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<b>13. ABSTRACT (Maximum 200 Words)</b> Extensive molecular, biochemical, cell biological, and cytogenetic studies of breast cancer cell lines and breast cancer biopsies have indicated that overexpression of cyclin D1 plays an important role in breast tumor oncogenesis. Particularly, D-type cyclins are fundamental to cell cycle regulation of the tumor suppressor protein, Rb. However, cyclin D1 has been reported being overexpressed to the same degree in both the early and late stages of breast cancer. Therefore, cyclin D1 overexpression alone may not be a good indicator of breast cancer. Preliminary data from our laboratory suggest that the cdk inhibitor p21 <sup>cip1</sup> can be upregulated by cyclin D1 overexpression in NIH3T3. Also, we are examining the role of PI3K pathway in cell cycle progression given its relevance in cyclin D1 expression and its correlation with the tumor suppressor gene, PTEN. We show in MEFs that LY294002 (LY), a PI3K inhibitor, is able to reduce cyclin D1 expression level and to block entry in S phase with little effect on the level of the cdk inhibitors, p21 and p27. However, overexpression of cyclin D1 is not sufficient to restore cyclin A expression in cells treated with LY. Lastly, we found that cdk2 kinase activity was rescued in p27-null MEFs treated with LY. However, cyclin A induction was still completely abolished suggesting that another mechanism controlled by PI3K, together with p27 downregulation, needs to be active in order to allow the transition from G1 to S phase.			
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## INTRODUCTION

Tumorigenesis is a multi-step process that leads to abnormal cell growth and proliferation. Transition from the G<sub>1</sub> phase of cell cycle to S phase entry is strictly regulated by cyclins and cyclin-dependent kinases (CDK<sub>s</sub>) complexes. D-type cyclins associate with cdk4 and cdk6 in response to stimulation by growth factors and extracellular matrix (ECM). This event is followed by phosphorylation of the tumor suppressor protein Rb. More recently, additional reports in literature supported the additional role of cyclin D/cdk4-6 complexes to sequester the cdk inhibitors, p21 and p27, from cyclin E/cdk2 complexes facilitating its activation, thereby completing phosphorylation of Rb and E2F release with consequent induction of E2F-regulated genes such as Cyclin A, whose expression is essential for S phase entry (1).

Cyclin D protein levels have been implicated in tumorigenesis as its aberrant overexpression is frequently observed in human cancer (3). Extensive molecular, biochemical, cell biological, and cytogenetic studies of breast cancer cell lines and breast cancer biopsies have indicated that overexpression of cyclin D1 plays an important role in breast tumor oncogenesis (4,5). However, increases in cyclin D1 are not sufficient to confer a full malignant phenotype to mammary epithelial cells, suggesting that cyclin D1 overexpression cooperates with other genetic lesions to promote tumorigenesis (6).

Interestingly, there is compelling evidence supporting the relevance of PI3K pathway in the expression and stability of cyclin D1. In particular Akt, a downstream molecule in phosphatidylinositol 3-kinase (PI3K) signaling, has been shown to be involved in controlling cyclin D1 levels through phosphorylation and inactivation of glycogen synthase kinase-3 $\beta$  (GSK), a serine-threonine kinase responsible of cyclin D1 proteosomal degradation and subcellular localization (7, 8). Further, a PI3K/Akt-dependent pathway is responsible for an increase in the translation of cyclin D mRNA in response to mitogenic stimuli (9).

In addition, the lipid phosphatase PTEN has been shown to negatively regulate PI3K signaling and consequently cell cycle progression (10, 11, 12). PTEN has been reported to be mutated in a high percentage of solid tumors, including breast cancer (13, 14, 15). Further support for a role of PTEN in breast cancer comes from genetic studies that report germ-lines mutations of PTEN in the cancer syndrome known as Cowden disease that is associated with breast cancer in ~50% of the cases (16). Moreover, PTEN overexpression has been shown to cause suppression of cell growth in breast cancer cell lines, such as MCF7, and to induce apoptosis and anoikis in breast cancer cells (17, 18). Similarly, treatment of tumor cell lines with PI3K inhibitors mimicked the effect of PTEN causing cell cycle arrest (19).

