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13. ABSTRACT (Maximum 200 Words)

Synucleins are a family of highly conserved small proteins that are normally expressed predominantly in neurons. Very little is known about the physiological functions of the synucleins. We have reported that g-synuclein (also known as BCSG1) is dramatically up-regulated in the vast majority (>70%) of late-stage breast and ovarian cancers (Bruening, et al., 2000). When over-expressed, g-synuclein significantly stimulates cell proliferation and metastasis in some breast cancer cell lines. We have shown that DNA hypomethylation is a common mechanism underlying the abnormal expression of this gene in tumor cells (Gupta et al., 2003) and hypothesize that g-synuclein may be a proto-oncogene and that abberant expression of this protein may contribute to the development and progression of ovarian cancer. We also found that g-synuclein can promote cancer cell survival and inhibit stress- and chemotherapy drug-induced apoptosis by modulating MAPKs. Specifically, over-expression of g-synuclein lead to constitutive activation of ERK1/2, and down-regulation of JNK1 in response to a host of environmental stress signals, including UV, heat shock, sodium arsenate, nitric oxide and chemotherapeutic drugs (Pan, Z-Z, et al., 2002). Because of its high frequency of expression in late-stage ovarian cancers, we hypothesized that g-synuclein may be a promising target for cancer therapy.

14. SUBJECT TERMS
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INTRODUCTION:

The synucleins (α, β, γ, synoretin) are a family of highly conserved small proteins that are normally expressed predominantly in neurons. Little is known about the normal functions of synucleins in physiological conditions. Of the synucleins, α-synuclein is the best characterized because of its potential significance in neurodegenerative diseases including Parkinson's Disease. Recently we and others have found that γ-synuclein is dramatically up-regulated in the vast majority of late-stage breast (70%) and ovarian (> 85%) cancers and that γ-synuclein over-expression can enhance tumorigenicity (Bruening et al., 2000; Ji et al., 1997; Liu et al., 2000). We also observed that expression of γ-synuclein induces a phenotype similar to that induced by activation of RhoA/Rac/CDC42, altering the appearance of focal adhesions and stress fibers, and enhancing motility and invasion in ovarian cancer cells (Pan et al, submitted 2004). Recent studies by have also shown that when γ-synuclein is overexpressed in a breast tumor derived cell line, the cells experience a dramatic augmentation in their capacity to metastasize *in vivo* (Jia et al., 1999). Based on these known data, we hypothesize that γ-synuclein may be a proto-oncogene and that abberant expression of this protein may contribute to the progression of ovarian cancer and that this tumor-associated protein may be promising targets for drug discovery. We further hypothesize that γ-synuclein may be promoting this phenotype in part by activating the RhoA signal transduction pathway and have shown that RAC as well as the mitogen-activated kinase, ERK1/2, are constitutively activated in cells that overexpress γ-synuclein, but not alpha or beta. Furthermore, we have found that the activation of JNK by stress signals is significantly down-regulated by γ-synuclein, suggesting a role in tumor cell survival and inhibition of apoptosis. If expression of γ-synuclein in tumor cells induces an invasive phenotype and promotes tumor cell survival, then understanding how γ-synuclein functions may lead to therapies for metastatic disease. Thus, drugs that block the action of γ-synuclein may inhibit the spread ovarian cancer, as well as other neoplasms. We propose to further define the function of γ-synuclein through studying the signaling pathways that it affects. Disrupting the interactions between γ-synuclein and its interactors may provide a means to limit tumor cell metastasis, while inducing limited or no toxicity among other cells in human adults.

BODY

Progress report year 1.

Task 1 (Months 1-24). Determine how γ-synuclein modulates JNK and ERK signaling transduction and its effects on cell survival, apoptosis and tumor progression.

We have made substantial progress in terms of the first task. A paper submitted to *Oncogene* (Pan et al., 2004) is currently under review. Results from this study and other ongoing studies are given below (Figures 1 through 12).

Task 2 (Months 3-36). Identify specific mutations, and small molecule agents, that specifically disrupt interactions between γ-synuclein and ERK1/2 and between γ-synuclein and JNK, and evaluate them in cell culture models, with a goal of developing them as targeted therapeutics.

We have made some significant progress in regards to this task; however, we have yet to identify a specific inhibitor of γ-synuclein through our screens. The state of these studies is also indicated below (Figures 13 and 14).

Task 1-Progress Report-"Determine how γ-synuclein modulates JNK and ERK signaling transduction and its effects on cell survival, apoptosis and tumor progression".

γ-Synuclein interacts with ERK and JNK MAP kinases in cancer cells - α-Synuclein has recently been reported to bind directly to the ERK2 kinase (Iwata et al., 2001b). Therefore, we evaluated whether γ-synuclein could also interact with the ERK kinases as well as other MAPKs. By co-immunoprecipitation

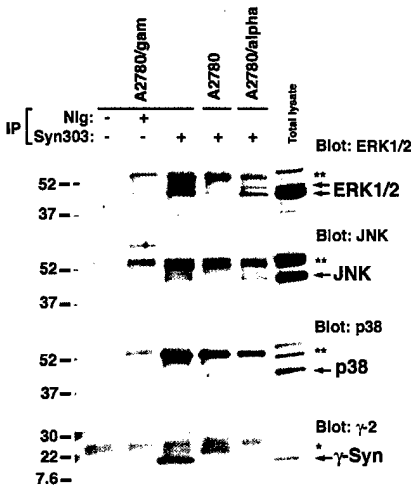
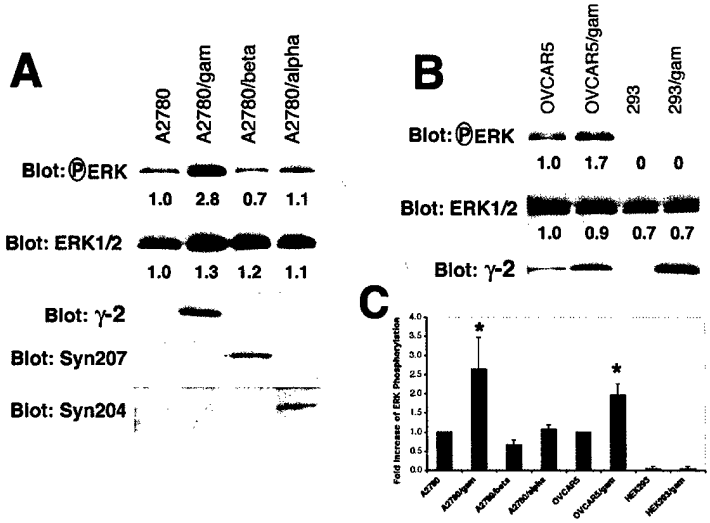


