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Identification and characterization of downstream effector protein(s) regulated by p53 and pRb

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IDENTIFICATION AND CHARACTERIZATION OF DOWNSTREAM EFFECTOR PROTEIN(S) REGULATED BY p53 AND pRb

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DEDICATION

My thesis is dedicated to my father, Millard. He is the foundation in my life in which I gather my strength. Without his love, support, and encouraging words none of this would have been possible.

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"The mediocre teacher tells. The good teacher explains. The superior teacher demonstrates. The great teacher inspires." ― William Arthur Ward

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AKT	—	V-akt murine thymoma viral oncogene homolog 1
Bak	—	Bcl-2-associated X protein
Bax	—	Bcl-2homologous antagonist killer
Bcl-2	—	B-cell lymphoma 2
BCL2L11	—	Bcl-2-like 11
BTG2	—	B-cell translocation gene 2
CDC42	—	Cell division cycle 42
CDKN2A	—	Cyclin dependent kinase inhibitor 2A
CMV	—	Cytomegalovirus
CxCR4	—	C-X-C chemokine receptor type 4
DMEM	—	Dulbecco's Minimal Essential Medium
E2F	—	E2F transcription factor
EGF	—	Epidermal growth factor
EGFR	—	Epidermal growth factor receptor
EMT	—	Epithelial to mesenchymal transition
ERK	—	Extracellular signal-regulated kinases
F-actin	—	Filamentous-actin
FBS	—	Fetal bovine serum
GAPDH	—	Glyceraldehyde-3-phosphate dehydrogenase
GFP	—	Green fluorescent protein
GPCR	—	G-protein coupled Receptor
HDAC	—	Histone deacetylases
HDM2	—	Human homolog of mouse double minute 2
HNSCC	—	Head and neck squamous cell carcinoma
IL-6	—	Interleukin-6
IPA	—	Ingenuity Pathway Analysis
MAPK	—	Mitogen-Activated Protein Kinases
MEM/EBSS	—	Minimum essential medium with Earle’s balanced salt solution
MOI	—	Multiplicity of infection
NR4A1	—	Nuclear receptor subfamily 4 group A member 1
NSCLC	—	Non-small cell lung cancer
p53	—	Tumor suppressor protein 53
PI3K	—	Phosphoinositide 3-kinase
pRb	—	Retinoblastoma protein
Rac1	—	Ras related C3 botulinum toxin substrate 1
RB1	—	Retinoblastoma 1 gene
RGS16	—	Regulator of G protein signaling 16
RhoA	—	Ras homolog family member A

RhoC	—	Ras homolog family member C
SASP	—	Senescence associated secretory phenotype
STAT4	—	Signal transducer and activator of transcription 4
TP53	—	Tumor suppressor 53 gene

ABSTRACT

A commonality among cancer types is the high frequency of mutations that inhibit or alter signaling of the p53 and pRb (Retinoblastoma) tumor suppressors. These genes regulate processes vital for cancer suppression such as apoptosis, senescence, and cell cycle arrest among others. Loss of both p53 and pRb promotes processes that support cancer progression and is associated with decreased patient survival and increased rates of tumor reoccurrence. Although data points to the ability of p53 and pRb to collaborate and to inhibit tumorigenesis, it remains unclear how p53 and pRb cooperate toward this task. Using RNA expression profiling, 179 p53 and pRb cross-talk candidates were identified in normal lung fibroblasts (WI38) cells exogenously coexpressing p53 and pRb. Regulator of G protein signaling 16 (RGS16) was among the p53 and pRb cross-talk candidates and reports suggest it inhibits the activation of several oncogenic pathways associated with proliferation, migration, and invasion of cancer cells.

RGS16 is downregulated in pancreatic cancer patients with metastases compared to patients without metastasized pancreatic cancer. The role of RGS16 in cancer cell metastasis is unknown; therefore I tested the hypothesis that RGS16 inhibits pancreatic cancer cell migration and invasion in vitro. Expression of RGS16 was decreased in the pancreatic cancer cell lines tested compared to control. Expression of RGS16 inhibited fetal bovine serum (FBS) and epidermal growth factor (EGF) induced migration of the BxPC-3 and AsPC-1 but not PANC-1 pancreatic cancer cells. It also inhibited EGF induced invasion of BxPC-3 and AsPC-1 cells with no impact on cell viability. Although RGS16 inhibited cell migration and invasion of BxPC-3 and AsPC-1 cells, there was no change in F-actin polymerization or the amounts of p-AKT, p-ERK and the epithelial mesenchymal transition (EMT) marker vimentin proteins, but there was a slight increase in E-cadherin protein expression in BxPC-3 cells. Our data suggests the

inhibitory effect of RGS16 on EGF induced pancreatic cancer cell migration is independent of the PI3K and MAPK pathways. To our knowledge, for the first time, we performed analyses to identify p53 and pRb cross-talk candidates and demonstrated a role for RGS16 in suppressing EGF and FBS induced pancreatic cancer migration and invasion.

CHAPTER 1.

INTRODUCTION

SECTION 1.1: p53 AND pRb COLLABORATORS IN THE FIGHT AGAINST CANCER

Background

The two most commonly mutated signaling pathways in cancer are those associated with tumor protein 53 (p53) and retinoblastoma protein (pRb). p53 and pRb are the first two tumor suppressors identified, however, years after identifying and cloning p53 (1979) and pRb (1986) we are still working to understand their function [1]. p53 and pRb regulate the same processes (such as; cell arrest, apoptosis, senescence, etc.) using different mechanisms and studies suggests these two tumor suppressors cooperate to inhibit cancer progression [2-5]. The ability of p53 and pRb to communicate in regulating cellular functions and determining cellular fate could be a reason mutations in these pathways often occur in cancer. The focus of this section is to give an overview of p53 and pRb functions and their regulation while presenting evidence and examples of the p53 and pRb cross-talk.

Brief overview of p53 function and regulation

The function of p53 has been extensively studied; this section will provide a brief overview of the function and regulation of p53. Originally thought to function as an oncogene, p53 is now known to induce a variety of processes in cells that inhibit tumorigenesis such as: cell cycle arrest, apoptosis, DNA repair, and senescence (reviewed in [6-10]). These processes aid in protecting the integrity of DNA supporting the use of p53's nickname as "Guardian of the Genome"[11]. The ability of p53 to regulate a wide variety of processes is due in part to its functions as either a transcriptional activator or a repressor. p53 forms a tetramer, binds to DNA,

and recruits co-activators inducing transcription of genes that are involved in DNA repair (*p53R2*, *MLH1*, *XPC*, *PCNA*, and *DDB2*), apoptosis (*Bax*, *Noxa*, *Bid*, *APAF-1*, *DR4*, *DR5* and *PUMA*), senescence (*plasminogen activator inhibitor 1*: *pai-1*, *p21*), G1 (G1: *BTG2*, *p21*), and G2 cell cycle arrest (*GADD45*, *14-3-3 sigma, and MCG10*) [12-15]. The p53 tumor suppressor promotes differentiation by repressing transcription of factors that promote pluripotency such as oct4 and nanog and inducing transcription of pRb to initiate myogenesis [16, 17]. In addition to its transcription regulatory activity, p53 translocates to the mitochondria and interacts with the outer membrane leading to mitochondria permeabilization by activating the pro-apoptotic Bax and Bak proteins and inhibiting the pro-survival Bcl-2 and Bcl-xl proteins [13]. These actions initiate mitochondria membrane permeabilization, the release of cytochrome c, and activation of caspases that trigger apoptosis (Figure 1.1).

In order to prevent the random induction of apoptosis and other p53-regulated processes, expression of p53 is normally sustained at low basal levels by the E3 ubiquitin ligase HDM2. HDM2 a transcriptional target of p53, binds and translocates p53 to the cytoplasm preventing p53 from binding to DNA and initiating transcription [12, 14]. HDM2 also poly-ubiquitinates p53 marking it for degradation by the proteasome. Stabilization of p53 occurs due to post-translational modifications by factors activated when the cell is under stress such as: DNA damage, hypoxia, loss of normal cell contacts, and activity of oncogenes [12, 14]. Post-translational modifications such as phosphorylation and acetylation stabilize p53 by preventing its poly-ubiquitination and degradation [12, 14]. The binding of p14ARF to HDM2 also leads to the stabilization of p53 preventing its polyubiquitination by HDM2. Figure 1.1 contains a summary of the function and regulation of p53 demonstrating the complexity of p53 signaling.

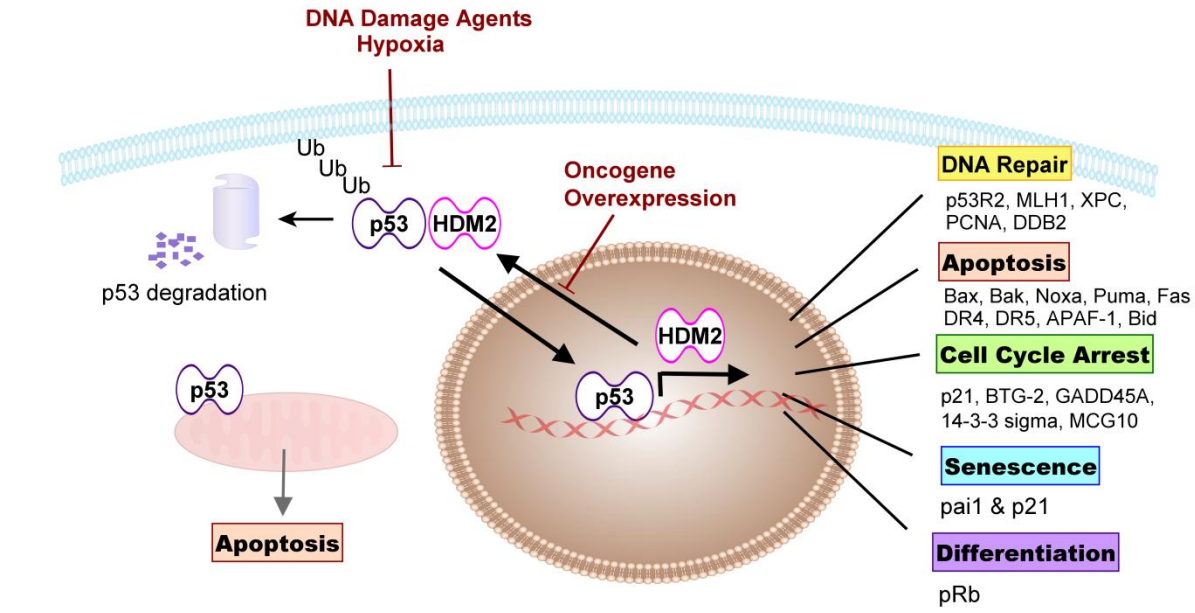


Figure 1.1: Schematic depicting regulation of p53 and downstream transcription targets. p53 initiates a variety of responses through its ability to regulate transcription of targets that are involved in DNA repair, apoptosis, cell cycle arrest, senescence, and differentiation. p53 expression is regulated by its own downstream transcriptional target, HDM2 which polyubiquitinates p53 marking it for degradation in the proteasomes. p53 expression is stabilized by signals initiated by DNA damaging, hypoxia, or overexpression of oncogenes. p53 also initiates apoptosis by binding and interacting with mitochondria outer membrane and proteins located there. Pathway was constructed using Qiagen's Ingenuity pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) Pathway Designer tools. Adapted from Levine, A. J. *et al.* 2006

Brief overview of pRb function and regulation

Known for its ability to induce cell cycle arrest at the G1/S checkpoint, pRb also regulates apoptosis, DNA repair, senescence, and differentiation. pRb is a transcription regulator that binds to co-factors and either functions as a co-repressor or co-activator of transcription. Classically, pRb is acknowledged for its role in binding to the cell cycle stimulatory E2F transcription factors and recruiting histone deacetylases and other transcription repressors and thereby inhibiting transcription of E2F target genes and initiating cell cycle arrest [18-20].

Cyclin-dependent kinases-4 and -6 phosphorylate pRb inhibiting binding of pRb with E2F resulting in the release of E2F to bind to DNA and its dimerization partner (DP2) resulting in the initiation of transcription of cell cycle stimulatory proteins [19, 21]. Activation of E2F transcription results in the expression of cyclin E, which binds to cyclin-dependent kinase 2 and functions in a negative feedback loop to promote phosphorylation and inactivation of pRb. Phosphorylation of pRb is inhibited by cyclin-dependent kinase inhibitors among which are the proteins p27, p16, and p21. Figure 1.2 summarizes the regulation and function of pRb to inhibit activation of E2F target genes.

The best known or studied function of pRb is its ability to act as a transcriptional repressor of E2F targets genes. However, pRb can regulate cellular processes or suppress cancer progression irrespective of its ability to bind to E2F family members. Studies using pRb mutants found E2F binding deficient pRb activated transcription and initiated differentiation and suppressed tumor progression *in vitro* and *in vivo* [22, 23]. pRb has been found to bind and enhance transcription of several transcription factors and nuclear receptors listed in Table 1.1. Enhanced transcriptional activity by pRb promotes differentiation and regulates hormone signaling (Table 1.1). However, there is still not much known regarding pRb's ability to function as a transcriptional co-activator indicating the need for more research to understand the full mechanisms of pRb suppression of cancer progression and promotion of differentiation processes.

Studies have implicated pRb in having a divergent role in either promoting or inhibiting apoptosis. pRb, like p53, can interact directly with the mitochondria mediating the induction of apoptosis triggered by tumor necrosis factor-α [24]. Ianari *et al.* found that pRb potentiates the induction of apoptosis triggered by genotoxic or oncogenic stress in proliferating cells [25]. The

ability of pRb to initiate apoptosis is context dependent, for example; in Ianari's study, pRb bound to E2F, enhanced the induction of apoptosis initiated by stress in proliferating cells [25]. However, in another study conducted using breast cancer cells, loss of pRb enhanced the activation of apoptosis mediated by E2F1 [26]. Furthermore, pRb inhibits induction of apoptosis by p53 in HeLa overexpressing exogenous p53 and pRb [27].

Table 1.1: Transcription factors that are regulated by pRb

Transcription Factor	Effect	Source
MyoD	Regulates muscle differentiation	[28]
C/EBP (CAAT/enhancer-binding protein) family	Regulates adipocyte and monocyte differentiation	[29, 30]
c-Jun	Regulates keratinocyte differentiation	[31]
AP-2	Binds and initiates activation of E-cadherin bcl-2 promoter	[32] [33]
CBFA1 (Runx2)	Regulates Osteogenic differentiation	[34, 35]
SP-1	Regulates differentiation	[36, 37]
Nuclear receptors (Glucocorticoid receptor; Androgen receptor; ERα & ERβ (Estrogen receptor α & β); HNF4 (hepatocyte nuclear factor-4); SF-1 (steroidogenic factor-1); and NGF1-B orphan nuclear receptor family members NGF1-B/Nurr 77/NR4A1, NOR1, & Nurr1)	Initiates transcription of genes that regulate hormone signaling and differentiation	[38-44]

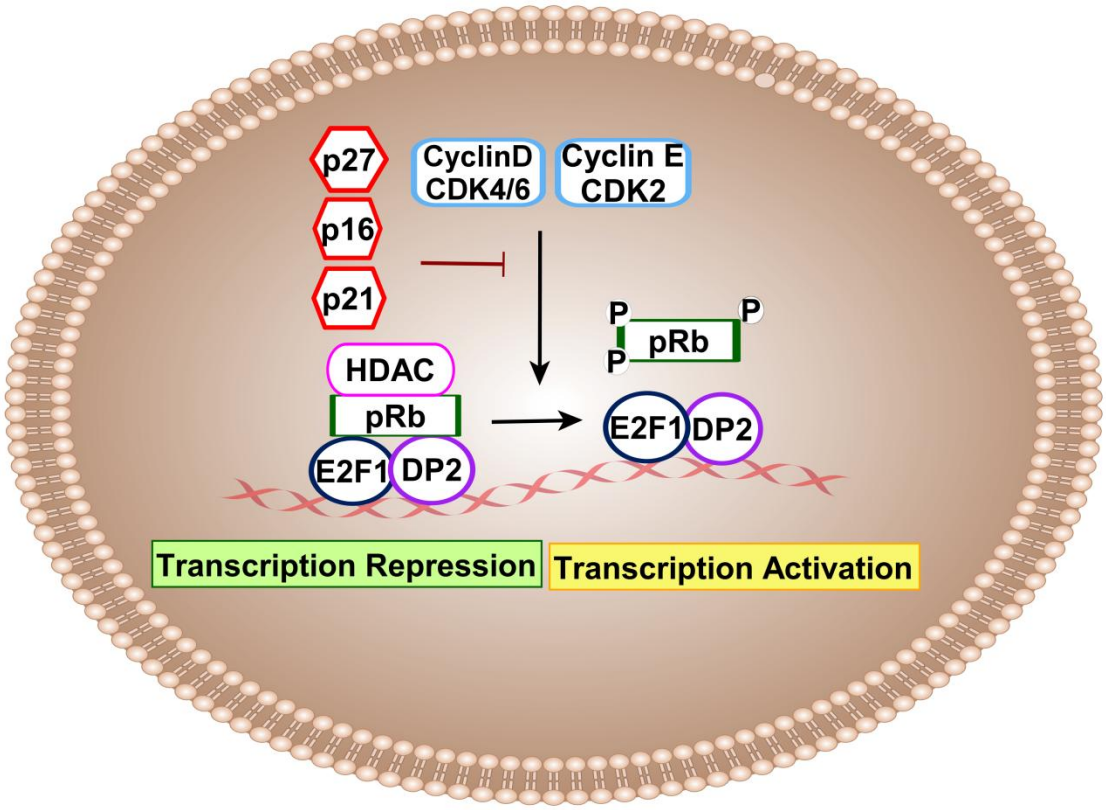


Figure 1.2: Schematic depicting pRb regulation and function as a transcriptional repressor. Pathway was constructed using Qiagen's Ingenuity pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) Pathway Designer tools.

p53 and pRb cross-talk

Evidence of p53 and pRb Cross-talk

The existence of a p53 and pRb cross-talk pathway is supported by evidence collected from tumor analyses and *in vitro* and *in vivo* experiments. Most tumors, including osteosarcomas and cancers of the breast, lung (small cell), cervix, and bladder [1, 4, 45, 46], have deficiencies in either the p53 or pRb pathway. Both p53 and pRb have a high frequency of mutations in osteosarcomas, lung (non-small cell), breast, and cervical carcinomas [1, 4, 46]. Approximately 60% of osteosarcomas have loss of both functional p53 and pRb, which is vital for osteosarcoma

(OS) development [1, 46]. Patients who have mutations in both *TP*53 and *RB1* have increased tumor recurrence and decreased survival compared to patients with only one inactive tumor suppressor genes [45, 47]. Deletions or mutations in the p16 locus, and overexpression of HDM2 or cyclin-D can also interfere with p53 and pRb activity. Table 1.2 describes examples (small representative) of mutations and alterations that prevent p53 and pRb activation.

Table 1.2: Examples of alterations that can occur in cancers that alter p53 and pRb signaling

Alteration	Cancer	Sources
p16 deletions/mutations	Pancreatic cancer, Head & Neck Squamous Cell Carcinomas (HNSCC), Non-Small Cell Lung Cancers (NSCLC), Glioblastomas (GBMs)-	[48-51]
Overexpression of HDM2	Sarcomas (soft tissue and osteosarcoma), retinoblastoma, & esophageal carcinomas	[52, 53]
Overexpression of cyclin-D	HNSCC, breast cancers, pancreatic cancers, and prostate cancers	[54-57]

Loss of both p53 and pRb promotes cancer by initiating tumorigenesis and increasing chromosome instability, and chemoresistance. Binding of DNA tumor virus proteins associated with adenovirus, certain strains of human papilloma virus (HPV), and simian virus 40 (SV-40) to p53 and pRb with resultant loss of activity is vital for viral induced transformation [4, 58]. Chromosome instability is associated with cancer initiation, promotion of tumor growth, and chemoresistance [59, 60]. In cell lines from two different cancer types, loss of both p53 and pRb synergizes to promote chromosome instability due to chromosome gains and losses during mitosis [60]. Loss of pRb leads to increased genetic alterations (gains or losses) and the loss of p53 cooperates to allow the continued proliferation of these cells [60].

A study examining p53 mediated responses to chemotherapy found a reduction in cellular response if there was a loss of pRb signaling [61]. Interestingly, inactivation of both p53 and pRb (through the use of a p53 dominant negative fragment and pRb siRNA) increased sensitivity of the cancer cells to chemotherapy compared to inactivation in p53 alone [61]. However, this was not seen in cells where p16 levels were decreased via siRNA, suggesting that p16 and p53 mutations are more advantageous for cancer progression [61]. Another study found loss of both p53 and pRb can induce multidrug resistance prior to transformation [62]. Accordingly, patients diagnosed with breast cancer and treated with adjuvant chemotherapy had a better response if they had functional p53 and pRb [61].

Several mouse models utilize inactivation of p53 and pRb to initiate tumor development that mimics the human disease. For example, inactivation of both *TP53* and *RB1* genes in bone marrow mesenchymal stem cells or dermal connective tissue cells resulted in the development of osteosarcomas or soft tissue sarcomas respectively [63, 64]. Mutations in both tumor suppressor genes work synergistically to promote cancer progression. Conditional inactivation of the *RB1* and *TP53* genes in lung epithelial cells of mice, led to the development of aggressive small cell lung tumors [65]. Also $p53^{-/-}$ mice that were also $RB1^{+/-}$ developed more tumors than mice with single mutations; i.e. heterozygous $TP53^{+/-}$ $RB1^{-/-}$ or *TP53* null mice [4]. Furthermore, inactivation of *TP53* and *RB1* genes in the prostate epithelium of mice led to the development of aggressive prostate cancers that were highly metastatic resulting in decreased survival compared to mice with only one in activated gene [5]. Likewise, inactivation of pRb by a fragment of the SV40 T-Antigen in mammary epithelium results in formation of adenocarcinomas that appear earlier in mice that are also $TP53^{+/-}$ [66].

Loss of functional p53 and pRb signaling happens with a high frequency in cancers and promotes processes involved in tumor progression. Loss of p53 or pRb promotes metastasis and both tumor suppressors inhibit processes such as migration and invasion needed for the spread of cancer [67-69, 70{Feakins, 2003 #7786]}. However, there is a lack of studies investigating whether the concomitant loss of p53 and pRb promotes migration and invasion more than inactivation of just one pathway. Although p53 and pRb are two of the most studied genes in cancer, there is a lack of understanding how they synergistically function to inhibit cancer formation and/or progression.

Examples of p53 and pRb cross-talk

Over the years evidence has accumulated painting a picture of the communication that exists between the p53 and pRb pathways. Proteins known to be implicated in the p53 and pRb cross-talk include E2F-1, HDM2, p21, BTG2 and the INK4a locus. Figure 1.3 is a schematic representing known examples of p53 and pRb cross-talk. Briefly, through mechanisms of alternate splicing, the CDKN2A locus codes for two genes, p16/INK4a and p14/ARF [71]. p14 binds to HDM2, inhibits p53 polyubiquitination its subsequent degradation by the proteasomes [71]. This allows p53 to initiate transcription of downstream target genes including p21, and BTG2 [72, 73]. The cyclin-dependent kinase inhibitors p16 and p21 together with BTG2 prevent the phosphorylation and inactivation of pRb and stop cell cycle progression [71-73]. Loss of pRb bound to E2F-1 due to inactivation of pRb or p16 can trigger p53 dependent apoptosis through E2F-1 stabilizing p53 by two methods, 1) activating proteins that phosphorylate or acetylate p53 thereby preventing HDM2 binding or by 2) inducing expression of p14 [74-78]. p53 induces expression of HDM2 which functions in a negative feedback loop to inhibit p53. However, pRb can bind to HDM2 and p53 complexes leading to the stabilization of p53 [47, 79].

p53 complexed to HDM2 and pRb is unable to bind to DNA and regulate transcription, however it can induce apoptosis by directly acting on the mitochondria membrane [47, 79].

The previous examples of p53 and pRb cross-talk mostly highlight the use of protein-protein interactions to determine cell fate. However, p53 and pRb also cross-talk using transcriptional regulatory mechanisms, for example, p21 and BTG-2, both transcribed by p53, can inhibit inactivation of pRb, illustrating a pathway by which p53 and pRb coordinate cell cycle arrest. p53 also binds to the *RB1* promoter and initiates transcription of pRb triggering pRb induced muscle differentiation [17]. Furthermore, both p53 and pRb bind to the promoter of the RNA polymerase III specific transcription factor, TFIIIB and suppress transcription of TFIIIB providing another example of how these proteins inhibit cell cycle progression [80]. However, management of p53 and pRb controlled processes such as apoptosis, cell cycle arrest or development may require p53 and pRb to regulate gene expression in an opposing rather than cooperative manner. Expression of an embryonic development gene, Placenta-specific 1 (PLAC1) is down-regulated by p53 and up-regulated by pRb demonstrating how p53 and pRb can play contrasting roles to regulate cellular processes [81].

Although proteins involved in the p53/pRb cross-talk have been identified, there is still a lack of information regarding how p53 and pRb communicate to regulate cell fate. p53 and pRb co-regulated genes were investigated to expanding on the current knowledge of the p53 and pRb cross-talk. We have identified 179 proteins co-regulated by p53 and pRb. Transcriptional regulation of the same genes by p53 and pRb may function as a failsafe mechanism if one pathway is inactivated, the other is able to prevent the accumulation of additional mutations that results in the development of cancer. Unraveling the complex interactions between p53 and pRb

will contribute to further understanding how p53 and pRb cooperate to prevent tumorigenesis, while also providing knowledge that can potentially be used to advance current cancer therapy.

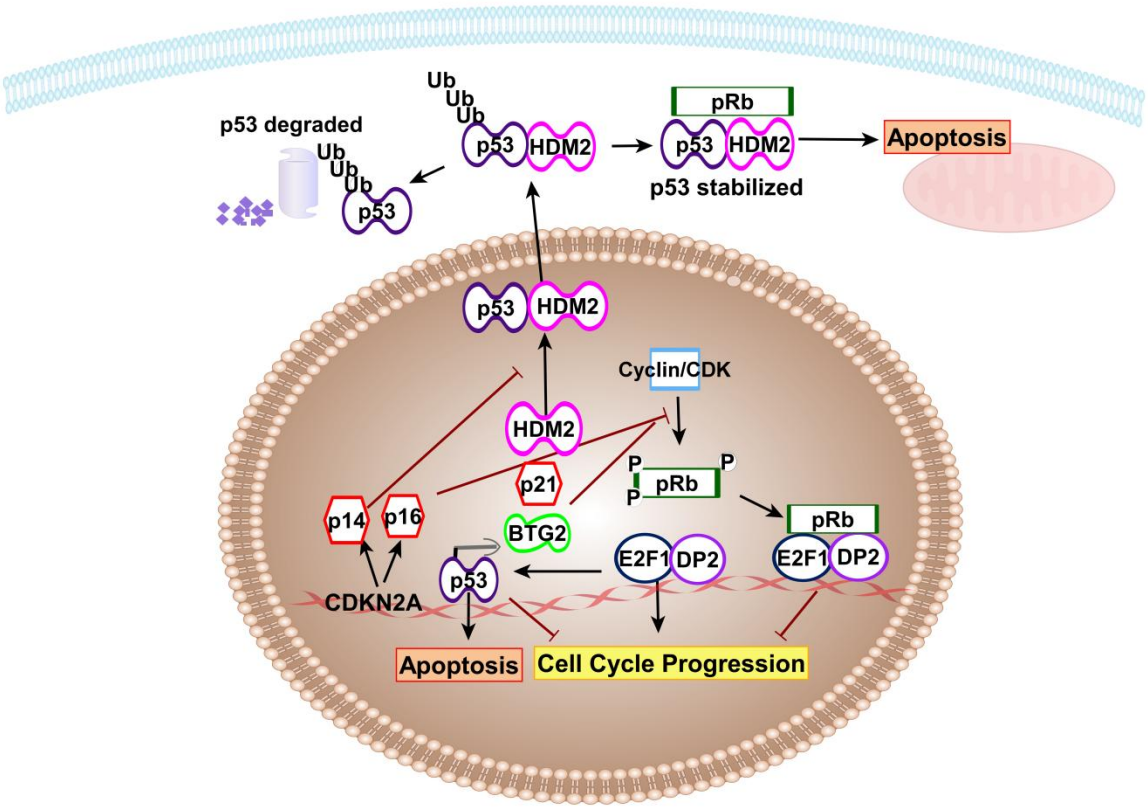


Figure 1.3: Schematic depicting proteins known to mediate p53 and pRb cross-talk. Pathway was constructed using Qiagen's Ingenuity pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) Pathway Designer tools.

SECTION 1.2. PANCREATIC CANCER

Epidemiology and characterization

Pancreatic ductal adenocarcinoma, also referred to as pancreatic cancer, accounts for 2.8% of all new cancer cases and is the 4th leading cause of cancer related death in the United States compared to the 8th and 9th cause of mortality for men and women respectively world-wide [82]. The American Cancer Society estimates that approximately 46,420 individuals will be diagnosed with pancreatic cancer and 39,590 will die from this disease in 2014 [83]. The one-year survival rate for all stages of pancreatic cancer is 20% and the five-year survival rate is 6% [83]. The percentage of cases and the five year survival rate are depicted in Table 1.3 for stage of cancer at time of diagnosis. A majority of patients (53%) present with metastatic disease at time of diagnosis and have a dismal 5-year survival rate of 2.3%. Only 9% of pancreatic cancer patients present with localized disease but their 5-year survival rate is still low at 25.8%. Most patients newly diagnosed with pancreatic cancer present with highly progressed and/or metastatic cancer that is resistant to treatment [84, 85]. This data reflects the great need for methods to prevent, and therapeutics to treat advanced pancreatic cancer.

Table 1.3: Percentages of survival and cases based on stage of cancer at time of diagnosis

Stage	% of Cases at Diagnosis	% Survival
Localized	9	25.8
Regional (regional lymph node metastasis)	28	9.9
Distant (metastasized)	53	2.3
Unstaged (unknown)	11	4.4

Information obtained from Howlader N et al.. SEER Cancer Statistics Review, 1975-2011, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2011/, based on November 2013 SEER data submission, posted to the SEER web site, April 2014.

Pancreatic cancer has a higher rate of incidence in industrialized areas and predominately affects individuals later in life with the average age of diagnosis being 71. Smoking, age, and chronic pancreatitis have been established as risk factors; whereas, obesity, diabetes, and family history of pancreatic cancer are associated with increased risk [86]. Approximately 5 to 10% of pancreatic cancer patients have a family history of pancreatic cancer and less than 20% of those are due to germline mutations [86-89].

Staging

Pancreatic cancers are commonly referred to pancreatic ductal adenocarcinomas (85%) however; pancreatic cancers can be classified as acinar cell carcinoma, pancreatoblastoma, solid pseudopapillary neoplasm, serous cystadenoma, and pancreatic endocrine tumors [86]. These cancers mostly affect the exocrine portion of the pancreas that consists of acinar and duct cells that are responsible for secretion of digestion enzymes [86]. The head of the pancreas is a favored location of tumor development and allows rapid infiltration into surrounding tissue [85]. There are four types of preneoplastic pancreatic lesions known as: pancreatic intraepithelial neoplasia (PanINs), mucinous cystic neoplasm, (MCN) and intraductal papillary mucinous neoplasm (IPMN) [85, 86]. PanINs are the most common preneoplastic lesion and can exist in three stages. Stage III PanINs develop into pancreatic cancer (pancreatic ductal adenocarcinomas) [86].

Pancreatic cancers are staged using the American Joint Committee on Cancer tumor-node-metastasis (TNM) classification described in Figure 1.4 & Table 1.4 Unknown TNM tumor classifications are designated with a X, (TX, NX, or MX). T*is* designates carcinoma *in situ* or preneoplastic lesions.

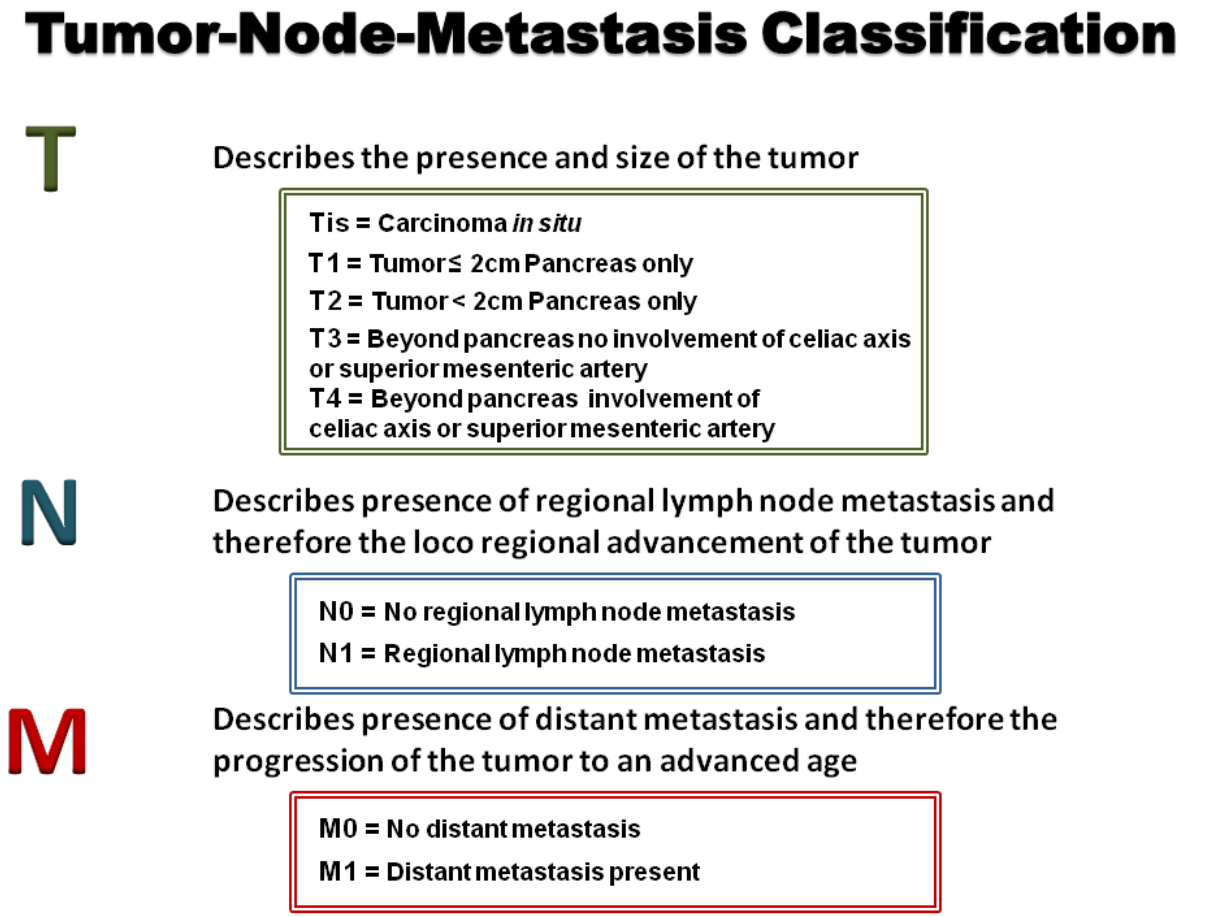


Figure 1.4: Describes the American Joint Committee on Cancer Tumor-Node-Metastasis classification system. Information adapted from the National Institute of Health

Table 1.4: Pancreatic Cancer Stages

Stage	Tumor	Node	Metastasis
0	Tis	N0	M0
IA	T1	N0	M0
IB	T2	N0	M0
IIA	T3	N0	M0
IIB	T1-3	N0	M0
III	T4	N0 or N1	M0
IV	Any T	N0 or N1	M1

Based on the American Joint Committee on cancer tumor-node-metastasis. Information adapted from the National Cancer Institute (2014)

Diagnosis and Treatment

Pancreatic cancer is rarely diagnosed during the early stages of carcinogenesis because of the lack of noticeable and distinct symptoms [85]. Many of the symptoms such as abdominal pain, nausea, and jaundice are unspecific and can be equated with other illnesses. Several imaging technologies including contrast enhanced multi-detector row computed tomography (MDCT), ultrasonography (US), endoscopic ultrasonography (EUS), and Magnetic resonance imaging (MRI) are used to diagnose pancreatic cancer [90]. Ultrasonograhy is often the first imaging modality used once a patient presents with jaundice or abdominal pain [90]. However, US is not the most reliable method for staging pancreatic cancer and can miss small pancreatic tumors (T*is* and T1) [91]. Multi-detector row computed tomography is the most accurate and used method for diagnosing and staging pancreatic cancer [85, 90]. Endoscopic ultrasonography can be used to rule out pancreatic cancer as a diagnosis therefore eliminating the need to use other diagnostic methods and is often chosen to visualize tissue extraction for diagnosis [90, 91]. Contrast enhanced MRI with magnetic cholangiopancreatography is often a secondary imaging tool that can more readily diagnose small pancreatic tumors and rule out other pancreatic abnormalities [90, 91].

Surgery, chemotherapy, and/or chemoradiation are the therapies used to treat pancreatic cancer. Due to late stage of diagnosis less than 20% of patients are eligible for the only possible curative pancreatic resection (tumor staging T1-T3). The presence of metastasis to the lymph nodes, peritoneal cavity, liver, or other sites is a negative prognostic factor [85, 92]. In fact patients who undergo surgery have decreased survival if there is presence of lymph node metastases. Treatments used for pancreatic cancer are based on tumor staging (Table 1.5). Surgery is the first treatment option for patients with stage I & II pancreatic cancer followed by chemoradiation therapy. Neoadjuvant therapy is controversial, however a recent study found that patients who underwent neoadjuvant therapy had increased survival and time to recurrence than those who underwent surgery first [93]. The majority of patients diagnosed with pancreatic cancer present with stage III or IV pancreatic cancer that is ineligible for surgery. However, a portion of patients (8 to 30%) with stage III cancer undergoing neoadjuvant chemoradiation therapy become eligible for pancreatic resection and have survival rates similar to patients who first undergo surgery [84]. An analysis of the five-year survival rates for various cancers over a span of thirty years found that pancreatic cancer had the least improvement from 2% (1975-1977) to 6% (2003 to 2009) [83]. The aggressive nature of pancreatic cancer, lack of early diagnosis, and therapy resistance of this deadly disease contribute to the lack of advancement in enhancing the survival rate [94].

Table 1.5: Pancreatic cancer treatment broken down based on tumor stage.

Tumor Stage	Treatment
I & II	Surgery
	Postoperative chemoradiation (5-fluorouracil (5-FU) chemo and radiation therapy)
	Postoperative chemotherapy (gemcitabine or 5-FU/leucovorin)
III	Chemoradiation 1. Chemoradiation followed by chemotherapy 2. Chemotherapy followed by chemoradiation (patients w/o metastasis)
	Chemotherapy (gemcitabine; gemcitabine & erlotinib; gemcitabine and nab-paclitaxel; or 5-FU, leucovorin, irinotecan, & oxaliplatin (FOLFIRINOX)
IV	Palliative therapy (pain relieving procedures and supportive care)
	Chemotherapy (gemcitabine; gemcitabine & erlotinib; or (FOLFIRINOX)

Information adapted from National Cancer Institute (2014)

Mutations and altered signaling pathways in pancreatic cancer

Pancreatic cancer is a heterogeneous disease that contains on average 63 genetic mutations affecting a set core of 12 pathways [95]. At least one of four genes listed in Table 1.6 is commonly mutated in pancreatic cancer and aids in proliferation, survival, migration, epithelial mesenchymal transition (EMT), and invasion. K-Ras mutations are the most common genetic alteration and occur early in low grade PanIN 1A lesions. K-Ras is commonly mutated at codon G12 and with less frequency, codons G13 and Q61. These mutations result in the constitutive activation of K-Ras [96]. Expression of mutant K-Ras (G12D) in mice induces PanIns that develop into pancreatic cancer [97]. K-Ras mutations promote pancreatic cancer proliferation, survival, invasion, migration and metastasis and exerts its cancer promotion via modulation of the Raf/Mek/Erk, PI3K/AKT, and Ral-A and Ral-B pathways [96]. K-Ras/PI3K signaling is critical for the initiation, progression, and maintenance of pancreatic cancer in mice

[98]. Activation of the Raf/ERK MAPK pathway using mice with conditional knock-in $BrafV^{600e}$ in the pancreas induced PanIns that later developed into pancreatic cancer [99]. K-Ras activates the Ral guanine nucleotide exchange factor (RalGEFs) leading to the subsequent activation of Ral-B and Ral-A that are linked to pancreatic cancer growth (Ral-A) and metastasis (Ral-B) [100].

Inactivating mutations in CDKN2A (affecting predominantly INK4a/p16 and to a lesser extent, ARF/p14), p53, and Smad4 occur in moderate or advanced PanIns suggesting they are late events [101]. CDKN2A is the most targeted tumor suppressor gene for mutations in pancreatic cancer and, as highlighted in section 1.1, it codes for the p16/INK41 and p14ARF proteins. However, most of the mutations decrease p16 expression, thereby negating its ability to inhibit proliferation and induce cell cycle arrest through preventing the phosphorylation and inhibition of pRb. The importance of p16 inactivation in pancreatic cancer can be seen in genetically modified mice. The conditional concomitant mutation knock-in of mutant $K\text{-}Ras^{G12D}$ and knock-out of $CDKN2A^{flox/flox}$ in the pancreas of mice induces more aggressive and metastatic pancreatic cancers than $K\text{-}Ras^{G12D}$ (only) mice models [102]. Mutations in p53 occur at a high frequency and aid in suppression of apoptosis and cell cycle arrest, while also increasing metastasis, proliferation and genomic instability [103]. Similar to p16 knock-out mice, knock-in mutations of mutant $p53^{R172H}$ and $K\text{-}Ras^{G12D}$ in the pancreas of mice decreases the time for tumor development and subsequent metastasis compared to mice with only mutant $K\text{-}Ras^{G12D}$ [104]. Smad4 is activated by transforming growth factor-β (TGF-β) and mediates some of its downstream signaling [86, 101]. TGF-β has both tumor promoting and suppressing mechanisms. Mutations affecting Smad4 expression results in the loss of TGF-β mediated cell cycle arrest and

cell motility[105]. Loss of Smad4 decreases migration and increases chemoresistance, and expression of EGFR and VEGF promoting pancreatic cancer progression [105-107].

Other genetic alterations result in the overexpression of the epithelial growth factor receptor (EGFR), AKT2, and RhoC in pancreatic cancers [108-110]. AKT2 is overexpressed in 10% of pancreatic cancers and promotes growth and invasion that was inhibited by the use of antisense AKT RNA [109]. RhoC is overexpressed in pancreatic cancers with the highest expression seen in tumors from patients who had the presence of metastasis [108]. EGFR and its fellow family member HER2/erbB2 are overexpressed in 85% or 10% respectively, of pancreatic cancers. EGFR activates a variety of pathways (see following section) that are responsible for proliferation, EMT, migration and survival.

Table 1.6: Common genetic mutations in pancreatic cancer and incidence

Gene	Effect of mutation	% Pancreatic Cancers with mutation	Source
K-Ras	↑ proliferation; ↓ apoptosis; ↑ migration; ↑ metastasis & evasion of immune response	> 90	[96]
CDKN2A (p16/INK4a)	↓ cell cycle control; ↑ proliferation;	85	[51, 101]
p53	↓ apoptosis; ↓ cell cycle arrest; ↑ metastasis; ↑ proliferation; ↑ genomic instability	50 to 70	[86, 103, 111]
SMAD4/DPC4	↑ epithelial mesenchymal transition (EMT); ↑ invasion; ↓cell cycle arrest;	50	[86, 101, 105, 112]

Epidermal Growth Factor (EGF) Signaling

There are four receptors in the EGF family designated as EGFR/erbB1/HER1, erbB2/HER2, erbB2/HER3, and erbB4/HER4. EGFR/erbB1 is overexpressed in pancreatic cancer and promotes metastasis, proliferation, angiogenesis, and survival [110, 113, 114]. Knockdown of EGFR inhibits the epithelial mesenchymal transition in pancreatic cancer and EGFR is needed for K-Ras induced pancreatic tumorigenesis [115, 116]. EGFR activity is stimulated by the binding of its ligands (epidermal growth factor (EGF), transforming growth factor-α (TGF-α), and amphiregulin), which initiate receptor hetero- or homo- dimerization and autophosphorylation [110, 113, 114]. As a result of this receptor modification adapter proteins are recruited, leading to the activation of PI3K/AKT, Ras/MAPK, and phospholipase Cγ (PLC-γ) pathways [110, 113, 114]. Figure 1.5 depicts four downstream pathways stimulated by EGFR signaling and the biological effects mediated by these pathways such as metastasis, migration, invasion, proliferation and chemoresistance [110, 113, 114, 117-122]. Ras pathway can also activate Rho GTPases and PI3K demonstrating cross-talk between these signaling pathways [123].

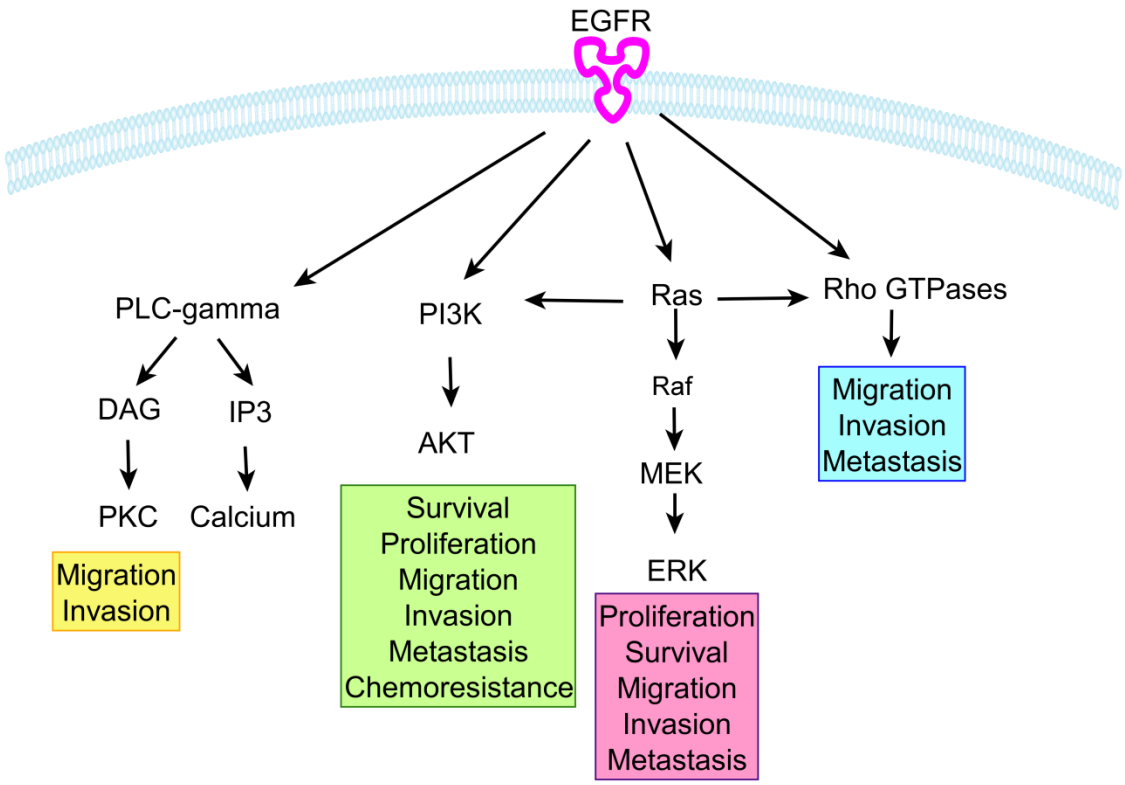


Figure 1.5: Summary of EGFR signaling and downstream effects upon cancer progression. Pathway was constructed using Qiagen's Ingenuity pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) Pathway Designer tools. Adapted from Robert Roskoski Jr. (2014)

Metastasis

Metastasis accounts for 90% of cancer related deaths and involves a series of steps that can be targeted for cancer therapy [124]. Table 1.7, describes the various steps, processes, and molecular signals involved for a cancer cell to metastasize. Briefly, a tumor cell needs to disassociate from the tumor and surrounding environment. Detachment is facilitated by the epithelial mesenchymal transition (EMT) in which cells adopt a mesenchymal phenotype [106, 125]. EMT results in a change in cell morphology, acquisition of motility and increased secretion of proteases [126]. A hallmark of EMT is the loss of E-cadherin and an increase in the expression of the mesenchymal markers N-cadherin, vimentin, fibronection, and $\alpha_v\beta_6$ integrin

[106, 125]. N-cadherin forms weaker cell-cell interactions than E-cadherin allowing the dissociation of the cancer cells [106, 125]. Growth factors [EGF, TGF-β, tumor necrosis factor-α (TNF-α), hepatocyte growth factor (HGF), and insulin-like growth factor-1 (IGF-1)] , secreted by stromal cells in the tumor microenvironment, act on the tumor cells to initiate EMT [125].

After acquisition of a mesenchymal phenotype cancer cells can disassociate, migrate and invade through surrounding tissues and, intravasate into the lymph or blood system [124]. Intravasation is a complex process mediated by multiple mechanisms including cell migration and invasion. Proteases are secreted, breaking down the extracellular matrix, creating a path for cells to migrate [127]. Once in the lymphatic or blood system, cells need to survive stresses that can induce apoptosis such as shear force, lack of adherence, and hypoxia [128]. Once the cell has migrated to a distant site, it extravasates out of the lymphatic or blood system. The migrated cell will divide to form a secondary tumor of up to 2 mm in diameter before initiating angiogenesis and other processes it needs to colonize the distant location [124, 129]. Angiogenesis provides the micro-metastasis with the nutrients it needs to further grow beyond 2 mm of diameter. Angiogenesis is promoted by the secretion of vascular endothelial growth factor (VEGF), platelet derived growth factor, (PDGF), chemokines, and cytokines by the tumor and stromal cells [129, 130].

Table 1.7 : Steps, processes and molecular signals involved in the metastasis of cancer cells

Steps of metastasis	Process	Molecular signals involved
Disassociation	EMT: cells obtain phenotype of mesenchymal cells that aids in metastasis. (↓ E-cadherin, ↑ N-cadherin, ↑ vimentin, ↑ fibronectin, ↑ αvβ6 integrin)	EGF, TGF-β, tumor necrosis factor-α (TNF-α), hepatocyte growth factor (HGF), and insulin-like growth factor-1 (IGF-1)
Intravasation & Extravasation	Cancer cells migrate and invade surrounding tissue and intravasate into lymph or blood vessels and extravasate into a new site. Intravasation and extravasation involve the ability of cancer cells to migrate and invade surrounding tissue and vessels.	Migration mediated by activation of Rho GTPases: RhoA, Cdc-42, Rac-1 (also associated with EMT).
		Invasion mediated by secretion of proteases that break down ECM: Matrix Metalloproteinases (MMP), urokinase plasminogen activator (uPA), and plasminogen.
Survival	Cells need to avoid cell death evoked by loss of cellular attachments and survive in vasculature	Activation of survival signals (Ras, AKT, & ERK) inhibition of apoptotic suppressors (p53, Bim) and upregulation of apoptosis inhibitors (BCL-2, BCL-XL)
Angiogenesis	Generation of blood vessels to supply metastasized cell(s) with nutrients needed to grow	Secretion of Vascular endothelial growth factor (VEGF), Platelet derived growth factor PDGF, chemokines, and cytokines.

Sources: [106, 124, 125, 127-129, 131]

SECTION 1.3. RGS16: EXPRESSION, REGULATION, AND FUNCTION

Background

The regulator of G protein coupled receptor (GPCR) signaling (RGS) family is comprised of 37 RGS proteins that regulate GPCR signaling [132]. GPCRs are the largest family of receptors, encoded by more than 2% of the genome [133]. GPCRs are overexpressed in cancer and promote proliferation, migration, metastasis, and angiogenesis [133]. GPCRs mediate cancer progression through activation and downstream signaling of small G proteins [133]. In their inactive state small G proteins consist of a heterotrimeric subunit complex (Gαβγ). Stimulation of GPCRs by ligand binding initiates the exchange of GDP to GTP on Gα by guanine nucleotide exchange factors (GEFs). Now in its active state, Gα dissociates from the Gβγ subunits and both Gα and Gβγ activate downstream signaling events. GPCR signaling is terminated by the hydrolysis of GTP to GDP on the Gα subunit. In the stimulated state the GTPase activity in Gα is lower than what is needed to mediate GPCR signals. RGS proteins function as GTP accelerating proteins (GAPs) by binding to Gα and enhancing the hydrolysis of GTP to GDP [132, 134]. All RGS proteins contain a ~120 amino acid conserved region called the RGS box that is responsible for accelerating GTPase activity of Gα proteins [132, 134].

RGS proteins are divided into 8 subfamilies based on homology and function. The R4 subfamily contains the highest number of members and consists of the smallest (molecular weight) RGS proteins. The R4 proteins contain one RGS box domain and a small number of amino acids on its N- and C- terminals. With the exception of RGS2 ($G\alpha_q$ only) R4 members modulate $G\alpha_i$ and $G\alpha_q$ proteins [132, 134]. The other subfamilies contain domains that assist in the stability, localization, and protein-protein interactions (reviewed [132, 134]). The induction of p53 levels through DNA damage was used to identify RGS16, an R4 family member, as a target gene in several cancer cell lines [135]. We have identified RGS16 as a p53 and pRb cross-

talk candidate using RNA expression profiling (Chapter 2) and expression of RGS16 inhibited the migration and invasion activity in several pancreatic cancer cell lines (Chapter 3). In this next section we will explore the expression, regulation, and function of RGS16.

RGS16 Expression

RGS16 expression is seen in a wide variety of normal and cancerous tissues as shown in Table 1. RNA tissue array analysis found RGS16 mRNA to be highly expressed in the kidney, brain, and lung; moderately expressed in the pancreas, colon, and small intestines, and ovaries; and weakly expressed in the skeletal muscle, liver, and heart [135]. Animal studies have also found wide spread expression of this G protein regulator corresponding to expression patterns seen in human tissue (Table 1.8). Recently aberrant expression of RGS16 has been found in colon (higher), breast (lower), metastatic pancreatic (lower), Burkitt's lymphomas and pediatric high hyperdiploid acute lymphoblastic leukemias (higher) (Table 1.8). Although RGS16 has been found to be aberrantly expressed in several cancer types, there are few reported studies focused on the function of RGS16 in cancer progression.

Table 1.8: Expression of RGS16 in normal and cancer tissue

Tissue with expression of RGS16	Species	Sources
Normal tissues		
Expressed in: kidney, brain, lung, pancreas, colon, small intestine, ovary, skeletal muscles, liver, and heart by RNA tissue array.	Human	[135]
Expressed in immune system: T-lymphocytes, auto immune B-cells,	Mouse	[136-138]
Expressed in Liver: periportal hepatocytes	Mouse	[139]
Expressed in progenitor cells: progenitor pancreatic cells during development and expression initiated in type I and II diabetes models and megakaryocytes	Mouse (pancreas) Human (megakaryocytes)	[140, 141]
Expressed in brain: superchiasmatic nucleus (SCN) and hypothalmus	Mouse	[142]
Expressed highly in retina	Mouse	[143]
Expressed in the heart myocardial myocytes	Rat	[144, 145]
Cancer		
Central neurocytomas: upregulated	Human	[146]
Burkitt's lymphomas and pediatric high hyperdiploid acute lymphoblastic leukemias: upregulated	Human	[147]
Colorectal cancers: upregulated	Human	[148]
Metastatic pancreatic cancer: downregulated	Human	[149]
Breast cancers with chromosomal breakpoints, promoter methylation, and allelic imbalances: downregulated	Human	[150]

RGS16 Regulation

RGS16 expression is modulated by GPCR signaling pathways and other stimuli

In the previous section, expression of RGS16 was shown to be widely expressed in several tissue types. But what regulates RGS16 expression? This section will highlight pathways and stimuli that induce expression of RGS16 and post-translational modifications that modulate its activity. RGS16 is upregulated by a variety of compounds including doxorubicin, retinoic acid, fetal bovine serum (FBS) and several ligands and signaling mediators of GPCRs (Table 1.9). The binding of Lysophosphatidic acid (LPA), Endothelin-1 (ET-1), and Sphingosine 1-phosphate (S1P) to their respective GPCRs increases transcription and expression of RGS16. Upregulation of RGS16 by ET-1 and SP-1 is dependent on RhoA and Rac-1

signaling respectively [145]. The transcription regulator Yin Yang inhibits transcription of RGS16 by induced by FBS, ET-1, and S1P [145].

Upregulation of RGS16 by PKC is hypothesized to induce increase expression of tumor necrosis factor- α (TNF-α) in an ERK dependent manner. TNF-α then signals through its receptor and upregulates RGS16. It is hypothesized that upregulation of RGS16 regulates the GPCRs responsible for T-cell response during inflammation [151]. Doxorubicin, retinoic acid, or histone deacetylase inhibitor (Vorinostat) plus lysine-specific demethylase1 (LSD1) knockdown induce expression of RGS16 in cancer cells. Doxorubicine increases expression of RGS16 through a p53 dependent mechanism, demonstrating for the first time that RGS16 is a transcriptional target for p53 [135].

