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## Glycogen Synthase Kinase 3 $\beta$ in Pancreatic Cancer and its Implications in Chemotherapy and Radiation Therapy

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### Abstract

Pancreatic cancer is a highly lethal disease with a poor prognosis characterized by local and systemic disease progression. Both radiation and chemotherapy play important roles in the management of this disease. However, in order to improve standard therapy many molecularly targeted agents are being developed. Glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) participates in a multitude of cellular processes and is a newly proposed therapeutic target in pancreatic cancer. This review will discuss both the oncogenic and tumor suppressor functions of GSK3 $\beta$  in pancreatic cancer with an emphasis on the roles of GSK3 $\beta$  in tumor cell survival and sensitivity to radiation and chemotherapy.

### Keywords

GSK3 $\beta$ ; Pancreatic cancer; c-Met; Radiation

### Introduction

Pancreatic cancer, the fourth leading cause of cancer-related death in the United States is characterized by both local and systemic disease progression [1,2]. Newer treatment strategies for metastatic disease include FOLFIRINOX and Abraxane in combination with the standard chemotherapy, gemcitabine [3-5]. In the locally-advanced disease setting chemoradiation is superior to chemotherapy (gemcitabine) alone [6,7]. Since surgical resection offers the only potential cure for this disease but is possible in a minority of

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patients, efforts are under way to improve therapy for both locally advanced and metastatic disease. Therefore, identification of novel therapeutic targets and development of mechanism-based targeted therapy in combination with existing treatment regimens is of great interest and has the potential to improve the efficacy of current therapy against this deadly disease.

GSK3 $\beta$  is being investigated as a therapeutic target for the treatment of a variety of human diseases including cancer. GSK3 $\beta$  regulates many important processes in cancer cells such as the cell cycle, proliferation, differentiation and apoptosis [8-10]. On the other hand, the mechanisms underlying GSK3 $\beta$  regulation of tumorigenesis and cancer progression are still obscure and to date, whether GSK3 $\beta$  is an oncogene or a tumor suppressor remains controversial. Given the multiple functions of GSK3 $\beta$ , it may act as both an oncogene and a tumor suppressor, depending upon cell properties and contexts as well as the stage of tumorigenesis. In this review, we will discuss both the oncogenic and tumor suppressor functions of GSK3 $\beta$  in the context of pancreatic cancer.

## GSK3 $\beta$

GSK3 $\alpha$  and GSK3 $\beta$  are encoded by two distinct genes and share 85% identity, especially in their kinase domains [8,9,11,12]. Interestingly, while these two isoforms do show redundancy in biological function, many studies have defined unique roles of GSK3 $\beta$  in physiological and pathological processes. GSK3 $\beta$  was first identified more than 30 years ago as one of the key kinases involved in glycogen metabolism [13,14]. GSK3 $\beta$  mediates insulin signaling by phosphorylating and inactivating glycogen synthase. Interestingly, GSK3 $\beta$  possesses its activity in unstimulated and resting cells, and extracellular signals diminish its activity by phosphorylation at Serine 9 (Ser9) mediated by AKT and other kinases as well as by a lack of phosphorylation at Tyrosine 216 (Tyr216) [8,15]. Insulin therefore promotes glycogen formation through inhibition of GSK3 $\beta$ .

Other functions independent of glycogen regulation occur as GSK3 $\beta$  phosphorylates many S/TPXXpS/T motif-containing proteins, targeting them for subsequent ubiquitin-mediated proteasomal degradation [11,16]. In most cases, GSK3 $\beta$  substrates require prior phosphorylation in the -4 position for subsequent GSK3 $\beta$ -mediated phosphorylation [16]. Phosphorylation of substrates by GSK3 $\beta$  and other kinases such as Casein Kinase (CK) and dual-specificity tyrosinephosphorylated and regulated kinase (DYRK) generates the optimal substrate conformation for binding of SCF (SKP1, Cullin and F-box protein complex)-type E3 ligases [11,17,18]. Several F-box proteins including SKP2, FBXW7, and  $\beta$ -TrCP, are able to recognize these GSK3 $\beta$ -phosphorylated motifs (degron) on the substrates for targeted degradation [19]. Through this cascade, GSK3 $\beta$  influences gene transcription by degradation of cytosolic transcription factors before nuclear translocation.

