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Study Of The Egfr, Src And Stat3 Pathway In Pancreatic Cancer

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STUDY OF THE EGFR, SRC, AND STAT3 PATHWAY IN PANCREATIC CANCER

by
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ABSTRACT

Cancer is associated with many molecular aberrations that support the malignant phenotype. In that regard, aberrant activation of epidermal growth factor receptor (EGFR), Src, and signal transducer and activator of transcription 3 (Stat3) occur concurrently in pancreatic cancer and are implicated in the disease phenotype. Notwithstanding, increasing evidence indicates that therapies that target only EGFR or Src are rather ineffective in modulating the cancer phenotype. The poor therapeutic outcome of the monotherapies targeting EGFR or Src may in part be due to the increased incidence of signaling cross-talks among aberrant signaling pathways in cancer. Molecular details of the signaling integration between EGFR, Src and Stat3, however, are lacking. Understanding how the aberrant EGFR, Src and Stat3 pathways are integrated in pancreatic cancer would facilitate the design of effective multiple-targeted, clinically feasible therapeutic modalities. Our study shows that in pancreatic cancer cell lines, aberrant Src activity promotes abnormal EGFR activation through the phosphorylation of the EGFR motifs, Tyr845, Tyr1068 and Tyr1086. Furthermore, aberrantly-active EGFR and Src together induce constitutive activation of Stat3 in pancreatic cancer cells. Evidence further shows that EGFR, Src and Stat3 physically associated into a heteromeric complex. Significantly, the EGFR, Src and Stat3 heteromeric complex is detectable in the nucleus and functions as a transcriptionally-active complex to induce the c-Myc gene. Of therapeutic significance, the concurrent inhibition of Stat3 and EGFR or Src promoted greater viability loss and apoptosis of pancreatic cancer cells in vitro, and induced stronger tumor growth inhibition in xenografts of human pancreatic cancer. Altogether, our studies suggest that the

heteromeric EGFR, Src, and Stat3 complex may serve as an additional novel mechanism of support of the pancreatic cancer phenotype. Furthermore, our studies provide evidence that the concurrent targeting of Stat3 and EGFR or Stat3 and Src could be a more effective therapeutic approach for human pancreatic cancer.

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ACRONYMS AND ABBREVIATIONS

Bcl-2 : B-cell Lymphoma 2

Bcl-xl : B-Cell Lymphoma-Extra Large

BSA : Bovine serum albumin

Cas : Crk-associated substrate

ChIP : Chromatin Immunoprecipitation

Con : Control

Das : Dasatinib

DLS : Dynamic Light Scattering

DMEM: Dulbecco's modified Eagle's medium

DOCK : Dedicator of cytokinesis

DTT : Dithiothreitol

EDTA : Ethylenediaminetetraaceticacid

EGFR : Epidermal growth factor receptor

EGTA : Ethylene glycol tetraacetic acid

EMSA: Electrophoretic Mobility Shift Assay

Erl : Erlotinib

FAK : Focal Adhesion Kinase

Gem : Gemcitabine

GNP : Gold nanoparticles

gp : Glycoprotein

HPDEC: Human pancreatic duct epithelial cell.

IgG : Immunoglobulin

IL : Interleukin

iNOS : inducible nitric oxide synthase

IP : Immunoprecipitation

i.v. : Intravenous

JAK : Janus kinase

MAP : Mitogen activated Protein

Mcl-1 : Myeloid cell leukemia sequence 1

MgcRacGAP : Male germ cell Rac GTPase-activating protein

NaCl : Sodium Chloride

NaF : Sodium Fluoride

NaHCO3 : Sodium bicarbonate

$Na_4P_2O_7$: Sodium Diphosphate

Na_3VO_4 : Sodium vanadate

NF-κB : Nuclear factor kappa-light-chain-enhancer of activated B cells

NP-40 : Nonidet P-40

PB: phosphate Buffer

PCR : Polymerase Chain Reaction

PD169540 (PD169) : Pan Erb inhibitor

PI3K : Phosphoinositol 3-Kinase

PMSF: Phenylmethylsulfonyl fluoride

pY : Phosphotyrosine

Ran GTPase : RAs-related Nuclear protein

S3I : Stat3 inhibitor

SDS- Sodium dodecyl Sulfate

SDS-PAGE: Sodium dodecyl Sulfate- Polyacrylamide gel electrophoresis

SEM : Standard Error Mean

SH2 : Src Homology domain 2

siRNA : Small Interfering Ribonucleic Acid

Stat3 : Signal transducer and activator of transcription

TGF : Transforming growth factor

VEGF : Vascular endothelial growth factor

ZD : Iressa (EGFR kinase inhibitor)

INTRODUCTION

Pancreatic cancer is one of the most lethal cancers. Nearly 37,680 new cases were diagnosed in 2008 and 34,290 deaths were reported. The one year relative survival is 20% and the five year rate is 4%[1]. The low survival rates are attributable to the fact that fewer than 10% of patient tumors are confined to the pancreas[2]. The symptoms are nonspecific and include weight loss, dyspepsia, nausea, vomiting and fatigue.

Not much is known regarding the etiological factors or the molecular mechanism behind the disease. Various signaling pathways have been linked to pancreatic cancer. K-ras mutation[3] and loss of p16[4] have been found in pancreatic cancer. Excessive activation of downstream signaling pathways such as Src, NFκB and Stat(signal transducer and activator of transcription)3 signaling pathways also occur[5]. These alterations enhance cancer cell proliferation, suppress pro-apoptotic effects and promote tumor spread and metastasis. There has been evidence of increased activity of growth factor receptors like epidermal growth factor, transforming growth factor, hepatocyte growth factor, vascular growth factor and fibroblast growth factor [6, 7]. Due to its significance, the epidermal growth factor receptor has been targeted for therapy against pancreatic cancer.

The epidermal growth factor receptors are activated in various cancers and high expression of EGFR is associated with poor prognosis[8]. The induction of EGFR promotes cell division and survival through the mitogen-activated protein kinase (MAPK) cascade, the phosphotidylinositol-3-kinase (PI3K) pathway and the signal transducer

and activator of transcription (STAT) pathway. EGFR belongs to the family of receptor tyrosine kinase, containing an extracellular region (ectodomain) that binds to the polypeptide ligands (EGF, TGF-α), a single pass transmembrane helix and a cytoplasmic domain with tyrosine kinase activity[9]. Upon binding of the ligand to the extracellular domain, the receptor forms a homo/heterodimers, leading to autophosphorylation of specific tyrosine residues. The tyrosine residues Y992, Y1068, Y1086, Y1148, and Y1173 are the autophosphorylation sites that serve as the docking sites for SH2- and phosphotyrosine- containing signaling molecules [10]. EGFR associates with and is phosphorylated by other receptor tyrosine kinase. c-Src, a non-receptor tyrosine kinase partly phosphorylates EGFR at the residues Y845, Y1101,Y992, Y1086[11-13]. EGFR and Src cooperate in both mitogenesis and transformation. Cellular Src functions as a co-transducer of various growth factor receptors[14]. c-Src regulates cell cycle progression, cell adhesion and stress responses. Src activation is shown to increase cell proliferation, cell migration and metastases [15-19]. Src is known to have a role in the production of pro-angiogenic factors such as VEGF[20]. EGFR and Src double expression increased MAPK activity and increased the number of aggressive tumors.

The concurrent activation of EGFR and Src in various cancers is suggestive of a possible crosstalk between the signaling molecules. In pancreatic cancer, initial results suggest that the aberrant Stat3 levels can be linked to constantly active EGFR and Src. Stat3 is a member of STAT family of proteins. Stat3 activation is induced by the

phosphorylation on tyrosine 705 mediated by tyrosine kinases including EGFR, c-Src and the Janus Kinase (JAK). Moreover, phosphorylation on serine 727 is reported to enhance Stat3 transcriptional activity. The phosphorylation on the Tyr705 mediates the formation of active Stat3:Stat3 dimers, which translocates to the nucleus and induces the transcription of genes important for cell growth and proliferation, survival and immune responses. While normal STAT activation is transient in keeping with the normal cellular requirements, aberrant Stat3 activation occurs and is associated with malignant transformation and tumorigenesis. In the context of malignant transformation, persistent Stat3 activation occurs with the hyper-activation of the IL-6/gp-130 and JAK pathway, or elevated EGFR and Src levels. At the molecular level it is known that aberrant Stat3 causes expression changes of critical genes that deregulate cell cycle and cell growth. Constitutive Stat3 is associated with induction of Cyclin D1/Cyclin D2, c-Myc expression, down regulation of expression of cyclin dependent Kinase inhibitor; p21. There is also evidence that suggests that abnormal Stat3 activity promotes tumorigenesis by up regulating anti-apoptotic proteins like Bcl-xl, Bcl-2, and Mcl-1. Constitutive Stat3 activation also promotes angiogenesis via induction of VEGF; hypoxia-inducing factor and also facilitates tumor migration, invasion and metastasis[21]. Stat3 has been known to interact with EGFR through its SH2 domain to the phospho-Tyrosine 1068 and 1086 on EGFR[22].

Our goal is thus to understand the interaction between EGFR, Src and Stat3 and to develop a molecular targeted therapy approach for the module as against for an

individual entity. Targeting EGFR alone or Src alone may not be sufficient and a combined therapy will provide a stronger basis to circumvent the diverse processes that promote and support the pancreatic cancer and progression of this disease.

Gemcitabine, the most active cytotoxic drug in advanced disease shows an improvement in symptoms in 20-30% of patients and a 1 year survival of 18%[5]. The knowledge of the molecular mechanisms underlying pancreatic cancer may improve the management of patients and development of new therapies. Erlotinib, an orally available EGFR inhibitor, significantly improved the survival of patients and was approved as a first line therapy with Gemcitabine[5]. However targeting EGFR alone has not been very effective, since there are other receptor and non-receptor kinases that show aberrant activation. A combination therapy approach has been used to target EGFR along with other growth factor receptors.

ENHANCED SENSITIVITY OF PANCREATIC CANCER CELLS TO CONCURRENT INHIBITION OF ABERRANT STAT3 AND EGFR OR SRC

EGFR, Src and Stat3 are known to be activated in pancreatic cancer. Targeting each of the proteins alone has not been very effective. A study of the correlation between the three proteins would help develop a better therapeutic approach for pancreatic cancer. The work here presents the mode of activation of the three proteins, their inter-relationship and targeting multiple proteins to determine if a combinatorial approach has a better therapeutic value.

Introduction:

Pancreatic cancer is a very lethal disease, with poor prognosis and mortality nearly identical to the rate of incidence. The disease also remains poorly understood. There are several genetic mutations and activated signal transduction proteins that occur during pancreatic ductal cell carcinogenesis. Understanding the critical molecular events that promote this disease and how they contribute to its maintenance and progression would facilitate the development of effective targeted therapeutic modalities. One of the major molecular abnormalities is the overexpression and/or activation of the epidermal growth factor receptor (EGFR) protein, with an incidence of 30 to 50% of pancreatic cancer cases [23]. Evidence indicates that the hyperactive epidermal growth factor (EGF)/EGFR pathway is important in the disease maintenance and progression[24]. Similarly, overexpression of the c-Src tyrosine kinase occurs in a large percentage of pancreatic adenocarcinoma and is observed to increase EGFR activities during tumorigenesis[25-27]. The overactivity of the Src family kinases leads to deregulation of tumor cell growth and survival, disruption of cell-to-cell contacts, promotion of migration and invasiveness, and induction of tumor angiogenesis [27]. Another molecular abnormality prevalent in pancreatic cancer and implicated in the disease is aberrant signal transducer and activator of transcription 3 (Stat3) [27-30]. Stat3 is a member of the STAT family of cytoplasmic transcription factors. As with the other STATs, Stat3 requires extrinsic tyrosine phosphorylation to become activated, and this event is induced by growth factor receptors and cytoplasmic tyrosine kinases, such as Src and Janus kinase (Jaks) families[31]. In contrast to normal STAT signaling that is

transient in accordance with the requirements for normal biological processes, tumor cells harbor aberrant Stat3 activation, which compelling evidence indicates dysregulates cell growth and survival, promotes tumor angiogenesis and tumor cell migration and invasion, and induces tumor immune tolerance[32, 33].

The concurrence of the hyperactive EGFR and Src tyrosine kinases, together with aberrant Stat3, in pancreatic cancer raises key questions about the contributory role of each entity to the disease. Deregulated signal transduction provides the framework for signaling cross-talk and functional cooperation that would not only support the malignant phenotype and the disease progression but also would influence drug responsiveness. Thus, a potential collaboration among hyperactive EGFR, Src, and Stat3 in support of the malignant phenotype and in regulating the response to monotargeted therapy is a reasonable model to propose. It is also a concept that would support the recent approval of the combined gemcitabine and EGFR inhibitor erlotinib for the treatment of pancreatic cancer patients[34]. An increased understanding of the integration, functional relationship, and the collective roles of the EGFR, Src, and Stat3 in supporting pancreatic cancer is needed to derive effective, multiple-targeted therapeutic modalities for this disease. We provide evidence that the concurrent inhibition of aberrant Stat3 and EGFR or Src is effective in inducing antitumor cell responses in vitro and in inhibiting human pancreatic tumor growth in xenograft models.

Materials and Methods

Cells and Reagents.
v-Src-transformed mouse fibroblasts (NIH3T3/v-Src), human pancreatic cancer (Panc-1), and leukemic (K562) lines have been described[35-37]. The human pancreatic cancer lines Colo-357 and Mia-PaCa-2 were gifts from Drs. J. M. Lancaster and M. P. Malafa (Moffitt Cancer Center, Tampa, FL). The immortalized human pancreatic duct epithelial cell (HPDEC) line was obtained from Dr. M. S. Tsao (Ontario Cancer Institute, University Health Network/Princess Margaret Hospital, Toronto, ON, Canada)[38]. Except for the HPDEC line that was grown in keratinocytes/serum-free medium supplemented with 0.2 ng of EGF, 30 μg/ml bovine pituitary extract and containing antimycol, and the K562 line cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin/streptomycin, all other cell lines were grown in Dulbecco's modified Eagle's medium containing 5% iron-supplemented bovine calf serum and 100 units/ml penicillin/streptomycin. Recombinant human EGF is from Creative Biolabs (Port Jefferson Station, NY), and gemcitabine is from Ely Lilly & Co. (Indianapolis, IN). Erlotinib, ZD1839 (ZD/Iressa), and dasatinib (Das) were purchased from ChemieTek (Indianapolis, IN).

Nuclear Extract Preparation and Gel-Shift Assays.
Nuclear extract preparation and DNA binding with electrophoretic mobility-shift assay (EMSA) were carried out as reported previously[35, 36]. The ^{32}P-labeled oligonucleotide probes used were high affinity *cis*-inducible element from the *c-fos* gene (m67 variant, 5′-AGCTTCATTTCCCGTAAATCCCTA) that binds Stat1 and Stat3 [39] and the

mammary gland factor element (from the bovine β-*casein* gene promoter, 5′-AGATTTCTAGGAATTCAA) that binds Stat1 and Stat5[40, 41].

SDS-Polyacrylamide Gel Electrophoresis/Western Blot Analysis.
Western blot analysis was performed as described previously[35, 42]. Primary antibodies used were anti-Stat3 (C20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-pY845EGFR (Millipore Corporation, Billerica, MA), and antibodies against pY705Stat3, Stat3, pY1068EGFR, pY1086EGFR, pY1173EGFR, EGFR, pY416Src, Src, c-Myc, and β-actin (Cell Signaling Technology Inc., Danvers, MA).

Small-Interfering RNA Transfection.
Small-interfering RNA (siRNA) sequences for EGFR and Src were ordered from Dharmacon RNAi Technologies, Thermo Scientific (Lafayette, CO). Sequences used were: EGFR sense strand, 5′-GAAGGAAACUGAAUUCAAAUU-3′; EGFR antisense strand, 5′-pUUUGAAUUCAGUUUCCUUCUU-3′; control siRNA sense strand, 5′-AGUAAUACAACGGUAAAGAUU-3′; and control siRNA antisense strand, 5′-pUCUUUACCGUUGUAUUACUUU-3′. The c-Src SMARTpool siRNA reagent (NM-005417; Millipore Corporation) was used for Src. Transfection into cells was performed using 20 nM EGFR siRNA or 25 nM Src siRNA and 8 μl of Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) in OPTI-MEM culture medium (Invitrogen).

Cell Proliferation Assay and Propidium Iodide Staining or Annexin V Binding with Flow Cytometry.
Proliferating cells in 6- or 96-well plates were treated once with 0.1 to 1 μM Iressa, 100 nM Das, 50 to 100 μM S3I-201, 1 μM gemcitabine, or combinations of inhibitors for up to 96 h. Viable cells were counted by trypan blue exclusion/phase-contrast microscopy or assessed by the CyQuant cell proliferation assay according to the manufacturer's instructions (Invitrogen), or cells were stained with propidium iodide for 30 min or subjected to annexin V binding (BD Biosciences, San Jose, CA) and analyzed by flow cytometry for cell cycle distribution or apoptosis, respectively.

Colony Survival Assay.
Single-cell suspension of Panc-1 and Colo-357 cells were seeded in 6-cm dishes (500 cells/dish) and assayed as reported previously [43]. Briefly, single-cell suspension of Panc-1 and Colo-357 cells were seeded in 6-cm dishes (500 cells/well), treated the next day with inhibitors for 48 h, and allowed to grow until large colonies were visible. Colonies were stained with crystal violet for 4 h and counted under a phase-contrast microscope.

Cell Migration and Matrigel Invasion Assays.
Cell migration and invasion experiments were carried out and quantified as described previously[44] using Bio-Coat migration chambers (BD Biosciences, Franklin Lakes, NJ) of 24-well companion plates with cell culture inserts containing 8-μm pore size filters, according to the manufacturer's protocol.

Mice and in Vivo Tumor Studies.
Studies with mice were performed under approved Institutional Animal Care and Use Committee procedures and guidelines. Six-week-old female athymic nude mice were purchased from Harlan (Indianapolis, IN) and maintained in the institutional animal

facilities approved by the American Association of Accreditation of Laboratory Animal Care. Athymic mice were inoculated (subcutaneously) in the left flank area with Colo-357 cells (3×10^6) in 100 µl of 1× phosphate-buffered saline or Panc-1 cells (8×10^6) in 100 µl of 1:1 (v/v) DMEM/Matrigel (Basement Membrane Matrix; BD Biosciences) suspension. Animals were monitored daily after inoculation. For Colo-357 cells, tumors of 60 mm^3 were established after 4 to 6 days, whereas tumors developed in Panc-1 cells took 10 to 15 days to reach 100 mm^3. Animals were grouped so that mean tumor sizes in all the groups were nearly identical. For the xenografts developed with the Colo-357 line, tumor-bearing mice were given ZD (75 mg/kg i.v.), Das (15 mg/kg i.v.), or S3I-201 (5 mg/kg i.v.) alone or in combination every 2 or 3 days for the first 2 weeks and daily for 5 days each week for the next 3 weeks. Mice bearing tumors developed using Panc-1 cells were given ZD (75 mg/kg i.v.), Das (15 mg/kg i.v.), or S3I-201 (5 mg/kg i.v.) alone or in combination every 3 to 5 days, or erlotinib (5 mg/kg i.v., every day) in combination with gemcitabine (100 mg/kg i.v., every 3 days) for 48 days. The doses and the treatment schedules used were inferred from literature reports. Tumor sizes were measured by calipers every 2 or 3 days and converted to tumor volumes by the formula $V = 0.52 \times a^2 \times b$, where a is the smallest superficial diameter and b is the largest superficial diameter. In addition, at the conclusion of the studies, weights of mice were recorded. For each treatment group, the tumor volumes for each set of measurements were statistically analyzed and compared with the control (nontreated) group.

Statistical and Data Analyses.

