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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Ovarian cancer is among the most common and deadly malignancies in women, accounting for nearly 15,000 deaths per year in the United States. We have developed a mouse model of ovarian cancer, which will allow for new studies into the biochemical changes that occur in the tumorigenic process. We have determined that Src tyrosine kinase is over expressed and constitutively activated in these mouse ovarian cancer cells. This leads to constitutive activation of downstream kinases such as phosphatidylinositol-3-kinase (PI3-kinase) and focal adhesion kinase (FAK). Pharmacologic inhibition of Src suppresses cell migration, alters localization of FAK, decreases protein tyrosine kinase phosphorylation and enhances the cell killing effects of the taxol, a commonly used chemotherapeutic agent in women with ovarian cancer. We have produced an inducible, epitope tagged Src dominant negative and are currently isolating clonal cell lines for characterization of the effects of Src dominant negative expression. These cells lines will be used for both in vitro and in vivo studies on the role of Src tyrosine kinase and its substrates in the growth of ovarian cancer cells and on the chemotherapeutic sensitivity.

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INTRODUCTION

Ovarian cancer is among the most common and deadly malignancies in women, accounting for nearly 15,000 deaths per year in the United States. The prognosis for ovarian cancer is generally very poor due to late presentation of patients and the aggressive nature of the disease. The biochemical changes that occur in the tumorigenic process in these cancers are not well defined but most undoubtedly include aberrant growth factor signaling cascades. A better understanding of signal transduction cascades inappropriately activated in ovarian cancer cells will provide insights into the pathophysiology of ovarian cancer and may ultimately provide targets for new therapeutic interventions. We have developed a mouse model of ovarian cancer, which will allow for new studies into the biochemical changes that occur in the tumorigenic process. Several cell lines derived from mouse ovarian surface epithelial cells that have spontaneously transformed *in vitro* have been developed (1). One such cell line (ID8) has been partially characterized. Intraperitoneal injection of ID8 cells in syngeneic, immune competent mice results in the formation of multiple abdominal mesotheliomas and accumulation of ascites fluid very reminiscent of what is seen in ovarian cancer in women. Src tyrosine kinase, an intracellular signaling molecule that is aberrantly activated in a large proportion of ovarian cancers in women, is constitutively activated in ID8 cells (manuscript submitted, see Appendix). In addition, we have found that several other signal transduction molecules important in regulating cell growth and survival (phosphatidylinositol-3-kinase, Akt/PKB) and morphology (focal adhesion kinase) and which directly interact with Src, are constitutively activated. Blockade of tyrosine kinase activity in ID8 tumor cells with the Src selective inhibitor, herbimycin A, is cytostatic. Furthermore, herbimycin A enhances the cytotoxic effects of Taxol. It is hypothesized that disruption of specific c-Src protein-protein interactions will change the growth, aggressiveness and phenotype of ID8 tumor cells. The research proposed within this project is aimed at determining the role of Src and its specific interactions in ID8 tumor cell malignancy. This will be accomplished by both general and specific disruption of Src protein-protein interactions and determining the effect on tumor cell growth, morphology and tumor characteristics. In Specific Aim 1, Src activity in ID8 cells will be disrupted through the expression of a dominant negative (kinase inactive) Src kinase. Cell growth rate, anchorage independence, and chemotherapeutic sensitivity will be determined. In addition, the ability to form tumors in syngeneic mice will be assessed. Growth rate, tumor morphology and metastatic potential will be determined. The second aim of the research is to determine if Src interacts with and phosphorylates tubulin, the target of the antineoplastic agent Taxol, and how blockade of this interaction enhances Taxol's cytotoxicity. This will be accomplished by *in situ* immunolocalization and expression of kinase inactive Src. The goal of Aim 3 is to disrupt specific Src protein-protein interactions in ID8 cells. Src - PI3-kinase and Src - FAK interactions will be disrupted through expression of Src binding domains. Cell growth and anchorage independence and metastatic potential will be assessed. The information gained may provide important and novel new insights into the roles of Src, phosphatidylinositol-3-kinase and focal adhesion kinase in the neoplastic transformation of ovarian surface epithelial cells. This may ultimately provide information regarding the utility of these kinases as targets for new therapeutic strategies.

BODY

Task 1. To determine the effect of expression of a Src dominant negative tyrosine kinase on ID8 tumor cell growth, anchorage independence, metastatic potential and chemotherapeutic resistance (months 1-18).

a. develop clonal cell lines derived from ID8 cells transfected with an expression vector encoding an epitope tagged Src dominant negative and a G418 resistance gene (this vector is already in hand; months 1-3).
b. determine FAK and PI3-kinase activity of ID8 cells expressing the Src dominant negative compared to control transfected cells (months 4-9).
c. determine *in vitro* growth characteristics of ID8 cells expressing the Src dominant negative compared to control transfected cells utilizing cell counts, ^{3}H-thymidine incorporation and colony formation in soft agar (months 4-12).
d. determine chemotherapeutic sensitivity of ID8 cells expressing the Src dominant negative compared to control transfected cells by way of *in vitro* cytotoxicity assays (months 13-18).
e. determine *in vivo* tumor formation of ID8 cells expressing the Src dominant negative compared to control transfected cells by intraperitoneal injection of cell lines into syngeneic mice (7 mice per cell line, 4 different cell lines) and monitoring tumor formation (months 4-18).

Development of an inducible Src dominant negative

A Src dominant negative (Srcdn) construct was obtained from Upstate Biotechnology (Waltham, MA). The Srcdn insert was excised from the plasmid and subcloned into a pCDNA3.1 *Myc-His* expression plasmid (Invitrogen, Carlsbad, CA) in order to attach an epitope tag to the Srcdn ($Srcdn^{Myc\text{-}His}$), providing a means to distinguish the Srcdn protein from endogenous Src protein. This construct was then used to transfect ID8 mouse ovarian surface epithelial cancer cells (ID8 MOSEC cells). Transient expression of the $Srcdn^{Myc\text{-}His}$ was achieved as demonstrated by immunofluorescence and immunoblot analysis (data not shown, see below for explanation)). Stable transfectants were selected with G418. Subsequent analysis failed to demonstrate expression of $Srcdn^{Myc\text{-}His}$. Thus we suspect that $Srcdn^{Myc\text{-}His}$ has been silenced. We are currently confirming that the $Srcdn^{Myc\text{-}His}$ construct was integrated into the genome. Additionally we are determining whether demethylation or histone acetylation will restore $Srcdn^{Myc\text{-}His}$ expression. While this has cost us a fair amount of time we do not see it in a negative light, it has forced us to pursue a strategy using an inducible system (see below) that in the end should prove to be more flexible and informative. In addition, if indeed the $Srcdn^{Myc\text{-}His}$ was silenced, this may suggest that cancer cells may be able to silence transgenes that are detrimental to their survival. This may have important implications with regard to the use of gene therapy in the treatment of cancers. We intend to pursue this further.

As a result of the inability to produce stable cells lines expressing the $Srcdn^{Myc\text{-}His}$ we are pursuing a strategy using an inducible system. The $Srcdn^{Myc\text{-}His}$ was excised from the pDNA 3.1 *Myc-His* vector and subcloned into an ecdysone inducible pInd vector (Invitrogen). This construct when cotransfected into cells with a vector encoding the ecdysone receptor and RXR (pVgRXR, Invitrogen) will be induced in the presence of the steroid hormone ponasterone. In the absence of ponasterone the $Srcdn^{Myc\text{-}His}$ is not expressed so there should be no selective pressure to silence the transgene. Initial transient transfections demonstrate the inducible system is working (Figure 1). We are currently in the process of selecting stable cell lines. Stable inducible lines will provide us with a very good *in vitro* and *in vivo* model system with a great deal of flexibility. We will be able to grow cells *in vitro* under certain conditions

and then induce expression of Srcdn$^{Myc-His}$ and determine the effect on tumor characteristics. Similarly, we will be able to use the cells *in vivo*, allow tumors to form and develop to different stages, induce expression of the Srcdn$^{Myc-His}$ by providing animals with ponasterone in their drinking water and monitor further tumor growth development and metastasis. Similarly we will be able to turn off Srcdn$^{Myc-His}$ expression by withdrawal of ponasterone. Thus we believe this system will prove to be far superior to our original attempt to produce lines which constitutively express the Srcdn$^{Myc-His}$. As soon as clonal cell lines have been established in vivo studies will be initiated.

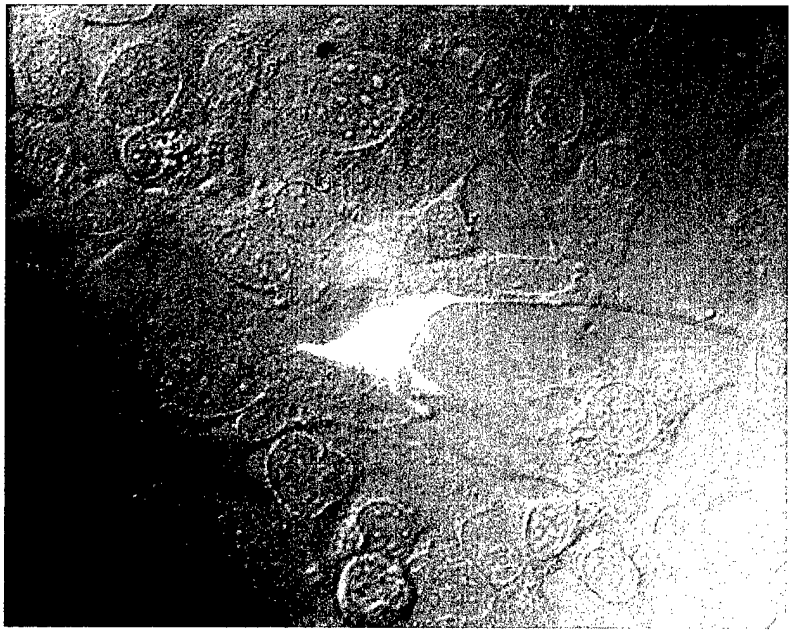

Figure 1. Expression of an inducible Srcdn$^{Myc-His}$. Srcdn was first subcloned into pCDNA Myc-His in order to provide an epitope tag. The resulting Srcdn$^{Myc-His}$ was then excised and subcloned into an ecdysone inducible construct. ID8 cells were cotransfected with a pVgRXR and the inducible Src. 48 hours post transfection cells were treated with ponasterone for eighteen hours in order to induce Srcdn$^{Myc-His}$ expression. Cells were fixed and immunofluorescence performed using an antibody directed against the His epitope tag. The image is a merged image of fluorescence and DIC optics.

