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14. ABSTRACT A summary is presented of research performed during the second year of a project to determine the role of Cdc25 phosphatases in human breast cancer. The research involves three specific aims. In the first aim, the role of Cdc25B in breast cancer proliferation will be determined. Examining whether alternative splicing of Cdc25C contributes to human breast cancer is the focus of the second aim. Finally, a potential novel breast cancer therapy involving altered expression of Cdc25C will be explored. The long term goals of this research are to validate a clear role for Cdc25B in breast tumor cell proliferation and to rigorously determine whether Cdc25C may contribute to human breast tumorigenesis in other ways besides its overexpression.					
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Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	8
References.....	9
Appendices.....	9

Introduction

Cell cycle progression is mediated by cyclin-dependent kinases. These kinases are, in turn, regulated by their own phosphorylation. The family of Cdc25 phosphatases is responsible for removing inhibitory phosphates from these kinases thereby triggering advancement through the cell cycle (Boutros et al., 2007). There are three members of this family. Two of these, Cdc25B and Cdc25C, have been implicated in entry of cells in to mitosis. Interestingly, only Cdc25B has been clearly shown to play a role in human cancer, although the precise mechanism remains unclear (Kristjansdottir and Rudolph, 2004; Rudolph, 2007). The focus of this research program is to validate the role of Cdc25B as playing an oncogenic role in human breast cancer and to explore whether Cdc25C may also be involved as well. Three specific aims are being actively pursued. In the first aim, the significance of overexpression of Cdc25B is being examined. In the second aim, oncogenic mechanisms involving Cdc25C are being studied. In the final aim, a novel therapeutic approach is being developed based upon preliminary studies which show that overexpression of Cdc25C sensitizes human tumor cells to chemotherapeutic agents which induce DNA damage. The studies reported here were performed during the second year of a three-year funding period.

Body

Task 1. Screen breast tumor cell lines for levels of Cdc25B and Cdc25C expression (Months 1-4)

This task was completed during the first year of funding.

Task 2. Abrogate Cdc25B expression in overexpressing breast tumor cell lines (Months 5-12)

During the previous funding year, methods to ablate expression of Cdc25B using an siRNA approach were established. The plan was to downregulate Cdc25B expression in tumor lines that have high levels to determine whether this affects cell proliferation. It was shown, however, that loss of Cdc25B was capable of inhibiting cell growth of cells with physiological levels of Cdc25B. This makes it difficult to assess the significance of Cdc25B overexpression using this approach. With this in mind, ongoing experiments are attempting to determine whether partial downregulation of Cdc25B may lead to a differential effect on normal versus tumor cells. This has been difficult, as it requires a method to carefully titrate the siRNA oligonucleotides. In the meantime, the effects of overexpression of Cdc25B were examined as a complement to the downregulation studies proposed in the original application.

Preliminary studies were performed to examine the effect of overexpression of wild-type Cdc25B in wild-type p53-expressing U2OS cells. Surprisingly, this caused premature entry into mitosis and cell cycle arrest. This was dependent upon the phosphatase activity of Cdc25B, as a catalytically dead mutant failed to exert these effects. p53 was induced and phosphorylated on ser15 but not on ser315. Increased phosphorylation of ATM as well as chk1 and chk2 was seen, implicating the ATM/ATR pathways in this response. The p53 target, the cyclin-dependent kinase inhibitor p21, was also upregulated in a manner requiring p53. Downregulation of either p53 or p21 using siRNA eliminated the cell cycle arrest. While 40% of cells showed detectable immunostaining for γ -H2AX, elimination of p53 caused 100% of the cells to stain positive. Consistent with these results, ectopic expression of Cdc25B inhibited long term growth in several different cell lines. As these effects are seen in cells that are null for p14^{ARF}, signaling via p14^{ARF} is not involved. These results show that premature entry into mitosis by overexpression of Cdc25B triggers DNA damage checkpoint responses, most likely through hyperactivation of cyclin-dependent kinases. It is proposed that inappropriate expression of Cdc25B transmits two distinct signals to p53. One stems from activation of DNA-damage pathways including ATM and ATR. The other is distinct from those pathways given that it may occur cells that are γ -H2AX-negative. These results further highlight the importance of p53 in modulating the cellular response to deregulated proliferation.