Fig. 1. Interaction between γ- and α-synucleins with ERK1/2 and JNK. Cell lysate from A2780, A2780/gam or A2780/alpha were immunoprecipitated with Syn303, NIg (normal IgG) or irrelevant antibodies (not shown here). The proteins in the immunoprecipitates were identified by immunoblotting with antibodies against ERK1/2, JNK1, p38, and γ-2 (a polyclonal antibody specific for γ-synuclein). Molecular mass standards (in kilodaltons) are indicated on the left. Non-specific bands around the IgG heavy (**) and light (*) chains are indicated by asterisks.

approaches, we were able to demonstrate a novel association of γ-synucleins with ERK1/2 and JNK1 kinase, but not with the p38 kinase (**Fig. 1**). We also confirmed that α-synuclein is associated with ERK1/2 as well with JNK1 (**Fig. 1**), which is consistent with the recently studies using neuro2a, a neuronal cell line (Iwata et al., 2001a). These data indicate that γ- and α-synuclein can interact with ERK1/2 and JNK1 in cancer cells that over-express these proteins.



Elevated activity of ERK in cells over-expressing γ-synuclein - We next evaluated whether these protein interactions would affect the activity of ERK1/2 and/or JNK1 (see below). In A2780 and OVCAR5 cancer cells over-expressing γ-synuclein, the activated ERK1/2 was increased 2-3 fold as evidenced by immunoblotting with an anti-phospho-ERK specific antibody (**Fig. 2**). In contrast, α- and β-synucleins appeared to have little or no effect on the activity of ERK1/2 (**Fig. 2A**) although α-synuclein was also found to be associated with ERK (as described above and shown in **Fig. 1**) in A2780 cells. In HEK 293

Figure 2. Activation of ERK in cells over-expressing γ-synuclein. A, ERK1/2 activation is enhanced in γ-synuclein over-expressing A2780 cells. Whole cell lysates (40 μg/lane) from A2780 cells (parent) or A2780 cells transfected with γ-, β-, or α-synuclein were separated by SDS-PAGE and blotted with appropriate antibodies. The levels of activated ERK or total ERK1/2 were determined using an anti-phospho-ERK specific antibody or ERK1/2 antibodies, respectively. The number beneath each band represents the arbitrary densitometry units of the corresponding band (the integrated densitometry reading number of A2780 or OVCAR5 was assigned to an arbitrary unit 1.0, and the other readings were normalized thereafter). The synucleins were evaluated by blotting with specific antibodies, i.e., γ-2 for γ-synuclein, Syn207 for β-synuclein, and Syn204 for α-synuclein, respectively. B, activation of ERK by γ-synuclein in OVCAR5 cells but not HEK 293 cells. Whole cell lysate (40 μg/lane) from parental or γ-synuclein over-expressing cells were separated and blotted as in A. Panels A and B are representative of at least three independent experiments with comparable results. C, fold increase of ERK activation. The data shown are the average ± S.E. of three independent experiments. Phosphorylated ERK was normalized to the protein level of total ERK. The basal levels of ERK phosphorylation in the parental A2780 or OVCAR5 cells were set as 1.0. (*) Represents significant difference compared to the parental cells ($p < 0.05$).

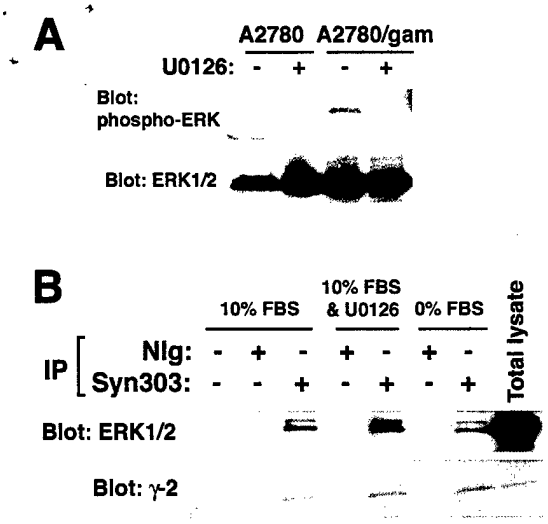


Fig. 3. Requirement of MEK1/2 for γ-synuclein enhanced ERK1/2 activation. A, A2780 and A2780/gam cells untreated or treated with the MEK1/2 inhibitor, U0126 (10 μM), were lysed and 30 μg of proteins were loaded into each lane. As in Fig. 2, anti- phospho-ERK1/2 specific antibody was used to detect activated ERK1/2; and the antibody against ERK1/2 was used to detect the protein level of total ERK1/2. B, the interaction between ERK and γ-synuclein is independent of the activation status of ERK1/2. A2780/gam cells in normal 10% FBS medium, 10% FBS medium with U0126 (10 μM), or serum-free medium were lysed and immunoprecipitated with Syn303 or control IgG. The proteins in the immunoprecipitates were detected with the antibodies against ERK1/2 or against human γ-synuclein (γ-2). The autoradiogram shown is the representative of three independent experiments with comparable results.

cells, the basal level of ERK activation is undetectable and γ-synuclein over-expression does not increase its activation level (**Fig. 2B**). Structural analysis indicates that γ-synuclein does not contain any kinase domain, suggesting that the activation of ERK is mediated by other kinase. Since MEK1/2 is required for the activation of ERK1/2 in response to many mitogens, we determined whether MEK1/2 is still required for γ-synuclein mediated activation of ERK1/2. When cells over-expressing γ-synuclein were treated with the MEK1/2 inhibitor U0126, the activation of ERK1/2 was suppressed (**Fig. 3A**). We further studied the relation of γ-synuclein-ERK interaction and the activation status of ERK1/2. In cells treated with U0126 or serum-starved, the association of γ-synuclein and ERK1/2 was still present (**Fig. 3B**). These data indicate that γ-synuclein may be constitutively associated with ERK1/2, which could facilitate the activation of ERK by MEK1/2 and lead to the constitutive activation of ERK1/2 in cells over-expressing γ-synuclein.

