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Kinase Activity is Required for Growth Regulation but not Invasion Suppression by Syk Kinase in Pancreatic Adenocarcinoma Cells

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1. Introduction

Syk (spleen tyrosine kinase) is a nonreceptor tyrosine kinase containing two tandem amino-terminal SH2 domains, followed by an extended linker region and a carboxy-terminal kinase domain (Sada et al., 2001). Tyrosine-352 (Y352) in the linker region is trans-phosphorylated by src family members, promoting the activation of syk (Kimura et al., 1996; Sada et al., 2001). Activation further involves the autophosphorylation of syk on tyrosines 525 and 526 (YY525/6) in the activation loop of the kinase domain, which promotes substrate-specific catalytic activity and is required for signaling by syk (Sada et al., 2001; Zhang et al., 1998); this modification is thus indicative of the functional enzyme. The active form of syk then localizes to appropriate substrates or bridging molecules primarily through interactions with its SH2 domains and linker tyrosines (Kimura et al., 1996; Sada et al., 2001).

Syk has been identified as a putative tumor suppressor in human breast cancer since reintroduction of syk retarded the growth of syk-negative breast cancer cells, and suppression of endogenous syk enhanced the tumorigenic phenotype of the resulting cells (Coopman et al., 2000). Loss of syk correlates with poor survival and metastasis of breast cancer in patients (Toyama et al., 2003), and syk regulates breast cancer cell mitosis (Zyss et al., 2005) and transcription (Wang et al., 2005). Previously we identified syk as being a pancreatic ductal adenocarcinoma (PDAC) tumor suppressor. Syk is uniformly expressed by normal pancreatic ductal epithelium and well-differentiated (grade1; G1) PDAC; however moderately-differentiated (grade2; G2) PDAC demonstrates progressive loss of syk, and high-grade (grade3; G3), poorly-differentiated lesions are essentially devoid of syk *in situ* (Layton et al., 2009). In fact, syk expression is a strong positive indicator of patient survival (Layton et al., 2009). Mechanistically, we demonstrated that syk is a central mediator of phenotypic changes regulating PDAC progression, including anchorage-independent growth, cellular invasion, and gene expression changes responsible for epithelial-mesenchymal transition (Layton et al., 2009). To assess the utilization of syk in pancreatic ductal cells, we examined phosphorylation states of syk that correspond to upstream

activation (tyrosine-352) and catalytic activity (tyrosines-525/6). We further examined the activity of syk and the requirement for syk kinase activity in regulating cell growth and invasion. Herein we show that although blockade of syk activity suppressed growth of endogenously syk-positive PDAC cells *in vitro*, kinase activity is not required for syk-dependent regulation of PDAC cell invasion. Phosphorylation of tyrosine-352 and tyrosines-525/6 is detectable in pancreatic ductal epithelial cells *in situ* and *in vitro*, and inhibition of syk kinase activity specifically retards the growth of endogenously syk-positive PDAC cells *in vitro*. In contrast to our previous demonstration that syk regulates the invasion of αvβ3-positive Panc1 PDAC cells by attenuation of the matrix metalloproteinase-2 axis (Layton et al., 2009), invasion of αvβ3-negative MIAPaCa2 and BxPC3 PDAC cells is metalloproteinase-independent and involves the urokinase/plasminogen system, which is regulated by syk in these cells. Thus we demonstrate that endogenous syk is active in pancreatic ductal epithelial cells, and that syk kinase activity is required for growth regulation, but not invasion suppression, by syk in this cell type.

2. Materials and methods

2.1 Cell lines and transfection

CAPAN2(G1), CFPAC1(G2), AsPC1(G2), BxPC3(G2), Panc1(G3) and MIAPaCa2(G3+) cells were originally from ATCC and cultured accordingly. pCDNA3.1/IH/syk^{wt} encoding myc-tagged human syk linked to a hygromycin phosphotransferase gene through an IRES was described previously (Layton et al., 2009). Kinase-dead (KD) syk was produced by site-directed mutagenesis of lysine-402 in the ATP-binding pocket of pCDNA3.1/IH/syk^{wt} using the following primers (K402R-FWD: 5′-GTGAAAACCGTGGCTGTGAGAATACTGAAAAACGAGGC-3′; K402R-REV: 5′-GCCTCGTTTTTCAGTATTCTCACAGCCACGGTTTTCAC-3′). MIAPaCa2 cells were transfected using Lipofectamine2000 (Invitrogen, Carlsbad, CA). BxPC3 cells were electroporated as described previously (Layton et al., 2009). Stable populations were hygromycin-selected and assessed for protein expression. For transient studies, pEF4-LacZ reporter (Invitrogen, Carlsbad, CA) was cotransfected, and cells stained with x-gal to identify and specifically quantitate transfected cells; average MIAPaCa2 transfection efficiency >95%. In all cases, serum-free (SF-) media consisted of all components except serum, as appropriate for the cell line, supplemented with 0.5% bovine serum albumin (BSA).

2.2 Antibodies and reagents

Anti-syk mAb 4D10 and anti-erk2 pAb C14 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-syk phospho-Y352 and anti-syk phospho-YY525/6 pAbs were from Cell Signaling (Beverly, MA). Anti-phosphotyrosine mAb 4G10 and anti-myc tag mAb 4A6 were from UBI (Lake Placid, NY). Function-blocking anti-integrin antibodies LM609 (αvβ3) and PIF6 (αvβ5) and the MMP inhibitor *N*-(*R*)-[2-(Hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-napthylalanyl-L-alanine, 2-aminoethylamide (TAPI-1) were from Chemicon/EMD (San Diego, CA). Function-blocking anti-uPA mAb 3689 was from American Diagnostica (Stamford, CT). HRP- and FITC-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). Nonspecific mouse IgG1 antibody MOPC-21, purified rabbit IgG, piceatannol (3,4,3′,5′- tetrahydroxy-*trans*-stilbene), Crystal Violet and other chemicals were from Fisher Scientific (Pittsburg, PA).

2.3 Immunohistochemistry

Patient tissue samples were obtained under approved UCSD Institutional Review Board protocol from the UCSD Dept. of Pathology archives and stained essentially as described previously (Layton et al., 2009). Briefly, samples were deparaffinized with xylenes, rehydrated through sequential alcohols (100%, 95%, 70%, 50%), and incubated with 1% H_2O_2 to inactivate endogenous peroxidases. Slides were quenched with 50 mM glycine, and blocked with 2% horse serum/5% BSA/phosphate-buffered saline (PBS), pH7.4, before renaturing for 20min in a steamer using Target Retrieval Solution (DAKO North America; Carpinteria, CA). Slides were allowed to cool and then incubated with the appropriate primary antibody overnight at 4˚C. Slides were washed and biotinylated-anti-rabbit applied according to the VectaStain Elite ABC Kit (Vector Labs; Burlingame, CA, USA). Sections were developed with DAB, counterstained with hematoxylin, dehydrated through sequential alcohols and mounted. Brightfield images were acquired on a Nikon TE600 microscope with a Model 3.2.0 CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA) using SpotBasic software at the Moores UCSD Cancer Center.