The linkage of the PI3K and PTEN pathways have been supported by several recent observations. First, PTEN phosphatase activity on PI3K-generated phospholipids compromises the function of the PI3K effector kinase Akt which has potent antiapoptotic activity (20). Fibroblasts and tumors derived from PTEN-null mice have constitutively activated Akt and are resistant to proapoptotic factors further supporting this linkage (21, 22). Lastly, reconstitution of

PTEN expression in these cells restores Akt regulation. These findings support the coupling of PTEN and PI3K and suggest that their deregulation participates in malignant transformation.

These results prompted us to investigate the role of PI3K signaling in cell cycle progression given its relevance in cyclin D1 expression and its correlation with the tumor suppressor gene, PTEN. We were specifically interested in identifying the cell cycle molecules whose expression is regulated by PI3K and that are essential for cell cycle progression. We show in MEFs that LY294002 (LY), a PI3K inhibitor, is able to reduce cyclin D1 expression levels and to block entry in S phase with little effect on the level of the cdk inhibitors, p21 and p27. However, overexpression of cyclin D1 is not sufficient to rescue cyclin A expression in cells treated with LY294002 (LY). Moreover, we found that PI3K pathway is required for the activation of cyclinE/cdk2 in a p27-dependent manner consistent with data reported in literature. However, in LY-treated MEFs p27-null rescue of cyclinE/cdk2 activity is not sufficient to rescue cyclin A induction. Therefore restoration of cdk2 kinase activity alone is not sufficient to restore S phase entry in G1 arrested cells, suggesting that another PI3K-dependent pathway is required for cell cycle progression that is independent from the level of the cdk inhibitor p27.

## BODY OF WORK

### Experimental procedures

**Reagents.** Wild type and p27-null mouse embryo fibroblasts (MEFs) overexpressing cyclin D1 under a Tetracycline(Tet)-regulated promoter were generated in our laboratory. p27-null MEFs were the gift of Jim Roberts. Rabbit polyclonal antibody against cyclin A was prepared in this laboratory and the antiserum to p21 was a gift from Claudio Schneider. All other antibodies were purchased: anti-cdk4 (sc-260), and anti-cyclin D1 (sc-8396) from Santa Cruz Biotechnology, anti-cdk2 (06-505) from UBI , anti-pRb (28-0007) from Zymed, anti-p27 (K25020), and anti-ERK from Transduction Laboratories.

**Cell Culture and Transfection.** Wild type or p27-null MEFs were stably transfected with the mouse cyclin D1 cDNA, subcloned into a Tet-operator vector, together with a plasmid containing the transactivator molecule to the Tet-promoter. Stable transfectants were selected in the presence of geneticin and hygromycin. Tet was added to growth media at 2 µg/ml to avoid ectopic expression of cyclin D1 during culturing. MEFs and all derivative cell lines were grown in DMEM + 10% FCS. For G0-synchronization, cells were brought to confluence in 150-mm dishes, washed once with serum-free DMEM, and then cultured in 20 ml serum-free DMEM + 1 mg/ml BSA for 48 hours. To stimulate entry into the cell cycle, G0-synchronized cells were trypsinized, and reseeded in tissue culture dishes in the presence of 10% FCS or purified growth factors (2nM EGF, 10 ng/ml bFGF , 1µM insulin, and 10 ng/ml PDGF BB). In the case of MEFs overexpressing cyclin D1, cells were starved and then stimulated in the presence or absence of Tetracycline. For all experiments, cells were washed 2-3 times with PBS, collected by scraping and extracted for immunoblotting, or immunoprecipitation and kinase assay.

