Identification of novel PTEN-regulated secreted factors

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ABSTRACT

PTEN, the most frequently mutated gene in human prostate cancer, is a tumour suppressor that inhibits angiogenesis by regulating the expression of several pro- and anti-angiogenic factors. The goal of this project was to identify novel angiogenic factors and other secreted proteins that are regulated by PTEN. Proteomics techniques were employed to identify proteins that were differentially expressed in the conditioned media of PTEN-null and PTEN-expressing human prostate cancer cells. 16 proteins were identified, 4 of which were up-regulated by PTEN and 12 of which were down-regulated by PTEN. Gene expression analysis and polysome profiling revealed that 2 proteins were transcriptionally regulated by PTEN and 5 proteins were regulated at the level of translation. Three of the PTEN-regulated factors identified in this study, spondin 2 (SPO2), Zn-alpha-2-glycoprotein (ZAG), and cystatin C (CST3) are up-regulated in various cancers and could be useful serum biomarkers for the diagnosis of prostate cancer.

RÉSUMÉ

PTEN est un gène supresseur de tumeur. Il est fréquemment muté chez les humains ayant un cancer de la prostate. Un de ses rôles est d'inhiber l'angiogénèse en régulant l'expression de plusieurs facteurs pro- et anti-angiogéniques. L'objectif de cette étude était d'identifier de nouveaux facteurs angiogéniques et de nouvelles protéines secretées qui sont régulés par cette protéine. L'utilisation de différentes techniques protéomiques, sur le milieu de culture de cellules prostatiques cancéreuses, nous a permis d'identifier 16 protéines ayant des niveaux d'expression différents selon que la protéine a l'étude, PTEN, était ou non exprimée. Parmi elles, 4 ont un niveau d'expression moindre en présence de la protéine PTEN alors que les 12 autres présentent un niveau d'expression plus élevé. L'analyse du profil de polysomes et de l'expression génique pour 7 d'entre elles montrent que la protéine PTEN en régule deux au niveau transcriptionnel et cinq au niveau traductionnel. La forte expression de trois protéines identifiées dans cette étude, spondin 2 (SPO2), Zn-alpha-2-glycoprotein (ZAG), et cystatin C (CST3), dans plusieurs cancers pourrait les qualifier comme nouveaux biomarqueurs dans le diagnostique du cancer de la prostate.

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2DE	Two-dimensional electrophoresis
2D-DiGE	Two-dimensional fluorescence difference electrophoresis
4E-BP1	4E-binding protein 1
A2M	Alpha-2-macroglobulin
ACE	Angiotensin-converting enzyme
ACTB	Beta-actin
ACTN4	Alpha-actinin 4
Ad	Adenovirus
Akt	(Also known as PKB – protein kinase B)
Ang	Angiotensin
ANG	Angiopoietin
AR	Androgen receptor
BAD	Bcl-2-associated death promoter
BRRS	Bannayan-Riley Ruvalcaba syndrome
CAM	Chorioallantoic membrane
CCT8	Chaperonin containing TCP1, subunit 8 (theta)
CDAF	Collagen-derived anti-angiogenic factor
CK2	Casein kinase 2
CLSTN1	Calsyntenin 1
CM	Conditioned media
COL6A1	Alpha-1 collagen VI
CS	Cowden syndrome
CST3	Cystatin C
CTC	Circulating tumour cell
DSG2	Desmoglein 2
ECM	Extracellular matrix
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOXO	Forkhead box, class O
FPDNA	Free plasma DNA
GAPDH	Glyceraldehyde 3-dehydrogenase
GSK3β	Glycogen synthase kinase 3 beta
HIF-1	Hypoxia-inducible factor-1
HT028	ATPase, H+ transporting lysosomal accessory protein 2
IGFBP-2	Insulin-like growth factor binding protein 2
IPG	Immobilized pH gradient
ITS	Insulin transferrin selenium
LacZ	β-galactosidase
LAR	Putative LAR preprotein
m^7G	7-methyl guanosine
MAPK	Mitogen-activated protein kinase

MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
NEDD4-1	Neural precursor cell expressed developmentally downregulated 4-1
NFκB	Nuclear factor kappa B
OPN	Osteopontin
pAkt	Phospho-Akt
PDGF	Platelet-derived growth factor
PDK1	Phosphatidylinositol-dependent kinase 1
PFU	Plaque forming unit
PHTS	PTEN hamartoma-tumour syndrome
PI3K	Phosphatidylinositol 3-kinase
PIN	Prostatic intraepithelial neoplasia
PIP_2	Phosphatidylinositol (4,5) bisphosphate
PIP_3	Phosphatidylinositol (3,4,5) triphosphate (PIP_3)
PlGF	Placental growth factor
PFU	Plaque forming unit
PLS	Proteus-like syndrome
PPAR	Peroxisome proliferator-activated receptor
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PS	Proteus syndrome
PSA	Prostate specific antigen
RAS	Renin-angiotensin system
RB	Retinoblastoma protein
RNH1	Ribonuclease/angiogenin inhibitor 1
RTK	Receptor tyrosine kinase
RT-PCR	Real-time PCR
SPO2	Spondin 2
SRP	Signal recognition particle
sqRT-PCR	Semi-quantitative real-time PCR
TGF-β	Transforming growth factor beta
TNFα	Tumour necrosis factor alpha
TSP-1	Thrombospondin 1
VCL	Vinculin isoform VCL
VEGF	Vascular endothelial growth factor
VHL	von-Hippel-Lindau protein
ZAG	Zn-alpha-2-glycoprotein

1. INTRODUCTION

1.1 Angiogenesis

During the process of tumour formation, mutations and epigenetic effects allow cancer cells to acquire capabilities that promote uncontrolled growth. Such capabilities include insensitivity to negative growth signals, resistance to apoptosis, uncontrolled cell cycle progression, and enhanced angiogenic potential[1]. Cancer cells are no different from normal tissues in that they require a vasculature system to provide oxygen and nutrients that are critical for survival and growth. As a general rule, most cell types in the human body exist within 100-200 μm of a capillary blood vessel – the diffusion limit of oxygen[2]. During embryogenesis, blood vessels are formed from endothelial precursor cells through the process of vasculogenesis. Subsequently, angiogenesis, the process of formation of new blood vessels from the existing vasculature, expands this network[2]. In adults, new blood vessels are produced exclusively through angiogenesis[3]. Physiological angiogenesis occurs in adults only in very specific situations such as during the female reproductive cycle and wound healing. However, several human diseases have been associated with inappropriate induction of angiogenesis including psoriasis, macular degeneration, and cancer[3].

Angiogenesis is a complex multi-step process involving several different cell types. First, the pericytes surrounding the existing blood vessel detach and the vessel dilates. Next, the basement membrane surrounding the existing blood vessel is degraded to allow endothelial cells to invade into the perivascular space. The endothelial cells then proliferate and form a migrating column that moves through the perivascular space toward the angiogenic stimuli produced by the tumour cells or stromal cells. Behind the migrating column, endothelial cells change shape and adhere to each other to form a new capillary tube. This is accompanied by basement membrane formation and pericyte attachment. Lastly, the newly formed sprouts fuse with other vessels, thereby beginning to circulate blood to the newly vascularized region[3,4].

1.2 Angiogenesis and Cancer

1.2.1 Tumour blood vessels

Pathological tumour angiogenesis is similar to that in normal tissues except that the resulting blood vessels are abnormal in terms of their structure and their function. In contrast to the highly ordered network of venules, arterioles and capillaries of the normal vasculature, the tumour vasculature is very disorganized[4]. Tumour vessels are dilated and tortuous, have uneven diameter, and exhibit excessive branching[2]. Furthermore, tumour blood vessels have high vascular permeability due to abnormalities in the vessel walls. Together, these abnormalities cause irregular blood flow through the tumour, which leads to hypoxic and acidic regions[2].

1.2.2 The angiogenic switch

Angiogenesis has been an important topic in cancer research since 1971, when Judah Folkman proposed that tumour growth is dependent on angiogenesis[5]. It is now well established that the "angiogenic switch" – the induction of tumour vasculature – is a necessary step in the progression from a small, avascular lesion of less than 2 mm in diameter to a large, vascularized tumour[4]. In the avascular phase, tumour cells proliferate, but this proliferation is counteracted by apoptosis, thus the tumour remains dormant[6]. The angiogenic switch is activated by a change in the balance of pro- and anti-angiogenic factors found in the extracellular space[3]. These endogenous pro- and anti-angiogenic factors signal to the endothelial cells to either promote or inhibit proliferation and migration. Changes in the balance of these factors can be induced by physiological stimuli such as hypoxia, by signaling through extracellular growth factors, by activating mutations of oncogenes such as Ras, Myc, and PI3K or by inactivating mutations of tumour suppressor genes including p53, RB, PTEN, and VHL[4,6]. Once the angiogenic switch has been activated, the tumour progresses to the vascular phase. Now that the tumour has its own blood supply, the cancer cells can acquire the oxygen and metabolites required for massive proliferation, and the tumour grows exponentially[6]. Therefore the angiogenic switch is a crucial step in cancer

progression as it allows tumours to advance from a dormant stage to a stage of exponential growth.

1.3 Major Regulators of Angiogenesis

The net balance of pro- and anti-angiogenic factors in the extracellular space controls angiogenesis. Today, we know of at least 22 activators and 29 inhibitors of angiogenesis[7-9]. Endogenous pro-angiogenic factors are mostly growth factors secreted by the tumour or the stroma. On the other hand, many of the endogenous anti-angiogenic factors are fragments derived from extracellular matrix (ECM) proteins. Here we present some of the best-characterized endogenous regulators of angiogenesis.

1.3.1 Pro-angiogenic factors

VEGF

Vascular endothelial growth factor (VEGF, or VEGF-A) is one of the most highly studied pro-angiogenic factors. VEGF is a member of the platelet derived growth factor (PDGF) family, whose other members include placental growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D[10]. VEGF is secreted from tumour or stromal cells, and binds to VEGF receptor-1 or VEGF receptor-2 on endothelial cells. These VEGF receptors are receptor tyrosine kinases, which undergo dimerization and autophosphorylation upon VEGF binding, thus triggering a downstream signaling cascade which promotes cell proliferation[10].

High VEGF levels are sufficient to induce angiogenesis in quiescent vessels[4]. VEGF promotes angiogenesis in several different ways. VEGF increases vascular permeability and promotes migration of endothelial cells[10]. Furthermore, VEGF protects endothelial cells from apoptosis by inducing expression of the anti-apoptotic proteins Bcl-2 and survivin[10]. Because VEGF is such a potent inducer of angiogenesis, several inhibitors of VEGF are being studied as anti-angiogenic cancer therapies.

VEGF gene expression is regulated by growth factors and by physiological stimuli such as hypoxia. PDGF, fibroblast growth factor (FGF) and

tumour necrosis factor (TNF) are some of the tumour-derived growth factors that promote VEGF expression[10]. Hypoxia is also known to regulate VEGF expression through hypoxia-inducible factor 1 (HIF-1). HIF-1 is a heterodimeric transcription factor composed of an oxygen-sensitive alpha subunit (HIF-1α), and a constitutively expressed beta subunit (HIF-1β)[11]. When stabilized by hypoxia, HIF-1α dimerizes with HIF-1β in the nucleus and binds to hypoxia response elements in the promoters of its target genes, including VEGF[11]. Loss of expression of tumour suppressor genes or activation of oncogenes can promote VEGF transcription by stabilizing HIF-1α.

Angiopoietins

The angiopoietins, ANG-1 and ANG-2, are secreted factors that influence tumour angiogenesis by binding to the endothelial cell-specific TIE-2 receptor[6]. ANG-1 promotes angiogenesis and tumour growth by recruiting perivascular cells to endothelial cells, thus promoting maturation of new blood vessels[12]. ANG-1 also prevents endothelial cell apoptosis by suppressing caspase activity[12]. Moreover, increased ANG-1 expression is associated with increased tumour malignancy in many types of cancer.

Conversely, ANG-2 blocks angiogenesis and tumour growth by antagonizing ANG-1-TIE-2 signaling, thus promoting detachment of perivascular cells from vessel endothelial cells[6]. Overexpression of ANG-2 in glioma cells results in delayed tumour growth and metastasis[12]. It has been proposed that the balance between ANG-1 and ANG-2 levels could be a major regulator of the angiogenic switch, with ANG-1 promoting angiogenesis and ANG-2 blocking angiogenesis[6].

1.3.2 Anti-angiogenic factors

Thrombospondins

The thrombospondins, TSP-1 and TSP-2 are large ECM-derived glycoproteins. TSP-1 was the first endogenous angiogenesis inhibitor to be discovered[13]. Overexpression of TSP-1 inhibits angiogenesis and tumour growth,

while loss of TSP-1 leads to increased angiogenesis[14]. Similarly, TSP-2 inhibits tumour growth by reducing the number and size of tumour blood vessels[14]. Furthermore, TSP-1-null mice and TSP-2-null mice both display enhanced tumour angiogenesis[14]. The anti-angiogenic activity of both TSP-1 and TSP-2 is localized to the N-terminus, in the pro-collagen-like domain and the globular domain, respectively[14].

Collagen-derived anti-angiogenic factors

The basement membrane of blood vessels is a specialized, collagen-rich extracellular matrix that provides structural support to the endothelium[8]. Several collagen isoforms in the basement membrane contain C-terminal peptides that, when proteolytically cleaved, have anti-angiogenic activity. These collagen-derived anti-angiogenic factors (CDAFs) include arresten, derived from the α1 collagen IV chain, tumstatin, derived from the α3 collagen IV chain, and endostatin, derived from the α1 collagen XVIII chain[15]. CDAFs have been shown to inhibit endothelial cell proliferation, migration, and tube formation as well as inducing apoptosis[15]. Through these complex inhibitory mechanisms, CDAFs have a potent anti-angiogenic effect.

1.4 The PI3K Pathway

The phosphatidylinositol 3-kinase (PI3K) pathway is a major driver of cell growth and survival (Figure 1). PI3K is a lipid kinase that is activated by various receptor tyrosine kinases (RTK) upon binding of their extracellular growth factor ligands[16]. PI3K is composed of two subunits: a p110 catalytic subunit and a p85 regulatory subunit[17]. PI3K converts the lipid second messenger phosphatidylinositol (4,5) bisphosphate (PIP_2) into phosphatidylinositol (3,4,5) triphosphate (PIP_3)[18]. PIP_3 then recruits phosphatidylinositol-dependent kinase 1 (PDK1) and Akt to the membrane, where Akt is phosphorylated[18].

Akt, also known as protein kinase B (PKB), is a serine/threonine kinase that is responsible for the phosphorylation of many downstream targets and thus acts as a central node in the PI3K pathway. Akt promotes cell growth by

activating protein synthesis. Akt phosphorylates mammalian target of rapamycin (mTOR), which in turn phosphorylates and activates the ribosomal protein S6 kinase and inactivates 4E-binding protein 1 (4E-BP1)[19]. Consequently, inhibition of eukaryotic initation factor 4E (eIF4E) is blocked, and cap-dependent protein translation is activated.

Moreover, active Akt enhances cell survival by inhibiting apoptosis. By phosphorylating and inactivating the forkhead (FOXO) transcription factors, Akt blocks transcription of genes involved in promoting apoptosis and cell cycle arrest[20]. Furthermore, Akt directly phosphorylates pro-apoptotic factors including caspase-9 and BAD, thereby inhibiting their pro-apoptotic function[20].

