THE ROLE OF THE TRANSCRIPTION FACTOR ETS-1 IN MITOCHONDRIAL METABOLISM AND OXIDATIVE STRESS

THE ROLE OF THE TRANSCRIPTION FACTOR ETS-1 IN MITOCHONDRIAL METABOLISM AND OXIDATIVE STRESS

BY

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Abstract

Normal cellular energy metabolism is fundamentally altered in cancer cells to facilitate rapid production of new cellular components, thereby enabling uncontrolled cell growth. Specifically, cancer cells rely on glycolysis and alternative pathways such as lipid and glutamine metabolism for energy, while diverting substrates away from oxidative metabolism regardless of the prevalence of oxygen in the microenvironment. This hallmark of cancer cells is referred to as the Warburg effect, the precise regulation of which is poorly understood despite several decades of research. In comparing the global gene expression profiles of ovarian cancer cells to those that overexpress Ets-1, we have revealed that this transcription factor is involved, at least in part, to this cancer-associated metabolic switch. To support the validity of these findings, we have shown that Ets-1 functionally regulates glycolytic dependence in ovarian and breast cancer cells, while concomitantly displaying a decreased capacity for oxidative phosphorylation. Reactive oxygen species are a normal byproduct of metabolism, and are produced excessively in cancer cells leading to oxidative stress. Interestingly, our genomic pathway analyses uncovered enrichments in antioxidant pathways associated with increased Ets-1 expression. Accordingly, we have also observed that Ets-1 regulates increased intracellular glutathione levels, and induces the activity of key antioxidant enzymes under oxidative stress. Sulfasalazine, an agent that restricts cystine uptake, was shown to be effective for decreasing these high glutathione levels during oxidative stress. These results are clinically relevant because high glutathione levels are associated with

therapeutic resistance in cancer cells. Collectively, the evidence presented has identified a novel role for the transcription factor Ets-1 in the regulation of cancer energy metabolism, as well as the response to oxidative stress. We have also described a mechanism for Ets-1-mediated therapeutic resistance, suggesting that this transcription factor may be a promising novel target to enhance conventional cancer therapies.

Preface

This doctoral thesis is presented as a "sandwich" thesis, and is consists of four manuscripts that were prepared for publication during the course of the author's doctoral work. Three manuscripts have been published (Chapters 2, 3 and 4), while one has been submitted for publication (Chapters 5) at the time this thesis was prepared. Beginning with a conceptual overview and comprehensive background information, each manuscript is presented as a separate chapter that underscores a specific theme within the overall context of the thesis, and includes a preface detailing each author's contributions and detailing the underlying context of the corresponding manuscript. In addition, unpublished data that supports and strengthens the materials and objective of each study may be included following the paper. Lastly, the final concluding chapter summarizes the major findings of the thesis as a whole, and discusses future directions for the continuation of the author's research.

The figures presented within this thesis are denoted as the chapter number first, followed by the figure number that corresponds to the appropriate manuscript (e.g. Figure 1 in Chapter 2 is presented at Figure 2.1). References present within the body of this thesis are formatted using the style of the journal to which they were submitted and refer only to the reference list within that manuscript. Literature cited within the body of the thesis use the American Psychological Association (6^{th} edition) style, and appears in the *References* section at the end of the dissertation. Appendices are included which describe in detail common methodologies used in this research.

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First and foremost I want to thank my supervisor, Dr. Gurmit Singh, for giving me the opportunity to work in his research laboratory, and for his ample support and guidance throughout my doctoral program. He has taught me to look at the "bigger picture", to keep my focus while moving forward with my research, and most importantly to think critically about science on a whole. He has been a wonderful mentor and I will be grateful for his role in my maturation as a scientist. I also would like to thank the members of my advisory committee, Dr. Richard Austin, Dr. Sandeep Raha, and Dr. Michael Trus, whose advice and insight have helped me focus and properly interpret my experimental work.

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Lastly, I want to thank my family and friends who have been incredibly supportive over this long journey, in particular my parents who have always supported me in every direction that life takes me. Most importantly of all I want to thank my little family Chris, Desmond, and our dog Odin. To my son Desmond, who came along right in

the middle of my PhD program, I want you to know how much being your mommy inspires me to work harder and be a better researcher. I want to show you through my example the value of a good education, and that with hard work and dedication you can achieve whatever you want in life. I hope that the sacrifices we have made as a family will only make our future brighter, and that Daddy and I inspire you to never stop learning. Regardless of what you choose to do with your life, we are always proud of you and promise to be there for every single graduation…even if you have as many as we have had! I also want to thank you for being the absolute brightest light in my life, and for making every second of it worth living. Finally, to my husband Chris, I cannot express in words the extent of my gratitude for your love and support over the last few years. It has been absolutely invaluable to me to have someone to come home to every single day that truly understands the many trials and tribulations inherent to scientific research. You are always there to encourage me when I need it, lifting my spirits when they've hit rock bottom, and celebrate my accomplishments. Thank you for keeping me grounded, and most of all thank you for loving me unconditionally. I feel so fortunate to have you in my life and I love you so much.

Table of Contents

Abstract iii

Preface v

Acknowledgements vi

List of Figures and Tables xii

List of Abbreviations and Symbols xiv

Chapter 1: Introduction 1

Cancer Metabolism 1

Healthy Cellular Metabolism *1*

Metabolic Alterations in Cancer Cells *5*

Glucose Metabolism: Cancer-associated Enzyme Expression and Regulation *5*

Glutamine *9*

Role of Mitochondria in Cancer *10*

Genomics of mitochondrial DNA: mutations and polymorphisms *11*

Mitochondrial proteomics *15*

Oxidative Stress in Cancer 17

Major Cellular Antioxidant Systems *18*

Enzymatic Antioxidant Alterations in Cancer Cells *22*

The Ets-1 Transcription Factor 23

Structure and Function of Ets-1 ..... *23*

Regulation of Ets-1 Expression *25*

Ets-1 Overexpression in Cancer *26*

Hypothesis and Objectives 29

Manuscript Plan *30*

Chapter 2: Ets-1 regulates energy metabolism in ovarian cancer 33

Context and background information 34

PAPER: Ets-1 regulates energy metabolism in cancer cells 37

Tables 65

Figures 70

Chapter 3: Global gene expression profile for Ets-1 overexpression 75

Context and background information 75

PAPER: Ets-1 global gene expression profile reveals associations with metabolism and oxidative stress in ovarian and breast cancers 79

Tables 115

Figures 116

Chapter 4: Mitochondrial-generated reactive oxygen species in cancer 126

Context and background 126

PAPER: Mitochondrial-generated reactive oxygen species in cancer 128

Tables 182

Figures....183

Chapter 5: The role of Ets-1 in cellular response to oxidative stress ...189

Context and background 189

PAPER: Ets-1 regulates intracellular glutathione levels: Key target for resistant ovarian cancer 193

Figures....214

Chapter 6: Conclusions and Future Directions....225

Future directions 229

Confirmation Of Ets-1 Target Genes....230

Delving Further Into Metabolism 237

Transcriptional Partners During Oxidative Stress....239

Validation of In Vitro Findings....241

Summary 243

APPENDIX 1: General materials and methods 281

Cell culture & treatments 281

Isolation of total RNA....281

Real-time qRT-PCR....284

Cell Lysate Collection....284

Bio-Rad protein assay 285

Western Blotting 285

CM_2-H_2DCFDA Intracellular ROS detection 287

Quantitative determination of GSH & GSSG 289

Glutamate release assay 292

Statistical Analyses 296

Appendix 2: Copyright License Agreements for Published Papers 297

List of Figures and Tables

Figure 1.1: Overview of cancer metabolism. 2

Figure 1.2: Overview of the role of mitochondrial alterations in cancer. 13

Figure 1.3: Major sources of cysteine for glutathione synthesis. 21

Table 2.1: Real time qRT-PCR validation of microarray findings 65

Table 2.2: Effect of Ets-1 over-expression on glycolysis, glycolytic feeder pathways, and lipid metabolism 66

Table 2.3: Effect of Ets-1 over-expression on oxidative stress 67

Table 2.4: Effect of Ets-1 over-expression on the ETC 68

Table 2.5: Primer sequences used for real time qRT-PCR 69

Figure 2.1: Generation of an ovarian cancer cell model for Ets-1 expression. 70

Figure 2.2: Ets-1 expression did not affect the expression of similar ETS family members. 71

Figure 2.3: Glycolytic dependence of Ets-1-expressing ovarian cancer cells. 72

Figure 2.4: Effect of Ets-1 expression on oxygen consumption. 74

Table 3.1: Functional annotation clustering of genes associated with Ets-1 overexpression 115

Figure 3.1: Global functional interaction network. 116

Figure 3.2: Enrichment map of GSEA analysis. 118

Figure 3.3: Integrating various bioinformatic analyses. 119

Figure 3.4: Ets-1 regulated oxidative stress in ovarian cancer cells. 121

Figure 3.5: Breast cancer cell model of Ets-1 expression knockdown. 123

Figure 3.6: Effect of Ets-1 knockdown on breast cancer cell metabolism. 124

Table 4.1: Association between Ets-1 expression and cancer 182

Figure 4.1: Mitochondrial alterations in cancer cells.....183

Figure 4.2: ROS as signaling molecules. 184

Figure 4.3: Mitochondria to nucleus crosstalk.....186

Figure 4.4: Ets-1 as a mediator of mitochondria to nucleus crosstalk. 187

Figure 5.1: Ets-1 decreases intracellular ROS, while increasing intracellular GSH and GPX activity.....215

Figure 5.2: Ets-1 increases System x_c^- expression and activity. 216

Figure 5.3: The transsulfuration pathway is a major GSH source in normoxic ovarian cancer cells.....218

Figure 5.4: Ets-1 recruits Sx_c^- to maintain glutathione pool under oxidative stress. 220

Figure 5.5: Ets-1 redox regulation involves changes in HIF-1 and GPX-2 protein levels. 221

Figure 5.6: Proposed mechanism for Ets-1 mediated drug resistance in ovarian cancer.224

Figure 6.1: Overview of the role of Ets-1 in mitochondrial metabolism and oxidative stress.....246

List of Abbreviations and Symbols

2-DG	2-Deoxy-D-glucose
2-HG	2-hydroxyglutarate
α-KG	α-ketoglutarate
ANOVA	Analysis of variance
AP-1	Activator protein 1
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BCAR1	Breast cancer anti-estrogen resistance 1
CBS	Cystathionine beta synthase
ChIP	Chromatic immunoprecipitation
CO2	Carbon dioxide
CoA	Coenzyme A
CPT1	Carnitine palmitoyltransferase
DNA	Deoxyribonucleic acid
EBS	ETS binding site
ECM	Extracellular matrix
Elf-3	E74-like factor 3
EMSA	Electrophoretic mobility shift assay
ERG	V-ets erythroblastosis virus E26 oncogene homolog
ERM	ETS-related molecule
ETC	Electron transport chain
FADH2	Flavin adenine dinucleotide, hydroquinone form
FADS	Fatty acid desaturase
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FERMT2	Kindlin-2
FH	Fumarate hydratase
Fli1	Friend leukemia virus integration 1
GAB1	GRB2-associated-binding protein 1
GLS	Glutaminase
GLUT	Glucose transporter
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
H2O2	Hydrogen peroxide
HGF	Hepatocyte growth factor

HIF-1	Hypoxia-inducible factor 1
HK	Hexokinase
HRE	Hypoxia response element
HRE	Hypoxia response element
HSP-70	Heat-shock protein 70
IDH	Isocitrate dehydrogenase
Keap1	Kelch-like ECH-associated protein 1
LCP1	L-plastin
LOX	Lysyl oxidase
LOXL	Lysyl oxidase-like
LPXN	Leupaxin
MAPK	Mitogen-activated protein kinase
MMP	Metalloproteinase
mtDNA	Mitochondrial deoxyribonucleic acid
N-CoR	Nuclear receptor co-repressor
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
Nrf2	Nuclear factor (erythroid-derived)-like-2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDEF	Pointed domain containing ETS transcription factor
PEA3	Polyomavirus enhancer activator-3
PEP	Phosphoenolpyruvate
PI3K	Phosphoinositide 3-kinase
PK	Pyruvate kinase
PKC	Protein kinase C
PNT	Pointed domain
PPP	Pentose phosphate pathway
PRX	Peroxidase
PTHrP	Parathyroid hormone-related protein
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Media
rRNA	Ribosomal ribonucleic acid
SAS	Sulfasalazine
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SO	Superoxide
SOD	Superoxide dismutase
Sxc-	System x(c)-
TBS	Tris buffered saline

TBS-T	Tris buffered saline-Tween 20
TCA	Tricarboxylic acid cycle
TGF-β	Transforming growth factor β
TIMP-1	Tissue inhibitor of metalloproteinase 1
tRNA	Transfer ribonucleic acid
UBC9	Ubiquitin conjugating enzyme 9
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
wHTH	Winged helix-turn-helix

Chapter 1: Introduction

Cancer Metabolism

One of the main hallmarks of cancer cell physiology is the ability to proliferate rapidly despite poor access to nutrients and unfavourable microenvironment conditions. Cancer cells achieve such high proliferative rates through genetic mutations and coordinated alterations in metabolic pathways that ultimately favour survival and rapid growth (Figure 1). Though cancer-associated metabolic changes have been studied for nearly 90 years, we still do not fully understand the complicated interactions between different metabolic pathways, nor precisely how they are regulated in cancer cells. Additionally, reactive oxygen species are major metabolic byproducts that are integral to signal transduction, redox state balance, and cellular response to stress whose role in cancer are not fully understood, further obscuring the overall picture of metabolic changes in cancer. The staggering complexity of cancer cell metabolism renders this an extremely exciting area for research, where the potential for novel therapeutic development is extensive.

Healthy Cellular Metabolism

The conversion of nutrients into useable cellular energy sources is a necessary function to maintain cellular homeostasis. Energy-requiring processes such as gene transcription, protein translation and turnover, deoxyribonucleic acid (DNA) repair, cytoskeletal function, and numerous biochemical processes all depend on cellular metabolism for

maintenance. Arguably the most important source for cellular energy is glucose, which is metabolized through glycolysis to pyruvate, which is further metabolized by the

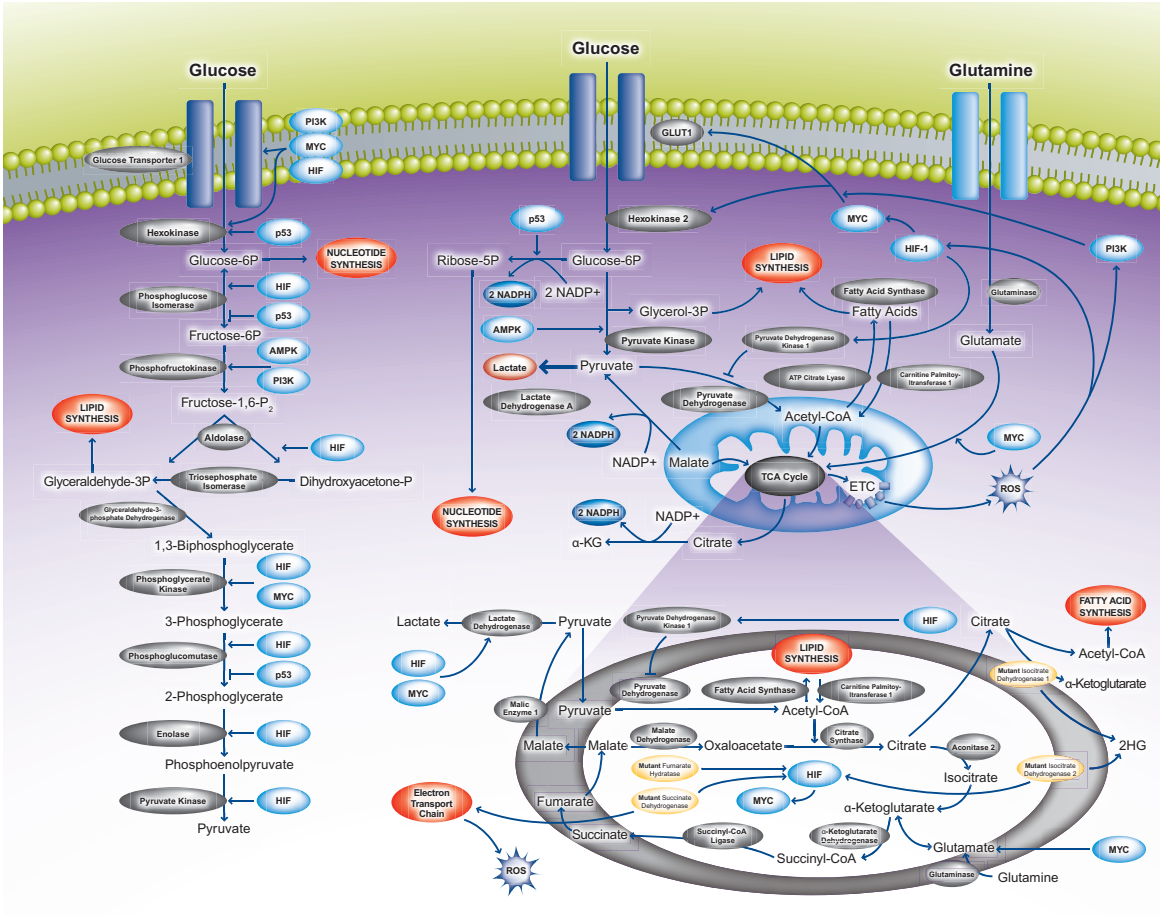

Figure 1.1: Overview of cancer metabolism.

Cancer cells display enhanced glycolytic flux while decreasing reliance on oxidative phosphorylation for energy metabolism, a characteristic known as the Warburg effect. I

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tricarboxylic acid (TCA) cycle during oxidative phosphorylation generating carbon dioxide (CO_2) and a high yield of 36 molecules of adenosine triphosphate (ATP). In addition to ATP generation, glucose is also used for a variety of metabolic processes including glycogen formation for glucose buffering, as well as nucleotide and nicotinamide adenine dinucleotide phosphate (NADPH) synthesis via the pentose phosphate pathway (PPP) (Herling, Konig, Bulik, & Holzhutter, 2011). When oxygen is limited, anaerobic glycolysis occurs where the pyruvate generated from glucose is converted into lactate, resulting in minimal ATP generation (2 mol ATP/mol glucose).

Fatty acids are another important energy source within the cell, yielding significant amounts of ATP when metabolized efficiently. Triacylglycerides, which are stored in intracellular lipid droplets, are converted to fatty acids and glycerol by lipase enzymes in the cytosol. Free-fatty acids are then coupled with coenzyme A (CoA) to generate acyl-CoA, and transported into the mitochondria via carnitine palmitoyltransferase (CPT1) (Santos & Schulze, 2012). In the mitochondria, acyl-CoA undergoes repeated oxidation and hydration reactions to eventually produce reduced nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (hydroquinone form) ($FADH_2$), and acetyl-CoA in a process called β-oxidation (Santos & Schulze,

2012). Both NADH and $FADH_2$ are used as electron donors in the electron transport chain, whereas acetyl-CoA can enter the TCA cycle to become fully oxidized and generate ATP.

In proliferating cells, growth signals stimulate a metabolic switch favouring glycolysis, regardless of oxygen availability, in order to facilitate the generation of the biocomponents necessary for making new cells(Vander Heiden, Cantley, & Thompson, 2009). This switch is at the expense of a low ATP yield, which is typically not of great consequence as proliferating cells have ample access to nutrients from the blood stream, and results in the creation and excretion of large amounts of lactate. In addition to generating ample amounts of fatty acid precursors, intermediates for amino acid synthesis, and ribose for the production of nucleotides, robust quantities of NADPH are also generated. Large amounts of NADPH are necessary for nucleotide, amino acid, and lipid biosynthesis, as well as cellular redox control, which are all important for proliferating cells (Thompson, 2009). Growth signals from factors such as phosphoinositide 3-kinase (PI3K) tightly regulate this form of metabolism in order to control the rate of cell division and ensure that excessive nutrients are not taken from the microenvironment (Vander Heiden et al., 2009). In most cancer cells, these growth signals are overactive and often mutated resulting in the uncontrolled cell proliferation that is key to cancer progression.

Metabolic Alterations in Cancer Cells

Cancer cells display a very similar metabolic profile to normal proliferating cells, although they do not rely on growth signals to facilitate aerobic glycolysis and high rates of proliferation. Nearly 90 years ago, Otto Warburg observed that cancer cells consume large quantities of glucose while concomitantly excreting high amounts of lactate, even in the presence of ample oxygen (Warburg, Wind, & Negelein, 1927). Termed the Warburg Effect, the aerobic glycolysis displayed in cancer cells but not in normal cells was assumed to be due to defective mitochondrial oxidative phosphorylation. However, in recent years several key studies have definitively shown that most cancer cells have completely functional mitochondria (Fantin, St-Pierre, & Leder, 2006; Moreno-Sanchez, Rodriguez-Enriquez, Marin-Hernandez, & Saavedra, 2007; Zu & Guppy, 2004). It is now evident that the Warburg Effect is associated with the activation of specific oncogenes, the loss of tumour suppressor expression, and the mutation of key enzymes in an elaborate gene expression network, the details of which have yet to be fully elucidated (Cairns, Harris, & Mak, 2011; Koppenol, Bounds, & Dang, 2011; Levine & Puzio-Kuter, 2010; Vander Heiden et al., 2009).

Glucose Metabolism: Cancer-associated Enzyme Expression and Regulation

Glucose import into cells is mainly facilitated by five transmembrane glucose transporters (GLUT), of which GLUT1 and GLUT3 are expressed ubiquitously, while GLUT2,

GLUT4, and GLUT5 expression is tissue-specific (Lunt & Vander Heiden, 2011). Both GLUT1 and GLUT3 are frequently overexpressed in a variety of cancer types (Au et al., 1997; Binder, Binder, Marx, Schauer, & Hiddemann, 1997; Medina & Owen, 2002; T. A. Smith, 1999; T. Suzuki et al., 1999; Yamamoto et al., 1990; Younes, Lechago, Somoano, Mosharaf, & Lechago, 1996), and GLUT1 in particular is associated with invasive, metastatic tumours (Grover-McKay, Walsh, Seftor, Thomas, & Hendrix, 1998; Oliver et al., 2004; Tateishi et al., 2006). Regulation of these transporters is mediated by hypoxia, where acute hypoxia leads to increased transporter availability, and prolonged chronic hypoxia leads to increased *GLUT* gene transcription facilitated by the binding of hypoxia-inducible factor 1α (HIF-1α) to hypoxia response elements (HRE) within the gene promoter (Behrooz & Ismail-Beigi, 1999; C. Chen, Pore, Behrooz, Ismail-Beigi, & Maity, 2001; J. Z. Zhang, Behrooz, & Ismail-Beigi, 1999).

Various studies have shown that nearly every enzyme in the glycolysis and oxidative phosphorylation pathways has been altered in either expression, or regulation in cancer cells (Zhao et al., 2011). For the sake of brevity, only the most prolific changes will be reviewed in this thesis.

The glycolytic flux of cancer cells is predominantly controlled by the activity of GLUT transporters, and the enzyme hexokinase (HK), which is also deregulated in cancer cells. HK enzymes are responsible for the conversion of glucose to glucose-6-phosphate, a reaction that traps glucose within the cell for further metabolic processing (Lunt & Vander Heiden, 2011). The predominant isoform of HK expressed in normal tissues is HK1, however, in cancer cells HK2 is the main isoform and is extremely overexpressed

(Mathupala, Ko, & Pedersen, 2006, 2009; Wolf et al., 2011). In a process mediated by Akt signaling, HK2 associates with the voltage-dependent anion channel (VDAC) on the outer mitochondrial membrane in order to garner preferential access to mitochondrial-generated ATP (Arora & Pedersen, 1988; Pastorino, Shulga, & Hoek, 2002). However, the degree to which the HK2-VDAC association is coupled with ATP generated from oxidative phosphorylation is unclear, especially considering the coupling has mainly been investigated *in vitro* (Herling et al., 2011).

Pyruvate kinase (PK) is another glycolytic enzyme with a predominant isoform expressed in cancer cells that is not commonly expressed in normal cells. There are four main forms of PK in humans: M1, M2, L, and R (Zhao et al., 2011). PKM1 is expressed in normal tissues and is a highly active form of the enzyme that catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate as the final step of glycolysis. PKM2, which is normally expressed during embryonic development, is expressed in proliferating cells and particularly overexpressed in tumour cells (Christofk, Vander Heiden, Harris, et al., 2008; Hacker, Steinberg, & Bannasch, 1998; Zhao et al., 2011). Unlike the other forms of PK, PKM2 is regulated by tyrosine-phosphorylated protein growth signals that cause the enzyme to breakdown from the active tetrameric form to the less active dimerized enzyme (Christofk, Vander Heiden, Wu, Asara, & Cantley, 2008; Vander Heiden et al., 2009). Since cancer cells display chronically active growth signaling, the low activity of PKM2 results in an accumulation of the upstream substrates in the glycolytic pathway, leading to the shuttling of these substrates to synthetic pathways such as the PPP. An important recent study by Christofk *et al* illustrates how important PKM2 is to the glycolytic shift in

cancer cells (Christofk, Vander Heiden, Harris, et al., 2008). In this report, a variety of cancer cell lines depleted of PKM2 expression were observed to have decreased glucose metabolism and proliferation, and these changes were rescued following PKM1 addition. They also conducted animal studies where the tumourigenicity of lung cancer cells was significantly decreased in PKM1-expressing tumours compared to mice expressing the PKM2 isoform. The aforementioned studies suggest that PKM2 may be an excellent target for therapeutic intervention.

Germline mutations to essential TCA cycle enzymes are common in several types of cancer, particularly succinate dehydrogenase (SDH), fumarate hydratase (FH), and isocitrate dehydrogenase (IDH) (Baysal et al., 2000; Bleeker et al., 2009; Parsons et al., 2008; Pollard, Wortham, & Tomlinson, 2003; Reitman & Yan, 2010; Yan et al., 2009). The inhibition of each of these enzymes leads to the stabilization of HIF-1, which in turn leads to increased glucose utilization (Selak et al., 2005; Zhao et al., 2011). In many human brain cancers, the mutant IDH enzyme gains the additional function of reducing α-ketoglutarate (α-KG) to 2-hydroxyglutarate (2-HG), an oncometabolite that results in increased carcinogenesis (Dang et al., 2009). The functional significance of 2-HG production has yet to be clearly defined, however it has been theorized that high amounts of 2-HG may cause hypoxic-like conditions within the cell (Herling et al., 2011).

Glutamine

Though glucose is arguably the most important nutrient for cancer cells, they are also dependent on glutamine for progression and growth. Glutamine is a conditionally essential amino acid that makes up a significant portion of the free fatty acid pool in various tissues. It is referred to as conditionally essential because cells are able to synthesize glutamine, however under conditions of rapid growth the demand for this nutrient becomes too great for the synthetic capacity of the cell (DeBerardinis & Cheng, 2010). Glutamine metabolism, referred to as glutaminolysis, is important for energy production, macromolecule synthesis, and redox balance in cancer cells, in addition to being important to several signal transduction pathways (Daye & Wellen, 2012). The role of glutamine in mitochondrial metabolism explains why cancer cells are addicted to this amino acid as an energy source.

As described previously, the glucose utilization in cancer cells is focused to glycolysis and biosynthetic pathways, largely bypassing oxidative pathways and generating little ATP. Additionally, the acetyl-CoA generated from the TCA cycle is predominantly used to fuel lipid synthesis, further leading to loss of TCA cycle intermediates. Glutamine is imported into the mitochondria, converted to glutamate by the enzyme glutaminase (GLS), and glutamate is subsequently converted to α-KG. In support of this concept, upon glutamine withdrawal cancer cells begin to die, whereby cell viability is returned following supplementation with α-KG and other TCA cycle intermediates (Gao et al., 2009; Wise et al., 2008; Yuneva, Zamboni, Oefner,

Sachidanandam, & Lazebnik, 2007). Recently, it was discovered that glutamine-derived α-KG also undergoes reductive carboxylation, where the TCA cycle reactions are reversed to generate isocitrate, which is then converted to citrate and used to fuel lipid synthesis (Metallo et al., 2012; Mullen et al., 2012; Wise et al., 2011). The utilization of glutamine as a main energetic source in cancer cells is largely driven by the oncogenic transcription factor c-Myc (Gao et al., 2009). Additionally, Myc-driven cancer cells are also dependent on glutamine intake for glutathione synthesis instead of glucose-derived carbon from glycolysis, highlighting the importance of glutamine in redox balance in cancer cells (Daye & Wellen, 2012).

Role of Mitochondria in Cancer

As the mitochondria are the central organelles involved in cellular metabolism, their role in tumourigenesis is predominantly centered on the Warburg effect and other alterations in metabolism. However, with recent advances in genomics and proteomics it is evident that the importance of mitochondria in cancer extends to the mitochondrial genome and proteome, and their crosstalk with the nucleus (Figure 2). The following sections discussing mitochondrial genomics and proteomics are excerpts from my review paper entitled "Mitochondria and Cancer: Past, Present, and Future" published in *BioMed Research International* January 2013 (Verschoor et al., 2013).

Genomics of mitochondrial DNA: mutations and polymorphisms

The mitochondrial genome is unique from that of the nucleus, containing 16,569 base pairs and organized in a double stranded circular structure (Anderson et al., 1981). The circular mitochondrial DNA (mtDNA) encodes 37 genes including several components of the electron transport chain (ETC), transfer ribonucleic acids (tRNA), and ribosomal RNAs (rRNA). Additionally, mtDNA contains a non-coding region comprised of three hypervariable regions and a displacement (D) loop, which is the location of the origin of replication and transcriptional promoters. Mutations in mtDNA are frequently observed in cancer, likely due to the lack of introns, lack of histone protection, and close proximity to damaging reactive oxygen species (ROS).

Each cell contains multiple copies of mitochondrial genes, giving rise to mitochondrial homoplasmy, where all the mitochondria of a cell have the same genomic composition, or heteroplasmy, where wild-type and mutant mtDNA coexist (Chatterjee, Dasgupta, & Sidransky, 2011). Thus is it possible for a mutation that confers a distinct advantage for cancer cells, such as accelerated growth or enhanced survival, to be clonally expanded to become a homoplasmic mutant and to predominate within a population of cancer cells. Alternatively, Coller and colleagues (Coller et al., 2001) used a mathematical model to show that random segregation of mitochondrial genomes during rapid tumour development could result in a mutant homoplasmic population without the need for a selective advantage. Regardless of the existence of background homoplasmic

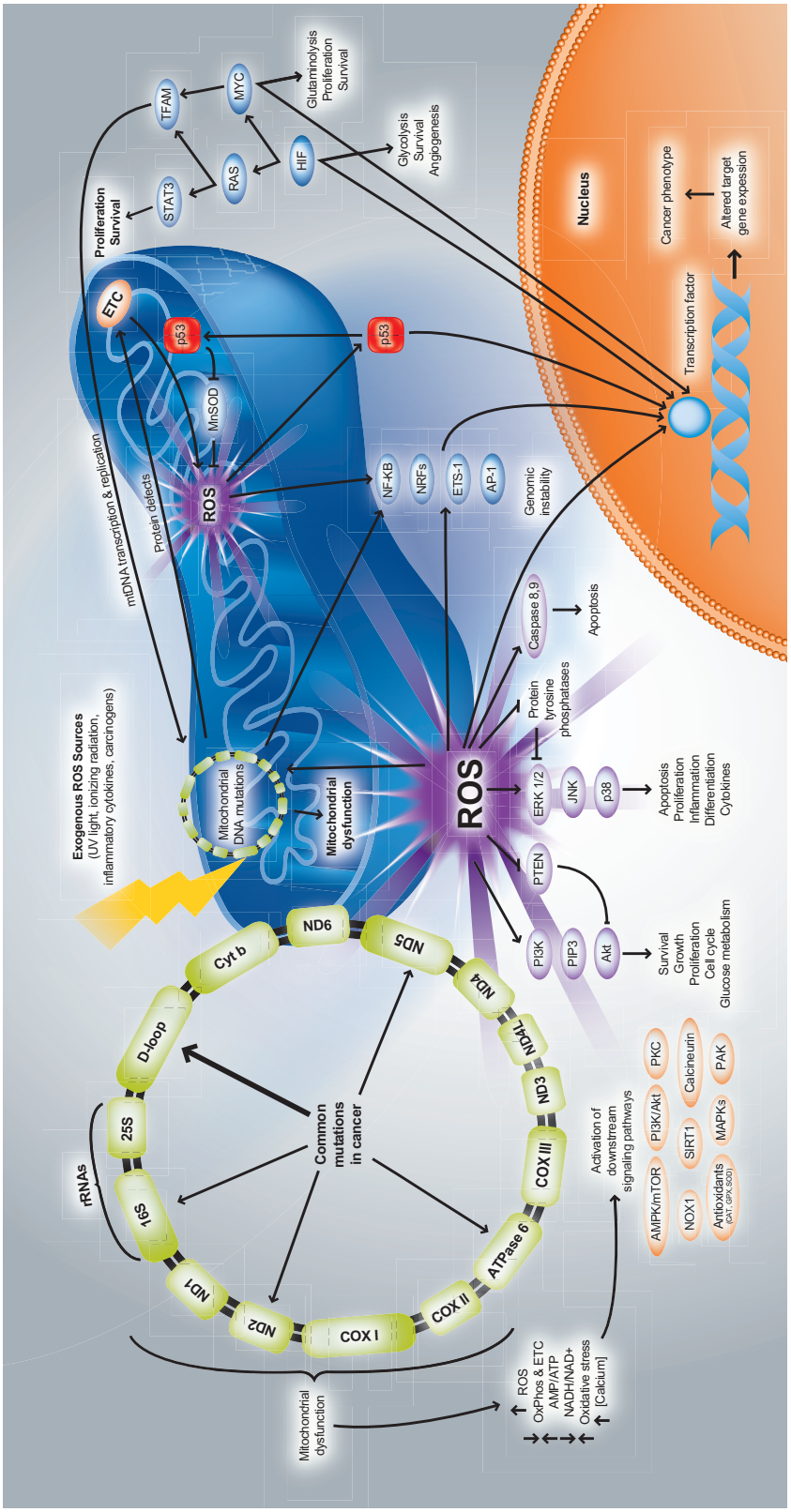
Exogenous ROS Sources
(UV light, ionizing radiation, inflammatory cytokines, carcinogens)
Mitochondrial DNA mutations
Mitochondrial dysfunction
ROS
Protein defects
mtDNA transcription & replication
ETC
p53
MnSOD
TFAM
STAT3
Proliferation Survival
RAS
MYC
HIF
Glutaminolysis Proliferation Survival
Glycolysis Survival Angiogenesis
NF-KB
NRFs
ETS-1
AP-1
Genomic instability
Nucleus
Transcription factor
Cancer phenotype
Altered target gene expession
Caspase 8,9
Apoptosis
Protein tyrosine phosphatases
ERK 1/2
JNK
p38
Apoptosis Proliferation Inflammation Differentiation Cytokines
PTEN
PI3K
PIP3
Akt
Survival Growth Proliferation Cell cycle Glucose metabolism
rRNAs
16S
25S
D-loop
Cyt b
ND6
ND5
ND4
ND4L
ND3
COX III
ATPase 6
COX II
COX I
ND2
ND1
Common mutations in cancer
Activation of downstream signaling pathways
AMPK/mTOR
PI3K/Akt
PKC
NOX1
SIRT1
Calcineurin
Antioxidants (CAT, GPX, SOD)
MAPKs
PAK
Mitochondrial dysfunction
ROS
OxPhos & ETC
AMP/ATP
NADH/NAD+
Oxidative stress
[Calcium]

Figure 1.2: Overview of the role of mitochondrial alterations in cancer.

Mutations in the mitochondria cause by exogenous sources of ROS lead to mitochondrial dysfunction and ROS production. Various signaling pathways are induced by ROS ultimately resulting in cancer development. I produced this diagram as resource material for Abcam Inc., UK, and have reproduced it here with permission from the publisher via personal communication.

mutations that confer no functional consequence, there are numerous mtDNA mutations that result in significant alterations in mitochondrial function that affect tumour development and progression.

Recent advances in high-throughput technologies, such as next-gen sequencing and Mitochip (Maitra et al., 2004), have allowed for the rapid and accurate detection of mtDNA mutations, polymorphisms or copy number variations in a variety of tissues and bodily fluids (Castle et al., 2010; Dasgupta et al., 2010; Fendt et al., 2011; Fliss et al., 2000; Hosgood et al., 2010; Nomoto, Yamashita, Koshikawa, Nakao, & Sidransky, 2002). In certain cancer tissues mtDNA mutations were more readily detectable and abundant than mutated nuclear p53 DNA, suggesting that mtDNA mutations could serve as excellent cancer biomarkers, particularly for early detection (Chatterjee et al., 2011).

The most commonly mutated or deleted region of mtDNA in cancer is within the D loop at the D310 tract, a mononucleotide cytidine repeat at position 310 (Sanchez-Cespedes et al., 2001). The D loop is involved in mitochondrial replication, thus

mutations in this region could also affect mtDNA copy number, though this theory has yet to be proven empirically. In one study, colorectal cancer patients with D loop mutations were found to have significantly lower overall survival rates and increased chemotherapeutic resistance compared to patients who's mtDNA did not harbour such mutations (Lievre et al., 2005). The high frequency of D loop deletion or insertion somatic mutations in cancer render these mutations unlikely to confer any functional impairment to mitochondria, and so the uncertain functional consequences of these mutations should remain an important area for mitochondrial research in cancer.

The importance of mitochondrial polymorphisms in cancer development and risk is intimately related to evolutionary haplogroups, and has recently been a contentious area of research. Haplogroups are characterized by a specific mutation that occurs widely within individuals of a particular population, and are further divided into haplotypes generally based on restriction fragment length polymorphisms (Singh & Kulawiec, 2009). Among the main European haplotypes, the A12308G mutation in $tRNA^{Leu2}$ common to haplotype U was associated with increased risk of both renal and prostate cancers (Booker et al., 2006). The NADH-ubiquinone oxioreductase chain 3 (ND3) substitution mutation at G10398A has been associated with increased breast cancer risk in both African American and Indian women (Canter, Kallianpur, Parl, & Millikan, 2005; Darvishi, Sharma, Bhat, Rai, & Bamezai, 2007; Kulawiec, Owens, & Singh, 2009; Mims et al., 2006; Setiawan et al., 2008). In European-American women the A10398G ND3 substitution conferred increased risk of breast cancer, as did the T16519C D loop polymorphism (Bai, Leal, Covarrubias, Liu, & Wong, 2007). A comprehensive study of

pancreatic cancer risk revealed associations with the A331T substitution in mitochondrial ND2 (Lam et al., 2012).

Despite these promising findings, because the majority of mtDNA polymorphisms are functionally inconsequential, associations with specific polymorphisms and cancer risk have been subject to heated debate. Several older studies involving association of specific polymorphisms with cancer risk have been heavily scrutinized due to erroneous experimental design, interpretation, and poor data quality (Chatterjee et al., 2011). However, due to the potential usefulness of somatic mtDNA mutational profiling as a diagnostic tool, the study of mitochondrial somatic mutations and associations with cancer should remain an important focus of cancer biomarker research pending proper study design, population stratification, and independent replication of results.

Mitochondrial proteomics

The majority of mitochondrial proteins are encoded by the nuclear genome and imported to the mitochondria to perform their specific functions. Thus the mitochondrial proteome is the result of complex crosstalk between both nuclear and mitochondrial programs, and is greatly influenced by pathological conditions including cancer. In the past decade, the mitochondrial proteome has been characterized from highly purified mitochondria resulting in a comprehensive list of over 1,000 mitochondrial proteins (as reviewed in (Bottoni, Giardina, Pontoglio, Scara, & Scatena, 2012)).