Statistical analysis was performed on mean values using Prism software (GraphPad Software, Inc., San Diego, CA). Cell proliferation data and effect of drug combinations

were analyzed with CalcuSyn software (Biosoft, Cambridge, UK) to determine additive or synergistic effects. The significance of differences between groups was determined by the paired *t* test at *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

Results

Aberrant EGFR, Src, and Stat3 in Pancreatic Cancer Lines.
Consistent with published reports[28, 29, 44], Stat3 activity, per DNA binding with EMSA analysis in nuclear extract preparations, is constitutive in Panc-1 and Colo-357, low in Mia-Paca-2, and undetectable in the normal HPDEC compared with aberrant levels in NIH3T3/v-Src[35] (Fig. 2Ai). Per supershift analysis, the DNA–protein complex contains Stat3 (Fig. 2Ai, lane 3). No Stat5 activity is detectable in pancreatic cancer cells (Fig. 2Aii) compared with aberrant levels in the K562 leukemic cells [37]. Similarly, immunoblotting analysis showed pY416c-Src levels are moderate in Mia-Paca-2 but elevated in Panc-1 and Colo-357 cells as reported previously[24, 27] and similar to the levels in NIH3T3/v-Src compared with low levels in HPDEC (Fig. 2B, top). The elevated pY416Src levels parallel the enhanced levels of the Src-sensitive pY845EGFR motif[26] in Panc-1 and Colo-357 cells compared with low levels of the same in HPDEC (Fig. 2B, bottom). The total Src or EGFR protein remained unchanged. Immunoblotting analysis further showed enhanced EGFR autophosphorylation motifs[45], pY1068EGFR (Fig. 1Ai, lanes 2 and 7), pY1086EGFR (Fig. 1Aii, lanes 2 and 7), and pY1173EGFR (Fig. 1Aiii, lanes 2 and 7) in Panc-1 and Colo-357 compared with basal levels of the same in HPDEC (Fig. 1Ai–iii, lane 1).

Functional Integration of EGFR and Src in Pancreatic Cancer Cells.

We examined the functional relationship between the activated EGFR and Src. Immunoblotting analysis showed treatment of cells with Das inhibited Src activity (pY416Src)[46] and induced an early (1 h) and a sustained (24 h) decrease in pY845EGFR levels (Fig. 1Bi). By contrast, no detectable changes in pY416Src and pY845EGFR levels were induced by treatment with the pan-ErbB inhibitor, PD169540 (PD169)[47] (data not shown) or the selective EGFR inhibitor, ZD[48] (Fig. 1Bi). In confirmation, the siRNA knockdown of c-Src abrogated pY845EGFR levels (Fig. 1Bii, Src siRNA), whereas the EGFR knockdown by siRNA had minimal effect on pY416Src levels (Fig. 1Biii, EGFR siRNA). Scrambled siRNA (con siRNA) has no effect (Fig. 1B, ii and iii, con siRNA). Thus, elevated pY845EGFR levels in pancreatic cancer cells are sensitive to Src activity.

Immunoblot analysis further showed that the treatment of Panc-1 and Colo-357 cells with ZD decreased pY1173EGFR levels (Fig. 1Aiii, lanes 3, 4, 8, and 9) by as early as 1 h and up to 24 h, with no effect on pY1068EGFR (Fig. 1Ai, lanes 3, 4, 8, and 9) or pY1086EGFR levels (Fig. 1Aii, lanes 3, 4, 8, and 9), suggesting that the EGFR kinase is essential for the induction of pY1173EGFR but not of pY1068EGFR or pY1086EGFR. By contrast, Das treatment decreased pY1068EGFR and pY1086EGFR levels (Fig. 1A, i and ii, lanes 5, 6, 10, and 11) with minimal effect on pYEGFR1173 levels (Fig. 1Aiii, lanes 5, 6, 10, and 11). Thus, Src activity additionally promotes pY1068EGFR and pY1086EGFR in pancreatic cancer cells.

Fig :1

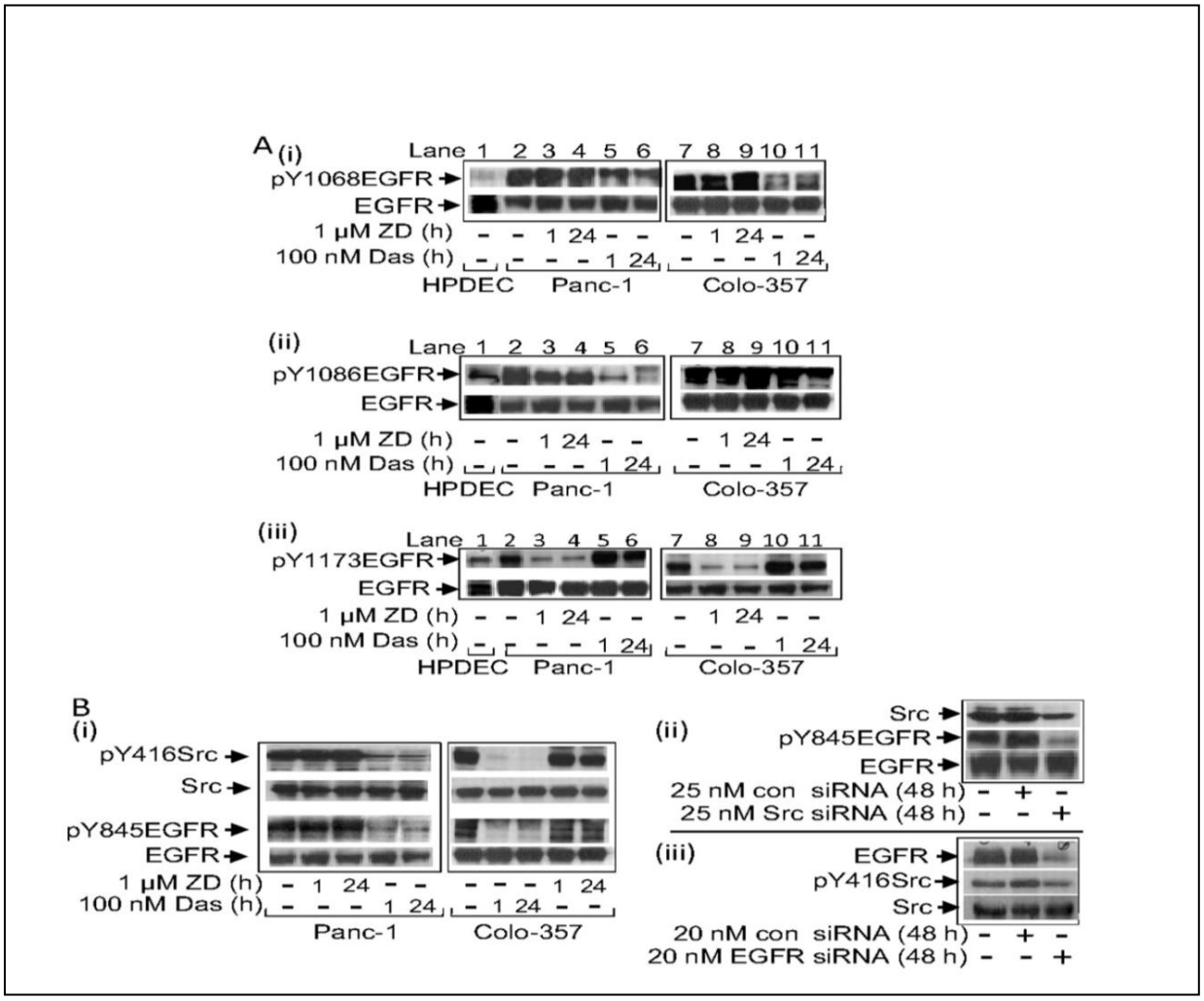


Figure 1:Immunoblotting analyses of Stat3, Src and EGFR activities for effects of inhibitors.
A and B, immunoblotting analysis of whole-cell lysates from cells untreated or treated with ZD or Das (Ai) or transfected with or without c-Src siRNA (Aii), EGFR siRNA (Aiii), or scrambled siRNA control (con) and probing for pY416c-Src (pY416Src), Src, pY845EGFR, and EGFR; and untreated or treated with ZD or Das and probing for pY1068EGFR (Bi), pY1086EGFR (Bii), and pY1173EGFR (Biii), and EGFR. Data are consistent with those obtained from four independent experiments.

Fig :2

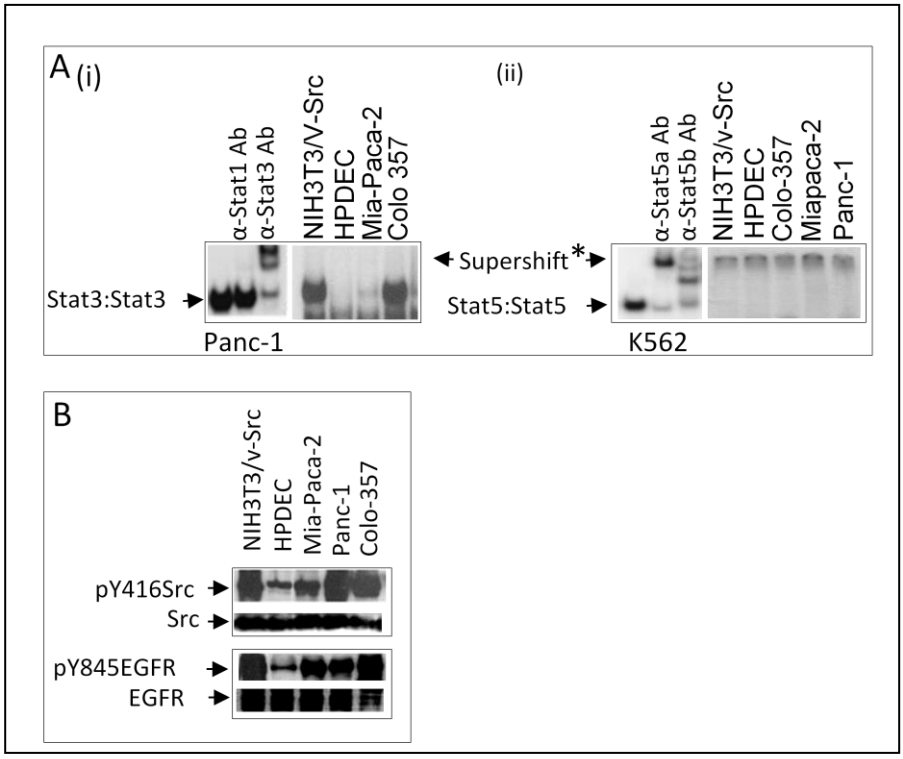


Figure 2: EMSA and immunoblot of activated Stat3, Stat5, pY416Src and pY845EGFR
A. (i) EMSA analysis of Stat3 binding activity in Panc-1, NIH3T3/v-Src, HPDEC, Mia-Paca-2, Colo-357 (ii) EMSA analysis of Stat5 binding activity in K562 , NIH3T3/v-Src, HPDEC, Colo-357, Mia-paca-2, Panc-1. B. Immunoblot analysis of pY416Src, Src, pY845EGFR,EGFR in NIH3T3/v-Src, HPDC, Mia-Paca-2, Panc-1 and Colo357 cell lines.

EGFR and Src Promote Aberrant Stat3 Activation but Do Not Induce Erk^{MAPK} or Akt Activity.

Both pY1068EGFR and pY1086EGFR levels are binding sites for Stat3 [22]. Given the concurrent EGFR and Src activation in Panc-1 and Colo-357 cells, we sought to define the regulation of aberrant Stat3 activation. By in vitro DNA-binding assay with EMSA analysis of nuclear extract preparations, we observed an early repression (in the first 30 min to 1 h of treatment) of constitutively active Stat3 by the pan-ErbB inhibitor, PD169, the ErbB2-selective inhibitor, AG879[28], ZD, or Das (3Ai, lanes 4, 5, 7, and 8, Aii, lanes 2, 4, 6, and 11, and B, 1 h), or by a combined PD169 and Das (Fig. 3Ai, lanes 10 and 11 and Aii, lane 8). However, the Stat3 activity in Panc-1 cells consistently rebounded after 24-h treatments with Das, ZD, or PD169 (Fig. 3A, i and ii, 24 h), even though EGFR or Src activity remained inhibited (Fig. 1, 24 h). Twenty-four-hour treatment with the AG879 moderately inhibited Stat3 activity (Fig. 3Aii, lane 12), which we speculate may be because of its widespread activity as a pan-ErbB inhibitor. By contrast, treatment with the Jak inhibitor AG490 for 1 h had no effect on constitutive Stat3 activity but abolished Stat3 activity at 24-h treatment (Fig. 3Aii, lanes 9 and 10). Moreover, the combined treatment with AG490 and ZD, Das, or PD169 for 24 h abolished constitutively active Stat3 (Fig. 3Aii, lanes 14–16) and prevented the previously observed rebound of Stat3 activity. In Colo-357, Stat3 activity was inhibited by ZD and Das, with the effect more striking for Das (Fig. 3B). These findings together reveal a constitutive Stat3 activation in pancreatic cancer cells that is mediated by both EGFR and Src, with a compensatory, long-term Jak-dependent Stat3 activation, a pattern similarly observed in head and neck squamous carcinoma, mesothelioma, squamous

cell skin carcinoma, and non–small cell lung cancer cell lines after the inhibition of Src[49]. Data also show that the concentration of S3I-201 (50 μM) used in the studies sufficiently inhibits aberrant Stat3 activation in Panc-1 and Colo-357 cells (Fig. 2C). In further support, the siRNA knockdown of EGFR (EGFR siRNA) or Src (Src siRNA) led to pStat3 suppression, as assayed by immunoblotting analysis (Fig. 3D), with no effect of scrambled siRNA (con). We confirm that treatment of Panc-1 cells with S3I-201 for 24 h inhibits aberrant Stat3 activity in a dose-dependent manner (Fig. 3Ai, lanes 13–16).

In contrast to the effects on Stat3 activity, immunoblotting analysis showed elevated pErk1/pErk2^{MAPK} and pAkt activities in Panc-1 and Colo-357 cells, which were not responsive to ZD or Das treatment (Fig. 4), suggesting that aberrant EGFR and Src kinases do not promote the baseline elevated Erk^{MAPK} and Akt activities in pancreatic cancer cells. Thus, Erk and Akt are not key mediators of EGFR and Src functions in pancreatic cancer.

Fig:3

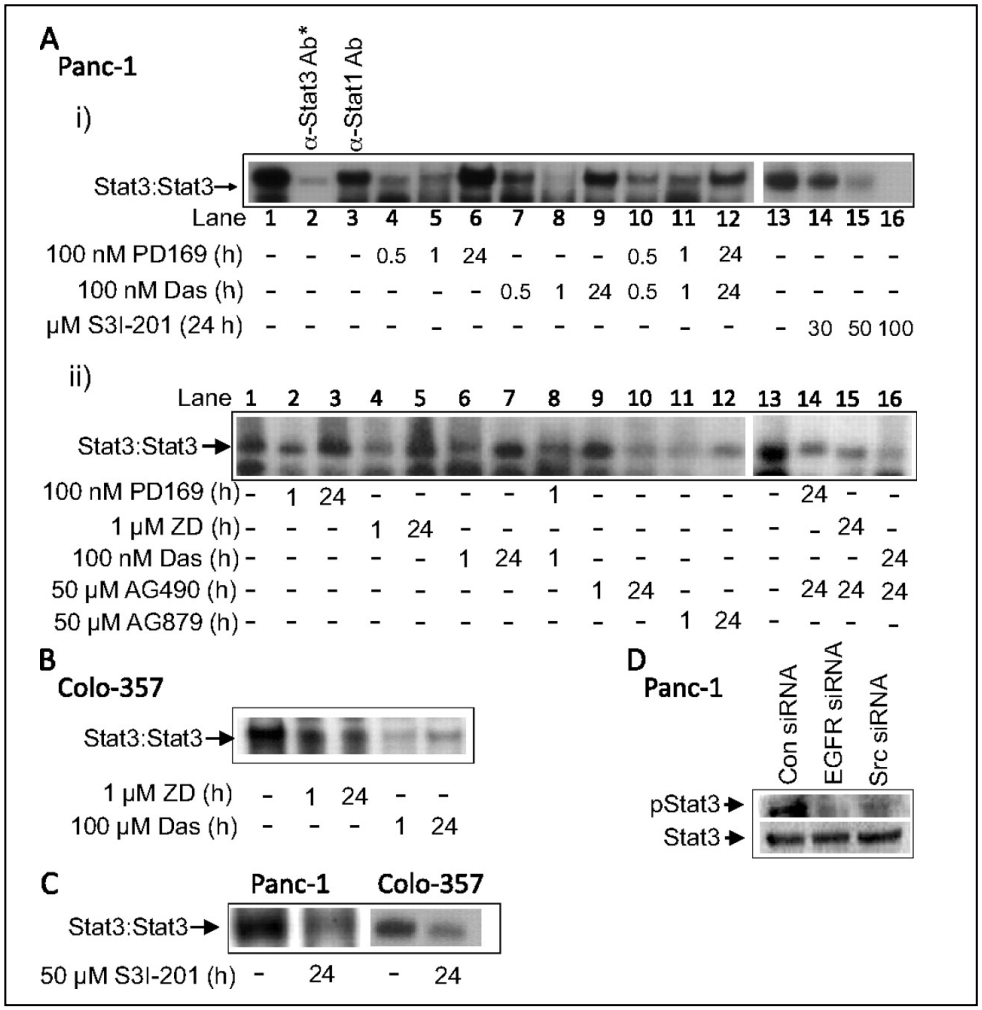


Figure 3: EMSA and immunoblotting analyses for effects of inhibitors on Stat3 activation.
EMSA analysis of Stat3 DNA-binding activity in Panc-1 (A) or Colo-357 (B) cells treated or untreated with the pan ErbB inhibitor PD169, ZD, Das, the Jak inhibitor AG490, the ErbB2- selective inhibitor AG879, or inhibitor combinations for the indicated times, or Panc-1 and Colo-357 cells treated for 24 h with S3I-201 (C), or immunoblotting analysis of whole-cell lysates from Panc-1 cells transfected with EGFR siRNA, Src siRNA, or scrambled siRNA (control) and probing for pStat3 or Stat3 (D).*, supershift analysis. Data are consistent with those obtained from three independent experiments.

Fig:4

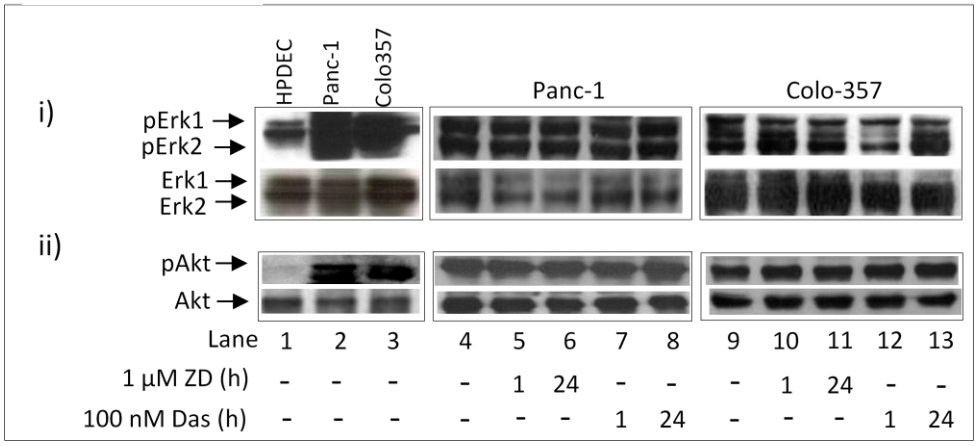


Figure 4: Immunoblotting analyses for effects of inhibitors on pERK1/2 and pAkt
Immunoblot analysis in Panc-1 cells and Colo-357 cells treated (for 1h or 24h) or untreated and probing for (i) pERK1/2, ERK1/2, and (ii) pAkt, Akt

Inhibition of Stat3 Sensitizes Pancreatic Cancer Cells to Effects of EGFR and Src Inhibitors.