Post translational modification regulate RGS16 activity and localization

RGS16 transcription is up-regulated by a variety of stimuli; however other mechanisms such as post-translational modification and proteasomal degradation regulate RGS16 activity and protein half-life. Palmitoylation, the addition of palmitic acid to a cysteine residue in the N-terminal domain of RGS16, is integral to the localization and activity of RGS16. Loss of the N-terminal region inhibits RGS16 localization to the membrane and as a consequence the Gα GTPase activity [152, 153]. RGS16 contains two palmitoylation sites at cysteines 2 and 12 of the N-terminus. Mutational studies inhibiting palmitoylation at either site impaired RGS16 GAP activity and prevented RGS16 localization to lipid rafts [154, 155]. Localization of RGS16 to lipid rafts promotes the palmitoylation of cysteine 98 in the RGS box by a protein acyltransferase resulting in the acceleration of RGS16 GAP activity demonstrating the necessity of palmitoylation for RGS16 function [155, 156].

RGS16 activity is also regulated by phosphorylation. Prevention of EGFR induced phosphorylation of Tyr168 in RGS16 diminished its GTPase activity by 30% [157]. Furthermore phosphorylation of RGS16 by Src inhibited RGS16 degradation [158]. However phosphorylation of RGS16 can also impair its function in regulating GPCRs. Phosphorylation of mouse RGS16 on Ser 58 and Ser194 subsequent to GPCR stimulation prevented its ability to accelerate hydrolysis of GTP [159].

RGS16 contains a destabilizing N-terminal residue called N-degron which targets RGS16 for degradation by the N-end rule pathway [160]. The N-end rule was developed to calculate a protein's half-life based upon its N-terminal amino acid sequence [161]. Recognition of the N-degron by the E3 ubiquitin ligases N-recognins marks RGS16 for degradation by the proteasomes [160, 161]. Degradation of RGS16 by the N-end rule pathway demonstrates another layer of RGS16 regulation.

Table 1.9: Regulation of RGS16 transcription

Protein / Stimulus	Effect on RGS16	Sources
Doxorubicin/p53 activation	Increases transcription	[135]
Retinoic acid	Increases expression (Neuroblastoma cell lines)	[162]
Concomitant treatment HDAC (Vorinostat) inhibitor and LSD1 (lysine-specific demethylase1) knockdown	Increased transcription (Triple negative breast cancer cells)	[163]
Carbachol	Increases transcription (Mouse fibroblast cells)	[135]
Fasting	Increases RGS16 transcription (in mouse liver)	[139]
IL-17	Increases transcription (Mice autoimmune B cells)	[138]
Lysophosphatidic acid (LPA)	Increases RGS16 transcription (MCF-7 breast cancer cells)	[164]
Endotoxin (LPS)	Increases transcription (Rat myocardial myocytes)	[144, 165]
FBS	Increases transcription (Rat myocardial myocytes)	[135, 145]
Sphingosine 1-phosphate (S1P)	Increases transcription (Rat myocardial myocytes)	[145]
Endothelin-1 (ET-1)	Increases transcription (Rat myocardial myocytes)	[145]
Yin Yang	Inhibits transcription induced by FBS, S1P, and ET-1 (Rat myocardial myocytes)	[145]
Runx2	inhibits transcription (mouse calvariae progenitors)	[166]

RGS16 Function

Pathways regulated by RGS16

There are 16 known Gα proteins that mediate GPCR signaling. The Gα proteins are broken down into four subfamilies based on function designated as $G\alpha_{i/o}$, $G\alpha_{q}$, $G\alpha_{12/13}$ and $G\alpha_{s}$ [167]. RGS16 regulates GPCR signaling mediated by $G\alpha_{i/o}$ and $G\alpha_{q/11}$ proteins by accelerating GTPase activity [134]. $G\alpha_{i/o}$ proteins are so named because they inhibit adenyl cyclase activation preventing generation of cAMP [167]. These proteins activate phospholipase Cβ

(PLCβ) which hydrolyzes PIP_2 (phosphoinositide 4,5-bisphospate) to DAG (diacyl glycerol) and IP3 (inositol 1,4,5 triphosphate) [167]. DAG and IP3 activation results in activation of PKC-ε (protein kinase C-ε) and mobilization of Ca^{2+} respectively [167]. However, the Gβγ complex also mediates downstream signaling that is deactivated by the hydrolysis of GTP on Gα by RGS16 [135]. An example of this is the loss of MAPK activation induced by Gβγ of M1 and M2 muscarinic receptors due to RGS16 GAP activity [135].

RGS16, as seen in Table 1.9, is regulated by mitogenic signals and analogous to other RGS proteins, functions in a negative feedback loop to inhibit GPCRs that induce their expression. Examples of this negative regulatory process can be seen for the GPCRs of LPA and ET-1 ligands. Binding of LPA to its receptor (LPA_1) increases expression of RGS16 which then inhibits activation of RhoA and serum response element dependent transcription induced by LPA [164]. ET-1 was previously shown to induce expression of RGS16, however this increased expression inhibits activation of PLCβ by ET-1 [145, 165]. Table 1.10 contains on overview of RGS16 targets and effects on cell signaling.

Table 1.10: Effect of RGS16 on downstream events

Target of RGS16	Effect	Sources
LPA	↓ RhoA activation & ↓ transcription of Serum response element	[164]
ET-1	↓ activation of PLCβ	[165]
Platelet Activating Factor	↓ activation of p38 MAPK	[168]
GPR39	↓ of survival & ↓ pigment epithelium-derived growth factor (PEDF)	[169]
Muscarinic receptor	↓ activation of ERK/MAPK	[135]
Chemokines (CxCR4, CCR10, CCR3, CCR5, and CCR4)	↓ migration in megakaryocytes, B cells, and T-lymphocytes	[136, 137, 141, 170]
EGF/EGFR	↓ proliferation & ↓ activation of PI3K/AKT pathway	[171]

Non-canonical functions of RGS16

RGS proteins also have non-canonical functions, ergo, regulation of cell signaling independent of the RGS box (Reviewed in [172]). Two examples of RGS16 functioning in a non-canonical fashion are the regulation of LPA induced activation of RhoA and inhibition of EGF/EGFR phosphorylation and activation of PI3K. LPA, as previously mentioned, stimulates expression of RGS16, which with a negative feedback loop inhibits LPA induced activation of RhoA. LPA is a GPCR that mediates its signaling through Gα12/13, which is not a target for RGS16 regulation.

RGS16 regulates LPA signaling events by binding to Gα13 independently of its RGS box and sequestering this subunit to the lipid rafts. The exile of Gα13 by RGS16 inhibits Gα13 mediated activation of RhoA and serum response elements [164].

RGS16 can also inhibit EGF/EGFR signaling. Knockdown of RGS16 in breast cancer cells increased proliferation induced by EGF or FBS and increased expression of RGS16 inhibited EGF and FBS induced proliferation [171]. RGS16 binds to the p85α subunit of PI3K preventing recruitment of PI3K to adapter proteins attached to EGFR thereby inhibiting PI3K mediated phosphorylation and activation of AKT [171]. GPCRs can activate tyrosine kinase receptors such as EGFR and together these two proteins induce MAPK activation [173]. Regulation of EGFR signaling by RGS16 is another mechanism by which RGS16 inhibits mitogenic signals initiated by GPCRs.

RGS16 and cell migration

Several studies have shown a role for RGS16 in inhibiting cell migration. Trafficking or migration of T lymphocytes, B cells, and megakaryocytes induced by chemokine GPCRs are inhibited by RGS16 [136, 137, 141, 170]. RGS16 inhibits migration of megakaryocytes and

activation of MAPK and AKT by the SDF-1/CxCR4 chemokine pathway [141]. Expression of RGS16 in the lymphocytes of a transgenic mice inhibited CxCR4, CCR3, and CCR5 allergen induced migration to the lung parenchyma [137]. Conversely, in RGS16 knockout mice there are decreases in T helper type 2 and 17 cell trafficking through regulation of CCR4 and CCR10 chemokine pathways [136]. These studies also show the importance of RGS16 in immune response and trafficking. Several studies have used knockout mice to delineate the function of RGS16, but it should be noted that there is no observable phenotype for mice lacking RGS16 [174].

RGS16 and Cancer

RGS16 is aberrantly expressed in some cancer types (Table 1.8). However, the role of RGS16 in cancer progression remains unclear. RGS16 is upregulated in colon, central neurocytomas, Burkitt's lymphomas, and pediatric high hyperdiploid acute lymphoblastic leukemia (Table 1.8). Upregulation of RGS16 is due to mitogenic signals. The question remains whether RGS16 is a reporter for GPCR signaling or does it indeed have a cancer promoting function in these cancer types? Studies investigating the function of RGS16 in these cancer types will determine if it promotes cancer progression or if post-translational modifications can affect its function. Furthermore post-translational modifications can affect the function of RGS16 and should also be investigated in these cancer types.

Recent research suggests RGS16 may specifically have tumor suppressor function in breast and pancreatic cancer. Figure 1.6 depicts the signaling pathways that RGS16 can regulate demonstrating its potential as a tumor suppressor. As previously mentioned RGS16 inhibited EGF and FBS induced cell proliferation by blocking the PI3K/AKT pathway. RGS16 knockout breast cancer cells are more resistant to tyrosine kinase inhibitor treatments [171]. Loss of

RGS16 occurs in metastatic pancreatic cancer and is associated with decreased patient survival suggesting RGS16 may inhibit the metastatic process [149]. We have found in our *in vitro* studies that RGS16 is co-regulated by p53 and pRb and inhibits pancreatic cancer cell migration and invasion (Chapters 2 & 3 and [175]). Furthermore, increased expression of RGS16 is induced by retinoic acid and the down regulation of RGS16 with another retinoic regulated protein (DUSP6) disrupted retinoid inhibition of neuroblastoma growth [162]. Combined treatment of triple negative breast cancer cells with a HDAC inhibitor and knockdown of LSD1 induced RGS16 expression that was vital for HDAC induced cytoxicity, downregulation of NF-κB, and expression of E-cadherin, ING1, and CDKN1C, all of which have tumor suppressor function [163].

Several of the GPCRs regulated by RGS16 (Table 1.10) have been implicated in promoting angiogenesis, proliferation, and metastasis including M1 and M2 muscarinic receptors, Platelet-activating factor (PAF), LPA, CxCR4, and ET-1 [133, 176, 177]. These pathways are prime targets for future research regarding the role of RGS16 in cancer and will aid in identifying possible tumor suppressive functions of RGS16.

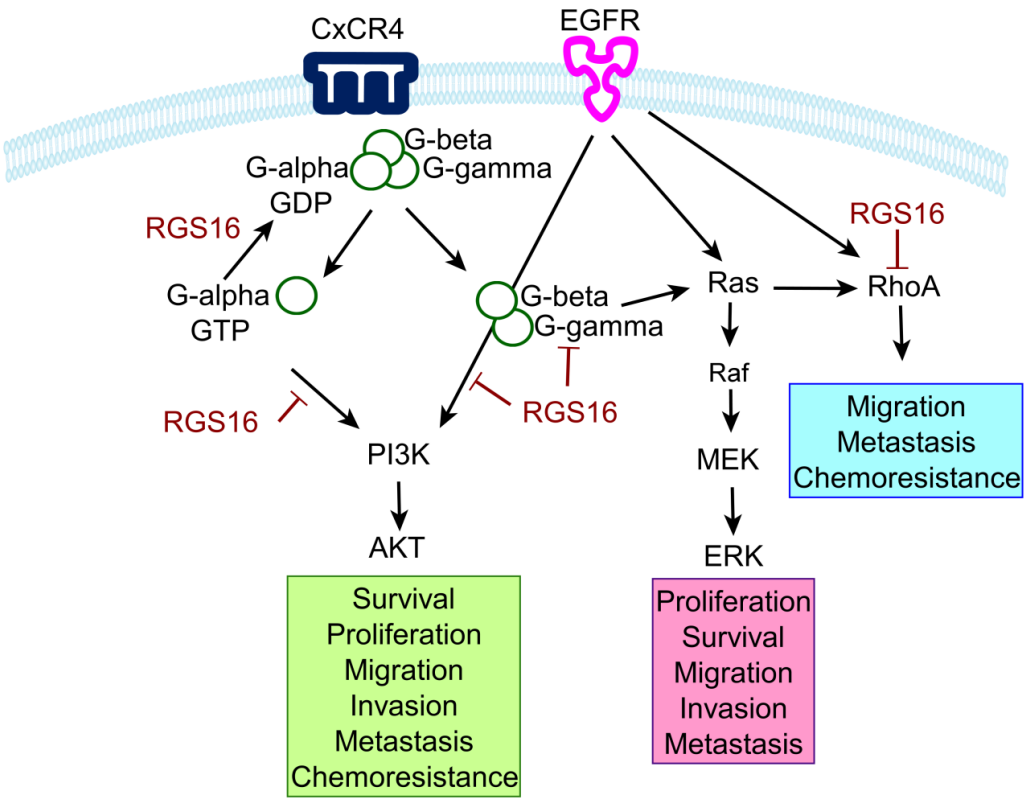


Figure 1.6: Known RGS16 regulation targets and downstream events that promote cancer progression. These pathways can be activated by a variety of signals including through EGFR which can also be regulated by RGS16. CxCR4 is a prototypical representation of a GPCR that is involved in promoting cancer and is regulated by RGS16. Pathway was constructed using Qiagen's Ingenuity pathway Analysis (IPA®, QIAGEN

CHAPTER 2.

EXPRESSION PROFILING ANALYSIS OF WI38 NORMAL LUNG FIBROBLASTS FOLLOWING CO-EXPRESSION OF p53 AND pRb[1]

SUMMARY

Mutations in p53 or *RB1* (Retinoblastoma) genes, their upstream regulators, or downstream effectors have been found in almost all human malignancies. Evidence suggests that p53 and pRb cooperate to suppress tumorigenesis, but does not elucidate the extent to which p53 and pRb cross-communicate to regulate cellular functions. RNA expression profiling was performed on normal human lung fibroblast WI38 cells following overexpression of p53 and/or pRb, to identify genes co-regulated and involved in mediating p53 and pRb tumor suppressor processes. The goals of the analyses were to investigate the cross-talk between p53 and Rb proteins and to find downstream effectors regulated by these two tumor suppressor genes. This knowledge might be used in the development of novel anti-cancer treatments. Microarray analyses performed on WI38 cells overexpressing p53, pRb, or both p53 and pRb identified 294-p53, 650-Rb, and 514-p53/Rb differentially regulated transcripts compared to vector control. By examining the intersecting genes, we generated lists of p53 and pRb cross-talk candidates. Several of the cross-talk candidates are known to be regulated by p53 and/or pRb. Five differentially expressed transcripts were chosen for validation by quantitative Real-Time PCR (qRT-PCR) in WI38 cells and in the p53 negative and *RB1* mutated SAOS-2 cells. Ingenuity Pathway Analysis (IPA) identified an enrichment of transcripts involved in cellular movement, development, cellular growth and proliferation among others in the WI38 cells overexpressing

[1] A portion of this work has been submitted and accepted for publication in the journal Genes and Cancer (see Appendix for draft) or [175]..

p53 and pRb. To our knowledge, this is the first time microarray analyses have been used to identify putative p53 and pRb cross-talk candidate genes. The identity of these genes can be used in future studies on specific cancer types to better understand how p53 and pRb regulate cellular functions to prevent tumorigenesis. Deeper knowledge of the p53 and pRb cross-talk pathway may aid in identifying new molecular targets, developing better therapies for cancer, and providing more information on the coordination of tumor suppressor mechanisms by p53 and pRb.

INTRODUCTION

The p53 and pRb tumor suppressors are two signaling molecules that are frequently altered during cancer progression. Mutations that disrupt the p53 and pRb function can occur in the gene sequences or in their upstream regulators and/or downstream effectors [178]. Both tumor suppressor genes are inactivated in a variety of malignancies including osteosarcoma, small cell lung, breast, and bladder carcinomas [1, 4, 45, 46]. Furthermore, alterations in expression or activity of proteins involved in p53 and pRb signaling pathways have been identified in retinoblastoma and cancers of the pancreas, colon, and head and neck among others [49, 51, 52, 111]. The large number of cancers that have defects in the p53 and pRb pathways demonstrates the importance of these signaling modules in preventing cancer development and progression.

Both p53 and pRb regulate processes vital for the suppression of cancer progression, such as cell cycle arrest, apoptosis, senescence, and differentiation [4, 73, 179]. Existing data suggests that p53 and pRb cooperate to prevent tumor progression. Examples of this cooperative interaction have been shown in various studies using human primary cancer samples and mouse models. Patients who have mutations in both p53 and *RB1* genes have increased tumor

recurrence and decreased survival compared to patients with a mutation in either p53 or *RB1* [45, 47, 180]. Studies conducted in primary tumor samples and established cell lines found that inactivation of both p53 and pRb signaling pathways promotes processes that support cancer progression such as chromosome instability, chemoresistance, and the Epithelial Mesenchymal Transition (EMT) [60, 62, 181]. Mice that are p53-/- and also heterozygous for *RB1* developed more tumors than mice with single mutations; i.e. heterozygous p53 or *RB1* null or p53 null with w.t. RB1 [4]. In another study, mice with conditional inactivation of both p53 and *RB1* in prostate epithelium developed highly metastatic tumors and had decreased survival time compared to mice with single inactivation of either p53 or *RB1* [5]. The accumulated evidence suggests p53 and *RB1* gene products have cooperative or synergistic effects for cancer suppression.

Considering the network of communication that exists within a cell, the rate of mutation of p53 and *RB1*, and the cellular processes these two proteins regulate, a natural hypothesis is that these two genes and respective gene products cross-communicate in order to determine cellular fate and prevent carcinogenesis. Over the years, data have accumulated that paints a picture of the communication that exists between the p53 and pRb pathways. The cell cycle stimulating transcription factor E2F protein provides a prime example of the link between the p53 and pRb pathways. In its hypophosphorylated form pRb binds to E2F family members (E2F1, E2F2, or E2F3) and acts as a transcriptional repressor to inhibit the transcription of genes needed for the continuation of the cell cycle [18-20]. Cyclin-dependent kinases-4 and -6 phosphorylate pRb resulting in the release of E2F from pRb binding thus allowing this factor to stimulate the transcription of genes encoding cell cycle stimulatory proteins [19, 21]. An increased amount of unbound E2F due to loss of *RB1* or alteration in the pRb pathway can

trigger a p53-dependent apoptotic response [74, 78]. The interaction between E2F1 and p53 to initiate apoptosis demonstrates a protective mechanism employed by the cell to prevent cancer development when pRb regulation is lost. E2F1 is not the only protein known to be involved in the convergent signaling between the p53 and pRb pathways; other proteins known to be implicated in p53 and pRb cross-talk are Hdm2, p21, and the INK4a locus (reviewed in [3, 47, 182]). Although several proteins that are involved in the p53 and pRb pathways have been identified, the full extent in which these two tumor suppressors interact along their pathways to regulate cellular fate is still unknown. Current data and analyses have only begun to elucidate the proteins involved between p53 and pRb mediated cancer suppression. The focus of this study was to identify signaling molecules involved in the p53 and pRb cross-talk pathway and to provide leads to the nature of downstream effector molecules responsible for inducing p53 and pRb mediated cancer suppression. Identification of p53 and pRb downstream effectors will provide new targets for future anti-cancer therapies.

Materials and Methods

Cell culture and virus transductions

The human lung fibroblast WI38 cell line and the osteosarcoma cell line SAOS-2 (p53 null and truncated *RB1*) were purchased from the American Type Culture Collection (Manassas, VA, USA). WI38 cells were grown in Hyclone MEM/EBSS (ThermoFisher Scientific, Waltham, MA) media supplemented with 10% research grade fetal bovine serum (FBS) (ThermoFisher Scientific, Waltham, MA) and 1% Penicillin Streptomycin (Corning, Corning, NY) and SAOS-2 cells were grown in Hyclone High Glucose DMEM (ThermoFisher Scientific, Waltham, MA) supplemented with 10% FBS and 1% Penicillin Streptomycin. Cells were cultured at 37°C in a humidified 5% CO_2 incubator.

Ad.CMV (adenovirus with CMV promoter) and Ad.CMV.p53 (Adenovirus containing wild-type p53 gene under control of CMV promoter) viral vectors were generated using the AdEasy system (Carlsbad, CA). The Ad.CMV.pRb (Adenovirus containing *RB1 gene* cDNA under control of CMV promoter) vector was provided by Dr. Juan Fueyo (M.D. Anderson Cancer Center, The University of Texas). Viruses were amplified and tittered as previously described [183-185].

Microarray expression profiling

For expression profiling, WI38 cells were transduced with each of the following vectors or vector combination: (1) adenovirus vector with no insert (Adenoviral CMV-vector ctrl), (2) Ad.CMV.p53, (3) Ad.CMV.pRb, and (4) both Ad.CMV.p53 and Ad.CMV.pRb. Vectors were added at a multiplicity of infection (MOI) of 50 to confluent WI38 cells (500,000 cells / 100mm plate) in MEM/EBSS media supplemented with 2% heat-inactivated FBS. Culture media were replaced with 10% FBS and 1% Penicillin/Streptomycin supplemented MEM/EBSS medium 16 hours after vector addition; cells were collected after 48 hours. Four biological replicates were performed for each of the four expression studies. Immunoblots were used to verify increased expression of p53 and/or pRb in the WI38 samples prior to microarray analysis.

Total RNA was isolated from transduced WI38 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according the manufacturer's protocol. Using a universal reference design, two RNAs (transduced WI38 cells + Agilent (Santa Clara, CA) human universal reference RNA) were hybridized to Agilent 44K whole human genome expression arrays. Total RNAs were labeled with either cyanine (Cy)-3-CTP and Cy5-CTP (Perkin Elmer, Waltham, MA) using Agilent QuickAmp cRNA labeling kits. Following purification, Cy3- and Cy5-labeled cRNAs

were combined and hybridized for 17 hours at 65ºC in an Agilent hybridization oven. Microarrays were then washed and scanned using Agilent DNA Microarray Scanner.

Statistical Analysis of Expression Profiling Data

Lowess-normalized feature intensities were extracted from the scanned image using Feature Extraction (Agilent). These data were exported as tab-delimited files (one file per sample) to Microsoft Excel® for filtering. For each feature, data were removed if both channels reported values not well-above background according to default Feature Extraction Criteria. For each comparison, log base-2 ratios of each sample to universal reference RNA were collated into a single table. Features for which fewer than 50% of all samples had a present value were removed from further analysis.

The resulting tables were imported into Multiple Experiment Viewer (MEV) v4.3. Log base 2 ratios were compared between each of three sample sets (p53 overexpressed samples. *RB1* overexpressed samples and p53 and *RB1* overexpressed samples) and the adenovirus vector control samples by Significance Analysis of Microarrays [186]. We used a conservative threshold whereby only genes for which MEV reported a false discovery rate of 0% were considered significantly differentially expressed.

Data extracted using Feature Extraction was uploaded to the NCBI's Gene Expression Omnibus (GEO) public database and is available via access number GSE59660.

Ingenuity Pathway Analysis

The functional analyses were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). The accession number and fold change of differentially expressed mRNA identified by RNA expression profiling were uploaded into Ingenuity Pathway Analysis (IPA) software for functional analysis.

The gene transcripts identified in the microarray were categorized based on their molecular and cellular function in order to identify pathways that are being altered or enriched by p53 and pRb signaling, however, information regarding disease and physiological system development were also reported (Doc. SI2 or Doc. SI3). Benjamini-Hochberg multiple correction p-values were computed for the null hypotheses that genes with altered mRNA levels are independent of molecular and cellular functions, and functions with a corrected p-value less than 0.05 were considered statistically significant. Pathways examining known relationships between the cross-talk candidates and p53, pRb, and E2F1-3 were generated using IPA Knowledge Base and Pathway designer tools (grow and connect).

Quantitative Real-time PCR analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA (2ug) was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit from Applied Biosystems (Foster City, CA) according to the manufacturer's protocol. Real-Time PCR was performed using the Applied Biosystems TaqMan Gene Expression Assays in the ABI 7000 detection system (Foster City, CA). TaqMan probes were purchased from Applied Biosystems (Foster City, CA) IL-6 (HS00197982_m1), BCL2L11 (BCL2L11) (HS00197982_m1), RGS16 (HS00892674_m1), BTG2 (HS00198887), STAT4 (HS00231372_ml) and GAPDH (HS02758991). The relative fold change for each marker was calculated using the $2^{-\Delta\Delta CT}$ analysis according to Livak *et.al* and statistical significance was determined using a one way ANOVA with a Dunnett's post-hoc test, using Prism V6.0c (GraphPad Software, Inc., La Jolla, CA) *[187]*.

Western blot analysis

WI38 or Saos-2 cells were lysed in whole cell lysis buffer containing 50mM Tris-HCl (pH7.4), 5mM EDTA 250mM NaCl, 50mM NaF, 0.1mM Na_3VO_4, 0.1% Triton X-100 and protease inhibitors (Pierce Protease inhibitor Tablets 88661; Thermo Scientific, Rockford, IL). Protein extracts (50ug, measured using Bradford protein assay) were loaded onto 8% polyacrylamide gels and proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Blots were blocked 1 hour in 5% dry non-fat milk diluted in Tris-buffered saline solution containing 0.1% Tween-20 pH 7.6 (TBS-T). Membranes were probed overnight at 4°C with mouse anti-p53 (SC-DO1, 1: 1000) or mouse anti-pRb (SC-IF8, 1:500) antibodies from Santa Cruz Biotechnology (Dallas, TX). Following primary antibody incubation the membranes were washed using TBS-T (three times; 5 minutes) and probed with Horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:5000) secondary antibodies (Rockland, Gilbertsville, PA) for 1 hour at room-temperature. Primary and Secondary antibodies were diluted in TBS-T. Blots were washed 5 minutes in TBS-T three times and Amersham ECL prime western blotting detection reagent was added in order visualize the protein bands (RPN 2232, GE Life Sciences, Pittsburgh, PA). Western blot images were captured using FOTODYNE FOTO/Analyst FX (Hartland, WI) imaging camera. Membranes were normalized using mouse anti-actin (1:1000).

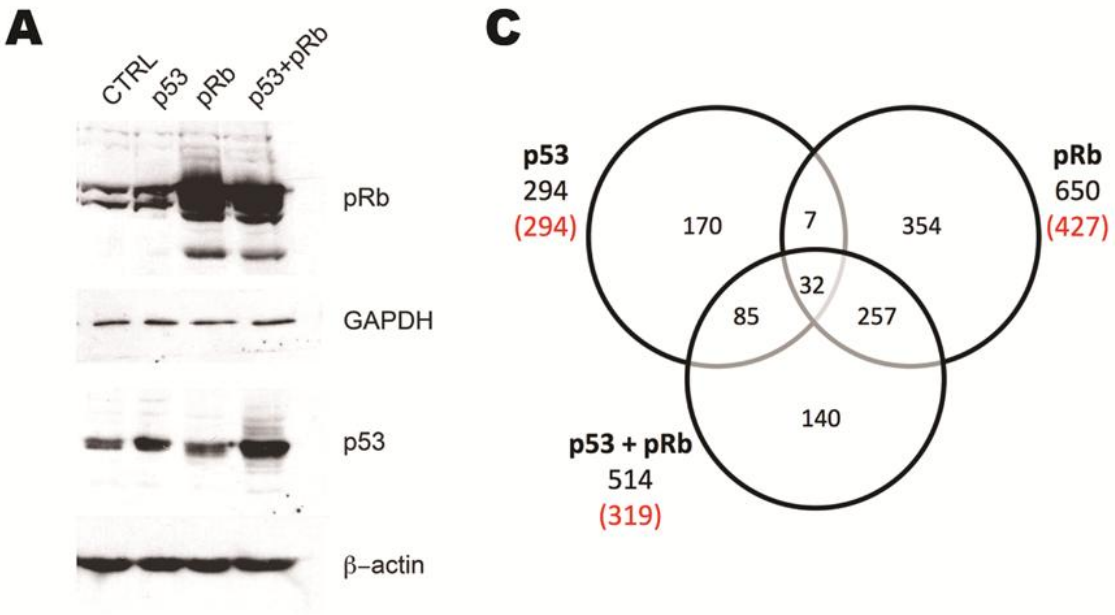
RESULTS

Identification of p53 and pRb cross-talk candidates in WI38 cells following exogenous expression of p53 and/or pRb.

Studies have shown that p53 and pRb cooperate to prevent tumorigenesis. Currently, the molecules that function in the p53 and pRb cross-talk pathway to regulate cellular fate are not

known thus expression profiling by microarray was performed to find genes co-regulated by p53 and pRb. Normal human lung WI38 fibroblast cells were transduced with adenoviral vectors expressing the p53 and/or RB1 genes under the control of a cytomegalovirus (CMV) promoter. The WI38 cell line was used because it is from non-cancerous tissue and lacks mutations or viral transformations that could disrupt the p53 and pRb pathways. Four experimental conditions were used in which WI38 cells were transduced with adenovirus vector control (cond. 1, Adenoviral CMV-vector control, Ad.CMV.p53 (cond. 2), Ad.CMV.pRb (cond. 3), or both Ad.CMV.p53 and Ad.CMV.pRb (cond. 4). RNA and protein from WI38 cells was collected 48 hours after adenoviral infection. Immunoblots verified increased expression of p53 (fold change compared to Ad.CMV control = 2.80, 1.54, and 2.77) and/or hypophosphorylated (active form) pRb (hypophosphorylated/total pRb fold change compared to Ad.CMV control = 0.94, 5.48, 5.02) in the WI38 cells treated with adenoviruses containing p53, pRb, or both p53 and pRb respectively (Figure 2.1A). Fold change values for p53 and hypophosphorylated pRb coincided with previously reported results in experiments that activated endogenous p53 and pRb [30, 31]. Microarray data from the adenovirus vector control (empty vector with CMV promoter) was used as a reference to determine genes that were differentially expressed as a consequence of p53, pRb, and p53 + pRb expression. Analysis of the microarray data identified 294-p53, 650-pRb, and 514-p53 + pRb differentially expressed genes (Figure 2.1B). Of the differentially expressed genes, 294/294 genes were upregulated in cells with p53 expression, 427/650 genes were upregulated in cells with pRb expression, and 319/514 genes were up-regulated in cells with p53 + pRb coexpression (Figure 2.1B). Consistent with protein measurements, increased expression of p53 and/or RB1 mRNAs were also found in the appropriate groups (data not shown).

A Venn diagram shows the number of differentially expressed genes shared between the experimental groups (Figure 2.1B). By looking at the common genes between the three experimental groups, we were able to generate two lists of genes that may be involved in the p53 and pRb cross-talk pathway. The first list of cross-talk candidates (designated as the p53 and pRb common gene set) consisted of 39 genes found to be commonly up-regulated in cells expressing either p53 or pRb. The second list of possible cross-talk members (designated as the p53 and pRb interaction gene set) contained 140 genes that were found to be differentially expressed only when p53 and pRb were overexpressed together (see Addendum for Chapter 2 for p53 and pRb interaction gene set). Thirty-two of the 39 common gene set cross-talk candidates were found to be up-regulated in the interaction gene set, while the remaining 7 were commonly up-regulated in cells that overexpress either p53 or pRb (Table 2.1). By focusing on the common and interaction gene sets, we were able to remove transcripts that were up- or down-regulated by only p53 or pRb and focus on candidates that may be involved in the p53 and pRb cross-talk pathway.



B

Normalized fold change

	CTRL	p53	pRb	p53 + pRb
p53	1.00	2.80	1.54	2.77
hypophosphorylated pRb/total pRb	1.00	0.94	5.48	5.02

Figure 2.1: Identification of differentially expressed transcripts in WI38 cells overexpressing p53 and/or pRb. WI38 cells were transduced with adenoviruses carrying the transgenes p53, or *RB1*/p105; a MOI of 50 was used in each case. A) Western blot analysis was used to test for p53 and pRb expression prior to microarray analysis. B) Fold change of protein expressions compared to CMV control. C) A Venn diagram shows the differentially expressed transcripts and intersects identified during the microarray analysis. The numbers in red denote transcripts that were up-regulated due to p53, pRb, or p53 and pRb expression.

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Table 2.1: Fold change of p53 and pRb common gene set cross-talk candidates

Gene Symbol	Name	FC-p53	FC-Rb	FC-p53+Rb
LOC387763	hypothetical LOC387763	30.62	159.25	297.07
A_24_p775812	Unknown	15.65	199.36	252.80
RGS16	Regulator of G-protein signaling 16	18.84	30.82	149.75
AREG	Amphiregulin	8.15	46.41	81.91
CCL3	Chemokine (c-c motif ligand 3)	3.78	8.18	56.12
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	10.68	69.61	53.34
IL-1B	Interleukin-1 beta	4.82	22.53	43.79
OLFM2	Olfactomedin 2	10.60	37.76	27.31
NR4A1	Nuclear receptor subfamily 4 group A member 1	11.56	20.73	27.08
POSTN	Periostin	2.91	21.051	25.66
D4S234e	D4S234e (NSG1; neuron specific gene family member 1)	21.20	7.86	22.06
IL-6	Interleukin-6	6.04	12.37	21.99
DMN	Desmuslin	4.57	27.42	21.25
EPPK1	Epiplakin	32.06	8.27	20.04
IQSEC3	IQ motif and Sec7 domain 3	7.29	20.37	19.95
PLAC2	Placenta specific 2	21.60	4.63	19.00
L3MBTL2	Lethal(3)malignant brain tumor-like protein 2	18.33	11.82	16.00
LHX6	LIM homeobox 6	10.11	7.11	15.15
AKR1B10	Aldo-keto reductase family 1 member B10	11.61	13.92	13.30
RRAD	Ras associated with diabetes	5.98	7.80	12.61
c10orf58	chromosome 10 open reading frame 58	4.86	9.20	11.74
BCL2L11	Bcl2-like 11 (apoptosis facilitator)	9.58	6.50	11.34
COL7A1	Collagen, type VII, alpha 1	5.97	10.65	10.94
JUP	Junction plakoglobin	7.60	16.61	9.92
VCAN	Versican proteoglycan	5.61	9.17	9.73
CRISPLD2	Cystein-rich secretory protein 11	10.11	5.38	9.55
STOX2	Storkhead-box 2	14.48	8.70	9.33
BTG-2	B-cell translocation gene 2	3.85	5.07	7.46
P2RY2	purinergic receptor P2Y, G-protein coupled, 2	2.38	19.53	6.91
TSKU	Tsukusi,small leucine rich proteoglycan	5.28	5.42	5.97
C4B	Complement component 4B	3.38	7.64	2.22
RTN4R	Reticulon 4 receptor	8.01	6.19	N/A
STAT4	Signal transducer and activator of transcription 4	5.98	7.80	N/A
AK124344	cDNA FLJ42353 fis, clone UTERU2007520	5.21	7.13	N/A
KLHL20	Kelch like 20	4.88	4.46	N/A
NOTCH3	Notch homolog 3	4.68	3.96	N/A
KSR1	Kinase suppressor of RAS	3.86	4.08	N/A
GDF15	Growth/differentiation factor 15	3.24	3.40	N/A
LOC654346	similar to galectin 9 short isoform (LOC654346)	2.81	4.77	N/A

FC = Fold change

N/A= Fold change not available. Gene was not found to be significantly differentially expressed in WI38 cells overexpressing p53 and pRb.

qRT-PCR validation of microarray data in WI38 and SAOS-2 cells

The ultimate goal in performing the microarray analysis was to determine molecules involved in the p53 and pRb cross-talk pathway in order to identify and study downstream effector molecules that can be expressed to induce a p53 and/or pRb tumor suppressive function. Because of our interest in identifying downstream effector molecules, we chose five mRNA transcripts (IL-6, BTG-2, STAT4, RGS16, BCL2L11) from the set of 39 commonly up-regulated transcripts by p53 and pRb for validation via qRT-PCR. IL-6, BTG-2, STAT4, RGS16, and BCL211 were chosen for validation because of vaying function, known regulation by p53 and pRb, and fold change values of the expression profiling assay. WI38 cells were plated and transduced with adenoviral expression vectors via the same methods used for the microarray analysis. Relative fold change was calculated for IL-6, BTG-2, STAT4, RGS16, and BCL2L11 in WI38 cells expressing p53 and/or pRb as shown in Figure 2.2. Statistically significant up-regulation of all transcripts tested except BCL2L11 was found in WI38 cells expressing p53 and pRb confirming the microarray results. Expression of p53 and pRb in WI38 cells increased mRNA expression for some of the transcripts (for example, RGS16 and BTG-2) to a greater extent than single expression of either p53 or pRb. This suggests p53 and pRb are working together resulting in an additive (i.e. BTG-2) or synergistic (i.e. RGS16) effect on mRNA expression for some of the transcripts.

To further support the RNA expression profiling results, we repeated the expression of p53 and pRb in a p53 null, *RB1* mutant osteosarcoma cell line (SAOS-2) and performed qRT-PCR analysis of IL-6, BTG-2, STAT4, RGS16, and BCL2L11. The expression of all five transcripts including IL-6 and BCL2L11 were found to be significantly increased by one-way ANOVA compared to vector control in SAOS-2 cells expressing p53 and/or pRb (Figure 2.3).

Dunnett's test for multiple comparison found BCL2L11 expression to be significantly increased in cells expressing p53, pRb, and both p53 and pRb and IL-6 was found to be significantly increased in cells expressing pRb and p53+pRb. Expression of IL-6 was not found to be statistically significant in SAOS-2 cells expressing p53 due to variation between replicates (fold change= 2.86). All five transcripts were found to be up-regulated when p53 and/or pRb were expressed in the microarray analysis and qRT-PCR analysis showed similar results in WI38 and SAOS-2 cells.

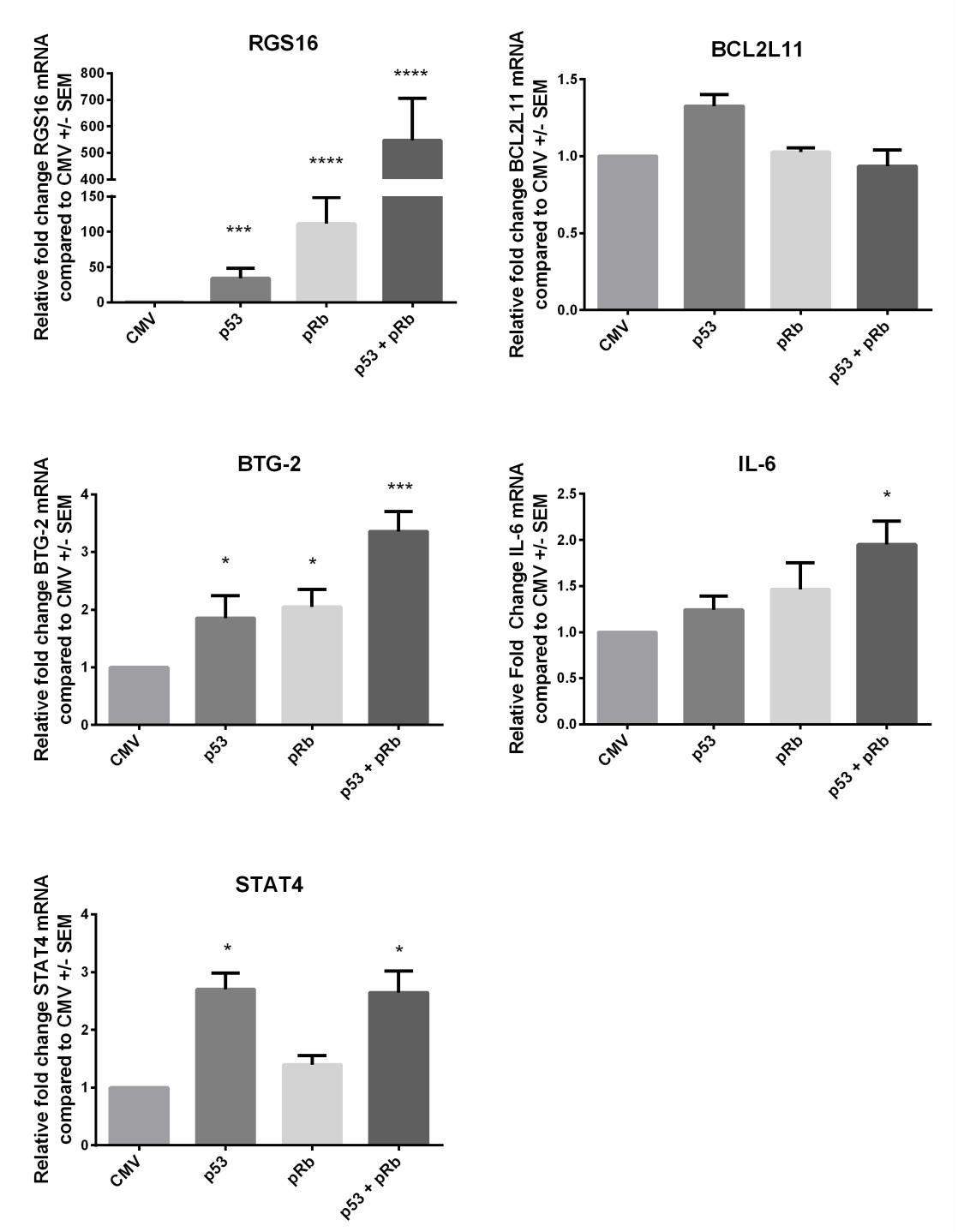

Figure 2.2: Validation of microarray data using qRT-PCR in WI38 cells.
Five transcripts RGS16, BCL2L11, BTG2, IL-6 and STAT4 from the p53 and pRb intersect were chosen for validation by qRT-PCR in WI38 cells overexpressing p53, pRb, or both p53 and pRb. The vector control (Ad.CMV) was used to calculate the fold change for each transcript. One-way ANOVA with Dunnett's test for multiple comparison were used to test for statistical significance * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, and **** p-value < 0.0001.

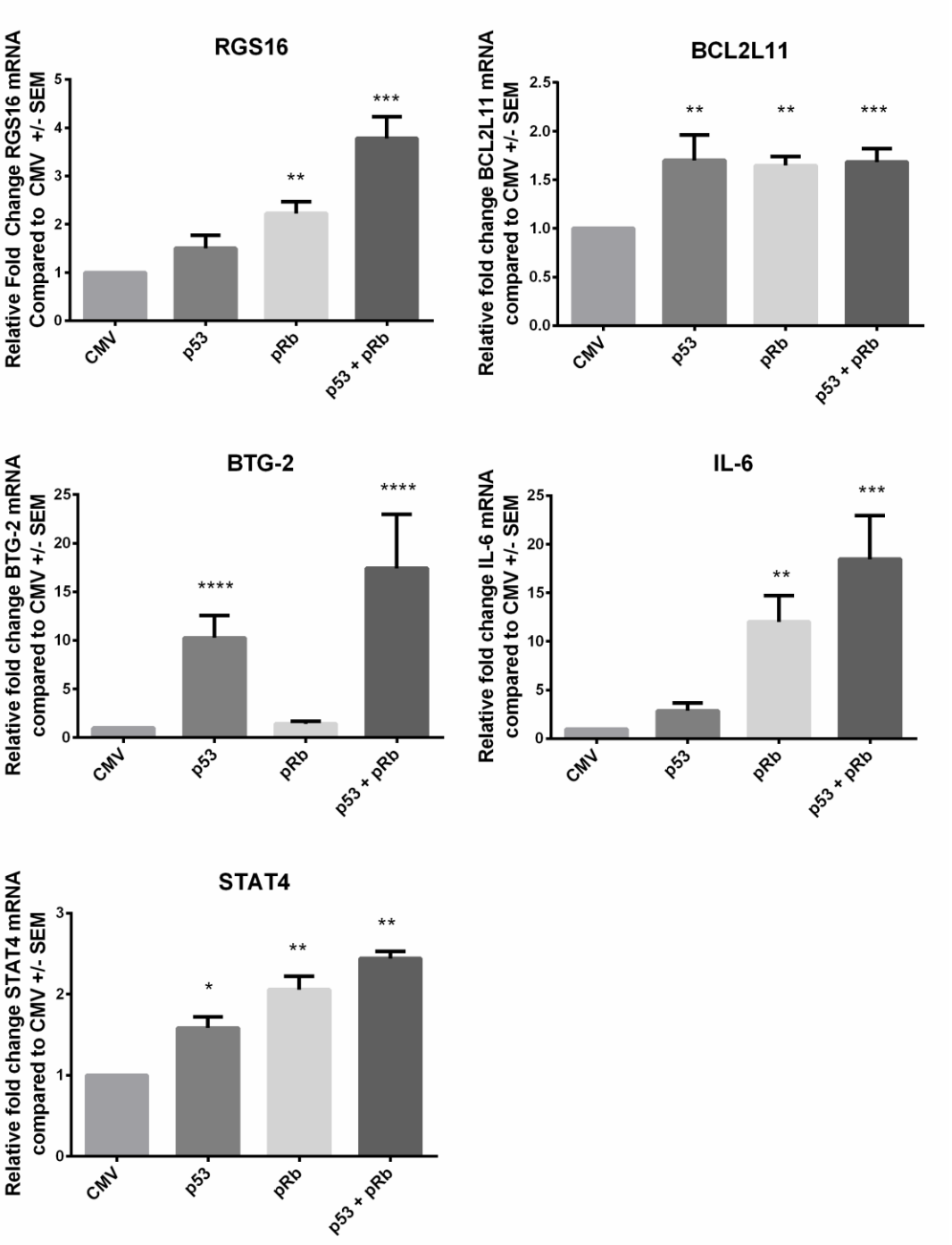

Figure 2.3: Validation of microarray data using qRT-PCR in SAOS-2 cells.
Five transcripts RGS16, BCL2L11, BTG2, IL-6 and STAT4 from the p53 and pRb intersect were chosen for validation by qRT-PCR in SAOS-2 cells overexpressing p53, pRb, or both p53 and pRb. The vector control (Ad.CMV) was used to calculate the fold change for each transcript. One-way ANOVA with Dunnett's test for multiple comparison were used to test for statistical significance * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, and **** p-value < 0.0001.

Ingenuity Pathway Analysis of differentially expressed transcripts in WI38 cells following overexpression of p53 and/or pRb.

The web-based Ingenuity Pathway Analysis software (IPA) was used to perform functional analysis on the WI38 expression profiles. Differentially expressed mRNA transcripts with known functions are eligible for IPA analysis. Eligible transcripts for each overexpression were as follows: p53: 263/294, pRb: 533/650, and p53 + pRb: 441/514). These gene sets were loaded into IPA for global functional analysis to predict biological functions that may be activated or inhibited due to p53 and pRb signaling. Lists of the significant molecular and cellular functions found to be statistically overrepresented by *p*-value (Benjamini-Hochberg correction after Fisher's exact test) due to overexpression of p53, pRb, and both p53 and pRb based on IPA annotations are shown in Figures 2.4-2.6. Cellular development was the top overrepresented molecular and cellular function in WI38 cells overexpressing p53 followed by cellular movement and cell-to-cell signaling and interaction (Figure 2.4). Transcripts associated with cell cycle regulation functions such as cell cycle, cellular assembly and organization, and DNA replication, recombination, and repair were enriched in WI38 cells overexpressing pRb (Figure 2.5). WI38 cells overexpressing both p53 and pRb showed enrichment in transcripts associated with cellular growth and proliferation, cell cycle, cell death and survival, and development (Figure 2.6). Similar cell and molecular functions were found to be overrepresented in WI38 cells overexpressing p53, pRb, and p53 + pRb. Transcripts involved in cellular development, cellular growth and proliferation, cell death and survival, and cellular movement were found to be differentially expressed in all three experimentally conditions with differences in order of significance between groups. Genes involved in DNA replication, recombination and repair biological functions were not found to be significantly differentially expressed in WI38

cells overexpressing p53 but were significantly enriched in cells overexpressing pRb and p53 + pRb. There was also enrichment in genes involved in cell cycle regulation in all three experimental conditions, however, in WI38 cells overexpressing p53, cell cycle was the twelfth significant biological pathway. The differences in biological functions found to be enriched by IPA highlights the various pathways regulated by p53 or pRb.

Because of my interest in discovering p53/pRb cross-talk mechanisms, IPA functional analysis was also performed on IPA-eligible genes from both cross-talk candidate gene subsets (p53 and pRb common (36/39) and interaction (104/140) gene sets combined and separately) identified by RNA expression profiling. IPA functional analysis on all cross-talk candidates (combined cross-talk gene subsets) revealed statistically significant enrichment in functions involved in cellular growth and proliferation, cellular development, cell death and survival, cellular movement, and cell cycle (Figure 4.7). Interestingly, when examining the cross-talk gene sets separately; different functions are significantly enriched between groups. Only annotations associated with cellular growth and proliferation, cell cycle, and cellular development were found to be statistically significant in the interaction gene set (Figure 4.8). In the common gene set, 25 functions were found to be statistically overrepresented including cellular movement, cellular development, cellular growth and proliferation, cell cycle, and lipid metabolism (Figure 4.9).

Each category of cell and molecular functions is broken down into subcategories. Analysis of these sub categories in combination with examination of the top annotations associated with disease in IPA, revealed an enrichment of transcripts involved in immune response (not a category for cell and molecular function) in cells expressing p53, pRb, and p53 + pRb. Interestingly, annotations associated with immune response were among the top

statistically significant functions in the IPA analysis of p53 and p53 + pRb common gene sets. Biological processes associated with inflammation of organ (2.29E-03), development of leukocytes (2.29E-03), accumulation of leukocytes (2.75E-03), and movement of lymphocytes (1.10E-02) were found to be statistically significant in WI38 cells that overexpress p53 (Data not shown). Generation of T lymphocytes (3.04E-04), inflammation of organ (6.41E-04), TH1 immune response (5.36E-03), development of TH17 cells (5.70E-03), and mobilization of phagocytes (8.25E-03) among others were statistically significant in the p53 and pRb common gene set (data not shown). Annotations associated with immune response were also statistically enriched in the other gene sets (WI38 overexpressing pRb, p53 + pRb, collective cross-talk candidates). These data demonstrate an increase in proteins associated with immune response.

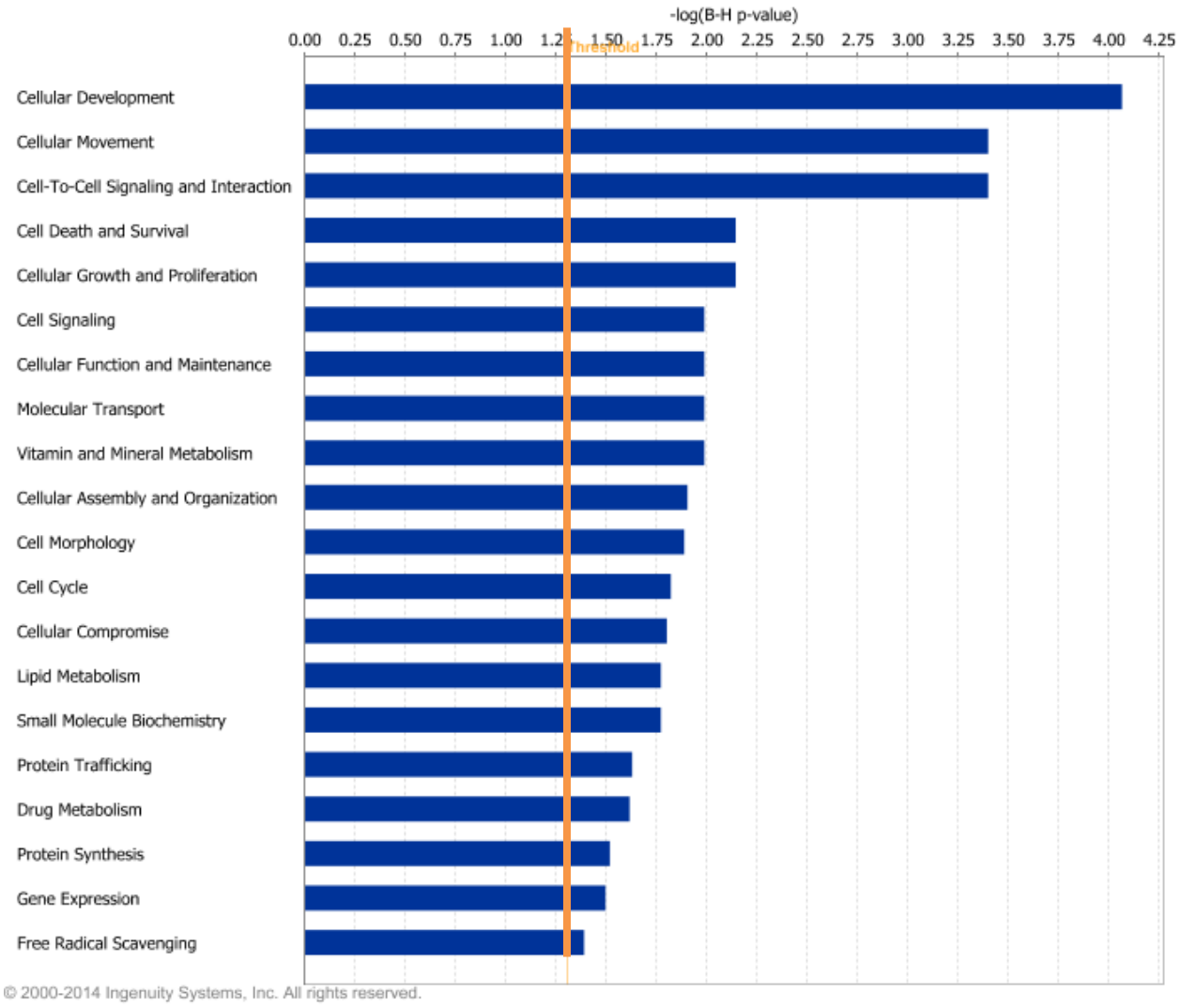

Figure 2.4: Cell and molecular functions statistically overrepresented in WI38 cells overexpressing p53.

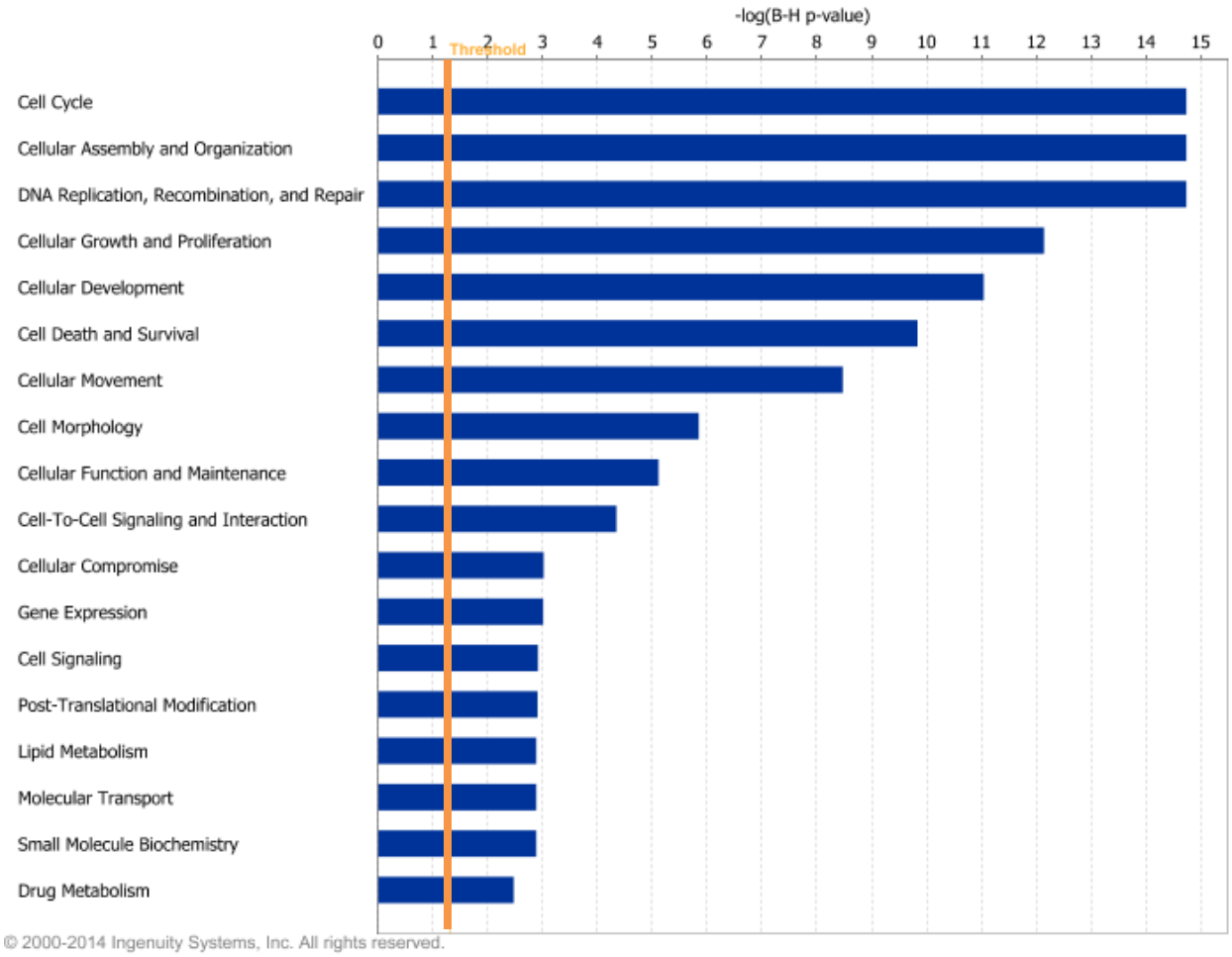

Figure 2.5: Cell and molecular functions statistically overrepresented in WI38 cells overexpressing pRb.