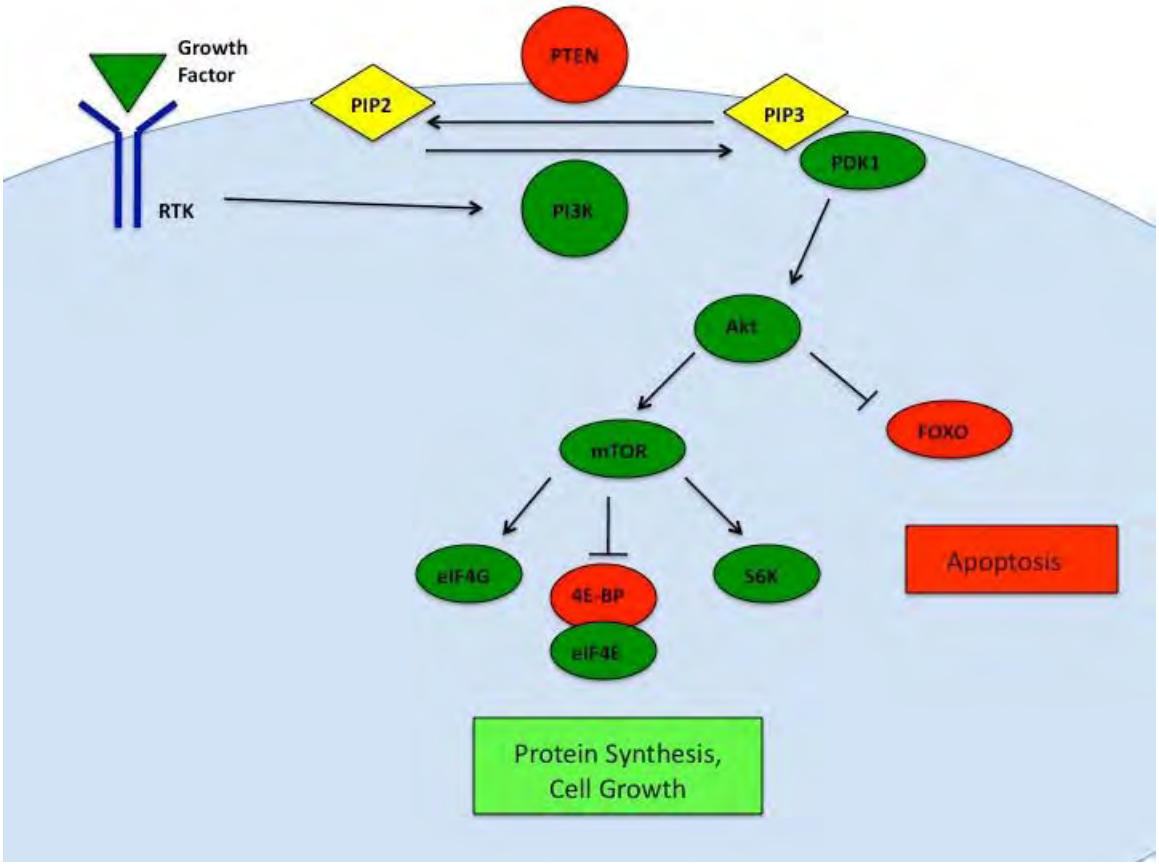

Figure 1. The PI3K pathway.

PI3K is activated by the binding of various extracellular growth factors to their cell-surface RTKs. PI3K catalyzes the phosphorylation of the membrane lipid PIP2 to form PIP3. PDK1 is then recruited to the cell membrane, where it phosphorylates Akt, which, in turn, phosphorylates many downstream targets. Akt phosphorylates and inactivates the FOXO transcription factors, thereby inhibiting apoptosis. Phosphorylation by Akt activates mTOR, which then activates eIF4G and S6K, and inhibits the respressive factor 4E-BP in order to initiate protein synthesis. PTEN, a direct antagonist of PI3K, dephosphorylates PIP3, and thereby inactivates downstream signaling, thus inhibiting protein synthesis and cell growth and promoting apoptosis.

1.5 PTEN

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is the second most frequently mutated gene in human cancer. PTEN, also known as MMAC1 or TEP1, was first identified on human chromosome 10q23 as a region that is homozygously mutated in many clinical cancer samples and tumour cell lines[21,22]. Functionally, the PTEN protein acts as both a lipid and protein phosphatase. The major lipid substrate for PTEN is PIP_3[23]. This lipid phosphatase activity, which directly antagonizes PI3K, is the main tumour suppressive function of PTEN[24,25].

1.5.1 PTEN mutations

PTEN is mutated in 30% of human cancers[26]. Many different types of mutations occur, including point mutations leading to catalytic inactivity, and structural rearrangements of the PTEN gene including inversions, insertions, deletions and duplications[27]. Moreover, the PTEN promoter has been shown to be methylated in many cancers[28]. Two common sites of point mutations are cysteine 124, the catalytic residue, and glycine 129. Mutations in C124 abolish lipid and protein phosphatase activity, while G129 mutations affect only the lipid phosphatase activity of PTEN[29].

In addition to inactivating mutations of PTEN, activating mutations of PI3K subunits or Akt also lead to enhanced PI3K activity. Akt1 and Akt2 gene amplifications have been observed in a small percentage of cancers[27]. Activating mutations in PIK3CA, the gene which encodes the p110α catalytic subunit of PI3K, are common in colon, breast, lung, and gastric cancers[27].

1.5.2 Genetic disorders associated with PTEN loss

There are several autosomal dominant disorders associated with germline mutations in PTEN, including Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome (BRRS), Proteus syndrome (PS) and Proteus-like syndrome (PLS)[30]. Cowden syndrome patients develop multiple hamartomas and have a predisposition to breast, thyroid and endometrial cancer[31]. Similarly, BRRS

patients develop lipomas and hemangiomas[32]. Often these four disorders are grouped together as the PTEN Hamartoma-Tumour Syndrome (PHTS) due to their common etiology and similar symptoms[30].

1.5.3 PTEN mouse models

Many mouse models have been created to study the effects of loss of PTEN. PTEN is required for embryonic development since homozygous Pten knockout mice die by E7.5[33]. Mice with heterozygous deletion of Pten spontaneously develop tumours in numerous organs, including the thyroid, colon, endometrium, prostate, breast and liver[33-35]. Furthermore, prostate-specific deletion of Pten leads to development of advanced prostate cancer and metastasis[36,37]. Many other tissue-specific Pten knockout mouse models have been created, most of which show an increased susceptibility to cancer. Interestingly, these Pten-deficient mouse models mimic the human disease, further highlighting the importance of PTEN in tumour suppression.

1.5.4 Regulation of PTEN

PTEN was initially thought to be a constitutively active phosphatase, however recent studies have shown that PTEN is regulated by transcription factors, protein degradation, and post-translational modifications. PI3K represses transcription of PTEN through modulation of nuclear factor κB (NF-κB), peroxisome proliferator-activated receptors β and γ (PPARβ/γ), and tumour necrosis factor α (TNFα)[16]. Furthermore, PTEN is transcriptionally repressed by Ras and JNK signaling pathways[16]. Conversely, transcription of PTEN is activated by the tumour suppressor p53[38].

Protein levels of PTEN are controlled by post-translational modifications. PTEN can be phosphorylated at several sites on its C-terminal tail, which stabilizes the protein[39]. Several kinases, including casein kinase 2 (CK2) and glycogen synthase kinase 3β (GSK3β) have been reported to mediate the phosphorylation of PTEN[26]. PTEN is also controlled by the E3 ubiquitin ligase neural precursor cell expressed developmentally downregulated-4-1 (NEDD4-1),

which targets PTEN for degradation[40]. Monoubiquitination of PTEN induces its transport into the nucleus, while polyubiquitination promotes its proteasomal degradation in the cytoplasm[41].

1.5.5 Protein phosphatase activity of PTEN

In addition to its important tumour-suppressive role as a lipid phosphatase, PTEN is also known to be a protein phosphatase. PTEN has been shown to interact with and dephosphorylate focal adhesion kinase (FAK) in vivo[42]. PTEN's protein phosphatase activity also regulates MAP kinase (MAPK) phosphorylation and cyclin D1 expression[43,44]. Interestingly, it appears that nuclear PTEN regulates these processes as a nuclear localization-defective PTEN mutant was not able to block MAPK and cyclin D1 activity[45]. This suggests that PTEN may dephosphorylate lipid substrates in the cytoplasm, while dephosphorylating proteins in the nucleus.

1.6 The Role of PTEN in Angiogenesis

Over the last ten years, it has become clear that PI3K and PTEN signaling regulate tumour angiogenesis. Loss of function of PTEN in clinical prostate cancer samples was found to be associated with increased microvessel number and density[46]. Jiang and colleagues were the first to directly link PI3K signaling to angiogenesis. Their 2000 study showed that overexpression of PI3K in the chorioallantoic membrane (CAM) of the chicken embryo induced led to sprouting of new blood vessels and enlargement of exisiting vessels[47]. Furthermore, overexpression of PTEN or dominant negative mutants of PI3K inhibited angiogenesis[47]. More recently, PTEN has been shown to regulate several pro- and anti-angiogenic factors and to prevent endothelial cell proliferation through inhibition of PI3K.

1.6.1 PTEN & VEGF

PTEN and PI3K regulate the pro-angiogenic factor VEGF by controlling protein levels of its transcription factor, HIF-1α. Reintroduction of PTEN into

PTEN-null U373 glioblastoma cells inhibited the stabilization of HIF-1α, and thereby blocked the transcription of VEGF and other HIF-1α-regulated genes[48]. PTEN and PI3K were shown to affect protein levels of HIF-1α, but not mRNA levels[49]. HIF-1α-dependent transcriptional activity was inhibited by expression of wild-type PTEN or dominant negative mutants of PI3K in prostate cancer cells[49]. Moreover, treatment of prostate cancer cells with the PI3K inhibitor LY294002 or the mTOR inhibitor rapamycin inhibited transcription of VEGF[49,50]. Overexpression of PTEN inhibited VEGF transcription, whereas overexpression of a lipid phosphatase mutant of PTEN increased VEGF transcription[50].

More recently, two studies have demonstrated that PTEN's ability to inhibit VEGF transcription mediates its anti-angiogenic activity. Knockdown of the PI3K p110α catalytic subunit using siRNAs decreased HIF-1α levels and VEGF expression, also resulting in decreased tumour growth and angiogenesis, as shown in the CAM model[51]. Moreover, introduction of PTEN into PTEN-null prostate cancer cells blocked angiogenesis by decreasing expression levels of HIF-1α and VEGF[52]. Altogether these studies suggest that PI3K promotes angiogenesis by regulating VEGF levels through HIF-1α, and that the lipid phosphatase activity of PTEN reverses this effect.

1.6.2 PTEN & TSP-1

Conflicting evidence exists regarding the role of PI3K signaling in regulating the expression of TSP-1, a secreted glycoprotein that suppresses angiogenesis and tumour growth. PTEN has been shown to both activate and inhibit TSP-1. Reconstitution of PTEN-null U87MG glioblastoma cells with wild-type PTEN increased mRNA and protein levels of TSP-1[53]. However, re-introduction of PTEN into PTEN-null thyroid cancer cells resulted in decreased expression of TSP-1[54].

Moreover, PI3K and Akt have been shown to both activate and inhibit TSP-1 expression. Treatment of endothelial cells and Ras-expressing kidney-derived cells with the PI3K inhibitor LY294002 resulted in increased expression of TSP-1, suggesting that PI3K represses TSP-1 expression[55,56]. Conversely,

tumour and endothelial cells from an Akt-deficient mouse model were shown to have decreased protein levels of TSP-1 compared to cells from wild-type mice, suggesting that PI3K and Akt activate TSP-1 expression[57]. It is possible that PTEN and PI3K affect TSP-1 expression differently in different cell types, however more studies are required to conclude the role of the PI3K pathway in regulating TSP-1.

1.6.3 Other PTEN-regulated factors

Maspin is a secreted protein previously shown to have anti-angiogenic and anti-metastatic activity. PTEN reconstitution in PTEN-null glioblastoma cells resulted in increased protein levels of maspin in response to hypoxia[58]. PTEN controls maspin expression through its complex network with p53[58]. Cytoplasmic PTEN activity blocks Akt-induced phosphorylation of Mdm2, thereby preventing its translocation to the nucleus[59]. Mdm2 is a ubiquitin ligase which degrades p53 only in the nucleus, thus by preventing its translocation to the nucleus, PTEN stabilizes p53[59]. In turn, p53 transcriptionally regulates PTEN, thus forming a feedback loop[59]. Interestingly, both PTEN and p53 activity were necessary to activate maspin expression in glioblastoma cells[58].

Pleiotrophin is a secreted growth factor with oncogenic and pro-angiogenic activity[60]. Pleiotrophin mRNA and protein levels were increased in PTEN-null mouse embryonic fibroblasts[61]. Expression of constitutively active mutants of Akt increased pleiotrophin levels, while treatment with the PI3K inhibitor LY294002 or expression of PTEN decreased pleiotrophin levels[61]. Together these results suggest that PTEN inhibits pleiotrophin expression through its ability to antagonize PI3K signaling.

Osteopontin (OPN) is a secreted glycophosphoprotein that is commonly overexpressed in human cancers[62]. OPN is known to promote tumour angiogenesis and metastasis[62]. OPN mRNA levels were elevated in PTEN-null prostate cancer and colon cancer cells[37,63]. Additionally, PTEN has been shown to decrease mRNA and protein levels of OPN in melanoma cells[64]. Treatment

with the PI3K inhibitor LY294002 produced a similar effect, suggesting that PTEN's lipid phosphatase activity mediates its inhibitory effects on OPN[64].

Adrenomedullin is a secreted peptide that promotes tumour angiogenesis[65]. PTEN decreases adrenomedullin mRNA levels in glioma cells and endometrial cancer cells[66,67]. Adrenomedullin expression is induced by hypoxia, and its promoter has several putative hypoxia response elements[65], thus PTEN may control adrenomedullin expression through its effects on HIF-1α.

1.6.4 PTEN/PI3K signaling in endothelial cells

In addition to its important function in tumour cells, PI3K signaling also plays a role in endothelial cells to regulate angiogenesis. PI3K is activated in endothelial cells by binding of growth factors such as VEGF and angiopoietins, and subsequently promotes endothelial cell proliferation and migration[68]. Conversely, overexpression of PTEN in cultured endothelial cells inhibits vessel sprouting and tube formation induced by VEGF[69].

Evidence suggests that downstream signaling through Akt and mTOR mediates the pro-angiogenic effects of PI3K in endothelial cells. Mice expressing an endothelial cell-specific constitutively active mutant of Akt have leaky, tortuous vessels which mimic those observed in human tumours[70]. Furthermore, the mTOR inhibitor rapamycin has been shown to block angiogenesis and tumour growth[71]. Rapamycin treatment rescues the vascular permeability defect seen in Akt mutant mice[70], inhibits VEGF-dependent endothelial cell proliferation, and decreases VEGF secretion by tumour cells[71]. Moreover, rapamycin has been shown to inhibit proliferation and differentiation of endothelial precursor cells[72]. Together, these results indicate that mTOR may be acting downstream of PI3K and Akt to control endothelial cell proliferation and tumour angiogenesis.

Several mouse models have explored the effects of the loss of PTEN and PI3K in endothelial cells on angiogenesis. In an endothelial cell-specific Pten knockout mouse model, mice with a homozygous deletion of Pten died by embryonic day 11.5 due to excessive bleeding and cardiac failure[73]. In heterozygotes, partial loss of Pten made the endothelial cells hypersensitive to

growth factors including VEGF, and led to enhanced angiogenesis and more rapid tumour growth[73]. Similarly, mice with endothelial cell-specific knockout of the p85 regulatory subunit of PI3K also died during embryonic development due to excessive bleeding[74]. However, mice with a heterozygous deletion of PI3K-p85 in endothelial cells had smaller vessel size, and decelerated tumour growth compared to control mice[74]. Together these two mouse models indicate that expression of both PI3K and PTEN in endothelial cells is essential for vessel formation during embryogenesis, but that the two proteins play opposite roles during tumour progression.