## GSK3 $\beta$ as a Tumor Suppressor

GSK3 $\beta$  substrate proteins play crucial roles in a wide spectrum of cellular processes, including the cell cycle, transcription, and signal transduction. Most of them are functionally inhibited by GSK3 $\beta$ -mediated phosphorylation (Figure 1) and some key oncoproteins are well known substrates of GSK3 $\beta$ , such as  $\beta$ -catenin, c-Myc, Cyclin D, Cyclin E, and c-Jun

[20-22].  $\beta$ -catenin is the prototypical example of an oncoprotein that is negatively regulated by GSK3 $\beta$ . It is well-known that GSK3 $\beta$  is a negative regulator of Wnt/ $\beta$ -catenin signaling pathway such that in the absence of Wnt ligand, the scaffold protein Axin facilitates GSK3 $\beta$ -mediated phosphorylation of  $\beta$ -catenin resulting in  $\beta$ -catenin degradation by  $\beta$ -TrCP [23]. Activation of Wnt signaling causes disruption of the GSK3 $\beta$ -Axin-APC complex and inhibits  $\beta$ -catenin degradation, which in turn binds the T-cell factor (TCF)/Lymphoid Enhancer Factor (LEF) family of transcription factors and positively regulates transcription of oncogenes such as Cyclin D and c-Myc. Thus, GSK3 $\beta$  suppresses  $\beta$ -catenin activation through proteasome targeting while inhibition of GSK3 $\beta$  allows accumulation of  $\beta$ -catenin followed by nuclear translocation and  $\beta$ -catenin-dependent gene transcription.

Connections between GSK3 $\beta$  and other oncoproteins, such as K-RAS are emerging. In the context of Wnt pathway regulation, GSK3 $\beta$  was found to phosphorylate RAS, ultimately leading to ubiquitination by  $\beta$ -TrCP-E3 ligase and subsequent degradation of RAS in colon cancer models [24]. Since K-RAS mutations occur in more than 90% of pancreatic carcinomas, it is important for future studies to determine whether GSK3 $\beta$  negatively regulates K-RAS stability in pancreatic cancer. The action of GSK3 $\beta$  to degrade oncogenic proteins such as K-RAS, c-Myc, and Cyclin D suggests that GSK3 $\beta$  possesses some tumor suppressor properties.

### **GSK3 $\beta$ as an Oncoprotein**

In contrast to these tumor suppressor properties, GSK3 $\beta$  oncogenic properties are largely thought to be mediated by inhibition of apoptosis. GSK3 $\beta$  is required for cell survival during development, as *GSK3 $\beta$ <sup>-/-</sup>* mice die at embryonic day 13.5 due to hepatocyte apoptosis [25]. This phenotype is attributable to the function of GSK3 $\beta$  in activation of NF- $\kappa$ B to inhibit tumor necrosis factor (TNF)-induced apoptosis [25]. GSK3 $\beta$  was also found to prevent apoptosis in a number of human cancer cell lines, further supporting an oncogenic role of GSK3 $\beta$  in cancer [26-28]. Moreover, in cancers such as those of the colon [27], pancreas [29], and kidney [30]. GSK3 $\beta$  is often overexpressed, suggesting its oncogenic association. Finally, it has been suggested that the effects of GSK3 $\beta$  on glycogen production may elicit positive effects on cancer cell proliferation and survival, further suggesting an oncogenic function [31].

### **Role of GSK3 $\beta$ In Pancreatic Cancer**

The functions of GSK3 $\beta$  in the normal pancreas are seemingly distinct from the functions of GSK3 $\beta$  in pancreatic cancers which, in the vast majority of cases arise from epithelial cells within the exocrine system. In contrast, in the normal pancreas GSK3 $\beta$  plays an important role in the endocrine system, specifically in the beta cells of the islets. Here, GSK3 $\beta$  is known to negatively regulate beta cell DNA synthesis, cell cycle progression, and cell mass, as conditional knockout or pharmacological inhibition of GSK3 $\beta$  promotes these activities [32,33]. In pancreatic cancers GSK3 $\beta$  protein expression is increased relative to non-neoplastic pancreatic tissues, which varies throughout disease progression. Both nuclear and cytoplasmic GSK3 $\beta$  are overexpressed in pancreatic cancer cells as compared to normal pancreatic ductal and acinar cells. Specifically, increased expression of cytoplasmic GSK3 $\beta$  occurs in early PanIN lesions and in well/moderately -differentiated pancreatic cancer