We then compared ectopic expression of Cdc25B with that of Cdc25C and found that Cdc25B but not Cdc25C inhibits cell proliferation in long-term assays. Chimeric proteins generated from the two phosphatases show that the anti-proliferative activity is associated with the C-terminal end of Cdc25B. Indeed, the catalytic domain of Cdc25B is sufficient to suppress cell viability, in a manner dependent upon its C-terminal 26 amino acids. This region of Cdc25B is shown to be critical for substrate recognition and affinity. These results demonstrate key differences in the biological activities of Cdc25B and Cdc25C due to differential substrate affinity and recognition. This also argues that the antiproliferative activity of Cdc25B needs to be overcome in order for it to act as an oncogene during tumorigenesis. Although these studies were not planned as part of the original statement of work and fall outside of the plans in the research application the findings are intriguing and have led to one accepted publication (Varmeh-Ziaie and Manfredi, 2007) and a second that is under

revision (Varmeh-Ziaie and Manfredi, in revision). The U2OS (osteosarcoma) and HCT116 (colon carcinoma) cell lines were used initially as they have been well characterized in terms of cell cycle responses and are technically amenable to the approaches used here. These results are now being validated in wild-type p53 expressing breast cells, both the non-transformed MCF-10A and the tumorigenic MCF-7. It should be noted that use of the breast lines has been technically challenging, but optimization of methods for adenoviral infection and siRNA transduction now appear to be in place.

Task 3. Compare ability of *cdc25B* and *cdc25C* to cooperate with activated *ras* in transformation of mammary epithelial cells (Months 13-26)

It was proposed to determine whether overexpression of Cdc25B or Cdc25C is capable of transforming primary cells. Although it was originally planned to use a mouse cell line C127I, a recent publication highlighted the utility of the non-transformed human breast line MCF-10A for such assays. Importantly, it was shown that a third member of the Cdc25 family, Cdc25A was required for transformation of MCF-10A cells by activated Ras and a dominant-negative p53 (Ray et al., 2007). With this in mind, conditions have been established to perform transformation assays using these human cells. The use of human cells for these studies will provide findings that are much more relevant to the goal of determining the role of Cdc25 family members in human breast tumorigenesis.

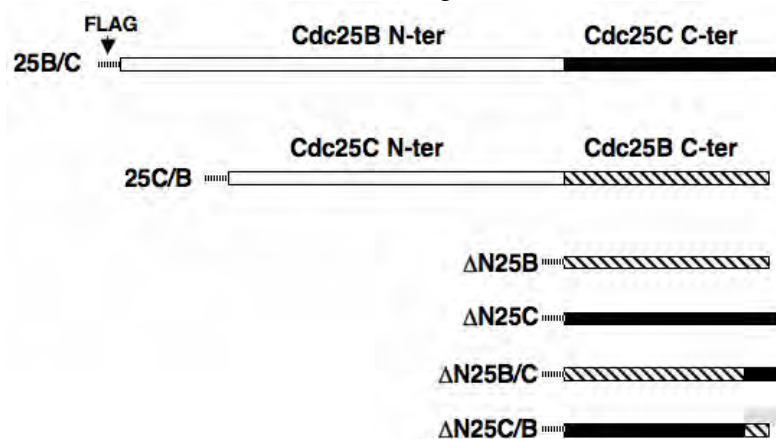


Figure 1. Expression constructs for FLAG-tagged chimeras of Cdc25B and Cdc25C and their isolated C-terminal regions were generated.

Sequences corresponding to the N- and C- terminal ends of Cdc25B and Cdc25C were PCR amplified. The N-terminal end of Cdc25B encompasses residues 1-390 and that of Cdc25C residues 1-280. The C-terminal end of Cdc25B (shaded) and Cdc25C (black) consists of residues 391-580 and residues 281-473 respectively. The constructs expressing 25B/C and 25C/B were generated by blunt-end ligation of the indicated parts of the proteins. ΔN25B and ΔN25C each expresses the C-terminal ends of Cdc25B and Cdc25C respectively. The constructs expressing ΔN25B/C and ΔN25C/B were generated by fusion PCR. ΔN25B/C expresses residues 391-554 of Cdc25B fused to residues 445-473 of Cdc25C. ΔN25C/B expresses residues 281-444 of Cdc25C fused to residues 555-580 of Cdc25B. All the constructs were FLAG tagged at their N-terminal ends. The position of the FLAG epitope is indicated.

