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Abstract

The purpose of this project is to study the effect of constitutive cyclin E expression on mitotic division and to ultimately identify the mechanism through which cyclin E leads to chromosome instability. Cyclin E functions in timing the G1/S phase transition and centrosome duplication, but when cyclin E expression is deregulated cells enter S phase and exit more slowly and exhibit a moderate level of chromosome instability. We propose that cyclin E may be interfering with mitotic division leading to chromosome instability and eventual tumorigenesis. In the second year of funding, I have accomplished my goals including, finishing documentation of mitotic delays using immunofluorescence and live cell microscopy, quantification of centrosome aberrations, and using biochemical assays to analyze expression of mitotic regulators in order to help in the identification of possible substrates of cyclin E/Cdk2 phosphorylation in mitosis. Finally, important progress has been made toward understanding how cyclin E delays mitosis. Using siRNA against BubR1 to disrupt the spindle checkpoint, the effects previously observed with deregulated cyclin E expression were reversed, showing that cyclin E is working through the spindle checkpoint to delay mitotic cells.

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Introduction

Deregulation of the cell cycle is a critical step in tumorigenesis. The cell cycle is regulated through the periodic expression of cyclins. Cyclin E expression is normally limited to the G1/S boundary where it functions to initiate DNA replication and centrosome duplication. Deregulated cyclin E in cell culture causes a premature but elongated S phase (Resnitzky *et al.* 1994). In cyclin E transgenic mice, tumor incidence is increased (Bortner and Rosenberg 1997), especially with expression of a hyperstable cyclin E mutant (Smith *et al.* submitted). The link between cyclin E and tumorigenesis can be explained by the presence of chromosome instability following constitutive cyclin E expression (Spruck *et al.* 1999). The purpose of this study is to investigate the effects of constitutive cyclin E expression on mitotic division and to ultimately identify the mechanism through which cyclin E leads to chromosome instability. We show that one pathway might be through a delay in mitosis and failure to properly segregated chromosomes. This report describes the observations of this mitotic delay in cyclin E expressing cells, shows that cyclin E deregulation leads to an unusually high accumulation of mitotic regulating proteins, and works through the mitotic spindle checkpoint to delay cells.

Body

Task 1 (reviewed) - completed in first year: Characterization of cells transduced with cyclin E retrovirus and adenovirus (1-9 months)

a. Cyclin E retrovirus was produced and used to stably express cyclin E in IME cells

b. Cyclin E adenovirus was produced and used to transiently express cyclin E in IME, U2OS, and KB cells

c. Cell cycle analysis: FACS analysis was used to observe S phase and G2/M phase delays in cyclin E expressing cells

d. Western blots were used to show cyclin E overexpression

e. Laser scanning cytometry experiments – this method proved to be difficult and was replaced by using immunofluorescence with fixed cells (task 2)

*To address comments from the previous annual report concerning cell type: IME cells or immortalized mammary epithelial cells are derived from normal breast tissue and were initially used for a more accurate model of 'normal' breast epithelial cells. IME cells are immortalized with telomerase, untransformed, and have normal tumor suppressor expression, unlike most cancer cell lines. In all tasks completed in the second year I use KB cells, a cervical cancer cell line (p53+ and derived from HeLa cells). These cells grow well, infect well with adenovirus, and the protocol for thymidine block and release was previously worked out for synchronization.

Task 2 – (completed in first and second years): Observation of aberrant mitoses with fixed and live cell microscopy (Months 8-17)

The majority of this objective was accomplished and discussed in the first annual report. In summary, asynchronous cells were infected with cyclin E adenovirus and then fixed and processed for immunofluorescence. The number of cells in each phase of mitosis was manually counted and the exact mitotic phase was determined by the position of the chromosomes and the orientation of the mitotic spindle. These and subsequent experiments revealed that the mitotic delay caused by cyclin E deregulation occurred in early mitosis, before all chromosomes were aligned at the metaphase plate. This data provided the insight that cyclin E was perhaps inhibiting cells from exiting the spindle checkpoint.