Using the wealth of knowledge from such studies, numerous databases have been created such as MitoInteractome (Reja et al., 2009), MitoP2 (Elstner, Andreoli, Klopstock, Meitinger, & Prokisch, 2009), HMPDb, and MitoMiner (A. C. Smith, Blackshaw, & Robinson, 2012). The MitoInteractome database contains 6,549 protein sequences derived from multiple databases (SwissProt, MitoP, MitoProteome, HPRD, GO) from several different species creating a comprehensive protein-protein interaction network. Certainly one of the most extensive databases, MitoP2 contains data from a wide breadth of mitochondrial proteomic studies spanning from single protein studies to extensive proteome-wide mapping and expression studies. The HMPDb (Human Mitochondrial Protein Database) provides consolidated information on mitochondrial DNA sequences, polymorphisms, disease-related proteins, and 3-D mitochondrial protein structures. Collectively these databases serve as wonderful utilities for the discovery and characterization of novel mitochondrial biomarkers for diagnosis and molecular targets for drug treatments.

Extensive protein expression differences have been found in mitochondrial glycolytic enzymes, heat-shock proteins, cytoskeleton proteins, and antioxidant enzymes through comparative proteomic analysis. In regards to metabolism, proteins of the glycolytic and pentose phosphate pathways tend to be induced, along with reductions in oxidative phosphorylation pathways (Bottoni et al., 2012; Scatena, 2012). Recently, Chen et al. (2011) performed 2D-DIGE and MALDI-TOF mass spectrometry to compare the proteomic profile of purified mitochondria from normal breast cells (MCF10A), non-invasive breast cancer cells (MCF7), and invasive breast cancer cells (MDA-MB-231) (Y.

W. Chen et al., 2011). The most differentially expressed mitochondrial proteins between normal and cancerous cells included cytochrome oxidase subunit 5B, malate dehydrogenase, and elongation factor Tu. Several proteomic studies have shown a significant correlation between high levels of heat-shock protein 70 (HSP-70) in a variety of cancers including gastric adenocarcinoma, hepatocarcinoma, and esophageal cancer (Bottoni et al., 2012). HSP-70 functions as a mediator of cell proliferation, cellular senescence, and cellular immortalization, and when concentrated in to cytoplasm sequesters p53 and activates Ras-Raf signaling which controls cell proliferation (Wadhwa et al., 2002; Wadhwa, Yaguchi, Hasan, Taira, & Kaul, 2003).

Oxidative Stress in Cancer

Oxidative stress refers to an imbalance in the production of ROS and the system's ability to detoxify these reactive intermediates and repair the resulting damage they cause. ROS are capable of damaging all cellular components, including nucleic acids, proteins, and lipids, leading to cell death when present at high levels (Poli, Leonarduzzi, Biasi, & Chiarpotto, 2004). However, ROS have many beneficial roles including involvement in the defense against pathogen infection, mitogenic response, cellular stress response, and are integral to several signaling pathways. In the context of cancer, oxidative damage to nucleic acids is recognized as a first step in the mutagenesis that initiates carcinogenesis (Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006).

ROS can be produced through exogenous means, including exposure to UV light, X-rays, or radiation, and atmospheric pollutants. Endogenous ROS production occurs through metal-catalyzed reactions, from immune cells during inflammation, and from normal metabolism (Gupta-Elera, Garrett, Robison, & O'Neill, 2012; Valko et al., 2006). The predominant ROS in cancer cells is hydrogen peroxide (H_2O_2) generated by the mitochondria as a necessary byproduct of oxidative phosphorylation (Hyoudou et al., 2009; Petit et al., 2009; Szatrowski & Nathan, 1991). The mechanisms associated with mitochondrial-generated ROS in cancer will be discussed in great detail in Chapter 4. Thus a review of the role of cellular antioxidant systems in cancer, the other side of the oxidative stress axis, will be discussed further here.

Major Cellular Antioxidant Systems

Due to the diverse, important and sometimes deleterious effects of ROS, cells must have an effective means of detoxifying these molecules. Antioxidants are factors that inhibit the oxidation of other molecules, and therefore are capable of reducing oxidative damage caused by ROS. Exogenous antioxidants such as carotenoids, tocopherols, and ascorbic acid are consumed in the diet, whereas endogenous antioxidant enzymes include thiols, superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (Crawford et al., 2012). This interacting network of antioxidants protect cells against oxidative stress by converting the superoxide (SO) produced form oxidative metabolism to H_2O_2, then

further reducing H_2O_2 into water. SOD enzymes perform the first step in this antioxidant cascade, whereas catalase and peroxidases are responsible for H_2O_2 reduction.

SOD enzymes are a family of three mammalian members, including CuZn-SOD (SOD1) present in the cytoplasm, Mn-SOD (SOD2) found exclusively in the mitochondria, and EC-SOD (SOD3) found in extracellular spaces (Zelko, Mariani, & Folz, 2002). It is likely that the mitochondrial SOD2 is the most important to normal physiology, as mice that lack this enzyme develop severe neuropathy and die shortly after birth (Melov et al., 1998). In contrast, mice deficient in cytoplasmic SOD1 develop normally with only a vulnerability to neuron loss following injury (Reaume et al., 1996). Catalases also require metal cofactors in the form of either iron or manganese, and are localized to peroxisomes within the cell (del Rio, Sandalio, Palma, Bueno, & Corpas, 1992). Though catalases specifically reduce H_2O_2, thereby rendering these enzymes essential for H_2O_2 detoxification, humans with acatalasemia that lack catalase in the blood and other tissues are often asymptomatic, or merely suffer ulceration of oral issues (Ogata, 1991).

Peroxiredoxins are a family of antioxidant enzymes responsible for the detoxification of H_2O_2 produced from cytokine induction (Rhee, Chae, & Kim, 2005). There are six mammalian peroxidase enzymes (PRX1-6), all of which contain a conserved cysteine that is oxidized during H_2O_2 reduction. Peroxiredoxins are relatively abundant in mammalian cells, though PRX1 and PRX2 seem to be the most important physiologically as evidenced by *in vivo* knockout studies (Muller, Lustgarten, Jang, Richardson, & Van Remmen, 2007; Neumann et al., 2003; Rhee, Kang, et al., 2005).

Involved in antioxidant defense and redox signaling, thioredoxin and thioredoxin reductase are ubiquitous enzymes necessary for mammalian physiology (Nordberg & Arner, 2001). Not unlike the peroxiredoxins, thioredoxin is dependent on two cysteine residues in order to reduce other substrates, while thioredoxin reductase maintains the reduced state of thioredoxin in an NADPH-dependent reaction (Mustacich & Powis, 2000; Nordberg & Arner, 2001). Transgenic mice that overexpress thioredoxin are significantly more resistant to oxidative stress and display a longer life span than normal mice, illustrating the importance of this system in redox regulation (Yoshida, Nakamura, Masutani, & Yodoi, 2005).

The glutathione antioxidant system is critically important to a variety of cellular processes, mostly notably cellular redox status regulation, and consists of selenium-dependent GPX, glutathione reductase (GR), and selenium-independent glutathione S-transferase (GST) enzymes (Ballatori et al., 2009). Cellular glutathione levels are dependent on cysteine availability (Figure 3), and the redox status of glutathione serves as an indicator of oxidative stress. Reduced glutathione (GSH) is oxidized to glutathione disulfide (GSSG) through the action of GPX enzymes, which catalyze the reduction of H_2O_2 to water, and then returns to a reduced state via GR in an NADPH-dependent reaction. GST enzymes are responsible for the conjugation of compounds with electrophilic centers to GSH (Oakley, 2011). Under normal conditions, nearly all of the total intracellular glutathione pool is in the form of GSH, thus an increase in the ratio of GSH/GSSG is indicative of oxidative stress. In addition to being the predominant cellular

antioxidant, glutathione also plays a role in the nitric oxide cycle (Chiueh & Rauhala, 1999), and iron metabolism (Kumar et al., 2011).

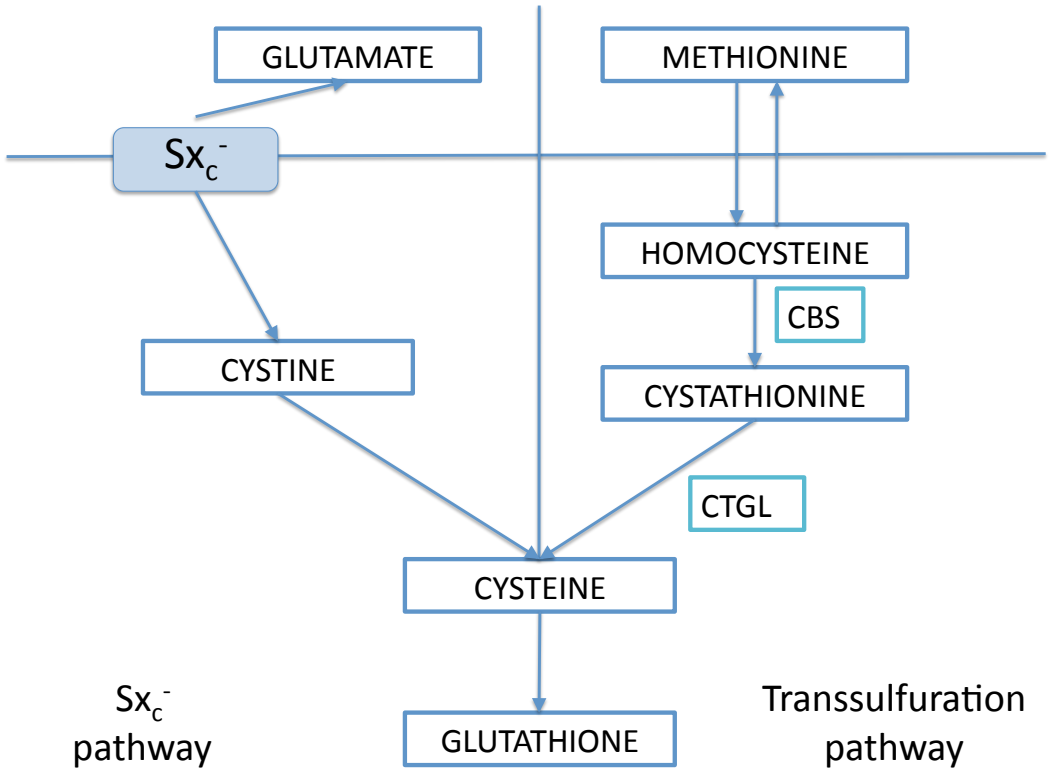

Figure 1.3: Major sources of cysteine for glutathione synthesis.

The availability of intracellular cysteine is the rate-limiting step for glutathione synthesis. Cysteine levels are determined mainly by the activities of the transsulfuration pathway and the membrane antiporter Sx_c^-, as detailed in this diagram.

Enzymatic Antioxidant Alterations in Cancer Cells

The expression and activity of most antioxidant enzymes are altered in at least one form of cancer, and several cancer-specific single nucleotide polymorphisms (SNPs) have been identified in antioxidant enzymes (Crawford et al., 2012). However, the impacts of these changes are not well understood. Interestingly, decreases in antioxidant enzymes leads to increased mutagenesis and cancer initiation, and increase proliferation in later stages of cancer, however, increased antioxidant levels leads to improved survival in cancer cells. Thus, the role and consequence of antioxidant expression and activity in cancer is highly context-dependent, and most likely varies for different cancer types as well. SOD2 is a very effective anti-tumour agent, and the overexpression of this antioxidant leads to decreased tumour growth (Behrend, Henderson, & Zwacka, 2003). However, high levels of SOD2 are also associated with poor prognosis and invasive tumours, likely due to the observation that SOD2 leads to matrix metalloproteinase (MMP) activation (Y. Jiang, Goldberg, & Shi, 2002). The role of GPX enzymes in cancer development is equally blurry, where many cancers display decreased GPX levels that lead to increased tumour growth (Cullen, Mitros, & Oberley, 2003; Gladyshev, Factor, Housseau, & Hatfield, 1998). However, the overexpression of GPX1 has been found to improve cancer cell survival, and even increase the growth of some cancer cell lines (Kim et al., 2009; J. Liu et al., 2004; Lu et al., 1997). Clearly, the specific roles of antioxidant enzymes in cancer development and progression are dependent on the overall redox state of the tumour cell and the consequences of the resulting stress responses.

The master regulator of cellular redox state is arguably the transcription factor nuclear factor (erythroid-derived)-like-2 (Nrf2), which initiates the antioxidant response pathway primarily responsible for cellular defense against oxidative stress. Normally sequestered in the cytoplasm and degraded by Kelch-like ECH-associated protein 1 (Keap1) ubiquination, under oxidative stress Nrf2 is stabilized and translocates to the nucleus to alter target gene transcription (Itoh et al., 1999). Nrf2 prominently regulates the expression of antioxidant enzymes, but is also involved in the regulation of NADPH levels, heat-shock proteins, and growth factors (Hayes & McMahon, 2009). The expression of Nrf2 is notably increased in cancers, and mutations that cause permanent stabilization of this factor are frequent in lung, head and neck, and gall bladder cancers (Hayes & McMahon, 2009). A recent landmark study found that Nrf2 was regulated by the oncogenes *K-Ras*, *Braf*, and *c-Myc* in pancreatic cancer, leading to altered ROS levels during early cancer development (DeNicola et al., 2011). Additionally, this study also showed that early-stage tumours deficient in Nrf2 displayed increased ROS levels and decreased growth rates, an effect that was abrogated by antioxidant treatment. Therefore, the oxidative stress response is active during early tumour development, and resulting tumourigenesis is potentially coupled with Nrf2 stabilization.

The Ets-1 Transcription Factor

Structure and Function of Ets-1

Ets-1 is a member of the ETS family of transcription factors, and was first characterized as a proto-oncogene of the retroviral *v-ets* oncogene in avian leukaemia retrovirus E26 (Leprince et al., 1983). This family of transcription factors is currently comprised of 28 members, many of which are known to be elevated in various cancers (T. Oikawa & Yamada, 2003; Seth & Watson, 2005) including Ets-2 (Baldus et al., 2004; Foos, Garcia-Ramirez, Galang, & Hauser, 1998; Foos & Hauser, 2000; Santoro et al., 1992; Sapi, Flick, Rodov, & Kacinski, 1998; Sementchenko, Schweinfest, Papas, & Watson, 1998), friend leukemia virus integration 1 (Fli1) (Poppe et al., 2004), v-ets erythroblastosis virus E26 oncogene homolog (ERG) (Baldus et al., 2004), polyomavirus enhancer activator-3 (PEA3) (Kurpios, Sabolic, Shepherd, Fidalgo, & Hassell, 2003; Shepherd, Kockeritz, Szrajber, Muller, & Hassell, 2001), ETS-related molecule (ERM) (Kurpios et al., 2003), pointed domain containing ETS transcription factor (PDEF) (Ghadersohi & Sood, 2001), and E74-like factor 3 (Elf-3) (Kurpios et al., 2003).

All known ETS family members contain a core double-stranded DNA binding element that recognizes the consensus sequence GGAA/T (Dittmer, 2003; Graves & Petersen, 1998). DNA binding facilitates their function as either transcription factors or as regulatory proteins that control the initiation of target gene transcription (Dittmer, 2003; Graves & Petersen, 1998). The ETS binding site (EBS) is a winged helix-turn-helix (wHTH) fold that binds target DNA as a monomer (Seth & Watson, 2005; Verger & Duterque-Coquillaud, 2002). The ETS domain itself contains 85 amino acids, including three α-helix wHTH regions and a four-stranded β-sheet, where the recognition site of the α-3 helix makes groove contacts with the two guanines in the EBS (Seth & Watson, 2005;

Verger & Duterque-Coquillaud, 2002). Additionally, a smaller pointed domain (PNT) containing 65-85 amino acids can impact the function of protein-protein interactions and oligomerisation in certain ETS family members, including Ets-1 (Seth & Watson, 2005). Ets-1 and Ets-2 contain an activation domain before the PNT, and auto-inhibitory domains flanking the ETS domain (T. Oikawa & Yamada, 2003). The auto-inhibitory domains ensure correct ETS binding, as the core EBS is highly conserved and generic the binding of correct ETS family members and binding partners cover these domains allowing for DNA binding and thus transcriptional activation (Seth & Watson, 2005).

Regulation of Ets-1 Expression

ETS domain activation may also be controlled by post-transcriptional modifications or protein-protein interactions. For example, Ets-1 binding activity is enhanced following acetylation through specific signaling pathways such as Ets-1 acetylation by transforming growth factor β (TGF-β) signaling (Czuwara-Ladykowska, Sementchenko, Watson, & Trojanowska, 2002). Although not confirmed, sumoylation could also affect Ets-1 binding activity as Ets-1 interacts with the sumoylation factor ubiquitin conjugating enzyme 9 (UBC9) (Seth & Watson, 2005). However, the most significant effectors of Ets-1 activity are the interaction between other proteins, especially other transcription factors.

Transcription factor binding adjacent to EBS sites typically results in increased binding affinity, and synergistic activation or repression of gene targets (Seth & Watson, 2005). Physical interaction between the activator protein 1 (AP-1) subunits Jun and Fos,

and ETS proteins has been studied well, whereby tyrosine residues in the α-3 helix (Y395 in Ets-1) interact directly with the N-terminal basic domain of Jun (Dittmer, 2003; Verger & Duterque-Coquillaud, 2002; Wood, Irvin, Nucifora, Luce, & Hiebert, 2003). Specific targets of AP-1/EBS interaction include MMP-1, urokinase plasminogen activator (uPA), granulocyte macrophage colony-stimulating factor (GM-CSF), maspin, and tissue inhibitor of metalloproteinases 1 (TIMP-1), each important genes in the context of cancer (Seth & Watson, 2005).

In invasive breast cancer cells, Ets-1 and Ets-2 activation of parathyroid hormone-related protein (PTHrP), which promotes survival, metastasis and proliferation, can be initiated by protein kinase C (PKC) phosphorylation (Lindemann, Braig, Ballschmieter, et al., 2003; Lindemann, Braig, Hauser, Nordheim, & Dittmer, 2003). While mitogen-activated protein kinase (MAPK), which is involved in proliferation, survival and differentiation in cancer, can bind near the PNT domain of Ets-1 to increase transcriptional activity (Seth & Watson, 2005). Nuclear receptor co-repressor (N-CoR) interacts with Ets-1 in breast cancer and is a positive prognostic indicator, where decreased N-CoR is associated with increased malignancy and decreased hormone responsiveness (Girault et al., 2003). Additionally, the expression of Ets-1 was associated with reduced disease-free survival further suggesting an important role for Ets-1 in the progression and therapeutic resistance in cancer, particularly breast cancer.

Ets-1 Overexpression in Cancer

Ets-1 overexpression appears to correlate specifically with more advanced, invasive tumours in breast and ovarian carcinomas (Buggy et al., 2004; Davidson, Reich, et al., 2001; Davidson, Risberg, et al., 2001; Fujimoto et al., 2004; Katayama, Nakayama, Ito, Naito, & Sekine, 2005; Span et al., 2002). Increasing evidence indicates that a correlation between Ets-1 expression and tumour progression is a general phenomenon as observed in prostate, gastric, lymphoma, oral and thyroid cancers, as well as in melanoma and meningioma. Moreover, Ets-1 itself is thought to be involved in oncogenesis due to its ability to transform NIH3T3 cells, and to promote the transformed phenotype of a human epithelial tumour cell line (HeLa cells)(Hahne et al., 2005; Seth & Papas, 1990). In the latter study, HeLa cells with increased Ets-1 expression were characterized by enhanced migration, invasion, and anchorage-independent growth (Hahne et al., 2005).

The link between Ets-1 and cancer can further be explained by the increasing list of known target genes regulated by this transcription factor. Several MMP genes, uPA, and integrin β3, which are known mediators of extracellular matrix degradation and cell migration, are all targets for Ets-1 (Lefter et al., 2009; Okuducu, Zils, Michaelis, Michaelides, & von Deimling, 2006; Park, Jung, Ahn, & Im, 2008; Rothhammer et al., 2004). The normal expression of Ets-1 in lymphoid and vascular tissues as well as data obtained from a targeted Ets-1 knockout, suggest that target genes may be generally grouped in two functional groups, lymphoid cell development and angiogenesis (Bories et al., 1995; Naito et al., 1998). There is a growing list of lymphoid tissue-related gene products regulated by Ets-1 which include T cell receptors, cytokines, and cytokine receptors (Aringer et al., 2003; Sementchenko & Watson, 2000). Thus it is apparent that

the products of several Ets-1-regulated genes are generally involved in the degradation of the extracellular matrix, the promotion of migration, and angiogenesis, thus suggesting key roles in mediating tumour progression (Lefter et al., 2009; Okuducu et al., 2006; Park et al., 2008; Rothhammer et al., 2004; Sementchenko & Watson, 2000).

Ets-1 is widely expressed by tumour cells, endothelial cells, and tumour-associated fibroblasts where it contributes predominantly to tumour angiogenesis and cancer cell invasion (Adam, Schmidt, Wardelmann, Wernert, & Albers, 2003; Behrens et al., 2003; Behrens, Rothe, Wellmann, Krischler, & Wernert, 2001; Bolon et al., 1995; Fujimoto et al., 2004; Hahne et al., 2006; Hahne et al., 2005; Hahne et al., 2008; Khatun, Fujimoto, Toyoki, & Tamaya, 2003; Lefter et al., 2009; Nakada, Yamashita, Okada, & Sato, 1999; Okuducu et al., 2006; Park et al., 2008; Rothhammer et al., 2004; Sahin et al., 2005; Sahin et al., 2009; Sakaguchi et al., 2004; Shimizu et al., 2004; Watabe et al., 1998; Wernert et al., 1999; Yasuda et al., 1999; Zhan et al., 2005). Metastasis remains one of the most pathologically important processes in cancer, and involves the stromal compartment surrounding the tumour containing fibroblasts, endothelial cells, immune cells and infiltrating blood cells, which contribute significantly to tumour progression (Bissell, Radisky, Rizki, Weaver, & Petersen, 2002; Holliday, Brouilette, Markert, Gordon, & Jones, 2009; Seth & Watson, 2005). The primary processes involved in the development of metastatic potential are growth via angiogenesis, invasion via proteolytic extracellular matrix (ECM) breakdown, cytoskeleton remodeling, and alterations in cell adhesion. As the tumour stromal compartment is responsible for the production and remodeling of ECM components, interactions between cancer cells and surrounding

stromal cells are necessary to achieve new blood vessel growth, and the acquisition of metastatic potential (Seth & Watson, 2005). ETS transcription factors are increasingly associated with such interactions between tumour and stromal cells, particularly in the context of ECM remodeling. Numerous ETS proteins are aberrantly expressed in both tumour and stromal cells resulting in the overexpression of tumour-promoting factors such as MMP-1, MMP-3, MMP-9, urokinase plasminogen activator (PLAU/uPA), vascular endothelial growth factor (VEGF), and endothelium-specific tyrosine kinase 2 (Tie2) (Behrens et al., 2003; Behrens et al., 2001; Bolon et al., 1995; Hahne et al., 2006; Nakada et al., 1999; Oda, Abe, & Sato, 1999; Park et al., 2008; Sementchenko & Watson, 2000; Seth & Watson, 2005; Watabe et al., 1998).

Hypothesis and Objectives

The central hypothesis of this project is stated as:

> ***"Increased expression of Ets-1 mediated by oxidative stress in ovarian cancer causes an alteration in metabolic gene expression pathways. Specifically, Ets-1 is involved in the regulation of glycolysis, oxidative phosphorylation, and antioxidant defenses in ovarian cancer cells."***

The major objectives formulated to test the above stated hypothesis are:

1) Examine the role of Ets-1 in the regulation of mitochondrial metabolism in an ovarian cancer cell model.

2) Generate a global gene expression profile of Ets-1 overexpression in an ovarian cancer cell model to further clarify the role of this factor in cancer metabolism and the response to oxidative stress.
3) Investigate the impact of Ets-1 on the glutathione antioxidant system, and evaluate the effectiveness of glutathione-depleting agents in the Ets-1 overexpression model.

Manuscript Plan

To facilitate a better understanding of how the papers are organized in this thesis, the following is a list of the titles of each manuscript and a brief description of their contents.

- **Chapter 2:** *Ets-1 regulates energy metabolism in cancer cells.* In this study, the role of the transcription factor Ets-1 in the regulation of mitochondrial function and metabolism was investigated. Microarray analysis shows that Ets-1 up-regulates key enzymes involved in glycolysis, fatty acid metabolism, and antioxidant defense, while downregulating genes involved in oxidative metabolism. At the functional level, Ets-1 overexpression confers glycolytic dependence and decreased reliance on oxidative phosphorylation, suggesting the important of Ets-1 regulation in the Warburg effect.
- **Chapter 3:** *Ets-1 global gene expression profile reveals associations with metabolism and oxidative stress in ovarian and breast cancers*. In this study,

functional interaction and enrichment analyses on microarray data were performed to clarify the role of Ets-1 in an ovarian cancer model, and these results were validated with preliminary functional assays. Enrichments in oxidoreductase activity and various metabolic pathways were observed upon integration of the different bioinformatics analyses. Functionally, Ets-1 overexpression resulted in an altered cellular redox balance with low ROS levels, and high antioxidant capacity. The importance of Ets-1 in metabolism was extended to a knockdown breast cancer model, where decreased glycolytic dependence and increased oxygen consumption were observed confirming the findings from Chapter 2.

- **Chapter 4:** *Mechanisms associated with mitochondrial-generated reactive oxygen species in cancer.* Invited review paper discussing mitochondrial-generated ROS in cancer and their role as signaling molecules. ROS are a major byproduct of metabolism, which is fundamentally altered in cancer cells, thus the role of ROS in cancer physiology is of paramount importance. This review details how mitochondria produce ROS, mitochondria-to-nucleus crosstalk, and how ROS activates several important signaling pathways involved in proliferation, survival and apoptosis.

- **Chapter 5:** *Ets-1 regulates intracellular glutathione levels: Novel target for resistant ovarian cancer.* In this paper, a role for proto-oncogene Ets-1 in the regulation of glutathione levels was identified, and the effects of the anti-inflammatory drug sulfasalazine on glutathione depletion was investigated. Overexpression of Ets-1 was associated with decreased intracellular ROS,

concomitantly with an increased glutathione antioxidant system in the ovarian cancer cell model. The findings from this study show that Ets-1 mediates enhanced cystine import to increase glutathione levels under oxidative stress, suggesting that Ets-1 could be a promising putative target to enhance conventional therapeutic strategies.

Chapter 2: Ets-1 regulates energy metabolism in ovarian cancer

In this chapter, an author-generated version of the manuscript entitled "Ets-1 regulates energy metabolism in cancer cells", published in *PLOS ONE* in October 2010. The paper is reproduced with permission from the **Public Library of Science (PLOS)**, as stated on the copyright agreement form:

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For this paper, I performed the cell culture, RNA isolations, microarray, real-time qRT-PCR, and Western blotting analyses. Furthermore, I created all of the figures, and both wrote and revised the manuscript. Dr. Leigh Wilson completed the glycolytic dependency and oxygen consumption assays, the cell culture required for the aforementioned assays, and aided in the interpretation of the experimental results. The statistical analyses of the microarray experiment were performed in collaboration with Dr. Chris Verschoor. Dr. Gurmit Singh provided intellectual direction and revised the manuscript.

Context and background information

It is well established that Ets-1 plays a key role in cancer progression and metastasis, however little is known about the importance of this transcription factor in the context of cancer metabolism. The foundation for my project was based on the work of Dr. Leigh Wilson, a former Ph.D. student in the Dr. Gurmit Singh laboratory, whose research investigated the role of Ets-1 in crosstalk between the mitochondria and nucleus in ovarian cancer cells. Her first metabolically relevant observations regarding Ets-1 were found in a preliminary microarray experiment where several genes related to various metabolic processes were increased in response to Ets-1 overexpression (Wilson, Yamamoto, & Singh, 2004). In addition, Dr. Wilson also showed that Ets-1 is transcriptionally upregulated by H_2O_2 via Nrf2 direct binding, in an ovarian cancer cell model, suggesting that oxidative stress regulates Ets-1 levels in ovarian cancer (Wilson, Gemin, Espiritu, & Singh, 2005). These observations are relevant as oxidative stress is an initiating factor for mitochondrial mutations that likely cause the characteristic metabolic alterations in cancer cells, as well as a major byproduct of metabolism.

To further clarify these observations I performed a more comprehensive whole genome microarray experiment comparing the gene expression profiles of 2008 ovarian cancer cells and their Ets-1 overexpressing variant 2008-Ets1. I isolated RNA from both cell lines following 24 hours of treatment with tetracycline to induce Ets-1 expression in 2008-Ets1 cells. Control 2008 cells were treated with tetracycline as well in order to negate any potential confounding effects of the treatment. Following purification, the RNA was hybridized to the GeneChip® Human Gene 1.0 ST Array and gene expression

was detected by The Centre for Applied Genomics at the Hospital for Sick Children in Toronto, Ontario. The results from the microarray were validated using real time qRT-PCR based on the expression of 10 randomly chosen target genes of varying degree of differential expression. The expression of several genes involved in glycolysis, glycolytic feeder pathways, oxidative phosphorylation, lipid metabolism, and antioxidant defense were altered in response to Ets-1 overexpression. To examine the specificity of the Ets-1 cell model, I used Western blotting to show that the expression of Ets-2 and PEA3, two factors similar to Ets-1, were not changed in 2008-Ets1 cells. Although there are several more ETS transcription factors that could have expression differences in the 2008/2008-Ets1 ovarian cancer cell model, because changes in their gene expression were not observed in the microarray analysis, I deemed the gene expression differences to be most relevant to differences in Ets-1.

To investigate whether the gene expression changes I observed translate to functional changes to metabolism, the glycolytic dependence and oxygen consumption of 2008 and 2008-Ets1 cells were measured. In the context of glycolytic dependence, cells were treated with the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) and cellular growth was measured. Cellular proliferation was also measured in a separate assay following culture in the absence of glucose. Both of these assays showed that cells expressing Ets-1 have a significantly greater reliance on glycolysis for cellular growth, as evidenced by decreased cell numbers following either 2-DG treatment or a glucose-free culture environment. An important characteristic of the Warburg effect in cancer cells is the dependence on aerobic glycolysis with a corresponding decreased in oxidative

phosphorylation regardless of the presence of oxygen (Warburg, 1956; Warburg et al., 1927). Thus, the oxygen consumption of 2008 and 2008-Ets1 cells was determined using an Oroboros oxygraph machine, where the total oxygen concentration and flux was recorded at regular intervals, and the basal oxygen consumption measured following stabilization of the readings. In accordance with our observations of increased glycolytic use, cellular oxygen consumption was significantly decreased in 2008-Ets1 cells compared to their parental counterparts suggesting less oxidative capacity in these cells.

Based on these findings, we have shown for the first time that Ets-1 is involved in the regulation of cancer metabolism in ovarian cancer cells. The proposed mechanism begins with mitochondrial malfunction resulting in excessive ROS production, which in turn leads to the upregulation of Ets-1. A complex regulatory network is then initiated by Ets-1 that encourages dependence on glycolysis for cellular energy requirements, while decreasing the flux through oxidative phosphorylation. In defining this new role for Ets-1, we have gained a better understanding of this important transcription factor and its role in ovarian tumourigenesis.

PAPER: Ets-1 regulates energy metabolism in cancer cells

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Running Title: Ets-1 regulates cancer cell metabolism

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. Microarray analysis of the effects of Ets-1 over-expression in 2008 ovarian cancer cells show that Ets-1 up-regulates key enzymes involved in glycolysis and associated feeder pathways, fatty acid metabolism, and antioxidant defense. 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 pronounced 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 cancer cells.

Keywords: Mitochondria, cancer, Ets-1 transcription factor, metabolism, microarray

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 H_2O_2, 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 O_2 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, H_2O_2 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 ($p \leq 0.001$) (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).

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 IC_{50} 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, with an IC_{50} of 0.75 mM, which was significantly lower than the parental 2008 cells, IC_{50} of 4.29 mM ($p \leq 0.01$). C13* cells, a cisplatin-resistant variant of 2008 cells, had an IC_{50} of 2.04 mM, which was also significantly lower than parental 2008 cells ($p \leq 0.05$). To discount any treatment effect, parental 2008 cells were treated with tetracycline, and showed no significant growth inhibition by 2-DG with an IC_{50} of 3.67 mM (data not shown). Growth of cells in normal or glucose-free media (supplemented with sodium pyruvate) was compared over 96 hrs, after which it was observed that the proliferation of cells with increased expression of Ets-1 was notably slower compared to parental 2008 cells (Figure 3B). Treatment with glucose-free media resulted in a decreased proliferation rate for all cells tested in comparison to normally supplemented media (data not shown).

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 O_2 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 $O_2/1\times10^6$ cells/sec; $p\leq0.05$) compared to 2008 cells (40.60 pmoles $O_2/1\times10^6$ cells/sec, $p\leq0.05$) (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 (32). 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 (15, 33). Subsequent treatment of these cells with H_2O_2 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_2O_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 at the transcriptional level (15) suggest that the importance of this transcription factor in cancer initiation and progression extends beyond angiogenesis and metastasis alone.

We hypothesized that Ets-1 may be involved in the regulation of mitochondrial metabolism in cancer cells because mitochondrial stress from both increased reactive oxygen species production and electron transport chain malfunction result in increased Ets-1 mRNA and protein (15). In order to determine the functional importance of Ets-1 expression in cancer cell metabolism, we generated the tetracycline-inducible Ets-1 over-expressing ovarian cancer cell line 2008-Ets1. Parental 2008 cells do not express detectable levels of Ets-1 protein endogenously. To analyze the genomic consequences of Ets-1 over-expression in these ovarian cancer cells, we conducted a human gene microarray. Our findings indicate that Ets-1 is either directly or indirectly involved in regulating the expression of more than 3,000 of the over 28,000 human genes examined. Interestingly, our findings suggest that Ets-1 could act as a transcriptional repressor of more than half (1,665) of these genes in ovarian carcinoma cells.

Although Ets-1 has been studied extensively in both physiological and pathological processes (17), it is rarely referred to as a transcriptional repressor. In the context of

mitochondrial dysfunction and metabolism, Ets-1 was found to at least partially down-regulate several components of the electron transport chain. Complex I, the most prominent site for electron leakage leading to excessive reactive oxygen species production in the electron transport chain, is composed of 39 nuclear encoded subunit genes, of which Ets-1 down-regulates 11. Another important site for 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 metabolism transitions in 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), and overexpressed 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 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 potently 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 O_2 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 H_2O_2. The polarographic system used in this study to measure O_2 consumption includes sensors that yield a current proportional to the partial pressure of O_2 in cell containing media by consuming O_2 in a cathode half reaction, thus the signal responds exponentially to changes in O_2 pressure within the sample. In the absence of specific complex substrates and ADP, thereby simulating respiration, the basal rate of O_2 consumption can be measured. We have observed significant decreases in O_2 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 O_2 consumption, and Ets-1 down-regulated cells displayed a very prominent increase in O_2 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 H_2O_2-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 H_2O_2 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_2O_2 (15). However, it is important to note that Ets-1 also down-regulated genes encoding certain H_2O_2-detoxifying enzymes, suggesting that these cancer cells likely require and maintain a certain level of H_2O_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_2O_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, QC, Canada), and blocked for 1 hr in 5% skim milk TBS-T. Membranes were incubated overnight with primary antibody in 0.5% skim milk TBS-T. Following primary antibody incubation, membranes were washed and incubated for 1 hr with horseradish peroxidase-linked IgG secondary antibody (1:10,000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Proteins were detected using ECL chemiluminescence reagent (Amersham), and exposed to film. Antibodies against Ets-1 (1:100), G6PD (1:1000), and PDHA (1:500) were from Abcam; antibodies against Ets-2 (1:500), PEA3 (1:100), and HK (1:250) were from Santa Cruz Biotechnology; and the antibody against CYC (1:250) was from BD Biosciences.

RNA isolation and quantitative real-time PCR

Total RNA was isolated using Trizol reagent as indicated by the manufacturer (Invitrogen). RNA samples were DNase treated using Turbo DNA-free™ as per the manufacturer's directions (Ambion), and 3ug of total cellular RNA was reverse-transcribed using poly-T primers and the Superscript III First Strand Synthesis System (Invitrogen). Quantitative real-time PCR was conducted using a DNA Engine® thermal cycler (Bio-Rad) and Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) with primer sequences listed in Table 5. Target gene expression was normalized to the pooled

gene expression values of β-actin (ACTB), B-2 macroglobulin (B2M), glyceraldehyde-3-phosphdate dehydrogenate (GAPDH), and RNA polymerase II (RPII) 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 Log_2 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 2×10^3 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 Cytofluor series 4000 multiwell plate reader (excitation 350 nm, emission 460 nm) (PerSeptive Biosystems, Framingham, MA). Cell number was standardized to fluorescence 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 mg/L pyruvate was added to cells following overnight adherence. Plates of cells were frozen every 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 3×10^6 cells were used per experiment at a density of 1×10^6 cells/100 μL. Freshly harvested cells were added to the oxygraph chambers containing 1.8 mL of KCl medium (80 mM KCl, 10 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate, pH 7.2). The total O_2 concentration and flux were recorded at 1 sec intervals throughout the experiment. Once the oxygen concentration stabilized, the basal O_2 consumption was determined for each cell line and condition, where OROBOROS software was used for data acquisition and analysis.

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 ($p\leq0.05$ = *, $p\leq0.01$ = **, $p\leq0.001$ = ***).

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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.

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Tables

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

Table 2.1: Real time qRT-PCR validation of microarray findings

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_033500	HK1	+1.71 ***
	Aldolase C, fructose-bisphosphate	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	Phosphorylase, 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 ***
	Transketolase	NM_001135055	TKT	+2.07 ***
Lipid metabolism	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 ***
	Lysophosphatidylcholine 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 ***

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

Table 2.2: Effect of Ets-1 over-expression on glycolysis, glycolytic feeder pathways, and lipid metabolism

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 ***
	Scavenger 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 ***

$p \leq 0.05 = *$, $p \leq 0.01 = **$, $p \leq 0.001 = ***$.
doi:10.1371/journal.pone.0013565.t003

Table 2.3: Effect of Ets-1 over-expression on oxidative stress

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 α subcomplex 1	NM_004541	NDUFA1	−1.60 ***
	NADH dehydrogenase (ubiquinone) 1 α subcomplex 9	NM_005002	NDUFA9	−1.59 ***
	NADH dehydrogenase (ubiquinone) 1, α/β subcomplex 1	NM_005003	NDUFAB1	−2.23 ***
	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5	NM_002492	NDUFB5	−1.50 ***
	NADH dehydrogenase (ubiquinone) 1 β subcomplex 11	NR_024234	NDUFB11	−1.48 ***
	NADH dehydrogenase (ubiquinone) 1 α subcomplex assembly factor 1	NM_016013	NDUFAF1	−1.71 ***
	NADH dehydrogenase (ubiquinone) Fe-S protein 4	NM_002495	NDUFS4	−1.58 ***
Complex III	Cytochrome *c*-1	NM_001916	CYC1	−1.46 ***
	Ubiquinol-cytochrome *c* reductase, Rieske iron-sulfur polypeptide 1	NM_006003	UQCRFS1	−1.50 ***
	Ubiquinol-cytochrome *c* reductase, complex III subunit VII	NM_014402	UQCRQ	−1.50 ***
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 associated factors	Cytochrome *c*, somatic	NM_018947	CYCS	−1.51 ***
	Holocytochrome c synthase (cytochrome c heme-lyase)	NM_005333	HCCS	−1.76 ***

$p \leq 0.05 = *$, $p \leq 0.01 = **$, $p \leq 0.001 = ***$.
doi:10.1371/journal.pone.0013565.t004

Table 2.4: Effect of Ets-1 over-expression on the ETC

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

Gene name (acronym)	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-actin (ACTB)	cctccctggagaagagctac	gatgtccacgtcacacttca
B-2 macroglobulin (B2M)	gctatccagcgtactccaaag	tcacacggcaggcatactc
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	atcatcagcaatgcctcctg	ctgcttcaccaccttcttga
RNA polymerase II (RPII)	gaaacggtggacgtgcttat	tctccatgccatacttgcac
Cytochrome *c* 1 (CYC1)	ccaatgaagatccccacatg	ccaggaaagtaggggttgaagt
V-ets erythroblastosis virus E26 oncogene homolog 1 (ETS1)	tgtattttgcatccctggtt	aacgacatcgattcaggact
Glucose-6-phosphate dehydrogenase (G6PD)	tggaaccgggacaacatc	caacaccttgaccttctcatcac
Glutathione peroxidase 2 (GPX2)	cccctacccttatgatgacc	gttgatggttgggaaggtg
Hexokinase 1 (HK1)	ggcgtttccacaagactcta	cttggtgaggtggaaatgag
Matrix metallopeptidase 13 (MMP13)	cagtctttcttcggcttagagg	cagaggagttacatcggacca
NADH dehydrogenase 1, α/β subcomplex 1 (NDUFAB1)	cgtctcgtcgtcctttcagc	cctggatgccctctaacg
Pyruvate dehydrogenase α 1 (PDHA)	cacagaccatctcatcacagt	ggcagacctcatcttttcca
Peroxisome proliferator-activated receptor γ (PPARG)	cctattgacccagaaagcgatt	cattacggagagatccacgga
Succinate dehydrogenase complex, subunit B (SDHB)	atcttgttcccgatttgagc	gtctccgttccaccagtacg

doi:10.1371/journal.pone.0013565.t005

Table 2.5: Primer sequences used for real time qRT-PCR

Figures

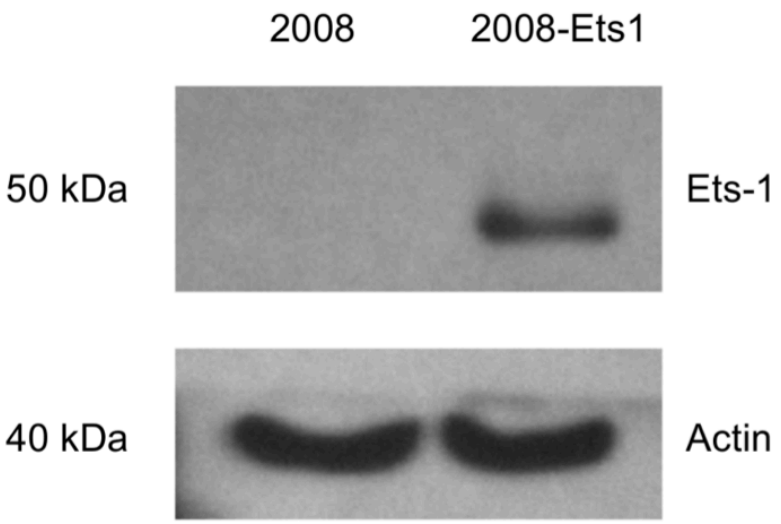

Figure 2.1: Generation of an ovarian cancer cell model for Ets-1 expression.

