Ets-1 Regulates Energy Metabolism in Cancer Cells

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Abstract

Cancer cells predominantly utilize glycolysis for ATP production even in the presence of abundant oxygen, an environment that would normally result in energy production through oxidative phosphorylation. Although the molecular mechanism for this metabolic switch to aerobic glycolysis has not been fully elucidated, it is likely that mitochondrial damage to the electron transport chain and the resulting increased production of reactive oxygen species are significant driving forces. In this study, we have investigated the role of the transcription factor Ets-1 in the regulation of mitochondrial function and metabolism. Ets-1 was over-expressed using a stably-incorporated tetracycline-inducible expression vector in the ovarian cancer cell line 2008, which does not express detectable basal levels of Ets-1 protein. Microarray analysis of the effects of Ets-1 overexpression in these ovarian cancer cells shows that Ets-1 up-regulates key enzymes involved in glycolysis and associated feeder pathways, fatty acid metabolism, and antioxidant defense. In contrast, Ets-1 down-regulates genes involved in the citric acid cycle, electron transport chain, and mitochondrial proteins. At the functional level, we have found that Ets-1 expression is directly correlated with cellular oxygen consumption whereby increased expression causes decreased oxygen consumption. Ets-1 over-expression also caused increased sensitivity to glycolytic inhibitors, as well as growth inhibition in a glucose-depleted culture environment. Collectively our findings demonstrate that Ets-1 is involved in the regulation of cellular metabolism and response to oxidative stress in ovarian cancer cells.

Introduction

Over 50 years ago, Otto Warburg first proposed that mitochondrial injury that leads to depressed electron transport chain function and respiratory defects is an important step in the development and progression of carcinogenesis [1, 2]. Over the past two decades, many studies have shown that tumours preferentially use glycolysis for energy production over oxidative phosphorylation, a characteristic that has become a hallmark of tumour pathophysiology [3–5]. Known as the Warburg effect, cancer cells predominantly utilize glycolysis for ATP production even in the presence of abundant oxygen, an environment that would normally result in energy production through oxidative phosphorylation [2, 6]. Alterations in mitochondrial DNA correspond to increased production of ROS and impaired oxidative phosphorylation, resulting in decreased ATP production and increased glycolytic dependence [5, 7]. The increased production of reactive oxygen species, particularly H2O2, by cancer cells is likely the result of mitochondrial dysfunction, as mitochondria are considered to be the predominant cellular source of reactive oxygen species [8]. Four to five percent of the O2 consumed by oxidative phosphorylation in the mitochondria is normally converted to reactive oxygen species; therefore defects to the electron transport chain system in cancer cells would result in excessive reactive oxygen species formation [8–10]. Excessively produced, H2O2 can act as a signaling molecule by oxidizing cysteine molecules on proteins, activating several signaling pathways including MAPK, as well as several transcription factors including AP-1, p53, NF-κB, and Ets-1 [11–15].

Ets-1, a member of the Ets protein family of transcription factors, regulates the expression of a diverse set of proteins through its interaction with specific consensus sequences upstream of target genes [16]. The over-expression of Ets-1 has been associated with a multitude of different cancers, specifically with regards to tumour progression and invasion [16–19]. Additionally, over-expression of Ets-1 has also been associated with poor prognosis in breast [20], ovarian [21], and cervical carcinomas [22]. Traditionally, Ets-1 is thought to function as a transcriptional activator and its high expression in endothelial and stromal cells correlates with tumour cell invasiveness and unfavourable outcome in ovarian and breast cancer [23–25]. Our laboratory and that of others have highlighted the importance of Ets-1 in the regulation of different aspects of cancer cell behaviour, including extracellular matrix remodeling, invasion, angiogenesis [26], and drug resistance [27]. The link between Ets-1 and cancer invasiveness can potentially be explained by the list of known target genes regulated by this transcription factor. Several MMPs and integrin genes, as well as urokinase plasminogen activator (uPA), which are all known mediators of extracellular matrix degradation and cell migration, are known targets for Ets-1 [28–31]. Many genes crucial to angiogenesis and extracellular matrix remodeling, such as matrix metalloproteinases (MMP-1, MMP-3, MMP-9), uPA and Integrinβ3, are under the direct regulation of Ets-1 [26]. This transcription factor is thus considered to be an important mediator of cancer cell development and tumour progression.

In this study we have demonstrated that Ets-1 plays a role in the regulation of energy metabolism in ovarian cancer cells. Using
high throughput genomic analysis, we have found that Ets-1 regulates, either directly or indirectly, several important genes involved in mitochondrial metabolic and antioxidant defense pathways in our Ets-1 over-expression ovarian cancer cell model. Functionally, we have shown that glycolysis, oxidative phosphorylation, and cellular respiratory systems are altered in response to changes in Ets-1 expression. Taken together, our findings indicate that Ets-1 is a key transcription factor involved in regulating metabolic and oxidative stress in cancer cells.

**Results**

**Cancer cell model of Ets-1 gene expression**

Ets-1 was over-expressed in 2008 ovarian cancer cells using a tetracycline-inducible system, generating 2008-Ets1 cells. Ets-1 protein expression in tetracycline-treated 2008 and 2008-Ets1 was examined via Western blotting (Figure 1). Ets-1 protein expression was not detectable in 2008 whole cell lysates, but was readily detected in the induced 2008-Ets1 lysate. Increased Ets-1 expression was found to be a specific effect as the protein levels of two similar Ets family members, Ets-2 and PEA3 were not altered in this model of Ets-1 over-expression (Figure 2).