In addition, the first report discussed results from live microscopy experiments that supported the fixed cell data. Cells containing an H2B-GFP construct were filmed on a deconvolution microscope as

they progressed through mitosis. The times of each mitotic phase were assessed with delays found in early mitosis during prometaphase and early metaphase. Furthermore, some cells expressing cyclin E were observed to completely fail mitosis and re-attach to the coverslip to become polyploidy cells.

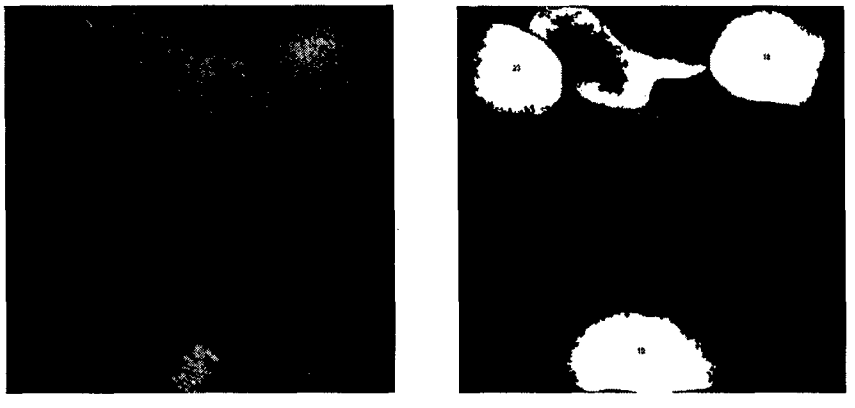
Task 2 was completed during my second year of funding and the results are shown below:

a. *Performed synchrony experiments with thymidine: released from S phase block to enhance for mitotic cells and analyze protein expression with immunofluorescence*

This assay was used to enrich mitotic populations for immunofluorescence experiments. Cyclin B is an essential mitotic regulator. We chose to analyze cyclin B expression in single cells in order to assess whether cyclin E not only delayed mitosis but also affected proteins expression during mitosis. Cells were fixed and processed for immunofluorescence to quantify cyclin B levels in prometaphase cells (cells with unaligned chromosomes) and metaphase cells (cells with aligned chromosomes). Indeed, cyclin E-deregulated cells expressed higher levels of cyclin B in both prometaphase and metaphase cells. Interestingly, among the cyclin E cells, a small population of metaphase cells did not degrade cyclin B even when chromosomes were aligned at the metaphase plate. Cyclin B is a substrate of the anaphase promoting complex (APC). The APC is important for regulating the levels of many mitotic proteins. This observation led us to investigate other known substrates of the APC, discussed further in Task 4.

Figure 1: Cyclin B levels increase in mitotic cells upon constitutive expression of cyclin E. (A) Representative immunofluorescence image of KB cells transduced with either CyE or Control adenovirus and stained for cyclin B (green) and centromeres (red). Images were captured with a deconvolution microscope. Mitotic cells were enriched using a single thymidine block and release. Image J was used to measure average pixel intensities for single cells. The image on the right shows the mask applied by Image J to quantify average intensities of cyclin B. (B) Graphs comparing pixel intensities for cyclin B. Analysis was performed on cells containing either unaligned or aligned chromosomes. The X axis is divided into arbitrary groups of pixel intensities.

A.)