2008 ovarian cancer cells were stably transfected to over- express Ets-1 in a tetracycline inducible system. Protein expression of 2008 and 2008-Ets1 cells was measured via Western blot following induction with tetracycline (n = 3).

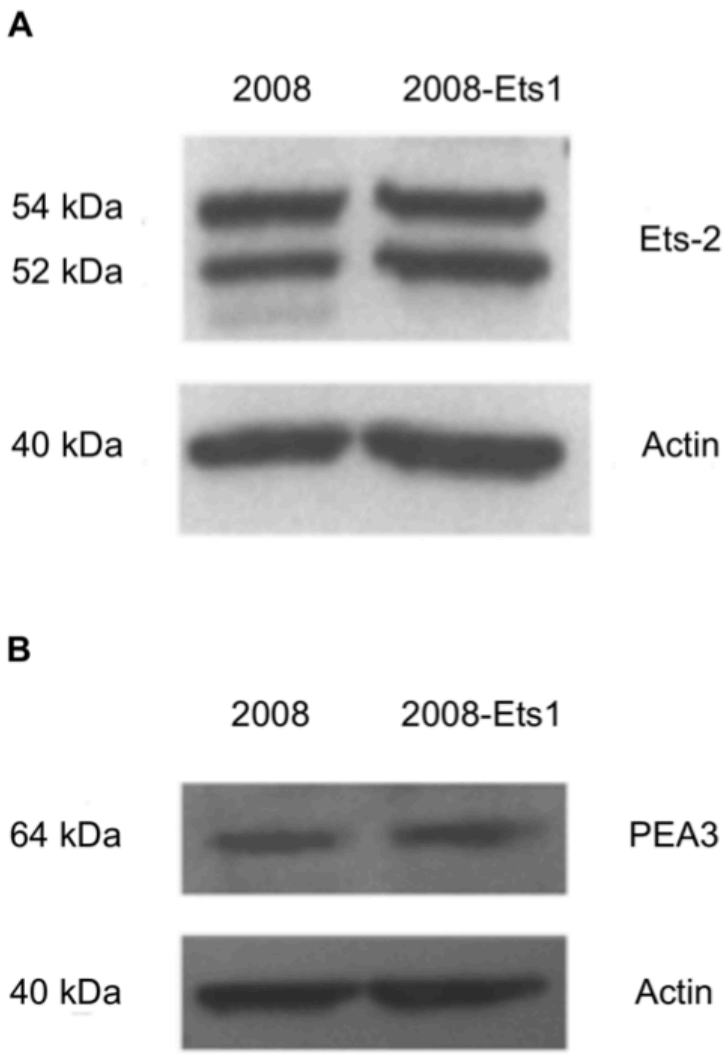

Figure 2.2: Ets-1 expression did not affect the expression of similar ETS family members.

The protein expression of ETS family transcription factors (A) Ets-2 and (B) PEA3 were compared in 2008 and 2008-Ets1 ovarian cancer cells to determine the specificity of our Ets-1 expression model. Western blot analysis showed that neither of these transcription factors was affected by Ets-1 expression in 2008 cells.

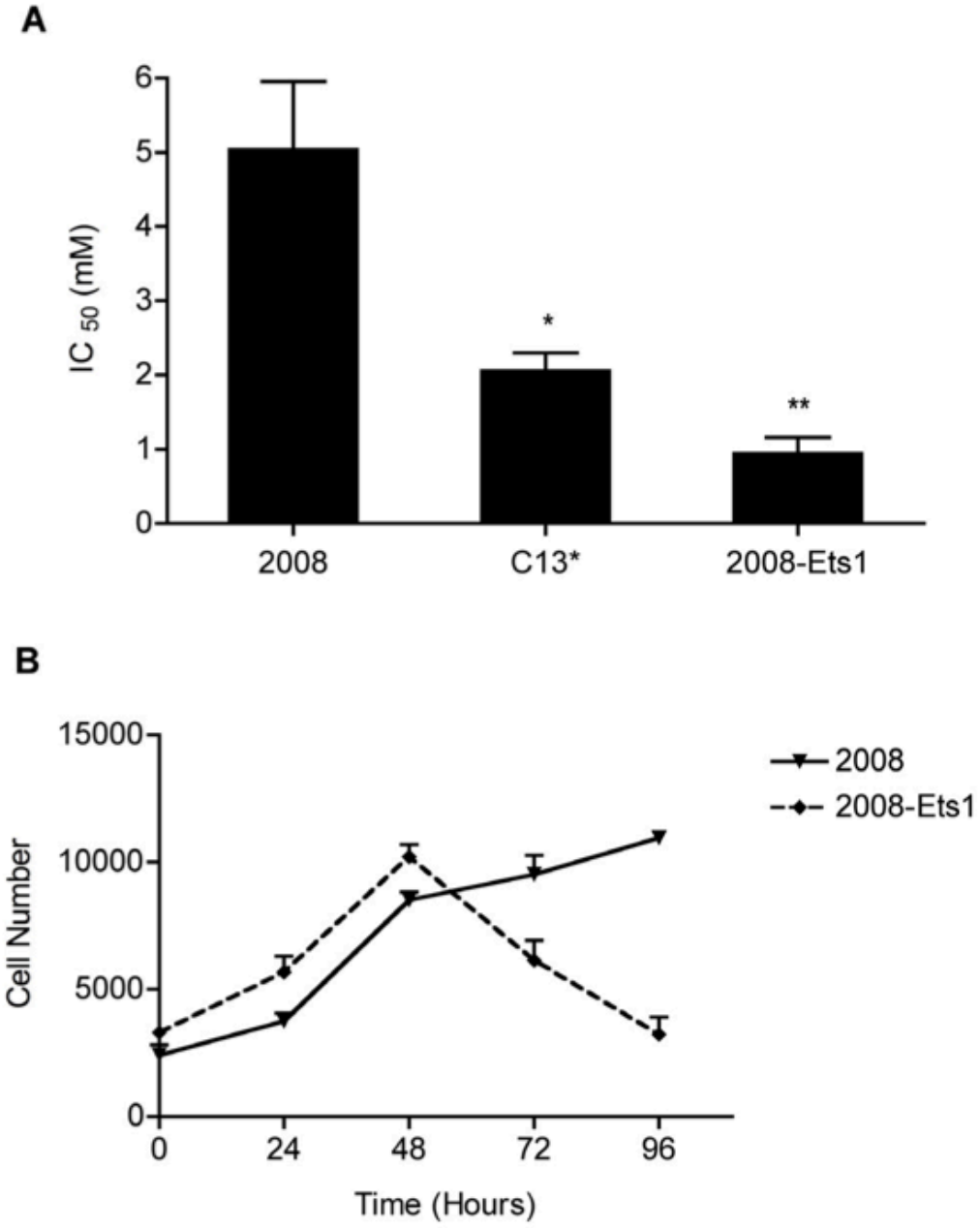

Figure 2.3: Glycolytic dependence of Ets-1-expressing ovarian cancer cells.

(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).

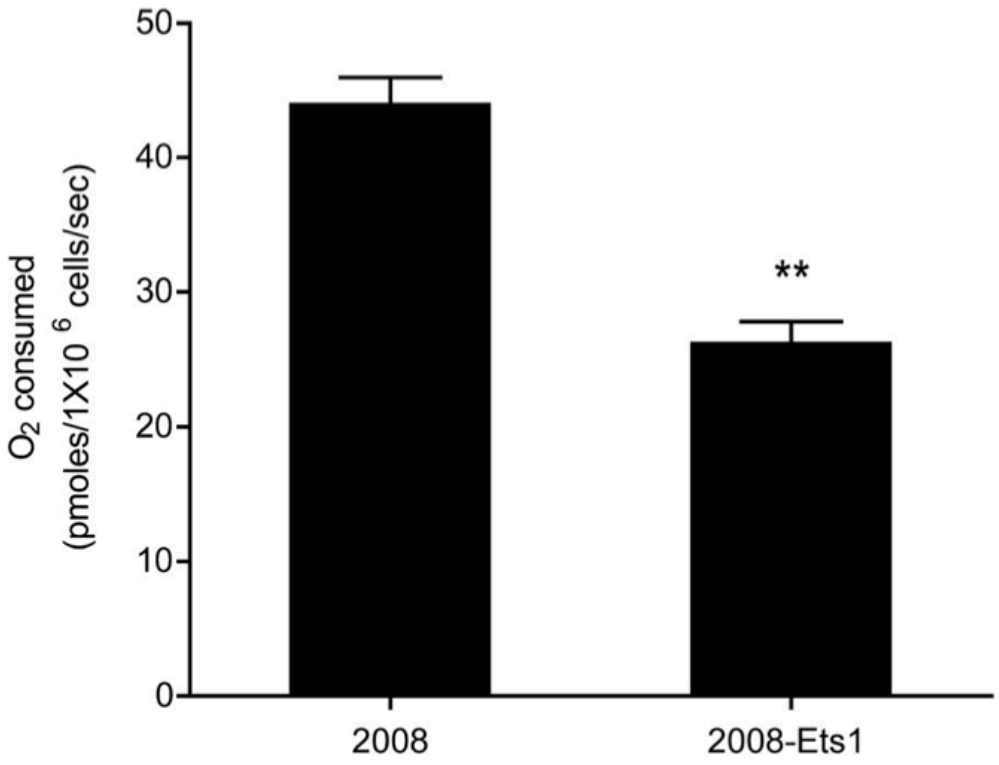

Figure 2.4: Effect of Ets-1 expression on oxygen consumption.

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.

Chapter 3: Global gene expression profile for Ets-1 overexpression

In this chapter, an author-generated version of the manuscript entitled "Ets-1 global gene expression profile reveals associations with metabolism and oxidative stress in ovarian and breast cancers", published in *Cancer & Metabolism* in July 2013. The paper is reproduced with permission from **Biomed Central**, as state on the copyright agreement form attached in Appendix 2.

For this paper, I performed all of the cell culture, Western blotting, RNA work, functional annotation, bioinformatic pathway-based network analyses, intracellular GSH determination, and GPX activity assays. Furthermore, I created all of the figures, and both wrote and revised the manuscript. The statistical analyses of the microarray experiment were performed in collaboration with Dr. Chris Verschoor, and he participated in manuscript revisions. Former students of the Singh lab, Darren Hammerlick and Stephanie Marra, generated the breast cancer Ets-1 knockdown cell line and performed the functional assays on those cells. Dr. Gurmit Singh provided intellectual direction and revised the manuscript.

Context and background information

In the previous chapter, a whole genome microarray was performed that showed gene expression changes relating to metabolism in an ovarian cancer cell model that expresses high levels of Ets-1. As would be expected when altering the expression of a transcription

factor, the differences I observed between 2008 and 2008-Ets1 cells were diverse and numerous, extending to many important functions beyond metabolism and the known role of Ets-1 in metastasis. Though it is tempting to examine individual target gene expression changes within the large volume of data we generated, a more logical and realistic approach is to examine enrichments in groups of genes involved in established functional pathways. This type of analysis also overcomes some of the major analytical challenges inherent to microarray analysis when a large number of significant genes are differentially expressed, such as interpretation bias based on the researcher's area of expertise, and missing a group of genes that may act in concert by focusing only on large expression changes in individual genes (Subramanian et al., 2005). Thus, for this paper I have used various bioinformatic pathway analysis methods to identify the key functional pathways that are enriched in the 2008 ovarian cancer cell model for Ets-1 overexpression.

A global human functional interaction network was created in Cytoscape based on the gene expression changes observed in the microarray using the Reactome Functional Interaction database. The major pathway enrichments included cell cycle regulation, RNA splicing, Wnt signaling, antigen presentation, and metabolic pathways. Functional ontology was examined using DAVID, an online software that identifies enriched pathways and gene groups in order to define the biological meaning behind large sets of differentially expressed genes (Huang da, Sherman, & Lempicki, 2009). Using the highest classification stringency, 8 clusters of enriched genes were identified, notably including MMP activity, chymotrypsin activity, antigen presentation, oxidoreductase activity, and metabolic pathways. Gene Set Enrichment Analysis (GSEA) was used in

conjunction with the Enrichment Map and MCODE plugins in Cytoscape to generate a network of enriched gene sets. GSEA analysis compares the differentially expressed genes within a microarray data set to known gene set data from published pathway information, leading to correlations with known pathways or phenotypic distinctions (Subramanian et al., 2005). The major findings from this analysis include enrichments in several important signaling pathways, inflammatory pathways, angiogenesis, oxidoreductase activity, and metabolic pathways. Upon integrating the results from each of the aforementioned analyses, the pathways involved in antigen presentation, oxidoreductase activity, and particularly metabolism were most commonly associated with Ets-1 overexpression in the 2008 ovarian cancer cell model.

Oxidoreductases are enzymes that catalyze the transfer of electrons between molecules, and can function as oxidases, dehydrogenases, peroxidases, hydroxylases, oxygenases, or reductases. The most important role for this class of enzymes is in cellular metabolism, where they are involved in glycolysis, TCA cycle, oxidative phosphorylation, and the maintenance of cellular redox state. To functionally examine the association with oxidoreductases and Ets-1 overexpression, I measured GPX expression and activity, as well as intracellular ROS levels. In accordance with the pathway analyses, GPX expression and activity were observed to increase concomitantly with decreased intracellular ROS in correlation with high levels of Ets-1 expression. Though these findings represent a preliminary investigation into the association of Ets-1 with redox state regulation, they do provide compelling evidence that warrants further study into this area with respect to Ets-1 overexpression in ovarian cancer.

The previous chapter detailed the importance of Ets-1 in metabolic regulation of 2008 ovarian cancer cells, and thus in this paper I sought to extend these findings to another type of cancer cells to determined how generalized these phenomena are. I decided to focus on breast cancer in particular because Ets-1 is commonly overexpressed in tumours of the breast, and is associated with poor prognosis and lower survival rates in this cancer type (Buggy et al., 2004; Katayama et al., 2005; Span et al., 2002; Y. Zhang et al., 2011). Ets-1 was stably knocked down in MDA-MB-231 breast cancer cells using shRNA specific for Ets-1, generating the MDA-Ets1KD cell line variant that expresses 77% less Ets-1 than parental cells. Differences in the expression of several metabolic genes were measured in this breast cancer Ets-1 model, and were found to be in accordance with results from the 2008 ovarian cancer model of Ets-1 overexpression. Likewise, MDA-Ets1KD cells were shown to be less reliant on glycolysis for energy than parental MDA-MB-231 cells, and also to consume significantly more oxygen. These findings are particularly encouraging as they confirm the major results from Chapter 2, and suggest that the role of Ets-1 in metabolic regulation is a generalized phenomenon in various cancer cells.

PAPER: Ets-1 global gene expression profile reveals associations with metabolism and oxidative stress in ovarian and breast cancers

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ABSTRACT

Background

The Ets-1 proto-oncogene is frequently upregulated in cancer cells, with known involvement in cancer angiogenesis, metastasis, and more recently energy metabolism. In this study we have performed various bioinformatic analyses on existing microarray data to further clarify the role of Ets-1 in ovarian cancer, and validated these results with functional assays.

Methods

Functional pathway analyses were conducted on existing microarray data comparing 2008 and 2008-Ets1 ovarian cancer cells. Methods included over-representation analysis, functional class scoring and pathway topology, and network representations were visualized in Cytoscape. Oxidative stress regulation was examined in ovarian cancer cells by measuring protein expression and enzyme activity of glutathione peroxidases, as well as intracellular reactive oxygen species using dichlorofluorescin fluorescence. A stable Ets-1 knockdown MDA-MB-231 cell line was created using short hairpin RNA, and glycolytic dependence of these cells was measured following treatment with 2-deoxy-D-glucose and Hoechst nuclear staining to determine cell number. High-resolution respirometry was performed to measure changes in basal oxygen flux between MDA-MB-231 cells and MDA-Ets1KD variants.

Results

Enrichments in oxidoreductase activity and various metabolic pathways were observed upon integration of the different analyses, suggesting that Ets-1 is important in their regulation. As oxidative stress is closely associated with these pathways, we functionally validated our observations by showing that Ets-1 overexpression resulted in decreased reactive oxygen species with increased glutathione peroxidase expression and activity, thereby regulating cellular oxidative stress. To extend our findings to another cancer type, we developed an Ets-1 knockdown breast cancer cell model, which displayed decreased glycolytic dependence and increased oxygen consumption following Ets-1 knockdown, confirming our earlier findings.

Conclusions

Collectively, this study confirms the important role of Ets-1 in the regulation of cancer energy metabolism in ovarian and breast cancers. Furthermore, Ets-1 is a key regulator of oxidative stress in ovarian cancer cells by mediating alterations in glutathione antioxidant capacity.

KEYWORDS:
Ets-1
Ovarian cancer
Breast Cancer
Metabolism
Oxidative Stress

BACKGROUND

Ets-1 is a member of the E-26 (Ets) family of transcription factors, and was first characterized as a proto-oncogene of the retroviral *v-ets* oncogene in avian leukemia retrovirus E26 [1]. This family of transcription factors currently comprises 28 members, many of which are known to be elevated in various cancers [2,3] including Ets-2 [4-9], Friend leukemia integration 1 [10], Ets-related gene [4], Polyomavirus enhancer activator 3 homolog [11,12], Ets-related molecule [11], Prostate epithelium-specific Ets transcription factor [13] and E74-like factor-3 [11]. All known Ets family members contain a core double-stranded DNA binding element that recognizes the consensus sequence GGAA/T [14,15]. Because the Ets binding element is simple and generic, there is significant functional redundancy among Ets factors, allowing for complex transcriptional networks depending on which factors are bound to a specific promoter. The diverse functional roles of these factors include differentiation, proliferation, apoptosis, angiogenesis, malignant transformation and metastasis, which are all processes relevant to the study of cancer.

High levels of Ets-1 expression are observed in a wide variety of cancer types including those of the breast, prostate and ovary; this suggests that the association between Ets-1 expression and tumor progression is a generalized phenomena [16]. Ets-1 upregulation appears to associate specifically with more advanced, invasive tumors in breast and ovarian carcinomas [17-22], and is positively correlated with the enhanced metastatic potential of numerous cancers [17,23-26]. Indeed, there are many well-

established target genes for Ets-1 that are closely linked to cancer progression, particularly mediators of extracellular matrix degradation, cancer cell migration and angiogenesis [16,25,27-31]. Thus, the consequences of Ets-1 overexpression are particularly relevant to the study of ovarian cancer as this type of malignancy is very difficult to detect, and is most commonly diagnosed at advanced stages of disease progression that include metastases. Comparing the transcriptional programs of cancer cells that express low levels of Ets-1 protein to those that express Ets-1 protein in abundance will create a gene expression profile illustrating some of the key differences between invasive and non-invasive ovarian cancer cells.

Recently, our laboratory showed the importance of Ets-1 as a regulator of cellular metabolism in ovarian cancer cells, where Ets-1 overexpression resulted in increased glycolysis while suppressing oxidative phosphorylation, a phenomena known as the Warburg effect [32]. The objective of the present study was to examine the functional interactions of the potential downstream targets of Ets-1 identified in the microarray analysis from our previous work. In our previous study, we used a stable Ets-1 overexpression model in 2008 ovarian cancer cells to conduct whole genome microarray analysis, which we have more comprehensively examined here to further clarify the role of Ets-1 in ovarian tumorigenesis. We have utilized three different approaches of bioinformatic pathway analysis, and compared them to identify the pathway associations that are common to each method in order to delineate the most important pathways represented following Ets-1 overexpression. The findings from our pathway-based

network analyses illustrate the importance of Ets-1 expression in cancer-associated metabolic regulation in ovarian cancer.

The most novel finding among other commonly enriched functional pathways we identified was likely that of pathways involving the regulation of cellular redox status. To provide some validation for this finding, we examined the protein expression of elevated targets involved in the regulation of cellular redox status, and measured intracellular reactive oxygen species (ROS) production in ovarian cancer cells overexpressing Ets-1. Additionally, to investigate the ability of our findings to extend to other types of human cancer, we developed an Ets-1 knockdown model in MDA-MB-231 breast cancer cells, as aggressive breast cancer is frequently associated with the overexpression of Ets-1 [17,21,22,33]. Because metabolic pathways were the most common pathway association with changes to Ets-1, and because we have previously established an association between energy metabolism and Ets-1 in ovarian cancer cells, we used this model to examine the significance of Ets-1 knockdown on glycolysis and oxidative phosphorylation. We observed that Ets-1 regulation of cancer-specific metabolic changes is a phenomenon that also exists in breast cancer cells, thus complementing our previous work and suggesting that Ets-1 regulation of metabolism may be a generalized phenomenon.

METHODS

Cell culture

The human ovarian carcinoma cell line 2008 was kindly provided by Dr Paul Andrews (Georgetown University, Rockville, MD, US) [34]. MDA-MB-231 human mammary epithelial adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC# HTB-26). The 2008 cells were maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin. MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin. Stable cell lines 2008-Ets1 and MDA-Ets1KD were maintained in growth medium as described with the addition of 200 ng/ml selective antibiotic. 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, ONT, Canada), and fetal bovine serum from Fisher Scientific (Ottawa, ONT, Canada). All reagents were purchased from Sigma (Oakville, ONT, Canada).

Microarray pathway analysis

Ontological analysis was performed using the online software DAVID [35]. Gene lists composed of probe sets showing absolute linear fold-change differences ≥2 and q-values ≤0.05 were compared against the HuGene-1_0-st-v1 background list supplied by DAVID. Functional annotation charts were produced for ontological categories of interest comprising at least 10 genes, and functional annotation clustering was performed at high classification stringency. Ranked gene set enrichment analysis (GSEA) was performed to

identify significantly enriched gene sets between 2008 and 2008-Ets1 gene expression profiles as described previously [36,37].

Complex network analysis and visualization was performed using Cytoscape, an open source bioinformatics software package [38]. The Network analyzer plugin was used to perform statistical calculations and visualizations of topological parameters and centrality measures for the biological networks generated from the various analyses [39]. Normalized gene expression data from the microarray was used to create a large, comprehensive network using Reactome Function Interaction (FI) using a size of 10 and an average correlation minimum of 0.75 [40]. This plugin utilizes the Reactome FI network of human protein interactions to create a specific sub-network based on the differentially regulated genes in the microarray. The Enrichment Map plugin [41] was used to generate networks derived from the GSEA results, and the MCODE plugin [42] was used to further cluster the GSEA enrichment map.

Protein isolation and western blot analysis

Whole cell lysates were collected, 30 μg of protein was separated by 10% SDS-PAGE electrophoresis, transferred to polyvinylidene difluoride membrane, and blocked for 1 h in 5% skim milk Tris-buffered saline with Tween. Membranes were incubated overnight with antibody reactive to Ets-1 (Abcam, Boston, MA, US), glutathione peroxidases 1 and 2 (GPX1 and 2; Abcam) or Actin (Cell Signaling, Danvers, MA, US) in 0.5% Tris-buffered saline with Tween. Following primary antibody incubation, membranes were washed and incubated for 2 h with horseradish peroxidase-linked anti-mouse or anti-

rabbit immunoglobulin G secondary antibody (Cell Signaling). Proteins were detected by ECL chemiluminescence reagent (Amersham Biosciences, Baie D'Urfe, QC, Canada), and exposed to film. Densitometry analysis was performed using ImageJ software developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA [43].

Intracellular reactive oxygen species and glutathione peroxidase activity assays

Intracellular ROS levels were measured using CM_2-H_2DCFDA reagent (Invitrogen), which is cleaved once inside the cell allowing the DCF dye to bind to ROS species, resulting in fluorescence. Cells were plated in 96-well plates and grown to 70% to 90% confluency in phenol red-free medium. CM_2-H_2DCFDA reagent was reconstituted in dimethyl sulfoxide, and 10 μM was added to each experimental well using phenol red-free medium containing 10% fetal bovine serum. Following a 30 min incubation to allow the dye to load into cells, plates were washed twice with phosphate-buffered saline, and allowed to recover in phenol red-free medium for 10 minutes. Plates were then treated with 250 μM H_2O_2 and read in a Cytofluor fluorescent plate reader at 485 nm excitation and 530 nm emission for 1 h. Plates were then stained with crystal violet, dried overnight, solubilized with SDS, and read at 570 nm. Arbitrary fluorescent values were normalized to crystal violet absorbance values, and reported as arbitrary fluorescent units (AFU). Glutathione peroxidase enzyme activity was measured using the GPX activity kit from Enzo Life Sciences (Farmingdale, NY, USA) according to the manufacturer instructions.

Ets-1 knockdown breast cancer model

MDA-MB-231 cells were plated in 6-well plates at 1.5×10^5 cells per well, incubated for 24 h, then transfected with 1 μg of the pSM2 retroviral vector (Open Biosystems, Huntsville, AL, US) containing a pre-designed short hairpin RNA sequence targeting Ets-1 (SH2588-A-5) or a non-silencing control vector (RHS1703) using Arrest-In^{to} (Open Biosystems). Plates were exposed to selection medium containing 2 μg/ml Puromycin over 3 weeks, and surviving clones were isolated, expanded and tested for Ets-1 protein expression via western blotting. The clone with the lowest Ets-1 protein expression was expanded and will be referred to as MDA-Ets1KD cells in this study.

RNA isolation and quantitative real-time PCR

Total RNA was isolated using Trizol reagent as indicated by the manufacturer (Invitrogen). RNA samples were DNase treated using Turbo DNA-free^{TM} as per the manufacturer's directions (Invitrogen), and 3 μg of total cellular RNA was reverse-transcribed using the Superscript III First Strand Synthesis System (Invitrogen). Quantitative real-time PCR was conducted using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) with primers sequences listed in Additional file 1. All target gene expression was normalized to the pooled gene expression values of β-actin, B-2 macroglobulin, glyceraldehyde-3-phosphate dehydrogenase and RNA polymerase II as housekeeping controls. Data were normalized and efficiency corrected using the ΔΔCt method of relative quantification, where statistical significance and standard error were determined from ΔCt values.

Measurement of glycolytic dependency

The glycolytic dependency assays were performed as described previously [32]. Briefly, cells were treated with 2-deoxy-D-glucose (2-DG), which was administered at concentrations ranging from 1 to 7.5 mM over 96 h, at which time a Hoechst DNA content assay (Invitrogen) was performed. Cell number was standardized to fluorescence for each cell type by comparison with a standard curve of known cell numbers.

Oxygen consumption assay

The oxygen consumption assay was performed as described previously [32]. Briefly, harvested cells were added to the oxygraph chambers containing 1.8 mL of KCl medium and the total O_2 concentration and flux was recorded at one second intervals throughout the duration of the experiment. Once the oxygen concentration stabilized, cells were permeabilized with digitonin and treated with various respiratory substrates and inhibitors. OROBOROS software was used for data acquisition and analysis (OROBOROS Instruments GmbH, Innsbruck, Austria).

Statistical analysis

Data is presented as the mean ± SD from at least three independent experiments. Statistically significant differences between sample groups were determined using a Student's t-test or analysis of variance where applicable, with a P-value ≤ 0.05 considered to be statistically significant.

RESULTS

Global gene expression analysis

We previously performed comparative microarray analysis of 2008 and 2008-Ets1 ovarian cancer cells [32,44], which revealed a large amount of differentially regulated genes. Subsequent real-time qRT-PCR validation was examined, and gene expression changes greater than 1.5-fold were deemed valid and included in this study. The complete raw data is available via the GEO database under accession number [GEO:GSE21129].

Reactome FI was used to create a global human interaction network for the differentially regulated genes in our microarray data set (Figure 1). This plugin was specifically created to find network patterns pertaining to cancer, by building a network of highly interacting gene groups, referred to as modules. To decrease the complexity of the network generated, the module size was set to a minimum of 10 interacting genes, average correlation set to 0.75, and false discovery rate (FDR) set to 0.25 [40]. The resulting network contained 275 nodes of curated and predicted protein interactions clustered into 16 functional modules as delineated by different node coloring (Figure 1). Functional pathway enrichment analysis (FDR <0.01) was performed on the large network to identify the major pathways and ontological terms associated with each module of interacting genes. The module functions with the highest gene set ratio, and thereby the most important pathways annotated, included cell cycle regulation, RNA splicing, wingless-type (WNT) signaling and metabolic pathways, as well as insulin synthesis and secretion (identified by colored outlines in Figure 1). The gene set ratio is a

measure of the number of enriched genes present in a data set compared to the total number of genes within the curated gene set, and thus a good indicator of the relative importance of each pathway within the data set. The individual genes contained in the most important outlined modules are detailed in Additional file 2.

Gene ontology

Functional annotation clustering of the differentially regulated genes was completed using DAVID, an online database of functional annotation tools. This software identifies which annotation groups are enriched in a list of differentially expressed genes by grouping functionally similar terms associated with these genes into annotation clusters. Meaningful functional relationships can then be inferred from the functional categories that are significantly over-represented within the list of enriched genes. Under the highest classification stringency, eight annotation clusters were identified as significantly enriched in our microarray data set (Table 1). Clusters relevant to our functional pathway enrichment analysis (Figure 1) included oxidoreductase activity relating to fatty acid desaturation (cluster 1), unsaturated fatty acid metabolism (cluster 2), D-Aspartate:2-oxoglutarate aminotransferase activity (cluster 3), and dihydrodiol dehydrogenase activity (cluster 4). We have reported the *P*-value and fold enrichment for each annotation cluster. The *P*-value represents the threshold of EASE Score, which is a modified Fisher exact *P*-value used for gene-enrichment analysis to determine the significance of the gene set enrichment. The measure of fold enrichment represents a ratio of the proportion of input genes present within a gene set compared to the number of genes that term represents in

the human genome background data set [35]. Thus, a low *P*-value combined with a high fold enrichment together indicate a high magnitude of enrichment for that particular ontology term. Our findings indicated that the best enrichments relating to Ets-1 overexpression included fatty acid desaturation (annotation clusters 1 and 2) and dihydrodiol dehydrogenase activity (annotation cluster 4).

Gene set enrichment analysis

To examine the biological pathways enriched in the data set in an unbiased, systematic method, GSEA was performed [36]. This software examines gene expression data at the level of gene sets, which are based on existing biological pathway or co-expression data from published research within the Molecular Signature Database. GSEA results were also applied to Enrichment Map in Cytoscape to generate a large network of enriched gene sets, then the large network was clustered using MCODE to generate five sub-networks of interrelated gene sets (Figure 2). The largest cluster included several signaling pathways, most notably extracellular-signal-regulated kinase 5 (ERK5), mitogen-activated protein kinase (MAPK), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), MET proto-oncogene and G protein-coupled receptor (GPCR) pathways. Relevant to our other analyses, clusters containing gene sets involved in mitochondrial metabolism and fatty acid metabolic processes were also identified (gene lists provided in Additional file 3). More detailed description of these gene sets can be found in the MsigDB database [36].

Comparison of bioinformatic data

Pathway analysis of microarray data is a powerful tool for the identification of the underlying biological significance of a large gene expression lists, allowing for improved understanding of high-throughput data. There exist three distinct methods for conducting pathway analyses, including over-representation analysis, functional class scoring and pathway topology analysis. Each approach generates a list of significantly enriched pathways present within the input gene list, however there are limitations and advantages to each of these methods as reviewed by Khatri *et al.* [45]. For this study, we chose to analyze our gene expression data using each of these approaches, and then compared the results to determine the most important pathway associations present following Ets-1 overexpression in 2008 ovarian cancer cells. Oxidoreductase activity was a common pathway association between DAVID (over-representation analysis) and GSEA (functional class scoring) methods, whereas antigen presentation was enriched in both GSEA (functional class scoring) and Reactome (pathway topology analysis) analyses (Figure 3). Interestingly, metabolic pathway associations were found using all analysis methods, suggesting that this observation is the most significant finding within our Ets-1 overexpression model of ovarian cancer.

Ets-1 overexpression affects oxidative stress in cancer cells

Because enrichments in various metabolic pathways and oxidoreductase activity were observed in each of the bioinformatic analyses we examined (Figure 3), we investigated the association between oxidative stress and Ets-1 expression. The upregulation of GPX-1

and GPX-2 was validated via western blot, where the protein expression of both factors was increased in response to Ets-1 induction by 3.10-fold and 2.25-fold respectively (Figure 4A). Functionally, intracellular ROS levels were significantly lower in 2008-Ets1 cells (1233.99 AFU) compared to 2008 cells (1872.73 AFU) (Figure 4B). The activity of GPX enzymes was significantly higher in 2008-Ets1 cells (7725.66 U/mL/mg) than in 2008 cells (3944.22 U/mL/mg) (Figure 4C).

Breast cancer cell metabolism is regulated by Ets-1

As we have previously shown that Ets-1 regulates energy metabolism in ovarian cancer cells, for this study we endeavored to extend those findings to breast cancer cells. The Ets-1 knockdown cell line MDA-Ets1KD was generated, because the aggressive MDA-MB-231 breast cancer cell line expresses Ets-1 in abundance, where Ets-1 expression is decreased to 22.7% of parental levels (Figure 5A). To determine whether this cell model expressed the same differences in metabolic genes as we observed in our previous microarray analysis [32], we performed real-time qRT-PCR on the breast cancer cell model. The gene expression of *PDHA*, *CYC1*, *NDUFAB1* and *SDHB* were increased in MDA-Ets1KD cells, whereas the expression of *G6PD* was downregulated (Figure 5B). The inverse relationship observed between the expression levels of these genes in our ovarian and breast cancer cell models suggest that Ets-1 regulates the expression of these factors in a similar manner in breast cancer cells.

To examine the functional consequences of decreased Ets-1 expression in breast cancer cells, we examined glycolytic dependence and oxygen consumption in MDA-

Ets1KD cells. Cells were treated with various amounts of the glycolytic inhibitor 2-DG, and representative growth curves were generated for each cell line. When inhibited with 2-DG, the growth of MDA-Ets1KD cells was decreased to a lesser extent than that of MDA-MB-231 cells (Figure 6A). The dose at which 50% of cells had stopped proliferating, or 2-DG IC_{50}, was greater in MDA-Ets1KD cells with an IC_{50} of 3.94 mM compared to an IC_{50} of 2.03 mM for MDA-MB-231 cells. Basal oxygen consumption was measured using high-resolution respirometry, and MDA-Est1KD cells were observed to consume significantly more oxygen (41.80 pmol/10^6 cells/s) than parental MDA-MB-231 cells (21.07 pmol/10^6 cells/s) (Figure 6B).

DISCUSSION

Our analysis shows that the expression of Ets-1 in cancer cells results in a transcriptional program that confers enhanced cancer progression and development through the alteration of metabolism and redox status. Ets-1 is widely expressed by tumor cells, endothelial cells and tumor-associated fibroblasts, where it is known to contribute to tumor angiogenesis and cancer cell invasion [20,29,30,46-64]. Ets transcription factors are increasingly associated with such interactions between tumor and stromal cells, particularly in the context of extracellular matrix remodeling. Numerous Ets proteins are aberrantly expressed in both tumor and stromal cells, resulting in the overexpression of tumor-promoting factors such as matrix metalloproteinase (MMP)-1, MMP-3, MMP-9, urokinase plasminogen activator (PLAU/uPA), vascular endothelial growth factor and

endothelium-specific tyrosine kinase 2 [3,30,31,47-50,55,61,65]. The functional interaction analyses in this study further strengthen the importance of Ets-1 in regulating cancer metastasis due to the pathway associations we have observed in WNT, vascular endothelial growth factor, and MMP signaling, as well as ERK5, MAPK, EGF, PDGF, MET and GPCR pathways.

We have previously shown that our Ets-1 ovarian cancer expression model results in changes to cellular metabolism, particularly in the context of glucose utilization and oxygen consumption [32]. Several genes involved in glycolysis, the pentose phosphate pathway and other glycolysis feeder pathways were increased following the overexpression of Ets-1, while key enzymes in the oxidative phosphorylation pathway and the electron transport chain were repressed. Functionally, these cells are unable to grow effectively when glucose is depleted or blocked, suggesting that Ets-1 expression results in a greater reliance on glycolysis for energy generation. In accordance with these results, cellular oxygen consumption was significantly decreased in cancer cells expressing Ets-1, suggesting that electrons are passing through the electron transport chain and generating ROS at a decreased rate. In this study, we were able to repeat these findings in a breast cancer Ets-1 knockdown model, giving more strength to our theory that Ets-1 is integral in regulating cancer cell metabolism. In further support of this role, each of the different pathway analysis approaches used identified enrichments in metabolic pathways, as represented in Figure 3.

Cancer cells display enhanced anabolic nutrient processing, leading to increased rates of protein, nucleic acid and lipid biosynthesis and metabolism. Fatty acid

metabolism affects several important pathways involved in cancer progression including cellular signaling and energy processing [66]. The bioinformatics analyses employed here showed enrichments in various fatty acid metabolic pathways, particularly relating to fatty acid desaturation. Fatty acid desaturation by the delta-5 and delta-6 desaturases upregulated by Ets-1 in this study, fatty acid desaturase-1 and −2, result in the production of arachidonic acid, which is associated with breast cancer progression, metastasis and angiogenesis [67-69]. The metabolism of arachidonic acid by lipoxygenases and cyclooxygenases leads to increased ROS production, likely through the stimulation of nicotinamide adenine dinucleotide phosphate-oxidases, leading to the activation of the MAPK pathway, which in turn leads to increased cell proliferation [70]. Though we have not validated this association functionally, the upregulation of fatty acid desaturation by Ets-1 represents another potential mechanism by which this transcription factor enhances cancer progression, and warrants further investigation.

