associated protein profile have a poor survival outcome (less than 90 months compared to 200 months observed otherwise, p = 0.016, Figure 6C). Altogether it appears that the O2-insensitive associated protein profile suggests that unrestricted G2/M accompanies a substantial proportion of ovarian cancer cells and primary tumor samples. Further, this O2-insensitive profile is associated with poor prognosis for this disease.

Elevated 14-3-3 σ expression in metastatic ovarian tumors

Having observed that the O2-insensitive associated protein profile (high P-RB with either high 14-3-3 σ or high CDC2) is both relatively common in ovarian cancer and associated with poor prognosis, we went on to determine directly whether metastatic ovarian tumors exhibit an overt 14-3-3 σ signature. Of note, the ovarian tumors represented in the ovarian tumor RPPA are from primary sites and thus do not necessarily provide an accurate representation of the protein profile in the metastatic cancer. We therefore expect that metastatic tumors or primary tumors that give rise to metastatic tumors will exhibit a more overt 14-3-3 σ signature than primary tumors. In fact, an increased expression of 14-3-3 σ has been previously reported with other tumors [44] and a functional involvement for 14-3-3 σ in metastatic disease is known [45,46]. We analyzed 14-3-3 σ expression using immunohistochemistry on paraffin embedded tissues obtained from 10 different metastatic ovarian tumors and their corresponding primary site tumors. We consistently observed intense immunostaining of 14-3-3 σ in 8/10 metastatic tumors and the corresponding primary tumors (Figure 7j–l). In contrast, the primary tumors without metastasis at diagnosis showed moderate immunostaining for 14-3-3 σ, and occasionally intense staining was also noted (Figure 7i). Borderline tumors showed a mild to moderate staining pattern for 14-3-3 σ, while in normal tissues, protein levels were absent or diffusely present (Figure 7a–c). Increased expression of 14-3-3 σ in the metastatic primary tumors compared to normal tissue or malignant tumors without metastasis were observed to be statistically significant by the Fisher’s exact test (Figure 7, Bar Graph). The high level of 14-3-3 σ expression offers the first indication of the manner in which regulation of G2/M may be dysfunctional in these tumors.

Over-expression of 14-3-3 σ in metastatic disease is not unexpected and has been previously noted [45,46,47]. However, we speculate the reason for this association is due to a loss of O2-sensitivity and that this provides a selective advantage for metastatic progression. Our conclusion is that O2-sensitive and insensitive patterns of 14-3-3 σ and CDC2 expression are readily detectable and common to cancer cells, regardless of whether they are grown in vivo or in vitro. Further, these expression patterns may have prognostic implications, but additional experiments will be required to confirm the mechanistic relevance of O2-sensitivity in the clinical progression of cancer.

