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

A recent study showed that about 50% of small breast cancers have higher expression of CDC25A protein than normal breast tissues, suggesting that CDC25A overexpression plays an important role in development of breast cancer. The ***long-term goal*** of this proposed program is to understand how CDC25A participates in cellular transformation to cancer. The ***objective of this application*** is to determine whether the expression level of CDC25A is rate-limiting for development of breast cancer. Our working ***hypothesis*** is that CDC25A plays dual functions of promoting cell divisions and suppressing cell death by activating cyclin-dependent kinases and inhibiting apoptosis signal-regulating kinase-1 (ASK1), respectively. This proposed study is ***innovative*** because the interaction of CDC25A and ASK-1 is our novel discovery, and it is a good candidate for coordinated control of cell division and death during cancer development, according to the known functions of these two proteins. It is directly ***related to the biology of breast cancer***, addressing a mechanism of breast cancer development. The study will also generate novel mouse models for breast cancer, which could be used for future studies to develop cancer therapies. We expect that this program will provide significant insight into the mechanism of breast cancer development.

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## Introduction

The subject of this IDEA award is the role of CDC25A in breast cancer development. CDC25A is a phosphatase that activates G1-regulatory cyclin-dependent kinases (Cdks) (Jinno et al., 1994) and also inhibits stress-responsive Apoptosis Signal-regulating Kinase-1 (ASK1) (Zou et al., 2001). About 50% of small breast cancers exhibit overexpression of CDC25A protein (Cangi et al., 2000). The purpose of this project is to determine whether increased expression of CDC25A is responsible for development of breast cancer, using human non-transformed mammary epithelial cells and transgenic/knockout mice. We expect that information generated from the project will provide insight into the mechanism of breast cancer formation and establish a scientific basis for therapeutic intervention against CDC25A overexpression.

## Body (Progress during the first year, March 2002 – March 2003)

The central hypothesis examined in this project is that increased expression of CDC25A is a critical step for transformation of the mammary epithelium, leading to deregulated G1/S progression with CDK2 activated, and diminished apoptotic responses to cellular stresses with the ASK1/JNK/p38 pathways inhibited.

To evaluate this hypothesis, the three specific aims are being pursued:
(1) Determine whether the ASK1-inhibitory action of CDC25A plays a role in transformation of human breast epithelial cells and mouse embryonic fibroblasts
(2) Determine whether induced overexpression of CDC25A in the breast epithelium of transgenic mice results in development of breast tumors.
(3) Determine whether the level of CDC25A expression is rate-limiting for development of breast tumors in MMTV-Ras transgenic mice

As described in the STATEMENT OF TASK in the original application, we are in the process of three tasks, which correspond to the three specific aims.

***Task 1.*** Determine whether the ASK1-inhibitory action of CDC25A plays a role in transformation of human breast epithelial cells and mouse embryonic fibroblasts (months 1-30)

We are examining the effects of CDC25A overexpression on nontransformed human breast epithelial cell line MCF10. Our hypothesis is that CDC25A overexpression induces transformation and/or cooperates with activated Ras to transform the cells. Specific procedures proposed in the original grant application and actual progress in each procedure are outlined as follows:

- Construct retrovirus vectors encoding Ha-Ras plus CDC25A (wild type or C430S mutant), Ha-Ras only or CDC25A only. Also construct expression vectors for GFP-ASK1(961-1020) fusion protein (months 1-3)

  We have made the retrovirus vectors for CDC25A(wt, C430S) with and without Ha-Ras, as proposed. A viral vector for Ha-Ras only has been provided by Dr. Andrei Gudkov, Cleveland Clinic, and used for other studies in our lab (Zou et al., 2002). Construction of GFP-ASK1(961-1020) has not been completed yet, but we expect to finish it within 3 months.

- Transfect Phoenix *ampho* and *eco* packaging cells with the retrovirus vector and harvest virus-containing supernatants (months 4-5)

  We have produced virus containing supernatants for Ha-Ras, CDC25A(wt, C430S), and both, as planned.