An inevitable consequence of cellular metabolism is the production of ROS, an effect that is amplified in cancer cells due to their altered metabolism, in addition to mitochondrial dysfunction and alterations in antioxidant pathways [16]. As a result of elevated levels of ROS, cancer cells are under high amounts of oxidative stress, leading to enhanced tumor progression. As a signaling molecule, ROS can activate several proliferative signaling pathways including MAPK/ERK, PI3K/Akt and NF-κB [16]. Thus, ROS encourage cancer proliferation, metastasis and angiogenesis, while also inducing apoptosis when left unchecked by cellular antioxidant systems. The pathway analyses in this study have shown common enrichment in metabolic and oxidoreductase pathways in

response to Ets-1 overexpression in ovarian cancer cells. These pathways are all important in regulating oxidative stress and cellular redox state, thus it is not surprising that we also observed decreased ROS levels and increased GPX activity in Ets-1 overexpressing cancer cells. GPXs are integral to the control of H_2O_2 in cells, and as such have dual pro- and anti-carcinogenic roles depending on the stage of tumor development.

Cancer initiation is facilitated by low levels of GPX enzymes leading to the failure to protect against DNA damage, resulting in genomic instability [71]. In established tumors, loss of GPX activity may promote proliferation and metastasis by allowing the ROS-mediated activation of associated signaling pathways. However, high levels of GPX activity would prevent cellular oxidative damage due to excessive ROS levels leading to improved cancer cell survival through the inhibition of ROS-induced apoptosis. In this study, we have observed the upregulation of GPX-1 and -2 protein expression, increased GPX enzyme activity, and decreased intracellular ROS in response to Ets-1 overexpression in ovarian cancer cells. In addition to being relevant to cell apoptosis, the upregulation of GPX1 is also associated with chemotherapeutic resistance in breast cancer [72,73]. GPX2 overexpression is associated with increased cancer cell proliferation through WNT signaling, resistance to apoptosis by reducing ROS, and enhanced growth in breast and intestinal cancers [74-77]. These findings suggest that Ets-1 is important in regulating cellular ROS levels, and thereby regulating cellular redox status and the response to oxidative stress in cancer cells. In further support of this theory, our laboratory has previously shown that Ets-1 transcription can be induced by nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in 2008 ovarian cancer cells [78]. As a master

regulator of redox state, Nrf2 initiates key antioxidant pathways to defend against oxidative stress. Notably, this factor is also increased in many types of cancer, and mutations that confer permanent stabilization of Nrf2 are frequently observed [79]. Considering the findings presented in this study, it is possible that Nrf2-mediated induction of Ets-1 is central to the regulation of antioxidant capacity in cancer cells, and thus this may be a promising focus for future studies.

CONCLUSIONS

In this study we have identified some novel pathway associations for Ets-1 transcriptional control, though further study is required to confirm the nature of the gene interactions therein. Interestingly, we have found that several enriched metabolic and oxidative stress pathways are differentially expressed in an ovarian cancer cell model of Ets-1 overexpression. The study of cancer metabolism is a rapidly emerging field in the context of cancer research, though the groundwork for this research was built almost half a century ago [80]. One of the main byproducts of metabolism is ROS, which are produced in excess in cancer cells due to high metabolic rates and mitochondrial dysfunction. We have previously shown that Ets-1 is transcriptionally activated by H_2O_2 in ovarian cancer cells via Nrf2 antioxidant response element binding within the Ets-1 promoter [78]. Aggressive cancer cells are often chronically exposed to high levels of oxidative stress, which could explain why Ets-1 is commonly upregulated in these cancers. Therefore, we suggest that increased levels of ROS produced from cancer cells result in the induction of

Nrf2 and subsequently Ets-1, which is then involved in a largely undefined transcriptional network that confers metabolic reliance on glycolysis and fat metabolism to fulfill the cancer cell's high energy needs. As ROS have such integral roles in cancer progression, an intimate understanding of the underlying mechanisms that achieve elevated levels of ROS will open the door to novel molecular avenues for drug development.

ABBREVIATIONS

2-DG, 2-Deoxy-D-glucose; AFU, Arbitrary fluorescent units; EGF, Epidermal growth factor; ERK, Extracellular-signal-regulated kinase; Ets, E-26; FDR, False discovery rate; FI, Function interaction; GPCR, G protein-coupled receptor; GPX, Glutathione peroxidases; GSEA, Gene set enrichment analysis; IC_{50}, Dose at which 50% of cells stop proliferating; MAPK, Mitogen-activated protein kinase; MMP, Matrix metalloproteinase; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; PDGF, Platelet-derived growth factor; qRT-PCR, Quantitative reverse-transcription polymerase chain reaction; ROS, Reactive oxygen species; WNT, Wingless-type

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

MV carried out bioinformatic analyses, all functional assays, and drafted the manuscript. CV participated in the gene ontology analysis and aided in statistical analysis. GS participated in the study design and coordination, and revised the manuscript. All authors read and approved the final manuscript.

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Tables

Annotation Cluster	Index	Term	Count	PValue	Fold Enrichment
1					
	INTERPRO	IPR010257:Fatty acid desaturase, type 1, N-terminal	4	0.001202952	15.75206612
	GOTERM_MF_ALL	GO:0016717~oxidoreductase activity (O or H2O as acceptor)	4	0.002076948	13.62682804
	INTERPRO	IPR005804:Fatty acid desaturase, type 1	4	0.00231522	13.12672176
	PFAM	PF00487:FA_desaturase	4	0.002376597	13.0085934
2					
	PANTHER_FAMILY	PTHR19353:FATTY ACID DESATURASE 2	3	0.006947572	20.39866667
	INTERPRO	IPR012171:Fatty acid/sphingolipid desaturase	3	0.007446662	19.69008264
	PIR_SUPERFAMILY	PIRSF015921:fatty acid desaturase/sphingolipid desaturase	3	0.007774128	19.21752266
	EC_NUMBER	1.14.99.25	3	0.009923174	16.91794872
	EC_NUMBER	1.14.19.-	3	0.009923174	16.91794872
	GOTERM_BP_ALL	GO:0006636~unsaturated fatty acid biosynthetic process	3	0.014165978	14.88516746
	GOTERM_BP_ALL	GO:0033559~unsaturated fatty acid metabolic process	3	0.014165978	14.88516746
3					
	KEGG_REACTION	R05053:D-ASPARTATE + D-4-HYDROXY-2-OXOGLUTARATE <=> OXALOACETATE + L-ERYTHRO-4-HYDROXYGLUTAMATE	9	0.011923332	2.778904665
	KEGG_COMPOUND	C05947:L-ERYTHRO-4-HYDROXYGLUTAMATE	9	0.012186929	2.759558824
	KEGG_COMPOUND	C05946:D-4-HYDROXY-2-OXOGLUTARATE	9	0.012186929	2.759558824
	KEGG_COMPOUND	C00036:OXALOACETATE	9	0.012186929	2.759558824
	KEGG_COMPOUND	C00402:D-ASPARTATE	9	0.012186929	2.759558824
4					
	GOTERM_MF_ALL	GO:0047115~trans-1,2-dihydrobenzene-1,2-diol dehydrogenase activity	3	0.006916259	20.44024206
	EC_NUMBER	1.1.1.213	3	0.009923174	16.91794872
	EC_NUMBER	1.3.1.20	3	0.009923174	16.91794872
	CYTOBAND	10p15-p14	3	0.039066046	9.172582619
5					
	PIR_SUPERFAMILY	PIRSF001191:matrix metalloproteinase, stromelysin type	5	0.012200314	5.338200739
	INTERPRO	IPR002477:Peptidoglycan binding-like	5	0.016599595	4.922520661
	PFAM	PF01471:PG_binding_1	5	0.017111321	4.878222524
	PANTHER_FAMILY	PTHR10201:MATRIX METALLOPROTEINASE	5	0.024008203	4.434492754
	INTERPRO	IPR000585:Hemopexin	5	0.031041653	4.102100551
	PFAM	PF00045:Hemopexin	5	0.031952698	4.065185436
	INTERPRO	IPR006026:Peptidase, metallopeptidases	5	0.040347894	3.786554355
	GOTERM_BP_ALL	GO:0000270~peptidoglycan metabolic process	5	0.049792017	3.544087491
6					
	INTERPRO	IPR001314:Peptidase S1A, chymotrypsin	11	0.022004661	2.279904306
	INTERPRO	IPR001254:Peptidase S1 and S6, chymotrypsin/Hap	11	0.03597926	2.10282436
	PANTHER_FAMILY	PTHR19355:SERINE PROTEASE-RELATED	11	0.036738405	2.09705919
	PFAM	PF00089:Trypsin	11	0.037915127	2.083900884
7					
	INTERPRO	IPR001039:MHC class I, alpha chain, alpha1 and alpha2	5	0.019701837	4.688114915
	PFAM	PF00129:MHC_I	5	0.023834409	4.434747749
	PANTHER_FAMILY	PTHR16675:MHC CLASS I-RELATED	5	0.031765674	4.079733333
	INTERPRO	IPR011161:MHC class I-like antigen recognition	5	0.035518071	3.938016529
	GOTERM_CC_ALL	GO:0042612~MHC class I protein complex	5	0.036360752	3.907922272
8					
	INTERPRO	IPR010579:MHC class I, alpha chain, C-terminal	3	0.014393244	14.76756198
	PFAM	PF06623:MHC_I_C	3	0.014647426	14.63466757

Table 3.1: Functional annotation clustering of genes associated with Ets-1 overexpression

Figures

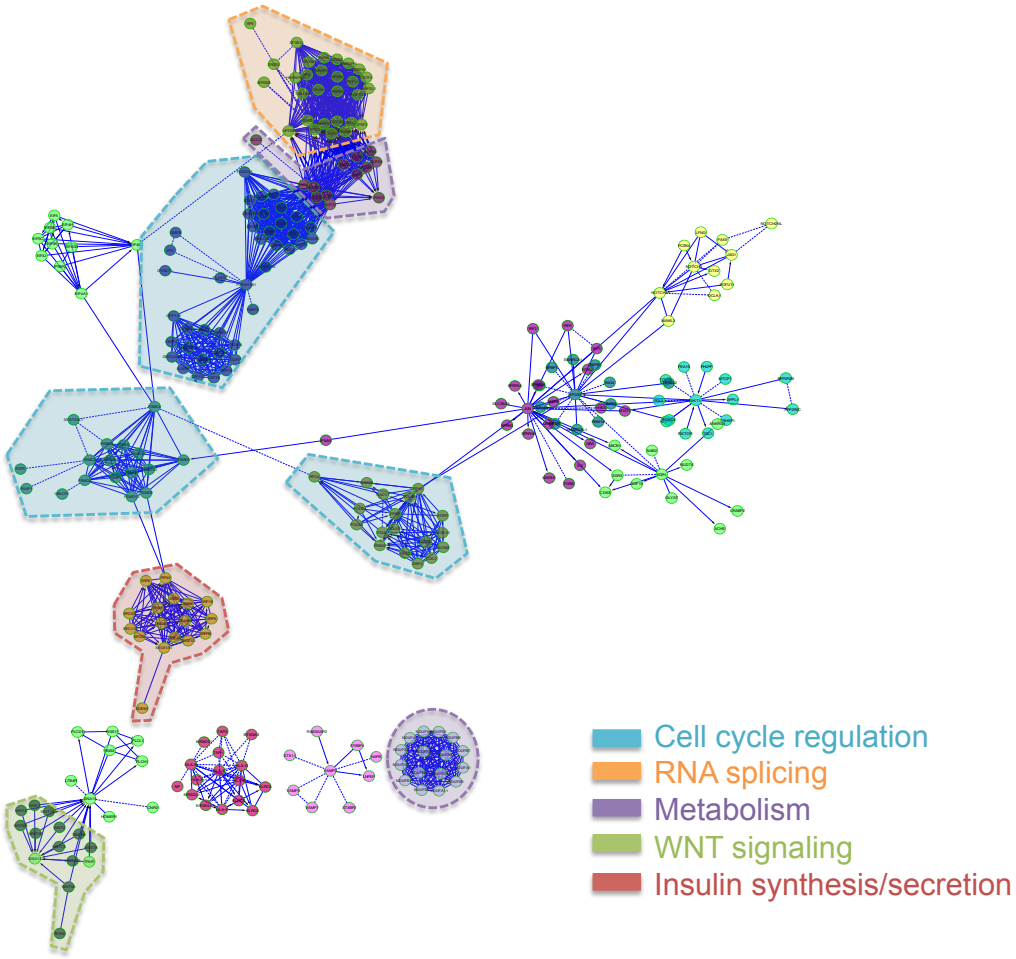

Figure 3.1: Global functional interaction network.

Reactome FI was used to create a functional interaction network, which is divided into modules as defined by node colouring, and further delineated by functional pathway enrichments into outlined, colour-coded groups. Major associations among the curated and predicted interactions include cell cycle regulatory, RNA slicing, metabolism, WNT

signaling, and insulin-related pathways (FDR <0.01). Network was generated in Cytoscape.

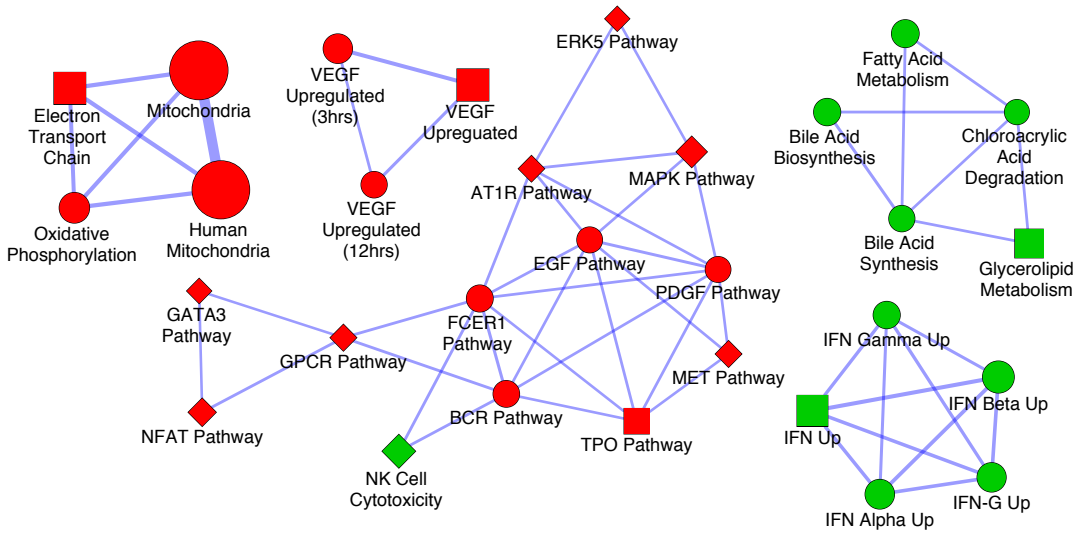

Figure 3.2: Enrichment map of GSEA analysis.

The map shows enriched gene sets in 2008 vs. 2008-Ets1 ovarian cancer cells clustered by MCODE to generate subnetworks of the interrelated gene sets. Red nodes indicate enrichment (upregulation) in 2008 cells, while green nodes represent enrichment (upregulation) in 2008-Ets1 cells. Node size is representative of the number of enriched genes in the gene set. The largest cluster includes the signaling pathways of ERK5, MAPK, EGF, PDGF, MET, and GPCR. Notably, clusters with gene sets involved in mitochondrial metabolism and fatty acid metabolic processes were also identified. Network was generated in Cytoscape.

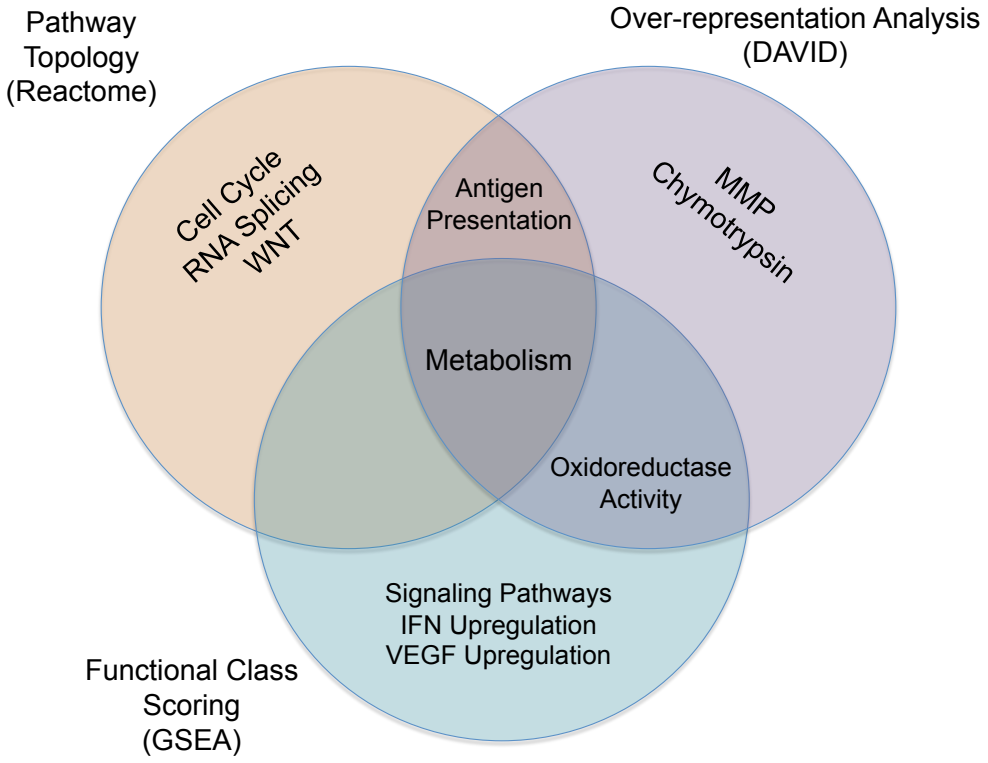

Figure 3.3: Integrating various bioinformatic analyses.

Venn diagram representing the overlapping enrichments from the various bioinformatic pathway analyses (Figures 1 and 2, Table 1) employed on the microarray expression data. The functional interaction network and ontological analyses both included enrichments in antigen presentation, while the ontological analysis shared oxidoreductase activity

enrichment with the gene set enrichment analysis. All three analyses found enrichments in various metabolic pathways.

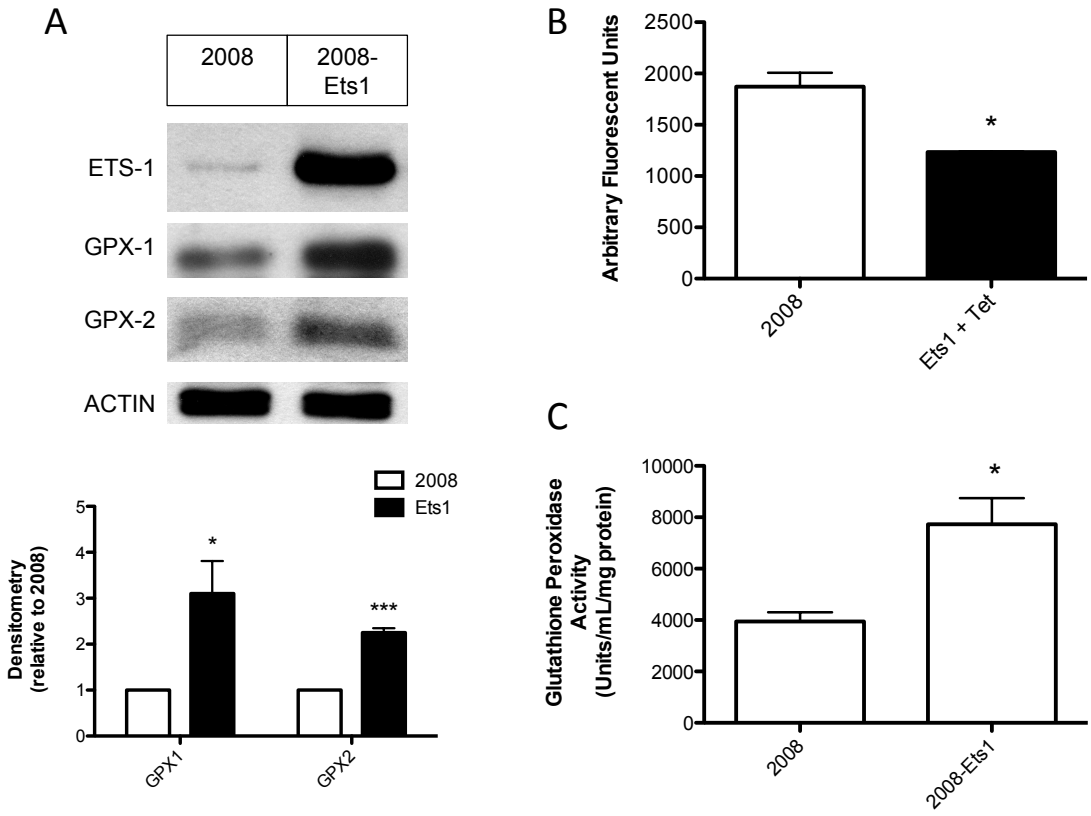

Figure 3.4: Ets-1 regulated oxidative stress in ovarian cancer cells.

(A) The protein expression of Ets1, GPX-1, and GPX-2 were examined via Western blot, and normalized to Actin expression by densitometry analysis. Ets-1 overexpressing cells show 3.10-fold and 2.25-fold inductions in GPX-1 and GPX-2 protein levels, respectively (n=3). (B) Intracellular ROS levels were measured using the fluorescent CM_2-H_2DCFDA reagent in ovarian cancer cells. 2008-Ets1 cells contain lower ROS levels than 2008 cells (1233.99 AFU and 1872.71 AFU, respectively). (C) The activity of glutathione peroxidase enzymes was measured using a colourimetric assay, where Ets-1

overexpressing cells were observed to have significantly higher activity than parental cells (7725.66 U/mL/mg and 3944.22 U/mL/mg, respectively).

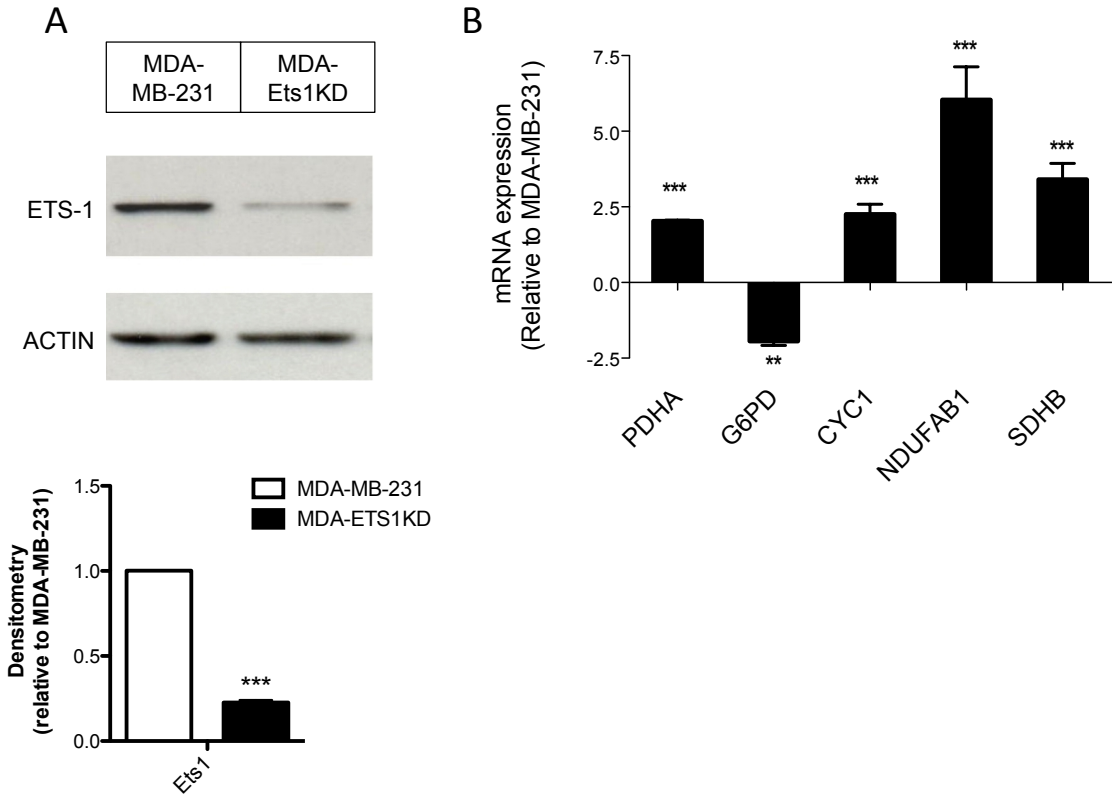

Figure 3.5: Breast cancer cell model of Ets-1 expression knockdown.

MDA-MB-231 breast cancer cells were stably depleted of Ets-1 expression via targeted shRNA knockdown. (A) The Ets-1 knockdown cell line MDA-Ets1KD expresses Ets-1 protein at 22.7% of parental protein levels in MDA-MB-231 cells (n=3). (B) Real time qRT-PCR of the breast cancer Ets-1 expression model. The gene expression of PDHA, CYC1, NDUFAB1, and SDHB were increased in MDA-Ets1KD cells, while the expression of G6PD was downregulated in MDA-Ets1KD cells.

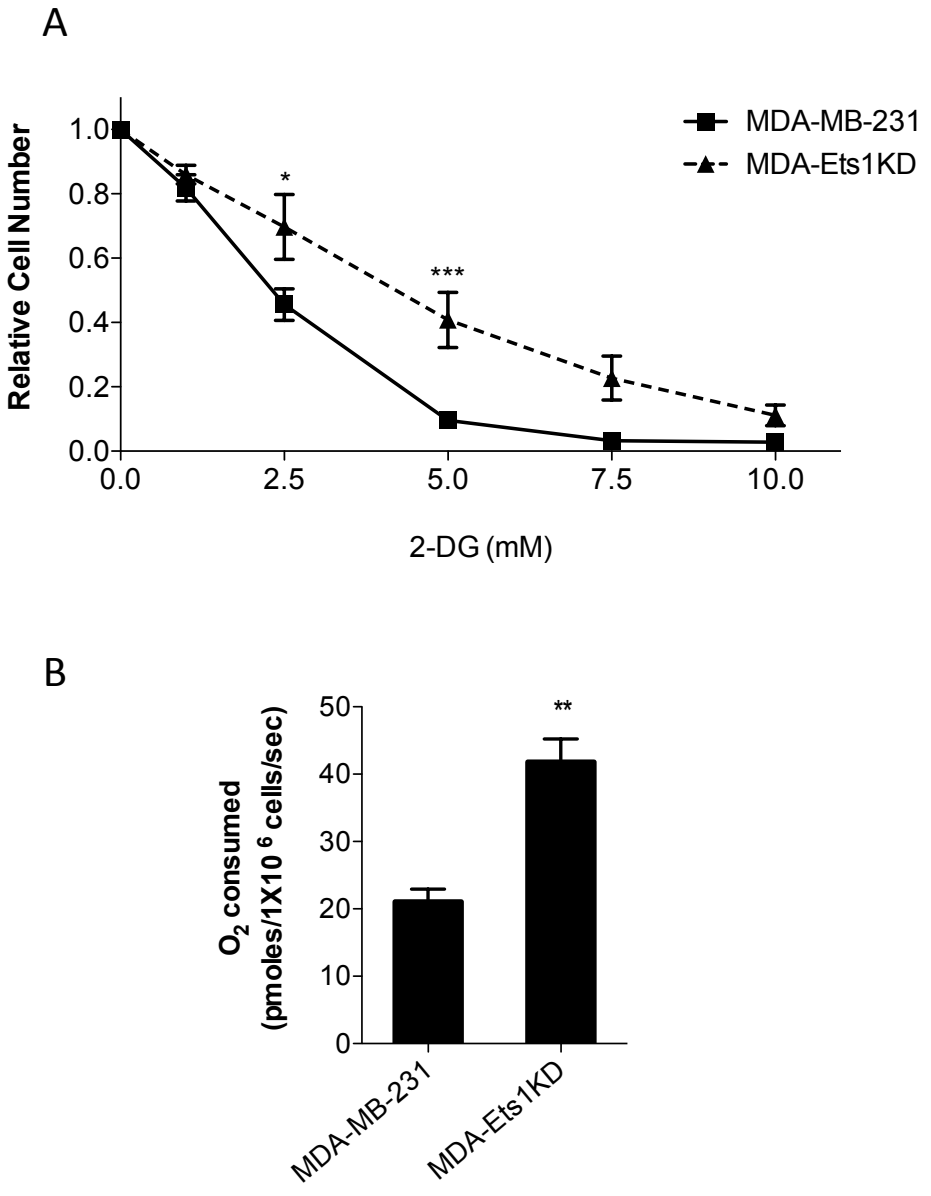

Figure 3.6: Effect of Ets-1 knockdown on breast cancer cell metabolism.

(A) MDA-MB-231 and MDA-Ets1KD cells were treated with various amounts the glycolytic inhibitor 2-DG, and representative growth curves were generated for each cell

line. The 2-DG IC_{50} was greater in MDA-Ets1KD cells with an IC_{50} of 3.94mM compared to the IC_{50} 2.03mM for MDA-MB-231 cells. (B) Basal oxygen consumption was measured using high-resolution respirometry, and MDA-Est1KD cells were observed to consume significantly more oxygen (41.80 pmoles/10^6 cells/sec) than parental MDA-MB-231 cells (21.07 pmoles/10^6 cells/sec).

Chapter 4: Mitochondrial-generated reactive oxygen species in cancer

This chapter includes an author-generated version of the review paper entitled "Mechanisms associated with mitochondrial-generated reactive oxygen species in cancer" published in the *Canadian Journal of Physiology & Pharmacology*. The paper is reproduced with permission from the **NRC Press**, as stated on the copyright agreement form:

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For this manuscript, I performed the literature review, generated diagrams and wrote the text in equal collaboration with Dr. Leigh Wilson. The manuscript sections written by Dr. Leigh Wilson were extracted from her doctoral thesis entitled "The role of the transcription factor Ets-1 in mitochondria to nucleus signaling" (2005), which I also updated with recent references and thoroughly revised. Dr. Gurmit Singh provided intellectual direction and revised the manuscript.

Context and background

The focus of this review paper is the importance of the mitochondria with respect to ROS in cancer cells, with a specific focus on nuclear crosstalk and transcriptional regulation.

My novel observation that Ets-1 regulates metabolism in 2008 ovarian cancer cells (Chapter 2) led to the further characterization of the role of Ets-1 in these cancer cells (Chapter 3), which illustrated the great importance of Ets-1 in cancer metabolism. The major byproducts of metabolism are ROS generated by the mitochondria, and thus developing a more in depth understanding of the mechanisms associated with mitochondrial ROS was my next logical step.

Though the mitochondria possesses it's own genome, coordinated transcription and translation with the nucleus are necessary for the transcription of several ETC components, as well as for mitochondrial DNA replication and transcription (Butow & Avadhani, 2004; Scarpulla, 2002). The crosstalk between the mitochondria and nucleus, called retrograde regulation, is achieved through ROS that activate key signaling pathways. In cancer cells, mitochondrial signaling is altered due to mutations in the mitochondrial genome resulting in changes to major signaling pathways and leading to increased glycolytic dependence. Mitochondrial-generated ROS affect proliferation and survival via the MAPK pathway, and profoundly alter the activities of several transcription factors, including Ets-1. All of these actions combine to greatly enhance the cancer phenotype, and highlight the significant importance of these molecules in cancer-associated signaling. Furthermore, by developing a better understanding of how ROS regulate transcription factor signaling and the feedback between transcriptional regulation and ROS levels, novel preventative and therapeutic strategies could be developed.

PAPER: Mitochondrial-generated reactive oxygen species in cancer

Title: Mechanisms associated with mitochondrial-generated reactive oxygen species in cancer

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Abstract

The mitochondria are unique cellular organelles that contain their own genome and, in conjunction with the nucleus, are able to transcribe and translate genes encoding components of the electron transport chain (ETC). In order to do so, the mitochondria must communicate with the nucleus via the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), which are produced as a byproduct of aerobic respiration within the mitochondria. Mitochondrial signaling is proposed to be altered in cancer cells, where the mitochondria are frequently found to harbor mutations within their genome and display altered functional characteristics leading to increased glycolysis. As signaling molecules, ROS oxidize and inhibit MAPK phosphatases resulting in enhanced proliferation and survival, an effect particularly advantageous to cancer cells. In terms of transcriptional regulation, ROS affect the phosphorylation, activation, oxidation, and DNA binding of transcription factors such as AP-1, NF-κB, p53, and HIF-1α, leading to changes in target gene expression. Increased ROS production by defective cancer cell mitochondria also results in the upregulation of the transcription factor Ets-1, a factor that has been increasingly associated with aggressive cancers.

Keywords: Mitochondria, reactive oxygen species, cancer, nuclear crosstalk, transcription factors, hypoxia, mitochondrial signaling, ETS

Introduction

Several important functional changes to cancer cell mitochondria have been observed which implicate this organelle in tumour formation, including the increased production of reactive oxygen species (ROS), decreased oxidative phosphorylation and a corresponding increase in glycolysis (Carew and Huang, 2002; Chen et al., 2009) (Figure 1). The increased production of ROS, including superoxide (SO) and hydrogen peroxide (H_2O_2), by cancer cells is likely a result of mitochondrial dysfunction, as the mitochondria are the major cellular source of ROS. Four to five percent of the oxygen (O_2) consumed by oxidative phosphorylation in the mitochondria is converted to ROS under normal conditions, therefore defects to the electron transport system in cancer cells would likely result in increased levels of ROS formation (Konstantinov et al., 1987; Szatrowski and Nathan, 1991; Wallace, 2008). The elevation of ROS in several different cancer cell types can be inhibited by catalase, a specific H_2O_2 detoxifying enzyme, implying that H_2O_2 is the predominant ROS over-produced in cancer cells (Hyoudou et al., 2009; Petit et al., 2009; Szatrowski and Nathan, 1991).

An imbalance of antioxidant enzymes has been observed in many cancer cell types, which would result in added oxidative stress within the tumour environment (Gokul et al., 2009; Oberley and Oberley, 1997; Sinha et al., 2009). In both mouse and human cancer cell lines, high levels of malignancy were associated with increased H_2O_2-generating superoxide dismutase activity, as well as decreased catalase and H_2O_2-detoxifying glutathione peroxidase activities (Policastro et al., 2004). Similar antioxidant

enzyme imbalances have been found in melanoma (Picardo et al., 1996), lung cancer (Chung-man Ho et al., 2001), prostate cancer (Arsova-Sarafinovska et al., 2009), thyroid cancer (Akinci et al., 2008), and breast cancer (Policastro et al., 2004)

Considering the sheer number and variability of the alterations seen in cancer cell mitochondria (summarized in Figure 1), the role of mitochondria in tumourigenesis warrants further investigation. Results from the studies mentioned above highlight the importance of mitochondrial DNA (mtDNA) mutations and mitochondrial dysfunction in the progression of tumourigenesis, therapeutic resistance, and prominently the enhancement of invasiveness and metastatic potential. However, the key question that remains unanswered is whether these mitochondrial changes are a secondary effect of tumourigenesis, or if they play key roles in tumour promotion and initiation. Thus future studies should focus on the molecular mechanisms that facilitate these changes, as well as precisely how these dysfunctions affect tumour formation and progression.

Mitochondrial ROS

The increased production of ROS observed in tumour cells may be linked to mitochondrial injury as this organelle represents the main cellular source of ROS in the majority of mammalian cells (Klaunig and Kamendulis, 2004). The production of ROS is an unavoidable consequence of aerobic metabolism, where an estimated 4-5% of O_2 consumed in aerobic reactions is converted to ROS (Klaunig and Kamendulis, 2004). This conversion occurs when electrons escape from the electron transport chain (ETC) to

react with O_2 and produce SO, which is converted to H_2O_2 by superoxide dismutase (Barber and Harris, 1994; Betteridge, 2000; Murphy, 2009). Though there are at least nine possible mitochondrial sites of electron leakage and consequent ROS production, the most well described sites are complexes I, and III of the ETC (Cadenas and Davies, 2000; Andreyev et al., 2005; Murphy, 2009).

Complex I, or NADH-ubiquinone oxidoreductase, is located within the inner mitochondrial membrane where it oxidizes NADH derived from the tricarboxylic acid cycle (TCA) and utilizes coenzyme Q as an electron acceptor (Andreyev et al., 2005). The generation of SO and other ROS from complex I is considered to be the most physiologically relevant mitochondrial site of ROS production via electron leakage (Liu et al., 2002; Murphy, 2009; Sipos et al., 2003). By examining the production of H_2O_2 from mitochondria isolated from rat liver in the presence of various respiratory substrates and inhibitors, it was observed that succinate, a FAD-linked substrate which enters the ETC at complex II, was responsible for high H_2O_2 production in the absence of inhibitors (Liu et al., 2002). Originally, this succinate-driven H_2O_2 production was thought to be via electron leakage at complex III, however it was found that rotenone, a specific inhibitor of complex I, abolished this effect. Therefore it was determined that the succinate-driven H_2O_2 production was due to reverse electron transfer along the ETC in the absence of adenosine diphosphate (ADP), thus resulting in electron leakage at complex I (Liu et al., 2002). Additionally, the inhibition of both complexes I (rotenone), and III (antimycin) led to increases in H_2O_2 production in synaptic mitochondria (Sipos et al., 2003).

Other sources of ROS

Although the mitochondria represents the major source of cellular ROS, there are alternative sources of ROS production present elsewhere within the cell. In the endoplasmic reticulum, unsaturated fatty acids and xenobiotics are oxidized by cytochrome P450 and b_5 enzymes to produce O_2^- and H_2O_2 (Csordas and Hajnoczky, 2009; Timothy et al., 2007). Peroxisomes oxidize intracellular NADPH to reduce O_2 to O_2^- via membrane-associated NADPH oxidases, as well as toxic molecules including ethanol to H_2O_2 by peroxisomal catalase, although most of the ROS generated here does not leave the peroxisome (Bonekamp et al., 2009; Timothy et al., 2007). Catalytic cycling within the cytoplasm may also generate ROS by various soluble oxidases, dehydrogenases, and dioxygenases (Timothy et al., 2007).

ROS as nuclear signaling molecules

Mitochondrial-generated ROS are proposed to behave as initiation factors for many important signaling pathways in which a mitochondrial signal induces changes in nuclear gene expression, thus influencing overall cellular functioning (Scarpulla, 2002). Though the mitochondria contain their own genome, which encodes several components of the ETC, several tRNAs and some rRNAs needed for gene transcription and translation, this organelle has a functional requirement to coordinate with nuclear encoded

genes. Important nuclear genes involved in mitochondrial-nuclear communications, or retrograde regulation, include those that encode several other ETC component genes, as well as factors involved in mitochondrial DNA replication and transcription (Scarpulla, 2002; Butow and Avadhani, 2004). This type of compensatory gene regulation by the nucleus would be necessary following damage to or inhibition of the mitochondria itself, and likely occurs in cancer cells with damaged mitochondria.

In order to investigate the genes involved in retrograde regulation, cells lacking mtDNA called rho^0 cells were developed and have been extensively studied (Higuchi, 2007; Wang and Morais, 1997). Clear evidence for the occurrence of some form of mitochondria to nucleus communication in rho^0 breast cancer cells was obtained from microarray analysis comparing nuclear gene expression in a variant of MDA-MB-435 breast cancer cells lacking mtDNA to that in parental cells (Delsite et al., 2002). Several groups of genes were noted to be altered in the rho^0 variant including an up-regulation of genes involved in cell cycle arrest, transcription factors and signaling molecules such as phospholipase c, whereas genes involved in fatty acid signaling, cytochrome p450 were downregulated (Delsite et al., 2002). Human breast cancer cells depleted of mtDNA have also been found to display an enhanced tumourigenic phenotype both *in vitro* and in xenograft models, likely due to differentially regulated proteins in the fibronectin and p53 gene networks (Kulawiec et al., 2008). Recently, human breast and prostate cancer cells with depleted mtDNA were found to exhibit hormone-independent growth, thus inducing the epithelial-mesenchymal transition of cancer cells and ultimately resulting in more aggressive cancer phenotypes (Naito et al., 2008).