In addition, small interfering RNA's (siRNAs) are being designed and synthesized as an alternative method to specifically inhibit Src activity (2). Cells will be transfected with the double stranded siRNA complementary to Src mRNA in order to block or knockdown Src protein expression. This provides a very specific and reversible way of blocking or knocking down translation of message. We plan to perform growth and anchorage independence assays in ID8 cells that have been transfected with the Src siRNAs.

Inhibition of Src inhibits ID8 cell migration

Our previous studies had demonstrated that Src activation appears to be associated with activation of PI3-kinase. PI3-kinase plays an important role in cell motility and migration. Thus we undertook experiments to determine the role of Src and PI3-kinase in ID8 MOSEC cell migration. ID8 cells were plated on 5 μm tissue culture treated transwell inserts (5, 000 cells/insert) in serum free media. Cells were allowed to attach for 1.5 hours and then pretreated with the following kinase inhibitors; PP2 (Src specific inhibitor, 5 μM), LY294002 (PI3-kinase inhibitor, 50 μM) and PD98059 (MEK inhibitor, 50 μM). Following a 30-minute incubation with the inhibitors, serum-containing media was added to the bottom of the transwells. Cells were then allowed to culture overnight. At the end of the culture period cells were fixed with methanol and stained with Giemsa stain. Cell migration was determined by first counting the total number of cells attached to the membranes and then scraping the cells off the top of

the membrane (ie. the side on which the cells were initially plated). The remaining cells (those that migrated through the membrane) were then counted. As represented in Figure 2 both the Src inhibitor PP2 and the PI3-kinase inhibitor LY294002 suppressed ID8 cell migration. The MEK inhibitor PD98059 had little effect on ID8 cell migration. This finding indicates that both PI3-kinase and Src can stimulate cell migration. As inhibition of Src inhibits PI3 kinase activity as measured by Akt phosphorylation we believe that Src is acting to stimulate cell motility via activation of PI3-kinase.

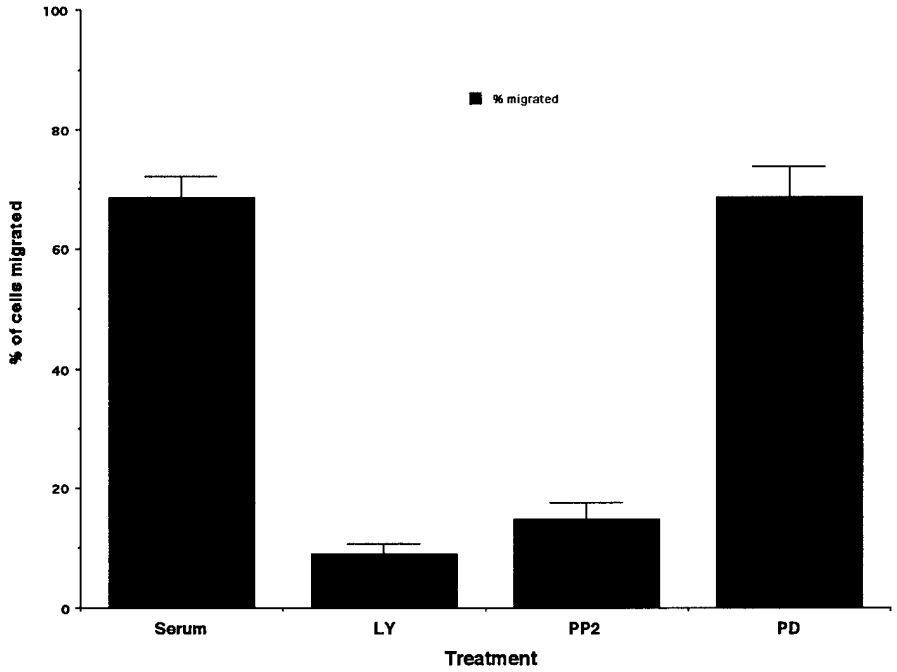

Figure 2. ID8 cell migration is blocked by Src or PI3-kinase inhibition. ID8 cells were plated on 5 μm tissue culture treated transwell inserts (5,000 cells/ insert) in serum free media. Cells were allowed to attach for 1.5 hours and then pretreated with the following kinase inhibitors; PP2 (5 μM), LY294002 (50 μM) and PD98059 (50 μM). Following a 30-minute incubation with the inhibitors, serum-containing media was added to the bottom of the transwells. Cells were then allowed to culture overnight. At the end of the culture period cells were fixed with methanol and stained with Giemsa stain. Cell migration was determined by first counting the total number of cells attached to the membranes and then scraping the cells off the top of the membrane (ie. the side on which the cells were initially plated). The remaining cells (those that migrated through the membrane) were then counted. The data provided is the mean ± the SEM of 4 replicates from a representative experiment. The experiment was repeated three times with consistent results.

Inhibition of Src disrupts peripheral focal adhesions and protein tyrosine phosphorylation

We have found that Src will co-immunoprecipitate focal adhesion kinase (FAK), an important regulator of cell growth and metastatic potential. Over expression of activated FAK has been associated with loss of anchorage dependence and resistance to anoikis (apoptosis resulting from loss of contact with extracellular matrix), a hallmark of cell transformation. Phosphorylation of tyrosine 397 of FAK, which

serves as the binding site for the SH2 domain of Src, is necessary for this effect (3). Therefore the constitutive activation of FAK may be a very important event in ID8 cell transformation. In order to determine whether inhibition of Src will alter focal adhesions, cells were treated with PP2, a Src specific inhibitor, and then cells were fixed and analyzed for FAK expression and localization by immunofluorescence microscopy. We found that treatment of ID8 cells with PP2 led to a loss of peripheral membrane FAK localization (Figure 3). In addition there was an increase in the number of small rounded, presumably proapoptotic cells. Treatment with PP2 also led to an overall decrease in protein tyrosine phosphorylation. These results will be confirmed in cells expressing the inducible Src dominant negative once clonal lines have been isolated.