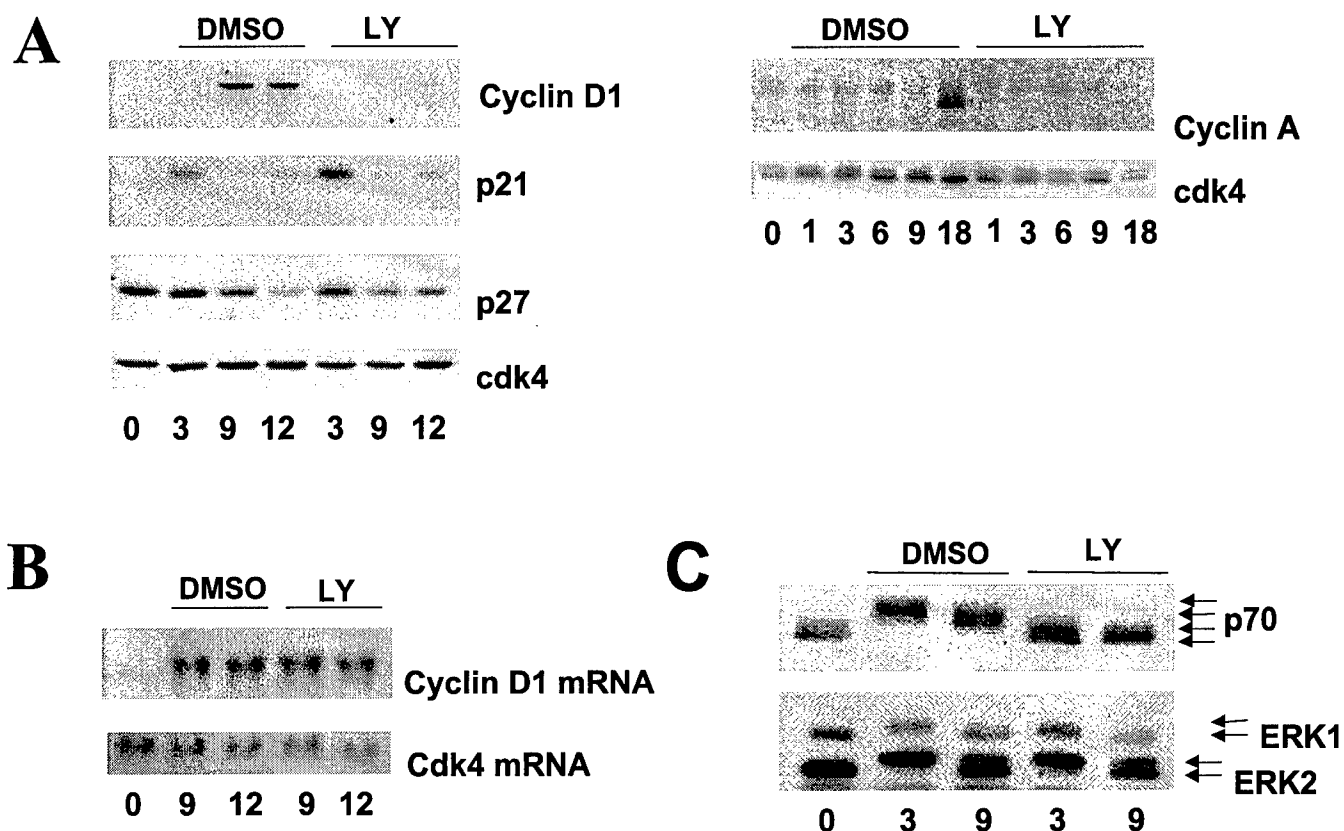
**Extractions for immunoblotting, immunoprecipitation, and kinase assay.** Cells were collected and lysed in 50 µl TNE (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 2 mM EDTA, 1% NP-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, 50 mM sodium fluoride and 10 mM sodium orthovanadate) and analyzed by immunoblotting using enhanced chemiluminescence. Protein concentrations were determined by Coomassie binding (Bio-Rad Protein Assay). Equal amounts of protein from each cell lysate were fractionated on reducing SDS-gels containing 12% acrylamide and then analyzed by immunoblotting. Alternatively, 7.5% acrylamide gels were used for analysis of ERK, p70 and Rb shifts. For cdk2 and cdk4 immunoprecipitations, cell lysates (500 µg) were brought to 200 µl with TNE and incubated (1 h at 4°C with rocking) with ~5 µg of antibody. 25 µl of the immunocomplexes were then processed for kinase assay while the rest were used for western blot analysis. The volume of all samples were brought to 200 µl with TNE and the immunocomplexes were collected (1 h, 4°C with rocking) with 50 µl anti-mouse IgG-agarose (Sigma). Collected immunoprecipitates were washed twice with cold TNE and suspended in 100 µl SDS-sample buffer, fractionated on reducing SDS-gels containing 12% acrylamide and analyzed by immunoblotting with cdk2,