Similar results have been observed in cells treated with specific inhibitors of various ETC components, including antimycin A that specifically inhibits complex III. Inhibition of complex III by antimycin A causes an up-regulation of both mitochondrial and nuclear encoded ETC components, suggesting that mitochondrial stress causes coordinated changes in both nuclear and mitochondrial gene expression (Suzuki et al., 1998). These coordinated alterations in nuclear gene expression as a result of depletion of mtDNA or inhibition of ETC function indicate that there is a mitochondrial-derived signal driving these nuclear changes. Mitochondrial-generated ROS are a likely candidate for this retrograde regulation as increased ROS production is a major result of inefficient electron transport (Finley and Haigis, 2009; Suzuki et al., 1998). ROS can be further implicated as the stimulus for retrograde regulation because genes involved in antioxidant defense are upregulated in both rho^0 and respiratory deficient cells (Brambilla et al., 1997; Miceli and Jazwinski, 2005).

Mechanism of retrograde regulation

Regardless of the nature of mitochondrial-generated signal, there remains a requirement for factors that regulate the expression of both nuclear and mitochondrial genes if retrograde regulation is to effectively modulate mitochondrial biogenesis in response to environmental changes. Such a system has been well described in yeast, particularly with regard to the regulation of citrate synthase isoform CIT2, whose gene expression is increased in cells with damaged mitochondria (Iurina and Odintsova, 2008).

CIT2 upregulation involves Rtg positive regulatory factors, as well as a group of negative regulatory factors (Mks1, Lst8p, Bmh1p, Bmh2p) (Iurina and Odintsova, 2008). Rtg2p is a sensor of mitochondrial dysfunction, that acts to dephosphorylate an Rtg1p/3p heterodimer and allow its nuclear localization, resulting in activation of target genes with R-box promoter elements (Butow and Avadhani, 2004). In mammalian cells however, the pathway for retrograde regulation has yet to be clearly elucidated, although several factors are thought to be important in mediating a nuclear response to mitochondrial stress (Finley and Haigis, 2009). Nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2/GABPα), as well as co-activator peroxisome-proliferator activated receptor γ co-activator-1 (PGC-1) may regulate a coordinated nuclear and mitochondrial response given that these transcription factors regulate the expression of several ETC component nuclear genes (Scarpulla, 2008; Scarpulla, 2002; Vercauteren et al., 2009; Vercauteren et al., 2008). Additionally, factors involved in mediating mitochondrial DNA replication and transcription could be important in controlling the nuclear response to mitochondrial stress (Scarpulla, 2002).

NRF-1 was first identified following analysis of the *cytochrome c* promoter where a novel factor was found to bind to an enhancer region required for maximal promoter activity (Evans and Scarpulla, 1989). This binding factor was recognized to be unique in humans, and generated from a single copy gene in vertebrates with no known family members. However, NRF-1 is related to developmental regulatory proteins in sea urchins and homologues in drosophila, chicken, zebra fish and mouse have been identified (Scarpulla, 2002). NRF-1 binds its recognition site as a homodimer, and acts as a

transcription factor via a transactivation domain within its carboxy terminus (Virbasius et al., 1993). NRF-1 consensus binding sites have been found in several other genes encoding either components of the ETC or factors involved in mtDNA replication and transcription including succinate dehydrogenase subunits, several cytochrome oxidase subunits, ATP synthase γ and c subunits, mitochondrial transcription factors mtTFA and mtTFB, and mitochondrial ribosomal subunit S12 (Dhar et al., 2008; Piantadosi and Suliman, 2006; Scarpulla, 2002). The regulation of mtTFA as well as several nuclear encoded respiratory subunits indicates that NRF-1 may be an important factor in leading a coordinated response of the nucleus and the mitochondria to environmental stress.

Shortly after the discovery of NRF-1, a second respiratory factor responsible for directing expression of several key respiratory subunits was identified when the NRF-1 independent promoter for the cytochrome oxidase subunit IV gene was analyzed (Virbasius and Scarpulla, 1991). Several Sp1 binding sites are located in the 5' promoter region of the cytochrome oxidase subunit IV enhancer, in addition to a repeated sequence that was critical for activity and bound a novel nuclear factor, NRF-2. This repeated sequence corresponded to a core E26 transformation specific (ETS) binding sequence, and the newly identified NRF-2 was determined to be capable of binding a well characterized EBS sequence, as well as the ETS sequence within the promoter, indicating that NRF-2 is an ETS family member (Virbasius and Scarpulla, 1991). NRF-2 has since been identified as the human homologue of mouse GABP (GA binding protein), GABPα, a unique two-protein obligate heterotetramer ETS transcription factor involved in cell cycle control, protein synthesis, and cellular metabolism (Gugneja et al., 1995; Kang et

al., 2008; Rosmarin et al., 2004). Genes regulated by GABPα/NRF-2 include several cytochrome oxidase subunits, ATP synthase β, succinate dehydrogenase subunits, mtTFA, mtTFB, and mitochondrial ribosomal S12. Most genes regulated by GABPα/NRF-2 also contain an NRF-1 recognized site, however there are some examples of uniquely GABPα/NRF-2 regulated genes (Scarpulla, 2002). GABPα/NRF-2 and NRF-1 knockout models are embryonic lethal, indicating they are essential factors and cannot be replaced by other ETS proteins (Ristevski et al., 2004; Scarpulla, 2008). NRF-1 and GABPα/NRF-2 regulate a myriad of other genes besides those involved in respiratory chain function, including enzymes involved in purine nucleotide biosynthesis, chemokine, neural and poliovirus receptors, as well as myeloid specific genes, interleukin-1 (IL-1) receptor enhancer, interleukin-16 (IL-16), and the nicotine acetylcholine receptor (Scarpulla, 2002; Rosmarin et al., 2004).

The coordinated nuclear and mitochondrial response to mitochondrial stress may be directed by NRFs, suggesting that the expression and/or activity of these factors is responsive to mitochondrial-generated signals. In the case of NRF-1, phosphorylation is required for translocation to the nucleus and may be induced by increased oxidative stress generated by dysregulated mitochondria (Gugneja and Scarpulla, 1997). Lipopolysaccharide (LPS), an agent that depletes glutathione, stimulates ROS generation and has a deleterious effect on hepatic mitochondria, was found to increase NRF-1 protein expression and binding activity in hepatic cells (Suliman et al., 2003). Rho^0 cells derived from NRF-1-expressing cells displayed downregulated NRF-1 and mitochondrial-generated ROS (Suliman et al., 2003). The loss of both mitochondrial ROS generation

and NRF-1 expression were restored following oxidant stimulation via PI3K/Akt activation, suggesting mitochondrial ROS-mediated regulation was responsible for this effect (Suliman et al., 2003). The induction of mitochondrial ROS by estrogen in several human breast cancer cell lines has also been linked to the phosphorylation and activation of NRF-1 via PI3K/Akt activation, illustrating the importance of NRF-1 in cancer (Suliman et al., 2003; Felty et al., 2005).

In contrast to the documented induction of NRF-1, mitochondrial ROS appear to have an opposite effect on the activity of GABPα/NRF-2. Treatment of mouse fibroblasts with oxidizing agents led to a diminished binding of GABPα/NRF-2 to target gene promoters, an effect rescued with antioxidant treatment (Martin et al., 1996). This loss of binding was also seen following administration of the oxidized form of glutathione, and both effects were linked to a cysteine residue within the DNA binding domain of GABPα/NRF-2 that was defined as the redox sensitive site (Martin et al., 1996). Additional regulators of GABPα/NRF-2 activity include depolarizing stimulation by potassium chloride (KCl) in neurons that leads to an up-regulation of both GABP subunits, as well as thyroid hormone that transcriptionally up-regulates NRF-2 but not NRF-1 (Zhang and Wong-Riley, 2000; Rodríguez-Peña et al., 2002). Though these two nuclear respiratory factors may react in opposite ways, they are both able to respond to mitochondrial stress in the form of ROS to affect changes in nuclear gene expression.

Effect of ROS on signaling pathways

The basic mechanism by which cells are induced to proliferate involves the interaction of growth factors with receptors on the cell membrane. Upon interaction, downstream protein tyrosine kinases or proximal membrane phospholipases are activated, leading to mitogen-activated protein kinase (MAPK) stimulation, and finally the activation of proteins directly involved in proliferative control (Burdon, 1995; Wagner and Nebreda, 2009). The mitogenic effect of SO and/or H_2O_2 has been observed in a range of cell types, where low concentrations of these ROS stimulate growth of cells including rat and human fibroblasts, various mouse cell lines, human leukemia and smooth muscle cells (Burdon, 1995; Day and Suzuki, 2005). Our research group has previously showed that the growth of rat fibroblast cells is inhibited by the scavenging of H_2O_2 by catalase, implicating mitochondrial ROS in the proliferative response (Preston et al., 2001). The concept that H_2O_2 behaves as a growth factor is now widely accepted and studied, where examples include interactions with receptors, tyrosine and serine/threonine kinases, phosphatases, as well as transcription factors (Burdon, 1995; Day and Suzuki, 2006; Lander, 1997; Kamata and Hirata, 1999).

H_2O_2 can also directly regulate receptor-mediated effects by acting on downstream proteins. The platelet derived growth factor (PDGF) receptor (PDGFR) is an example of this response where vascular smooth muscle cells *in vitro* display spikes in H_2O_2 levels that occur following PDGF addition, which are required to initiate PDGF-induced downstream events (Sundaresan et al., 1995). The role of H_2O_2 as an activator of the PDGFR has also been demonstrated in knockout mouse embryonic fibroblasts using peroxiredoxin II (perII), which has a high affinity for H_2O_2 and is capable of eliminating

this ROS in response to PDGF stimulation (Choi et al., 2005). In the absence of perII, PDGF stimulation caused a two-fold increase in H_2O_2 production, in addition to increased tyrosine phosphorylation of two distinct PDGFR residues when compared to wild type cells (Choi et al., 2005). H_2O_2 administration to the PDGF, epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) receptors, stimulates growth factor receptor transactivation and subsequent activation of downstream events independent of the receptor ligands (Chen et al., 2004). Interestingly, this outcome appears to be dependent on mitochondrial function because inhibition of the ETC abolishes H_2O_2-induced transactivation of target receptors (Chen et al., 2004). These findings suggest that ROS create a feedback loop that stimulates the generation of mitochondrial ROS, which directly manipulates growth factor receptors.

The main method by which H_2O_2 affects key proteins involved in signaling pathways is likely through the oxidation and modification of cysteine residues within these target proteins. ROS can cause the formation of intra- or inter-molecular disulfide bonds between cysteine residues leading to altered protein conformation, which may then affect association with other proteins (Adler et al., 1999; Han et al., 2009). The mechanism by which increased levels of H_2O_2 increase phosphorylation at PDGFR sites appears to be via the oxidation and resulting inactivation of PDGF-specific phosphatases within the membrane fraction of cells (Lee and Esselman, 2002). Generally, protein tyrosine phosphatases (PTPs) contain an essential catalytic cysteine residue subject to inhibition by H_2O_2, which may explain the observed H_2O_2-induced activation of MAPK enzymes (Lee and Esselman, 2002).

The effect of H_2O_2 on MAPK enzymes was clearly demonstrated in a study done using mouse fibroblast cells where H_2O_2 treatment led to a rapid, transient phosphorylation of several cellular proteins prompting an evaluation of cellular kinase activity (Guyton et al., 1996a). Consequent kinase activity assays indicated that the activity of MAPK family members c-Jun-NH2 terminal kinase (JNK) and p38 kinase were increased by 3-5 fold following H_2O_2 treatment, while extracellular signal-related kinase (Erk) activity showed a striking 25 fold increase (Guyton et al., 1996a). These observations were further confirmed in a range of cell lines, and have been attributed to an inhibition of phosphatase activity. T lymphocytes treated with H_2O_2 had reduced activity of T cell specific PTPs (CD45, SHP-1 and HePTP0), where SHP-1 was involved in the inhibition of Erk and JNK phosphorylation (Lee and Esselman, 2002). Additionally, HePTP was involved in the inhibition of Erk and p38 phosphorylation in response to H_2O_2, suggesting distinct roles for phosphatases in the regulation of H_2O_2-induced activation of different MAPK enzymes (Lee and Esselman, 2002). The serine/threonine protein phosphatase PP2A has also been implicated as an H_2O_2 target involved in MAPK activation in rat brain fractions, where MAPK phosphatase activity was largely attributable to PP2A and reversibly inhibited by H_2O_2 (Foley et al., 2004).

However, MAPK enzymes are not the only kinase targets activated by H_2O_2. Growth factor stimulation also leads to the activation of PI3K resulting in PI 3,4,5-triphosphate (PIP3) production, which in turn activates downstream targets like the protein kinase Akt. In growth factor-stimulated cells containing enzyme-inactivated phosphatase and tensin homologue detected chromosome ten (PTEN), inhibition of PIP3

was observed (Kwon et al., 2004). The H_2O_2 produced by these cells caused PIP3 production and Akt activation, an effect that was later confirmed to occur by means of mitochondrial-generated ROS (Kwon et al., 2004; Connor et al., 2005). Additionally, cell lines that over-express mitochondrial superoxide dismutase (MnSOD), and thus produced elevated levels of H_2O_2, exhibit enhanced PTEN oxidation. Enhanced PTEN oxidation results in the accumulation of PIP3 and activation of Akt, effects which were reversible following the administration of the anti-oxidant catalase (Figure 2A)(Connor et al., 2005).

Effects of ROS on transcription factors

In addition to influencing phosphatase activity, the signaling pathways activated by H_2O_2 ultimately effect the phosphorylation, activation, oxidation and DNA binding ability of various transcription factors including activator protein 1(AP-1), nuclear factor kappa B (NF-κB), p53, and hypoxia-inducible factor-1 alpha (HIF-1α).

AP-1, a heterodimer composed of Jun and Fos protein family members, is a redox-sensitive transcription factor with many gene targets capable of inducing cell proliferation (Mattie et al., 2008; Shaulian and Karin, 2001). H_2O_2-induced activation of MAPK enzymes results in AP-1-induced proliferation due to MAPK-mediated c-Jun phosphorylation, a key event in AP-1 activation (Klaunig and Kamendulis, 2004). The DNA binding of the c-Jun/c-Fos heterodimer is however negatively regulated by oxidation, as a highly conserved cysteine residue modulates DNA binding (Abate et al., 1990; Cosse et al., 2009). Recently a study using chemically disrupted breast cancer cells

that display enhanced ROS generation, cellular motility and invasiveness, discovered a novel pathway of ROS-mediated activation of AP-1 that promotes cancer cell motility (Pelicano et al., 2009). The highly malignant behaviour of these cells was inhibited by the administration of the antioxidant NAC, suggesting that ROS play a critical role in this aggressive phenotype. Subsequent gene expression analysis revealed an increased expression of a novel chemokine, CXCL14, which was enhanced by exogenous ROS and attenuated by an antioxidant (NAC)(Pelicano et al., 2009). Additionally, a CXCL14-specific DNA decoy and the JNK inhibitor SP00125 greatly decreased CXCL14 expression, indicating that the expression of this gene is induced by ROS-mediated activation of AP-1 (Pelicano et al., 2009). The enhanced expression of CXCL14 was found to induce Ca^{2+} leakage from the endoplasmic reticulum, causing increased cytosolic Ca^{2+} and stimulating cytoskeleton assembly, and ultimately enhancing cancer cell motility and metastasis (Pelicano et al., 2009).

NF-κB has long been recognized as an oxidant-sensitive transcription factor, however there are conflicting views about whether the overall effect of an oxidative environment will activate or inhibit the activity of this factor. NF-κB is an inducible transcription factor dimer composed of Rel proteins p65 and p50, and is involved in the activation of genes required for cell survival, differentiation, inflammation, and growth (Chen et al., 2001; Shen and Tergaonkar, 2009). This transcription factor is typically sequestered in the cytoplasm by an inhibitory complex, inhibitory factor kappa B (IκB), which may be dissociated via phosphorylation in response to H_2O_2. Dissociation of the IκB/NF-κB complex reveals the nuclear localization signal of NFκB, allowing nuclear

localization and activation of NF-κB target genes (Pahl, 1999). The activation of NF-κB target genes can be blocked with several antioxidant treatments, indicating that NF-κB activity is stimulated by H_2O_2 (Nomura et al., 2000; Song et al., 2009). A recent study demonstrated that IL-1β induction of NF-κB, an important signaling pathway in the promotion of inflammation in cancer, was dependent on IL-1β-mediated stimulation of ROS, which activates NF-κB-inducing kinase (NIK) and consequently NF-κB (Li and Engelhardt, 2006). However, the ROS-mediated induction of NF-κB only occurred between 1-10 μM concentrations of H_2O_2, suggesting that this method of NF-κB activation is highly dependent on a specific ROS threshold.

The mechanism of NF-κB activation by H_2O_2 was observed not to be through the expected serine phosphorylation and subsequent degradation of IκB, but through the activation of a Syk protein-tyrosine kinase, leading to tyrosine phosphorylation of IκBα (Takada et al., 2003). Phosphorylation at this site of IκB resulted in the activation of NF-κB by means of translocation to the nucleus without an accompanying degradation of IκB (Takada et al., 2003). However, contradictory reports indicate that ROS inhibit the activation of NF-κB target genes mainly due to the oxidation on key cysteine residues within the NF-κB DNA binding region (Han et al., 2009; Matthews et al., 1993). The overall effect of an oxidizing environment on NF-κB activity is therefore unclear because nuclear localization of this factor from the cytoplasm is favored following H_2O_2-induced phosphorylation events, however once inside the nucleus oxidation will inhibit DNA binding (Martindale and Holbrook, 2002). Future studies that focus on the conditions

under which H_2O_2 activates NF-κB activity in the context of various cancer cell types would be particularly helpful in further deciphering the complex role that this pathway has on gene expression in cancer.

The tumour suppressor and transcription factor p53 is a key player in regulating the expression of genes involved in growth arrest and/or cell death under conditions of cellular stress (Martindale and Holbrook, 2002; Menendez et al., 2009). This transcription factor contains a critical cysteine residue within its DNA binding domain not unlike that of AP-1, which is responsive to ROS levels where oxidation negatively affects the ability of p53 to bind DNA (Sun et al., 2003). Although, the regulation of p53 and its downstream targets is mainly via post-translational modifications, genotoxic stresses such as oxidative stress can induce phosphorylation, stabilization of the protein, and the prevention of nuclear export (Colman et al., 2000; Zhang and Xiong, 2001). H_2O_2 is capable of inducing p53 phosphorylation in endothelial cells, resulting in increased p53 levels within cells, as well as an increase in the expression of downstream p53 targets (Chen et al., 2003). Various kinase inhibitors were tested to identify the key enzyme involved in p53 phosphorylation under these circumstances, and the DNA- dependent kinase ataxia telangiectasia mutated protein (ATM) kinase, which is activated via the PDGFβ receptor, was found to be uniquely involved (Chen et al., 2003). A recent study using p53 null mice and p53 knockout human fibroblasts found that the removal of p53 resulted in mtDNA depletion, decreased mitochondrial mass, and a reduction in mtTFA (Lebedeva et al., 2009). Interestingly, these p53 knockouts also displayed reduced mitochondrial superoxide levels and significantly increased cellular H_2O_2 levels.

Collectively, these results suggest that p53 is involved in mtDNA depletion and alterations in ROS homeostasis, thereby implicating the H_2O_2/p53 axis in tumourigenesis, and in the metabolic switch from oxidative phosphorylation to aerobic glycolysis known as the Warburg effect (Lebedeva et al., 2009; WARBURG, 1956) (Figure 2B).

The major factor controlling oxygen homeostasis and the cellular response to hypoxic conditions is hypoxia-inducible factor-1, (HIF-1). HIF-1 is a heterodimer composed of α and β subunits, where HIF-1β is ubiquitously expressed, and HIF-1α is tightly controlled by O_2 levels (Semenza, 2003; Semenza, 2009). The stability and transcription of HIF-1α are controlled by hypoxic conditions, and thus increased mitochondrial ROS production, providing a hypoxia-inducible mechanism to appropriately control target gene expression (Semenza, 2009). Under hypoxic conditions, the proline hydroxylase enzymes are inhibited by low O_2 and increased mitochondrial ROS, preventing sufficient hydroxylation of HIF-1α, increasing HIF-1α stability, and inducing target gene transcription (Ivan et al., 2001; Jaakkola et al., 2001; Semenza, 2009) (Figure 3). However, the involvement of mitochondrial-generated ROS in the stabilization of HIF-1α has not been well defined and remains controversial. An important hallmark of cancer cells is the Warburg effect, or the metabolic switch from oxidative phosphorylation to aerobic glycolysis in response to a hypoxic tumour environment (WARBURG, 1956). This switch is most likely mediated through coordinated HIF-1α transcriptional activity as achieved by the stabilization of this transcription factor, perhaps through mitochondrial ROS. However, the conditions under which this stabilization occurs remains a major controversy as there have been many

studies showing that ROS induces HIF-1α stabilization (Chandel et al., 2000; Guzy et al., 2005; Jung et al., 2008; Kietzmann et al., 1996; Simon, 2006), while there are other studies whose findings suggest that ROS destabilizes HIF-1α expression (Chang et al., 2005; Huang et al., 1996; Srinivas et al., 2001). Clearly the mechanisms by which ROS regulates HIF-1α stabilization and transcriptional activity are very complex, and are related to hypoxic conditions, ROS source, and cellular environment (Qutub and Popel, 2008).

Regulation of Ets-1 by mitochondrial ROS

Ets-1 is the prototype of a group of transcription factors that comprise the E26 transformation specific sequence (ETS) family. The promoters of all ETS family members contains a core DNA binding element, GGAA/T, which can independently bind to DNA, to facilitate their function as either transcription factors or as regulatory proteins that control the initiation of target gene transcription (Dittmer, 2003; Graves and Petersen, 1998). Ets-1 is over-expressed in several different cancer tissues and appears to correlate specifically with more advanced, invasive tumours in breast, ovarian and colorectal carcinomas (Summarized in Table 1). Increasing evidence indicates that a correlation between Ets-1 expression and tumour progression is a general phenomenon as evidence for such a correlation has also been found in prostate, gastric, oral and thyroid cancers as well as melanoma and meningioma (Summarized in Table 1). Moreover, Ets-1 itself is thought to have oncogenic potential due to its ability to transform NIH3T3 cells, and to

promote the transformed phenotype of a human epithelial tumour cell line (HeLa cells) (Hahne et al., 2005; Seth and Papas, 1990). In the latter case, HeLa cells with increased Ets-1 expression displayed enhanced migration, invasion, and anchorage-independent growth (Hahne et al., 2005).

The link between Ets-1 and cancer can potentially be explained by the list of known target genes regulated by this transcription factor. Several matrix metalloproteinase (MMP) genes, urokinase plasminogen activator (uPA), and integrin β3, which are all known mediators of extracellular matrix degradation and cell migration, are all targets for Ets-1 (Lefter et al., 2009; Okuducu et al., 2006; Park et al., 2009; Rothhammer et al., 2004). The normal expression of Ets-1 in lymphoid and vascular tissues as well as data obtained from a targeted Ets-1 knockout, suggest that target genes may be generally grouped in two functional groups, lymphoid cell development and angiogenesis (Bories et al., 1995; Naito et al., 1998). There is a growing list of lymphoid tissue-related gene products regulated by Ets-1 which include T cell receptors, cytokines, and cytokine receptors (Aringer et al., 2003; Sementchenko and Watson, 2000). However, it has become apparent that the products of several Ets-1-regulated genes are generally involved in the degradation of the extracellular matrix, the promotion of migration, and angiogenesis, thus suggesting key roles in mediating tumour progression (Lefter et al., 2009; Okuducu et al., 2006; Park et al., 2009; Rothhammer et al., 2004; Sementchenko and Watson, 2000).

Mitochondrial ROS is a proposed regulator of Ets-1 up-regulation, a theory that is based mainly on angiogenic studies using bovine aortic endothelial cells (Shimizu et al., 2004; Yasuda et al., 1999). In this model, angiogenesis is initiated by H_2O_2 and nitric oxide (NO), where both of these agents led to increased Ets-1 mRNA expression. Additionally, Ets-1 antisense oligonucleotides prevented ROS-mediated vessel formation indicating that angiogenesis was activated by Ets-1 (Shimizu et al., 2004; Yasuda et al., 1999). There is also evidence that Ets-2, a closely related family member of Ets-1, is transcriptionally up-regulated by H_2O_2 treatment in NIH 3T3 fibroblasts, and that cells with inherent defects to their antioxidant defense systems display endogenously increased level of Ets-2 (Sanij et al., 2001). Hypoxic induction of Ets-1 promoter activity has been shown in a model where HIF-1α protein complex was bound to a putative hypoxia response element (HRE) within the Ets-1 promoter in a hypoxia-induced fashion (Oikawa et al., 2001). Additionally, Ets-1 expression can be induced under hypoxic conditions in a rat adjuvant-induced arthritis model where joint inflammation was found to be associated with increased HIF-1α and Ets-1 protein expression, suggesting that hypoxia and HIF-1α mediate Ets-1 up-regulation in these tissues (Peters et al., 2004).

Among the known and putative regulators of Ets-1 transcription, H_2O_2 is a tumour-derived factor that may cause transcriptional upregulation of Ets-1, a response whose mediators could include AP-1, HRE and antioxidant response (ARE) elements. A functional AP-1 binding site was identified within the Ets-1 enhancer region that could be responsible for an H_2O_2-mediated Ets-1 promoter induction since the components of the AP-1 heterodimer, c-*jun* and c-*fos*, are MAPK responsive genes, a pathway which is

activated by H_2O_2 (Oka et al., 1991). A specific increase in AP-1 binding following H_2O_2 treatment has been seen in various AP-1 target gene promoters, where pro-oxidant treatment leads to direct activation of reporter constructs containing an AP-1 binding element (Duvoix et al., 2004; Guyton et al., 1996b; Zhou et al., 2001). NF-κB target genes are also activated by oxidative stress, however NF-κB is an unlikely candidate for the mediator of oxidant-induced expression of Ets-1 as no consensus NF-κB site has been identified within the promoter (Zhou et al., 2001). H_2O_2 induction of Ets-1 through an HRE has been proposed as a functional HRE capable of binding HIF-1α in a hypoxia-inducible fashion lies within the Ets-1 enhancer, and because HIF-1α is a target of H_2O_2 (Chandel et al., 1998; Oikawa et al., 2001; Salnikow et al., 2008).

Another proposed element involved in the H_2O_2-mediated induction of Ets-1 is the ARE. Our analysis of the Ets-1 promoter led to the identification of an ARE that proved to be pivotal in regulating the expression of Ets-1 under both basal and H_2O_2-induced conditions (Wilson et al., 2005). The transcription factor c-Jun has been noted to be under such regulation, however the ARE described in this case did not match the perfect consensus sequence and appeared to bind unique factors under induced conditions as compared to a control ARE (Radjendirane and Jaiswal, 1999). A second example is NF-E2 related factor 2 (NRF2/NFE2L2), a key factor involved in regulating the ARE that is likely auto-regulated given that an ARE within the NRF2 gene promoter is critical in regulating gene expression (Kwak et al., 2002). This second example is logical, as a feedback loop to enhance the expression of signaling proteins may be required to maintain a response to oxidative stress, although the regulation of Ets-1 via an ARE is not

so intuitive. Array analysis of Ets-1 over-expressing cells indicates that genes involved in antioxidant defense were upregulated, representing a protective regulatory mechanism when Ets-1 levels are increased by oxidative stress (Wilson et al., 2004). However, as discussed earlier, several genes involved in extracellular matrix degradation, migration, and angiogenesis are well-described Ets-1 targets, and thus would likely be upregulated in response to ARE-inducing stress (Behrens et al., 2001; Nakada et al., 1999; Watabe et al., 1998). Products of most ARE target genes are considered to be protective against carcinogens, therefore targeting the ARE as a cancer preventive therapy could prove to be rewarding, although clearly the upregulation of Ets-1 would not be desired in such a scenario (Hayes and McMahon, 2001). The ARE is therefore a plausible promoter element involved in a specific H_2O_2-mediated gene upregulation. Thus AP-1, HRE and potentially ARE sequences within the promoter region of Ets-1 may all be involved in the proposed H_2O_2-mediated regulation of this transcription factor.

Our laboratory has focused on H_2O_2 generated by the dysfunctional mitochondria of cancer cells as a possible mediator of increased Ets-1 expression. We have observed that Ets-1 mRNA and protein expression are specifically elevated in C13* ovarian cancer cells, which are cisplatin-resistant with observable mitochondrial differences as compared to parental 2008 ovarian cancer cells, suggesting that H_2O_2 causes an up-regulation of Ets-1 (Wilson et al., 2004). These mitochondrial differences were hypothesized to elicit a resulting increase in H_2O_2 production by C13* cells which was confirmed via intracellular H_2O_2 analysis (Wilson et al., 2005). The increased production of H_2O_2 by cells with compromised mitochondria in correlation with the observed enhanced

expression of Ets-1 suggests that this transcription factor can be specifically upregulated by H_2O_2. H_2O_2-mediated Ets-1 upregulation was found to occur at the transcriptional level as the administration of a transcriptional inhibitor prevented this response (Wilson et al., 2005). To further confirm these findings, C13* cells were treated with the antioxidant NAC which was found to reduce Ets-1 expression levels (Wilson et al., 2005). The observed up-regulation of Ets-1 by oxidative stress and increased protein levels in cells with disrupted mitochondria, and a resulting elevated production of ROS, indicate that the induction of mitochondrial stress likely leads to an upregulation of Ets-1.

To address this possibility, the specific electron transport chain inhibitors rotenone and oligomycin B were administered to C13* cells resulting in increased Ets-1 levels, likely due to increased byproduct production of H_2O_2 from inefficient electron transport (unpublished results). Though any conclusions regarding the direct effect of these treatments on H_2O_2 levels could not be made, there have been several studies indicating that ETC inhibitors will cause increased ROS production. Complex I is considered a major site of electron leakage along the ETC, thus inhibition of this site with rotenone would be expected to elicit an increase in H_2O_2 production (Liu et al., 2002). Several independent studies have confirmed that rotenone administration causes increased intracellular or extracellular H_2O_2 production using fluorescent dyes to detect H_2O_2 levels (Liu et al., 2002; Sipos et al., 2003; Vanden Hoek et al., 1997). The effects of oligomycin B on H_2O_2 production are less well described, although this drug is typically used to lower cellular ATP levels (Gong and Agani, 2005). However, this effect is not necessarily achieved following drug administration to cancer cells, given that these cells

display an enhanced glycolytic phenotype. Therefore, it is not clear whether both ETC inhibitors can elicit increases in Ets-1 via increased ROS production. However, a plausible conclusion is that mitochondrial stress in the form of blocked electron flow along the ETC or in the production of ATP by the ETC, will lead to increased Ets-1 protein levels. Ets-1 is then responsive at the transcriptional level, to changes in intracellular H_2O_2 levels as well as to the general malfunctioning of the mitochondria (Figure 4).

Concluding Remarks

Mitochondria function as the metabolic centre of the cell, performing cellular respiration through the ETC and ultimately generating cellular energy in the form of ATP. Over 50 years ago, Warburg first proposed that the mitochondrial injury frequently seen in cancer cells could lead to decreased ETC function and thus deficient respiration (WARBURG, 1956). Such mitochondrial dysfunction would cause a metabolic switch in cancer cells from oxidative phosphorylation to aerobic glycolysis, a phenomenon known as the Warburg effect that has been well defended in recent literature. In addition to altered metabolism, other mitochondrial dysfunctions such as mutations in mtDNA, increased ROS production, and altered metabolic enzyme expression have been closely associated with cancer, suggesting that this organelle is critical to the initiation, maintenance, and progression of the neoplastic phenotype.

Mitochondria are the predominant source of cellular ROS that are increased in cancer cells, and function as complex signaling molecules for mitochondria-nucleus crosstalk called retrograde regulation. The ways in which ROS regulate signaling pathways are extensive and diverse, and represent one of the most significant roles of these molecules. Generated by cell membrane receptors following ligand binding and by the mitochondria, mitochondrial-generated ROS can directly activate receptors further enhancing ROS production independent of ligand binding. Intracellular ROS act to oxidize and inhibit MAPK-specific PTPs, which thus fail to dephosphorylate kinase enzymes such as Erk 1/2 that therefore remain in active conformations. Additionally, intracellular ROS oxidize and inactivate PTEN, which then fails to dephosphorylate Akt resulting in the maintenance of an active conformation. These activated kinase pathways lead to downstream gene expression changes that ultimately result in enhanced proliferation and survival, qualities that are particularly advantageous to cancer cells.

In addition to their effects on phosphatase activation, the signaling pathways activated by H_2O_2 ultimately effect the phosphorylation, activation, oxidation and DNA binding ability of cancer-associated transcription factors. Evident from the findings presented, the complexity of ROS signaling on transcription factors is significant and generally not well understood, especially in the context of cancer. Intracellular ROS has the capacity to induce the translocation and activation of NF-κB, as well as the phosphorylation of c-Jun and p53 resulting in nuclear translocation, ultimately activating target gene expression. Additionally, intranuclear ROS can oxidize NF-κB, c-Jun, c-Fos, and p53 DNA binding residues resulting in the downregulation of target gene expression.

HIF-1α, which can either be activated or repressed by H_2O_2, initiates a nuclear-encoded mitochondrial response to oxidative stress, and therefore acts as a mediator of mitochondria-nucleus crosstalk. Ets-1 represents a more novel example of ROS-mediated induction, where increased H_2O_2 production by defective cancer cell mitochondria results in upregulation of Ets-1 at the transcription level likely through an ARE-mediated mechanism. Clearly, the regulation of transcription factors by ROS is complex and has yet to be fully elucidated, however it is clear that the manipulation of these regulatory mechanisms could provide potential novel cancer therapies and preventative strategies.

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Tables

Table 1. Association between Ets-1 expression and cancer.

Type of cancer	Ets-1 expression findings	Association between Ets-1 expression and clinical outcomes	Reference
Breast	Protein expression found in >60% primary cancer samples	Correlation between expression and poor survival	Span et al. 2002
Breast	mRNA and protein expression high in primary carcinomas	—	Buggy et al. 2004
Breast	High protein levels in invasive carcinoma	—	Katayama et al. 2005
Ovarian	mRNA detected in 42% carcinomas tested	Associated with poor survival	(Davidson et al. 2001
Ovarian	High protein and mRNA detected in metastatic carcinoma	Lower than 24 month survival rate	Fujimoto et al. 2004
Colorectal	Increased levels in stroma of invasive carcinoma	—	Behrens et al. 2003
Colorectal	48% of all specimens tested positive for Ets-1	Associated with lower survival rate	Tokuhara et al. 2003
Melanoma	Higher levels in invasive melanoma	—	Keehn et al. 2003
Prostate	Protein expressed in 77% of malignant cases tested	—	Alipov et al. 2005
Oral squamous cell carcinoma	Normal tissue negative, 58% malignant tissue positive	Advanced stage tumors most frequently positive	Pande et al. 1999
Esophageal squamous cell carcinoma	Over-expression in 80% malignant samples	Expression correlated with poor survival	Mukherjee et al. 2003
Gastric	Protein detected in 52% of primary cancers	Associated with poor survival	Tsutsumi et al. 2005
Thyroid	Increased protein in papillary thyroid carcinomas	—	Fuhrer et al. 2005
Meningioma (tumors of the CNS)	High protein levels correlate with higher tumor grade	Infiltration to the brain correlates with Ets-1 expression	Kitange et al. 2000
Testicular germ cell tumors	Expression higher and more frequently observed in metastatic tumors	—	Adam et al. 2003

Table 4.1: Association between Ets-1 expression and cancer

Figures

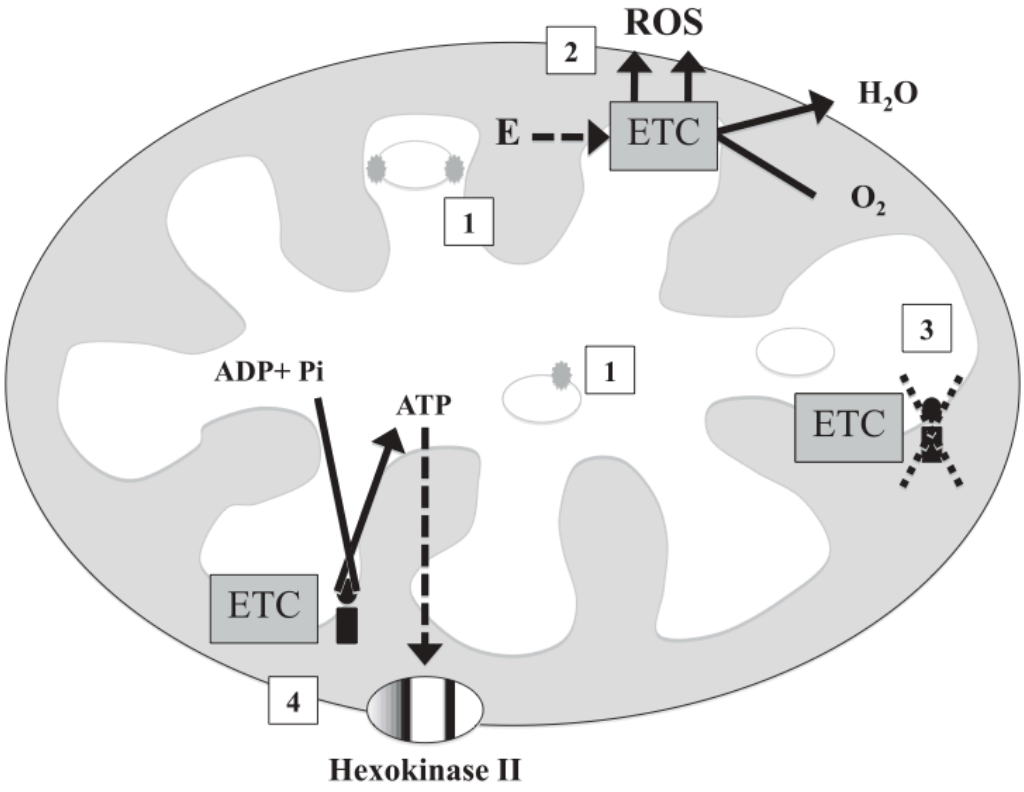

Figure 4.1: Mitochondrial alterations in cancer cells.

1) Mutations to circular mitochondrial genome are frequently observed, particularly in control D loop region. 2) Elevated levels of ROS result from inefficient electron transfer along the ETC. 3) Downregulation of ATPsynthaseβ, a subunit involved in oxidative phosphorylation. 4) Translocation of hexokinase II, an enzyme involved in the first phase of glycolysis, to the outer mitochondrial membrane where it has easy access to ATP generated by oxidative phosphorylation.