1.6.5 Summary

PTEN inhibits angiogenesis through its effects on both tumour cells and the endothelial compartment. PTEN inhibits the expression of pro-angiogenic factors including VEGF, pleiotrophin, osteopontin, and adrenomedullin in tumour cells. Moreover, PTEN increases expression of anti-angiogenic factors including thrombospondin-1 and maspin. Interestingly, the ability of PTEN to antagonize PI3K signaling through its lipid phosphatase activity seems to mediate its ability to control expression of these angiogenic factors.

Furthermore, PTEN suppresses the activation of PI3K in endothelial cells, thus preventing formation of new blood vessels. In the future it will be important to continue to elucidate the mechanism through which PTEN inhibits angiogenesis, as well as addressing possible roles of PTEN's protein phosphatase activity.

1.7 Prostate Cancer

Prostate cancer is one of the leading causes of cancer mortality in men. The prostate gland is located at the base of the bladder, surrounding the urethra. Its main function is to secrete proteins that liquefy the semen, including prostate specific antigen (PSA). The typical process of prostate cancer progression begins with a precancerous lesion called prostatic intraepithelial neoplasia (PIN). Over time, the PIN progresses to a localized adenocarcinoma, which can be treated with

androgen ablation therapy. Eventually, the tumour becomes insensitive to androgen ablation, and metastasizes to other organs[75].

1.7.1 Treatment of prostate cancer

Prostate cancer treatment generally consists of radiation, chemotherapy, or surgical removal of the prostate (radical prostatectomy). Upon relapse, patients are treated with androgen ablation therapy, which can consist of surgical castration and/or treatment with anti-androgens. Androgen ablation is generally successful, however, the tumour may eventually undergo hormone relapse and develop the ability to grow in the absence of testosterone[76]. Androgen-insensitive prostate cancer almost inevitably results in death within two years of recurrence[77]. There are very few targeted therapies approved for the treatment of prostate cancer.

1.7.2 Prostate cancer biomarkers

Levels of some of the factors secreted by the prostate become altered in prostate cancer patients. These prostate-derived proteins can serve as biomarkers to diagnose and monitor prostate cancer. For many years, the serine protease PSA has been the gold standard of diagnostic biomarkers for prostate cancer. Normally, PSA levels are high in semen, but relatively low in the blood. However, blood PSA levels are increased by up to 10^5-fold in men with prostate cancer compared to healthy individuals[78]. High PSA levels correlate with larger tumour size and clinical stage of disease, as men with metastatic prostate cancer have higher PSA levels than men with localized disease[78]. In addition to its usefulness as an early predictor of prostate cancer, PSA also serves as a predictor of recurrence after radical prostatectomy.

More recently a few other prostate cancer biomarkers have been introduced. Free plasma DNA (FPDNA) is DNA derived from tumour cells that circulates through the bloodstream. High FPDNA levels correlate with tumour stage in men with prostate cancer[79]. Another prostate cancer biomarker is circulating tumour cells (CTCs). CTCs can be detected in blood by PCR for

prostate-specific transcripts such as PSA. Increased levels of CTCs are associated with tumour stage in prostate cancer[79].

1.7.3 PTEN and prostate cancer

PTEN is the most frequently mutated gene in metastatic prostate cancer[80], and is mutated in up to 80% of patients[81]. Consequently, Akt is highly activated in prostate cancer[82]. Many prostate cell lines, including PC-3 and LNCaP, are null for PTEN expression. Furthermore, PTEN loss is associated with resistance of prostate cancer to chemo- and radiotherapy (reviewed in [83]). PTEN is also known to protect from cancer recurrence after radical prostatectomy [84]. Together, these studies indicate that PTEN is critical for the inhibition of prostate cancer initiation and progression.

1.8 Secreted Proteins

1.8.1 The secretome

The secretome is the full complement of proteins found in the conditioned media of cultured cells[85]. This includes a mixture of classical secreted proteins, proteins secreted through non-classical pathways, and exosomal proteins[85]. Studying the secretome has become increasingly popular in recent years. Many groups have studied the secretome of tumour cell lines in order to identify novel cancer biomarkers. Studying plasma and other bodily fluids is very challenging because these samples contain a complex mixture of proteins and growth factors derived from various sources (tumour cells, stromal cells, and blood cells)[86]. Therefore examining the secretome of cultured cells greatly simplifies the study of extracellular proteins.

1.8.2 The secretory pathway

The traditional secretory pathway consists of the endoplasmic reticulum (ER), the Golgi apparatus and vesicular transport between the two compartments. Most secreted proteins contain an N-terminal signal sequence, which is 20-30 residues in length[87]. A ribonucleoprotein known as the signal recognition particle

(SRP) binds to the signal sequence of a protein as it is being translated by a ribosome and targets the ribosome-polypeptide complex to the ER surface[87]. Upon termination of translation, the protein migrates through a translocon into the ER lumen, where molecular chaperones assist in folding the protein[88]. In the ER, post-translational modifications, including proline hydroxylation and glycosylation occur[88]. The protein is then packaged into a vesicle and transported to the Golgi apparatus[88]. In the Golgi, glycosylation continues and proteolytic processing (removal of the signal sequence), sulfation and phosphorylation occur[89]. The fully processed protein is then packed into a vesicle and transported to the plasma membrane. The vesicle fuses with the plasma membrane thus releasing the enclosed proteins into the extracellular space[90].

Many secreted factors, including FGF-1 and FGF-2, do not contain an N-terminal signal sequence and are not processed through the ER-Golgi network[90]. These "non-classical" secreted proteins are synthesized on free ribosomes and folded by cytosolic chaperone proteins[90]. These factors can exit the cell by direct translocation, by a lysosome-dependent mechanism, or by exosomal transport[90]. Exosomes are membrane-derived microvesicles that are used to transport factors including RNA, proteins and lipids from one cell to another[91].

1.9 Proteomics

Proteomics, the study of all proteins synthesized by a certain cell at a given time, is a valuable tool to obtain a complete and unbiased answer to a biological question. Instead of seeking the answer to a specific question such as "is protein x regulated by protein y?" proteomics allows us to answer broader questions such as "which proteins are regulated by protein y?" Many different techniques exist to analyze the proteome. This section will focus on 2D electrophoresis, the technique applied in this study.

1.9.1 2D Electrophoresis

Two-dimensional electrophoresis (2DE) is a technique that was developed in the 1970s in order to separate a complex mixture of proteins (from a

cytoplasmic extract, for example)[92]. First, proteins are separated on a pH gradient, where each protein equilibrates at its isoelectric point. Then the proteins are further resolved by SDS-PAGE, which separates based on molecular weight. Finally, the gel is stained with a protein-binding dye, such as silver nitrate, and two gels can be compared to determine if a protein is more highly expressed in one experimental condition than the other. For many years, 2DE served as the gold standard for proteomics studies. However, some fundamental issues remained unsolved, including poor reproducibility and a lack of sensitivity[93]. Furthermore, gel-to-gel variations, caused by differences in acrylamide polymerization, fluctuations in electricity or temperature during electrophoresis, and differential precipitation of proteins during the isoelectric focusing step, made it very difficult to draw reliable conclusions[94].

1.9.2 2D-DiGE

A variation on 2DE called 2D fluorescence difference electrophoresis (2D-DiGE) was developed by Unlu and colleagues in 1997 to reproducibly detect differences in protein levels between two samples[95]. In 2D-DiGE, two or three samples are labeled with fluorescent dyes, and then run on the same 2D gel. The DiGE dyes are N-hydroxysuccinimidyl ester derivatives of the cyanine dyes Cy3 and Cy5, which react with primary amine groups such as the N-terminus of proteins and lysine residues[93]. Both dyes have a single positive charge, and differ in mass by only 2 Da[93]. Furthermore, the emission spectra of Cy3 and Cy5 are very different (590 nm and 680 nm respectively), so there is little crosstalk between the fluorescent signals[94]. A third dye, Cy2 was later added in order to incorporate an internal standard into each gel. Cy2 is a less ideal match to Cy3 and Cy5 since its mass is somewhat lower and its emission spectrum overlaps slightly with that of Cy3[94]. Generally a minimal labeling strategy is used, wherein a small amount of dye is incubated with a protein sample such that only 1-2% of the total lysine residues are labeled[93]. Thus generally only a single dye molecule is associated with each protein, and the shift in electrophoretic mobility of the dye-conjugated protein compared to the unlabeled protein is minimized[94]. Once

the minimal labeling is complete, free lysine is added to quench the reaction by binding all unincorporated dye.

The internal standard, which is a pool of all samples from all replicates of the experiment, allows for accurate quantification of changes in protein levels between samples. The Cy3 and Cy5 fluorescent signals (from the two experimental samples on each gel) are compared to the Cy2 internal standard signal in order to detect a change in the level of a specific protein spot in one sample compared to the total level of that protein. This also allows us to assign statistical significance to the observed changes in protein levels between samples.

For a typical 2D-DiGE experiment, equal amounts of two protein samples are incubated with Cy dyes and then the reaction is quenched with free lysine. Next, the protein samples are mixed together and separated by isoelectric focusing and SDS-PAGE, as in traditional 2DE. The gels are then imaged using a scanner compatible with fluorescent dyes. The resulting gel images are then compared using sophisticated computer software. Once the significantly different protein spots have been identified, the gel is stained with a visible dye such as Coomassie blue or silver nitrate, or a fluorescent total protein stain such as Sypro Ruby or Deep Purple. The interesting protein spots are then cut from the gel and analyzed by mass spectrometry to identify the differentially regulated proteins.

In conclusion, DiGE offers several key advantages over traditional 2DE. DiGE minimizes gel-to-gel variation by allowing multiple protein samples to be run on a single gel. Moreover, the use of fluorescent dyes improves the range of protein detection from 40-fold (the range for silver-stained 2D gels) to 20 000-fold[93]. Finally, the pooled internal standard allows for accurate and reproducible quantification of differences in protein expression between samples.

1.10 Rationale and Research Objective

PTEN is an important tumour suppressor that inhibits angiogenesis by regulating the expression of several pro- and anti-angiogenic factors. However, the study of PTEN in angiogenesis is a fairly new field, and much more can be learned regarding the mechanism by which PTEN inhibits this process.

The objective of this project was to identify novel anti-angiogenic factors and other secreted proteins that are regulated by PTEN. 2D-DiGE was used to identify proteins that were differentially expressed in the conditioned media of PTEN-null and PTEN-expressing human prostate cancer cells. 16 proteins were identified, 4 of which were up-regulated by PTEN and 12 of which were down-regulated by PTEN. Gene expression analysis and polysome profiling were employed to examine whether these newly identified factors are regulated by PTEN at the level of transcription or translation.

The identification of novel PTEN-regulated secreted factors in this study has expanded our knowledge of downstream targets in the PI3K signaling pathway and led to a better understanding of the molecular mechanism by which PTEN inhibits tumour angiogenesis. Moreover, the identified PTEN-regulated secreted factors could serve as blood biomarkers to diagnose prostate cancer. As of right now, the only way to monitor PI3K pathway activation in patients is to take a tissue biopsy and perform immunohistochemistry to examine levels of pAkt or other downstream PI3K targets. The PTEN-regulated secreted proteins identified in this study could provide a valuable, non-invasive diagnostic tool to monitor PTEN status in prostate cancer patients.

2. MATERIALS AND METHODS

2.1 Western Blotting

Whole cell lysates were prepared by lysing cells in Laemmli buffer and boiling samples for 5 minutes. Protein concentrations of lysates were measured using the RC DC protein assay (BioRad). 25-30 μg of total protein from each lysate were run on 10% polyacrylamide gels, and then transferred to 0.45 μm nitrocellulose membranes (BioRad). Antibodies used were rabbit anti-PTEN (Cell Signaling #9559), rabbit anti-phospho-Akt (Cell Signaling #4058), rabbit anti-Akt pan (Cell Signaling #4685), rabbit anti-actin (Sigma), rabbit anti-SPON2 (Sigma-Aldrich AV52336), mouse anti-ZAG [1E2] (Santa Cruz sc-21720), and goat-anti Cystatin C (R&D Systems AF1196). Horse radish peroxidase-coupled secondary antibodies (anti-rabbit, anti-mouse, or anti-goat; Jackson ImmunoResearch) and Western Lightning Plus enhanced chemiluminescence substrate (Perkin Elmer) were used to visualize proteins on autoradiography film.

2.2 Cell Culture and Adenovirus Infection

LNCaP were cultured in RPMI-1640 1x (Wisent Bioproducts) supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Wisent Bioproducts). Adenoviruses expressing wild-type PTEN, a catalytically inactive mutant of PTEN (C124S), and β-galactosidase (LacZ) were amplified in 293 cells. The construction of the adenoviruses has been previously described[69,96]. The adenoviruses were purified by ultracentrifugation on a cesium chloride gradient, and titered using the $TCID_{50}$ method.

At least 24 hours prior to adenoviral infection, LNCaP were seeded onto 15 cm dishes at a density of 6.5×10^6 cells per dish. The next day, cells from one extra dish were trypsinized and counted to determine the number of cells per dish. This value was then used in order to calculate the volume of adenovirus required to infect the cells at a multiplicity of infection (MOI) of 100 plaque forming units per cell (unless a different MOI is stated). The adenovirus was diluted in 2 mL RPMI per plate. Media was aspirated from the cells, then 2 mL of diluted

adenovirus was added to each plate, and the cells were incubated for 1 hour at 37°C. The virus mix was aspirated from the cells, and 20 mL RPMI + 15% FBS was added to each plate. For 2D-DiGE experiments, 24 hours after infection, cells were washed twice in PBS, then 12 mL of RPMI + 1% insulin transferrin selenium (ITS) was added to each plate and left to condition for 24 hours.

2.3 Protein Purification from Conditioned Media

Conditioned media (CM) was collected from adenovirus-infected LNCaP (12 mL per 15 cm dish) and centrifuged at 1500 rpm for 5 minutes to pellet cell debris. The supernatant was filtered into a 50 mL tube using a 0.45 μm syringe filter. A scaled-up variation of chloroform-methanol protein precipitation was used to precipitate the proteins from the conditioned media[97]. 4 volumes of methanol, 1 volume of chloroform, and 3 volumes of water were added to each sample, and vortexed to mix. Samples were then centrifuged for 40 minutes at 4750 rpm in a table-top centrifuge at 4°C. The upper aqueous phase was discarded, while the proteins remained in the interface between the lower and upper phases. 3 volumes of methanol were added and the samples were vortexed. Samples were centrifuged for 20 minutes at 4750 rpm and 4°C. The supernatant was removed, and the pellet was washed with 25 mL of methanol, and centrifuged once more for 20 minutes at 4750 rpm and 4°C. The supernatant was removed, and the pellets were left to air-dry for 30-45 minutes. 300 μL of DiGE lysis buffer (7M Urea, 2M Thiourea, 4% CHAPS, 30 mM Tris, 5 mM DTT, pH 8.5) was added to each protein pellet, and samples were left to overnight on a thermal shaker at 22°C and 750 rpm to resuspend pellets. Samples were then stored at -20°C.

2.4 2D-DiGE

2.4.1 DiGE Cy Dye Labeling

LNCaP CM proteins were collected and precipitated from four independent experiments. Protein concentrations were measured using the 2D quant kit (GE Healthcare). Proteins were labeled with CyDye DiGE Fluor

minimal dyes (GE Healthcare) following standard protocol (see "Ettan DiGE System User Protocol", GE Healthcare, 2005). 50 μg of protein from each sample (PTEN and LacZ) of each replicate was incubated with 400 pmol of either Cy3 or Cy5 dye. A pooled internal standard, consisting of 25 μg of protein from each sample of each replicate (200 μg total) was incubated with 1600 pmol of Cy2 dye. Samples were incubated with the dyes for 30 minutes, and then 1 μL of 10 mM lysine solution was added to each reaction to quench the dye.