We investigated the biological and therapeutic implications of the unique inter-relationship between EGFR, Src, and Stat3 activation. Das and ZD were used at 100 nM and 0.1 to 1 μM, respectively, as in the literature reports [46, 47], whereas the Stat3 inhibitor S3I-201 was used at the suboptimum, 50 μM, or at the 100 μM required to inhibit Stat3 activation[21]. Cell growth was measured as viable cell count by trypan blue exclusion/phase-contrast microscopy. Cells treated with 1 μM ZD, 100 nM Das, or 50 μM S3I-201 alone for 24 h showed minimal effect on growth (Fig. 5A, day 1). Treatment for 48 to 96 h with Das or S3I-201 alone appeared to slow down cell growth, whereas treatment for the same period with ZD showed minimal effect (Fig. 5A, i and ii, days 2–4). The concurrent treatment with both S3I-201 and ZD or S3I-201 and Das for 24 h seemed to decrease growth (Fig. 5A, day 1). By contrast, the concurrent treatment with both S3I-201 and ZD or S3I-201 and Das for 48 to 96 h induced larger decreases in the number of viable cells (Fig. 5A, days 2–4). These data were confirmed by the CyQuant cell proliferation assay (Invitrogen). The CyQuant assay showed that, to some extent, the 48-h treatment with each inhibitor alone decreased cell growth in a dose-dependent manner (Fig. 5B, ZD, Das, and S3I-201). However, the observed effects of any single agent were significantly weaker than the effects of the concurrent treatment with a Stat3 inhibitor and an inhibitor of EGFR or Src. Thus, treatment with 50 μM S3I-201 increased the sensitivity of Panc-1 and Colo-357 cells to ZD and Das, shifting the dose-response curves to the left (Fig. 5B, ZD + S3I-201 and Das + S3I-201; insert shows the effect of 50 μM S3I-201 alone on growth). Cotreatment with S3I-201

significantly decreased the IC_{50} values from 17 to 0.4 μM and 100 to 6 nM, respectively, for ZD and Das against Panc-1 growth (Fig. 5B, i and ii), and from 6.5 to 2.4 μM and 90 to 8 nM, respectively, for ZD and Das against Colo-357 growth (Fig. 5B, iv and v). Analysis, per Chou and Talalay equation, using CalcuSyn (Biosoft) indicated synergism for the combination of S3I-201 and ZD and S3I-201 and Das, with combination indexes of <1. The CyQuant cell proliferation assay also showed that Das but not ZD increased the sensitivity of both cell lines to S3I-201, decreasing the IC_{50} value from 40 to 15 μM and 45 to 20 μM, respectively, for effects on Panc-1 and Colo-357 cells (Fig. 5B, iii and iv). Thus, treatment with S3I-201 sensitized pancreatic cancer cells to ZD and Das.

Fig:5

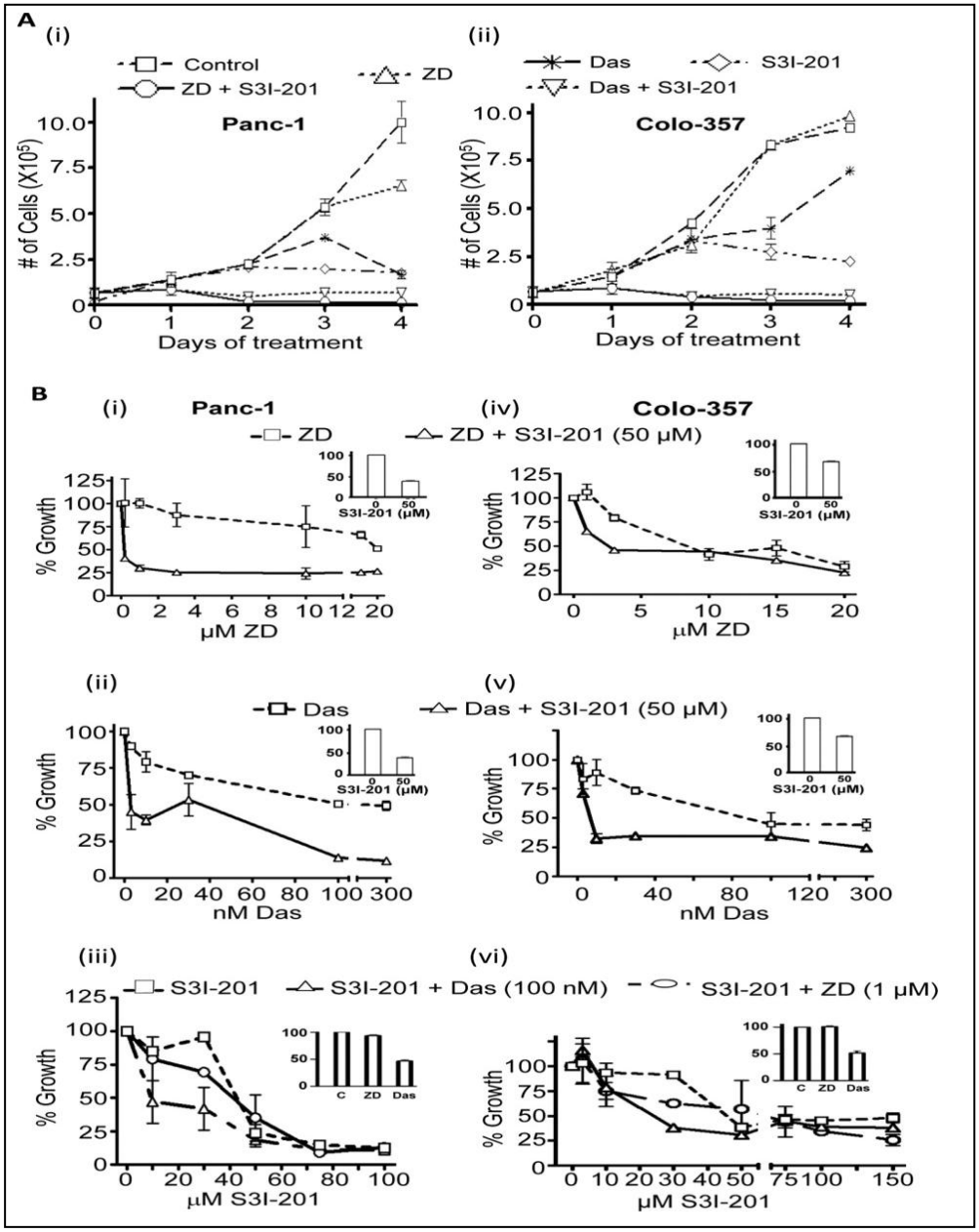


Figure 5:Cell proliferation and viability studies for the effects of inhibitors
A, trypan blue exclusion/phase-contrast microscopy for viable Panc-1 or Colo-357 cells after treatment for 0 to 96 h with 1 µM ZD, 100 nM Das, or 50µM S3I-201, or combinations. B, CyQuant cell proliferation assay for the viability of Panc-1 (left) or Colo-357 (right) cells in response to 48-h treatments with the designated concentrations of ZD, Das, and S3I-201, alone and in combinations. Values, mean and S.D. *n* = 4 experiments, each in triplicate.

Given the potential that the concurrent treatment with agents slowed down growth of pancreatic cancer cells, propidium iodide staining with flow cytometry was performed for cell cycle profile analysis. Treatment of Panc-1 or Colo-357 cells with ZD, Das, or S3I-201 alone for 24 h had no significant impact on the cell cycle distribution, with nearly identical distribution at G_0/G_1, S, and G_2/M phases as in the control, dimethyl sulfoxide-treated cells (Fig. 6A, ZD, Das, and S3I-201). Similarly, the combined treatment with S3I-201 and ZD or S3I-201 and Das for 24 h appeared to have no dramatic effect on the cell cycle distribution of Colo-357 cells (Fig. 6Aii). For Panc-1, a noticeable decrease in the population in the S phase, with no dramatic increase in G_0/G_1, and an increased sub-G_0 population occur in response to the combined treatment with ZD and S3I-201 or Das and S3I-201 (Fig. 6Ai, ZD + S3I and Das + S3I). We deduce that the combined treatment with the inhibitors of Stat3 and Src or EGFR induces some degree of apoptosis in Panc-1 cells at 24 h, with no dramatic change in the cell cycle profile in Colo-357 cells.

Given the clinical implications of these findings, we extended these studies to examine the effect of inhibitors of the EGFR, Src, and Stat3 pathway on the sensitivity of pancreatic cancer lines to gemcitabine, the antimetabolite agent used for treating pancreatic cancer. The rationale for this study is the approval of erlotinib and gemcitabine combination therapy for locally advanced metastatic pancreatic cancer. CyQuant cell proliferation studies showed that inhibition of EGFR, Src, or Stat3 alone did induce some sensitization of Panc-1 and Colo-357 cells to gemcitabine (Fig. 6B).

More importantly, the combined inhibition of Stat3 and EGFR or Src induced a higher sensitization of cells to gemcitabine (Fig. 6B), further supporting the ability to substantially suppress the malignant phenotype by the combined inhibition of Stat3 and EGFR or Src.

To further explore the sensitization potential of Stat3 inhibitors, we performed colony survival assay[43] . Generally, colonies were formed by day 21 after the single, 48-h treatment, and there were no differences between control (untreated) and treated samples in the length of time to form colonies and the sizes of colonies. Panc-1 cells showed decreased sensitivity to inhibitors, and the treatment with single inhibitors had minimal effect on colony numbers compared with the combined treatment with S3I-201 (S3I) and Das or S3I-201 and ZD (Fig. 6Ci). Colo-357 cells were less sensitive to Das or ZD but showed a high sensitivity to S3I-201 and the combined S3I-201 and Das or ZD (Fig. 6Cii). Except for the Colo-357 response to S3I-201, overall the results indicate that Panc-1 and Colo-357 cells are more sensitive to the concurrent treatment with S3I-201 and Das or S3I-201 and ZD. Furthermore, annexin V binding/flow cytometric analysis showed higher percentages of Panc-1 and Colo-357 cells undergo apoptosis in response to the concurrent inhibition of Stat3 and EGFR or Src than to the treatment with any single agent alone. Treatment of Panc-1 and Colo-357 cells with S3I-201, ZD, or Das alone induced lower percentage of apoptosis (Fig. 6D, ii and iii). By contrast, combined treatment with S3I-201 and Das or S3I-201 and ZD induced apoptosis in both Panc-1 and Colo-357 cells at 24 h and a higher percentage of apoptosis at 48 h (Fig.

6D, ii and iii). Panc-1 cells showed higher sensitivity to the combined treatment with S3I-201 and ZD or Das, with up to 60% apoptosis, compared with 25% apoptosis in Colo-357 cells (Fig. 6D, ii and iii). Similar results were obtained for the concurrent treatments with AG490 and ZD or Das (Fig. 6D, ii and iii), whereas similar treatments of normal HPDEC cells showed no significant apoptosis in response to combination treatments (Fig. 6Di). Altogether, our findings indicate that pancreatic cancer cells have a higher sensitivity to the combined inhibition of Stat3 and EGFR or Stat3 and Src than to the inhibition of a single entity.

Fig 6

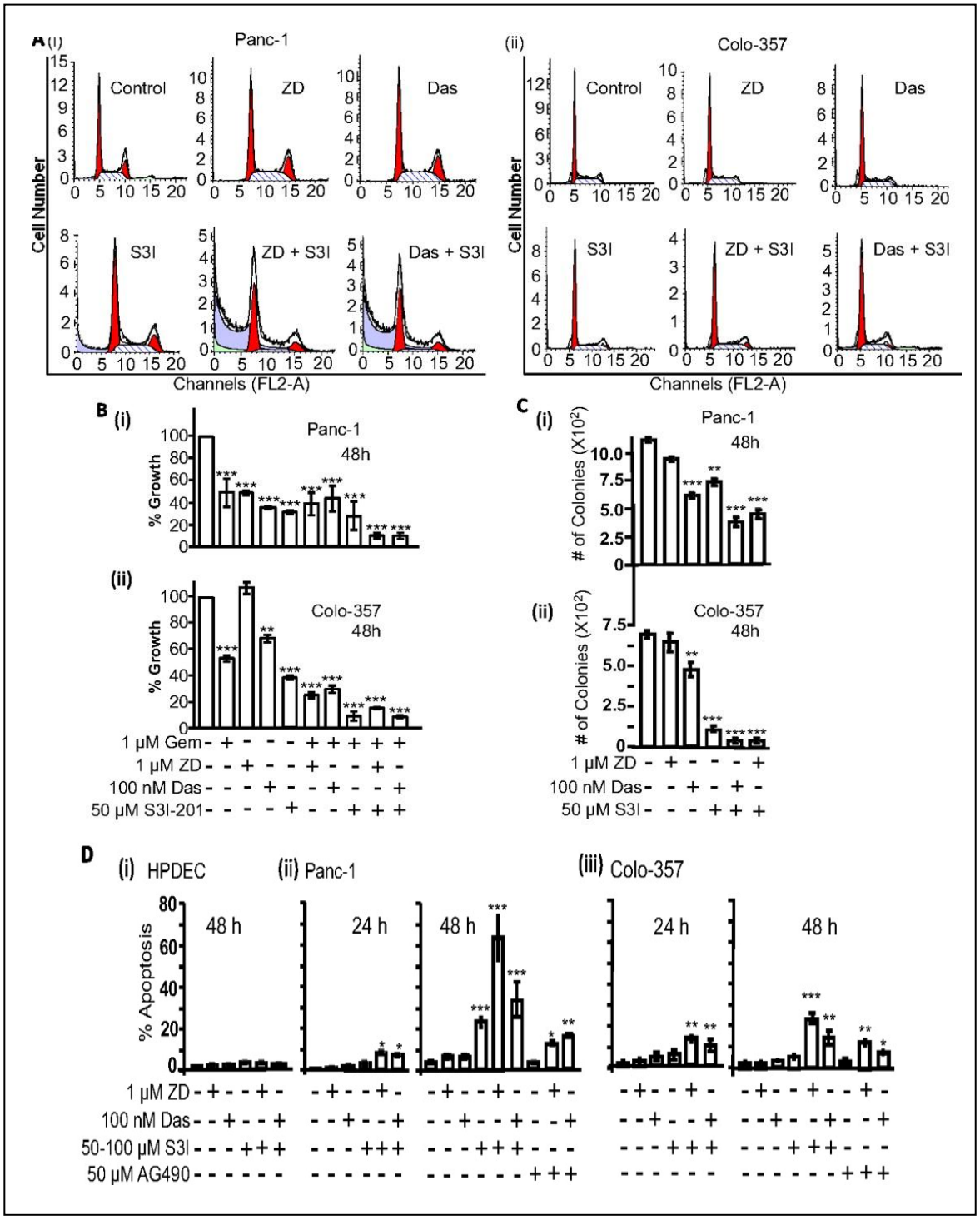


Figure 6: Cell cycle distribution, cell gowth and colony survival and apoptosis studies for the effects of inhibitors

A, cells were untreated (control) or treated with ZD (1 μM), Das (100 nM), S3I-201 (50 μM), ZD +S3I-201, or Das +S3I-201 for 24 h, stained with propidium iodide, and analyzed by flow cytometry for cell cycle distribution. B, CyQuant cell proliferation assay for the viability of Panc-1 (i) and Colo-357 (ii) cells after treatments for 48 h with the designated concentrations of ZD, Das, S3I-201, and gemcitabine (Gem), alone and in combinations. C, number of colonies emerging from cells in culture (500/6-cm dish) untreated or treated once with ZD, Das, S3I- 201 (S3I), or combinations and allowed to culture. D, annexin V binding/flow cytometry analysis of normal HPDEC, Panc-1, or Colo-357 cells treated or untreated with inhibitors or combinations. Values, mean and S.D. n = 4 experiments each in triplicate. *, p <0.05; **, p <0.01; ***, p < 0.001.

EGFR, Src, and Stat3 Together Promote Pancreatic Cancer Cell Migration and Invasion.

Consistent with roles for Src and Stat3 in tumor cell motility, migration, invasion, and metastasis[27, 46]; in vitro Matrigel assay showed that Das or S3I-201 alone suppresses migration and invasion (Fig. 7A). However, the concurrent inhibition for 24 h of Stat3 and EGFR or Src induced stronger effects on Colo-357 migration and Panc-1 invasion, except for the similar effect of Src inhibition on Panc-1 migration (Fig. 7A). At the 24-h treatment when these studies were done, there is no significant effect on the growth of Panc-1 and Colo-357 cells (Fig. 5), although for Panc-1 cells, cell cycle analysis revealed some evidence of increased sub-G_0 population after 24-h treatment with ZD and S3I-201 or Das and S3I-201 (Fig. 6Ai), which may contribute to the reduced number of migrating Panc-1 cells.

EGFR, Src, and Stat3 Module Regulates c-Myc Overexpression in Pancreatic Cancer Cells.

For insight into the underlying molecular mechanisms, we studied the regulation of key cancer-relevant genes and observed that c-Myc is overexpressed in pancreatic cancer lines compared with normal HPDEC, and its expression was consistently more responsive to the 24-h treatment with both S3I-201 and ZD or S3I-201 and Das than the inhibition of Stat3, Src, or EGFR alone (Fig. 7B). These data raise the potential that Stat3, EGFR, and Src functionally cooperate to induce c-Myc overexpression in pancreatic cancer cells.

Fig 7

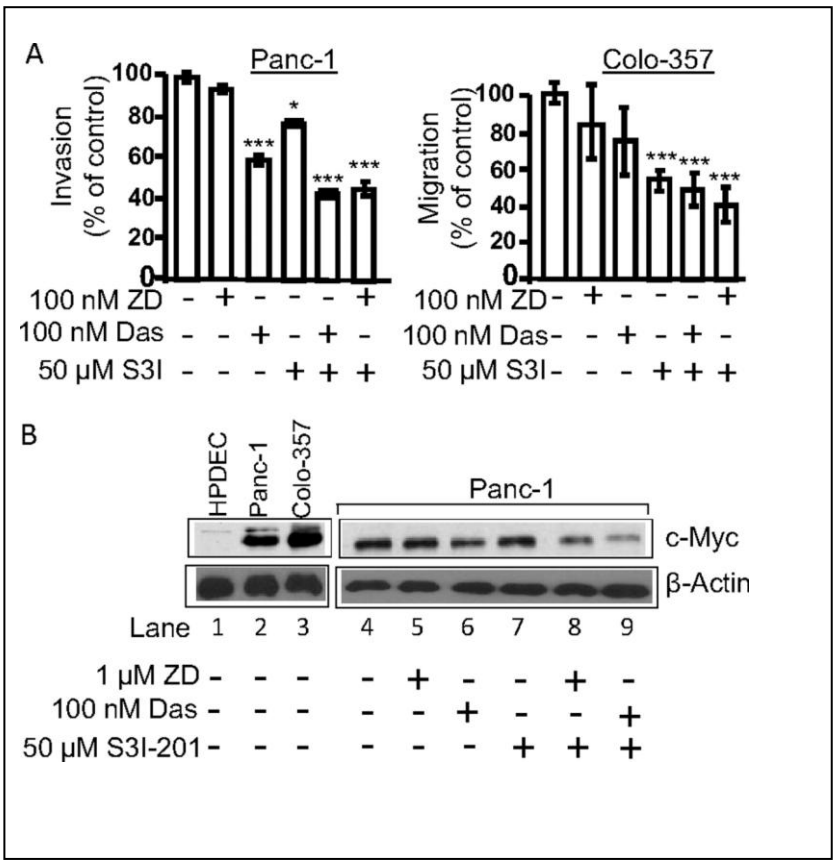


Figure 7: Concurrent inhibiton of Stat3 and EGFR or Src inhibits migration and invasion and suppresses c-Myc expression

A, effects of 24-h treatment with ZD, Das, and/or S3I-201 (S3I) on migration and invasion. B, immunoblotting analysis of whole-cell lysates for c-Myc and β-actin expression in Panc-1 cells after treatment with inhibitors for 24 h. Values, mean and S.D. n = 3 or 4 experiments each in triplicate. *, p <0.05; **, p< 0.01; ***, p <0.001.

Concurrent Inhibition of Stat3 and EGFR or Src Inhibits Human Pancreatic Tumor Growth in Xenografts.