Discussion

There is an increasing interest to study cell biology under the context of physiological O2 levels. Investigations with primary mouse embryonic fibroblasts comparing the effects of physiological (3%) and ambient (21%) oxygen, show that 21% O2 causes increased oxidative stress and induces senescence [4]. Several studies conducted with embryonic stem (ES) cells reported that characteristic stem cell properties are preserved only when ES cells are maintained under physiological O2– ES cells otherwise differentiate under ambient O2 as reviewed in [2]. This prompted us to investigate the effects of physiological (3%) and ambient (21%) oxygen in the context of cancer. With A2780 ovarian cancer cells grown under 21% or 3% O2, a 20% growth suppression was observed with 21% O2 by three days (Figure 2) and although the proportional changes to cell cycle profile appear small, they were significant (Table 1). The accumulated effect of these differences in proliferation and cell cycle resulted in a 2.6 fold difference to the growth of the cancer cells by 12 days in the presence of different O2 concentrations (Figure 1). This observation demonstrates that standard tissue culture conditions may adversely impact the in vitro proliferation of cancer, which is primarily a disease of proliferation. Previous studies compared the growth of primary mouse embryonic fibroblast cells [4], adult human fibroblasts [48] and human cancer cells [8] grown under physiological (3–5%) or ambient (21%) O2 and observed increased cell proliferation under physiological O2. In this study, we observed similar effects with ovarian cancer cells (A2780, OVCAR3, OVCAR8 and HOCE8 - Figure 2), however other cell lines failed to respond to O2 concentration (HeyA8 and SKOV3) (Figure 2). These proliferative responses to O2 seem to affect all phases of the cell cycle, particularly the G1 and S phases of cell cycle, in all cell lines. However, only the G2 phase was affected in cell lines which displayed proliferative response to 3% O2 (Table 1), suggesting the possibility that the G2 phase transition of the cell cycle is crucial for regulating proliferation in response to differences in 3% O2 levels. A change in the G2 phase in response to O2 levels was reported in only one other study performed with Fanconi anemia (FA) cell lines [49]. Analogous to our study, the experiments with FA cells demonstrated a characteristic G2 delay with standard tissue culture conditions (20% O2), but a reduced proportion of cells in G2 and increased proliferation when cultured at 5% O2 [49]. Furthermore, growth of different human fibroblast cells under physiological O2 has also been observed to be accompanied by a reduction in the G2 cell population [27,48]. Overall, it appears that the G2 phase is the most O2-sensitive phase of the cell cycle. Exploring the possible molecular mechanisms that render ovarian cancer cells either sensitive or insensitive to oxygen has
clearly demonstrated that it is 14-3-3 \( \sigma \) and its inability to control CDC2 dependent G2/M transition in response to O\(_2\) levels that results in oxygen-insensitive cell lines. Although expression of 14-3-3 \( \sigma \) is regulated by p53 [25], we observed no difference in the levels of p53 expression under different oxygen concentrations (Figure S1), suggesting that the involvement of 14-3-3 \( \sigma \) in O\(_2\)-sensitivity is independent of p53. If the decrease in 14-3-3 \( \sigma \) is associated with oxygen-sensitive increase in proliferation, then silencing the expression of 14-3-3 \( \sigma \) in oxygen-insensitive cell lines should restore proliferative sensitivity to oxygen. In fact, our experiments show that RNAi mediated silencing of 14-3-3 \( \sigma \) in HeyA8 cells restored oxygen sensitivity (Figure 5E) and in a converse experiment, over-expression of 14-3-3 \( \sigma \) abolished oxygen sensitivity in the A2780 cell line (Figure 5D). This suggests that high levels of 14-3-3 \( \sigma \) protein is sufficient to restrict the regulation of CDC2 mediated G2/M progression. The cytoplasmic restriction of overexpressed 14-3-3 \( \sigma \) in the O\(_2\)-insensitive HeyA8 cells provides the first indication for the possible mechanistic basis of this dysregulation (Figure 5A). Other reports also show preferential changes to cellular localization of 14-3-3 \( \sigma \) during different phases of the cell cycle [30], suggesting that cell cycle changes observed with oxygen could be relevant to the 14-3-3 \( \sigma \) localization and pattern in our experiments. Furthermore, 14-3-3 \( \sigma \) is actively exported out of nucleus by CRM1, [51], a nuclear

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**Figure 6. Reverse phase protein array data analysis.** (A) Hierarchical clustering of normalized RPPA data over Phospho-RB (Ser 807/811), 14-3-3 \( \sigma \), CDC2 and p53 across 57 ovarian cancer cell lines. (B) Hierarchical clustering of normalized RPPA data over Phospho-RB (Ser 807/811), 14-3-3 \( \sigma \), CDC2 and p53 across 205 ovarian tumors. The color codes for overall survival represents overall survival >24 months (blue) and overall survival <24 months (pink). The color codes for tumor stage represent stage I (red), stage II (green), stage III (light-blue) and stage IV (dark-blue). (C) Kaplan-Meier survival curve for the RPPA results comparing the group of ovarian tumors with high Phospho-RB and high 14-3-3 \( \sigma \) or CDC2 (blue line) with other expression profiles (red line).

