# PHENETHYL ISOTHIOCYANATE (PEITC) DECREASES SPECIFICITY PROTEIN (SP) TRANSCRIPTION FACTORS THROUGH AN ROS-DEPENDENT MECHANISM

A Thesis

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## ABSTRACT

Isothiocyanates (ITCs) are phytochemicals highly expressed in cruciferous vegetables and these compounds are associated with the decreased incidence of cancers in populations consuming high levels of cruciferous vegetables. Several individual ITCs including phenethyl isothiocyanate (PEITC) inhibit tumor growth and angiogenesis and their anticancer activity has been linked to inhibition of cancer cell growth, survival and inflammation (NFκB). It has also been demonstrated that PEITC induces reactive oxygen species (ROS) and that ROS is largely responsible for PEITC-induced cell death. To confirm PEITC-induced cancer cell death we have investigated the mechanism of action of PEITC in pancreatic cancer cell lines and PEITC induces ROS and inhibits growth and induces apoptosis (PARP cleavage). In addition, PEITC downregulates expression of several gene products including vascular endothelial growth factor (VEGF), cyclin D1 (CD1), Bcl2 and survivin and these have previously been reported in other studies. However, since these gene products are all regulated by specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4, which are overexpressed in cancer cells and tumors, we investigated the effects of PEITC on Sp proteins and observed that PEITC decreased expression of Sp1, Sp3 and Sp4 in pancreatic cancer cells. These results demonstrate for the first time that an important underlying mechanism of action of ITCs likely involves targeting Sp transcription factors through an ROS-mediated mechanism and the pathways required for ITC-induced Sp downregulation were investigated and the results are presented in this paper.

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# INTRODUCTION

## Part one

### Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are chemically reactive, oxygen-containing species and ROS can be classified into two groups: 1) radical ROS and 2) non-radical ROS. Radical ROS include species that contain one or more unpaired electrons in their outer molecular orbital and include superoxide ($O_2^{\cdot-}$), nitric oxide ($NO^{\cdot}$) and hydroxyl ($^{\cdot}OH$) radicals. Non-radical ROS do not have any unpaired electrons in their outer orbitals, however they are also chemically reactive and can be converted into radical ROS. Examples of non-radical ROS include hydrogen peroxide ($H_2O_2$), ozone ($O_3$), peroxynitrite ($ONOO^-$) and hydroxide (OH) [1].

ROS normally exist in all cells that undergo aerobic respiration. Endogenous sources of ROS in cells includes mitochondria, peroxisomes and cytochrome P450 enzymes and it has also been reported that membrane-bound NADPH oxidases can contribute to endogenous ROS production [2, 3]. Exogenous sources include UV light, ionizing radiation, inflammatory cytokines and pathogens. Although ROS can be generated via multiple pathways the majority of cellular ROS comes from mitochondria.

Mitochondria provide approximately 90% of the energy that cells need to function through the generation of ATP from oxidative phosphorylation and most ROS produced in mitochondria come from the electron transport chain as a byproduct of respiration [4-6]. During oxidative phosphorylation a proton gradient is established across the inner mitochondrial membrane by the constant transport of electrons through

the electron transport chain. It has been demonstrated *in vitro* that NADH dehydrogenase, in Complex I of the electron transport chain, is a major entry point for electrons and thus is a major site of ROS production [7]. Another major site of ROS production in the mitochondria is in Complex III of the electron transport chain. The bc1 complex (ubiquinone: cytochrome c reductase) on the inner side of the inner mitochondrial membrane in Complex III generates ROS through the Q-cycle [8]. There are other non-respiratory chain mitochondrial enzymes that have been shown to generate ROS, however it is difficult to estimate their total contribution of ROS production within the mitochondria [9]. It is interesting to note that approximately 2% of the oxygen consumed by mitochondria is converted into superoxide and superoxide generated by mitochondria can be converted into $H_2O_2$ and other ROS [10]. Although mitochondria are the primary source of ROS in cells there are also other pathways in the cell that generate ROS.

Peroxisomes are organelles found in nearly all eukaryotes and their name is based on the large number of $H_2O_2$-producing oxidases found within this organelle. Peroxisomes have been identified as critical organelles that contain greater than 50 enzymes involved in cellular metabolism. Peroxisomes participate in such metabolic pathways as β-oxidation of very long and long chain fatty acids, leukotrienes, and prostaglandins; biosynthesis of cholesterol, bile acids, dolichol and ether lipids; oxidation of D-amino acids, polyamines and uric acid; and detoxification of xenobiotics, glyoxylate and ROS [11-14]. Peroxisomes are largely responsible for producing $H_2O_2$, however peroxisomes also produce superoxide and nitric oxide radicals. For example,

$H_2O_2$ is produced by acyl-CoA oxidase, an enzyme involved in the β-oxdiation of fatty acids [15] and nitric oxide is produced in peroxisomes by nitric oxide synthase which catalyzes the oxidation of L-arginine to nitric oxide. Once formed, nitric oxide can combine with superoxide to form peroxynitrite, a strong radical [11]. One study showed that approximately 35% of all $H_2O_2$ produced in rat liver was produced by peroxisomal oxidases and this accounts for roughly 20% of all oxygen consumption [16, 17]. Mitochondria and peroxisomes are therefore largely responsible for the generation of ROS in cells.

It has also been reported that cytochrome P450 enzymes are capable of producing ROS. Cytochrome P450s (CYPs) are membrane-bound enzymes found in the endoplasmic reticulum and these enzymes are involved in the oxygenation of drugs, xenobiotics, carcinogens and endogenous substrates [18]. CYPs are primarily located in the liver, however they are also expressed in tissues such as lung, kidney, brain and various other organs [19]. CYPs require the input of electrons from the electron donor, NADPH, in order to activate oxygen and catalyze substrate oxidation. The input of electrons from NADPH leading to the activation of oxygen is the first step leading to the production of ROS. The catalytic cycle of CYPs, which inserts oxygen into its substrate, is a multi-step process and ROS can be generated at three different points in the catalytic cycle. CYP catalyzed addition of oxygen to its substrate relies on the coordination of oxygen around the iron atom in the heme group and coordination of oxygen associated with the iron atom is critical for ROS production. The first part of the cycle leading to the production of ROS is the release of a superoxide anion due to the decay of the one-

electron-reduced ternary complex. The second point of ROS generation is the production of $H_2O_2$ coming from the protonation of peroxycytochrome P450. The final part of the cycle giving rise to ROS involves the addition of two more electrons following the decay of peroxycytochrome P450 resulting in the release of the second oxygen atom in the form of water [20]. A schematic representation of ROS production by CYPs is illustrated in Figure 1.

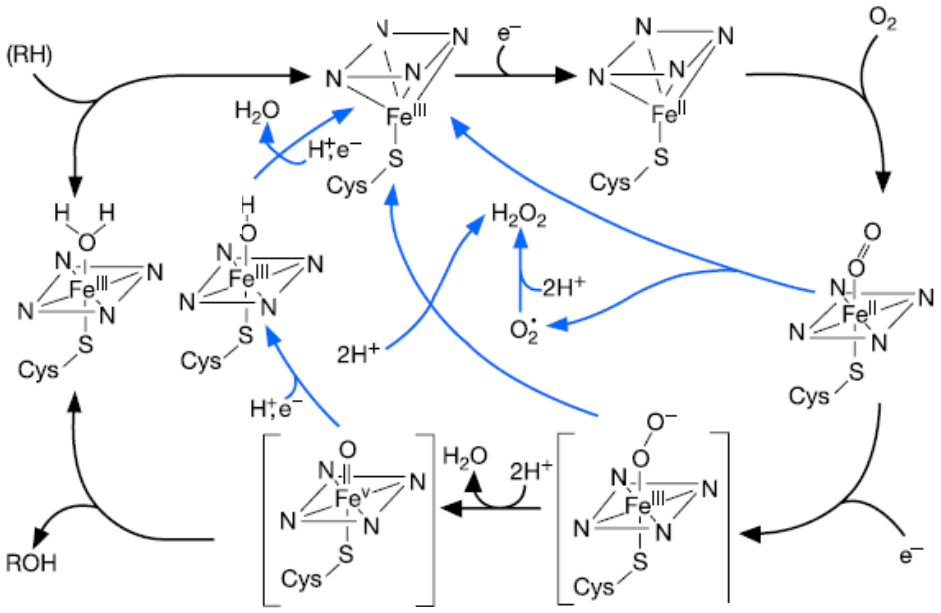


Figure 1. Schematic of ROS production within the catalytic cycle of cytochrome P450. Sites of ROS production can be seen in blue [20].

The release of ROS from CYP-mediated oxygenation of substrates is an ongoing and continual process. ROS are generated as a result of substrate oxygenation, however the amount of ROS produced depends on many factors including the type of substrate, pH of

the solution, ionic strength and oxygen concentration [21]. Mitochondria, peroxisomes and cytochrome P450s are the primary cellular locations for generation of ROS which are highly reactive and can cause cell damage.

**Targets of ROS**

ROS can directly damage DNA, protein and lipid membranes and this can result in disruption of cellular structure and function [22]. The most common type of DNA damage is that induced by ROS. Not surprisingly, mitochondria are very susceptible to injury from their endogenous production of ROS [23]. Mitochondrial DNA (mtDNA) is located on the inner mitochondrial membrane adjacent to the respiratory chain, the primary site of mitochondrial ROS production and mtDNA is particularly susceptible to damage from ROS. MtDNA lacks introns and histones and lacks the high quality DNA repair machinery present in nuclear DNA [24]. Both the base and sugar in DNA are susceptible to ROS mediated damage and this can result in oxidized bases, DNA strand breaks, DNA intra-strand adducts and DNA-protein crosslinks and hydroxylation of pyrimidines at C5 and C6 and purines at C4, C5 and C8 [25, 26]. 8-Hydroxy-deoxyguanosine (8-OHDG) is a reliable marker of ROS-induced effects [27, 28]. Damage to mtDNA can potentially lead to disruption of the mitochondrial respiratory chain which could ultimately lead to increased formation of ROS [29].

Mitochondrial ROS not only targets mtDNA but also targets proteins and lipids within mitochondria. ROS can cause oxidative damage to proteins thereby inducing the formation of protein carbonyls which are commonly detected in aged animal tissues and cells. Oxidative damage to proteins in mitochondria can occur on amino acids such as

arginine, lysine, threonine and proline and peroxynitrite oxidizes tryptophan, cysteine and tyrosine residues. Carbohydrates are also susceptible to oxidative damage, which can lead to the formation of glycation end products [30].

It is well known that the mitochondrial respiratory chain generates large amounts of superoxide anions due to the large input of electrons needed to generate ATP. ROS can directly affect the respiratory chain by oxidizing iron-sulfur clusters present in Complexes I, II and III; heme groups in Complexes II, III and IV; and copper centers in Complex IV [31]. Cytochrome c, a component of oxidative phosphorylation, can undergo nitration on its tyrosine residues ultimately leading to the disruption of electron flow through the respiratory chain [32]. Overall, the damaging effects of ROS on the respiratory chain can impair the production of ATP and leads to generation of more ROS by oxidizing subunits of oxidative phosphorylation [9].

Another target of ROS in the mitochondria is the mitochondrial permeability transition pore (mPTP). The mPTP is a multi-protein complex consisting of a voltage-dependent anion channel (VDAC), adenine dinucleotide translocase (ANT) and cyclophilin D located in the inner and outer mitochondrial membrane and the mitochondrial matrix [33]. It is well documented that oxidation of thiol residues in ANT leads to the opening of the mPTP and subsequently the collapse of mitochondrial membrane potential, mitochondrial swelling and cytochrome c release, all of which can potentially induce apoptosis [34].

Finally, lipids and lipid membranes are targets of mitochondria-induced ROS. Unsaturated fatty acids present in mitochondrial membranes are also susceptible to

oxidation by hydroxyl radicals which are produced in the mitochondria by converting $H_2O_2$ into the hydroxyl radical using metal ions in what is known as the Fenton reaction [35, 36]. The hydroxyl radical is highly reactive and is known to be one of the strongest oxidants in nature [9]. Hydroxyl radicals oxidize the unsaturated fatty acids present in both mitochondrial lipid membranes and cellular membranes forming lipid hydroperoxides which are converted into highly reactive unsaturated aldehydes [35, 36]. Once formed, lipid hydroperoxides can induce a chain reaction forming new lipid hydroperoxides. Lipid peroxidation affects membrane fluidity by leading to an increase in membrane permeability to protons and leading to the uncoupling of oxidative phosphorylation [36]. In addition, lipid peroxides are able to diffuse through mitochondrial membranes and covalently modify proteins as well as cause oxidative damage to mtDNA [37]. Just as proteins and DNA are affected by ROS in mitochondria, these same biomolecules can be affected by ROS outside of the mitochondria.

Superoxide anion radicals generated by mitochondria are converted into $H_2O_2$, which is membrane permeable and the relatively stable $H_2O_2$ molecule enters the nucleus. Upon entering the nucleus $H_2O_2$ can be converted into the highly reactive hydroxyl radical in the presence of an iron catalyst (Fenton reaction) and the hydroxyl radical causes severe oxidative damage to nuclear DNA resulting in mutations that are generally found in the form of base pair mismatching during DNA replication or in the form of nucleotide insertions [35, 38]. It should also be noted that nuclear DNA is not as susceptible to oxidative damage as mtDNA as indicated previously.