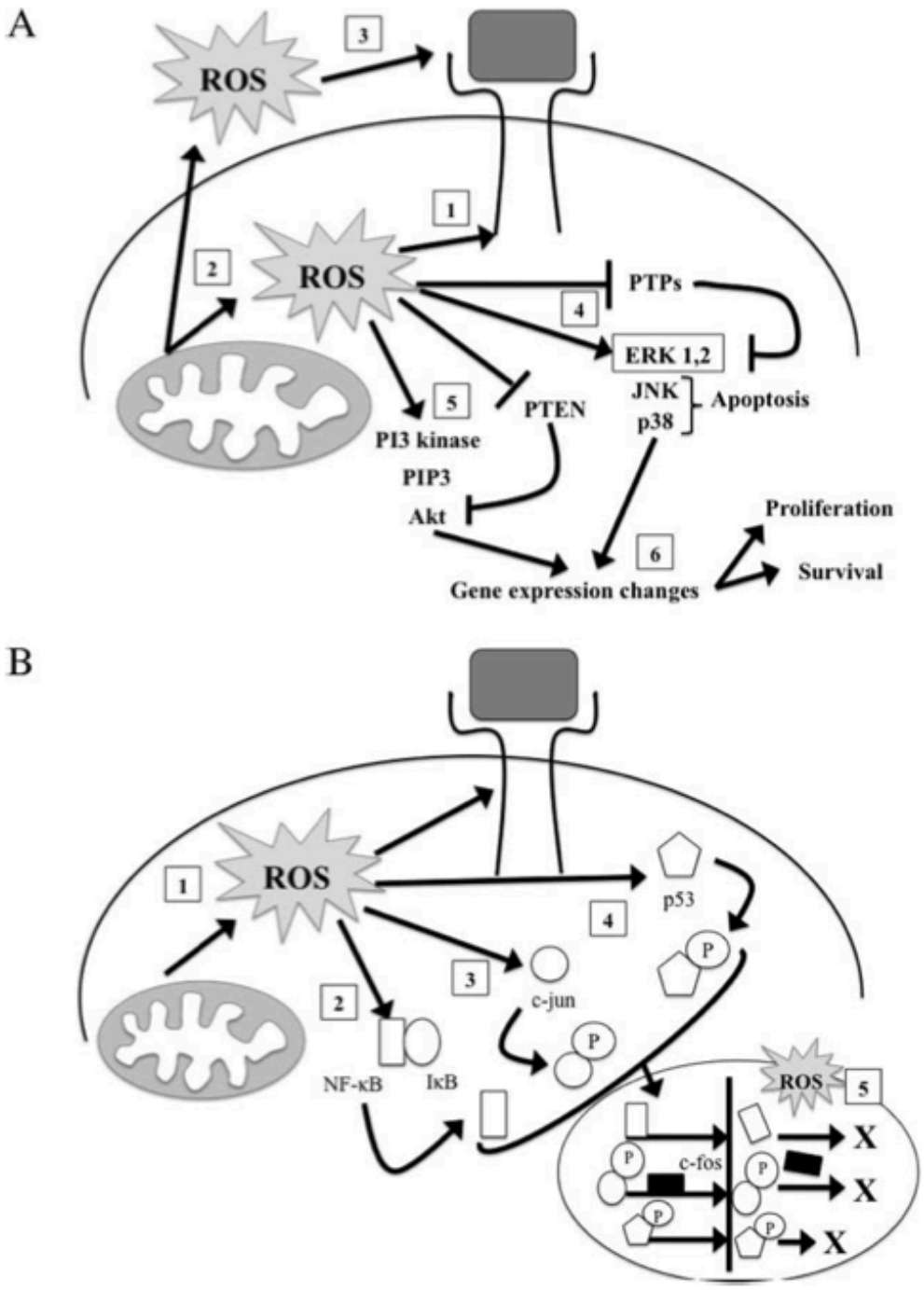

Figure 4.2: ROS as signaling molecules.

A) The effect of ROS on kinase pathways: 1) ROS is generated by cell membrane receptors following ligand binding. 2) ROS generated by mitochondria. 3) Mitochondrial

ROS can directly activate further production of ROS. 4) Intracellular ROS act to oxidize and inhibit MAPK specific PTPs causing dephosphorylation of kinase enzymes, which remain in active conformations. Activated JNK and p38 may also trigger to apoptosis. 5) Intracellular ROS oxidize and inactivate PTEN, failing to dephosphorylate Akt, which remains in active conformation. 6) Active kinase pathways lead to downstream gene expression changes resulting in enhanced proliferation and survival. B) The effect of ROS on transcription factors: 1) ROS are generated by mitochondria and bound receptors. 2) Intracellular ROS leads to dissociation of NF-κB causing free translocation to the nucleus, activating target gene expression. 3,4) Intracellular ROS cause phosphorylation of factors such as c-Jun and p53, causing translocation to the nucleus, activating target gene expression. 5) Intranuclear ROS oxidize NF-κB, c-Jun, c-Fos and p53 DNA binding residues, reducing DNA binding of these factors, and down-regulating target gene expression.

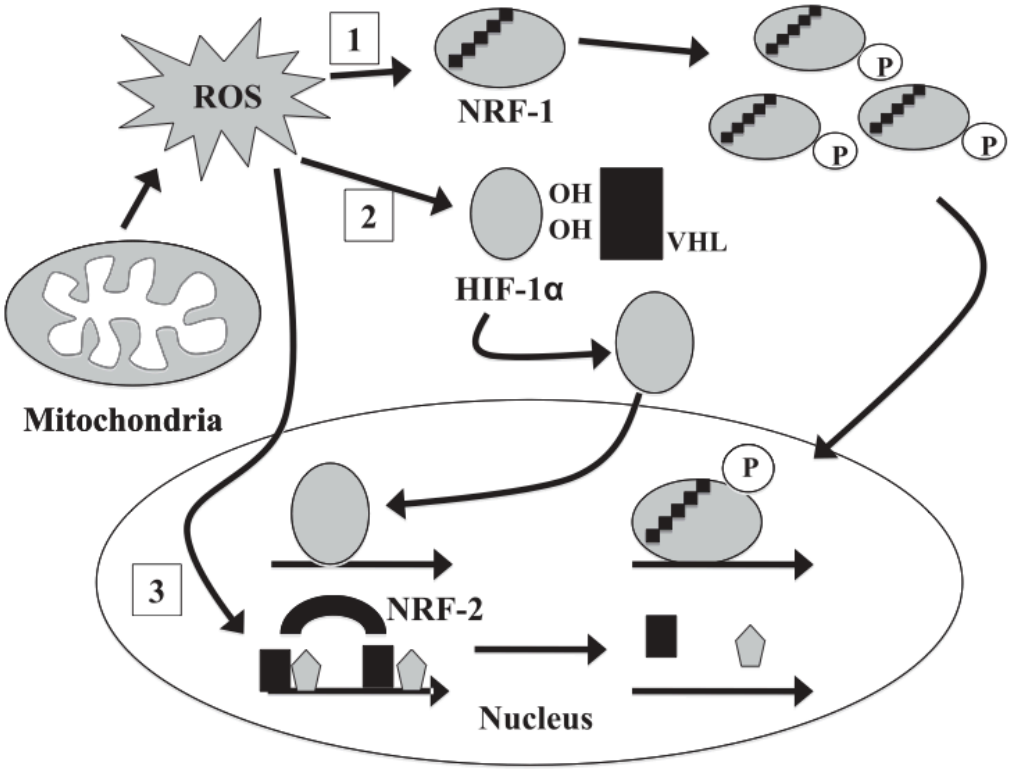

Figure 4.3: Mitochondria to nucleus crosstalk.

Mitochondrial generated ROS act as signaling molecules that can affect nuclear gene transcription. 1) Mitochondrial ROS lead to upregulation of NRF-1 expression and phosphorylation, promoting nuclear translocation. As a result, NRF-1 target mitochondrial proteins are upregulated. 2) Mitochondrial ROS stabilize HIF-1α by inhibiting hydroxylation, thus preventing VHL recognition and degradation. Stabilized HIF-1α is able to translocate to the nucleus and activate hypoxic response genes. 3) ROS directly oxidize cysteine residues in the DNA binding domain of NRF-2, inhibiting binding and decreasing target mitochondrial protein expression.

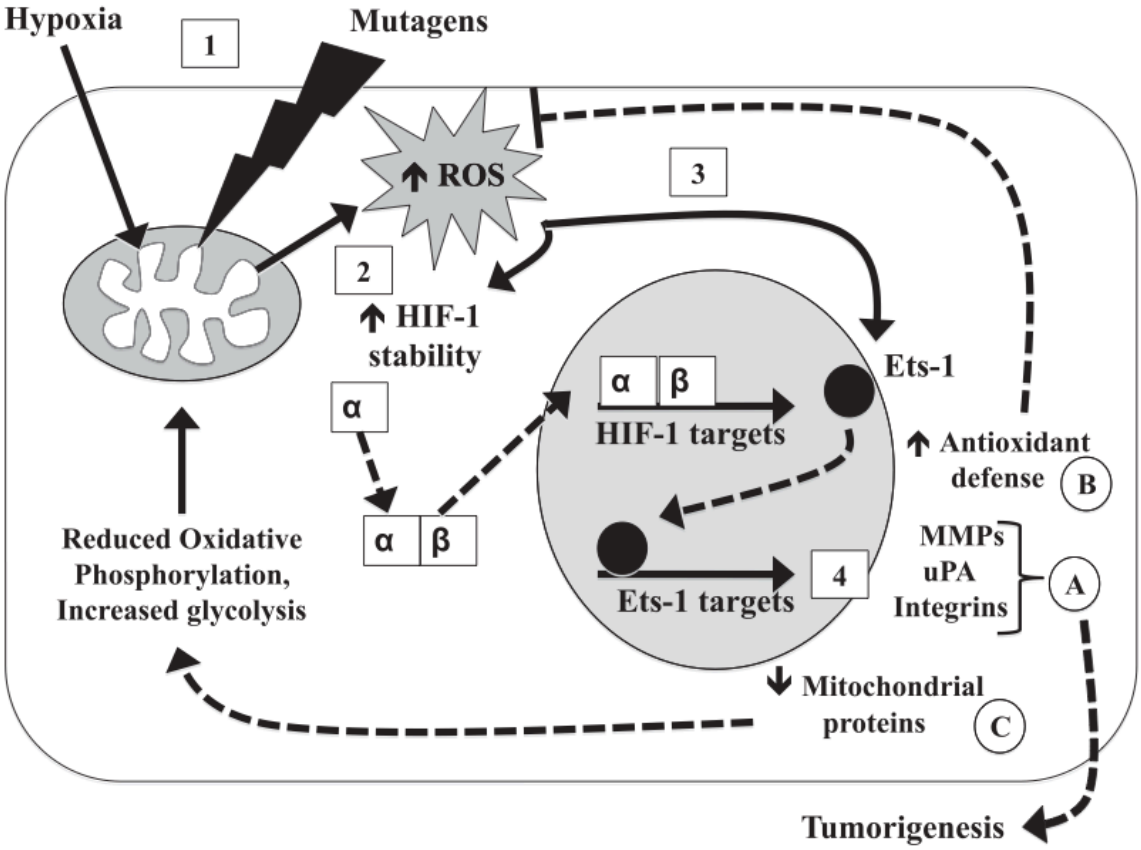

Figure 4.4: Ets-1 as a mediator of mitochondria to nucleus crosstalk.

1) Damage to the mitochondria, provoking mtDNA damage and mutation, as well as hypoxic conditions promote ROS production. 2) ROS stabilize the expression of HIF-1α, thereby upregulating target gene expression, including Ets-1. 3) Ets-1 is directly upregulated by ROS, via the activation of an ARE within the promoter. 4) Upregulation of Ets-1 leads to downstream effects: A) Upregulation of MMPs, uPA and integrins ultimately lead to enhanced ECM degradation, migration, angiogenesis, and increased tumourigenesis. B) Upregulation of antioxidant defense genes result in a feedback

protective effect countering increased levels of ROS. C) Downregulation of genes encoding mitochondrial proteins and metabolic enzymes involved in the ETC leads to an overall inhibition of oxidative phosphorylation and an increased reliance on glycolysis.

Chapter 5: The role of Ets-1 in cellular response to oxidative stress

In this chapter, an author-generated version of the manuscript entitled "Ets-1 regulates intracellular glutathione levels: Key target for resistant ovarian cancer" prepared for submission to *Molecular Cancer* is presented. As this is a manuscript in submission, no copyright license documentation is required.

For this paper, I performed the cell culture, CM_2-DCFDA ROS detection assays, quantitative glutathione determinations, glutathione peroxidase enzyme assays, and western blotting. Additionally, I generated all of the manuscript figures, wrote and revised the manuscript. Natalie Zacal completed two replicates of the glutamate release assay included in this manuscript. Dr. Gurmit Singh provided intellectual direction and revised the manuscript.

Context and background

Based on my observations that Ets-1 is associated with oxidoreductase activity in the 2008 Ets-1 overexpression model (Chapter 3), and evidence that this transcription factor is upregulated by ROS in cancer cells (Chapter 4), I became interested in examining the importance of Ets-1 in the regulation of cellular antioxidant capacity in cancer cells. I chose to continue working with my ovarian cancer model because ovarian cancer is particularly associated with high reccurence, metastatic, and therapeutic resistance rates. Notably, previous work by Dr. Leigh Wilson, *et al.* discovered the involvement of Ets-1 in chemotherapeutic resistance in a variant of 2008 ovarian cancer cells (Wilson et al.,

2004). Many chemotherapeutic agents kill cancer cells by inducing oxidative stress, and tumours that are resistant to such onslaught often display enhanced antioxidant defenses. The major cellular antioxidant defence is the glutathione system, where glutathione levels are notably higher in ovarian tumours compared to healthy ovarian tissue, and even higher in patients who are resistance to conventional therapies (Cheng et al., 1997; Gamcsik, Kasibhatla, Teeter, & Colvin, 2012). Interestingly, I found that Ets-1 overexpressing 2008 cells upregulate several genes involved in the glutathione antioxidant system, thus making this avenue a potentially promising one to examine further.

When compared to parental 2008 ovarian cancer cells, 2008-Ets1 cells display decreased intracellular ROS levels, along with increased intracellular glutathione levels, glutathione enzyme activity, and Sx_c^- transporter activity. This transporter functions to import cystine while exporting glutamate from the cell, and thereby influencing the intracellular cysteine pool upon which glutathione synthesis is dependent. Another pathway that contributes significantly to intracellular cysteine levels is the transsulfuration pathway that creates cysteine from homocysteine. To determine the relative importance of both of these pathways with respect to Ets-1 regulation, I used specific blocking agents for either pathway. Sulfasalazine (SAS) is an anti-inflammatory drug classically used in the treatment of inflammatory bowel diseases and rheumatoid arthritis that is now known to potently inhibit Sx_c^- (Gout, Buckley, Simms, & Bruchovsky, 2001). Propargylglycine (PPG) inhibits the enzyme cystathionine γ-lyase irreversibly, thus blocking one of the essential steps of the transsulfuration pathway.

Treatment with PPG resulted in decreased glutathione levels in both 2008 and 2008-Ets1 cells, whereas SAS treatment only decreased intracellular glutathione in 2008-Ets1 cells.

Oxidative stress is a very common occurrence in the tumour microenvironment, and as mentioned previously, is the mode of action of many conventional cancer therapeutic modalities. Since I observed that Ets-1 overexpression induces high intracellular glutathione levels, I was interested in measuring glutathione levels, GPX activity, and protein levels of redox factors during oxidative stress. To induce oxidative stress tried several methods, but found that glucose oxidase treatment was the most consistent and effective means of induction. I treated cells with glucose oxidase over 24 hours with or without simultaneous treatment with SAS or PPG. Under these conditions, both PPG and SAS achieved decreases in glutathione levels, with the efficiency of treatment with SAS inversely correlating with Ets-1 levels. These results show that ovarian cancer cells that overexpress Ets-1 recruit the Sx_c^- transporter to bolster the cellular cysteine pool, making these cells particularly sensitive to agents that block this transporter.

The glutathione system plays a key role in cellular antioxidant capacity, and high levels of glutathione provide cancer cells with protection against conventional therapies that induce oxidative stress. Thus, the development and use of glutathione-depleting agents presents a particularly promising are for therapeutic enhancement. Glutathione depletion can be achieved through inhibition of transport, enhancement of export from cells, or as I have shown in this paper by limiting intracellular cysteine availability. My data suggest that Ets-1 is an important regulator of this antioxidant system in ovarian

cancer cells, although my conclusions are limited as only one cell model was used for this study. I believe that further study into different cell types and eventually in patient samples would show that tumours that express high levels of Ets-1 would benefit from pretreatment with Sx_c^- blocking agents prior to chemotherapy as a means to enhance therapeutic effectiveness.

PAPER: Ets-1 regulates intracellular glutathione levels: Key target for resistant ovarian cancer

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ABSTRACT

Ovarian cancer is characterized by high rates of metastasis and therapeutic resistance. Many chemotherapeutic agents rely on the induction of oxidative stress to cause cancer cell death, thus targeting redox regulation is a promising strategy to overcome drug resistance. Glutathione, a major regulator of redox balance, is present at high levels in resistant cancer cells and is dependent on cysteine availability for synthesis. In this study we have identified a role for proto-oncogene Ets-1 in the regulation of glutathione levels, and examined the effects of the anti-inflammatory drug sulfasalazine on glutathione depletion. Overexpression of Ets-1 was associated with decreased intracellular ROS, concomitantly with increased intracellular GSH, GPX antioxidant activity, and Sx_c^- transporter activity. Under basal conditions, inhibition of the transsulfuration pathway resulted in decreased GSH levels and GPX activity in all cell lines, whereas inhibition of Sx_c^- by sulfasalazine decreased GPX activity in Ets-1-expressing cells only. However, under oxidative stress the intracellular GSH levels decreased significantly in correlation with increased Ets-1 expression following sulfasalazine treatment. The findings from this study show that Ets-1 mediates enhanced Sx_c^- activity to increase glutathione levels under oxidative stress, suggesting that Ets-1 could be a promising putative target to enhance conventional therapeutic strategies.

INTRODUCTION

Ovarian cancer, much like several types of human cancer, remains extremely challenging to treat in advanced stages despite prolific therapeutic advances. Patients with ovarian cancer generally have a poor prognosis, as most women are not diagnosed until the disease has reached advanced stages. Surgical intervention is the most successful treatment, however, due to the close proximity of other peritoneal structures to the ovary, recurrence of the disease within nearby organs is very common [1]. As recurrent ovarian cancer remains a largely incurable state of disease, it is vitally important to examine the characteristic differences between early and late stage ovarian cancers, and mechanisms of therapeutic resistance. Our research group has previously established that the transcription factor Ets-1 is associated with chemotherapeutic resistance in ovarian cancer cells [2]. More recently, we have established that Ets-1 regulates energy metabolism in ovarian cancer cells by enhancing glycolytic dependence [3]. Additionally, the increased glycolytic utilization observed was paired with increases in the expression of several pentose phosphate pathway genes. This pathway is one of the major sources of NADPH that is required for glutathione reduction, which is a major factor in therapeutic resistance. Therefore, because Ets-1 overexpression leads to increased glycolytic flux, it is possible that Ets-1 is also involved in the regulation of cellular redox state since these processes are intrinsically linked.

The balance between cell death and proliferation is tightly controlled in healthy cells, whereas in cancer cells this equilibrium is shifted towards a proliferative state with enhanced survival. Imbalances in cellular redox state are frequently observed in cancer

cells, and contribute significantly to cancer progression and apoptotic resistance. Reduced glutathione (GSH) is the most abundant non-protein thiol in mammalian cells, and tightly regulates redox state through its antioxidant and reducing activities. Additionally, GSH in involved in the control of cell cycle regulation, proliferation, apoptosis, and therapeutic resistance in cancer cells [4-7]. Many chemotherapeutic agents and radiation treatments depend on the alteration of redox state through the induction of oxidative stress via reactive oxygen species (ROS) generation to kill cancer cells. Changes in GSH levels affect mitochondrial pore permeability, where depletion of GSH leads to the release of cytochrome c and cell death, thus rendering GSH an attractive target for cancer therapy that could ameliorate the success of conventional therapies [8]. Interestingly, glutathione levels are higher in ovarian tumours than in healthy ovarian tissue, and increase in patients who are non-responsive to therapeutic intervention [9, 10].

In this study, we reveal that Ets-1 regulates cellular redox balance in ovarian cancer cells, which could account for the therapeutic resistance associated with this factor. We have shown that ovarian cancer cells that overexpress Ets-1 display decreased intracellular ROS with increased intracellular GSH and glutathione peroxidase (GPX) antioxidant activity. The availability of intracellular cysteine is rate-limiting for GSH synthesis, and is determined by the activities of the transsulfuration pathway and the membrane antiporter System x_c^- (Sx_c^-), which imports cystine in exchange for glutamate release. We have used inhibitors of each of these systems to determine whether Ets-1 plays a role in the regulation of either pathway, thus explaining the changes in GSH that we have observed. Our results show that Ets-1 mediates enhanced Sx_c^- activity to increase

glutathione recycling in ovarian cancer cells, and that this effect was enhanced during oxidative stress. This is the first study to report that Ets-1 regulates redox balance in cancer cells, suggesting that Ets-1 could be a promising putative target for the enhancement of therapeutic strategies dependent on GSH depletion.

MATERIALS AND METHODS

Cell culture and treatments

The human ovarian carcinoma cell line 2008 was kindly provided by Dr. Paul Andrews (Georgetown University, Rockville, MD, United States) [11], and were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin. Stable cell line 2008-Ets1 was maintained in growth medium as described with the addition of 200ng/ml 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 (ONT, Canada). When cells reached 40-50% confluency, the cultures were exposed to either propargylglycine (PPG) or sulfasalazine (SAS) at 2.5mM and 50uM respectively for 24 hours prior to isolation or analysis. To induce oxidative stress in cultures, cells were treated with 10mU/mL glucose oxidase and/or PPG/SAS for 24 hours prior to isolation or analysis.

Western Blotting and densitometry analysis

Whole cell lysates were collected, 30µg of protein was separated by 10% SDS-PAGE electrophoresis, transferred to PVDF membrane, and blocked for 1 hr in 5% skim milk TBS-T. Membranes were incubated overnight with antibody reactive to Ets-1 (Abcam, MA, United States), HIF-1 (Cell Signaling, MA, United States), xCT (Abcam), GPX1 (Abcam), GPX2 (Abcam), or Actin (Cell Signaling) in 0.5% TBS-T. Following primary antibody incubation, membranes were washed and incubated for 2 hrs with horseradish peroxidase-linked anti-mouse or anti-rabbit IgG secondary antibody as appropriate (Cell Signaling). Proteins were detected by ECL chemiluminescence reagent (Amersham, NJ, United States), and exposed to film. Densitometry analysis was performed using ImageJ software available at http://rsb.info.nih.gov/ij and developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA.

Glutamate Release and Cell Viability Assays

Glutamate levels in the culture medium was evaluated using the AMPLEX red® glutamic acid assay kit, which was optimized for higher glutamate concentrations by the omission of L-alanine and L-glutamate pyruvate transaminase from the reaction [12]. Cell viability was measured using the Crystal Violet staining with all cell lines seeded at 10,000 cells per well, and normalized to a standard curve of cell numbers.

Intracellular ROS assay

Intracellular ROS levels were measured using CM_2-H_2DCFDA reagent (Invitrogen), which is cleaved once inside the cell allowing the DCF dye to bind to ROS species resulting in fluorescence. Cells were plated in 96-well plates and grown to 70-90% confluency in phenol red-free medium. CM_2-H_2DCFDA reagent was reconstituted in DMSO, and 10μM was added to each experimental well using phenol red-free medium containing 10% FBS. Following a 30min incubation to allow the dye to load into cells, plates were washed twice with PBS, and allowed to recover in phenol red-free medium for 10 minutes. Plates were then treated with 250μM H_2O_2 and read in a Cytofluor fluorescent plate reader at 485nm excitation and 530nm emission for 1 hour. Plates were then stained with Crystal Violet, dried overnight, solubilized with SDS, and read at 570nm. Arbitrary fluorescent values were normalized to Crystal Violet absorbance values, and reported as Arbitrary Fluorescent Units (AFU).

Determination of intracellular GSH concentration

Metabolite cell extracts were prepared as described by Rahman *et al.* [13]. Briefly, cultures were grown to 70-80% confluency, washed with PBS, and pelleted twice. Extracts were resuspended in cold extraction buffer containing 0.06% sulfosalicylic acid, homogenized using a 26.5 gauge needle, lyzed via freeze/thaw at -70°C, pelleted, and the supernatant was retained as used for GSH measurement. Intracellular GSH content of the metabolite extracts was measured as described [13]. The concentration of total GSH was normalized to the protein concentration of each extract.

GPX activity assay

Glutathione peroxidase enzyme activity was measured using the glutathione peroxidase activity kit from Enzo Life Sciences (NY, USA) according to the manufacturer instructions. The GPX activity was normalized to the protein concentration of the each cell extract.

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$\leq$0.05 considered to be statistically significant ($p\leq0.05$=*, $p\leq0.01$=**, $p\leq0.001$=***).

RESULTS

Ets-1 decreases intracellular ROS, while increasing intracellular GSH and GPX activity

To examine the role of Ets-1 in the regulation of cellular antioxidant capacity, we measured intracellular ROS levels, total cellular GSH, and GPX enzyme activity. Intracellular ROS was measured using CM_2-H_2DCFDA in 2008, 2008-Ets1, and induced 2008-Ets1 cells (Figure 1A). The amount of ROS present in induced 2008-Ets1 cells

(1264.4 AFU) was significantly less than their parental 2008 cells (1885.5 AFU), while non-induced 2008-Ets1 cells were not significantly different. Quantitative total GSH was measured via colourimetric assay under basal conditions, and both 2008-Ets1 and induced 2008-Ets1 cells showed increased amounts of total GSH relative to 2008 cells (11.11- and 22.14-fold respectively)(Figure 1B). GPX enzyme activity was assessed by measure of the oxidation of NADPH, and induced 2008-Ets1 cells were found to have a higher GPX activity rate (1.96-fold) than 2008 cells (Figure 1C).

Ets-1 increases System x_c^- expression and activity

The protein expression of xCT, the subunit of Sx_c^- responsible for transporter activity of the antiporter, and of CBS, which catalyzes the first step of the transsulfuration pathway, were examined in 2008 and induced 2008-Ets1 cells (Figure 2A). The expression of xCT was found to be 4.6-fold higher in induced 2008-Ets1 cells compared to parental 2008 cells via densitometry analysis (n=3, data not shown). No significant difference in the expression of CBS was found between 2008 and induced 2008-Ets1 cells. The amount of glutamate released into the culture medium was measured, and used to test the effectiveness of SAS in blocking Sx_c^- transporter activity. Both non-induced and induced 2008-Ets1 cells released more glutamate into the culture environment than parental 2008 cells (2.04-fold and 2.53-fold respectively)(Figure 2B). Treatment with SAS, an inhibitor of Sx_c^-, resulted in a significant reduction of extracellular glutamate for all cell lines. Treatment with SAS, an inhibitor of Sx_c^-, resulted in a significant reduction of

extracellular glutamate for all cell lines. Additionally, SAS treatment resulted in significantly decreased cell viability in all cell types of our model system (Figure 2C), a finding that is in accordance with previously published work by Lo *et al* [14].

The transsulfuration pathway is a major GSH source in normoxic ovarian cancer cells

To identify the role of Ets-1 in regulating either of the transsulfuration or Sx_c^- sources of GSH, we have used specific inhibitors of both pathways. PPG irreversibly inhibits the cystathionine γ-lyase enzyme (CGL), thus preventing cysteine synthesis. SAS is a pharmacological inhibitor Sx_c^- that prevents the import of cystine from the transporter. Following treatment with inhibitors of the transsulfuration pathway (PPG) or Sx_c^- (SAS), we measured intracellular GSH levels and GPX activity in order to examine the importance of each pathway in cellular redox regulation. Total cellular GSH was decreased by PPG treatment in all cell lines, and SAS did not have any significant effect (Figure 3A). Transsulfuration pathway inhibition with PPG resulted in decreased GPX activity in all cell lines, and Sx_c^- inhibition with SAS caused decreased GPX activity in both non-induced and induced 2008-Ets1 cells (Figure 3B). The protein expression of several factors involved in redox state regulation was examined via Western blot (Figure 3C). Ets-1 is very lowly expressed in 2008 cells compared to 2008-Ets1 cells and their induced counterparts. In addition, the protein levels of xCT, GPX-1, and GPX-2 were increased with overexpression of Ets-1. In 2008 cells, both PPG and SAS treatments caused a decreased in HIF-1 protein. In contrast, PPG treatment resulted in the increase of

both GPX-1 and GPX-2 protein levels. In 2008-Ets1 cells, inhibition of the transsulfuration pathway with PPG decreased the protein expression of HIF-1, xCT, and GPX-1, while SAS decreased the expression of GPX-1. Induced 2008-Ets1 cells showed decreases in HIF-1, xCT, and GPX-2 protein following PPG treatment, and decreased GPX-2 after treatment with SAS.

Ets-1 recruits Sx_c^- to maintain glutathione pool under oxidative stress

Glucose oxidase treatment was used to induce oxidative stress, resulting in increased intracellular ROS in all cell lines (Figure 4A). Intracellular GSH levels were decreased in response to transsulfuration inhibition in all cell types, but only Ets-1 overexpressing cells displayed decreased GSH levels following inhibition of Sx_c^- (Figure 4B). The decrease in GSH levels following sulfasalazine treatment was inversely correlated with increased Ets-1 expression.

Ets-1 redox regulation involves changes in HIF-1 and GPX-2 protein levels

Following the induction of oxidative stress, the protein expression of the redox related proteins HIF-1, xCT, GPX-1, and GPX-2 were determined via Western blot (Figure 5A). When comparing 2008, 2008-Ets1, and induced 2008-Ets1 cells, the expression of HIF-1 is decreased, while the expression of xCT and GPX-1 are increased in response to Ets-1 overexpression (Figure 5B). In response to inhibitor treatment, PPG decreased HIF-1 and

GPX-2 expression in all cell lines, in addition to increasing Ets-1 and GPX-1 protein levels in 2008 cells (Figure 5C-D). SAS treatment resulted in similar observations in Ets-1 overexpressing cells where inhibition of Sx_c^- decreased GPX-2 in both Ets-1-expressing lines, and also decreased HIF-1 protein levels in induced 2008-Ets1 cells (Figure 5C-D).

DISCUSSION

In the present study, we have identified Ets-1 as a mediator of cellular redox state in an ovarian cancer model of Ets-1 overexpression, shown that Ets-1 recruits the membrane antiporter Sx_c^- to increase intracellular GSH levels, and described a possible mechanism to overcome drug resistance in ovarian cancer cells. The importance of Ets-1 in cancer has been investigated extensively with regards extracellular matrix remodeling and angiogenesis [15, 16]. Our laboratory recently identified Ets-1 as a key regulator of cancer metabolism by encouraging glycolytic dependence and decreasing oxidative phosphorylation [3]. To our knowledge, this is the first report to define a role for Ets-1 in redox state regulation of intracellular glutathione levels in cancer cells.

Cellular redox balance is an important regulator of cancer cell proliferation, apoptotic evasion, and therapeutic resistance. Thus, Ets-1-mediated alterations in redox state likely account, at least in part, for the associations observed with overexpression of this factor and poor prognosis, advanced malignancy, and enhanced metastatic potential in several types of cancer [17-26]. We have chosen to further characterize the function of Ets-1 in ovarian cancer based on our previous work using the 2008/C13* ovarian cancer

model of cisplatin resistance [2]. C13* cells are a variant of 2008 adenocarcinoma cells that were generated from 2008 cells subjected to 13 consecutive rounds of cisplatin treatment. These cells display elevated mitochondrial membrane potential, enhanced DNA repair mechanisms, and altered transcription factor expression, which all contribute to therapeutic resistance [2]. As Ets-1 is also overexpressed in C13* cells, we generated a stable overexpression model of Ets-1 in 2008 cells and have since characterized novel roles for Ets-1 in ovarian cancer using this model [2, 3, 27]. In this study, we have shown that overexpression of Ets-1 leads to decreased intracellular ROS levels, concomitantly with increased intracellular GSH levels and GPX activity. These findings are significant as they suggest a mechanism for the increased tolerance to oxidative stress observed in aggressive, drug-resistant ovarian cancer cells (Figure 6).

GSH plays a key role in the antioxidant capabilities of cancer cells, and high levels confer protection against oxidative stress-inducing chemotherapy and ionizing radiation. The development of targeted agents that deplete GSH would improve the ability of these agents to induce cancer cell death, as low levels of GSH trigger mitochondrial apoptosis, necrosis, and autophagy [7, 8, 28, 29]. Depletion of GSH can be achieved through the inhibition of GSH transport, enhancement of efflux from cells, or by limiting cysteine availability causing impaired GSH synthesis. We have reported here that the overexpression of Ets-1 induces increased expression and activity of Sx_c^-, suggesting that GSH depletion by limiting intracellular cysteine might be a viable therapy in these cells. Treatment with SAS, an anti-inflammatory drug that blocks Sx_c^- transport, resulted in a significant decrease in GPX activity but not total GSH levels under basal conditions.

However, decreases in GSH levels and GPX activity were achieved following treatment with PPG, which blocks cysteine synthesis by the transsulfuration pathway. Therefore, we suggest that under basal conditions ovarian cancer cells rely predominantly on the transsulfuration pathway to maintain GSH levels irrespectively of Ets-1 expression level.

However, since many therapeutic agents function by inducing oxidative stress, we also examined the effects of SAS and PPG on ovarian cancer cells exposed to such conditions by glucose oxidase treatment. Interestingly, we have shown that GSH depletion following SAS treatment correlates with Ets-1 overexpression levels, where cancer cells that express high levels of Ets-1 display greatly decreased GSH levels following Sx_c^- blockade. Protein expression of the Sx_c^- catalytic subunit xCT and glutathione peroxidase enzyme GPX-1 were increased in correlation with increasing Ets-1 expression suggesting that they may be target genes of Ets-1. Interestingly, the protein expression of HIF-1 and GPX-2 were decreased concomitantly in response to transsulfuration pathway inhibition in all cell lines, an effect that was mimicked with SAS treatment in Ets-1 overexpression lines only. These findings not only suggest that HIF-1 and GPX-2 are involved in the maintenance of intracellular GSH levels, but also that cellular redox state can regulate the expression of HIF-1. In agreement with our findings, the ability of redox state fluctuations to affect HIF-1 expression was recently observed in hepatocellular carcinoma cells following inhibition of GSH synthesis by the chemotherapeutic agent buthionine sulphoximine [30].

Our results suggest that agents that deplete GSH levels may be effective sensitizing agents in aggressive, drug-resistant ovarian cancers when used as a pre-

treatment prior to conventional oxidative stress-inducing therapies. There is precedence for such a therapeutic strategy, as SAS specifically has shown therapeutic promise in pancreatic [14, 31], lung [32, 33], hepatocellular [34], prostate [35], and breast cancers [36]. These studies illustrated that SAS has the ability to decrease tumour growth *in vivo* in several types of cancer, and can also enhance the efficacy of the chemotherapeutic agents etoposide [31], gemcitabine [14, 31], and doxorubicin [33, 36]. Gemcitabine and cisplatin are commonly used in combination together, and since we have previously shown that Ets-1 is involved in cisplatin resistance, further study of the validity of pre-treatment of GSH-depleting agents with gemcitabine and cisplatin combination therapy for ovarian cancer is warranted [2]. The diverse functional roles of Ets-1 in a variety of cancer types truly illustrate the potential use of Ets-1 inhibition as an effective therapeutic target, although the limitations to such an approach are significant. Several other Ets factors share sequence homology with Ets-1, and thus direct inhibition of Ets-1 specifically is unreasonable. Therefore, further examination of the regulation of Ets-1 and the functional consequences of its overexpression are of particular interest to the development of novel therapeutic approaches for ovarian cancer as we have suggested in this study.

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Figures

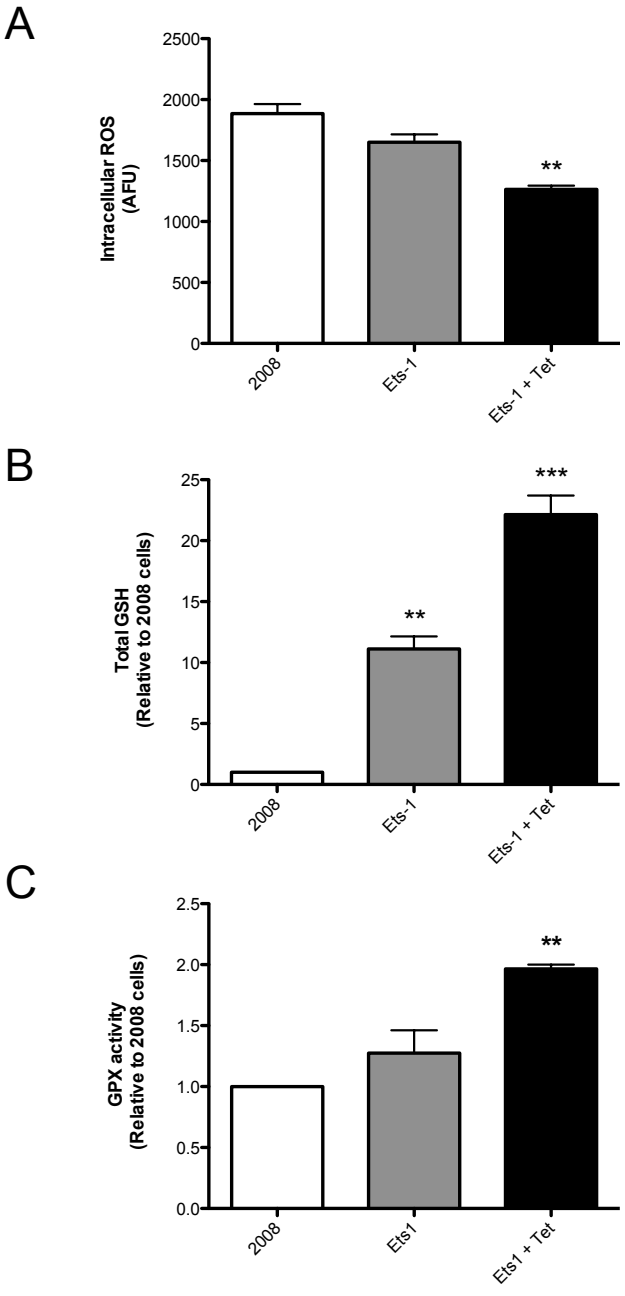
A
Intracellular ROS (AFU)
2500
2000
1500
1000
500
0
**
2008
Ets-1
Ets-1 + Tet
B
Total GSH (Relative to 2008 cells)
25
20
15
10
5
0
***
**
2008
Ets-1
Ets-1 + Tet
C
GPX activity (Relative to 2008 cells)
2.5
2.0
1.5
1.0
0.5
0.0
**
2008
Ets1
Ets1 + Tet

Figure 5.1: Ets-1 decreases intracellular ROS, while increasing intracellular GSH and GPX activity.

A) Basal intracellular ROS levels were measured in 2008, 2008-Ets1 (Ets-1), and tetracycline-induced 2008-Ets1 (Ets-1 + Tet) cells using CM_2-H_2DCFDA. There is a trend for decreased ROS levels in correlation with increased Ets-1 expression (n=3). B) Total intracellular amounts of GSH were measured using an enzymatic recycling method as described by Rahman *et al* (12). Metabolite extracts from 2008, Ets-1 and induced Ets-1 cells contained increased GSH levels concomitantly with increased Ets-1 expression (n=4). C) Glutathione peroxidase activity also displayed a trend towards increased activity in association with higher levels of Ets-1 expression (n=4).