tissues [29]. Intriguingly, with disease progression to less differentiated phenotypes, the GSK3 $\beta$  expression pattern shifts from cytoplasmic to nuclear with GSK3 $\beta$  kinase activity contributing to its nuclear localization [29]. This dynamic expression pattern of GSK3 $\beta$  in pancreatic cancer development implicates different functions of GSK3 $\beta$  during disease progression, with GSK3 $\beta$  acting in the cytoplasm during the early stage of tumorigenesis (initiation, proliferation and transformation) and in the nucleus during later stages of disease (proliferation, survival and metastasis) [29]. Furthermore, overexpression of GSK3 $\beta$  was found to be partially attributable to K-RAS-mediated transcriptional upregulation of GSK3 $\beta$  [34]. Subsequent studies have found that pancreatic tumor tissues have higher levels of active GSK3 $\beta$  (pGSK3 $\beta$  Y216), but lower levels of inactive GSK3 $\beta$  (pGSK3 $\beta$  S9) as compared to non-neoplastic tissues [35,36]. However, whether GSK3 $\beta$  expression correlates with patient survival remains to be determined and will be an important aspect for further defining the role of GSK3 $\beta$  in pancreatic cancer. Despite these seemingly oncogenic properties of GSK3 $\beta$  in pancreatic cancer, established tumor suppressor functions in other cancers [12] as well as recent data regarding the role of GSK3 $\beta$  in radiation survival (described below) [37] suggest that GSK3 $\beta$  may have some tumor suppressor functions in pancreatic cancer as well.

### Promotion of Chemotherapy Sensitivity by GSK3 $\beta$ Inhibition

The aberrant expression and disease-promoting activity of GSK3 $\beta$  indicates that it may serve as a therapeutic target in pancreatic cancer. Indeed, several studies have found that GSK3 $\beta$  inhibition reduces cell proliferation, increases cell apoptosis and sensitizes cells to death by different stimuli (Table 1) [26,38,39]. These phenotypes resulting from GSK3 $\beta$  inhibition are attributable to a variety of mechanisms including disruption of NF- $\kappa$ B-mediated survival [26,40] activation of tumor suppressors (p53 and Rb) [41], increased expression of the proapoptotic factor Bim [42], and inhibition of human telomerase reverse transcriptase and telomerase [35]. It has been reported that GSK3 $\beta$  is involved in NF- $\kappa$ B mediated cell survival and acts as a tumor promoter [26,40]. Consistent with this observation, a decrease in cellular GSK3 $\beta$  or pharmacologic inhibition of GSK3 $\beta$  activity reduced tumor cell survival and proliferation. GSK3 $\beta$  inhibition by the small molecule AR-A014418 sensitized pancreatic cancer cells to apoptosis induced by TRAIL (tumor necrosis factor-related apoptosis inducing ligand) both *in vitro* and in tumor xenografts [38]. In addition, AR-A014418 was shown to sensitize the Panc-1 pancreatic cell line model to gemcitabine both *in vitro* and *in vivo* [36,39]. The role of GSK3 $\beta$  in chemotherapy resistance, however, is controversial, as Mamaghani et al. [43] reported an overall lack of synergy and in some cases even antagonism between GSK3 $\beta$  inhibition (by AR-A014418) and gemcitabine in a panel of 6 pancreatic cancer cell lines. Further investigation is needed to determine the nuances of GSK3 $\beta$  inhibition as a means of sensitizing pancreatic cancer cells to chemotherapy.

### Promotion of Radiation Therapy Resistance by GSK3 $\beta$ Inhibition

Given the importance of radiation therapy in the management of locally advanced pancreatic cancer, the role of GSK3 $\beta$  in radiation survival has been investigated [37]. Our previous work has shown that radiation inhibits GSK3 $\beta$  activity by inducing Ser9 phosphorylation, while protein kinase C $\beta$  (PKC $\beta$ ) inhibition by enzastaurin prevents radiation-induced Ser9