2.4.2 First Dimension Isoelectric Focusing

Following CyDye labeling, PTEN and LacZ samples were combined together, according to the randomized experimental design (Table 1), and 50 μg of pooled internal standard was added to each strip. This mixture was then combined with 4.5 μL of 1% immobilized pH gradient (IPG) buffer (GE Healthcare), 5.4 μL of DeStreak Reagent (GE Healthcare), and a variable volume of rehydration buffer (7M Urea, 2M Thiourea, 4% CHAPS, 0.002% Bromophenol blue), to bring the total volume for each IPG strip up to 450 μL. Samples were then loaded onto four 24 cm pH 3-11 non-linear Immobiline DryStrips (GE Healthcare). Active rehydration and isoelectric focusing (IEF) were then performed using the Ettan IPGphor IEF system (GE Healthcare). The program used is demonstrated in Table 2.

Table 1. Randomized sample combination for 2D-DiGE. Samples were labeled with the indicated Cy dye and then combined and run on polyacrylamide gels.

Gel #	Cy3	Cy5
1	LacZ I	PTEN II
2	LacZ II	PTEN IV
3	PTEN VII	LacZ IV
4	PTEN I	LacZ VII

Table 2. Isoelectric focusing program. Step 1 is the active rehydration step, and steps 2-6 are IEF steps.

Step	Voltage Mode	Voltage (V)	Time (h)	kVh
1	Step and Hold	50	10	
2	Step and Hold	500		0.5
3	Gradient	1000		0.8
4	Gradient	8000		13.5
5	Step and Hold	8000		25
6	Step and Hold	500	2	

2.4.3 Second Dimension SDS-PAGE

Immediately following completion of the IEF protocol, the IPG strips were removed from the strip holders and equilibrated in two different equilibration buffers (100mM Tris, 6M Urea, 30% Glycerol, 2% SDS, 0.002% Bromophenol blue). First, each strip was equilibrated in 10 mL of Equilibration Buffer + 5 mg/mL DTT for 15 minutes. Next, the strips were equilibrated in Equilibration Buffer + 45 mg/mL iodoacetamide. IPG strips were then loaded into the wells of 12.5% SDS-PAGE gels, and sealed in place using a 1% agarose solution. The four gels were then loaded into the Ettan DALTsix electrophoresis unit. The gels were run overnight at 10W, until the bromophenol blue dye front reached the bottom of the gel. When the gels finished running, they were immediately scanned using the Typhoon Trio+ scanner (GE Healthcare). Scanning voltage for each channel was optimized at low resolution, and then high-resolution scans were obtained of all channels (Cy3, Cy5, and Cy2) for each gel. Immediately following scanning, the gels were removed from their glass plates and placed in fixative solution (40% methanol, 5% acetic acid) overnight. Following fixation, the gels were left in double-distilled water for long-term storage.

2.4.4 Data Analysis

2D gels were analyzed using DeCyder image analysis software (GE Healthcare). The Difference In-gel Analysis (DIA) module was used to detect around 2000 spots on each of the four gels. These spot maps were then imported into the Biological Variation Analysis (BVA) module for further analysis. Using the BVA module, spots from all four gels were matched to one "master gel", and protein spot intensities were quantified and compared across gels and between sample groups. The software calculated the ratio of protein abundance between PTEN and LacZ sample groups for each gel, and then calculated the average. These data were then statistically analyzed by independent student's T-test. Proteins were filtered according to the following criteria:

- Student's T-test p-value less than 0.05
- Average ratio PTEN/LacZ greater than 1.3 or less than -1.3

- Protein must appear on at least 3 of 4 gels

A total of 60 proteins fit these criteria, and were flagged for further analysis. Next, the master gel was silver stained according to standard procedure. The 60 significant spots were cut with a scalpel and temporarily stored in 1.5 mL tubes at -20°C. The protein spots were later analyzed by mass spectrometry.

2.5 Real-Time PCR

RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. DNase treatment and cDNA synthesis were performed with the QuantiTect reverse transcription kit (Qiagen) using 1 μg of RNA as a template. Real-time PCR (RT-PCR) was performed using a Realplex-2 Mastercycler (Eppendorf) and QuantiFast SYBR green master mix (Qiagen) supplemented with 1 μL of cDNA and 0.5 μM final concentration of primers per reaction. Primer sequences are listed in Table 3. Fold change PTEN/LacZ was calculated using the delta-delta Ct method relative to the housekeeping gene glyceraldehyde 3-dehydrogenase (GAPDH).

Table 3. RT-PCR primer sequences.

Gene Studied	Primer Name	Sequence (5'-3')
Insulin-like growth factor binding protein 2	IGFBP2 Fwd	GGAGGAGCCCAAGAAGCTGCG
Insulin-like growth factor binding protein 2	IGFBP2 Rev	CCCGTTCAGAGACATCTTGCACTG
ATPase, H+ transporting, lysosomal accessory protein 2	HT028 Fwd	GACACCTCCCTCATTAGGAAGAC
ATPase, H+ transporting, lysosomal accessory protein 2	HT028 Rev	GATCATTATCCAAAGTACCATGTTG
Calsyntenin 1 (CLSTN1)	CLSTN2 Fwd	GGAACACCGCTCCTTTGTTGACCTG
Calsyntenin 1 (CLSTN1)	CLSTN2 Rev	CCGCATGGTCCGCCGATGTG
Vinculin	VCL2 Fwd	CTACTAGGCAAAATGAGAGGGCAG
Vinculin	VCL2 Rev	GAAGGAAGGATGTTCAGGCAAGG
PTEN	PTENa FWD	CAAGTTCATGTACTTTGAGTTCCC
PTEN	PTENa REV	GCACGCTCTATACTGCAAATGC
Protein tyrosine phosphatase, receptor type, F	LAR Fwd	ACGCTCTGCCAACTACACC
Protein tyrosine phosphatase, receptor type, F	LAR Rev	CACAAGATCAATCGGAGGCTT
Desmoglein 2	DSG2 Fwd	TGATGCTGCTGCTTAGGTGCC
Desmoglein 2	DSG2 Rev	CCTGGGTTGCTTTACTGGACGGC
Chaperonin containing TCP1, subunit 8 (theta)	CCT8 Fwd	AGGAGGGAGCGAAACACTTTT
Chaperonin containing TCP1, subunit 8 (theta)	CCT8 Rev	GCAGGATGCTGTACTTCTAGTTC
Ribonuclease/Angiogenin Inhibitor 1	RNH1 Fwd	CCTGCTGCTGCTCTCCCTGG
Ribonuclease/Angiogenin Inhibitor 1	RNH1 Rev	GGCAGAAATAAGCGGATCTGAGCGT
Spondin 2	SPO2 Fwd	GAGGGCACAGGGGGTTTCGC
Spondin 2	SPO2 Rev	AGAGCAGACGGGACACGGGG
Zn-alpha-2-glycoprotein	ZAG Fwd	ACGACAGTAACGGGTCTCAC
Zn-alpha-2-glycoprotein	ZAG Rev	AGGCTGGGATTTCTTTGTTGAA
Prostate specific antigen precursor	PSA Fwd	TGAACCATGTGCCCTGCCCG
Prostate specific antigen precursor	PSA Rev	AGGGGTGCTCAGGGGTTGGC
Cystatin C	CST3 Fwd	TGCGGCGTGCACTGGACTTT
Cystatin C	CST3 Rev	GCTACGATCTGCTTGCGGGCG
Alpha-1 collagen VI	COL6A1 Fwd	ACACCGACTGCGCTATCAAG
Alpha-1 collagen VI	COL6A1 Rev	CACCGAGAAGACTTTGACGC
Alpha actinin 4	ACTN4 Fwd	TGGAGGTCATATCAGGGGAGC
Alpha actinin 4	ACTN4 Rev	CTTCTGCCCCGATGGAGAC
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH Fwd	AGGGCTGCTTTTAACTCTGGT
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH Rev	CCCCACTTGATTTTGGAGGGA

2.6 Polysome Profiling

LNCaP cells were grown in 15 cm dishes and infected with 100 PFU/cell of Ad-PTEN or Ad-LacZ. 48 hours post-infection, cells were treated with 100 μg/mL cycloheximide for 3 minutes. The cells were then washed twice in cold PBS containing 100 μg/mL cycloheximide and collected by centrifuging at 1200 rpm for 5 minutes at 4°C. Pelleted cells were lysed in hypotonic buffer [5 mM Tris-HCl (pH 7.5), 2.5 mM MgCl2, 1.5 mM KCl, 100 μg/mL cycloheximide, 2 mM DTT, 0.5% Triton X-100, 0.5% sodium deoxycholate, and 200 U/mL RNasin RNase inhibitor (Promega)], and cell debris was removed by centrifuging the lysates at 12 000 rpm for 5 minutes. The RNA concentration of each lysate was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Equal amounts of RNA from each lysate were then loaded onto sucrose gradients containing 10-50% density sucrose [200mM HEPES (pH7.6), 1M KCl, 50 mM MgCl2, 100 μg/mL cycloheximide, 200 U/mL RNasin] in centrifuge tubes (Beckman #331374). The samples were then centrifuged at 37 000 rpm for 2 hours at 4°C in an SW40Ti rotor (Beckman). Following centrifugation, the gradients were fractionated (14 fractions, 750 μL volume) and the absorbance at 254 nm was recorded using an ISCO fractionator (Teledyne Isco). Fractions were stored at -80°C.

2.6.1 RT- PCR of polysome fractions

RNA was isolated from the polysome fractions by adding 750 μL of TRIzol reagent (Invitrogen) to a 500 μL volume of each fraction, and then proceeding according to the manufacturer's protocol. cDNA was prepared for each fraction using 1 μg of RNA as a template. Quantitative RT-PCR was performed as described above. The relative amount of transcript in each fraction was determined by normalizing the Ct values to the first LacZ fraction, which was set at a value of 1. Semi-quantitative real-time PCR (sqRT-PCR) was performed on cDNA obtained from each individual polysome fraction using a Realplex-2 Mastercycler (Eppendorf) and QuantiFast SYBR green master mix (Qiagen). RT-PCR reactions were stopped in the linear range of amplification, and PCR

products were run on agarose gels containing ethidium bromide and imaged under ultraviolet light.

3. RESULTS

3.1 Optimization of conditions for 2D-DiGE

The goal of this project was to use 2D-DiGE in order to identify secreted proteins that are regulated by PTEN. To accomplish this, an experimental system was set up wherein an adenoviral vector was used to re-introduce wild-type PTEN expression in a PTEN-null cell line. After testing several PTEN-null cell lines, LNCaP, a PTEN-null prostate cell line, was chosen. Various conditions were optimized, including adenovirus multiplicity of infection (MOI), collection time point and growth media (Figure 2). First, LNCaP were infected with adenoviruses expressing wild-type PTEN (Ad-PTEN) or the negative control β-galactosidase (Ad-LacZ) at a range of MOIs. Western blotting was used to determine which MOI yielded the highest PTEN expression and activity, as assessed by decreased phospho-Akt (pAkt) levels (Figure 2A). A dose-dependent increase in PTEN levels and concomitant decrease in pAkt levels were observed, up to an MOI of 100 PFU/cell, which was selected as the optimal MOI.

Next, a time course experiment was performed to determine which time point post-infection resulted in the highest PTEN levels and lowest pAkt levels (Figure 2B). At a time point of 48 hours post-infection, PTEN levels were maximal while pAkt levels were minimal. Therefore the final conditions selected for 2D-DiGE used an adenovirus MOI of 100 PFU/cell and collection at 48 hours post-infection.

Finally, a conditioning media was selected. The ultimate goal of these experiments was to separate proteins from the conditioned media on a 2D gel and identify the spots by mass spectrometry. In order to minimize contamination of the 2D gel with bovine proteins, it was important to remove fetal bovine serum (FBS) from the growth media. Insulin transferrin selenium (ITS) is a minimal supplement commonly added to media in the absence of FBS. Because insulin is a major activator of the PI3K signaling pathway, there was concern that adding ITS to the growth media might mask the inhibitory effects of PTEN on the PI3K pathway. To assess this, Ad-PTEN and Ad-LacZ infected cells were conditioned

in RPMI alone, or RPMI containing ITS, and pAkt levels were monitored by Western blotting (Figure 2C). PTEN-expressing cells showed an equal decrease in pAkt levels when conditioned with or without ITS, therefore ITS does not interfere with PTEN's inhibitory effect on the PI3K pathway.

When cell death occurs, intracellular proteins, including actin and tubulin, are released into the growth media. To ensure that intracellular contamination of the media was minimized, extracellular levels of tubulin were examined (Figure 2D). Under the conditions chosen for further experiments (conditioning in RPMI supplemented with ITS and collecting 48 hours post-infection), extracellular tubulin levels were minimial.

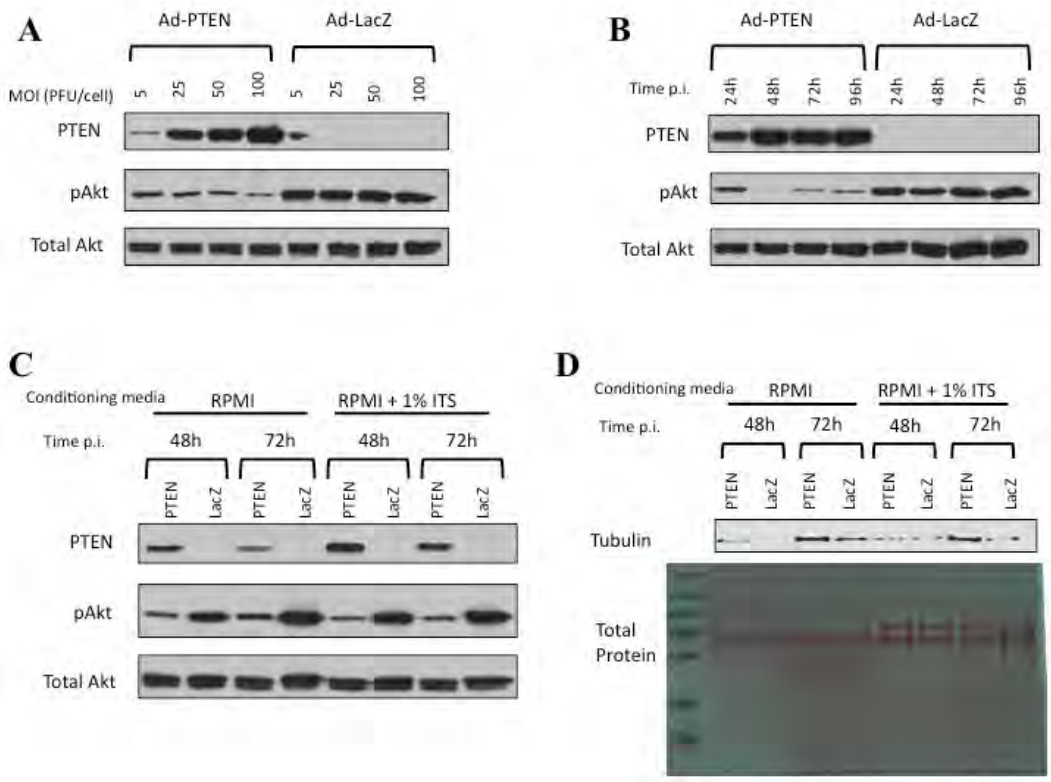

Figure 2. Optimization of 2D-DiGE conditions.