**Ets-1 regulates metabolic gene expression**

Microarray analysis of 2008 and 2008-Ets1 cells revealed that 3,131 genes of the 28,869 genes probed were up-regulated or down-regulated in response to Ets-1 over-expression by at least 1.45 fold (p≤0.001) (GEO database accession #GSE21129). For this study, we have chosen to report and examine changes in selected mRNAs whose functions are relevant to mitochondrial activity, cellular metabolism, and oxidative stress. Real time qRT-PCR validation was conducted using 10 target genes representing various fold change values, and results were normalized to 4 separate housekeeping genes. Although both PPARG and SDHB gene expression in 2008-Ets1 cells were not significantly different from 2008 cells, the fold changes determined from real time qRT-PCR were associated with the microarray fold changes by a correlation coefficient of 0.99 (Table 1). Therefore, fold changes greater than 1.45 were deemed to be valid results and were included in this study.

The expression of G6PD, PDHA, HK, and CYC were examined by Western blotting to determine whether the gene expression differences observed were also present at the protein level. We did not find any significant differences in protein expression between 2008 and 2008-Ets1 cells for any of the enzymes examined (data not shown).

**Table 1. Real time qRT-PCR validation of microarray findings.**

| Gene name | Microarray fold change | Real time PCR fold change | Valid? |
|-----------|------------------------|--------------------------|--------|
| ETS1      | 12.70 ***              | 10.14 **                 | Y      |
| GPX2      | 8.80 ***               | 9.95 ***                 | Y      |
| PPARG     | 2.58 ***               | 2.07                     | N      |
| G6PD      | 1.74 ***               | 1.91 *                   | Y      |
| HK1       | 1.71 ***               | 2.18 **                  | Y      |
| SDHB      | –1.30 ***              | –2.07                    | N      |
| CYC1      | –1.46 ***              | –2.87 *                  | Y      |
| PDHA      | –2.05 ***              | –1.99 *                  | Y      |
| NDUFAB1   | –2.23 ***              | –2.76 ***                | Y      |
| MMP13     | –11.00 ***             | –11.26 **                | Y      |

Correlation Coefficient = 0.99; p≤0.05 = *, p≤0.01 = **, p≤0.001 = ***. doi:10.1371/journal.pone.0013565.t001
The glycolytic capability of cancer cells is regulated by Ets-1

Microarray analysis of 2008-Ets1 ovarian cancer cells revealed that Ets-1 is involved in the regulation of mitochondrial stress and dysfunction as metabolic genes, including those involved in glycolysis, glycolytic feeder pathways, the TCA cycle, and lipid metabolism (Table 2), and genes involved in antioxidant defense (Table 3) were altered in Ets-1 over-expressing cells. To evaluate whether cells with stable over-expression of Ets-1 favour glycolysis over oxidative phosphorylation for energy, as predicted by our microarray analysis, cells were grown in glucose-free media supplemented with the glycolytic inhibitor 2-DG, an analog of glucose. Cells were grown in the presence of varying amounts of 2-DG, and representative growth curves were generated for each cell line. The growth of cells in media containing 2-DG was inhibited to a greater extent in cells with a higher Ets-1 expression than in parental cells (Figure 3A). The 2-DG IC50 doses, or doses where 50% of the cells had stopped proliferating, were calculated from 4 independent experiments. Our results indicated that 2008-Ets1 cells induced with tetracycline were the most sensitive to 2-DG, an IC50 of 0.75 mM, which was significantly lower than the parental 2008 cells, IC50 of 4.29 mM (p<0.01). C13* cells, a cisplatin-resistant variant of 2008 ovarian carcinoma cells, correlated with intracellular reactive oxygen species in C13* cells, a cisplatin-resistant variant of 2008 cells, and did not affect oxygen consumption in our model (data not shown).

Mitochondrial reactive oxygen species activate several key signaling pathways involved in tumourigenesis and up-regulate the expression of important oncotrophic transcription factors including Ets-1 [92]. We have previously shown that increased production of intracellular reactive oxygen species in C13* cells, a cisplatin-resistant variant of 2008 ovarian carcinoma cells, correlated with an increase in Ets-1 mRNA and protein expression [13,35]. Subsequent treatment of these cells with H2O2 increased Ets-1

Table 2. Effect of Ets-1 over-expression on glycolysis, glycolytic feeder pathways, and lipid metabolism.