2.4 Immunoblotting

Immunoblotting was performed essentially as described previously (Chen et al., 2010). Briefly, cells were lysed on the plate in NP40 lysis buffer (50mM Tris pH7.4, 150mM NaCl, 1% NP-40) containing Complete™ Protease Inhibitor Cocktail (Roche, Indianapolis, IN) supplemented with 10mM PMSF, 1mM NaF and 10mM Na_3VO_4. Samples were separated by SDS-PAGE under reducing conditions and electroblotted to a PVDF membrane. Membranes were blocked with 10% nonfat dry milk in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and incubated 2h to overnight in 2% milk/TBS-T with the indicated primary antibody. Primary antibody was detected with HRP-conjugated secondary antibody, and complexes were visualized by enhanced chemiluminescence with PS-3 (Lumigen, Inc.; Southfield, MI).

2.5 Flow cytometry (FACS)

FACS was performed on a FACScalibur (BD Biosciences, Bedford, MA) at the Moores UCSD Cancer Center Flow Cytometry Shared Resource as described previously (Chen et al., 2010). Cells were harvested with 0.1% trypsin/versene, inactivated with 0.1% soybean trypsin inhibitor and resuspended in FACS buffer (1mM $MgCl_2$, 1mM $CaCl_2$, 0.1% NaN_3 0.5% BSA in PBS pH 7.4) before sequential labelling with primary and FITC-conjugated secondary antibodies. Gates were set with secondary alone, and 5µg/ml propidium iodide was included to exclude dead and dying cells.

2.6 Proliferation assays

Cell growth was assessed with the CellTiter96 Aqueous One Solution Cell Proliferation Assay (MTS) kit (Promega, Madison, WI), or as follows: cells ($5x10^2$/well) were seeded into a 48-well plate. After 24 hours (and every 72 hours thereafter), fresh growth medium was replaced and the initial time point fixed with 1% paraformaldehyde/PBS, pH7.4. Additional triplicate wells were fixed at 24 hour intervals. All wells were stained with 1% Crystal Violet, which was subsequently extracted with 10% acetic acid, quantitated at 550nm and

compared to a standard curve of cells. For proliferation assays in the presence of the syk inhibitor, piceatannol, a titration of cells was plated and allowed to adhere for 24 hours before fresh growth medium (full serum) containing the indicated concentrations of piceatannol or DMSO vehicle was replaced. Medium was replenished in the same manner every other day for a total of 3 treatments, and the cells were fixed and stained with Crystal Violet 24h after the last treatment. Dye was extracted with 10% acetic acid and quantitated at 550nm.

2.7 Anchorage-independent growth

Anchorage-independent growth was assessed as described previously (Layton et al., 2009). Briefly, a top layer containing $5x10^3$ cells in 0.5% agar/DMEM/10%FBS was seeded onto a base layer of 0.7% agar/DMEM containing 10% FBS in a 6 well plate. Cultures were incubated at 37°C, media was replaced every 3rd day, and the assay stopped on day 10. Cultures were stained with 0.01% Crystal Violet and colonies were enumerated on a Bio-Rad GelDocXR using QuantityOne Software (Sensitivity=8.1, Average=5).

2.8 Invasion assay

Invasion assays were performed as described previously (Layton et al., 2009) using BioCoat Growth Factor-Reduced Matrigel Invasion Chambers (BD Biosciences, Bedford, MA, USA). Briefly, cells were applied in SF-media to the upper chamber with or without 15min preincubation with TAPI1 (40µg/mL), aprotinin (100µg/mL), anti-uPA monoclonal antibody 3689 (25µg/mL), nonspecific IgG_1 control antibody MOPC-21 (25µg/ml), or an equal volume of DMSO or PBS control included in both chambers. SF-media or growth media was provided in the lower chamber and cells were allowed to invade for 24h before removal of uninvaded cells, and enumeration of invaded cells.

2.9 Reverse Transcription (RT)-PCR

cDNA was synthesized from 1µg of total RNA using oligo-dT primer. PCR was performed on 1µL of resulting cDNA using primers described previously (Layton et al., 2009; Leissner et al., 2006). GAPDH primers were from Stratagene (San Diego, CA) and served as internal controls. Densitometry was performed on unadjusted images using NIH *Image* 1.61 software and GAPDH as reference control.

2.10 Statistics

Experiments were performed in triplicate and independently repeated at least twice. Data shown are mean $\pm$ standard deviation unless otherwise indicated. Colony-formation and cellular invasion were analyzed by two-tailed Students *t*-Test.

3. Results

3.1 Endogenously–expressed syk is functional in pancreatic ductal cells in situ and in vitro

Previously we demonstrated the expression of syk in normal pancreatic ductal epithelial cells *in situ* (Layton et al., 2009). In order to assess whether this endogenous syk is

functionally active, we performed immunohistochemistry with antibodies specific for the phosphorylated form of Y352, which is representative of syk activation by upstream mediators, or YY525/6, which is indicative of substrate-specific activity of the syk kinase domain (Kimura et al., 1996; Sada et al., 2001; Zhang et al., 1998). These antibodies react with both isoforms of syk and do not recognize more distantly related src family members (Cell Signaling Technology, Beverly, MA). Strong staining for both phospho-Y352 (Fig.1A-C) and phospho-YY525/6 (Fig.1D-F) was observed in individual cells of independent ducts as well as ductules associated with acinar clusters. No staining was observed in the absence of primary antibody or when purified rabbit IgG was used as a control (not shown).

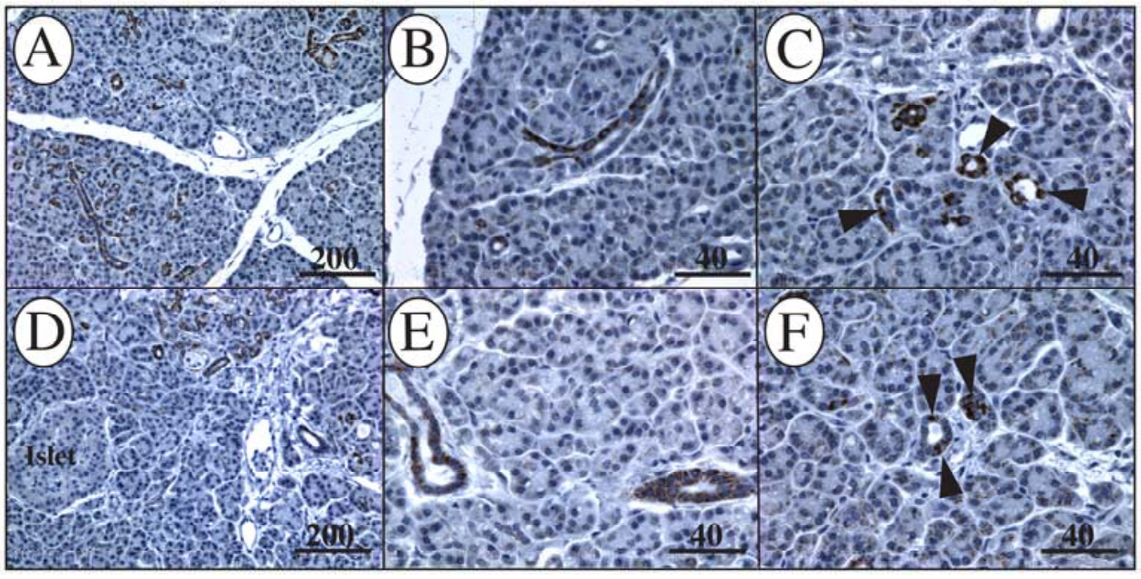

Fig. 1. **Syk is functional in pancreatic ductal cells *in situ*.** Immunohistochemical staining (brown-black) of normal pancreas with phospho-Y352 **(A-C)** and phospho-YY525/6 **(D-F)**. Arrowheads in (**C,F**) denote nuclear staining. Scale bars in μm.