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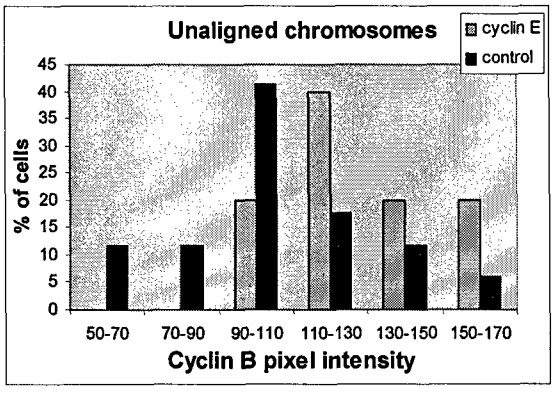
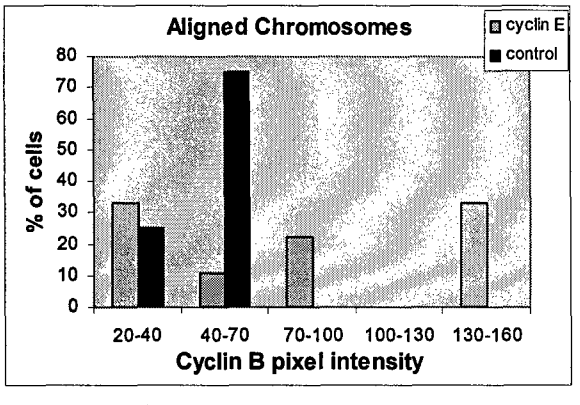




Task 3: (completed in second year) - Quantification of centrosomes in cells transduced with cyclin E (Months 17-23)

Centrosomes were quantified in fixed cells using immunofluorescent analysis. Centrosomal markers, such as gamma tubulin were used to count the number of centrosomes in cells transduced with cyclin E or an empty vector adenovirus. Results showed that 8% of cyclin E cells contained more than 2 centrosomes (normally four). This accumulation of centrosomes is most likely due to the failure of mitosis observed in cyclin E cells since most often there were four centrosomes, indicating that the cells did not divide into daughter cells.

Task 4: (on-going) - Investigate potential targets of cyclin E/Cdk2 phosphorylation in mitosis (Months 24-36)

This task was initiated during my first year of funding and is now the focus of my project for the remaining term. The task of investigating the mechanism by which cyclin E leads to a mitotic delay is the most interesting and crucial to this project. My first objective was to identify which mitotic proteins were upregulated in response to cyclin E deregulation. In the previous experiments discussed above, cyclin B was shown to accumulate to a greater extent in mitotic cells expressing cyclin E. Therefore, additional substrates of the APC were investigated. Similar to the immunofluorescence experiments, cells were first synchronized using a thymidine block in S phase, followed with a release into mitosis and collection of samples for Western blot analysis as cells proceed through mitosis. Cells were not perturbed by drugs inhibiting mitosis such as nocodazole, so to not alter protein expression. Indeed, an accumulation of several important mitotic regulators such as cyclin B, cyclin A, and Pds1/securin was observed (shown in Figure 3). This is further proof that the APC complex is inhibited in the presence of cyclin E/Cdk2. However, the direct target of cyclin E/Cdk2 phosphorylation in mitosis is yet to be identified. Preliminary data in my first annual report focused on Emi1, an upstream inhibitor of the APC. Initially, Emi1 appeared to accumulate in the presence of deregulated cyclin E. However, as this biochemical assay was perfected and cells were synchronized and released more efficiently, several experiments demonstrated that Emi1 protein levels and phosphorylation status appeared to be unaffected by cyclin E deregulation, as shown in the representative experiment below in Figure 2. Therefore, the focus of my studies is now downstream of Emi1 to direct interacting partners of the APC, such as proteins from the mitotic checkpoint complex (MCC), namely BubR1 and Mad2.

Figure 2. Emi1 protein levels are not affected by the expression of cyclin E in mitosis. Cells were synchronized by thymidine block and released through mitosis. Samples were collected at 7.5, 8.5, and 9.5hrs following release. In addition samples from thymidine and nocodazole arrest were collected. No changes in the levels of Emi1 or the phosphorylation status (determined by mobility shift) were observed.