cdk4 and p27 antisera. For kinase assay, samples were further washed and resuspended in 30  $\mu$ l of the kinase buffer (50 mM Tris-HCl pH 7.5 and 10mM MgCl<sub>2</sub> ). The kinase reaction was performed at 30<sup>0</sup> C for 30' using histone H1 as a substrate (1  $\mu$ g), <sup>32</sup>P- $\gamma$ ATP (10  $\mu$ Ci) and cold ATP (25  $\mu$ M). SDS-sample buffer was added to stop the reaction and samples were boiled and resolved into a 12% acrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membrane and then exposed to X-ray film.

## Results

We concluded our previous report showing that treatment of MEFs with the PI3K inhibitor LY294002 (LY) leads to downregulation of cyclin D1 protein level with a minor effects on the cdk inhibitors p21 and p27. However block of PI3K signaling completely suppressed the induction of cyclin A (Fig. 1 panel A). LY did not block the induction of cyclin D1 mRNA indicating that the downregulation of cyclin D1 was mainly due to posttranscriptional regulation by the PI3K pathway (Fig.1 panel B).

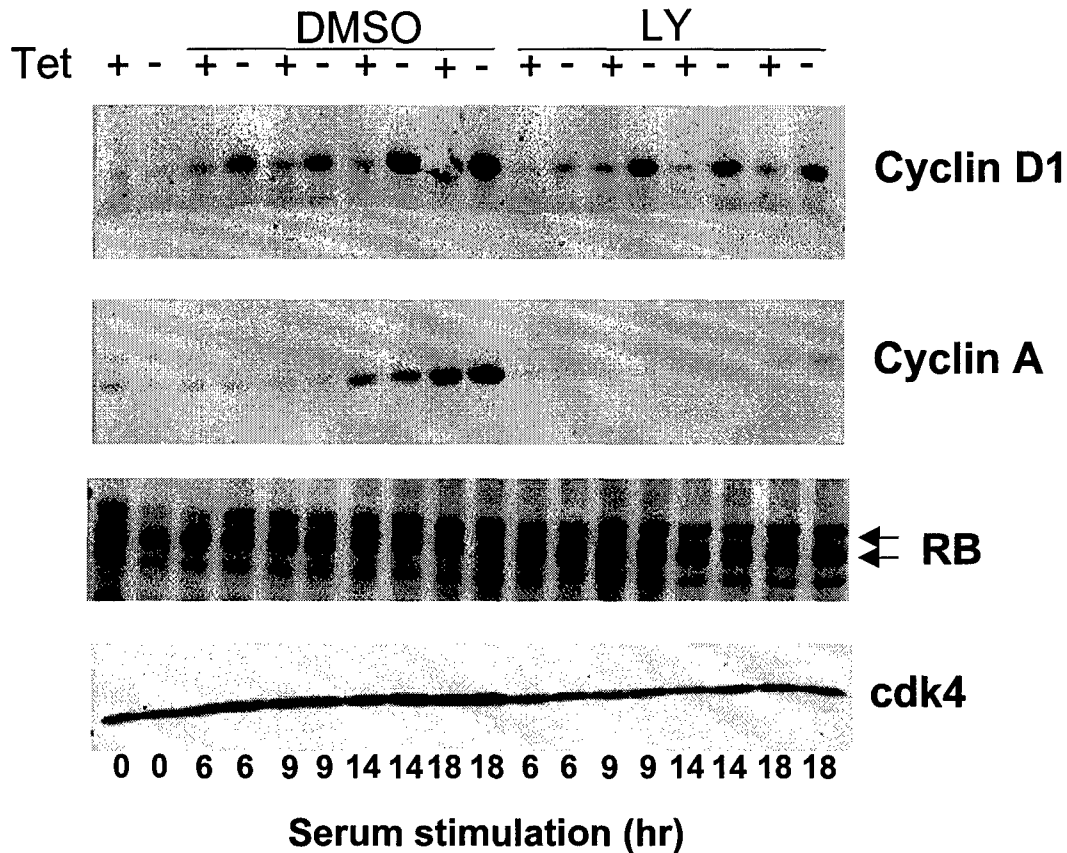