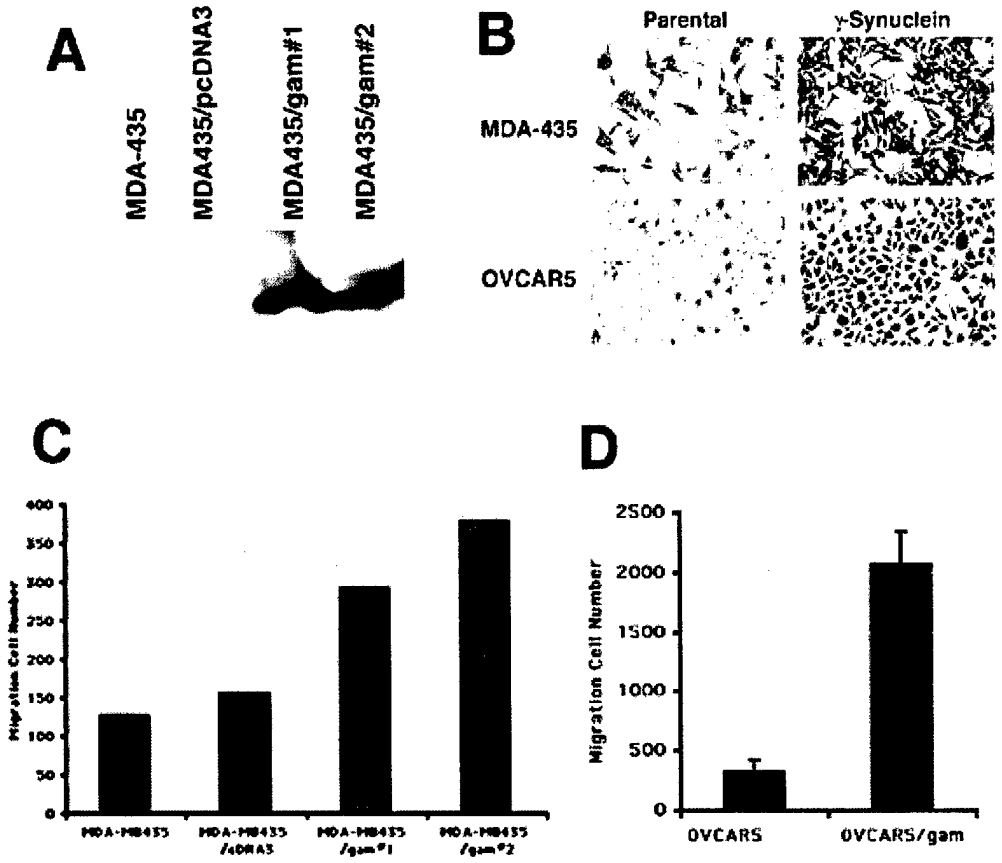


Fig.4. γ-Synuclein enhance cell migration. A, γ-synuclein expression in parental MDA-MB-435 cells, and those stably transfected with pcDNA3 vector alone, or with CMV-γ-synuclein (two clones were shown here). B throuhg D, Boyden chamber assay for cell migration. Cells migrated to the lower chamber were stained (B) and counted (C, D).

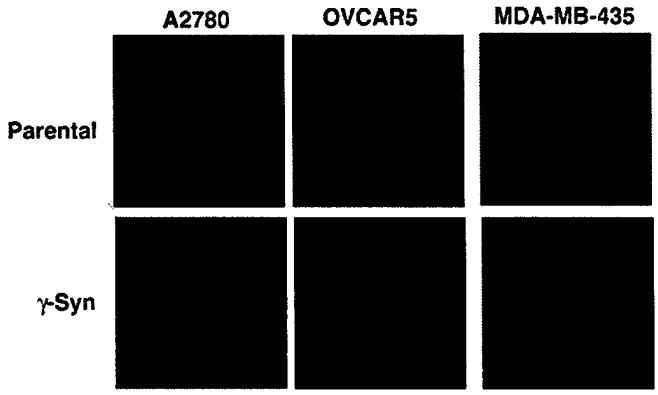


Figure 5. Induction of stress fiber formation by γ-synuclein. Ovarian tumor (A2780, OVCAR5) and breast tumor (MDA-MB-435) cells with (γ-Syn) or without (parental) exogenously expressed γ-synuclein were grown on glass coverslips and were fixed and stained with Rhodamin-phalloidin for stress fibers. Images were taken with a laser scanning confocal microscope.

Over-expression of γ-synuclein leads to increased cell motility- Recent stuidies by Jia and colleagues indicated that γ-synuclein might increase cell migration and metastasis (Jia et al., 1999). We also established several stable cancer cell lines that over-express γ-synuclein (Fig. 4A, and data not shown) and found that γ-synuclein can enhance cell motility as analyzed by Boyden chamber assay (Fig. 4B, 4C, 4D, and data not shown). Consistent with their role in cell migration, more stress fibers were found in cells over-expressing γ-synuclein (Fig. 5)

Over-expression of γ-synuclein leads to activation of at least one member of the Rho family GTPase- Cell migration and invasion involves a series of coordinated complex processes, including focal adhesion formation in the front and release of adhesion in the back, polarized stress fiber formation and disassembly and contraction (Ridley, 2000). Several protein kinases are known to regulate these processes, including Rho/Rac/Cdc42 small GTPase proteins, ERK, Crk, and Akt (Krueger et al., 2001; Ridley, 2001; Ridley et al., 1999; Wicki and Niggli, 2001). Among these effector kinases, Rho family members play a pivotal role in regulating stress fiber and focal adhesion formation. In cells over-expressing γ-synuclein, we found at least one major Rho/Rac/Cdc42 member is activated although the protein levels of these proteins were not affected (**Figure 6**).