phosphorylation and causes radiosensitization of pancreatic cancers both *in vitro* and *in vivo* [44]. Furthermore, inhibition of GSK3 $\beta$  by genetic or pharmacological inhibitors (shRNA or Lithium Chloride, respectively) causes radioresistance of pancreatic cancer cells and pancreatic tumor xenografts (BxPC-3, Panc-1) [37]. This observation has been confirmed in additional studies which demonstrate that siRNA-mediated inhibition of GSK3 $\beta$  causes radiation resistance in Panc-1 and to a lesser extent in AsPC-1 pancreatic cancer cells (Figure 2). In addition, introduction of a non-degradable point mutant of  $\beta$ -catenin (S33Y) mimicked the effects of GSK3 $\beta$  inhibition on radiation survival in pancreatic cancer cells [37]. Mechanistic studies revealed that radioresistance modulated by GSK3 $\beta$  inhibition is partly attributable to activation of the Wnt/ $\beta$ -catenin pathway as radioresistance is associated with stabilization of  $\beta$ -catenin and upregulation of  $\beta$ -catenin target genes [37]. These results suggest that radiosensitization of pancreatic cancer would be achieved by the activation of the Wnt/GSK3 $\beta$  axis to trigger  $\beta$ -catenin degradation, rather than by the inhibition of GSK3 $\beta$  activity. In contrast, another study found that inhibition of GSK3 $\beta$  by AR-A014418 produced modest radiosensitization in pancreatic cancer cells (Panc-1 and MiaPaCa-2) although this effect was not confirmed by an independent method of GSK3 $\beta$  inhibition or in animal models [36]. Taken together, it appears that radiation resistance in response to pharmacological inhibition of GSK3 $\beta$  is variable and may be dependent on the specific GSK3 $\beta$  inhibitor used. However, there is general agreement that genetic inhibition of GSK3 $\beta$  produces radiation resistance warranting detailed future investigations, including the use of genetically modified mouse models (e.g. Kras<sup>G12D</sup> pancreatic ductal adenocarcinoma model [45]) with targeted manipulation of GSK3 $\beta$ .

### Mechanisms Contributing to Radiation Survival Following GSK3 $\beta$ Inhibition

To further elucidate the mechanism by which GSK3 $\beta$  inhibition confers radiation resistance, we conducted a DNA microarray screen and found that the hepatocyte growth factor/scatter factor (HGF/SF), the ligand for the c-MET receptor was significantly upregulated after GSK3 $\beta$  silencing (Figure 3). While others have shown that c-MET is transcriptionally regulated by  $\beta$ -catenin [46], there are no prior reports of GSK3 $\beta$ / $\beta$ -catenin modulation of HGF. Upon HGF binding, c-MET forms a homodimer and undergoes auto-phosphorylation at tyrosine residues 1234 and 1235 leading to activation of c-MET and downstream effectors such as RAS, focal adhesion kinase (FAK), and AKT, ultimately stimulating proliferation, survival, motility and invasion [47,48]. Deregulation of the HGF/c-MET axis occurs frequently in cancers, including those of the pancreas as a result of activating mutations, overexpression, as well as aberrant secretion (both autocrine and paracrine) of the HGF ligand [47,49]. While c-MET is not frequently mutated in pancreatic cancer, it is overexpressed at the protein level in a subset of pancreatic cancers and its activity is further promoted by HGF secreted from tumor-associated fibroblasts [50]. In addition, we found that pancreatic cancer cells are capable of autocrine-mediated c-Met activation which is increased upon GSK3 $\beta$  silencing (Figure 4A).

While c-MET has been shown to regulate radiation survival in other types of cancer [51,52], its role in pancreatic cancer has not previously been identified although irradiated fibroblasts were shown to induce c-Met activity in pancreatic cancer cells [53]. Consistent with HGF upregulation, we found phosphorylation of c-MET to be increased in response to GSK3 $\beta$

depletion in AsPC-1 pancreatic cancer cells (Figure 4A). Taken together, these findings warrant further investigation of the interactions between GSK3 $\beta$  and HGF/c-MET as well as their effects on radiation survival in pancreatic cancer.