(A) Optimization of adenovirus multiplicity of infection (MOI). LNCaP were infected with Ad-PTEN or Ad-LacZ at MOIs ranging from 5-100 PFU/cell and lysed 48 hours post-infection. PTEN, pAkt and total Akt levels were assessed by autoradiography. **(B)** Optimization of time point. LNCaP were infected with 100 PFU/cell of Ad-PTEN or Ad-LacZ, and lysed 24 – 96 hours post-infection. PTEN, pAkt and total Akt levels were determined by autoradiography. **(C)** Optimization of conditioning media. LNCaP were infected with 100 PFU/cell of Ad-PTEN or Ad-LacZ. 24 hours post-infection, cells were washed with PBS and then conditioned in RPMI or RPMI + 1% ITS for 24 – 48 hours before being lysed. PTEN, pAkt and total Akt levels were visualized by autoradiography. **(D)** Assessment of intracellular protein contamination in the media. LNCaP were treated as in (C). Conditioned media was collected and ethanol-precipitated. Equal amounts of protein were run on a 10% polyacrylamide gel. Extracellular tubulin levels were examined by autoradiography.

3.2 Identification of PTEN-regulated proteins

Now that the conditions were optimized, we were ready to use 2D-DiGE to identify proteins that were differentially expressed in PTEN-expressing and PTEN-null cells. For the 2D-DiGE experiment, conditioned media and lysates were collected from four replicates of LNCaP infected with Ad-PTEN and Ad-LacZ. High PTEN and low pAkt levels were confirmed for all four replicates (Figure 3A). Cell death was also monitored for each replicate, and no significant difference in percentage cell death was observed between LacZ- and PTEN-infected cells ($P > 0.05$) (Figure 3B). Proteins from the conditioned media were incubated with Cy dyes, and then run on four 2D gels (representative image shown in Figure 3C). Two large aggregates, bovine serum albumin and transferrin, were observed in the low pH, high molecular weight region of the gels.

The 2D gels were then analyzed using image analysis software. Spots from all four gels were matched to one "master gel", and protein spot intensities were quantified and compared across gels and between sample groups. The ratio of protein abundance between PTEN and LacZ sample groups was calculated for each gel. The average ratios of protein abundance between PTEN and LacZ were then statistically analyzed by independent student's T-test. Proteins that had a student's T-test P-value less than 0.05, showed at least a 1.3-fold change between PTEN and LacZ, and appeared on at least 3 of the 4 gels were considered significant. A total of 60 proteins spots fit these criteria, and were picked and sent for mass spectrometry analysis. 56 of the 60 significant proteins were down-regulated by PTEN.

33 of the protein spots were identified by mass spectrometry (Figure 4). These corresponded to only 16 different proteins, 4 of which were up-regulated by PTEN and 12 of which were down-regulated by PTEN (Table 4). Three of the identified proteins, Spondin 2 (SPO2) and Zn-alpha-2-glycoprotein (ZAG), and cystatin C (CST3) were confirmed to be down-regulated by PTEN, as measured by Western blotting (Figure 5).

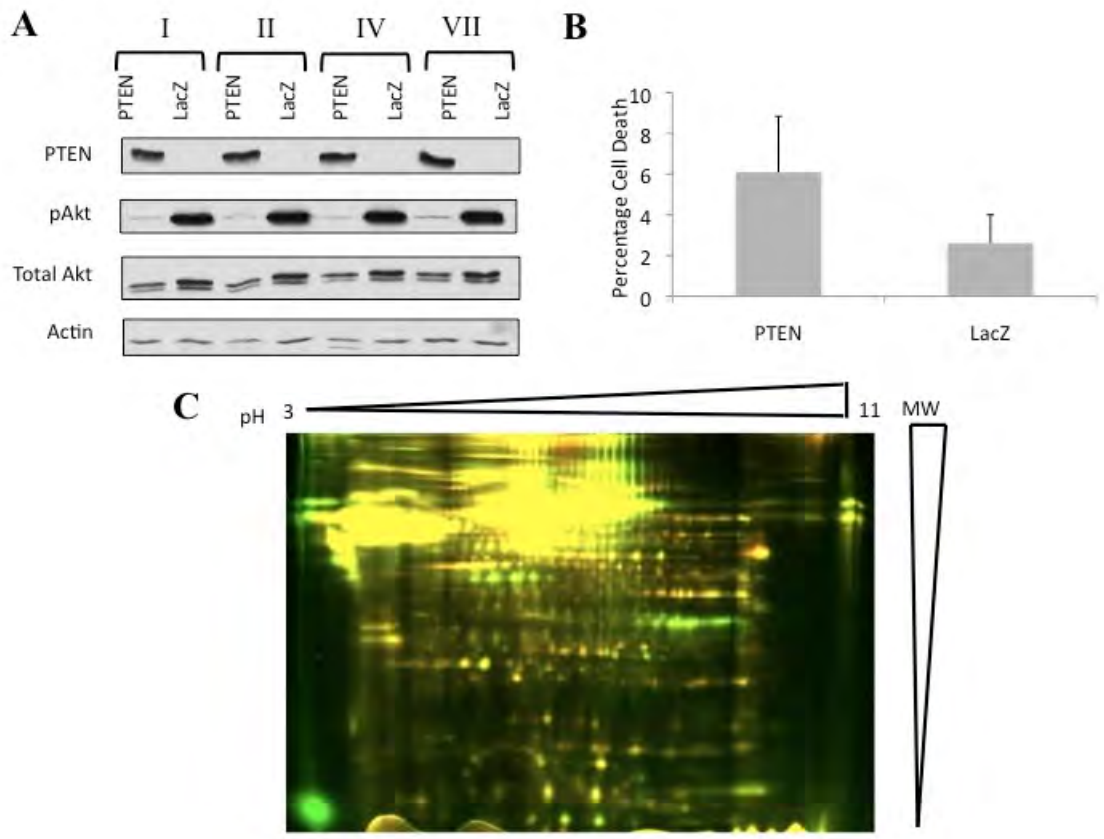

Figure 3. 2D-DiGE results.

(A) Western blots of whole cell lysates collected from 2D-DiGE experiment. LNCaP were infected with 100 PFU/cell of Ad-PTEN or Ad-LacZ. 24 hours post-infection, cells were washed with PBS and then conditioned in RPMI + 1% ITS. After 24 hours of conditioning, cells were lysed. PTEN, pAkt and total Akt levels were assessed in all four replicates by autoradiography. **(B)** Cell death was measured using Trypan blue exclusion. Percentage cell death is the mean of 4 replicates. Cell death was not significantly different between PTEN and LacZ ($P > 0.05$). **(C)** Representative image of a 2D-DiGE gel. Extracellular proteins collected from Ad-LacZ and Ad-PTEN-infected LNCaP were labeled with Cy3 (LacZ; green) and Cy5 (PTEN; red) dyes. Proteins were equilibrated across a 3-11 non-linear immobilized pH gradient, and then separated by SDS-PAGE.

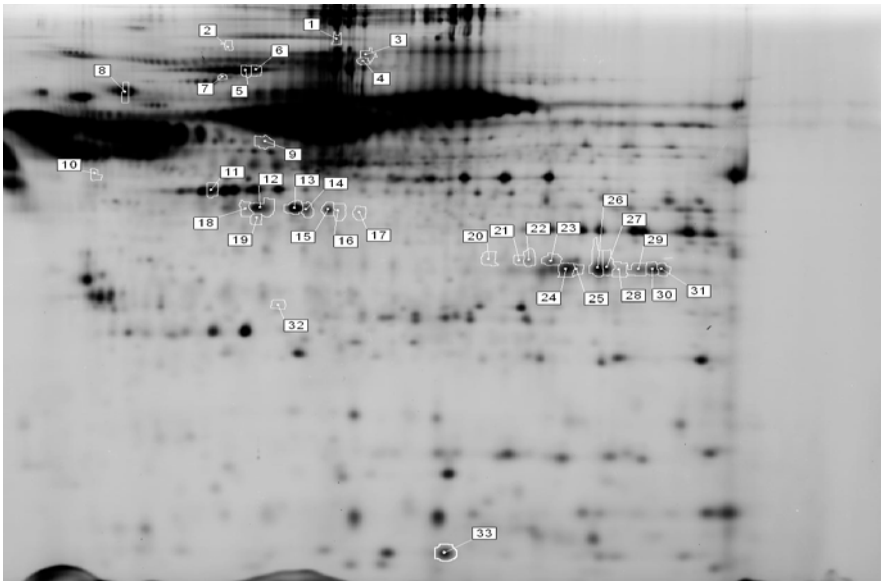

Figure 4. PTEN-regulated proteins identified by mass spectrometry.
The localization of the identified proteins on a representative 2D gel is indicated.

Table 4. PTEN-regulated proteins identified by mass spectrometry
The average ratio of protein abundance between PTEN and LacZ samples for each protein was calculated using DeCyder software. Protein levels were significantly different between PTEN and LacZ for all 16 proteins identified ($P < 0.05$). Spot numbers correspond to Figure 4.

Protein name	Abbreviation	Average ratio PTEN/LacZ	Number of peptides	Spot numbers
Alpha-2-macroglobulin precursor	A2M	-1.35	1	1
Alpha-1 collagen VI	COL6A1	-1.46	6	2
Putative LAR preprotein	LAR	-1.43	6	3
Vinculin isoform VCL	VCL	-1.61	2	4
Calsyntenin 1	CLSTN1	-1.38	27-29	5, 6
Alpha actinin 4	ACTN4	1.37	24	7
Desmoglein 2 preprotein	DSG2	1.43	57	8
Chaperonin containing TCP1, subunit 8 (theta)	CCT8	1.52	3	9
Ribonuclease/angiogenin inhibitor 1	RNH1	-1.45	4	10
Beta-actin	ACTB	1.42	15	11
Spondin 2 precursor	SPO2	-1.76	1-15	12-17
Insulin-like growth factor binding protein 2	IGFBP-2	-1.64	7-17	18, 20-23
Zn-alpha-2-glycoprotein	ZAG	-1.83	3-15	19
Prostate specific antigen precursor	PSA	-2.14	3-17	24-31
ATPase, H+ transporting lysosomal accessory protein 2	HT028	-2.10	5	32
Cystatin C	CST3	-1.48	14	33

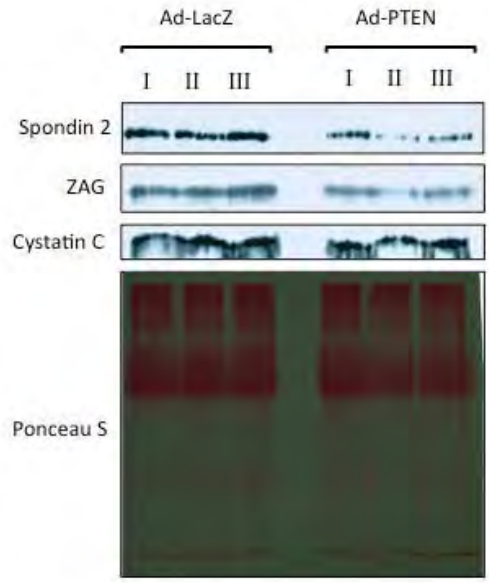

Figure 5. PTEN regulates SPO2, ZAG, and CST3 protein levels.

LNCaP were infected with Ad-PTEN or Ad-LacZ, and conditioned in RPMI + 1% ITS media. 48 hours post-infection, conditioned media was collected and ethanol-precipitated. Equal amounts of protein from 3 replicates were run on polyacrylamide gels. SPO2, ZAG, and CST3 protein levels were assessed by autoradiography.

3.3 Effect of PTEN on transcription of the identified proteins

Since PTEN can affect gene expression at the transcriptional level, we assessed whether PTEN affected the mRNA levels of the identified factors. mRNA levels of 14 of the factors identified by mass spectrometry were measured in LacZ- and PTEN-infected cells by RT-PCR (Figure 6A). Only two of the factors tested, prostate specific antigen (PSA) and SPO2 were significantly down-regulated by PTEN at the mRNA level ($P < 0.05$).

To test whether the lipid phosphatase activity of PTEN mediates the observed repression of PSA and SPO2 transcription, cells were infected with an adenovirus expressing an active site mutant of PTEN. The C124S active site mutation abrogates the lipid phosphatase activity of PTEN. Cells infected with Ad-PTEN-C124S had elevated pAkt levels when compared to cells infected with wild-type PTEN (Ad-PTEN-WT) (Figure 6B). The transcriptional repression of PSA and SPO2 observed in cells expressing PTEN-WT was abolished in cells expressing PTEN-C124S (Figure 6C). These data suggest that the ability of PTEN to antagonize the PI3K signaling pathway is necessary to repress transcription of PSA and SPO2.

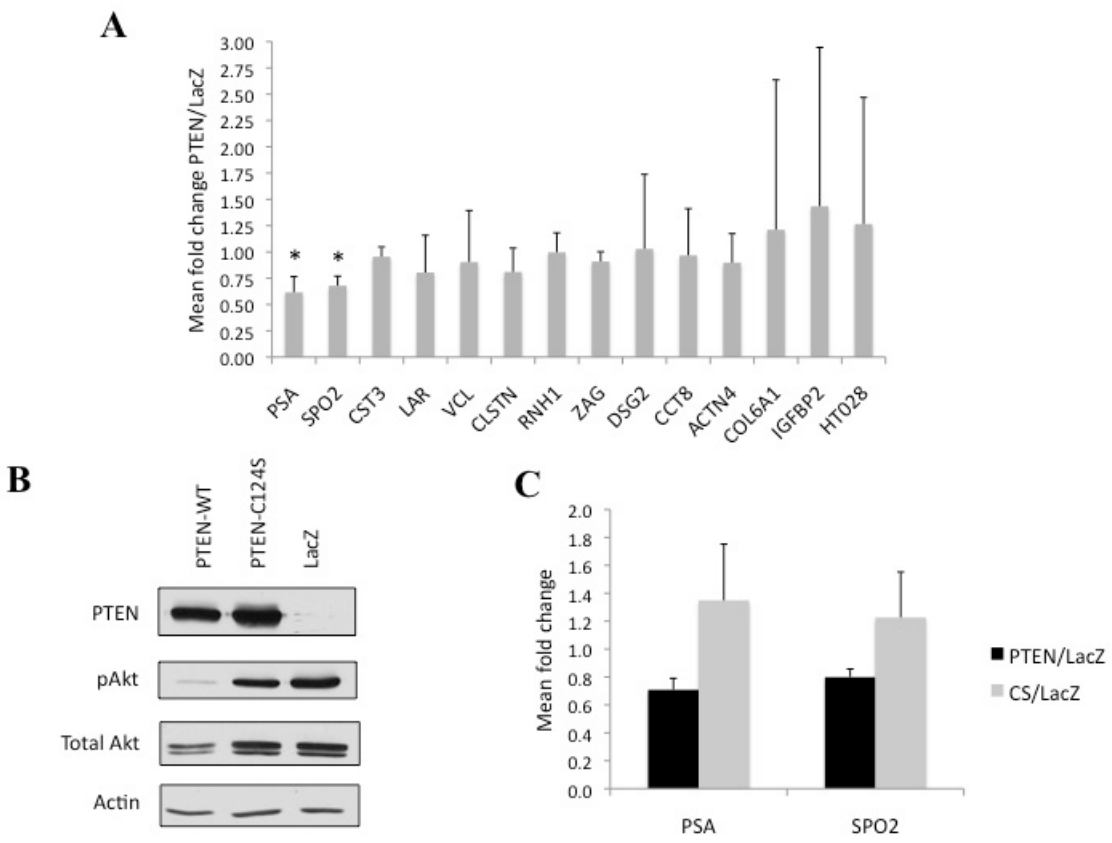

Figure 6. PTEN regulates transcription of PSA and Spondin 2.