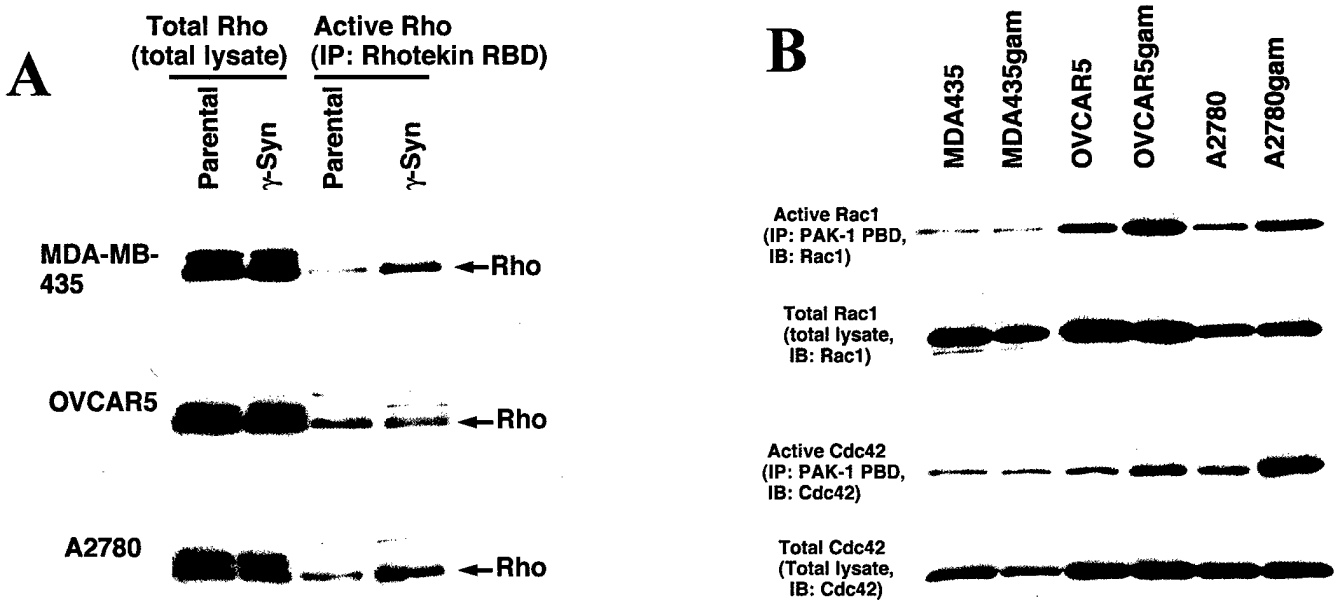


Fig. 6. Enhanced activation of Rho (A) and Rac/Cdc42 (B) in cancer cells over-expressing γ-synuclein. A, whol3 cell lysate (2 mg protein) were immunoprecipitated with Rhotekin Rho binding domain and analyzed by Western blot with anti-Rho antibody. B, who cell lysate (2 mg protein) were immunoprecipitated with PAK1 PBD and analyzed by Western blot with anti-Rac and anti-Cdc42 antibodies, respectively.

Requirements of Rho/Rac/Cdc42 and ERK for γ-synuclein-enhanced cell motility-
As described above, over-expression of γ-synuclein also leads to enhanced activation of ERK. To determine whether and to what extent Rho/Rac/Cdc42 or ERK kinase contribute to cell motility and invasiveness, parental cancer cells and their corresponding γ-synuclein over-expressing cells were treated with specific inhibitors. When treated with *C. difficile* toxin B which can inactivate most, if not all, members of the Rho family, and U0126, an inhibitor of ERK activation, the cell migration in both parental and γ-synuclein over-expressing cells were almost completely blocked (Fig. 7). These data indicate that both Rho and ERK pathways are involved in the parental basal level cell migration and the enhanced cell migration induced by γ-synuclein.

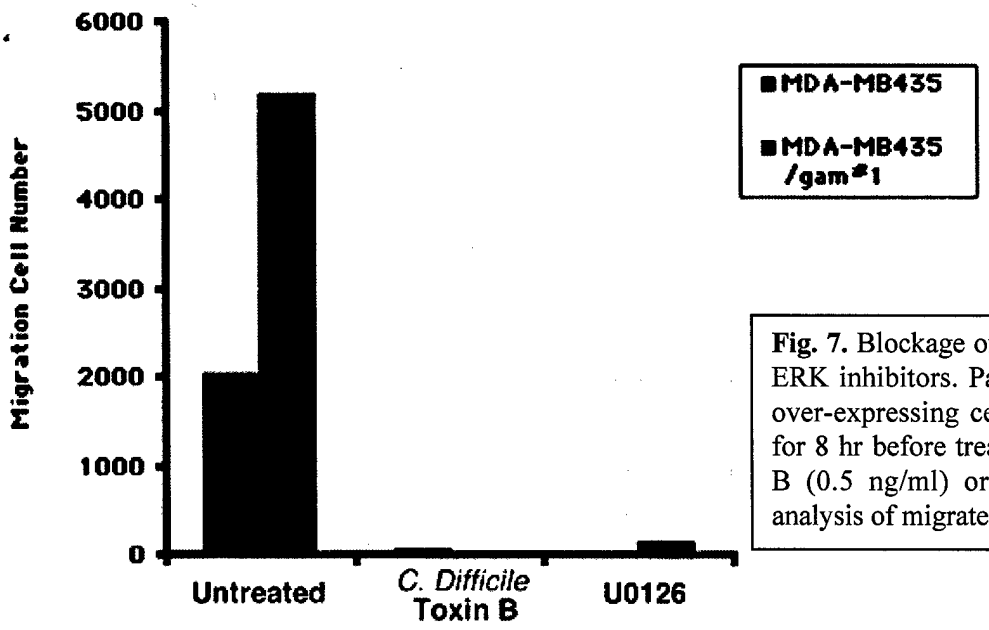


Fig. 7. Blockage of cell migration by Rho and ERK inhibitors. Parental cells or γ-synuclein over-expressing cells were allowed to attach for 8 hr before treated with *C. difficile* Toxin B (0.5 ng/ml) or U0126 (10 μM) before analysis of migrated cells at 52hr.

Down-regulation of JNK activation by γ-synuclein in response to UV - JNK is activated by stress signals including UV which leads to mitochondria mediated apoptosis (Davis, 2000). The basal level of JNK activity in A2780 and OVCAR5 ovarian cancer cells is very low in untreated cells whether γ-synuclein was over-expressed or not (**Fig. 8**). JNK was highly activated in the parental cells when treated with UV (**Fig. 8**). In cells over-expressing γ-synuclein, the activation of JNK was almost completely blocked in A2780/gam cells ($p < 0.05$) and was down-regulated by approximately 50% in OVCAR5/gam cells when treated with UV (**Fig. 8**) or heat-shock (data not shown). The blockage of JNK activation by UV appears to be γ-synuclein specific since over-expression of α- and β-synucleins appeared to have little or no effect on the activation of JNK by UV (**Fig. 8**, and data not shown). The inhibition of JNK activation in OVCAR5/gam cells is much less than that in A2780/gam cells. The cause of this difference could either be cell-type specific, or it could be the high endogenous γ-synuclein expression in OVCAR5 cells. Collectively, these data indicate that stress-induced activation of JNK can be blocked by γ-synuclein over-expression in a variety of cell lines.