Subcutaneous xenografts of Colo-357, a metastatic pancreatic adenocarcinoma line, and the epithelioid carcinoma, Panc-1, line were used to study the therapeutic implication of the Stat3, EGFR, and Src inter-relationships and evaluate the in vivo antitumor effects of concurrent inhibition of Stat3 and EGFR or Src. Data showed that, in general, xenografts of Colo-357 cells showed low responsiveness to treatment with inhibitor of EGFR, Src, or Stat3 alone, although as the therapy progressed, tumors treated with only one inhibitor alone appeared to show reduced growth, which was statistically not significant from control, nontreated tumors (Fig. 8A). On the other hand, xenografts of Panc-1 cells showed greater sensitivity to the Src or the Stat3 inhibitor, which induced significant ($p < 0.05$) inhibition of tumor growth (Fig. 8B, Das and S3I-201). Importantly, for both Colo-357 and Panc-1 lines, xenograft tumors were much more sensitive to the combined inhibition of Stat3 and EGFR or Stat3 and Src than to the inhibition of either one alone, with tumors consistently showing reduced growth and smaller sizes in response to the combined treatment throughout the entire study (Fig. 8, ZD + S3I-201 and Das + S3I-201). Results also showed that xenograft tumors of Panc-1 showed increased sensitivity to the concurrent treatment with the approved combination therapy, gemcitabine and erlotinib, similarly to the treatment with Das and S3I-201 (Fig. 8B, gemcitabine + erlotinib compared with Das + S3I-201). Studies also showed that xenografts of Panc-1 were more sensitive to drug combinations than xenografts derived from Colo-357 cells (Fig. 8B, compare with Fig. 8A), which is consistent with the in vitro cell growth/viability and apoptosis data (Figs. 5 and 6D). Thus, significant differences (*p*

< 0.05) between the tumor volumes (sizes) for Colo-357 tumors in mice treated with the combination inhibitors and the control tumors occurred at day 20 and onward posttreatment, compared with the significant differences that occurred at day 10 posttreatment between the tumor volumes for xenografts of Panc-1 that were treated with the combination of Das and S3I-201 or gemcitabine and erlotinib and the tumor volumes for control tumors (Fig. 8). The in vivo antitumor effects of the combination treatment with S3I-201 and Das or S3I-201 and ZD are consistent with the in vitro antitumor cell data and together indicate that aberrant Stat3 cooperates with hyperactive EGFR or Src to sustain human pancreatic cancer. There were no obvious signs of toxicity of any of the drugs or drug combinations, and the average weights of mice for control and treated mice were 23 to 26 mg at the end of the studies.

Fig 8

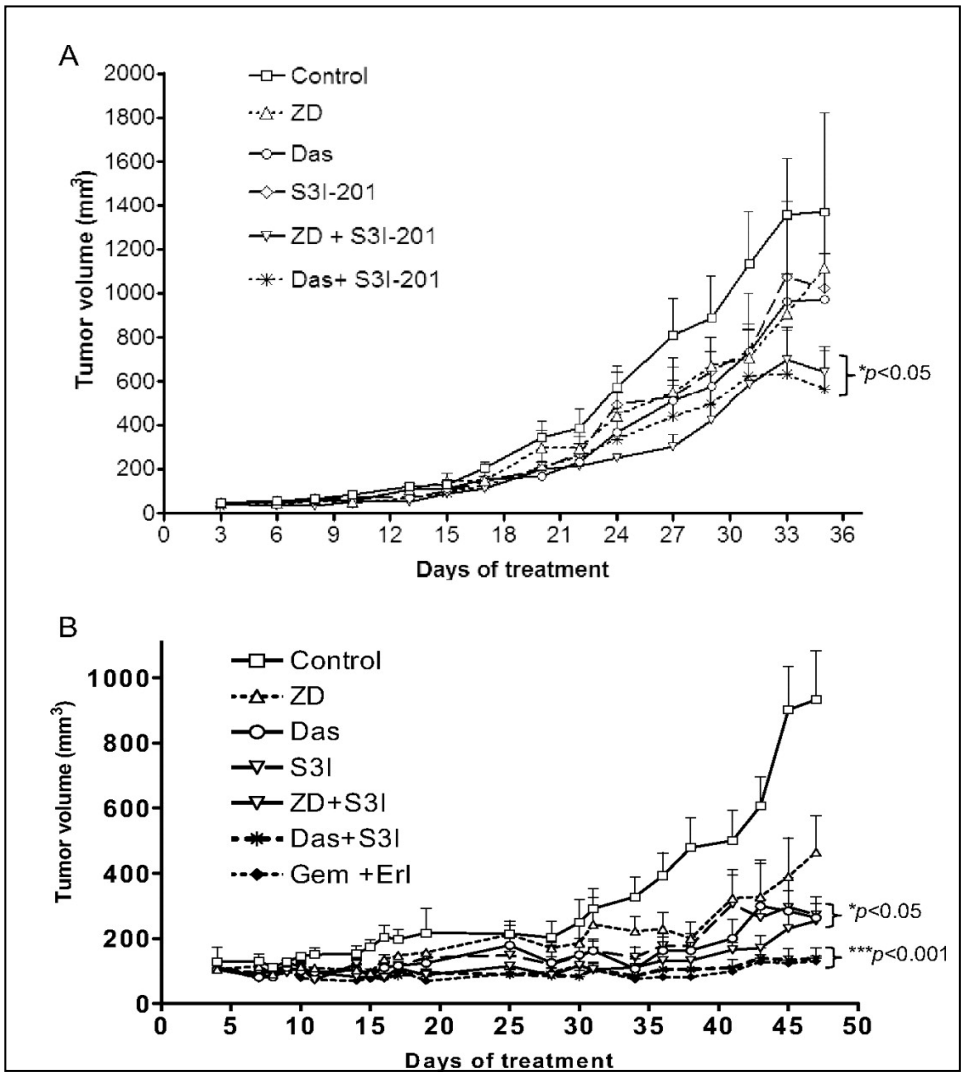


Figure 8: In vivo study of growth inhibiton of human pancreatic cancer xenografts
Human pancreatic Colo-357 tumorbearing mice were given ZD (75 mg/kg i.v.), Das (15 mg/kg i.v.), and S3I-201 (5 mg/kg i.v.) alone or in combination every 2 or 3 days for the first 2 weeks and then daily for 5 days each week for the next 3 weeks (A), or Panc-1 tumorbearing mice were given ZD (75 mg/kg i.v.), Das (15 mg/kg i.v.), and S3I-201 (5 mg/kg i.v.) alone or in combination every 2 or 3 days, or given erlotinib (5 mg/kg i.v., every day) in combination with gemcitabine (100 mg/kg i.v., every 3 days) for 48 days (B). Tumor sizes, measured at 2- to 5-day intervals, were converted to tumor volumes and plotted against days. Values, mean and S.E.M. *n*= 7 or 8 mice per group. *, *p* <0.05; ***, *p* <0.001.

Discussion

Pancreatic cancer is a lethal disease that has remained a challenge to treatments. The lack of understanding of the disease has also limited the chances of designing effective therapeutic modalities. We present evidence from in vitro and in vivo studies that the multiple targeting of Stat3 and EGFR or Stat3 and Src has the potential to induce strong antitumor responses in pancreatic cancer. Our study shows that the concurrent treatment with the Stat3 inhibitor, S3I-201, and the EGFR inhibitor, ZD, or S3I-201 and the Src inhibitor, Das, induced an enhanced tumor growth inhibition, with reduced tumor sizes in xenograft models developed from the metastatic pancreatic cancer line, Colo-357, and the epithelioid carcinoma line, Panc-1. The robust antitumor effects of the combination therapy contrast the weaker antitumor responses to the inhibition of EGFR, Src, or Stat3 alone in the xenografts of Colo-357 and Panc-1, suggesting the monotargeting of EGFR, Src, or Stat3 alone might not be sufficient to suppress the malignant phenotype and induce significant antitumor effects. These findings are generally consistent with the reports of dismal responses in molecular targeted monotherapy, such as the anti-EGFR monotherapy[34, 50]. Although the combined therapy of gemcitabine and the EGFR inhibitor, erlotinib, for locally advanced/metastatic pancreatic cancer[6, 51] may be justified by the same argument as the multiple-targeted therapy in our study, it is not without toxicities[34]. In our study, the treatment with Das and S3I-201 showed a similar antitumor efficacy. Given that the Das and S3I-201 combination therapy is based on molecular targeting and is mechanism-based, this therapeutic modality presents advantages over the approved gemcitabine and erlotinib

combination. We also note from our in vitro study that the concurrent inhibition of Stat3 and EGFR or Src promotes a higher sensitization of pancreatic cancer cells to gemcitabine than the inhibition of EGFR or Src alone. The present in vitro data also provide evidence that the combined inhibition of Stat3 and Src or EGFR induces stronger cell growth inhibition and apoptosis, mechanisms that would contribute to the decreased tumor growth in vivo. Molecularly, the enhanced antitumor effects of the concurrent inhibition of Stat3 and EGFR or Src against pancreatic cancer could in part be the result of the increased potential to down-regulate critical tumor-relevant genes. Altogether, the present study shows the in vivo efficacy of the concurrent targeting of aberrant Stat3 and EGFR or Src and provides support for the use of this multiple-targeting modality as a therapeutic approach for pancreatic cancer.

In vitro studies provide the biochemical and biological insights into the enhanced antitumor effects in vivo of the multiple targeting of Stat3 and EGFR or Src in pancreatic cancer. Our study provides strong evidence for a complex signaling cross-talk and a functional cooperation (Fig. 9), which would undercut the biological response of targeting only the EGFR, Src, or Stat3 protein. In the context of this signal integration (Fig. 9), data presented reveal a strong role for Src in increasing the aberrant EGFR activity in pancreatic cancer cells by promoting the phosphorylation of multiple tyrosine residues, including the Y1068EGFR and Y1086EGFR motifs. The induction of aberrant Stat3 activation is promoted by both hyperactive EGFR and Src activities. In this context, the induction of pY1068EGFR and pY1086EGFR by Src would serve to further

promote Stat3 activation, given that those two EGFR tyrosine motifs are essential sites for the binding and the activation of Stat3[22]. We also note that apart from the baseline, constitutive Stat3 activation in pancreatic cancer cells, which is caused primarily by EGFR and Src, there is a contributory role for Jak tyrosine kinases in the induction of Stat3 activation (Fig. 9). However, Jaks are unlikely to be the predominant mediators of the baseline, constitutive Stat3 activity, given that their inhibition by AG490 did not abolish aberrant Stat3 activation at the earliest time point, whereas the EGFR or Src inhibition completely attenuated the baseline, aberrant Stat3 activity. Instead, Jaks could provide a secondary, compensatory mechanism for Stat3 activation, as a part of a two-phase model for the induction of Stat3 activity in pancreatic cancer cells. A similar secondary induction of Stat3 activation via Jak activities has previously been observed in the head and neck squamous cell carcinoma line[49] and is suggested to be caused by the growth-stimulatory factors that are released from tumor cells[52] which in turn induce Jaks activity and thereby promote Stat3 activation. In this complex signaling structure (Fig. 9), the inhibition of EGFR or Src alone is unlikely to abolish the functional effects of abnormal Stat3 signaling. Instead, combining the direct inhibition of aberrant Stat3 with the EGFR or Src inhibition serves to provide the framework for substantially thwarting the oncogenic events with measureable beneficial outcome. Our data would suggest that the inhibition of Stat3 enhances the sensitivity of pancreatic cancer cells to the antitumor effects of ZD and Das.

Fig 9

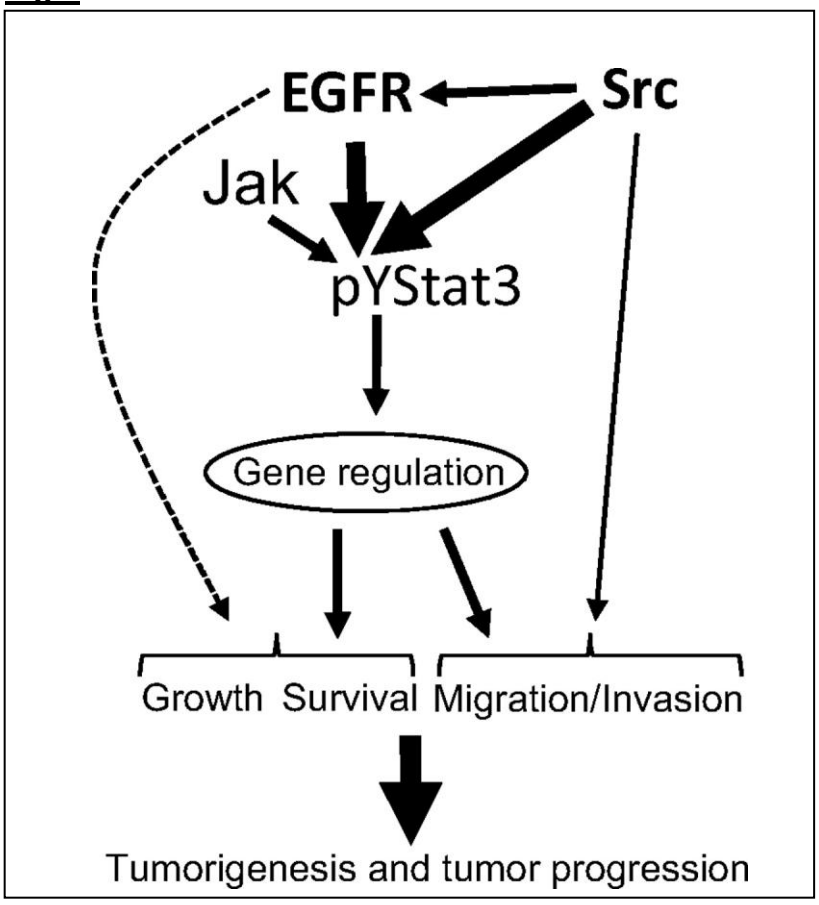


Figure 9: A model of collabrative function of hyperactive EGFR, Src and Stat3 in support of human pancreatic cancer

Hyperactive c-Src contributes to promoting the aberrant EGFR activation. Both hyperactive EGFR and Src induce constitutive Stat3 activation, whereas Jak activity contributes to promoting Stat3 signaling by serving as a compensatory mechanism. Aberrantly active Stat3 dysregulates the induction of target genes and, together with other potential mechanisms induced by the hyperactive EGFR and Src, induces cell growth, survival, and migration/ invasion, thereby promoting pancreatic tumorigenesis and progression.

Additional in vitro biological data further indicate a collective role of EGFR, Src, and Stat3 in supporting pancreatic cancer. Although the inhibition of the activity of each of the three proteins induced some degree of antitumor cell response in vitro, albeit minimal, data are convincing that the simultaneous targeting of Stat3 and EGFR or Src has a higher potential to be more effective in inducing diverse antitumor cell effects against pancreatic cancer cells than the inhibition of EGFR or Src alone. The hyperactivation of EGFR signaling has been deemed a prognostic indicator of low survival among pancreatic cancer patients[53, 54]. In addition, there is evidence to indicate that the concurrence of hyperactive Src signaling potentiates the effects of aberrant EGFR[26]. Such increase of effect or the cooperation of activities and/or functions between the critical molecular entities that supports the pancreatic cancer phenotype can undermine the efficacy of a targeted monotherapy in pancreatic cancer. Indeed, the present data that the simultaneous inhibition of Stat3 and EGFR or Src induced greater antitumor cell effects and a higher sensitization to gemcitabine strongly supports this viewpoint. We infer from our study that the more robust tumor growth inhibition in vivo by simultaneous inhibition of Stat3 and EGFR or Src is the result of the concomitant suppression of the abnormal functions of both Stat3 and EGFR or Stat3 and Src, suggesting that the concurrent targeting of Stat3 and EGFR or Stat3 and Src could be a more effective, multitargeted therapeutic modality for pancreatic cancer than the inhibition of EGFR, Src, or Stat3 alone.

A FUNCTIONAL NUCLEAR EPIDERMAL GROWTH FACTOR RECEPTOR, SRC AND STAT3 HETEROMERIC COMPLEX IN PANCREATIC CANCER CELLS

Our study showed that Src regulated the activation of EGFR and both EGFR and Src regulated the activation of Stat3. However it is not known if the three proteins associate. A study of whether the proteins remain in a complex after activation, to exhibit a significant biological effect, would help understand the complex signaling mechanism in the pancreatic cancer cells. This study, thus aims to address intermolecular interaction between the three proteins.

Introduction

Many intracellular biochemical processes are triggered by the assembly of proteins into macromolecular complexes. The association between proteins or of proteins with other molecular entities modulates protein conformation, providing a means to regulate the myriad of biochemical processes for the efficient management of vital biological responses. Moreover, protein dynamics and trafficking, and protein stability are also processes that can be modulated by the association of proteins with others. In the broader sense, inter-molecular associations allow specialty proteins, such as receptors, adapters, enzymes, and transcription factors to differentially modulate intracellular events, thereby creating the diversity in physiological responses and promoting context dependency.

During the induction of signal transduction, there is assembly of different proteins, each of which has specific functions important for the signal transduction and for the accompanying biological response. The traditional epidermal growth factor receptor (EGFR) signal transduction pathway incorporates the activation of the mitogen-activated protein kinase kinase (MEK)-mitogen-activated protein kinase/extracellular signal-regulated kinase (ErkMAPK) and promotes mitogenic responses [55, 56]. Moreover, EGFR induction also promotes the activation of the Signal Transducer and Activator of Transcription (STAT) family of proteins, which similarly have a central role in EGF-induced biological responses[56]. The STAT proteins are latent cytoplasmic transcription factors that are activated in response to cellular stimulation by cytokines

and growth factors[31] via the phosphorylation of a critical tyrosyl residue (Tyr705 for Stat3). The tyrosine phosphorylation of STATs is mediated by tyrosine kinases of growth factor receptors and by cytoplasmic tyrosine kinases, such as Src and Janus kinase (Jaks) families. Activated STATs as dimers in the nucleus bind to specific DNA response elements in the promoters of target genes to induce gene transcription. The nuclear translocation mechanism for STATs has been the subject of recent intense investigation. Stat3 nuclear translocation has been reported to be mediated by mechanisms, including the recognition and transport by importin-α and the Ran-GTPase [57], and the transport via chaperoning by MgcRacGAP[58], and by EGF receptor mediated endocytosis [59] and plasma membrane-associated lipid rafts trafficking mechanisms[60].

The prevalence of many hyperactive signal transduction pathways that support the cancer phenotype is a major challenge to therapy. Further to the classical way of promoting crosstalks among multiple signaling pathways, macro-molecular protein assemblies provide additional unique mechanisms for inducing events that would support the malignant phenotype. Such a non-traditional signaling mechanism has been identified for the EGFR, which has been detected in the cell nucleus and observed to function as a transcription factor[61, 62]. Studies further revealed the nuclear EGFR complexes with Stat3 in breast cancer cells, and this complex induces specific genes, including the inducible nitric oxide synthase (iNOS) [63]. The additional EGFR function would compound its role as a mitogen and a promoter of cell survival, which all favor

cancer. In that regard, the concurrent aberrant activation of EGFR and downstream signal mediators, including Src and Stat3, which occur with high frequencies in human cancers reflects an overall signaling complexity that supports the cancer phenotype. For example, with reference to pancreatic cancer, aberrant activation of EGFR occurs in 30-50% of cases[24], activated c-Src is noted in more than 70% of cases, and frequently accompanies EGFR overexpression[64], while aberrant Stat3 activation is also highly prevalent [27, 29, 32]. Importantly, our recent report that pancreatic cancer is more sensitive to the concurrent inhibition of aberrant Stat3 and EGFR or Src [65] show the dependence on multiple aberrant signaling pathways for the maintenance of the cancer phenotype and the responsiveness to therapy. To extend our earlier studies [65], we sought to probe the molecular and functional interplay between Stat3, EGFR and Src and the underlying mechanisms of support of the pancreatic cancer phenotype. We herein provide evidence for a functional nuclear heteromeric EGFR, Src and Stat3 complex in pancreatic cancer cells, which promotes the induction of the c-Myc gene. During the revision of this paper, a study was reported on the detection of EGFR and Stat3 and EGFR and Src complexes following EGF stimulation of breast cancer cells[66], although our report is the first on the identification of a nuclear EGFR, Src and Stat3 heteromeric complex that promotes the c-Myc gene induction. Understanding the dynamics of the EGFR, Src and Stat3 molecular interactions in pancreatic cancer would provide basis to design novel effective multiple-targeted therapy approaches for pancreatic cancer.

Experimental Procedures

Cells and Reagents.
The human pancreatic cancer, Panc-1 and Colo-357 lines have all been previously described [65, 67]. The immortalized human pancreatic duct epithelial cell (HPDEC) line was a kind gift of Dr. Tsao, OCI, UHN-PMH, Toronto)[38]. HPDEC were grown in Keratinocyte-SFM media supplemented with 0.2 ng EGF, 30 μg/mL bovine pituitary extract and containing antimycol. All other cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% iron-supplemented bovine calf serum and 100 units/ml penicillinstreptomycin.