- Establish cultures of breast epithelial MCF-10 cells. Also, harvest mouse embryos at day 12.5 of gestation and culture MEFs (months 4-5)

  Optimizing the culture conditions for MCF-10 cells has been taking much longer than expected. This is because of the complexity of the culture medium and the unstable nature of this cell line. Recently, Dr. Brian Schlegel joined our university as a faculty member of Department of Pathology. He has extensive experience in MCF-10 cells (Schlegel et al., 2003), and currently assisting us to optimize it. Therefore, we should soon be able to initiate retroviral infection described below. MEFs has been routinely prepared and cultured in the laboratory.

- Infect MCF-10F cells and MEFs with the retroviruses (Ras+CDC25A, Ras alone, CDC25A alone, mock virus with GFP for infection efficiency, and DNp53+Ras as a positive control), culture for 14-28 days with 5% fetal bovine serum and count foci after staining with crystal violet (months 6-18)

  While we have not initiated viral infection in MCF-10 cells, we went ahead and performed infection of MEFs with the constructs. Infection efficiency in MEFs was quite high, about 80% of cells infected with undiluted mock GFP viral supernatant displayed the fluorescent signal. We have optimized foci assay for MEFs, as demonstrated in our recent publication (Zou et al., 2002). Once MCF-10F cultures are stabilized, we should have no major problem to optimize foci assay using these cells.

- Harvest clones from foci, and test them for colony formation in medium containing 0.3% agar (months 9-18)
- Transplant $10^6$ cells of each clone from transformed foci into the flank of nude mice, monitor for tumorigenesis every 3 days until tumors reach 5-10 mm, harvest tumors for histological and biochemical analyses. Approximately 30 mice will be used (months 9-20)
- Perform immunohistochemistry, western blotting and kinase assays using tumors developed in nude mice (months 20-30)

  These procedures have not been performed yet.

***Task 2.*** Determine whether induced overexpression of CDC25A in the breast epithelium of transgenic mice results in development of breast tumors (months 1-36)

To pursue the Specific Aim 2, we planned to generate transgenic mice with inducible overexpression of CDC25A in the mammary gland. In the original application, the task was outlined as follows:

- Construct the transgenic vector for the MMTV-rtTA line and the CMV-lox-STOP-lox CDC25A line (months 1-4)
- Injection of the vectors into one-cell stage FVB mouse embryos and transfer to uteri of pseudopregnant CD-1 females. Approximately 50 FVB mice and 10 CD-1 mice will be used for development of each line (months 5-6)
- Breeding of founder transgenic mice and confirm germline transgenic progeny (months 7-9)
- Crossbreed the two strains with the CMV-tet-O-Cre line provided by Dr. Whitsett. Also use the lox-STOP-lox LacZ reporter line instead of the lox-STOP-lox CDC25A line to examine recombination efficiency of the Cre/lox system in the mammary gland. Approximately 640 mice will be produced with expectation that 40 female mice with the triple transgenes will be found (months 10-22)
- Inject 20 mice with the triple transgenes with doxycycline to induce expression of CDC25A and monitor them for formation of breast tumors (months 13-36)
- Histological and biochemical analyses of tumors developed (months 13-36)

As the reviewers of the original application pointed out, this plan was over-ambitious in terms of the time frame. Generating the MMTV-rtTA x CMV-lox-STOP-lox CDC25A double transgenic mice and optimizing the inducible conditions for CDC25A overexpression should take more than the 3-year funding period. Therefore, to examine CDC25A overexpression in mice within a reasonable time frame, we decided to modify our priority in the strategy and generate MMTV-CDC25A mice, which is simpler and less time-consuming than the inducible double transgenic line or WAP-CDC25A mice requiring multiple rounds of pregnancy for transgene overexpression.

In collaboration with Dr. Sophia Y. Tsai, Baylor College of medicine, we have already generated MMTV-CDC25A mice and obtained encouraging preliminary results. Founder FVB/N mice with the MMTV-CDC25A transgene are currently being crossbred with MMTV-Ras mice in the same genetic background. The first group of the offspring has been observed for spontaneous tumorigenesis for 7 months. So far, none of MMTV-25A single transgenic mice have developed obvious tumors (n=6, 3 females and 3 males). In contrast, we have confirmed that MMTV-Ras mice develop salivary and mammary tumors visible around 4 and 5 months of age, respectively (n=5, 3 females and 2 males), consistent with previously published studies (Sinn et al., 1987). Intriguingly, all MMTV-Ras x MMTV-CDC25A double transgenic females (n=3) developed salivary and mammary tumors within 2-3 months. All double transgenic males (n=3) also developed salivary tumors in 2 months. These data suggest that the MMTV-CDC25A transgene is insufficient to trigger tumorigenesis, but can cooperate with the MMTV-Ras transgene in mammary and salivary tumorigenesis. We are now actively breeding the mice to obtain sufficient numbers of mice in each group, for statistical analyses.