| Pathway                  | Gene                              | GenBank ID  | Gene name               | Fold change |
|--------------------------|-----------------------------------|-------------|-------------------------|-------------|
| Glycolysis               | Enolase 2                         | NM_001975   | ENO2                    | +2.38 ***   |
|                          | Hexokinase1                       | NM_033501   | HK1                     | +1.71 ***   |
|                          | Aldolase C, fructose-bisphosphatase| NM_005165   | ALDOC                   | +2.66 ***   |
|                          | Phosphoglucomutase 1              | NM_002633   | PGM1                    | +1.59 ***   |
| Galactose                | UDP-galactose-4-epimerase          | NM_000403   | GALE                    | +1.57 ***   |
|                          | Galactose mutarotase (aldose 1-epimerase) | NM_138801 | GALM                    | -3.54 ***   |
| Glycogen                 | Phosphorlyase, glycogen, liver    | NM_002863   | PYGL                    | +2.00 ***   |
| Citric acid cycle        | Citrate synthase                  | NM_004077   | CS                      | -1.63 ***   |
|                          | Fumarate hydratase                | NM_000143   | FH                      | -1.48 ***   |
| Pentose phosphate        | Glucose-6-phosphate dehydrogenase | NM_000402   | G6PD                    | +1.74 ***   |
|                          | Phosphoglucomutase 1              | NM_002633   | PGM1                    | +1.59 ***   |
| Lipid metabolism         | Transketolase                     | NM_00113505 | TKT                     | +2.07 ***   |
|                          | Apolipoprotein B mRNA editing catalytic polypeptide-like 3G | NM_021822 | APOBEC3G               | +2.25 ***   |
|                          | Fatty acid desaturase 1           | NM_013402   | FADS1                   | +6.47 ***   |
|                          | Fatty acid desaturase 2           | NM_004265   | FADS2                   | +5.29 ***   |
|                          | Fatty acid synthase               | NM_004104   | FASN                    | +1.61 ***   |
|                          | Lysophosphatidylycholine acyltransferase 2 | NM_017839 | LPCAT2                  | +2.56 ***   |
|                          | Phospholipase C, beta 3           | NM_000932   | PLCB3                   | +2.75 ***   |
|                          | Prostaglandin-endoperoxide synthase 1 | NM_000962 | PTGS1                   | +3.88 ***   |
|                          | Protein kinase, AMP-activated, beta 2 | NM_005399 | PRKAB2                  | +2.05 ***   |

p<0.05 = *, p<0.01 = **, p<0.001 = ***. doi:10.1371/journal.pone.0013565.t002

Ets-1 regulates cellular oxygen consumption in cancer cells

Our microarray analyses suggest that Ets-1 over-expression resulted in an overall down-regulation of genes that encode electron transport chain components, suggesting that these cell lines would likely display decreased O2 consumption (Table 4). This assumption was evaluated using high-resolution respirometry, where basal oxygen consumption was measured following the addition of cells to an oxygraph. Basal oxygen consumption was significantly lower in induced 2008-Ets1 cells (26.23 pmoles O2/1×10⁶ cells/sec; p<0.05) compared to 2008 cells (40.60 pmols O2/1×10⁶ cells/sec, p<0.05) (Figure 4). No significant tetracycline treatment effect on basal oxygen consumption was found following induction of 2008 cells, confirming that tetracycline did not affect oxygen consumption in our model (data not shown).

Discussion

Mitochondrial reactive oxygen species activate several key signaling pathways involved in tumourigenesis and up-regulate the expression of important oncogenic transcription factors including Ets-1 [92]. We have previously shown that increased production of intracellular reactive oxygen species in C13* cells, a cisplatin-resistant variant of 2008 ovarian carcinoma cells, correlated with an increase in Ets-1 mRNA and protein expression [13,35]. Subsequent treatment of these cells with H2O2 increased Ets-1...
expression in a dose-dependent manner, suggesting that this transcription factor is highly responsive to tumour-derived signals.

Our previous analysis of the Ets-1 promoter led to the identification of an antioxidant response element (ARE) that proved to be pivotal in regulating the expression of Ets-1 under both basal and H$_2$O$_2$-induced conditions [15]. Traditionally, the functional importance of Ets-1 over-expression in cancer has been associated with the regulation of matrix-degrading proteases and angiogenic factors [26,34]. However, our recent findings that mitochondrial reactive oxygen species potently affects Ets-1 expression in a dose-dependent manner, suggesting that this transcription factor is highly responsive to tumour-derived signals.

Table 3. Effect of Ets-1 over-expression in oxidative stress.

| Pathway               | Gene                               | GenBank ID | Gene name | Fold change |
|-----------------------|------------------------------------|------------|-----------|-------------|
| Antioxidant defense   | Glutathione peroxidase 1           | NM_000581.2| GPX1      | +1.98 ***   |
|                       | Glutathione peroxidase 2           | NM_005333  | GPX2      | +8.81 ***   |
|                       | Glutathione peroxidase 3           | NM_002084  | GPX3      | +4.19 ***   |
|                       | Peroxiredoxin 5                   | NM_012094  | PRDX5     | +1.54 ***   |
| ROS metabolism        | Arachidonate 12-lipoxygenase       | NM_000697  | ALOX12    | −1.81 **    |
|                       | GTF2I repeat domain containing 2   | NM_173537  | GTF2IRD2  | +1.92 ***   |
|                       | GTF2I repeat domain containing 2B  | NM_001003795| GTF2IRD2B| +2.19 ***   |
|                       | Neutrophil cytosolic factor 2      | NM_000433  | NCF2      | −2.24 ***   |
|                       | Superoxide dismutase 2 (mitochondrial)| NM_001024465| SOD2    | −1.49 ***   |
|                       | ATX1 antioxidant protein 1         | NM_004045  | ATOX1     | +1.53 ***   |
|                       | Prion protein                      | NM_000311  | PRNP      | −1.60 ***   |
|                       | Scaavenger receptor class A, member 3| NM_016240  | SCARA3    | +1.48 **    |
|                       | Selenoprotein P, plasma, 1         | NM_005410  | SEPP1     | −2.04 ***   |
|                       | Aldehyde oxidase 1                 | NM_001159  | AOX1      | +2.81 ***   |
|                       | BCL2/adenovirus E1B interacting protein 3| NM_004052  | BNIP3     | +3.15 ***   |

p≤0.05 = *, p≤0.01 = **, p≤0.001 = ***.
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Thus, our gene expression findings suggest that Ets-1 is a key transcription factor involved in the metabolism of cancer cells, and particularly important in the metabolic shift towards glycolysis and electron leakage and reactive oxygen species production is Complex III, which is composed of 10 subunits, of which Ets-1 down-regulates 3. Ets-1 also represses components of Complex IV, Complex V ATP synthases, as well as some electron transport associated factors. Taken together, these repressive functions suggest that Ets-1 is prominently involved in the decreased reliance on oxidative phosphorylation frequently associated with cancer cells. Consequently, genes encoding mitochondrial proteins involved in oxidative phosphorylation would be down-regulated, and cells would require alternate methods of ATP production including glycolysis and fatty acid oxidation. Given that components of almost every complex of the electron transport chain, several key enzymes of the TCA cycle, and ultimately the reducing equivalents needed for electron transport were similarly down-regulated, Ets-1 over-expressing cells appear to have a decreased capacity to generate ATP via oxidative phosphorylation at the gene expression level.