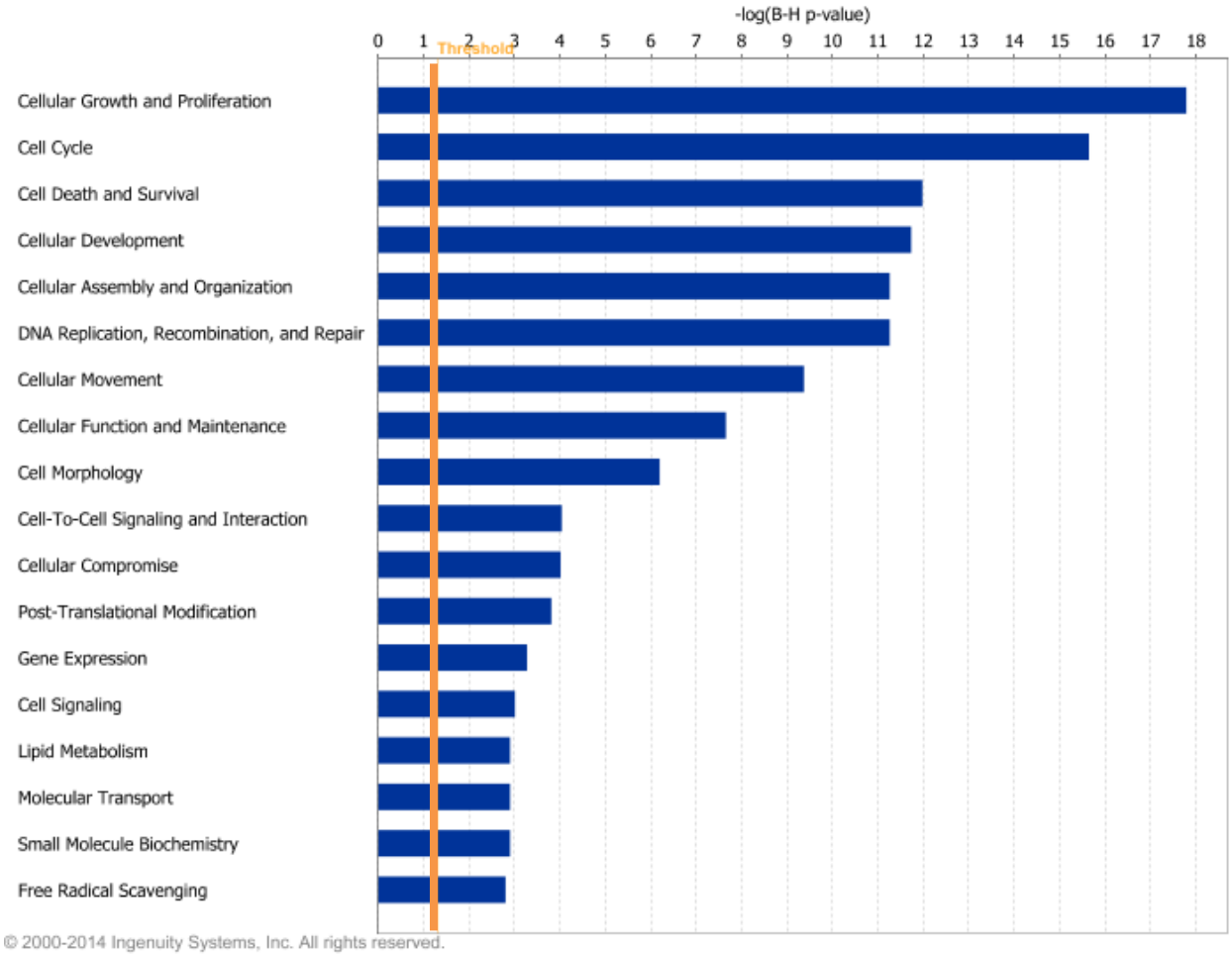

Figure 2.6: Cell and molecular functions statistically overrepresented in WI38 cells overexpressing p53 + pRb.

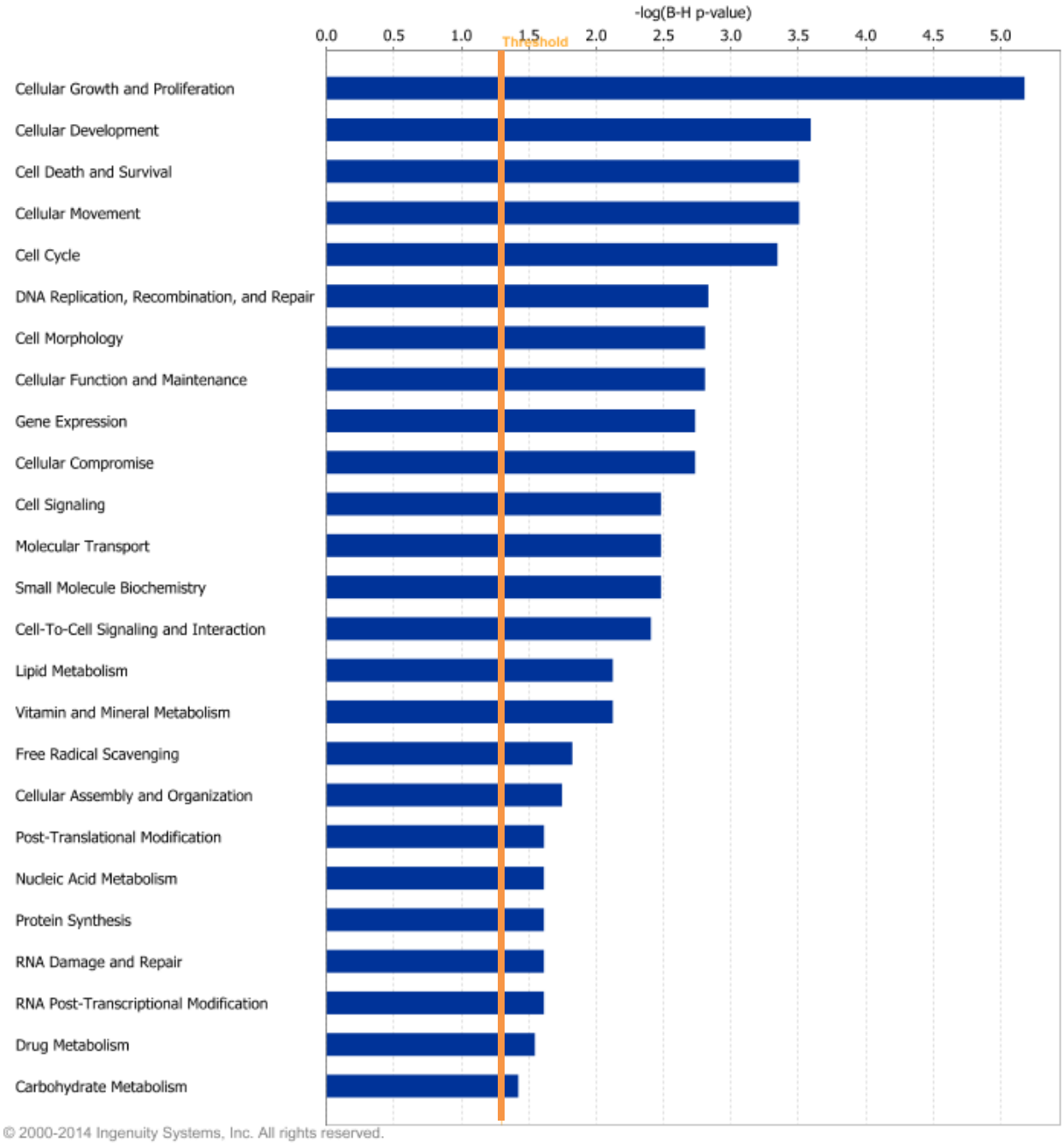

Figure 2.7: Cell and molecular functions statistically overrepresented in collective p53 and pRb common and interaction cross-talk candidates.

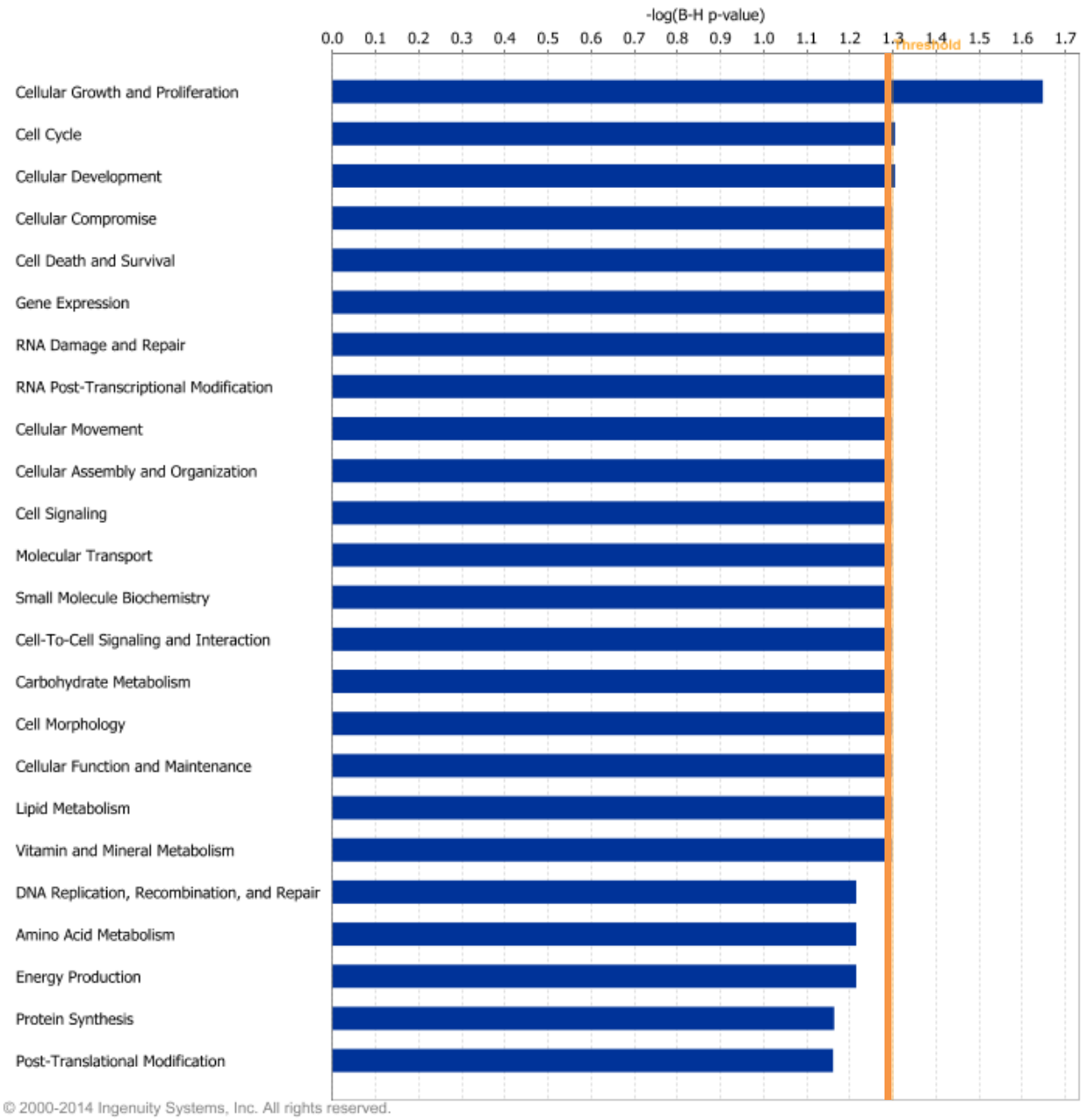

Figure 2.8: Cell and molecular functions statistically overrepresented in the interaction gene set p53 and pRb cross-talk candidates

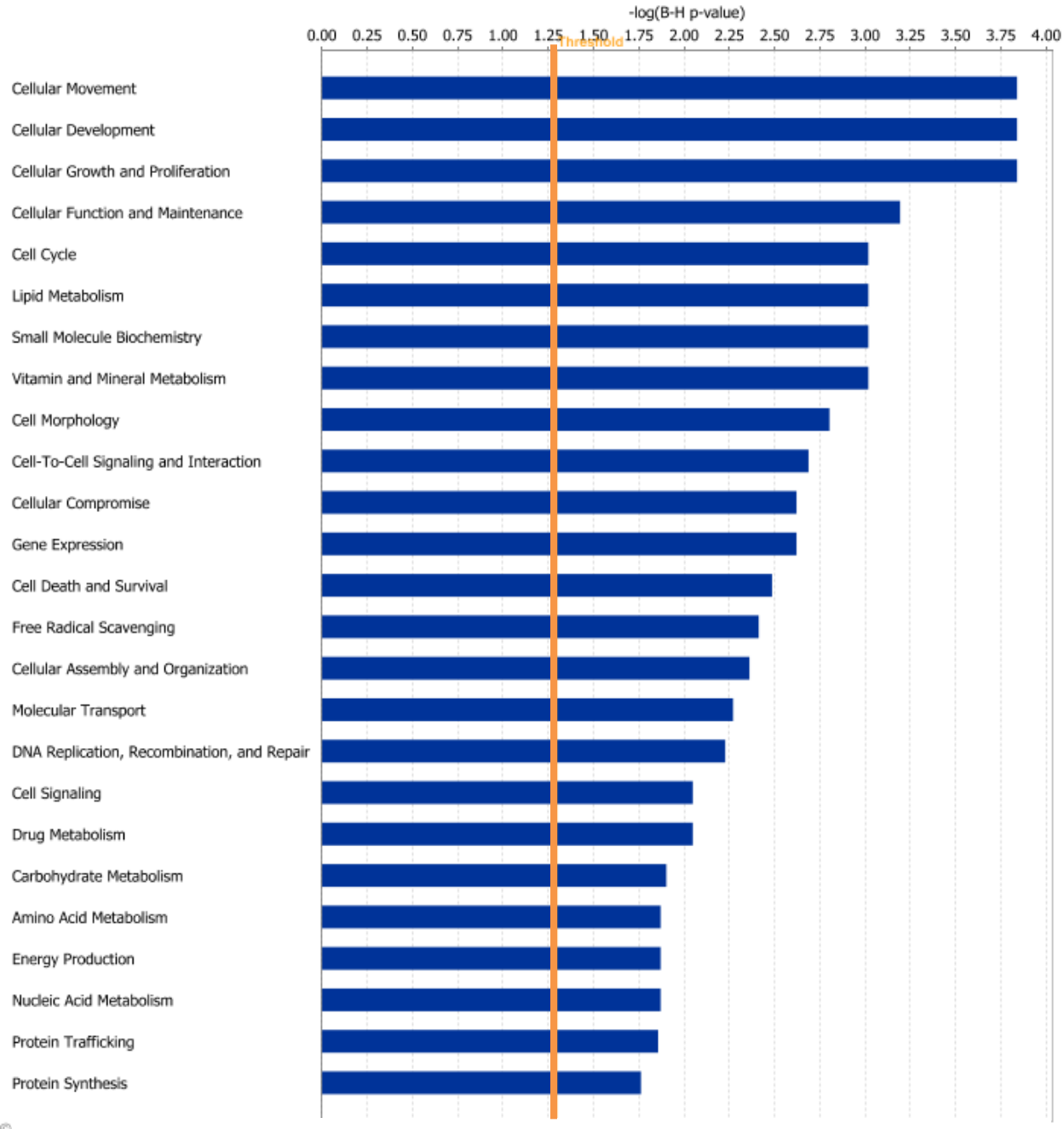

Figure 2.9: Cell and molecular functions statistically overrepresented in the common gene set p53 and pRb cross-talk candidates.

IPA pathway generation of known interactions between p53, pRb, E2F family members (E2F1-3), and cross-talk candidates identified by RNA expression profiling

By using RNA expression profiling, we were able to identify transcripts that may be co-regulated by p53 and pRb and involved in regulating tumor progression. In order to gain more understanding of p53 and pRb cross-talk, pathways were generated examining known direct and indirect relationships between the cross-talk candidates and p53, pRb, and E2F1-3 using IPA's Ingenuity Knowledge Base (Ingenuity Systems). The p53 and Rb common and interaction cross-talk candidate gene sets were loaded into IPA and pathways were generated using the Ingenuity Knowledge Base and IPA pathway tools ("Grow, Connect, and Pathway Designer") to examine upstream and downstream relationships between p53, pRb, E2F1-3 (E2F family members regulated by pRb) and the differentially expressed cross-talk gene sets (Figure 2.10-2.12). A pathway comprised of known interactions between all of the cross talk candidates (designated as the collective p53 and pRb cross-talk candidate pathway) made using the p53 and pRb common and interaction gene sets is depicted in Figure 2.12. Separate pathways for each cross-talk gene set, designated as p53 and pRb common gene set and interaction gene set pathways, respectively, were also generated (Figures 2.10-2.11). Pathway generation by IPA identified the common gene set candidates RGS16, D4s234e/NSG1, BTG-2, GDF-15, VCAN, AKR1B10 and AREG and the interaction gene set candidates: F11R, TNFRSF10C, CERS6, HDM2, SESN1, RBM38 and PMAIPI/NOXA as targets of p53 transcriptional activation (Figures 2.10-2.12). The interaction gene set cross-talk candidates BUB1, CDT1, and MCM3 are targets for transcriptional repression by p53, whereas, VRK1, MCM3, and CDT1 are known to be down-regulated by pRb (Figure 2.11-2.12). Interactions between E2F and the cross-talk candidates were assessed because of regulation of E2F gene expression by pRb. IPA identified FGFR3,

MCM3, and KRT14 interaction gene set candidates as transcriptional targets of E2F1-3 family members (Figures 2.11-2.12). Interestingly, IPA's database identified only 17 out of 39 of the p53 and pRb common and 26 out of 140 of the interaction cross-talk candidates as having known up- or down-stream relationships with p53, pRb, or E2F1. The low number of cross-talk signaling molecules known to have a direct and indirect relationship with p53 and pRb highlights the need for more research to understand the functions of these tumor suppressors.

Known interactions between the p53 and pRb cross-talk candidates are also depicted. Connections can be seen between cross-talk members that are centrally located in the IPA pathway. In the common gene set pathway, IL-1β, IL-6, BCL2L11, STAT4, BTG-2, and NR4A1 all have multiple connections (4 or more) with p53, pRb, E2Fs, or other cross-talk candidates (Figure 2.10). Centrally located signaling molecules or nodes are also present in the p53 and pRb interaction pathway and consist of HDM2, BMP-2, and IL-12A (Figure 2.11). Furthermore, the same cross-talk candidates (IL-1β, IL-6, BCL2L11, STAT4, BTG-2, NR4A1, HDM2, BMP-2, and IL-12A) appear to function as signaling nodes in the collective cross-talk pathway signifying their possible role in mediating p53 and pRb functions.

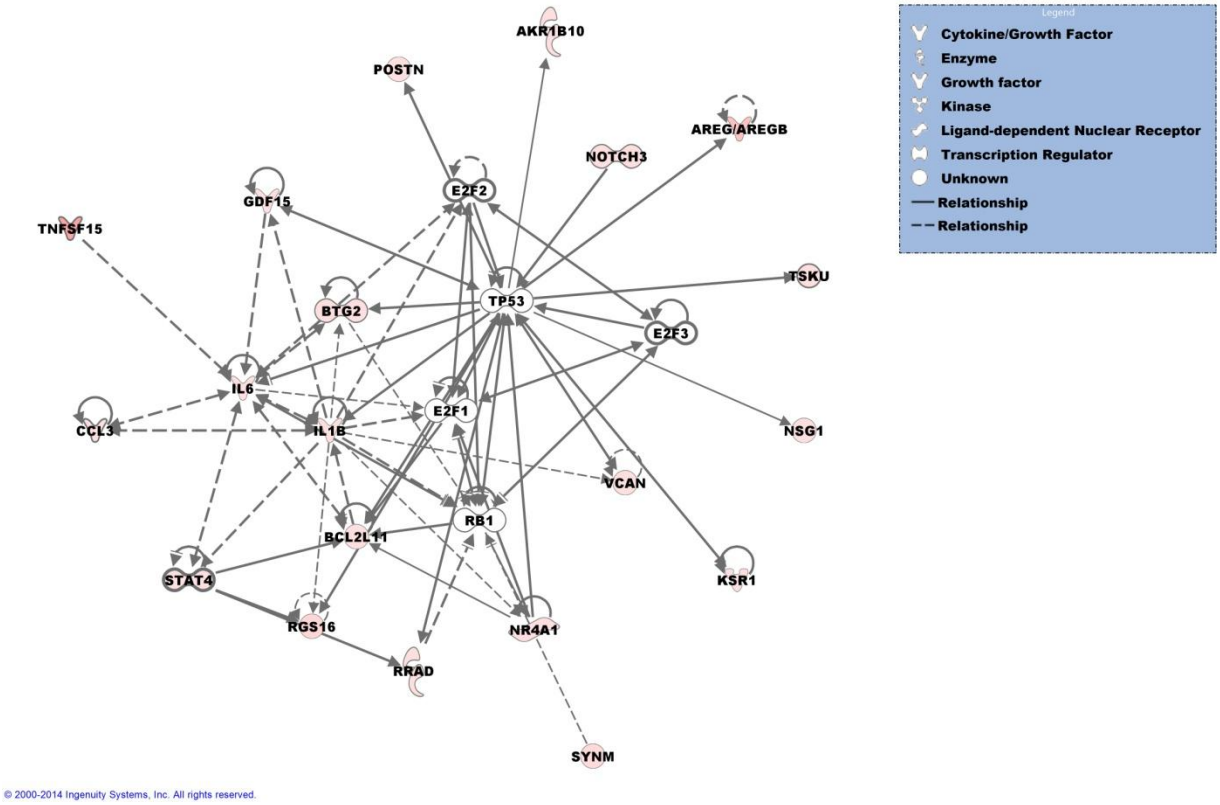

Figure 2.10: Known interaction network of common gene set cross-talk candidates with p53, pRb, and E2F1-3. IPA Ingenuity Knowledge base pathway tools and pathway designer were used to generate a pathway showing known direct (solid lines) and indirect (dashed lines) relationships between the p53 and pRb common gene set and p53, pRb, and E2F1-3. Each shape represents a different type of signaling molecule. Color intensity is associated with the degree in which the transcripts were up- (red) or down-regulated (green).

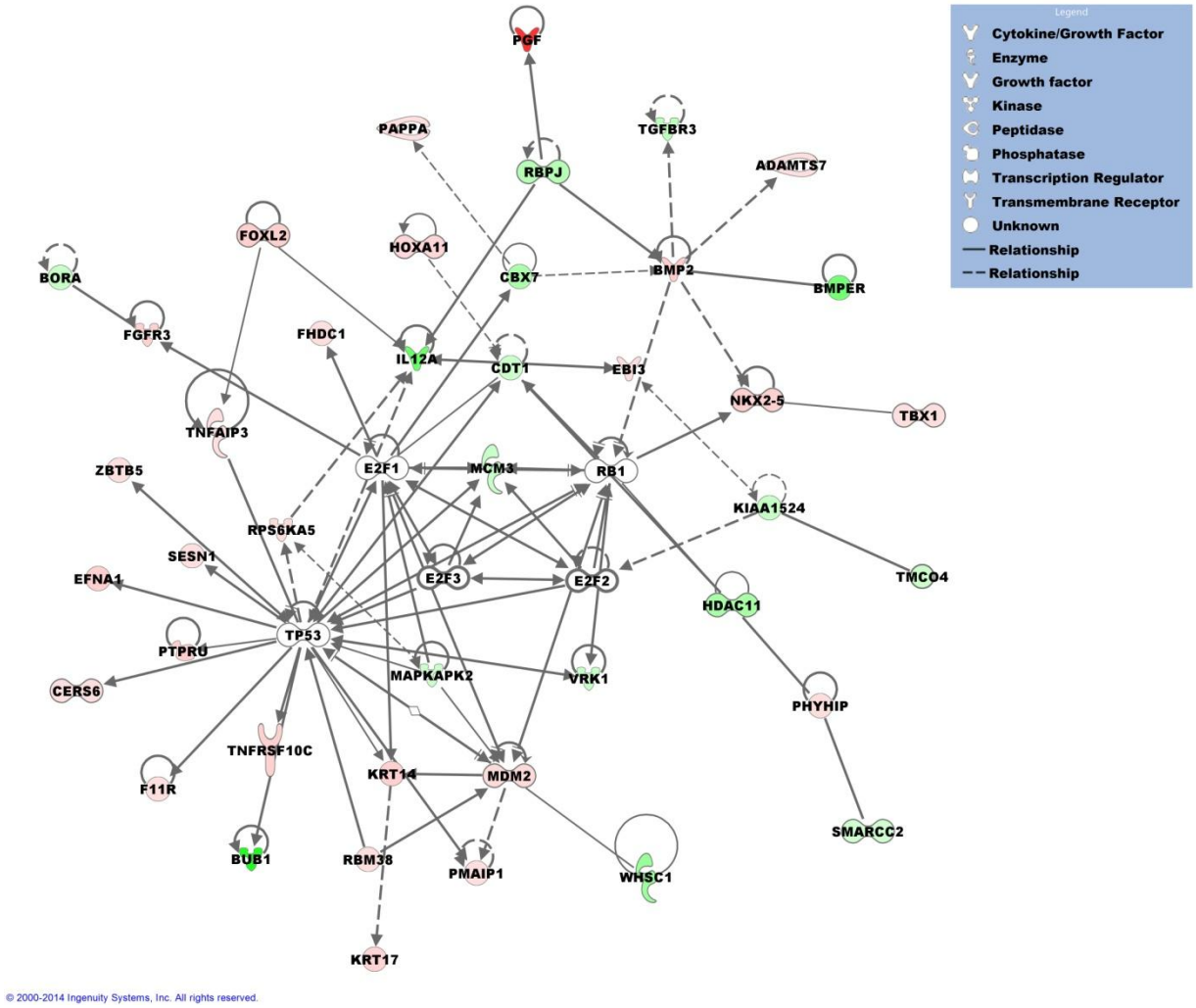

Figure 2.11: Known interaction network of p53 and pRb interaction gene set cross-talk candidates with p53, pRb, and E2F1-3. IPA Ingenuity Knowledge base pathway tools and pathway designer were used to generate a pathway showing known direct (solid lines) and indirect (dashed lines) relationships between the p53 and pRb interaction gene set cross-talk candidates and p53, pRb, and E2F1-3. Each shape represents a different type of signaling molecule. Color intensity is associated with the degree in which the transcripts were up- (red) or down-regulated (green).

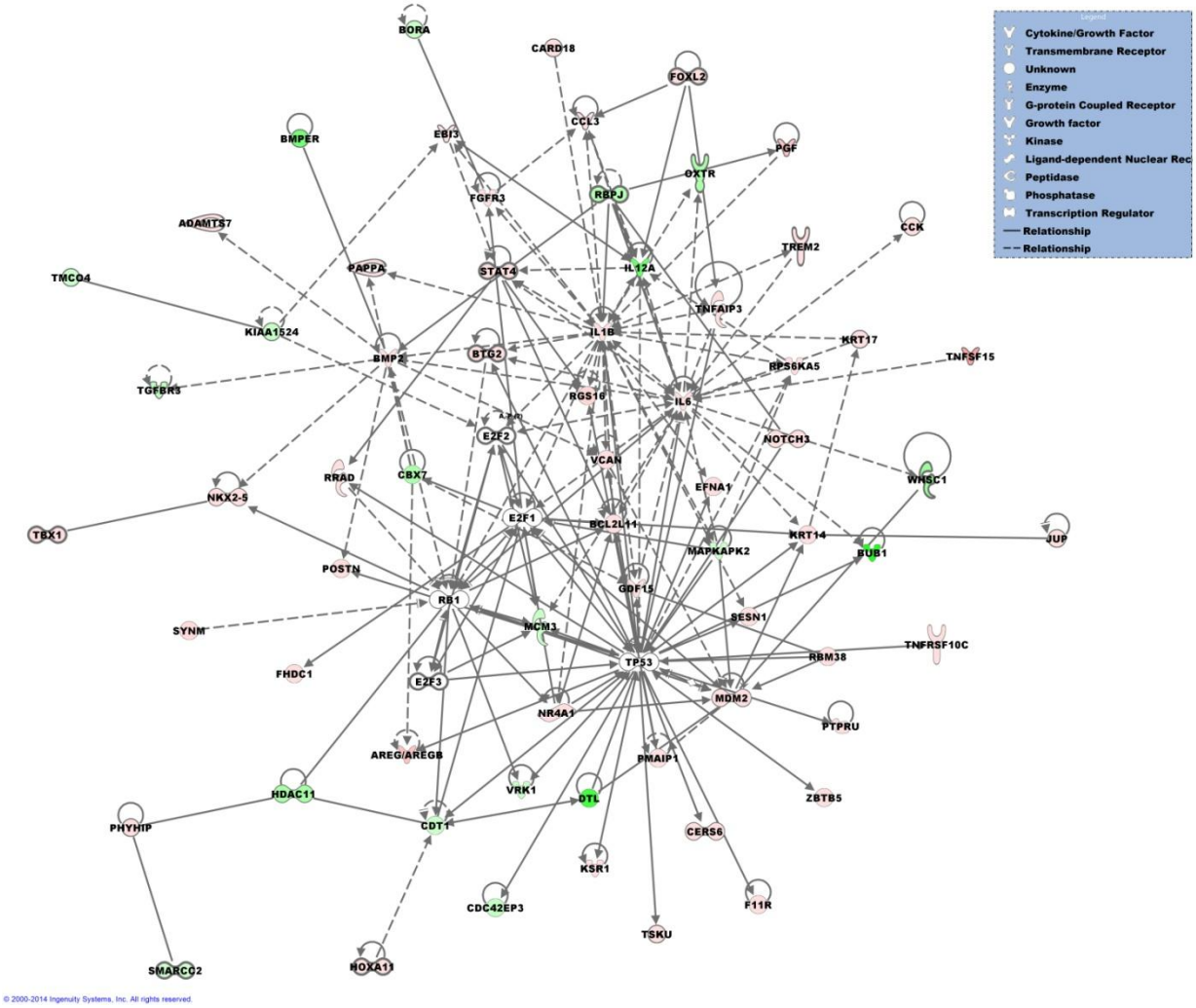

Figure 2.12: Known interaction network of colectiveollective p53 and pRb cross-talk candidates with p53, pRb, and E2F1-3. IPA Ingenuity Knowledge base pathway tools and pathway designer were used to generate a pathway showing known direct (solid lines) and indirect (dashed lines) relationships between all of the p53 and pRb cross-talk candidates and p53, pRb, and E2F1-3. Each shape represents a different type of signaling molecule. Color intensity is associated with the degree in which the transcripts were up- (red) or down-regulated (green).

DISCUSSION

Significance of investigating p53 and pRb cross-talk

Historically, investigations of p53 and pRb regulated transcription have focused on identifying the individual downstream targets of p53 and pRb. However, cell fate is not determined solely by one signaling pathway but by many pathways that communicate through a network of signaling molecules. Cross-communication between pathways allows the integration of the exogenous and endogenous signals in a cell to aid in the determination of cell fate. Co-expression of p53 and pRb in cancer cells with compromised p53 and pRb activity inhibited p53 mediated apoptosis and promoted cell cycle arrest suggesting that p53 and pRb cross-talk to regulate cellular fate [188, 189]. Furthermore, data from previous studies suggests p53 and pRb may also cooperate to inhibit cancer progression. Patients diagnosed with breast cancer and treated with adjuvant chemotherapy had a better prognosis to adjuvant chemotherapy if they had functional p53 and pRb [61].

To our knowledge this is the first study that examined altered gene expression when p53 and pRb are overexpressed together or separately with the purpose of finding genes co-regulated by both tumor suppressor genes. How p53 and pRb cross-communicate to regulate cellular functions or cooperate to inhibit cancer progression still remains largely unknown. The p53 and pRb pathways are commonly altered during tumorigenesis. The study of genes dually regulated by p53 and pRb will provide a valuable insight into the collaborative cancer preventative properties of these two tumor suppressor proteins.

Transcriptional regulation may be one method used by p53 and pRb to coordinate cellular functions. For example, the cyclin kinase inhibitor p21 is a down-stream target gene of p53 that inhibits phosphorylation and inactivation of pRb [25]. Transactivation of p21 demonstrates a mechanism by which p53 can coordinate with pRb to initiate cell cycle arrest. During

myogenesis, p53 increases the expression of Rb (mRNA and protein), which later leads to pRb and MyoD initiation of muscle differentiation [17]. This provides an example by which p53 directly enhances pRb expression leading to muscle differentiation. However, this one example is likely an indicator of large number of interactions that regulate complex cellular programs.

Change in RNA expression profiles of WI38 cells overexpressing both p53 and pRb compared to expression of p53 or pRb alone and identification of cross-talk candidates.

In this study, we identified genes that may be regulated by p53 and pRb and compiled two lists of p53 and pRb cross-talk candidates by overexpressing p53 and/or pRb in WI38 cells. Although p53 has transcriptional repression activity, our microarray analysis did not detect any down-regulated transcripts in the WI38 cells overexpressing p53 [190, 191]. The deficit of p53 down-regulated transcripts in our microarray analysis compared to previous work, could be due to our method of p53 activation, cell type, or p53 levels, which have previously been found to induce a distinct p53 response with a small set of overlapping genes [192, 193]. Our expression profiling analyses were conducted in normal lung fibroblasts cells instead of cancer epithelial cells. Lack of p53 down-regulated genes in the p53 overexpressing WI38 cells could also be attributed to the ability of p53 and pRb to alter each other's transcriptional activation or repression functions. Previous studies that discovered p53 down-regulated targets using expression profiling were done in cancer cells with mutated or null p53 and wild-type *RB1* such as PC-3, HCT116, and H1299 cells [190, 194].

There were 319 upregulated transcripts when p53 and pRb were expressed together compared to 427 and 295 in the WI38 cells expressing pRb and p53 respectively. The change in upregulated genes suggests p53 and pRb can alter one another's ability to regulate gene expression. Management of p53 and pRb processes may require these transcription factors to

regulate gene expression in an opposing manner. Expression of an embryonic development gene, Placenta-specific 1 (PLAC1), has recently been found to be down-regulated by p53 and up-regulated by pRb demonstrating how p53 and pRb can play contrasting roles to regulate cellular processes [81].

pRb is most associated with transcriptional repression of E2F target genes. However, binding of E2F by pRb is not needed to promote transcription, suppress tumor growth and induce cellular differentiation or senescence [22, 23]. In fact, pRb acts as a co-activator for several transcription factors including Sp-1, RUNX-2, MyoD, and several nuclear receptors (including NR4A1) resulting in cellular differentiation [22, 39]. We found more upregulated transcripts in WI38 cells overexpressing pRb demonstrating its function as a transcription co-activator. There is still a lack of information regarding pRb regulation, therefore, this study could contribute to identifying genes up-regulated by pRb and understanding the function of pRb as a transcriptional co-activator.

Candidates for the p53 and pRb cross-talk pathway were chosen based on whether (1) the transcripts were differentially expressed in both WI38-p53 overexpressing cells and WI-38-pRb-overexpressing cells (the common gene set), or (2) only in WI38 cells that simultaneously overexpress p53 and pRb (interaction gene set). By focusing on the p53 and pRb common and unique genes, we were able to remove from our analysis genes regulated by p53 or pRb alone.

RNA expression profiling validation

Validation of microarray data was performed using qRT-PCR for five (RGS16, BCL2L11, BTG-2, IL-6 and STAT4) of the p53 and pRb common gene set cross-talk candidates. Up-regulation of all transcripts tested except BCL2L11 was found to be statistically significant in WI38 cells overexpressing p53 and pRb confirming the microarray results. The analysis was

performed using a normal cell line in order to avoid any mutations that could be present up- or downstream of p53 and pRb that could hinder identification of downstream targets of both genes. Although p53 and pRb were expressed using adenoviruses in normal cells, the fold change of p53 and hypophosphorylated pRb proteins compared to CMV control were equivalent to or less than fold change values in WI38 cells incubated in serum free media to induce quiescence (fold change p53 after 24 hours in serum free media = 5.5) or MCF7 cells undergoing confluence induced cell growth arrest (fold change hypophosphorylated pRb/total pRb = 6.00) [195, 196]. This data suggests the concentration of virus used did not induce protein expression exceeding endogenous protein expression of p53 and the active hypophosphorylated form of pRb. The use of a normal cell line with wild-type p53 and *RB1* could make it difficult to identify cross-talk molecules due to possible interactions between endogenous and exogenous p53 and pRb. Expression of RGS16, BCL2L11, BTG-2, IL-6, and STAT4, were measured using qRT-PCR in the p53 null and pRb mutated osteosarcoma cell line SAOS-2 to investigate if exogenous and endogenous p53 and pRb interactions could influence expression profiles. Expression of all transcripts in the p53 and pRb overexpressing SAOS-2 cells was found to be increased with differences in magnitude of expression similar to the WI38 microarray data and qRT-PCR results. Differential expression of BCL2L11 was not statistically significant in WI38 cells but was in SAOS-2 cells overexpressing p53, pRb, or p53 + pRb. Replication of the qRT-PCR analysis in SAOS-2 cells provided additional information that supports the hypothesis that the chosen transcripts are involved in the p53 and pRb cross-talk pathway. Interestingly, in the microarray data, STAT4 was found to be differentially expressed in WI38 cells overexpressing p53 or pRb but not in cells that overexpressing both genes. However, qRT-PCR analysis found a statistically significant increase in STAT4 expression in WI38 and SAOS-2 cells overexpressing

p53 and pRb. The statistical analyses of expression profiling data or the sensitivity of microarray signal detection could account for the failure to observe differential expression of STAT4 in WI38 cells overexpressing p53 and pRb.

IPA functional analysis identified enriched cell and molecular functions in p53 and/or pRb overexpressed WI38 cells

p53, pRb, and p53 + pRb overexpressing cells

Co-expression of p53 and pRb in bladder and cervical cancer cells with mutations or inactivation of both proteins induced cell cycle arrest and inhibited p53 induced apoptosis [188, 189]. The ability of pRb to inhibit p53-mediated apoptosis suggests co-expression of both genes can alter gene regulation and ultimately cellular fate. IPA functional analysis was performed on all three experimental groups plus cross-talk candidate gene sets to determine if co-expression of p53 and pRb would differentially induce expression of transcripts associated with different biological functions compared to cells with overexpression of either p53 or pRb. IPA software cross-references genes and expression changes against Ingenuity Knowledge Base to identify known connections, diseases, and biological functions associated with a large dataset. The biological functional analysis information provided by IPA is broken down into molecular and cellular functions and physiological system development and functions. In the analysis, I was interested in the biological functional analysis information pertaining to molecular and cellular functions enriched due to overexpression of p53 and/or pRb. Interestingly, IPA found significant enrichment in molecular and cellular functions such as cellular development, cellular movement, cell death and survival, and cellular growth and proliferation in WI38 cells overexpressing p53 and/or pRb with differences in order of significance between groups. Furthermore, cell cycle regulation and DNA replication recombination and repair did not make the top significant

molecular cellular functions in cells overexpressing p53 but did in the pRb and p53 + pRb groups. Also noteworthy, biological functions overrepresented in cells overexpressing p53 + pRb are a cross between the results obtained from the cells overexpressing p53 or pRb suggesting activation of both p53 and pRb alters gene expression and cellular fate more than if one tumor suppressor was activated. This data supports the hypothesis that p53 and pRb coordinate to regulate cellular functions.

Cross-talk candidate gene sets

Both cross-talk candidate gene sets are differentially expressed when p53 and pRb are overexpressed together suggesting these candidates could interact with one another. Because of the possible interaction between the p53 and pRb common and interaction gene sets, we performed IPA analysis collectively on all of the cross-talk candidate gene sets and separately to identify enriched cell and molecular functions. Even though it was the larger gene set, IPA analysis of the interaction gene set, using Benjamini-Hochberg multiple testing correction p-value, yielded only three IPA annotations significantly enriched (cellular growth and proliferation, cell cycle, and cellular development) compared to 25 significantly enriched biological functions in the common gene set. Collective IPA analysis of all of the cross-talk candidates increased the amount of cell and molecular functions found to be statistically significant compared to separate IPA analyses performed on common and interaction gene sets. In summary IPA identified a significant enrichment in transcripts responsible for development, cell cycle, apoptosis, and immune response in WI38 cells overexpressing p53 and/or pRb and in the cross-talk gene sets. Enrichment in similar biological functions in all three experimental conditions and in the cross-talk gene sets supports the hypothesis that p53 and pRb communicate to regulate cell outcomes. This study provides information regarding the p53 and pRb cross-talk

that can be used to better understand how p53 and pRb regulate processes that inhibit cancer progression and are those vital for organism development.

IPA pathway generation

Identification of known interaction of cross-talk candidates with p53, pRb, and E2F1-3

IPA pathway tools were used to construct pathways to aid in identifying known up- and down-stream interactions (i.e. expression, regulation, activation, and protein-protein interaction) between the cross-talk candidates, p53, pRb, and E2F1-3. These interactions were chosen because of pRb's known function as a transcriptional repressor of E2F target genes and the known involvement of E2F-1 in p53 and pRb cross-talk [18]. From the IPA derived pathways we were able to 1) determine which cross-talk candidates have previously been found to be regulated by p53 or pRb, and 2) uncover known interactions between the cross-candidates. Using IPA software we were able to identify which of the cross-talk candidates were previously found to be regulated by p53 or pRb, providing a validation of the microarray results [135, 193, 197-205]. Only a few of the downregulated p53 and pRb cross-talk candidates have previously been found by other studies to be downregulated by p53 (MCM3, BUB1, and CDT1) or pRb individually (VRK1, MCM3, and CDT1) [190, 206-209]. There are no down-regulated p53 and pRb commonly expressed cross-talk candidates due to lack of p53 down-regulated transcripts in the microarray data. Although several of the p53 and pRb cross-talk candidates have previously been found to be regulated by p53, regulation of these transcripts by pRb has not been reported. In fact, very few of the p53 and pRb cross-talk candidates have been identified as down-stream targets of pRb regulation by the IPA database. This lack in knowledge regarding pRb

transcriptional control can be attributed to lack of studies on the ability of the Rb protein to act as a transcriptional co-factor. Furthermore, function and regulation of several of the cross-talk candidates in the common and interaction gene sets remains unknown demonstrating the need for more research to understand the function of these unknown genes.

Identification of possible signaling nodes in WI38 cells overexpressing p53 and pRb

Also of interest, several cross-talk candidates were found to be centrally located in the IPA pathways denoting relationships with other cross-talk candidates and the transcription factors of interest (p53, pRb, or E2F1-3). This observation suggests these candidates may function as signaling nodes to mediate p53 and pRb downstream effects. The cross-talk candidates IL-1β, IL-6, BCL2L11, IL12A, STAT4, BTG-2, NR4A1, HDM2, and BMP-2 were all found to have a number of interactions between other cross-talk candidates or p53, pRb, or E2F1-3. NR4A1, STAT4, and BTG-2 all have transcription regulation activities and are involved in controlling processes such as differentiation, immune response, and cell cycle arrest [72, 210, 211].

Several of the signaling nodes (IL12A, IL-1β, and IL-6) are known to play a role in regulation of immune responses and have been linked to cancer [212, 213]. Contrary to the expression profiling and qRT-PCR findings, the cytokine IL-6 is transcriptionally repressed by p53 and pRb in HeLa cells and is usually associated with aiding cancer progression [214]. Despite its role in cancer promotion, IL-6 has also been found to inhibit phosphorylation of pRb and to aid in cell cycle arrest in growth-sensitive hematopoietic cells [215]. Furthermore, *RB1* negative murine fibroblasts have decreased expression of chemokines and cytokines including IL-6 [216]. With these functions in mind, the role of IL-6 in cells overexpressing p53 and/or pRb is unclear. Expression of IL-6 and other immune associated factors could be due to p53 and

pRb induced senescence [217]. Senescence induced by these two tumor suppressors causes an immune response through secretion of factors collectively referred to as the senescence associated secretory phenotype (SASP) [218, 219]. In fact several of the immune regulatory p53 and pRb cross-talk candidates upregulated in the microarray data (IL-6, IL-1β, CCL3, and AREG) are associated with SASP and their expression could be due to the increased levels of p53 and pRb. [217].

CONCLUSIONS

p53 and pRb are two of the most studied tumor suppressors and function to regulate many of the same processes. Therefore, it is not surprising similar cell and molecular functions are enriched in cells overexpressing p53 and pRb. The different order of significance when both genes are overexpressed suggests that the combination of p53 and pRb alters outcome of the cell compared to activation of only one tumor suppressor.

By utilizing microarray expression profiling, p53 and pRb regulated candidates or genes involved in coordinating cancer suppression processes and determining cell fate were identified. p53 and pRb are not the only pathways involved in cancer suppression and determination of cell fate, but they are currently the most targeted for mutations in cancer. The investigation of p53 and pRb cross-talk focused on transcriptional regulation as a mechanism for p53 and pRb to coordinate cell functions and provides a frame work to study the cooperation between p53 and pRb in determining cell fate. Further studies are required to identify new molecular targets that in turn could lead to the development of more effective anti-cancer therapies.

Addendum for Chapter 2

p53 and pRb Interaction Significant Differentially Expressed Gene Set

Systematic Name	Gene Name	Description	Average Fold Change
AI911302	AI911302	AI911302 wd14e10.x1 Soares_NFL_T_GBC_S1 Homo sapiens cDNA clone IMAGE:2328138 3', mRNA sequence [AI911302]	86.74
NM_002632	PGF	Homo sapiens placental growth factor, vascular endothelial growth factor-related protein (PGF), mRNA [NM_002632]	46.762
NM_018965	TREM2	CYP4F2	24.856
A_24_P878366	A_24_P878366	Unknown	21.512
NM_198173	GRHL3	Homo sapiens grainyhead-like 3 (Drosophila) (GRHL3), transcript variant 2, mRNA [NM_198173]	21.126
NM_017565	FAM20A	Homo sapiens family with sequence similarity 20, member A (FAM20A), mRNA [NM_017565]	20.308
THC2668267	THC2668267	Q3MAK1_ANAVT (Q3MAK1) Phosphoglucomutase/phosphomannomutase partial (4%) [THC2668267]	19.498
THC2565393	THC2565393	Q5VT28_HUMAN (Q5VT28) Family with sequence similarity 27, member B (Family with sequence similarity 27, member A) (Family with sequence similarity 27, member C), partial (81%) [THC2565393]	19.246
A_32_P168727	A_32_P168727	Unknown	17.802
BC073976	BC073976	Homo sapiens cDNA clone IMAGE: 6018774, partial cds. [BC073976]	17.16
NM_002590	PCDH8	Homo sapiens protocadherin 8 (PCDH8), transcript variant 1, mRNA [NM_002590]	16.226
CD511705	CD511705	AGENCOURT_14360862 NIH_MGC_187 Homo sapiens cDNA clone IMAGE:30405414 5', mRNA sequence [CD511705]	16.062
NM_138344	C14orf152	Homo sapiens chromosome 14 open reading frame 152 (C14orf152), mRNA [NM_138344]	15.977

NM_001080468	SYCN	Homo sapiens syncollin (SYCN), mRNA [NM_001080468]	15.905
NM_021571	ICEBERG	Homo sapiens ICEBERG caspase-1 inhibitor (ICEBERG), mRNA [NM_021571]	14.305
NM_001082	CYP4F2	Homo sapiens cytochrome P450, family 4, subfamily F, polypeptide 2 (CYP4F2), mRNA [NM_001082]	12.569
NM_000729	CCK	Homo sapiens cholecystokinin (CCK), mRNA [NM_000729]	12.407
NM_001200	BMP2	Homo sapiens bone morphogenetic protein 2 (BMP2), mRNA [NM_001200]	11.436
NM_133177	PTPRU	Homo sapiens protein tyrosine phosphatase, receptor type, U (PTPRU), transcript variant 2, mRNA [NM_133177]	11.168
THC2642537	THC2642537	Q2Q5T5_MOUSE (Q2Q5T5) Embryonic stem cell-and germ cell-specific protein ESGP, complete [THC2642537]	10.941
NM_023067	FOXL2	Homo sapiens forkhead box L2 (FOXL2), mRNA [NM_023067]	10.62
NM_003991	EDNRB	Homo sapiens endothelin receptor type B (EDNRB), transcript variant 2, mRNA [NM_003991]	10.585
NM_004428	EFNA1	Homo sapiens ephrin-A1 (EFNA1), transcript variant 1, mRNA [NM_004428]	10.517
NM_004387	NKX2-5	Homo sapiens NK2 transcription factor related, locus 5 (Drosophila) (NKX2-5), mRNA [NM_004387]	10.47
NM_000526	KRT14	Homo sapiens keratin 14 (epidermolysis bullosa simplex, Dowling-Meara, Koebner) (KRT14), mRNA [NM_000526]	10.436
NM_003841	TNFRSF10C	Homo sapiens tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain (TNFRSF10C), mRNA [NM_003841]	10.17
NM_000142	FGFR3	Homo sapiens fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism) (FGFR3), transcript variant 1, mRNA [NM_000142]	10.133
NM_152404	UGT3A1	Homo sapiens UDP glycosyltransferase 3 family, polypeptide A1 (UGT3A1), mRNA [NM_152404]	10.083

NM_152670	C2orf51	Homo sapiens chromosome 2 open reading frame 51 (C2orf51), mRNA [NM_152670]	9.5914
NM_005268	GJB5	Homo sapiens gap junction protein, beta 5 (GJB5), mRNA [NM_005268]	9.5447
NM_000422	KRT17	Homo sapiens keratin 17 (KRT17), mRNA [NM_000422]	9.5142
NM_033120	NKD2	Homo sapiens naked cuticle homolog 2 (Drosophila) (NKD2), mRNA [NM_033120]	8.9252
BC063385	TRAα	Homo sapiens T cell receptor alpha locus, mRNA (cDNA clone MGC:71411 IMAGE:4853814), complete cds. [BC063385]	8.706
NM_015879	ST8SIA3	Homo sapiens ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 3 (ST8SIA3), mRNA [NM_015879]	8.6842
NM_005523	HOXA11	Homo sapiens homeobox A11 (HOXA11), mRNA [NM_005523]	8.521
NM_024989	PGAP1	Homo sapiens GPI deacylase (PGAP1), mRNA [NM_024989]	8.3487
NM_018558	GABRQ	Homo sapiens gamma-aminobutyric acid (GABA) receptor, theta (GABRQ), mRNA [NM_018558]	8.2783
AK125985	AK125985	Homo sapiens cDNA FLJ43997 fis, clone TESTI4021456. [AK125985]	8.1169
CB852325	CB852325	UI-CF-FN0-afp-n-21-0-UI.s1 UI-CF-FN0 Homo sapiens cDNA clone UI-CF-FN0-afp-n-21-0-UI 3', mRNA sequence [CB852325]	7.8293
NM_002392	MDM2	Homo sapiens Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) (MDM2), transcript variant MDM2, mRNA [NM_002392]	7.8157
ENST00000377525	ENST00000377525	Protein FAM27E1. [Source:Uniprot/SWISSPROT;Acc:Q5T7N7] [ENST00000377525]	7.7762
NM_004755	RPS6KA5	Homo sapiens ribosomal protein S6 kinase, 90kDa, polypeptide 5 (RPS6KA5), transcript variant 1, mRNA [NM_004755]	7.7329
NM_015404	DFNB31	Homo sapiens deafness, autosomal recessive 31 (DFNB31), mRNA [NM_015404]	7.657

NM_153268	PLCXD2	Homo sapiens phosphatidylinositol-specific phospholipase C, X domain containing 2 (PLCXD2), mRNA [NM_153268]	7.6269
AF334945	FKSG43	Homo sapiens FKSG43 (FKSG43) mRNA, complete cds. [AF334945]	7.4397
NM_005755	EBI3	Homo sapiens Epstein-Barr virus induced gene 3 (EBI3), mRNA [NM_005755]	7.3997
NM_014759	PHYHIP	Homo sapiens phytanoyl-CoA 2-hydroxylase interacting protein (PHYHIP), mRNA [NM_014759]	7.3405
NM_017495	RBM38	Homo sapiens RNA binding motif protein 38 (RBM38), transcript variant 1, mRNA [NM_017495]	7.3358
NM_002581	PAPPA	Homo sapiens pregnancy-associated plasma protein A, pappalysin 1 (PAPPA), mRNA [NM_002581]	7.2992
THC2532504	THC2532504	Q9BX12_HUMAN (Q9BX12) GTP binding protein 2 (Fragment), partial (26%) [THC2532504]	7.0938
BC085019	GDF5OS	Homo sapiens hypothetical LOC554250, mRNA (cDNA clone MGC:99835 IMAGE:6650156), complete cds. [BC085019]	6.7892
AF140675	ADAMTS7	Homo sapiens zinc metalloprotease ADAMTS7 (ADAMTS7) mRNA, complete cds. [AF140675]	6.4441
NM_004097	EMX1	Homo sapiens empty spiracles homeobox 1 (EMX1), transcript variant 1, mRNA [NM_004097]	6.4091
NM_033393	KIAA1727	Homo sapiens KIAA1727 protein (KIAA1727), mRNA [NM_033393]	6.2283
NM_014872	ZBTB5	Homo sapiens zinc finger and BTB domain containing 5 (ZBTB5), mRNA [NM_014872]	6.0517
ENST00000327026	ENST00000327026	coiled-coil domain containing 57 [Source:RefSeq_peptide;Acc:NP_932348] [ENST00000327026]	6.0306
NM_017434	DUOX1	Homo sapiens dual oxidase 1 (DUOX1), transcript variant 1, mRNA [NM_017434]	5.9386
NM_007021	C10orf10	Homo sapiens chromosome 10 open reading frame 10 (C10orf10), mRNA [NM_007021]	5.8409
NM_144503	F11R	Homo sapiens F11 receptor (F11R), transcript variant 4, mRNA [NM_144503]	5.7322

NM_182608	ANKRD33	Homo sapiens ankyrin repeat domain 33 (ANKRD33), mRNA [NM_182608]	5.7262
NM_152361	EID2B	Homo sapiens EP300 interacting inhibitor of differentiation 2B (EID2B), mRNA [NM_152361]	5.7078
NM_021176	G6PC2	Homo sapiens glucose-6-phosphatase, catalytic, 2 (G6PC2), transcript variant 1, mRNA [NM_021176]	5.7053
NM_018897	DNAH7	Homo sapiens dynein, axonemal, heavy chain 7 (DNAH7), mRNA [NM_018897]	5.6555
NM_014454	SESN1	Homo sapiens sestrin 1 (SESN1), mRNA [NM_014454]	5.5047
NM_080647	TBX1	Homo sapiens T-box 1 (TBX1), transcript variant C, mRNA [NM_080647]	5.4698
NM_203463	LASS6	Homo sapiens LAG1 homolog, ceramide synthase 6 (S. cerevisiae) (LASS6), mRNA [NM_203463]	5.4147
BU602485	BU602485	AGENCOURT_10015118 NIH_MGC_142 Homo sapiens cDNA clone IMAGE:6496567 5', mRNA sequence [BU602485]	5.2332
NM_148959	HUS1B	Homo sapiens HUS1 checkpoint homolog b (S. pombe) (HUS1B), mRNA [NM_148959]	5.2101
NM_006290	TNFAIP3	Homo sapiens tumor necrosis factor, alpha-induced protein 3 (TNFAIP3), mRNA [NM_006290]	5.1853
NM_203286	PVRL1	Homo sapiens poliovirus receptor-related 1 (herpesvirus entry mediator C; nectin) (PVRL1), transcript variant 3, mRNA [NM_203286]	5.0026
NM_002283	KRT85	Homo sapiens keratin 85 (KRT85), mRNA [NM_002283]	4.9741
ENST00000292729	USP41	Ubiquitin carboxyl-terminal hydrolase 41 (EC 3.1.2.15) (Ubiquitin thioesterase 41) (Ubiquitin-specific-processing protease 41) (Deubiquitinating enzyme 41) (Fragment). [Source:Uniprot/SPTREMBL;Acc:Q3LFD5] [ENST00000292729]	4.8886
BP871540	BP871540	BP871540 Sugano cDNA library, embryonal kidney Homo sapiens cDNA clone HKR00303, mRNA sequence [BP871540]	4.6823
A_24_P767699	A_24_P767699	Unknown	4.5259

NM_004443	EPHB3	Homo sapiens EPH receptor B3 (EPHB3), mRNA [NM_004443]	4.03
NM_021127	PMAIP1	Homo sapiens phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1), mRNA [NM_021127]	3.9596
THC2660977	THC2660977	Unknown	3.9263
NM_017654	SAMD9	Homo sapiens sterile alpha motif domain containing 9 (SAMD9), mRNA [NM_017654]	0.2496
NM_139067	SMARCC2	Homo sapiens SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2 (SMARCC2), transcript variant 2, mRNA [NM_139067]	0.232
NM_001280	CIRBP	Homo sapiens cold inducible RNA binding protein (CIRBP), mRNA [NM_001280]	0.2308
NM_182616	C15orf38	Homo sapiens chromosome 15 open reading frame 38 (C15orf38), mRNA [NM_182616]	0.2277
NM_004627	WRB	Homo sapiens tryptophan rich basic protein (WRB), mRNA [NM_004627]	0.2273
NM_020422	TMEM159	Homo sapiens transmembrane protein 159 (TMEM159), mRNA [NM_020422]	0.2263
NM_022071	SH2D4A	Homo sapiens SH2 domain containing 4A (SH2D4A), mRNA [NM_022071]	0.2172
NM_004759	MAPKAPK2	Homo sapiens mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), transcript variant 1, mRNA [NM_004759]	0.2136
THC2586959	THC2586959	1PK0_D Chain D, Crystal Structure Of The Ef3-Cam Complexed With Pmeapp. {Homo sapiens} (exp=-1; wgp=0; cg=0), partial (70%) [THC2586959]	0.212
NM_033219	TRIM14	Homo sapiens tripartite motif-containing 14 (TRIM14), transcript variant 2, mRNA [NM_033219]	0.211
NM_003130	SRI	Homo sapiens sorcin (SRI), transcript variant 1, mRNA [NM_003130]	0.21
NM_007203	PALM2-AKAP2	Homo sapiens PALM2-AKAP2 protein (PALM2-AKAP2), transcript variant 1, mRNA [NM_007203]	0.2083
NM_181719	TMCO4	Homo sapiens transmembrane and coiled-coil domains 4 (TMCO4), mRNA [NM_181719]	0.2044

THC2638360	THC2638360	Q6STG2_HUMAN (Q6STG2) DNA polymerase-transactivated protein 3, partial (13%) [THC2638360]	0.2044
AF256215	ARNTL2	Homo sapiens cycle-like factor CLIF mRNA, complete cds. [AF256215]	0.2043
NM_030928	CDT1	Homo sapiens chromatin licensing and DNA replication factor 1 (CDT1), mRNA [NM_030928]	0.2038
NM_001008224	UACA	Homo sapiens uveal autoantigen with coiled-coil domains and ankyrin repeats (UACA), transcript variant 2, mRNA [NM_001008224]	0.2023
A_24_P927230	A_24_P927230	Unknown	0.2015
NM_020964	KIAA1632	Homo sapiens KIAA1632 (KIAA1632), mRNA [NM_020964]	0.1988
NM_024808	C13orf34	Homo sapiens chromosome 13 open reading frame 34 (C13orf34), mRNA [NM_024808]	0.1938
NM_003384	VRK1	Homo sapiens vaccinia related kinase 1 (VRK1), mRNA [NM_003384]	0.1937
NM_002388	MCM3	Homo sapiens MCM3 minichromosome maintenance deficient 3 (S. cerevisiae) (MCM3), mRNA [NM_002388]	0.1906
AB040957	KIAA1524	Homo sapiens mRNA for KIAA1524 protein, partial cds. [AB040957]	0.1875
AK055915	AK055915	Homo sapiens cDNA FLJ31353 fis, clone MESAN2000264. [AK055915]	0.1793
NM_203284	RBPJ	Homo sapiens recombination signal binding protein for immunoglobulin kappa J region (RBPJ), transcript variant 4, mRNA [NM_203284]	0.1661
NM_014857	RABGAP1L	Homo sapiens RAB GTPase activating protein 1-like (RABGAP1L), transcript variant 1, mRNA [NM_014857]	0.1656
NM_004695	SLC16A5	Homo sapiens solute carrier family 16, member 5 (monocarboxylic acid transporter 6) (SLC16A5), mRNA [NM_004695]	0.1612
NM_004494	HDGF	Homo sapiens hepatoma-derived growth factor (high-mobility group protein 1-like) (HDGF), mRNA [NM_004494]	0.1605
NM_198969	AES	Homo sapiens amino-terminal enhancer of split (AES), transcript variant 1, mRNA [NM_198969]	0.1605

NM_032294	CAMKK1	Homo sapiens calcium/calmodulin-dependent protein kinase kinase 1, alpha (CAMKK1), transcript variant 1, mRNA [NM_032294]	0.1596
AK090664	AK090664	Homo sapiens cDNA FLJ33345 fis, clone BRACE2003713. [AK090664]	0.1593
NM_012140	SLC25A10	Homo sapiens solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10 (SLC25A10), mRNA [NM_012140]	0.1588
NM_052853	ADCK2	Homo sapiens aarF domain containing kinase 2 (ADCK2), mRNA [NM_052853]	0.1581
NM_003243	TGFBR3	Homo sapiens transforming growth factor, beta receptor III (TGFBR3), mRNA [NM_003243]	0.1574
AK021837	AK021837	Homo sapiens cDNA FLJ11775 fis, clone HEMBA1005891. [AK021837]	0.156
NM_016052	RRP15	Homo sapiens ribosomal RNA processing 15 homolog (S. cerevisiae) (RRP15), mRNA [NM_016052]	0.1462
ENST00000307662	SYNPO	Synaptopodin. [Source:Uniprot/SWISSPROT;Acc:Q8N3V7] [ENST00000307662]	0.144
D14041	RBPJ	Homo sapiens mRNA for H-2K binding factor-2, complete cds. [D14041]	0.1431
NM_173636	WDR62	Homo sapiens WD repeat domain 62 (WDR62), mRNA [NM_173636]	0.1393
ENST00000359236	FLJ20674	CDNA FLJ20674 fis, clone KAIA4450. [Source:Uniprot/SPTREMBL;Acc:Q9NWQ7] [ENST00000359236]	0.1383
NM_018199	EXDL2	Homo sapiens exonuclease 3'-5' domain-like 2 (EXDL2), mRNA [NM_018199]	0.1366
THC2607337	THC2607337	Unknown	0.1354
ENST00000374851	C9orf125	Uncharacterized protein C9orf125. [Source:Uniprot/SWISSPROT;Acc:Q9BRR3] [ENST00000374851]	0.1276
NM_175709	CBX7	Homo sapiens chromobox homolog 7 (CBX7), mRNA [NM_175709]	0.1259
NM_000916	OXTR	Homo sapiens oxytocin receptor (OXTR), mRNA [NM_000916]	0.1242
A_32_P171043	A_32_P171043	Unknown	0.1202
NM_024827	HDAC11	Homo sapiens histone deacetylase 11 (HDAC11), mRNA [NM_024827]	0.1186

A_32_P36709	A_32_P36709	Unknown	0.1158
NM_024693	ECHDC3	Homo sapiens enoyl Coenzyme A hydratase domain containing 3 (ECHDC3), mRNA [NM_024693]	0.1099
AK125077	AK125077	Homo sapiens cDNA FLJ43087 fis, clone BRTHA3019105. [AK125077]	0.1041
NM_007331	WHSC1	Homo sapiens Wolf-Hirschhorn syndrome candidate 1 (WHSC1), transcript variant 8, mRNA [NM_007331]	0.103
NM_152913	TMEM130	Homo sapiens transmembrane protein 130 (TMEM130), mRNA [NM_152913]	0.1005
NM_017915	C12orf48	Homo sapiens chromosome 12 open reading frame 48 (C12orf48), mRNA [NM_017915]	0.0998
THC2677783	THC2677783	Unknown	0.0937
NM_005498	AP1M2	Homo sapiens adaptor-related protein complex 1, mu 2 subunit (AP1M2), mRNA [NM_005498]	0.0867
NM_024490	ATP10A	Homo sapiens ATPase, Class V, type 10A (ATP10A), mRNA [NM_024490]	0.0818
NM_133468	BMPER	Homo sapiens BMP binding endothelial regulator (BMPER), mRNA [NM_133468]	0.0763
NM_145251	STYX	Homo sapiens serine/threonine/tyrosine interacting protein (STYX), mRNA [NM_145251]	0.0724
NM_000882	IL12A	Homo sapiens interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35) (IL12A), mRNA [NM_000882]	0.0702
BC047636	BC047636	Homo sapiens cDNA clone IMAGE:4822429. [BC047636]	0.0677
NM_000891	KCNJ2	Homo sapiens potassium inwardly-rectifying channel, subfamily J, member 2 (KCNJ2), mRNA [NM_000891]	0.0657
NM_016448	DTL	Homo sapiens denticleless homolog (Drosophila) (DTL), mRNA [NM_016448]	0.0554
NM_004336	BUB1	Homo sapiens BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast) (BUB1), mRNA [NM_004336]	0.0461

CHAPTER 3.