Nuclear staining was observed in a subset of the duct cells (arrowheads in Fig.1C,F), consistent with the detection of nuclear syk in pancreatic ductal cells *in situ* (Layton et al., 2009) and previous reports of syk nuclear translocalization (Wang et al., 2003) and regulation of cell division (Zyss et al., 2005) and transcription (Wang et al., 2005). Of the 20 samples analyzed, all showed evidence of syk activation in a subset of ductal cells and none showed widespread constitutive phosphorylation at either site, demonstrating the active regulation of syk activation and activity in ductal cells of the normal human pancreas and suggesting an active role for syk in regulating the phenotype of this cell type.

We also previously established the expression of syk in well- to moderately-differentiated PDAC cells (CAPAN2-G1, CFPAC1-G2, BxPC3-G2, AsPC1-G2), to the exclusion of poorly-differentiated PDAC cells (Panc1-G3, MIAPaCa2-G3+)(Layton et al., 2009). In order to establish the utilization of syk by these endogenously syk-positive PDAC cells, we analyzed the activation surrogate readout of Y352 and YY525/6 phosphorylation in BxPC3 cells. Serum-starved cells were stimulated with insulin and then lysates immunoblotted with the phospho-syk-specific pAbs used for IHC. Interestingly, both Y352 and YY525/6 are

constitutively phosphorylated under starvation conditions, and dephosphorylated in response to insulin (Fig.2). Similar results were obtained with serum stimulation (not shown), and with endogenously syk-positive CAPAN2 cells (not shown). These data demonstrate the active regulation of syk activity in response to specific signalling pathways in endogenously syk-positive, well-differentiated PDAC cells, consistent with the restricted syk activation observed *in situ* and suggesting that syk is an active participant in regulating the phenotype of these cells.

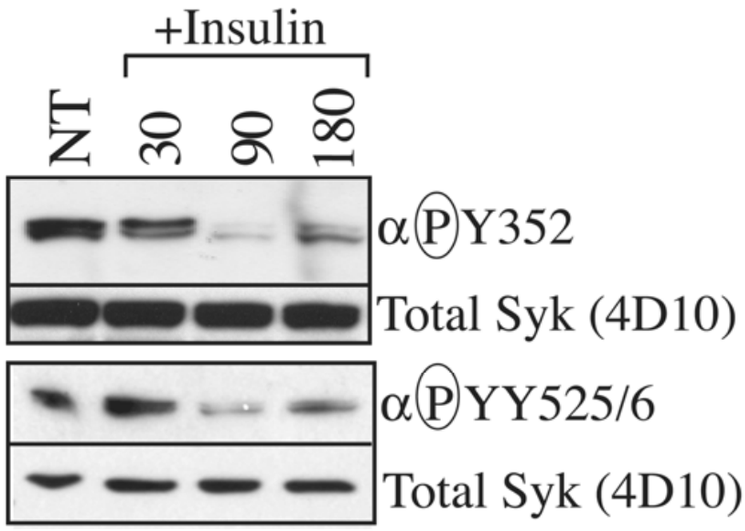

Fig. 2. **Syk is functional in PDAC cells *in vitro*.** Immunoblot of phospho-Y352 and phospho-YY525/6 in BxPC3 cells that had been serum-starved for 24h prior to stimulation with 10ng/ml insulin for the indicated times. NT, no treatment. Total syk levels determined with the 4D10 mAb are shown as a control.

3.2 Syk-dependent regulation of cell growth requires syk kinase activity

Syk regulates proliferation of immune cells in response to specific receptor activation events (Kimura et al., 1996; Sada et al., 2001; Wieder et al., 2001), and ectopic expression of an RFP-tagged syk caused anomalous cell division and mitotic catastrophe in breast cancer cells, where it was observed to interact with γ-tubulin of the mitotic spindle (Zyss et al., 2005). We observed localization of syk to the perinuclear region of dividing PDAC cells (not shown), therefore we assessed whether syk might be involved in regulating the proliferation of endogenously syk-positive PDAC cells in a manner analogous to that described in breast cancer (Coopman et al., 2000). Consistent with such a role, the *in vitro* growth of endogenously syk-positive CAPAN2, CFPAC1, BxPC3 and AsPC1 cells was suppressed in a dose-dependent manner by piceatannol, a phytochemical that inhibits the kinase activity of syk, suppressing downstream phosphorylation events by a largely unknown mechanism (Geahlen et al., 1989; Ferrigni et al., 1984; Wieder et al., 2001)(Fig.3A). Significantly, the only G1 cells in this group, CAPAN2, were completely eradicated by the highest dose of inhibitor, suggesting cytotoxicity in addition to any cytostatic effect of this compound in these highly differentiated cells.

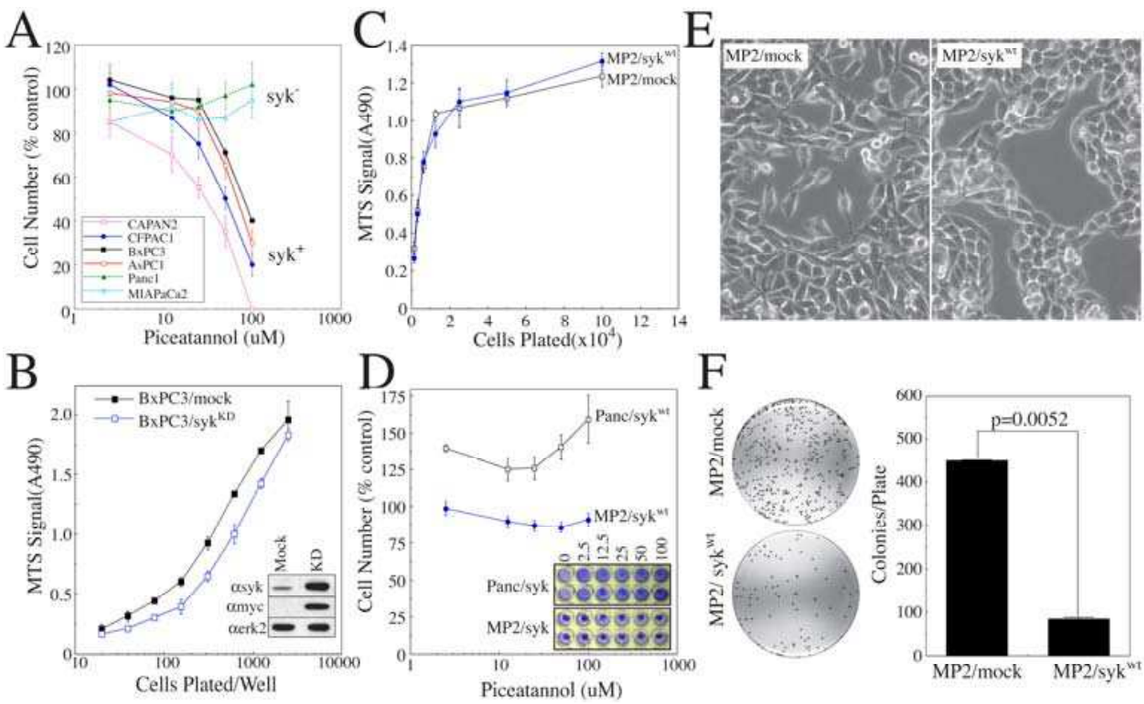

Fig. 3. **Kinase activity is required for syk-dependent regulation of cell growth. (A)** Cell growth of syk-positive versus syk-negative PDAC cell lines after 7 days of piceatannol treatment, plotted as percent of untreated control. **(B)** Proliferation of stable BxPC3/mock and BxPC3/syk^{KD} cells after 5 days. **(B,inset)** Immunoblot of total syk (syk) and the myc-tag (myc) of syk^{KD}. Erk2, loading control. **(C)** Proliferation of MP2/mock and MP2/syk^{wt} cells after 5 days. **(D)** MP2/syk^{wt} and Panc1/syk cell growth assay after 7 days of piceatannol treatment, as in (A). **(E)** Phase contrast images demonstrating the effect of stable syk expression on MIAPaCa2 cell morphology. Both populations were maintained under identical culture conditions including maintenance doses of the selectable marker hygromycin. **(F)** Effect of syk on anchorage-independent growth of MIAPaCa2 cells. A representative example of replicate plates is shown with quantitation points superimposed.