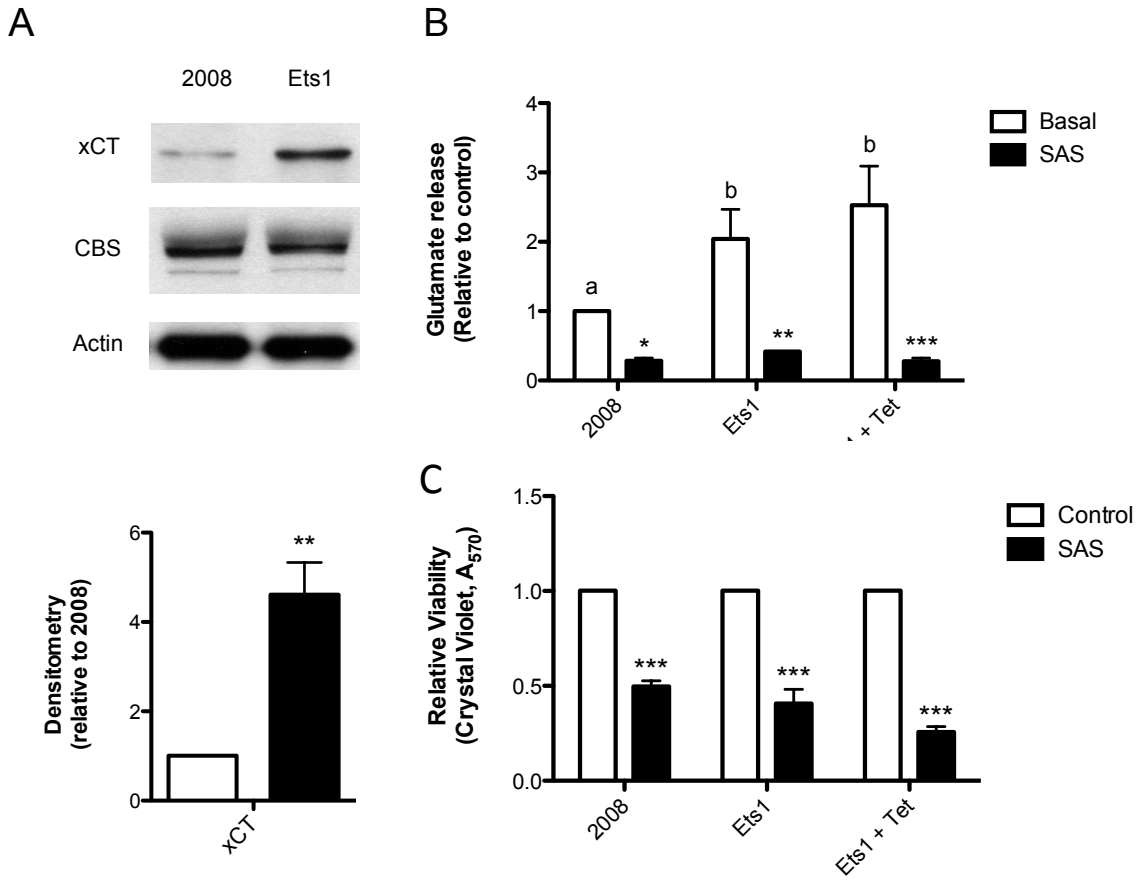

Figure 5.2: Ets-1 increases System x_c^- expression and activity.

A) The protein expression of the catalytic subunit of Sx_c^-, xCT, and the transsulfuration pathway enzyme CBS were compared in 2008 and induced 2008-Ets1 cells via Western Blot. Though the expression of CBS was not different, xCT protein levels were increased in response to Ets-1 overexpression (n=3). B) Glutamate release was measured by AMPLEX red® fluorescence in 2008 and 2008-Ets1 cells under basal or SAS-treated conditions. Cells that overexpress Ets-1 release more glutamate into the culture medium,

and SAS was an effective inhibitor of glutamate release (n=3). C) Cell viability was measured via Crystal Violet assay following SAS treatment, which was found to decrease viability in all cell lines (n=3).

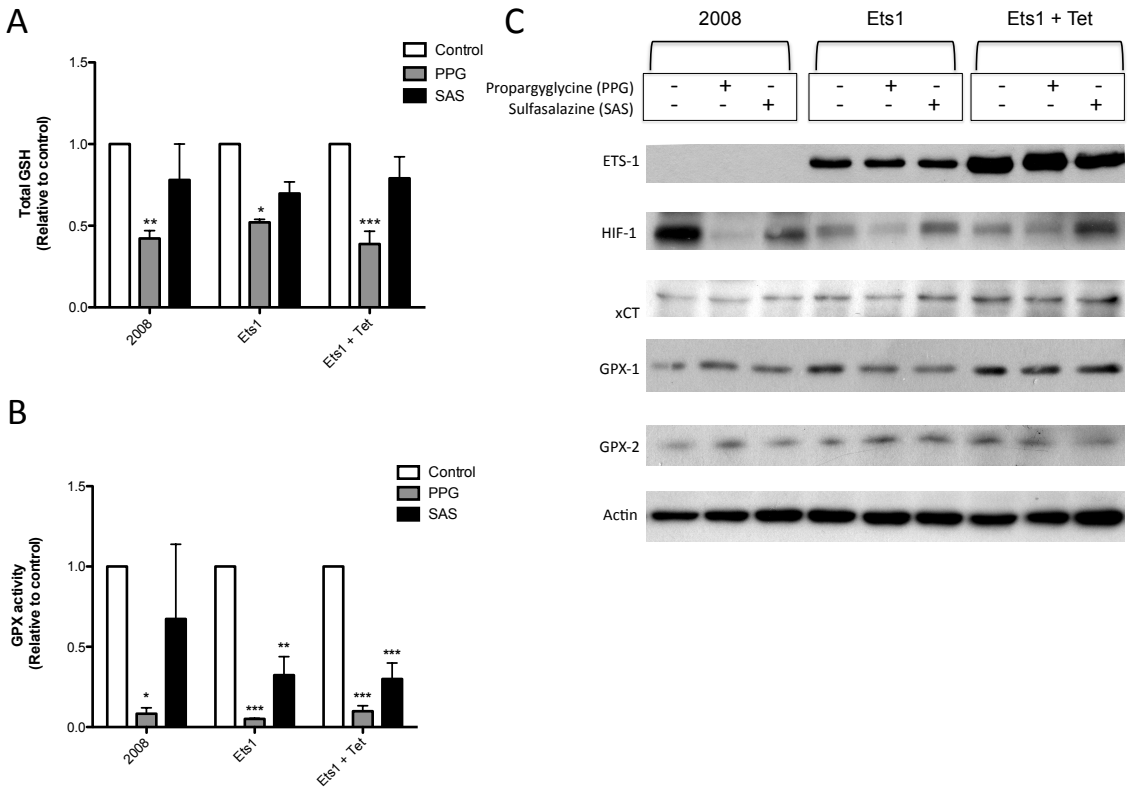

Figure 5.3: The transsulfuration pathway is a major GSH source in normoxic ovarian cancer cells.

2008, 2008-Ets1 and induced 2008-Ets1 ovarian cancer cells were treated with PPG or SAS for 24hrs, and the redox capacity was examined. A) Quantitative total GSH levels were significantly decreased in response to PPG treatment in all cell lines, while SAS did not significantly affect GSH levels (n=3). B) Like total GSH levels, enzyme activity of GPX was significantly decreased by PPG-mediated blockade of the transsulfuration pathway. SAS treatment resulted in decreased GPX activity only in Ets-1 overexpression

cell lines (n=3). C) The protein expression of factors involved in redox regulation was measured via Western blot, including Ets-1, HIF-1, xCT, GPX-1, and GPX-2, with actin as a loading control (n=3).

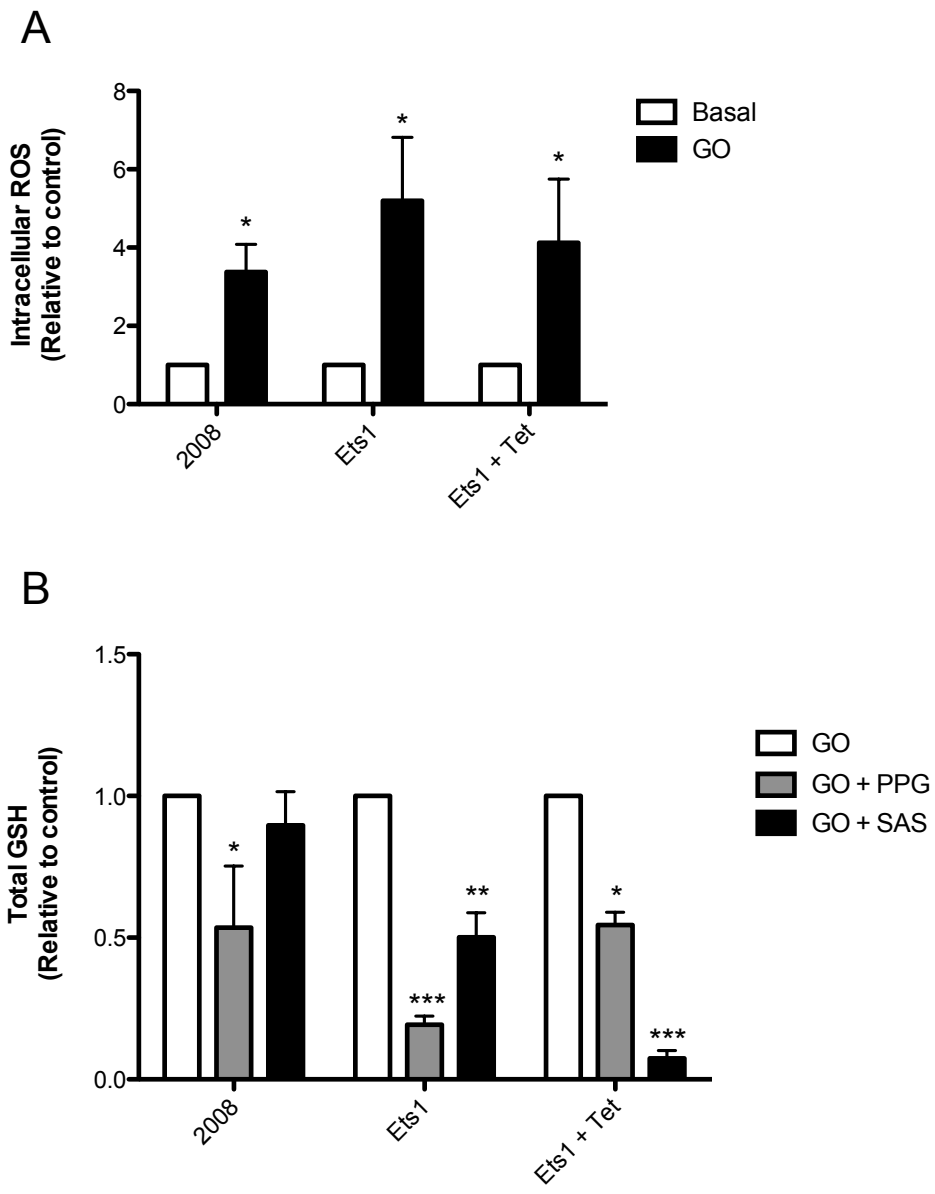

Figure 5.4: Ets-1 recruits Sx_c^- to maintain glutathione pool under oxidative stress.

Glucose oxidase was used to induce oxidative stress in cultures, and successfully increased intracellular ROS levels in all cell lines (A). B) Under oxidative stress, PPG treatment resulted in decreased intracellular GSH levels. The amount of GSH was decreased by SAS in only ovarian cancer cells that express Ets-1 in abundance.

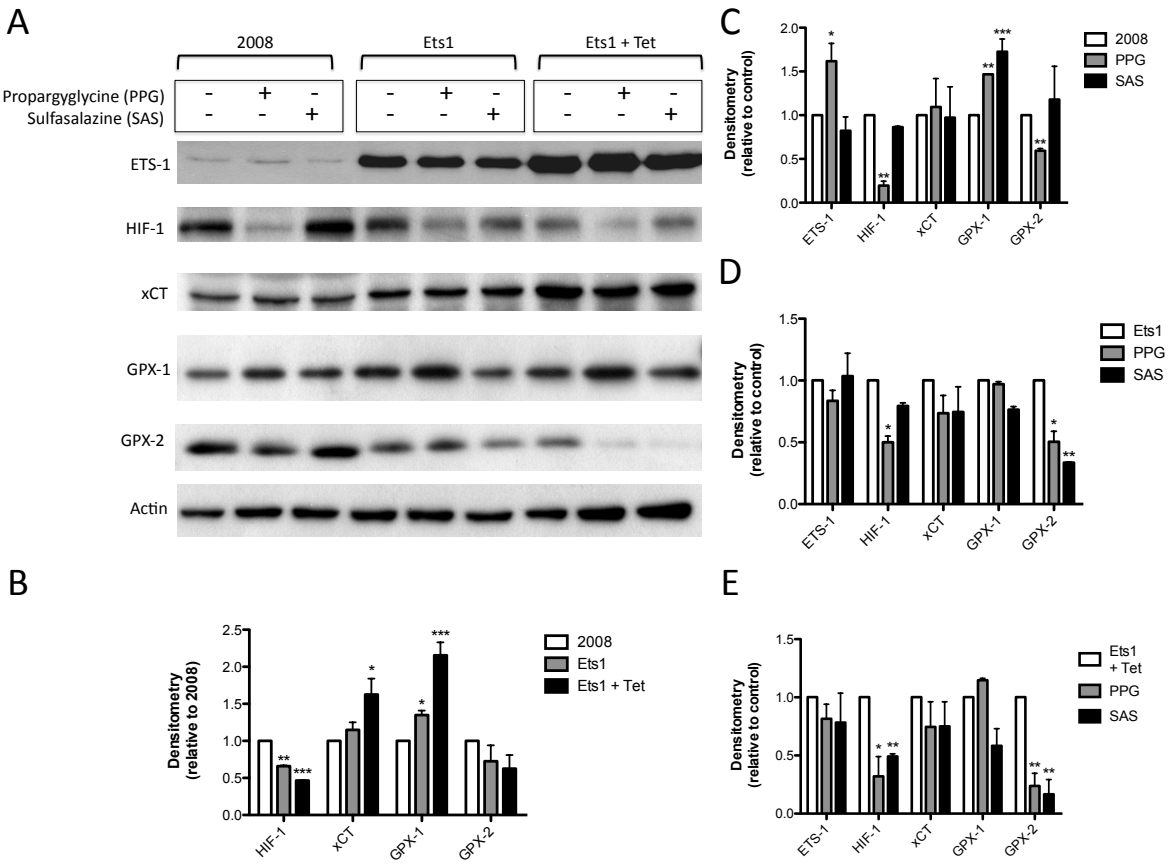

Figure 5.5: Ets-1 redox regulation involves changes in HIF-1 and GPX-2 protein levels.

A) The protein expression of redox balance-related factors was examined by Western blot. B) Both xCT and GPX-1 protein are increased in correlation with Ets-1 overexpression, while HIF-1 and GPX-2 showed a trend to decreased with higher levels of Ets-1. C) In 2008 cells, HIF-1 and GPX-2 were decreased in response to PPG treatment, while GPX-1 was increased with both PPG and SAS. D) In 2008-Ets1 cells, decreased protein expression was observed for HIF-1 and GPX-2, while SAS also

decreased GPX-2 protein levels. E) Similarly, tetracycline-induced 2008-Ets1 cells displayed decreased protein expression of HIF-1 and GPX-2 following PPG and SAS treatment.

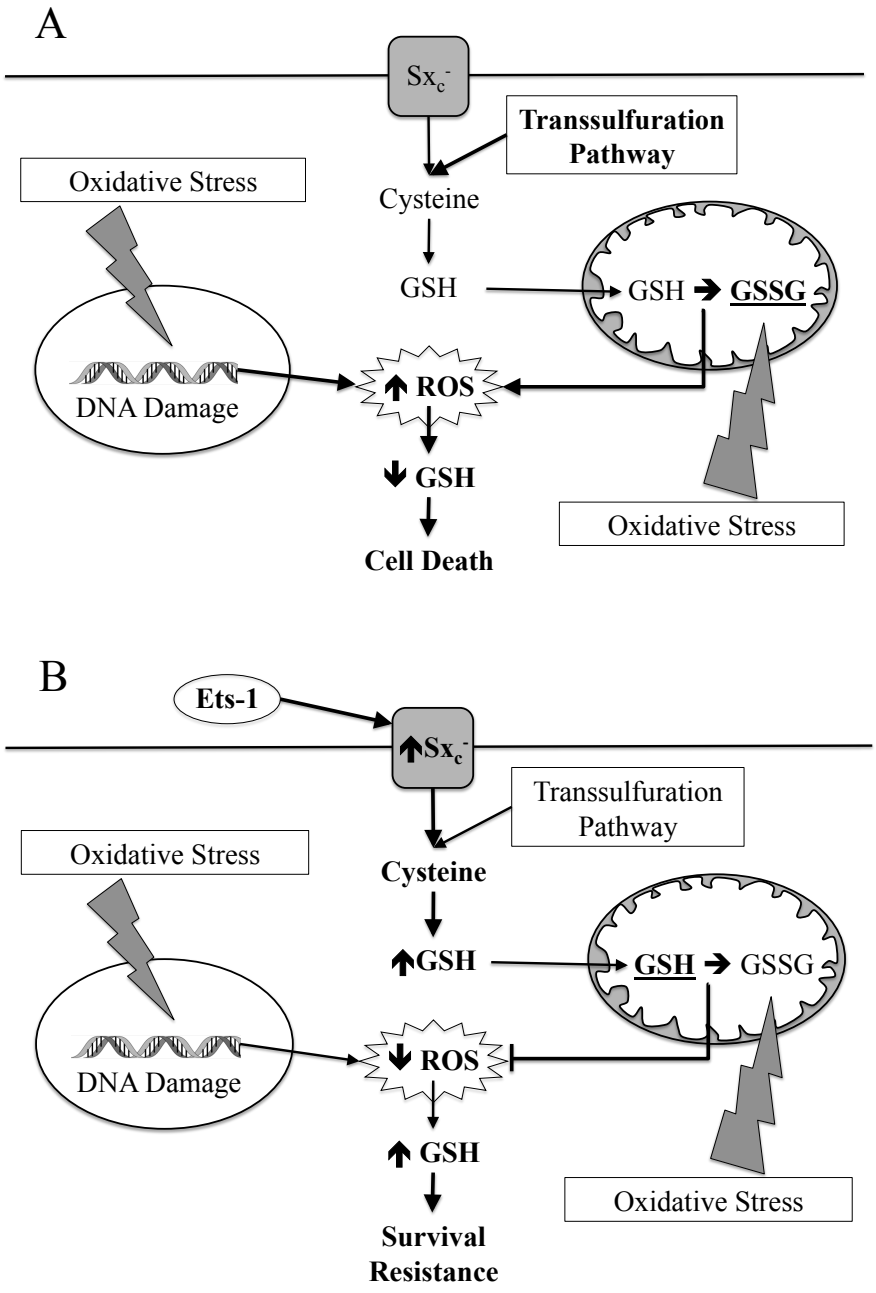
A
Sx$_c^-$
Transsulfuration Pathway
Oxidative Stress
Cysteine
GSH
GSH → GSSG
DNA Damage
↑ ROS
↓ GSH
Oxidative Stress
Cell Death
B
Ets-1
↑Sx$_c^-$
Transsulfuration Pathway
Oxidative Stress
Cysteine
↑GSH
GSH → GSSG
DNA Damage
↓ ROS
↑ GSH
Oxidative Stress
Survival Resistance

Figure 5.6: Proposed mechanism for Ets-1 mediated drug resistance in ovarian cancer.

A) In the absence of abundant Ets-1 expression, the transsulfuration pathway is the main cysteine source for glutathione synthesis. Treatment with chemotherapeutic agents causes DNA damage and GSH depletion by increasing intracellular ROS, leading to cell death. B) Under the control of Ets-1 expression, Sx_c^- activity is increased to bolster cysteine stores thereby increasing intracellular glutathione. High levels of glutathione prevent oxidative stress-inducing therapies from causing an accumulation of ROS. Thus, Ets-1 overexpression may play an important role in the drug resistance often observed in aggressive ovarian cancer.

Chapter 6: Conclusions and Future Directions

In this thesis I have tested the hypothesis that:

> ***"Increased expression of Ets-1 mediated by oxidative stress in ovarian cancer causes an alteration in metabolic gene expression pathways. Specifically, Ets-1 is involved in the regulation of glycolysis, oxidative phosphorylation, and antioxidant defenses in ovarian cancer cells."***

From this hypothesis, several experimentally testable objectives were formulated that represent the main body of the experimental work presented in this dissertation. Each of these specific objectives will be address individually in this final chapter, and the major results from each objective will be summarized. Major conclusions will be drawn for each body of experimental work to determine whether the results support the overall hypothesis.

> Objective 1: Examine the role of Ets-1 in the regulation of mitochondrial metabolism in an ovarian cancer cell model.

The corresponding null hypothesis to this statement would be that Ets-1 is not involved in the regulation of ovarian cancer metabolism. However, as our research group has previously established, Ets-1 expression is increased by high levels of the metabolic byproduct H_2O_2, as well as through malfunctions in oxidative phosphorylation (Wilson et al., 2005). Thus, it is reasonable to theorize that Ets-1 is involved in the regulation of mitochondrial metabolism in ovarian cancer cells.

The experimental evidence provided in Chapter 2 of this thesis clearly demonstrates that the induction of Ets-1 overexpression leads to changes in the metabolic phenotype of the 2008/2008-Ets1 ovarian cancer cell model. To investigate this objective, a tetracycline-inducible, stable Ets-1 overexpression cell line was used, which is derived from 2008 ovarian cancer cells (Wilson et al., 2004). The major hallmark of the Warburg effect in cancer cells is the utilization of glycolysis for energy metabolism, regardless of the presence or absence of oxygen (Warburg, 1956; Warburg et al., 1927). The findings from glycolytic dependency assays performed in Chapter 2 clearly demonstrate that 2008 ovarian cancer cells that express high levels of Ets-1 show a distinct dependence on glucose for proliferation and survival. Furthermore, the gene expression microarray analysis shows that genes involved in glycolysis and associated glycolytic feeder pathways were increased in response to Ets-1 overexpression. Concomitantly, genes related to oxidative phosphorylation were downregulated, illustrating a rare example of Ets-1 acting as a transcriptional repressor. In correlation with these genomic results, cellular oxygen consumption of these cells was also decreased compared to 2008 parental cells, indicating a decreased oxidative capacity. Overall, the findings presented support the rejection of the null hypothesis, leading to the conclusion that Ets-1 is involved in the regulation of ovarian cancer cell energy metabolism.

Objective 2: Generate a global gene expression profile of Ets-1 overexpression in an ovarian cancer model to further clarify the role of this factor in cancer metabolism and the response to oxidative stress in ovarian and breast cancers.

The null hypothesis for this statement is that the global gene expression profile for Ets-1 overexpression in 2008 ovarian cancer cells does not contain enrichments in metabolic or oxidative stress pathways. The bioinformatic data presented in Chapter 3 show that metabolic and oxidative stress pathways are prominently enriched across various means of analysis. Indeed, comprehensive gene set enrichment, functional ontology, and functional interaction networking analyses all displayed enrichments in various metabolic pathways. Though the expression of several genes involved in these pathways were already reported in Chapter 2, the bioinformatic confirmation of these results gives more weight to the conclusions drawn from objective 1. Importantly, the Ets-1 deficient breast cancer cell model generated in this study displayed the same relationship as in the previous objective where increased Ets-1 expression contributes to glycolytic dependency.

Additionally, both gene set enrichment and functional ontology analyses showed enrichments in oxidoreductase activity, suggesting that Ets-1 is important in the regulation of redox balance in our ovarian cancer cell model. Furthermore, functional assays were performed that validated this assumption showing that Ets-1 overexpression is associated with decreased ROS levels and increased activity of glutathione peroxidase antioxidants in the model system. Collectively, the evidence presented is sufficient to reject the null hypothesis and further supports the role of Ets-1 in the regulation of energy metabolism and the response to oxidative stress in ovarian and breast cancer cells.

Objective 3: Investigate the impact of Ets-1 on the glutathione antioxidant system, and evaluate the effectiveness of glutathione-depleting agents in the Ets-1 overexpression model.

In this case, the null hypothesis would be that differences in Ets-1 expression do not affect cellular antioxidant capacity in our model system. The experiments conducted in Chapter 5 address this hypothesis by examining differences in the glutathione antioxidant system, a major regulator of redox balance, in the 2008-Ets-1 overexpression ovarian cancer cell model. High expression of Ets-1 was associated with decreased intracellular ROS, increased intracellular GSH, enhanced GPX enzyme activity, and increased Sx_c^- transporter activity. The amount of glutathione available for ROS detoxification is limited by the availability of cysteine for synthesis. Under basal conditions, the transsulfuration pathway was the main source of cysteine regardless of Ets-1 expression; however, during oxidative stress Ets-1 overexpressing cells recruited the Sx_c^- transporter to bolster glutathione stores. These observations all imply that Ets-1 is involved, at least in part, in the regulation of cellular antioxidant systems and redox balance in ovarian cancer cells.

Many chemotherapeutic interventions, as well as radiation therapies rely on the induction of massive amounts of oxidative stress to kill cancer cells. Despite this, cancer cells often develop resistance to these therapeutic modalities, particularly in aggressive or recurrent disease. These cancer cells often display increased levels of glutathione, which allows them to survive and be resistant to these methods of treatment. Agents that deplete intracellular GSH, including those that limit cysteine availability, could therefore combat this type of therapeutic resistance. Since Ets-1 overexpression was associated with

increased GSH in the ovarian cancer model tested in this study, the effects of inhibitors of both the transsulfuration pathway and the Sx_c^- transporter were investigated. Interestingly, the Sx_c^- inhibitor sulfasalazine significantly decreased GSH levels and cell viability in 2008-Ets1 cells, particularly under conditions of oxidative stress. Clearly further study is warranted, however, the results from the experiments in Chapter 5 suggest that pre-treatment with sulfasalazine prior to conventional therapies might enhance the efficiency of these treatments for Ets-1-overexpressing ovarian cancers.

Future directions

Collectively, the findings from the objectives presented in this dissertation support the hypothesis that Ets-1 is an important regulator of glycolysis, oxidative phosphorylation, and antioxidant defense in ovarian cancer cells. Despite compiling compelling evidence, the studies presented here are only part of the investigation necessary to determine the extent of the metabolic regulation of this transcription factor. Though the study of Ets-1 and glycolytic dependence was extended to a breast cancer cell model, the experiments investigating antioxidant capacity were performed in only one cell model. Thus, these studies require further validation in more ovarian cancer cell lines, such as SKOV-3, OVAR-3, and primary patient tissues. In addition to the study of other metabolic pathways, the binding activity of Ets-1, as well as the identification of the transcriptional partners involved in the gene expression program defined in Chapter 3 also require further study. Additionally, because *in vitro* modeling cannot accurately represent the complex pathology of ovarian cancer, another logical future direction will be to examine

the role of Ets-1 overexpression on metabolism in an appropriate animal model. Furthermore, the effectiveness of GSH-depletion therapy to abrogate chemotherapeutic resistance in Ets-1 overexpressing ovarian tumours will need to be evaluated *in vivo* to determine the usefulness of such a strategy.

Confirmation Of Ets-1 Target Genes

The whole genome microarray analysis included in Chapters 2 and 3 identified a large number of potentially novel target genes and pathways for Ets-1 in ovarian cancer cells. Several of these factors were also altered at the protein level in the Ets-1 expression model, and furthermore were shown to have altered functional activity as a result of changes to Ets-1 expression levels. Taken together, these observations strongly suggest that at least some of these factors are target genes of Ets-1, particularly the Sx_c^- transporter, and GPX enzymes. In order to truly evaluate this, transcription factor binding assays should be performed. Chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift (EMSA) assays have been performed successfully in the literature to show Ets-1 promoter binding, and thus would be the ideal methods to use (Kamoshida et al., 2012; Kumari, Mukhopadhyay, & Tyagi, 2012; J. Liu et al., 2012). Indeed, several potential Ets-1 binding sites exist in the promoters of both Sx_c^- and GPX1 genes further implicating them as Ets-1 target genes. Additionally, as the ovarian cancer cell line SKOV-3 expresses Ets-1 protein, the effect of Ets-1 knockdown via small interfering

RNA (siRNA) on Sx_c^- and GPX gene expression, protein expression, and functional activities would also gain insight into the relationship between these factors.

Ets-1 overexpression appears to correlate specifically with more advanced, invasive tumours in breast and ovarian carcinomas (Buggy et al., 2004; Davidson, Reich, et al., 2001; Davidson, Risberg, et al., 2001; Fujimoto et al., 2004; Katayama et al., 2005; Span et al., 2002). Thus comparing the transcriptional programs of cancer cells that express low levels of Ets-1 protein to those that have been engineered to express Ets-1 protein in abundance provides a gene expression profile of some of the key differences between invasive and non-invasive ovarian cancer cells. My microarray analysis from Chapters 2 and 3 shows that the expression of Ets-1 in ovarian cancer cells results in a different transcriptional program for gene expression and many of these differences relate closely to various aspects of cancer progression and development.

The Wnt/β-catenin pathway promotes the proliferation and survival of endothelial cells, while the non-canonical Wnt signaling pathway can promote angiogenesis (Zerlin, Julius, & Kitajewski, 2008). In particular the expression of WNT5A is associated with the metastatic potential of cancer cells, however whether WNT5A is increased or decreased in association with cancer metastasis depends on both cell type and context-dependent signaling. WNT5A silencing is more associated with invasive colon cancer cells lines compared to non-invasive lines, as well as poor prognosis in hepatocellular cancer (Geng et al., 2012; Q. Li & Chen, 2012). In epithelial ovarian cancer both *in vitro* and *in vivo* the loss of WNT5A correlated with advanced tumour stage and shorter overall survival times, and WNT5A expression promoted cellular senescence (Bitler et al., 2011). In correlation

with these studies, *wnt5a* is downregulated in ovarian cancer cells expressing Ets-1 suggesting increased angiogenic non-canonical Wnt signaling in this cell type. Additionally, the expression of potent angiogenic factor *vegfc* was increased in 2008-Ets1 cells, which is interesting as the loss of WNT signaling was recently shown to activate VEGFC lymphangiogenic activity (Niederleithner et al., 2012; Zerlin et al., 2008). Thus, an investigation of the relationship between Ets-1, WNT5A, and VEGFC would be yet another interesting avenue to pursue.

GRB2-associated-binding protein 1 (GAB1) is a GRB2-related scaffolding protein that has been studied extensively in the context of PI3K signaling, and correlates with increased Ets-1 in 2008/2008-Ets1 cell model. GAB1 positively regulates the survival and proliferation of fibroblasts, colorectal cancer, squamous cell carcinoma, and melanoma cells, where hepatocyte growth factor (HGF)-mediated GAB1 amplification of PI3K signaling was associated with oxidative stress signaling (Holgado-Madruga & Wong, 2003; Kiyatkin et al., 2006; Oka, Kikkawa, & Nishigori, 2008; Seiden-Long et al., 2008). GAB1 has also been found to increase endothelial cell migration, survival, and VEGF-mediated capillary formation, strongly suggesting an important role for this factor in angiogenesis (Caron et al., 2009; Laramee et al., 2007).

Proteolytic breakdown of the ECM is an essential process to facilitate cancer metastasis. My comprehensive pathway analyses in Chapter 3 uncovered multiple enrichments in invasion-associated proteolytic pathways including associations with the ECM in my global human interaction network, and the downregulation of extracellular protein groups in the ontological analysis. Cancer cell invasion is facilitated by the

coordinated actions of key proteolytic enzymes including uPA, uPA receptor (uPAR), cathepsins, and collagenases. My results suggest that high levels of Ets-1 expression in ovarian cancer cells would result in enhanced cancer invasion due to the increased gene expression of *uPA, uPAR, cathepsins O* and *L1, MMPs 14, 15, 19,* and *28* observed in these cells.

The MMP family of proteases is responsible for the degradation of extracellular matrix proteins, therefore facilitating the migration and invasion of cancer cells. In HeLa cells, the downregulation of Ets-1 results in decreased expression of MMP-1, MMP-3, uPA, and integrin-β2, suggesting that Ets-1 is a direct regulator of invasive potential (Hahne et al., 2005; Rothhammer et al., 2004). The expression of Ets-1 in breast cancer cells is correlated to increased invasion and metastatic potential, in addition to the expression of MMP-1, MMP-3, uPA, and vimentin (Hahne et al., 2008). Recently, Ets-1 protein was found to regulate VEGF-mediated expression of MMP-9 and MMP-13 in SKOV-3 ovarian cancer cells (Ghosh, Basu, & Roy, 2012). In contrast, MMP13 gene expression in Ets1 cells is downregulated suggesting that Ets-1-mediated induction of MMP13 is regulated through growth factor signaling, particularly VEGF.

My findings also suggest that Ets-1 is important in the regulation of MMP-14, MMP-15, and MMP-28, three genes that have not been associated with Ets-1 to my knowledge. MMP-14 and MMP15, also known as MT1-MMP and MT2-MMP respectively, are membrane-inserted proteins involved in the degradation of ECM components. MMP-14 is involved in the induction of cancer cell invasion through the activation of pro-gelatinase A on the surface of cancer cells, thus causing the degradation

of type IV collagen macro-molecules in the ECM (Sato et al., 1994). In addition to the well-established role of MMP-14 in cell migration, a recent 3-D breast cancer model showed that MMP-14 expression was required in endothelial cells and fibroblasts, but not cancer cells, for new blood vessel sprouting (Correa de Sampaio et al., 2012). Although MMP-15 has not been as extensively studied as MMP-14 in the context of cancer metastasis, this membrane protein likely has an equally as important role in cancer cell proliferation and tumour growth (Ito et al., 2010). MMP-15 also confers apoptotic resistance in pancreatic, lung, and cervical cancer cells, an effect that is regulated by HIF-1α under hypoxic conditions (Abraham et al., 2005; Zhu et al., 2011). The relatively new MMP-28 degrades casein and is expressed in pancreatic, ovarian, and colon cancers (Marchenko & Strongin, 2001). Interestingly, when expressed in lung cancer cells MMP-28 resulted in epithelial-to-mesenchymal transition (EMT) of cell phenotype, increased active TGF-β, and the upregulation of MMP-9 and MMP-14 (Illman, Lehti, Keski-Oja, & Lohi, 2006).

In recent years lysyl oxidase-like 2 (LOXL2), a family member of the well-studied lysyl oxidase (LOX), has been the focus of some promising research relating to metastasis (Brekhman & Neufeld, 2009; Hollosi, Yakushiji, Fong, Csiszar, & Fong, 2009; Kirschmann et al., 2002; Schietke et al., 2010; Sebban, Davidson, & Reich, 2009). In breast cancer cells, both LOXL2 RNA and protein are over-expressed in MDA-MB-231, but not in MCF-7 cells suggesting a link to invasive potential and EMT (Hollosi et al., 2009; Kirschmann et al., 2002). Additionally, LOXL2 protein seems to be alternatively processed and cleaved in cancer cells to form a secreted instead of membranous protein,

facilitating LOXL2 interaction with ECM components such as fibronectin, a factor known regulate invasion and cell adhesion that is one of the functional groups downregulated in 2008-Ets1 cells.

Breast cancer anti-estrogen resistance 1 (BCAR1), induced 2.0-fold in 2008-Ets1 cells, is a well-studied mediator of increased migration in a number of cancers including breast cancer, glioblastoma, lymphoma, melanoma, hepatocellular carcinoma and prostate cancer (Guo, Liu, Yang, Zhang, & Yao, 2008; Tikhmyanova, Little, & Golemis, 2010; van Agthoven et al., 2009). Relevant to metastatic potential, BCAR1 is involved in the structural and functional alteration of E-cadherin and β-catenin to increase invasion and metastasis in hepatocellular carcinoma cells (Guo et al., 2008). In breast cancer the expression of BCAR1 is associated with chemotherapeutic resistance and tumour aggressiveness, likely through the regulation of TGF-β where BCAR1 increased MAPK coupling to increase tumourigenesis (van Agthoven et al., 2009; Wendt, Smith, & Schiemann, 2009).

Cell motility is essential for invasive cancer cells, thus cytoskeleton remodeling and altered cell adhesion are necessary for cancer metastasis. Actin cytoskeleton organization was a significantly enriched functional group within the upregulated gene set in ovarian cancer cells expressing Ets-1. The most highly upregulated genes within this functional group included *lcp1, fermt2, limch1, add2,* and *coro2b.* LCP1, also known as L-plastin, is and actin-bundling protein necessary to freely moving cells that is expressed in most epithelial-derived cancer cells (Lin, Park, Chen, & Leavitt, 1993). The ectopic expression of L-plastin has been observed in many types of human cancer including

ovarian, breast, prostate, and colorectal cancers (Shinomiya, 2012). FERMT2, also known as kindlin-2, is a focal adhesion protein that mediates integrin activation, induces tumour cell invasion, and has recently been identified as a regulator of Wnt/β-catenin signaling (Yu et al., 2012).

The cell junction functional group was significantly enriched within the gene set of downregulated genes in 2008-Ets1 cells in the microarray performed. Cell-cell adhesion dictates cell polarity, a characteristic that cancer cells often reduce allowing for invasion and metastasis. Cell adhesion is mediated largely through the actions of cadherins, selectins, integrins, epidermal growth factor receptor, and TGF-β receptor. Cadherins such as E-cadherin, along with catenins that undercoat cadherin protein and function to connect cadherins to the actin cytoskeleton, establish strong cell-cell adhesion, particularly on adherens junctions (Hirohashi & Kanai, 2003). Although Ets-1 did not alter the expression of *e-cadherin* in my expression analysis, increased expression of Ets-1 did result in a significant decrease in *α-catenin* gene expression. Reduced α-catenin expression is associated with increased metastasis and poor prognostic rates in many human cancers due to its role in facilitating the E-cadherin-β-catenin complex that strengthens cell-cell adhesion (Carico et al., 2010; Ji, Wang, Fang, Fang, & Lu, 2011; T. Jiang et al., 2012; Morrogh et al., 2012; Silvis et al., 2011). Increased Ets-1 expression also resulted in potent downregulation of *gap junction protein β 2 and 6* (connexin 26 and 30 respectively), suggesting that Ets-1 is an important regulator for these factors. Both of these factors are known tumour suppressors that are often decreased in aggressive cancer phenotypes in breast, gastric, head and neck, and prostate cancers (McLachlan, Shao,

Wang, Langlois, & Laird, 2006; Ozawa et al., 2007; Sentani et al., 2010; M. Tanaka & Grossman, 2004). Leupaxin (LPXN) is a close relative of paxillin that is known to influence cancer cell invasiveness (D. Li, Ding, Wang, Wang, & Wu, 2009), which has only recently been the focus of cancer research. This LIM domain-containing adaptor was found to localize in focal adhesions and regulate cell adhesion and spreading in leukemic cells (T. Tanaka, Moriwaki, Murata, & Miyasaka). In prostate cancer cells, LPXN was shown to induce motility and invasiveness through the downregulation of p120catenin expression and the redistribution of β-catenin (Kaulfuss et al., 2009).

Clearly the findings from the gene expression microarray analysis describe a wide variety of potential target genes for Ets-1, illustrating the usefulness for high-throughput studies as tools for hypothesis generation. Despite thorough statistical analysis and subsequent validation of my results, further validation at the protein and functional levels are required to confirm these results. The aforementioned upregulated and downregulated genes represent some of the most interesting targets for future study, the study of which could generate several productive research projects. However, confirmation of differential expression via PCR and Western blot, preliminary transcription factor binding site analysis, and subsequent Ets-1 binding confirmation of the identified sites should be conducted prior to pursuing functional studies.

Delving Further Into Metabolism

As described in Chapter 1, cancer cells rely primarily on glycolysis for cellular energy due to their high rates of proliferation. In addition to favouring aerobic glycolysis, cancer

cells also display enhanced anabolic nutrient processing leading to increased rates of protein, nucleic acid, and lipid biosynthesis, as well as lipid metabolism. Fatty acid metabolism affects several important pathways involved in cancer progression including cellular signaling and energy processing (Y. Liu, 2006). One of the key players in cancer fatty acid synthesis, fatty acid synthase (FASN), was upregulated in 2008-Ets1 cells and has extensive and emerging roles in not only cancer metabolism by also increased malignancy (Menendez & Lupu, 2007). Fatty acid desaturase genes *fads-1, fads-2, and fads-3* were also upregulated in 2008-Ets1 cells suggesting a role for Ets-1 in unsaturated fatty acid biosynthesis. Several genes upregulated by Ets-1 expression in 2008-Ets1 cells are involved in fatty acid β-oxidation including acetyl-CoA acyltransferases, acyl-CoA dehydrogenases, oxidases, and sythetases. Carnitine palmitoyltransferases *cpt1a*, *cpt1c,* and *cpt2*, are also upregulated in response to increased Ets-1 expression, which are responsible for facilitating the transport of fatty acids across the mitochondrial membrane. β-oxidation results in the production of acetyl-CoA that is utilized to fuel the TCA cycle, providing an alternative means for TCA cycle-driven ATP production from pyruvate that is predominantly converted to lactate in cancer cells. Considering these preliminary findings, an examination β-oxidation through measuring individual enzyme activity and flux through the pathway would be an interesting avenue for future Ets-1 metabolism work.