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protein that is frequently over-expressed in ovarian cancer [52]. A host of other factors such as, BRCA1, p53 and estrogen induced zinc finger protein (EFP) are also known to regulate the levels of 14-3-3 σ [53]. Therefore, it is possible that 14-3-3 σ expression and its cellular distribution could be influenced by several factors, independent of p53 (as must be the situation in the O2 insensitive p53 null cell line SKOV3).

The differences in O2-sensitivity and, consequently, cell proliferation is most important when trying to recapitulate in vivo responses where physiological O2 tensions vary from 2.7–5% in the interstitial space (where many cancer cells reside) to 14.7% in the arterial circulation and lung [18]. Thus, it is reasonable to predict that if O2-sensitive cancer cells were to dislodge from a primary interstitial space and migrate to the lungs via blood circulation, the increased O2 concentration would restrict proliferation. In contrast, we speculate that oxygen insensitive cancer cells would have a selective advantage compared to sensitive ones, being better able to thrive in the conditions of increased oxygen concentration. In fact, 14-3-3 σ is frequently over-expressed in many thyroid [54], colorectal [55] and prostate [56] tumors, and is also a potential target for therapeutic modulation [55,56]. Our results provide one rationale for selecting the cancers best suited for 14-3-3 σ targeted therapy. Oxygen insensitivity observed in HeyA8 or SKOV3 is less likely an adaptation to in vivo growth conditions because transient over-expression of 14-3-3 σ renders O2-sensitive A2780 cell line insensitive to increased levels of O2 (Figure 5D), and over-expression of 14-3-3 σ is observable in primary tumors with metastatic potential (Figure 7). Oxygen sensitivity could therefore be an important factor in the context of metastatic spread of cancer because over-expression of 14-3-3 σ is frequently observed in metastatic cancers, including this study (Figure 7) and others [gastric [57], endometrial [58] and pancreatic [59]]. However, epigenetic inactivation of 14-3-3 σ by gene methylation has also been reported to correlate with decreased expression of 14-3-3 σ in cancer progression [60] and metastasis of certain types of tumors [61]. Further, a correlation with a functional role for 14-3-3 σ in promoting tumor invasion and metastasis has also been demonstrated [45,47,62]. Taken together, there is ample evidence to support that over-expression of 14-3-3 σ is relevant to tumor metastasis and therefore, it is likely that O2 insensitivity associated with over-expression of 14-3-3 σ may have a pivotal role in metastatic dissemination of tumors. Further support to demonstrate the explicit role of 14-3-3 σ in in vivo O2 sensitivity and its relevance to metastasis would require experiments with animal models.

In conclusion, there are many advantages to studying cancer biology under physiological O2. In fact, compared to cell propagation under physiological O2, ambient O2 levels are expected to result in oxidative stress [4], mutation proneness and persistence of transformation [63]. In this context, we have demonstrated that growing cancer cells in vitro at low physiological O2 (not hypoxia), compared with ambient (21%) O2 is a prudent approach to identify and understand some of the behavioral diversity observed in cancer.

**Materials and Methods**

**Cell culture and Transfection**

Ovarian cancer cells were grown in RPMI 1640 (A2780, OVCAR3, OVCAR8, SKOV3) or DMEM (HeyA8 and HOC8)
supplemented with 10% heat inactivated Fetal Bovine Serum (Sigma Aldrich, St.Louis, MO, Cat#: F6178) and 200 units of penicillin/streptomycin and 0.5 µg amphotericin-B. Transfection was performed using AmazaX nucleofector technology (Lonza) as described previously [64]. Plasmid pcDNA 3.0 HA 14-3-3 σ was obtained from Addgene (plasmid 11946 [65]) and pcDNA 3.0 HA empty vector was a gift from Dr. Y. Shiio, UTHSCSA. 14-3-3 σ siRNA and non-targeting dsRNA were purchased from Dharmacon. For oxygen exposures we used Forma Series II 3110 water-jacketed multigas incubator (Thermo Fisher scientific, Waltham, MA) with built-in CO2 and O2 monitors and controllers. To maintain 3% O2, the incubator received an additional supply of nitrogen gas.