Cancer cells commonly have a decreased reliance on oxidative phosphorylation for energy generation, and they likewise have an increased dependence on glycolysis and fat metabolism for cellular energy [35]. In further support that Ets-1 is involved in the regulation of altered cancer metabolism, Ets-1 is associated with the increased expression of many genes involved in glycolysis, glycolytic feeder pathways, and the pentose phosphate pathway. Additionally, the expression of Ets-1 is also correlated with increases in the expression of many genes involved with lipid metabolism and biosynthesis. Taken together, these trends suggest that Ets-1 is an important transcriptional regulator not only in the catabolic, but also anabolic metabolic transitions of cancer cells that ultimately promote tumourigenesis [35].

It was almost half a century ago when the up-regulation of fatty acid synthase (FASN) was first described in cancer [36], which is now known to be over-expressed in the majority of cancers. Interestingly, Ets-1 was also found to be involved in the regulation of increased FASN gene expression in our ovarian cancer model. Thus, our gene expression findings suggest that Ets-1 is a key transcription factor involved in the metabolism of cancer cells, and particularly important in the metabolic shift towards glycolysis and...
reliance on glycolysis for energy (n = 3).

2008-Ets1 ovarian cancer cells were grown in the absence of glucose, and proliferation assays were conducted at 24 hour intervals. 2008-Ets1 compared to parental 2008 cells (n = 4). (B) 2008 and 2008-Ets1 cells were grown in the absence of glucose, and proliferation assays were conducted at 24 hour intervals. 2008-Ets1 ovarian cancer cells expressing high levels of Ets-1 showed a decreased ability to grow in glucose-free culture medium, suggesting an increased reliance on glycolysis for energy (n = 3).

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Figure 3. Glycolytic dependence of Ets-1-expressing ovarian cancer cells. (A) 2008, C13*, and 2008-Ets1 cells were treated with the glycolytic inhibitor 2-DG, and cellular growth was measured following 96 hours of incubation. 2008-Ets1 and C13* cells which express Ets-1 showed significantly decreased growth following glycolytic inhibition in 2008-Ets1 compared to parental 2008 cells (n = 4). (B) 2008 and 2008-Ets1 cells were grown in the absence of glucose, and proliferation assays were conducted at 24 hour intervals. 2008-Ets1 ovarian cancer cells expressing high levels of Ets-1 showed a decreased ability for growth in glucose-free culture medium, suggesting an increased reliance on glycolysis for energy (n = 3).

Ets-1 Regulates Metabolism

anabolic means of energy production. Although it is important to note that Ets-1-mediated metabolic regulation is likely achieved by a large consortium of different transcription factors, a more complex regulatory network of transcription factors influenced by mitochondrial dysfunction have yet to be elucidated. We have demonstrated that the over-expression of Ets-1 in our ovarian cancer model did not affect protein levels of two closely related ETS family members; however, it is possible that other ETS transcription factors from this very large family also influence cancer metabolism. Considering that these transcription factors recognize almost identical consensus sequences, the repetition of the experiments within this study following the over-expression of other ETS family members could yield similar results. In addition, such experiments would further characterize the potentially large transcriptional network involved in the specialized metabolism of cancer cells.

To determine the functional relevance of our genomic results, we have examined the glycolytic capability of our Ets-1 over-expression model. We have indirectly evaluated the oxidative phosphorylation capacity of these cells through treatment with the glycolytic inhibitor 2-DG. Ovarian C13* and induced 2008-Ets1 cells, which both express Ets-1, showed prominent growth inhibition in response to 2-DG, suggesting that these cells are more reliant on glycolysis for ATP production. Additionally, the Ets-1 over-expressing ovarian cancer cells displayed significantly decreased growth following glucose deprivation, further emphasizing their glycolytic reliance. The growth rate of all cell types in glucose-free media was decreased as compared to normally supplemented media, particularly after 48 hrs when a distinct divergence in cell growth was consistently observed, likely due to decreased glucose availability. Over-expression of Ets-1 potentially exacerbated this divergence as the growth of induced 2008-Ets1 cells drastically decreased after 48 hrs. However at the protein level, we did not find any significant differences in the expression of glucose-6-phosphate dehydrogenase, pyruvate dehydrogenase, cytochrome c, or hexokinase, which are all enzymes involved in glycolysis or oxidative phosphorylation. Importantly, we did see repeatable and significant differences in glycolytic dependence associated with Ets-1 expression, and the lack of changes in protein expression could therefore be due to post-translational modifications mediated by Ets-1. For example, differences within the catalytic region of an enzyme would result in functional differences, but would not necessarily be detectable via Western blot, as this technique is dependent on the specific epitope targeted by the antibody used. Thus, we plan to examine the enzyme activity of these metabolic enzymes in future studies to determine if any differences exist between 2008 and 2008-Ets1 cells that could account for the functional differences we have demonstrated in this study.