In order to examine the role of the N-terminal ends of Cdc25B and Cdc25C in their activity, it was proposed to make hybrid proteins. To this end, chimeras in which the N-termini of Cdc25B and Cdc25C were exchanged were generated (25B/C and 25C/B) (Fig. 1). 25B/C consists of residues 1-390 of Cdc25B fused to residues 281-473 of Cdc25C (Fig. 1). The N-terminus of 25C/B encompasses residues 1-280 of Cdc25C and it was fused to residues 391-580 of Cdc25B (Fig. 1). The chimeras were generated by blunt-end cloning to avoid possible influence of irrelevant sequences. As controls, constructs expressing only the catalytic domains of Cdc25B (ΔN25B) and Cdc25C (ΔN25C) and their corresponding catalytically inactive mutants were generated (Fig. 1). ΔN25B contains residues 391-580 of Cdc25B and ΔN25C residues 281-473 of Cdc25C.

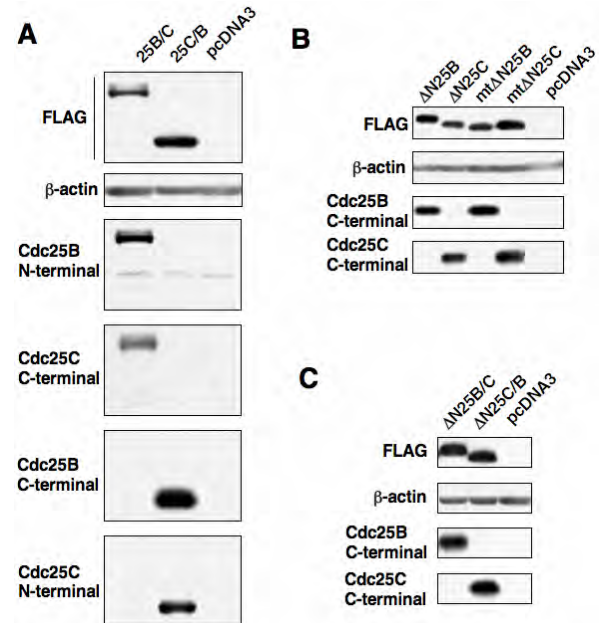


Figure 2. Various Cdc25B and Cdc25C proteins are expressed at comparable levels.

pcDNA3 vectors expressing the indicated FLAG-tagged proteins or empty vector were transfected into cells. Twenty-four hours later cells were lysed and expression levels of the proteins were analyzed by immunoblotting using anti-FLAG antibody or specific antibodies against the N- or C-terminus of Cdc25B or Cdc25C. β-actin is loading control. (A) 25B/C and 25C/B chimeras. (B) ΔN25B and ΔN25C and their corresponding catalytically inactive mutants (C) ΔN25B/C and ΔN25C/B.

All constructs were FLAG-tagged at the N-terminus. Each of these constructs or empty vector was cotransfected with a puromycin-resistant plasmid into HCT116 cells. Twenty-four hours post-transfection, cell samples were lysed and immunoblotted with FLAG antibody to verify similar expression levels and size of the proteins (Fig. 2). To further validate these constructs, the immunoblots were then stripped and subsequently probed with specific antibodies against the N-terminus or C-terminus of Cdc25B or Cdc25C (Fig. 2). These constructs will be used in the planned studies in MCF-10A cells to examine their transforming activity.

Task 4. Screen breast tumor cell lines for alternatively spliced forms of cdc25C (Months 1-6)

This task was completed during the first year of funding.

Task 5. Determine the role of individual splice forms of cdc25C (Months 10-30)

This task is to be performed in the next funding year.

Task 6. Confirm effect of cdc25C overexpression in human breast tumor cells (Months 16-36)

Downregulation of Cdc25C by p53 or its phosphorylation by Chk1 and subsequent cytoplasmic sequestration have been shown to contribute to the G2 arrest caused by genotoxic stress (St Clair et al., 2004). To confirm this effect in breast epithelial cells, MCF-10A cells were infected a recombinant adenovirus expressing Cdc25C (Ad-25C) 24 hrs prior to treatment with doxorubicin or empty vector with the same multiplicity of infection (Fig. 3). At this time point more than 95% of cells were infected as judged by microscopic evaluation of green-fluorescent protein (GFP) expression. Uninfected cells were included as control. The cell-cycle profile of these cells were examined 48 hrs after drug treatment by flow-cytometry analysis (Fig. 3). Parental MCF-10A cells or those infected with empty adenovirus were able to arrest with a 4N DNA content representing a G2 arrest (Fig. 3). MCF-10A cells overexpressing Cdc25C, however, were unable to maintain the G2 arrest and undergo apoptosis as evidenced by an increase in cells with a hypodiploid DNA content (Fig. 3).