Nuclear Extract Preparation and Gel Shift Assays.
Nuclear extract preparation from cells was carried out as previously described [67].

SDS-PAGE/Western Blot Analysis.
Western blotting analysis was performed as previously described [35, 42] on whole-cell lysates, nuclear extracts, and on cytosolic and membrane fractions. Primary antibodies used are against pY845EGFR (Upstate Biotech, Millipore, Billerica, MA), pY705Stat3, Stat3, pY1068EGFR, pY1086EGFR, pY1173EGFR, EGFR, pY416Src, Src, and β-Actin (Cell Signaling Technology, Danvers, MA), and Tata-binding protein (TBP) (Santa Cruz Biotechnology, Santa Cruz, CA).

Small-interfering RNA (siRNA) Transfection.
siRNA sequences for EGFR and Src were ordered from Dharmacon RNAi Technologies, Thermo Scientific (Lafayette, CO). Sequences used are: EGFR sense

strand, 5′- GAAGGAAACUGAAUUCAAAUU-3′; EGFR antisense strand, 5′-pUUUGAAUUCAGUUUCCUUCUU-3′; control siRNA sense strand, 5′-AGUAAUACAACGGUAAAGAUU-3′; and control siRNA antisense strand, 5′-pUCUUUACCGUUGUAUUACUUU-3′. The c-Src SMARTpool siRNA reagent (NM-005417, Catalog # M-003175-01-05) was used for Src. Transfection into cells was performed using 20 nM of EGFR siRNA or 25 nM of Src siRNA and 8 μl Lipofectamine RNAiMAX (Invitrogen Corporation, Carlsbad, CA) in OPTI-MEM culture medium (GIBCO, Invitrogen Corporation).

Immunostaining with laser-scanning confocal imaging.
Details are provided in Supplementary Information, “Experimental Procedures”. Confocal analysis was performed by the examination of slides under Leica TCS SP5 confocal microscope (Germany) at appropriate wavelengths. Images were captured and processed using the Leica TCS SP 5 software. Phenylarsine oxide was purchased from Sigma-Aldrich (St. Louis, MO).

Immunoprecipitation (IP), and Sequential Immunoprecipitation Studies.
Immunoprecipitation and sequential IP from whole-cell lysate or nuclear extracts and immunoblotting (250 μg total protein) were performed as previously described[44], using 5 μL of anti-EGFR or anti-Src polyclonal antibody or the monoclonal anti-Stat3 antibody (Cell Signaling Technology). For the test of specificity, immunoblotting analysis using anti-EGFR, anti-Src and anti-Stat3 antibody was performed in the presence of the respective blocking peptide. For the sequential IP studies, nuclear extracts, prepared as

previously described[44], were subjected to similar Immunoprecipitation studies with regard to the first primary antibody by incubating with anti-EGFR antibody or IgG (for no antibody) (Santa Cruz) at 4°C overnight. The complexes were then collected with 20 µL protein A/G agarose beads (Santa Cruz), washed three times using wash buffer A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 20 mM Tris-HCl, pH 8.0) and two times with wash buffer B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0), and then eluted with freshly prepared elution buffer (1% SDS, 100 mM NaHCO3). The eluted complexes were then subjected to a second immunoprecipitation by incubating with anti-Src antibody or IgG (for no antibody) (Santa Cruz). The complexes were then precipitated, washed, eluted with lamelli buffer and then subjected to SDS-PAGE and Western blotting for Stat3.

Chromatin Immunoprecipitation (ChIP) and Sequential ChIP Analyses.

For ChIP assay, cells in culture were treated with formaldehyde at a final concentration of 1%, for 10 min at room temperature followed by treatment with glycine at a final concentration of 0.125 M for 5 min at room temperature for cross-linking. Subsequently, cells were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in and lysed with lysis buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM Na3VO4, 1 mM Na4P2O7, 1 mM dithiothreitol (DTT), 1X TLA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5% Nonidet P-40 [NP-40], and centrifuged. Then nuclear pellet was resuspended in nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS and protease inhibitors) (Roche, Indianapolis, IN). The nuclear lysates were sonicated (Omni International, Kennesaw, GA) at 30% power for 3 pulses for 10 s

intervals on ice to shear DNA. The chromatin solution was pre-cleared with protein A/G agarose beads for 1 h at 4°C with rocking. Then the pre-cleared lysates were immunoprecipitated by incubating with anti-EGFR, anti-Src, or anti-Stat3 antibodies or with IgG (for no antibody) (Santa Cruz) at 4°C overnight with rocking. The complexes were collected with 20 µL protein A/G agarose beads (Santa Cruz), washed three times using wash buffer A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 20 mM Tris-HCl, pH 8.0) and two times with wash buffer B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 500 mM NaCl, 20 mM Tris- HCl, pH 8.0). Then complexes were eluted with freshly prepared elution buffer (1% SDS, 100 mM NaHCO3). Cross-links were reversed by heating at 65°C in the presence of NaCl followed by proteinase K treatment (20 µl of a 20 mg/ml) for 6 h. The DNA was recovered and purified using DNA purification kit from Qiagen (Valencia, CA). The purified chromatin immunoprecipitated DNA was next used as a template for the polymerase chain reaction (PCR) amplification of the c-Myc promoter using the primers, Forward, 5'AAAAGGGGAAAGAGGACCTGG-3', and Reverse, 5'-TAAAAGGGGCAAGTGGAGAGC-3' (Invitrogen). The 133 bp product was resolved on 2% agarose gel. In the case of the sequential ChIP studies, the primary and the secondary immunoprecipitation followed the sequential IP studies described. The recovered complexes after the secondary immunoprecipitation were eluted with the elution buffer and then subjected to ChIP assay, as described.

Preparation of anti-EGFR and mouse IgG1- GNP probes.

Gold nanoparticles (GNPs), 0.1 nM, with a diameter of 40 nm were purchased from Ted

Pella Inc. (Redding, CA). Mouse monoclonal anti-EGFR [F4] antibody was purchased from Abcam (cat. no. ab62, conc. 1.2 mg/ml), and non-specific mouse monoclonal IgG1 was purchased from Sigma (cat. no. M9629, conc. 1 mg/ml). Polyclonal anti-Stat3 (conc. 0.2 mg/ml), polyclonal anti- Src (0.1 mg/ml), polyclonal anti-EGFR (0.2 mg/ml) and non-specific rabbit IgG (0.4 mg/ml) were purchased from Santa Cruz. All other chemicals and buffer ingredients for the assay development were purchased from Sigma. The anti-EGFR-GNP probe was prepared by adding 10 µl of mouse monoclonal anti-EGFR antibody to 1 ml of GNPs. After incubation for 15 min at room temperature, the probe was blocked with 2.5 mg BSA for 30 min. After the centrifugation at 10,000 rpm for 5 min, the supernatant was discarded and the nanoparticle residue was redispersed in 0.5 ml of 0.25% BSA in 10 mM phosphate buffer (PB). The probe was then used in the assay. The negative control mouse IgG1-GNP probe was prepared by adding 10 µl of mouse monoclonal IgG1 antibody to 1 ml GNPs, and following the procedure identical to the one used for the EGFR probe preparation. Mouse IgG1 was used here to prepare the control probe, because the anti- EGFR monoclonal antibody is a mouse IgG1 type antibody.

Detection and kinetic binding study of EGFR from nuclear extract to the GNP probes.

The Panc-1 nuclear extract sample was diluted in phosphate buffer (PB) to 1 mg/ml of total protein. In a sample cell for Dynamic Light Scattering (DLS) measurement (Hellma cuvette QS 3 mm), 20 µl of the anti-EGFRGNP probe was mixed with 2 µl of the sample, and the particle size increase was read with a DLS instrument (Zetasizer Nano

ZS90 DLS system, Malvern Instruments Ltd, England) at exactly 1, 6, 11, 16, and 30 min after the mixing. The same experiment was also performed using the mouse IgG1-GNP probe. In order to confirm the specificity of the anti-EGFR-GNP probe in the detection of EGFR from nuclear extract, an inhibition experiment was conducted by treating 5 µl of the sample with 1 µl of monoclonal anti- EGFR antibody at room temperature for 7 min and 24 min prior to using the sample in the assay. After this incubation, 20 µl of the anti- EGFR-GNP probe was mixed with 2 µl of the treated sample, and the particle size was read at 1, 6 and 11 min after the mixing.

Protein complex binding partner study using polyclonal antibody.

In a 1.5 ml microcentrifuge tube, 80 µl of the anti-EGFRGNP probe was mixed with 8 µl of the sample. After incubation for 30 min at room temperature, this solution was divided into four 20 µl portions. After transferring into the sample cell, the particle size of each of these portions was read with a DLS instrument. After this reading, the solution was spiked with a polyclonal antibody: either with 1 µl of anti-Stat3 or 2 µl of anti-Src or 1 µl of anti- EGFR or 0.5 µl of rabbit IgG. The particle size increase was read at exactly 5 min and 10 min after the start of the first reading.

DLS measurements.

The DLS measurements of all sample solutions were conducted using a Zetasizer Nano ZS90 DLS system equipped with a red (633 nm) laser and an Avalanche photodiode detector (APD) (quantum efficiency>50% at 633 nm) (Malvern Instruments Ltd). DTS applications 5.10 software was used to analyze the data. The average particle size (Z-

average) of the solution was obtained using a Cumulant method. For each sample, ten DLS measurements were conducted with one run, and each run lasted for 10 seconds. All measurements were done at a 90° detection angle.

Results

Detection of EGFR, Src and Stat3 heterocomplex using coimmunoprecipitation and immunoblotting analysis.

Our previous work[65, 68] and others[28, 29] showed that Stat3, EGFR and Src are constitutively-activated in human pancreatic cancer cells, including Panc-1 and Colo-357 cells. We sought to probe the assembly of the three entities. In coimmunoprecipitation (co-IP) with immunoblotting analysis, EGFR immunecomplex from Panc-1 or Colo-357 whole-cell lysates contained both Src and Stat3 (Fig. 10A(i)), Src immunecomplex contained both EGFR and Stat3 (Fig. 10A(ii)), while Stat3 immunoprecipitate contained EGFR and Src (Fig. 10A(iii)). To verify the specificity of the immunoreagents, nonspecific rabbit IgG was used to prepare immunoprecipitates and immunoblotted for Src, Stat3 or EGFR, each of which showed no detectable protein (Fig. 10A, IgG, and data not shown). Moreover, immunoblotting analysis performed on immune precipitates in the presence of the respective blocking peptides (BP) showed a complete or near complete block of the immune detection of Stat3, Src or EGFR, compared to the levels detected in the absence of the blocking peptides (Fig. 10A(iv)-(vi), compare + BP, lower panels to – BP, upper panels). These results further demonstrate the specificity of the antibody-based detection in the present study. We were interested in determining the effect of siRNA knockdown of EGFR or Src on the

complex formation. Coimmunoprecipitation with immunoblotting studies of whole-cell lysates of Panc-1 cells showed that when EGFR is knockdown by siRNA (Fig.10B(i) and (ii), upper band), Src immunecomplex remains associated with Stat3 (Fig. 10B(i), IP:Src), and vice-versa (Fig. 10B(ii), IP:Stat3). Likewise, when Src is knockdown by siRNA (Fig. 10B(iii) and (iv), upper band), EGFR immunecomplex remains associated with Stat3 (Fig. 10B(iii), IP:EGFR), and vice-versa (Fig. 10B(iv), IP:Stat3). Scrambled (con) siRNA has no effect. These findings together indicated EGFR, Src and Stat3 form a heteromeric complex in Panc-1 and Colo-357 cells, and that Stat3 remains associated with Src or EGFR, respectively, upon the siRNA knockdown of EGFR or Src.

Given the reported nuclear EGFR and Stat3 complex in breast cancer cells [63], we asked the question whether the observed heteromeric complex was present in the nucleus. Nuclear extracts were prepared and subjected co-IP with immunoblotting analysis. Results show that EGFR immunecomplex contained both Stat3 and Src (Fig. 10C(i), IP:EGFR), Src immunecomplex contained both Stat3 and EGFR (Fig. 10C(ii), IP:Src), while Stat3 immunecomplex contained both EGFR and Src (Fig. 10C(iii), IP:Stat3). These data demonstrated that the presence of the EGFR, Src and Stat3 heteromeric complex in the nucleus. For studies of the specificity of the immunoreagents, the non-specific rabbit IgG pull-down samples were similarly immunoblotted and showed no detectable EGFR, Src or Stat3 (Fig. 10C, IgG and data not shown). The heteromeric complex was validated by performing sequential immunoprecipitation analysis, whereby EGFR immunecomplex (IP:EGFR) was further

subjected to a secondary Immunoprecipitation using anti-Src antibody ((IP:EGFR/IP:Src) and then immunoblotted for EGFR and Stat3. The results of these studies showed the presence of EGFR and Stat3 in the sequential immunoprecipitates (Fig. 10C(iv), IP:EGFR/IP:Src). By contrast, IgG pull-down that was subjected immunoblotting for EGFR, Src or Stat3 showed no detectable levels (Fig. 10C(iv), IgG), further confirming the specificity of the immunoreagents used. Moreover, immunoblotting analysis probing for the Tata-binding protein (TBP) (Fig. 10C(vi)) confirmed the extracts used in these studies are of nuclear origin.

Results so far show the EGFR, Src and Stat3 complex is detected in both whole-cell and nuclear lysates. We were therefore interested to determine the relative levels of EGFR, Src and Stat3 in the different sub-cellular fractions and to assess whether size of each of these proteins in the different sub-cellular locations. Membrane and cytosolic fractions, and nuclear extracts were prepared from Panc-1 cells according to established protocol using NP-40 lysis and low salt (100 mM NaCl) for cytosolic extract, high salt (400 mM NaCl) extraction for nuclear extracts, and 1% Triton X-100 buffer for membrane fraction. Samples of equal total protein were then subjected to SDS-PAGE and immunoblotting analysis. Immunoblotting analysis of sub-cellular fractions of equal total protein shows the size of EGFR, Src, or Stat3 obtained in the membrane or cytosolic fractions, or in nuclear extracts are the same (Fig. 10D). Results further show that the levels of total EGFR protein are highest in the membrane, and are higher in the cytosolic fraction than in the nuclear extract (Fig. 10D (i)). By contrast, results show that

Stat3 levels are highest in the cytosol fraction, and higher in the nuclear extract, compared to the levels associated with the cell membrane (Fig. 10D(ii)). The results for Src were also different and showed the membrane-associated levels were higher than the levels detected in the cytosolic fraction and nuclear extracts, which were nearly idetnifcal (Fig. 10D(iii)). These results together reveal for the first time the detection of c-Src in the nucleus, together with the presence of EGFR and Stat3.

In the stimulation of the EGFR signaling, key phospho-tyrosine (pY) peptide motifs of the receptor are induced, which recruit a variety of signaling proteins, including Stat3[22, 69]. The Stat3 protein, via its SH2 domain binds to the receptor, engaging in pY-SH2 domain interactions[70]. We therefore sought to define the observed nuclear assembly of the EGFR, Src and Stat3 complex by using the known Stat3-binding EGFR motifs, pY1068 peptide and pY1086 peptide, and the newly reported Stat3 peptide inhibitor, SPI, which is derived from the Stat3 SH2 domain and is capable to block the binding of the Stat3 SH2 domain to pY peptide motifs[71]. Nuclear extract preparations from Panc-1 cells were first incubated with or without peptides for 2 h at room temperature, subjected to immunoprecipitation analysis using anti-Stat3 antibody, and the Stat3 immunecomplex subjected to SDS-PAGE and immunoblotting analysis for EGFR and Src. Results showed no detectable EGFR protein in the Stat3 immunecomplex from nuclear extracts pre-incubated with the three peptides (Fig. 10E(i)), suggesting the EGFR-Stat3 interaction is facilitated by pY1068 and pY1086EGFR, and requires the key amino acid residues, 588-615[71] that make up the

Stat3 SH2 domain peptide inhibitor SPI. By contrast, similar incubation with the SPI peptide has minimal effect on the detectable Src protein associated with the Stat3 immunecomplex, suggesting a direct interaction between Src and Stat3, which is independent of the amino acid residues 588-615 of the Stat3 SH2 domain, and the detection of such a complex in the nucleus. These findings together indicate that there is a key requirement for the Stat3 SH2 domain and the EGFR peptide motifs, pY1068 and pY1086 in the assembly of the nuclear EGFR, Src and Stat3 complex. We further sought to determine the importance of EGFR or Src in the assembly of the nuclear complex by examining the effect of siRNA knockdown. Immunoblotting analysis of immunecomplexes of Stat3 (IP:Stat3), EGFR (IP:EGFR) or Src (IP:Src) prepared from nuclear extracts showed that the siRNA knockdown of Src (Fig. 1F(i), upper panel) has little effect on the association of Stat3 with EGFR in the nucleus (Fig. 1F(i), IP:Stat3, IP:EGFR), while similarly, the knockdown of EGFR (Fig. 1F(ii), upper panel) did not alter the binding of Stat3 with Src (Fig. 1F(ii), IP:Stat3, IP:Src). Scrambled (con) siRNA has no effect. These data parallel the findings of the whole-cell lysates analysis in Figure 10B on the lack of effect of the knockdown of EGFR or Src on the association of Stat3 with the remaining partner. These data indicate that the knockdown of one protein does not preclude the interaction between the other two protein partners.

Given the results of the siRNA knockdown studies, we sought to determine if the heteromeric complex formation is dependent on the tyrosine kinase activities of EGFR and Src. Panc-1 cells were first treated with the selective EGFR inhibitor, Iressa

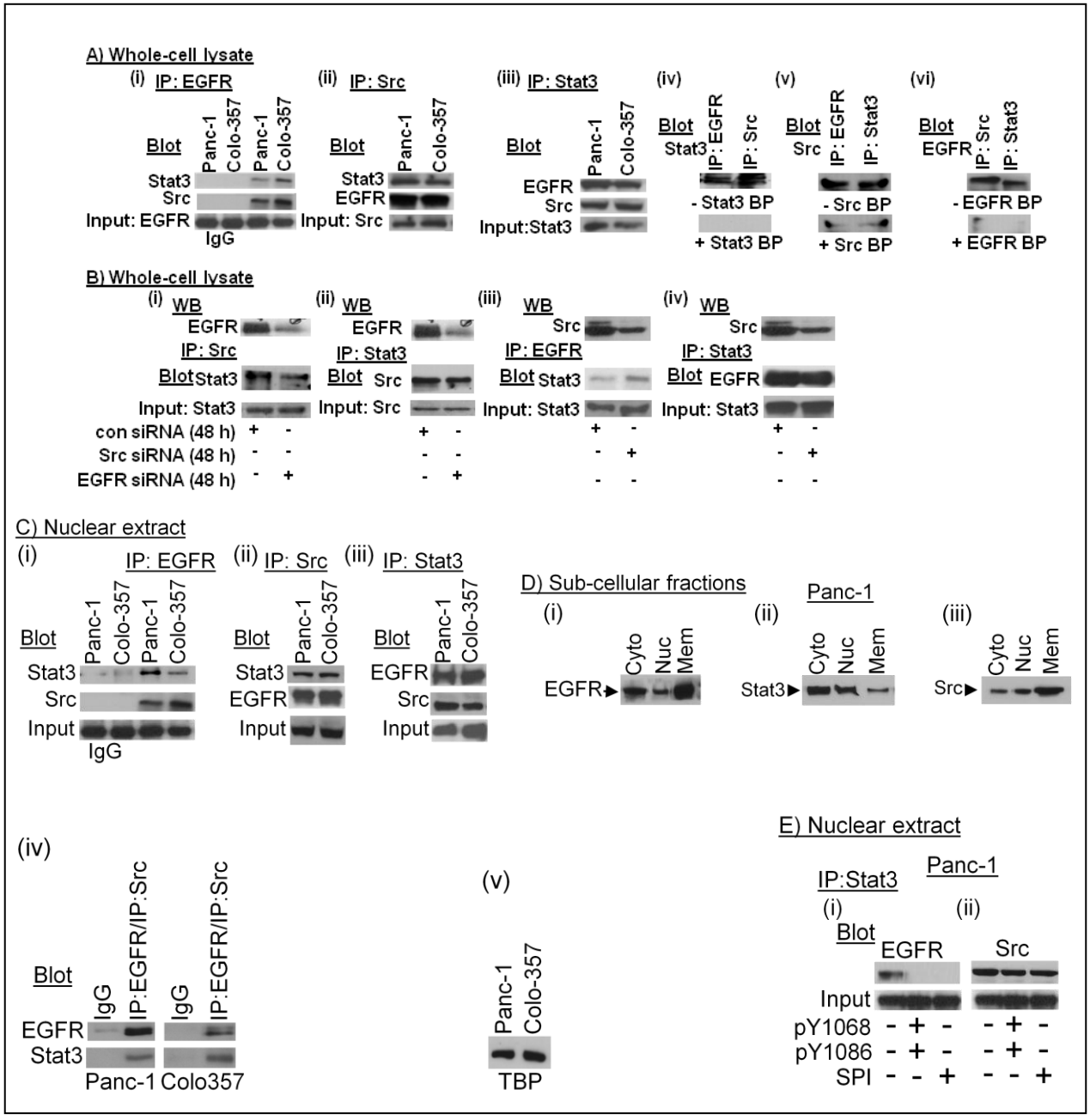
(ZD1839, ZD), the Src inhibitor, Dasatinib (Das), or the Stat3 dimerization disrupting inhibitor, S3I-201[21] prior to co-IP and immunoblotting analyses. Compared to IgG, immunoblotting of immunecomplex of EGFR showed no effect of treatment of cells for 1 h with any of the inhibitors (Fig. 1G, IP:EGFR, lanes 3, 6, and 8, compared to lane 2). On the other hand, immunoblotting analysis of EGFR immunecomplex showed decreased levels of associated Stat3 protein, but not Src in cells treated with ZD, Das, or S3I-201 for 24 (Fig. 1G, lanes 4, 7, and 9). We surmise that both the EGFR and Src kinase activities promote the association of Stat3 with EGFR in the nuclear heteromeric complex, while the inhibition of EGFR or Src kinase alone does not preclude EGFR/Src interaction. Furthermore, a Stat3 dimerization disrupting inhibitor blocks Stat3 association in the complex; however, the disruption of Stat3 dimerization, and hence its binding to EGFR does not preclude EGFR/Src interaction. The observation that the nuclear heteromeric EGFR, Src, and Stat3 complex was not completely dissociated and that EGFR/Src complex persisted by 24-h inhibition of EGFR (ZD) or Src (Das) (Fig. 1G, lanes 4, and 9) has important clinical implications in terms of the responsiveness of pancreatic cancer cells to a monotherapy targeting EGFR or Src.