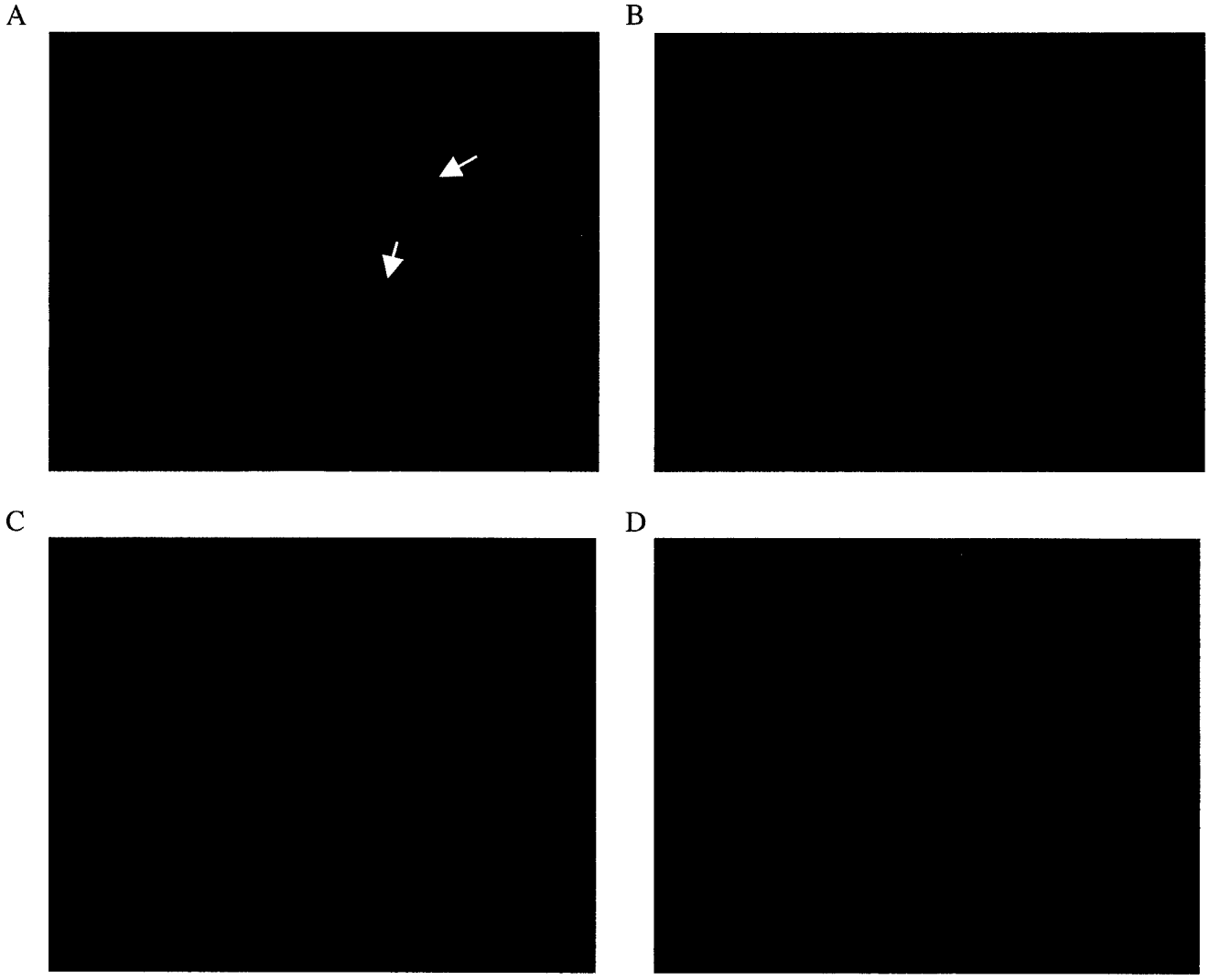

Figure 3. Loss of peripheral focal adhesion kinase and protein tyrosine phosphorylation following treatment with the Src specific inhibitor PP2. Cells were treated overnight with either vehicle or PP2 (10 μM), then fixed and analyzed for focal adhesion kinase (A and B) or tyrosine phosphoproteins (C and D) by immunofluorescence. Arrows indicate peripheral membrane focal adhesions.

Task 2. To determine how disruption of Src activity enhances Taxol cytotoxicity of ID8 cells (months 1-24).

a. determine tubulin and Src co-precipitation and tubulin tyrosine phosphorylation state in ID8 cells and ID8 cells treated with the Src selective inhibitors herbimycin A and PP2 (months 1-6).
b. determine tubulin and Src co-precipitation and tubulin tyrosine phosphorylation state in ID8 cells expressing the Src dominant negative compared to control transfected cells (months 6-12).
c. determine Src and tubulin co-localization and how disruption of Src activity alters Src and tubulin localization utilizing confocal immunofluorescence (months 12-24).

Inhibition of Src does not alter microtubule dynamics in ID8 mouse ovarian cancer cells

We have shown that inhibition of Src activity synergizes with both Taxol and colchisine induced ID8 MOSEC cell death. Although there is literature to suggest that Src and tubulin directly interact, to date we have been unable to detect any effect of Src inhibition on microtubule dynamics. In order to test the effects of Src inhibition on microtubule disruption cells were treated with the microtubule disrupting agent nocodozole with or without the Src specific inhibitor PP2. There appeared to be no effect of PP2 on the time course of microtubule destabilization. Conversely PP2 had no effect on the time course of microtubule repolymerization following nocodozole washout (data not shown). In addition we have been unable to convincingly demonstrate that Src will co-precipitate with tubulin. However, we have consistently observed that taxol or colchisine and PP2 have a synergistic cell killing effect. Therefore we plan to continue to pursue the mechanism by which this occurs.

Task 3. To determine how specific disruption of Src - FAK and Src - PI3-kinase interactions alters ID8 cell growth, anchorage independence, metastatic potential and chemotherapeutic resistance (months 13-36).

a. Subclone sequences corresponding to the region of Src that interacts with FAK and the region that binds with PI3-kinase into expression vectors containing a G418 resistance gene (months 13-15).
b. develop clonal cell lines derived from ID8 cells transfected with the above expression vectors (months 16-18).
c. determine *in vitro* growth characteristics of ID8 cells expressing the FAK and PI3-kinase binding sequences compared to control transfected cells utilizing cell counts, ^{3}H-thymidine incorporation and colony formation in soft agar (months 19-27).
d. determine chemotherapeutic sensitivity of ID8 cells expressing the FAK and PI3-kinase binding sequences compared to control transfected cells by way of *in vitro* cytotoxicity assays (months 28-36).
e. determine *in vivo* tumor formation of ID8 cells expressing the FAK and PI3-kinase binding sequences compared to control transfected cells by intraperitoneal injection of cell lines into syngeneic mice (7 mice per cell line, 4 different cell lines) and monitoring tumor formation (months 15-36).

Work has only just begun on task 3 thus there are no results at this time to report.

KEY RESEARCH ACCOMPLISHMENTS:

- Production of mouse model of ovarian cancer (in collaboration with Paul Terranova, University of Kansas Medical Center).
- Construction of an inducible Src dominant negative.
- Identification of aberrant signaling networks contributing to tumor characteristics in mouse ovarian cancer cells.
- Identification of signaling pathways involved in cell migration in mouse ovarian cancer cells.
- Identification of a possible chemotherapeutic target for ovarian cancer (Src tyrosine kinase).

REPORTABLE OUTCOMES:

1. Development of spontaneously transformed mouse ovarian surface epithelial cell line (Ovarian cancer cells).
2. Development of an inducible Src dominant negative system.

OVERALL CONCLUSIONS

The results obtained from the performed research extend our previous findings with regard to Src – FAK and Src – PI3-kinase. Inhibition of PI3 kinase inhibits ID8 cell migration. Inhibition of Src also inhibits ID8 cell migration and Akt phosphorylation. Akt is a PI3-kinase target, thus it appears that Src may activate PI3-kinase in ID8 cells, potentially stimulating cell migration. In addition, inhibition of Src activity appears to alter focal adhesion kinase localization. Focal adhesions play an important role in cell migration and attachment to the extracellular matrix. Therefore, Src activation may play a role in cell migration (metastasis?) by multiple mechanisms, ie. by activating PI3-kinase, and by stimulating assembly of new focal adhesions. With the development of the inducible Src dominant negative we will be able to confirm and extend these results, using both *in vitro* and *in vivo* models. Finally, much of the work that is performed in the mouse ovarian cells is being replicated in human ovarian cancer cell lines. To date, the results from the human cells are consistent with what we have found in the mouse. The great advantage with the mouse cell line is that we can use these in immune intact animals.

Unfortunately the work has not progressed at the pace I would have liked. The reasons are two fold. First the inability to produce stable cell lines constitutively expressing a Src dominant negative slowed the research dramatically. However, as mentioned in the body of the text this may have pushed us to a

superior and more flexible model, namely an inducible expression system. The second factor was the inability to identify and hire a research associate until 8 months into the project, although several candidates were interviewed. Thus, much of the work was performed by myself, between teaching, committee work, writing and other tasks. I expect the work will progress at a faster and more consistent rate now that a research associate has been hired.

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APPENDIX

Submitted manuscript:

Taylor, C.C., Huang, H., Roby, K.F. and Terranova, P.F. Src tyrosine kinase signaling in a mouse model of ovarian cancer. Submitted to Oncogene, September 2001

Src Tyrosine Kinase Signalling in a Mouse Model of Ovarian Cancer[1]

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[3] Abbreviations used are: MOSEC, mouse ovarian surface epithelial cells; PI3-kinase, phosphatidylinositol-3-kinase; FAK, focal adhesion kinase; HBSS, Hank's Balanced Salt Solution; EGF, epidermal growth factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MTT, 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

[3] Taylor and Terranova, unpublished observations

Running title: Src signaling in mouse ovarian cancer cells

5

Key words: Src, PI3-kinase, FAK, ovary, cancer

Abstract

The vast majority of ovarian cancers, including those that contribute most to mortality, originate in the ovarian surface epithelium. One difficulty of research in ovarian cancer has been the lack of a good animal model. Multiple clonal cell lines were derived from C57 Black 6 mouse ovarian surface epithelial cells, which have spontaneously transformed *in vitro* with repeated passage. One such cell line (ID8) has been partially characterized. Protein preparations from ID8 cell lysates show high levels of tyrosine phosphorylation of several proteins (≈ 60, 120 and 200 Kd), even under serum starvation conditions. ID8 cells express large amounts of c-Src protein, a proportion of which is constitutively active. Both focal adhesion kinase and phosphatidylinositol-3-kinase (PI3-kinase) co-precipitate with Src and also show a level of constitutive activation. In addition, Akt/protein kinase B (PKB), a serine-threonine kinase activated by PI3-kinase, is phosphorylated in ID8 cells, suggesting Akt/PKB activation. Blockade of Src activity blocks ID8 cell migration, alters focal adhesion localization, inhibits Akt phosphorylation and enhances the cytotoxicity of taxol, a compound widely used chemotherapeutically in ovarian cancer. Thus the constitutive activation of Src appears to play multiple roles in the tumor characteristics of ID8 mouse ovarian cancer cells.

Introduction

In the United States, ovarian cancer accounts for nearly 15,000 deaths per year (1). The prognosis for ovarian cancer is generally very poor with high mortality rates. This is largely the result of late presentation of patients due to clinically silent symptoms until metastatic disease has been well established.

The vast majority of ovarian cancers, including those that contribute most to mortality, originate in the ovarian surface epithelium (2), the single layer of cells covering the surface of the ovary. As with many other epithelial derived neoplasms most of these ovarian epithelial cancers are thought to arise from a single clonal cell (3).

The pathogenesis of ovarian cancer is not well understood but probably requires multiple genetic alterations, including activation of oncogenes and loss of function of tumor suppresser genes. One hypothesis as to the etiology of ovarian cancer is that of "persistent ovulation", in which the chances of ovarian cancer increase with the number of ovulations (4). With each ovulation the normally quiescent surface epithelial cells must proliferate to cover and repair the ruptured ovulation site. With increased ovulations there is the possible accumulation of genetic mutations, karyotype instability, activation of oncogenes and loss of function of tumor suppresser genes.