Other potential mechanisms which may contribute to radioresistance in response to GSK3 $\beta$  inhibition include accumulation of GSK3 $\beta$  substrates such as RAS,  $\beta$ -catenin, and Snail which undergo ubiquitin-mediated degradation following GSK3 $\beta$ -mediated phosphorylation. Oncogenic activation or overexpression of RAS confers intrinsic radiation resistance in many human cancer cells [54]. Recently, a study indicated oncogenic K-RAS induces expression of an oncogenic kinase, Pim-1 and thus modulates radioresistance in pancreatic cancer [55]. Snail is a key transcription factor involved in Epithelial-Mesenchymal Transition (EMT) [56] which promotes EMT by transcriptional repression of E-Cadherin [57]. Through a mechanism similar to  $\beta$ -catenin destruction, GSK3 $\beta$  phosphorylates the degron of Snail, promoting subsequent  $\beta$ -TrCP-mediated degradation [56]. Therefore, inhibition of GSK3 $\beta$  blocks the phosphorylation and degradation of Snail, which in turn suppresses E-Cadherin expression thus promoting EMT [56]. Indeed, we found that depletion of GSK3 $\beta$  in Panc-1 cells results in the decreased expression of E-Cadherin and also consistent with EMT, a modest increase in N-cadherin (Figure 4B). Since EMT is associated with cancer stem cell-like properties and resistance to chemo- and radiation therapy [58], downregulation of E-cadherin in response to GSK3 $\beta$  depletion may represent yet another potential mechanism for radiation resistance.

## Conclusions

The aberrant expression and elevated kinase activity of GSK3 $\beta$  in pancreatic cancer implicate its role as an oncogene. However, GSK3 $\beta$  also possesses many well-established tumor suppressor properties. While GSK3 $\beta$  is emerging as a therapeutic target in pancreatic cancer, some issues remain unresolved regarding the use of GSK3 $\beta$  inhibitors as a therapeutic strategy for the treatment of pancreatic cancer. First, activation of oncogenic signaling pathways (e.g.  $\beta$ -catenin, K-RAS) upon GSK3 $\beta$  inhibition may have detrimental effects in both tumor and normal cells, the latter of which could potentially result in transformation of normal cells. Second, our studies strongly suggest that targeting GSK3 $\beta$  renders pancreatic cancer cells resistant to radiation. This raises concerns in the context of combining GSK3 $\beta$  inhibitors with chemoradiation in pancreatic cancer. As GSK3 $\beta$  participates in a complex signaling network in which our current understanding is still incomplete, the development of GSK3 $\beta$  targeted agents for pancreatic cancer should progress with careful evaluation of both the potential oncogenic and tumor suppressor roles of GSK3 $\beta$ .