Cell proliferation

Cell proliferation was determined using Celltitre-Glo (Promega, Madison, WI) per manufacturer instructions, as described previously [64]. Cells were seeded to a final density of 100, 200 or 400 cells per well in a 384 well plate containing 40 µl of growth medium consisting of 2%, 6% or 10% FBS and antibiotics. Plates were then placed in a humid chamber and returned to the incubators of appropriate oxygen pressure. After 3 days of incubation, the number of cells present per well was measured using Celltitre-Glo reagent, as described previously [64]. The number of cells per well was determined using a standard curve based on ATP concentration, as recommended by the manufacturer.

Mitotic Index

The number of mitotic cells were quantified based the method as described [66]. Briefly, 96 well collagen coated plates were used to seed cells at a final concentration of 1000 cells/well in their respective media. Cells were then incubated for three days at 37°C in 3% or 21% O₂. Finally, cells were washed, resuspended in phosphate buffered saline and stained with DAPI, as described [66]. Images of stained cells were acquired using a Zeiss Axivert 200M inverted fluorescent microscope using 10X magnification and Openlab (PerkinElmer) image acquisition software. Using Image J, a set threshold for staining intensity was used to count the brightly stained nuclei, with obvious chromatin condensation and the mitotic index was determined based on the ratio of number of mitotic cells present in 1000 cells, as described [66].

Protein isolation and Western blot analysis

Protein lysates and western blot analysis were performed as previously described [64]. The immunoblots were probed with the appropriate dilutions of primary antibody and visualized using either Lumiglo (Cell signaling technology) or the ECL plus system (Millipore) and Ultraherm (Amersham). Protein lysates from 57 cancer cell lines or 205 primary ovarian cancer cell lines. We then examined all replicated representations from the same source as annotated to reduce down to 57 ovarian cancer cell lines or 205 patient samples (from each source) by taking the median protein expression level of all replicates. An additional cell-line specific normalization step was performed in which median expression levels for each protein was

Flow Cytometry

Cells were trypsinized and seeded to a final density of 1×10⁶ cells per well in a 10 cm dish containing growth medium, antibiotics and appropriate concentrations of FBS. Dishes were then returned to the incubators set for the different oxygen conditions. Following three days of incubation, cells were harvested and prepared for FACS analysis as described previously [67]. Experiments were performed in triplicate. Stained cells were analyzed using a FACS Canto I (BD Biosciences) flow cytometer using an argon laser at 488 nm wavelength. Cell cycle analysis was performed using ModFit LT (version 3.2) software (Verity Software House).

Quantification of M phase cells

The number of cells in M phase were quantified based on mitosis-specific histone H3 phosphorylation in the ovarian cancer cell lines using the Cellomics® Cell Cycle Kit I (Thermo Scientific) as per the manufacturer’s recommended protocol. Briefly, 96 well collagen coated plates were used to seed cells at a final concentration of 1000 cells/well in their respective media. Cells were then incubated for three days at 37°C in 3% or 21% O₂. Control wells were treated with 1.5 µg/ml nocodazole (Sigma Aldrich) for 16 hours, fixed with 16% formaldehyde, permeabilized, blocked and stained with reagents consisting anti-phospho-histone H3 primary antibody, as per instructions provided in the kit. Stained cells were analyzed with a Zeiss Axivert 200M inverted fluorescent microscope using 10X magnification and Openlab (PerkinElmer) image acquisition software. 100–250 cells per replicate were counted for phospho-histone H3 positive cells.