Extra-mitochondrial proteins are affected by ROS in the same manner as mitochondrial proteins and undergo oxidative modification of amino acid side chains and cleavage of peptide bonds [39]. Proteins can undergo nitrosylation by ROS and peroxynitrite and this can affect the signaling of such proteins as NF-κB, AP-1 and p53 resulting in damage to cell function and structure [40].

**Cellular defense against ROS**

All cells undergoing aerobic respiration, inevitably, will generate ROS as byproducts of cellular metabolism and when these natural byproducts of metabolism bioaccumulate the resulting oxidative stress induces apoptosis or necrosis. Cells have developed many genes/pathways for regulating ROS levels and these include both enzymatic and non-enzymatic mechanisms. Enzymatic mechanisms include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). Non-enzymatic mechanisms include the use of ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids and others [41]. Both enzymatic and non-enzymatic mechanisms are critical for maintaining a healthy redox balance to ensure cellular survival. A brief overview of the antioxidant enzymatic mechanisms will be discussed below.

***Enzymatic mechanisms***

The primary enzymes responsible for maintaining the redox balance within the cell are SOD, catalase and GPx. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide [42]. There are three forms of SOD found in humans: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD and extracellular SOD (EC-SOD) [43,

44]. SOD catalyzes the dismutation of superoxide by oxidation and reduction of the transition metal ion at the active site [45]. Mn-SOD contains one Mn atom per subunit that cycles between $Mn^{2+}$ [46] and $Mn^{3+}$ [46] [47]. It has been demonstrated that cytokines play a pivotal role in induction and repression of Mn-SOD expression and that oxidants play only a minor role in regulating the expression of this enzyme [48]. In addition, knockdown of Mn-SOD in mice resulted in dilated cardiomyopathy and neonatal lethality thus indicating its importance for cellular survival [49].

Unlike Mn-SOD, Cu/Zn-SOD is not essential for cellular survival. Knocking out Cu/Zn-SOD in mice has no deleterious effects except after traumatic injury [49]. Although Cu/Zn-SOD is not an essential enzyme it is believed to play a role in the first line of antioxidant defense. In fact, calves that were fed milk supplemented with 25 ppm Cu and 100 ppm Zn exhibited a stronger immune response and showed higher SOD activity [43, 50].

EC-SOD is a glycoprotein containing both Cu and Zn and has a high affinity for glycosaminoglycans including heparin and heparan sulfate. EC-SOD accounts for the majority of SOD activity in plasma and is primarily regulated by cytokines and not oxidants [44, 51, 52].

Catalase is an enzyme responsible for regulating hydrogen peroxide levels within the cell. Catalase catalyzes hydrogen peroxide cleavage to form water and molecular oxygen. In addition, catalase reacts with various hydrogen donors, such as methanol, ethanol, formic acid and phenols, using peroxidase activity. It has been demonstrated that catalase is not essential in some cell types, however it is well understood that

catalase plays a critical role in the cellular response to oxidative stress from hydrogen peroxide and thus is a critical enzyme for maintaining a redox balance within the cell [53].

Glutathione peroxidase (GPx) is a seleno-containing enzyme consisting of one selenocysteine residue in each of its four subunits that is essential for its enzyme activity [54]. GPx uses glutathione as a cofactor to catalyze the reduction of lipid and other organic hydroperoxides and is a major enzyme responsible for protection against oxidative stress. There are five GPx isoenzymes found in mammals and although GPx expression is ubiquitous the levels of each isoform vary depending upon tissue type. GPx1 is found within erythrocytes, kidney and liver and is responsible for reducing fatty acid hydroperoxides and hydrogen peroxide using glutathione as a cofactor [55, 56]. Levels of GPx2 and GPx3 are low and are only expressed in the gastrointestinal tract and kidney, respectively [43, 56]. GPx4 has been detected in the cytosolic and membrane fraction from renal epithelial cells and testes and catalyzes reduction of phospholipid hydrperoxides, fatty acid hydroperoxides and cholesterol hydroperoxides found in peroxidized membranes and oxidized lipoproteins [43, 55, 56]. GPx5 has only recently been discovered and is located exclusively in mouse epididymis and is selenium-independent [56].

***Non-enzymatic mechanisms***

Glutathione (GSH) is one of the most essential intracellular defense mechanisms against ROS-mediated damage [57]. GSH is expressed in high concentrations in various cellular compartments including the cytosol (1-11 mM), nuclei (3-15 mM) and

mitochondria (5-11 mM) [58]. There are two forms of GSH: reduced GSH (GSH) and oxidized GSH (GSSG). To protect cells from oxidative stress GSH donates an electron to an oxidant from a sulfhydryl group resulting in the formation of a disulphide bond with another GSH molecule creating an oxidized form (GSSG). The oxidized GSSG does not participate in regulating redox balance until is converted back into its reduced form (GSH) [59]. High a concentrations of GSSG can increase the amount of protein mixed disulphides within a cell and if the concentration of protein mixed disulphides are too high then critical proteins such as receptors, protein kinases and transcription factors can be inactivated [41]. Because the amount of GSH and GSSG in the cell is critical for maintaining cellular homeostasis the ratio of GSH/GSSG is an important measure of oxidative stress [60]. A higher value of reduced GSH is considered to be healthy while a higher value of oxidized GSH is unhealthy [61]. GSH is involved in protecting cells from oxidative stress in a variety of ways including its use as a cofactor for detoxifying enzymes such as GPx and glutathione transferase. In addition, GSH aids in amino acid transport through the plasma membrane and scavenges hydroxyl radicals and singlet oxygen using GPx, thereby clearing hydrogen peroxide and lipid peroxides from the cell. Moreover, GSH regenerates vitamin C and E into their active forms and GSH can reduce the tocopherol radical of vitamin E directly or indirectly [41, 62].

Other non-enzymatic mechanisms for defense against oxidative stress include vitamin E and C and less established molecules that exhibit antioxidant activity including carotenoids and related plant pigments, flavonoids and other phenolics. α-Tocopherol is the main component of vitamin E and is the most important antioxidant

located in membranes and lipoproteins involved in stopping the chain reaction of lipid peroxidation [63]. α-Tocopherol inhibits the lipid peroxidation chain reaction by scavenging peroxyl radical intermediates according to the following reaction : αTH + $LOO^{\cdot} \longrightarrow \alpha T^{\cdot} + LOOH$ [64]. One of the products of the above reaction is an α-tocopherol radical, however this radical is much less efficient at attacking fatty acid side chains that the peroxyl radical [65].

Vitamin C is water soluble and one of the most important antioxidants present in extracellular fluids [66]. It has been demonstrated that vitamin C scavenges superoxide, hydrogen peroxide, hypochlorite, the hydroxyl radical, peroxyl radicals and singlet oxygen [67]. Studies with human plasma lipids have shown that vitamin C is more efficient as an inhibitor lipid peroxidation initiated by peroxyl radicals than other plasma components including α-tocopherol [68]. In addition, it was shown that vitamin C protects human sperm from oxidative DNA damage and vitamin C protects membranes from peroxidation by increasing α-tocopherol activity [69, 70]. In fact, vitamin C increases α-tocopherol activity by reducing the tocopherol radical generated when tocopherol participates in chain breaking of the lipid peroxidation reaction. By reducing the tocopherol radical vitamin C restores the antioxidant activity of α-tocopherol [69, 71].

Carotenoids can deactivate singlet oxygen and inhibit free radical reactions [72, 73]. The antioxidant activity of carotenoids is due to their extended system of conjugated double bonds. β-Carotene and lycopene are among the most effective carotenoids that quench singlet oxygen [74]. In addition to quenching singlet oxygen β-

carotene and related carotenoids also inhibit free radical reactions by reduction of free radicals [72].

Flavonoids are involved in a variety of antioxidant defenses *in vitro* including inhibition of ROS formation by suppression of enzymes or by chelating trace elements involved in ROS production, scavenging ROS directly and upregulating antioxidant defenses [75]. Flavonoids inhibit enzymes involved in ROS formation including cyclooxygenases, lipoxygenases, microsomal monooxygenases, glutathione *S*-transferases, mitochondrial succinoxidases, and NADH oxidase; flavonoids also chelate iron and copper, two pro-oxidant metals directly involved in the production of ROS [76, 77]. Due to their low redox potentials flavonoids can also reduce oxidizing free radicals, such as superoxide, peroxyl, alkoxyl and hydroxyl radicals. Overwhelming *in vitro* data suggests that flavonoids represent an essential class of natural compounds which function as cellular antioxidants, however a lack of *in vivo* data and inconsistent results concerning bioavailability question the utility of these phytochemicals and their use as neutriceuticals as "antioxidants".

**Positive roles of ROS**

Although ROS, as described previously, damage proteins, lipids and nucleic acids it has also been demonstrated that ROS are involved in cell signaling and gene regulation [78]. In fact, at low cellular concentrations ROS can stimulate cell proliferation, while at high concentrations ROS can lead to growth arrest and cell death. Studies have shown that low concentrations of superoxide radicals and hydrogen peroxide stimulate cell proliferation in many cell types demonstrating that ROS acts as

secondary messengers in signal transduction pathways [79]. In addition, ROS mediates the function of a variety of intracellular signaling molecules including cytokines, non-receptor tyrosine kinases, Ras, protein tyrosine phosphatases, serine threonine kinases and nuclear transcription factors including AP-1, NF-κB, p53, NFAT and HIF-1 [41]. ROS are both beneficial and harmful to cells and therefore cellular antioxidants play a critical role in maintaining appropriate levels of ROS so that the cell may function normally.

**ROS and cancer**

The production of ROS within the cell is inevitable due to normal metabolic functions and there is a defined range of ROS levels associated with cellular homeostasis. When levels of ROS become too high cells have increased susceptibility to damage. There is *in vitro* and *in vivo* evidence suggesting that cancer cells intrinsically have higher levels of ROS than normal cells [80-82]. In fact, increased stress in cancer cells due to ROS is correlated with aggressiveness of tumors and poor patient prognosis [83]. Studies have shown that solid tumors have increased levels of oxidized DNA bases (e.g. 8OHdG); and lipid peroxidation products and malignant cells have lower levels of antioxidant enzymes such as SOD, GPx and peroxiredoxin [84]. There is no definitive mechanism to explain the increased levels of ROS within cancer cells, however there are many different mechanisms that contribute to the production of ROS in cancer cells such as the activation of oncogenes, unregulated metabolism, mitochondrial dysfunction, and loss of p53 [85-88]. In addition, a variety of genes, such as Ras, Bcr-Abl and c-Myc, whose expression is associated with tumor transformation can induce ROS production

[89]. Increased ROS stress in cells is known to lead to the initiation and progression of cancer and is thus viewed as a deleterious cellular effect, however excess levels of ROS in cancer cells can also be toxic and thus cancer cells exposed to high levels of ROS are more susceptible to damage and cellular death. Therefore, manipulating ROS levels in cancer cells but not in normal tissues can be a potential mechanism to selectively kill cancer cells while causing minimal toxicity to non-cancer cells [90].

**Drugs that induce ROS**

There are a variety of anti-cancer drugs that induce ROS levels either by directly generating ROS or by depleting antioxidants thus leading to elevated levels of ROS. Examples of anti-cancer drugs that induce ROS production in cancer cells include arsenic trioxide, motexafin gadolinium, daunorubicin, doxorubicin, paclitaxel and elesclomol [91-93]. Compounds that target cellular antioxidants, such as GSH, include arsenic trioxide, celastrol, ascorbic acid, 2-cyano-3,12-dioxo-oleana-1,9-dien-28-oic acid methyl ester (CDDO-Me), isothiocyanates (ITCs) and buthionine sulfoxamine (BSO) [94-99]. In addition, there are many more compounds that target antioxidants such as SOD and thioredoxin. Most of the above-mentioned drugs have been in pre-clinical or clinical trials and some have been approved for certain forms of cancer. For instance, arsenic trioxide was approved for treatment of relapsing acute promyelocytic leukemia [1]. It is evident that the effective use of ROS-inducing compounds for treating a disease when, especially cancer, will require selective killing of the diseased cell and this must not be accompanied by acquired drug resistance. Although it has been proven that

the compounds listed above regulate ROS production or through antioxidant depletion their overall mechanism of action as anti-cancer agents is not well defined.

Understanding the mechanism of action of a drug is critical for selective application of the agent to target critical pathways and is also essential for developing combined therapies. Recently there have been many significant advances in elucidating the mechanism of action of some of the high profile ROS-inducing anti-cancer agents, such as arsenic trioxide, celastrol, ascorbic acid and CDDO-Me. In fact, all four of these compounds have been shown to downregulate a family of transcription factors known as specificity protein (Sp) transcription factors through an ROS dependent mechanism [94-97]. Given that these compounds are drug candidates or are being used in clinical applications it is possible that other ROS-inducing compounds will share a comparable mechanism of action.