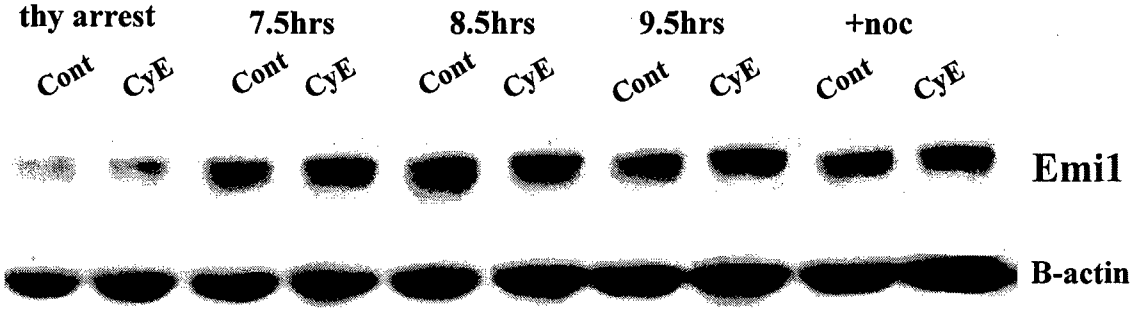
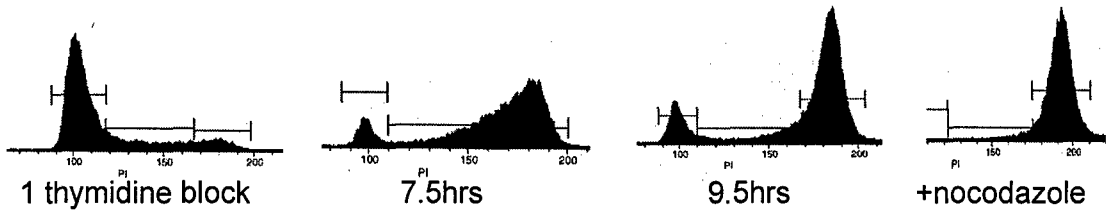


Figure 3. Cyclin E deregulation leads to accumulation of several APC substrates during mitosis. A.) Representative images of FACS data (PI staining) from control adenovirus samples collected as cells progressed through mitosis to confirm the percentage of mitotic cells, this was performed on both control and cyclin E samples for comparison to ensure equal percentages of cells in G2/M. B.) A representative Western blot experiment showing the accumulation of mitotic proteins in cyclin E-transduced cells. Cyclin B, cyclin A, Cdc20, and Pds1/securin are shown to accumulate to a greater extent in each time point throughout mitosis. β-actin is used as a loading control.

A.)



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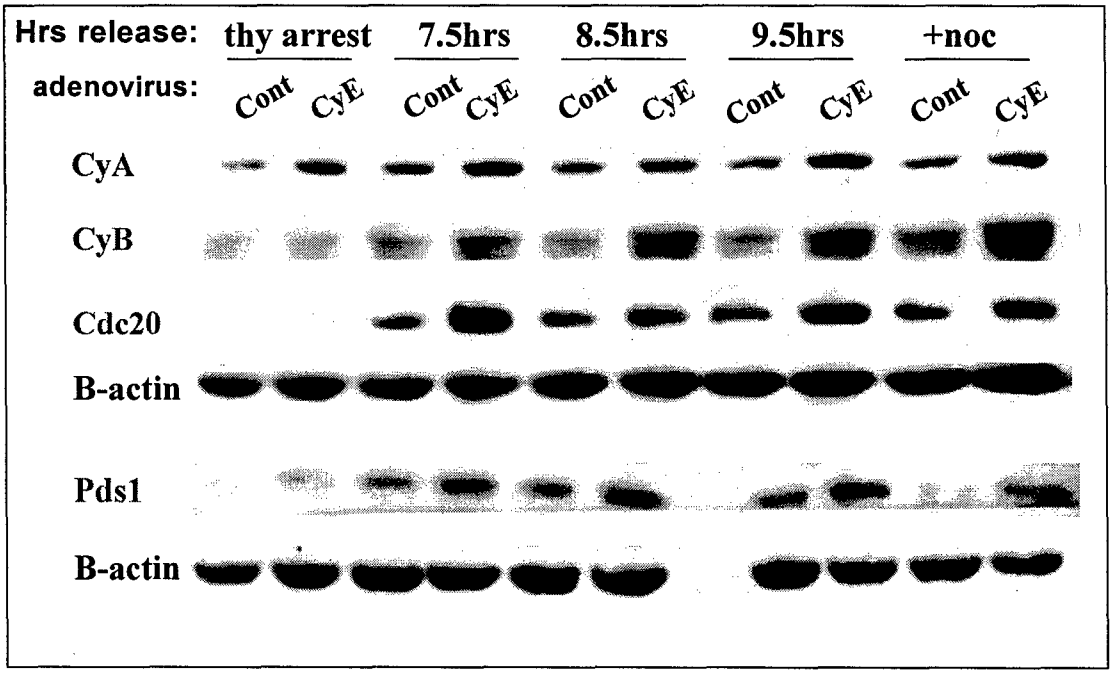
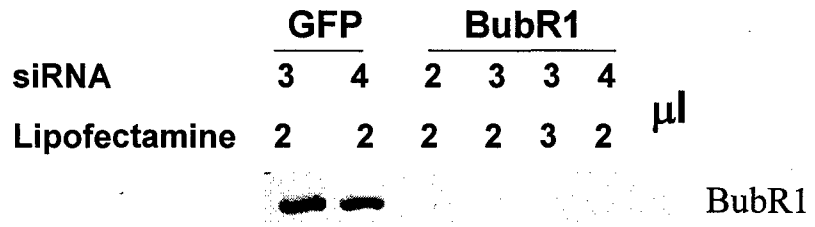