IDENTIFYING THE ROLE OF RGS16 IN PANCREATIC CANCER CELL MIGRATION AND INVASION[2]

SUMMARY

Pancreatic cancer is ranked as the fourth highest cause of cancer-related deaths in the United States with a five-year survival rate <5%. Pancreatic cancer is associated with early systemic dissemination resulting in the majority of newly diagnosed patients having aggressive non-localized cancer and non-eligibility for curative treatments. New therapies are needed to inhibit and treat metastatic pancreatic cancer. A study aimed at identifying markers for pancreatic cancer metastasis found that Regulator of G protein Signaling 16 (RGS16) is downregulated in patients with lymph node metastases compared to patients with non-lymph node metastasized pancreatic cancer. RGS16 belongs to a large family of proteins that play a role in swiftly shutting down G protein-coupled receptor pathways and is implicated in turning off signaling of several oncogene pathways that are involved in proliferation, migration, and invasion of cancer cells. Currently, the role of RGS16 in pancreatic cancer is unknown.

We found that the expression of RGS16 mRNA was downregulated in established pancreatic cancer cell lines compared to mRNA extracted from normal human pancreatic tissue. We exogenously expressed RGS16 and/or GFP (control) using adenoviral vectors in established pancreatic cancer cell lines (BxPC-3, PANC-1, or AsPC-1) and measured the impact of RGS16 expression on cell migration, invasion and cell viability. Expression of RGS16 inhibited migration and invasion of BxPC-3 and AsPC-1 cells but did not modify PANC-1 cell migration.

[2] A portion of this work has been submitted and accepted for publication in the journal Genes and Cancer (see Appendix for draft) or [175].

RGS16 expression did not alter BxPC-3, PANC-1, or AsPC-1 cell growth stimulated by FBS or EGF (epidermal growth factor). Experiments investigating the mechanism behind RGS16 suppression of migration and invasion found no change in the levels of phosphorylated AKT and ERK suggesting RGS16 inhibits pancreatic migration and invasion independent of the PI3K/AKT and MAPK pathways.

For the first time we have shown that RGS16 inhibits EGF induced migration and invasion of pancreatic cancer cells. Further studies are needed to elucidate the mechanism(s) used by RGS16 to inhibit pancreatic cancer cell migration and invasion.

INTRODUCTION

In 2014, the American Cancer Society estimates that approximately 46,420 individuals will be diagnosed with pancreatic cancer and 39,590 will die from this disease [83]. The one-year survival rate for all stages of pancreatic cancer is 20% and the five-year survival rate is 6% [83]. An analysis looking at the five-year survival rates of various cancers over a span of thirty years found that pancreatic cancer had the least improvement from 2% (1975-1977) to 6% (2003 to 2009) [83]. The majority of patients newly diagnosed with pancreatic cancer present with highly progressed and/or metastatic cancer that is resistant to treatment [84, 85]. Due to the late stage of diagnosis and the aggressive nature of this disease, less than 20% of pancreatic cancer patients are eligible for the potentially curative surgery [85, 220]. Therefore, there is a great need for more effective drugs aimed at treating or preventing metastatic pancreatic cancer.

The presence of lymph-node metastases is regarded as a negative prognostic factor for patients who have undergone pancreatic surgery [85, 92]. In order to find biomarkers for lymph node metastasis to aid in patient prognosis, cDNA microarrays were used to analyze gene expression in local vs. lymph-node metastasized pancreatic cancer [149]. Microarray analysis

revealed decreased expression of Regulator of G-protein signaling 16 (RGS16) in pancreatic tumors with lymph-node metastases compared to non-metastasized pancreatic cancer [149]. Immunohistological analysis revealed only 5.7% (1 out of 17) of the pancreatic tumors with lymph-node metastases had expression of the Regulator of G-protein signaling 16 (RGS16) compared to 70.6% (12 out of 17) of pancreatic tumors with non-metastasized pancreatic cancer [149]. Furthermore, decreased expression of RGS16 was associated with poor pancreatic cancer patient survival indicating the potential of RGS16 as a pancreatic cancer prognostic marker [149].

RGS16, a signaling molecule we identified as a p53 and pRb cross-talk candidate belongs to a large family of proteins that play a role in swiftly shutting down G protein-coupled receptor (GPCR) signaling pathways [134, 135]. RGS16 is a GTPase activating protein (GAP) that enhances GTPase activity of the α-subunit of G proteins associated with G-protein coupled receptors (GPCR). RGS16 has been implicated in negatively regulating the MAPK, AKT/PI3K, RhoA, and SDF-1/CxCR4 oncogene pathways in normal or cancer cell lines [135, 141, 164, 171]. These oncogene pathways have been implicated in cancer progression processes (such as: proliferation, survival, chemoresistance, migration, invasion, and metastasis) in a variety of malignancies including pancreatic cancer [221-225].

Few reports have been published that describe the impact of RGS16 on cancer cell signaling and progression. Among these are: increased expression of RGS16 in pediatric high hyperdiploid acute lymphoblastic leukemia (ALL) and colon cancer; however, functional analysis has not been performed to identify any oncogenic function of RGS16 in these cancers [147-149]. Functional and expression analysis of RGS16 has been performed in breast cancers. The RGS16 promoter is located at a site that is vulnerable to allelic imbalances that can result in

promoter methylation of RGS16 in 10% of breast cancers with these genomic instabilities [150]. Liang *et al.* (2009) found that RGS16 overexpression in breast cancer cell lines decreased EGF induced proliferation and AKT activation by binding to the p85-alpha subunit of PI3K preventing the phosphorylation of AKT [171]. RGS16 has also been associated with the anti-proliferative effect of retinoic acid in neuroblastoma cells and the cytotoxity effect of the histone deacetylase inhibitor Vorinostat and lysine-specific demethylase 1 knock-down in triple negative breast cancers [162, 163]. The current data suggest RGS16 plays a role in cancer signaling, however, more research is needed to delineate the function of RGS16 in cancer cells.

Due to pre-existing data on RGS16 expression and function, we hypothesized that exogenous expression of RGS16 would inhibit migration and proliferation of pancreatic cancer cells *in vitro*. We chose to focus our investigations on EGF induced migration and invasion because 1) RGS16 inhibits EGF signaling in breast cancer cells, and 2) the epidermal growth factor receptor (EGFR/ERBB-1) is overexpressed in ~85% of pancreatic cancers, and is linked with development, invasion, and decreased survival in this deadly disease [110, 115, 226, 227]. Currently, RGS16 has not been linked with inhibition of cancer cell metastasis nor has its function been investigated to understand the consequences of its downregulation in metastasized pancreatic cancer.

MATERIALS AND METHODS

Cell culture and virus transductions

The pancreatic cancer cell lines BxPC-3, AsPC-1, MIA PaCa-2, and PANC-1 (described in Table 4.1) were purchased from the American Type Culture Collection (Manassas, VA, USA). BxPC-3, AsPC-1, MIA PaCa-2 and PANC-1 cells were grown in complete media (RPMI supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Dartmouth, MA) and 1% Penicillin Streptomycin (P/S) (Mediatech, Inc. A Corning Subsidiary, Manassas, VA), RPMI supplemented with 15% FBS and 1% P/S or DMEM with 10% FBS and 1% P/S respectively). All cell lines were cultured at 37°C in a humidified 5% CO_2 incubator.

Ad.GFP (adenovirus containing GFP) and Ad.GFP.RGS16 (Adenovirus containing RGS16 and reporter gene GFP) viruses were purchased from Vector Biolabs (Philadelphia, PA). Viruses were amplified and tittered as previously described [183-185]. For each type of virus, concentration of 50 MOI (multiplicity of infection) were added to pancreatic cancer cells in media supplemented with 2% heat-inactivated Hyclone FBS (GE Healthcare Life Sciences, Pittsburgh, PA).

Quantitative Real-time PCR analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA (2ug) was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit from Applied Biosystems (Foster City, CA) according to the manufacturer's protocol. Real-Time PCR was performed using the Applied Biosystems TaqMan Gene Expression Assays in the ABI 7000 detection system. TaqMan probes were purchased from Applied Biosystems [RGS16 (HS00892674_m1), and GAPDH (HS02758991)]. The relative fold change for each marker was calculated using the $2^{-\Delta\Delta CT}$ analysis according to Livak *et.al* and statistical significance was determined using a one

way ANOVA with a Tukey's post-hoc test using Prism V6.0c (GraphPad Software, Inc., La Jolla, CA) *[187]*.

Wound Healing Assay

Pancreatic cancer cells (BxPC-3, AsPC-1 and PANC-1) were placed in a 6 well plate at approximately 70% confluency. The following day, 50 Multiplicity of Infection (MOIs) of Ad.GFP (control) or Ad.GFP.RGS16 were added to the cells in media containing 2% heat-inactivated FBS and incubated for 24 hours. The media was then changed to complete media (10% FBS for BxPC-3 and PANC-1 or 15% for AsPC-1) and cells incubated for 24hrs. Forty eight hours after the addition of the virus the media was changed from complete media to media supplemented with 0.5% FBS and 1% P/S and the cells incubated for additional 24-hours. The media was replaced with PBS and three wounds or scratches were made per well using a p200 pipette. The cells were washed three times with PBS and incubated for 16-24 hours in complete media or media supplemented with 100ng/ml of EGF. EGF was added to induce cell migration at a concentration previously described in [228-230]. Wound widths were measured and images taken at 0, 16, or 24 hrs after addition of media supplemented with FBS or EGF at 100x magnification using an Olympus DP71 microscope (Center Valley, PA). Efficacy of virus transduction was confirmed using fluorescent microscopy to examine GFP expression prior to the start of the experiment. Percent wound healing was determined using the following equation; % wound-healing = ([initial scratch width _ final scratch width]/ initial scratch width) X 100. Three independent replicates were performed for each cell line.

MTT Assay

BxPC-3, AsPC-1, and PANC-1 cells (5,000) were plated in quadruplicate in a 96 well tissue culture dish and incubated for 24 hours at 37°C. The pancreatic cancer cells were treated as described in the wound healing assay section. After the cells were serum starved (media supplement with 0.5% FBS and 1% P/S) for 24-hours complete media or media supplemented with 100ng/ml of EGF were added to the cells. A 1:10 dilution of MTT (thiazolyl blue tetrazolium bromide; MP Biomedicals; Santa Ana, CA) stock solution (5mg/ml diluted in PBS), was added to the media of the cells followed by a 2 hour incubation at 37°C. The media was removed and DMSO was added to the cells to solubilize the purple formazan crystals. Absorbance (560nm) was determined at 0, 24, 48, and 72 hours after addition of supplemented media using the SpectraMax M2^{e} Molecular Devices (Sunnyvale, CA). Cell viability fold change was calculated using the average absorbance for each treatment group at 24, 48, or 72 hours divided by the initial absorbance at time zero. Percent viability was calculated using the average absorbance for each treatment divided by the average absorbance for the cells not treated with virus and multiplied by 100. Statistical significance was assessed using Student's *t*-test by Prism V6.0c. The experiment was repeated 3 independent times.

Invasion Assay

BD Bio Coat Matrigel coated polycarbonate invasion chambers (Bedford, MA) containing membranes with 8um pores were used to assess the ability of RGS16 to inhibit pancreatic cancer cell migration and invasion. BxPC-3 cells were plated into 6-well dish at 70% confluency, 24 hours later 50 MOIs of Ad.GFP or Ad.GFP.RGS16 virus were added to the cells followed by 24 hour incubation in complete media and 24 hours in low-serum media as described in the wound-healing section. Chambers were re-hydrated in RPMI containing 1% P/S

and 0.1 % Bovine serum albumin (BSA, Fisher Scientific, Waltham, MA) for 2 hours at 37°C. BxPC-3 and AsPC-1 cells were collected and 25 x 10^4 cells were added to the top of the chambers in RPMI supplemented with 1% P/S and 0.1% BSA. RPMI supplemented with 100ng/ml EGF, 1% P/S, 0.1% BSA was added to lower portion and the chambers were incubated for 18- (AsPC-1) or 20- (BxPC-3) -hours at 37°C. Time points were determined by literature and previous preliminary experiments to optimize conditions of the assay. The non-migrating cells were removed using a cotton swab and the invaded cells were fixed using 100% methanol (MeOH) for 5 minutes and stained using 0.5% crystal violet plus 20% MeOH (10-15 mins). Invaded cells were counted using 200x magnification with 12 different views. Percentage of invasion compared to GFP control was calculated for each cell line [(# of invaded $cells_{treated}$ / # of invaded $cell_{control}$) x 100]. Three replicates were performed for each cell line.

Western Blot Analysis

AsPC-1 and BxPC-3 cells were treated to express GFP and/or RGS16 using adenoviruses as described in the wound healing assay section. Cells were collected after 0 min, 15 mins, 1 hour and 24 hours after addition of EGF. Cells were lysed in RIPA (Radioimmunoprecipitation assay) buffer plus protease inhibitors (Pierce Protease inhibitor Tablets 88661; Thermo Scientific, Rockford, IL). Protein extracts (35-60ug) were loaded onto 10% or 12% polyacrylamide gels and proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Blots probed with mouse anti-panAKT (40D4, 1:2000), rabbit anti-phospho-AKT (p-AKT, S473 D9E, 1:2000), rabbit anti-phospho-p44/42 MAPK (p-ERK1/2, Thr202, Tyr204, D13.14.4E, 1:2000), rabbit anti-p44/42 MAPK (total-ERK1/2, 137F5, 1:1000) antibodies from Cell Signaling Technology (Danvers, MA) were blocked in 5% BSA in Tris-buffered saline solution pH 7.6 containing 0.1% Tween-20 (TBS-T). Blots probed in rabbit

anti-E-cadherin (Santa Cruz Technologies, Dallas, TX, 1:1000), rabbit anti-RGS16 (Proteintech, Chicago, IL, 1:250-1:500, band detected at 23kDa) mouse anti-GAPDH (Chemicon International, Temecula, CA, 1:1000) or mouse anti-vimentin (AF-14b, 1:500) were blocked an hour in 5% dry non-fat milk diluted in TBS-T. The anti-vimentin (AMF14b), monoclonal antibody developed by Fulton, A.B. was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. Membranes were probed in primary antibody overnight at 4°C on a rocker. Following primary antibody incubation the membranes were washed and probed with Horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:5000) or goat anti-rabbit (1:10000) secondary antibodies (Rockland, Gilbertsville, PA) for 1 hour at room-temperature. Primary and Secondary antibodies were diluted in TBS-T. Amersham ECL prime western blotting detection reagent was added to visualize the protein bands (RPN 2232, GE Life Sciences, Pittsburgh, PA). Western blot images were captured using a FOTODYNE FOTO/Analyst FX (Hartland, WI) imaging camera. Densitometry was performed using TotalLab Quant software (TotalLab Ltd, UK).

Phalloidin F-actin staining

BxPC-3 and AsPC-1 cells were plated into a six well dish at 70% confluency for adenovirus treatment and after 24 hours the cells were treated with 50 MOIs of Ad.GFP or Ad.RGS16 for 24 hours. Cells were washed with PBS and plated into a Lab-TekII chamber slide system (Thermo Fisher Scientific, Waltham, MA) with a concentration of 50,000 cells/well and incubated for 24 hours in complete media. Cells were then incubated in low serum (0.5% FBS) supplemented media for 24 hours. Media supplemented with EGF (100ng/ml) was added to the cells for 15 min (BxPC-3) or 30 min (AsPC-1) to induce cytoskeleton rearrangement. Time

points were determined by literature and previous preliminary experiments to optimize conditions of the assay. Cells were fixed in 4% paraformaldehyde in PBS (Fisher Scientific) for 15 min and permeabilized using 0.1% Triton X-100 in PBS containing 1% BSA at room temperature. Cells were blocked for 30 min in PBS containing 1% BSA at room temperature followed by 30 min incubation of a final concentration of 50nM of Acti-stain 555 Fluorescent Phalloidin (Cytoskeleton, Inc., Denvir, CO) at room-temperature in a dark humid chamber. Cells were washed 3x in PBS + 1% BSA for 5 minutes and the nuclei were stained with 300nM DAPI for 2 min and the cells washed 3x in PBS + 1% BSA for 5 minutes. F-actin was visualized at 400x and 1000x using an Olympus DP71 microscope with a mercury fluorescent light source U-LH100HG (Center Valley, PA). The staining for phalloidin was performed three independent times in the AsPC-1 cells.

Statistical Analysis

All data were expressed as the mean ± SEM and represented using Graph Pad Prism V6.0c software (GraphPad Software, Inc., La Jolla, CA). Differences between control and treated samples for the MTT (viability fold change), wound healing and invasion assay were analyzed by using Student's *t*-test (Graph PadPrism V6.0c). Differences for percent viability determined by MTT assay was analyzed by two-way ANOVA with Dunnet's post-hoc test for multiple comparisons. Data from real-time PCR were analyzed by one-way ANOVA followed by Tukey post-hoc test. Additional statistical analysis used for qRT-PCR analyses are listed in the respective section. Data was considered significant when the p value was less than 0.05.

Statistical Analysis tests used for qRT-PCR analyses are listed in the respective section.

Table 3.1: Characterization of pancreatic cancer cell lines [51, 111, 231, 232]

	p53	p16	Ras	EGFR	Differentiation	Origin	Metastasis
BxPC-3	mt	del	wt	high	moderate	primary	no
PANC-1	mt	del	mt	high	poor	primary	yes
AsPC-1	mt	mt	mt	high	poor	metastatic (ascites)	yes
MiaPaCa-2	mt	del	mt	low	poor	primary	no

mt = mutant, del = deleted, wt = wild-type

RESULTS

mRNA expression of RGS16 is decreased in pancreatic cancer cell lines.

We first investigated the relative expression of RGS16 mRNA in the BxPC-3, MiaPaCa-2, PANC-1, and AsPC-1 pancreatic cancer (pancreatic ductal adenocarcinomas) cell lines in order to characterize the endogenous expression of RGS16. Expression of RGS16 was measured by qRT-PCR analysis and the relative RGS16 mRNA fold change was calculated in the four pancreatic cancer cell lines compared to total RNA from normal human pancreatic tissue. Expression of RGS16 was decreased in all four pancreatic cancer cell lines compared to control with BxPC-3 having the highest expression of RGS16 mRNA (Figure 3.1). Expression of RGS16 varied between the four pancreatic cancer cell lines with BxPC-3 and MIA PaCa-2 having significantly higher expression of RGS16 than PANC-1 and the metastatic derived AsPC-1 cells. Higher RGS16 expression was found in the more differentiated and less aggressive cell line, while less RGS16 expression correlated with the more aggressive and/or metastatic cell lines (Table 3.1).

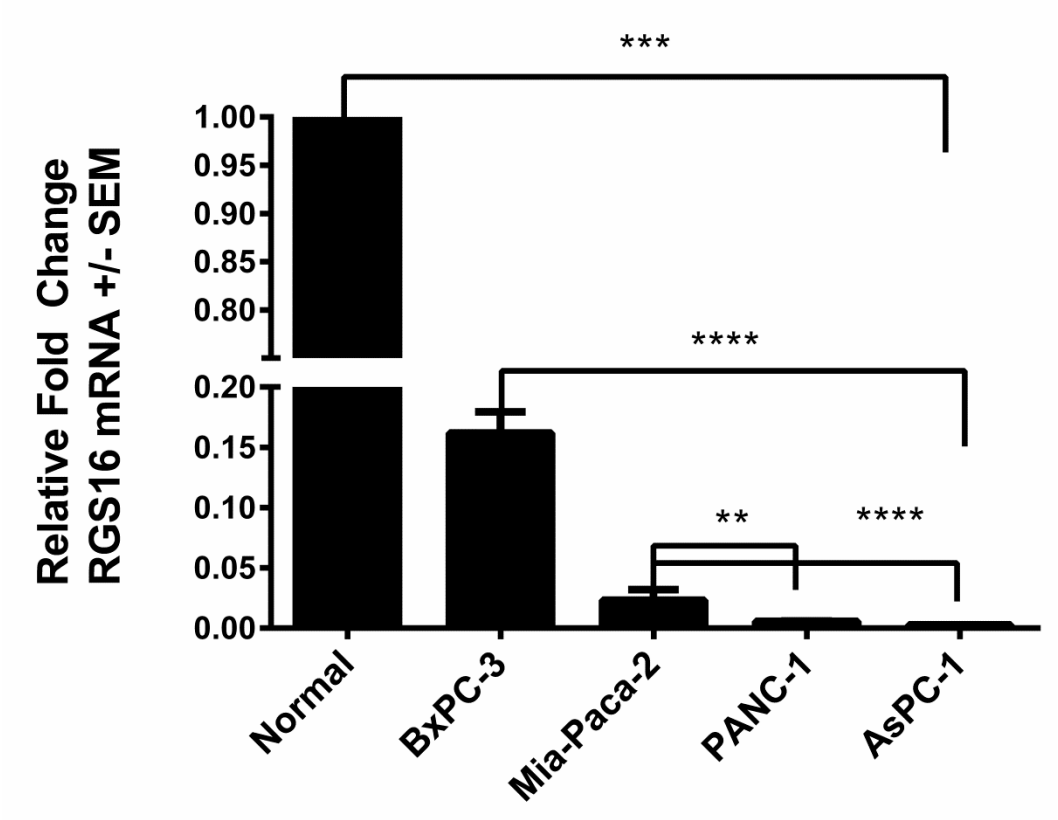

Figure 3.1: Decreased expression of RGS16 mRNA relative to total RNA extracted from normal human pancreatic tissue. Expression of RGS16 was measured using qRT-PCR in BxPC-3, MiaPaCa-2, PANC-1, and AsPC-1 cells. Relative fold change was measured using total RNA extracted from normal human pancreatic tissue as the control. One-way ANOVA with Tukey's test for multiple comparison were used to test for statistical significance between the cell lines and control * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, and **** p-value < 0.0001.

Expression of RGS16 inhibited migration of BxPC-3 and AsPC-1 pancreatic cancer cells but not PANC-1.

To test the hypothesis that RGS16 inhibits pancreatic cancer cell migration, we exogenously expressed RGS16 in BxPC-3, PANC-1, and AsPC-1 cells with an adenoviral vector and used wound-healing assays to measure cell migration. We chose BxPC-3, PANC-1, and AsPC-1 because these three cell lines are derived from tumors with varying expression of RGS16, differentiation status, mutations, presence of metastases, and expression of Epidermal Growth Factor Receptor (Table 3.1). EGF was used to stimulate cell migration therefore; we did not use the MiaPaCa-2 cell line because of the lower levels of EGFR (Table 3.1). We RGS16 was expressed using Adenoviruses that contain RGS16 plus a GFP reporter (Ad.GFP.RGS16). An Adenovirus expressing GFP (Ad.GFP) was used as the vector control. Expression of RGS16 protein correlated with GFP expression in cells treated with Ad.GFP.RGS16 (Figure 3.2). Fluorescent microscopy was used to determine viral transductions prior to experiment (Figures 3.3a, 3.4a, and 3.5a). Overexpression of RGS16 significantly inhibited FBS and EGF induced migration of BxPC-3 cells and FBS induced migration of AsPC-1 cells, but had no effect on FBS and EGF induced migration of PANC-1 cells (Figures 3.3-3.5). Interestingly, expression of RGS16 in BxPC-3 cells incubated in media supplemented with EGF caused an increase in wound width compared to control 16 hours after the start of the experiment.

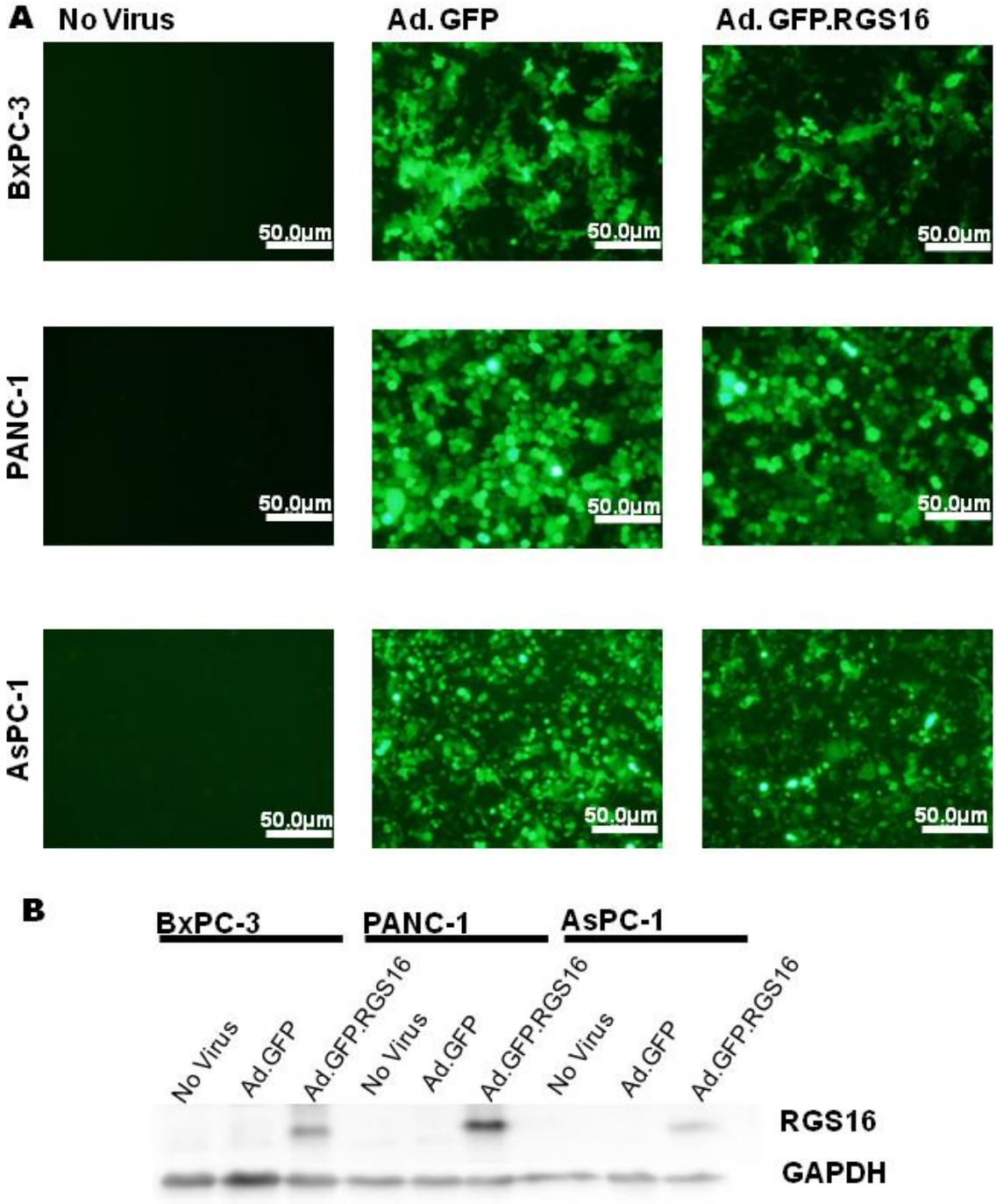

Figure 3.2: Fluorescence of GFP associated with RGS16 protein expression in cells treated with Ad.GFP.RGS16. Cells were plated and received the following treatment: 1) untreated cells, 2) Ad.GFP and 3) Ad.GFP.RGS16 treated cells. Cells were incubated in complete and low serum media 24 hours each. A) Images of GFP fluorescence were taken 72 hours after treatment and B) protein expression for RGS16 and GAPDH was assessed by western blot analysis.

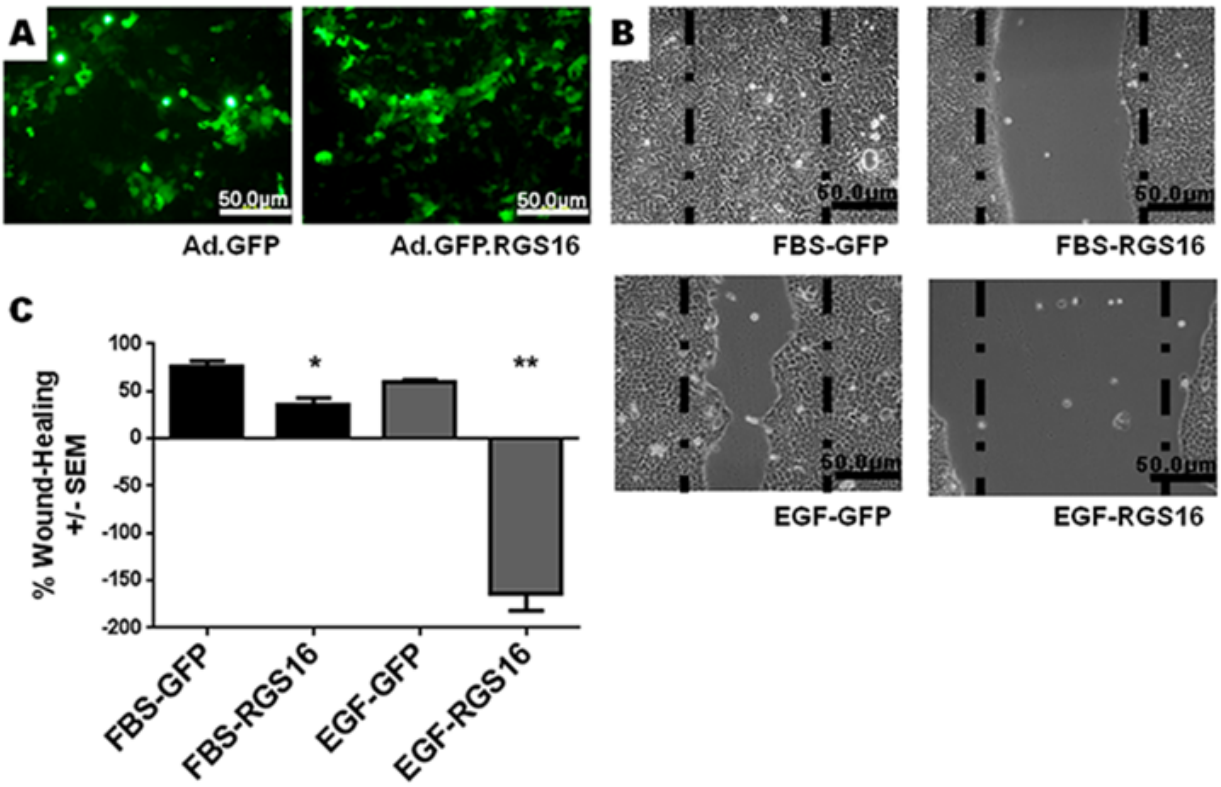

Figure 3.3: Expression of RGS16 inhibited migration of BxPC-3 cells.
BxPC-3 cells were transduced with 50 MOI of Ad.GFP (CTRL) or Ad.GFP.RGS16. A) Virus transduction was verified by fluorescent microscopy. B) Images (100x) and measurements of wounds were taken prior and 16 hours after addition of media supplemented with FBS (10%) or EGF (100ng/ml). The dashed lines represent size of scratch at time 0. C) Mean Percentage of wound healing ± SEM of three separate experiments (three scratches/well) was determined. Student's t-test was used to determine statistical significance compared to control * p-value < 0.05, ** p-value < 0.01.

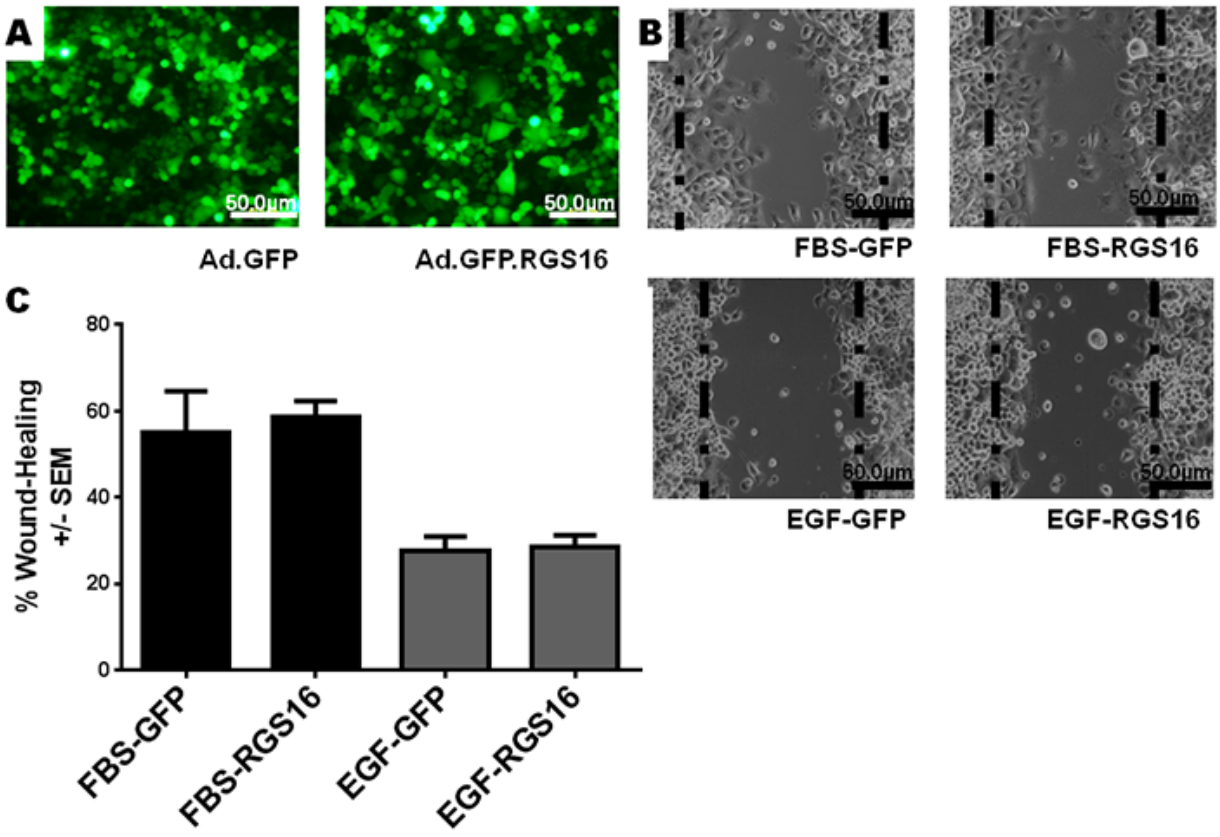

Figure 3.4: Expression of RGS16 did not inhibit migration of PANC-1 cells.
Wound healing assays were performed as described in Figure 3.3 A) Fluorescent microscopy was used to verify virus transductions: B) Images (100X) were taken and C) percentages of wound healing were calculated at 24 hrs after the addition of media supplemented with FBS or EGF.

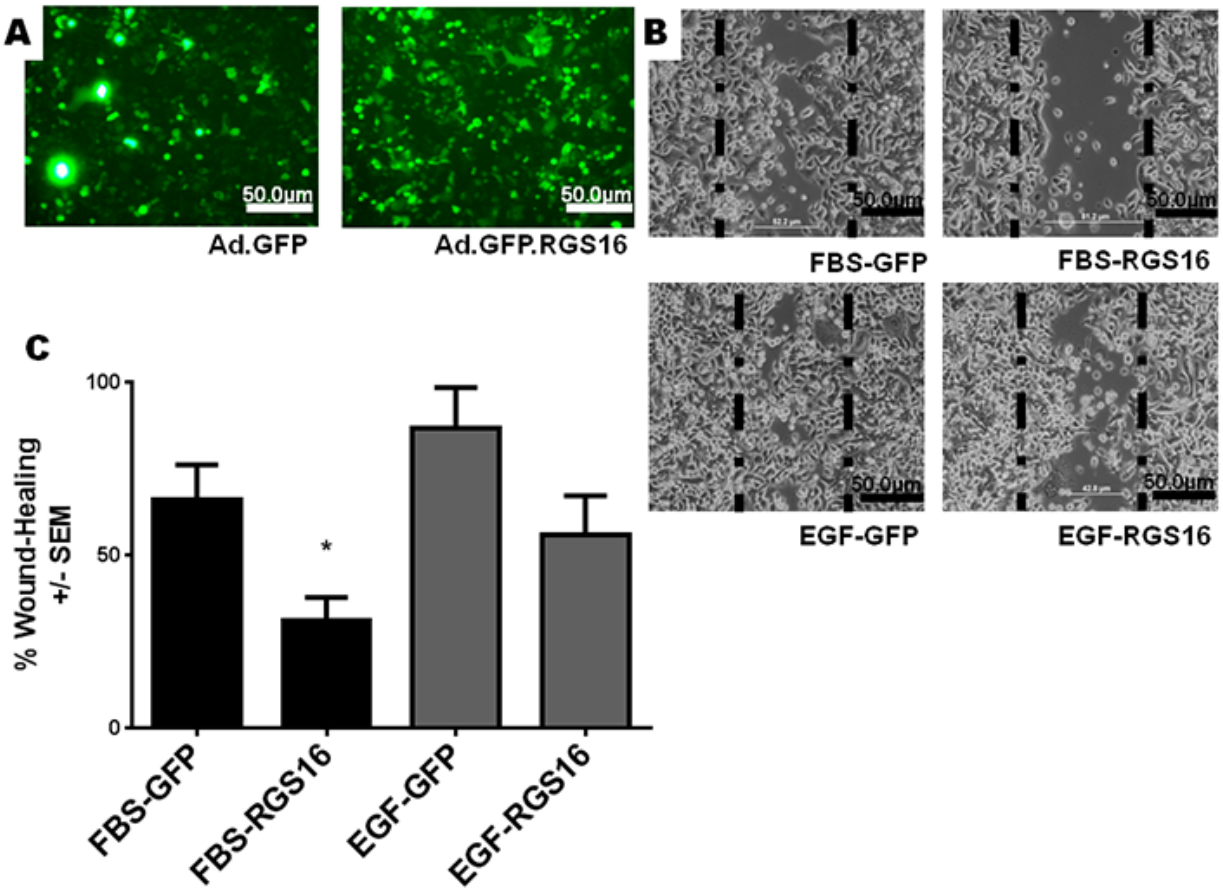

Figure 3.5: Expression of RGS16 inhibited migration of AsPC-1 cells.
Wound healing assays were performed as described in Figure 3.3. A) Fluorescent microscopy was used to verify virus transductions: B) Images (100X) were taken and C) percentages of wound healing were calculated at 24 hrs after the addition of media supplemented with FBS or EGF, * p-value < 0.05.

Expression of RGS16 inhibited EGF induced invasion of BxPC-3 and AsPC-1 cells.

Since RGS16 inhibited EGF induced migration of BxPC-3 and AsPC-1 cells, matrigel invasion chambers were used to investigate whether RGS16 could also inhibit EGF induced invasion of these pancreatic cancer cells. Media supplemented with EGF was used as the chemoattractant to induce migration and invasion of BxPC-3 and AsPC-1 cells expressing GFP and or RGS16. Compared to control cells. RGS16 expression significantly inhibited EGF induced invasion of the BxPC-3 and AsPC-1 cells by 35.73% and 66% respectively(Figure 3.7).

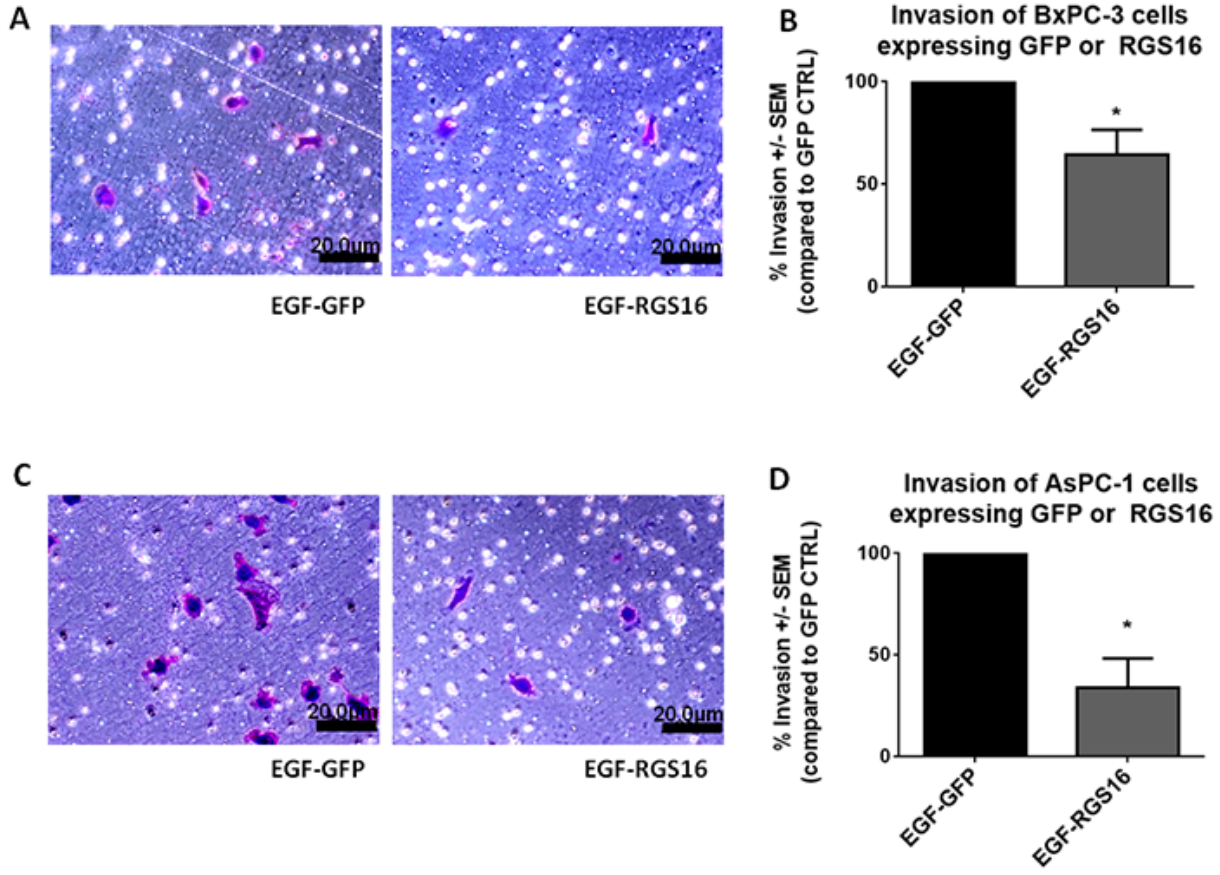

Figure 3.6: Expression of RGS16 inhibited invasion of BxPC-3 and AsPC-1 cells.
Matrigel invasion chambers were used to measure cell migration and invasion of GFP and/or RGS16 expressing BxPC-3 (A & B) and AsPC-1 (C & D) cells using EGF as a chemoattractant. Migrated cells were stained with Crystal Violet and counted at 200x magnification (A &C). Percent invasion was calculated for each cell line (B & D) * p-value < 0.05.

Expression of RGS16 using adenoviruses did not modify cell viability

The results from the wound healing assays demonstrated an increase in the wound width of BxPC-3 cells treated with Ad.GFP.RGS16 and incubated in media supplemented with EGF. This effect could be due to a decrease in the number of viable cells caused by expression of RGS16 or by a cytolytic effect of adenoviruses. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were used to determine if expression of RGS16 or infection with the adenoviruses affects the viability of the pancreatic cancer cell lines. The cells were serum starved for 24 hours to remove any pre-existing growth factors. Complete media containing FBS (10 or 15%) or EGF (100 ng/ml) was added to induce growth of the pancreatic cancer cells. Absorbance was measured at 0, 24, 48, and 72 hours following the addition of FBS or EGF. The fold change of viable cells after 24, 48, and 72 hours compared to initial viable cell number was calculated for BxPC-3, AsPC-1, and PANC-1 cells treated with Ad.GFP or Ad.GFP.RGS16. Expression of RGS16 did not alter the number of viable BxPC-3, PANC-1 or AsPC-1 cells stimulated with FBS or EGF (Figure 3.7A – 3.7C). The percentage of viable BxPC-3 and AsPC-1 cells treated with Ad.GFP or Ad.RGS16 was not significantly different compared to no virus treated controls (NV FBS or NV EGF) for any of the time points (Figure 3.7D and 3.7E). There was a significant 20-25% mean drop in cell viability for PANC-1 cells treated with Ad.GFP or Ad.GFP.RGS16 48 and 72 hours after the addition of FBS to serum starved cells (Figure 3.7F). However, all migration and invasion experiments were completed at a time in which there was no significant change in the percentage of viable cells (experiment completion $\leq$ 24 hours). The results from the MTT assays suggest that RGS16 expression does not have an impact on BxPC-3, PANC-1, and AsPC-1 cell viability and the concentration of virus used to transduce RGS16 was tolerated by the pancreatic cancer cells.

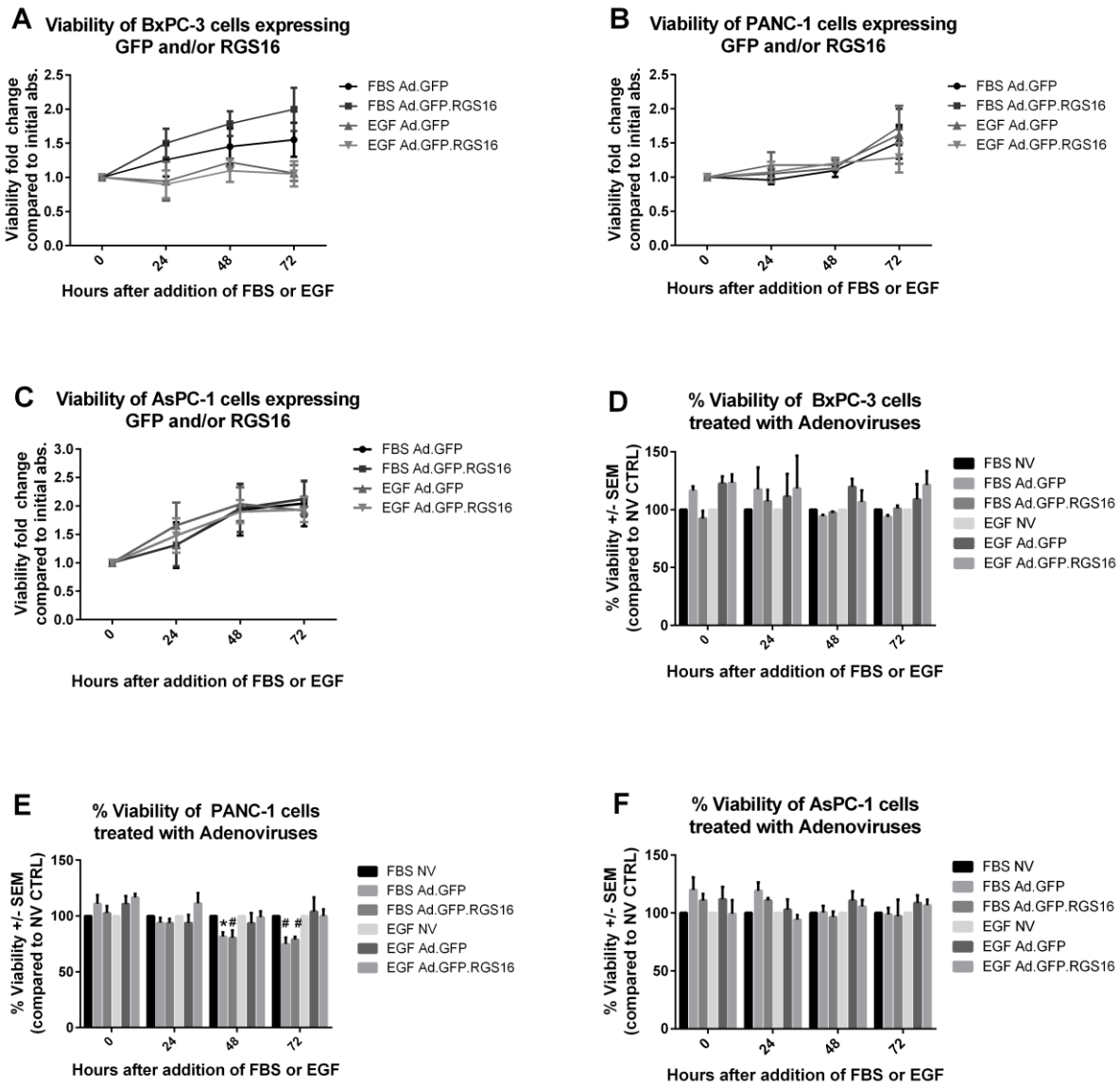

Figure 3.7: Expression of RGS16 did not alter number of viable cells and concentration of Adenoviruses used was tolerated by BxPC-3, PANC-1, and AsPC-1 pancreatic cancer cells. A-C) The fold change of viable cells was calculated by comparing the initial absorbance (0HR) compared to absorbance for 24, 48, and 72 hours after the addition of complete media or media supplemented with 100ng/ml of EGF (mean fold change of viable cells +/- SEM of three separate experiments) Statistical significance was tested using paired Student's t-test. D-F) Effect of Adenoviruses on number of viable pancreatic cancer cells was determined by calculating the % viability by comparing the number of viable cells treated with Ad.GFP or Ad.GFP.RGS16 to cells that were not treated with viruses. Data represents mean % of viable cells +/- SEM of three separate experiments. Statistical significance was tested using two-way ANOVA with Dunnet's post-hoc test for multiple comparisons. *P<0.05, # P<0.01 (NV = No virus)

Expression of RGS16 did not alter EGF induced expression of p-AKT (phosphorylated AKT), p-ERK (phosphorylated ERK).