**Sp transcription factors**

Sp proteins belong to a family of zinc finger transcription factors known as Sp/Kruppel-like nuclear proteins [100]. Sp1-4 contain many structurally similar features including an activation domain, a C-terminal zinc finger DNA binding region, and an inhibitory domain in Sp3. Sp5-8 are structurally similar to Sp1-4, however they possess an N-terminal truncation [101]. Sp proteins bind to and recognize DNA through GC/GT rich sequences using their C-terminal zinc finger DNA binding domain [102]. Because Sp proteins recognize GC rich sequences of DNA it has been demonstrated that Sp proteins are an important family of transcription factors that regulate a wide variety of mammalian genes [103]. In fact, gene knockout studies in mice showed that loss of Sp1

resulted in significant abnormalities including, retarded development and embryolethality on day 11 of gestation [104]. Gene knockout studies of Sp2 have not been reported and Sp3 knockout mice also exhibit growth retardation, tooth defects and death at birth [105]. Sp4 knockout mice die shortly after birth or survive with significant growth retardation [106]. Sp proteins regulate a wide variety of mammalian genes including several pro-oncogenic factors and not surprisingly these transcription factors play a critical role in tumor development, growth and metastasis [101]. Knockdown of Sp1, Sp3, Sp4 or their combination by RNA interference has shown that Sp proteins regulate genes involved in growth (cyclin D1 (CD1)), survival (survivin), angiogenesis (vascular endothelial growth factor (VEGF), and their receptors, VEGFR1 and VEGFR2) and anti-apoptosis (Bcl2) [46, 107-112]. In clinical studies it was shown that Sp1 expression was high in gastric tumor cells compared to normal glandular cells within or surrounding the tumor and that patients with high Sp1 expression had decreased survival compared to patients with low levels of Sp1 expression [113]. In addition, Sp1 expression was elevated in tumors from patients with pancreatic, breast, thyroid and colon cancer compared to normal tissues [114-117]. In many different forms of cancer high Sp expression is known to be a negative prognostic factor and thus Sp proteins represent a potential drug target for anti-cancer therapy [101].

**Phenethyl isothiocyanate (PEITC) as a ROS-inducing anti-cancer agent**

As mentioned above, there are numerous compounds that are known to induce ROS. Moreover, the mechanism of action of four of these compounds, arsenic trioxide, celastrol, ascorbic acid and CDDO-Me, have been elucidated in this laboratory in which

ROS is induced and subsequently results in downregulation of Sp1, Sp3 and Sp4 proteins. Isothiocyanates (ITCs) represent a class of anti-cancer drugs that have been studied extensively. ITCs are a class of dietary phytochemical compounds generated from the hydrolysis of glucosinolates found in cruciferous vegetables, such as cabbage, kale, broccoli, cauliflower, brussels sprouts, kohlrabi, rape, black and brown mustard, and root crops including turnips and rutabagas [118, 119]. Studies in laboratory animals have shown that ITCs are effective in preventing various types of cancers including breast, lung, colon and prostate and epidemiological studies demonstrated that dietary consumption of ITCs in the form of cruciferous vegetables is associated with a reduced risk of cancer [120, 121]. In addition, ITCs are known to act not only as chemopreventitive agents but have also been demonstrated to act chemotherapeutically. Many different mechanisms of action have been proposed for ITC-induced cancer cell death, however no definitive mechanism has been identified.

ITCs are highly electrophilic having a general structure of R-N=C=S. The electron deficient central carbon atom in ITCs exposes them to nucleophilic attack by a variety of molecules including DNA/RNA, proteins and peptides [122]. Studies using $^{14}C$ labeled ITCs in A549 human non-small cell lung cancer cells showed no binding of ITCs to DNA/RNA suggesting that the primary target of ITCs are proteins [123]. Thiol groups of proteins and peptides are the primary target of the electrophilic center in ITCs and for this reason ITCs are able to conjugate with many different cellular proteins and one of the most significant targets of ITCs is glutathione. ITCs readily bind to and conjugate with glutathione forming the glutathione conjugate, S-(N-

alkyl/arylthiocarbamoyl) glutathione. Once formed the glutathione conjugate proceeds through the mercapturic acid pathway and is excreted in the urine [118]. By conjugating to and thus depleting glutathione levels in the cell, ITCs induce ROS thereby causing oxidative stress and cell death. Based on the results in this laboratory regarding the mechanism of action of arsenic trioxide, celsastrol, ascorbic acid and CDDO-Me, we hypothesize that compounds that induce ROS in turn downregulate Sp proteins and this mechanism is due to ROS-dependent downregulation of microRNA-27a (miR-27a) and/or miR-20a/miR-17-5p and this is accompanied by induction of the miR-regulated Sp repressors ZBTB10 and ZBTB4, respectively

**Part two**

Reactive oxygen species (ROS) includes radicals such as superoxide, nitric oxide and hydroxyl radicals and non-radical species including hydrogen peroxide, ozone and peroxynitrate and these molecules function in normal cells to maintain homeostasis via redox pathways [124-128]. In some cancer cell lines a modest increase in forms of ROS can enhance cell proliferation, survival and drug resistance, however further increases in ROS that cannot be attenuated by intracellular redox systems can lead to cell death [128]. ROS levels are higher in cancer vs. non-cancer cells and drug-induced elevation of ROS is a "way to selectively kill cancer cells without causing toxicity to normal cells" [128]. Drug-induced ROS in cancer cells may be due to inhibition or inactivation of redox pathway enzymes or by direct effects on mitochondria which includes opening of the permeability transition pore complex, decreased mitochondrial membrane potential (MMP) and release of ROS and proapoptotic factors [128-133]. Many clinically used

anticancer agents such as arsenic trioxide induce ROS as a primary mechanism of action and other highly effective or promising drugs including curcumin and other phytochemicals and synthetic analogs such as 2-cyano-3,12-dioxo-oleana-1,9-dien-28-oic acid (CDDO) induce ROS in some cancer cells and tumors and this response contributes to their anticancer activities [134-150].

Several studies demonstrate that consumption of cruciferous vegetables is associated with a decreased incidence of some cancers [151-155] and the expression of various isothiocyanates (ITCs) has been linked to cancer chemoprevention by cruciferous vegetables. ITCs including 1-isothiocyanato-4-(methylsulfinyl) butane (sulforaphane), benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) and allyl isothiocyanate (AITC) have been extensively investigated as anticancer agents, and ITCs inhibit several pathways associated with cancer cell growth, survival, inflammation and angiogenesis/metastasis [156-175]. Modulation of these pathways by ITCs has been linked to downregulation of total and activated STAT3 protein [165] suppression of NFκB (and p65 downregulation) [166-168], decreased expression of epidermal growth factor receptor (EGFR) [169], migrations/invasion factors [171, 172], Bcl-2 [175], cyclins [160,175, 176] and modulation of kinase activities. Many of these ITC-induced responses in pancreatic and other cancer cell lines are also accompanied by decreased mitochondrial membrane potential (MMP) and/or induction of (ROS); and the effects can be attenuated, in part, by cotreatment with antioxidants [156-164, 177]. The potential clinical applications of ITCs for cancer chemotherapy will depend on a more

detailed understanding of their underlying mechanisms of action which is necessary for development of effective drug combinations.

Recent studies in this laboratory have demonstrated that several anticancer drugs that induce ROS including arsenic trioxide the methyl ester of CDDO (CDDO-Me), betulinic acid, a synthetic nitro-non-steroidal antiinflammatory drug (GT-094) and celastrol also downregulate specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 and pro-oncogenic Sp-regulated genes [178-181]. Similar results have also been observed for $H_20_2$, t-butylhydroperoxide and pharmacologic doses of ascorbate that induce $H_20_2$ [180, 182-184] and the effects of ROS inducers and the prooxidants on downregulation of Sp proteins, Sp-regulated genes, growth inhibition and induction of apoptosis are attenuated after cotreatment with antioxidants. Moreover, induction of ROS by CDDO-Me, GT-094, betulinic acid and celastrol downregulated Sp transcription factors through downregulation of microRNA-27a (miR-27a) or miR-17-5/miR-20a and induction of the miR-regulated Sp repressors ZBTB10 and ZBTB4 respectively [178-181]. The relationship between drug-induced ROS and disruption of miR-ZBTB interactions observed for these compounds suggests general mechanisms of action for other ROS-inducing anticancer agents and this concept was further investigated using PEITC as a model. The mechanisms of action PEITC has been extensively studied in cancer cell lines. Ras transformed ovarian cells PEITC decreases intracellular GSH and also directly inhibits glutathione peroxidase resulting in the induction of ROS and decreased MMP [163]. In this study we have investigated the anticancer activity of PEITC in pancreatic cancer cells and show that like other ROS inducers PEITC

decreased expression of Sp1, Sp3 and Sp4 through disruption of miR-ZBTB interactions demonstrating the important contributions of this pathway to their anticancer activity of PEITC.

## MATERIALS AND METHODS

### Cell lines, reagents and antibodies

The Panc-28 cell line was a generous gift from Dr. Paul Chiao (University of Texas M.D. Anderson Cancer Center, Houston, TX). The L3.6pL cell line was developed at the M.D. Anderson Cancer Center (Houston, TX) and was kindly provided by Dr. I.J. Fidler. Panc-1 cells were obtained from the American Type Culture Collection (Manassas, VA). All above-mentioned cells were maintained in Dulbecco's modified/Ham's F-12 (Sigma Aldrich, St. Louis, MO) with phenol red supplemented with 0.22% sodium bicarbonate, 5% fetal bovine serum and 10 ml/L 100X antibiotic/antimycotic solution (Sigma). The cells were grown in 150 $cm^2$ culture plates in an air/$CO_2$ (95:5) atmosphere at 37°C and passaged approximately every 3-5 days. Survivin and CD1 was purchased from Epitomics (Burlingame, CA); VEGF from Rockland (Gilbertsville, PA); CPARP and C-myc from Cell Signaling (Boston, MA); and VEGFR1 from Abcam (Cambridge, MA). All other antibodies were purchased from Santa Cruz (Santa Cruz, CA). Glutathione, 98% (γ-Glu-Cys-Gly, GSH) and PEITC (99% pure) were purchased from Sigma Aldrich. Chemiluminescence reagents (Immobilon Western) for western blot imaging were purchased from Millipore (Billerica, MA).

### Cell proliferation assay

Pancreatic cancer cells ($4 \times 10^4$ per well) were plated in 12-well plates and allowed to attach for 24 h. The medium was then changed to DMEM/ Ham's F-12 medium without phenol red containing 2.5% charcoal-stripped FBS, and either vehicle

(DMSO) or different concentrations of compounds were added. Cells were then trypsinized and counted after 24, 48 and 72 h using a Coulter Z1 cell counter. Pancreatic cancer cells (1.5 X $10^5$ per well) were plated in 12-well plates and allowed to attach for 24 h. The medium was changed to DMEM/Ham's F-12 medium without phenol red containing 2.5% charcoal-stripped FBS and cells were pre-treated with GSH for 45 min. After pre-treatment cells were either dosed with PEITC alone or co-treated with PEITC and GSH. Cells were trypsinized and counted after 24 h using the Coulter Z1 cell counter. Each experiment was done in triplicate, and results were expressed as means ± SE for each set of experiments.

**Western blot analyses**

Pancreatic cancer cells were seeded in DMEM/ Ham's F-12 medium supplemented with 2.5% fetal bovine serum. After 24 h, cells were pre-treated with GSH for 45 min. After pre-treatment cells were dosed with vehicle (DMSO) and PEITC alone or co-treated with PEITC and GSH for the indicated time. The tumor tissues from the L3.6pL pancreatic cancer xenograft study were also processed similarly and probed for proteins ofinterest and β-actin served as the loading control. Cells were lysed using high-salt lysis buffer containing 50 mmol/l *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 0.5 mol/l sodium chloride, 1.5 mmol/l magnesium chloride, 1 mmol/l ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 10% (vol/vol) glycerol, 1% Triton X-100 and protease inhibitor cocktail, 1:1000 (Sigma). Lysates were collected and vortexed every 15 sec for 1 h. The lysates were then centrifuged at 20,000 X g for 10 min at 4° C and quantified with Bradford reagent. Equal amounts of protein from each

treatment group were separated on 7.5 and 7.5/12% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) at 150 V for 5 h. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (Biorad, Hercules, CA) by wet electroblotting and the membranes were blocked with 5% milk in buffer containing 1.576 g/l Tris, 8.776 g/l sodium chloride and 0.5 ml/l Tween 20. The PVDF membranes were then probed with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies as indicated. Membranes were then incubated with Immobilon Western chemiluminescence substrates (Millipore, Billerica, MA) and images were captured on a Kodak 4000 MM Pro image station.

**Measurement of MMP**

The MMP was measured with a Mitochondrial Membrane Poten-tial Detection Kit (Stratagene,CedarCreek,TX). Briefly, cells were seeded on Lab-Tek Coverglass system (NUNC,NY) and treated with PEITC alone or co-treated with GSH for the indicated time. They were then incubated with 1×JC-1 cationic dye at 37°C for 30 min. according to the manufacturer's instructions. After washing with 1×JC-1 assay buffer twice, cells were subjected to microscopic analysis using a confocal instrument (Carl Zeiss, Thornwood, NY). J-aggregates are detected as red fluorescence and J-monomers are detected as green fluorescence.