The observation of increased mitotic protein accumulation provided further evidence that the presence of cyclin E/Cdk2 was delaying cells in early mitosis. Next, siRNA experiments were preformed to knockout the spindle checkpoint and observe whether cyclin E continued to delay cells in early mitosis. The spindle checkpoint acts as a 'wait signal' to ensure that all chromosomes are aligned on the metaphase plate before sister chromatids separate and migrate toward opposite poles. The mitotic checkpoint complex is made up of proteins such as BubR1 and Mad2. These proteins bind to APC/Cdc20 and inhibit activity until all chromosomes are aligned. Upon release of the 'wait signal', BubR1 and Mad2 disassociate from APC/Cdc20, allowing for activation of this ubiquitin ligase complex.

To test whether cyclin E delays cells in mitosis by interfering with the spindle checkpoint, siRNA against either BubR1 or Mad2 was used in order to knockout the spindle checkpoint. Figure 4 shows that BubR1 was effectively decreased with siRNA, as detected by Western blot, and that cells no longer arrested in mitosis with nocodazole, proving that the spindle checkpoint was no longer intact.

Figure 4. BubR1 siRNA effectively decreases protein levels and bypasses the spindle checkpoint induced by nocodazole. A.) Optimization of BubR1 knockdown using various amounts of BubR1 siRNA and lipofectamine. GFP siRNA is used as a control in the first two lanes, BubR1 protein is still detectable by Western blot. However, with all concentrations of BubR1 siRNA, the protein is no longer expressed at detectable levels (even upon overexposure of the blot). B.) FACS data (PI staining) showing loss of the spindle checkpoint in cells transfected for 48hrs with BubR1 siRNA and treated with nocodazole. With GFP siRNA, cells are able to maintain a G2/M arrest after 12hrs of nocodazole.

A.)