The evaluation of O2 consumption of a population of cells is a functional evaluation of oxidative capacity, as it represents a good estimate of the rate at which electrons are passing along the electron transport chain and being reduced to H2O2. The polarographic system used in this study to measure O2 consumption includes sensors that yield a current proportional to the partial pressure of O2 in cell containing media by consuming O2 in a cathode half reaction, thus the signal responds exponentially to changes in O2 pressure within the sample. In the absence of specific complex substrates and ADP, thereby simulating respiration, the basal rate of O2 consumption can be measured. We have observed significant decreases in O2 consumption in cells with increased Ets-1 expression. Our results indicate that Ets-1 is directly involved in the regulation of cellular oxidative capacity, where Ets-1 over-expression led to significantly decreased O2 consumption, and Ets-1 down-regulated cells displayed a very prominent increase in O2 consumption. These results suggest that up-regulated Ets-1 expression promotes a decreased dependence on oxidative phosphorylation for energy, and provides further evidence towards the functional importance of Ets-1 in cancer cell metabolism.

High levels of oxidative stress are typically observed in the tumour microenvironment as a result of imbalances in antioxidant defense factors, and impaired DNA repair mechanisms [37–39]. In breast cancer cells lines, increased malignancy is associated with high levels of reactive oxygen species-producing superoxide dismutase activity, in combination with decreased levels of reactive oxygen species-detoxifying glutathione peroxidases and the H2O2-detoxifying enzyme catalase [40]. Similar antioxidant enzyme imbalances have been found in melanoma [41], as well as lung [42], prostate [43], and thyroid cancers [44]. Our microarray analysis determined that Ets-1 is a regulator of antioxidant gene expression in ovarian cancer cells, particularly glutathione peroxidases, which preferentially target H2O2 and lipid hydroperoxides for detoxification. This increased expression of antioxidants is in response to mitochondrial oxidative stress in the form of excessive reactive oxygen species production, and we have previously shown that Ets-1 gene expression increases in response
to H$_2$O$_2$ [15]. However, it is important to note that Ets-1 also down-regulated genes encoding certain H$_2$O$_2$-detoxifying enzymes, suggesting that these cancer cells likely require and maintain a certain level of H$_2$O$_2$ to encourage the high growth rates inherent to tumour progression.

A novel function for Ets-1 was elucidated in this study following genomic and functional analysis of an ovarian cancer Ets-1 expression model. To our knowledge, this is the first study to show a role for this transcription factor in metabolism. Numerous down-regulated genes were identified in Ets-1 over-expressing cells, including those encoding several mitochondrial proteins involved in oxidative phosphorylation, as well as important metabolic enzymes that are responsible for the generation of required substrates of the electron transport chain. Functional assays confirmed that Ets-1 over-expressing cancer cells displayed reduced oxidative phosphorylation capabilities, as well as enhanced reliance on glycolysis for cellular energy. Additionally, Ets-1 was shown to be important in the regulation of cellular O$_2$ consumption further suggesting a reduced usage of oxidative phosphorylation in cancer cells expressing Ets-1.

Therefore, damage to the mitochondria results in increased production of H$_2$O$_2$ and consequent up-regulation of Ets-1, which then participates in an active regulatory network that encourages reliance on glycolysis and lipid metabolism for cellular energy requirements. Thus, Ets-1 may be grouped with other transcription factors that have been observed to up-regulate the expression of mitochondrial proteins (NRFs) or genes involved in glycolysis (HIF-1) in response to specific stresses [45,46]. In summary, our findings demonstrate a novel role for Ets-1 in the regulation of cellular metabolism in response to mitochondrial stress.

### Materials and Methods

#### Cell culture

The human ovarian carcinoma cell lines, 2008 and C13*, were kindly provided by Dr. Paul Andrews (Georgetown University, Rockville MD) [33]. The 2008 and C13* cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin. The stable cell line 2008-Ets1 [27] was maintained in growth medium as described with the addition of 200 ng/ml of the selective antibiotic Zeocin. All cells were kept at 37°C in a humidified atmosphere of 5% CO$_2$. Media and supplements were purchased from Invitrogen Life Technologies (Burlington, ON, Canada), and FBS from Fisher Scientific (Ottawa, ON, Canada). All reagents were purchased from Sigma (Oakville, ON, Canada).

#### Protein isolation and Western blot analysis

Whole cell lysates were collected, 30 µg of protein were separated by 10% SDS-PAGE electrophoresis, transferred to nitrocellulose membrane (Amersham Biosciences, Baie D’Urfe,}