Our next step was the investigation of the mechanism(s) employed by RGS16 to regulate EGF induced cell migration and invasion. Activation of EGFR by EGF initiates a signaling cascade that is most commonly associated with activation of the PI3K/AKT and mitogen activated protein kinase (MAPK) pathways [110, 114]. Phosphorylation and activation of AKT or ERK1/2 (MAPK) are both known to phosphorylate downstream signaling molecules and transcription factors that are responsible for promoting cell migration and invasion [119, 233-235]. Protein expression of p-AKT and p-ERK in BxPC-3 and AsPC-1 cells treated with EGF were determined in order to delineate if one or both of these pathways is regulated by RGS16. Resulting in the inhibition of EGF induced migration and invasion. There was no change detected in the protein levels of p-AKT or p-ERK following 15 minutes of EGF treatment of BxPC-3 or AsPC-1 cells expressing RGS16 compared to GFP controls (Figure 3.8). This data suggests RGS16 inhibits migration and invasion of the pancreatic cancer cells independently of AKT and ERK activity.

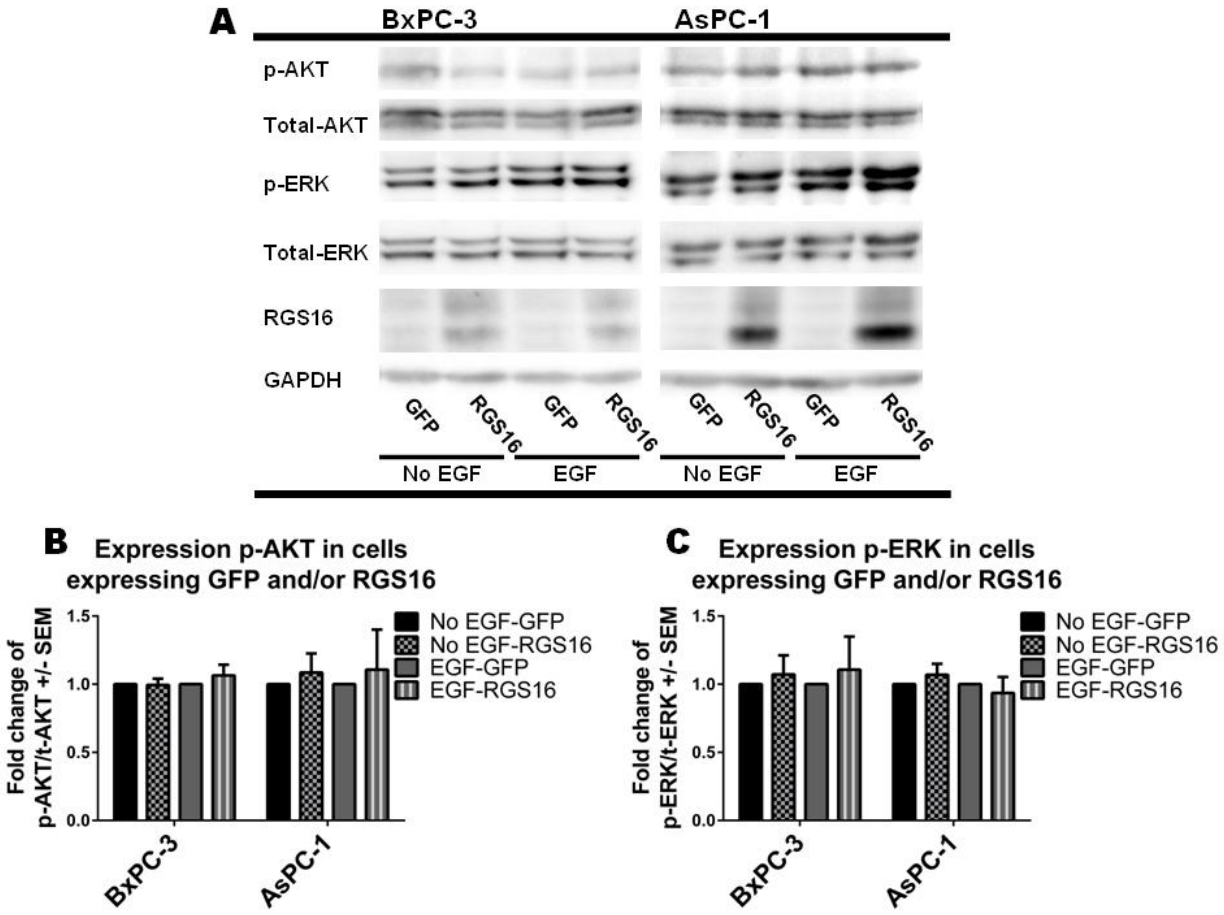

Figure 3.8: RGS16 did not alter expression of phosphorylated AKT (p-AKT) or phosphorylated ERK (p-ERK). Western blot analyses were used to examine levels of phosphorylated AKT and ERK in BxPC-3 and AsPC-1 cells expressing GFP and/or RGS16 treated with or without EGF for 15 min (A). The mean fold change +/- SEM of p-AKT/total AKT (t-AKT) and p-ERK/total ERK (t-ERK) was calculated using densitometry analysis (B & C).

Investigation of RGS16's Efect on epithelial mesenchymal transition markers and cytoskeleton rearrangement

Next we examined if expression of RGS16 inhibits cell migration and invasion by preventing epithelial mesenchymal transition (EMT) in BxPC-3 and AsPC-1 cells. EGF promotes EMT by decreasing the expression of the epithelial cell-cell adhesion molecule E-cadherin and increasing the expression of mesenchymal phenotype markers such as vimentin, fibronectin, and n-cadherin; these mesenchymal proteins allow cell migration and invasion [116, 126]. Using western blot analysis, we examined the expression of vimentin and E-cadherin 1 hour and 24 hours following EGF treatment. Expression of RGS16 up-regulated E-cadherin protein levels in BxPC-3 but not in the metastatic AsPC-1 cells (Figure 4.10). Furthermore, vimentin expression did not change in either BxPC-3 or AsPC-1 cells expressing RGS16 compared to the GFP control (Figure 3.9).

Rearrangement of the actin cytoskeleton is an integral part of cancer cell migration and invasion and it is regulated by members of the Rho GTPase family. Activation of the EGF/EGFR pathway initiates actin cytoskeleton rearrangement through activation of Rho GTPases (Cdc42, RhoA, Rac1) that aide in the migration of cancer cells [121, 122, 236]. We treated serum starved cells expressing GFP and or RGS16 with EGF for 15- (BxPC-3) or 30- (AsPC-1) minutes and stained the F-actin of the cells using phalloidin. There was no obvious difference in the arrangement of the F-actin in BxPC-3 and AsPC-1 cells expressing RGS16 compared to GFP controls (Figure 3.10). Both cell lines contain a heterogeneous population making it difficult to quantify a difference in the actin organization between cells expressing GFP and/or RGS16.

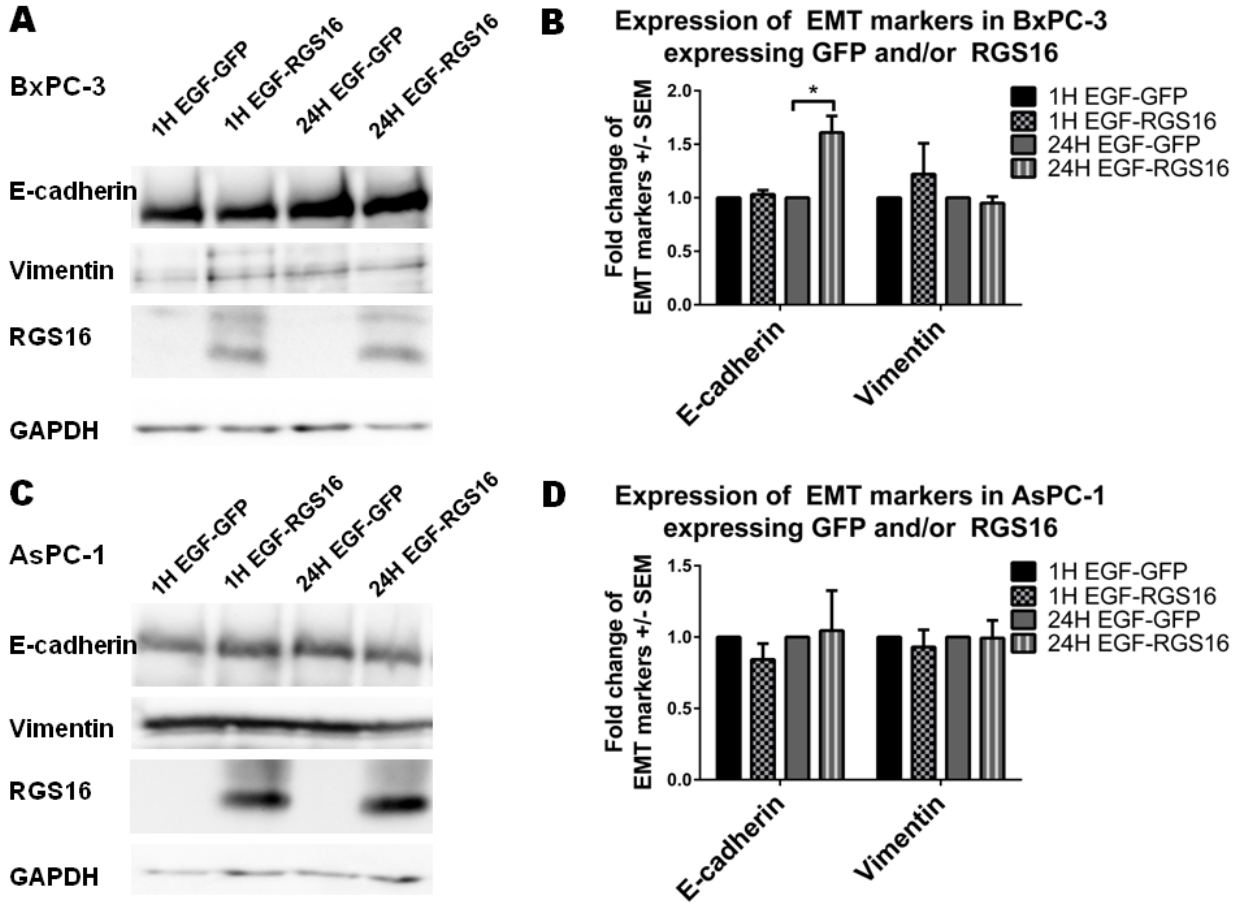

Figure 3.9: RGS16 increased expression of E-cadherin in BxPC-3 cells but not AsPC-1 and did not alter vimentin expression in either cell line. Western blots were used to determine E-cadherin and Vimentin expression in BxPC-3 and AsPC-1 expressing GFP and/or RGS16 (A & C) and treat with EGF for 24h. Mean fold change +/- SEM of EMT markers was calculated using densitometry analysis for both EMT markers (B & D). * p-value < 0.05

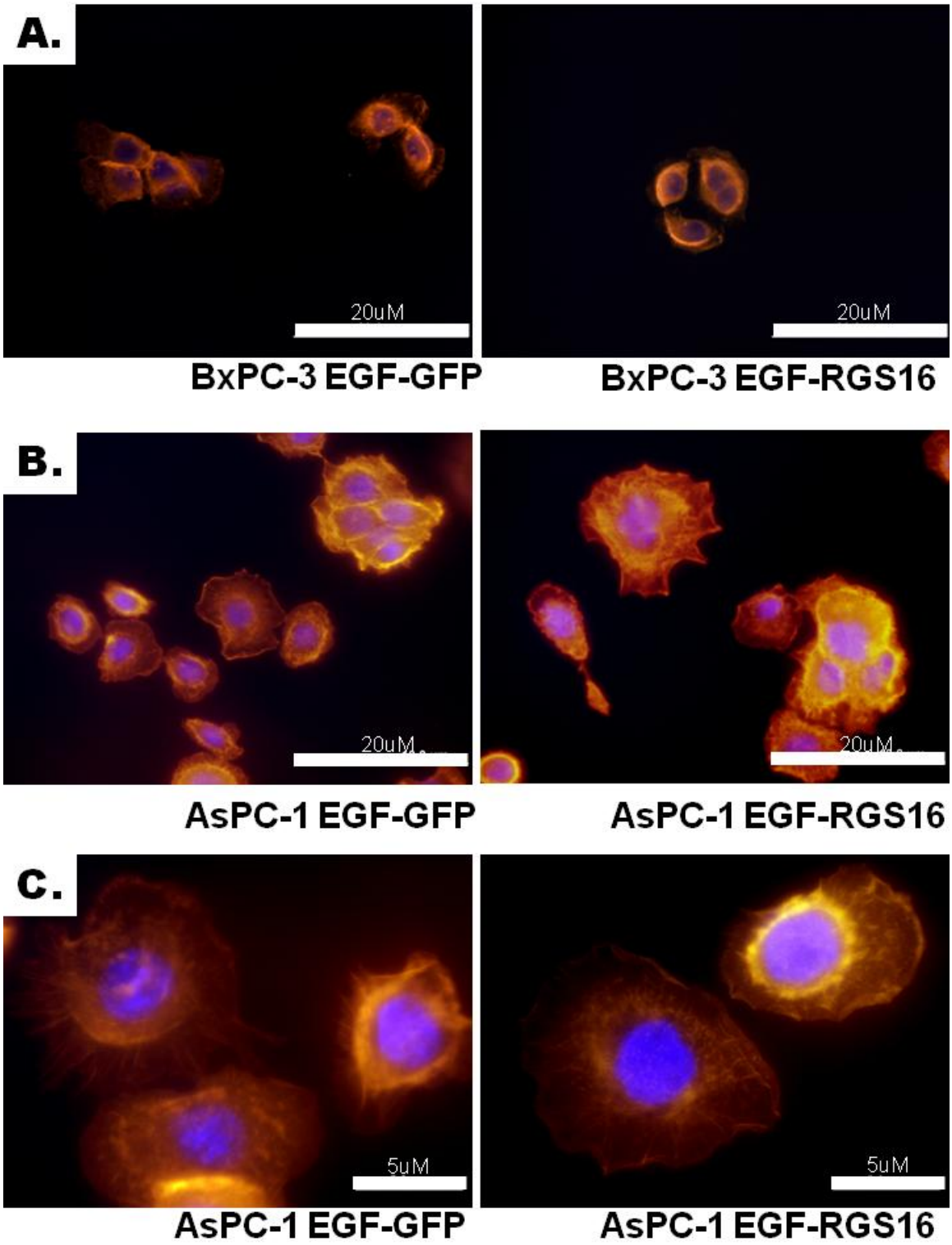

Figure 3.10: F-actin of BxPC-3 and AsPC-1 cells did not show obvious reorganization in cells expressing GFP and/or RGS16. BxPC-3 and AsPC-1 cells expressing GFP and/or RGS16 were treated with EGF for 15(BxPC-3) or 30 (AsPC-1) minutes, fixed, and stained using Phalloidin conjugated to rhodamine and Dapi cells were imaged at (A & B) 400x or (C) 1000x

DISCUSSION

Pancreatic cancer and RGS16 significance

A large portion of patients newly diagnosed with pancreatic cancer present with metastatic or locally advanced disease resulting in their ineligibility for curative pancreatic surgical resection [237]. Early dissemination and aggressiveness of this disease contributes to the dismal survival rates of pancreatic cancer [237, 238]. Loss of RGS16 has been identified as a possible marker for lymph node metastasis in pancreatic cancer; however, the function of RGS16 is unknown [149]. We, for the first time, showed that RGS16 expression inhibits pancreatic cancer cell migration and invasion suggesting that loss of RGS16 could be important in the metastatic process.

New treatments are needed to prevent or treat advanced or metastatic cancer. However, in order to develop new treatments we need a better understanding of the molecular changes that occur in pancreatic cancer. We identified RGS16 as a p53 and pRb cross-talk candidate (Chapter 2). RGS16 has been of interest to further our studies for two reasons: 1) RGS16 regulates GPCRs, which are common targets for deregulation in cancer and 2) RGS16 has been linked to regulating the MAPK/RAS, PI3K/AKT, RhoA, and SDF-1/CxCR4 oncogene pathways [133, 135, 141, 164, 171]. Investigations have found that oncogene pathways can feed into one another and bypass or overcome the inhibitory effects of monoclonal antibodies or other targeted inhibitors. For example, in melanoma, increased production of VEGF or increased expression or activation of the platelet-derived growth factor receptor-β or insulin-like growth factor-1 receptor is associated with resistance to BRAF inhibitors, demonstrating mechanisms cancer cells use to overcome single target modalities [239]. Therefore investigation of RGS16, a protein known to modulate several oncogene pathways will aid in understanding mechanisms by which cells alter

multiple signaling pathways to prevent carcinogenesis. This information could then be used for future drug development.

RGS16 and cell migration and invasion

RGS16 has been linked with inhibition of cell migration in canonical (through regulation of GPCR signaling) and non-canonical pathways in normal cells. RGS16 inhibits megakaryocytes and T lymphocyte migration by regulating the activation of the GPCR CxCR4 and decreasing T helper type 2 and 17 cell trafficking through regulation of CCR4 and CCR10 chemokine pathways representing the canonical form of RGS signaling [136, 137, 141]. The activation of RhoA, a small GTPase involved in reorganizing actin cytoskeleton and mediating EGF induced pancreatic cell invasion is inhibited in MCF-7 cells by RGS16 preventing the relocation of Gα13 to the plasma membrane thus blocking Gα13 mediated activation of RhoA [164, 240]. The regulation of RhoA activation by RGS16 is an example of a non-canonical, non-GPCR-mediated mechanism. There are no published reports demonstrating inhibition or activation of cell invasion by RGS16. However, another member of the R4 subfamily of RGS proteins, RGS4, suppresses breast cancer migration and invasion *in vitro* and *in vivo* by regulating PAR-1, CxCR4 signaling, and RAC-1 lamellipodia formation [241].

Ours is the first report demonstrating a role of RGS16 in inhibiting EGF induced cell migration and invasion. The GPCR CxCR4 is aberrantly expressed in malignant and cancer stem cells and contributes to pancreatic cancer progression by aiding in gemcitabine resistance, cell migration, and invasion [173, 222, 242, 243]. Although our studies focused on EGF induced cell migration and invasion, RGS16 may be able to inhibit other pathways that mediate cell migration and invasion such as CxCR4.

EGF and pancreatic cancer signaling

RGS16 and EGF induced activation of the PI3K and AKT pathways

Pancreatic carcinomas have overexpression or amplification of the tyrosine kinase receptors EGFR/erbB1 (~85%) or HER2/erbB2 (2%) [110, 244]. Activation of EGFR by its ligands EGF or TGFα induces or enhances activation of a variety of signaling pathways including PI3K/AKT, MAPK, SRC, PLC, and the small Rho GTPases (Rac1,RhoA, RhoC, and Cdc42) [114, 121, 122]. These pathways are involved in activating processes that enhance cell proliferation, migration, invasion, EMT, and metastasis in pancreatic cancer [119, 126, 233]. For example, both AKT and ERK can aid in cell migration and invasion through regulating production of matrix metalloproteinase 9 [233, 245]. Mutations downstream of EGFR resulting in AKT2 amplification or constitutive activation of K-Ras (MAPK pathway) also occur in pancreatic cancer, making EGFR and its downstream components popular targets for pancreatic cancer treatments [96, 109]. We investigated whether RGS16 can inhibit EGF induced migration and invasion via regulation of AKT and ERK (MAPK). A study conducted using breast cancer cells found that RGS16 knockdown resulted in an increase rate of EGF induced proliferation in these cells due to increase in p-AKT [171], suggesting that RGS16 may regulate proliferation by preventing the phosphorylation and activation of AKT. In our studies, RGS16 had no effect on the number of viable pancreatic cancer cells and did not decrease the protein levels of p-AKT compared to control. Our conflicting results could be due to two possibilities, 1) RGS16 targets different signaling pathways in pancreatic cancer compared to breast cancer or 2) mutations in pancreatic cancer (for example K-Ras or AKT2) overcome the inhibitory effect of RGS16 on pancreatic cancer viability or activation of AKT.

RGS16 can inhibit MAPK activation via regulation of G proteins associated with GPCRs [135]. However, other RGS molecules are known to directly interact and regulate localization and activity of members in the MAPK cascade. We examined the expression of phosphorylated ERK and found no change in BxPC-3 and AsPC-1 cells expressing RGS16 relative to the GFP controls. Our data suggests RGS16 inhibits EGF induced migration and invasion independently of the PI3K/AKT and MAPK pathways.

RGS16 and EGF induced activation of EMT and F-actin rearrangement

Cancer cells can adapt a mesenchymal phenotype aiding in their dissociation and migration to a distant site [126]. EGF can induce EMT in a variety of cancer cells by decreasing expression or localization of E-cadherin and increasing the expression of EMT markers such as N-cadherin, vimentin, and fibronectin [116, 246]. Knockdown of EGFR in the pancreatic cancer cell line PANC-1 inhibited EMT and decreased cell migration and invasion [116]. RGS16 may play a role in regulating E-cadherin. Triple negative breast cancer cells that were knocked-down for lysine-specific demethylase1 (LSD1) and treated with the histone deacetylase inhibitor (SAHA) had increased expression of RGS16 that was vital for growth suppression and increased expression of E-cadherin [163]. Our studies showed increased expression of E-cadherin in BxPC-3 cells expressing RGS16 compared to GFP control. We did not see a change in the expression of E-cadherin in the metastatic cell line AsPC-1. This could be due to the differences in the status of K-Ras in AsPC-1 (mutant) vs. BxPC-3 (wild-type) cells. Studies in prostate cancer cells overexpressing K-Ras found that increased hypermethylation of E-cadherin promoter mediated by K-Ras prevented E-cadherin expression [247]. Promoter methylation or other processes regulated by mutated K-Ras could be responsible for prevention of E-cadherin expression in AsPC-1. Our data suggests RGS16 may have a role in regulating E-cadherin

expression, however, further studies are needed to determine if increased expression of E-cadherin aids in the inhibition of BxPC-3 cell migration and invasion. We did not find a change in vimentin expression. However in addition to the amount of this protein, phosphorylation of vimentin regulates its organization, localization, and function [248, 249]. We did not examine the localization or phosphorylation status of vimentin following RGS16 expression, and this is something to consider for future studies.

EGF can also lead to the activation of RhoA, Rac1 and other Rho GTPases that are also associated with EMT and initiate rearrangement of filamentous actin (F-actin) [121, 122]. Rac1, RhoA and Cdc42 are activated by oncogenic Ras and activation of these proteins is vital for Ras transformation and/or cell motility [250, 251]. Activation of Rac1, RhoA, and Cdc42 results in reorganization of the actin cytoskeleton causing lamellipodia, stress fiber, or filopodia formation respectively that aids in different aspects of cell migration [126, 131]. Activation of Rho GTPases contributes to cell motility, invasion, and metastasis [131]. RhoA and RhoC have both been shown to be important signaling molecules required for migration, invasion, and metastasis of pancreatic cancer. Quantitative *in vivo* fluorescence lifetime imaging of mice with p53 and K-Ras mutant pancreatic cancer found increased RhoA activity in certain subcellular locations that are important for invasion [252]. Fukumoto and colleagues found that the Rho GTPase RhoC is overexpressed in human pancreatic cancer and is associated with metastases and decreased survival [108]. Inhibition of EGF induced activation of RhoA alone or in combination with inhibition of RhoB and RhoC by p190 RhoGap (converts active RhoGTP to inactive RhoGTP) in pancreatic cancer cells repressed EGF induced invasion and metastasis [240, 253].

RGS16 can inhibit activation of RhoA by binding and preventing the small G protein, Gα13 from mediating the activation of RhoA in MCF-7 cells [164]. Although commonly associated

with GPCR signaling, Gα13 was identified as a mediator of growth factor induced migration and was found to bind and aid in the localization of active Rac1 to sites of lamellipodia formation [254]. The significance of Gα13 binding to Rac1 following growth factor stimulation in unclear, but this interaction may aid in the deactivation of Rac1 resulting in the turnover of actin to be used for formation of stress fibers or lamellipodia in the cytoskeleton [254]. Rac1 and RhoA work in an antagonistic fashion and prolonged activation of Rac1 may inhibit RhoA induced formation of stress fibers needed for cell motility [255, 256].

Although only RGS16 has been directly linked to regulation of RhoA, based on literature reports other Rho GTPases could be targets for RGS16 regulation. We examined F-actin organization in BxPC-3 and AsPC-1 cells expressing GFP and/or RGs16 in order to 1) determine if there is a change in cytoskeleton, and 2) narrow down possible targets for future study. We were unable to detect any significant change between cells treated with Ad.GFP or Ad.GFP.RGS16. This was due in part to the heterogeneity of the cell population making it difficult to quantify differences between control and RGS16 expressing cells. Also any changes to the organization of the cytoskeleton could be subtle and not detectable using phalloidin staining. Measurement of Rho GTPases' activation state will be required to test the hypothesis that RGS16 is regulating cytoskeleton rearrangement through modulation of RhoA, RhoC, Rac1, or Cdc42.

FUTURE STUDIES AND CONCLUSIONS

RGS16 is downregulated in metastatic pancreatic cancer and for the first time we have delineated a function of RGS16 in inhibiting EGF induced pancreatic cancer cell migration and invasion *in* vitro. Phosphorylation is an important mechanism that regulates RGS16 GTPase, accelerating its activity and stability [157-159]. Tyrosine 168 is located in the RGS box and its

phosphorylation by EGF/EGFR enhances the GTPase activity of RGS16. In contrast, phosphorylation by Src slowed degradation of RGS16 [157, 158]. Our data suggests the enhancement of RGS16 activity by EGF/EGFR functions in a negative feedback loop to inhibit pancreatic cancer migration and invasion. Our study focused on examining migration and invasion mediated by the EGF/EGFR pathway. However, a single RGS protein can interact and regulate signaling of multiple pathways ([134, 172]). Future studies are needed to determine if RGS16 can inhibit cell migration and invasion through other pathways such as the SDF-1/CxCR4 pathway. The mechanism behind RGS16 inhibition of migration and invasion still remains unclear, however, our studies show it is independent of the PI3K/Akt and MAPK pathways. More experiments are needed to determine the mechanism governing RGS16 inhibition of EGF induced migration and invasion. Examination of E-cadherin activity in BxPC-3 cells and activation of Rho GTPases in cells expressing RGS16 will help narrow down possible mechanisms. Our study did not directly investigate whether RGS16 inhibits invasion by preventing the secretion of factors (such as matrix metalloproteinases) involved in the breakdown of the extracellular matrix and basement membrane required for the infiltration of cancer cell into the vasculature. However, this is the first report to our knowledge demonstrating a relationship between an RGS protein and invasion. Further studies examining the expression and activity of matrix metalloproteinases (MMPs) could determine whether RGS16 may inhibit invasion by decreasing the amount, activity or secretion of MMPs.

CHAPTER 4.

DISCUSSION

Conclusions

The objectives of our research were twofold: first to identify p53 and pRb cross-talk candidates by examining p53 and pRb co-regulated genes; and second to characterize the function of a p53 and pRb cross-talk candidate genes in cancer development. As described in Chapter 1, p53 and pRb are vital for cancer suppression and studies suggest p53 and pRb cross-talk to regulate cellular fate and prevent cancer progression. By utilizing microarray expression profiling, we have identified 179 p53 and pRb regulated cross-talk candidates that may be involved in coordinating cancer suppression processes and determining cell fate (Chapter 2). Ingenuity Pathway Analysis (IPA) identified molecular and cellular functions that could be modulated by the cross-talk candidate genes and in cells exogenously expressing p53, pRb, and both p53 and pRb (Chapter 2). This IPA analysis will be useful for generating future hypotheses aimed at identifying mechanisms employed by p53 and pRb to regulate cellular processes. We chose to further study RGS16 a common gene set cross-talk candidate because of its known regulation of several oncogene pathways that are depicted in Figure 1.4 and its ability to regulate GPCR pathways that are commonly deregulated in cancer. Loss of RGS16 in metastatic pancreatic cancer suggests it functions to inhibit processes that aid in metastasis. Our results support our hypothesis that RGS16 inhibits pancreatic cancer cell migration and invasion *in vitro* and suggests that loss of RGS16 may provide to pancreatic cancer a metastatic advantage.

Although it is known that RGS16 is regulated by p53, we are the first to report the regulation of RGS16 by pRb and its ability to inhibit EGF induced migration and invasion with no impact on cell viability (Chapter 2 & 3). The findings from our study suggest RGS16 is regulated by p53

and pRb and functions to inhibit pancreatic cancer cell migration and invasion, however this effect was cell line dependent. Although not commonly associated with p53 and pRb signaling, regulation of cellular migration and invasion by both tumor suppressors has become evident over the course of the past several years. p53 regulates cell polarization and migration of cells predominately by inhibiting Rho signaling [257] and also inhibits cancer cell invasion by suppressing the activity or expression of matrix metalloproteinases (MMPs) [258-261]. pRb's role in cell migration has recently come to light. pRb has been implicated as an important factor in regulating neuronal cell migration and was recently found to inhibit CD44 induced collective cell migration of breast cancer cells [68, 262]. pRb is linked to regulating invasion through its ability to bind and inhibit E2F induced transcriptional activation of the matrix metalloproteinases (MMPs) 9, 14, and 15 [67]. Knock-down of E2F1 and E2F3 inhibited migration and invasion of non-small cell lung cancer cells [67]. RGS16 may be another mechanism employed to regulate cell migration and invasion by p53 and pRb. However, future studies are needed to verify if RGS16 regulation by p53 and pRb mediates suppression of cancer cell migration and invasion.

While the exact mechanisms employed by RGS16 to regulate pancreatic cancer cell migration and invasion remains unknown,, our studies suggests RGS16 regulation is independent of the PI3K/AKT, and MAPK pathways and increases RGS16 in Ras wild-type cells. Critique of the data and future directions will be explored below in the following sections outlining the next steps that can be taken to understand the role of RGS16 in pancreatic cancer.

Critique and Data analysis

Expression profiling analysis was used to identify p53 and pRb cross-talk candidates using normal lung fibroblast cells (WI38) expressing p53, pRb, or p53 + pRb. Our studies focused on transcriptional regulation as a cross-talk mechanism employed by p53 and pRb. However,

downstream protein-protein interactions are another mechanism of communication and were not investigated in our study. To avoid mutations that can alter p53 and pRb signaling pathways, normal WI38 cells were used to study p53 and pRb cross-talk. Our experimental design used an exogenous system to express p53 and pRb in cells that already express both tumor suppressors. This method does raise the concern that the total expression of p53 and pRb (exogenous + endogenous) exceeds normal activation levels of these tumor suppressors inducing irregular responses in the WI38 cells. This is a valid concern, however using western blot and densitometry analysis (Figure 2.1), we found that the fold change in p53 and hypophosphorylated pRb (active form) to total pRb was equivalent to those seen endogenously in cells undergoing quiescence or cell cycle arrest respectively [195, 196]. Pathways that tightly control p53 and pRb expression and activity (highlighted in Chapter 1) are present in WI38 cells and regulate the expression of these two proteins.

p53 and pRb activity is tightly controlled, so in order to study their transcriptional regulation functions, we had to induce their expression and/or activity. We used adenoviruses to induce expression of p53 and pRb overcoming regulatory pathways and activating p53 and pRb transcriptional responses. There are alternate methods that could have been used to induce p53 and pRb transcriptional activity and will be briefly discussed. p53 and pRb can both be activated by a variety of compounds or stimuli that induce DNA damage such as doxorubicin, cisplatin, or UV [263-265] . However, it is hard to isolate activation of just p53 and/or pRb using chemicals or other stimuli that induce DNA damage. Mouse embryonic fibroblasts (MEF) negative for p53 or pRb were also considered for studying p53 and pRb cross-talk. However, we were unable to find $p53^{-/-}$ and $pRb^{-/-}$ MEFs. Furthermore both p53 and pRb induce cell cycle arrest and initiate DNA repair. Loss of both p53 and pRb would make the cells vulnerable to mutations that could

alter the p53 and pRb signaling pathways hindering identification of valid cross-talk candidate genes. siRNA knockdown of p53 and/or pRb could have also been employed. Levels of p53 are kept at low levels in normal cells by MDM2, and depending on the stage of the cell cycle pRb could be in its inactive form. Additional stimuli to siRNA treated samples may have been required to initiate p53 and pRb transcriptional activity to compare changes in gene expression between controls and siRNA treated cells. We chose to use adenoviruses to express p53 and pRb because it offered a simple method to induce one or both of these tumor suppressor genes without initiating other cellular pathways.

We identified 179 cross-talk candidate genes that were broken down into two gene sets; the common (39) and interaction (140) cross-talk candidates (Figure 2.1). Our interest in the common set cross-talk candidate RGS16 was first peaked when qRT-PCR analysis revealed p53 and pRb combined expression in WI38 cells synergistically increased RGS16 mRNA. Analysis of the literature demonstrated that RGS16 not only regulated GPCR receptor signaling but is also associated with regulating oncogene pathways that are altered in cancer signaling. RGS16 has been shown to regulate pathways that are associated with cell migration and invasion. However, no studies have investigated whether RGS16 can inhibit migration and invasion. Primary pancreatic tumors from patients with lymph node metastasis had decreased expression of RGS16 that was associated with decreased survival. Since a majority of pancreatic cancer patients present with metastases at time of diagnosis, we used pancreatic cancer as a model to study RGS16 migration and invasion.

When we began the migration and invasion studies, we chose to use adenoviruses to express RGS16 because of the decreased expression of RGS16 in clinical data. Western blot analysis later showed a faint endogenous band in the BxPC-3 cells (highest expression of RGS16 mRNA)

(chapter 3) further supporting our decision to express RGS16. However, repeating the migration and invasion studies using RGS16 siRNA would complement our data and further support our hypothesis, however due to low basal levels of RGS16, it would be difficult to detect RGS16 knockdown.

We saw decreased EGF induced migration of BxPC-3 and AsPC-1 but not in the PANC-1 cells. The difference in behavior between cell lines could be due to alternate mutations that are preventing inhibition of PANC-1 cell migration by RGS16. However, this does not mean that RGS16 could still not inhibit migration of these cells. We examined the ability of RGS16 to inhibit EGF induced migration and invasion; however, RGS proteins canonically regulate GPCR signaling [134]. The RGS16 targeted GPCR CxCR4 is expressed in pancreatic cancer and promotes migration and invasion of the PANC-1 cells [141, 222, 242].

We explored the mechanism behind RGS16 suppression of migration and invasion. EGF/EGFR activates a variety of pathways that can contribute to cell migration and invasion including PI3K/AKT, Ras/MAPK, and Rho GTPases. We did not see a change in expression of phosphorylated AKT or ERK suggesting RGS16 does not modulate EGF induced migration and invasion through the PI3K/AKT or MAPK pathways. Examinations of EMT markers E-cadherin and vimentin were inconclusive. We did find an increase in E-cadherin expression in the K-Ras wild-type BxPC-3 cells but there was no change in vimentin for either BxPC-3 or AsPC-1 cells. Although there was an increase in E-cadherin expression in BxPC-3 cells by RGS16, we did not test the activity of E-cadherin in these cells. Induced upregulation of RGS16 increased expression of E-cadherin in triple negative breast cancer cells [163]. E-cadherin, often referred to as the master regulator of EMT, is important for the formation of tight cell-cell contact [125]. RGS16 may regulate E-cadherin in a Ras wild-type dependent manner. K-Ras mutations in

prostate cancer inhibit E-cadherin expression by promoting methylation of the E-cadherin promoter [247]. Future studies are needed to test the hypothesis that K-Ras mutations inhibit increased expression of E-cadherin by RGS16.

Phosphorylation of vimentin regulates its activity and localization. Our studies only focused on mechanisms that regulate vimentin by increasing or decreasing its expression. Investigation of the phosphorylation state and localization of vimentin will determine if RGS16 regulates cell migration and invasion by regulating the post-translational modifications of vimentin. We cannot say definitively if RGS16 regulates EMT due to the use of only two EMT markers and differences seen between the two cell lines. Examination of other EMT markers such as fibronectin and N-cadherin will provide a more complete picture that will aid in forming more conclusive results regarding RGS16 and EMT.

Activation of Rho GTPases is also associated with EMT and aids in cell migration and invasion [126]. Although RGS16 is only directly linked with inhibiting activation of RhoA in MCF-7 cells there are other Rho GTPases that are involved in the reorganization of the cytoskeleton and that are integral to motility [164]. We used phalloidin to stain and visualize F-actin in BxPC-3 and AsPC-1 cells. We were expecting that phalloidin staining would identify distinct changes in the organization of F-actin that would pinpoint alterations in signaling to a Rho GTPase. We could not distinguish changes quantitatively in the F-actin that forms lamellipodia, filopodia, or stress fibers; this is likekly due in part to the heterogenous population of cells. Furthermore phalloidin staining is not a very good quantitative assay and changes in F-actin may be subtle in these cells. Activation kits examining RhoA, RhoC Rac1, Cdc42 would provide more quantifiable assays that would help determine whether RGS16 causes changes to expression of activated Rho GTPases.

Our mechanistic investigations concentrated on pathways that are predominately known to mediate cell migration. However, both AKT and ERK (MAPK) can aid cell invasion by increasing expression of MMP-9 [233, 245]. Activation of Rho GTPases does stimulates cell invasion *in vitro* and *in vivo*, however, this is predominately linked with rearrangement of the actin cytoskeleton, changes in cell-cell contact, and cell adhesion dynamics that mediate cell movement [126]. Remodeling of the extracellular matrix is an important step for invasion and is mediated by extracellular proteases such as matrix metalloproteases (MMPs). The mesenchymal phenotype adopted by cancer cells that underwent EMT is associated with an increase in the secretion of MMP 2 & 9 [125]. We did not examine the activity or expression of MMPs, but since we are found an inhibition of invasion, it is possib inhibits the activity or expression of MMPs.

Future studies

Our studies have only begun to uncover the function and role of RGS16 in pancreatic cancer progression. There are still several questions that remain unanswered. These questions will be explored along with future studies that can be performed to fill in the gaps.

How does RGS16 inhibit pancreatic cancer cell migration and invasion *in vitro*?

We were unable to identify the mechanism used by RGS16 to inhibit pancreatic cancer cell migration and invasion. However there are several steps that can be taken to further understand the function of RGS16 in pancreatic cancer that are outlined in Table 4.1. All proposed experiments are for BxPC-3 and AsPC-1 cells expressing GFP and/or RGS16 and treated with EGF following previous protocols as described in Materials and Methods of Chapter 3.

E-cadherin binds to β-catenin and other proteins to form a complex that connects cell-cell adhesion complexes with the F-actin [126, 266]. Loss of E-cadherin liberates β-catenin that can

be stabilized by the Wnt signaling pathway in the cytosol leading to the translocation of β-catenin to the nucleus where it can initiate transcription of factors that promote proliferation, migration, and invasion [126]. Examination of β-catenin expression in the nucleus will determine if the increased expression of E-cadherin in BxPC-3 translates to increased expression of E-cadherin at the membrane.

The second proposed experiment is the investigation of the localization and phosphorylation of vimentin. The post-translational modification of vimentin regulates its organization, localization, and function [248, 249]. Although the role of phosphorylated vimentin is not well known it appears to aid in cell migration [249, 267] Phoshorylation of vimentin causes disassembly of the intermediate filament resulting in increase of soluble vimentin. Investigation of vimentin localization (still in filamentous fibers) and phosphorylation will show whether RGS16 regulates this protein through post-translational modifications.

The Third experiment will test the hypothesis that mutant K-Ras prevents the upregulation of E-cadherin by mediating the promoter methylation of its gene. A demethylating agent (decitabine and azacitidine) will be used in cells with or without adenoviruses expressing GFP and/or RGS16. The hypothesis will be supported if the data shows increased expression of E-cadherin in cells expressing RGS16 and treated with the demethylating agent.

The last three proposed experiments focus on examining expression or activation of other EMT markers, MMPs, and Rho GTPases. These proteins can be examined using western blots or PCR arrays to obtain a more global view of cellular changes due to RGS16 expression. Completion of these experiments will provide data demonstrating whether RGS16 inhibits expression of EMT markers, activation of Rho GTPases involved in cytoskeleton rearrangement, or activation of MMPs.

Table 4.1: Proposed future *in vitro* studies to examine mechanisms governing RGS16 inhibition of pancreatic cancer cell migration and invasion.

Experiment Type	Method/hypothesized outcome
1. E-cadherin activity	Examine β-catenin localization in nucleus (western blots or immunofluorescence / ↑ E-cadherin activity ↓ localization of β-catenin in the nucleus
2. Vimentin localization and phosphorylation	Examine localization (immunofluorescence) & phosphorylation (western blot using anti-phosphorylated vimentin and/or extract soluble and filamentous proteins followed by western blot using anti-vimentin / determine if localization or spread of vimentin changes (for example, change in amount of filamentous fibers) and if vimentin is phosphorylated (phosphorylated vimentin can break down and become soluble)
3. Test if RGS16 induced expression of E-cadherin is dependent on wild-type K-Ras	Treat cells with a DNA demethylating agent with or without concomitant treatment with adenoviruses expressing GFP and/or RGS16. Measure E-cadherin expression / determine if K-Ras mutations inhibit up-regulation of E-cadherin by methylation and inhibition of E-cadherin promoter.
4. Examine expression of other EMT markers	Examine expression of other EMT markers such as N-cadherin, fibronectin, αvβ6 integrin, or E-cadherin transcriptional repressors (Snail, slug, ZEB1, or twist (by western blot or PCR array) / more conclusively show if RGS16 regulates EMT.
5. Activation of RhoGTPases	Rho GTPase activation kits (immunoprecipitation followed by western blot analysis) for RhoA, RhoC, Rac1, and Cdc42 / determine if RGS16 inhibits activation of select Rho GTPases
6. Matrix Metalloproteinase (MMPs) activity	Examine Matrix metalloproteinase activity using fluorometric activation kit / determine if and which MMPs are active

Does RGS16 inhibit cell migration and invasion induced by other factors?

A single RGS protein can interact and regulate signaling of multiple pathways [134, 172]. While our studies focused on EGF induced migration and invasion, RGS16 may inhibit migration and invasion induced by other factors such as CxCR4. Repeating the migration and invasion experiments using other factors to stimulate cell migration and invasion will show if RGS16 can regulate multiple mechanism of pancreatic cancer cell migration and invasion.

These experiments can be completed by two methods: the first, is to use single ligands to induce activation of certain signaling pathways (for example SDF-1 ligand for CxCR4). The second option is to use fibroblast conditioned media that contains multiple factors providing a partial mimic of the microenvironment that cancer cells are exposed to *in vivo*. Identification of mechanisms will be easier to identify using single ligands to induce migration and invasion. However, using fibroblast conditioned media is more representative of the growth factors and ligands that a cancer cell is exposed to in the microenvironment.

Does RGS16 inhibit pancreatic cancer cell metastasis *in vivo*?

Pancreatic cancer has a low survival rate that is due in part to the highly metastatic nature of this disease. By understanding the underlying mechanisms of pancreatic cancer progression, we will be able to better prevent or treat advanced and metastatic pancreatic cancer. Although RGS16 was found to inhibit pancreatic cancer cell migration and invasion *in vitro* (Chapter 3) this does not guarantee that RGS16 can inhibit pancreatic cancer metastasis. The process of metastasis is dynamic and complex involving multiple interactions between the tumor and the surrounding microenvironment that can be better studied using *in vivo*. There are several mouse models that can be employed to test the hypothesis that RGS16 inhibits pancreatic cancer cell metastasis such as genetically engineered mouse (GEM) and mice xenograft models. There are multiple advantages of GEM over xenograft or carcinogen induced pancreatic cancer models: 1) GEM models more closely mimic human pancreatic cancer, 2) can be used to study pancreatic cancer progression from initiation to metastasis, 3) and allows investigation of any interactions between the tumor and immune response [268, 269]. Using GEM models we can induce targeted knock-out of RGS16 in the pancreas of mice with knock-in of mutant KRasG12D and mutant p53R172H (Pdx1-Cre;LSL-$KRas^{G12D/+}$;LSL-$p53^{R172H/+}$) or mice with knock-in of mutant

KRasG12D and knock-out of Ink4a/ARF (Pdx1-Cre;LSL-KRas$^{G12D/+}$;Ink4a/Arf$^{lox/lox}$). Both the Pdx1-Cre;LSL-KRas$^{G12D/+}$;LSL-p53$^{R172H/+}$ and Pdx1-Cre;LSL-KRas$^{G12D/+}$;Ink4a/Arf$^{lox/lox}$ models develop pancreatic intraepithelial neoplasias (PANINs) that later progress to pancreatic cancer with the presence of metastases [102, 104]. By combining RGS16 knock-out with well characterized pancreatic cancer GEM models, we can determine if loss of RGS16 increases metastases.

There are a couple of disadvantages to using GEM models; one, is expense and the second, is that the signaling molecules in mice may not have the same mechanisms or effect as in humans. Wilkie and colleagues (2010) using RGS16::GFP BAC transgenic mice found that RGS16 is expressed in pancreatic progenitor cells during development [140]. However, after birth, expression of RGS16::GFP remained in pancreatic cells associated with ducts and veins for 3-4 weeks but was lost in adult mice. In an abstract published earlier this year, the authors found expression of GFP tagged RGS16 throughout the different stages of tumorigenesis in mice with pancreatic cancer induced by knock-in mutant KRas and knock-out p16 and p19 targeted to pancreatic acinar cells (p48^{CRE};LSL-KRas^{G12D};CDKN2A$^{f/f}$) [270]. In our studies, we found that the expression of RGS16 mRNA was lower in established pancreatic cancer cells compared to normal human pancreatic RNA (Chapter 3). Immunohistochemical analysis of RGS16 in primary samples showed a decrease in RGS16 staining in tumors extracted from patients with lymph-node metastases [149]. This data suggests RGS16 expression is present in human pancreas tissue and expression is lost during progression of pancreatic cancer contradicting the findings of the mice studies. Human and mouse RGS16 share 85% homology, however, that 15% difference could be sufficient to elicit different functions of RGS16 in the pancreas of these two species [154]. The function of RGS16 in mice with pancreatic cancer has not yet been

investigated and experiments using RGS16 knock-out mice will increase our understanding of the role of RGS16 in mouse pancreatic cancer.

A xenograft orthotopic pancreatic tumor mouse model can used to determine if expression of RGS16 inhibits pancreatic cancer cell metastasis. In orthotopic models the pancreatic cancer cells are injected directly into the pancreas of an immunodeficient (nude) mouse and up to 65% of mice develop metastases [271]. This model can only be used to study the endpoint of metastasis (in our case) as opposed to GEM models that can be used to study the different stages of tumorigenesis or metastasis. Using this model, pancreatic cancer cells (AsPC-1) will be injected into the pancreas of a nude mouse. Mice will be treated with adenoviruses expressing GFP and/or RGS16 encapsulated in microbubbles for targeted delivery to the tumor site. Ultrasound will be used to target the delivery of the bubble to the pancreas of the mouse. Metastasis and tumor growth will be measured. Orthotopic models are invasive and rely on imaging modalities to measure size of the tumor [272]. However, ultrasound guided tumor injection protocols for pancreatic cancer has been developed to limit injury to the mice [273]. Use of xenograft mice prevents endogenous investigation of RGS16 function on pancreatic cancer development and progression. However, this model may bypass problems associated with RGS16 having different functions in mice vs. humans.

References

1. Friend, S.H., et al., *A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma.* Nature, 1986. **323**(6089): p. 643-6.
2. McClendon, A.K., et al., *RB and p53 cooperate to prevent liver tumorigenesis in response to tissue damage.* Gastroenterology, 2011. **141**(4): p. 1439-50.
3. Sherr, C.J. and F. McCormick, *The RB and p53 pathways in cancer.* Cancer Cell, 2002. **2**(2): p. 103-12.
4. Williams, B.O., et al., *Tumorigenic and developmental effects of combined germ-line mutations in Rb and p53.* Cold Spring Harb Symp Quant Biol, 1994. **59**: p. 449-57.
5. Zhou, Z., et al., *Synergy of p53 and Rb deficiency in a conditional mouse model for metastatic prostate cancer.* Cancer Res, 2006. **66**(16): p. 7889-98.
6. Amaral, J.D., et al., *The role of p53 in apoptosis.* Discov Med, 2010. **9**(45): p. 145-52.
7. Vogiatzi, P., et al., *The limitless role of p53 in cell cycle machinery: good news or bad news?* Cancer Biol Ther, 2006. **5**(9): p. 1090-3.
8. Agarwal, M.L., et al., *The p53 network.* J Biol Chem, 1998. **273**(1): p. 1-4.
9. Rufini, A., et al., *Senescence and aging: the critical roles of p53.* Oncogene, 2013. **32**(43): p. 5129-43.
10. Sengupta, S. and C.C. Harris, *p53: traffic cop at the crossroads of DNA repair and recombination.* Nat Rev Mol Cell Biol, 2005. **6**(1): p. 44-55.
11. Lane, D.P., *Cancer. p53, guardian of the genome.* Nature, 1992. **358**(6381): p. 15-6.
12. Beckerman, R. and C. Prives, *Transcriptional regulation by p53.* Cold Spring Harb Perspect Biol, 2010. **2**(8): p. a000935.
13. Speidel, D., *Transcription-independent p53 apoptosis: an alternative route to death.* Trends Cell Biol, 2010. **20**(1): p. 14-24.
14. Shu, K.X., B. Li, and L.X. Wu, *The p53 network: p53 and its downstream genes.* Colloids Surf B Biointerfaces, 2007. **55**(1): p. 10-8.
15. Wei, C.L., et al., *A global map of p53 transcription-factor binding sites in the human genome.* Cell, 2006. **124**(1): p. 207-19.
16. Qin, H., et al., *Regulation of apoptosis and differentiation by p53 in human embryonic stem cells.* J Biol Chem, 2007. **282**(8): p. 5842-52.
17. Porrello, A., et al., *p53 regulates myogenesis by triggering the differentiation activity of pRb.* J Cell Biol, 2000. **151**(6): p. 1295-304.
18. Chen, H.Z., S.Y. Tsai, and G. Leone, *Emerging roles of E2Fs in cancer: an exit from cell cycle control.* Nat Rev Cancer, 2009. **9**(11): p. 785-97.
19. Claudio, P.P., T. Tonini, and A. Giordano, *The retinoblastoma family: twins or distant cousins?* Genome Biol, 2002. **3**(9): p. reviews3012.
20. Genovese, C., et al., *Cell cycle control and beyond: emerging roles for the retinoblastoma gene family.* Oncogene, 2006. **25**(38): p. 5201-9.
21. Claudio, P.P., et al., *Functional analysis of pRb2/p130 interaction with cyclins.* Cancer Res, 1996. **56**(9): p. 2003-8.
22. Sellers, W.R., et al., *Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth.* Genes Dev, 1998. **12**(1): p. 95-106.

23. Sun, H., et al., *E2f binding-deficient Rb1 protein suppresses prostate tumor progression in vivo.* Proc Natl Acad Sci U S A, 2011. **108**(2): p. 704-9.
24. Hilgendorf, K.I., et al., *The retinoblastoma protein induces apoptosis directly at the mitochondria.* Genes Dev, 2013. **27**(9): p. 1003-15.
25. Ianari, A., et al., *Proapoptotic function of the retinoblastoma tumor suppressor protein.* Cancer Cell, 2009. **15**(3): p. 184-94.
26. Sun, B., et al., *Absence of pRb facilitates E2F1-induced apoptosis in breast cancer cells.* Cell Cycle, 2010. **9**(6): p. 1122-30.
27. Haupt, Y., S. Rowan, and M. Oren, *p53-mediated apoptosis in HeLa cells can be overcome by excess pRB.* Oncogene, 1995. **10**(8): p. 1563-71.
28. Gu, W., et al., *Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation.* Cell, 1993. **72**(3): p. 309-24.
29. Chen, P.L., et al., *Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs.* Genes Dev, 1996. **10**(21): p. 2794-804.
30. Chen, P.L., et al., *Retinoblastoma protein directly interacts with and activates the transcription factor NF-IL6.* Proc Natl Acad Sci U S A, 1996. **93**(1): p. 465-9.
31. Nead, M.A., et al., *Rb binds c-Jun and activates transcription.* EMBO J, 1998. **17**(8): p. 2342-52.
32. Batsche, E., et al., *RB and c-Myc activate expression of the E-cadherin gene in epithelial cells through interaction with transcription factor AP-2.* Mol Cell Biol, 1998. **18**(7): p. 3647-58.
33. Decary, S., et al., *The retinoblastoma protein binds the promoter of the survival gene bcl-2 and regulates its transcription in epithelial cells through transcription factor AP-2.* Mol Cell Biol, 2002. **22**(22): p. 7877-88.
34. Lee, J.S., et al., *HES1 cooperates with pRb to activate RUNX2-dependent transcription.* J Bone Miner Res, 2006. **21**(6): p. 921-33.
35. Thomas, D.M., et al., *The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation.* Mol Cell, 2001. **8**(2): p. 303-16.
36. Udvadia, A.J., et al., *Sp-1 binds promoter elements regulated by the RB protein and Sp-1-mediated transcription is stimulated by RB coexpression.* Proc Natl Acad Sci U S A, 1993. **90**(8): p. 3265-9.
37. Guo, C.S., et al., *Regulation of MyoD activity and muscle cell differentiation by MDM2, pRb, and Sp1.* J Biol Chem, 2003. **278**(25): p. 22615-22.
38. Balasenthil, S. and R.K. Vadlamudi, *Functional interactions between the estrogen receptor coactivator PELP1/MNAR and retinoblastoma protein.* J Biol Chem, 2003. **278**(24): p. 22119-27.
39. Batsche, E., et al., *Rb enhances p160/SRC coactivator-dependent activity of nuclear receptors and hormone responsiveness.* J Biol Chem, 2005. **280**(20): p. 19746-56.
40. Batsche, E., et al., *Retinoblastoma and the related pocket protein p107 act as coactivators of NeuroD1 to enhance gene transcription.* J Biol Chem, 2005. **280**(16): p. 16088-95.
41. Lu, J. and M. Danielsen, *Differential regulation of androgen and glucocorticoid receptors by retinoblastoma protein.* J Biol Chem, 1998. **273**(47): p. 31528-33.

42. Martens, C., et al., *Protein-protein interactions and transcriptional antagonism between the subfamily of NGFI-B/Nur77 orphan nuclear receptors and glucocorticoid receptor.* Mol Endocrinol, 2005. **19**(4): p. 885-97.
43. Singh, P., S.W. Chan, and W. Hong, *Retinoblastoma protein is functionally distinct from its homologues in affecting glucocorticoid receptor-mediated transcription and apoptosis.* J Biol Chem, 2001. **276**(17): p. 13762-70.
44. Singh, P., J. Coe, and W. Hong, *A role for retinoblastoma protein in potentiating transcriptional activation by the glucocorticoid receptor.* Nature, 1995. **374**(6522): p. 562-5.
45. Cordon-Cardo, C., et al., *Cooperative effects of p53 and pRB alterations in primary superficial bladder tumors.* Cancer Res, 1997. **57**(7): p. 1217-21.
46. Toguchida, J. and T. Nakayama, *Molecular genetics of sarcomas: applications to diagnoses and therapy.* Cancer Sci, 2009. **100**(9): p. 1573-80.
47. Yap, D.B., et al., *mdm2: a bridge over the two tumour suppressors, p53 and Rb.* Oncogene, 1999. **18**(53): p. 7681-9.
48. Gazzeri, S., et al., *Mechanisms of p16INK4A inactivation in non small-cell lung cancers.* Oncogene, 1998. **16**(4): p. 497-504.
49. Mathew, R., et al., *Alterations in p53 and pRb pathways and their prognostic significance in oesophageal cancer.* Eur J Cancer, 2002. **38**(6): p. 832-41.
50. Ueki, K., et al., *CDKN2/p16 or RB Alterations Occur in the Majority of Glioblastomas and Are Inversely Correlated.* Cancer Research, 1996. **56**(1): p. 150-153.
51. Caldas, C., et al., *Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma.* Nat Genet, 1994. **8**(1): p. 27-32.
52. Laurie, N.A., et al., *Inactivation of the p53 pathway in retinoblastoma.* Nature, 2006. **444**(7115): p. 61-6.
53. Momand, J., et al., *The MDM2 gene amplification database.* Nucleic Acids Res, 1998. **26**(15): p. 3453-9.
54. Drobnjak, M., et al., *Overexpression of Cyclin D1 Is Associated with Metastatic Prostate Cancer to Bone.* Clinical Cancer Research, 2000. **6**(5): p. 1891-1895.
55. Gansauge, S., et al., *Overexpression of Cyclin D1 in Human Pancreatic Carcinoma Is Associated with Poor Prognosis.* Cancer Research, 1997. **57**(9): p. 1634-1637.
56. Gillett, C., et al., *Amplification and Overexpression of Cyclin D1 in Breast Cancer Detected by Immunohistochemical Staining.* Cancer Research, 1994. **54**(7): p. 1812-1817.
57. Michalides, R., et al., *Overexpression of Cyclin D1 Correlates with Recurrence in a Group of Forty-seven Operable Squamous Cell Carcinomas of the Head and Neck.* Cancer Research, 1995. **55**(5): p. 975-978.
58. Levine, A.J., *The common mechanisms of transformation by the small DNA tumor viruses: The inactivation of tumor suppressor gene products: p53.* Virology, 2009. **384**(2): p. 285-93.
59. Bakhoum, S.F. and D.A. Compton, *Chromosomal instability and cancer: a complex relationship with therapeutic potential.* J Clin Invest, 2012. **122**(4): p. 1138-43.
60. Manning, A.L., C. Benes, and N.J. Dyson, *Whole chromosome instability resulting from the synergistic effects of pRB and p53 inactivation.* Oncogene, 2013.
61. Derenzini, M., et al., *The p53-mediated sensitivity of cancer cells to chemotherapeutic agents is conditioned by the status of the retinoblastoma protein.* J Pathol, 2009. **219**(3): p. 373-82.