Another important factor in the pathogenesis of these neoplasms may be the overexpression and overactivation of growth factor signal transduction cascades (3) which may result from continual reactivation to repair ovulation sites. Overexpression of various growth factor receptors and intracellular tyrosine kinases is a common occurrence in ovarian cancer. *c-erbB2/her2/neu*, a gene encoding a member of the epidermal growth factor receptor (EGFR) family, is amplified in roughly 30% of epithelial ovarian cancers

(5), and is overexpressed in an additional 10% (6). Overexpression and over-activity of platelet-derived growth factor receptor alpha and colony stimulating factor-1 receptor have also been implicated in reproductive tract cancers (7). Non-receptor tyrosine kinases then transmit the signal from the above mentioned receptors in a cascade until the signal reaches the nucleus, inducing genes involved in cell growth and regulation of the cell cycle. Many of the non-receptor tyrosine kinases have been found to be proto-oncogenes. One such non-receptor tyrosine kinase, pp60 c-Src, has been found to have elevated activity in many human cancers, including a large proportion of ovarian cancers (8). In addition to tyrosine kinases, overexpression of tyrosine phosphatases, which activate kinases such as c-Src, also have been associated with ovarian epithelial cancers (9).

One difficulty of research in ovarian cancer has been the lack of a good animal model. We have utilized an *in vitro* model of repeated passage of mouse ovarian surface epithelial cells (MOSEC[3]) to mimic the effect of repeated ovulation and wound healing as reported previously for rats (10). Following repeated passage MOSEC showed characteristics of spontaneous neoplastic transformation and tumor formation in syngeneic mice (11). Several clonal cell lines have been produced from these initial primary cultures (11). The present study was undertaken to determine aberrant signal transduction cascades in one such cell line (ID8). The results of the present study reveals that the ID8 cell line overexpresses and shows constitutive activation of c-Src. In addition, both PI3-kinase and FAK directly associate with Src and also show constitutive activation. Blockade of tyrosine kinase activity with herbimycin A enhances the cytotoxicity of taxol, a chemotherapeutic agent commonly used in ovarian cancer. Thus

the ID8 ovarian surface epithelial tumor cell line provides a new and important model for the study of ovarian cancer.

Methods:

Reagents

All liquid media, G418 sulfate and lipofectamine were purchased from Life Technologies (Grand Island, NY). A Src-specific antibody (clone 327) was purchased from Oncogene Science (Cambridge, MA). PI3-kinase and FAK antibodies were from Upstate Biotechnology (Lake Placid, NY). Akt and phospho-Akt were from New England Biolabs (Beverly, MA). A phosphotyrosine antibody (PY20) and protein AG + agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cy3-conjugated seconday antibodies were from Jackson Immunolabs. Herbimycin A, PP2, LY294002 and PD 98059 were obtained from Calbiochem (La Jolla, CA). [^{32}P]-ATP (1 mCi/ml) was obtained from ICN (Costa Mesa, CA). ECL Western blotting detection reagents were purchased from Amersham Life Science Incorporated (Arlington Hieghts, IL). Taxol (paclitaxel) and all other reagents were purchased from Sigma (St. Louis, MO).

Development of ID8 MOSEC cells and cell culture

The development of ID8 cells from the ovarian surface epithelium of C57BL6 mice has previously been described (11). For experimental purposes, unless otherwise stipulated, subcultures of ID8 cells were grown to confluency in serum containing media.

Upon reaching confluency media were changed to fresh serum free media for 16 hours prior to harvesting the cells for analysis of kinase expression and activity.

MA10 Leydig tumor cells and an MA10 cell line genetically engineered to overexpress Src were produced and cultured as previously described (12).

Human ovarian surface epithelial cells were collected by gentle brushing of the ovarian surface after the ovaries from the body cavity from patients undergoing total abdominal hysterectomy with salpingo- oophorectomy or total bilateral salpingo-oophorectomy. Ovaries were being removed for reasons unrelated to ovarian pathology, and were thus considered normal. This procedure was approved by the Institutional Human Studies Committee of the University of Kansas Medical Center and was given exemption status due to the discarded nature of the tissue (ovaries). Human cells were placed in a 15 ml polypropylene centrifuge tube containing 6 mls growth media composed of MCDB-105, M199 (with Earl's salts) 1:1, 15% FBS and 2 ng/ml EGF. Cells were transported to the cell culture facility at room temperature and transferred to a T25 culture flask. The tube was then rinsed with 2-ml growth media which was then added to the culture flask. The human cells were left undisturbed and were allowed to attach during a 48-hour incubation at 37° C, after which media were replaced and cells were allowed to grow until subconfluency. Cells were subcultured to T75 tissue culture flasks for further growth and experimental procedures.

Immunoprecipitation and immunoblotting

ID8 cells were grown to confluency then lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P40, 0.1% SDS, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM

EGTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 μg aprotinin/ml, 1 μg leupeptin/ml, 1 μg pepstatin /ml) for 20 min at 4° C. Insoluble material was cleared by centrifugation (14,000 x g for 20 minutes at 4° C). For immunoprecipitation, 500 μg of soluble protein was incubated with specific antibodies (1 μg) with gentle rocking at 4° C for 1 hour following which 20 μl of protein A/G plus agarose was added. Samples were further incubated for 1 hour (kinase assays) or overnight (immunoblots). Immunoprecipitates were collected by centrifugation and washed five times with RIPA buffer, then resuspended in 20 μl RIPA buffer. For immunoblotting, total soluble or immunoprecipitated proteins were mixed with an equal volume of 2x Laemmli sample buffer. Samples were heated to 95° C for 5 minutes and then subjected to SDS-PAGE. Protein was electrotransferred to PVDF membranes. Membranes were blocked with TBST-5% milk (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 5% non fat dry milk) for 1 hour at room temperature prior to incubation with the appropriate specific antibody in TBST-5% milk (1:1000 dilution) for 1 hour. Membranes were washed extensively with TBST, incubated with the appropriate peroxidase conjugated secondary antibody in TBST-5% milk for 1 hour and then washed with TBST. Proteins were visualized by enhanced chemiluminesence. For phosphotyrosine immunoblots, membranes were blocked with TBST-3% BSA, incubated with antiphosphotyrosine antibody in TBST-1% BSA and washed with modified TBST (50 mM Tris-HCl, 200 mM NaCl, 0.25% Tween-20).

Src and PI3-kinase immune complex kinase assays

Cells were grown, collected, lysed and immunoprecipitated with the appropriate antibody as outlined above. For determination of Src activity, Src immune complexes were washed three times in RIPA and once with 20 mM Hepes, 150 mM NaCl. Immune complexes were then resuspended in 40 μl reaction buffer (20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 μM ATP) containing 0.25 mg acid denatured enolase/ml as a Src substrate. Src kinase activity was determined by the addition of 10 μCi [γ^{32}P]-ATP to each immune complex kinase reaction mixture. Samples were incubated in a water bath at 30° C for 15 minutes and then the reactions were stopped by the addition of an equal volume of 2x Laemmli sample buffer. Samples were heated to 95° C for 5 minutes and centrifuged in order to remove the agarose beads. Supernatants were subjected to PAGE on a 10% SDS gel, proteins were electro-transferred to PVDF membranes, membranes were washed with 1N KOH for 1 hour at 60° C and subsequently analyzed for phosphorylation by autoradiography. Relative activity (% of control) was determined by densitometry.

For determination of PI3-kinase activity, PI3-kinase immune complexes were washed multiple times with RIPA, then the immunoprecipitated pellet was resuspended in 50 μl TNE buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA) containing 20 μg phosphatidylinositol and 25 mM $MgCl_2$. PI3-kinase activity was determined by ^{32}P incorporation into phosphatidylinositol. The reaction was started by the addition of 1 mM cold ATP and 30 μCi [γ^{32}P]-ATP and allowed to proceed for 10 minutes with constant agitation. The reaction was stopped by the addition of 20 μl of 6 N HCl. $CHCl_3$:MeOH (1:1, 160 μl) was then added to extract the lipid. The $CHCl_3$ phase was

spotted on silicon thin layer chromatrography plates treated with oxalate and the lipids resolved by thin layer chromatography in $CHCl_3$:MeOH:water:ammonium hydroxide (60:47:11.3:2). $\gamma^{32}P$ incorporation into phospholipid was visualized by autoradiography.

Cell migration assays

ID8 cells were plated on 5 μm tissue culture treated transwell inserts (5, 000 cells/insert) in serum free media. Cells were allowed to attach for 1.5 hours and then pretreated with the following kinase inhibitors; PP2 (Src specific inhibitor, 5 μM), LY294002 (PI3-kinase inhibitor, 50 μM) and PD98059 (MEK inhibitor, 50 μM). Following a 30-minute incubation with the inhibitors, serum-containing media was added to the bottom of the transwells. Cells were then allowed to culture overnight. At the end of the culture period cells were fixed with methanol and stained with Giemsa stain. Cell migration was determined by first counting the total number of cells attached to the membranes and then scraping the cells off the top of the membrane (ie. the side on which the cells were initially plated). The remaining cells (those that migrated through the membrane) were then counted.

Immunofluorescence

ID 8 cells were seeded on glass cover slips in six-well culture plates. After the treatment period (described in the) cells were fixed with ice-cold methanol for ten minutes at -20°C. Cover slips were allowed to air dry and then cells were permeabilized with 0.1% NP40 in PBS for 20 minutes at room temperature. Coverslips were blocked with 10% preimmune serum (in PBS) from the species in which the secondary antibody

was raised. Following blocking coverslips were incubated with primary antibody (anti FAK, 1:200; anti phosphotyrosine, 1:100) in PBS – 1% normal serum at room temperature for 2 hours. Coverslips were then washed extensively with PBS, incubated with a Cy3 conjugated secondary (1:200) antibody for 2 hours at room temperature, washed in PBS, counterstained with DAPI (0.1 μg/ml PBS) for 15 minutes and then washed with PBS. Coverslips were mounted with Vectamount mounting media.