(A) RNA was collected from LNCaP 48 hours post-infection with Ad-PTEN or Ad-LacZ. RNA levels of the indicated transcripts were assessed by RT-PCR and normalized to the housekeeping gene GAPDH. The fold change represents the mean of four replicates. * indicates a statistically significant difference between PTEN and LacZ ($P < 0.05$). **(B)** PTEN-C124S mutant is inactive. LNCaP were infected with wild-type Ad-PTEN (Ad-PTEN-WT), an active site PTEN mutant adenovirus (Ad-PTEN-C124S), or Ad-LacZ. Protein levels of PTEN, pAkt, total Akt and actin were assessed by autoradiography. **(C)** LNCaP were infected with Ad-PTEN-WT, Ad-PTEN-C124S, or Ad-LacZ. RNA was collected 48 hours post-infection. RNA levels of PSA and SPO2 were assessed by RT-PCR and normalized to GAPDH. The fold change relative to LacZ represents the mean of three replicates.

3.4 Effect of PTEN on translation of the identified proteins

Since the ability of PTEN to repress transcription accounted for the observed decrease of only two of the 14 factors identified, the remaining factors must be regulated by PTEN through some other mechanism. It is well established that PI3K activates protein translation through signaling downstream of Akt and mTOR. mTOR activates eIF4G and the ribosomal protein S6K, and inactivates the translation inhibitor 4E-BP1, ultimately resulting in increased protein synthesis. Since PTEN inhibits this pathway, we hypothesized that PTEN might regulate some of the identified proteins at the level of translation. Polysome profiling was used to assess translation in LacZ- and PTEN-infected LNCaP cells. Cytoplasmic extracts were equilibrated on sucrose gradients, and then fractions were collected. During fraction collection, the absorbance at 254 nm was measured to determine the localization of RNA across the gradient. RNA peaks corresponding to 40S and 60S ribosomal subunits, 80S monosomes, and polysomes can be visualized in the polysome profile. The peaks closest to the heavy (50% sucrose) region of the gradient correspond to heavily translated mRNAs that are associated with many ribosomes. When translation is inhibited, the monosomal peak is higher and the polysomal peaks are lower.

Polysome profiles of LacZ- and PTEN-infected cells were very similar, suggesting that PTEN does not inhibit global protein translation (Figure 7A). To assess the effect of PTEN on the translation of individual mRNAs, RT-PCR was performed on RNA collected from fractions of the polysome gradient. IGFBP-2, PSA, SPO2, CST3, and HT028 transcripts were shifted toward the monosomal population in PTEN-expressing cells compared to control cells (Figure 7B). This shift was confirmed by semi-quantitative RT-PCR (Figure 7C). These data suggest that PTEN specifically inhibits translation of IGFBP-2, PSA, SPO2, CST3, and HT028 mRNAs without having an appreciable effect on the overall rate of protein synthesis.

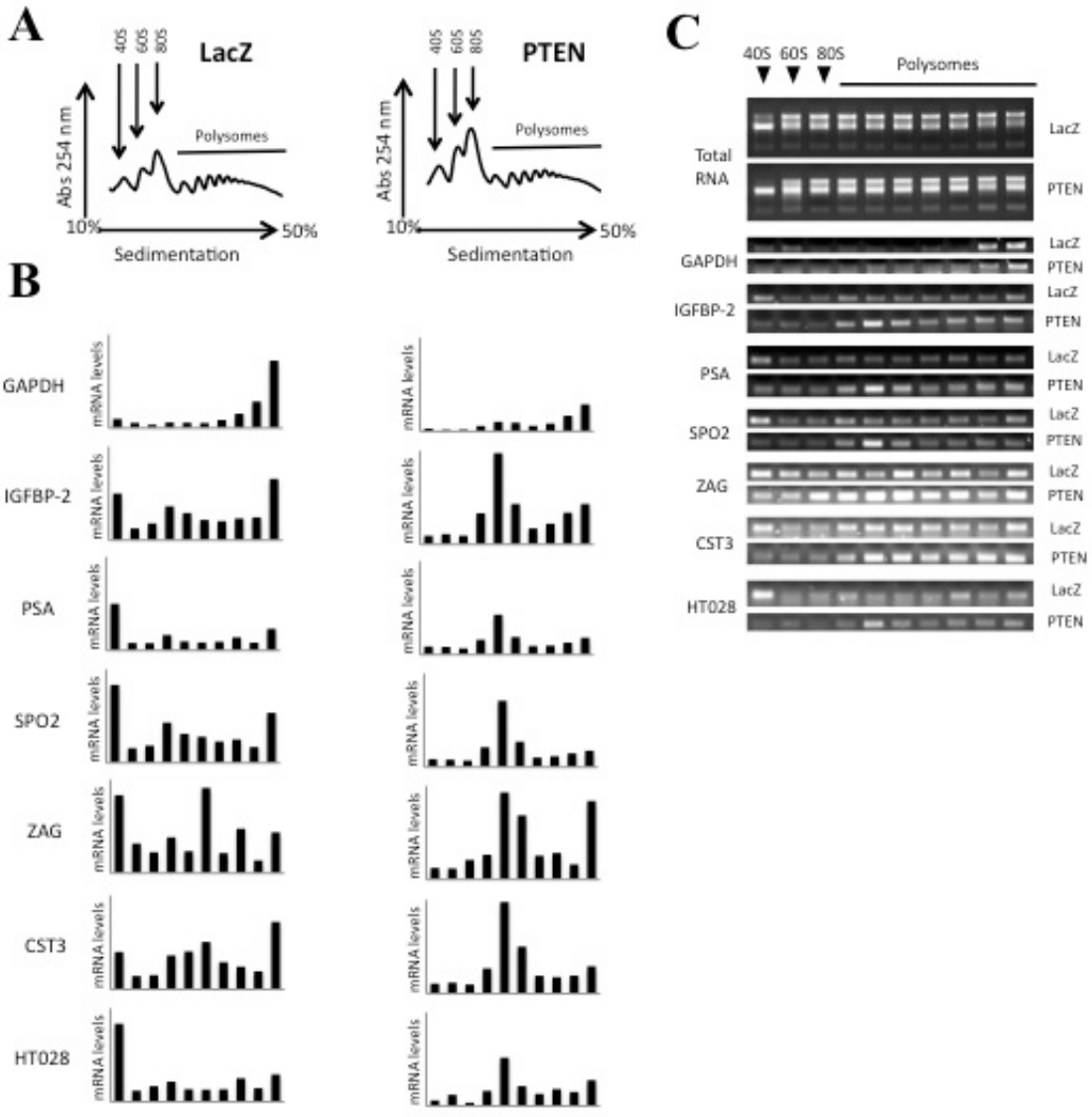
A
40S
60S
80S
LacZ
Abs 254 nm
Polysomes
10%
50%
Sedimentation
PTEN
B
GAPDH
IGFBP-2
PSA
SPO2
ZAG
CST3
HT028
mRNA levels
C
Polysomes
Total RNA
LacZ
PTEN

Figure 7. PTEN regulates translation of IGFBP-2, PSA, SPO2, CST3, and HT028. **(A)** Sucrose gradient absorbance profiles (A254 nm) of cytoplasmic extracts from LNCaP infected with Ad-LacZ and Ad-PTEN. The positions of 40S and 60S ribosomal subunits, 80S monosomes, and polysomes are indicated. **(B)** Quantitative analysis of mRNA levels across the polysome profile. RNA was isolated from each fraction of the polysome profile, and the levels of the indicated transcripts were determined by RT-PCR. Values in each fraction were normalized to the first LacZ fraction, which was set at 1. The data presented are representative of three replicates. **(C)** Semi-quantitative analysis of mRNA levels across the polysome profile. The levels of the indicated transcripts in each fraction of the polysome profile were determined by semi-quantitative RT-PCR. RT-PCR reactions were stopped in the linear range of amplification, and the products were run on agarose gels containing ethidium bromide and imaged under ultraviolet light.

4. DISCUSSION

In this study, we identified 16 proteins that are controlled by PTEN, 4 of which are up-regulated and 12 of which are down-regulated by PTEN. Two of the factors are regulated by PTEN at the level of transcription, and 5 are regulated at the level of protein translation.

4.1. Factors previously shown to be PTEN-regulated

Two of the identified factors, PSA and insulin-like growth factor binding protein-2 (IGFBP-2) have previously been shown to be regulated by PTEN. The fact that we were able to identify these two proteins as differentially regulated between LacZ- and PTEN-infected cells in this study validates the experimental system that we used. As was previously mentioned, PSA is a widely used prostate cancer biomarker. PSA transcription is regulated by PTEN through its inhibition of the androgen receptor (AR) transcription factor[98]. We confirmed that PSA is transcriptionally down-regulated by PTEN, and demonstrated that PTEN also down-regulates PSA at the level of protein translation.

IGFBP-2 is a member of the IGFBP superfamily of proteins, a group of at least 15 proteins found in serum and other biological fluids. IGFBPs function as inhibitors of insulin signaling by binding insulin and insulin-like growth factors, and preventing them from binding to their receptors at the cell membrane[99]. Interestingly, IGFBP-2 has been proposed as a prostate cancer biomarker as serum IGFBP-2 levels are elevated in patients with prostate cancer relative to healthy individuals[100]. Prior to this study, IGFBP-2 protein levels were known to be decreased by PTEN, however the mechanism of this down-regulation remained unknown[101]. In this study, we demonstrated that PTEN controls its expression by decreasing translation of IGFBP-2 mRNA.

4.2 Intracellular proteins and other artifacts

Although the goal of this study was to identify secreted proteins that are regulated by PTEN, some of the identified factors are intracellular proteins. Such

factors include vinculin (VCL), alpha actinin 4 (ACTN4), chaperonin containing TCP1, subunit 8 (CCT8), ribonuclease/angiogenin inhibitor 1 (RNH1), and β-actin (ACTB). Some of the other proteins identified in this study are transmembrane proteins, including putative LAR preprotein (LAR), calsyntenin 1 (CLSTN1), and desmoglein 2 (DSG2). Because one of the rationales for this project was to identify potential PTEN-regulated prostate cancer serum biomarkers, we chose to focus only on the extracellular proteins for subsequent analysis.

Alpha-2-macroglobulin (A2M) is an extracellular glycoprotein that inactivates various proteases, including plasmin, urokinase-type plasminogen activator, and matrix metalloproteases[102]. Because only one A2M peptide was identified by mass spectrometry in this study, we cannot be confident that it is truly a PTEN-regulated protein. Moreover, we were unable to detect A2M mRNA in LNCaP cells by RT-PCR, suggesting that A2M is either poorly expressed or not expressed at all in this cell line. A2M is an abundant protein in bovine serum, so this may explain the source of the A2M peptide that was detected by mass spectrometry[103].

Alpha-1 collagen VI (COL6A1) is a collagen isoform present in the ECM. COL6A1 is over-expressed in patients with astrocytoma and glioblastoma[104]. COL6A1 was also identified as a protein that is highly expressed in six different cancer cell lines[105]. Moreover, treatment of LNCaP cells with the anti-cancer agent Se-methylselenocysteine decreased COL6A1 expression[106]. In this study, COL6A1 was identified as a factor that is down-regulated by PTEN. However, because COL6A1 mRNA levels and translational profiles did not appear to change appreciably between PTEN-positive and PTEN-null conditions, we chose not to conduct follow-up experiments on COL6A1 at this point.

4.3 Cancer biomarkers regulated by PTEN

Three of the PTEN-regulated factors identified in this study, SPO2, ZAG, and CST3, are bona fide secreted proteins that are known to be up-regulated in cancer. Spondin 2 (SPO2), also known as DIL-1 and mindin, is a member of the

mindin-F-spondin family of ECM proteins. Like all other classically secreted proteins, SPO2 contains an N-terminal signal sequence[107]. SPO2 is implicated in immunological functions including microbial pathogen recognition and inflammatory cell recruitment[108,109]. It is well known that SPO2 is up-regulated in cancer, however, very little is known about its function in tumorigenesis. SPO2 could be a promising new biomarker for various cancers, as SPO2 levels are increased in the serum of patients with ovarian and prostate cancer compared to healthy individuals[110,111]. In this study, SPO2 was shown to be down-regulated by PTEN both at the level of mRNA transcription and protein translation. This data corroborates with these previous studies, and suggests that the down-regulation of SPO2 may be a newly defined tumour suppressive function of PTEN.

Zn-alpha-2-glycoprotein (ZAG) is an extracellular protein that is secreted by prostate epithelial cells. ZAG has many functions, including lipolysis, which mediates its ability to promote cancer cachexia[112,113]. Several studies show that ZAG levels are elevated in the serum of prostate cancer patients relative to healthy individuals or men with non-malignant prostate disease[114,115]. ZAG expression is also increased in breast[116,117] and cervical[118] cancers. The data from this study indicate that ZAG expression is down-regulated by PTEN in prostate cancer cells. Together with the published data indicating that ZAG is commonly overexpressed in cancer, these results suggest that ZAG may have tumour-promoting activity, and that PTEN blocks tumour progression by inhibiting its synthesis.

Cystatin C is a cysteine protease inhibitor that is found in almost all bodily fluids. Cystatin C specifically inhibits the cysteine protease cathepsin B by forming a high-affinity enzyme-inhibitor complex[119]. Cathepsin B promotes tumour metastasis by helping to degrade the ECM, thereby enabling circulating tumour cells to exit the bloodstream and invade new tissues. Due to its ability to inhibit this pro-metastatic protease, cystatin C has been implicated as an inhibitor of invasion and metastasis[120-122]. On the other hand, many clinical studies suggest that cystatin C is highly expressed in human cancer. Elevated cystatin C

levels have been reported in serum and other bodily fluids collected from patients with various cancers, including ovarian[123], melanoma[124], colorectal[125], lung[126], head and neck[127], breast[128], and Non-Hodgkin B-cell lymphoma[129]. Moreover, three different proteomics studies have identified cystatin C as an abundant protein in the conditioned media of breast cancer cell lines[130-132]. Cystatin C expression is controlled by transforming growth factor beta (TGF-β), a protein that is known to have both tumour-promoting and tumour-suppressive effects[133]. Altogether, these studies suggest that cystatin C might act as both an oncogene and a tumour suppressor, like its regulator TGF-β. Interestingly, cystatin C has previously been linked to the PI3K signaling pathway, as a factor whose expression is decreased in cells that are null for PDK1[134]. In this study, we determined that cystatin C is down-regulated by PTEN. Thus deletion of PDK1 and overexpression of PTEN both cause a decrease in cystatin C protein levels, suggesting that PTEN controls cystatin C expression through its antagonistic effects on the PI3K pathway.

4.4 Down-regulation of a potential pro-angiogenic protein by PTEN?

ATPase, H+ transporting lysosomal accessory protein 2 (HT028) is a transmembrane protein that functions as the cell-surface receptor for renin. The renin-angiotensin system (RAS) is a key regulator of blood pressure. Renin, the rate-limiting enzyme in the RAS, is synthesized as an inactive precursor called prorenin. Maturation of prorenin into renin occurs in the juxtaglomerular cells of the kidney. Once secreted into the bloodstream, renin acts as an aspartyl protease whose only known substrate is angiotensinogen. Renin cleaves angiotensinogen to form angiotensin I (Ang I), which is later converted into angiotensin II (Ang II) by angiotensin-converting enzyme (ACE). Ang II binds to its receptor on vascular smooth muscle cells to induce vasoconstriction[135]. Chronic activation of the RAS and the resulting increase in Ang II levels contribute to cardiovascular diseases including hypertension. Recently, Nguyen and colleagues demonstrated that HT028 is the cell-surface receptor for renin, and that binding of renin to HT028 greatly increases the rate of conversion of angiotensinogen to Ang I[136].