**ROS estimation**

Cellular ROS levels were evaluated with the cell permeable probe CM-H2DCFDA(5-(and-6)-chloromethyl-2′,7′ dichlorodihydrofluorescein diacetate acetyl ester). CM-H2DCFDA is non-fluorescent until removal of the acetate groups by

intracellular esterases and subsequent oxidation occurs within the cell. Cells were seeded at a cellular density of 1 X $10^5$ cells/mL in 6 well plates and were allowed to attach for 24 h. After 24 h cells were pre-treated with GSH for 45 min. After pre-treatment cells were treated with vehicle (DMSO) or with PEITC alone or co-treated with PEITC and GSH for the indicated time. After the indicated amount of time cells were trypsinized and neutralized with Dulbecco's modified/Ham's F-12 medium supplemented with 5% fetal bovine serum. Cells were collected in tubes and were spun for 5 min at 0.1 rcf to pellet the cells. The supernatant was discarded and cells were resuspended in Dulbecco's modified/Ham's F-12 medium without 0% serum with 10uM CM-H2DCFDA. Cells were incubated with dye for 30 min. at 37°C with 5% $CO_2$. After incubation, cells were spun down at 0.1 rcf for 5 min. The supernatant was removed and cells were washed three times with Dulbecco's modified/Ham's F-12 medium. Immediately before analysis 100 nM propidium iodide was added to the cells to measure the number of dead cells compared to live cells. Cells were analyzed by flow cytometric analysis using a BD Accuri-C6 Flow Cytometer (BD Biosciences, San Jose, CA). Cells were collected at a fast fluidics rate at a count of 30,000 cells. Data was analyzed using Cflow and FloJo analysis software.

**Annexin V staining**

Pancreatic cancer cells ($1 \times 10^5$) were seeded in 2 well chambered glass slides and left to attach overnight. The apoptotic and necrotic assay kit was obtained from Biotium, Inc.(Hayward, CA) and contained FITC-Annexin V, ethidium homodimer III, and Hoechst 3342. Cells were pre-treated with GSH for 45 min. After pre-treatment

cells were dosed with vehicle and PEITC alone or PEITC with GSH for the indicated time. The apoptotic, necrotic, and healthy cell detection kit was used according to the instructions provided by the manufacturer for analysis of adherent cells.

**Transmission electron microscopy**

Cells were seeded at a cellular density of 1 X $10^5$ cells/mL in Dulbecco's modified/Ham's F-12 medium with 2.5% fetal bovine serum in Permanox 2-well chambered slides. Cells were pre-treated with GSH for 45 min. After pre-treatment cells were treated with vehicle (DMSO) and PEITC alone or co-treated with PEITC and GSH for the indicated time. After dosing, cell cultures were fixed in 2% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M sodium cacodylate buffer for 1 hr at room temperature. After washing in buffer the cell monolayers were post stained with 1% osmium tetroxide reduced by 0.2% potassium ferrocyanide for 1 h then dehydrated in an ascending alcohol series and embedded in epoxy resin. Thin sections were cut *en face*, stained with 2% uranyl acetate and Reynold's lead citrate and then examined with an FEI Morgagni 268 transmission electron microscope (FEI, Hillsoboro, OR) at an accelerating voltage of 80 kV. Digital images were acquired with a MegaViewIII camera operated with iTEM software (Olympus Soft Imaging Systems, Munster, Germany) then post-processed with Adobe Photoshop.

**Real-time PCR**

Total RNA was isolated using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. RNA was eluted with 100 µl of RNase-free water and stored at -80˚C. RNA was reverse transcribed using Superscript II

reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was prepared from the pancreatic cancer cell lines using a combination of oligodeoxythymidylic acid (Applied Biosystems, Foster City, CA), dNTP mix and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). All microRNA's were purchased from Applied Biosystems.

**SiRNA interference assay**

Pancreatic cancer cells were seeded ($1 \times 10^5$ per well) in six-well plates in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS without antibiotic and left to attach for 1 day. C-myc (Sigma Aldrich SASI _Hs01_00222676 and SASI_Hs01_00222677) along with iLamin as a control was performed using Lipofectamine 2000 as the carrier reagent according to the manufacturer's instructions. Small inhibitory RNAs were prepared by Sigma-Aldrich.

**Statistical analysis**

Statistical significance of differences was determined by an analysis of variance and student t-test, and the levels of probability were noted.

## RESULTS

Initial studies showed that PEITC inhibited growth of Panc1, L3.6pL and Panc28 cells after treatment for 1, 2 or 3 days and growth inhibition after treatment for 24 hr was observed for 20 μM PEITC in all cell lines and 10 μM PEITC also significantly inhibited growth in L3.6pL and Panc28 cells (Fig. 1A).

The concentrations of PEITC required for inhibition of pancreatic cell growth were slightly higher than previously reported in prostate and bladder cancer cells and this was also confirmed in this study (data not shown). Subsequent in vitro cell culture experiments primarily used 20 μM PEITC since the major focus of this study was on the primary mechanism of action of PEITC and early events that occur (within 24 hr) after treatment. Induction of ROS by 20 μM PEITC was investigated in Panc1, L3.6pL and Panc28 cells by FACS analysis using the cell permeant probe carboxy-$H_2$DCFDA (Figs. 1B) ROS were induced by PEITC after treatment for 3 or 6 hr and in cells co-treated with PEITC plus the antioxidant GSH induction of ROS by PEITC was significantly inhibited.

Not surprisingly the magnitude of ROS induction varied among the 3 cell lines. The rapid induction ROS by PEITC is consistent with a previous report in oncogenically transformed cells where PEITC rapidly depleted intracellular GSH and inhibited glutathione peroxidase enzyme activity in cells and a cell-free system [163] and these effects contributed to induction of ROS by this ITC.

The effects of PEITC on mitochondrial structure and integrity in pancreatic cancer cells were examined by transmission electron microscopy (TEM). Initial studies

with L3.6pL cells treated with 20 μM PEITC for 1, 3 or 6 hr showed that significant loss of mitochondrial architecture and cristae structure which was observed only after treatment for 6 hr and in L3.6pL cells co-treated with GSH the mitochondrial damage was reversed (Fig. 2A). A similar approach was used for Panc1 (Fig. 2B) and Panc28 (Fig. 2C) cells and the results showed that mitochondrial structural damage was observed after induction of ROS (3 hr) and this damage was also attenuated after co-treatment with GSH (TEM time course results for L3.6pL, Panc1 and Panc28 cells coming).

The ROS-mediated mitochondrial damage in pancreatic cancer cells treated with PEITC was further investigated by determine the effects of PEITC on loss of mitochondrial membrane potential (MMP) using the JC-1 dye and by determining the JC-aggregates (red fluorescence)/JC-monomer (green fluorescence) ratios. Several different time points were investigated and MMP was not decreased after treatment for 3 hr (data not shown) results in Figures 2D-2F show results in L3.6pL (6 hr), Panc1 (12 hr) and Panc28 (12 hr) respectively at the earliest time points for PEITC-mediated effects on MMP. PEITC clearly decreased MMP in all 3 cell lines and co-treatment with the antioxidant GSH significantly inhibited this response further demonstrating the important role of PEITC-induced ROS in subsequent mitochondrial damage. The cytotoxic effects of PEITC-induced ROS was further examined by determining the effects of PEITC in the absence or presence of GSH on Annexin V staining (apoptosis) and cell proliferation. PEITC clearly induced Annexin V staining in Panc1, L3.6pL and Panc28 cells (Figs. 3A-3C) and this response was significantly blocked in cells co-

treated with GSH; moreover, GSH also attenuated PEITC-induced growth inhibition (Fig. 3D) confirming that PEITC-induced ROS plays an essential role in the anticancer activity of this compound in pancreatic cancer cells. Previous studies in this laboratory show that ROS-inducing anticancer agents BA, celastrol and CDDO-Me (all triterpenoids) and the NO-NSAID GT-094 decrease expression of Sp transcription factors [178-181] and therefore the effects of PEITC or expression of Sp1, Sp3, Sp4 and Sp-regulated gene products were also determined. \Treatment of L3.6pL, Panc1 and Panc28 cells with 10 or 20 μM PEITC for 24 hr decrease levels of Sp1, Sp3 (low and high molecular weight forms) and Sp4 proteins (Fig. 4A) and this was consistent with results of previous studies with ROS inducers [178-181]. Moreover, the effects on Sp proteins were accompanied by downregulation of survival genes products Bcl-2 and survivin and induction of PARP cleavage (Fig. 4B) and downregulation of growth promoting (cyclin DL and EGFR) and angiogenic (VEGF and VEGFR1) proteins (Fig. 4C). We also observed that PEITC-induced downregulation of Sp1, Sp3 and Sp4 proteins was reversed in L3.6pL, Panc1 and Panc28 cells co-treated with PEITC plus GSH (Fig. 4D) confirming a role for ROS in mediating repression of Sp transcription factors and this correlated with the role of ROS in PEITC-induced apoptosis and growth inhibition in pancreatic cancer cells (Fig. 3).

The role of ROS-dependent disruption of miR-ZBTB interactions was investigated in pancreatic cancer cells treated with 20 μM PEITC for 24 hr. Figure 5A shows that in L3.6pL, Panc28 and Panc1 cells treatment with PEITC decreased expression of miR-27a, miR-17-5p and miR-20 and in cells treated with PEITC plus the

cantioxidant GSH significantly attenuated downregulation of the miRs (Fig. 5B). Similar results were previously observed for celastrol in bladder cancer cells [181]. Preliminary studies in Panc28 cells showed minimal induction of ZBTB10 or ZBTB4 gene expression and after screening several related transcriptional repressors we identified ZBTB34 as a PEITC-inducible gene. Figure 5C demonstrates that PEITC induced expression of ZBTB34 (results coming) on L3.6pL, ZBTB10 and ZBTB34 in Panc28 and ZBTB4 and ZBTB10 in Panc1 cells. Moreover, in cells co-treated with PEITC plus GSH, induction of the transcriptional repressors is attenuated (Fig. 5D). Thus like celastrol GT-094, betulinic acid and CDDO-Me in bladder, colon and pancreatic cancer cells [178-181], PEITC also induced ROS-dependent downregulation of miRs in pancreatic cancer cells.

A recent study showed that ROS-induced genome wide shifts in repressor complexes in colon cancer cells resulting in decreased expression of Myc [185] which regulates expression of multiple miRs including miR-27a and miR-20a/miR-17-5p which are members of the miR-17-92 cluster [186-188]. L3.6pL cells were used as a model and treatment with PEITC decreased expression of Myc protein within 3 hr after treatment and the decrease was observed for up to 12 hr (Fig. 6A). Surprisingly we also observed similar effects of PEITC on Sp1 (but not Sp3 or Sp4) and downregulation of Myc and Sp1 were reversed in cells co-treated with PEITC plus glutathione. The role of Myc in downregulation of Sp1, Sp3 and Sp4 was confirmed by RNAi in L3.6pL cells; knockdown of c-Myc by two oligonucleotides (i-c-Myc1/i-c-Myc2) showed that

decreased expression of the oncogene resulted in downregulation of Sp1, Sp3 (high MW form only) and Sp4 proteins (Fig. 6B).

Current studies are focused on knockdown of c-Myc and Sp1 by RNAi to determine their effects on Sp proteins miR-27a, miR-20a, miR-17-5p, ZBTB10 and ZBTB4 and also PEITC-mediated changes in histone methylation marks associated with regulation of c-Myc and Sp.

The effects of PEITC on pancreatic tumor growth were determined in athymic nude mice bearing L3.6pL cells as xenografts. PEITC (60 mg/kg/d) decreased tumor growth and weight (Fig. 7A).

## CONCLUSIONS

Sp1, Sp3 and Sp4 transcription factors are highly expressed in cancer cells and tumors [178-184], and studies focused on Sp1 show that this protein is a negative prognostic factor for pancreatic and gastric cancer patient survival [189-191] and Sp1 expression is critical for malignant transformation of human fibroblast cells [192]. Expression of Sp transcription factors in rodent and human tissues decreases with age [153-155] and the high tumor/non-tumor ratio of Sp1 suggests that Sp proteins are important drug targets for cancer chemotherapy. Knockdown of Sp1, Sp3 and Sp4 alone or in combination by RNAi also decreased expression of several genes involved in cancer cell growth (cyclin D1, c-MET, EGFR), survival (bcl-2 and survivin), angiogenesis (VEGF and VEGF receptors), and inflammation (p65, NFκB), and many of these genes are themselves targets for anticancer drugs. Moreover, knockdown of Sp transcription factors in cancer cells alone also induces apoptosis and growth inhibition [180-182], and carcinogen-induced transformed fibrosarcoma cells that form tumors in nude mice lose their tumorigenicity after knockdown of Sp1 [192]. Several clinically used anticancer agents and experimental drugs downregulated Sp1, Sp3 and Sp4 proteins and pro-oncogenic Sp-regulated gene products in cancer cell lines through multiple pathways and these are dependent on the drug and cell context. For example curcumin induces proteasome-dependent downregulation of Sp proteins in bladder cancer cells whereas in pancreatic cancer cells the effects of curcumin are ROS-dependent and are attenuated after co-treatment with antioxidants [182, 193].