Relative Cell Viability

Relative viable cell number was determined as previously described (13) by incubation of cells with MTT and measuring the production of formazan at an absorbance at 600 nm, which occurs only in the mitochondria of viable cells. Briefly, ID8 cells were seeded at 5,000 cells/well in 96-well plates and allowed to attach overnight. After attachment, media were removed and replaced with fresh serum containing media and treatments (taxol ± herbimycin A) were initiated. Following the treatment period, relative cell number was assessed by adding MTT (1mg/ml) and measuring formazan production following a three hour incubation. For statistical analyses, control values were set to 100%. Treatment effects were analyzed by an analysis of variance with differences between individual means compared by Fisher's protected least significant differences test (Statview 512+, Agoura Hills, CA).

Results

ID8 cells express large amounts of Src tyrosine kinase

Because high levels of constitutive tyrosine kinase activity are associated with cell transformation, ID8 cells lysates were probed for protein tyrosine phosphorylation. ID8 cells were grown to confluency, serum starved for 16 to 20 hours then lysed in RIPA buffer. Soluble protein was analyzed for tyrosine phosphorylated proteins by immunoblotting with an antibody directed against phosphotyrosine. As it was not possible to collect enough freshly isolated mouse ovarian surface epithelial cells for analysis, lysates from a gonadal tumor cell line (MA10 cells) and early passage human ovarian surface epithelial (HOSE) cells were included for comparison. Several proteins (≈ 60, 120 and 200 Kd) were heavily tyrosine phosphorylated (Figure 1) in ID8 cell lysates. In contrast, cell lysates from MA10 cells and HOSE cells showed no such constitutive tyrosine phosphorylation. As Src tyrosine kinase is over expressed in a large proportion of ovarian cancers, and the molecular weight of Src (60 Kd) corresponds to the molecular weight of one of the tyrosine phosphorylated proteins in ID8 cells, Src protein expression was examined by immunoblot analysis. ID8 cells express large amounts of c-Src protein, in fact the level of expression appears comparable to that of MA10 cells that have been previously transfected with and over express c-Src (Figure 2a) and roughly 5 fold greater levels than Src expression in HOSE cells (H1 and H2; Figure 2b) as determined by image analysis.

As Src is highly expressed in ID8 tumor cells and overexpression is often associated with constitutive activation, the activation state of Src in resting ID8 cells was determined. Cells grown to confluency and serum starved continue to display Src

activity by an immune complex kinase activity assay (Figure 2c), indicating at least a proportion of c-Src in ID8 cells is constitutively active. Furthermore, immunoprecipitation of cell lysates with an antibody to phosphotyrosine precipitates a large amount of c-Src (Figure 2d), again indicating active Src (tyrosine 527 phosphorylated inactive Src is not recognized by phosphotyrosine antibodies). Thus, ID8 cells express a large amount of constitutively active Src.

Src Interactions

Src is known to interact with many other signaling molecules, several of which have important implications in regulation of cell growth and morphology. PI3-kinase and FAK are two such molecules. Therefore, whether PI3-kinase and FAK would co-precipitate with Src was investigated. Cell lysates were immunoprecipitated with an antibody directed against Src. The resulting immunoprecipitate was subjected to PAGE and then probed by immunoblot analysis for either FAK or PI3-kinase. As shown in Figure 3, both FAK and PI3-kinase co-precipitate with Src, indicating a direct interaction between Src and FAK, and Src and PI3-kinase.

To determine if FAK was tyrosine phosphorylated, an indication of FAK activation, ID8 cell lysates were immunoprecipitated with a phosphotyrosine antibody and the resulting immunoprecipitate was analyzed by immunoblot analysis with an antibody specific for FAK. Tyrosine phosphorylated FAK was present in ID8 cell lysates prepared from confluent, serum starved cultures (Figure 4a), indicating constitutive FAK activation.

In order to determine if PI3-kinase was activated, ID8 cell lysates were immunoprecipitated with an antibody directed against the regulatory subunit of PI3-kinase, which co-precipitates the catalytic subunit. A PI3-kinase activity assay was performed on the resulting immunoprecipitate. As with Src and FAK, constitutive PI3-kinase activity was detected in ID8 cell lysates prepared from confluent, serum starved cultures, as indicated by the substantial production of phosphatidylinositol-3,4,5-triphosphate (PIP3) in the *in vitro* kinase reaction (Figure 4b).

PI3-kinase activation is known to be associated with activation of Akt/protein kinase B (PKB). As PI3-kinase is constitutively activated in ID8 cells, the level of Akt/PKB phosphorylation, an indicator of Akt/PKB activation, was investigated. ID8 total cell lysates were subjected to immunoblot analysis with an antibody directed against phosphoserine 473 of Akt/PKB. Serine 473 phosphorylated Akt/PKB is present in ID8 cell lysates (Figure 4c), indicating activation of a downstream effector of PI3-kinase in ID8 cells cultured *in vitro*. In contrast, virtually no serine 473 phosphorylated Akt/PKB is detectable in early passage HOSE cells (H1 and H2).

Inhibition of Src Activity

As Src is at a point of integration of signals it was of interest to determine the effects of blockade of Src activity on ID8 cell characteristics. In order to determine the effect of Src inhibition on ID8 MOSEC cell migration, ID8 cells were plated on 5μm transwell inserts, pretreated with various inhibitors . Following the pretreatment serum containing medium were added to the bottom of the transwells. Cells were allowed to migrate overnight. Serum stimulated approximately 7)% of ID8 cells to migrate across the

transwell membranes (Figure 5). Pretreatment of cells with either and the PI3-kinase inhibitor LY294002 or the Src inhibitor PP2 suppressed ID8 cell migration. In contrast the MEK inhibitor PD98059 had little effect on ID8 cell migration.

As PI3-kinase associates with Src and is constituively activated in ID8 MOSEC cells the effect of Src inhibition on the PI3-kinase substrate Akt was determined. Immunoblot analysis demonstrates that Akt was constitutively phosphorylated in ID8 MOSEC cells (Figure 6). Treatment of cells with the PI3-kinase inhibitor decreased Akt phosphorylation. Similarly, treatment of ID8 cells with PP2, the Src inhibitor, was associated with a decrease in Akt phosphorylation, suggesting that Src may activate PI3-kinase in ID8 cells.

Focal adhesion kinase may play an important role in metastasis and resistance to anoikis, program cell death resulting from loss of attachment to the extracellular matrix. FAK is a well known substate for Src and FAK co-precipitates with Src in ID8 cells, therefore the effect of Src inhibition on Fak was determined. Inhibition of Src with PP2 resulted in a decrease in peripheral membrane FAK localization (Figure 7).

Herbimycin A and Taxol

Taxol is a microtubule stabilizing agent widely used chemotherapeutically in women with ovarian cancer. As all of the above signaling molecules (i.e. Src, PI3-kinase, FAK and Akt/PKB) are intimately associated with the cytoskeleton and that at least Src and FAK are known to modify cytoskeletal elements, the effects of taxol on ID8 cell growth were determined. Herbimycin, a relatively selective inhibitor of the Src family of tyrosine kinases was also tested in order to determine the effect of tyrosine kinase

blockade on ID8 tumor cell growth. In order to determine the effect of these agents 5,000 cells were seeded per well in 96 well plates in serum containing media. Cells were treated with various concentrations of taxol, herbimycin or both for 24, 48 and 72 hours, then relative cell number was determined by an MTT cell viability assay. Taxol dose dependently inhibited ID8 cell growth at all time points examined (Figure 8; the 48 hour time point is not shown due to space limitations). Herbimycin (1 μM) alone appeared to be cytostatic, especially at 72 hours where it reduced growth to approximately 40% of controls. Interestingly, addition of herbimycin increased the sensitivity of ID8 cells to the cytostatic/cytotoxic effects of taxol. The maximal effect of taxol alone was 0.04 and 0.2 μM at 24 and 72 hours, respectively. Addition of herbimycin was associated with a continued decrease in cell survival with increasing taxol concentration. Similar results were obtained with PP2.

Discussion

The studies outlined above provide a partial characterization of aberrant signal transduction cascades in the mouse ID8 ovarian surface epithelial tumor cell line. This cell line displays a high degree of constitutive tyrosine kinase activity, at least a portion of which can be attributed to high expression and constitutive activation of the proto-oncogene product, c-Src. This high level of Src expression and activation is associated with a constitutive direct interaction with both focal adhesion kinase and PI3-kinase, as demonstrated by the ability of Src to co-precipitate both of these factors. In addition to a direct physical association, both PI3-kinase and FAK also display constitutive activation. Furthermore, the serine-threonine kinase Akt/PKB, a PI3-kinase substrate, is

constitutively phosphorylated and thus presumably activated in ID8 cells. All of these signaling molecules are associated with stimulation of cell growth and resistance to apoptosis, thus providing a framework for the neoplastic transformation of ID8 cells.

Whether freshly isolated mouse ovarian surface epithelial cells express this high level of activated Src remains a lingering and important question. Unfortunately, due to the very small number of cells initially collected it is not feasible to perform the analyses required for the answer. However, the observation that Src remains highly expressed and active under culture conditions that should render the cells quiescent and do not favor Src activity argues strongly that this is truly a transformation event. Furthermore, human ovarian surface epithelial cells at a similar early passage stage, but showing no signs of transformation as determined by contact inhibition and failure to produce tumors in athymic mice (data not shown), express minimal amounts of Src protein and show little constitutive tyrosine kinase activity. Finally, preliminary studies utilizing immunocytochemistry fail to demonstrate high Src expression in intact mouse ovarian surface epithelial cells[4].