In addition to its role in regulating blood pressure, the RAS has also been implicated as an important regulator of tumour angiogenesis. Several enzymes that are responsible for the downstream cleavage of Ang II to produce smaller, active proteins, have pro-angiogenic activity and are up-regulated in human cancer (reviewed in [137]). Furthermore, Ang II is known to stabilize HIF-1α, leading to increased VEGF production[138]. Moreover, treatment of patients with ACE inhibitors has been shown to decrease the incidence of cancer[137]. In this study, PTEN was found to down-regulate the renin receptor, HT028, at the level of protein translation. By blocking HT028 synthesis, PTEN could decrease the efficiency of cleavage of angiotensinogen into its downstream cleavage products, thereby inhibiting tumour angiogenesis.

4.5 Transcriptional effects of PTEN

Only 2 of the 16 proteins examined in this study were transcriptionally regulated by PTEN. In a previous proteomics study of PTEN-expressing and PTEN-null Jurkat T cells, only 1 of the 22 identified proteins was regulated by PTEN at the mRNA level[139]. Moreover, in a comparative study of cells expressing wild-type or mutant Akt, the effect of Akt on protein translation was significantly greater than its effect on transcription[140]. Thus it is not surprising that PTEN had a minimal effect on transcription of the factors identified in this study.

So far, PTEN has never been shown to directly affect mRNA transcription. However, PTEN is known to indirectly regulate transcription through modulation of various transcription factors, including p53, NF-κB, the forkhead (FOXO) family, AR, and HIF-1α[141]. In this study, we confirmed that PSA and SPO2 are transcriptionally down-regulated by PTEN. PSA is already known to be AR-responsive[98], however little is known about SPO2. Further experiments could be conducted to determine if PTEN regulates SPO2 transcription through its effects on one of the above-mentioned transcription factors.

4.6 Translational effects of PTEN

In this study, we showed that PTEN does not have an appreciable effect on the overall rate of protein synthesis, but it affects the rate of translation of five specific mRNAs: IGFBP-2, PSA, SPO2, CST3, and HT028.

The rate of protein synthesis is controlled by the rate at which the ribosome binds to the mRNA. Various eukaryotic initiation factors (eIFs) help to guide the ribosome to the 5' end of the vast majority of mRNAs, which contain a 7-methylguanosine (m^7G) cap. The eIF4F complex is composed of the cap-binding protein eIF4E, the scaffolding protein eIF4G, and the RNA helicase eIF4A. eIF4E recognizes and binds the m^7G cap, and then eIF4A begins to unwind the mRNA to facilitate binding of the 40S ribosomal subunit. Another factor, eIF4B, promotes the helicase activity of eIF4A, and is necessary for efficient unwinding of the mRNA[19]. mTOR controls the rate of protein synthesis by controlling the phosphorylation of three key proteins: eIF4B, eIF4G, and the 4E-binding proteins (4E-BPs). mTOR phosphorylates the ribosomal protein S6K, which in turn phosphorylates and activates eIF4B. mTOR directly phosphorylates and activates the scaffolding protein eIF4G. Phosphorylation of eIF4G by mTOR is thought to trigger a conformational change in the scaffolding protein that increases its affinity for its binding partners. In their hypophosphorylated form, the 4E-BPs competitively bind eIF4E and prevent it from binding eIF4G. However, upon phosphorylation by mTOR, the 4E-BPs lose their affinity for eIF4E, thus allowing eIF4E to bind to eIF4G and eIF4A to form the eIF4F complex and activate cap-dependent protein synthesis[19]. PTEN inhibits cap-dependent protein translation by preventing the activation of mTOR and its downstream signaling factors.

PTEN/PI3K signaling affects the rate of translation of a group of "eIF4E-sensitive" mRNAs, including VEGF and FGF, which function to control cell growth and proliferation[19]. Not all m^7G-containing mRNAs are considered eIF4E-sensitive as some mRNAs maintain a high rate of translation, even in the absence of eIF4E. These constitutively translated mRNAs include housekeeping genes such as GAPDH and β-actin[142]. Therefore although we showed that PTEN

does not affect the overall rate of protein synthesis, the ability of PTEN to specifically affect the rate of translation of IGFBP-2, PSA, SPO2, CST3, and HT028 might help to inhibit cell growth and proliferation.

IGFBP-2 is known to be translationally regulated in response to androgen treatment of LNCaP cells[143], however, ours is the first study to show that PTEN decreases IGFBP-2 translation. It is well known that PTEN decreases PSA mRNA levels, and that this results in decreased protein levels of PSA. However, ours is the first study to directly show that PTEN regulates PSA by affecting its rate of protein synthesis. This is the first study to show that PTEN decreases translation of SPO2 and HT028. In this study, we showed that CST3 translation is decreased by PTEN, which confirms the results of an earlier study by Tominaga and colleagues. Translation of CST3 was decreased in PDK1-null mouse embryonic stem cells relative to wild-type cells[134]. Thus deletion of PDK1 and overexpression of PTEN have a similar effect on the translation of CST3.

4.7 Future directions

Future experiments will aim to define the role of spondin 2, Zn-alpha-2-glycoprotein, and cystatin C in angiogenesis and tumorigenesis. First, SPO2, ZAG, and CST3 cDNAs will be cloned into mammalian expression vectors. These genes will be overexpressed in mammalian cells, and their effect on cell growth, migration and invasion will be monitored. We will also test whether these factors promote angiogenesis by growing endothelial cells in conditioned media from cells overexpressing SPO2, ZAG, or CST3. Increased endothelial cell proliferation will indicate a pro-angiogenic effect. Moreover, we will inject nude mice with cells overexpressing SPO2, ZAG, and CST3 to determine whether these factors accelerate tumour growth *in vivo*. To further clarify the effects of these factors on tumorigenesis, SPO2, ZAG, and CST3 will also be expressed in bacteria. The resulting recombinant proteins will then be injected into mice, and tumour growth will be monitored.

Finally, a tumour bank containing tissue from patients with varying stages of prostate cancer will be analyzed. Immunohistochemistry for SPO2, ZAG, and

CST3 will determine whether these factors are overexpressed in human prostate cancer. Furthermore, tumours can be probed for PTEN and pAkt staining in order to ascertain whether high SPO2, ZAG, and CST3 levels correlate with loss of PTEN in patient samples. This study will provide further evidence that PTEN regulates these factors, and will highlight the possibility of monitoring blood levels of SPO2, ZAG, and CST3 to diagnose prostate cancer.

5. REFERENCES

1. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
2. Carmeliet, P. & Jain, R.K. Angiogenesis in cancer and other diseases. *Nature* **407**, 249-257 (2000).
3. Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-364 (1996).
4. Bergers, G. & Benjamin, L.E. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* **3**, 401-410 (2003).
5. Folkman, J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* **285**, 1182-1186 (1971).
6. Ribatti, D., Nico, B., Crivellato, E., Roccaro, A.M. & Vacca, A. The history of the angiogenic switch concept. *Leukemia* **21**, 44-52 (2007).
7. Hall, K. & Ran, S. Regulation of tumor angiogenesis by the local environment. *Front Biosci* **15**, 195-212 (2010).
8. Nyberg, P., Salo, T. & Kalluri, R. Tumor microenvironment and angiogenesis. *Front Biosci* **13**, 6537-6553 (2008).
9. Folkman, J. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov* **6**, 273-286 (2007).
10. Carmeliet, P. VEGF as a key mediator of angiogenesis in cancer. *Oncology* **69 Suppl 3**, 4-10 (2005).
11. Rankin, E.B. & Giaccia, A.J. The role of hypoxia-inducible factors in tumorigenesis. *Cell Death Differ* **15**, 678-685 (2008).
12. Shim, W.S., Ho, I.A. & Wong, P.E. Angiopoietin: a TIE(d) balance in tumor angiogenesis. *Mol Cancer Res* **5**, 655-665 (2007).
13. Good, D.J.*, et al.* A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc Natl Acad Sci U S A* **87**, 6624-6628 (1990).
14. Nyberg, P., Xie, L. & Kalluri, R. Endogenous inhibitors of angiogenesis. *Cancer Res* **65**, 3967-3979 (2005).
15. Assadian, S. & Teodoro, J.G. Regulation of collagen-derived antiangiogenic factors by p53. *Expert Opin Biol Ther* **8**, 941-950 (2008).
16. Carracedo, A. & Pandolfi, P.P. The PTEN-PI3K pathway: of feedbacks and cross-talks. *Oncogene* **27**, 5527-5541 (2008).
17. Engelman, J.A., Luo, J. & Cantley, L.C. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* **7**, 606-619 (2006).
18. Cully, M., You, H., Levine, A.J. & Mak, T.W. Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat Rev Cancer* **6**, 184-192 (2006).
19. Hay, N. & Sonenberg, N. Upstream and downstream of mTOR. *Genes Dev* **18**, 1926-1945 (2004).

20. Manning, B.D. & Cantley, L.C. AKT/PKB signaling: navigating downstream. *Cell* **129**, 1261-1274 (2007).
21. Li, J.*, et al.* PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**, 1943-1947 (1997).
22. Steck, P.A.*, et al.* Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* **15**, 356-362 (1997).
23. Maehama, T. & Dixon, J.E. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **273**, 13375-13378 (1998).
24. Myers, M.P.*, et al.* The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proc Natl Acad Sci U S A* **95**, 13513-13518 (1998).
25. Stambolic, V.*, et al.* Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**, 29-39 (1998).
26. Stiles, B.L. Phosphatase and tensin homologue deleted on chromosome 10: extending its PTENtacles. *Int J Biochem Cell Biol* **41**, 757-761 (2009).
27. Keniry, M. & Parsons, R. The role of PTEN signaling perturbations in cancer and in targeted therapy. *Oncogene* **27**, 5477-5485 (2008).
28. Tamguney, T. & Stokoe, D. New insights into PTEN. *J Cell Sci* **120**, 4071-4079 (2007).
29. Leslie, N.R., Gray, A., Pass, I., Orchiston, E.A. & Downes, C.P. Analysis of the cellular functions of PTEN using catalytic domain and C-terminal mutations: differential effects of C-terminal deletion on signalling pathways downstream of phosphoinositide 3-kinase. *Biochem J* **346 Pt 3**, 827-833 (2000).
30. Eng, C. PTEN: one gene, many syndromes. *Hum Mutat* **22**, 183-198 (2003).
31. Liaw, D.*, et al.* Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat Genet* **16**, 64-67 (1997).
32. Marsh, D.J.*, et al.* Germline mutations in PTEN are present in Bannayan-Zonana syndrome. *Nat Genet* **16**, 333-334 (1997).
33. Di Cristofano, A., Pesce, B., Cordon-Cardo, C. & Pandolfi, P.P. Pten is essential for embryonic development and tumour suppression. *Nat Genet* **19**, 348-355 (1998).
34. Podsypanina, K.*, et al.* Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *Proc Natl Acad Sci U S A* **96**, 1563-1568 (1999).
35. Stambolic, V.*, et al.* High incidence of breast and endometrial neoplasia resembling human Cowden syndrome in pten+/- mice. *Cancer Res* **60**, 3605-3611 (2000).
36. Trotman, L.C.*, et al.* Pten dose dictates cancer progression in the prostate. *PLoS Biol* **1**, E59 (2003).
37. Wang, S.*, et al.* Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. *Cancer Cell* **4**, 209-221 (2003).

38. Stambolic, V.*, et al.* Regulation of PTEN transcription by p53. *Mol Cell* **8**, 317-325 (2001).
39. Vazquez, F.*, et al.* Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. *J Biol Chem* **276**, 48627-48630 (2001).
40. Wang, X.*, et al.* NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN. *Cell* **128**, 129-139 (2007).
41. Trotman, L.C.*, et al.* Ubiquitination regulates PTEN nuclear import and tumor suppression. *Cell* **128**, 141-156 (2007).
42. Tamura, M.*, et al.* Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science* **280**, 1614-1617 (1998).
43. Weng, L.P., Brown, J.L. & Eng, C. PTEN coordinates G(1) arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model. *Hum Mol Genet* **10**, 599-604 (2001).
44. Weng, L.P., Brown, J.L., Baker, K.M., Ostrowski, M.C. & Eng, C. PTEN blocks insulin-mediated ETS-2 phosphorylation through MAP kinase, independently of the phosphoinositide 3-kinase pathway. *Hum Mol Genet* **11**, 1687-1696 (2002).
45. Chung, J.H. & Eng, C. Nuclear-cytoplasmic partitioning of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) differentially regulates the cell cycle and apoptosis. *Cancer Res* **65**, 8096-8100 (2005).
46. Giri, D. & Ittmann, M. Inactivation of the PTEN tumor suppressor gene is associated with increased angiogenesis in clinically localized prostate carcinoma. *Hum Pathol* **30**, 419-424 (1999).
47. Jiang, B.H., Zheng, J.Z., Aoki, M. & Vogt, P.K. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc Natl Acad Sci U S A* **97**, 1749-1753 (2000).
48. Zundel, W.*, et al.* Loss of PTEN facilitates HIF-1-mediated gene expression. *Genes Dev* **14**, 391-396 (2000).
49. Jiang, B.H.*, et al.* Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. *Cell Growth Differ* **12**, 363-369 (2001).
50. Zhong, H.*, et al.* Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res* **60**, 1541-1545 (2000).
51. Xia, C., Meng, Q., Cao, Z., Shi, X. & Jiang, B.H. Regulation of angiogenesis and tumor growth by p110 alpha and AKT1 via VEGF expression. *J Cell Physiol* **209**, 56-66 (2006).
52. Fang, J., Ding, M., Yang, L., Liu, L.Z. & Jiang, B.H. PI3K/PTEN/AKT signaling regulates prostate tumor angiogenesis. *Cell Signal* **19**, 2487-2497 (2007).
53. Wen, S.*, et al.* PTEN controls tumor-induced angiogenesis. *Proc Natl Acad Sci U S A* **98**, 4622-4627 (2001).