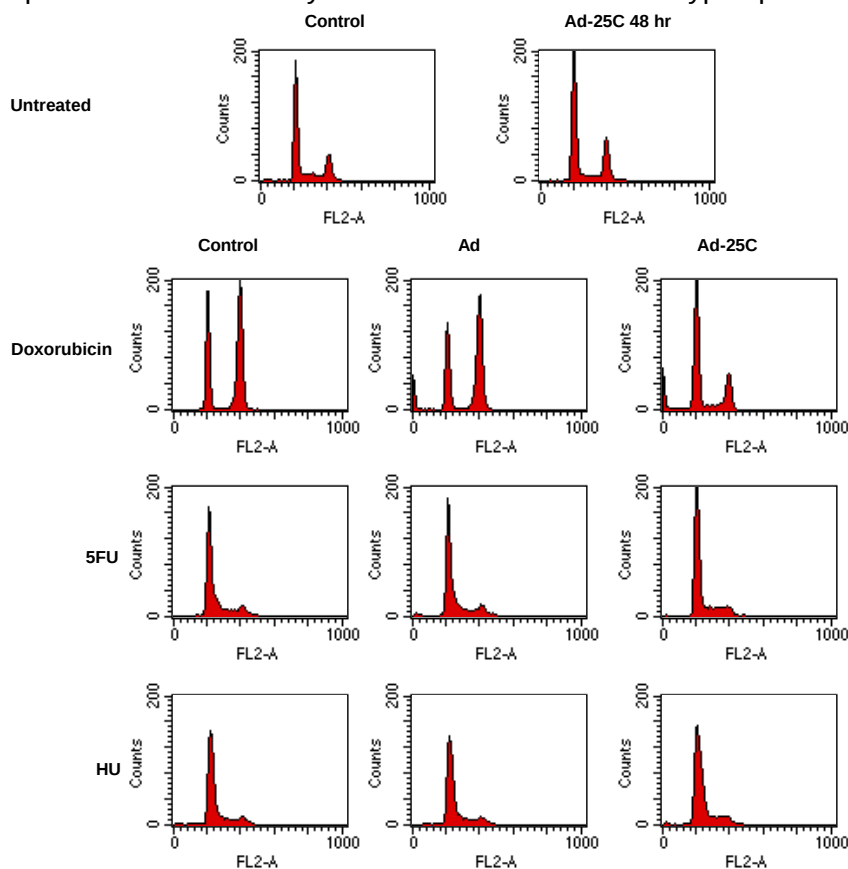


Figure 3. Adenovirus-mediated overexpression of Cdc25C abrogates the cell cycle arrest and sensitizes breast cells to doxorubicin, but does not affect the cellular response to 5-fluorouracil or hydroxyurea.

MCF-10A cells were infected with the indicated adenoviruses for 24 hr and then treated with doxorubicin, 5-fluorouracil, or hydroxyurea for 48 hr. Cells were stained with propidium iodide prior to flow-cytometry analyses.

In order to investigate the effect of excess amount of Cdc25C on cell-cycle arrest in G1/S or S-phase of the cell cycle, Cdc25C-overexpressing cells were treated with either of two chemotherapeutic agents, 5-fluorouracil (5-FU, 50 μ M), an inhibitor of pyrimidine synthesis, or hydroxyurea (HU, 2mM), an inhibitor of both purine and pyrimidine synthesis. Cell-cycle profiles of these cells at the indicated time points were measured by flow-cytometry analyses (Fig. 3). These results indicate that excess Cdc25C only overrides the G2 arrest

caused by doxorubicin and has no effect on the G1/S arrest resulting from 5-FU and HU. These findings confirm that the ability of Cdc25C to sensitize cells to doxorubicin occurs in breast epithelial cells.

