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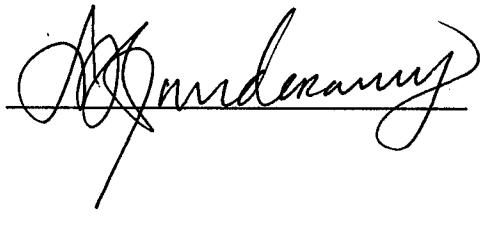

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**13. ABSTRACT** *(Maximum 200 Words)*
Extensive molecular, biochemical, cell biological, and cytogenetic studies of breast cancer cell lines and breast cancer biopsies have indicated that overexpression of cyclin D1 plays an important role in breast 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 in NIH3T3. We also show in MEFs that LY294002, a PI3K inhibitor, is able to reduce cyclin D1 expression level and to block entry in S phase without affecting the level of the cdk inhibitors, p21 and p27. Therefore we want to study the mechanism underlying these events using a Tet-regulated cell model by modulating the expression of cyclin D1.

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Paula Costopoulos

PI - Signature Date

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## Introduction

Overexpression of cyclin D1 is a frequent event in breast tumorigenesis. However, increases in cyclin D1 are not sufficient to confer full malignant phenotype to mammary epithelial cells, suggesting that cyclin D1 overexpression cooperates with other genetic lesions to promote tumorigenesis. We have generates a cyclin D1 inducible system to examine effects of controlled cyclin D1 expression on p21 protein expression in monolayer and suspension culture. Ectopic elevation of cyclin D1 protein in NIH-3T3 fibroblasts results in an increase in p21 protein levels. Therefore the aim of this proposal is to investigate the mechanism by which changes in the level of expression of cyclin D1 can account for a deregulation in cell cycle progression. We also show that LY294002, a PI3K inhibitor, partially inhibits cyclin D1 expression and blocks S phase entry. We will use our cyclin D1-inducible cell model to determine if the inhibition of S phase entry is solely because of the reduction of cyclin D1 expression.

Body of work

The induction of cyclin D1 and the phosphorylation of pRb require both growth factor and cell adhesion to the extracellular matrix (ECM). To manipulate the expression of cyclin D1 in both monolayer and suspension, we generated NIH-3T3 cells overexpressing cyclin D1 under a tetracycline-regulated promoter. In this mammalian expression system the presence of tetracycline in regular growth media prevents the binding of the transactivator molecule to the Tet-promoter, while removal of tetracycline allows transcription of the gene of interest under the Tet-promoter. Two independent plasmids, one expressing the transactivator molecule and the other cyclin D1, are required to form the Tetracycline-Off mammalian expression system. The cyclin D1 construct used in the transfection of NIH-3T3 cell lines was engineered as follows: a 1 kb Eco RV/Sma I fragment, containing the mouse cyclin D1 cDNA, was subcloned into the Xba I site of the Tet-operator vector in the sense orientation and named Tet-D1. This construct contains the full-length cyclin D1 cDNA and the 5' UTR but lacks most of the 3' UTR. NIH-3T3 cells were stably transfected using Lipofectamine (GIBCO) with prcTA and Tet-D1 plasmids carrying resistance to geneticin and hygromicin, respectively. Stable transfectants were selected in the presence of hygromicin (0.3mg/ml) and geneticin (G418, 0.5mg/ml). Tetracycline (Tet) was added to these cultures at a final concentration of 2 μg/ml to avoid the ectopic expression of cyclin D1 cDNA during the drug selection.