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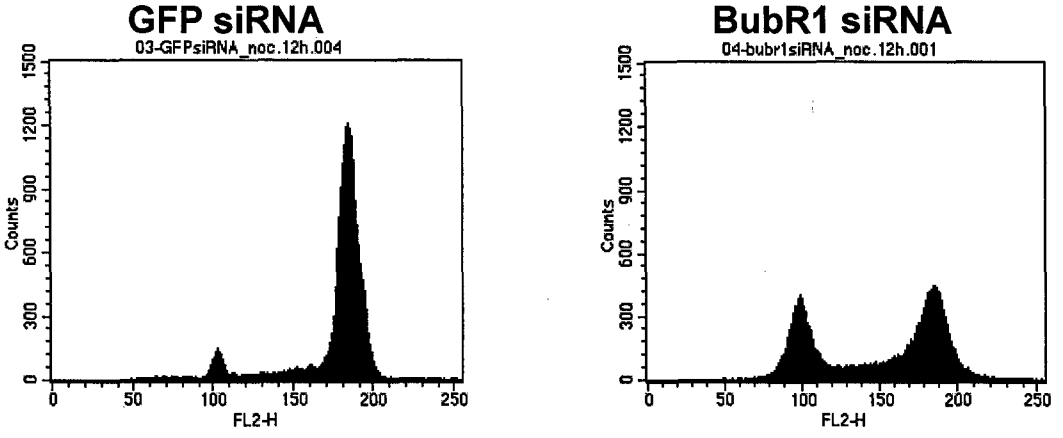
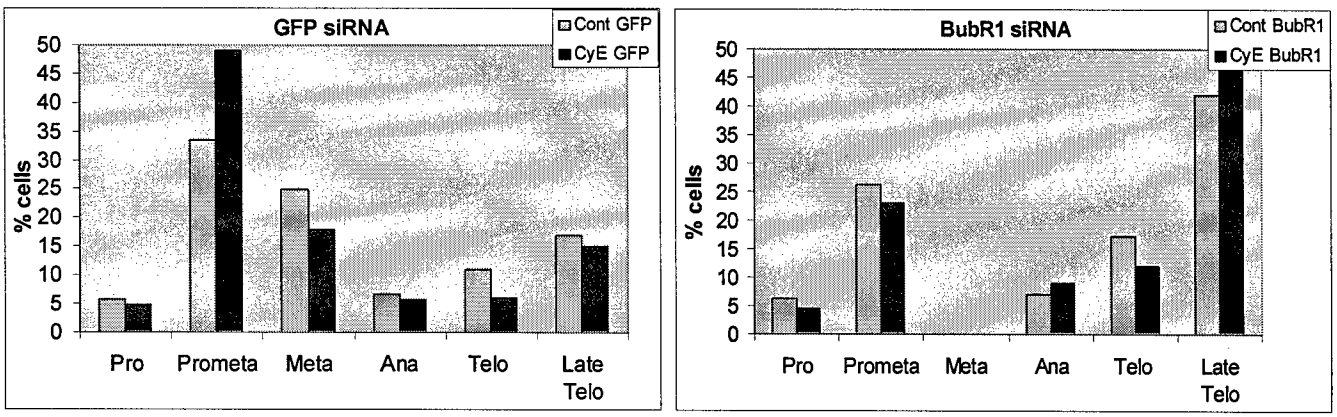


Once the BubR1 siRNA experiment was optimized, experiments were performed in combination with deregulated cyclin E expression to test whether cells would still delay in mitosis without an intact spindle checkpoint. I chose to use the lowest possible concentration of BubR1 siRNA and lipofectamine (40µm siRNA and 2µl of lipofectamine). In the first experiment, the percentages of each mitotic phase was determined in fixed asynchronous cells transduced with control or CyE adenovirus in combination with GFP or BubR1 siRNA. Two hundred mitotic cells were counted per slide. In cells transfected with GFP siRNA, the results were reproducible from previous experiments. Cyclin E deregulated cells delayed in early mitosis near prometaphase. Interestingly, 48hrs following transfection of BubR1 siRNA, cells did not delay in early mitosis. Both CyE and control adenoviral-transduced cells went through mitosis rapidly and without delay.