The specificity of this effect to the inhibition of syk is demonstrated by the following facts. First, *trans*-stilbene, which is the base molecule from which piceatannol is derived, had no effect on the proliferation of any of these cells (not shown). Second, syk-negative MIAPaCa2 and Panc1 cells demonstrated no growth response to piceatannol in this assay (Fig. 3A). Third, although piceatannol has been reported to inhibit FAK, src, PI3K and IκBα/NF-κB kinases (Ashikawa et al., 2002; Choi et al., 2010; Law et al., 1999), inhibition of these kinases has been shown to retard the proliferation of both syk-positive and syk-negative lines used in this study (Hering et al., 2007; Hochwald, et al., 2009; Ito et al., 2003; Perugini et al., 2000). Together, these points demonstrate that the antiproliferative effect of piceatannol reported here is likely restricted to the inhibition of syk itself. It should be noted that the concentrations of piceatannol used in this study are based on prior established parameters designed to minimize non-specific effects (Ashikawa et al., 2002; Choi et al., 2010; Geahlen et al., 1989; Law et al., 1999; Seow et al., 2002; Wieder et al., 2001).

To extend these pharmacological inhibitor studies, we stably transfected endogenously syk-positive BxPC3 cells with a kinase-dead syk construct harbouring a point mutation (K402R) in the ATP binding site of the kinase domain (BxPC3/KD cells). This construct has been shown to act as a dominant-negative in endogenously syk-positive cells through an incompletely understood mechanism (Coopman et al., 2000). BxPC3/KD cells demonstrated a consistently reduced growth rate versus mock-transfectants (Fig.3B). Stable expression of wildtype syk did not affect the proliferation rate of these cells (not shown). It should be noted that these engineered cells are non-clonal populations, being the result of a bicistronic system that links transgene expression to drug resistance through an IRES sequence. Moreover, repeated generation of stable populations using this system resulted in stable "lines" that behaved similarly, demonstrating that these data are not artifactual in nature.

Since stable reexpression of syk in G3 Panc1 cells reduced their growth rate *in vitro* (Layton et al., 2009), we questioned whether stable reexpression of syk in MIAPaCa2 (MP2/syk^{wt}) cells would have a similar effect on these G3+ PDAC cells. Unlike Panc1 cells, no growth rate difference was observed between MP2/mock and MP2/syk^{wt} cells in culture (Fig.3C). Moreover, piceatannol did not suppress the growth of MIAPaCa2 or Panc1 cells stably reexpressing syk (Fig.3D), suggesting that PDAC cells that have progressed to syk-negative status are able to bypass the growth requirement for endogenous syk when it is ectopically reintroduced. It should be noted, however, that piceatannol was functional in this assay, since it caused a slight dose-dependent increase in the growth rate of Panc1/syk cells (Fig.3D) that is commensurate with overcoming the minor growth rate reduction that resulted from the original stable reexpression of syk in these cells (Layton et al., 2009). In contrast, however, we did observe an effect of stable syk reexpression on overall MIAPaCa2 morphology similar to that observed in Panc1/syk cells (Layton et al., 2009). As such, MP2/syk^{wt} cells demonstrate increased cell-cell interactions, resulting in more of a traditional monolayer characteristic of epithelial cells in culture, and a reduced propensity for cells to remain separate in between cell clusters (Fig.3E). This suggests the reestablishment of a more differentiated phenotype by syk in these cells. Accordingly, MP2/syk^{wt} cells demonstrate a dramatically reduced ability to grow in an anchorage-independent growth assay (Fig.3F), exhibiting a plating efficiency of 1.7%, versus 9.0% for MP2/mock cells, less dramatic but similar to the effect observed previously in Panc1/syk cells (Layton et al., 2009).

3.3 Stable expression, but not kinase activity, is required for syk-dependent invasion suppression

We previously observed a negative regulation of Panc1 invasion by stable reexpression of syk (Layton et al., 2009). Therefore, we assessed the *in vitro* invasion capabilities of MP2/mock and MP2/syk^{wt} cells. Stable MP2/syk^{wt} cells exhibit 90% less invasion towards serum-containing media compared to MP2/mock, and essentially no invasion in the absence of serum attractant (Fig.4A). To determine whether the kinase activity of syk is required for this effect, we stably expressed the K402R kinase-dead (KD) mutant syk in MIAPaCa2 cells (MP2/syk^{KD}); both wildtype and KD proteins were expressed at equal levels (Fig.4A, inset). Importantly, stable MP2/syk^{KD} cells exhibit nearly identical invasion suppression to MP2/syk^{wt} cells (Fig.4A), demonstrating that kinase activity is not required for syk's invasion-suppressor function in these cells. Consistent with this finding, 48h

pretreatment with piceatannol did not affect the invasion of either endogenously syk-positive BxPC3 cells or stable MP2/syk^{wt} cells (Fig.4B). Additionally, transient transfection with syk^{wt} or syk^{KD} did not retard MIAPaCa2 invasion (Fig.4C), suggesting that long-term expression is required for syk to influence invasion, likely through gene expression changes as described previously in Panc1 cells (Layton et al., 2009).

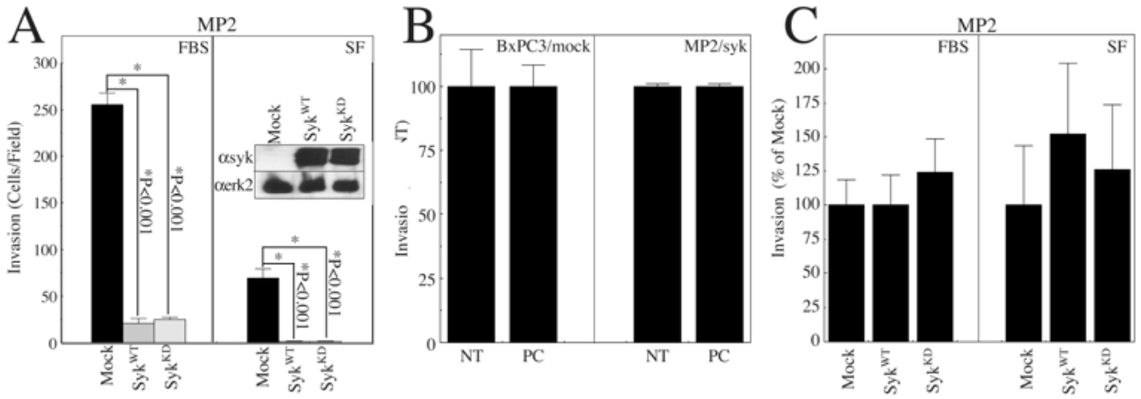

Fig. 4. **Stable expression, but not kinase activity, is required for invasion suppression by syk. (A)** The indicated stable MIAPaCa2 populations were provided serum-containing (FBS) or serum-free (SF) medium in the lower compartment of invasion chambers and allowed to invade for 24h before removal of uninvaded cells and enumeration of invaded cells. **(A,inset)** Immunoblot of ectopic syk expression with the 4D10 mAb (αsyk). Erk2, loading control. **(B)** Cells were pretreated for 48h with 25μM piceatannol (PC) or DMSO vehicle (NT) prior to seeding into invasion chambers with serum-containing media in the lower compartment. **(C)** MIAPaCa2 cells were transiently cotransfected with *lac*Z reporter and empty vector (mock), or wildtype (WT) or kinase-dead (KD) syk and 48h later seeded into invasion chambers. After 24 hours cells were stained with x-gal and invaded transfected cells (blue) were enumerated and plotted as percent of mock.