Previous studies with PEITC and related ITCs show that the compounds inhibit cancer cell growth and angiogenesis/invasion, induced apoptosis and also decreased expression of several gene products such as p65 (NFκB0, bcl-2, cyclin and EGFR that are also Sp-regulated genes [166-176]. The mechanisms of action of PEITC are both ROS-dependent and independent in different cancer cell lines and the ROS-mediated effects have been extensively investigated [158, 159, 163, 177]. For example, PEITC rapidly depleted intracellular GSH and inhibited glutathione peroxidase activity and the subsequent induction of ROS decreased MMP which was accompanied by induction of apoptosis [163]. PEITC also induced ROS and decreased MMP in bladder and prostate cancer cells [159, 161, 162, 169] through slightly different pathways which involved cytochrome c release and changes in mitochondrial proteins. In prostate cancer cells it was suggested that PEITC directly affected mitochondrial oxidative phosphorylation [162]. In pancreatic cancer cells PEITC rapidly induced ROS (within 3 hr) (Figs. 1B-1D) and using TEM we also showed that PEITC did not significantly affect mitochondrial ultrastructure until 6 hr after treatment (Fig. 2). Similar results were observed for MMP which were only decreased after $\geq$ 6 hr (Fig. 2). These results coupled with the protective effects of GSH are consistent with an initial mechanism of extramitochondrial-induced ROS by PEITC as observed in H-Ras transformed ovarian epithelial cells [163].

The central role of ROS in the anticancer activity of PEITC in pancreatic cancer cells was confirmed by the effects of GSH on reversing PEITC-induced cell death and cell proliferation (Fig. 3). Previous studies with PEITC, related ITCs and many other

ROS inducers have demonstrated that induction of extra- or intra-mitochondrial ROS is associated with mitochondrial damage which is linked to activation of apoptosis. In contrast, several ROS inducers, $H_20_2$ and other pro-oxidants also induce downregulation of Sp1, Sp3, Sp4 and pro-oncogenic Sp-regulated genes and gene products in cancer cell lines [178-185]. In this study we have observed similar PEITC-induced effects which are attenuated by cotreatment with glutathione (Fig. 4). Since knockdown of Sp1 and other Sp proteins by RNA interference in pancreatic cancer and other cancer cell lines results in growth inhibition, induction of cell death and inhibition of tumor growth in vivo [180-182, 192] then the anticancer activity of PEITC on pancreatic cancer cells is due not only to apoptosis resulting from mitochondrial damage but also to downregulation of Sp transcription factors and Sp-regulated genes.

The high expression of Sp1, Sp3 and Sp4 in cancer cell lines is due to microRNA-dependent suppression of transcriptional repressors ZBTB10 and ZBTB4 which competitively bind GC-rich promoter sites to displace Sp proteins [194]; this results in decreased transcription since ZBTB10 and ZBTB4 do not express transactivation domains. ZBTB10 expression is repressed by miR-27a [195] and ZBTB4 is repressed by miR-20a and miR-17-5p (part of the miR-17-92 cluster) and other paralogs [195]. PEITC/ROS-mediated downregulation of miR-27a and/or miR-20a/miR-17-5p results in induction of ZBTB10 and ZBTB4 and subsequent downregulation of Sp proteins [178-181]. Similar results were observed using the antagomirs or overexpression of ZBTB10 or ZBTB4 [194,195]. Figure 5 illustrates that

PEITC also disrupts the miR-ZBTB circuits through an ROS-dependent pathway in which the effects of ROS are attenuated by antioxidants.

These results demonstrate that PEITC, other ROS inducers (e.g. betulinic acid, GT-094, celastrol, CDDO-Me and curcumin) and prooxidants, activate a common pathway in cancer cells resulting in the induction of ZBTB10 and/or ZBTB4 through ROS-dependent downregulation of miR-27a and miR-20a/miR-17-5p. This is an important mechanism of action for PEITC and other ROS-inducing anticancer agents since this also activates downregulation of Sp transcriptions which play a role in cancer cell growth, survival, metastasis and inflammation. The key unknown link between induction of ROS and downregulation of miRs is the mechanism associated with miR downregulation. A recent study reported that treatment of colon cancer cells with $H_20_2$ results in relocation of large chromatin-associated repressor complexes from non-GC rich to GC-rich sites and that genes with GC-rich promoters such as Myc were downregulated [185]. Using L3.6pL cells as a model (Fig. 6) we showed that PEITC downregulated c-Myc and Sp1 proteins as early as 3 hr after treatment and this response was also reversed in cells co-treated with PEITC plus GSH. These data coupled with the effects of c-Myc knockdown on Sp proteins (and induction of ZBTB4) (Fig. 6) suggest that in pancreatic cancer cells ROS may induce comparable shifts in repressor complexes observed in colon cancer cells [185]. Moreover, the decreased expression of Myc is consistent with downregulation of miR-27a and miR-20a/miR-17-5p which are members of 2 Myc-regulated miR clusters [186-188].

Therefore, we demonstrate for the first time that an important component of the anticancer activity of ROS inducers, namely downregulation of Sp1, Sp3, Sp4 and pro-oncogenic Sp-regulated gene products is due to ROS-induced epigenetic effects which results in repression of the c-Myc oncogene. Currently we are further analyzing methylation marks associated with ROS-medicated regulation of c-Myc and evaluating the role of c-Myc, Sp1 and other factors as essential downstream responses to ROS-induction by anticancer agents in different cancer cell lines.

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**APPENDIX**

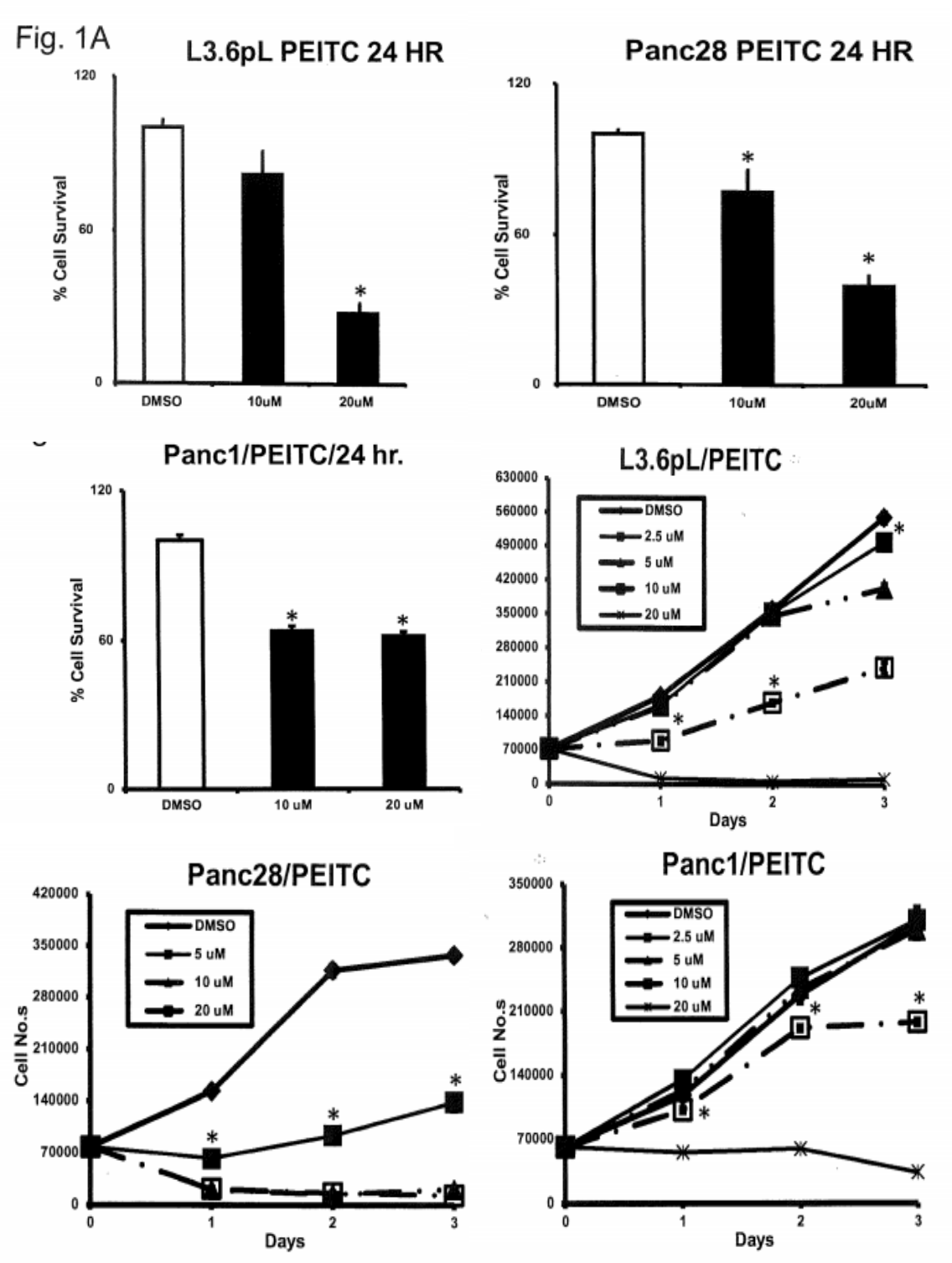


Fig. 1A
L3.6pL PEITC 24 HR
% Cell Survival
120
60
0
DMSO
10uM
20uM
Panc28 PEITC 24 HR
% Cell Survival
120
60
0
DMSO
10uM
20uM
Panc1/PEITC/24 hr.
% Cell Survival
120
60
0
DMSO
10 uM
20 uM
L3.6pL/PEITC
630000
560000
490000
420000
350000
280000
210000
140000
70000
0
DMSO
2.5 uM
5 uM
10 uM
20 uM
0
1
2
3
Days
Panc28/PEITC
Cell No.s
420000
350000
280000
210000
140000
70000
0
DMSO
5 uM
10 uM
20 uM
0
1
2
3
Days
Panc1/PEITC
Cell No.s
350000
280000
210000
140000
70000
0
DMSO
2.5 uM
5 uM
10 uM
20 uM
0
1
2
3
Days