**Figure 1. Effects of the PI3K inhibitor (LY) in growth factors-stimulated MEFs.**

*G<sub>0</sub>*-synchronized MEFs were stimulated with purified growth factors for the indicated times (hr) with or without LY294002 (25  $\mu$ M). In A, cell extracts were analyzed by with antibodies for cyclin D1, p21, p27 (left) and cyclin A (right). Cdk4 was used as a loading control. In B, the effect of LY on cyclin D1 transcript level were analyzed by Northern Blot. C shows the specificity of the LY treatment on p70, downstream effector molecule in PI3K signaling.

To test if the block of S phase entry was due to the downregulation of cyclin D1, we used the cyclin D1 Tet-regulated MEFs described in our previous report. Briefly, induction of cyclin D1 is obtained by removal of Tet from growing media. In our experiment, cells were

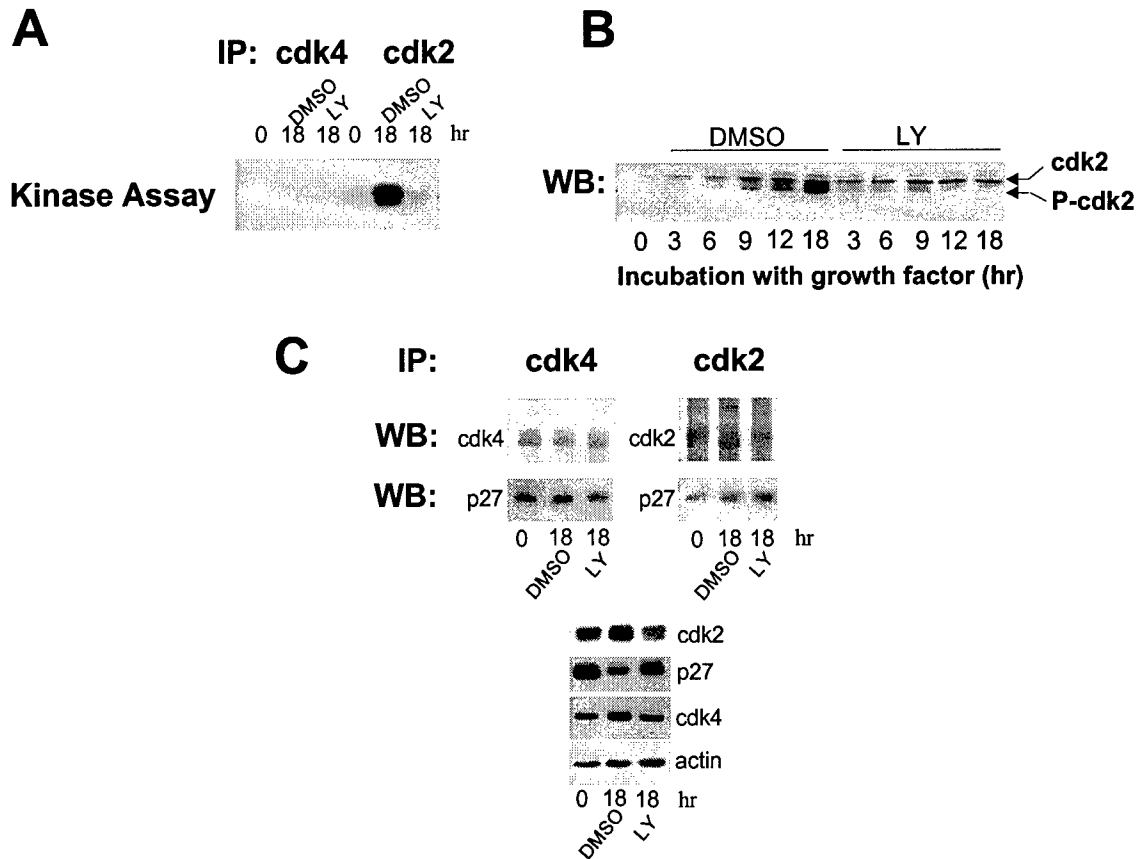
starved for 48 hrs with or without Tet and then serum-stimulated for 18 hrs in the presence or absence of LY. Western blot analysis of cell lysates showed that forced expression of cyclin D1 in the presence of LY is not sufficient to rescue cyclin A induction (fig. 2). Moreover Rb phosphorylation was partially impaired by LY despite the expression of cyclin D1.