3.4 MIAPaCa2 invasion is regulated by urokinase–type plasminogen activators

We previously demonstrated that Panc1 invasion is dependent upon the matrix metalloproteinase (MMP)-2 axis, and that syk specifically attenuates the expression of MMP2 and its inhibitor, TIMP2, in these cells (Layton et al., 2009). In contrast to Panc1 cells, the MMP inhibitor TAPI1 did not suppress MIAPaCa2/mock invasion (Fig.5A), and RT-PCR (Fig.5B) and zymography (not shown) showed that MIAPaCa2 cells do not produce MMP2 in culture. MMP2 and MMP9 products were detected in parallel reactions run at the same time on unrelated samples (not shown), demonstrating that lack of signal in these samples is not the result of failed amplification reactions. These results demonstrate that MIAPaCa2 invasion is MMP-independent *in vitro*.

Another key regulator of epithelial cell invasion is the urokinase-type plasminogen activator (uPA)/uPA receptor (uPAR) axis (Leissner et al., 2006; McMahon and Kwaan, 2009), whose components are expressed by MIAPaCa2 cells in culture (Fig.5B). Consistent with the potential involvement of this system, MP2/mock invasion was completely suppressed by the serine protease inhibitor aprotinin (Fig.5C). This effect was not due to toxicity, as

aprotinin-treated cells remained 100% viable after 24h (Fig.5C, inset), which is the duration of the invasion assay. Specifically demonstrating the involvement of the uPA/uPAR axis, MP2/mock invasion was almost completely inhibited by a function-blocking anti-uPA antibody (Fig.5C). An isotype-matched (IgG_1) control antibody (MOPC-21) had no effect (not shown). Importantly, densitometry demonstrated >75% reduction in uPA mRNA levels by both syk^{wt} and syk^{KD} in MIAPaCa2 cells (Fig.5B), suggesting a potential mechanism for syk's effect on MIAPaCa2 invasion. Interestingly, expression of the uPA inhibitor, PAI-1, was also suppressed >30% by both wildtype and kinase-dead syk (Fig.5B). However, the uPA receptor, uPAR, was actually increased ~50% by syk^{wt}, but suppressed by >60% by syk^{KD}, suggesting that kinase activity may be required for appropriate regulation of uPAR, but not uPA or PAI-1.

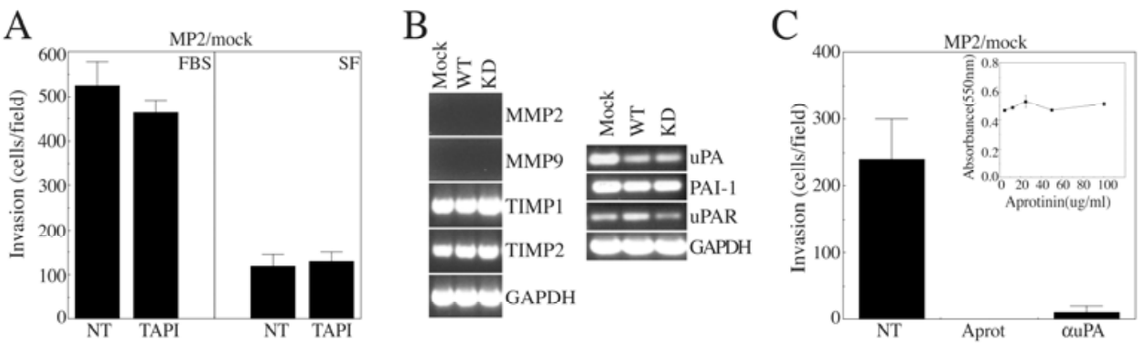

Fig. 5. **MIAPaCa2 invasion is mediated by the uPA/uPAR axis. (A)** MP2/mock invasion towards serum-containing (FBS) or serum-free (SF) medium +/- the MMP inhibitor TAPI1**. (B)** Semi-quantitative RT-PCR from the indicated stable MIAPaCa2 transfectants for the indicated products. GAPDH, control. **(C)** MP2/mock invasion +/- the serine protease inhibitor aprotinin or function-blocking anti-uPA antibody. (**C,inset**) Viability of aprotinin-treated cells after 24h.

Previous studies have demonstrated the regulation of the MMP2 axis by the αvβ3 integrin (Deryugina et al., 2001; Nisato et al., 2005), and an association of the uPA/uPAR axis with the αvβ5 integrin (Yebra et al., 1995; 1996). Moreover, MMP2 activation has been linked to αvβ3 in PDAC (Hosotani et al., 2002), and αvβ3 engagement suppresses the expression of uPA/uPAR components (Hapke et al., 2001). Therefore, to assess whether the different protease dependencies observed in this and our former study (Layton et al., 2009) are related to differential integrin expression, FACS analysis was performed on live cells from standard culture. Consistent with MMP2-dependence, Panc1 cells proved to be strongly αvβ3-positive (Fig.6A). In contrast, MIAPaCa2 cells are αvβ3-negative (Fig.6B), commensurate with their MMP2-independent/uPA-dependent phenotype. These results on cell surface integrin expression were corroborated at the total expression level by immunoblotting of whole cell lysates (not shown). Further demonstrating the integrin-dependent phenotypes of these cells, MIAPaCa2 invasion could be fully suppressed by a αvβ5 integrin-specific function-blocking mAb, while Panc1 invasion also involves αvβ3 and could only be fully blocked by combination of αvβ5- and αvβ3-specific function blocking mAbs (Fig.6C).

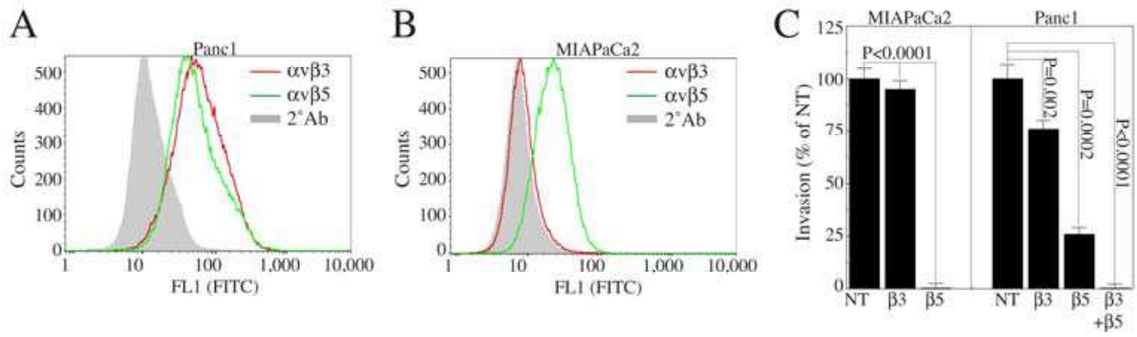

Fig. 6. **The invasion mechanism of MIAPaCa2 and Panc1 cells is related to their integrin expression profiles. (A,B)** FACS analysis of integrin αvβ3 and αvβ5 surface expression in Panc1 (**A**) and MIAPaCa2 cells (**B**). **(C)** Invasion of MIAPaCa2 (left) and Panc1 (right) cells in the presence or absence of 50μg/ml of function-blocking antibodies directed against the indicated integrins.