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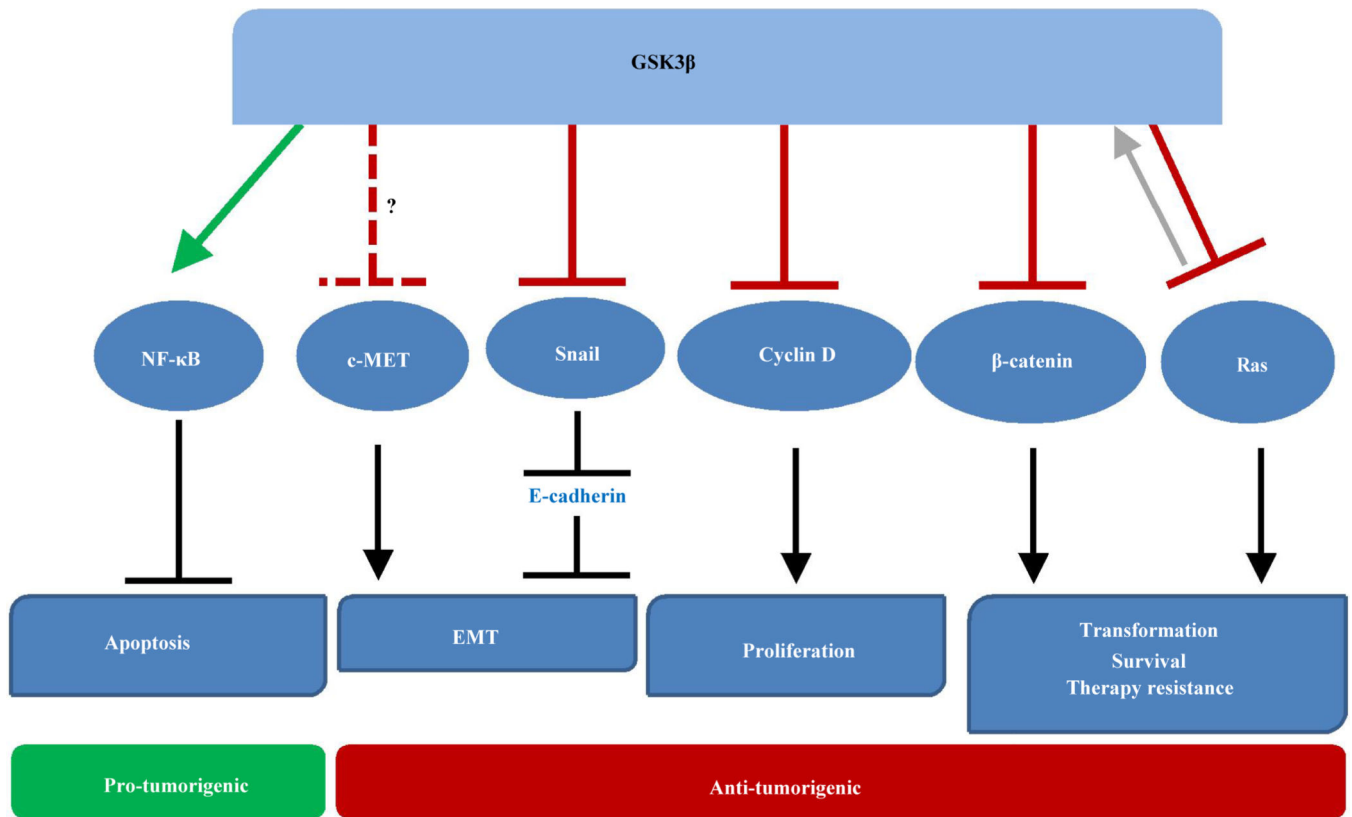
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