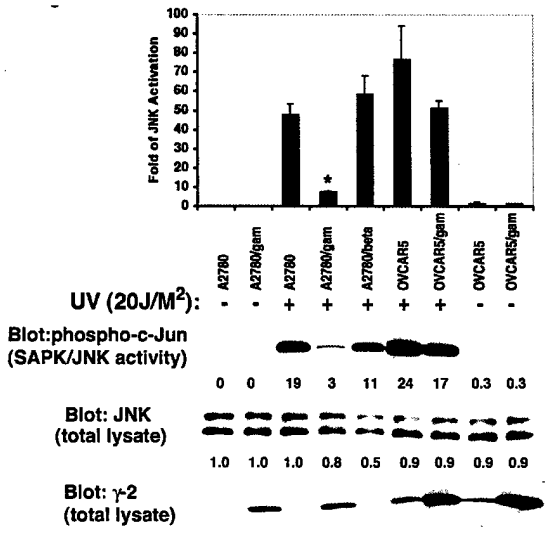


Fig. 8. Inhibition of JNK activation by γ-synuclein in response to UV treatment. A2780, A2780/gam, A2780/beta, OVCAR5, OVCAR5/gam cells were un-treated or treated with UV (20 J/M^2) and cells were lysed at 30 min. JNK activities were analyzed by an immunocomplex kinase assay using GST-c-JUN as substrate. The phosphorylated GST-c-JUN by activated JNK was evaluated by immunoblotting with anti-phospho-c-JUN specific antibody. The protein levels of JNK and γ-synuclein were determined by immunoblotting with anti-JNK and γ-2 antibody, respectively. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. JNK activities were measured by levels of phosphorylated c-JUN and normalized to levels of total JNK proteins. The graph above the blots is the average ± S.E. of three independent experiments. (*) Represents significant inhibition of JNK activation compared to that in the parental cells ($p < 0.05$).

γ-Synuclein may protect paclitaxel (Taxol) induced cell death by regulating JNK and ERK activities.

Based on the data presented above, we hypothesized that γ-synuclein may contribute to cancer cell survival by up-regulating the ERK cell survival pathway and by suppressing the JNK apoptosis pathway under adverse conditions. However, when we treated γ-synuclein over-expressing cells with UV, significant differences in cell survival between A2780 and A2780/gam cells were not observed (data not shown). The reason for this lack of difference is not readily apparent. However, the counter-balance between the survival factors and the apoptotic signaling pathways regulates cell survival and cell death. Since UV treatment also activates the cell survival pathways ERK [(Rosette and Karin, 1996), and data not shown] and PI3K-AKT (Krasilnikov et al., 1999; Nomura et al., 2001), the initiation of the mitochondria associated caspase pathway may be blocked by the activation of ERK or AKT in A2780 cells. In support of these findings we did not observe the cleavage of the caspase-3 substrate PARP in A2780 cells when treated with UV (data not shown).

We next evaluated the survival of γ-synuclein over-expressing cells in response to Taxol, a commonly used chemotherapeutic drug. In addition to its role in affecting microtubule assembly, Taxol is known to lead to apoptosis via the mitochondria by activating the JNK signaling pathway and Taxol-induced apoptosis can be enhanced by MEK inhibition (Lee et al., 1998; Mandlekar et al., 2000; Wang et al., 1999; Wang et al., 2000). In A2780 cells, Taxol did not affect the basal level activity of ERK or the activation of ERK by γ-synuclein (**Fig. 9A**). To test the effect of γ-synuclein on cell survival, cells were treated with Taxol for varying lengths of time. At 48hr after treatment, 45-60% of A2780 cells had died, while only 7-15% of A2780/gam cells were dead indicating that Taxol induced cell death can be suppressed by γ-synuclein over-expression (**Fig. 9B**). When ERK activation was inhibited using the MEK1/2 inhibitor U0126, the cell death was reduced by ~25% in A2780 cells but was nearly doubled in A2780/gam cells (**Fig. 9B**). These data suggest that enhanced cell survival in γ-synuclein over-expressing cells is partially mediated by activation of ERK.

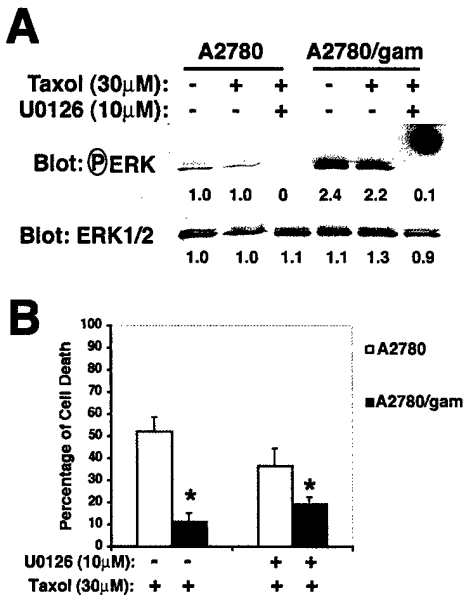


Fig. 9. γ-Synuclein protects cells from paclitaxel (Taxol) induced cell death and is partially mediated by ERK activation. A, Taxol does not affect ERK activity or γ-synuclein mediated ERK activation. A2780 or A2780/gam cells pre-treated with or without U0126 (10 μM) were treated with Taxol (30 μM) in the absence or presence of U0126 for 30 min. Whole cell lysate were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. **B**, cell death induced by Taxol was significantly reduced in cells over-expressing γ-synuclein. A2780 and A2780/gam cells treated with Taxol for 48hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining (shown) and WST-1 assays (not shown). The graph represents the average ± S.E. of three independent experiments. (*) Represents significant difference in cell death between A2780 and A2780/gam cells ($p < 0.05$).