### Table 4. Effect of Ets-1 over-expression on the ETC.

| Component               | Gene                                      | GenBank ID | Gene name   | Fold change |
|-------------------------|------------------------------------------|------------|-------------|-------------|
| Complex I               | NADH dehydrogenase (ubiquinone) 1         | NM_004541  | NDUFA1      | −1.60 ***   |
|                         | 1 β subcomplex 1                          | NM_005002  | NDUFA9      | −1.59 ***   |
|                         | NADH dehydrogenase (ubiquinone) 1         | NM_005003  | NDUFA1B     | −2.23 ***   |
| Complex II              | NADH dehydrogenase (ubiquinone) 1 β      | NM_002492  | NDUF8S      | −1.50 ***   |
|                         | subcomplex 5                              |            |             |             |
| Complex III             | Cytochrome c                            | NM_001916  | CYC1        | +1.46 ***   |
|                         | Ubiquinol-cytochrome c reductase, Rieske | NM_006003  | UQCRFS1     | −1.50 ***   |
|                         | iron-sulfur polypeptide 1                |            |             |             |
| Complex IV              | Cytochrome c oxidase subunit 7b          | NM_001866  | COX7B       | −2.08 ***   |
| ATP Synthases           | ATP synthase, H+ transporting, mitochondrial F1 complex, α subunit 1, cardiac muscle | NM_001001937 | ATP5A1 | −1.53 *** |
|                         | ATPase, H+ transporting, lysosomal accessory protein 2 | NM_005765 | ATP6AP2 | −1.50 *** |
| Electron transport      | Cytochrome c, somatic                     | NM_018947  | CYCS        | −1.51 ***   |
| associated factors      | Holocytochrome c synthase (cytochrome c heme-lyase) | NM_005333 | HCCS       | −1.76 ***   |

p ≤ 0.05 = *, p ≤ 0.01 = **, p ≤ 0.001 = ***.

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Figure 4. Effect of Ets-1 expression on oxygen consumption.
Basal oxygen consumption was determined for 2008 and 2008-Ets1 ovarian cancer cells. Ets-1 expression was associated with a significant decrease in basal oxygen consumption suggesting a decreased reliance on oxidative phosphorylation in 2008-Ets1 cells.

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error were determined from relative quantification, where statistical significance and standard expression and efficiency corrected using the DD housekeeping controls. Data was normalized to housekeeper gene (ACTB), B-2 macroglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and RNA polymerase II (RPRI) as housekeeping controls. Data was normalized to housekeeper gene expression and efficiency corrected using the ΔΔCt method of relative quantification, where statistical significance and standard error were determined from ΔCt values.

Microarray analysis

Total RNA was isolated from tetracycline-induced 2008 and 2008-Ets1 cells as described, purified using the RNeasy purification kit (Qiagen), and hybridized to the GeneChip® Human Gene 1.0 ST Array (28,869 human gene probes). RNA quality analysis via bioanalyzer, microarray preparation, hybridization, and detection were performed by The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, Canada. All low-level analyses, calculation of differential expression, and statistical adjustments were computed using R version 2.9.2 [47]. Assessment of RNA quality post-hybridization, and low-level analysis were performed using the package ‘affy’ [48]. RNA degradation plots revealed little 5' to 3' bias and all arrays were within 1.35 standard deviations of the average slope. Background correction and normalization was performed using the robust multi-array average (RMA) algorithm. To reduce the number of differential expression tests downstream, thereby increasing overall power, 50% of the lowest normalized Log2 probe-set intensities were removed according to recommendations by Hackstadt and Hess (2009) [49]. Differential expression estimates were computed using the adjusted Local Pooled Error test in the package ‘LPEadj’ [50]. The Benjamini-Hochberg procedure for controlling false discovery rate (FDR) was applied to comparison-wise p-values using the package ‘multtest’. Reported q-values represent the minimum FDR at which a particular test can be considered significant. Fold change expression values of 10 random target genes of varying fold change magnitude were validated using real time PCR. Corresponding relative fold changes were determined using the ΔΔCt method of relative quantification, and correlation coefficients were calculated to determine the relationship between real time PCR and microarray findings.

Glycolytic dependency assays

Cellular growth rate and the inhibition of proliferation were evaluated by assessing total cell numbers, where 2 x 10^5 cells were plated onto 96-well tissue culture plates and allowed to adhere. Cells were treated with 2-deoxy-D-glucose (2-DG; Sigma) administered at concentrations ranging from 1–7.5 mM over 96 hrs, at which time a Hoechst DNA content assay (Invitrogen) was performed. Following treatment, cells were washed and lysed in MilliQ water and 2 μg/mL Hoechst 33258 stain diluted in TNE buffer (10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.4). Fluorescence was evaluated using a Cytoflour series 4000 multi-well plate reader (excitation 350 nm, emission 460 nm) (PerSeptive Biosystems, Framingham, MA). Cell number was standardized to fluoresce for each cell type by comparison with a standard curve of known cell numbers. To determine cell growth in glucose free media, glucose free RPMI and DMEM media supplemented with 110 μg/mL pyruvate was added to cells following overnight adherence. Plates of cells were frozen every

**Table 5. Primer sequences used for real time qRT-PCR.**

| Gene name (acronym) | Forward primer (5'–3') | Reverse primer (5'–3') |
|---------------------|------------------------|-----------------------|
| β-actin (ACTB)      | ccccccgctgggaagactac   | gatgcaccgctacactcga  |
| B-2 macroglobulin (B2M) | gctctacgctactccaaag  | tccacctcagctacctcga  |
| Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | atctagaagttctctcttg | cttgctacacccctgta |
| RNA polymerase II (RPRI) | gaaacgggtggacggctaat | tccctatgccatcatctgac |
| Cytochrome c1 (CYC1) | caaatgaagatcctaccatg | ccagtaagatgggtagtgaag |
| V-ets erythroblastosis virus E26 oncogene homolog 1 (ETS1) | tgtattttgcctggag | aacagtcatcctagactc |
| Glucose-6-phosphate dehydrogenase (G6PD) | tggaccagggcagacatc | caaccatcttgctttcatc |
| Glutathione peroxidase 2 (GPX2) | cccctacccattgtgacc | gttgtgtggggaaaggtg |
| Hexokinase 1 (HK1) | gccggttgacacagctctctct | ctgggtgaggtggaaatag |
| Matrix metallopeptidase 13 (MMP13) | cagccctctctccgctagag | cagggaggtcatccgacca |
| NADH dehydrogenase 1, s/β subcomplex 1 (NDUFAB1) | cgctctgcgcctggccg | cttgctacacccctgta |
| Pyruvate dehydrogenase 1 (PDHA) | cagccacccatcatcaaggt | ggccagactcacttcctca |
| Peroxisome proliferator-activated receptor γ (PPARG) | cattttagcggagagcgcatt | cattttacgagacagttcag |
| Succinate dehydrogenase complex, subunit B (SDHB) | cttgtggccagtgggagct | gtttccgttccacctagtg |

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24 hrs up to and including the 96 hr time point and Hoechst assays were performed at each time point.