Twelve independent resistant clones were isolated and maintained in 5% CS-DMEM in the presence of hygromicin, geneticin and tetracycline. The selected clones were then screened for the level of expression of cyclin D1 and $p21^{cip1}$ by Western blot analysis. Briefly, cells were grown to confluence in monolayer in the presence of tetracyline and subsequently serum-starved for 36-48 hours in the absence of tetracycline. Starved cells were trypsinized and reseeded in monolayer or suspension in the presence or absence of tetracycline for different times corresponding to transit through G1 phase. Cells were washed in PBS, scraped from the dishes and collected by centrifugation. Cell pellets were lysed and equal amounts of protein were analyzed on a 10% SDS-polyacrylamide gel. Western blot analysis showed that the induction of cyclin D1 led to increased expression of $p21^{cip1}$ and that this effect occurred whether or not cells were cultured in suspension (Sp) or monolayer (Mn) (Figure 1). However, the effect was most pronounced in suspended cells (Sp), presumably because p21 is downregulated by integrin signals in adherent cells (Bottazzi et al., 1999).

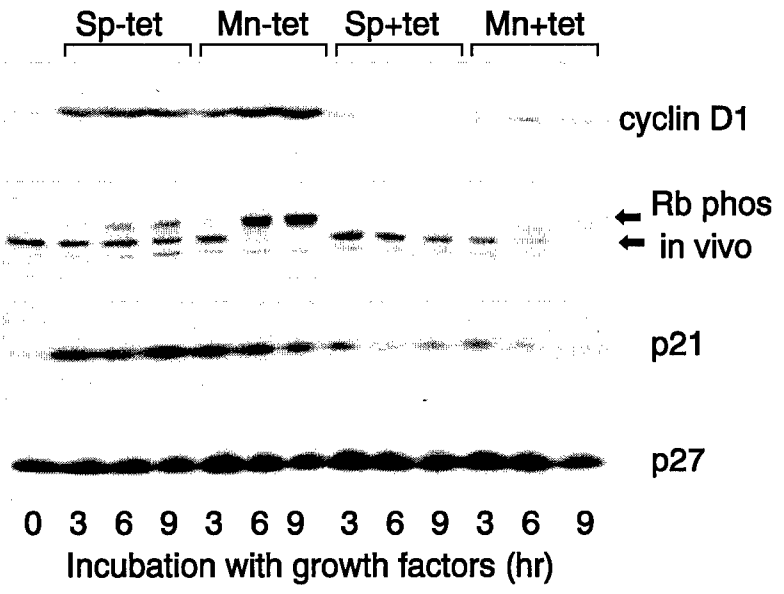


*Figure 1. Forced expression of cyclin D1 induces p21cip1. Quiescent cells were replated in monolayer (Mn) and suspension (Sp) and stimulated with 5% FCS in the presence and absence of tetracycline. Western blot analysis was performed with cell lysates prepared at the indicated times after serum stimulation.*

An analogous strategy was used to transfect mouse embryo fibroblasts (MEFs) with the same Tet-D1 plasmid described above. Stable transfectants were selected in 10% FCS-DMEM in the presence of hygromicin (0.25mg/ml), geneticin (G418 1mg/ml) and Tet at 2 μg/ml. Eighteen independent clones were isolated and maintained in the medium described above. Analysis of cyclin D1 expression by western blotting identified three clones that overexpressed cyclin D1 in a Tet-repressible manner. These were designated YDM14, YDM15 and YDM18.

Control MEFs and MEFs stably expressing tet-regulated cyclin D1 were then $G_0$ synchronized by a 48 hr serum-starvation. Cells were then trypsinized, resuspended in 5% FCS-DMEM and replated in monolayer (Mn), or in suspension (Sp) in the presence or absence of tetracycline for 18 hours. Cells were collected, lysed and the amount of cyclin D1 and p21 were determined by Western Blot analysis (Figure 2). The results indicated that the three clones differed in the basal level of cyclin D1 expression observed when the cells were cultured in the presence of tetracycline. Additionally, clones M14 and M18 do not show the usual adhesion requirement for the expression of endogenous cyclin D1 while clone M15 does. Importantly, all three clones showed strong induction of cyclin D1 upon removal of tetracycline. p21 expression, as expected from data previously generated in our laboratory (Zhu et al., 1996, Bottazzi et al., 1999) was upregulated by culturing the cells in the absence of a substratum in all three clones. However, p21 expression was not altered by the induction of cyclin D1, and the lack of a cyclin D1 effect was seen in both adherent and non-adherent cells from all three clones (Figure 2). Thus, analysis in MEFs did not show the same cyclin D-p21 effect observed in NIH-3T3 cells.