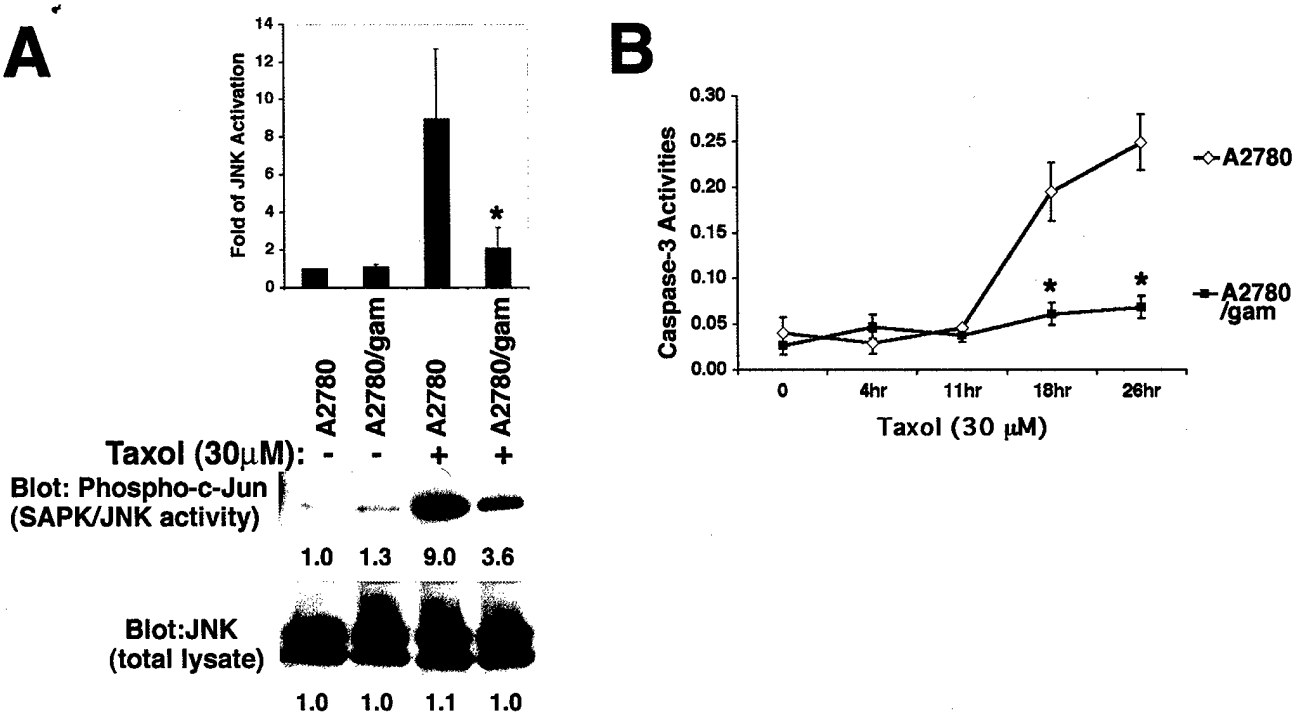


Fig. 10. Taxol activated JNK and caspase-3 apoptotic pathway is blocked by γ-synuclein. A, down-regulation of JNK activation by Taxol in cells over-expressing γ-synuclein. Cell lysates from A2780 and A2780/gam cells treated with or without Taxol (30 μM) for 60 min were assayed for JNK activity (see the legend for **Fig. 8** for experimental details). The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. JNK activities were measured by levels of phosphorylated c-JUN and normalized to levels of total JNK proteins. The graph above the blots is the average ± S.E. of three independent experiments. **B,** down-regulation of caspase-3 activity activated by Taxol in A2780/gam cells. A2780 and A2780/gam cells treated with Taxol (30 μM) for different time lengths were lysed, and approximately 20 μg protein were incubated with the caspase-3 substrate Ac-DEVD-pNA for 4hr at 37°C. Cleavage of the substrate to release pNA was monitored at 405 nm using a microplate reader. The graph represents the average ± S.E. of three independent experiments. In panels A and B, (*) represents significant difference compared to that in the parental cells ($p < 0.05$).

To determine whether the protective role of γ-synuclein on cell survival is also mediated through down-regulating JNK associated apoptotic pathway(s), caspase-3 activity was monitored at different time points after Taxol treatment. Consistent with the data in other ovarian and breast cancer cell lines (Lee et al., 1998; Wang et al., 1999), JNK was activated in A2780 cells when treated with 30 μM Taxol. The caspase-3 substrate PARP was found cleaved in cells treated with Taxol (data not shown). In A2780/gam cells, the activity of JNK was significantly inhibited following treatment with Taxol ($p < 0.05$) (**Fig. 10A**). In the parental A2780 cells, caspase-3 was highly activated following drug treatment. By contrast, the activation of caspase-3 by Taxol treatment was significantly reduced in γ-synuclein over-expressing ovarian cancer cells ($p < 0.05$) (**Fig. 10B**). These data indicate that Taxol activated JNK mediated caspase apoptotic pathway was significantly attenuated in cells over-expressing γ-synuclein. Taken together, our results indicate that the cell death in ovarian cancer cells induced by Taxol may be protected by γ-synuclein and that this may be mediated by regulating the JNK and ERK signaling pathways.

γ-Synuclein over-expression leads to protection from vinblastine but not etoposide induced cell death.

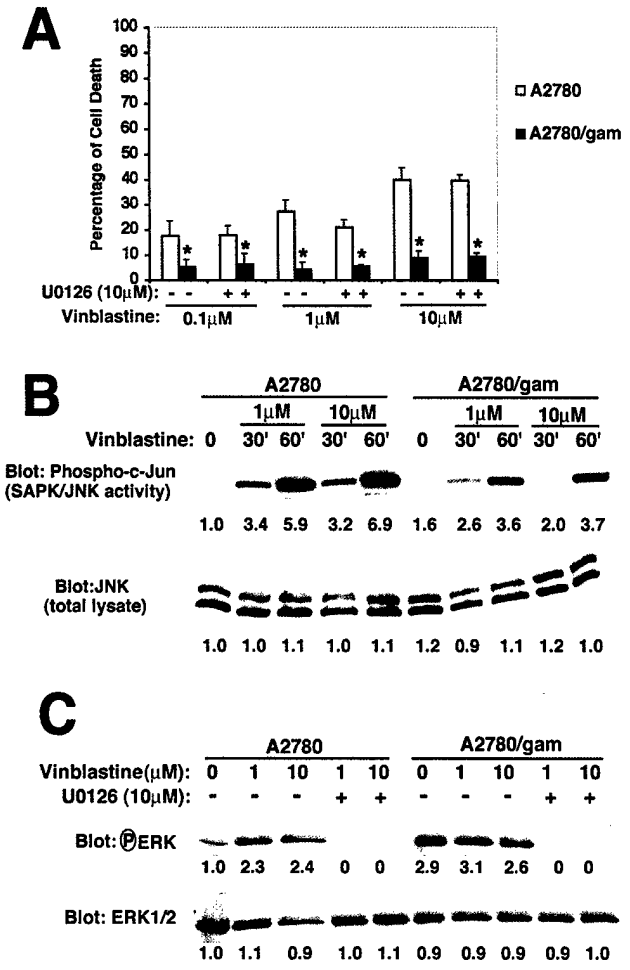


Fig. 11. Vinblastine induced cell death and activation of the MAPK pathways in γ-synuclein over-expressing cells. A, the cell death induced by vinblastine was significantly reduced in cells over-expressing γ-synuclein. A2780 and A2780/gam cells treated with vinblastine (0.1, 1, or 10 μM) for 30 hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining. The graph represents the average ± S.E. of three independent experiments. (*) Represents significant difference in cell death between A2780 and A2780/gam cells ($p < 0.05$). **B,** inhibition of JNK activation by γ-synuclein in response to vinblastine treatment. A2780 and A2780/gam cells were un-treated or treated with vinblastine (1μM, and 10μM) for 30 min and 60 min before lysis for JNK activity assay. JNK activities were analyzed as described in the legend for Fig. 5. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. **C,** the effect of vinblastine on ERK phosphorylation. A2780 or A2780/gam cells pre-treated with or without U0126 were treated with vinblastine (1 μM and 10 μM) in the absence or presence of U0126 (10 μM) for 30 min. Whole cell lysates were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown are representative of three independent experiments with comparable results.