54. Soula-Rothhut, M., *et al.* The tumor suppressor PTEN inhibits EGF-induced TSP-1 and TIMP-1 expression in FTC-133 thyroid carcinoma cells. *Exp Cell Res* **304**, 187-201 (2005).
55. Niu, Q., *et al.* Inhibition of Tie-2 signaling induces endothelial cell apoptosis, decreases Akt signaling, and induces endothelial cell expression of the endogenous anti-angiogenic molecule, thrombospondin-1. *Cancer Biol Ther* **3**, 402-405 (2004).
56. Watnick, R.S., Cheng, Y.N., Rangarajan, A., Ince, T.A. & Weinberg, R.A. Ras modulates Myc activity to repress thrombospondin-1 expression and increase tumor angiogenesis. *Cancer Cell* **3**, 219-231 (2003).
57. Chen, J., *et al.* Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. *Nat Med* **11**, 1188-1196 (2005).
58. Eitel, J.A., *et al.* PTEN and p53 are required for hypoxia induced expression of maspin in glioblastoma cells. *Cell Cycle* **8**, 896-901 (2009).
59. Mayo, L.D. & Donner, D.B. The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. *Trends Biochem Sci* **27**, 462-467 (2002).
60. Mikelis, C. & Papadimitriou, E. Heparin-binding protein pleiotrophin: an important player in the angiogenic process. *Connect Tissue Res* **49**, 149-152 (2008).
61. Li, G., *et al.* PTEN deletion leads to up-regulation of a secreted growth factor pleiotrophin. *J Biol Chem* **281**, 10663-10668 (2006).
62. Rangaswami, H., Bulbule, A. & Kundu, G.C. Osteopontin: role in cell signaling and cancer progression. *Trends Cell Biol* **16**, 79-87 (2006).
63. Shao, J., Washington, M.K., Saxena, R. & Sheng, H. Heterozygous disruption of the PTEN promotes intestinal neoplasia in APCmin/+ mouse: roles of osteopontin. *Carcinogenesis* **28**, 2476-2483 (2007).
64. Packer, L., *et al.* Osteopontin is a downstream effector of the PI3-kinase pathway in melanomas that is inversely correlated with functional PTEN. *Carcinogenesis* **27**, 1778-1786 (2006).
65. Nikitenko, L.L., Fox, S.B., Kehoe, S., Rees, M.C. & Bicknell, R. Adrenomedullin and tumour angiogenesis. *Br J Cancer* **94**, 1-7 (2006).
66. Betchen, S.A., Musatov, S., Roberts, J., Pena, J. & Kaplitt, M.G. PTEN inhibits adrenomedullin expression and function in brain tumor cells. *J Neurooncol* **79**, 117-123 (2006).
67. Matsushima-Nishiu, M., *et al.* Growth and gene expression profile analyses of endometrial cancer cells expressing exogenous PTEN. *Cancer Res* **61**, 3741-3749 (2001).
68. Jiang, B.H. & Liu, L.Z. PI3K/PTEN signaling in angiogenesis and tumorigenesis. *Adv Cancer Res* **102**, 19-65 (2009).
69. Huang, J. & Kontos, C.D. PTEN modulates vascular endothelial growth factor-mediated signaling and angiogenic effects. *J Biol Chem* **277**, 10760-10766 (2002).
70. Phung, T.L., *et al.* Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. *Cancer Cell* **10**, 159-170 (2006).
71. Guba, M., *et al.* Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat Med* **8**, 128-135 (2002).

72. Butzal, M.*, et al.* Rapamycin inhibits proliferation and differentiation of human endothelial progenitor cells in vitro. *Exp Cell Res* **300**, 65-71 (2004).
73. Hamada, K.*, et al.* The PTEN/PI3K pathway governs normal vascular development and tumor angiogenesis. *Genes Dev* **19**, 2054-2065 (2005).
74. Yuan, T.L.*, et al.* Class 1A PI3K regulates vessel integrity during development and tumorigenesis. *Proc Natl Acad Sci U S A* **105**, 9739-9744 (2008).
75. Jeet, V., Russell, P.J. & Khatri, A. Modeling prostate cancer: a perspective on transgenic mouse models. *Cancer Metastasis Rev* **29**, 123-142 (2010).
76. Lee, J.T.*, et al.* Targeting prostate cancer based on signal transduction and cell cycle pathways. *Cell Cycle* **7**, 1745-1762 (2008).
77. Harris, K.A. & Reese, D.M. Treatment options in hormone-refractory prostate cancer: current and future approaches. *Drugs* **61**, 2177-2192 (2001).
78. Lilja, H., Ulmert, D. & Vickers, A.J. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. *Nat Rev Cancer* **8**, 268-278 (2008).
79. Fiorentino, M., Capizzi, E. & Loda, M. Blood and tissue biomarkers in prostate cancer: state of the art. *Urol Clin North Am* **37**, 131-141, Table of Contents (2010).
80. Zhao, H., Dupont, J., Yakar, S., Karas, M. & LeRoith, D. PTEN inhibits cell proliferation and induces apoptosis by downregulating cell surface IGF-IR expression in prostate cancer cells. *Oncogene* **23**, 786-794 (2004).
81. Pesche, S.*, et al.* PTEN/MMAC1/TEP1 involvement in primary prostate cancers. *Oncogene* **16**, 2879-2883 (1998).
82. Kreisberg, J.I.*, et al.* Phosphorylation of Akt (Ser473) is an excellent predictor of poor clinical outcome in prostate cancer. *Cancer Res* **64**, 5232-5236 (2004).
83. Uzoh, C.C.*, et al.* PTEN-mediated pathways and their association with treatment-resistant prostate cancer. *BJU Int* **104**, 556-561 (2009).
84. Bedolla, R.*, et al.* Determining risk of biochemical recurrence in prostate cancer by immunohistochemical detection of PTEN expression and Akt activation. *Clin Cancer Res* **13**, 3860-3867 (2007).
85. Volmer, M.W.*, et al.* Differential proteome analysis of conditioned media to detect Smad4 regulated secreted biomarkers in colon cancer. *Proteomics* **5**, 2587-2601 (2005).
86. Xue, H., Lu, B. & Lai, M. The cancer secretome: a reservoir of biomarkers. *J Transl Med* **6**, 52 (2008).
87. Hegde, R.S. & Bernstein, H.D. The surprising complexity of signal sequences. *Trends Biochem Sci* **31**, 563-571 (2006).
88. Lee, M.C., Miller, E.A., Goldberg, J., Orci, L. & Schekman, R. Bi-directional protein transport between the ER and Golgi. *Annu Rev Cell Dev Biol* **20**, 87-123 (2004).
89. Marie, M., Sannerud, R., Avsnes Dale, H. & Saraste, J. Take the 'A' train: on fast tracks to the cell surface. *Cell Mol Life Sci* **65**, 2859-2874 (2008).

90. Nickel, W. The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur J Biochem* **270**, 2109-2119 (2003).
91. Simpson, R.J., Lim, J.W., Moritz, R.L. & Mathivanan, S. Exosomes: proteomic insights and diagnostic potential. *Expert Rev Proteomics* **6**, 267-283 (2009).
92. O'Farrell, P.H. High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250**, 4007-4021 (1975).
93. Minden, J. Comparative proteomics and difference gel electrophoresis. *Biotechniques* **43**, 739, 741, 743 passim (2007).
94. Timms, J.F. & Cramer, R. Difference gel electrophoresis. *Proteomics* **8**, 4886-4897 (2008).
95. Unlu, M., Morgan, M.E. & Minden, J.S. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* **18**, 2071-2077 (1997).
96. Bacchetti, S. & Graham, F.L. Inhibition of cell proliferation by an adenovirus vector expressing the human wild type p53 protein. *Int. J. Oncology* **3**, 781-788 (1993).
97. Wessel, D. & Flugge, U.I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem* **138**, 141-143 (1984).
98. Nan, B.*, et al.* The PTEN tumor suppressor is a negative modulator of androgen receptor transcriptional activity. *J Mol Endocrinol* **31**, 169-183 (2003).
99. Ruan, W. & Lai, M. Insulin-like growth factor binding protein: a possible marker for the metabolic syndrome? *Acta Diabetol* **47**, 5-14 (2010).
100. Shariat, S.F.*, et al.* Association of preoperative plasma levels of insulin-like growth factor I and insulin-like growth factor binding proteins-2 and -3 with prostate cancer invasion, progression, and metastasis. *J Clin Oncol* **20**, 833-841 (2002).
101. Levitt, R.J., Georgescu, M.M. & Pollak, M. PTEN-induction in U251 glioma cells decreases the expression of insulin-like growth factor binding protein-2. *Biochem Biophys Res Commun* **336**, 1056-1061 (2005).
102. Lindner, I.*, et al.* Alpha2-macroglobulin inhibits the malignant properties of astrocytoma cells by impeding beta-catenin signaling. *Cancer Res* **70**, 277-287 (2010).
103. Ma, H., Li, R., Zhang, Z. & Tong, T. mRNA level of alpha-2-macroglobulin as an aging biomarker of human fibroblasts in culture. *Exp Gerontol* **39**, 415-421 (2004).
104. Fujita, A.*, et al.* Identification of COL6A1 as a differentially expressed gene in human astrocytomas. *Genet Mol Res* **7**, 371-378 (2008).
105. Lawlor, K., Nazarian, A., Lacomis, L., Tempst, P. & Villanueva, J. Pathway-based biomarker search by high-throughput proteomics profiling of secretomes. *J Proteome Res* **8**, 1489-1503 (2009).
106. Hurst, R., Elliott, R.M., Goldson, A.J. & Fairweather-Tait, S.J. Se-methylselenocysteine alters collagen gene and protein expression in human prostate cells. *Cancer Lett* **269**, 117-126 (2008).

107. Manda, R.*, et al.* Identification of genes (SPON2 and C20orf2) differentially expressed between cancerous and noncancerous lung cells by mRNA differential display. *Genomics* **61**, 5-14 (1999).
108. He, Y.W.*, et al.* The extracellular matrix protein mindin is a pattern-recognition molecule for microbial pathogens. *Nat Immunol* **5**, 88-97 (2004).
109. Jia, W., Li, H. & He, Y.W. The extracellular matrix protein mindin serves as an integrin ligand and is critical for inflammatory cell recruitment. *Blood* **106**, 3854-3859 (2005).
110. Simon, I.*, et al.* Evaluation of the novel serum markers B7-H4, Spondin 2, and DcR3 for diagnosis and early detection of ovarian cancer. *Gynecol Oncol* **106**, 112-118 (2007).
111. Sardana, G., Jung, K., Stephan, C. & Diamandis, E.P. Proteomic analysis of conditioned media from the PC3, LNCaP, and 22Rv1 prostate cancer cell lines: discovery and validation of candidate prostate cancer biomarkers. *J Proteome Res* **7**, 3329-3338 (2008).
112. Todorov, P.T.*, et al.* Purification and characterization of a tumor lipid-mobilizing factor. *Cancer Res* **58**, 2353-2358 (1998).
113. Bing, C.*, et al.* Zinc-alpha2-glycoprotein, a lipid mobilizing factor, is expressed in adipocytes and is up-regulated in mice with cancer cachexia. *Proc Natl Acad Sci U S A* **101**, 2500-2505 (2004).
114. Hale, L.P., Price, D.T., Sanchez, L.M., Demark-Wahnefried, W. & Madden, J.F. Zinc alpha-2-glycoprotein is expressed by malignant prostatic epithelium and may serve as a potential serum marker for prostate cancer. *Clin Cancer Res* **7**, 846-853 (2001).
115. Bondar, O.P., Barnidge, D.R., Klee, E.W., Davis, B.J. & Klee, G.G. LC-MS/MS quantification of Zn-alpha2 glycoprotein: a potential serum biomarker for prostate cancer. *Clin Chem* **53**, 673-678 (2007).
116. Bundred, N.J., Miller, W.R. & Walker, R.A. An immunohistochemical study of the tissue distribution of the breast cyst fluid protein, zinc alpha 2 glycoprotein. *Histopathology* **11**, 603-610 (1987).
117. Diez-Itza, I.*, et al.* Zn-alpha 2-glycoprotein levels in breast cancer cytosols and correlation with clinical, histological and biochemical parameters. *Eur J Cancer* **29A**, 1256-1260 (1993).
118. Abdul-Rahman, P.S., Lim, B.K. & Hashim, O.H. Expression of high-abundance proteins in sera of patients with endometrial and cervical cancers: analysis using 2-DE with silver staining and lectin detection methods. *Electrophoresis* **28**, 1989-1996 (2007).
119. Rivenbark, A.G. & Coleman, W.B. Epigenetic regulation of cystatins in cancer. *Front Biosci* **14**, 453-462 (2009).
120. Konduri, S.D.*, et al.* Modulation of cystatin C expression impairs the invasive and tumorigenic potential of human glioblastoma cells. *Oncogene* **21**, 8705-8712 (2002).
121. Tian, M. & Schiemann, W.P. Preclinical efficacy of cystatin C to target the oncogenic activity of transforming growth factor Beta in breast cancer. *Transl Oncol* **2**, 174-183 (2009).

122. Wegiel, B.*, et al.* Cystatin C is downregulated in prostate cancer and modulates invasion of prostate cancer cells via MAPK/Erk and androgen receptor pathways. *PLoS One* **4**, e7953 (2009).
123. Kolwijck, E.*, et al.* Cathepsins B, L and cystatin C in cyst fluid of ovarian tumors. *J Cancer Res Clin Oncol* **136**, 771-778 (2010).
124. Kos, J.*, et al.* Cathepsins B, H, and L and their inhibitors stefin A and cystatin C in sera of melanoma patients. *Clin Cancer Res* **3**, 1815-1822 (1997).
125. Kos, J.*, et al.* Cysteine proteinase inhibitors stefin A, stefin B, and cystatin C in sera from patients with colorectal cancer: relation to prognosis. *Clin Cancer Res* **6**, 505-511 (2000).
126. Naumnik, W., Niklinska, W., Ossolinska, M. & Chyczewska, E. Serum cathepsin K and cystatin C concentration in patients with advanced non-small-cell lung cancer during chemotherapy. *Folia Histochem Cytobiol* **47**, 207-213 (2009).
127. Strojan, P., Svetic, B., Smid, L. & Kos, J. Serum cystatin C in patients with head and neck carcinoma. *Clin Chim Acta* **344**, 155-161 (2004).
128. Decock, J.*, et al.* Cathepsin B, cathepsin H, cathepsin X and cystatin C in sera of patients with early-stage and inflammatory breast cancer. *Int J Biol Markers* **23**, 161-168 (2008).
129. Mulaomerovic, A.*, et al.* Cystatin C as a potential marker for relapse in patients with non-Hodgkin B-cell lymphoma. *Cancer Lett* **248**, 192-197 (2007).
130. Kulasingam, V. & Diamandis, E.P. Proteomics analysis of conditioned media from three breast cancer cell lines: a mine for biomarkers and therapeutic targets. *Mol Cell Proteomics* **6**, 1997-2011 (2007).
131. Mbeunkui, F., Metge, B.J., Shevde, L.A. & Pannell, L.K. Identification of differentially secreted biomarkers using LC-MS/MS in isogenic cell lines representing a progression of breast cancer. *J Proteome Res* **6**, 2993-3002 (2007).
132. Jacobs, J.M.*, et al.* The mammary epithelial cell secretome and its regulation by signal transduction pathways. *J Proteome Res* **7**, 558-569 (2008).
133. Sokol, J.P. & Schiemann, W.P. Cystatin C antagonizes transforming growth factor beta signaling in normal and cancer cells. *Mol Cancer Res* **2**, 183-195 (2004).
134. Tominaga, Y., Tamguney, T., Kolesnichenko, M., Bilanges, B. & Stokoe, D. Translational deregulation in PDK-1-/- embryonic stem cells. *Mol Cell Biol* **25**, 8465-8475 (2005).
135. Chaudhary, K., Nistala, R. & Whaley-Connell, A. Is there a future for direct renin inhibitors? *Expert Opin Investig Drugs* **19**, 653-661 (2010).
136. Nguyen, G.*, et al.* Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest* **109**, 1417-1427 (2002).
137. Khakoo, A.Y., Sidman, R.L., Pasqualini, R. & Arap, W. Does the renin-angiotensin system participate in regulation of human vasculogenesis and angiogenesis? *Cancer Res* **68**, 9112-9115 (2008).

138. Richard, D.E., Berra, E. & Pouyssegur, J. Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. *J Biol Chem* **275**, 26765-26771 (2000).
139. Huang, Y.*, et al.* Modulation of specific protein expression levels by PTEN: identification of AKAP121, DHFR, G3BP, Rap1, and RCC1 as potential targets of PTEN. *Oncogene* **24**, 3819-3829 (2005).
140. Rajasekhar, V.K.*, et al.* Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. *Mol Cell* **12**, 889-901 (2003).
141. Downward, J. PI 3-kinase, Akt and cell survival. *Semin Cell Dev Biol* **15**, 177-182 (2004).
142. Dowling, R.J.*, et al.* mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. *Science* **328**, 1172-1176 (2010).
143. Degraff, D.J.*, et al.* Androgen mediated translational and postranslational regulation of IGFBP-2 in androgen-sensitive LNCaP human prostate cancer cells. *Am J Transl Res* **2**, 200-208 (2010).