**Figure 2. Forced expression of cyclin D1 does not rescue induction of cyclin A.** Cyclin D1 tet-regulated MEFs were starved for 48 hr in the presence or absence of Tet. Cells were trypsinized, seeded in monolayer in the presence or absence of LY and stimulated with 10% FCS with or without Tet. Samples were collected at the indicated times and analyzed by Western Blot for expression of cyclin D1, cyclin A and activation of RB. CDK4 was used as loading control.

Our next question was to determine if PI3K signaling was involved in the activation of cyclinE/cdk2 complex, the G1 phase cyclin/cdk required for Rb phosphorylation and cyclin A induction. We first asked if there was any differences in the activation state of cdk2 in serum-stimulated MEFs in the presence or absence of LY by mobility shift and kinase assay. As shown in Fig.3 panel A we were able to detect kinase activity only in the control cells (DMSO). This result was consistent with western blot analysis revealing the presence of the activated form of

cdk2 only in control cells while in the LY-treated MEFs cdk2 were predominantly in the inactive form as shown by gel shift in Fig. 3 panel B.



**Figure 3. Inhibition of cdk2 phosphorylation and kinase activity by LY.**

In A (upper), immunoprecipitates of serum-stimulated MEFs (-/+LY) were analyzed by Western Blot for the levels of cdk4, cdk2 and p27. (Lower) the whole-cell extracts were immunoblotted for cdk2 activation and total p27. In B, the same immunocomplexes were tested for kinase activity. In C, the activation state of cdk2 was determined by Western Blot analysis on cell lysates. Note that the CAK phosphorylated (P-cdk2) form of cdk2 has a greater mobility.

It has been reported in literature that block of PI3K signaling by specific inhibitors such as LY and Wortmannin lead to upregulation of the cdk inhibitor p27 and cell cycle arrest. Therefore we wanted to determine if the inhibitory effect of LY on cdk2 activity was dependent on the level of associated p27. We were able to detect a moderate increase of p27 in the cdk2 immunocomplexes from the LY-treated samples as shown in Fig. 3 panel C. However, it was

technically difficult to quantify the exact amounts of p27 in the immunoprecipitates. Therefore, we decided to examine the activation state of cdk2 using p27-null MEFs to better. In this experiment, we starved the cells for 48 hours and then we reseeded and stimulated with 10% FCS for 18 hours. We were able to partially rescue cdk2 kinase activity suggesting that the suppression of cdk2 activity we observed in MEFs was dependent by the levels of p27. However, we were still unable to detect cyclin A induction after 18 hrs serum-stimulation suggesting that this event, although is PI3K-dependent, might require a different mechanism that is independent from p27 (data not shown).