To demonstrate whether the effects of γ-synuclein on cell survival were specific to Taxol or were a general mechanism of drug resistance, we evaluated two additional chemotherapeutic agents, i.e., vinblastine and etoposide. Both Taxol and vinblastine are microtubule-interfering agents; Taxol binds to microtubule polymers while vinblastine binds to monomers and dimers. When treated with vinblastine (either 0.1, 1.0, or 10 μM for 30 hr), cell death in A2780/gam cells was significantly lower ($p < 0.05$ for all the three concentrations tested) as compared to the parental cells (**Fig. 11A**). Consistent with other studies using a variety of tumor cell lines (Fan et al., 2000; Osborn and Chambers, 1996; Stone and Chambers, 2000; Wang et al., 1999), vinblastine strongly activates JNK in A2780 cells. However, this activation of JNK by vinblastine was significantly inhibited by γ-synuclein over-expression (**Fig. 11B**). Furthermore, we observe that treatment with vinblastine results in a two-fold increase in phosphorylated ERK1/2 in A2780 cells. This enhancement in activated ERK levels was not observed in the A2780/gam cells (**Fig. 11B**). Unlike our results for Taxol, inhibition of ERK phosphorylation by U0126 did not significantly affect the cell death in the parental cells or A2780/gam cells (**Fig. 11A & C**). These data

indicate that suppression of vinblastine-induced cell death by γ-synuclein may be mediated by inhibition of JNK activation.

We also evaluated etoposide, a DNA damage agent that has also been shown to induce JNK activation in some cell lines (Anderson et al., 1999; Gibson et al., 1999; Jarvis et al., 1999; Osborn and Chambers, 1996). When treated with 1, 10, or 100 μM of etoposide for 56 hrs, there was no significant difference in cell survival between A2780 and A2780/gam cells (**Fig. 12A**). As might be predicted, JNK was not activated in response to etoposide treatment (**Fig. 12B**). Furthermore, etoposide treatment did not result in ERK activation in A2780 cells. However, surprisingly the constitutive phosphorylated ERK levels observed in A2780/gam cells were significantly down regulated within 30 min of treatment with 10 or 100 μM of etoposide (**Fig. 12C**). In the presence of the MEK inhibitor U0126, cell death induced by etoposide was reduced in both the parental and γ-synuclein over-expression cells, but statistical analysis indicated these differences were not statistically significant.

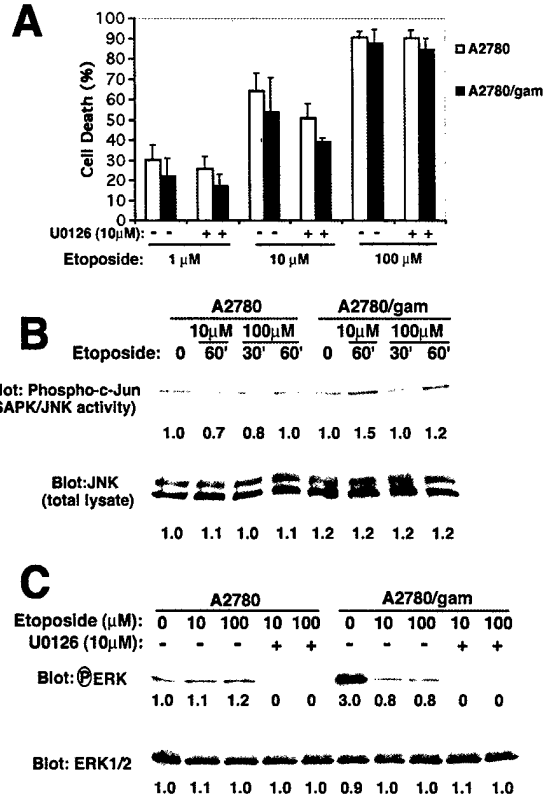


Fig. 12. Effects of γ-synuclein over-expression on etoposide induced cell death and activation of the MAPK pathways. A, cell death induced by etoposide was not significantly altered in cells that over-express γ-synuclein. A2780 and A2780/gam cells treated with etoposide (1 μM, 10 μM, 100 μM) for 56 hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining. The graph represents the average ± S.E. of three independent experiments. B, vinblastine treatment does not induce JNK activation. A2780 and A2780/gam cells treated with or without etoposide (10 μM, and 100 μM) for 30 min and/or 60 min before lysis for JNK activity assay. JNK activities were analyzed as described in Fig. 5. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. C, effect of etoposide on ERK phosphorylation. A2780 or A2780/gam cells pre-treated with or without U0126 were treated with etoposide (10 μM and 100 μM) in the absence or presence of U0126 (10 μM) for 30 min. Whole cell lysate were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results.

Task 2-Progress Report-"Identify specific mutations, and small molecule agents, that specifically disrupt interactions between γ-synuclein and ERK1/2 and between γ-synuclein and JNK, and evaluate them in cell culture models, with a goal of developing them as targeted therapeutics".

Many approaches have been used to screen for small molecules with desirable bioactive properties in treatment of disease. One recent focus of interest has been to exploit the burgeoning resources for genomics and proteomics, to develop drugs or peptides that are capable of specifically disrupting the interaction between proteins whose association is necessary for disease-related signaling. The ultimate goal of such an approach is to devise agents with very narrowly defined activities that might be successful

in blocking a specific disease process -for instance, acquisition of metastatic properties by cancerous cells - without demonstrating broad toxicity. We hypothesize that the protein associations between γ-synuclein and ERK1/2 and potentially other molecules such as JNK is likely to be critical for cellular migration in metastasis and cell survival following chemotherapy (as described above), but may be dispensable for normal cellular viability in human adults. Thus, identification of molecules that can block such interactions is of significant interest as a source for novel therapeutics. Using the scheme shown in figure 13, we have already screened >1 x10^7 peptide aptamer clones. A total of 324 γ-synuclein bait strain/peptide aptamer cDNA library derived yeast clones were identified through the first screens. Of these 324 potential γ-synuclein interacting peptides, 53 (16%) demonstrated reproducible interactions with γ-synuclein as assayed in **Figure 13**. A second round of yeast two-hybrid based screening yielded similar results. Surprisingly, most γ-synuclein interacting peptides had early translational stop codons in close proximity to the thioredoxin coding sequence, which raised the question as to whether or not thioredoxin itself may be a potential γ-synuclein interactor (**Figure 14**). Thioredoxin is a component of the peptide aptamer/transcriptional activation domain fusion protein, which consists of a nuclear localization sequence, a transcriptional activation domain, a haemagglutinin epitope tag, and an *E. coli* derived thioredoxin A scaffold, which holds the aptamer. If these early stop codon aptamers still interact with γ-synuclein, it may be due to an association between γ-synuclein and regions of thioredoxin. This question is currently being investigated.