Fig. 1B

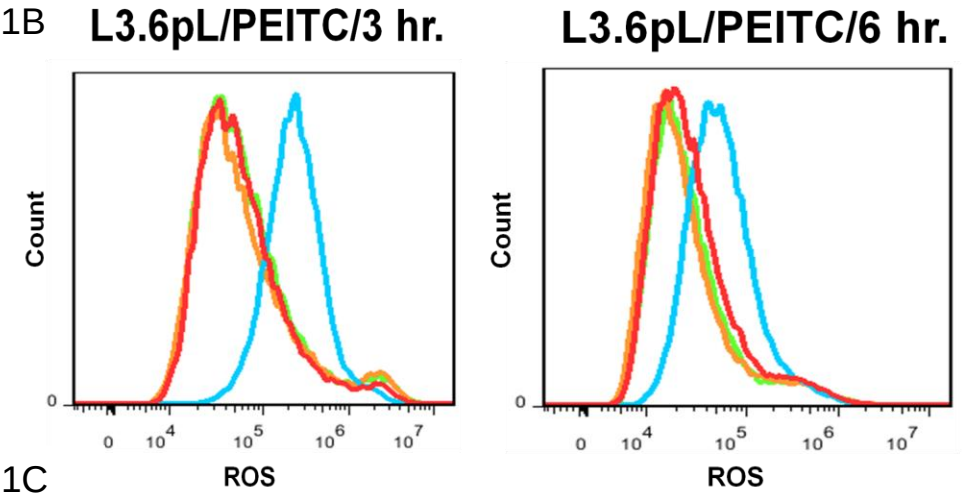



Fig. 1C

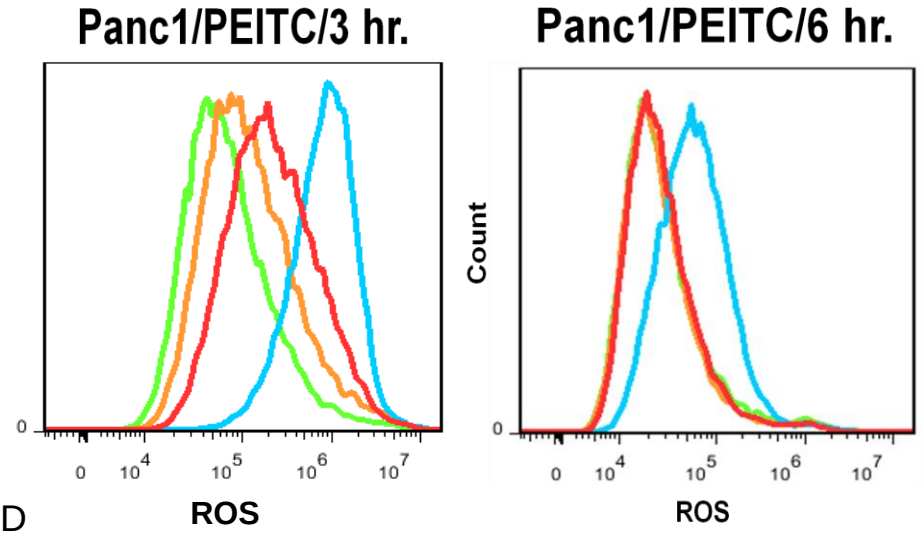



Fig. 1D

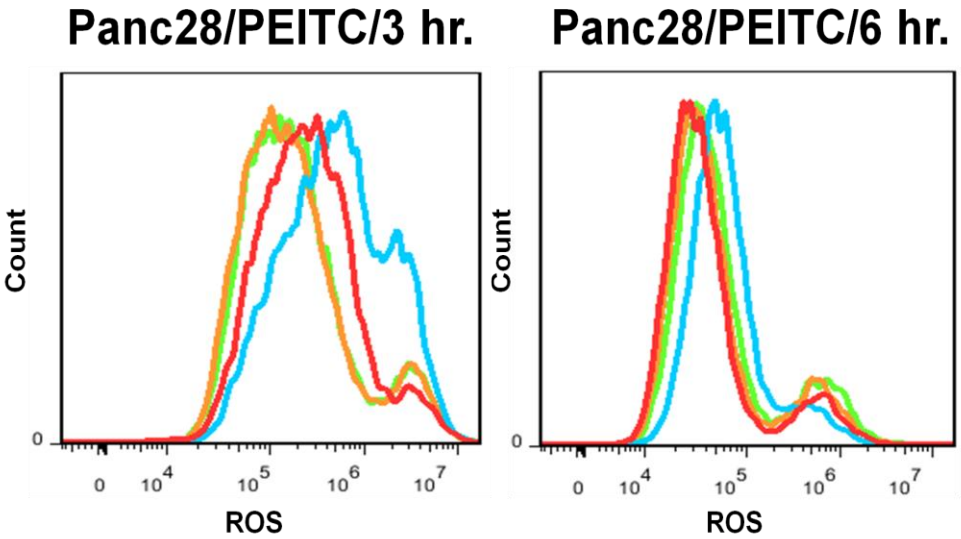


**Figure 1**. PEITC inhibits pancreatic cancer cell growth and induces ROS. A. L3.6pL, Panc28 and Panc1 cells were treated with different concentrations of PEITC for up to 72 hr and cells were counted as outlined in the Materials and Methods. Panc1, L3.6pL and Panc28` cells were treated with 20 μM PEITC, GSH or their combination for 3 and 6 hr and ROS was determined by FACS analysis using the cell permeant CM-H2DCFDA as described in the Materials and Methods. The colors represent treatment as follows: red=DMSO, blue=20uM PEITC, orange=GSH, green=co-treatment. Results in A are means ± SE (3 replicates) and significant ($p < 0.05$) inhibition is indicated (*).

Fig. 2A

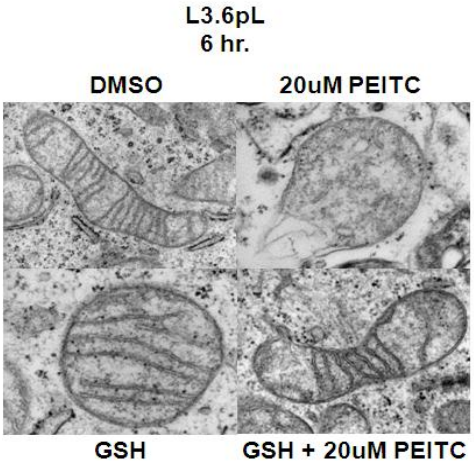



Fig. 2B

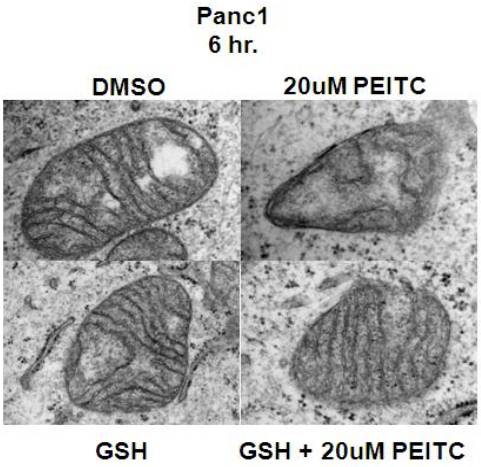



Fig. 2C

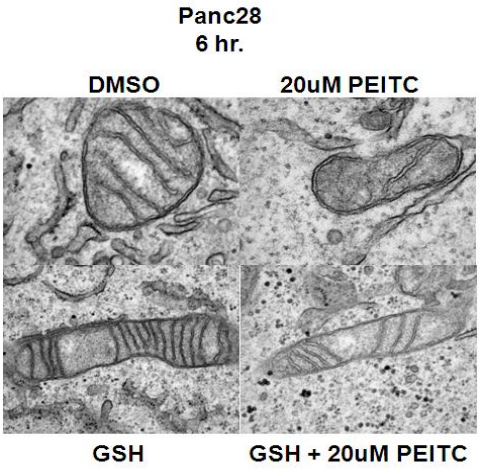


Fig. 2D

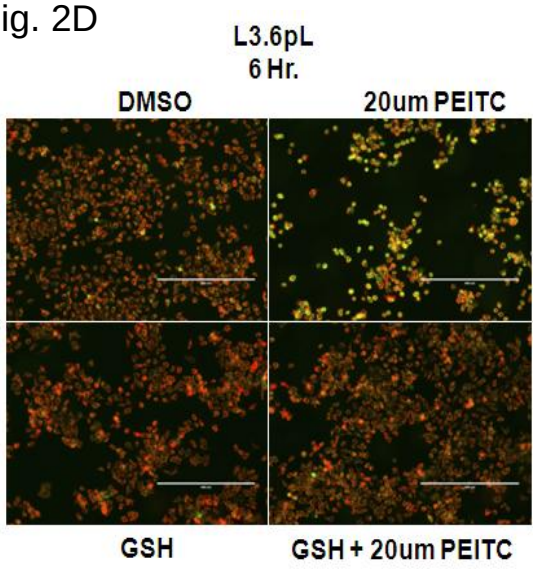
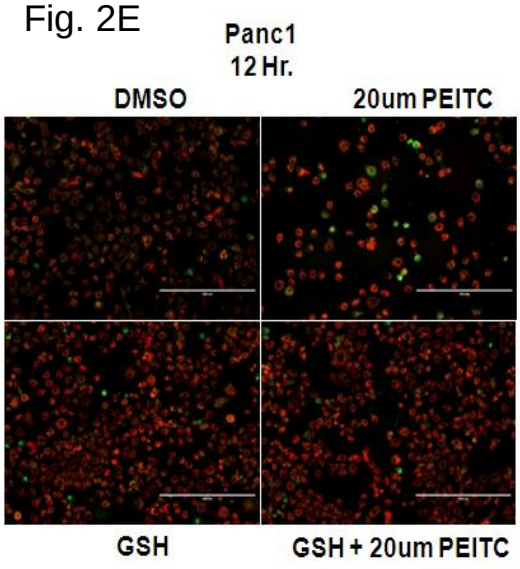
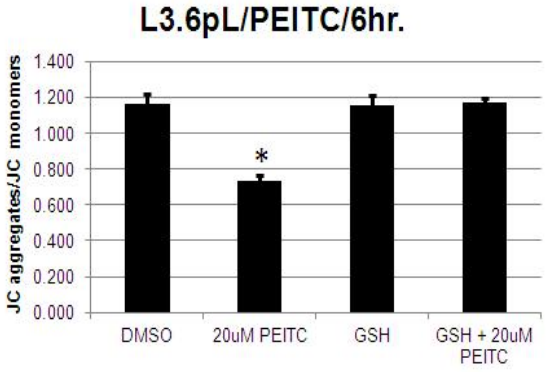



Fig. 2E



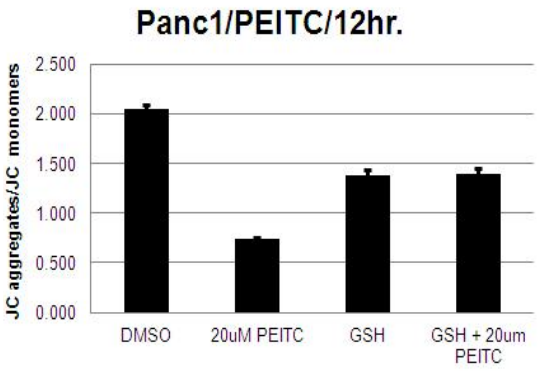
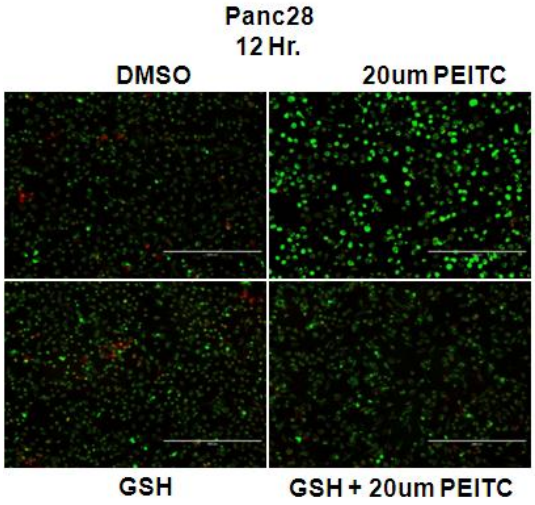




Fig. 2F



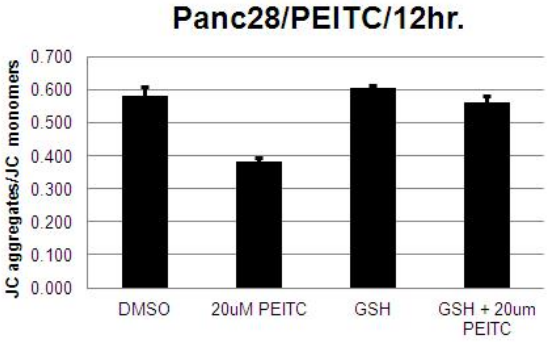



**Figure 2.** PEITC disrupts mitochondrial structure and decreases MMP. L3.6pL (A), Panc1 (B) and Panc28 (C) cells were treated with 20uM PEITC for different times and mitochondrial structure was determined by TEM as outlined in the Materials and Methods. D. Cells were treated with DMSO (solvent), 20 uM PEITC, 5 mM GSH or in combination for 6 hr and mitochondrial structure was determined as outlined in Materials and Methods. The effects of PEITC, GSH alone or in combination on MMP was determined L3.6pL (E), Panc1 (F) and Panc28 (G) cells by JC-1 staining as outlined in the Materials and Methods. Results (E-G) are means ± SE (3 replicates per data point) and significant ($p < 0.05$) inhibition (*) or reversal of the effect by GSH (**) is indicated.

Fig. 3A

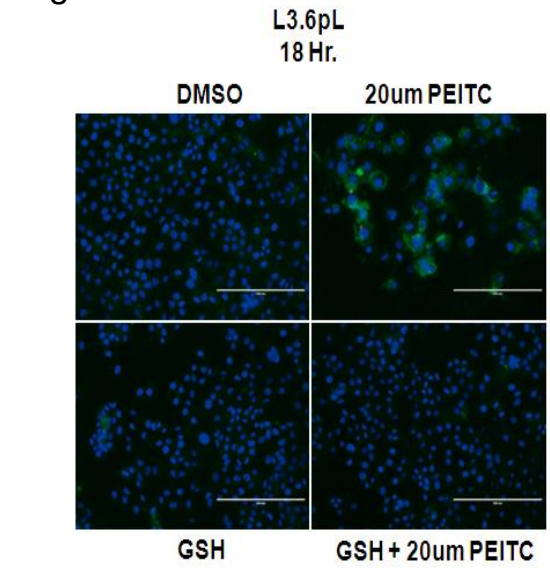
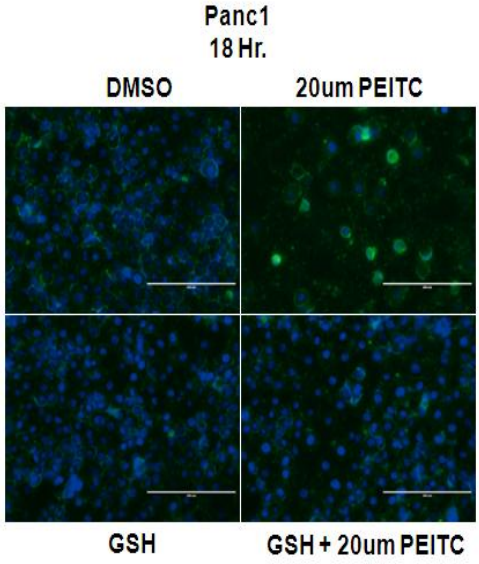
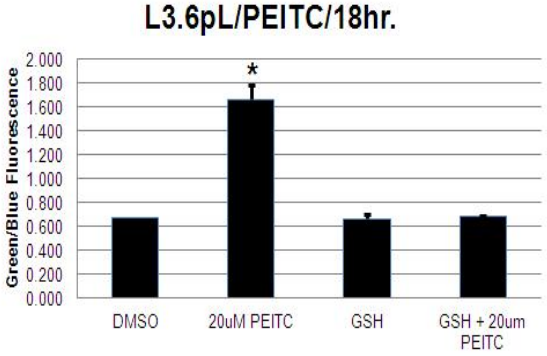