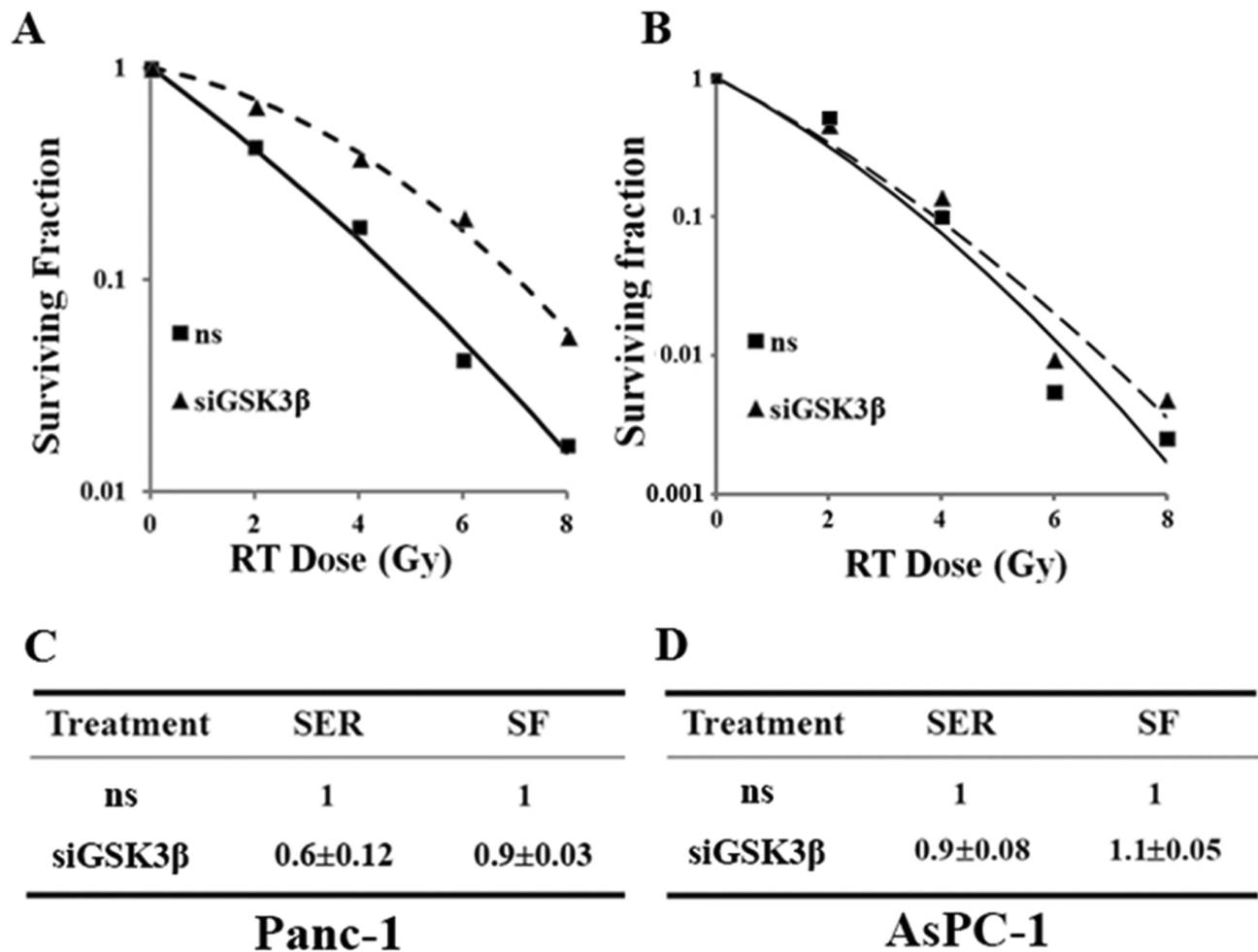
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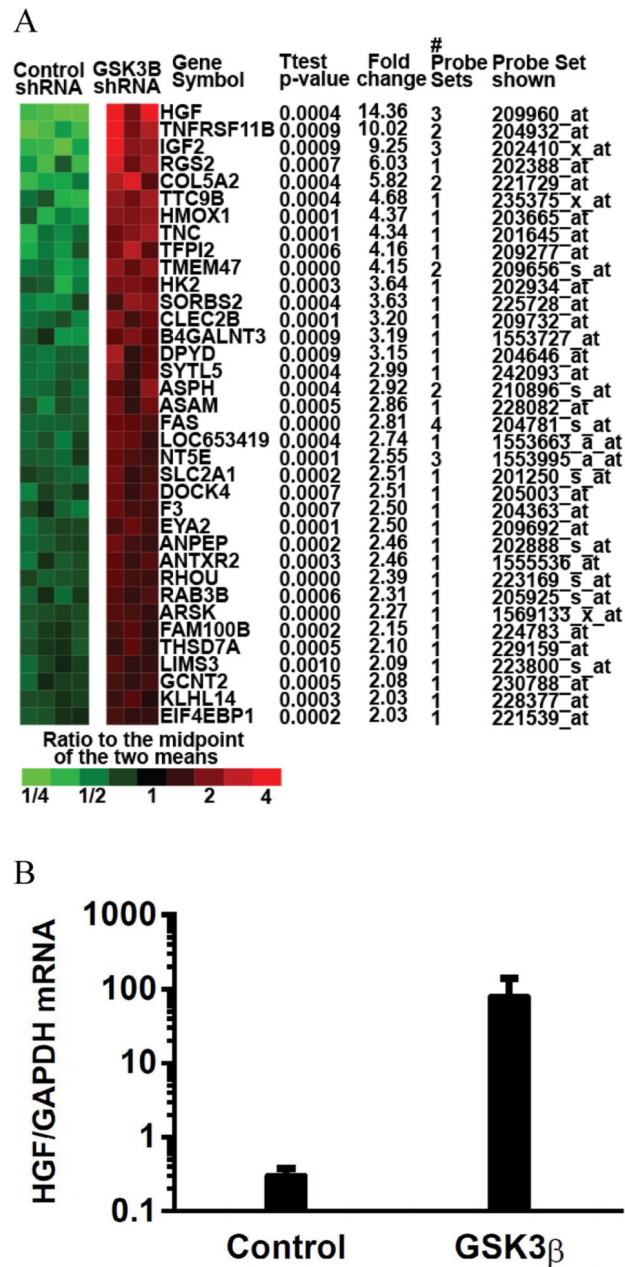
**Figure 1.**