62. Yague, E., et al., *Ability to acquire drug resistance arises early during the tumorigenesis process.* Cancer Res, 2007. **67**(3): p. 1130-7.
63. Choi, J., et al., *Local mesenchymal stem/progenitor cells are a preferential target for initiation of adult soft tissue sarcomas associated with p53 and Rb deficiency.* Am J Pathol, 2010. **177**(5): p. 2645-58.
64. Rubio, R., et al., *The differentiation stage of p53-Rb-deficient bone marrow mesenchymal stem cells imposes the phenotype of in vivo sarcoma development.* Oncogene, 2013. **32**(41): p. 4970-80.
65. Meuwissen, R., et al., *Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model.* Cancer Cell, 2003. **4**(3): p. 181-9.
66. Simin, K., et al., *pRb inactivation in mammary cells reveals common mechanisms for tumor initiation and progression in divergent epithelia.* PLoS Biol, 2004. **2**(2): p. E22.
67. Johnson, J.L., et al., *Regulation of matrix metalloproteinase genes by E2F transcription factors: Rb-Raf-1 interaction as a novel target for metastatic disease.* Cancer Res, 2012. **72**(2): p. 516-26.
68. Kim, K.J., et al., *Rb suppresses collective invasion, circulation and metastasis of breast cancer cells in CD44-dependent manner.* PLoS One, 2013. **8**(12): p. e80590.
69. Hui, A.M., et al., *Over-expression and lack of retinoblastoma protein are associated with tumor progression and metastasis in hepatocellular carcinoma.* Int J Cancer, 1999. **84**(6): p. 604-8.
70. Powell, E., D. Piwnica-Worms, and H. Piwnica-Worms, *Contribution of p53 to metastasis.* Cancer Discov, 2014. **4**(4): p. 405-14.
71. Voorhoeve, P.M. and R. Agami, *The tumor-suppressive functions of the human INK4A locus.* Cancer Cell, 2003. **4**(4): p. 311-9.
72. Lim, I.K., *TIS21 (/BTG2/PC3) as a link between ageing and cancer: cell cycle regulator and endogenous cell death molecule.* J Cancer Res Clin Oncol, 2006. **132**(7): p. 417-26.
73. Vattemi, E. and P.P. Claudio, *Tumor suppressor genes as cancer therapeutics.* Drug News Perspect, 2007. **20**(8): p. 511-20.
74. Polager, S. and D. Ginsberg, *p53 and E2f: partners in life and death.* Nat Rev Cancer, 2009. **9**(10): p. 738-48.
75. Qin, X.Q., et al., *Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis.* Proc Natl Acad Sci U S A, 1994. **91**(23): p. 10918-22.
76. Rogoff, H.A., et al., *E2F1 induces phosphorylation of p53 that is coincident with p53 accumulation and apoptosis.* Mol Cell Biol, 2002. **22**(15): p. 5308-18.
77. Rogoff, H.A., et al., *Apoptosis associated with deregulated E2F activity is dependent on E2F1 and Atm/Nbs1/Chk2.* Mol Cell Biol, 2004. **24**(7): p. 2968-77.
78. Wu, X. and A.J. Levine, *p53 and E2F-1 cooperate to mediate apoptosis.* Proc Natl Acad Sci U S A, 1994. **91**(9): p. 3602-6.
79. Hsieh, J.K., et al., *RB regulates the stability and the apoptotic function of p53 via MDM2.* Mol Cell, 1999. **3**(2): p. 181-93.
80. Felton-Edkins, Z.A., et al., *Direct regulation of RNA polymerase III transcription by RB, p53 and c-Myc.* Cell Cycle, 2003. **2**(3): p. 181-4.
81. Chen, Y., D. Schlessinger, and R. Nagaraja, *T antigen transformation reveals Tp53/RB-dependent route to PLAC1 transcription activation in primary fibroblasts.* Oncogenesis, 2013. **2**: p. e67.
82. Jemal, A., et al., *Global cancer statistics.* CA Cancer J Clin, 2011. **61**(2): p. 69-90.

83. Siegel, R., et al., *Cancer statistics, 2014.* CA Cancer J Clin, 2014. **64**(1): p. 9-29.
84. Mian, O.Y., et al., *Management options in locally advanced pancreatic cancer.* Curr Oncol Rep, 2014. **16**(6): p. 388.
85. Hidalgo, M., *Pancreatic cancer.* N Engl J Med, 2010. **362**(17): p. 1605-17.
86. Hezel, A.F., et al., *Genetics and biology of pancreatic ductal adenocarcinoma.* Genes Dev, 2006. **20**(10): p. 1218-49.
87. Bartsch, D.K., et al., *Prevalence of familial pancreatic cancer in Germany.* Int J Cancer, 2004. **110**(6): p. 902-6.
88. Fernandez, E., et al., *Family history and the risk of liver, gallbladder, and pancreatic cancer.* Cancer Epidemiol Biomarkers Prev, 1994. **3**(3): p. 209-12.
89. Klein, A.P., et al., *Familial pancreatic cancer.* Cancer J, 2001. **7**(4): p. 266-73.
90. Lee, E.S. and J.M. Lee, *Imaging diagnosis of pancreatic cancer: a state-of-the-art review.* World J Gastroenterol, 2014. **20**(24): p. 7864-77.
91. Schima, W., et al., *Pancreatic adenocarcinoma.* Eur Radiol, 2007. **17**(3): p. 638-49.
92. Geer, R.J. and M.F. Brennan, *Prognostic indicators for survival after resection of pancreatic adenocarcinoma.* Am J Surg, 1993. **165**(1): p. 68-72; discussion 72-3.
93. Roland, C.L., et al., *Neoadjuvant Therapy is Associated with a Reduced Lymph Node Ratio in Patients with Potentially Resectable Pancreatic Cancer.* Ann Surg Oncol, 2014.
94. Schober, M., et al., *Desmoplasia and chemoresistance in pancreatic cancer.* Cancers (Basel), 2014. **6**(4): p. 2137-54.
95. Jones, S., et al., *Core signaling pathways in human pancreatic cancers revealed by global genomic analyses.* Science, 2008. **321**(5897): p. 1801-6.
96. Eser, S., et al., *Oncogenic KRAS signalling in pancreatic cancer.* Br J Cancer, 2014. **111**(5): p. 817-22.
97. Hingorani, S.R., et al., *Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse.* Cancer Cell, 2003. **4**(6): p. 437-50.
98. Eser, S., et al., *Selective requirement of PI3K/PDK1 signaling for Kras oncogene-driven pancreatic cell plasticity and cancer.* Cancer Cell, 2013. **23**(3): p. 406-20.
99. Collisson, E.A., et al., *A central role for RAF-->MEK-->ERK signaling in the genesis of pancreatic ductal adenocarcinoma.* Cancer Discov, 2012. **2**(8): p. 685-93.
100. Lim, K.H., et al., *Divergent roles for RalA and RalB in malignant growth of human pancreatic carcinoma cells.* Curr Biol, 2006. **16**(24): p. 2385-94.
101. McCleary-Wheeler, A.L., R. McWilliams, and M.E. Fernandez-Zapico, *Aberrant signaling pathways in pancreatic cancer: a two compartment view.* Mol Carcinog, 2012. **51**(1): p. 25-39.
102. Aguirre, A.J., et al., *Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma.* Genes Dev, 2003. **17**(24): p. 3112-26.
103. Morton, J.P., et al., *Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer.* Proc Natl Acad Sci U S A, 2010. **107**(1): p. 246-51.
104. Hingorani, S.R., et al., *Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice.* Cancer Cell, 2005. **7**(5): p. 469-83.
105. Levy, L. and C.S. Hill, *Alterations in components of the TGF-beta superfamily signaling pathways in human cancer.* Cytokine Growth Factor Rev, 2006. **17**(1-2): p. 41-58.

106. Kalluri, R. and R.A. Weinberg, *The basics of epithelial-mesenchymal transition.* J Clin Invest, 2009. **119**(6): p. 1420-8.
107. Chen, Y.W., et al., *SMAD4 loss triggers the phenotypic changes of pancreatic ductal adenocarcinoma cells.* BMC Cancer, 2014. **14**: p. 181.
108. Suwa, H., et al., *Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas.* Br J Cancer, 1998. **77**(1): p. 147-52.
109. Cheng, J.Q., et al., *Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA.* Proc Natl Acad Sci U S A, 1996. **93**(8): p. 3636-41.
110. Oliveira-Cunha, M., W.G. Newman, and A.K. Siriwardena, *Epidermal growth factor receptor in pancreatic cancer.* Cancers (Basel), 2011. **3**(2): p. 1513-26.
111. Barton, C.M., et al., *Abnormalities of the p53 tumour suppressor gene in human pancreatic cancer.* Br J Cancer, 1991. **64**(6): p. 1076-82.
112. Rozenblum, E., et al., *Tumor-suppressive pathways in pancreatic carcinoma.* Cancer Res, 1997. **57**(9): p. 1731-4.
113. Normanno, N., et al., *Epidermal growth factor receptor (EGFR) signaling in cancer.* Gene, 2006. **366**(1): p. 2-16.
114. Roskoski, R., Jr., *The ErbB/HER family of protein-tyrosine kinases and cancer.* Pharmacol Res, 2014. **79**: p. 34-74.
115. Ardito, C.M., et al., *EGF receptor is required for KRAS-induced pancreatic tumorigenesis.* Cancer Cell, 2012. **22**(3): p. 304-17.
116. Chang, Z.G., et al., *Suppression of the epidermal growth factor receptor inhibits epithelial-mesenchymal transition in human pancreatic cancer PANC-1 cells.* Dig Dis Sci, 2012. **57**(5): p. 1181-9.
117. Kassis, J., et al., *A role for phospholipase C-gamma-mediated signaling in tumor cell invasion.* Clin Cancer Res, 1999. **5**(8): p. 2251-60.
118. Price, J.T., et al., *Epidermal growth factor promotes MDA-MB-231 breast cancer cell migration through a phosphatidylinositol 3'-kinase and phospholipase C-dependent mechanism.* Cancer Res, 1999. **59**(21): p. 5475-8.
119. Dhillon, A.S., et al., *MAP kinase signalling pathways in cancer.* Oncogene, 2007. **26**(22): p. 3279-90.
120. Kim, D., et al., *AKT/PKB signaling mechanisms in cancer and chemoresistance.* Front Biosci, 2005. **10**: p. 975-87.
121. Schiller, M.R., *Coupling receptor tyrosine kinases to Rho GTPases--GEFs what's the link.* Cell Signal, 2006. **18**(11): p. 1834-43.
122. Tu, S., et al., *Epidermal growth factor-dependent regulation of Cdc42 is mediated by the Src tyrosine kinase.* J Biol Chem, 2003. **278**(49): p. 49293-300.
123. Bos, J.L., *ras oncogenes in human cancer: a review.* Cancer Res, 1989. **49**(17): p. 4682-9.
124. Chaffer, C.L. and R.A. Weinberg, *A perspective on cancer cell metastasis.* Science, 2011. **331**(6024): p. 1559-64.
125. Thiery, J.P., *Epithelial-mesenchymal transitions in tumour progression.* Nat Rev Cancer, 2002. **2**(6): p. 442-54.
126. Yilmaz, M. and G. Christofori, *EMT, the cytoskeleton, and cancer cell invasion.* Cancer Metastasis Rev, 2009. **28**(1-2): p. 15-33.

127. Friedl, P. and K. Wolf, *Tumour-cell invasion and migration: diversity and escape mechanisms.* Nat Rev Cancer, 2003. **3**(5): p. 362-74.
128. Mehlen, P. and A. Puisieux, *Metastasis: a question of life or death.* Nat Rev Cancer, 2006. **6**(6): p. 449-58.
129. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
130. Benelli, R., et al., *Cytokines and chemokines as regulators of angiogenesis in health and disease.* Curr Pharm Des, 2006. **12**(24): p. 3101-15.
131. Vega, F.M. and A.J. Ridley, *Rho GTPases in cancer cell biology.* FEBS Lett, 2008. **582**(14): p. 2093-101.
132. Willars, G.B., *Mammalian RGS proteins: multifunctional regulators of cellular signalling.* Semin Cell Dev Biol, 2006. **17**(3): p. 363-76.
133. Dorsam, R.T. and J.S. Gutkind, *G-protein-coupled receptors and cancer.* Nat Rev Cancer, 2007. **7**(2): p. 79-94.
134. De Vries, L., et al., *The regulator of G protein signaling family.* Annu Rev Pharmacol Toxicol, 2000. **40**: p. 235-71.
135. Buckbinder, L., et al., *The p53 tumor suppressor targets a novel regulator of G protein signaling.* Proc Natl Acad Sci U S A, 1997. **94**(15): p. 7868-72.
136. Shankar, S.P., et al., *RGS16 attenuates pulmonary Th2/Th17 inflammatory responses.* J Immunol, 2012. **188**(12): p. 6347-56.
137. Lippert, E., et al., *Role of regulator of G protein signaling 16 in inflammation-induced T lymphocyte migration and activation.* J Immunol, 2003. **171**(3): p. 1542-55.
138. Xie, S., et al., *IL-17 activates the canonical NF-kappaB signaling pathway in autoimmune B cells of BXD2 mice to upregulate the expression of regulators of G-protein signaling 16.* J Immunol, 2010. **184**(5): p. 2289-96.
139. Huang, J., et al., *Feeding and fasting controls liver expression of a regulator of G protein signaling (Rgs16) in periportal hepatocytes.* Comp Hepatol, 2006. **5**: p. 8.
140. Villasenor, A., et al., *Rgs16 and Rgs8 in embryonic endocrine pancreas and mouse models of diabetes.* Dis Model Mech, 2010. **3**(9-10): p. 567-80.
141. Berthebaud, M., et al., *RGS16 is a negative regulator of SDF-1-CXCR4 signaling in megakaryocytes.* Blood, 2005. **106**(9): p. 2962-8.
142. Hayasaka, N., et al., *Attenuated food anticipatory activity and abnormal circadian locomotor rhythms in Rgs16 knockdown mice.* PLoS One, 2011. **6**(3): p. e17655.
143. Snow, B.E., et al., *Cloning of a retinally abundant regulator of G-protein signaling (RGS-r/RGS16): genomic structure and chromosomal localization of the human gene.* Gene, 1998. **206**(2): p. 247-53.
144. Patten, M., et al., *Interleukin-1beta mediates endotoxin- and tumor necrosis factor alpha-induced RGS16 protein expression in cultured cardiac myocytes.* Naunyn Schmiedebergs Arch Pharmacol, 2003. **368**(5): p. 360-5.
145. Stuebe, S., et al., *Sphingosine-1-phosphate and endothelin-1 induce the expression of rgs16 protein in cardiac myocytes by transcriptional activation of the rgs16 gene.* Naunyn Schmiedebergs Arch Pharmacol, 2008. **376**(5): p. 363-73.
146. Vasiljevic, A., et al., *Molecular characterization of central neurocytomas: potential markers for tumor typing and progression.* Neuropathology, 2013. **33**(2): p. 149-61.
147. Davidsson, J., et al., *Tiling resolution array comparative genomic hybridization, expression and methylation analyses of dup(1q) in Burkitt lymphomas and pediatric high*

hyperdiploid acute lymphoblastic leukemias reveal clustered near-centromeric breakpoints and overexpression of genes in 1q22-32.3. Hum Mol Genet, 2007. **16**(18): p. 2215-25.
148. Miyoshi, N., et al., *RGS16 is a marker for prognosis in colorectal cancer.* Ann Surg Oncol, 2009. **16**(12): p. 3507-14.
149. Kim, J.H., et al., *RGS16 and FosB underexpressed in pancreatic cancer with lymph node metastasis promote tumor progression.* Tumour Biol, 2010. **31**(5): p. 541-8.
150. Wiechec, E., J. Overgaard, and L.L. Hansen, *A fragile site within the HPC1 region at 1q25.3 affecting RGS16, RGSL1, and RGSL2 in human breast carcinomas.* Genes Chromosomes Cancer, 2008. **47**(9): p. 766-80.
151. Fong, C.W., et al., *Specific induction of RGS16 (regulator of G-protein signalling 16) mRNA by protein kinase C in CEM leukaemia cells is mediated via tumour necrosis factor alpha in a calcium-sensitive manner.* Biochem J, 2000. **352 Pt 3**: p. 747-53.
152. Chen, C., et al., *The membrane association domain of RGS16 contains unique amphipathic features that are conserved in RGS4 and RGS5.* J Biol Chem, 1999. **274**(28): p. 19799-806.
153. Chen, C. and S.C. Lin, *The core domain of RGS16 retains G-protein binding and GAP activity in vitro, but is not functional in vivo.* FEBS Lett, 1998. **422**(3): p. 359-62.
154. Druey, K.M., et al., *Amino-terminal cysteine residues of RGS16 are required for palmitoylation and modulation of Gi- and Gq-mediated signaling.* J Biol Chem, 1999. **274**(26): p. 18836-42.
155. Hiol, A., et al., *Palmitoylation regulates regulators of G-protein signaling (RGS) 16 function. I. Mutation of amino-terminal cysteine residues on RGS16 prevents its targeting to lipid rafts and palmitoylation of an internal cysteine residue.* J Biol Chem, 2003. **278**(21): p. 19301-8.
156. Osterhout, J.L., et al., *Palmitoylation regulates regulator of G-protein signaling (RGS) 16 function. II. Palmitoylation of a cysteine residue in the RGS box is critical for RGS16 GTPase accelerating activity and regulation of Gi-coupled signalling.* J Biol Chem, 2003. **278**(21): p. 19309-16.
157. Derrien, A. and K.M. Druey, *RGS16 function is regulated by epidermal growth factor receptor-mediated tyrosine phosphorylation.* J Biol Chem, 2001. **276**(51): p. 48532-8.
158. Derrien, A., et al., *Src-mediated RGS16 tyrosine phosphorylation promotes RGS16 stability.* J Biol Chem, 2003. **278**(18): p. 16107-16.
159. Chen, C., et al., *Multiple phosphorylation sites in RGS16 differentially modulate its GAP activity.* FEBS Lett, 2001. **504**(1-2): p. 16-22.
160. Lee, M.J., et al., *RGS4 and RGS5 are in vivo substrates of the N-end rule pathway.* Proc Natl Acad Sci U S A, 2005. **102**(42): p. 15030-5.
161. Varshavsky, A., *The N-end rule pathway and regulation by proteolysis.* Protein Sci, 2011.
162. Liu, T., et al., *The retinoid anticancer signal: mechanisms of target gene regulation.* Br J Cancer, 2005. **93**(3): p. 310-8.
163. Vasilatos, S.N., et al., *Crosstalk between lysine-specific demethylase 1 (LSD1) and histone deacetylases mediates antineoplastic efficacy of HDAC inhibitors in human breast cancer cells.* Carcinogenesis, 2013. **34**(6): p. 1196-207.
164. Johnson, E.N., et al., *RGS16 inhibits signalling through the G alpha 13-Rho axis.* Nat Cell Biol, 2003. **5**(12): p. 1095-103.

165. Patten, M., et al., *Endotoxin induces desensitization of cardiac endothelin-1 receptor signaling by increased expression of RGS4 and RGS16.* Cardiovasc Res, 2002. **53**(1): p. 156-64.
166. Teplyuk, N.M., et al., *Runx2 regulates G protein-coupled signaling pathways to control growth of osteoblast progenitors.* J Biol Chem, 2008. **283**(41): p. 27585-97.
167. Milligan, G. and E. Kostenis, *Heterotrimeric G-proteins: a short history.* Br J Pharmacol, 2006. **147 Suppl 1**: p. S46-55.
168. Zhang, Y., et al., *RGS16 attenuates galphaq-dependent p38 mitogen-activated protein kinase activation by platelet-activating factor.* J Biol Chem, 1999. **274**(5): p. 2851-7.
169. Dittmer, S., et al., *The constitutively active orphan G-protein-coupled receptor GPR39 protects from cell death by increasing secretion of pigment epithelium-derived growth factor.* J Biol Chem, 2008. **283**(11): p. 7074-81.
170. Hsu, H.C., et al., *Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice.* Nat Immunol, 2008. **9**(2): p. 166-75.
171. Liang, G., et al., *RGS16 inhibits breast cancer cell growth by mitigating phosphatidylinositol 3-kinase signaling.* J Biol Chem, 2009. **284**(32): p. 21719-27.
172. Sethakorn, N., D.M. Yau, and N.O. Dulin, *Non-canonical functions of RGS proteins.* Cell Signal, 2010. **22**(9): p. 1274-81.
173. Billadeau, D.D., et al., *Characterization of the CXCR4 signaling in pancreatic cancer cells.* Int J Gastrointest Cancer, 2006. **37**(4): p. 110-9.
174. Bansal, G., K.M. Druey, and Z. Xie, *R4 RGS proteins: regulation of G-protein signaling and beyond.* Pharmacol Ther, 2007. **116**(3): p. 473-95.
175. Carper, M.B., Denvir, J., Boskovic, G., Primerano, D.A., Claudio, P. P., *RGS16, a novel p53 and pRb cross-talk candidate inhibits migration and invasion of pancreatic cancer cells.* Genes and Cancer, 2014. **5**(11-12): p. 420-435.
176. Shah, N., et al., *Muscarinic receptors and ligands in cancer.* Am J Physiol Cell Physiol, 2009. **296**(2): p. C221-32.
177. Tsoupras, A.B., et al., *The implication of platelet activating factor in cancer growth and metastasis: potent beneficial role of PAF-inhibitors and antioxidants.* Infect Disord Drug Targets, 2009. **9**(4): p. 390-9.
178. Rutka, J.T., et al., *Alterations of the p53 and pRB pathways in human astrocytoma.* Brain Tumor Pathol, 2000. **17**(2): p. 65-70.
179. Sherr, C.J., *Principles of tumor suppression.* Cell, 2004. **116**(2): p. 235-46.
180. Dong, Y., et al., *Reduced expression of retinoblastoma gene product (pRB) and high expression of p53 are associated with poor prognosis in ovarian cancer.* Int J Cancer, 1997. **74**(4): p. 407-15.
181. Jiang, Z., et al., *RB1 and p53 at the crossroad of EMT and triple-negative breast cancer.* Cell Cycle, 2011. **10**(10): p. 1563-70.
182. Yin, Y., et al., *Differential regulation of p21 by p53 and Rb in cellular response to oxidative stress.* Mol Carcinog, 1999. **24**(1): p. 15-24.
183. Su, Z.Z., et al., *The cancer growth suppressor gene mda-7 selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice.* Proc Natl Acad Sci U S A, 1998. **95**(24): p. 14400-5.

184. Greco, A., et al., *Eradication of therapy-resistant human prostate tumors using an ultrasound-guided site-specific cancer terminator virus delivery approach.* Mol Ther, 2010. **18**(2): p. 295-306.
185. Nande, R., et al., *Targeting a newly established spontaneous feline fibrosarcoma cell line by gene transfer.* PLoS One, 2012. **7**(5): p. e37743.
186. Tusher, V.G., R. Tibshirani, and G. Chu, *Significance analysis of microarrays applied to the ionizing radiation response.* Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5116-21.
187. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
188. Shinohara, H., et al., *Retinoblastoma protein-initiated cellular growth arrest overcomes the ability of cotransfected wild-type p53 to induce apoptosis.* Br J Cancer, 2000. **83**(8): p. 1039-46.
189. Ip, S.M., et al., *pRb-expressing adenovirus Ad5-Rb attenuates the p53-induced apoptosis in cervical cancer cell lines.* Eur J Cancer, 2001. **37**(18): p. 2475-83.
190. Spurgers, K.B., et al., *Identification of cell cycle regulatory genes as principal targets of p53-mediated transcriptional repression.* J Biol Chem, 2006. **281**(35): p. 25134-42.
191. Ho, J. and S. Benchimol, *Transcriptional repression mediated by the p53 tumour suppressor.* Cell Death Differ, 2003. **10**(4): p. 404-8.
192. Zhao, R., et al., *Analysis of p53-regulated gene expression patterns using oligonucleotide arrays.* Genes Dev, 2000. **14**(8): p. 981-93.
193. Hammond, E.M., et al., *Genome-wide analysis of p53 under hypoxic conditions.* Mol Cell Biol, 2006. **26**(9): p. 3492-504.
194. Kannan, K., et al., *DNA microarrays identification of primary and secondary target genes regulated by p53.* Oncogene, 2001. **20**(18): p. 2225-34.
195. Itahana, K., et al., *A role for p53 in maintaining and establishing the quiescence growth arrest in human cells.* J Biol Chem, 2002. **277**(20): p. 18206-14.
196. Marchesini, N., et al., *Role for mammalian neutral sphingomyelinase 2 in confluence-induced growth arrest of MCF7 cells.* J Biol Chem, 2004. **279**(24): p. 25101-11.
197. Osada, M., et al., *A p53-type response element in the GDF15 promoter confers high specificity for p53 activation.* Biochem Biophys Res Commun, 2007. **354**(4): p. 913-8.
198. Rouault, J.P., et al., *Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway.* Nat Genet, 1996. **14**(4): p. 482-6.
199. Taira, N., et al., *Induction of amphiregulin by p53 promotes apoptosis via control of microRNA biogenesis in response to DNA damage.* Proc Natl Acad Sci U S A, 2014. **111**(2): p. 717-22.
200. Yoon, H., et al., *Gene expression profiling of isogenic cells with different TP53 gene dosage reveals numerous genes that are affected by TP53 dosage and identifies CSPG2 as a direct target of p53.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15632-7.
201. Hoeferlin, L.A., et al., *Folate stress induces apoptosis via p53-dependent de novo ceramide synthesis and up-regulation of ceramide synthase 6.* J Biol Chem, 2013. **288**(18): p. 12880-90.
202. Jeong, B.S., et al., *Differential levels of transcription of p53-regulated genes by the arginine/proline polymorphism: p53 with arginine at codon 72 favors apoptosis.* FASEB J, 2010. **24**(5): p. 1347-53.

203. Sheikh, M.S., et al., *The antiapoptotic decoy receptor TRID/TRAIL-R3 is a p53-regulated DNA damage-inducible gene that is overexpressed in primary tumors of the gastrointestinal tract.* Oncogene, 1999. **18**(28): p. 4153-9.
204. Ohashi, T., et al., *AKR1B10, a transcriptional target of p53, is downregulated in colorectal cancers associated with poor prognosis.* Mol Cancer Res, 2013. **11**(12): p. 1554-63.
205. Kudoh, T., et al., *D4S234E, a novel p53-responsive gene, induces apoptosis in response to DNA damage.* Exp Cell Res, 2010. **316**(17): p. 2849-58.
206. Jiang, H., et al., *pRB and p107 have distinct effects when expressed in pRB-deficient tumor cells at physiologically relevant levels.* Oncogene, 2000. **19**(34): p. 3878-87.
207. Vernell, R., K. Helin, and H. Muller, *Identification of target genes of the p16INK4A-pRB-E2F pathway.* J Biol Chem, 2003. **278**(46): p. 46124-37.
208. Bourgo, R.J., et al., *RB restricts DNA damage-initiated tumorigenesis through an LXCXE-dependent mechanism of transcriptional control.* Mol Cell, 2011. **43**(4): p. 663-72.
209. Daoud, S.S., et al., *Impact of p53 knockout and topotecan treatment on gene expression profiles in human colon carcinoma cells: a pharmacogenomic study.* Cancer Res, 2003. **63**(11): p. 2782-93.
210. Wurster, A.L., T. Tanaka, and M.J. Grusby, *The biology of Stat4 and Stat6.* Oncogene, 2000. **19**(21): p. 2577-84.
211. Hanna, R.N., et al., *The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes.* Nat Immunol, 2011. **12**(8): p. 778-85.
212. Apte, R.N., et al., *The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions.* Cancer Metastasis Rev, 2006. **25**(3): p. 387-408.
213. Lippitz, B.E., *Cytokine patterns in patients with cancer: a systematic review.* Lancet Oncol, 2013. **14**(6): p. e218-28.
214. Santhanam, U., A. Ray, and P.B. Sehgal, *Repression of the interleukin 6 gene promoter by p53 and the retinoblastoma susceptibility gene product.* Proc Natl Acad Sci U S A, 1991. **88**(17): p. 7605-9.
215. Resnitzky, D., et al., *Interferons and interleukin 6 suppress phosphorylation of the retinoblastoma protein in growth-sensitive hematopoietic cells.* Proc Natl Acad Sci U S A, 1992. **89**(1): p. 402-6.
216. Markey, M.P., et al., *Loss of the retinoblastoma tumor suppressor: differential action on transcriptional programs related to cell cycle control and immune function.* Oncogene, 2007. **26**(43): p. 6307-18.
217. Freund, A., et al., *Inflammatory networks during cellular senescence: causes and consequences.* Trends Mol Med, 2010. **16**(5): p. 238-46.
218. Iannello, A., et al., *p53-dependent chemokine production by senescent tumor cells supports NKG2D-dependent tumor elimination by natural killer cells.* J Exp Med, 2013. **210**(10): p. 2057-69.
219. Kansara, M., et al., *Immune response to RB1-regulated senescence limits radiation-induced osteosarcoma formation.* J Clin Invest, 2013. **123**(12): p. 5351-60.
220. Ghaneh, P., E. Costello, and J.P. Neoptolemos, *Biology and management of pancreatic cancer.* Postgrad Med J, 2008. **84**(995): p. 478-97.

221. Teicher, B.A. and S.P. Fricker, *CXCL12 (SDF-1)/CXCR4 pathway in cancer.* Clin Cancer Res, 2010. **16**(11): p. 2927-31.
222. Marchesi, F., et al., *Increased survival, proliferation, and migration in metastatic human pancreatic tumor cells expressing functional CXCR4.* Cancer Res, 2004. **64**(22): p. 8420-7.
223. Ng, S.S.W., et al., *Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells.* Cancer Res, 2000. **60**(19): p. 5451-5.
224. Schlieman, M.G., et al., *Incidence, mechanism and prognostic value of activated AKT in pancreas cancer.* Br J Cancer, 2003. **89**(11): p. 2110-5.
225. Osaki, M., M. Oshimura, and H. Ito, *PI3K-Akt pathway: its functions and alterations in human cancer.* Apoptosis, 2004. **9**(6): p. 667-76.
226. Zhao, S., et al., *Expression of oncogenic K-ras and loss of Smad4 cooperate to induce the expression of EGFR and to promote invasion of immortalized human pancreas ductal cells.* Int J Cancer, 2010. **127**(9): p. 2076-87.
227. Yamanaka, Y., et al., *Coexpression of epidermal growth factor receptor and ligands in human pancreatic cancer is associated with enhanced tumor aggressiveness.* Anticancer Res, 1993. **13**(3): p. 565-9.
228. Lo, H.W., et al., *Epidermal growth factor receptor cooperates with signal transducer and activator of transcription 3 to induce epithelial-mesenchymal transition in cancer cells via up-regulation of TWIST gene expression.* Cancer Res, 2007. **67**(19): p. 9066-76.
229. Dittmar, T., et al., *Induction of cancer cell migration by epidermal growth factor is initiated by specific phosphorylation of tyrosine 1248 of c-erbB-2 receptor via EGFR.* FASEB J, 2002. **16**(13): p. 1823-5.
230. Giehl, K., et al., *Growth factor-dependent activation of the Ras-Raf-MEK-MAPK pathway in the human pancreatic carcinoma cell line PANC-1 carrying activated K-ras: implications for cell proliferation and cell migration.* Oncogene, 2000. **19**(25): p. 2930-42.
231. Deer, E.L., et al., *Phenotype and genotype of pancreatic cancer cell lines.* Pancreas, 2010. **39**(4): p. 425-35.
232. Ali, S., et al., *Simultaneous targeting of the epidermal growth factor receptor and cyclooxygenase-2 pathways for pancreatic cancer therapy.* Mol Cancer Ther, 2005. **4**(12): p. 1943-51.
233. Kim, D., et al., *Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production.* FASEB J, 2001. **15**(11): p. 1953-62.
234. Tanno, S., et al., *AKT activation up-regulates insulin-like growth factor I receptor expression and promotes invasiveness of human pancreatic cancer cells.* Cancer Res, 2001. **61**(2): p. 589-93.
235. Tan, X., et al., *Involvement of MMP-7 in invasion of pancreatic cancer cells through activation of the EGFR mediated MEK-ERK signal transduction pathway.* J Clin Pathol, 2005. **58**(12): p. 1242-8.
236. Malliri, A., et al., *The transcription factor AP-1 is required for EGF-induced activation of rho-like GTPases, cytoskeletal rearrangements, motility, and in vitro invasion of A431 cells.* J Cell Biol, 1998. **143**(4): p. 1087-99.
237. Keleg, S., et al., *Invasion and metastasis in pancreatic cancer.* Mol Cancer, 2003. **2**: p. 14.

238. Yachida, S. and C.A. Iacobuzio-Donahue, *The pathology and genetics of metastatic pancreatic cancer.* Arch Pathol Lab Med, 2009. **133**(3): p. 413-22.
239. Jang, S. and M.B. Atkins, *Treatment of BRAF-mutant melanoma: the role of vemurafenib and other therapies.* Clin Pharmacol Ther, 2014. **95**(1): p. 24-31.
240. Kusama, T., et al., *Inhibition of epidermal growth factor-induced RhoA translocation and invasion of human pancreatic cancer cells by 3-hydroxy-3-methylglutaryl-coenzyme a reductase inhibitors.* Cancer Res, 2001. **61**(12): p. 4885-91.
241. Xie, Y., et al., *Breast cancer migration and invasion depend on proteasome degradation of regulator of G-protein signaling 4.* Cancer Res, 2009. **69**(14): p. 5743-51.
242. Mori, T., et al., *CXCR4 antagonist inhibits stromal cell-derived factor 1-induced migration and invasion of human pancreatic cancer.* Mol Cancer Ther, 2004. **3**(1): p. 29-37.
243. Singh, S., et al., *CXCL12-CXCR4 signalling axis confers gemcitabine resistance to pancreatic cancer cells: a novel target for therapy.* Br J Cancer, 2010. **103**(11): p. 1671-9.
244. Chou, A., et al., *Clinical and molecular characterization of HER2 amplified-pancreatic cancer.* Genome Med, 2013. **5**(8): p. 78.
245. Lakka, S.S., et al., *Downregulation of MMP-9 in ERK-mutated stable transfectants inhibits glioma invasion in vitro.* Oncogene, 2002. **21**(36): p. 5601-8.
246. Lu, Z., et al., *Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion.* Cancer Cell, 2003. **4**(6): p. 499-515.
247. Kwon, O., et al., *Modulation of E-cadherin expression by K-Ras; involvement of DNA methyltransferase-3b.* Carcinogenesis, 2010. **31**(7): p. 1194-201.
248. Nagasawa, R., et al., *Unique phosphorylation of vimentin filaments in glomerular epithelial cells in culture and in disease.* Nephron, 1997. **77**(4): p. 373-7.
249. Ivaska, J., et al., *Novel functions of vimentin in cell adhesion, migration, and signaling.* Exp Cell Res, 2007. **313**(10): p. 2050-62.
250. Khosravi-Far, R., et al., *Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation.* Mol Cell Biol, 1995. **15**(11): p. 6443-53.
251. Qiu, R.G., et al., *Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation.* Mol Cell Biol, 1997. **17**(6): p. 3449-58.
252. Timpson, P., et al., *Spatial regulation of RhoA activity during pancreatic cancer cell invasion driven by mutant p53.* Cancer Res, 2011. **71**(3): p. 747-57.
253. Kusama, T., et al., *Inactivation of Rho GTPases by p190 RhoGAP reduces human pancreatic cancer cell invasion and metastasis.* Cancer Sci, 2006. **97**(9): p. 848-53.
254. Shan, D., et al., *The G protein G alpha(13) is required for growth factor-induced cell migration.* Dev Cell, 2006. **10**(6): p. 707-18.
255. Nimnual, A.S., L.J. Taylor, and D. Bar-Sagi, *Redox-dependent downregulation of Rho by Rac.* Nat Cell Biol, 2003. **5**(3): p. 236-41.
256. Wildenberg, G.A., et al., *p120-catenin and p190RhoGAP regulate cell-cell adhesion by coordinating antagonism between Rac and Rho.* Cell, 2006. **127**(5): p. 1027-39.
257. Roger, L., G. Gadea, and P. Roux, *Control of cell migration: a tumour suppressor function for p53?* Biol Cell, 2006. **98**(3): p. 141-52.

258. Liu, J., et al., *Wild-type p53 inhibits nuclear factor-kappaB-induced matrix metalloproteinase-9 promoter activation: implications for soft tissue sarcoma growth and metastasis.* Mol Cancer Res, 2006. **4**(11): p. 803-10.
259. Sun, Y., et al., *Human metalloproteinase-1 (collagenase-1) is a tumor suppressor protein p53 target gene.* Ann N Y Acad Sci, 1999. **878**: p. 638-41.
260. Sun, Y., et al., *p53 down-regulates human matrix metalloproteinase-1 (Collagenase-1) gene expression.* J Biol Chem, 1999. **274**(17): p. 11535-40.
261. Zhu, H., et al., *A role for p53 in the regulation of extracellular matrix metalloproteinase inducer in human cancer cells.* Cancer Biol Ther, 2009. **8**(18): p. 1722-8.
262. Andrusiak, M.G., et al., *Rb/E2F regulates expression of neogenin during neuronal migration.* Mol Cell Biol, 2011. **31**(2): p. 238-47.
263. Knudsen, K.E., et al., *RB-dependent S-phase response to DNA damage.* Mol Cell Biol, 2000. **20**(20): p. 7751-63.
264. Amundson, S.A., et al., *Stress-specific signatures: expression profiling of p53 wild-type and -null human cells.* Oncogene, 2005. **24**(28): p. 4572-9.
265. Harrington, E.A., et al., *pRB plays an essential role in cell cycle arrest induced by DNA damage.* Proc Natl Acad Sci U S A, 1998. **95**(20): p. 11945-50.
266. Orsulic, S., et al., *E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation.* J Cell Sci, 1999. **112 (Pt 8)**: p. 1237-45.
267. Barberis, L., et al., *Leukocyte transmigration is modulated by chemokine-mediated PI3Kgamma-dependent phosphorylation of vimentin.* Eur J Immunol, 2009. **39**(4): p. 1136-46.
268. Herreros-Villanueva, M., et al., *Mouse models of pancreatic cancer.* World J Gastroenterol, 2012. **18**(12): p. 1286-94.
269. Becher, O.J. and E.C. Holland, *Genetically engineered models have advantages over xenografts for preclinical studies.* Cancer Res, 2006. **66**(7): p. 3355-8, discussion 3358-9.
270. Ocal, O., et al., *Rgs16 is an early marker of pancreatic ductal adenocarcinoma (842.7).* The FASEB Journal, 2014. **28**(1 Supplement).
271. Qiu, W. and G.H. Su, *Challenges and advances in mouse modeling for human pancreatic tumorigenesis and metastasis.* Cancer Metastasis Rev, 2013. **32**(1-2): p. 83-107.
272. Qiu, W. and G.H. Su, *Development of orthotopic pancreatic tumor mouse models.* Methods Mol Biol, 2013. **980**: p. 215-23.
273. Huynh, A.S., et al., *Development of an orthotopic human pancreatic cancer xenograft model using ultrasound guided injection of cells.* PLoS One, 2011. **6**(5): p. e20330.

APPENDIX

OFFICE OF RESEARCH AND INTEGRITY IRB APPROVAL

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

October 10, 2014

Miranda Carper
Marshall University School of Medicine
Biomedical Sciences
Department of Biochemistry/Molecular Biology
McKown Translation Genomic Research Institute

Dear Ms. Carper:

This letter is in response to the submitted thesis abstract to test the hypothesis that the Regulator of G protein signaling 16 (RGS16) inhibits pancreatic cancer cell migration and invasion *in vitro*. After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

CURRICULUM VITAE

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Education

Current Ph.D. Graduate Candidate Aug 2008-Dec 2014
Marshall University, Huntington WV
- Major: Biomedical Sciences

Bachelor of Science 2007
Marietta College, Marietta OH
- Major: Biochemistry

Associates of Arts 2004
Washington State Community College, Marietta OH
- Major: Liberal Arts, Social Science

High school Diploma 2004
- Marietta High school, Marietta Ohio

Employment

Analytical Products Group November 19, 2007-July 25, 2008
Quality Control Chemist/Technical Support- I tested products prior to shipping to customers. I was also a part of the technical support team where I aided customers with any problems they may have had in running the company's products. I specialized in operating HPLC and the Total Organic Carbon Analyzer, and aided in running IC, titrations, pH, and UV- VIS Spectrometer analyses

Ely Chapman Education Foundation
I was assistant Karate instructor till October 2005. In 2005, I became head Karate instructor where I was in charge of running the SUCCESS AND HONOR karate program, which was part of an after school outreach program for children. I was in charge of attendance, promoting classes, tuition, belt testing, and teaching classes ranging in ages from four year olds to adults. I was also involved in teaching summer karate classes to children enrolled in a summer program through the YMCA and Ely Chapman Education Foundation.

Skills

- Immunofluorescence
- qPCR
- PCR
- Western blot
- Cell culture
- Adenovirus culture, expansion, and titering
- Microscopy
- Immunoprecipitation
- Accuri Flow Cytometer
- Fotodyne imaging system

Research Experience

- September 2006 to May 2007, I conducted research at Marietta College to form a guided inquiry organic laboratory experiment focused on investigating the relationship between the structure of organic compounds and their HOMO (highest occupied molecular orbital) LUMO (lowest unoccupied molecular orbital) gap. The goal of the research was to create an organic laboratory experiment that better relates to students how a compound's properties impact color.
- January 2009 to present, I have been working in Dr. Pier Paolo Claudio's lab investigating the cross-talk pathway between p53 and pRb. This has included analyzing Microarray data in order to find proteins that are co-regulated by both p53 and pRb. Currently, I am examining the role of Regulator of G protein Signaling 16 (RGS16) (a protein regulated by both p53 and pRb in our microarray and qPCR data) in modulating pancreatic cancer cell migration.

Grants

- Received NASA WV Space Grant Consortium (WVSGC) grant. **Characterization of RGS16: a player in the crossroad between the p53 and pRb tumor suppressors.** August 2011 to May 2012
- Received NASA WV Space Grant Consortium (WVSGC) grant. **Identifying the role of RGS16 in pancreatic cancer cell migration and invasion.** August 2012 to May 2013.

Awards Received

- Marshall University Graduate Student Organization Award for Outstanding Leadership and Academic Excellence, August 2012
- American Society of Biochemistry and Molecular biology Travel Award, April 2013
- Marshall University Biomedical Sciences Best Overall Graduate Student Award, August 2014

Extracurricular Achievement / committees

- Trained in Karate for seven years and was awarded a second degree blackbelt and teaching certificate. Volunteered as an assistant children's karate instructor at the Ely Chapman Education Foundation from November, 2002 to February 2003. In February of 2003, I became a part-time assistant karate instructor at the Ely Chapman Education Foundation teaching karate to children and adults. November, 2005 became head karate instructor and managed karate school until May, 2008.

- Secretary/Treasurer of the Marshall University School of Medicine Graduate Student Organization (MUSOM GSO) from May 2010-May 2011. Duties include managing finances, aiding in fundraising activities, and helping with the Marshall University Biomedical Sciences open house (fall) and Ph.D. interview activities (Spring)

- Elected as MUSOM GSO President for May 2011-May 2012. Duties include keeping students informed on current program events, coordinating fundraisers, holding monthly meetings, organized meetings to help students apply for grants, volunteering to aid in local community events and aid in the planning of the Marshall University Biomedical Sciences open house and Ph.D. interview activities. I was also instrumental in enacting the first student scholarship to be give to Biomedical sciences Master and Ph.D. students by the Marshall Graduate Student Organization.

- Graduate student representative for the MUSOM Women in Medicine and Science Executive Council (March 2012 to June 2014). Duties include helping to organize events, getting graduate student involvement, and vocalizing graduate students' concerns and suggestions to council.

- Appalachian Regional Cell Conference secondary Marshall representative for October 2013 conference. Worked with Allison Wolf and other representatives from Ohio University, Kentucky University, and West Virginia University to plan a student driven conference. This conference has helped boost collaboration and interaction with other graduate students from regional universities.

Presentations and Publications

- Poster Presentation: 233rd ACS National Meeting. Chicago, IL. March 25-29, 2007. Investigating color: a guided inquiry experiment for the sophomore organic chemistry laboratory. **Carper, M.**, Pate, K. L.
- Poster Presentation: Annual American Institute for Cancer Research on Food Nutrition, Physical Activity, and Cancer. Washington D.C. November 5, 2009. Omega-3 and -6 fatty acids select, proliferate, and sensitize colorectal cancer stem-like cells to chemotherapy. Kelly, S.E., **Carper, M**., Valluri, J., Claudio, PP.
- Poster Presentation: Marshall University School of Medicine Resesarch Day, Huntington, WV. March 20, 2012. Finding a bridge that connects the p53 and pRb tumor suppressor pathways. **Carper M.**, Boskovic G., Denvir J., Primerano D., and Claudio PP.
- Poster presentation: CCTS Spring Conference at the Lexington Convention Center, Lexington WV. March 29, 2012. Finding a bridge that connects the p53 and pRb tumor suppressor pathways. **Carper M**., Boskovic G., Denvir J., Primerano D., and Claudio PP.
- Poster Presentation: STaR Symposium at West Virginia State University Institute, WV. April 21, 2012. Finding a bridge that connects the p53 and pRb tumor suppressor pathways. **Carper M**., Boskovic G., Denvir J., Primerano D., and Claudio PP.
- Poster Presentation: Marshall University School of Medicine Research Day, Huntington, WV. March 19, 2013. RGS16 mediated inhibition of pancreatic cancer cell migration. **Carper M.**, Boskovic G., Denvir J., Primerano D., and Claudio PP.
- Poster Presentation: Experimental Biology Conference Boston, MA. April 19& 20, 2013. RGS16 mediated inhibition of pancreatic cancer cell migration. **Carper M.**, Boskovic G., Denvir J., Primerano D.A., and Claudio PP.
- Oral Presentation: Marshall University School of Medicine Research Day, Huntington, WV. March 25, 2014. RGS16 inhibits migration and invasion of pancreatic cancer.
- Publication: Nande, R., DiBenedetto, A., Aimolla PP., DeCarlo F., **Carper M.,** Claudio C., Duncan G., Claudio, PP.Gene therapy in a newly established spontaneous feline fibrosarcoma cell line. PLoS ONE
- Publication: **Carper M.B**., Denvir J., Boskovic G, Primerano D, and Claudio PP. (2014) RGS16, a novel p53 and pRb cross-talk candidate inhibits migration and invasion of pancreatic cancer cells. Genes and Cancer 5: 420-435

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RGS16, A NOVEL P53 AND PRb CROSS-TALK CANDIDATE INHIBITS MIGRATION AND INVASION OF PANCREATIC CANCER CELLS

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Short title: RGS16 inhibits pancreatic cancer cell migration

Keywords: p53, pRb, RGS16, EGF, migration, pancreatic cancer.

Abstract

Data collected since the discovery of p53 and pRb/RB1 suggests these tumor suppressors cooperate to inhibit tumor progression. Patients who have mutations in both p53 and RB1 genes have increased tumor reoccurrence and decreased survival compared to patients with only one tumor suppressor gene inactivated. It remains unclear how p53 and pRb cooperate toward inhibiting tumorigenesis. Using RNA expression profiling we identified 179 p53 and pRb cross-talk candidates in normal lung fibroblasts (WI38) cells exogenously coexpressing p53 and pRb. Regulator of G protein signaling 16 (RGS16) was among the p53 and pRb cross-talk candidates and has been implicated in inhibiting activation of several oncogenic pathways associated with proliferation, migration, and invasion of cancer cells.

RGS16 has been found to be downregulated in pancreatic cancer patients with metastases compared to patients without metastasis. Expression of RGS16 mRNA was decreased in the pancreatic cancer cell lines tested compared to control. Expression of RGS16 inhibited migration of the BxPC-3 and AsPC-1 but not PANC-1 cells and inhibited invasion of BxPC-3 and AsPC-1 cells with no impact on cell viability. We have identified for the first time p53 and pRb cross-talk candidates and a role for RGS16 to inhibit pancreatic cancer migration and invasion.

Introduction

The p53 and pRb tumor suppressors are two signaling pathways that are frequently altered during cancer progression. Mutations that disrupt the p53 and pRb pathways can occur in the gene sequences or in their upstream regulators and/or downstream effectors. Results of studies have found that both tumor suppressor genes are inactivated in a variety of malignancies including osteosarcoma, small cell lung, breast, and bladder carcinomas [1, 4, 45, 46]. Furthermore, alterations in expression or activity of proteins involved in p53 and pRb signaling pathways have been identified in retinoblastoma and cancers of the pancreas, colon, and head and neck among others [49, 51, 52, 111]. The large number of cancers that have defects in the p53 and pRb pathways demonstrates the importance of these genes in preventing cancer development and progression.

Existing data suggests that p53 and pRb cooperate to prevent tumor progression. Examples of this cooperative interaction have been shown by various studies using human primary cancer samples and mouse models. Patients who have mutations in both p53 and *RB1* genes have increased tumor recurrence and decreased survival compared to patients with a mutation in either p53 or *RB1* [45, 47, 180]. A study conducted in mice found that p53 null mice who were also heterozygous for *RB1* were susceptible to developing more tumors than mice with single mutations; i.e. heterozygous p53 or *RB1* null or p53 null mice [4]. In another study, mice with conditional inactivation of both p53 and *RB1* in prostate epithelium developed highly metastatic tumors and had decreased survival time compared to mice with single p53 or *RB1* inactivation [5]. The accumulated evidence suggests p53 and *RB1* gene products have cooperative or synergistic effects for cancer suppression.

Considering the network of communication that exists within a cell, the rate of mutation of p53 and *RB1*, and the cellular processes these two proteins regulate, a natural hypothesis is that these two genes and respective gene products cross-communicate in order to determine cellular fate and prevent carcinogenesis. In fact, there are known examples of genes and proteins that are involved in the convergent signaling between the p53 and pRb pathways; such as Hdm2, p21, E2F-1 and the INK4a locus (reviewed in [3, 47, 74, 182]). Although several proteins that are involved in the p53 and pRb pathways have been identified, the full extent in which these two tumor suppressors interact along their pathway to regulate cellular fate is still unknown. To identify downstream targets of both p53 and pRb regulation and to elucidate mechanisms of p53 and pRb cross-talk, we coexpressed p53 and pRb in normal human lung fibroblasts cells (WI38) and used RNA expression profiling to identify up- or down-regulated genes. We identified Regulator of G protein Signaling 16 (RGS16) as a p53 and pRb cross-talk candidate.

RGS16, previously found to be induced by doxorubicin in cells expressing wild-type p53, belongs to a large family of proteins that plays a role in swiftly shutting down G protein-coupled receptor (GPCR) signaling pathways [134, 135]. RGS16 is a GTPase activating protein (GAP) that aids GTPase activity of the α-subunit of G proteins associated with G-protein coupled receptors (GPCR). RGS16 has been implicated in negatively regulating the MAPK, AKT/PI3K, RhoA, and SDF-1/CXCR4 oncogene pathways in normal or cancer cell lines [135, 141, 164, 171]. These oncogene pathways have been implicated in cancer progression processes (such as proliferation, survival, chemoresistance, migration, invasion, and metastasis in a variety of malignancies including pancreatic cancer [221-225]. Recently, evidence has demonstrated a role of RGS16 in cancer signaling. RGS16 locus is a site of genomic instability in (50% of 222) primary breast tumors and knockdown of RGS16 in breast cancer cell lines increases Epidermal

Growth Factor (EGF) and Fetal Bovine Serum (FBS) initiated proliferation [150, 171]. A previous report using tissue microarray analysis revealed decreased expression of Regulator of G-protein signaling 16 (RGS16) in pancreatic tumors with lymph-node metastases compared to non-metastasized pancreatic cancer and this loss was associated with decreased patient survival [149]. Based upon the link of RGS16 regulating several oncogenic pathways and the decreased expression of RGS16 in metastasized pancreatic cancer, we chose to further study the function of RGS16 in pancreatic cancer in order to identify the role it has in the p53 and pRb signaling pathways. Currently, RGS16 has not been linked with inhibition of cancer cell metastasis nor has its function been investigated to understand it's downregulation in metastasized pancreatic cancer. The majority of patients newly diagnosed with pancreatic cancer present with highly progressed and/or metastatic cancer that is resistant to treatment [84, 85]. Due to the late stage of diagnosis and the aggressive nature of this disease, less than 20% of pancreatic cancer patients are eligible for the potentially curative surgery [85, 220]. Therefore, there is a great need for more effective drugs aimed at treating or preventing metastatic pancreatic cancer. Pancreatic cancer is associated with p53 mutations and p16 (pRb activator) deletions resulting in the crippling of both the p53 and pRb pathways. By investigating the p53 and pRb cross-talk and the role of RGS16 in pancreatic cancer cell migration, we have uncovered a novel regulator of metastasis processes that could be a future target in developing treatments for metastatic pancreatic cancer.

Results

Identification of p53 and pRb cross-talk candidates in WI38 cells following coexpression of p53 and/or pRb.

Studies have shown that p53 and pRb cooperate to prevent tumorigenesis. Currently, the molecules that function in the p53 and pRb cross-talk pathway to regulate cellular fate are not known thus expression profiling by microarray was performed to find genes co-regulated by p53 and pRb. Normal human lung WI38 fibroblast cells were transduced with adenoviral vectors expressing the p53 and/or RB1 genes under the control of a cytomegalovirus (CMV) promoter. The WI38 cell line was used because it is from non-cancerous tissue and lacks mutations or viral transformations that could disrupt the p53 and pRb pathways. Four experimental conditions were used in which WI38 cells were transduced with adenovirus vector control (cond. 1, Adenoviral CMV-vector control, Ad.CMV.p53 (cond. 2), Ad.CMV.pRb (cond. 3), or both Ad.CMV.p53 and Ad.CMV.pRb (cond. 4). RNA and protein from WI38 cells was collected 48 hours after adenoviral infection. Immunoblots verified increased expression of p53 (fold change compared to Ad.CMV control = 2.80, 1.54, and 2.77) and/or hypophosphorylated (active form) pRb (hypophosphorylated/total pRb fold change compared to Ad.CMV control = 0.94, 5.48, 5.02) in the WI38 cells treated with adenoviruses containing p53, pRb, or both p53 and pRb respectively (Figure 1A and 1B). Fold change values for p53 and hypophosphorylated pRb coincided with previously reported results in experiments that activated endogenous p53 and pRb [195, 196]. Microarray data from the adenovirus vector control (empty vector with CMV promoter) was used as a reference to determine genes that were differentially expressed as a consequence of p53, pRb, and p53 + pRb expression. Analysis of the microarray data identified 294-p53, 650-pRb, and 514-p53 + pRb differentially expressed genes (Figure 1C; see Supplementary Document 1) for full list of differentially expressed genes. Of the differentially expressed genes, 294/294 genes were upregulated in cells with p53 expression, 427/650 genes were upregulated in cells with pRb expression, and 319/514 genes were up-regulated in cells with p53 + pRb

coexpression (Figure 1C). Consistent with protein measurements, increased expression of p53 and/or *RB1* mRNAs were also found in the appropriate groups (Supplementary Document 1).

A Venn diagram shows the number of differentially expressed genes shared between the experimental groups (Figure 1C). By looking at the common genes between the three experimental groups, we were able to generate two lists of genes that may be involved in the p53 and pRb cross-talk pathway. The first list of cross-talk candidates (designated as the p53 and pRb common gene set) consisted of 39 genes found to be commonly up-regulated in cells expressing either p53 or pRb. The second list of possible cross-talk members (designated as the p53 and pRb interaction gene set) contained 140 genes that were found to be differentially expressed only when p53 and pRb were overexpressed together (see Supplementary Document 1). Thirty-two of the 39 common gene set cross-talk candidates were found to be up-regulated in the interaction gene set, while the remaining 7 were commonly up-regulated in cells that overexpress either p53 or pRb (Table 1). By focusing on the common and interaction gene sets, we were able to remove transcripts that were up- or down-regulated by only p53 or pRb and focus on candidates that may be involved in the p53 and pRb cross-talk pathway.

qRT-PCR validation of microarray data in WI38 and SAOS-2 cells.