Several mechanisms have been reported for EGFR nuclear translocation, including receptor endocytosis, endosomal sorting machinery, importins alpha1/beta1, and exportin CRM1[72, 73]. Previous studies have shown that Stat3 nuclear translocation is facilitated by EGFR-mediated endocytosis[59]. However, the siRNA knockdown of EGFR did not affect the nuclear presence of Src/Stat3 (Fig. 10F(ii)), suggesting EGFR-

mediated mechanisms may not be essential for the nuclear presence of Stat3 or Src.

To determine whether endocytosis is required for the nuclear presence of the heteromeric complex, cells were treated with the pharmacological inhibitor of endocytosis, phenylarsine oxide[59]. In contrast to PAO induced inhibition of nuclear Stat3 DNAbinding activity induced by EGF [59], immunoblotting analysis showed treatment with PAO has no effect on the nuclear presence of EGFR, Src and Stat3 heterocomplex in pancreatic cancer cells (Fig. 10H), suggesting the nuclear presence of the heteromeric complex is not dependent on endocytosis. Altogether, these findings extend the previously reported nuclear EGFR and Stat3 complex in breast cancer cells[63] by showing that EGFR, Src and Stat3 associate into a complex in a manner where all three proteins interact. However, our study does not exclude the possibility that other proteins could be present in the complex.

Fig. 10


A) Whole-cell lysate
(i) IP: EGFR
(ii) IP: Src
(iii) IP: Stat3
(iv)
(v)
(vi)
Blot
Panc-1
Colo-357
Stat3
Src
Input: EGFR
IgG
EGFR
Input: Src
Input:Stat3
IP: EGFR
IP: Src
IP: Stat3
- Stat3 BP
+ Stat3 BP
- Src BP
+ Src BP
- EGFR BP
+ EGFR BP
B) Whole-cell lysate
WB
IP: Src
IP: Stat3
IP: EGFR
con siRNA (48 h)
Src siRNA (48 h)
EGFR siRNA (48 h)
C) Nuclear extract
D) Sub-cellular fractions
Cyto
Nuc
Mem
E) Nuclear extract
IP:Stat3
IP:EGFR/IP:Src
TBP
pY1068
pY1086
SPI

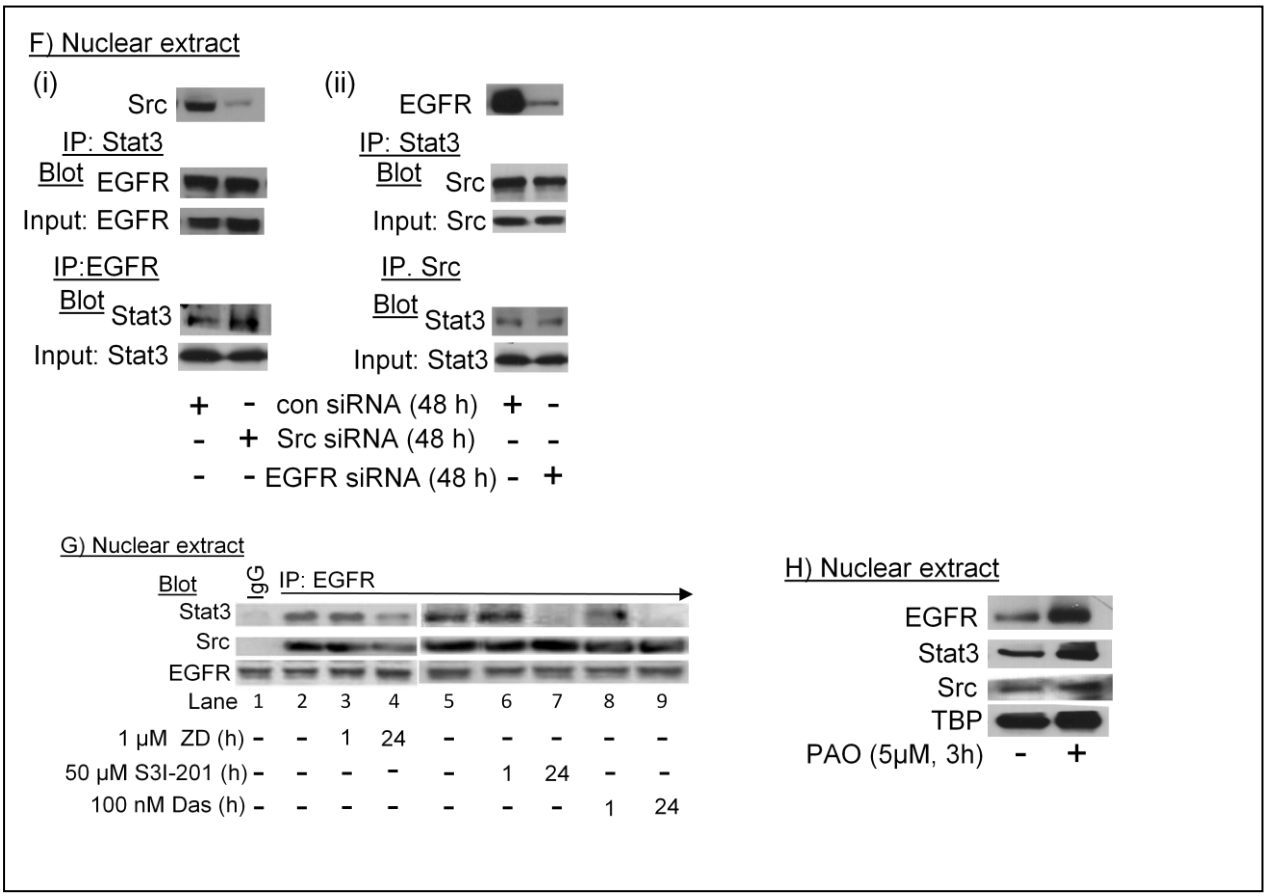


Figure 10: Co-immunoprecipitation analysis of EGFR, Src and Stat3 asscoiation in Panc-1 and Colo-357 cells

(A) and (B), immunoblotting analyses of immunecomplexes of EGFR (IP:EGFR), Src (IP:Src), and Stat3 (IP:Stat3), or of non-specific IgG non-immunoprecipitate prepared from whole-cell lysates of Panc-1 and Colo-357 cells untransfected (A) or of Panc-1 cells transfected with EGFR siRNA, Src siRNA, or control (con) siRNA (B) and probing for Src, Stat3 and EGFR in the presence or absence of Stat3 blocking peptide (Stat3 BP), Src blocking peptide (Src BP) or EGFR blocking peptide (EGFR BP); (C), immunoblotting analyses of immunecomplexes of EGFR (IP:EGFR), Src (IP:Src), Stat3 (IP:Stat3), or of non-specific IgG non-immuneprecpitate prepared from nuclear extracts of Panc-1 or Colo-357 cells and probing for Stat3, EGFR, Src, or the Tata-binding protein (TBP); (D), immunoblotting analysis of membrane (mem) and cytosolic (cyto) fractions and nuclear (nuc) extracts from Panc-1 cells probing for (i) EGFR, (ii) Stat3 and (iii) Src; (E), immunoblotting analyses of Stat3 immunecomplex (IP:Stat3) prepared from nuclear extracts pre-incubated for 2 h with 100 μM each of the peptides, pY1068, pY1086, or SPI; (F), and (G), immunoblotting analyses of immunecomplexes of EGFR (IP:EGFR), Src (IP:Src), Stat3 (IP:Stat3), or of non-specific IgG non-immunecomplex prepared from nuclear extracts of Panc-1 or Colo-357 cells untransfected (E) or transfected (F) and (G) with Src siRNA, EGFR siRNA, or control (con) siRNA, or treated with the EGFR inhibitor (ZD1839, ZD), Src inhibitor (Dasatinib, Das), or Stat3 inhibitor (S3I-201) for 1 or 24 h (G) and probing for EGFR, Src, Stat3; or (H) immunoblotting analysis of nuclear extracts prepared from Panc-1 cells treated or untreated with phenylarsine oxide (PAO) and probing for Src, Stat3, EGFR, and the Tata-binding protein (TBP); bands corresponding to proteins in gel are shown; input: except where indicated, represents the respective immunoprecipitated protein probed in the same amount of lysate or nuclear extract used in the assay; IP:EGFR/IP:Src, sequential immunoprecipitation

with anti-EGFR and then anti-Src antibody; M, molecular weight marker, of which 75kDa protein marker is shown; Data are representative of 3 independent studies.

Detection and Analysis through Nanoparticle Sizing (DANS) technology with Dynamic Light Scattering to detect EGFR, Src and Stat3 heterocomplex.

The EGFR, Src and Stat3 association was further probed using DANS (Detection and Analysis through Nanoparticle Sizing) technology[74-78]. The principle of DANS technology for protein complex detection and binding partner analysis is a single-step solution immunoassay based on gold nanoparticle (GNP) immunoprobes coupled with dynamic light scattering (DLS) detection. Details of this technology are provided as part of Supplemental Information, “Results”. Based on this general principle, the assay is designed to detect and identify protein binding partners of a protein complex. The kinetic binding assay revealed and confirmed that the anti- EGFR-GNP probe detects EGFR from the sample specifically (Fig. 11A(i)). The increase in particle size obtained with the anti-EGFRGNP probe was much larger than the one obtained with the non-specific mouse IgG1 control probe (Fig. 11A(i)). The approximate net increase of the average nanoparticle size of the assay solution was about 70 nm, after deducting the size increase caused by nonspecific interactions. For the mouse IgG1- GNP probe, the particle size increase was less than 10 nm after incubation with the sample for 30 min. Furthermore, the steepness of the kinetic curve for the EGFR probe suggested specific interactions in the assay system, while the shallow curve for the mouse IgG1 probe indicated the small size increase of the nanoparticle probes was due to non-specific interactions. To further confirm the specificity of anti-EGFR-GNP probe in the detection of EGFR from nuclear extract, an inhibition experiment was conducted in which the extract was first treated with the monoclonal anti-EGFR antibody. It is expected that the binding between the monoclonal antibody and the EGFR protein from the sample will

block the subsequent binding of EGFR (in the sample) to the GNP immunoprobes, therefore, leading to decreased nanoparticle size increase during the kinetic assay. Indeed, data shown in Figure 11A(ii) confirmed the inhibition effect of the monoclonal anti-EGFR antibody. The magnitude of this inhibition was correlated to the sample treatment time: the 24-min treatment inhibited the particle size increase in the assay stronger than the 7 min- reatment (Fig. 11A(ii)).

After using the anti-EGFR-GNP probe to “catch” the EGFR protein or protein complex from the nuclear extract sample, a second polyclonal antibody (anti-Stat3, anti-Src, anti8 EGFR antibodies, and non-specific rabbit polyclonal IgG) was then added to the assay solution to identify the binding partner of the complex. In negative control studies using the mouse IgG1-GNP probe, particle size remains nearly unchanged after the addition of the four polyclonal antibodies (Fig. 11B(i)). This result confirmed the kinetic binding study that the non-specific mouse IgG1-GNP probe did not bind with the EGFR protein or protein complex from the sample. The addition of a polyclonal antibody itself to the assay solution did not cause significant nanoparticle size change.

By contrast, with the anti-EGFR-GNP probe, significant particle size increase was observed when anti-Stat3 or anti-Src antibody was added to the assay solution (Fig. 11B(ii), anti- Stat3, Src). A smaller size increase was observed from anti-EGFR antibody (Fig. 11B(ii), anti-EGFR) and the rabbit IgG (Fig. 11B(ii), IgG). Per the assay principle (see Fig. S1 in Supplemental Information, “Results”), the substantial particle

size increases observed from the addition of anti-Stat3 or anti-Src antibody to the assay solution can only explained by the presence of EGFR in complex with Stat3 and Src in the nuclear extract. EGFR was specifically bound to the nanoparticle immunoprobes, bringing along the Stat3 and Src proteins to the nanoparticle surface, and the subsequent incubation of this assay solution with anti-Stat3 or anti-Src antibody led to further increase of the nanoparticle size. Furthermore, it appears that there is an equal amount of Stat3 and Src proteins in the EGFR, Src, and Stat3 complex. In multiple assays conducted so far, the size increase caused by anti-Src antibody is always just slightly lower than the case of anti-Stat3 antibody. Src is a smaller protein (60 KDa) than Stat3 (80-90 KDa). Therefore, with the binding of the same amount of antibody to the protein complex-GNP conjugates, the particle size should be just slightly smaller in the case of anti-Src assay solution than anti-Stat3 assay solution.

Compared anti-Stat3 or anti-Src antibody, the particle size increase upon addition of anti- EGFR antibody to the assay solution is much smaller, only slightly higher than the nonspecific rabbit IgG. This is explained by the fact that Stat3 and Src are located on the surface of the nanoparticle-bound EGFR protein, therefore, block the binding of anti-EGFR antibody to the bound EGFR proteins. The effectiveness of polyclonal anti-EGFR antibody with the EGFR protein in the sample was confirmed in a different experiment (data not shown). This result indirectly suggested that the EGFR protein detected by the nanoparticle immunoprobe from the nuclear extract exists as a complex with Stat3 and Src protein.

Fig.11

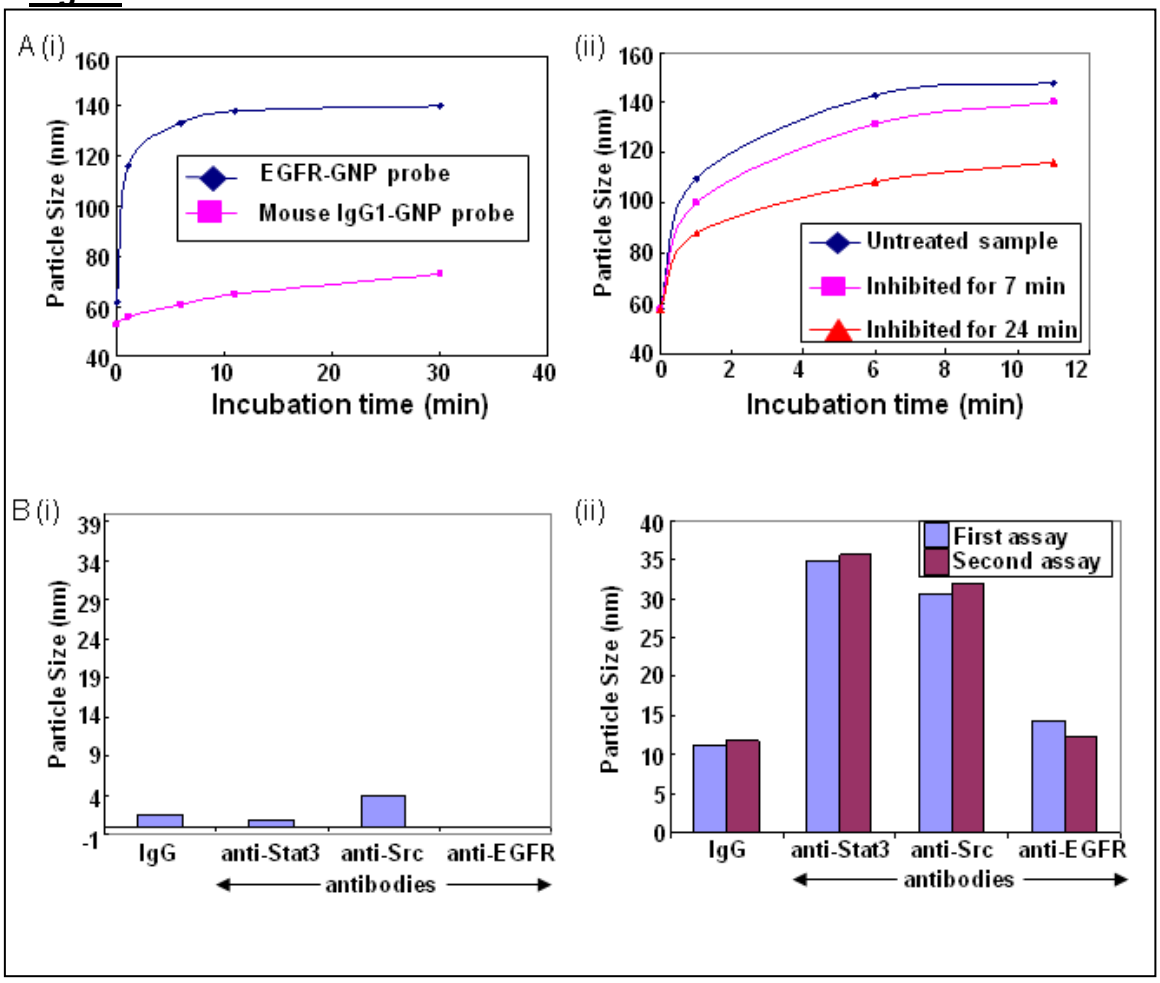


Figure 11: Studies of protein complex and protein binding partners using the Detection and Analysis through Nanoparticle Sizing technology

(A) Kinetic binding assay of EGFR-gold nanoparticle (GNP) probe (or mouse IgG1-GNP probe as negative control) binding to (i) EGFR protein and its complex from Panc-1 nuclear extracts, and the (ii) inhibitory effect of the mouse monoclonal anti-EGFR antibody on the EGFR-GNP probe binding to the EGFR protein; and (B) Protein complex binding partner analysis whereby the polyclonal anti-Stat3, anti-Src or anti-EGFR antibody or the non-specific rabbit IgG (negative control) is added to the assay solution prepared from the (i) non-specific mouse IgG1-GNP probe (negative control), or (ii) anti-EGFR-GNP probe; Data are representative of 4 independent studies.

Detection of EGFR, Src and Stat3 heterocomplex by Immunofluorescence with laser-scanning confocal microscopy.