Oxygen consumption assay
All oxygen consumption assays were performed on the OROBOROS oxygraph (Oroboros, Innsbruck, Austria) at 37°C. Cells were pelleted, resuspended in growth medium, and 3 x 10^6 cells were used per experiment at a density of 1 x 10^6 cells/100 μL. Freshly harvested cells were added to the oxygraph chambers containing 1.5 mL of KCl medium (80 mM KCl, 10 mM Tris-HCl, 3 mM MgCl2, 1 mM EDTA, 5 mM potassium phosphate, pH 7.2). The total O2 concentration and flux were recorded at 1 sec intervals throughout the experiment. Once the oxygen consumption stabilized, the basal O2 consumption was determined for each cell line and condition, where OROBOROS software was used for data acquisition and analysis.

References
1. Schatz G (1995) Mitochondria: beyond oxidative phosphorylation. Biochimica et biophysica acta 1271: 123-126.
2. Warburg O (1956) On the origin of cancer cells. Science 123: 309-314.
3. Wahl RL, Kaminski NS, Edsper SF, Hutchins GD (1990) The potential of 2-deoxy-2-[14C]fluoro-D-glucose (FDG) for the detection of tumour involvement in lymph nodes. J Nucl Med 31: 1831-1835.
4. Avril N, Dose J, Janicke F, Bense S, Ziegler S, et al. (1996) Metabolic characterization of breast tumors with positron emission tomography using F-18 labeled deoxyglucose. J Clin Oncol 14: 1849-1857.
5. Lee HC, Wei YH (2009) Mitochondrial DNA instability and mitochondrial shift in human cancers. Int J Mol Sci 10: 674-701.
6. Petros JA, Baumann AK, Ruiz-Pesini E, Amin MB, Sun COQ, et al. (2003) mtDNA mutations increase tumorigenicity in prostate cancer. Proc Natl Acad Sci U S A 100: 719-724.
7. Carew JS, Huang P (2002) Mitochondrial defects in cancer. Mol Cancer 1: 9.
8. Klaunig JE, Kamendulis LM (2004) The role of oxidative stress in carcinogenesis. Annu Rev Pharmacol Toxicol 44: 239-267.
9. Konnoeuzenas AA, Perkin AV, Popovska Y, Komotinos GB, Raage RK (1987) Superoxide generation by the respiratory chain of mitochondrial tumour. Biochim Biophys Acta 894: 1-10.
10. Szturwiova TP, Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 51: 794-798.
11. Chen K, Altman A, He A, Keaney JF (2003) Activation of p53 by oxidative stress involves platelet-derived growth factor-beta receptor-mediated ataxia telangectasia mutated (ATM) kinase activation. J Biol Chem 278: 39327-39335.
12. Marindale JL, Holbrook NJ (2002) Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 192: 1-15.
13. Nomura M, Ma W, Chen N, Bode AM, Dong Z (2000) Inhibition of 12-O-tetradecanoylphorbol-13-acetate-induced NF-kappaB activation by tea polyphenols, (-)-epigallocatechin gallate and theaflavins. Carcinogenesis 21: 1805-1809.
14. Pelciano H, Lu W, Zhou Y, Zhang W, Chen Z, et al. (2009) Mitochondrial dysfunction and reactive oxygen species imbalance promote breast cancer cell motility through a CXCL14-mediated mechanism. Cancer Res 69: 2375-2383.
15. Wilson LA, Gennia A, Espiritu R, Singh G (2005) eto-1 is transcriptionally up-regulated by H2O2 via an antioxidant response element. PASEB J 19: 2083-2097.
16. Dittmer J (2003) The biology of the Ets1 proto-oncogene. Mol Cancer 2: 29.
17. Hahne JC, Okuducu AF, Sahin A, Fafeur V, Kiriakidis S, et al. (2008) The biology of the Ets1 proto-oncogene. Mol Cancer 2: 29.
18. Martindale JL, Holbrook NJ (2002) Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 192: 1-15.
19. Nakayama T, Ito M, Ohnura A, Naito S, Nakashima M, et al. (1996) Expression of the Ets-1 proto-oncogene in human gastric carcinoma: correlation with tumor invasion. Am J Pathol 149: 1931-1939.
20. Watabe T, Yoshida K, Shindoh M, Kaya M, Fujikawa K, et al. (1998) The Ets-1 transcription factor is involved in the development and invasion of malignant melanoma. Carcinogenesis 21: 1835-1890.
21. Pelciano H, Lu W, Zhou Y, Zhang W, Chen Z, et al. (2009) Mitochondrial dysfunction and reactive oxygen species imbalance promote breast cancer cell motility through a CXCL14-mediated mechanism. Cancer Res 69: 2375-2383.
22. Wilson LA, Gennia A, Espiritu R, Singh G (2005) eto-1 is transcriptionally upregulated by H2O2 via an antioxidant response element. PASEB J 19: 2083-2097.
23. Davidson B, Reich R, Goldberg I, Godleb WH, Kopelow J, et al. (2001) Ets-1 messenger RNA expression is a novel marker of poor survival in ovarian carcinoma. Clin Cancer Res 7: 551-557.
24. Davidson B, Ruiberg B, Goldberg I, Nesland JM, Benner A, et al. (2001) Ets-1 mRNA expression in effusions of ovarian carcinoma patients is a marker of poor outcome. Am J Surg Pathol 25: 1493-1500.
25. Span PN, Manders P, Heuvel JJ, Thomas CM, Bossh RR, et al. (2002) Expression of the transcription factor Ets-1 is an independent prognostic marker for relapse-free survival in breast cancer. Oncogene 21: 8596-8599.
26. Oda N, Abe M, Sato Y (1999) ETS-1 converts endothelial cells to the angiogenic phenotype by inducing the expression of matrix metalloproteases and integrin beta3. J Cell Physiol 176: 121-132.
27. Wilson LA, Yamamoto H, Singh G (2004) Role of the transcription factor Ets-1 in cisplatin resistance. Mol Cancer Ther 3: 833-839.
28. Lefer LP, Dima S, Sunamura M, Furukawa T, Sato Y, et al. (2009) Transcriptional silencing of ETS-1 efficiently suppresses angiogenesis of pancreatic cancer. Cancer Gene Ther 16: 137-148.
29. Okuducu AF, Zils U, Michaelis SA, Michaelides S, von Deimling A (2006) Ets-1 is up-regulated together with its target gene products matrix metalloprotease-2 and matrix metalloproteinase-9 in anaplastic and anaplastic meningiomas. Histopathology 48: 836-845.
30. Park YH, Jung HH, Ahn JS, Im HY (2008) Ets-1 upregulates HER2-induced MMP-1 expression in breast cancer cells. Biochem Biophys Res Commun 377: 389-394.
31. Rothhammer T, Hahne JC, Florin A, Poser J, Sencic F, et al. (2004) The Ets-1 transcription factor is involved in the development and invasion of malignant melanoma. Cell Mol Life Sci 61: 118-128.
32. Hutter E, Unterluggauer H, Gareavel A, Janso-Durr P, Gnaiger E (2006) High-resolution respirationmetry-a modern tool in aging research. Exp Gerontol 41: 103-109.
33. Andrews PA, Albright KD (1992) Mitochondrial defects in cis-diamminedi- chloroplatinum(II)-resistant human ovarian carcinoma cells. Cancer Res 52: 1895-1901.
34. Trojanowska M (2000) Eto factors and regulation of the extracellular matrix. Oncogene 19: 6646-6647.
35. Yecies JL, Manning BD (2010) Chewing the fat on cell tumor metabolism. Cell 140: 28-30.
36. Medes G, Thomas A, Weinhouse S (1955) Metabolism of neoplastic tissue. IV. A study of lipid synthesis in neoplastic tissue slices in vitro. Cancer Res 13: 27-29.
37. Gokul S, Patil V, Jailkhani R, Hallikeri K, Kattappagari K (2009) Oxidant-antioxidant status in blood and tumor tissue of oral squamous cell carcinoma patients. Oral Dis 15: 103-109.
38. Oberley TD, Oberley LW (1997) Antioxidant enzyme levels in cancer. Histol Histopathol 12: 525-535.
39. Sinha S, Singh SN, Saha M, Kain TC, Tyagi AK, et al. (2009) Antioxidant and oxidative stress responses in psoriasis patients at high altitude. J Invest Dermatol 107: 322-326.
40. Chauhan HO, Zheng S, Coimbra SA, Farver C, Erramus SC (2001) Differential expression of manganese superoxide dismutase and catalase in lung cancer. Cancer Res 61: 8578-8585.