Src is at a focal point of several signal transduction cascades, linking cell surface receptors to the cell growth and survival machinery (14). Src has been shown to activate Ras, leading to activation of the MAP kinase pathway and cell proliferation. As demonstrated in the present study, and in other systems (15, 16) Src also directly interacts with focal adhesion kinase. This interaction is thought to lead to an increase in the kinase activity of FAK as well as phosphorylating other FAK tyrosine residues, thereby promoting new interactions with SH2 containing proteins. It has been demonstrated that FAK activation promotes a Grb2-Shc association and the subsequent activation of Ras

and that this interaction is mediated by Src (17). Thus constitutive activation of Src and FAK in ID8 cells potentially leads to activation of the Ras-MAP kinase pathway and cell proliferation. Another important activity of FAK may be to regulate "anoikis", or apoptosis resulting from loss of association with the extracellular matrix. Overexpression of activated FAK has been associated with loss of anchorage dependence and resistance to anoikis (18), a hallmark of cell transformation. Phosphorylation of tyrosine 397 of FAK, which serves as the binding site for the SH2 domain of Src, is necessary for this effect. Therefore the constitutive activation of FAK may be a very important event in ID8 cell transformation.

A well known substrate for Src is PI3-kinase (14). Activation of PI3-kinase results in accumulation of phosphoinositides phosphorylated at the D-3 position of the inositol ring (19). The most abundant and active of these phosphoinositides is PIP3, which in turn leads to activation of Akt/PKB. Activated Akt/PKB inhibits apoptosis through multiple mechanisms including phosphorylation and deactivation BAD (20, 21), a pro-apoptotic member of the Bcl-2 family, phosphorylation of pro-caspase 9, blocking its processing to an active caspase (22) and phosphorylation of forkhead family of transcription factors, leading to their sequestration in the cytoplasm, thus blocking their ability to activate forkhead responsive genes, which include IGF-binding proteins and Fas ligand (23).

Dephosphorylation of PIP3 by the tumor suppressor, PTEN, suppresses cell growth and increases sensitivity to apoptotic stimuli (24, 25). The present study demonstrates that components of this signaling pathway (PI3-kinase - Akt/PKB) are constitutively activated in ID8 tumor cells, thus providing a potential block to normal

apoptotic stimuli. At present the status of PTEN in these cells is unknown, however it is clear that dephosphorylation of the phosphotidylinositides in ID8 tumor cells cannot keep up with, or overcome the phosphotidylinositide phosphorylation events. Furthermore, pharmacologic inhibition of Src with PP2 blocks ID8 cell migration and inhibits Akt phosphorylation. These findings when coupled with the observation that PI3 kinase co-precipitates with Src suggests that Src is activating PI3-kinase in ID8 MOSEC cells.

Taxol is an antineoplastic agent that has been shown to have some efficacy in the treatment of ovarian cancer (26). Its main effect is to bind to tubulin, enhancing tubulin polymerization and inhibiting microtubule disassembly, thus preventing completion of mitosis and producing a G_2/M block (27, 28). The mechanism by which taxol induces its cytotoxicity is presently not clearly understood, although it is known to be an apoptotic process (29). The present study demonstrates that the cytotoxic effects of taxol on ID8 tumor cells are enhanced by blockade of tyrosine kinase activity with herbimycin A. How blockade of tyrosine kinase activity enhances the effects of taxol is unknown but could be at several levels: Decreased tyrosine phosphorylation of alpha tubulin leading to increased taxol-induced tubulin polymerization and stabilization (30, 31), decreased phosphorylation of microtubule associated proteins leading to increased microtubule stability (32), or a decrease in taxol resistance (33, 34). Finally, the combination of taxol and herbimycin completely blocked phosphorylation of Akt/PKB at serine 473, indicating inhibition of activation of this important cell survival promoting kinase, ultimately removing a potential block to apoptosis.

In summary, the present study demonstrates the expression of a number of constitutively active growth promoting - apoptosis inhibiting signaling kinases in ID8

ovarian surface epithelial tumor cells. The constitutive activation of Src, PI3-kinase, FAK and Akt/PKB as seen in ID8 cells set in motion a series of signals promoting rampant cell growth while inhibiting apoptosis (see Figure 8). These same signaling components are disregulated in a large proportion of ovarian cancers in women. As intraperitoneal injection of ID8 cells into syngeneic mice results in development of numerous peritoneal tumors and accumulation of ascitic fluid in a manner very consistent with human ovarian cancer, the ID8 cell line provides a unique and important new model by which to study the initiation, development and progression of ovarian cancer in mice with intact immune systems.

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Fig. 1. ID8 tumor cells derived from primary cultures of mouse ovarian surface epithelium contain constitutively tyrosine phosphorylated proteins. ID8, MA10 Leydig tumor cells (a) and two independent primary human ovarian epithelial cell cultures (b; H1, H2) from confluent, serum-starved cultures were lysed and 30 μg total soluble protein was subjected to polyacrylamide gel electrophoresis followed by immunoblot analysis with an antibody specific for phosphotyrosine (αpY). Molecular weight markers in kilodaltons are indicated on the left of each figure.

Fig. 2. ID8 tumor cells express large amounts of constitutively active Src protein. Protein expression was determined by immunoblot analysis, and (a) compared with wild type (wt) MA10 tumor cells, MA10 cells transfected with and overexpressing Src ($Srck^+$) and (b) two independent human ovarian surface epithelial cell cultures (H1, H2). Src activity (c) in confluent control (C) and confluent - serum starved (SS) ID8 cell cultures was determined by an immune complex kinase activity assay (c; see Materials and Methods for details). Numbers below the figure refer to relative activity (% of C) as determined by image analysis (Gel Pro). Src tyrosine phosphorylation (d) was determined by immunoprecipitating with an antibody directed against phosphotyrosine followed by immunoblot analysis with an antibody directed against Src.

Fig. 3. Src directly interacts with focal adhesion kinase, FAK (a), and PI3-kinase PI3-K (b), in ID8 tumor cells. Soluble ID8 cell protein (500 μg) was immunoprecipitated (IP) with preimmune IgG or an antibody specific for Src (αSrc). Immunoprecipitates were

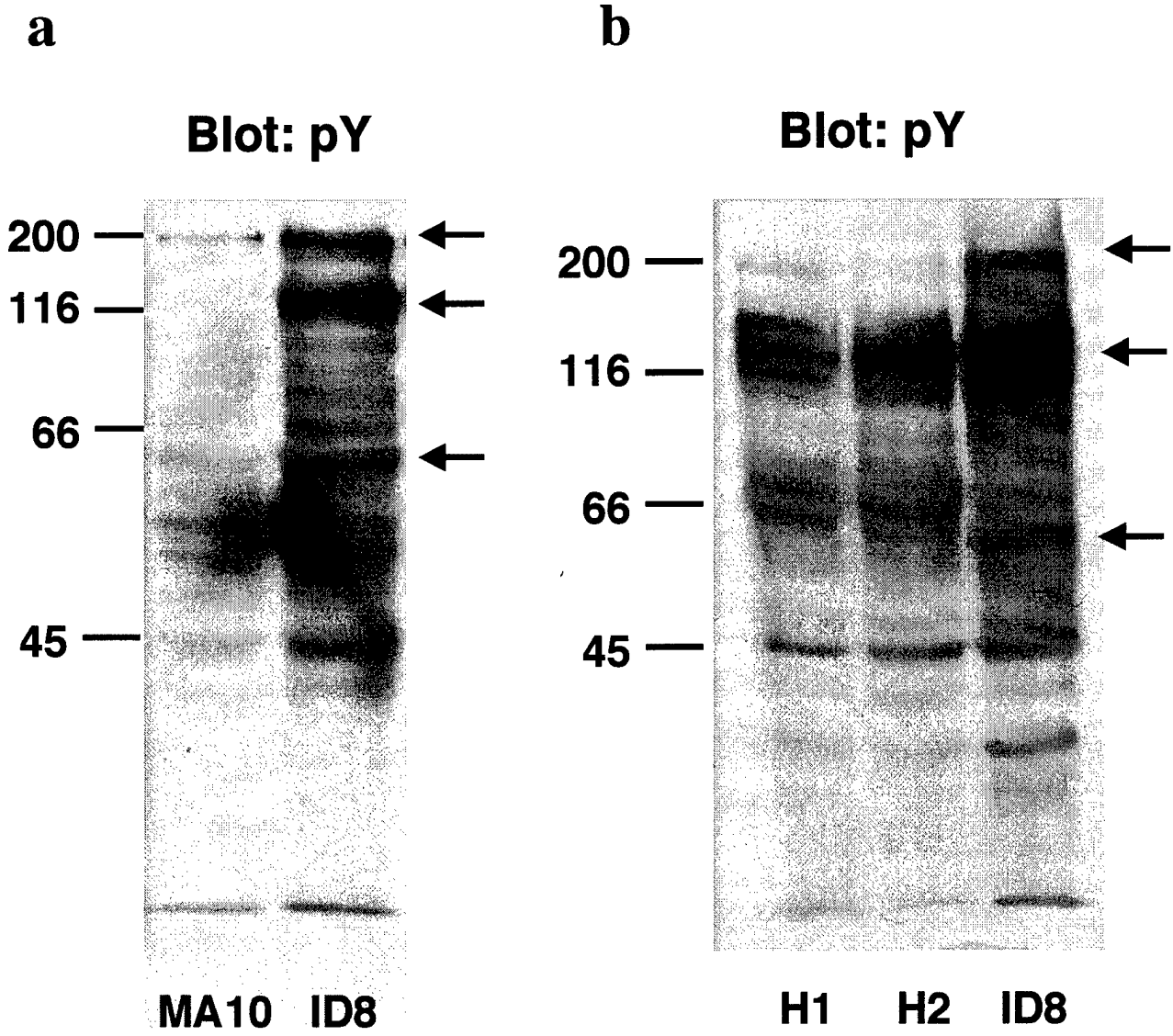
then analyzed by PAGE and subsequent immunoblot analysis with antibodies specific for either FAK or PI3-kinase.