Immunofluorescence with laser-scanning confocal microscopy allowed visualization of the intracellular distribution and localization patterns of EGFR, Src and Stat3. Immunofluorescence with laser-scanning confocal microscopy confirmed the localization patterns and showed that in contrast to the negative staining (Fig. 12B(i), left panel), EGFR appearance is punctuate (red) at the plasma membrane, and in the cytoplasm and the nucleus (Fig. 12B(i), single). Similar localizations for Src (blue) and Stat3 (green) were observed, but with greater presence in the nucleus (Fig. 12B(i), single). There are stainings for colocalization of EGFR and Src (magenta, see arrows), EGFR and Stat3 (yellow, see arrows), Src and Stat3 (cyan, see arrows), and of all three entities (pale yellow/white, see white arrows) at the plasma membrane, cytoplasm, perinuclear and nuclear spaces (Fig. 12B(i), merge). These studies confirm previous reports of the association of EGFR and Stat3 in similar intracellular spaces[59, 63]. Importantly, the data show for the first time the presence of a heteromeric EGFR, Src and Stat3 complex in the nucleus, as observed by the co-IP and immunoblotting analyses, and confirmed by DANS/DLS analysis (Figures 10 and 11). These data contrast the results observed for the normal human pancreatic duct epithelial cells (HPDEC) (Fig. 12A). Immunofluorescence staining with laser scanning confocal microscopy analysis of HPDEC shows a homogeneous distribution of EGFR (red), Src (blue) and Stat3 (green), all of which are strongly outside of the nucleus, with little evidence of co-localization (Fig. 12A).

Visualization of the intracellular distribution patterns of EGFR, Src and Stat3 upon inhibition of EGFR or Src tyrosine kinase by immunofluorescence with laser-scanning confocal microscopy indicated that as with the co-IP studies (Fig. 12E, the inhibition of Src or EGFR tyrosine kinase activity alone did not completely eliminate complex formation. However, Src or EGFR inhibition disrupted the localization patterns. Data shows EGFR, Src and Stat3 distribution in the cytoplasm and the nucleus following 1- or 24-h treatment with EGFR or Src inhibitor (Fig. 12B(ii) and (iii), 1 h, and 24 h, single). Nuclear EGFR levels are diminished, but not abolished (Fig. 12B(ii), single). Thus, EGFR, Src and Stat3 remain localized in the nucleus following tyrosine kinase inhibition. Results further showed persistent EGFR and Src (magenta), and Src and Stat3 (cyan) associations in both the nucleus and cytoplasm following treatment for 1 h with EGFR or Src inhibitor (Fig. 12B(ii) and (iii), 1 h), similar to the data in Figure 12E (1 h). Furthermore, a nuclear EGFR and Stat3 association (yellow) is detected upon 24-h treatment with the EGFR inhibitor (Fig. 12B(ii), 100 nM ZD, 24 h), while nuclear associations of EGFR and Src (magenta), and of Src and Stat3 (cyan) are detected following treatment with Src inhibitor for 24 h (Fig. 12B(iii), 100 nM Das, 24 h). The moderate differences in the observed patterns of complex formation between the co-IP (Fig. 10E) and confocal data may be due to the sensitivity differences between the two approaches. Overall, the findings are consistent with the co-IP data (Fig. 10E) in showing that the inhibition of the kinase activity of EGFR or Src alone is insufficient to completely disrupt all the proteins from the complex.

Fig.12

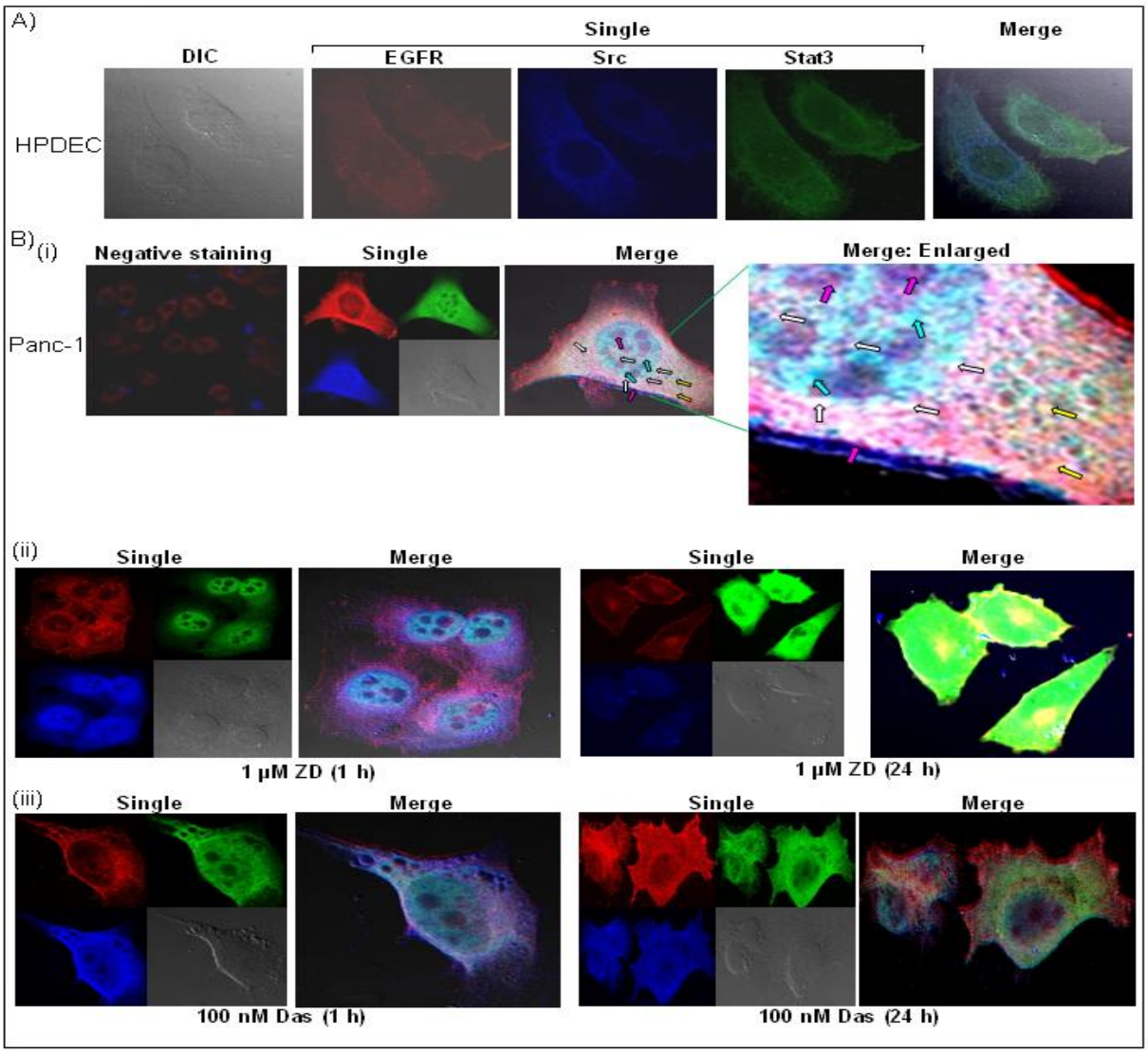


Figure 12: Immunofluorescence with laser scanning confocal microscopy of EGFR, Src and Stat3 association in Panc-1 Cells

Cultured normal human pancreatic duct epithelial cells (HPDEC) (A) or pancreatic cancer, Panc-1 cells (B) were fixed, stained with primary antibodies against EGFR, Src and Stat3 and their corresponding secondary antibodies, ALexaFLuor405 (goat anti-mouse, EGFR, red), AlexaFluor488 (donkey anti-rabbit, Src, blue) and AlexaFluor546(goat anti-rat, Stat3, green) and analyzed by laser-scanning confocal microscopy for localization (single) and colocalization (merge) studies of EGFR (red), Src (blue) and Stat3 (green) and the effects of treatment (i) without or (ii) with ZD1839 (ZD) or (iii) Dasatinib (Das) for the indicated times. Confocal images were collected using Leica TCS SP5 microscopes; Cyan, magenta, yellow and white/pale yellow arrows denote merged colors; single, one color capture, merged, three-color capture. Data are representative of 3 independent studies.

EGFR, Src and Stat3 heteromeric complex regulates gene expression.

Previous report identified the presence of a nuclear EGFR and Stat3 complex in breast cancer cells, which showed that EGFR/Stat3 complex induces specific genes, including inducible nitric oxide synthase (iNOS)[59, 63]. It is therefore reasonable to propose that the nuclear EGFR, Src and Stat3 observed in the present study in pancreatic cancer lines could be responsible for the induction of a set of genes. In our previous work, we showed that the expression of c-Myc was refractory to the inhibition of EGFR, Src or Stat3 alone and that the concurrent inhibition of aberrant Stat3 activity together with EGFR or Src inhibition strongly suppressed c-Myc expression [65], suggesting the regulatory mechanisms promoting cancer supporting genes, such as c-Myc are rather complex. Thus, studies were performed to assess the functional significance of the heteromeric complex and we focused on the c- Myc gene. We pursued a chromatin immunoprecipitation (ChIP) analysis to determine whether c-Myc promoter is associated with the heteromeric complex. PCR amplification of DNA fragment using a primer against the c-Myc gene promoter and agarose gel electrophoresis showed that each of the anti- EGFR, Src, or Stat3 antibody-chromatin DNA immunoprecipitate contained the c-Myc gene (Fig. 13A(i), EGFR, Src and Stat3). To further confirm this finding, we pursued a modified sequential Immunoprecipitation similar to the one performed in Fig. 11C(iv) in the context of a ChIP assay. The sequential ChIP assay in which EGFR chromatin immunecomplex was subjected to a second immunoprecipitation using anti-Src antibody and similarly analyzed by PCR amplification and agarose gel electophoresis similarly detected the presence of the c-Myc gene (Fig. 13B(ii), EGFR/Src).

To verify the specificity of the immunoreagents, the non-specific IgG was similarly used in ChIP assay, and the IgG immunoprecipitate, similarly analyzed by PCR for the c-Myc gene, showed no detectable levels (Fig. 13A, IgG). Taken together with the sequential immunoprecipitation data in Figure 10C(iv), these studies demonstrate that EGFR, Src and Stat3 form a detectable heterocomplex in pancreatic cancer cells that is associated with the expression of c-Myc. These findings support the complex regulation of genes in a manner that would confer a reduced sensitivity to the inhibition of EGFR, Src or Stat3 alone. In that context, immunoblotting analysis of whole-cell lysates showed a degree of suppression, but not an elimination of the c- Myc gene in response to EGFR or Src knockdown alone (Fig. 13B), consistent with our previously published study[65]. Similarly, immunoblotting analyses further show no observable changes in the Cyclin D1 expression by the EGFR or Src knockdown (Fig. 13B), a decrease in iNOS expression by the knockdown of EGFR, but not Src, and a decreased expression of VEGF in response to the knockdown of Src, but not EGFR (Fig. 13B). Given that none of the genes studied here showed a consistent pattern of regulation by any one of EGFR or Src, we surmise they could be under the complex regulation by mechanisms involving the EGFR, Src and Stat3 heteromeric complex. To further investigate the relevance of the EGFR, Src and Stat3 complex in the regulation of c-Myc, we pursued a more extensive analysis of its expression in response to modulation of EGFR, Src and Stat3. Immunoblotting analysis showed that by contrast to siRNA knockdown of EGFR or Src alone, or the pharmacological inhibition of only Src or aberrant Stat3 activity,

which only moderately suppresses c-Myc expression, the concurrent knockdown of EGFR with Stat3 inhibition by S3I-201[21] or knockdown of Src with Stat3 inhibition, or the EGFR knockdown with Src inhibition (by Das) results in strong suppression of c-Myc expression (Fig. 13C(ii)). Bands corresponding to levels of expression were quantified, analyzed by ImageQuant, and the expression levels are represented as percent of control (in parenthesis). Results show over 73% suppression of c-Myc expression (Fig. 13C(ii) following the concurrent modulation of any two of EGFR, Src and Stat3. Together, these studies indicate that the c-Myc gene is more responsive to the concurrent modulation of any two of the EGFR, Src and Stat3 proteins. The moderate change in the c-Myc expression in response to the concurrent transfection with EGFR siRNA and c-Src siRNA could be due to the fact that we could achieve only partial knockdown of Src (Fig. 13C(i)), although EGFR is significantly suppressed by the EGFR siRNA (Fig. 13C(i)), compared to the use of the pharmacological inhibitor, Das, which would strongly suppress Src activity.

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Fig. 13

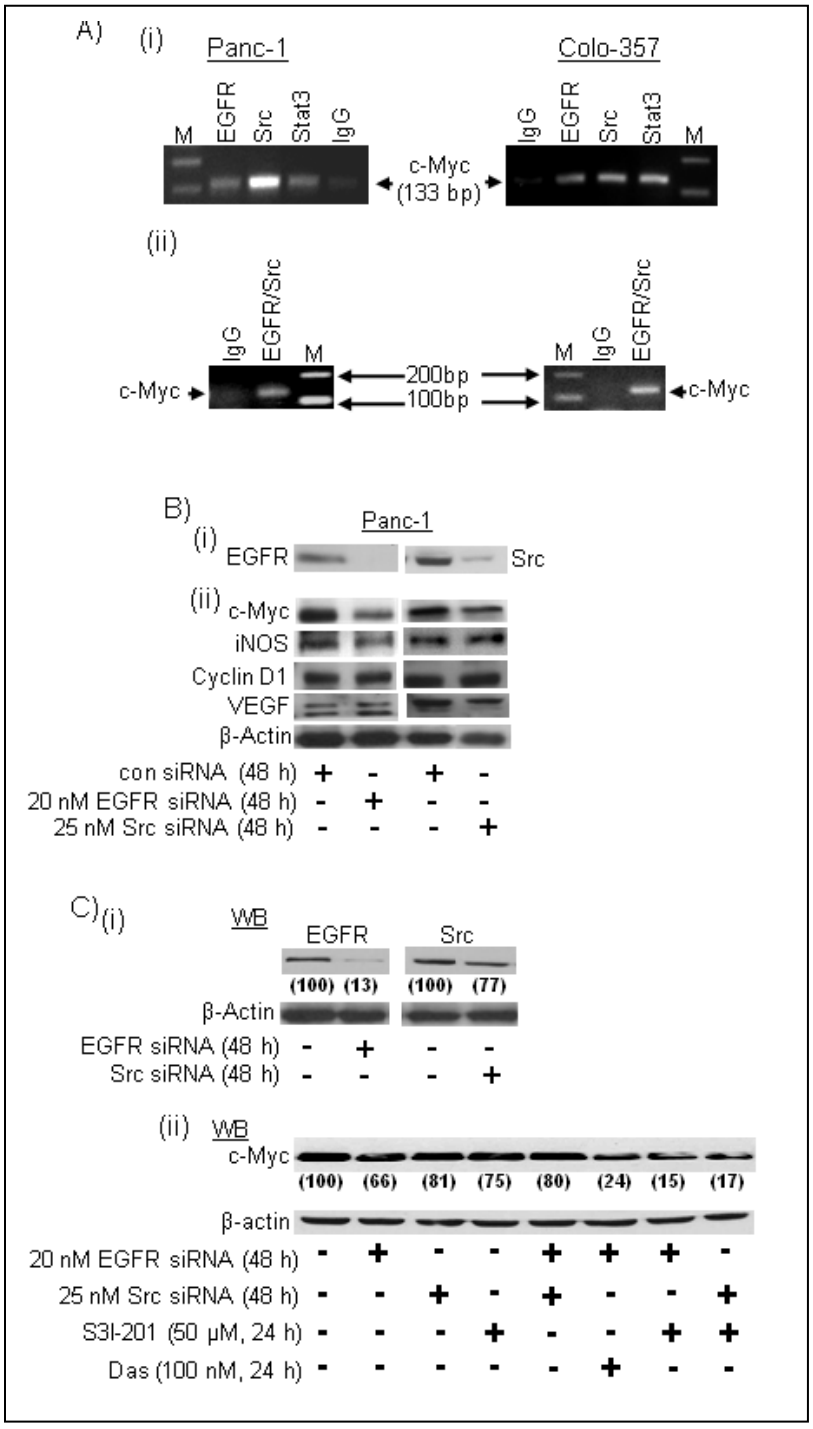

Figure 13: Chromatin Immunoprecipitation assay and Western blotting analysis of c-Myc, iNOS, Cyclin D1 and VEGF expression in Panc-1 and Colo-357 cells

(A), Agarose gel electrophoresis of Polymerase Chain Reaction (PCR)-amplified c-Myc gene fragment from the chromatin DNA precipitated with antibody against EGFR, Src, Stat3, or with the non-specific IgG; and (B) and (C), Immunoblotting analysis of whole-cell lysates probing for EGFR or Src (B(i) and C(i)) or c-Myc, iNOS, Cyclin D1 or VEGF (B(ii) and C(ii)), and the effects of siRNA knockdown of EGFR (EGFR

siRNA), Src (Src siRNA) or control (con) siRNA, or S3I-201 or Das); bands corresponding to proteins or c-Myc gene in gel are shown; M, molecular weight marker, EGFR/Src, sequential immunoprecipitation with anti-EGFR and then anti-Src antibody; Data are representative of 3 independent studies.

Discussion

Aberrations in the EGFR, c-Src and Stat3 signaling pathways occur with a high frequency in many human cancers[29, 32, 33, 79-81] and are associated with poor prognosis. Thus, there is increasing effort to gain further understanding of the role of these aberrant pathways in human tumors in order to derive novel, more effective treatment modalities. We present evidence for a nuclear EGFR, Src and Stat3 heteromeric complex. The presence of the nuclear EGFR, Src and Stat3 complex was identified using a variety of approaches, including co-immunoprecipitation (co-IP) with immunoblotting analysis, sequential co- IP analysis, Detection and Analysis through (Gold) Nanoparticle Sizing with Dynamic Light Scattering, and by Immunofluorescence with laser-scanning confocal microscopy. In contrast to pancreatic cancer lines, normal human pancreatic duct epithelial cells did not show any evidence of EGFR, Src and Stat3 heteromeric complex formation, placing importance to the presence of the complex in the nucleus of cancer cells.

De-regulation of gene expression is one of the hallmarks of cancer and is promoted by multiple mechanisms. It is well established that aberrant Stat3 activity promotes dysregulation of gene expression, and although a set of Stat3-responsive genes have been identified, there could still be others that are yet to be discovered. The exact mechanisms of how Stat3 would promote the expression of genes in the context of

supporting the malignant phenotype are not fully understood. Recent evidence of a nuclear EGFR pathway[61, 62] in which nuclear EGFR associates with Stat3, forming a transcriptionally-active EGFR/Stat3 complex that promotes the induction of iNOS in breast cancer cells [63] is evidence of how complex the Stat3-mediated regulation of gene expression could be in cancer cells. Other reports have shown a cooperation between Stat3 and NF-κB[82, 83] in promoting the transcription of genes. In that context, the present evidence extends our earlier report [65] in showing that the heteromeric EGFR, Src and Stat3 complex is associated with the induction of the c-Myc gene. There is likely to be other genes promoted by the EGFR, Src and Stat3 nuclear complex, and iNOS, VEGF, and Cyclin D1, which have been identified as induced by the EGFR/Stat3 complex or as Stat3-regulated genes[63, 84, 85] may be candidates for the regulation by the EGFR, Src and Stat3 complex. Taken together, these studies provide novel mechanistic insight into the de-regulation of gene expression that is a feature of cancer cells. The observation that the EGFR, Src and Stat3 complex is also detected in the cytoplasm raises the possibility it is formed extra-nuclear and transported into the nuclear space. However, it does not preclude the complex being assembled *de novo* in the nucleus. For Stat3, several nuclear translocation mechanisms have been proposed, including EGFR-mediated endocytosis [59, 73].However, the data that nuclear Src and Stat3 complex remain detectable under the conditions of EGFR knockdown, or when cells are treated with the general endocytosis inhibitor, phenylarsine oxide, suggests potential other nuclear translocation mechanisms could be involved. It has also been previously reported that EGFR has inherent nuclear

localization mechanisms, including endosomal sorting machinery, and interaction with importins α1/β1 [59, 73]. Other reported Stat3 nuclear translocation mechanisms include the Ran- GTPase [57], Sec61 translocon [86], and chaperoning by MgcRacGAP [58]. While we exclude endocytosis, any combination of these other mechanisms could facilitate the nuclear transport of the complex. Present data also suggest that only a portion of the levels of EGFR, Src and Stat3 are utilized in the formation of the nuclear complex, raising the possibility that there may be diverse cellular pools of EGFR, Src, or Stat3 with different accessibility limitations. There also could be different pools of pre-associated complexes of the three proteins, a possibility that will be consistent with the report that cytoplasmic Stat3 exists as complexes with accessory scaffolding proteins [87]. Such pre-formed complexes would not only facilitate the signal induction[88], but may also serve to stabilize proteins.