Statistical analysis
Data is presented as the mean +/- standard deviation from at least three independent experiments. Statistically significant differences between sample groups were determined using a Student’s t-test or ANOVA where applicable, with a p-value ≤0.05 considered to be statistically significant (p≤0.05 = *, p≤0.01 = **, p≤0.001 = ***).

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Author Contributions
Conceived and designed the experiments: MLV LAW GS. Performed the experiments: MLV LAW. Analyzed the data: MLV LAW CPV. Contributed reagents/materials/analysis tools: MLV LAW CPV. Wrote the paper: MLV.
43. Arsova-Sarafinovska Z, Eken A, Matevska N, Erdem O, Sayal A, et al. (2009) Increased oxidative/nitrosative stress and decreased antioxidant enzyme activities in prostate cancer. Clin Biochem 42: 1228–1235.
44. Akinci M, Kosova F, Cetin B, Sepici A, Altan N, et al. (2008) Oxidant/antioxidant balance in patients with thyroid cancer. Acta Cir Bras 23: 551–554.
45. Scarpulla RC (2002) Nuclear activators and coactivators in mammalian mitochondrial biogenesis. Biochim Biophys Acta 1576: 1–14.
46. Semenza GL (2003) Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3: 721–732.
47. R Development Core Team (2005) R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria.
48. Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) affy–analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20: 307–315.
49. Hackstadt AJ, Hess AM (2009) Filtering for increased power for microarray data analysis. BMC Bioinformatics 10: 11.
50. Murie C, Nadon R (2008) A correction for estimating error when using the Local Pooled Error Statistical Test. Bioinformatics 24: 1735–1736.