Figure 5. BubR1 siRNA alleviates the delay previously observed in cyclin E transduced cells. A.) Asynchronous KB cells were transfected with control or BubR1 siRNA on day one and transduced with CyE or control adenovirus on day two. Cells were then fixed with 2% PFA and stained histone3-phosphorylated (to identify mitotic cells) and α-tubulin (to label the mitotic spindle) on day three. Cells were counted in the various phases of mitosis. As shown below, cyclin E cells no longer delay in early mitosis when transfected with BubR1 siRNA. B.) Thymidine block experiment performed as previously described. siRNA was transfected on day one and cells were transduced and arrested on day two. Cells were collected 7, 8, and 9hrs following release. In cells transfected with BubR1 siRNA, mitotic regulators no longer accumulate in cyclin E deregulated cells. In contrast, cells transfected with GFP siRNA continue to accumulate mitotic regulators in response to cyclin E deregulation.

A.)



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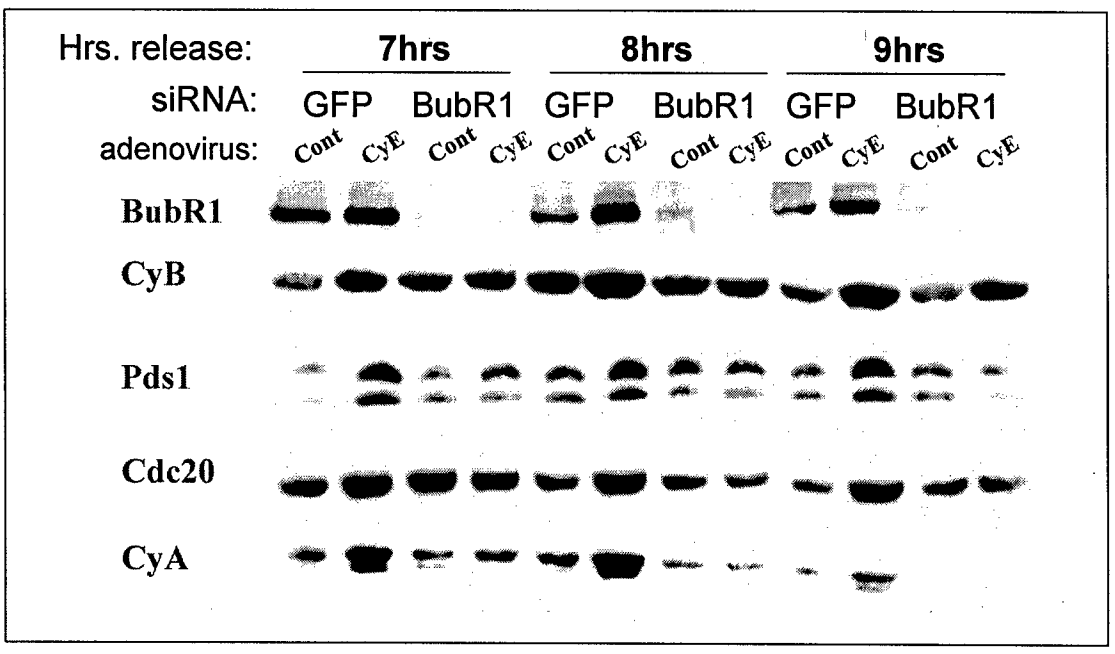
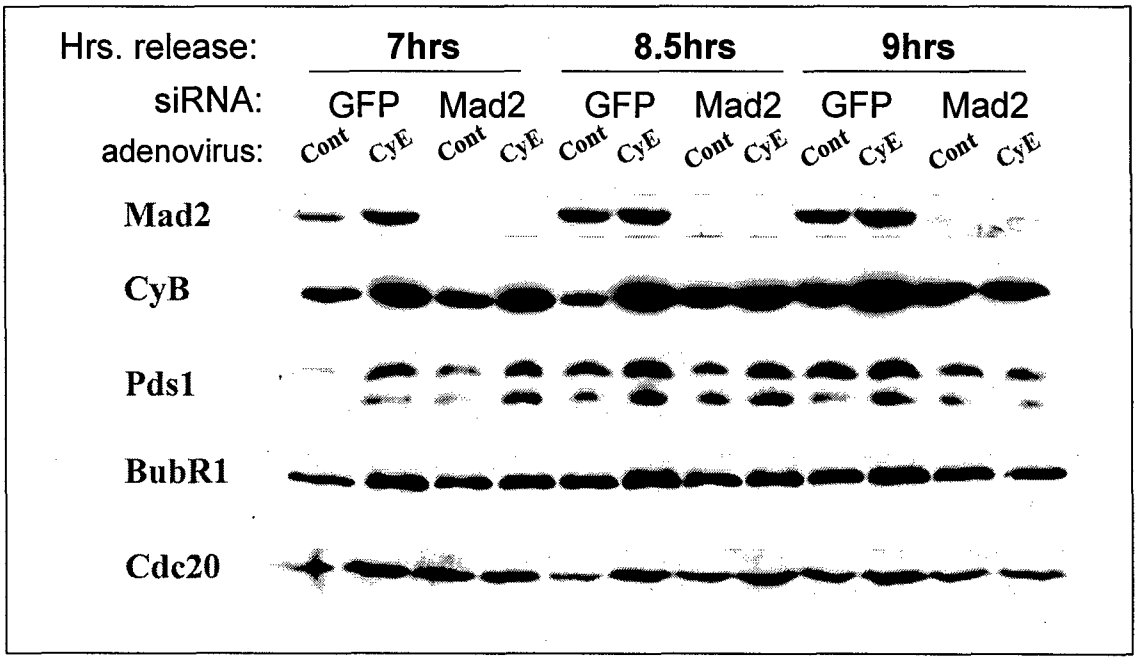