Fig. 4. FAK, PI3-kinase and Akt/PKB are constitutively activated in ID8 tumor cells. Soluble protein (500 μg) was immunoprecipitated with an antibody directed against either phosphotyrosine (a) or PI3-kinase (b). The resulting immunoprecipitates were either subjected to PAGE and subsequent immunoblot analysis with an antibody specific for FAK (a) or analyzed by an *in vitro* kinase assay utilizing phosphatidylinositol as substrate (see Materials and Methods) followed by thin layer chromatography (b). For Akt/PKB (c, d) total soluble protein was subjected to PAGE and then immunoblot analysis with antibodies specific for Akt and serine 473 phosphorylated Akt. ID8 cell Akt phosphorylation was compared to two independent human ovarian epithelial cell cultures (H1, H2). Cells were treated for 1 hour with either LY294002 (10 μM) or PP2 (10 μM) and then analyzed for Akt phosphorylation (d).

Fig. 5. Herbimycin enhances taxol cytotoxicity (a) and inhibits Akt/PKB serine473 phosphorylation in ID8 tumor cells. ID8 cells were grown in serum containing media and exposed to various doses of taxol (0 to 10 μM), with or without herbimycin A (Herb A, 1 μM) for 24 or 72 hours. Controls receoved no Herbimycin A or Taxol. Relative cell number was determined by an MTT cell viability assay (see Materials and Methods for details). Each data point represents the mean ± SEM of 8 replicates in a representative experiment. For determination of Akt/PKB phosphorylation determination ID8 cells were treated for 24 hours with vehicle (C; 0.1% DMSO), taxol (Tx; 0.04 μg/ml),

herbimycin A (H; 1 μM) or both taxol and herbimycin (Tx/H). Total cellular protein (50 μg) was subjected to PAGE and then immunoblot analysis was performed with antibodies directed against Akt or serine 473 phosphorylated Akt.

Fig. 6. Disregulated pathways possibly contributing to neoplastic transformation in ID8 tumor cells. Arrows and perpindicular bars indicate indicate stimulation and inhibition, respectively.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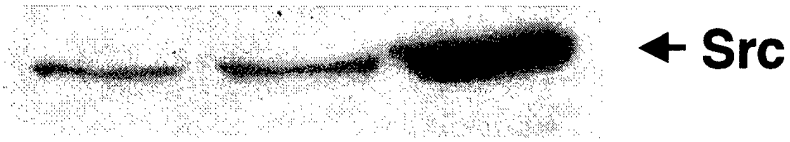

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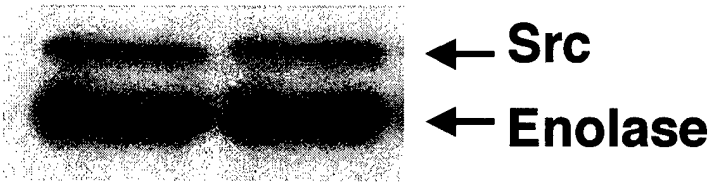
c