Our ultimate goal in performing the microarray analysis was to determine molecules involved in the p53 and pRb cross-talk pathway in order to identify and study downstream effector molecules that can be expressed to induce a p53 and/or pRb tumor suppressive function. Because of our interest in identifying downstream effector molecules, we chose five mRNA transcripts (IL-6, BTG-2, STAT4, RGS16, BCL2L11) from the set of 39 commonly up-regulated transcripts by p53 and pRb for validation via qRT-PCR. IL-6, BTG-2, STAT4, RGS16, and

BCL2L11 were chosen for validation because of varying function, known regulation by p53 and pRb, and fold change values expression profiling assay. WI38 cells were plated and transduced with adenoviral expression vectors via the same methods used for the microarray analysis. Relative fold change was calculated for IL-6, BTG-2, STAT4, RGS16, and BCL2L11 in WI38 cells expressing p53 and/or pRb as shown in Figure 2. Statistically significant up-regulation of all transcripts tested except BCL2L11 was found in WI38 cells expressing p53 and pRb confirming the microarray results. Expression of p53 and pRb in WI38 cells increased mRNA expression for some of the transcripts (for example, RGS16 and BTG-2) to a greater extent than single expression of either p53 or pRb. This suggests p53 and pRb are working together resulting in an additive (i.e. BTG-2) or synergistic (i.e. RGS16) effect on mRNA expression for some of the transcripts.

To further support the RNA expression profiling results, we repeated the expression of p53 and pRb in a p53 null, *RB1* mutant osteosarcoma cell line (SAOS-2) and performed qRT-PCR analysis of IL-6, BTG-2, STAT4, RGS16, and BCL2L11. The expression of all five transcripts including IL-6 and BCL2L11 were found to be significantly increased by one-way ANOVA compared to vector control in SAOS-2 cells expressing p53 and/or pRb (Figure 3). Dunnett's test for multiple comparison found BCL2L11 expression to be significantly increased in cells expressing p53, pRb, and both p53 and pRb and IL-6 was found to be significantly increased in cells expressing pRb and p53+pRb. Expression of IL-6 was not found to be statistically significant in SAOS-2 cells expressing p53 due to variation between replicates (fold change= 2.86). All five transcripts were found to be up-regulated when p53 and/or pRb were expressed in the microarray analysis and qRT-PCR analysis showed similar results in WI38 and SAOS-2 cells.

mRNA expression of RGS16 is decreased in pancreatic cancer cell lines.

RGS16 was identified as a p53 and pRb cross-talk candidate in our expression profiling analysis that was validated by qRT-PCR. We chose to study the role of RGS16 in pancreatic cancer cell migration due in part to its down-regulation in patients with metastasized pancreatic cancer and the high rate of p53 mutations (50-70%) and p16 deletions (85%) affecting both the p53 and pRb pathways in this disease [51, 111, 149]. We first investigated the relative expression of RGS16 mRNA in four pancreatic cancer cell lines (BxPC-3, MIA PaCa-2, PANC-1, and AsPC-1) in order to characterize the endogenous expression of RGS16. Expression of RGS16 was measured by qRT-PCR analysis and the relative RGS16 mRNA fold change was calculated in the four cell lines compared to total RNA from normal human pancreatic tissue. Expression of RGS16 was decreased in all four lines compared to control with BxPC-3 having the highest expression of RGS16 mRNA (Figure 4). Expression of RGS16 varied between the four lines with BxPC-3 and MIA PaCa-2 having significantly higher expression of RGS16 than PANC-1 and the metastatic derived AsPC-1 cells. RGS16 expression corresponded with the more differentiated and less aggressive cell lines having higher levels of RGS16 than the more aggressive and/or metastatic cell lines (Table 2).

RGS16 inhibited migration of BxPC-3 and AsPC-1 pancreatic cancer cells but not PANC-1.

To test the hypothesis that RGS16 inhibits pancreatic cancer cell migration, we exogenously expressed RGS16 in BxPC-3, PANC-1, and AsPC-1 cells with an adenoviral vector and used

wound healing assays to measure cell migration. We chose BxPC-3, PANC-1, and AsPC-1 because these three cell lines are derived from tumors with varying expression of RGS16, differentiation status, mutations, presence of metastases, and expression of Epidermal Growth Factor Receptor (EGRF, Table 2). We expressed RGS16 using adenoviral vector that contains RGS16 plus a GFP reporter (Ad.GFP.RGS16) and used a vector expressing only GFP (Ad.GFP) as the control. Expression of RGS16 protein correlated with GFP expression in cells treated with Ad.GFP.RGS16 (Supplementary Figure 1). Fluorescent microscopy was used to determine viral transduction prior to experiment (Figures 5a, 6a, and 7a). EGF was used to stimulate cell migration because EGFR is overexpressed in pancreatic cancer and is linked with development, invasion, and decreased survival in pancreatic cancer [115, 226, 227]. RGS16 significantly inhibited FBS- and EGF-induced migration of BxPC-3 cells and FBS-induced migration of AsPC-1 cells, but had no effect on FBS and EGF induced migration of PANC-1 cells (Figures 5-7).

Interestingly, expression of RGS16 in BxPC-3 cells incubated in media supplemented with EGF caused an increase in wound width compared to control 16 hours after the start of the experiment. However, MTT assay revealed that there was no statistically significant change in cell viability of FBS or EGF treated BxPC-3, PANC-1 or AsPC-1 following expression of RGS16 compared to control cells expressing GFP (Supplementary Figure 2).

Expression of RGS16 inhibited EGF induced invasion of BxPC-3 and AsPC-1 cells.

RGS16 inhibited EGF induced migration of BxPC-3 and AsPC-1 cells, we further investigated if RGS16 can inhibit EGF induced invasion of these pancreatic cancer cells using matrigel invasion chambers. Media supplemented with EGF was used as the chemoattractant to induce migration and invasion of BxPC-3 and AsPC-1 cells expressing GFP and or RGS16.

Expression of RGS16 significantly inhibited EGF induced invasion of the BxPC-3 and AsPC-1 cells by 35.73% and 66% respectively, compared to control (Ad.GFP) (Figure 8).

Discussion

Significance of investigating p53 and pRb cross-talk

Historically, investigations of p53 and pRb regulated transcription have focused on identifying the individual downstream targets of p53 and pRb. However, cell fate is not determined solely by one signaling pathway but by many pathways that communicate through a network of signaling molecules. Cross-communication between pathways allows the integration of the exogenous and endogenous signals in a cell to aid in the determination of cell fate. Previous studies have found that co-expression of p53 and pRb in cancer cells with compromised p53 and pRb activity inhibited p53 mediated apoptosis and promoted cell cycle arrest suggesting p53 and pRb cross-talk to regulate cellular fate [188, 189]. Furthermore, data from previous studies suggests p53 and pRb may also cooperate to inhibit cancer progression. Patients diagnosed with breast cancer and treated with adjuvant chemotherapy had a better prognosis to adjuvant chemotherapy if they had functional p53 and pRb [61].

To our knowledge this is the first study that examines altered gene expression when p53 and pRb are expressed together or separately with the purpose of finding genes co-regulated by both tumor suppressor genes. How p53 and pRb cross-communicate to regulate cellular functions or cooperate to inhibit cancer progression still remains largely unknown. The p53 and pRb pathways are commonly altered during tumorigenesis. Due to the dynamic properties of cell signaling, the study of genes dually regulated by p53 and pRb will provide a valuable insight into the collaborative cancer preventative properties of these two tumor suppressor proteins.

Transcriptional regulation may be one method used by p53 and pRb to coordinate cellular functions. For example, the cyclin kinase inhibitor p21 is a down-stream target gene of p53 that inhibits phosphorylation and inactivation of pRb [25]. Transactivation of p21 demonstrates a mechanism by which p53 can coordinate with pRb to initiate cell cycle arrest. However, this only begins our understanding of the complex regulation of cellular programs.

Change in RNA expression profiles of WI38 cells expressing both p53 and pRb compared to expression of p53 and pRb alone, identification of cross-talk candidates, and validation by qRT-PCR

In this study, we identified genes that may be regulated by p53 and pRb and compiled two lists of p53 and pRb cross-talk candidates by expressing p53 and/or pRb in WI38 cells. Although p53 has transcriptional repression activity, our microarray analysis did not detect any down-regulated transcripts in the WI38 cells expressing p53 [190, 191]. The deficit of p53 down-regulated transcripts in our microarray analysis compared to previous studies could be due to our method of p53 activation, cell type, or p53 levels, which have previously been found to induce a distinct p53 response with a small set of overlapping genes [192, 193]. Our expression profiling analyses were conducted in normal lung fibroblasts cells instead of cancer epithelial cells. Absence of p53 down-regulated genes in the p53 expressing WI38 cells could also be attributed to the ability of p53 and pRb to alter each other's transcriptional activation or repression functions in normal cells that contain intact pathways. Previous studies that discovered p53 down-regulated targets using expression profiling were done in cancer cells with mutated or null p53 and wild-type *RB1* such as PC-3, HCT116, and H1299 cells [190, 194].

There were 319 upregulated transcripts when p53 and pRb were expressed together compared to 427 and 295 in the WI38 cells expressing pRb and p53 respectively. The change in upregulated genes suggests p53 and pRb can alter one another's ability to regulate gene expression. Management of p53 and pRb processes may require p53 and pRb to regulate gene expression in an opposing manner. Expression of an embryonic development gene, Placenta-specific 1 (PLAC1), has recently been found to be down-regulated by p53 and up-regulated by pRb demonstrating how p53 and pRb can play contrasting roles to regulate cellular processes [81].

pRb is most associated with transcriptional repression of E2F target genes preventing transcription of genes needed for the continuation of the cell cycle [18-20]. However, binding of E2F by pRb is not needed to promote transcription, suppress tumor growth and induce cellular differentiation or senescence [22, 23]. In fact, pRb has been found to act as a co-activator for several transcription factors including Sp-1, RUNX-2, MyoD, and several nuclear receptors (including NR4A1) resulting in cellular differentiation [22, 39]. We found more transcripts that were up-regulated in WI38 cells expressing pRb than downregulated demonstrating its function as a transcription co-activator. There is still a lot not known about pRb regulation, therefore, this study could contribute to the identification of genes up-regulated by pRb and understanding of the function of pRb as a transcriptional co-activator.

Candidates for the p53 and pRb cross-talk pathway were chosen based on whether (1) the transcripts were differentially expressed in both WI38-p53 and WI38-pRb-expressing cells (the common gene set), or (2) only in WI38 cells that simultaneously expressed p53 and pRb (interaction gene set). By focusing on the p53 and pRb common and unique genes, we were able to remove from our analysis genes regulated by p53 or pRb alone. Several of the p53 and pRb

common gene set (RGS16, BTG-2, GDF15, VCAN, D4s234e/NSG1, AKR1B10 and AREG) and interaction gene set (F11R, TNFRSF10C, CERS6, HDM2, SESN1, RBM38 and PMAIP1/NOXA) cross-talk candidates have been previously found to be up-regulated by p53, and this data is in agreement with our microarray results [135, 193, 197-205]. Only a few of the downregulated p53 and pRb cross-talk candidates have previously been found by other studies to be downregulated by p53 (MCM3, BUB1, and CDT1) or pRb individually (VRK1, MCM3, and CDT1) [190, 206-209]. Although several of our p53 and pRb cross-talk candidates have previously been found regulated by p53, regulation of these transcripts by pRb is not known.

Our expression profiling analysis was performed using a normal cell line in order to avoid any mutations that could be present up- or downstream of p53 and pRb that could hinder identification of downstream targets of both genes. Although we expressed p53 and pRb using adenoviruses in normal cells, the fold change of p53 and hypophosphorylated pRb proteins compared to CMV control were equivalent to or less than fold change values in WI38 cells incubated in serum free media to induce quiescence (fold change p53 after 24 hours in serum free media = 5.5) or MCF7 cells undergoing confluence induced cell growth arrest (fold change hypophosphorylated pRb/total pRb = 6.00) [195, 196]. This data suggests the concentration of virus used did not exceed endogenous protein expression of p53 and the active hypophosphorylated form of pRb. However, the use of a normal cell line with wild-type p53 and *RB1* could make it difficult to identify cross-talk molecules due to possible interactions between endogenous and exogenous p53 and pRb. To investigate if exogenous and endogenous p53 and pRb interactions could influence expression profiles expression of RGS16, BCL2L11, BTG-2, IL-6, and STAT4, were measured using qRT-PCR in the p53 null and pRb mutated osteosarcoma cell line SAOS-2. Expression of all transcripts in the p53 and pRb expressing SAOS-2 cells were

found increased with differences in magnitude of expression as they did in our WI38 microarray data and qRT-PCR results. Interestingly, in the microarray data, STAT4 was found to be differentially expressed in WI38 cells expressing p53 and pRb but not in cells expressing both genes. However qRT-PCR analysis found a statistically significant increase in STAT4 expression in WI38 and SAOS-2 cells expressing p53 and pRb. The statistical analyses of expression profiling data or the sensitivity of microarray signal detection could account for the failure to observe differential expression of STAT4 in WI38 cells expressing p53 and pRb.

RGS16 significance and signaling in cancer

RGS16 was of interest to our study for two reasons: 1) RGS16 regulates GPCRs, which are common targets for deregulation in cancer and 2) RGS16 has been linked to regulating the MAPK/RAS, PI3K/AKT, RhoA, and SDF-1/CxCR4 oncogene pathways [133, 135, 141, 164, 171]. Investigations have found that oncogene pathways can feed into one another and bypass or overcome the inhibitory effects of monoclonal antibodies or other targeted inhibitors. For example, in melanoma, increased production of VEGF or increased expression or activation of the platelet-derived growth factor receptor β or insulin like growth factor 1 receptor is associated with resistance to BRAF inhibitors demonstrating mechanisms cancer cells use to overcome single target modalities [239]. Therefore investigation of RGS16, a protein known to modulate several oncogene pathways will aid in understanding mechanisms by which cells alter multiple signaling pathways to prevent carcinogenesis that could be used for future drug development.

We chose to study the function of RGS16 in pancreatic cancer because only 5.7% (1 out of 17) of pancreatic tumors with lymph-node metastases had expression of RGS16 compared to 70.6% (12 out of 17) of pancreatic tumors with non-metastasized pancreatic cancer [149].

Furthermore, decreased expression of RGS16 was associated with poor pancreatic cancer patient survival indicating the potential of RGS16 as a pancreatic cancer prognostic marker [149].

Few reports have been published that describe the impact of RGS16 on cancer cell signaling and progression. Although increased expression of RGS16 has been found in pediatric high hyperdiploid acute lymphoblastic leukemia (ALL) and colon cancer, functional analysis of RGS16 has not been performed to identify any oncogenic function in these cancers [147-149]. Functional and expression analysis of RGS16 has been performed in breast cancers. The RGS16 promoter is located at a site that is vulnerable to allelic imbalances in a subset of breast cancers that can result in promoter methylation of RGS16 in 10% of these cancers [150]. Liang *et al.* (2009) found that RGS16 overexpression in breast cancer cell lines decreased EGF induced proliferation and AKT activation by binding to the p85-alpha subunit of PI3K preventing the phosphorylation of AKT [171]. RGS16 has also been associated in the anti-proliferative effect of retinoic acid in neuroblastoma cells and the cytotoxic effect of histone deacetylase inhibitor Vorinostat in triple negative breast cancers [162, 163]. The current data suggests RGS16 plays a role in cancer signaling, however, more research is needed to delineate the function of RGS16 in cancer cells.

RGS16 and cell migration

RGS16 has been linked with inhibition of cell migration in a canonical (through regulation of GPCR signaling) and non-canonical pathways in normal cells. RGS16 inhibits megakaryocytes and T lymphocyte migration by regulating the activation of the GPCR CxCR4 and decreases T helper type 2 and 17 cell trafficking through regulation of CCR4 and CCR10 chemokine pathways representing the canonical form of RGS signaling [136, 137, 141]. The activation of

RhoA, a small GTPase involved in reorganizing actin cytoskeleton and a mediator of EGF induced invasion of pancreatic cancer cell lines is inhibited in MCF-7 cells by the relocation of Gα13 to the plasma membrane by RGS16 preventing Gα13 mediated activation of RhoA [164, 240]. The regulation of RhoA activation by RGS16 is an example of a non-canonical mechanism used to regulate signaling. These studies show mechanisms by which RGS16 can regulate cell migration. To date, this is the first report demonstrating RGS16 induced inhibition of cancer cell invasion.

The findings from our study suggest RGS16 is regulated by p53 and pRb and functions to inhibit pancreatic cancer cell migration and invasion; however this effect was cell line dependent. PANC-1 cell migration induced by FBS or EGF was not inhibited by RGS16, this could be due to different mutations in PANC-1 compared to the other cell lines that prevent RGS16 inhibition of FBS or EGF induced cell migration. Although not commonly associated with p53 and pRb signaling, regulation of cellular migration and invasion by both tumor suppressors has become evident over the course of the past several years. p53 has been found to regulate cell polarization and migration of cells predominately by inhibiting Rho signaling [257]. p53 also inhibits cancer cell invasion by inhibiting activity or expression of matrix metalloproteinases (MMPs) [258-261]. pRb's role in cell migration has recently come to light. pRb has been implicated as an important factor in regulating neuronal cell migration and was recently found to inhibit CD44 induced collective cell migration of breast cancer cells [68, 262]. pRb is linked to regulating invasion through its ability to bind and inhibit E2F induced transcriptional activation of the MMPs 9, 14, and 15 [67]. Knock-down of E2F1 and E2F3 inhibited migration and invasion of non-small cell lung cancer cells [67]. RGS16 may be another mechanism employed to regulate cell migration and invasion by p53 and pRb.

Future studies and conclusions

This is the first report of regulation of RGS16 pRb and RGS16-mediated inhibition of EGF-induced migration and invasion in normal and cancer cells. This study focused on examining migration and invasion mediated by the EGF/EGFR pathway. However, a single RGS protein can interact and regulate signaling of multiple pathways ([134, 172]). Future studies are needed to determine if RGS16 can inhibit cell migration and invasion through other pathways such as the SDF-1/CxCR4 pathway which is deregulated in pancreatic cancer ([222]).

By utilizing microarray expression profiling, we have 1) identified p53 and pRb regulated candidates or genes involved in coordinating cancer suppression processes and determining cell fate, 2) and identified a possible role for the cross-talk candidate RGS16 in inhibiting pancreatic cancer cell migration and invasion. Our study suggests that the loss of RGS16 promotes pancreatic cancer metastasis by removing the inhibitory function of RGS16 on cell migration and invasion. Our study further supports the use of RGS16 as a prognostic marker for predicting pancreatic cancer metastasis previously described by Kim *et a.l* that can be used to asses eligibility of patient for surgery [149]. By investigating the p53 and pRb cross-talk and the role of RGS16 in pancreatic cancer cell migration, we have uncovered a novel regulator of metastatic processes that could be a future target in developing treatments for late stage pancreatic cancer.

Materials and Methods

Cell culture and virus transductions

The human lung fibroblast WI38 cell line, osteosarcoma cell line SAOS-2 (p53 null and truncated *RB1*), and the pancreatic cancer cell lines, BxPC-3, AsPC-1, MIA PaCa-2, and PANC-1 were purchased from the American Type Culture Collection (Manassas, VA, USA). WI38

cells were grown in Hyclone MEM/EBSS (ThermoFisher Scientific, Waltham, MA) media supplemented with 10% research grade fetal bovine serum (FBS) (PAA Laboratories, Dartmouth, MA) and 1% Penicillin Streptomycin (Corning, Corning, NY) and SAOS-2, MIA PaCa-2, and PANC-1 cells were grown in Hyclone High Glucose DMEM (ThermoFisher Scientific, Waltham, MA) supplemented with 10% FBS and 1% Penicillin Streptomycin. BxPC-3 and AsPC-1 were cultured in RPMI supplemented with 10% or 15% FBS (respectively) and 1% Penicillin Streptomycin. Cells were cultured at 37°C in a humidified 5% CO_2 incubator.

Ad.CMV (adenovirus with CMV promoter) and Ad.CMV.p53 (Adenovirus containing wild-type p53 gene under control of CMV promoter) viral vectors were generated using the AdEasy system (Carlsbad, CA). The Ad.CMV.pRb (Adenovirus containing *RB1 gene* cDNA under control of CMV promoter) vector was provided by Dr. Juan Fueyo (M.D. Anderson Cancer Center, The University of Texas). The Ad.GFP and Ad.GFP.RGS16 viruses were purchased from Vector Biolabs (Philadelphia, PA). Viruses were amplified and tittered as previously described [183-185].

Microarray expression profiling

For expression profiling, WI38 cells were transduced with each of the following vectors or vector combination: (1) adenovirus vector with no insert (Adenoviral CMV-vector ctrl), (2) Ad.CMV.p53, (3) Ad.CMV.pRb, and (4) both Ad.CMV.p53 and Ad.CMV.pRb. Vectors were added at a multiplicity of infection (MOI) of 50 to 80% confluent WI38 cells in MEM/EBSS supplemented with 2% heat-inactivated FBS. Culture media were replaced with 10% FBS and 1% Penicillin/Streptomycin supplemented MEM/EBSS medium 16 hours after vector addition; cells were collected after 48 hours. Four biological replicates were performed for each of the

four expression studies. Immunoblots were used to verify increased expression of p53 and/or pRb in the WI38 samples prior to microarray analysis.

Total RNA was isolated from transduced WI38 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according the manufacturer's protocol. Using a universal reference design, two RNAs (transduced WI38 cells + Agilent (Santa Clara, CA) human universal reference RNA) were hybridized to Agilent 44K whole human genome expression arrays. Total RNAs were labeled with either cyanine (Cy)-3-CTP and Cy5-CTP (Perkin Elmer, Waltham, MA) using Agilent QuickAmp cRNA labeling kits. Following purification, Cy3- and Cy5-labeled cRNAs were combined and hybridized for 17 hours at 65ºC in an Agilent hybridization oven. Microarrays were then washed and scanned using Agilent DNA Microarray Scanner.

Statistical Analysis of Expression Profiling Data

Lowess-normalized feature intensities were extracted from the scanned image using Feature Extraction (Agilent). These data were exported as tab-delimited files (one file per sample) to Microsoft Excel for filtering. For each feature, data were removed if both channels reported values not well-above background according to default Feature Extraction Criteria. For each comparison, log base-2 ratios of each sample to universal reference RNA were collated into a single table. Features for which fewer than 50% of all samples had a present value were removed from further analysis.

The resulting tables were imported into Multiple Experiment Viewer (MEV) v4.3. Log base 2 ratios were compared between each of three sample sets (p53 expressed samples. *RB1* expressed samples and p53 and *RB1* coexpressed samples) and the adenovirus vector control samples by Significance Analysis of Microarrays [186]. We used a conservative threshold

whereby only genes for which MEV reported a false discovery rate of 0% were considered significantly differentially expressed.

Data extracted using Feature Extraction was uploaded to the NCBI's Gene Expression Omnibus (GEO) public database and is available via access number GSE59660.

Real-time PCR analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit from Applied Biosystems (Foster City, CA) according to the manufacturer's protocol. Real-Time PCR was performed using the Applied Biosystems TaqMan Gene Expression Assays in the ABI 7000 detection system. TaqMan probes were purchased from Applied Biosystems (Foster City, CA) IL-6 (HS00197982_m1), BCL2L11 (BCL2L11) (HS00197982_m1), RGS16 (HS00892674_m1), BTG2 (HS00198887), STAT4 (HS00231372_ml) and GAPDH (HS02758991). Human pancreatic total RNA used for comparing the expression of RGS16 mRNA was purchased from Agilent Technologies (Cedar Creek, TX). The relative fold change for each marker was calculated using the $2^{-\Delta\Delta CT}$ analysis according to Livak *et.al* and statistical significance was determined using a one way ANOVA with a Dunnett's or Tukey (pancreatic cancer cell lines) post-hoc test, using Prism V6.0c (GraphPad Software, Inc., La Jolla, CA) *[187]*.

Western blot analysis

WI38 or Saos-2 cells were lysed in whole cell lysis buffer containing 50mM TRIS (pH7.4), 5mM EDTA 250mM NACL, 50mM NaF, 0.1mM Na_3VO_4, 0.1% Triton X-100 and protease inhibitors (Pierce Protease inhibitor Tablets 88661; Thermo Scientific, Rockford, IL). Protein extracts (50ug) were loaded onto 8% polyacrylamide gels and proteins were separated using

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Blots were blocked 1 hour in 5% dry non-fat milk diluted in Tris-buffered saline solution containing 0.1% Tween-20 (TBS-T). Membranes were probed overnight at 4°C with mouse anti-p53 (SC-DO1, 1: 1000) or mouse anti-pRb (SC-IF8, 1:500) antibodies from Santa Cruz Biotechnology (Dallas, TX). Following primary antibody incubation the membranes were washed and probed with Horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:5000) secondary antibodies (Rockland, Gilbertsville, PA) for 1 hour at room-temperature. Primary and Secondary antibodies were diluted in TBS-T. Blots were washed 5 minutes in TBS-T three times and Amersham ECL prime western blotting detection reagent was added in order visualize the protein bands (RPN 2232, GE Life Sciences, Pittsburgh, PA). Western blot images were captured using FOTODYNE FOTO/Analyst FX (Hartland, WI) imaging camera. Membranes were normalized using mouse anti-actin (1:1000). Densitometry was performed using TotalLab Quant software (TotalLab Ltd, UK).

Wound healing Assay

Pancreatic cancer cells (BxPC-3, AsPC-1 and PANC-1) were placed in a 6 well plate at approximately 70% confluency. The following day, 50 Multiplicity of Infection (MOIs) of Ad.GFP (control) or Ad.GFP.RGS16 were added to the cells in media containing 2% heat-inactivated FBS for 24 hours. The media was changed to complete media (10% FBS for BxPC-3 and PANC-1 or 15% for AsPC-1) for 24hrs. 48 hours after the addition of the virus the media was changed from complete media to media supplemented with 0.5% FBS and 1% P/S for 24hours. Three wounds or scratches were made per well using a p200 pipette tip in PBS. The cells were washed three times with PBS and incubated for 16-24 hours in complete media or

media supplemented with 100ng/ml of EGF. FBS or EGF was added to induce cell migration at a concentration previously described in [228-230]. Wound widths were measured and images taken at 0, 16, or 24 hrs after addition of media supplemented with FBS or EGF at 100x magnification using an Olympus DP71 microscope (Center Valley, PA). Efficacy of virus transduction was confirmed using fluorescent microscopy to examine GFP expression prior to the start of the experiment. Percent wound healing was determined using the following equation; % wound healing = ([initial scratch width _ final scratch width]/ initial scratch width)*100. Three replicates were performed for each cell line.

Invasion Assay

BD Bio Coat Matrigel Invasion chambers (Bedford, MA) containing membrane with 8um pores were used to assess the role of RGS16 to inhibit pancreatic cancer cell migration and invasion. BxPC-3 cells were plated into 6-well dish, 24 hours later 50 MOIs of Ad.GFP or Ad.GFP.RGS16 virus were added to the cells followed by 24 hour incubation in complete media and 24 hours in low-serum media as described in the wound healing section. Chambers were re-hydrated in RPMI containing 1% P/S and 0.1 % BSA for 2 hours at 37°C. BxPC-3 and AsPC-1 cells were collected and 25 x 10^4 cells were added to the top of the chambers in RPMI supplemented with 1% P/S and 0.1%BSA. RPMI supplemented with 100ng/ml EGF, 1% P/S, 0.1% BSA was added to lower portion and the chambers were incubated for 18 (AsPC-1) or 20 (BxPC-3) hours at 37°C. The non-migrating cells were removed using a cotton swab and the invaded cells were fixed using 100% methanol (MeOH) for 5 minutes and stained using 0.5% crystal violet plus 20% MeOH (10-15 mins). Invaded cells were counted using 200x magnification with 12 different views. Percentage of invasion compared to GFP control was

calculated for each cell line [(# of invaded $cells_{treated}$ / # of invaded $cell_{control}$) *100]. Three replicates were performed for each cell line.

Statistical Analysis

Statistical significance for the wound healing and invasion assays was calculated using Student's *t*-test using Prism V6.0c (GraphPad Software, Inc., La Jolla, CA). Statistical Analysis tests used for expression profiling and qRT-PCR analyses are listed in their respective sections.

Abbreviations

qRT-PCR: quantitative real-time PCR; pRb: retinoblastoma protein; *RB1*: retinoblastoma gene; CMV: Cytomegalovirus; MOI: multiplicity of infection; RGS16: regulator of G protein signaling 16; EGF: epidermal growth factor; EGFR; epidermal growth factor receptor; GPCR: G protein coupled receptor.

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Competing interests

The authors declare that they have no competing interests.

References

References

Uncategorized References

1. Friend, S.H., et al., *A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma.* Nature, 1986. **323**(6089): p. 643-6.
2. McClendon, A.K., et al., *RB and p53 cooperate to prevent liver tumorigenesis in response to tissue damage.* Gastroenterology, 2011. **141**(4): p. 1439-50.
3. Sherr, C.J. and F. McCormick, *The RB and p53 pathways in cancer.* Cancer Cell, 2002. **2**(2): p. 103-12.
4. Williams, B.O., et al., *Tumorigenic and developmental effects of combined germ-line mutations in Rb and p53.* Cold Spring Harb Symp Quant Biol, 1994. **59**: p. 449-57.
5. Zhou, Z., et al., *Synergy of p53 and Rb deficiency in a conditional mouse model for metastatic prostate cancer.* Cancer Res, 2006. **66**(16): p. 7889-98.
6. Amaral, J.D., et al., *The role of p53 in apoptosis.* Discov Med, 2010. **9**(45): p. 145-52.
7. Vogiatzi, P., et al., *The limitless role of p53 in cell cycle machinery: good news or bad news?* Cancer Biol Ther, 2006. **5**(9): p. 1090-3.
8. Agarwal, M.L., et al., *The p53 network.* J Biol Chem, 1998. **273**(1): p. 1-4.
9. Rufini, A., et al., *Senescence and aging: the critical roles of p53.* Oncogene, 2013. **32**(43): p. 5129-43.
10. Sengupta, S. and C.C. Harris, *p53: traffic cop at the crossroads of DNA repair and recombination.* Nat Rev Mol Cell Biol, 2005. **6**(1): p. 44-55.
11. Lane, D.P., *Cancer. p53, guardian of the genome.* Nature, 1992. **358**(6381): p. 15-6.
12. Beckerman, R. and C. Prives, *Transcriptional regulation by p53.* Cold Spring Harb Perspect Biol, 2010. **2**(8): p. a000935.
13. Speidel, D., *Transcription-independent p53 apoptosis: an alternative route to death.* Trends Cell Biol, 2010. **20**(1): p. 14-24.
14. Shu, K.X., B. Li, and L.X. Wu, *The p53 network: p53 and its downstream genes.* Colloids Surf B Biointerfaces, 2007. **55**(1): p. 10-8.
15. Wei, C.L., et al., *A global map of p53 transcription-factor binding sites in the human genome.* Cell, 2006. **124**(1): p. 207-19.
16. Qin, H., et al., *Regulation of apoptosis and differentiation by p53 in human embryonic stem cells.* J Biol Chem, 2007. **282**(8): p. 5842-52.
17. Porrello, A., et al., *p53 regulates myogenesis by triggering the differentiation activity of pRb.* J Cell Biol, 2000. **151**(6): p. 1295-304.
18. Chen, H.Z., S.Y. Tsai, and G. Leone, *Emerging roles of E2Fs in cancer: an exit from cell cycle control.* Nat Rev Cancer, 2009. **9**(11): p. 785-97.
19. Claudio, P.P., T. Tonini, and A. Giordano, *The retinoblastoma family: twins or distant cousins?* Genome Biol, 2002. **3**(9): p. reviews3012.
20. Genovese, C., et al., *Cell cycle control and beyond: emerging roles for the retinoblastoma gene family.* Oncogene, 2006. **25**(38): p. 5201-9.

21. Claudio, P.P., et al., *Functional analysis of pRb2/p130 interaction with cyclins.* Cancer Res, 1996. **56**(9): p. 2003-8.
22. Sellers, W.R., et al., *Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth.* Genes Dev, 1998. **12**(1): p. 95-106.
23. Sun, H., et al., *E2f binding-deficient Rb1 protein suppresses prostate tumor progression in vivo.* Proc Natl Acad Sci U S A, 2011. **108**(2): p. 704-9.
24. Hilgendorf, K.I., et al., *The retinoblastoma protein induces apoptosis directly at the mitochondria.* Genes Dev, 2013. **27**(9): p. 1003-15.
25. Ianari, A., et al., *Proapoptotic function of the retinoblastoma tumor suppressor protein.* Cancer Cell, 2009. **15**(3): p. 184-94.
26. Sun, B., et al., *Absence of pRb facilitates E2F1-induced apoptosis in breast cancer cells.* Cell Cycle, 2010. **9**(6): p. 1122-30.
27. Haupt, Y., S. Rowan, and M. Oren, *p53-mediated apoptosis in HeLa cells can be overcome by excess pRB.* Oncogene, 1995. **10**(8): p. 1563-71.
28. Gu, W., et al., *Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation.* Cell, 1993. **72**(3): p. 309-24.
29. Chen, P.L., et al., *Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs.* Genes Dev, 1996. **10**(21): p. 2794-804.
30. Chen, P.L., et al., *Retinoblastoma protein directly interacts with and activates the transcription factor NF-IL6.* Proc Natl Acad Sci U S A, 1996. **93**(1): p. 465-9.
31. Nead, M.A., et al., *Rb binds c-Jun and activates transcription.* EMBO J, 1998. **17**(8): p. 2342-52.
32. Batsche, E., et al., *RB and c-Myc activate expression of the E-cadherin gene in epithelial cells through interaction with transcription factor AP-2.* Mol Cell Biol, 1998. **18**(7): p. 3647-58.
33. Decary, S., et al., *The retinoblastoma protein binds the promoter of the survival gene bcl-2 and regulates its transcription in epithelial cells through transcription factor AP-2.* Mol Cell Biol, 2002. **22**(22): p. 7877-88.
34. Lee, J.S., et al., *HES1 cooperates with pRb to activate RUNX2-dependent transcription.* J Bone Miner Res, 2006. **21**(6): p. 921-33.
35. Thomas, D.M., et al., *The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation.* Mol Cell, 2001. **8**(2): p. 303-16.
36. Udvadia, A.J., et al., *Sp-1 binds promoter elements regulated by the RB protein and Sp-1-mediated transcription is stimulated by RB coexpression.* Proc Natl Acad Sci U S A, 1993. **90**(8): p. 3265-9.
37. Guo, C.S., et al., *Regulation of MyoD activity and muscle cell differentiation by MDM2, pRb, and Sp1.* J Biol Chem, 2003. **278**(25): p. 22615-22.
38. Balasenthil, S. and R.K. Vadlamudi, *Functional interactions between the estrogen receptor coactivator PELP1/MNAR and retinoblastoma protein.* J Biol Chem, 2003. **278**(24): p. 22119-27.
39. Batsche, E., et al., *Rb enhances p160/SRC coactivator-dependent activity of nuclear receptors and hormone responsiveness.* J Biol Chem, 2005. **280**(20): p. 19746-56.

40. Batsche, E., et al., *Retinoblastoma and the related pocket protein p107 act as coactivators of NeuroD1 to enhance gene transcription.* J Biol Chem, 2005. **280**(16): p. 16088-95.
41. Lu, J. and M. Danielsen, *Differential regulation of androgen and glucocorticoid receptors by retinoblastoma protein.* J Biol Chem, 1998. **273**(47): p. 31528-33.
42. Martens, C., et al., *Protein-protein interactions and transcriptional antagonism between the subfamily of NGFI-B/Nur77 orphan nuclear receptors and glucocorticoid receptor.* Mol Endocrinol, 2005. **19**(4): p. 885-97.
43. Singh, P., S.W. Chan, and W. Hong, *Retinoblastoma protein is functionally distinct from its homologues in affecting glucocorticoid receptor-mediated transcription and apoptosis.* J Biol Chem, 2001. **276**(17): p. 13762-70.
44. Singh, P., J. Coe, and W. Hong, *A role for retinoblastoma protein in potentiating transcriptional activation by the glucocorticoid receptor.* Nature, 1995. **374**(6522): p. 562-5.
45. Cordon-Cardo, C., et al., *Cooperative effects of p53 and pRB alterations in primary superficial bladder tumors.* Cancer Res, 1997. **57**(7): p. 1217-21.
46. Toguchida, J. and T. Nakayama, *Molecular genetics of sarcomas: applications to diagnoses and therapy.* Cancer Sci, 2009. **100**(9): p. 1573-80.
47. Yap, D.B., et al., *mdm2: a bridge over the two tumour suppressors, p53 and Rb.* Oncogene, 1999. **18**(53): p. 7681-9.
48. Gazzeri, S., et al., *Mechanisms of p16INK4A inactivation in non small-cell lung cancers.* Oncogene, 1998. **16**(4): p. 497-504.
49. Mathew, R., et al., *Alterations in p53 and pRb pathways and their prognostic significance in oesophageal cancer.* Eur J Cancer, 2002. **38**(6): p. 832-41.
50. Ueki, K., et al., *CDKN2/p16 or RB Alterations Occur in the Majority of Glioblastomas and Are Inversely Correlated.* Cancer Research, 1996. **56**(1): p. 150-153.
51. Caldas, C., et al., *Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma.* Nat Genet, 1994. **8**(1): p. 27-32.
52. Laurie, N.A., et al., *Inactivation of the p53 pathway in retinoblastoma.* Nature, 2006. **444**(7115): p. 61-6.
53. Momand, J., et al., *The MDM2 gene amplification database.* Nucleic Acids Res, 1998. **26**(15): p. 3453-9.
54. Drobnjak, M., et al., *Overexpression of Cyclin D1 Is Associated with Metastatic Prostate Cancer to Bone.* Clinical Cancer Research, 2000. **6**(5): p. 1891-1895.
55. Gansauge, S., et al., *Overexpression of Cyclin D1 in Human Pancreatic Carcinoma Is Associated with Poor Prognosis.* Cancer Research, 1997. **57**(9): p. 1634-1637.
56. Gillett, C., et al., *Amplification and Overexpression of Cyclin D1 in Breast Cancer Detected by Immunohistochemical Staining.* Cancer Research, 1994. **54**(7): p. 1812-1817.
57. Michalides, R., et al., *Overexpression of Cyclin D1 Correlates with Recurrence in a Group of Forty-seven Operable Squamous Cell Carcinomas of the Head and Neck.* Cancer Research, 1995. **55**(5): p. 975-978.
58. Levine, A.J., *The common mechanisms of transformation by the small DNA tumor viruses: The inactivation of tumor suppressor gene products: p53.* Virology, 2009. **384**(2): p. 285-93.
59. Bakhoum, S.F. and D.A. Compton, *Chromosomal instability and cancer: a complex relationship with therapeutic potential.* J Clin Invest, 2012. **122**(4): p. 1138-43.

60. Manning, A.L., C. Benes, and N.J. Dyson, *Whole chromosome instability resulting from the synergistic effects of pRB and p53 inactivation.* Oncogene, 2013.
61. Derenzini, M., et al., *The p53-mediated sensitivity of cancer cells to chemotherapeutic agents is conditioned by the status of the retinoblastoma protein.* J Pathol, 2009. **219**(3): p. 373-82.
62. Yague, E., et al., *Ability to acquire drug resistance arises early during the tumorigenesis process.* Cancer Res, 2007. **67**(3): p. 1130-7.
63. Choi, J., et al., *Local mesenchymal stem/progenitor cells are a preferential target for initiation of adult soft tissue sarcomas associated with p53 and Rb deficiency.* Am J Pathol, 2010. **177**(5): p. 2645-58.
64. Rubio, R., et al., *The differentiation stage of p53-Rb-deficient bone marrow mesenchymal stem cells imposes the phenotype of in vivo sarcoma development.* Oncogene, 2013. **32**(41): p. 4970-80.
65. Meuwissen, R., et al., *Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model.* Cancer Cell, 2003. **4**(3): p. 181-9.
66. Simin, K., et al., *pRb inactivation in mammary cells reveals common mechanisms for tumor initiation and progression in divergent epithelia.* PLoS Biol, 2004. **2**(2): p. E22.
67. Johnson, J.L., et al., *Regulation of matrix metalloproteinase genes by E2F transcription factors: Rb-Raf-1 interaction as a novel target for metastatic disease.* Cancer Res, 2012. **72**(2): p. 516-26.
68. Kim, K.J., et al., *Rb suppresses collective invasion, circulation and metastasis of breast cancer cells in CD44-dependent manner.* PLoS One, 2013. **8**(12): p. e80590.
69. Hui, A.M., et al., *Over-expression and lack of retinoblastoma protein are associated with tumor progression and metastasis in hepatocellular carcinoma.* Int J Cancer, 1999. **84**(6): p. 604-8.
70. Powell, E., D. Piwnica-Worms, and H. Piwnica-Worms, *Contribution of p53 to metastasis.* Cancer Discov, 2014. **4**(4): p. 405-14.
71. Voorhoeve, P.M. and R. Agami, *The tumor-suppressive functions of the human INK4A locus.* Cancer Cell, 2003. **4**(4): p. 311-9.
72. Lim, I.K., *TIS21 (/BTG2/PC3) as a link between ageing and cancer: cell cycle regulator and endogenous cell death molecule.* J Cancer Res Clin Oncol, 2006. **132**(7): p. 417-26.
73. Vattemi, E. and P.P. Claudio, *Tumor suppressor genes as cancer therapeutics.* Drug News Perspect, 2007. **20**(8): p. 511-20.
74. Polager, S. and D. Ginsberg, *p53 and E2f: partners in life and death.* Nat Rev Cancer, 2009. **9**(10): p. 738-48.
75. Qin, X.Q., et al., *Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis.* Proc Natl Acad Sci U S A, 1994. **91**(23): p. 10918-22.
76. Rogoff, H.A., et al., *E2F1 induces phosphorylation of p53 that is coincident with p53 accumulation and apoptosis.* Mol Cell Biol, 2002. **22**(15): p. 5308-18.
77. Rogoff, H.A., et al., *Apoptosis associated with deregulated E2F activity is dependent on E2F1 and Atm/Nbs1/Chk2.* Mol Cell Biol, 2004. **24**(7): p. 2968-77.
78. Wu, X. and A.J. Levine, *p53 and E2F-1 cooperate to mediate apoptosis.* Proc Natl Acad Sci U S A, 1994. **91**(9): p. 3602-6.
79. Hsieh, J.K., et al., *RB regulates the stability and the apoptotic function of p53 via MDM2.* Mol Cell, 1999. **3**(2): p. 181-93.

80. Felton-Edkins, Z.A., et al., *Direct regulation of RNA polymerase III transcription by RB, p53 and c-Myc.* Cell Cycle, 2003. **2**(3): p. 181-4.
81. Chen, Y., D. Schlessinger, and R. Nagaraja, *T antigen transformation reveals Tp53/RB-dependent route to PLAC1 transcription activation in primary fibroblasts.* Oncogenesis, 2013. **2**: p. e67.
82. Jemal, A., et al., *Global cancer statistics.* CA Cancer J Clin, 2011. **61**(2): p. 69-90.
83. Siegel, R., et al., *Cancer statistics, 2014.* CA Cancer J Clin, 2014. **64**(1): p. 9-29.
84. Mian, O.Y., et al., *Management options in locally advanced pancreatic cancer.* Curr Oncol Rep, 2014. **16**(6): p. 388.
85. Hidalgo, M., *Pancreatic cancer.* N Engl J Med, 2010. **362**(17): p. 1605-17.
86. Hezel, A.F., et al., *Genetics and biology of pancreatic ductal adenocarcinoma.* Genes Dev, 2006. **20**(10): p. 1218-49.
87. Bartsch, D.K., et al., *Prevalence of familial pancreatic cancer in Germany.* Int J Cancer, 2004. **110**(6): p. 902-6.
88. Fernandez, E., et al., *Family history and the risk of liver, gallbladder, and pancreatic cancer.* Cancer Epidemiol Biomarkers Prev, 1994. **3**(3): p. 209-12.
89. Klein, A.P., et al., *Familial pancreatic cancer.* Cancer J, 2001. **7**(4): p. 266-73.
90. Lee, E.S. and J.M. Lee, *Imaging diagnosis of pancreatic cancer: a state-of-the-art review.* World J Gastroenterol, 2014. **20**(24): p. 7864-77.
91. Schima, W., et al., *Pancreatic adenocarcinoma.* Eur Radiol, 2007. **17**(3): p. 638-49.
92. Geer, R.J. and M.F. Brennan, *Prognostic indicators for survival after resection of pancreatic adenocarcinoma.* Am J Surg, 1993. **165**(1): p. 68-72; discussion 72-3.
93. Roland, C.L., et al., *Neoadjuvant Therapy is Associated with a Reduced Lymph Node Ratio in Patients with Potentially Resectable Pancreatic Cancer.* Ann Surg Oncol, 2014.
94. Schober, M., et al., *Desmoplasia and chemoresistance in pancreatic cancer.* Cancers (Basel), 2014. **6**(4): p. 2137-54.
95. Jones, S., et al., *Core signaling pathways in human pancreatic cancers revealed by global genomic analyses.* Science, 2008. **321**(5897): p. 1801-6.
96. Eser, S., et al., *Oncogenic KRAS signalling in pancreatic cancer.* Br J Cancer, 2014. **111**(5): p. 817-22.
97. Hingorani, S.R., et al., *Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse.* Cancer Cell, 2003. **4**(6): p. 437-50.
98. Eser, S., et al., *Selective requirement of PI3K/PDK1 signaling for Kras oncogene-driven pancreatic cell plasticity and cancer.* Cancer Cell, 2013. **23**(3): p. 406-20.
99. Collisson, E.A., et al., *A central role for RAF-->MEK-->ERK signaling in the genesis of pancreatic ductal adenocarcinoma.* Cancer Discov, 2012. **2**(8): p. 685-93.
100. Lim, K.H., et al., *Divergent roles for RalA and RalB in malignant growth of human pancreatic carcinoma cells.* Curr Biol, 2006. **16**(24): p. 2385-94.
101. McCleary-Wheeler, A.L., R. McWilliams, and M.E. Fernandez-Zapico, *Aberrant signaling pathways in pancreatic cancer: a two compartment view.* Mol Carcinog, 2012. **51**(1): p. 25-39.
102. Aguirre, A.J., et al., *Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma.* Genes Dev, 2003. **17**(24): p. 3112-26.
103. Morton, J.P., et al., *Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer.* Proc Natl Acad Sci U S A, 2010. **107**(1): p. 246-51.

104. Hingorani, S.R., et al., *Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice.* Cancer Cell, 2005. **7**(5): p. 469-83.
105. Levy, L. and C.S. Hill, *Alterations in components of the TGF-beta superfamily signaling pathways in human cancer.* Cytokine Growth Factor Rev, 2006. **17**(1-2): p. 41-58.
106. Kalluri, R. and R.A. Weinberg, *The basics of epithelial-mesenchymal transition.* J Clin Invest, 2009. **119**(6): p. 1420-8.
107. Chen, Y.W., et al., *SMAD4 loss triggers the phenotypic changes of pancreatic ductal adenocarcinoma cells.* BMC Cancer, 2014. **14**: p. 181.
108. Suwa, H., et al., *Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas.* Br J Cancer, 1998. **77**(1): p. 147-52.
109. Cheng, J.Q., et al., *Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA.* Proc Natl Acad Sci U S A, 1996. **93**(8): p. 3636-41.
110. Oliveira-Cunha, M., W.G. Newman, and A.K. Siriwardena, *Epidermal growth factor receptor in pancreatic cancer.* Cancers (Basel), 2011. **3**(2): p. 1513-26.
111. Barton, C.M., et al., *Abnormalities of the p53 tumour suppressor gene in human pancreatic cancer.* Br J Cancer, 1991. **64**(6): p. 1076-82.
112. Rozenblum, E., et al., *Tumor-suppressive pathways in pancreatic carcinoma.* Cancer Res, 1997. **57**(9): p. 1731-4.
113. Normanno, N., et al., *Epidermal growth factor receptor (EGFR) signaling in cancer.* Gene, 2006. **366**(1): p. 2-16.
114. Roskoski, R., Jr., *The ErbB/HER family of protein-tyrosine kinases and cancer.* Pharmacol Res, 2014. **79**: p. 34-74.
115. Ardito, C.M., et al., *EGF receptor is required for KRAS-induced pancreatic tumorigenesis.* Cancer Cell, 2012. **22**(3): p. 304-17.
116. Chang, Z.G., et al., *Suppression of the epidermal growth factor receptor inhibits epithelial-mesenchymal transition in human pancreatic cancer PANC-1 cells.* Dig Dis Sci, 2012. **57**(5): p. 1181-9.
117. Kassis, J., et al., *A role for phospholipase C-gamma-mediated signaling in tumor cell invasion.* Clin Cancer Res, 1999. **5**(8): p. 2251-60.
118. Price, J.T., et al., *Epidermal growth factor promotes MDA-MB-231 breast cancer cell migration through a phosphatidylinositol 3'-kinase and phospholipase C-dependent mechanism.* Cancer Res, 1999. **59**(21): p. 5475-8.
119. Dhillon, A.S., et al., *MAP kinase signalling pathways in cancer.* Oncogene, 2007. **26**(22): p. 3279-90.
120. Kim, D., et al., *AKT/PKB signaling mechanisms in cancer and chemoresistance.* Front Biosci, 2005. **10**: p. 975-87.
121. Schiller, M.R., *Coupling receptor tyrosine kinases to Rho GTPases--GEFs what's the link.* Cell Signal, 2006. **18**(11): p. 1834-43.
122. Tu, S., et al., *Epidermal growth factor-dependent regulation of Cdc42 is mediated by the Src tyrosine kinase.* J Biol Chem, 2003. **278**(49): p. 49293-300.
123. Bos, J.L., *ras oncogenes in human cancer: a review.* Cancer Res, 1989. **49**(17): p. 4682-9.
124. Chaffer, C.L. and R.A. Weinberg, *A perspective on cancer cell metastasis.* Science, 2011. **331**(6024): p. 1559-64.

125. Thiery, J.P., *Epithelial-mesenchymal transitions in tumour progression.* Nat Rev Cancer, 2002. **2**(6): p. 442-54.
126. Yilmaz, M. and G. Christofori, *EMT, the cytoskeleton, and cancer cell invasion.* Cancer Metastasis Rev, 2009. **28**(1-2): p. 15-33.
127. Friedl, P. and K. Wolf, *Tumour-cell invasion and migration: diversity and escape mechanisms.* Nat Rev Cancer, 2003. **3**(5): p. 362-74.
128. Mehlen, P. and A. Puisieux, *Metastasis: a question of life or death.* Nat Rev Cancer, 2006. **6**(6): p. 449-58.
129. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
130. Benelli, R., et al., *Cytokines and chemokines as regulators of angiogenesis in health and disease.* Curr Pharm Des, 2006. **12**(24): p. 3101-15.
131. Vega, F.M. and A.J. Ridley, *Rho GTPases in cancer cell biology.* FEBS Lett, 2008. **582**(14): p. 2093-101.
132. Willars, G.B., *Mammalian RGS proteins: multifunctional regulators of cellular signalling.* Semin Cell Dev Biol, 2006. **17**(3): p. 363-76.
133. Dorsam, R.T. and J.S. Gutkind, *G-protein-coupled receptors and cancer.* Nat Rev Cancer, 2007. **7**(2): p. 79-94.
134. De Vries, L., et al., *The regulator of G protein signaling family.* Annu Rev Pharmacol Toxicol, 2000. **40**: p. 235-71.
135. Buckbinder, L., et al., *The p53 tumor suppressor targets a novel regulator of G protein signaling.* Proc Natl Acad Sci U S A, 1997. **94**(15): p. 7868-72.
136. Shankar, S.P., et al., *RGS16 attenuates pulmonary Th2/Th17 inflammatory responses.* J Immunol, 2012. **188**(12): p. 6347-56.
137. Lippert, E., et al., *Role of regulator of G protein signaling 16 in inflammation-induced T lymphocyte migration and activation.* J Immunol, 2003. **171**(3): p. 1542-55.
138. Xie, S., et al., *IL-17 activates the canonical NF-kappaB signaling pathway in autoimmune B cells of BXD2 mice to upregulate the expression of regulators of G-protein signaling 16.* J Immunol, 2010. **184**(5): p. 2289-96.
139. Huang, J., et al., *Feeding and fasting controls liver expression of a regulator of G protein signaling (Rgs16) in periportal hepatocytes.* Comp Hepatol, 2006. **5**: p. 8.
140. Villasenor, A., et al., *Rgs16 and Rgs8 in embryonic endocrine pancreas and mouse models of diabetes.* Dis Model Mech, 2010. **3**(9-10): p. 567-80.
141. Berthebaud, M., et al., *RGS16 is a negative regulator of SDF-1-CXCR4 signaling in megakaryocytes.* Blood, 2005. **106**(9): p. 2962-8.
142. Hayasaka, N., et al., *Attenuated food anticipatory activity and abnormal circadian locomotor rhythms in Rgs16 knockdown mice.* PLoS One, 2011. **6**(3): p. e17655.
143. Snow, B.E., et al., *Cloning of a retinally abundant regulator of G-protein signaling (RGS-r/RGS16): genomic structure and chromosomal localization of the human gene.* Gene, 1998. **206**(2): p. 247-53.
144. Patten, M., et al., *Interleukin-1beta mediates endotoxin- and tumor necrosis factor alpha-induced RGS16 protein expression in cultured cardiac myocytes.* Naunyn Schmiedebergs Arch Pharmacol, 2003. **368**(5): p. 360-5.
145. Stuebe, S., et al., *Sphingosine-1-phosphate and endothelin-1 induce the expression of rgs16 protein in cardiac myocytes by transcriptional activation of the rgs16 gene.* Naunyn Schmiedebergs Arch Pharmacol, 2008. **376**(5): p. 363-73.

146. Vasiljevic, A., et al., *Molecular characterization of central neurocytomas: potential markers for tumor typing and progression.* Neuropathology, 2013. **33**(2): p. 149-61.
147. Davidsson, J., et al., *Tiling resolution array comparative genomic hybridization, expression and methylation analyses of dup(1q) in Burkitt lymphomas and pediatric high hyperdiploid acute lymphoblastic leukemias reveal clustered near-centromeric breakpoints and overexpression of genes in 1q22-32.3.* Hum Mol Genet, 2007. **16**(18): p. 2215-25.
148. Miyoshi, N., et al., *RGS16 is a marker for prognosis in colorectal cancer.* Ann Surg Oncol, 2009. **16**(12): p. 3507-14.
149. Kim, J.H., et al., *RGS16 and FosB underexpressed in pancreatic cancer with lymph node metastasis promote tumor progression.* Tumour Biol, 2010. **31**(5): p. 541-8.
150. Wiechec, E., J. Overgaard, and L.L. Hansen, *A fragile site within the HPC1 region at 1q25.3 affecting RGS16, RGSL1, and RGSL2 in human breast carcinomas.* Genes Chromosomes Cancer, 2008. **47**(9): p. 766-80.
151. Fong, C.W., et al., *Specific induction of RGS16 (regulator of G-protein signalling 16) mRNA by protein kinase C in CEM leukaemia cells is mediated via tumour necrosis factor alpha in a calcium-sensitive manner.* Biochem J, 2000. **352 Pt 3**: p. 747-53.
152. Chen, C., et al., *The membrane association domain of RGS16 contains unique amphipathic features that are conserved in RGS4 and RGS5.* J Biol Chem, 1999. **274**(28): p. 19799-806.
153. Chen, C. and S.C. Lin, *The core domain of RGS16 retains G-protein binding and GAP activity in vitro, but is not functional in vivo.* FEBS Lett, 1998. **422**(3): p. 359-62.
154. Druey, K.M., et al., *Amino-terminal cysteine residues of RGS16 are required for palmitoylation and modulation of Gi- and Gq-mediated signaling.* J Biol Chem, 1999. **274**(26): p. 18836-42.
155. Hiol, A., et al., *Palmitoylation regulates regulators of G-protein signaling (RGS) 16 function. I. Mutation of amino-terminal cysteine residues on RGS16 prevents its targeting to lipid rafts and palmitoylation of an internal cysteine residue.* J Biol Chem, 2003. **278**(21): p. 19301-8.
156. Osterhout, J.L., et al., *Palmitoylation regulates regulator of G-protein signaling (RGS) 16 function. II. Palmitoylation of a cysteine residue in the RGS box is critical for RGS16 GTPase accelerating activity and regulation of Gi-coupled signalling.* J Biol Chem, 2003. **278**(21): p. 19309-16.
157. Derrien, A. and K.M. Druey, *RGS16 function is regulated by epidermal growth factor receptor-mediated tyrosine phosphorylation.* J Biol Chem, 2001. **276**(51): p. 48532-8.
158. Derrien, A., et al., *Src-mediated RGS16 tyrosine phosphorylation promotes RGS16 stability.* J Biol Chem, 2003. **278**(18): p. 16107-16.
159. Chen, C., et al., *Multiple phosphorylation sites in RGS16 differentially modulate its GAP activity.* FEBS Lett, 2001. **504**(1-2): p. 16-22.
160. Lee, M.J., et al., *RGS4 and RGS5 are in vivo substrates of the N-end rule pathway.* Proc Natl Acad Sci U S A, 2005. **102**(42): p. 15030-5.
161. Varshavsky, A., *The N-end rule pathway and regulation by proteolysis.* Protein Sci, 2011.
162. Liu, T., et al., *The retinoid anticancer signal: mechanisms of target gene regulation.* Br J Cancer, 2005. **93**(3): p. 310-8.