## Conclusion

In this study we have investigated the role of PI3K signaling in cell cycle progression given the relevance of this pathway in tumor progression and its link with the tumor suppressor molecule PTEN that has been shown to be mutated in a high percentage of solid tumors, including breast cancer. We have shown that in LY-treated MEFs cyclin D1 is downregulated at the protein levels while there is not a significant effect neither on level of cyclin D1 mRNA nor on the expression of the cdk inhibitors p21 and p27. However, cyclin A induction, that is required for S phase entry is totally abolished by block of PI3K signaling. Next, we decided to use our cyclin D1 Tet-regulated MEFs to see if forced expression of cyclin D1 was sufficient to rescue entry into S phase. Western blot analysis demonstrated that in the LY-treated MEFs, although we forced cyclin D1 expression by removal of Tet from the media, cyclin A induction was still impaired and Rb was not fully phosphorylated. Our next question was to see if lack of cyclin A induction was due to the activation state of cdk2. Therefore we examined the kinase activity and the phosphorylation state of cdk2 in MEFs. We observed that indeed LY-treatment completely block the activation of cdk2, presumably by CAK (cdk activating kinase), and therefore its kinase activity. Next we want to investigate the role of p27 on cdk2 activation as it has been recently shown in literature that cell cycle arrest by LY is mediated by an increase in p27 protein levels. We found an increase in the amounts of p27 associated with cdk2 immunocomplexes in LY-treated MEFs. However, it was technically difficult to quantify the absolute levels of p27 and therefore to better answer this question we used p27-null MEFs. In this system we were able to partially rescue cdk2 phosphorylation and kinase activity, however cyclin A induction was still completely abolish, suggesting that upregulation of p27 alone might not be responsible for G1 arrest. These data suggested to us that another mechanism controlled by PI3K, together with p27 downregulation, needs to be active in order to allow the transition from G1 to S phase. Our future plans is to establish which PI3K-activated pathways are involved by using specific construct of downstream effector molecules such as Akt . Moreover, we want to find out if the G1 arrest caused by LY treatment is Rb-mediated using cyclin A-luciferase reporter construct. Lastly, as our lab showed that binding of CREB to the cyclin A promoter is required for transcription, we want to investigate the role of PI3K on the phosphorylation of CREB-family of transcription factors.

The goal of our study is to identify molecules required for cell cycle progression from G1 to S phase as deregulation of their cellular functions might contribute to malignant transformation and therefore they might represent potential targets for cancer treatment.

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## APPENDIX

- Effects of the PI3K inhibitor LY294002 (LY) on mRNA and protein levels of cyclin D1 and on the protein levels of cyclin A and cdk inhibitors in MEFs.
- Effect of LY on cyclin A induction in Tet-regulated cyclin D1 MEFs.
- Analysis of the activation state of cdk2/cyclin immunocomplexes in LY-treated MEFs by kinase assay and gel shift.
- Role of p27 on cdk2 kinase activity in wild type and p27-null MEFs.

Reportable outcomes:

a) manuscripts: none

b) abstracts/presentations: Castagnino, P., Oluwatosin, Y.E., and Assoian, R.K.  
“Overexpression of Cyclin D1 upregulates the cdk inhibitor p21<sup>cip1</sup>”  
Presented at the Era of Hope Meeting, 2000

b) patents and licenses applied for: none

d) degrees obtained: none

e) development of cell lines: none

f) funding applied for: none

g) employment opportunities applied for: Dr. Oluwatosin received an offer of employment from Astra Arcus USA, Inc and accepted that offer.

## Abstract

“Overexpression of Cyclin D1 upregulates the cdk inhibitor p21<sup>cip1</sup>”  
Presented at the Era of Hope Meeting, 2000

Extensive molecular, biochemical, cell biological, and cytogenetic studies of breast cancer cell lines and breast cancer biopsies have indicated that overexpression of cyclin D1 plays an important role in breast tumor oncogenesis. The connections between cyclin D and tumorigenesis are strengthened by compelling evidence that D-type cyclins are fundamental to cell cycle regulation of the tumor suppressor protein, Rb. However, overexpression of cyclin D1 fails to induce anchorage-independent cell proliferation and also fails to reproducibly stimulate growth in epithelial cell lines. Furthermore, analysis of breast cancer biopsies indicate that cyclin D1 is overexpressed to the same degree in both the early and late stages of breast cancer. Therefore, cyclin D1 overexpression alone may not be a good indicator of breast cancer. Preliminary data from our laboratory indicates that cyclin D1 overexpression results in upregulation of the cyclin-dependent kinase inhibitor p21. We propose that the compensatory increase in p21 expression negates the expected effects of cyclin D1 overexpression on cell proliferation and anchorage-independent growth and the induction of a full malignant phenotype requires both the overexpression of cyclin D1 and the loss of the cell's ability to upregulate the expression of cdk inhibitors in response to a cyclin D1 challenge.



DEPARTMENT OF THE ARMY  
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND  
504 SCOTT STREET  
FORT DETRICK, MD 21702-5012

REPLY TO  
ATTENTION OF

MCMR-RMI-S (70-1y)

15 May 03


MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.
2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

  
PHYLIS M. RINEHART  
Deputy Chief of Staff for  
Information Management

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