Fig. 3B



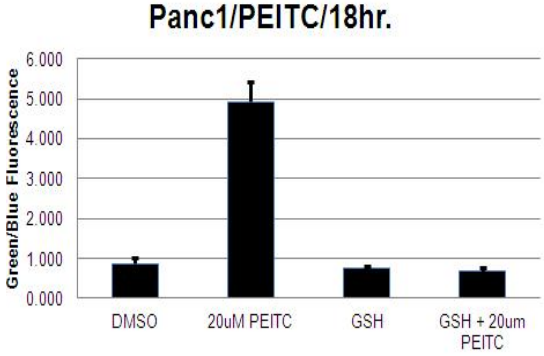
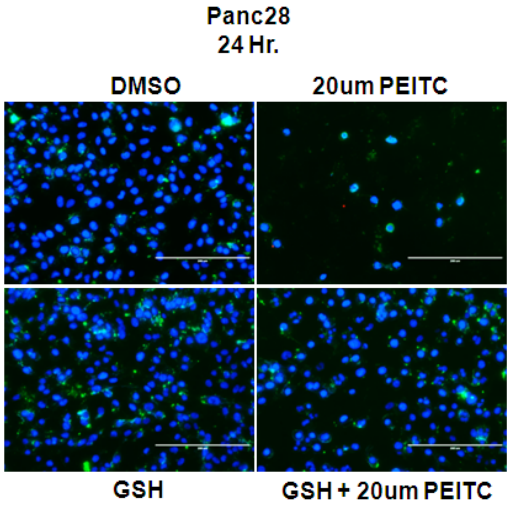




Fig. 3C



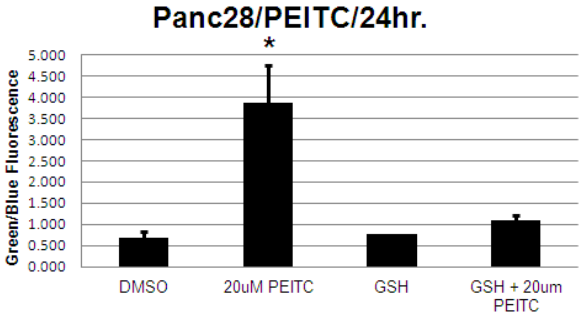



Fig. 3D

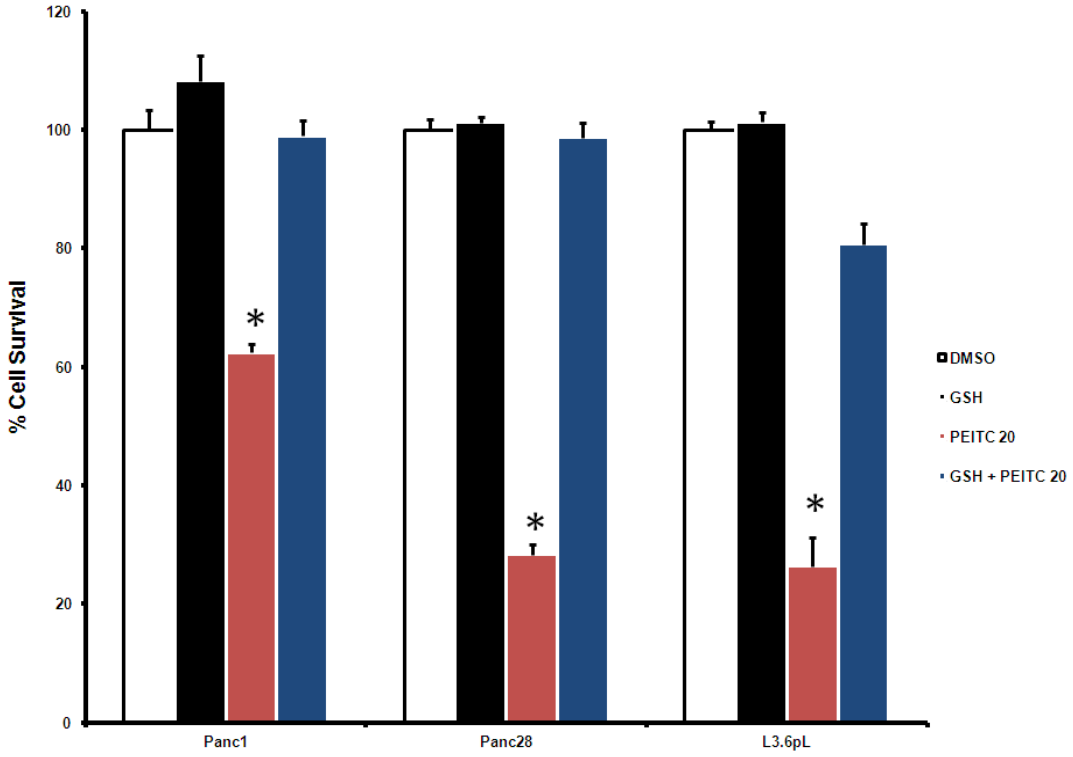



**Figure 3.** Glutathione inhibits PEITC-induced apoptosis and growth inhibition. Panc1 (A), L3.6pL (B) and Panc28 (C) cells were treated with 20 μM PEITC, GSH alone or in combination and Annexin V staining was determined as outlined in the Materials and Methods. D. Cells were treated for 24 hr and cells were counted as outlined in the Material and Methods. Results are means ± SE (3 replicates for each data point) and significant ($p < 0.05$) effects by PEITC ($^{*}$) and attenuation by cotreatment with GSH ($^{**}$) are indicated.

Fig. 4A

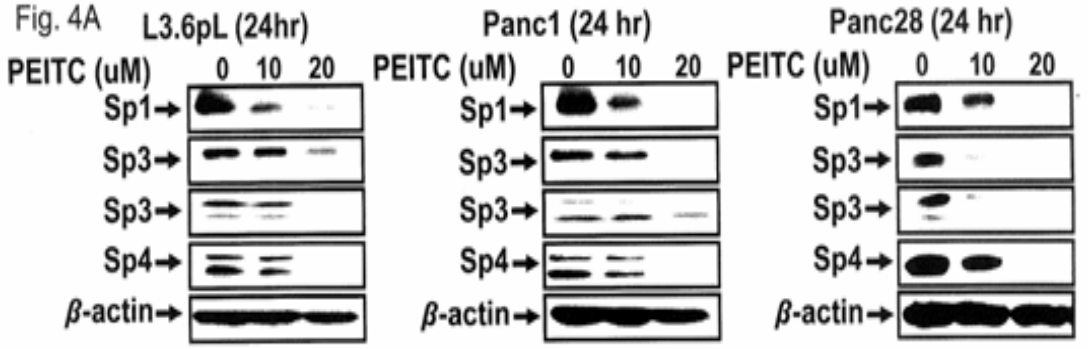



Fig. 4B

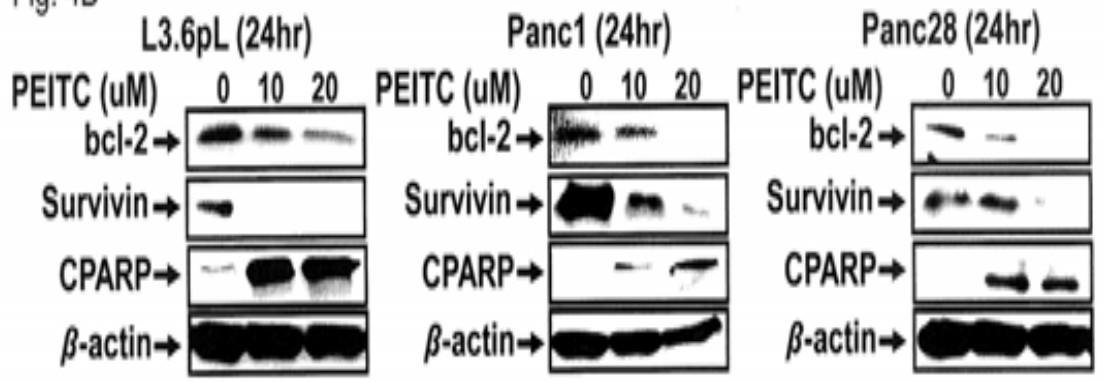
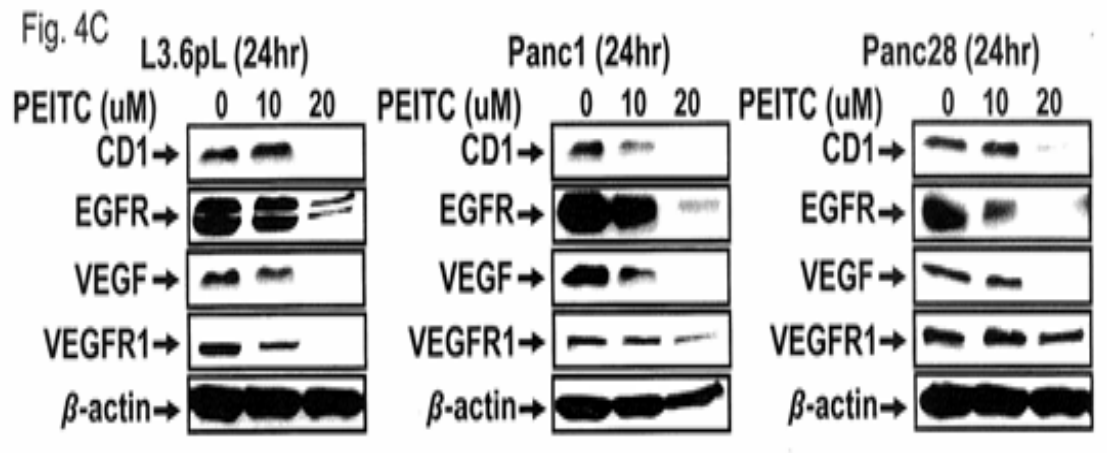



Fig. 4C



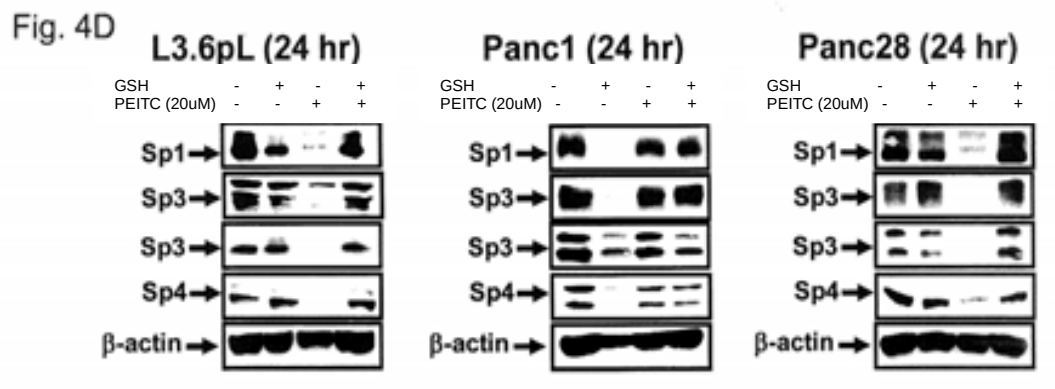



**Figure 4.** PEITC downregulates Sp1, Sp3, Sp4 and Sp-regulated genes. Pancreatic cancer cells were treated with different concentrations of PEITC for 24 hr and whole cell lysates were analyzed for Sp1, Sp3 and Sp4 proteins (A) prosurvival proteins (B), growth promoting and angiogenic proteins (C) by western blots as outlined in the Materials and Methods. D. Cells were treated with PEITC, GSH alone or in combination for 24 hr and cell lysates were analyzed by Western blots as indicated in A-C. Results in this figure are from one of at least duplicate analyses which gave comparable results.