To further test the role of syk in regulating PDAC invasion, we examined the BxPC3/KD cells, which stably express the dominant-negative kinase-dead syk in addition to their endogenous wildtype syk. While BxPC3/mock cells show marginal invasion towards serum-containing media and essentially no invasion in the absence of serum attractant, stable BxPC3/KD cells demonstrate a >75% increase in invasion towards serum-containing media and significant *de novo* invasion in the absence of serum attractant (Fig.7A). FACS demonstrated that BxPC3 cells are also αvβ3-negative (not shown) and, consistent with this finding, the MMP inhibitor TAPI1 had no effect on serum-free BxPC3/KD invasion (Fig.7B). However, both aprotinin and anti-uPA mAb treatment reduced serum-free BxPC3/KD invasion significantly (Fig.7B), demonstrating a role for the uPA/uPAR axis in the syk-regulated invasion of these endogenously syk-positive PDAC cells. An isotype-matched (IgG_1) control antibody (MOPC-21) had no effect (not shown). It should be stressed that BxPC3/mock cells do not invade under these conditions, thereby allowing us to assess the role of uPA in a phenotype that was dependent upon syk inhibition and allowing us to exclude confounding issues derived from the endogenous phenotype.

4. Discussion

Previously we identified syk as being expressed not only in normal pancreatic ductal epithelium, but also in well- to moderately-differentiated PDAC *in situ* (Layton et al., 2009). The expression of syk in normal ductal epithelium of the breast has also been reported, as has a role for syk as a tumor suppressor in that tissue (Coopman et al., 2000). As such, loss of syk expression in primary breast tumors is associated with a poor prognosis (Toyama et al., 2003). We found that syk similarly correlates with patient survival in PDAC patients (Layton et al., 2009). However, we observe consistent loss of syk expression in poorly-differentiated PDAC, and have not observed loss of syk in well-differentiated PDAC samples. This differs from breast cancer, where syk is absent from a subset of well-differentiated lesions and expressed normally in many poorly-differentiated samples (Toyama et al., 2003), suggesting potentially dramatic differences in the regulation or function of syk in the ductal epithelium of these two glandular tissues. Indeed, we present evidence that syk functions to facilitate growth of PDAC cells that express it. This is perhaps

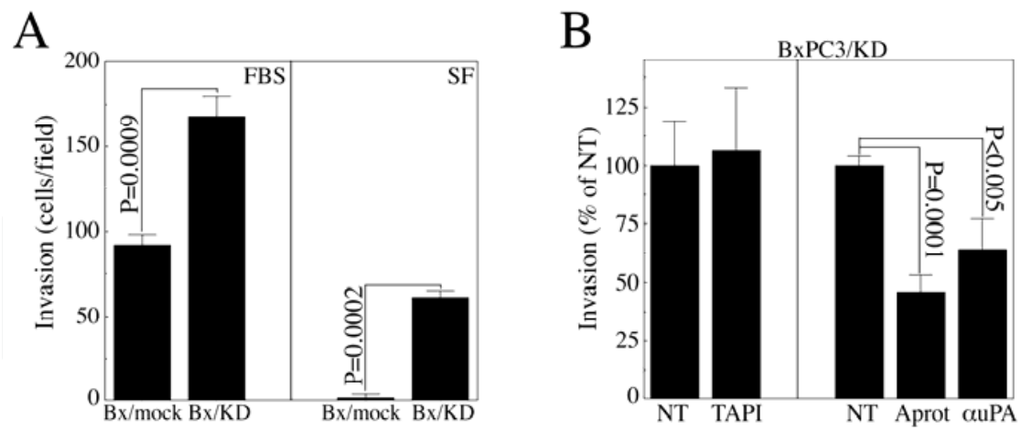

Fig. 7. **BxPC3 invasion is regulated by syk and mediated by the uPA/uPAR axis**.
(A) Invasion of stable BxPC3/mock (Bx/mock) and BxPC3/KD (Bx/KD) cells towards serum-containing (FBS) or serum-free (SF) medium. **(B)** Serum-free BxPC3/KD invasion +/- TAPI1 (left), or aprotinin or function-blocking anti-uPA antibody (right). BxPC3/mock cells were not assessed as they exhibit no invasion in the absence of serum in the lower chamber.

not entirely unexpected since syk promotes immune cell proliferation in response to antigenic stimulation (Kimura et al., 1996; Sada et al., 2001), and vascular defects observed in syk-deficient mice are attributable to a reduction in endothelial cell number (Yanagi et al., 2001). Indeed, syk was subsequently shown to be required for endothelial cell proliferation *in vitro* (Yanagi et al., 2001).

Mechanistically, syk has been shown to regulate mitosis through direct interactions with γ-tubulin and catalytic activity within the centrosome (Zyss et al., 2005). Morphological examination of piceatannol-treated PDAC cells stained with DAPI revealed that aberrant/dysfunctional mitosis may be responsible for the reduced cell numbers observed in this assay (not shown). This finding is consistent with prior ectopic expression studies that demonstrated localization of a carboxy-terminally RFP-tagged syk to centrosomes (Zyss et al., 2005); this chimera caused aberrant mitosis that likely resulted from steric interference of the RFP molecule on the directly adjacent kinase domain, further supporting our contention that syk kinase activity is required for syk-dependent regulation of cell division, and hence growth.

Similarly, since pharmacological inhibition of syk does not affect BxPC3 invasion, but stable expression of syk^{KD} does, this suggests that this effect of syk^{KD} is not due to direct suppression of endogenous syk signaling in these cells. As such, this construct may function by sequestering binding partners that would normally be phosphorylated by catalytically active syk, or a similar mechanism, since this construct can still be phosphorylated by upstream mediators such as src. Indeed, phosphorylation of Y352 couples syk to binding partners such as phospholipaseC and vav through the SH2 domains of these proteins (Sada et al., 2001). Mutation of Y352 impairs signaling in immune cells (Sada et al., 2001), demonstrating that Y352 is an important regulator of syk function in these cells. The presence of phosphorylated Y352 in pancreatic cells *in situ* and *in vitro* suggests that these or similar pathways are functional in syk-mediated processes in this cell type as well.