Task 7. Perform “proof of principle” studies (Months 8-24)

Previous studies have shown that DNA damage-induced downregulation of Cdc25C occurs in a p53-dependent manner (St. Clair et al., 2004). To confirm that this effect occurs in breast tumor cells, the MCF7 cell line was treated with doxorubicin. Induction of p53 protein and its target the cyclin-dependent kinase inhibitor p21 was observed. The levels of Cdc25C protein as well as that of the cell cycle regulators Cdc2 and Cyclin B1 were also decreased (Fig 4, left). RT-PCR analysis confirmed the downregulation of Cdc25C also occurred at the mRNA level (Fig. 4, right). To explore the p21-dependence of this, an siRNA approach was used to ablate p21 expression. Downregulation of p21 abrogated the decrease in Cdc25C, Cdc2, and Cyclin B1 protein levels as well as that of Cdc25C messenger RNA (Fig. 4). These results confirm and expand the published findings in cell lines from other tumor types. Thus, MCF7 cells are validated as a suitable cell line to perform the “proof of principle” studies. In the previous funding year, constructs were generated to restore Cdc25C expression to cells in which Cdc25C had been downregulated by an siRNA approach. The establishment of such clones using MCF7 cells is ongoing. It had been hoped that this would be completed in this funding year, however technical problems have arisen. Most notably, it became apparent that comparisons between different clones will be problematic if they contain differing copy numbers of the transfected plasmids. With this in mind, retroviral vectors are being generated which correspond to the plasmids described in the last progress report. Transduction recombinant retroviruses will allow for single copy uptake of the expression cassette and will avoid the confounding copy number problem.

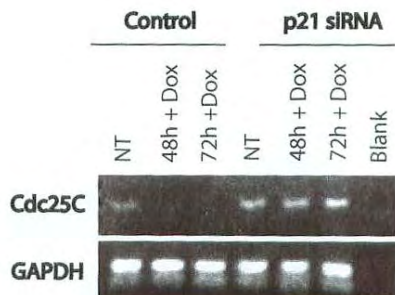
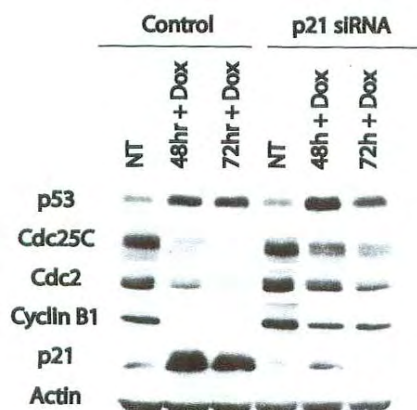


Figure 4. DNA damage induces downregulation of Cdc25 in a p21-dependent manner in the breast carcinoma cell line MCF7.

MCF7 cells were incubated with either control siRNA or siRNA directed against p21 for 24 hr, and were then treated with doxorubicin for either 48 or 72 hr. Cells were lysed and subjected to either immunoblot analysis (left panel) or RT-PCR to detect mRNA levels (right panel) as indicated.

Task 8. Identify factor involved in p53-dependent repression (Months 1-36)

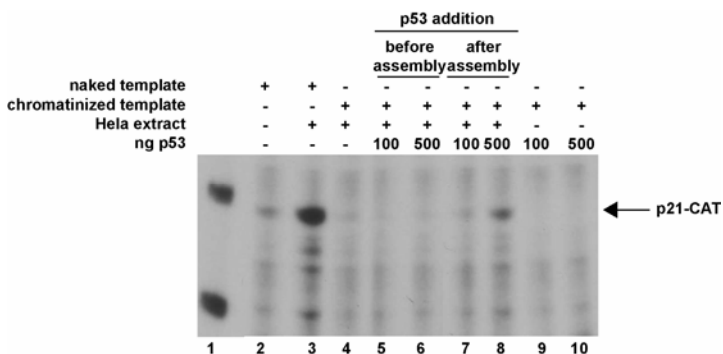
As was noted in the progress report for the previous funding year, the planned approach to ablate expression of candidate GC box binding proteins may be problematic if there is redundancy among the proteins in our list of likely candidates. We therefore have focused in a biochemical approach to identify this factor. Although it was proposed to perform DNA affinity chromatography, it became apparent that such an approach would likely identify the same proteins that were being screened in the siRNA approach and hence their validation would suffer from the same drawbacks. With this in mind, an in vitro transcription system has been established using chromatinized templates. Identification of a cooperating factor using a functional read-out is expected to be far more useful than relying solely on DNA binding to the GC box. Thus, instead of merely identifying GC box-binding proteins, it should be possible to purify factors which cooperate with p53 to induce transcriptional repression. Through the use of the appropriate reporters, it also will be possible to show that this occurs in a manner dependent upon the GC box.