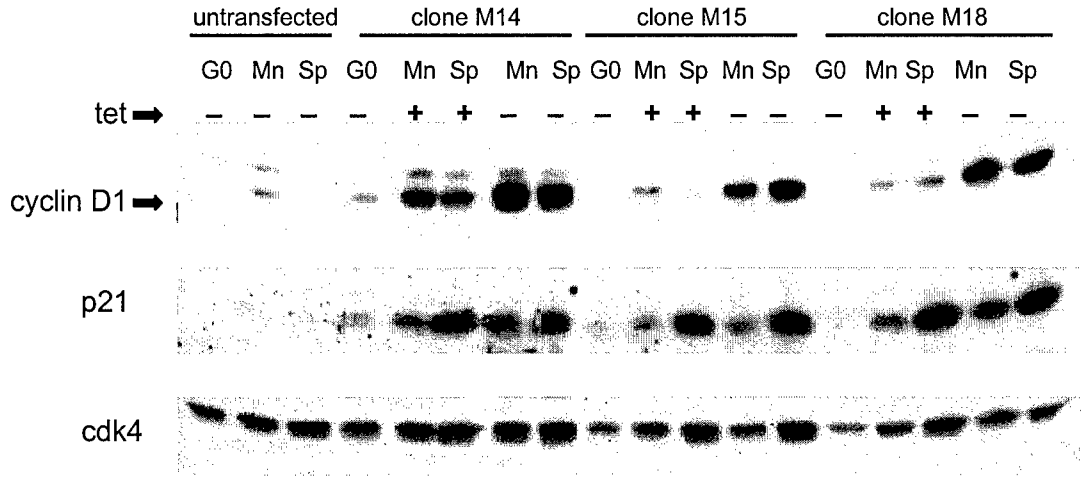


*Figure 2. Overexpression of cyclin D1 in MEFs does not alter the expression of p21cip1. This experiment was performed similarly to the procedure outlined in Figure legend 1.*

Given these results, the proposed follow-up experiment involving tet-regulated expression of cyclin D1 in MEFs from p21- and p27-null mice are no longer justified (aim 1 in the original application). We are considering the possibility that p21 is not being induced because the MEFs are acquiring p53 mutations as a consequence of the time they have been kept in culture. We plan to use the NIH-3T3 cell system to determine if the induction of p21 is due to changes in abundance of its mRNA. This result would support the idea that p53 mutations might be preventing us from seeing the cyclin D1 effect in long-term MEF culture.

Recent reports show that, besides being a potent inhibitor of cyclin E-cdk2, p21 acts as a positive regulator of cell cycle progression by promoting the assembly of cyclin D and cdk4 (LaBaer et al.,1997). Cyclin D-cdk4 also seems to be responsible for the sequestration of p21 and p27, and these effects lead to a complete activation of cyclinE-cdk2 and completion of G1 phase (Sherr and Roberts, 1995). On the basis of these new observations, our initial hypothesis for a strictly inhibitory role of p21 (in response to cyclin D upregulation) is no longer appropriate. Although we still intend to study cyclin D1, we will be placing increased emphasis on understanding how its expression is regulated. In particular, we have generated new data indicating that cyclin D1 protein expression is inhibited in growth factor-stimulated MEFs treated with LY294002, a phosphoinositide-3 kinase (PI-3K) specific inhibitor (Figure 3). Although we do not detect any effects of LY294002 on the expression of either cdk inhibitors, p21 or p27, the inhibition of PI-3 kinase completely blocked S phase entry as monitored by the induction of cyclin A (Figure 3). To determine if the inhibitory effect of PI-3 kinase on S phase entry results solely from its inhibitory effect on the expression of cyclin D1, we will use our Tet-Off cell models in which we can modulate the level of expression of cyclin D1 and ask if forced expression of cyclin D1 is sufficient to overcome the growth inhibitory effect of LY294002 in NIH3T3 cells and MEFs.