Proteolytic degradation of the extracellular matrix during tumor progression often involves uPA, its inhibitor, PAI-1, and its receptor, uPAR (Binder and Mihalay, 2008; McMahon and Kwaan, 2009). This system regulates the activation of the serine protease plasmin, and in the pancreas uPAR has been shown to regulate PDAC phenotype through interaction with integrin αvβ5 and PKC signalling (Yebra et al., 1995; 1996). More importantly, studies have shown a correlation between uPA and PAI-1 expression and tumor aggressiveness (Hansen et al., 2003), and PAI-1 has been shown to be necessary for tumor invasion (Binder and Mihaly, 2008). We recently reported that stable reexpression of syk dramatically reduced the invasion of endogenously syk-negative Panc1 cells at least partly by attenuating the MMP2 axis (Layton et al., 2009). Herein, we demonstrate that MIAPaCa2 and BxPC3 invasion is MMP-independent and mediated by the urokinase/plasminogen system, and that syk attenuates the expression of both uPA and PAI-1. This difference in protease utilization may be due to the differential expression and/or involvement of specific integrins since Panc1 cells are αvβ3-positive while MIAPaCa2 and BxPC3 cells are αvβ3-negative. Integrin αvβ3 suppresses uPA/uPAR expression in ovarian cancer cells (Hapke et al., 2001) and, as noted previously, the MMP2 axis has been associated with expression of the αvβ3 integrin and αvβ3 is required for MMP2 activation in some cell types (Deryugina et al., 2001); Nisato et al., 2005), including PDAC (Hosotani et al., 2002). Reciprocally, the uPA/uPAR axis is linked to αvβ5 (Yebra et al., 1995; 1996) and other integrins including α3β1 (Zhu et al., 2009), which is also expressed by both MIAPaCa2 and BxPC3 cells (S. Silletti, unpublished data). Importantly, the differential integrin utilization by these cells extended to the regulation of invasion as well. Therefore, this differential use of protease pathways in an integrin-specific manner by PDAC cells may be useful from a diagnostic and/or prognostic standpoint if further studies bear out this relationship in this tumor type.

In summary, we have further characterized the activity of syk in the growth and invasion regulation of pancreatic ductal epithelial cells. The expression of syk in these cells is associated with phosphorylation of tyrosines indicative of syk activation and activity, both *in vitro* and *in situ*, and syk activity is involved in regulating the proliferation/survival of syk-positive PDAC cells *in vitro*. This effect may be related to syk's role in transducing signals from growth factor receptors, or from syk's regulation of the mitotic spindle during mitosis. Irrespective, there has been a recent surge of interest in inhibitor-based strategies to target syk for conditions such as asthma, rheumatoid arthritis, and other immune disorders (Scott, 2011; Ulanova et al., 2005; Wong et al., 2004). Therefore, we propose that, aside from being a potential regulator of PDAC biology and biomarker of more differentiated PDAC tumors *in situ*, syk may be a viable target for therapeutic intervention in the clinic, since all grades of PDAC are uniformly fatal (NCI PDQ Database [http://www.cnacer.gov/cancertopics/pdq]; NCI-PANC-PRG) and syk expression is maintained in G1 and G2 PDAC *in situ* (Layton et al., 2009).

5. Conclusion

In this report we demonstrate that upstream activated and catalytically active syk kinase states can be detected in pancreatic ductal epithelial cells, both *in vitro* and *in situ*. The activity of syk is further demonstrated with regard to the regulation of cellular growth and invasion. Importantly, we have found that syk-dependent regulation of invasion is via modulation of the urokinase/plasminogen system in αvβ3-negative PDAC cells. This is in

contrast to the MMP-dependent invasion-suppressor effect of syk in αvβ3-positive PDAC cells, which are the exception, rather than the norm in this tumor type. These data provide further evidence of a central role for syk in regulating pancreatic ductal epithelial cell phenotype, and support the potential utility of targeting syk as a potential therapeutic modality in well- to moderately-differentiated PDAC patients, which are the majority of clinical cases. The significance of this is highlighted by the fact that PDAC is characterized by extensive dissemination at the time of diagnosis, irrespective of grade of disease.

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7. References

Ashikawa K, Majumdar S, Banerjee S, Bharti AC, Shishodia S, Aggarwal BB. (2002). Piceatannol inhibits TNF-induced NF-kappaB activation and NF-kappaB-mediated gene expression through suppression of IkappaBalpha kinase and p65 phosphorylation. *J Immunol,* 169, 6490-6497.

Binder BR, Mihaly J. (2008). The plasminogen activator inhibitor "paradox" in cancer. *Immunol Lett,* 118, 116-124.

Chen MM, Lee C-Y, Leland HA, Lin GY, Montgomery AM, Silletti S. (2010). Inside-out regulation of L1 conformation, integrin binding, proteolysis, and concomitant cell migration. *Mol Biol Cell*, 21, 1671-1685.

Choi KH, Kim JE, Song NR, Son JE, Hwang MK, Byun S, Kim JH, Lee KW, Lee HJ. (2010). Phosphoinositide 3-kinase is a novel target of piceatannol for inhibiting PDGF-BB-induced proliferation and migration in human aortic smooth muscle cells. *Cardiovasc Res,* 85, 836-844.

Coopman PJ, Do MT, Barth M, Bowden ET, Hayes AJ, Basyuk E, Blancato JK, Vezza PR, McLeskey SW, Mangeat PH, Mueller SC. (2000). The syk tyrosine kinase suppresses malignant growth of human breast cancer cells. *Nature,* 406, 742-747.

Deryugina EI, Ratnikov B, Monosov E, Postnova TI, DiScipio R, Smith JW, Strongin AY. (2001). MT1-MMP initiates activation of pro-MMP-2 and integrin αvβ3 promotes maturation of MMP-2 in breast carcinoma cells. *Exp Cell Res,* 263, 209-223.

Ferrigni NR, McLaughlin JL, Powell RG, Smith CR, Jr. (1984). Use of potato disc and brine shrimp bioassays to detect activity and isolate piceatannol as the antileukemic principle from the seeds of Euphorbia lagascae. *J Nat Prod,* 47, 347-352.

Geahlen RL, McLaughlin JL. (1989). Piceatannol (3,4,3′,5′-tetrahydroxy-trans-stilbene) is a naturally occurring protein-tyrosine kinase inhibitor. *Biochem Biophys Res Commun,* 165, 241-245.

Hansen S, Overgaard J, Rose C, Knoop A, Laenkholm AV, Andersen J, Sørensen FB, Andreasen PA. (2003). Independent prognostic value of angiogenesis and the level of plasminogen activator inhibitor type 1 in breast cancer patients. *Br J Cancer,* 88, 102-108.

Hapke S, Kessler H, Arroyo de Prada N, Benge A, Schmitt M, Lengyel E, Reuning U. (2001). Integrin αvβ3/vitronectin interaction affects expression of the urokinase system in human ovarian cancer cells. *J Biol Chem,* 276, 26340-26348.

Hering J, Garrean S, Dekoj TR, Razzak A, Saied A, Trevino J, Babcock TA, Espat NJ. (2007). Inhibition of proliferation by omega-3 fatty acids in chemoresistant pancreatic cancer cells. *Ann Surg Oncol,* 14, 3620-3628.

Hochwald SN, Nyberg C, Zheng M, Zheng D, Wood C, Massoll NA, Magis A, Ostrov D, Cance WG, Golubovskaya VM. (2009). A novel small molecule inhibitor of FAK decreases growth of human pancreatic cancer. *Cell Cycle,* 8, 2435-2443.

Hosotani R, Kawaguchi M, Masui T, Koshiba T, Ida J, Fujimoto K, Wada M, Doi R, Imamura M. (2002). Expression of integrin αvβ3 in pancreatic carcinoma: relation to MMP-2 activation and lymph node metastasis. *Pancreas,* 25, e30-35.

Ito H, Gardner-Thorpe J, Zinner MJ, Ashley SW, Whang EE. (2003). Inhibition of tyrosine kinase Src suppresses pancreatic cancer invasiveness. *Surgery,* 134, 221-226.

Kimura T, Sakamoto H, Appella E, Siraganian RP. (1996). Conformational changes induced in protein tyrosine kinase p72syk by tyrosine phosphorylation or by binding of phosphorylated immunoreceptor tyrosine-based activation motif peptides. *Mol Cell Biol*, 16, 1471-1478.