The incidence of signaling cross-talk has long been known, and the associations of EGFR with Src [89], Src with Stat3 [42, 90], and EGFR with Stat3 [22, 69] at the plasma membrane and perinuclear space [59] have all been reported. Specifically, Stat3 binds to pY1068 and pY1086 motifs of EGFR [22, 69], while Src binds to Y845EGFR. In cancer cells, aberrant Stat3 activation is promoted by hyperactive protein tyrosine kinases, including EGFR and Src [32, 33, 80], and evidence has indicated the phosphorylation of Y845EGFR, Y1068EGFR, and Y845EGFR motifs by c-Src in pancreatic cancer cells[22, 26, 65]. Data presented show the pY1068EGFR and pY1086EGFR motifs, and the Stat3 SH2 domain, specifically residues 588-615 are

essential for EGFR-Stat3 interaction within the EGFR, Src and Stat3 complex in nucleus. During the revision of this paper, a study was reported on the detection of EGFR/Stat3 and EGFR/Src complexes induced in breast cancer cells response to stimulation by EGF[66], While that study raises the potential that EGFR, Src and Stat3 could assemble into a heteromeric complex, our study is the first to report on the presence of Src in the nucleus and on the identification of a nuclear EGFR, Src and Stat3 heteromeric complex that promotes c-Myc gene induction. It is not yet determined the exact configuration of the heteromeric complex. Additionally, the present evidence does not exclude the possibility that other accessory proteins could be present in the complex together with EGFR, Src and Stat3.

Our more recent studies indicated a cooperation between EGFR, Src and Stat3 functions for promoting and supporting the pancreatic cancer phenotype[65], and that the expression of c-Myc was refractory to the inhibition of EGFR, Src or Stat3 alone. The latter events failed to induce significant antitumor cell effects. The present finding that the blockade of EGFR or Src tyrosine kinase alone or the siRNA knockdown of either protein is insufficient to completely disrupt the entire complex formation might in part explain the limited effect of the modulation of any one of the three proteins on the pancreatic cancer cell phenotype, while the cancer cells are rather more sensitive to the concurrent inhibition of EGFR and Stat3 or of Src and Stat3 [65] which parallels the present observation that the concurrent modulation strongly represses c-Myc expression. Altogether, our study extends the present understanding of the molecular

events mediated by EGFR, Src and Stat3 [22, 26, 81] in showing that their relationship goes farther than signal transduction. More significantly, evidence that nuclear EGFR serves as a prognostic indicator in breast and ovarian cancers[91, 92], raises the possibility that nuclear EGFR, Src and Stat3 complex could be a predictor of patient response to therapy and the overall patient survival.

ROLE OF SRC IN PANCREATIC CANCER MIGRATION, INVASION AND METASTASIS

Src is known to play a role in various tumor processes. However, our study indicates that Src plays a more significant role in pancreatic cell migration and invasion. The study here aims to understand the Src mediated regulation of cytoskeletal rearrangements and how they facilitate metastasis. A better understanding of the regulation would facilitate the development of effective therapeutic approach to reduce pancreatic cancer metastasis and hence the high mortality rate.

Introduction

Pancreatic cancer is one of the most malignant tumors. Its poor prognosis is owing to its aggressive and high metastatic potential and there are no effective therapeutic approaches. Aberrant Src signaling is prevalent in many human cancers, with the existing evidence suggesting numerous roles for Src including cell proliferation, survival, angiogenesis, adhesion, invasion and metastasis [93]. Thus, Src has emerged as potentially important target for novel molecular-targeted anticancer drug design. However, while the initial evidence raises the potential that treatments that target Src activity might be useful as new therapeutics, the exact molecular mechanisms by which Src might support the pancreatic cancer phenotype are not fully defined. Moreover, recent studies from our lab showed that the inhibition of Src alone by Dasatinib had only a moderate effect on pancreatic cancer cell proliferation and survival [65]. Thus understanding how Src promotes pancreatic cancer cell migration and invasion and thereby contributes to pancreatic cancer metastasis would provide basis for developing approach for treating pancreatic cancer.

There is some evidence that Src might regulate the invasion and migration in part through the induction of cytoskeletal rearrangements, although there is a little understanding of the molecular details. The activation of Src can be induced in response to the stimulation of various growth factor receptors, Integrins, or by stress[94]. In general the induction of Integrin signaling promotes focal adhesion kinase (FAK) autophosphorylation at Y397 and its aggregation at the focal adhesion site. The

activated FAK associates with Src, which further activates the two proteins. The complex of the two proteins further phosphorylates proteins downstream in the signaling pathways, including paxillin and p130Cas. On phosphorylation, p130CAS recruits Crk and DOCK180, which coordinate small GTPase activity required for cell migration and invasion [95, 96].

The localization of Src is also an important event that is required for its function, and the ability of Src to promote malignant transformation is dependent on its intracellular localization. Although evidence shows that Src can be distributed to the plasma membrane, adhesion plaques, cell-cell contact and perinuclear membranes[96], its presence at the plasma membrane is associated with transforming activity. Apart from phosphorylating the downstream proteins, Src also alters the cellular localization of certain downstream proteins such as β-catenin and p120catenin. Src is known to increase the cytoplasmic retention of β-catenin and increase the membrane localization of p120catenin.

During invasion, matrix metalloproteinases (MMPs) are localized to the membrane at the podosomes, membrane extensions to the extracellular matrix (ECM), allowing the accumulated, activated MMPs to actively degrade ECM fibres. Src kinases induce podosome formation and promote proteolytic degradation of ECM proteins [97], suggests Src plays a role in cell invasion.

Our previous studies showing the inhibition of Src reduced the migration and invasion in pancreatic cancer further suggest Src is important in pancreatic cancer metastasis. However the exact mechanism of how Src regulates the cytoskeletal rearrangement in pancreatic cancer cells, thereby promoting cancer metastasis is not well defined. A study delineating the molecular mechanism of the Src-mediated regulation and induction of cytoskeletal rearrangements would provide a better insight into developing a better therapy to reduce pancreatic cancer metastasis.

Experimental Procedures

Cells and Reagents.

The human pancreatic cancer, Panc-1 and Colo-357 lines have all been previously described ([65, 67].

Wound healing assay for migration.

Wounds were made using pipette tips in monolayer cultures of cells in 6-well plates. Cells were treated with or without increasing 100 nM Dasatinib and allowed to migrate into the denuded area over a 24-h period. The migration of cells was visualized at a 10x magnification using an Axiovert 200 Inverted Fluorescence Microscope (Zeiss, Gottingen, Germany), with pictures taken using a mounted Canon Powershot A640 digital camera[98] (Canon USA, Lake Success, NY). Distance migrated by the cells into the denuded area were quantified.

SDS-PAGE/Western Blot Analysis.

Western blotting analysis was performed as previously described[35, 42]. Primary antibodies used are against pY566/567FAK, FAK, pY410p130Cas, pY418Paxillin, Paxillin, pY421Cortactin, Cortactin (Cell Signaling Technology, Danvers, MA), and p130Cas (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoprecipitation (IP) Studies.

Immunoprecipitation from whole-cell lysates and immunoblotting analysis (250 µg total protein) were performed as previously described [44],using anti-Src monoclonal antibody (Santa Cruz, CA).

Results

Inhibition of Src reduces cell migration.

Wound healing assay was done to measure the effect of Src inhibition on cell migration in pancreatic cancer cells. Migration of Panc-1 cells was observed to be lower in cells treated with 100 nM Dasatinib following 24h treatment (Fig 14a and 14b.) suggesting the inhibition of Src reduced the cell migration.

Fig. 14

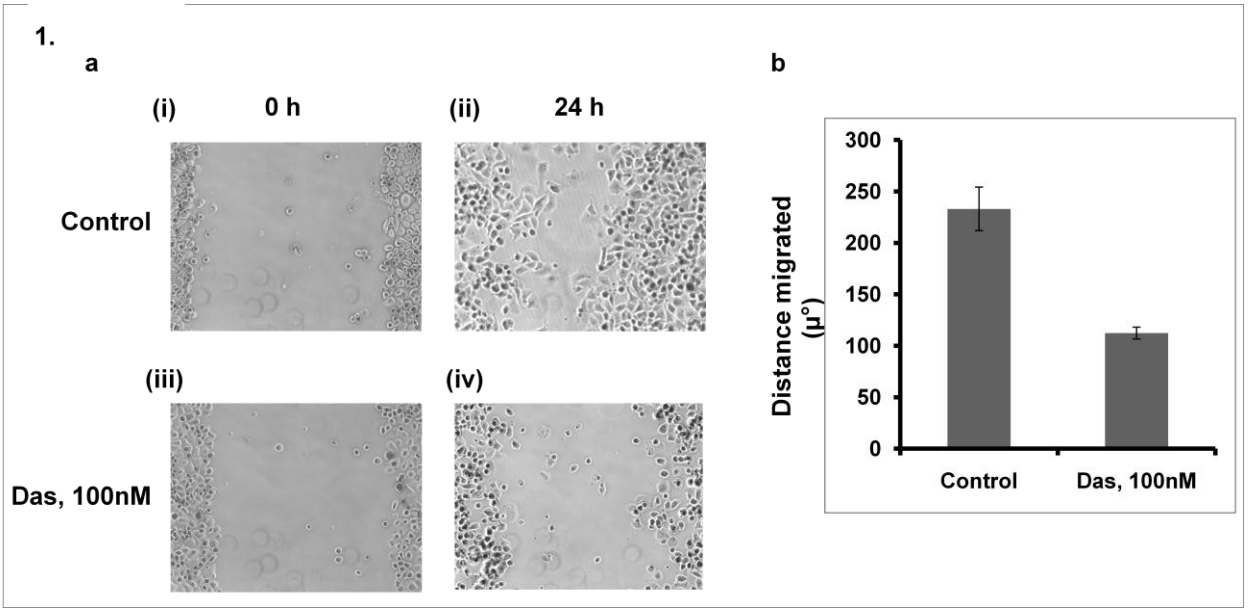

Figure 14: Inhibition of Src reduces cell migration in pancreatic cacner cell line,
Panc-1 in culture were wounded and treated with or without 100 nM Dasatinib for 24 h (11a. iv) and allowed to migrate into the denuded area in a wound healing assay. Cultures were visualized at 10x magnification by light microscopy (a) cultures were photographed and (b) distance migrated by the cells into the denuded area was measured and plotted Values are the mean and SD of 6 independent determinations, data are representative of 6 independent studies.

Inhibition of Src reduced the activation of FAK, p130Cas, paxillin, cortactin.

Src is known to phosphorylate proteins at adhesion sites and to facilitate cytoskeletal rearrangement, thereby contributing to cell migration. Immunoblotting assay was done to measure the phosphorylation of FAK, p130Cas, paxillin, cortactin. The levels of phosphorylated FAK, p130Cas, paxillin and cortactin were reduced in cells treated with 100 nM Dasatinib in Colo-357 (Fig 15a. Lane 2) and Panc-1 (Fig 15a. Lane 4) as compared to the untreated samples (Fig 15a. Lane 1 and 3). To further examine the role of Src in the regulation of key mediators of cytoskeletal rearrangement, Co-immunoprecipitation studies were performed. Immunoblotting analysis showed Src associates with paxillin and cortactin (Fig 15(b)) (Lanes 1 and 3). The inhibition of Src

activity disrupted the complex between Src and paxillin and between Src and Cortactin in Colo-357 cells (Fig 15b. Lane 2) and Panc-1(Fig 15b. Lane 4).

Fig. 15

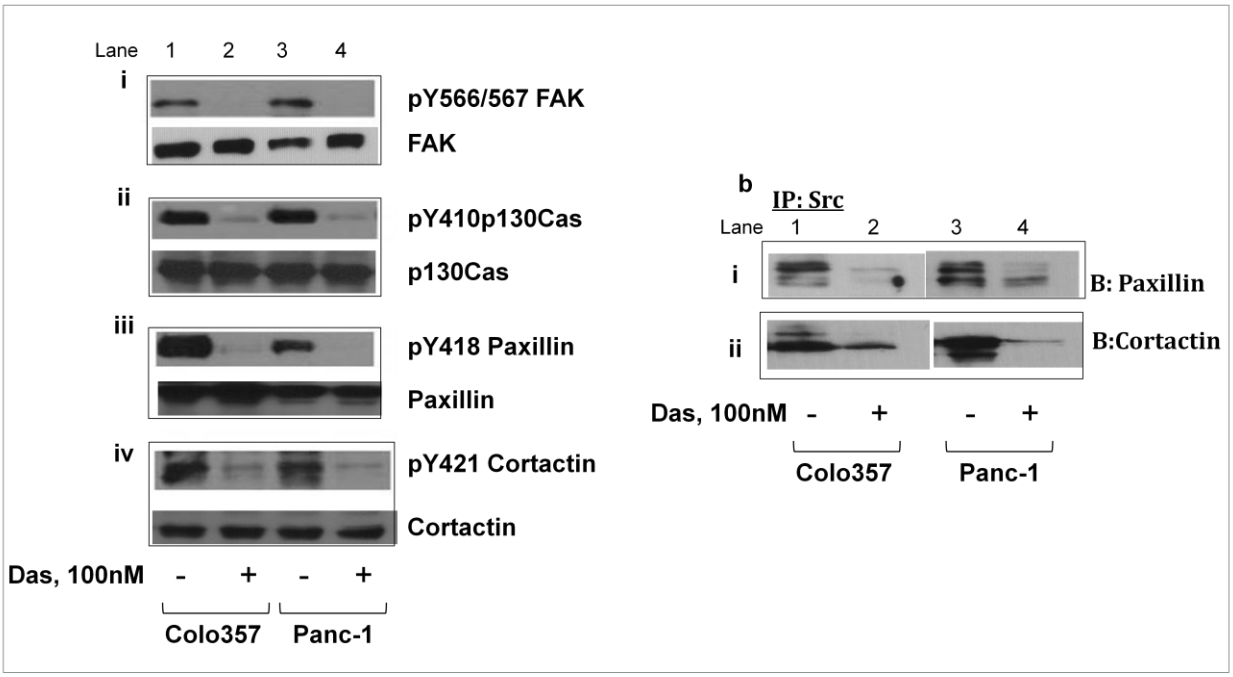

Figure 15: Effect of inhibition of Src on activation of FAK, p130 Cas, paxillin, cortactin
(a) SDS-PAGE and Western blotting analysis of whole-cell lysates of equal total protein prepared from dasatinib treated or untreated Panc-1 cells probing for pY566/567FAK, FAK, pY410p130Cas, p130Cas, pY418paxillin, paxillin, pY421cortactin, cortactin (b) Immunoblotting analyses of Src immunecomplexes (IP:Src) prepared from whole cell lysates of Colo-357 or Panc-1 cells treated with Src inhibitor dasatinib (Das), 24 h (Lanes 2 and 4) and probing for paxillin and cortactin.

Discussion

Existing reports have implicated Src in cell migration and invasion. Studies further show Src phosphorylates the downstream effectors at the adhesion site and forms complexes with the phosphorylated downstream proteins. These events contribute to promoting various cytoskeletal rearrangements. Our study shows that the inhibition of Src in

pancreatic cancer cells reduced the activated levels of FAK, p130Cas, paxillin and cortactin, and disrupted the complex between Src and paxillin and Src and cortactin. A further study of the effect that ensues after the activation of the effectors remain to be studied. Also a study of localization of these proteins at the focal adhesion site, would provide a better understanding of regulation of cell migration by Src.

Furthermore our study provides evidence that Src supports the migration of pancreatic cancer cells invitro. Altogether, our findings suggest Src activity is a critical mediator of the induction of paxillin, cortactin, FAK and p130Cas, as part of the mechanism promoting pancreatic cancer cell migration.

GENERAL CONCLUSION AND DISCUSSION

Cancer is characterized by multiple molecular aberrations, which support the malignant phenotype and moreover control the response to therapy. Accordingly, emerging evidence suggests targeting a single signal transduction molecule may not be sufficient to control cancer. By contrast, increasing evidence provides support for multiple-targeting of signaling molecules as a potentially more effective approach for treating cancer. The EGFR, Src and Stat3 proteins are concurrently activated in pancreatic cancer and implicated in the disease phenotype. Moreover, numerous studies have indicated signaling cross-talk between EGFR, c-Src, and Stat3. In that context, it is conceived that the signaling cross-talk between the three entities would in part account for the poor clinical outcome of therapies that target only EGFR or Src alone. Present studies were intended to delineate the details of the inter-relationship between the EGFR, Src and Stat3 proteins and define the basis by which they support pancreatic cancer. Ultimately, these studies were to provide a molecular basis for designing an effective multiple-targeting therapy against the EGFR, Src and Stat3 pathways for pancreatic cancer.

In defining the EGFR, Src, and Stat3 signaling and how it promotes and support pancreatic cancer, our studies show that Src activity contributes to the induction of EGFR activation, while both EGFR and Src activities promote constitutive Stat3 activation. Our studies further show surprisingly that prolonged inhibition of EGFR and Src, which induced initial inhibition of constitutively-active Stat3, results in the induction

of a JAK-Stat3 activation as a secondary event. Due to this complexity, the inhibition of EGFR, Src or Stat3 alone had moderate antitumor cell effects on pancreatic cancer cells in vitro. By contrast, our studies show that the concurrent inhibition of Stat3 and EGFR, Stat3 and Src, or EGFR and Stat3 was more effective and significantly suppresses pancreatic cancer cell proliferation and viability, induced apoptosis, and inhibited migration and invasion in vitro. Moreover, the combined inhibition of Stat3 and EGFR, or Stat3 and Src inhibited growth of human pancreatic cancer in xenografts models. Our study thus presents a novel, more effective therapeutic modality.

Additional studies to define details of the molecular interactions showed that the EGFR, Src and Stat3 proteins are physically associated into a heteromeric complex. Significantly, the EGFR, Src and Stat3 complex is detected in the nucleus of pancreatic cancer cells and not the normal human pancreatic duct epithelial cells. Moreover, our findings reveal that the EGFR, Src and Stat3 heteromeric complex is associated with and induces the c-Myc promoter, and the concurrent inhibition of Stat3 and EGFR, Stat3 and Src or EGFR and Src dramatically suppresses c-Myc expression. While present study is consistent with previous reports of an induction by EGF of a functional nuclear EGFR-Stat3 complex, it is the first report of presence of an EGFR-Src-Stat3 heteromeric complex in the nucleus of pancreatic cancer cells. The incidence of the EGFR, Src, and Stat3 heteromeric complex and its association with the c-Myc promoter suggests the heteromeric complex may represent a novel mechanism for regulating gene expression in support of the pancreatic cancer phenotype. The complexity in the

signaling pathways and its regulation of critical cancer-relevant genes would explains the poor responsiveness of pancreatic cancer cells to treatments that only inhibit EGFR, or Src, while providing the rationale and support for combination approaches that target Stat3 and EGFR, Stat3 and Src. Moreover, the presence of a functional EGFR, Src and Stat3 heteromeric complex in the nucleus defines a new paradigm of Stat3-mediated gene transcription in the context of cancer. Although the present studies identifies key peptide motifs of the EGFR and Stat3 that might important for the complex formation, the results also provide basis to pursue further characterize the heteromeric complex, in terms of determining the specific domains of each protein that mediates the interaction, determine whether the complex contains other proteins in addition to EGFR, Src and Stat3, and how the proteins are physically associated. Moreover, while the present since further provides data to indicate Src in important in promoting pancreatic cancer cell migration, the molecular details of how Src induces cytoskeletal rearrangement to promote the migration of pancreatic cancer cells remain to be determined.

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