The functions of GSK3 $\beta$  in pancreatic cancer. Active GSK3 $\beta$  has both tumor promoting and tumor inhibiting activities shown in green and red, respectively. GSK3 $\beta$  positively regulates NF- $\kappa$ B activity, resulting in inhibition of apoptosis and survival. On the other hand, GSK3 $\beta$  directly targets multiple proteins with oncogenic properties (RAS,  $\beta$ -catenin, Cyclin D, and Snail) for ubiquitin-mediated degradation. Data presented in this review also suggest a putative role for GSK3 $\beta$  in negatively regulating c-MET.



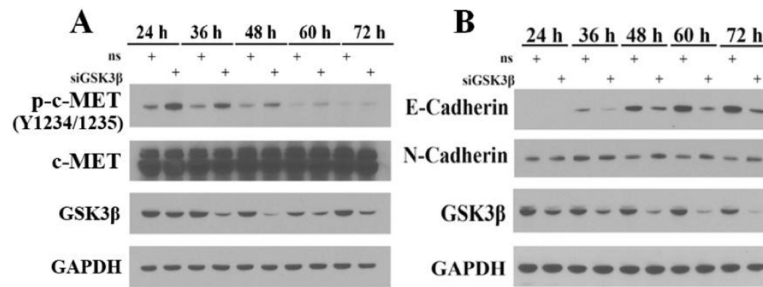
**Figure 2. GSK3β silencing causes radiation resistance in pancreatic cancer cells**

Panc-1 (A, C) and AsPC-1 (B, D) cells were transfected using Oligofectamine (Invitrogen) with ns (non-specific) or GSK3β siRNA (60 nM; Dharmacon) and 48 hours post-transfection were treated with ionizing radiation (RT; 0-8 Gy). Twenty-four hours later, cells were seeded in 60-mm dishes in triplicate for clonogenic survival. After 10 to 12 days of growth, colonies with more than 50 cells were scored. C, D, The Sensitization Enhancement Ratio (SER) was calculated from the radiation survival curves (A,B) as the ratio of the mean inactivation dose (according to the methods of Fertil et al. [59]) for ns siRNA-treated cells to siGSK3β-treated cells and are the mean ± SD of n=2 independent experiments. SER values <1 indicate radiation resistance. Cytotoxicity from siGSK3β treatment alone (in the absence of radiation) is expressed as the Surviving Fraction (SF).



**Figure 3.** Changes in gene expression in response to GSK3 $\beta$  silencing. A, Gene expression changes were determined in response to control or GSK3 $\beta$  shRNA in 3-4 Panc-1 tumors per treatment condition using an Affymetrix DNA microarray. Shown are distinct genes increased by an average of at least 2-fold relative to control shRNA with 2-sample t-test p-values of <0.001. The #probe sets indicate the number of probe sets used for each qualified gene. HGF was found to be the most up-regulated gene in response to GSK3 $\beta$  silencing. Microarray data, methods, and analysis have been deposited in the NCBI Gene Expression Omnibus [60] and are accessible through GEO Series accession number GSE35351 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35351>). B, Independent analyses using

quantitative PCR confirmed increased HGF expression in response to GSK3 $\beta$  shRNA in Panc-1 cells. Data are the mean of  $n=2 \pm SE$  independent experiments performed at least in duplicate..



**Figure 4.**

Inhibition of GSK3 $\beta$  induces c-MET activation and decreases E-Cadherin expression. AsPC-1 (A) and Panc-1 (B) cells transiently transfected with non-specific (ns) or GSK3 $\beta$  siRNA were collected at different time points following transfection and analyzed by immunoblotting. Membranes were probed with antibodies recognizing p-c-MET, GSK3 $\beta$ , c-MET, GAPDH (Cell Signaling), E-Cadherin, and N-Cadherin (BD Bioscience). Data are from a single representative experiment.

**Table 1**Consequences of GSK3 $\beta$  inhibition in pancreatic cancer cells.

Inhibitor	Cell type	Consequences	Reference
AR-A014418	Panc-1	Decreased proliferation and tumor growth, gemcitabine sensitization	39
AR-A014418	Panc-1, MiaPaCa-2, BxPC-3	Decreased proliferation, gemcitabine sensitization ( <i>in vitro</i> & <i>in vivo</i> ), radiosensitization, decreased migration & invasion	36
siGSK3 $\beta$ & AR-A014418	MiaPaCa-2, Panc-1	Decreased NF $\kappa$ B activity, caspase-3 cleavage, decreased proliferation	40
AR-A014418	BxPC-3, Panc-1, HPAC, PK-1, PK-8	Decreased proliferation, no gemcitabine sensitization (except Panc-1)	43
AR-A014418	Panc-1, BxPC-3	Increased TRAIL-induced apoptosis ( <i>in vitro</i> & <i>in vivo</i> )	38
AR-A014418, shGSK3 $\beta$ , SB216763	MiaPaCa-2, BxPC-3	Decreased proliferation, increased apoptosis	26
shGSK3 $\beta$ , LiCl	BxPC-3 & Panc-1	Resistance to radiation ( <i>in vitro</i> & <i>in vivo</i> )	37
siGSK3 $\beta$	Panc-1	Resistance to radiation, increased HGF, c-Met activation	[Figure 4]