Fig. 5A

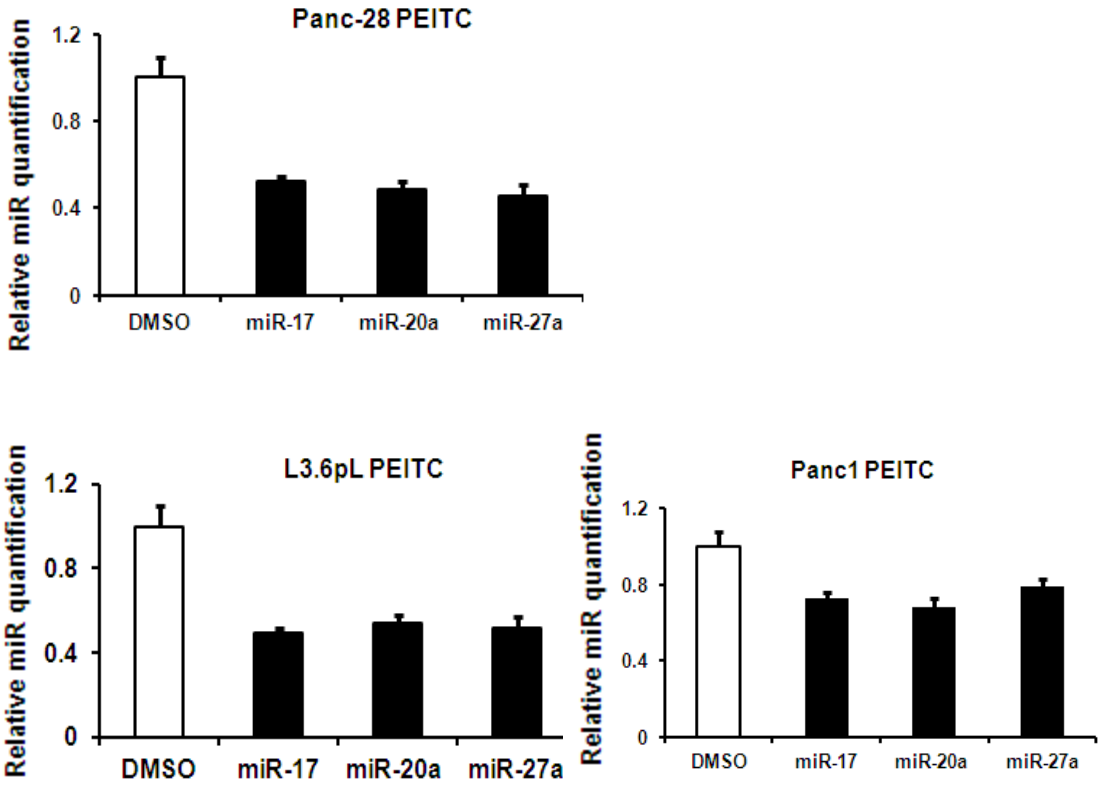


Fig. 5B

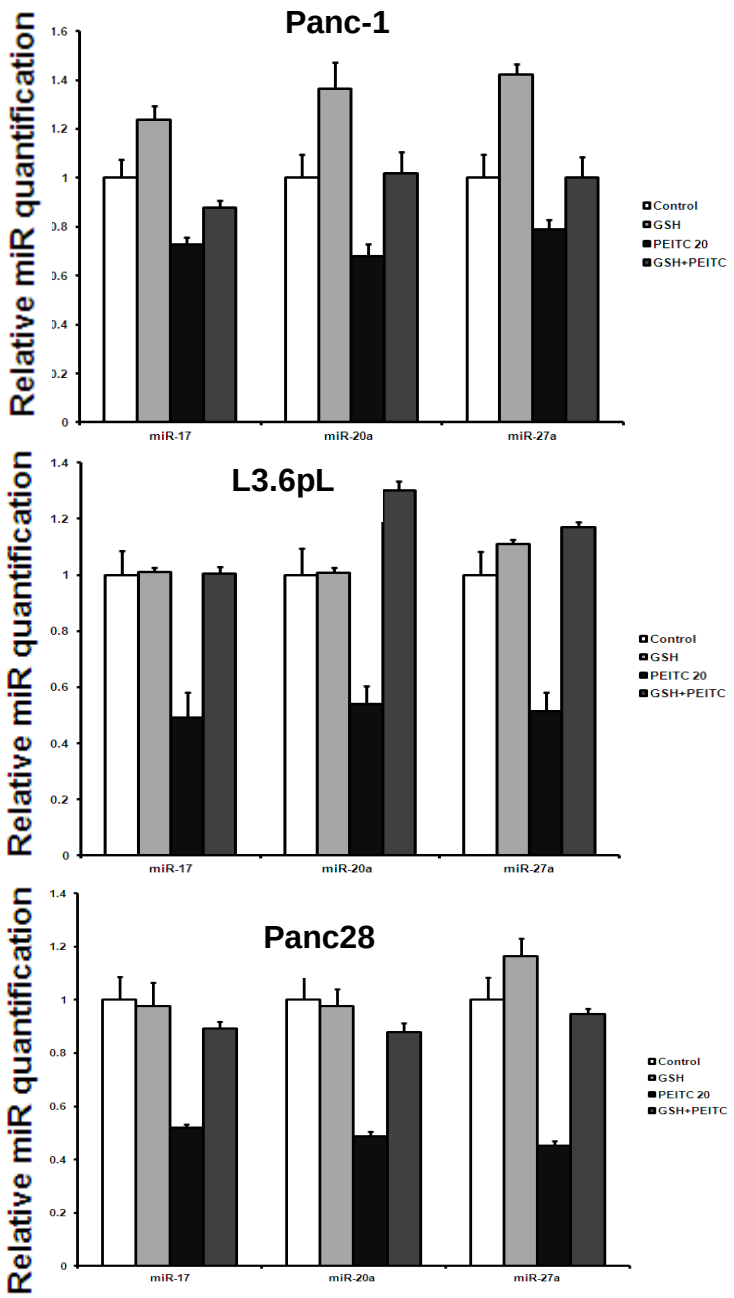

Panc-1
Relative miR quantification
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
miR-17
miR-20a
miR-27a
Control
GSH
PEITC 20
GSH+PEITC
L3.6pL
Relative miR quantification
1.4
1.2
1
0.8
0.6
0.4
0.2
0
miR-17
miR-20a
miR-27a
Control
GSH
PEITC 20
GSH+PEITC
Panc28
Relative miR quantification
1.4
1.2
1
0.8
0.6
0.4
0.2
0
miR-17
miR-20a
miR-27a
Control
GSH
PEITC 20
GSH+PEITC

Fig. 5C/D

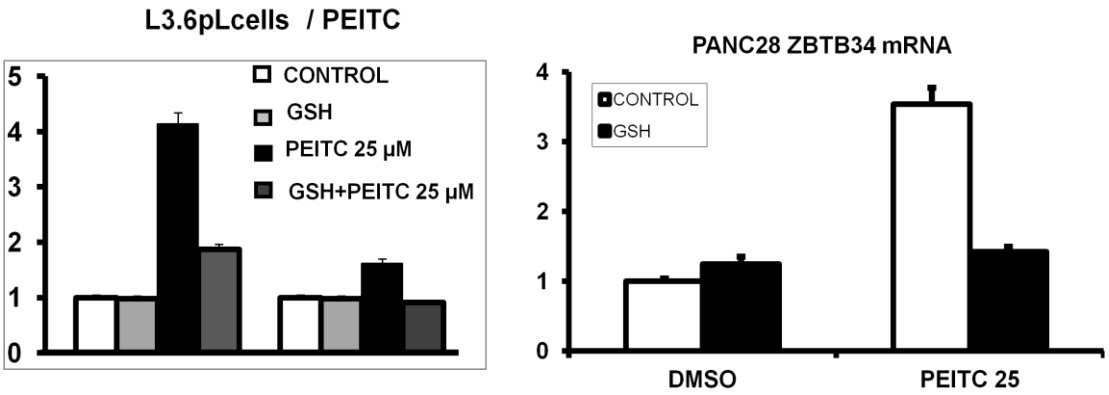



**Figure 5**. PEITC disrupts miR-ZBTB circuits. A. Pancreatic cancer cells were treated with PEITC for 24 hr and miR levels were determined as outlined in the Materials and Methods. B. Pancreatic cancer cells were treated with PEITC and GSH alone and in combination for 24 hr and miR levels were determined as described in the Materials and Methods. Using the same protocol as described in A/B the effects of PEITC alone (C) and PEITC plus GSH (D) on expression of ZBTB10, ZBTB4 and ZBTB34 mRNA levels were determined by real time PCR as described in the Materials and Methods. Results are means ± SE for at least 3 replicates per determination and significant ($p < 0.05$) effects of PEITC alone ($^{*}$) or attenuation of the effects by GSH ($^{**}$) are indicated.

Fig. 6A

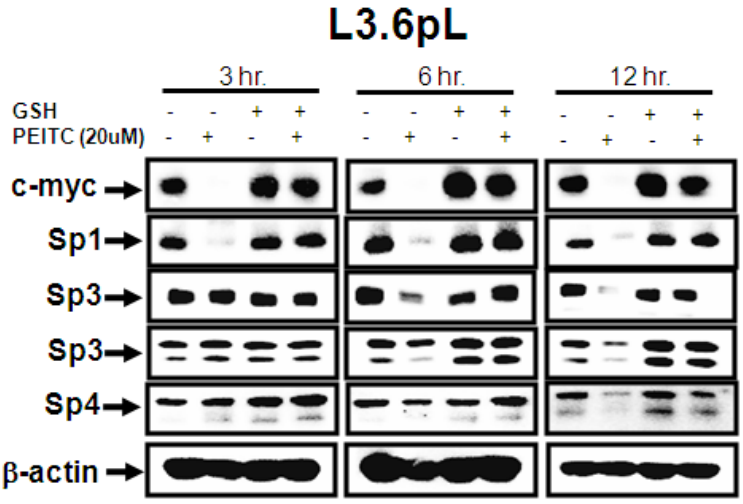


Fig. 6B

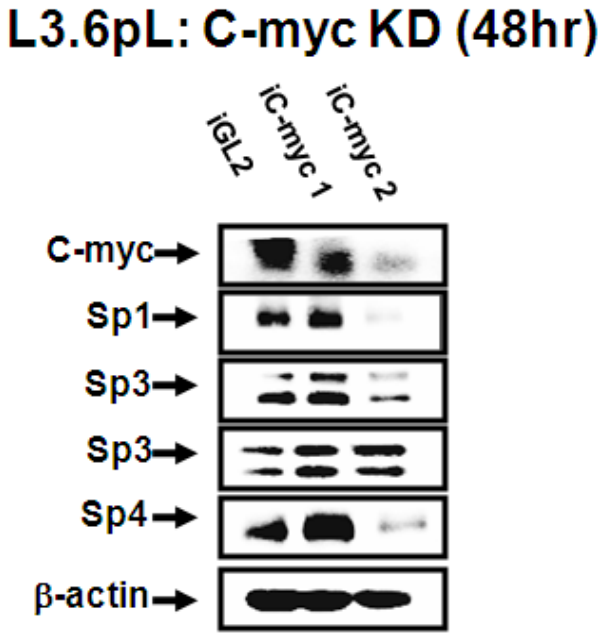
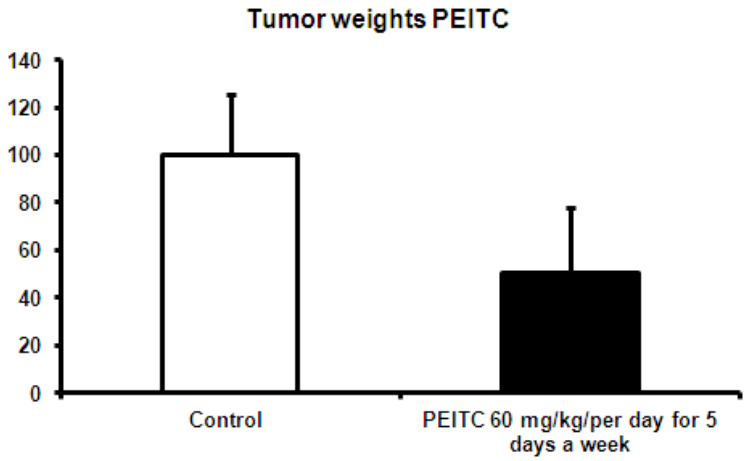



**Figure 6**. Role of c-Myc in PEITC-induce downregulation of Sp proteins. A. L3.6pL cells were treated with 20 μM PEITC alone or in combination with GSH for 3, 6 and 12 hr and whole cell lysates were analyzed by Western blots as described in the Materials and Methods. B. L3.6pL cells were transfected with oligonucleotides (i-c-Myc-1-i-c-Myc-2) targeted against c-Myc and whole cell lysates were analyzed by Western blots as outlined in the Materials and Methods.

Fig. 7A



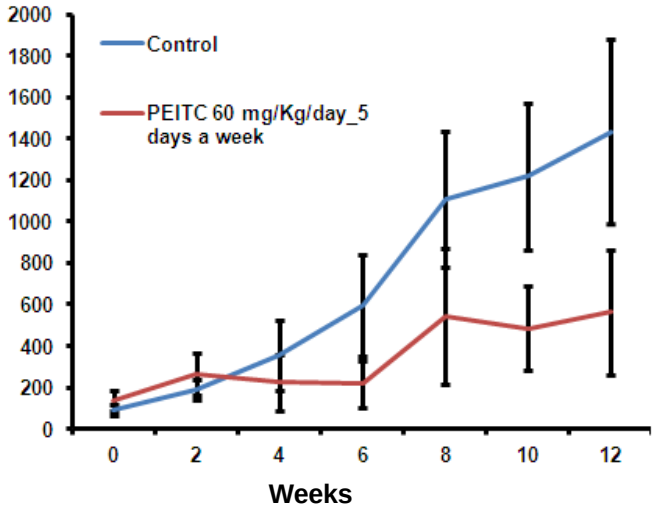



**Figure 7**. PEITC as an inhibitor of pancreatic tumor growth. A. Athymic nude mice bearing L3.6pL cells as xenografts were administered PEITC (60mg/kg/d) and after 12 days tumors and organs were excised and weighed as described in the Materials and Methods B.