Immunolocalization of 14-3-3 σ and CDC2

A2780 cells transfected with 14-3-3 σ cDNA expression construct or HeyA8 cells transfected with 14-3-3 σ siRNA were seeded at a final density of 10⁶ cells per fibronectin (Sigma) coated 12.5 mm² glass coverslip mounted in each well of a 12-well plate. Cells were maintained in complete growth medium supplemented with 10% fetal bovine serum and allowed to grow for three days in the presence of 21% or 3% oxygen. For the detection of 14-3-3 σ or CDC2 by immunofluorescence, cells were processed as described previously [66]. The primary antibodies used were mouse monoclonal 14-3-3 σ at 1.0 µg/ml (Upstate) and rabbit polyclonal total CDC2 at 1:1000 (Cell Signaling). Following a PBS wash, the cells were incubated with secondary antibodies, goat anti-mouse AlexaFluor 488 and goat anti-rabbit AlexaFluor 568 (Invitrogen) at 1:100 dilution in blocking buffer for 1 hour at room temperature. Cells were then counterstained with DAPI (1:3000 dilution in PBS) and mounted onto microscope slides using Fluoromount-G. Images were taken at 63X magnification using the Zeiss Axivert 200M inverted fluorescent microscope and Openlab software (PerkinElmer).

Reverse Phase Protein Array

Protein lysates from 57 cancer cell lines or 205 primary ovarian cancer tumors were spotted in RPPA slides and processed for expression analysis, as described previously [69,70]. Data acquisition and processing were performed as described previously [69]. Ovarian cancer specimens were obtained from Gynecology Tissue Bank at MD Anderson Cancer Center, following approval from the Institutional Review Board (BT).

Normalization and Clustering

log-transformed RPPA data was first examined to remove non ovarian cancer cell lines. We then examined all replicated representations from the same source as annotated to reduce down to 57 ovarian cancer cell lines or 205 patient samples (from each source) by taking the median protein expression level of all replicates. An additional cell-line specific normalization step was performed in which median expression levels for each protein was
first determined and then subtracted from individual RPPA experiments. The anchored heatmap (termed after anchored over/under-expression orientation) was generated by requiring RB, 14-3-3s and CDC2 to be arranged from over-expressed to under-expressed recursively from the given cell-line order, but exact positions of each protein was determined by hierarchical clustering algorithm with Euclidean distance as similarity measure and average linkage from all cell-lines, as shown in Figure 6 A&B. Raw data obtained from RPPA for the expression of Phospho RB, 14-3-3s and CDC2 is provided in the supplementary tables (for ovarian cancer cell lines, see Table S2, and for ovarian cancer patient specimens, see Table S3).

**Immunohistochemistry**

Tissue arrays (OV951-1) consisting of normal and malignant tissues from primary or metastatic sites were purchased from US Biomax Inc. Slides were processed for immunohistochemistry and analyzed, as described previously [71], 14-3-3s (Upstate) was used at 1:50 dilution for incubation with primary antibody and subsequent steps were performed using the Dako universal LSAB kit with DAB as described by the manufacturer.

**Statistical Analyses**

To determine significant differences to proliferation under 3% or 21% O2, a Student *t*-test was performed, and Dunn's was performed to compare different cell cycle profiles with the panel of ovarian cancer cell lines. Kaplan-Meier survival analysis with p-value determined with log-rank test was performed using MATLAB (Mathworks, Natick, MA) for RPPA data consisting patient specimens. For Kaplan-Meier survival analysis the data was censored based on patient's vital status. Statistical analysis for the correlation of 14-3-3s expression with the various pathological grades of ovarian tumors determined based on immunohistochemistry was analyzed by a Fisher's exact test using R.

**Supporting Information**

Figure S1 Western blot analysis of phospho and total p53, and p21, which are major upstream regulators of G2/M cell cycle progression and the relevance to 21% or 3% O2 in ovarian cancer cells. O2 insensitive cell lines are indicated by asterisk and italics. (EPS)

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