Glutamine represents another key nutrient source for cancer cells, where a majority of glutamine is used to produce lactate and alanine resulting in high NADPH levels, likely for use in redox regulation, fatty acid metabolism and other anabolic

processes (DeBerardinis et al., 2007). The other majority of glutamine is used to provide oxaloacetic acid for the TCA cycle through conversion of glutamate to α-ketoglutarate through anaplerosis. Recent studies have shown that hypoxic, proliferating cancer cells undergo isocitrate dehydrogenase-dependent carboxylation of α-ketoglutarate to citrate facilitating glutamine-driven lipid synthesis (Metallo et al., 2012; Mullen et al., 2012; Wise et al., 2011). Another use of glutamine is to provide glutamate for glutathione synthesis and recycling for use in glutathione-dependent antioxidant defense. Ets-1 expression also results in a decrease of *gls2*, suggesting that cells expressing Ets-1 display increased rates of proliferation as GLS2 prevalence is associated with cell quiescence and tumour growth inhibition (Perez-Gomez et al., 2005; S. Suzuki et al., 2010). The evidence presented in this thesis suggests that Ets-1 could play an important role in the regulation of glutamine metabolism and associated pathways in cancer cells, and warrants further investigation due to the general phenomena of glutamine addiction in these cells.

Transcriptional Partners During Oxidative Stress

Though the study of individual transcription factors can provide significant insight into their function, the complex nature of their regulation renders it necessary to also investigate the other transcription factors involved in any given response. The contents of this thesis have described the importance of Ets-1 in both metabolism and oxidative stress regulation; however, it is highly unlikely that this factor is acting alone. The results from Chapter 5 touched on the interplay between Ets-1 and HIF-1 in the response to metabolically driven oxidative stress. Hypoxic conditions increase Ets-1 expression

through the activity of HIF-1, which binds to a putative HRE in the *ets-1* promoter region (M. Oikawa et al., 2001; Peters et al., 2004). Additionally, there is some evidence that Ets-1 is capable of regulating hypoxia-inducible genes either in cooperation with HIF-1 or alone (Salnikow et al., 2008). In Chapter 5, the protein expression of HIF-1 was decreased in correlation with increasing Ets-1 expression, and also significantly decreased in response to transsulfuration pathway blockade. These findings are quite interesting, and certainly warrant further investigation to characterize the relationship between these two transcription factors.

Another relevant potential transcriptional partner would be Nrf2, a transcription factor discussed earlier in this thesis. As the master regulator of the oxidative stress response, this transcription factor is almost certainly involved in the regulation of antioxidant defense by Ets-1. Our laboratory has previously established that Ets-1 is transcriptionally regulated by ROS via an ARE within its promoter, a response that could be mediated by Nrf2 (Wilson et al., 2005). Additionally, since a recent study showed that Nrf2 is induced by several oncogenes in pancreatic cancer leading to decreased ROS levels, it is possible that there exists a feedback loop between Ets-1 and Nrf2 (DeNicola et al., 2011). In this thesis, I have shown that Ets-1 regulated several important factors in the glutathione antioxidant response, factors that are also known targets of Nrf2. Thus, a detailed investigation into the potential cooperation of Ets-1 and Nrf2 in ovarian cancer would be quite interesting. The relative importance of either factor could be determined using a siRNA-based approach, and the effect of GSH-depleting agents such as sulfasalazine could also be monitored. Further projects could be designed that examine

the role of Ets-1 in the stabilization of Nrf2, and also HIF-1, during oxidative stress in cancer cells.

Validation of In Vitro Findings

Though the *in vitro* evidence for Ets-1 metabolic and antioxidant regulation is compelling, these findings do not necessarily translate to what is truly happening in whole tumours. The cell culture environment is nearly completely controlled, and thus can only provide a preliminary indication of the transcriptional programs present in human cancer cells *in vivo*. Therefore, animal studies should be conducted that investigate the differences between Ets-1-expressing and non-expressing tumours with respect to glycolytic dependence, antioxidant capacity, and resistance to oxidative stress-inducing therapies. *In vitro* study of more ovarian cancer cell lines would be a necessary first step in this process, as 2008 ovarian cancer cells do not readily form tumours in animals. The most logical approach would be to use SKOV-3 cells that express Ets-1, and generate a stable Ets-1 knockdown variant to be used for both *in vitro* and *in vivo* work. Additionally, the use of the breast cancer MDA-Ets1KD model already generated in our laboratory would be appropriate to examine the extension of our findings to other types of cancer. Using these models, cells would be injected subcutaneously into nude mice, and the tumour growth kinetics monitored following the administration of the glycolytic inhibitor 2-DG. This experiment would indicate whether or not tumours with elevated Ets-1 expression confer increased glycolytic dependence *in vivo*.

Perhaps the most interesting future direction for research would be *in vivo* examination of the role of Ets-1 in chemotherapeutic resistance, and the impact of sulfasalazine on resistant cells. Previous work in our laboratory complement the experiments conducted in Chapter 5, and illustrate the important role of Ets-1 in chemotherapeutic resistance in ovarian cancer cells *in vitro* (Wilson et al., 2004). The cisplatin-resistant variant of 2008 ovarian cancer cells, C13* cells, express high levels of Ets-1, and the study of which has provided the basis for this thesis. Thus, an appropriate direction for further study would be comparing the cisplatin resistance levels of ovarian cancer cell models (ie C13*, 2008-Ets1, SKOV-3, SKOV-3 Ets-1 knockdown, MDA-MB-231, and MDA-Ets1KD cells) in nude mice. Any cells found to confer chemotherapeutic resistance could be pre-treated with sulfasalazine or other GSH-depleting agents, to determine the efficiency of these drugs to decrease cisplatin resistance. The results from such a study would have great therapeutic potential, as recurrent ovarian cancers are notoriously resistant to conventional therapies.

Summary

The central hypothesis of this dissertation proposes that the transcription factor Ets-1 is an important regulator of the Warburg effect in ovarian cancer cells, as well as a mediator of the oxidative stress response necessary to control metabolic byproduct ROS. The collective research presented shows that Ets-1 does indeed mediate glycolytic dependence in both ovarian, as well as breast cancer cells, and is involved in the regulation of cellular antioxidant capacity by the major redox regulator glutathione. These conclusions illustrate the potential utility of Ets-1 as a biomarker for metabolically altered ovarian tumours that have an increased resistance to conventional therapies that induce oxidative stress.

Beginning with a detailed overview of cancer metabolism, antioxidant systems, and the significance of Ets-1 in cancer cells, data in the form of individual manuscripts were presented to address the regulation of cancer metabolism and antioxidant systems in cancer cells. The first manuscript (Chapter 2) showed that ovarian cancer cells that overexpress Ets-1 display dependence on glycolysis concomitantly with decreased reliance on oxidative phosphorylation, results that were extended to a breast cancer model in my second manuscript (Chapter 3). The microarray performed in my first paper was further examined through pathway-based bioinformatic analyses in my second manuscript to clarify the role of Ets-1 in the 2008 ovarian cancer model system, further illustrating the importance of Ets-1 in ovarian cancer metabolism. Functional enrichments in redox-related factors were validated functionally where GPX protein levels and activity were increased along with decreased ROS levels in Ets-1 overexpressing 2008 ovarian cancer

cells. In a detailed review of the mechanisms associated with mitochondrial-derived ROS in cancer cells (Chapter 4), the importance of ROS in nuclear crosstalk, cellular signaling pathways, and transcription factor signaling were discussed. A connection was established between oxidative stress and Ets-1, where oxidative stress induces Ets-1 transcription leading to downstream increases in metastatic potential, angiogenesis, antioxidant defenses, and metabolic alterations. A final manuscript (Chapter 5) investigates the role of Ets-1 in the maintenance of cellular redox balance, where Ets-1 was shown to mediate increased intracellular glutathione levels in the 2008 ovarian cancer cell model. This manuscript also illustrates the potential for Ets-1 as a marker for therapeutic resistance, and suggests that glutathione-depleting therapy would be an appropriate avenue for combination therapy for the treatment of ovarian cancer. Finally, future directions for the study of Ets-1 in ovarian cancer were presented to define areas of Ets-1 function and signaling that require further investigation.

This dissertation has established the vital role of Ets-1 in ovarian cancer metabolism and redox balance regulation. An overall scenario may then be proposed where mitochondrial mutations caused by exogenous ROS sources result in mitochondrial dysfunction, leading to the excessive production of ROS from increased electron leakage during oxidative phosphorylation. Ets-1 is then upregulated by ROS leading to glycolytic dependence, enhanced antioxidant capacity, and the activation of pathways involved in ovarian cancer progression (Figure 6.1). Overexpression of Ets-1 results in high levels of intracellular glutathione, leading to resistance to oxidative stress-inducing cancer therapies and enhanced cell survival. Since patients with ovarian cancer suffer from high

rates of disease recurrence and therapeutic resistance, the expression status of Ets-1 is particularly relevant in this cancer type. Thus, by developing a better understanding of Ets-1 regulation and signaling in ovarian cancer, we may also discover novel therapeutic approaches that enhance conventional therapies and thereby improve ovarian cancer morbidity and mortality rates. Furthermore, because this transcription factor is overexpressed in a wide variety of cancer types, Ets-1 may be a putative target for therapeutic development in other malignancies, suggesting a generalized role for this transcription factor in cancer metabolism and oxidative stress.

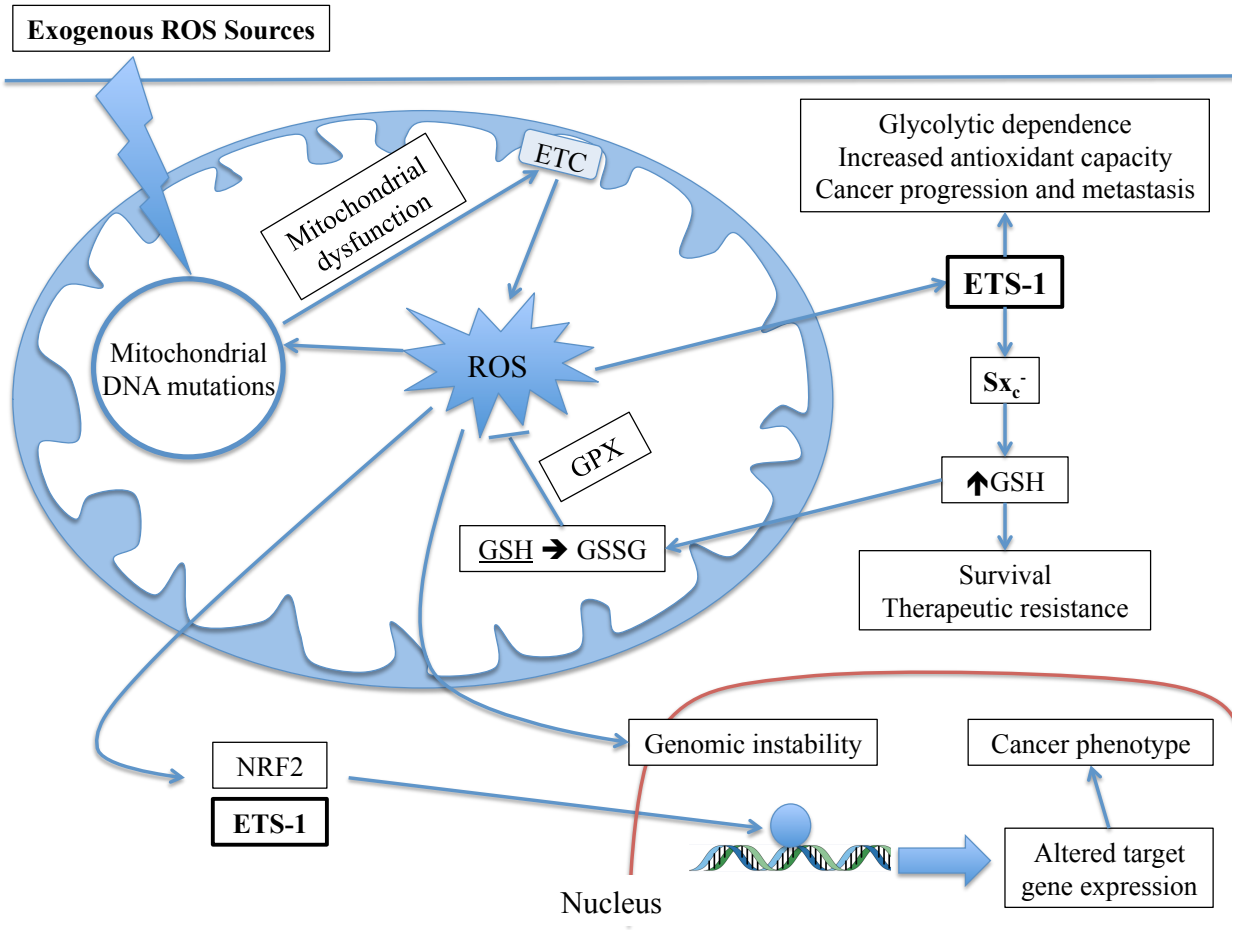

Figure 6.1: Overview of the role of Ets-1 in mitochondrial metabolism and oxidative stress.

Exogenous ROS cause mitochondrial DNA mutations leading to mitochondrial dysfunction and excessive intracellular ROS production. As a result, Ets-1 is transcriptionally induced leading to glycolytic dependence, increased antioxidant capacity, cancer progression, survival, and ultimately therapeutic resistance.

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APPENDIX 1: General materials and methods

This section describes the materials and methods commonly used in greater detail than was possible to include in the sandwiched manuscripts.

Cell culture & treatments

Ovarian and breast cancer cells were grown and maintained in T75 flasks in their recommended culture medium according to the American Tissue Culture Collection (ATCC) guidelines. All media was supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic-antimycotic solution (100 U/mL penicillin, 100 µg/mL streptomysin, and 250 ng/mL amphotericin B), and maintained in an atmosphere of 5% CO_2 at 37°C. Once confluent, cells were subcultured by washing with 10mL of PBS, followed by trypsinization with 2mL of 2X 0.1% EDTA-trypsin, and incubated for 10 minutes to facilitate cell lifting. New culture medium was then added to the flask to deactivate the trypsin, cells were moved to conical tubes, and an appropriate number of cells added to a new flask with fresh culture medium. For certain experiments cells were deprived of serum for 24 hours prior to experimentation, or cultured in different medium to prevent media component interaction with the methods as indicated.

Isolation of total RNA

1) Aspirate media from confluent, plated cells

2) For a 10cm plate add 1ml of Trizol and pipette up and down over plate until Trizol is running easily down the side of the plate (~20 times). Remove cells by gentle pipetting and transfer to an autoclaved 1.5ml tube
3) Incubate for 5mins at room temp (RT)
4) Add 200ul of chloroform for every 1ml of Trizol to the tube. Invert tube 10x fast or vortex briefly until homogenous (looks like Pepto)
5) Incubate at RT of 2-3 mins
6) Spin samples at 12,000g/rcf for 15mins at 4°C
7) Carefully harvest the supernant (clear layer only, be careful not to touch the white or pink layers) into new 1.5ml tubes. Dispose of pink layer & tube into proper waste in the fume hood.
8) Add 500ul of ice-cold 100% isopropanol per 1ml of Trizol originally added.
9) Incubate at RT for 5-10mins
10) Incubate at -20 for at least 1hr (preferably 2hrs)

***PAUSE POINT* Can leave in -20 until ready to continue**

11) Spin samples down at 12,000g/rcf for 10 mins at 4°C
12) Remove supernatant carefully and discard, keeping the pellet
13) Wash pellet with 1ml of 75% ethanol for every 1ml of Trizol originally added. Invert tubes several times and briefly vortex 3X
14) Spin samples down at 7,500g/rcf for 6mins at 4°C
15) Remove supernatant carefully, and allow ethanol to evaporate off in the culture hood for ~15mins or until it is all gone.
16) Dissolve pellet in 100ul of RNA-free water (or 50ul for small pellets)
17) Incubate for 10mins at 60°C in the heat block
18) Store total RNA samples at -70°C until further use (good for years)

DNase Treatment and RNA quantification

1) Thaw components of the Ambion DNA-free kit, and add 10% volume of 10X DNase I buffer to RNA. Add 1µL DNase I enzyme (2 U/mL). Mix and incubate at 37°C for 20-30 minutes.
2) Resuspend DNase inactivation reagent by vortex, and add 10% volume of slurry to sample. Flick tube to get reagent into solution, and incubate at room temperature for 2 minutes.
3) Vortex lightly, centrifuge at maximum speed for 1 minute to pellet the reagent. Put supernatant into a new tube on ice, and discard the pellet.
4) Prepare new tubes for quantification of RNA, and add 2µL of RNA to 98µL of MilliQ water.
5) Measure RV absorbance at 260nm and 280nm on a spectrophotometer.
6) Calculate the concentration of RNA in µg/mL.

cDNA Synthesis

Total RNA was reverse transcribed into cDNA using the Superscript III kit (Invitrogen) as per the manufacturer's instructions. Briefly, 3µg of RNA was diluted in DEPC water to a total volume of 8µL, and 1µL each of Oligo$(dT)_{20}$ and 10mM dNTP mix were added to each sample. Tubes were briefly centrifuged to ensure all contents were mixed, and heated at 65°C for 5 minutes in a MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories), and crashed on ice immediately following the incubation. PCR reaction mix containing 2µL 10X RT Buffer, 4µL 25mM $MgCl_2$, 2µL DTT, 1µL RNase OUT, and 1µL Superscript III was added to each sample tube, and gently mixed. Following brief centrifugation, samples were returned to the thermal cycler for incubation at 42°C for 55 minutes, and 70°C for 15 minutes. RNase H (1µL) was added to each reaction and incubated at 37°C for 20 minutes to remove any residual RNA.

Real-time qRT-PCR

All real-time qRT-PCR reactions were performed using a 10µL total reaction volume containing 5µL Platinum SYBR Green (Invitrogen), 0.5µL bovine serum albumin (BSA), 0.25µL each of sense and anti-sense 10µM primer, and 4µL of 1:10 diluted cDNA (prepared as described above). Each primer pair was optimized and amplification efficiency was determined via standard curve generation of serially diluted, pool cDNA, and products were examined via agarose gel electrophoresis to ensure proper size. Appropriate housekeeping genes were included in every real-time qRT-PCR experiment, with the average expression values of at least three different genes being pooled and averaged for normalization purposes. Products were amplified on a MiniOpticon System (Bio-Rad Laboratories) as follows: 50°C for 2 minutes, 95°C for 2 minutes, followed by 35 cycles of 95°C for 15 seconds, then 55-60°C (depending on primers in use) for 60 seconds. Gene expression fold changes were calculated based on an average of two analyses: the comparative threshold cycle method (ΔΔCt) and Pfaffl method (Pfaffl, 2001).

Cell Lysate Collection

Cells were grown in 55cm^2 dishes to confluency, mediate was aspirated, and cells were washed with 10mL PBS. Cells were lysed with 200µL of lysis buffer containing 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate in PBS, and scraped into Eppendorf tubes. Samples were boiled at 95°C for 5 minutes, then centrifuged at 13,000rpm at 4°C for 10 minutes. Supernatant was added to new tubes, and stored a -20C.

Bio-Rad protein assay

The protein concentration of whole cell lysates was determined by the Bio-Rad protein assay, using a standard curve of BSA dilutions (0.0313, 0.0625, 0.125, 0.250, 0.5, 1.0, and 2.0 mg/mL, respectively) and sample dilutions (1:1, 1:10, 1:100). The BioRad solution was prepared in a 1:4 dilution in MilliQ water, and added to each protein dilution. The absorbance was measured using a BioTek PowerWave XL plate reader at 570nm, and the final protein concentrations were calculated in Microsoft Excel using linear regression calculations from the BSA standard curve.

Western Blotting

Gel preparation:

10% Resolving Gel Recipe
11.6mL ddH_2O
10mL 30% Acrylamide
7.8mL 1.5M Tris-Hcl, pH 8.8
300µL 10% SDS
300µL 10% Ammonium persulfate
12µL TEMED

Stacking Gel Recipe
8.4mL ddH_2O
2mL 30% Acrylamide
1.5mL 1.5M Tris-Hcl, pH 6.8
120µL 10% SDS
120µL 10% Ammonium persulfate
12µL TEMED

SDS-PAGE Electrophoresis

1) Thaw protein samples, add appropriate volume of loading buffer, and boil at 95°C for 5 minutes.
2) Place prepared gels into the electrophoresis assembly, and add diluted running buffer between gel plates, allowing buffer to overflow into the main tank to 500mL.

3) Pipette appropriate quantity of volume-corrected protein sample (20-30µg protein/well is ideal).
4) Run apparatus at 100V through the stacking gel, then increase voltage to 120-150V until the samples have separated to the bottom of the gels.
5) Carefully remove gels from glass plates, and store in wet transfer buffer prior to transfer.

PVDF Membrane Transfer

Transfer Buffer Recipe
2.9g Glycine
5.8g Tris
200mL Methanol
H_2O to 1L

1) Assemble transfer apparatus, including ice packs, and place into a large container filled with ice.
2) Equilibrate PVDF membrane pieces in 100% methanol for 30 seconds, and then place in transfer buffer for 10 minutes.
3) Soak filter paper, sponge pads, membranes, and gels in transfer buffer thoroughly to discourage bubbles.
4) Assemble wet transfer sandwich as follows:
    a. BLACK – sponge pad – 2X filter paper – gel – membrane – 2X filter paper – sponge pad – RED/CLEAR
5) Run apparatus at 100V for 1.5 hours
6) Carefully removed and store membranes in TBS, and discard gels.

Membrane Blocking and Antibody Detection

1) Incubate PVDF membrane for 1 hour at room temperature on a rocker in 5% skim milk/TBS-T.
2) Rinse membranes with TBS-T, and incubate in primary antibody (typically 1:1000 dilution) in 0.5% skim milk/TBS-T overnight at 4°C in an airtight container.
3) Remove primary antibody (store at -20°C for subsequent use), and wash membranes with 15mL of TBS-T for 10 minutes on a rocker, repeat washing 3 times.
4) Incubate membranes in HRP-conjugated secondary antibody (typically 1:2000) in 0.05% skim milk/TBS-T for 2 hours at room temperature on a rocker.
5) Thoroughly wash membranes 3 times for 10 minutes with 15mL of TBS-T.

Exposure of Membranes to Film

1) Incubate membranes for 1 minute in 2mL of GE Healthcare ECL Detection Reagent at room temperature on saran wrap.
2) Drain off excess ECL reagent, and place in film cassette between two sheets of plastic.
3) Expose to autoradiography film and process in a film developer for exposure times appropriate to the protein being detected.

CM_2-H_2DCFDA Intracellular ROS detection

Reagent:

20x 50ug (Invitrogen C6827)

MW 577.8013

DCFDA Stock (100uM):

Add 86.5ul DMSO to 20ug tube of DCFDA reagent, and vortex lightly to ensure it is dissolved. In a new tube add 778.5ul of phenol red-free media with 10% FBS (or PBS+10%FBS), then add 86.5ul of resuspended DCFDA reagent.

(FBS is essential for the dye to effectively enter the cells)

Do this immediately before loading cells, not ahead of time

PROTOCOL

1) Seed cells in 96 well plate at an appropriate density for the cell type, cells should be 70-90% confluent the next day, ensure medium is phenol red-free (cell should be maintained in PR-free media for at least 24hr before assay)
2) If appropriate, expose cells to experimental treatment
3) Remove media, wash cells with PBS, add 100ul of phenol red-free culture medium (or PBS+10%FBS) to each well containing cells
4) To determine appropriate DCFDA concentration for the cell line, load cells with DCFDA dye at the following concentrations upon first assay:
    a. 20uM
    b. 10uM (typical concentration used)
    c. 5uM
    d. 2.5uM
    e. 1uM

5) Cover plate in aluminum foil and place in 37°C incubator for 5-60mins (30 mins optimal for most cell lines)
6) Remove dye and wash 3x PBS, add 100ul of phenol red-free culture medium (or PBS+10%FBS) to each well
7) If appropriate expose cells to experimental treatment
(using CM2-H2DCFDA should ensure stability of the dye within the cells for long-term treatments, though some cells will spit out the dye quickly)
8) Treat cells with 250uM H_2O_2 to spike ROS generation
9) Immediately read plate in fluorescent plate reader at 485nm excitation and 530nm emission every 5 mins for 1hr.

Controls

Negative:

1) Un-dyed cells for autofluorescence
2) Cell-free mixture of dye and media with and without experimental treatments
3) Untreated DCFDA-loaded cells in media
4) Cells treated with antioxidants (CAT or NAC)

Positive:

1) Cells treated with H2O2 (250uM)

Calculations

Arbitrary Fluorescent Unit method (most common):

Choose the time-point reading where absorbance is maximal (usually 45m-1hr), use these values to report as either relative measure compared to control or as AFU. You can also (recommended) do a crystal violet assay of the cells after the assay is finished reading, and normalize your values to the resulting absorbance reading, or run a standard curve of known cell numbers to normalize to and report findings as AFU/10^6 cells (or whatever is appropriate).

Kinetic method (most reproducible):

To determine ROS levels, generate a curve for each sample and control of AFU (arbitrary fluorescent units) per minute, to determine the rate of ROS generation. Subtract the ΔAbs/min for the background (DCFDA + H2O2 well, no cells) from that of the samples. Using a extinction coefficient of 36,295 M^{-1} (EC of DCF at 500nm (59,500 M^{-1} cm^{-1}), using a path length of 0.61cm) and the following equation:

ROS = (ΔAbs/min)/36,295 M^{-1}

REFERENCES

Extinction Coefficient of DCF from - Methods in Enzymology: Volume 301 (Nitric Oxide), 1999.

Quantitative determination of GSH & GSSG

GSH assay principle:

Assay is based on the colorimetric reaction of GSH with DTNB that produces a yellow colour upon formation of TNB as measured at 412nm, where the rate of TNB production is proportional to the concentration of GSH in the sample. This assay uses a recycling method where the GS-TNB product is reduced by GR back into GSH in the presence of NADPH. Thus the value measured represents the sum of reduced and oxidized glutathione in the sample:

[GSH]total = [GSH] + 2x[GSSG]

GSSG assay principle:

The measurement of GSSG in sample is challenging because the ratio of GSH:GSSG is only 1:10, and rapid oxidation of GSH will alter this ratio favouring GSSG levels. To avoid this issue, 2-vinylpyridine, which covalently binds GSH (but not GSSG) thus preventing oxidation, is added to the sample extracts. The measurement of GSSG is achieved by monitoring NADPH levels using Griffith's method (Griffith, 1980).

REAGENTS

-KH2PO4
-EDTA
-Sulfosalycylic acid
-Triton X-100
-DTNB (Sigma D-8130)
-β-NADPH (Sigma N-7505)
-Glutathione reductase (Sigma G-3664)
-Glutathione (reduced)/GSH (Sigma G-4251
-Glutathione (oxidized)/GSSG (Sigma G-6654)
-Triethanolamine (Sigma T1377)
-2-Vinylpyridine (Sigma 132292)

-PBS
-Culture media without phenol red

EQUIPMENT

-96 well plates
-Plate reader
-1ml syringes, 26 ½ gauge needles

REAGENT PREP

KPE Buffer A
6.8g KH_2PO_4 to 500ml dH_2O
Store at 4°C
KPE Buffer B
8.5g KH_2PO_4 to 500ml dH_2O
Store at 4°C
KPE Buffer *MAKE FRESH*
Add 16ml of KPE A
Add 84ml KPE B
Adjust pH to 7.5, and add 0.327g EDTA.
Extraction buffer *MAKE FRESH*
Add 20ul Triton X-100 and 120mg sulfosalicylic acid to 20ml KPE buffer
2-Vinylpyridine
CAUTION – this chemical is very nasty, use ONLY in fume hood, wear a mask when transferring to plate reader out of fume hood
-Dilute 1:10 with KPE in fume hood, store on ice
Triethanolamine
-Dilute 1:6 in KPE, store on ice
DTNB
4mg DTNB in 6ml KPE
Prepare FRESH immediately before assay and wrap in foil to prevent light exposure (causes non-specific colour intensity)
NADPH
4mg β-NADPH in 6ml KPE
Prepare FRESH immediately before assay and wrap in foil to prevent light exposure
Glutathione Reductase
80ul GR in 6ml KPE
Prepare FRESH immediately before assay

GSH Standards
Prepare GSH stock solution:
-1mg/ml GSH in KPE buffer (can store at -20 for several weeks)
Working solution:

-Add 8ul GSH stock solution to 992ul KPE to make top concentration of 26.4nM/ml
Standards:
Make two-fold serial dilutions for a series of standard concentrations:
26.4nM/ml, 13.2nM/ml, 6.6nM/nl, 3.3nM/ml, and so on to 0.103nM/ml

GSSG Standards
GSSG stock solution
-2.01mg/ml GSSG in KPE buffer (can store at -20 for several weeks)
Working solution:
-Add 4ul GSSG stock solution to 996ul KPE to make top concentration of 26.4nM/ml
Standards:
Make two-fold serial dilutions for a series of standard concentrations:
26.4nM/ml, 13.2nM/ml, 6.6nM/nl, 3.3nM/ml, and so on to 0.103nM/ml
*The concentration range of standard to be included in the assay can be varied so that the concentration of GSH/GSSG in the samples falls within the calibration curve. This needs to be determined for each cell line, for 2008 ovarian cells 13.2nM/ml -> 0.206nM/ml is best.

EXTRACT PREPARATION FOR ADHERENT CELLS

1. Grow cells in culture plates to 70-80% confluency
2. Wash twice with ice-cold PBS (Ca/Mg free)
3. Remove cells with scraper to 1ml of ice-cold PBS, transfer to a pre-chilled 1.5ml tube
4. Pellet cells at 2000g for 5mins at 4°C
5. Discard supernatant, resuspend pellet in 1ml ice-cold PBS, and cetrifuge again as in step 4
6. Discard supernatant and resuspend the pellet in 1ml ice-cold extraction buffer (0.1% Triton X-100, 0.6% sulfosalicylic acid in KPE)
7. Homogenize cell suspension by drawing through a 26 ½ gauge needle 3-5X
8. To further ensure proper lysis, freeze and thaw the cells (-70°C) 2X
9. Centrifuge cells at 3000g for 4min at 4°C
10. Immediately transfer the supernatant to pre-chilled 1.5ml tubes

Pause point – The extracts can be stored at -70°C for further use (up to 3 months)

MEASUREMENT OF TOTAL GSH

1. Add 20ul of KPE to well A1 (blank), add 20ul of each standard to wells B1-H1 (7 standards total), then add 20ul of your samples in duplicate (or triplicate if you have room) to well A-H in columns 2-6 maximum – DO NOT fill the whole plate as it is too time consuming and the assay is kinetic.
2. Mix 3ml of DTNB with 3ml of GR solutions together, and quickly add 120ul to each well. Avoid direct exposure to light, best to do this part of the assay in the culture hood without the light on or dim the lights in the lab
3. Allow 30sec for the conversion of GSSG to GSH

4. Quickly add 60ul of β-NADPH to each well
5. Immediately read the absorbance at 412nm in a microplate reader, and take kinetic measurements every 30sec for a total of 2mins (5 readings in total)
6. Calculate the rate of TNB formation, or the change in absorbance per minute. Determine the total GSH concentration by using linear regression from the calculated values obtained from the standard calibration curve. Express total GSH concentration as either uM or nM/mg protein or nM per 10^6 cells.

MEASUREMENT OF GSSG

1. Add 100ul of cell extract to a 1.5ml tube
2. Add 2ul of diluted 2-vinylpyridine, and vortex to derivatize GSH.
3. Incubate in the fume hood for 1hr at RT
4. Add 6ul of diluted triethanolamine to the side of the tube, and vortex rapidly and vigourously to ensure neutralization
5. Incubate in fume hood for 10min at RT
6. Assay the samples in a 96-well plate as described in steps 1-6 of GSH assay

Ensure that all GSSG standards and KPE blank contain 2-vinylpyridine and triethanolamine to control for any effects of those reagents

Glutamate release assay
(Originally created by Dr. Eric Seidlitz)

General protocol for quantification of L-Glutamic Acid (glutamate) in the 0.5 – 20 µM range using the Amplex® Red Glutamic Acid/Glutamate Oxidase Assay kit from Invitrogen (catalogue # A12221). Read kit instructions carefully first. Quantities or steps that will vary are in violet.

A. Stock solution preparation

1. Prepare 1X reaction buffer: Add 5 mL of 5X reaction buffer stock solution (Component E) to 20 mL deionized water. Store at 4°C. [If needed, 5X reaction buffer can be prepared by diluting 1M TRIS to 0.5 M then adjusting the pH to 7.5.]
2. Prepare a 200 mM solution of L-Glutamic Acid by dissolving the contents of the vial of Component H in l00 µL of 1X reaction buffer. Aliquot to 10 vials of 50 µL each and store at -20°C.

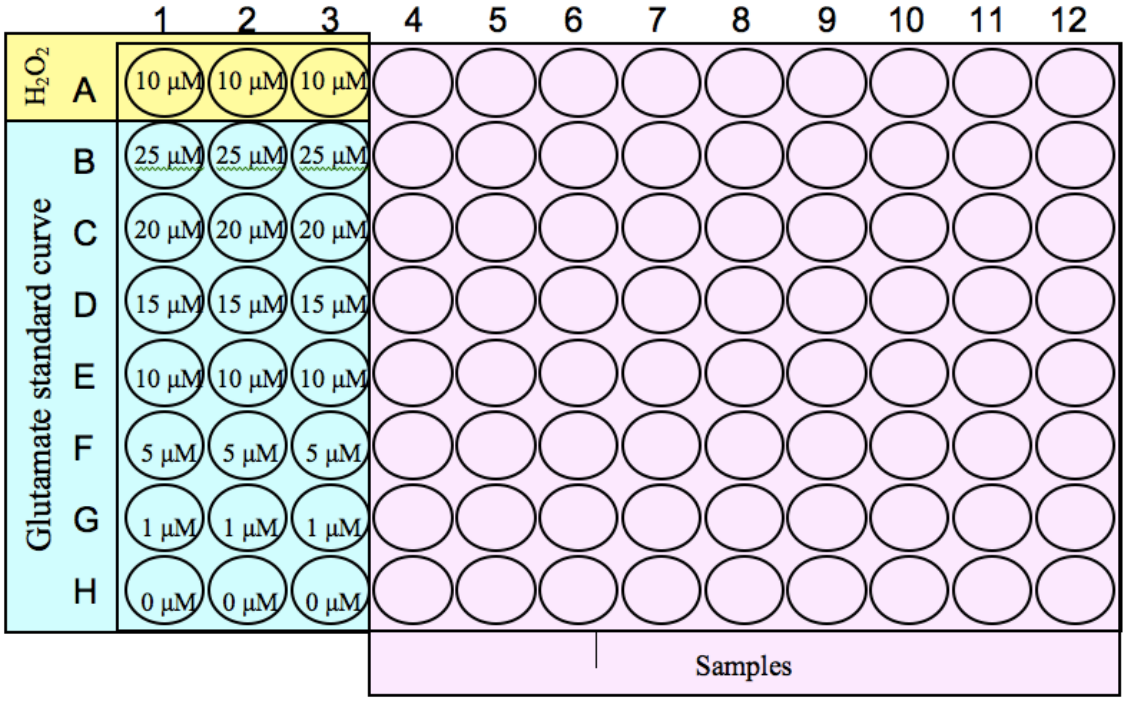
B. Standard curve preparation with points in triplicate: for 1 plate

1. Prepare 1 mM glutamate solution in a microtube: Add 2 µL of 200 mM glutamate stock to 398 µL of 1X reaction buffer.
2. Prepare a 25 µM glutamate solution in a microtube: Add 10 µL of 1 mM glutamate solution to 390 µL of 1X reaction buffer. Deliver 100 µL to well B1 of a preparatory 96-well plate.
3. Prepare rest of standard curve directly in the preparatory plate:

Well ID	Volume of 1X reaction buffer	Volume of 25 µM glutamate solution	Final concentration
B1	-	100 µL	25 µM
C1	20 µL	80 µL	20 µM
D1	40 µL	60 µL	15 µM
E1	60 µL	40 µL	10 µM
F1	80 µL	20 µL	5 µM
G1	96 µL	4 µL	1 µM
H1	100 µL	-	0 µM

4. Prepare a serial dilution of hydrogen peroxide: starting from the concentration of the stock, prepare a **first** tube of 20 mM H_2O_2 (e.g. 9.6 µL of a 3.6% stock + 490 µL of 1X reaction buffer). Prepare a **second** tube of 0.5 mM: 2 µL of 20 mM + 78 µL of 1X reaction buffer. Prepare a **third** tube of 10 µM: 4 µL of 0.5 mM + 196 µL of 1X reaction buffer. Deliver 100 µL of the 10 µM solution to well A1 of the preparatory plate.
5. Using a multichannel pipettor, dispense 25 µL of each of the eight wells A1 to H1 to three wells each of the assay plate, as per scheme:

Assay Scheme



C. Sample preparation

1. Map out sample location on plate, planning for duplicates or triplicates of each sample.
2. Prepare samples by diluting in 1X reaction buffer. A minimum of 1:5 dilution is recommended, with the aim to bring the sample concentration to within the range of the standard curve. Prepare at least 50 µL of each sample for duplicates and at least 75 µL of each sample for triplicates.
3. Deliver 25 µL to each well of the assay plate, as indicated on scheme above.

D. Assay preparation and initiation: for 1 plate

1. Prepare an assay mix for 100 reactions (2.5 mL) [NB. Protect from light once Amplex Red is added – add 30 µL DMSO to AR aliquot to make 2.6 µg/µL]:

Reagent	Volume to add	Final concentration in mix	Final concentration in assay
1X reaction buffer	2429 µL	-	-
HRP, 100 U/mL	6.25 µL	0.25 U/mL	0.125 U/mL
L-Glutamate Oxidase, 5 U/mL	40 µL	0.08 U/mL	0.04 U/mL
Amplex Red, 2.6 µg/µL	25 µL	0.026 µg/µL	0.013 µg/µL

2. Deliver 312 µL to each of 8 free consecutive wells of preparatory plate. Using multichannel pipettor, add 25 µL of mix to each well of the assay plate, mixing gently by pipetting.
3. Incubate at 37°C for 30 min (not in cell culture incubator with CO_2). Turn UV lamp on in plate reader 15 min before incubation is over.
4. Measure fluorescence using an excitation wavelength of 530-560 nm and emission wavelength of ~590 nm (530/25 nm and 580/50 nm in the CytoFluor in room 4-68, with the gain set at 40). Export the file to Excel – the Cytofluor computer only uses 3.5" floppy discs, so the file will need to be transferred to a computer with both a floppy drive and a USB port.

For each plate, you will need:

- 2 µL 200 mM glutamate stock
- 5 mL of 5x reaction buffer
- 9.6 µL of 3.6% H2O2
- 6.25 µL HRP
- 40 µL l-glutamate oxidase
- 25 µL AR reagent (most of the aliquot in which 30 µL DMSO was added)

Statistical Analyses

The statistical analysis methods used to determine statistical significance of experimental results were determined based on the variables and level of measurement for each analysis. The experimental data presented were normally distributed with independent groups, and so parametric statistical tests were predominantly used. Experimental data are presented as the mean and standard error (SE) of at least 3 independent experimental replicates, which were normalized to the appropriate controls. When two specific groups were compared, an unpaired student's T test was performed, whereas when more than two groups were compared an analysis of variance (ANOVA) test was performed. Differences between experimental groups were considered statistically significant at a p value of <0.05. These values were calculated using GraphPad Prism software or R where appropriate.

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