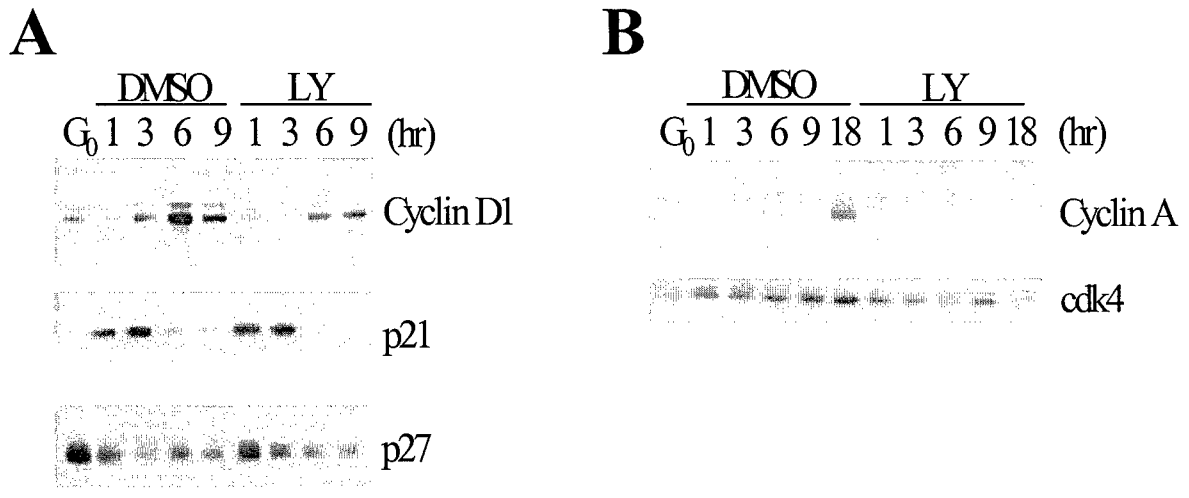


*Figure 3. Effects of the PI3K inhibitor (LY) in growth factor-stimulated MEFs. In A, $G_0$-synchronized MEFs were stimulated with purified growth factors for the indicated times with or without LY294002 (25μM). Cell extracts were analyzed by immunoblotting for the levels of cyclinD1, p21 and p27. In B, the expression of cyclin A in the same lysates was analyzed by immunoblotting, cdk4 was used as a loading control.*

Appendix

- Generation of stable transfected NIH3T3 with the full-length cDNA of cyclin D1 under a tetracycline suppressible system. Induction of cyclin D1 by removal of tetracycline from the media resulted in upregulation of p21.

- Transfection of mouse embryo fibroblasts (MEFs) with a tetracycline repressible cyclin D1 cDNA and characterization of different clones for the expression level of cyclin D1.

- Analysis of MEFs transfectants for p21 expression following induction of cyclin D1 in monolayer and suspension culture.

- Effects of LY294002, a PI3K inhibitor, in MEFs on the levels of expression of cdk inhibitors and cyclin D1 and cyclin A.

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 $p21^{cip1}$"
Presented at the Era of Hope Meeting, 2000

b) patents and licenses applied for: none

d) degrees obtained: none

e) development of cell lines: We produced two stable fibroblastic cell lines expressing tetracycline suppressible cyclin D1. The full-length cyclin D1 was stably expressed in NIH-3T3 cells and the coding domain of cyclin D1 was stably expressed in mouse embryo fibroblasts.

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 $p21^{cip1}$"
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
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