Law DA, Nannizzi-Alaimo L, Ministri K, Hughes PE, Forsyth J, Turner M, Shattil SJ, Ginsberg MH, Tybulewicz VL, Phillips DR. (1999). Genetic and pharmacological analyses of Syk function in αIIbβ3 signaling in platelets. *Blood,* 93, 2645-2652.

Layton T, Stalens C, Gunderson F, Goodison S, Silletti S. (2009). Syk tyrosine kinase acts as a pancreatic adenocarcinoma tumor suppressor by regulating cellular growth and invasion. *Am J Pathol,* 175, 2625-2637.

Leissner P, Verjat T, Bachelot T, Paye M, Krause A, Puisieux A, Mougin B. (2006). Prognostic significance of urokinase plasminogen activator and plasminogen activator inhibitor-1 mRNA expression in lymph node- and hormone receptor-positive breast cancer. *BMC Cancer,* 6, 216.

McMahon B, Kwaan HC. (2009). The plasminogen activator system and cancer. *Pathophysiol Haemost Thromb,* 236, 184-194.

National Cancer Institute PDQ Database [http://www.cancer.gov/cancertopics/pdq]

NCI-Pancreatic Cancer Progress Review Group: National Cancer Institute Strategic plan for addressing the recommendations of the Pancreatic Cancer Progress Review Group. (2002). *PANC-PRG Implementation Plan.* Bethesda, MD USA. National Cancer Institute.

Nisato RE, Hosseini G, Sirrenberg C, Butler GS, Crabbe T, Docherty AJ, Wiesner M, Murphy G, Overall CM, Goodman SL, Pepper MS. (2005). Dissecting the role of matrix metalloproteinases (MMP) and integrin αvβ3 in angiogenesis in vitro: absence of hemopexin C domain bioactivity, but membrane-Type 1-MMP and αvβ3 are critical. *Cancer Res,* 65, 9377-9387.

Perugini RA, McDade TP, Vittimberga FJ Jr, Callery MP. (2000). Pancreatic cancer cell proliferation is phosphatidylinositol 3-kinase dependent. J Surg Res. 90, 39-44.

Sada K, Takano T, Yanagi S, Yamamura H. 2001. Structure and function of syk protein-tyrosine kinase. *J Biochem,* 130, 177-186.

Scott, DL. (2011). Role of spleen tyrosine kinase inhibitors in the management of rheumatoid arthritis. *Drugs*, 71, 1121-1132.

Seow CJ, Chue SC, Wong WS. (2002). Piceatannol, a Syk-selective tyrosine kinase inhibitor, attenuated antigen challenge of guinea pig airways in vitro. *Eur J Pharmacol*, 443, 189-96.

Toyama T, Iwase H, Yamashita H, Hara Y, Omoto Y, Sugiura H, Zhang Z, Fujii Y. (2003). Reduced expression of the Syk gene is correlated with poor prognosis in human breast cancer. *Cancer Lett,* 189, 97-102.

Ulanova M, Duta F, Puttagunta L, Schreiber AD, Befus AD. (2005). Spleen tyrosine kinase (Syk) as a novel target for allergic asthma and rhinitis. *Expert Opin Ther Targets,* 9, 901-921.

Wang L, Duke L, Zhang PS, Arlinghaus RB, Symmans WF, Sahin A, Mendez R, Dai JL. (2003). Alternative splicing disrupts a nuclear localization signal in spleen tyrosine kinase that is required for invasion suppression in breast cancer. *Cancer Res,* 63, 4724-4730.

Wang L, Devarajan E, He J, Reddy SP, Dai JL. (2005). Transcription repressor activity of spleen tyrosine kinase mediates breast cancer suppression. *Cancer Res,* 65, 10289-10297.

Wieder T, Prokop A, Bagci B, Essmann F, Bernicke D, Schulze-Osthoff K, Dörken B, Schmalz HG, Daniel PT, Henze G. (2001). Piceatannol, a hydroxylated analog of the chemopreventive agent resveratrol, is a potent inducer of apoptosis in the lymphoma cell line BJAB and in primary, leukemic lymphoblasts. *Leukemia,* 15, 1735-1742.

Wong BR, Grossbard EB, Payan DG, Masuda ES. (2004). Targeting Syk as a treatment for allergic and autoimmune disorders. *Expert Opin Investig Drugs,* 13, 743-762.

Yanagi S, Inatome R, Takano T, Yamamura H. (2001). Syk expression and novel function in a wide variety of tissues. *Biochem Biophys Res Commun,* 288:495-498.

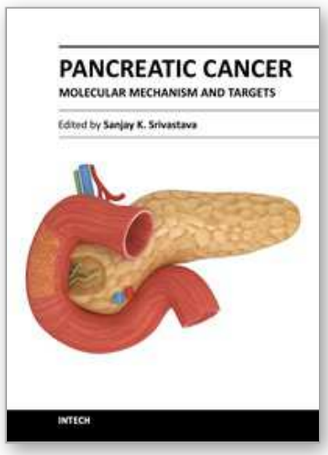
Yebra M, Parry GC, Strömblad S, Mackman N, Rosenberg S, Mueller BM, Cheresh DA. (1996). Requirement of receptor-bound urokinase-type plasminogen activator for integrin αvβ5-directed cell migration. *J Biol Chem.,* 271, 29393-29399.

Yebra M, Filardo EJ, Bayna EM, Kawahara E, Becker JC, Cheresh DA. (1995). Induction of carcinoma cell migration on vitronectin by NF-kappa B-dependent gene expression. *Mol Biol Cell,* 6, 841-50.

Zhang J, Kimura T, Siriganian RP. (1998). Mutations in the activation loop tyrosines of protein tyrosine kinase syk abrogate intracellular signaling but not kinase activity. *J Immunol,* 161, 4366-4374.

Zhu S, Gladson CL, White KE, Ding Q, Stewart J Jr, Jin TH, Chapman HA Jr, Olman MA. (2009). Urokinase receptor mediates lung fibroblast attachment and migration toward provisional matrix proteins through interaction with multiple integrins. *A J Physiol Lung Cell Mol Physiol,* 297, L97-L108.

Zyss D, Montcourrier P, Vidal B, Anguille C, Mérezègue F, Sahuquet A, Mangeat PH, Coopman PJ. (2005). The syk tyrosine kinase localizes to the centrosomes and negatively affects mitotic progression. *Cancer Res,* 65, 10872-10880.



Pancreatic Cancer - Molecular Mechanism and Targets

Edited by Prof. Sanjay Srivastava

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This book provides the reader with an overall understanding of the biology of pancreatic cancer, hereditary, complex signaling pathways and alternative therapies. The book explains nutrigenomics and epigenetics mechanisms such as DNA methylation, which may explain the etiology or progression of pancreatic cancer. Book also summarizes the molecular control of oncogenic pathways such as K-Ras and KLF4. Since pancreatic cancer metastasizes to vital organs resulting in poor prognosis, special emphasis is given to the mechanism of tumor cell invasion and metastasis. Role of nitric oxide and Syk kinase in tumor metastasis is discussed in detail. Prevention strategies for pancreatic cancer are also described. The molecular mechanisms of the anti-cancer effects of curcumin, benzyl isothiocyante and vitamin D are discussed in detail. Furthermore, this book covers the basic mechanisms of resistance of pancreatic cancer to chemotherapy drugs such as gemcitabine and 5-flourouracil.

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