We will soon start the histological and biochemical examinations of tumor samples collected.

We are still in the process of generating the MMTV-rtTA x CMV-lox-STOP-lox CDC25A mice, as described in the original Task2. The CMV-lox-STOP-lox CDC25A transgenic construct has been made, while the MMTV-rtTA plasmid is under construction.

***Task 3.*** Determine whether the level of CDC25A expression is rate-limiting for development of breast cancer in MMTV-Ras transgenic mice (months 1-36)

To pursue this aim, we planned to crossbreed CDC25A-heterozygous knockout mice and MMTV-Ras transgenic mice and compare Ras-induced mammary (and salivary) tumorigenesis between the CDC25A wild-type and heterozygous backgrounds.

- Continue backcross of CDC25A-heterozygous mutant mice into the FVB strain background. Approximately 60 mice will be produced (months 1-12)

  The backcross breeding is ongoing.

- Crossbreed CDC25A-heterozygotes with the MMTV-Ha-Ras transgenic mice obtained from Jackson Laboratory. Approximately 240 mice will be produced with expectation that 30 CDC25A+/- and 30 +/+ females with the transgene will be found (months 13-16)
- Monitor mice for formation of breast tumors (months 17-36)

  We have already started the proposed crossbreeding and obtained a few litters. The preliminary data regarding tumorigenesis is quite encouraging despite the limited numbers of mice. We have been able to observe 3 MMTV-Ras, CDC25A+/+ mice (2 females and 1 male) and 5 MMTV-Ras, CDC25A+/- mice (3 females and 2 males), for 9 months. All MMTV-Ras, CDC25A+/+ mice, with both sexes, developed visible breast tumors by 6 months of age. Strikingly, none of MMTV-Ras, CDC25A+/- mice has developed visible tumors during the 9-month observation. We are continuing to monitor these mice, as well as more new litters, for possible tumorigenesis. Salivary tumors were not observed in this group of mice for some reason, possibly associated with genetic backgrounds. This is an exciting finding, and we need to confirm the tumor-suppressive effect of hemizygous CDC25A loss, generating and observing more mice. We intend to produce and observe at least 30 mice per group (each sex), according to the original statistical estimations.

- Histological and biochemical analyses of tumors developed (months 13-36)

  We will soon start for histological and biochemical analyses of tumor samples collected.

**Key Research Accomplishments during the Year 1**

- Generated MMTV-CDC25A transgenic founder mice
- Obtained preliminary data suggesting that the MMTV-CDC25A transgene synergistically cooperates with MMTV-Ras
- Obtained preliminary data suggesting that hemizygous loss of CDC25A reduces or suppresses MMTV-Ras-induced mammary tumorigenesis

## Reportable Outcomes

None at this moment

## Conclusions

During the first year of the funding period, we have made significant progress in generating mouse models for evaluating the role of CDC25A in breast cancer development. The new MMTV-CDC25A transgene exhibits synergistic cooperation with MMTV-Ras. Moreover, hemizygous loss of CDC25A shows significant reduction in tumorigenesis in MMTV-Ras mice. Therefore, the expression level of CDC25A seems to be a critical rate-limiting factor for mammary tumorigenesis, which is consistent with our central hypothesis that increased expression of CDC25A is a critical step for transformation of the mammary epithelium.

We recommend to further characterize this model, as a more efficient alternative to the MMTV-rtTA x CMV-lox-STOP-lox CDC25A double transgenic mice for Task 2. The inducible double transgenic strain has an advantage to avoid possible deteriorating effects of CDC25A overexpression on mammary tissue development. However, our preliminary data shows the mammary gland in MMTV-Ras mice develops without major problems, and we have already obtained the encouraging data.

These studies will not only determine the role of CDC25A in breast cancer development but also provide unique and valuable animal models prone or resistant to breast tumorigenesis, in which various therapeutic strategies could be investigated.

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