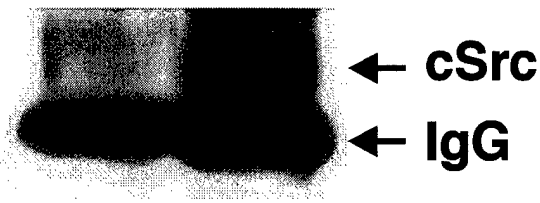
C SS



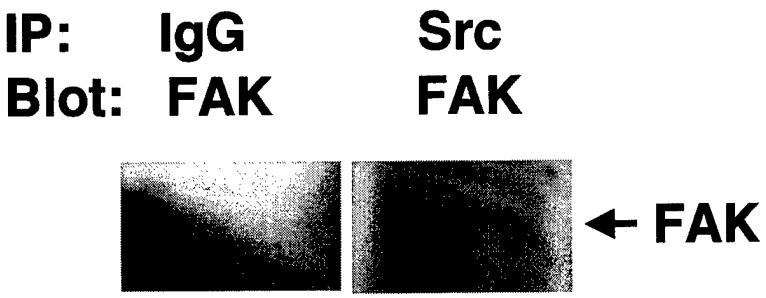
100% 44%
Activity relative
to control

d

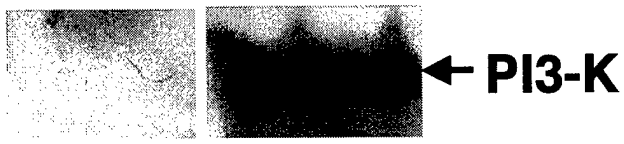
IP: IgG pY
Blot: Src Src



a

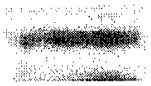


b



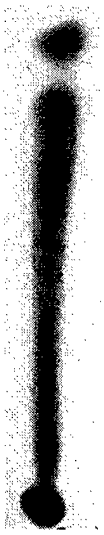
a

IP: pY
Blot: FAK



← FAK

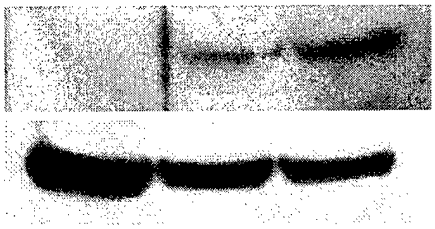
b



← PIP
← PIP2
← PIP3

← Origin

c

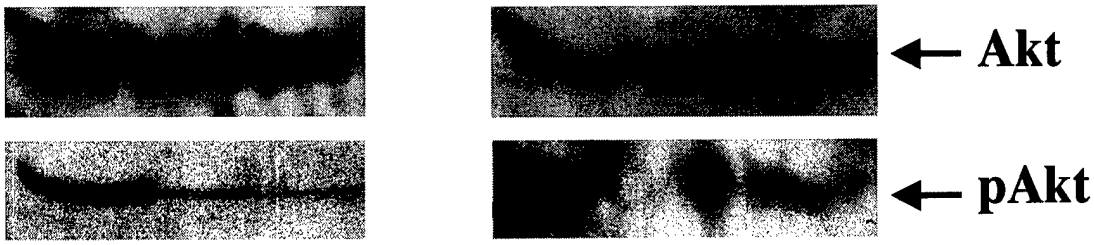


← S473 - Phospho - Akt

← Akt

H1 H2 ID8

d



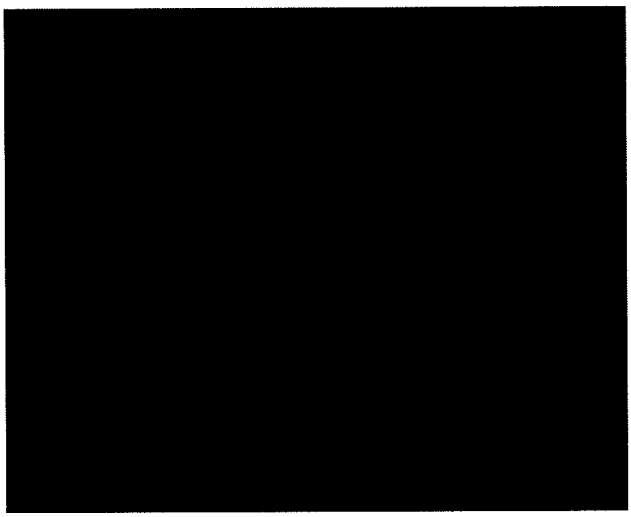
LY294002 PP2

C 15 30 Min C 15 30 Min

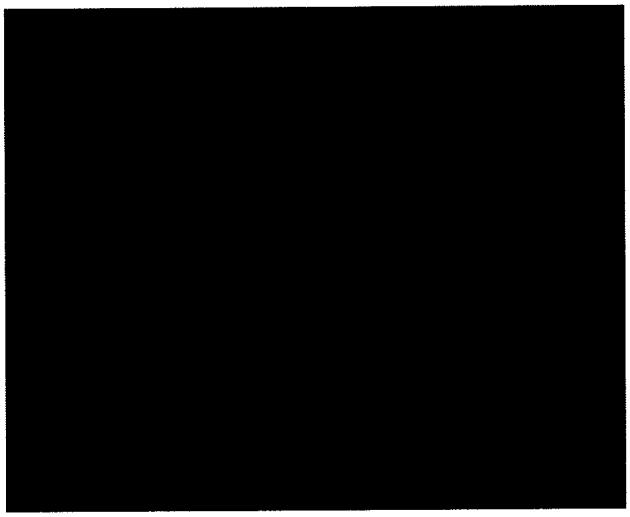
← Akt

← pAkt

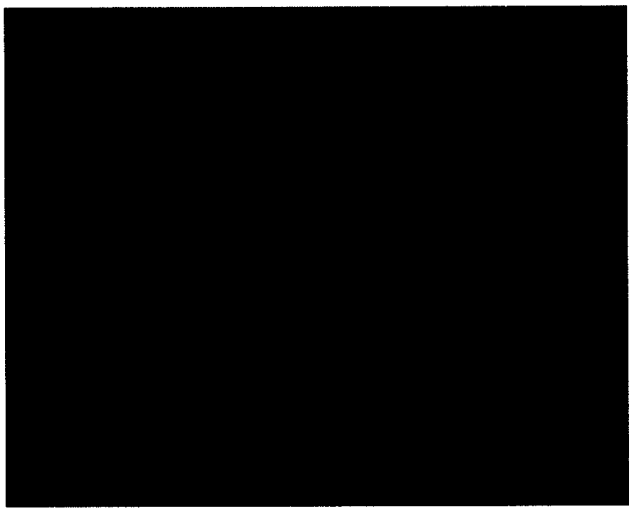
A



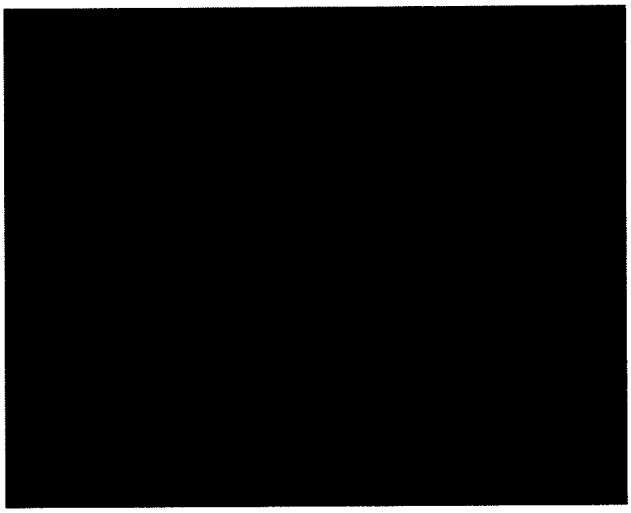
B



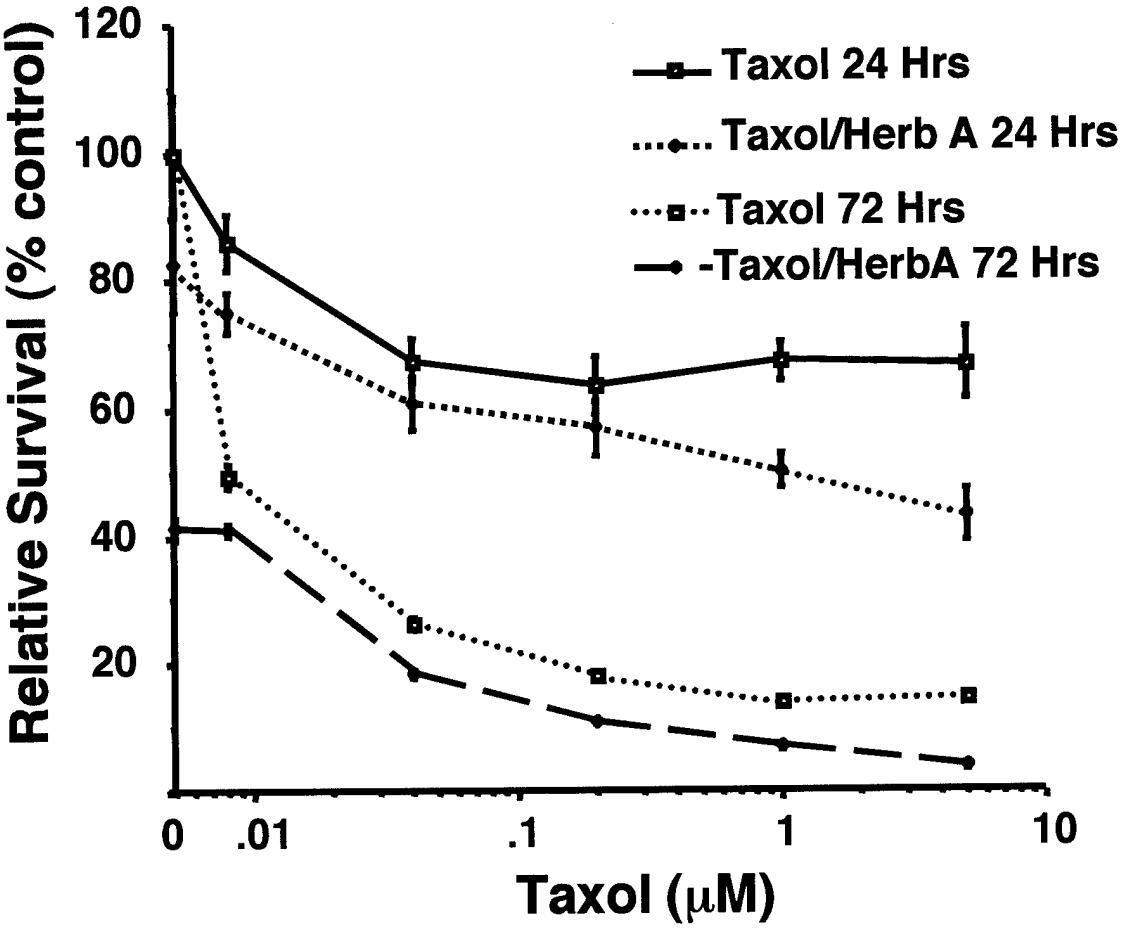
C



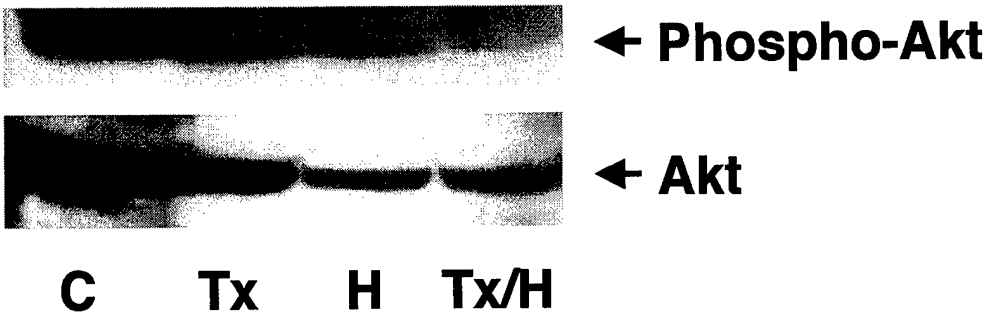
D

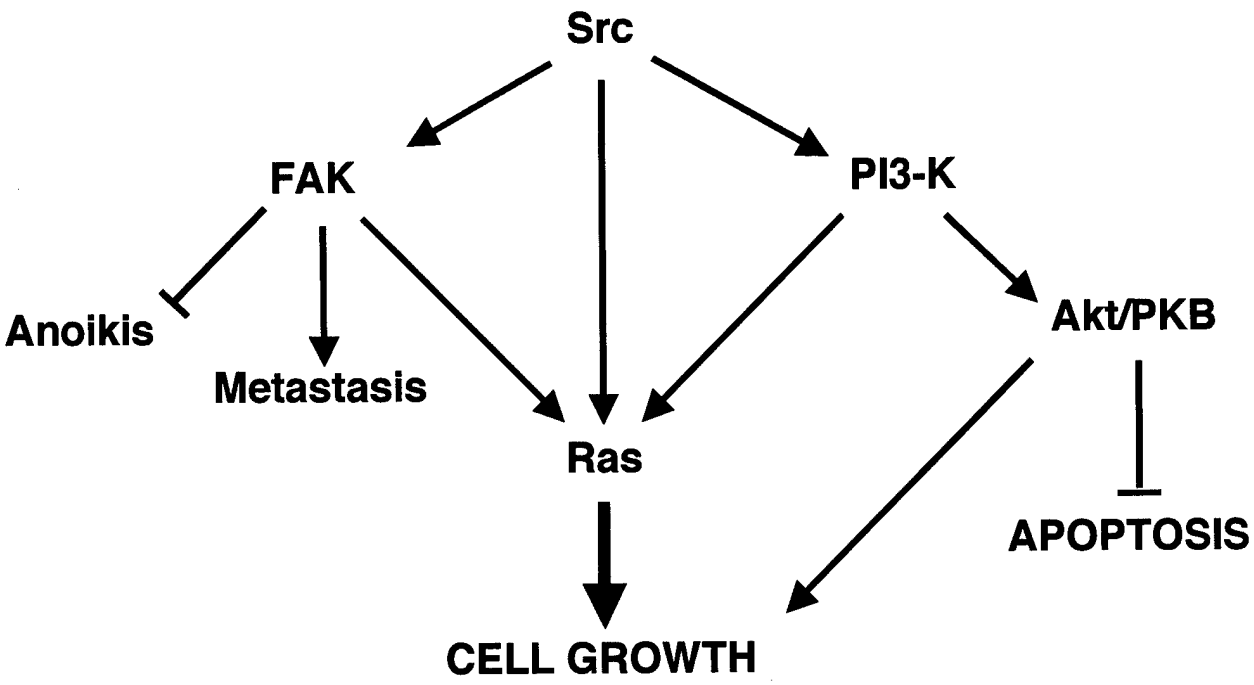


a


Relative Survival (% control)
120
100
80
60
40
20
0
.01
.1
1
10
Taxol (μM)
Taxol 24 Hrs
Taxol/Herb A 24 Hrs
Taxol 72 Hrs
-Taxol/HerbA 72 Hrs

b


Phospho-Akt
Akt
C
Tx
H
Tx/H


Src
FAK
PI3-K
Anoikis
Metastasis
Akt/PKB
Ras
APOPTOSIS
CELL GROWTH