In vitro transcription using chromatinized templates is technically challenging. Thus, a collaboration has begun with Dr. Michele Barton at the M.D. Anderson Cancer Center. Dr. Barton has extensive experience using chromatinized templates for the study of p53-dependent transcription. With her help, an in vitro transcription system has been established which demonstrates transcriptional activation that is strictly dependent upon addition of p53 (Fig. 5). The data in Fig. 5 demonstrates that a chromatinized template is essential for these studies. The in vitro transcription system will utilize a reporter containing the isolated site in the minimal promoter construct that is strictly dependent upon addition of p53 and that demonstrates transcriptional repression. Biochemical fractionation of the extract will be performed to identify the factor that is needed for p53-dependent repression. The first step is to use biotinylated DNA containing the response

element to attempt to clear the extract of this activity. The resulting extract should still be capable of supporting p53- dependent transcription of a minimal reporter containing the upstream p53 site from the *p21^{CIP1}* promoter. Proteins eluted from the DNA will then serve as candidates for further purification and analysis. Candidate factors can then be validated by use of siRNA to downregulate expression and abrogate p53-dependent repression in cells. If this is successful, the ability of such siRNA to affect the DNA damage response will be tested.

Figure 5. p53-dependent transcription of the p21 promoter in vitro requires a chromatinized template and is consistent with a role for p53 in chromatin remodeling.

Hela extract was incubated with either naked DNA (lanes 2-3) or a chromatinized template (lanes 4-10), either in the absence (lanes 2-4) or presence (lanes 5-8) of the indicated amounts of purified human p53. Transcription was also performed in the absence of Hela extract (lanes 9-10). Products were determined by primer-extension assay. p53 was added either prior (lanes 5-6) or after (lanes 7-8) chromatin assembly. A product of the correct size is only detected when p53 is added after assembly of chromatin. Lane 1 contains molecular weight markers.



Key Research Accomplishments

- Demonstrated that Cdc25B but not Cdc25C inhibits cellular proliferation in a manner dependent upon p53
- Showed that the ability of Cdc25B to inhibit cell proliferation involves activation of DNA damage checkpoints
- Showed that overexpression of Cdc25C sensitizes breast cells to treatment with chemotherapeutic agents which induce DNA damage
- Showed that DNA damage induces downregulation of Cdc25C protein and RNA in breast cancer cells

Reportable Outcomes

- Established expression constructs for hybrid Cdc25B/Cdc25C proteins and showed that they express the appropriately
- Established an in vitro transcription system which is p53-dependent and utilizes a chromatinized template
- Varmeh-Ziaie, S. and Manfredi, J.J. 2007. The dual-specificity phosphatase Cdc25B, but not the closely related Cdc25C, is capable of inhibiting cellular proliferation in a manner dependent upon its catalytic activity. J Biol Chem , in press. (<http://www.jbc.org/cgi/content/abstract/M703105200v1>)
- Varmeh-Ziaie, S. and Manfredi, J.J. Inappropriate cell cycle progression triggers a p53-dependent checkpoint in the absence of p14ARF: a role for DNA damage pathways in oncogenesis. Mol Cell Biol, in revision.
- Carvajal, L. and Manfredi, J.J. p53-dependent transcriptional repression of human Cdc25C requires a cooperating factor that interacts with an adjacent GC-rich sequence. In preparation.
- Varmeh-Ziaie, S. and Manfredi, J.J. Forced expression of Cdc25C sensitizes tumor cells to DNA damaging chemotherapeutic agents. In preparation.
-

Conclusions

Previous studies have implicated Cdc25B as an oncogene in a variety of human cancers, but an elaboration of the underlying molecular basis for this remains to be performed. Further, the role of Cdc25C as having oncogenic activity is an underexplored area as well. Ongoing studies will directly address each of these key issues in human breast cancer. Further, exploiting the finding that overexpression of Cdc25C sensitizes tumor cells to chemotherapeutic agents which cause genotoxic stress may lead to a novel approach to treat human breast carcinoma. While these studies are laboratory-based, it is hoped that they will provide a sound basis for further translational approaches that will impact the prognosis and treatment of this disease.

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Appendices

Varmeh-Ziaie, S. and Manfredi, J.J. 2007. The dual-specificity phosphatase Cdc25B, but not the closely related Cdc25C, is capable of inhibiting cellular proliferation in a manner dependent upon its catalytic activity. *J Biol Chem* , in press.