163. Vasilatos, S.N., et al., *Crosstalk between lysine-specific demethylase 1 (LSD1) and histone deacetylases mediates antineoplastic efficacy of HDAC inhibitors in human breast cancer cells.* Carcinogenesis, 2013. **34**(6): p. 1196-207.
164. Johnson, E.N., et al., *RGS16 inhibits signalling through the G alpha 13-Rho axis.* Nat Cell Biol, 2003. **5**(12): p. 1095-103.
165. Patten, M., et al., *Endotoxin induces desensitization of cardiac endothelin-1 receptor signaling by increased expression of RGS4 and RGS16.* Cardiovasc Res, 2002. **53**(1): p. 156-64.
166. Teplyuk, N.M., et al., *Runx2 regulates G protein-coupled signaling pathways to control growth of osteoblast progenitors.* J Biol Chem, 2008. **283**(41): p. 27585-97.
167. Milligan, G. and E. Kostenis, *Heterotrimeric G-proteins: a short history.* Br J Pharmacol, 2006. **147 Suppl 1**: p. S46-55.
168. Zhang, Y., et al., *RGS16 attenuates galphaq-dependent p38 mitogen-activated protein kinase activation by platelet-activating factor.* J Biol Chem, 1999. **274**(5): p. 2851-7.
169. Dittmer, S., et al., *The constitutively active orphan G-protein-coupled receptor GPR39 protects from cell death by increasing secretion of pigment epithelium-derived growth factor.* J Biol Chem, 2008. **283**(11): p. 7074-81.
170. Hsu, H.C., et al., *Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice.* Nat Immunol, 2008. **9**(2): p. 166-75.
171. Liang, G., et al., *RGS16 inhibits breast cancer cell growth by mitigating phosphatidylinositol 3-kinase signaling.* J Biol Chem, 2009. **284**(32): p. 21719-27.
172. Sethakorn, N., D.M. Yau, and N.O. Dulin, *Non-canonical functions of RGS proteins.* Cell Signal, 2010. **22**(9): p. 1274-81.
173. Billadeau, D.D., et al., *Characterization of the CXCR4 signaling in pancreatic cancer cells.* Int J Gastrointest Cancer, 2006. **37**(4): p. 110-9.
174. Bansal, G., K.M. Druey, and Z. Xie, *R4 RGS proteins: regulation of G-protein signaling and beyond.* Pharmacol Ther, 2007. **116**(3): p. 473-95.
175. Carper, M.B., Denvir, J., Boskovic, G., Primerano, D.A., Claudio, P. P., *RGS16, a novel p53 and pRb cross-talk candidate inhibits migration and invasion of pancreatic cancer cells.* Genes and Cancer, 2014. **5**(11-12): p. 420-435.
176. Shah, N., et al., *Muscarinic receptors and ligands in cancer.* Am J Physiol Cell Physiol, 2009. **296**(2): p. C221-32.
177. Tsoupras, A.B., et al., *The implication of platelet activating factor in cancer growth and metastasis: potent beneficial role of PAF-inhibitors and antioxidants.* Infect Disord Drug Targets, 2009. **9**(4): p. 390-9.
178. Rutka, J.T., et al., *Alterations of the p53 and pRB pathways in human astrocytoma.* Brain Tumor Pathol, 2000. **17**(2): p. 65-70.
179. Sherr, C.J., *Principles of tumor suppression.* Cell, 2004. **116**(2): p. 235-46.
180. Dong, Y., et al., *Reduced expression of retinoblastoma gene product (pRB) and high expression of p53 are associated with poor prognosis in ovarian cancer.* Int J Cancer, 1997. **74**(4): p. 407-15.
181. Jiang, Z., et al., *RB1 and p53 at the crossroad of EMT and triple-negative breast cancer.* Cell Cycle, 2011. **10**(10): p. 1563-70.
182. Yin, Y., et al., *Differential regulation of p21 by p53 and Rb in cellular response to oxidative stress.* Mol Carcinog, 1999. **24**(1): p. 15-24.

183. Su, Z.Z., et al., *The cancer growth suppressor gene mda-7 selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice.* Proc Natl Acad Sci U S A, 1998. **95**(24): p. 14400-5.
184. Greco, A., et al., *Eradication of therapy-resistant human prostate tumors using an ultrasound-guided site-specific cancer terminator virus delivery approach.* Mol Ther, 2010. **18**(2): p. 295-306.
185. Nande, R., et al., *Targeting a newly established spontaneous feline fibrosarcoma cell line by gene transfer.* PLoS One, 2012. **7**(5): p. e37743.
186. Tusher, V.G., R. Tibshirani, and G. Chu, *Significance analysis of microarrays applied to the ionizing radiation response.* Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5116-21.
187. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
188. Shinohara, H., et al., *Retinoblastoma protein-initiated cellular growth arrest overcomes the ability of cotransfected wild-type p53 to induce apoptosis.* Br J Cancer, 2000. **83**(8): p. 1039-46.
189. Ip, S.M., et al., *pRb-expressing adenovirus Ad5-Rb attenuates the p53-induced apoptosis in cervical cancer cell lines.* Eur J Cancer, 2001. **37**(18): p. 2475-83.
190. Spurgers, K.B., et al., *Identification of cell cycle regulatory genes as principal targets of p53-mediated transcriptional repression.* J Biol Chem, 2006. **281**(35): p. 25134-42.
191. Ho, J. and S. Benchimol, *Transcriptional repression mediated by the p53 tumour suppressor.* Cell Death Differ, 2003. **10**(4): p. 404-8.
192. Zhao, R., et al., *Analysis of p53-regulated gene expression patterns using oligonucleotide arrays.* Genes Dev, 2000. **14**(8): p. 981-93.
193. Hammond, E.M., et al., *Genome-wide analysis of p53 under hypoxic conditions.* Mol Cell Biol, 2006. **26**(9): p. 3492-504.
194. Kannan, K., et al., *DNA microarrays identification of primary and secondary target genes regulated by p53.* Oncogene, 2001. **20**(18): p. 2225-34.
195. Itahana, K., et al., *A role for p53 in maintaining and establishing the quiescence growth arrest in human cells.* J Biol Chem, 2002. **277**(20): p. 18206-14.
196. Marchesini, N., et al., *Role for mammalian neutral sphingomyelinase 2 in confluence-induced growth arrest of MCF7 cells.* J Biol Chem, 2004. **279**(24): p. 25101-11.
197. Osada, M., et al., *A p53-type response element in the GDF15 promoter confers high specificity for p53 activation.* Biochem Biophys Res Commun, 2007. **354**(4): p. 913-8.
198. Rouault, J.P., et al., *Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway.* Nat Genet, 1996. **14**(4): p. 482-6.
199. Taira, N., et al., *Induction of amphiregulin by p53 promotes apoptosis via control of microRNA biogenesis in response to DNA damage.* Proc Natl Acad Sci U S A, 2014. **111**(2): p. 717-22.
200. Yoon, H., et al., *Gene expression profiling of isogenic cells with different TP53 gene dosage reveals numerous genes that are affected by TP53 dosage and identifies CSPG2 as a direct target of p53.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15632-7.
201. Hoeferlin, L.A., et al., *Folate stress induces apoptosis via p53-dependent de novo ceramide synthesis and up-regulation of ceramide synthase 6.* J Biol Chem, 2013. **288**(18): p. 12880-90.

202. Jeong, B.S., et al., *Differential levels of transcription of p53-regulated genes by the arginine/proline polymorphism: p53 with arginine at codon 72 favors apoptosis.* FASEB J, 2010. **24**(5): p. 1347-53.
203. Sheikh, M.S., et al., *The antiapoptotic decoy receptor TRID/TRAIL-R3 is a p53-regulated DNA damage-inducible gene that is overexpressed in primary tumors of the gastrointestinal tract.* Oncogene, 1999. **18**(28): p. 4153-9.
204. Ohashi, T., et al., *AKR1B10, a transcriptional target of p53, is downregulated in colorectal cancers associated with poor prognosis.* Mol Cancer Res, 2013. **11**(12): p. 1554-63.
205. Kudoh, T., et al., *D4S234E, a novel p53-responsive gene, induces apoptosis in response to DNA damage.* Exp Cell Res, 2010. **316**(17): p. 2849-58.
206. Jiang, H., et al., *pRB and p107 have distinct effects when expressed in pRB-deficient tumor cells at physiologically relevant levels.* Oncogene, 2000. **19**(34): p. 3878-87.
207. Vernell, R., K. Helin, and H. Muller, *Identification of target genes of the p16INK4A-pRB-E2F pathway.* J Biol Chem, 2003. **278**(46): p. 46124-37.
208. Bourgo, R.J., et al., *RB restricts DNA damage-initiated tumorigenesis through an LXCXE-dependent mechanism of transcriptional control.* Mol Cell, 2011. **43**(4): p. 663-72.
209. Daoud, S.S., et al., *Impact of p53 knockout and topotecan treatment on gene expression profiles in human colon carcinoma cells: a pharmacogenomic study.* Cancer Res, 2003. **63**(11): p. 2782-93.
210. Wurster, A.L., T. Tanaka, and M.J. Grusby, *The biology of Stat4 and Stat6.* Oncogene, 2000. **19**(21): p. 2577-84.
211. Hanna, R.N., et al., *The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes.* Nat Immunol, 2011. **12**(8): p. 778-85.
212. Apte, R.N., et al., *The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions.* Cancer Metastasis Rev, 2006. **25**(3): p. 387-408.
213. Lippitz, B.E., *Cytokine patterns in patients with cancer: a systematic review.* Lancet Oncol, 2013. **14**(6): p. e218-28.
214. Santhanam, U., A. Ray, and P.B. Sehgal, *Repression of the interleukin 6 gene promoter by p53 and the retinoblastoma susceptibility gene product.* Proc Natl Acad Sci U S A, 1991. **88**(17): p. 7605-9.
215. Resnitzky, D., et al., *Interferons and interleukin 6 suppress phosphorylation of the retinoblastoma protein in growth-sensitive hematopoietic cells.* Proc Natl Acad Sci U S A, 1992. **89**(1): p. 402-6.
216. Markey, M.P., et al., *Loss of the retinoblastoma tumor suppressor: differential action on transcriptional programs related to cell cycle control and immune function.* Oncogene, 2007. **26**(43): p. 6307-18.
217. Freund, A., et al., *Inflammatory networks during cellular senescence: causes and consequences.* Trends Mol Med, 2010. **16**(5): p. 238-46.
218. Iannello, A., et al., *p53-dependent chemokine production by senescent tumor cells supports NKG2D-dependent tumor elimination by natural killer cells.* J Exp Med, 2013. **210**(10): p. 2057-69.
219. Kansara, M., et al., *Immune response to RB1-regulated senescence limits radiation-induced osteosarcoma formation.* J Clin Invest, 2013. **123**(12): p. 5351-60.

220. Ghaneh, P., E. Costello, and J.P. Neoptolemos, *Biology and management of pancreatic cancer.* Postgrad Med J, 2008. **84**(995): p. 478-97.
221. Teicher, B.A. and S.P. Fricker, *CXCL12 (SDF-1)/CXCR4 pathway in cancer.* Clin Cancer Res, 2010. **16**(11): p. 2927-31.
222. Marchesi, F., et al., *Increased survival, proliferation, and migration in metastatic human pancreatic tumor cells expressing functional CXCR4.* Cancer Res, 2004. **64**(22): p. 8420-7.
223. Ng, S.S.W., et al., *Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells.* Cancer Res, 2000. **60**(19): p. 5451-5.
224. Schlieman, M.G., et al., *Incidence, mechanism and prognostic value of activated AKT in pancreas cancer.* Br J Cancer, 2003. **89**(11): p. 2110-5.
225. Osaki, M., M. Oshimura, and H. Ito, *PI3K-Akt pathway: its functions and alterations in human cancer.* Apoptosis, 2004. **9**(6): p. 667-76.
226. Zhao, S., et al., *Expression of oncogenic K-ras and loss of Smad4 cooperate to induce the expression of EGFR and to promote invasion of immortalized human pancreas ductal cells.* Int J Cancer, 2010. **127**(9): p. 2076-87.
227. Yamanaka, Y., et al., *Coexpression of epidermal growth factor receptor and ligands in human pancreatic cancer is associated with enhanced tumor aggressiveness.* Anticancer Res, 1993. **13**(3): p. 565-9.
228. Lo, H.W., et al., *Epidermal growth factor receptor cooperates with signal transducer and activator of transcription 3 to induce epithelial-mesenchymal transition in cancer cells via up-regulation of TWIST gene expression.* Cancer Res, 2007. **67**(19): p. 9066-76.
229. Dittmar, T., et al., *Induction of cancer cell migration by epidermal growth factor is initiated by specific phosphorylation of tyrosine 1248 of c-erbB-2 receptor via EGFR.* FASEB J, 2002. **16**(13): p. 1823-5.
230. Giehl, K., et al., *Growth factor-dependent activation of the Ras-Raf-MEK-MAPK pathway in the human pancreatic carcinoma cell line PANC-1 carrying activated K-ras: implications for cell proliferation and cell migration.* Oncogene, 2000. **19**(25): p. 2930-42.
231. Deer, E.L., et al., *Phenotype and genotype of pancreatic cancer cell lines.* Pancreas, 2010. **39**(4): p. 425-35.
232. Ali, S., et al., *Simultaneous targeting of the epidermal growth factor receptor and cyclooxygenase-2 pathways for pancreatic cancer therapy.* Mol Cancer Ther, 2005. **4**(12): p. 1943-51.
233. Kim, D., et al., *Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production.* FASEB J, 2001. **15**(11): p. 1953-62.
234. Tanno, S., et al., *AKT activation up-regulates insulin-like growth factor I receptor expression and promotes invasiveness of human pancreatic cancer cells.* Cancer Res, 2001. **61**(2): p. 589-93.
235. Tan, X., et al., *Involvement of MMP-7 in invasion of pancreatic cancer cells through activation of the EGFR mediated MEK-ERK signal transduction pathway.* J Clin Pathol, 2005. **58**(12): p. 1242-8.
236. Malliri, A., et al., *The transcription factor AP-1 is required for EGF-induced activation of rho-like GTPases, cytoskeletal rearrangements, motility, and in vitro invasion of A431 cells.* J Cell Biol, 1998. **143**(4): p. 1087-99.

237. Keleg, S., et al., *Invasion and metastasis in pancreatic cancer.* Mol Cancer, 2003. **2**: p. 14.
238. Yachida, S. and C.A. Iacobuzio-Donahue, *The pathology and genetics of metastatic pancreatic cancer.* Arch Pathol Lab Med, 2009. **133**(3): p. 413-22.
239. Jang, S. and M.B. Atkins, *Treatment of BRAF-mutant melanoma: the role of vemurafenib and other therapies.* Clin Pharmacol Ther, 2014. **95**(1): p. 24-31.
240. Kusama, T., et al., *Inhibition of epidermal growth factor-induced RhoA translocation and invasion of human pancreatic cancer cells by 3-hydroxy-3-methylglutaryl-coenzyme a reductase inhibitors.* Cancer Res, 2001. **61**(12): p. 4885-91.
241. Xie, Y., et al., *Breast cancer migration and invasion depend on proteasome degradation of regulator of G-protein signaling 4.* Cancer Res, 2009. **69**(14): p. 5743-51.
242. Mori, T., et al., *CXCR4 antagonist inhibits stromal cell-derived factor 1-induced migration and invasion of human pancreatic cancer.* Mol Cancer Ther, 2004. **3**(1): p. 29-37.
243. Singh, S., et al., *CXCL12-CXCR4 signalling axis confers gemcitabine resistance to pancreatic cancer cells: a novel target for therapy.* Br J Cancer, 2010. **103**(11): p. 1671-9.
244. Chou, A., et al., *Clinical and molecular characterization of HER2 amplified-pancreatic cancer.* Genome Med, 2013. **5**(8): p. 78.
245. Lakka, S.S., et al., *Downregulation of MMP-9 in ERK-mutated stable transfectants inhibits glioma invasion in vitro.* Oncogene, 2002. **21**(36): p. 5601-8.
246. Lu, Z., et al., *Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion.* Cancer Cell, 2003. **4**(6): p. 499-515.
247. Kwon, O., et al., *Modulation of E-cadherin expression by K-Ras; involvement of DNA methyltransferase-3b.* Carcinogenesis, 2010. **31**(7): p. 1194-201.
248. Nagasawa, R., et al., *Unique phosphorylation of vimentin filaments in glomerular epithelial cells in culture and in disease.* Nephron, 1997. **77**(4): p. 373-7.
249. Ivaska, J., et al., *Novel functions of vimentin in cell adhesion, migration, and signaling.* Exp Cell Res, 2007. **313**(10): p. 2050-62.
250. Khosravi-Far, R., et al., *Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation.* Mol Cell Biol, 1995. **15**(11): p. 6443-53.
251. Qiu, R.G., et al., *Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation.* Mol Cell Biol, 1997. **17**(6): p. 3449-58.
252. Timpson, P., et al., *Spatial regulation of RhoA activity during pancreatic cancer cell invasion driven by mutant p53.* Cancer Res, 2011. **71**(3): p. 747-57.
253. Kusama, T., et al., *Inactivation of Rho GTPases by p190 RhoGAP reduces human pancreatic cancer cell invasion and metastasis.* Cancer Sci, 2006. **97**(9): p. 848-53.
254. Shan, D., et al., *The G protein G alpha(13) is required for growth factor-induced cell migration.* Dev Cell, 2006. **10**(6): p. 707-18.
255. Nimnual, A.S., L.J. Taylor, and D. Bar-Sagi, *Redox-dependent downregulation of Rho by Rac.* Nat Cell Biol, 2003. **5**(3): p. 236-41.
256. Wildenberg, G.A., et al., *p120-catenin and p190RhoGAP regulate cell-cell adhesion by coordinating antagonism between Rac and Rho.* Cell, 2006. **127**(5): p. 1027-39.
257. Roger, L., G. Gadea, and P. Roux, *Control of cell migration: a tumour suppressor function for p53?* Biol Cell, 2006. **98**(3): p. 141-52.

258. Liu, J., et al., *Wild-type p53 inhibits nuclear factor-kappaB-induced matrix metalloproteinase-9 promoter activation: implications for soft tissue sarcoma growth and metastasis.* Mol Cancer Res, 2006. **4**(11): p. 803-10.
259. Sun, Y., et al., *Human metalloproteinase-1 (collagenase-1) is a tumor suppressor protein p53 target gene.* Ann N Y Acad Sci, 1999. **878**: p. 638-41.
260. Sun, Y., et al., *p53 down-regulates human matrix metalloproteinase-1 (Collagenase-1) gene expression.* J Biol Chem, 1999. **274**(17): p. 11535-40.
261. Zhu, H., et al., *A role for p53 in the regulation of extracellular matrix metalloproteinase inducer in human cancer cells.* Cancer Biol Ther, 2009. **8**(18): p. 1722-8.
262. Andrusiak, M.G., et al., *Rb/E2F regulates expression of neogenin during neuronal migration.* Mol Cell Biol, 2011. **31**(2): p. 238-47.
263. Knudsen, K.E., et al., *RB-dependent S-phase response to DNA damage.* Mol Cell Biol, 2000. **20**(20): p. 7751-63.
264. Amundson, S.A., et al., *Stress-specific signatures: expression profiling of p53 wild-type and -null human cells.* Oncogene, 2005. **24**(28): p. 4572-9.
265. Harrington, E.A., et al., *pRB plays an essential role in cell cycle arrest induced by DNA damage.* Proc Natl Acad Sci U S A, 1998. **95**(20): p. 11945-50.
266. Orsulic, S., et al., *E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation.* J Cell Sci, 1999. **112 (Pt 8)**: p. 1237-45.
267. Barberis, L., et al., *Leukocyte transmigration is modulated by chemokine-mediated PI3Kgamma-dependent phosphorylation of vimentin.* Eur J Immunol, 2009. **39**(4): p. 1136-46.
268. Herreros-Villanueva, M., et al., *Mouse models of pancreatic cancer.* World J Gastroenterol, 2012. **18**(12): p. 1286-94.
269. Becher, O.J. and E.C. Holland, *Genetically engineered models have advantages over xenografts for preclinical studies.* Cancer Res, 2006. **66**(7): p. 3355-8, discussion 3358-9.
270. Ocal, O., et al., *Rgs16 is an early marker of pancreatic ductal adenocarcinoma (842.7).* The FASEB Journal, 2014. **28**(1 Supplement).
271. Qiu, W. and G.H. Su, *Challenges and advances in mouse modeling for human pancreatic tumorigenesis and metastasis.* Cancer Metastasis Rev, 2013. **32**(1-2): p. 83-107.
272. Qiu, W. and G.H. Su, *Development of orthotopic pancreatic tumor mouse models.* Methods Mol Biol, 2013. **980**: p. 215-23.
273. Huynh, A.S., et al., *Development of an orthotopic human pancreatic cancer xenograft model using ultrasound guided injection of cells.* PLoS One, 2011. **6**(5): p. e20330.

Figure Legends

Figure 1: Identification of differentially expressed transcripts in WI38 cells expressing p53 and/or pRb. WI38 cells were transduced with adenoviruses carrying the transgenes p53, or *RB1*/p105; a MOI of 50 was used in each case. A) Western blot analysis was used to test for p53 and pRb expression prior to microarray analysis. B) A Venn diagram shows the differentially expressed transcripts and intersects identified during the microarray analysis. The numbers in red denote transcripts that were up-regulated due to p53, pRb, or p53 and pRb expression.

Figure 2: Validation of microarray data using qRT-PCR in WI38 cells. Five transcripts RGS16, BCL2L11, BTG2, IL-6 and STAT4 from the p53 and pRb intersect were chosen for validation by qRT-PCR in WI38 cells expressing p53, pRb, or both p53 and pRb. The vector control (Ad.CMV) was used to calculate the fold change for each transcript. One-way ANOVA with Dunnett's test for multiple comparison were used to test for statistical significance * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, and **** p-value < 0.0001.

Figure 3: Validation of microarray data using qRT-PCR in SAOS-2 cells. Five transcripts RGS16, BCL2L11, BTG2, IL-6 and STAT4 from the p53 and pRb intersect were chosen for validation by qRT-PCR in SAOS-2 cells expressing p53, pRb, or both p53 and pRb. The vector control (Ad.CMV) was used to calculate the fold change for each transcript. One-way ANOVA with Dunnett's test for multiple comparison were used to test for statistical significance * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, and **** p-value < 0.0001.

Figure 4: Decreased expression of RGS16 mRNA relative to total RNA extracted from normal human pancreatic tissue. Expression of RGS16 was measured using qRT-PCR in BxPC-3, MIA PaCa-2, PANC-1, and AsPC-1 cells. Relative fold change was measured using total RNA extracted from normal human pancreatic tissue as the control. One-way ANOVA with Tukey's test for multiple comparison were used to test for statistical significance between the cell lines and control * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, and **** p-value < 0.0001.

Figure 5: Expression of RGS16 inhibited migration of BxPC-3 cells. BxPC-3 cells were transduced with 50 MOI of Ad.GFP (CTRL) or Ad.GFP.RGS16. A) Virus transduction was verified by fluorescent microscopy. B) Images (100x) and measurements of wounds were taken prior and 16 hours after addition of media supplemented with FBS (10%) or EGF (100ng/ml). The dashed lines represent size of scratch at time 0. C) Mean Percentage of wound healing ± SEM of three separate experiments (three scratches / well) was determined. Student's t-test was used to determine statistical significance compared to control * p-value < 0.05, ** p-value < 0.01.

Figure 6: Expression of RGS16 did not inhibit migration of PANC-1 cells. Wound healing assays were performed as described in Figure 2. A) Fluorescent microscopy was used to verify virus transductions: B&C Images (100X) were taken and percentages of wound healing were calculated at 24hrs.

Figure 7: Expression of RGS16 inhibited migration of AsPC-1 cells. Wound healing assays were performed as described in Figure 2. A) Fluorescent microscopy was used to verify virus transductions: B&C Images (100X) were taken and percentages of wound healing were calculated at 24hrs * p-value < 0.05.

Figure 8: Expression of RGS16 inhibited invasion of BxPC-3 and AsPC-1 cells. Matrigel invasion chambers were used to measure cell migration and invasion of GFP and/or RGS16 expressing BxPC-3 (A & B) and AsPC-1 (C & D) cells using EGF as a chemoattractant. Migrated cells were stained with Crystal Violet and counted at 200x magnification (A &C). Percent invasion was calculated for each cell line (B & D) * p-value < 0.05.

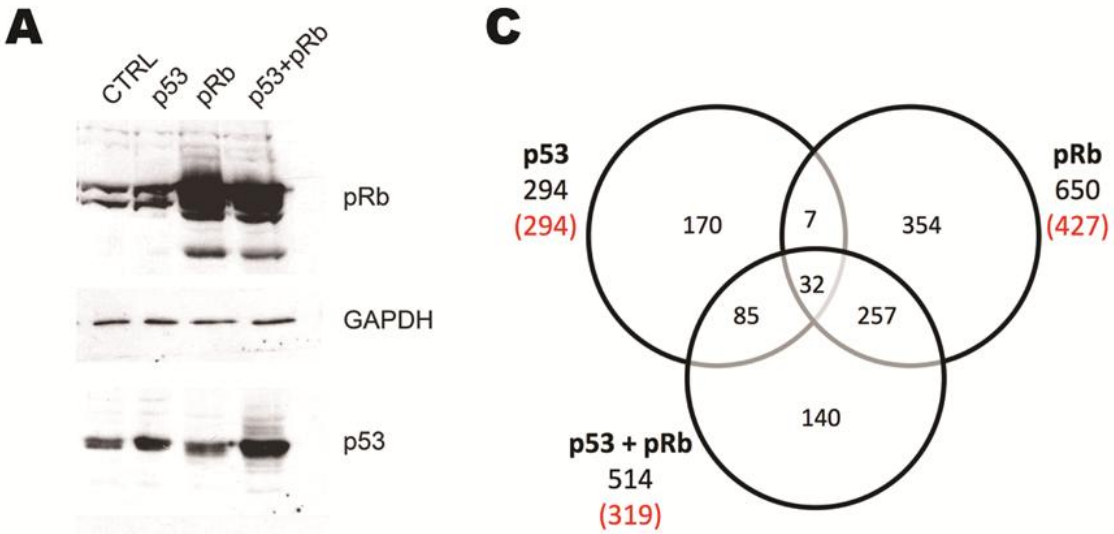
Table 1. Fold Change of p53 and pRb common gene set cross-talk candidates.

Gene Symbol	Name	FC-p53	FC-Rb	FC-p53+Rb
LOC387763	hypothetical LOC387763	30.62	159.25	297.07
A_24_p775812	Unknown	15.65	199.36	252.80
RGS16	Regulator of G-protein signaling 16	18.84	30.82	149.75
AREG	Amphiregulin	8.15	46.41	81.91
CCL3	Chemokine (c-c motif ligand 3)	3.78	8.18	56.12
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	10.68	69.61	53.34
IL-1B	Interleukin-1 beta	4.82	22.53	43.79
OLFM2	Olfactomedin 2	10.60	37.76	27.31
NR4A1	Nuclear receptor subfamily 4 group A member 1	11.56	20.73	27.08
POSTN	Periostin	2.91	21.051	25.66
D4S234e	D4S234e (NSG1; neuron specific gene family member 1)	21.20	7.86	22.06
IL-6	Interleukin-6	6.04	12.37	21.99
DMN	Desmuslin	4.57	27.42	21.25
EPPK1	Epiplakin	32.06	8.27	20.04
IQSEC3	IQ motif and Sec7 domain 3	7.29	20.37	19.95
PLAC2	Placenta specific 2	21.60	4.63	19.00
L3MBTL2	Lethal(3)malignant brain tumor-like protein 2	18.33	11.82	16.00
LHX6	LIM homeobox 6	10.11	7.11	15.15
AKR1B10	Aldo-keto reductase family 1 member B10	11.61	13.92	13.30
RRAD	Ras associated with diabetes	5.98	7.80	12.61
c10orf58	chromosome 10 open reading frame 58	4.86	9.20	11.74
BCL2L11	Bcl2-like 11 (apoptosis facilitator)	9.58	6.50	11.34
COL7A1	Collagen, type VII, alpha 1	5.97	10.65	10.94
JUP	Junction plakoglobin	7.60	16.61	9.92
VCAN	Versican proteoglycan	5.61	9.17	9.73
CRISPLD2	Cystein-rich secretory protein 11	10.11	5.38	9.55
STOX2	Storkhead-box 2	14.48	8.70	9.33
BTG-2	B-cell translocation gene 2	3.85	5.07	7.46
P2RY2	purinergic receptor P2Y, G-protein coupled, 2	2.38	19.53	6.91
TSKU	Tsukusi,small leucine rich proteoglycan	5.28	5.42	5.97
C4B	Complement component 4B	3.38	7.64	2.22
RTN4R	Reticulon 4 receptor	8.01	6.19	N/A
STAT4	Signal transducer and activator of transcription 4	5.98	7.80	N/A
AK124344	cDNA FLJ42353 fis, clone UTERU2007520	5.21	7.13	N/A
KLHL20	Kelch like 20	4.88	4.46	N/A
NOTCH3	Notch homolog 3	4.68	3.96	N/A
KSR1	Kinase suppressor of RAS	3.86	4.08	N/A
GDF15	Growth/differentiation factor 15	3.24	3.40	N/A
LOC654346	similar to galectin 9 short isoform (LOC654346)	2.81	4.77	N/A

FC = Fold change
N/A= Fold change not available. Gene was not found to be significantly differentially expressed in WI38 cells coexpressing p53 and pRb.

	p53	p16	Ras	EGFR	Differentiation	Origin	Metastasis
BxPC-3	mt	del	wt	high	moderate	primary	no
PANC-1	mt	del	mt	high	poor	primary	yes
AsPC-1	mt	mt	mt	high	poor	metastatic (ascites)	yes
MiaPaCa-2	mt	del	mt	low	poor	primary	no

Figure 1:



B

Normalized fold change

	CTRL	p53	pRb	p53 + pRb
p53	1.00	2.80	1.54	2.77
hypophosphorylated pRb/total pRb	1.00	0.94	5.48	5.02

Figure 2:

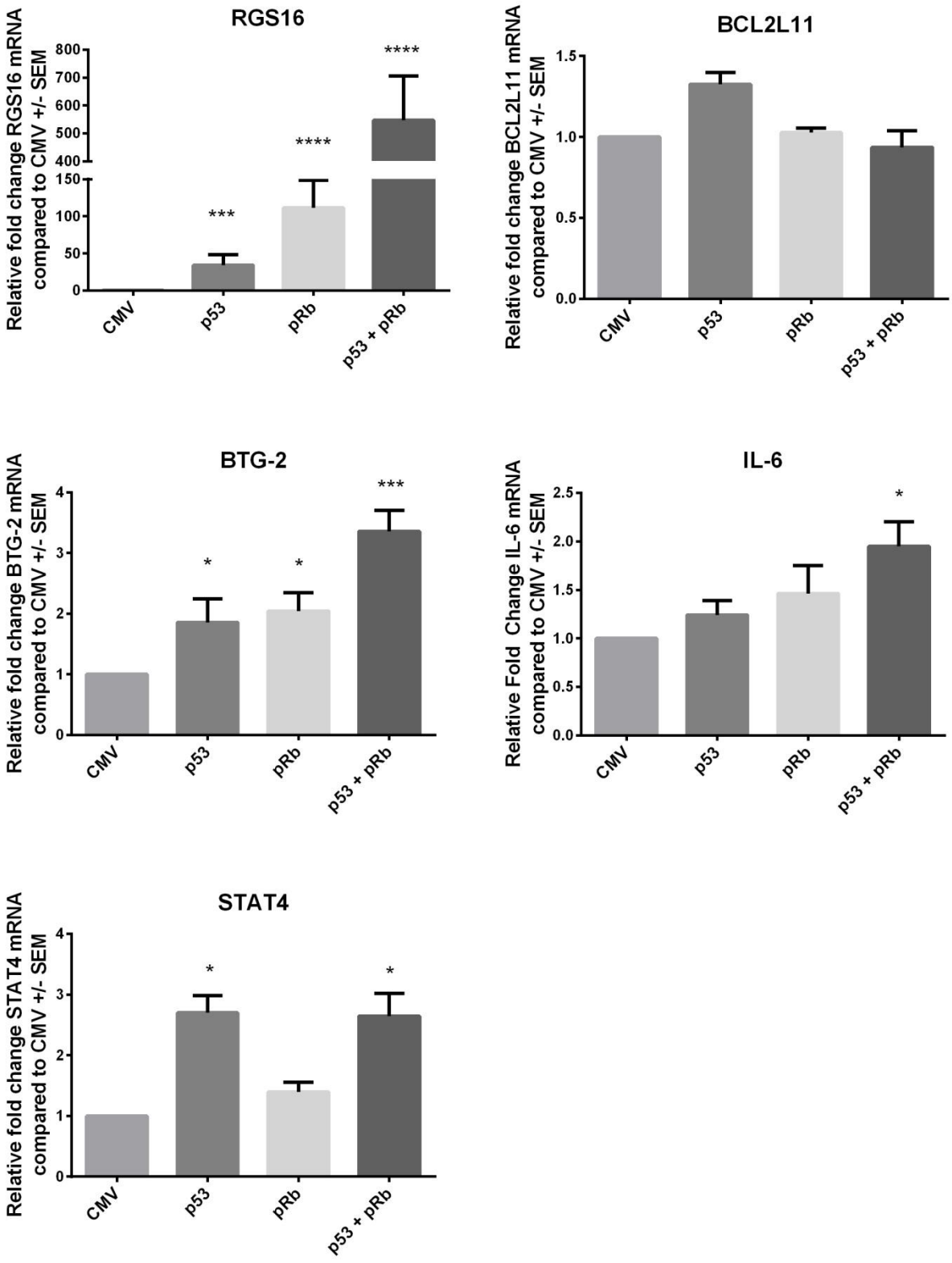

RGS16
Relative fold change RGS16 mRNA compared to CMV +/- SEM
800
700
600
500
400
150
100
50
0
***
****
****
CMV
p53
pRb
p53 + pRb
BCL2L11
Relative fold change BCL2L11 mRNA compared to CMV +/- SEM
1.5
1.0
0.5
0.0
CMV
p53
pRb
p53 + pRb
BTG-2
Relative fold change BTG-2 mRNA compared to CMV +/- SEM
4
3
2
1
0
*
*
***
CMV
p53
pRb
p53 + pRb
IL-6
Relative Fold Change IL-6 mRNA compared to CMV +/- SEM
2.5
2.0
1.5
1.0
0.5
0.0
*
CMV
p53
pRb
p53 + pRb
STAT4
Relative fold change STAT4 mRNA compared to CMV +/- SEM
4
3
2
1
0
*
*
CMV
p53
pRb
p53 + pRb

Figure 3:

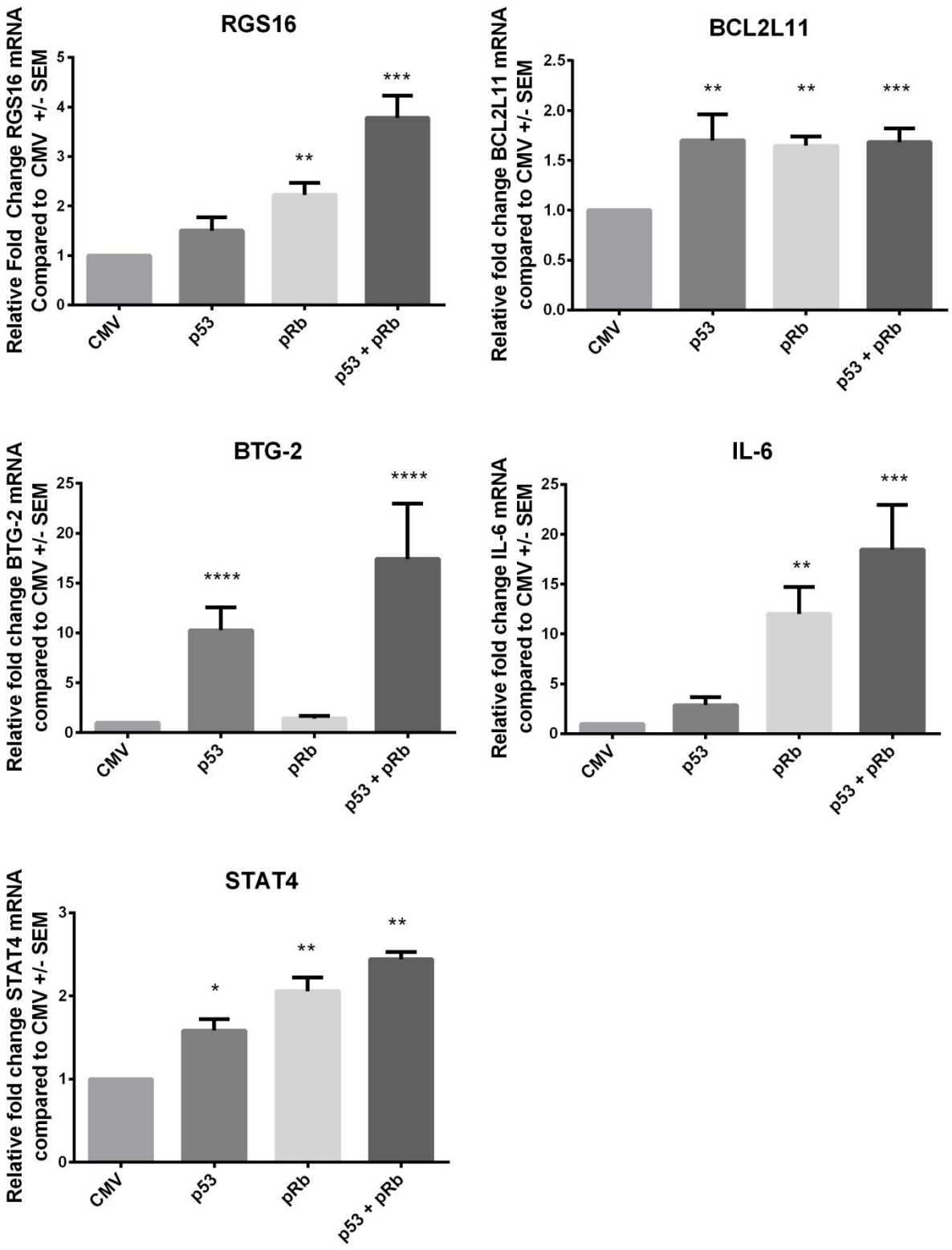

RGS16
Relative Fold Change RGS16 mRNA Compared to CMV +/- SEM
0
1
2
3
4
5
**
***
CMV
p53
pRb
p53 + pRb
BCL2L11
Relative fold change BCL2L11 mRNA compared to CMV +/- SEM
0.0
0.5
1.0
1.5
2.0
2.5
**
**
***
CMV
p53
pRb
p53 + pRb
BTG-2
Relative fold change BTG-2 mRNA compared to CMV +/- SEM
0
5
10
15
20
25
****
****
CMV
p53
pRb
p53 + pRb
IL-6
Relative fold change IL-6 mRNA compared to CMV +/- SEM
0
5
10
15
20
25
**
***
CMV
p53
pRb
p53 + pRb
STAT4
Relative fold change STAT4 mRNA compared to CMV +/- SEM
0
1
2
3
*
**
**
CMV
p53
pRb
p53 + pRb

Figure 4:

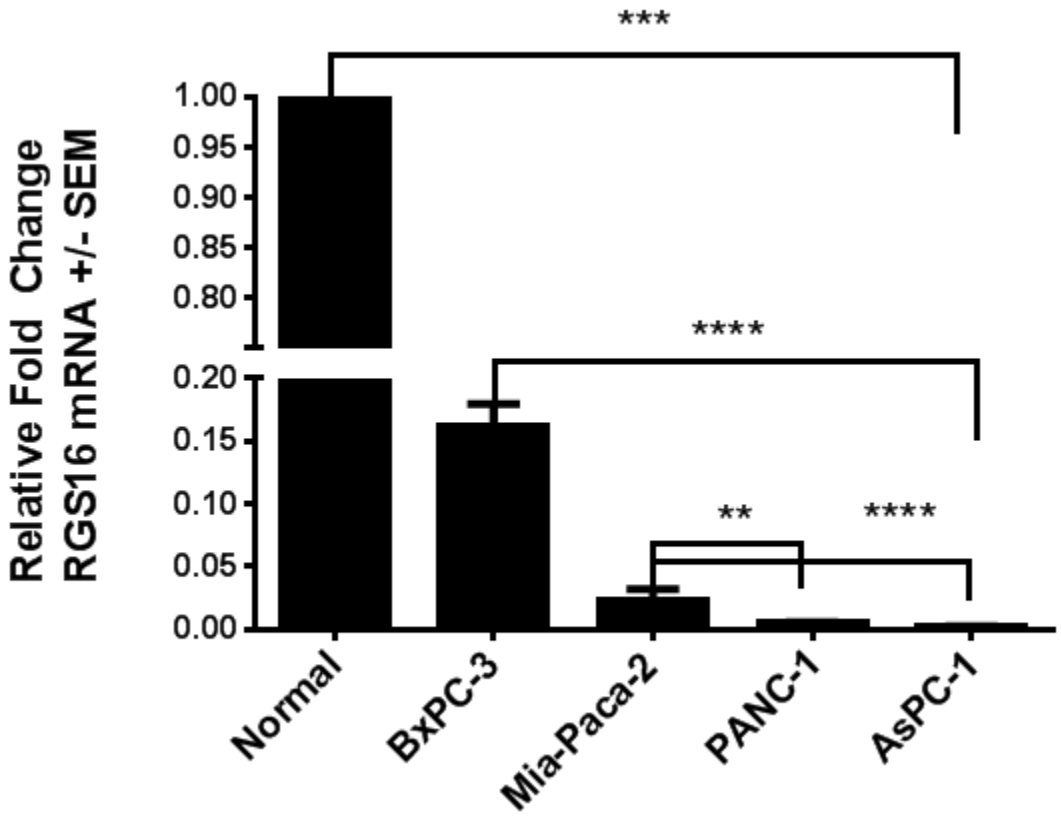


Figure 5:

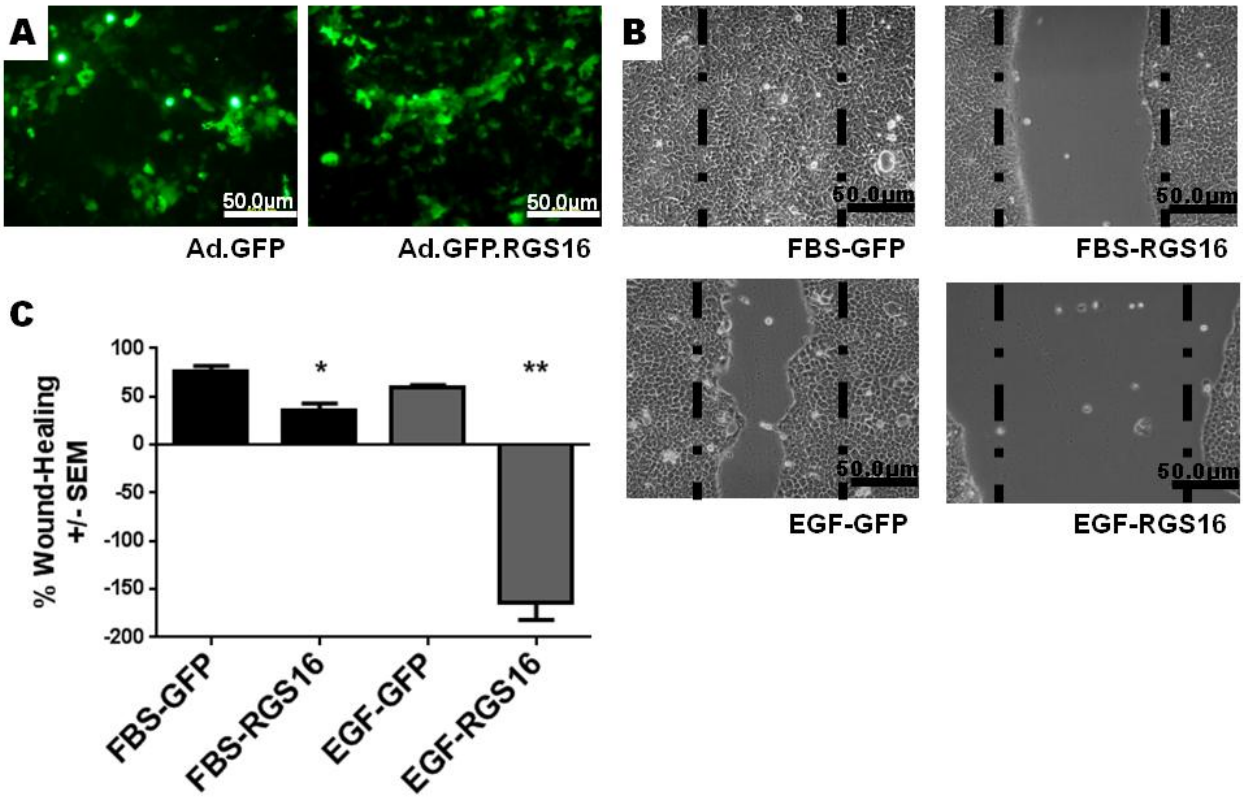

A
50.0µm
50.0µm
Ad.GFP
Ad.GFP.RGS16
B
50.0µm
50.0µm
FBS-GFP
FBS-RGS16
50.0µm
50.0µm
EGF-GFP
EGF-RGS16
C
% Wound-Healing +/- SEM
100
50
0
-50
-100
-150
-200
*
**
FBS-GFP
FBS-RGS16
EGF-GFP
EGF-RGS16

Figure 6:

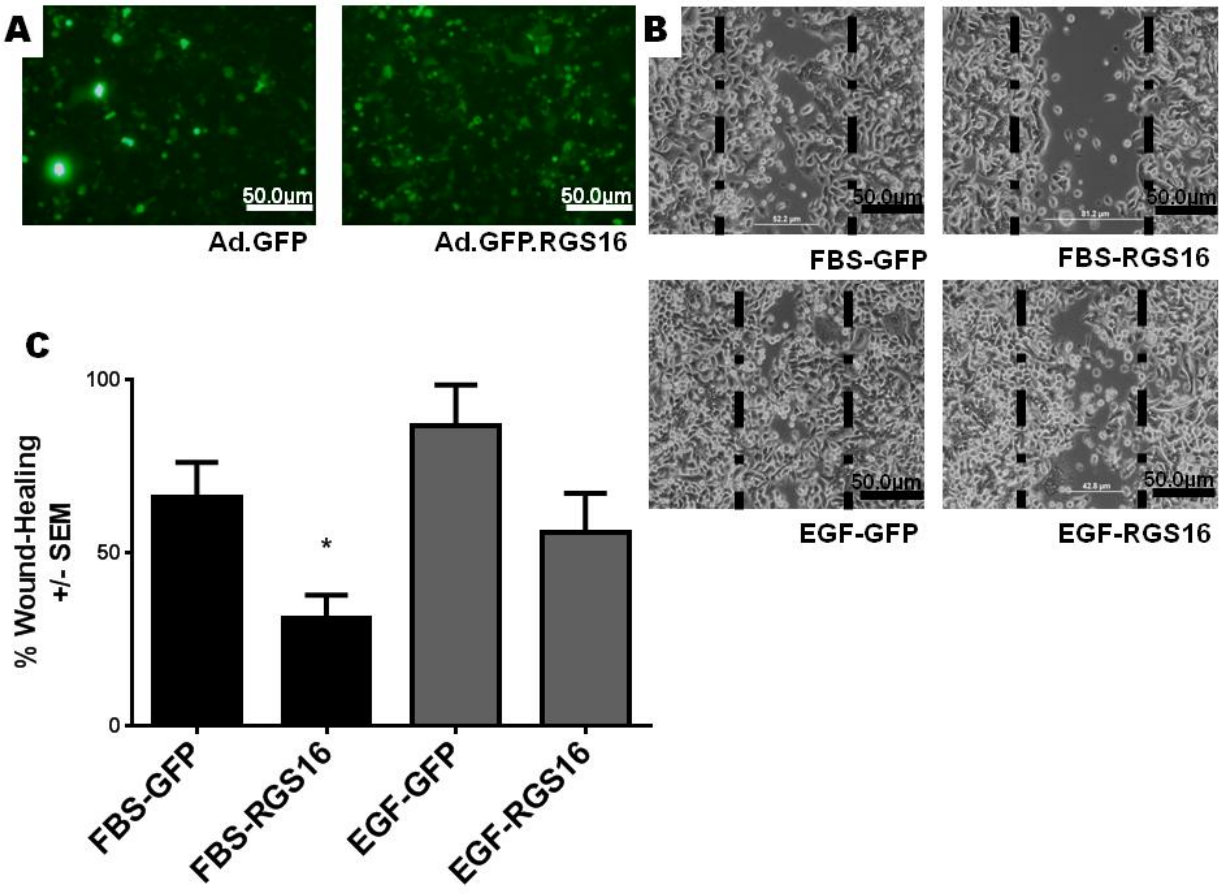

A
50.0µm
50.0µm
Ad.GFP
Ad.GFP.RGS16
B
50.0µm
50.0µm
FBS-GFP
FBS-RGS16
50.0µm
50.0µm
EGF-GFP
EGF-RGS16
C
% Wound-Healing
+/- SEM
100
50
0
*
FBS-GFP
FBS-RGS16
EGF-GFP
EGF-RGS16

Figure7:

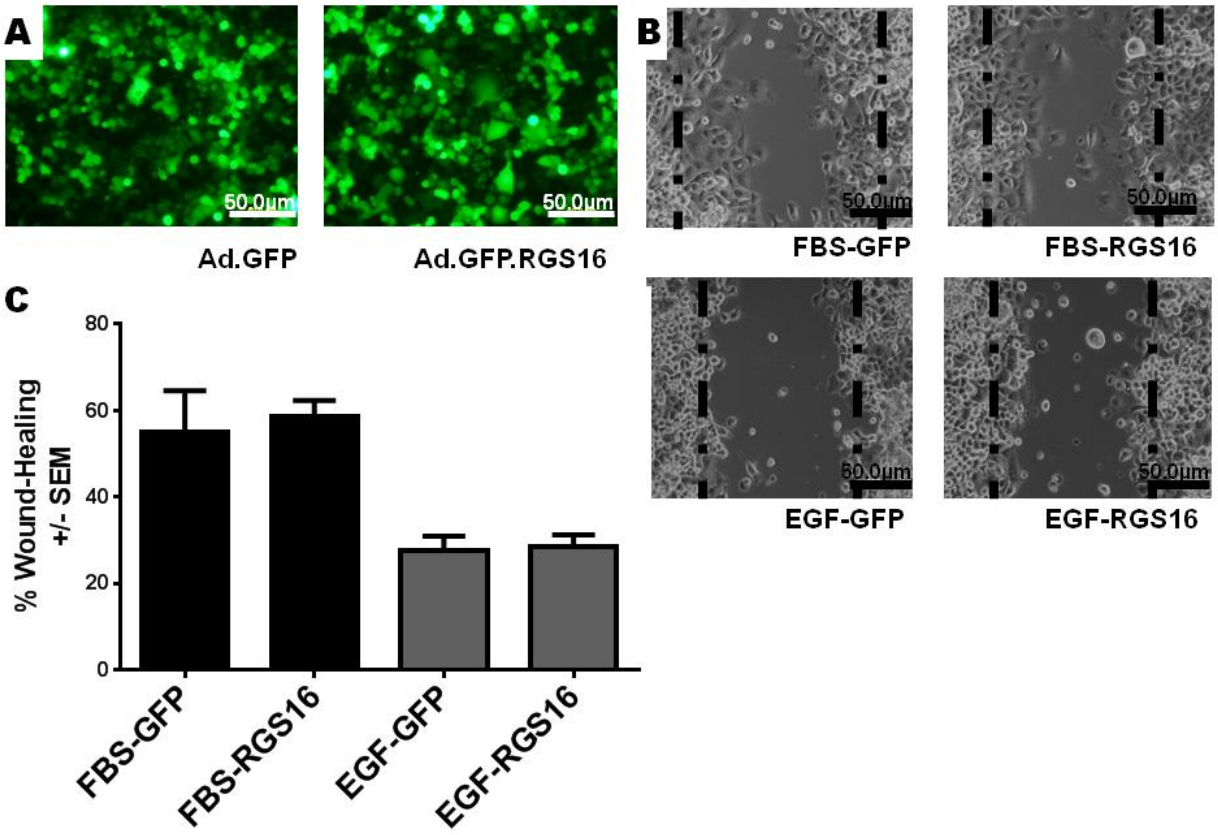
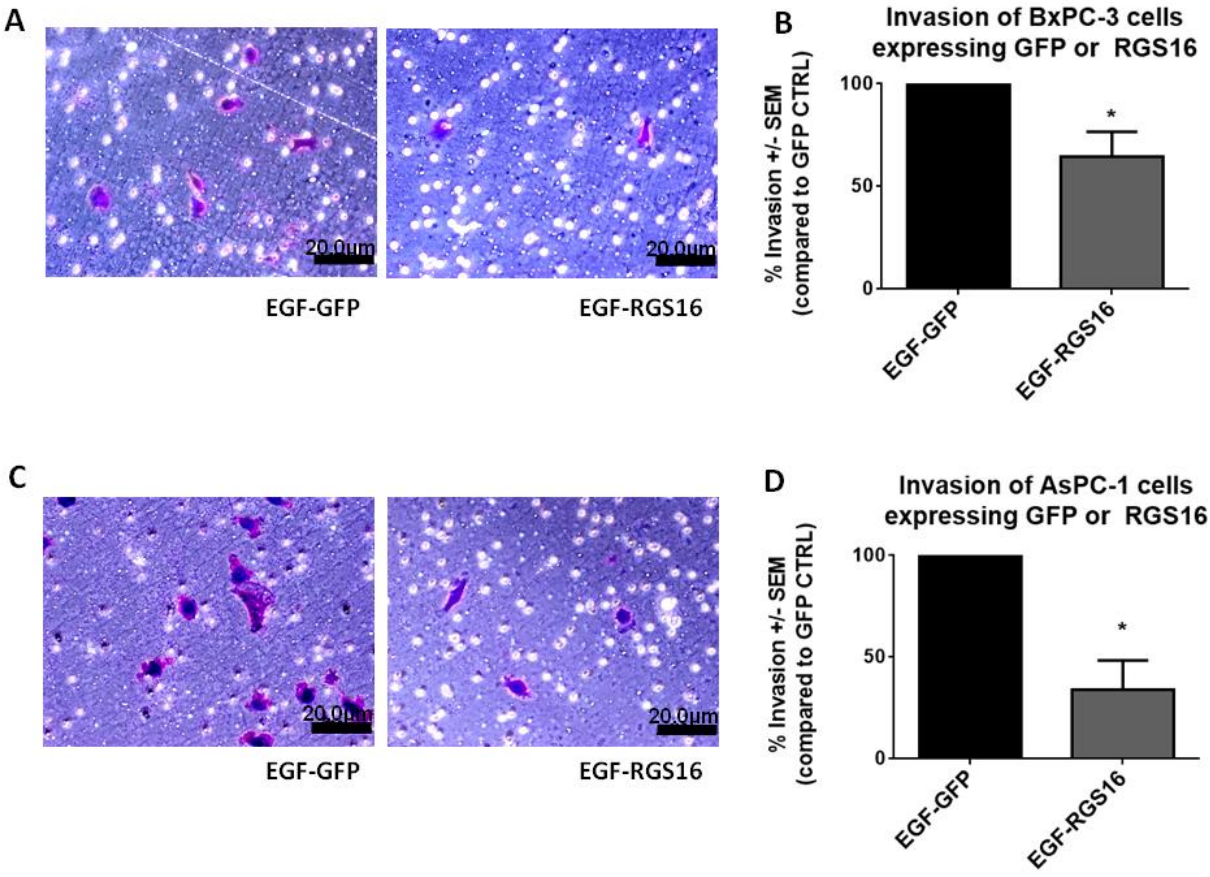

A
50.0µm
50.0µm
Ad.GFP
Ad.GFP.RGS16
B
50.0µm
50.0µm
FBS-GFP
FBS-RGS16
50.0µm
50.0µm
EGF-GFP
EGF-RGS16
C
% Wound-Healing +/- SEM
80
60
40
20
0
FBS-GFP
FBS-RGS16
EGF-GFP
EGF-RGS16

Figure 8:


A
20.0µm
20.0µm
EGF-GFP
EGF-RGS16
B
Invasion of BxPC-3 cells expressing GFP or RGS16
% Invasion +/- SEM (compared to GFP CTRL)
100
50
0
*
EGF-GFP
EGF-RGS16
C
20.0µm
20.0µm
EGF-GFP
EGF-RGS16
D
Invasion of AsPC-1 cells expressing GFP or RGS16
% Invasion +/- SEM (compared to GFP CTRL)
100
50
0
*
EGF-GFP
EGF-RGS16