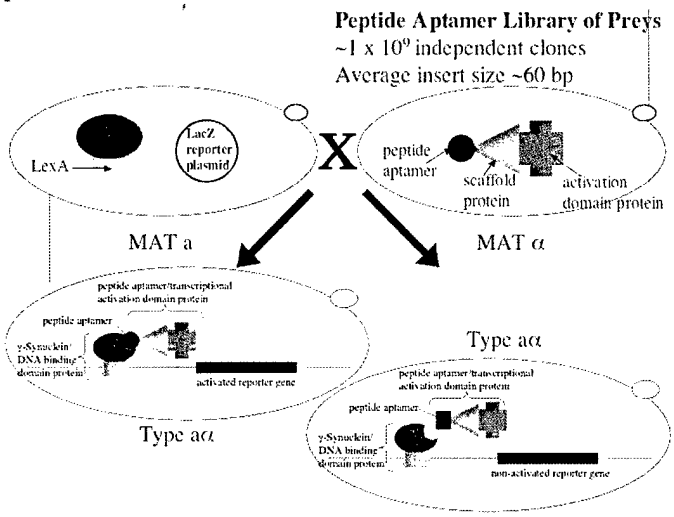


Figure 13. Schematic representation of the peptide screening approach. Yeast containing the synuclein bait are mated with yeast possessing one of the ~1x10^6 unique peptide prey expression constructs. Peptides that are able to interact with synuclein are identified and the sequence determined.

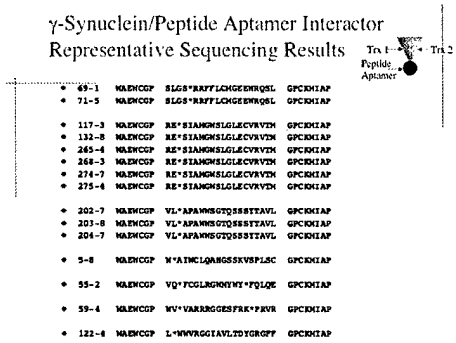


Figure 14. Examples of the sequence of the peptide aptamers found to interact with γ-synuclein through yeast two-hybrid screening approaches. Note that redundant clones were independently isolated during these screens, suggesting that they are likely to be genuine synuclein interactors. *=stop codon.

C- KEY RESEARCH ACCOMPLISHMENTS:

***C.1.* "Developing Inhibitors of Ovarian Cancer Progression by Targeted Disruption of the γ-Synuclein Activated Migratory and Survival Signaling Pathways ".**

1.a. Overexpression of γ-synuclein leads to constitutive activation of ERK and Rho/Rac/Cdc42 and down-regulation of JNK activation in response to stress signals or chemotherapy drugs.

1.b. Overexpression of γ-synuclein induces stress fiber formation and enhances cell migration. Both the basal level and the enhanced cell migration require the activities of both the ERK and Rho/Rac/Cdc42 kinases.

1.c. Overexpression of γ-synuclein may render cancer cells resistant to Taxol and vinblastine by modulating ERK cell survival pathway and JNK-mitochondria-Caspase 9/3 apoptotic pathway.

1.d. Identified 53 peptide aptamer clones coding for proteins that demonstrated reproducible interactions with γ-synuclein as assayed.

1.e. Determine that the vast majority of peptide aptamers contain a premature stop codon, suggesting that the N-terminal protion of thioredoxin may interact with γ-synuclein.

D-REPORTABLE OUTCOMES (4/2003 to present):

D.1. "Gamma synuclein promotes a metastatic phenotype in breast and ovarian tumor cells by modulating the Rho signal transduction pathway".

1.a. Abstracts

Zhong-Zong Pan and **A.K. Godwin**. γ-Synuclein May Render Cancer Cell Resistance to Paclitaxel by Activating AKT. Proceedings of American Association of Cancer Research, 44:2031, 2003.

Pan, Z.-Z., Vanderhyden, B. and **Godwin, A.K**. γ-Synuclein transgenic mouse model in cancer initiation and progression. Proceedings of American Association of Cancer Research, 45:5108, 2004.

1.b. Publications

Gupta, A., **Godwin, A.K.**, Vanderveer, L., Lu, A.P., and Liu, J. Hypomethylation of the synuclein γ gene CpG island promotes its aberrant expression in breast carcinoma and ovarian carcinoma. *Cancer Res.*, 63:664-673, 2003.

Pan, Z-Z., Bruening, W., Giasson, B. **Godwin, A.K**., Requirement of RHO and ERK Activation in γ-Synuclein Enhanced Breast and Ovarian Cancer Cell Migration and Invasion, *Oncogene*, under review, 2004.

Book chapters and review articles:

Ozols, R.F., Bookman, M., Connolly, D., Daly, M.B., **Godwin, A.K**. Schilder, R., Xu, X., Hamilton, T.C. Focus on epithelial ovarian cancer. *Cancer Cell*, 5:19-24, 2004.

Pan, Z-Z., and **Godwin, A.K**. Oncogenes, Cancer, and Targeted Therapy. Life and Analytical Science, accepted, 2004.

E-CONCLUSIONS:

E.1. "Developing Inhibitors of Ovarian Cancer Progression by Targeted Disruption of the γ-Synuclein Activated Migratory and Survival Signaling Pathways "

In these studies, we found that γ-synuclein can interact with two major MAPKs, i.e., ERK and JNK1. Over-expression of γ-synuclein may lead to enhanced activity of ERK and down-regulation of JNK activation in response to stress and chemotherapy drugs. Rho/Rac/Cdc42 pathway is also activated in cells over-expressing γ-synuclein. Activation of both the Rho/Rac/Cdc42 and ERK pathways are required for the enhanced cell migration in γ-synuclein over-expressing cells. Over-expression of γ-synuclein may render cancer cells resistant to Taxol and vinblastine by modulating ERK cell survival pathway and JNK-mitochondria-Caspase 9/3 apoptotic pathway. Taken together, these data indicate that γ-synuclein may promote tumorigenesis by enhancing cell motility through modulating Rho/Rac/Cdc42 and ERK pathways, and promoting cell survival and inhibiting apoptosis through modulating ERK cell survival and JNK-mitochondria-caspase9/3 apoptotic pathways. Since γ-synuclein is aberrantly expressed in the majority of late-stage ovarian cancers but is not expressed in normal ovarian epithelial cells, γ-synuclein may represent a very promising therapy target for these diseases. In this aspect we have uncovered a number of peptide aptamer sequences that appear to interact with γ-synuclein. Studies are currently underway to express these various peptides in mammalian cells and examine changes in the tumor cells' phenotype as described in Task 1.

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APPENDICES:

None