Next, cells were transfected with Mad2 siRNA, another component of the mitotic checkpoint complex, in order to reproduce the results found using BubR1 siRNA. Interestingly, although cells were found to progress through mitosis more quickly in both cyclin E and control-transduced cells, the Western blot experiments showed little change in accumulation of mitotic regulator proteins. Cyclin B,

Pds1/securin, and Cdc20 all continued to accumulate, especially in early time points, despite the lack of Mad2. This result provided insight in the mechanism of cyclin E deregulation. Perhaps BubR1 is more important for the mechanism of cyclin E-induced mitotic delays. Alternatively, BubR1 might simply be a more potent inhibitor of the APC.

Figure 6: Mad2 siRNA does not inhibit cyclin E-induced accumulation of mitotic proteins as does BubR1 siRNA. Western blot analysis of a thymidine block assay using Mad2 siRNA, performed in the same manner as described in the BubR1 siRNA experiments. In time points through early mitosis, such as 7 and 8.5hrs, mitotic regulators continue to accumulate in cyclin E cells, despite the lack of Mad2 protein.



Key Research Accomplishments

- Finished observations of mitotic delays using fixed cells to count mitotic phases and live cell microscopy to directly observe the mitotic delays and failures. Both assays pointed toward a delay in early mitosis before sister chromatid separation.
- Counted centrosomes and documented abnormal numbers of centrosomes in cyclin E cells.
- Used immunofluorescence for single cell analysis of increased cyclin B expression in mitotic cells.
- Synchronized cells and performed Western blot analysis of protein expression in mitosis. APC substrates were found to accumulate to a greater extent in cyclin E deregulated cells.
- siRNA experiments were optimized and performed to bypass the spindle checkpoint and observe whether cyclin E continued to delay cells in mitosis. BubR1 siRNA allowed cells to move through mitosis unperturbed by cyclin E expression.

Reportable Outcomes

- Keck J. Sept 2004. Oral presentation: The Effects of Deregulated CyclinE on Mitosis. The Scripps Research Institute Graduate Student Retreat, La Jolla, CA. Awarded best biology presentation.
- Keck J. April 2005. Oral presentation: The Effect of Deregulated Cyclin E on Mitosis. The American Association of Cancer Research (AACR) Annual Meeting, Anaheim, CA. Awarded a Scholar in-training fellowship for travels.
- Keck J. June 2005. Poster presentation: The Effects of Cyclin E on Mitosis. DOD Breast Cancer Research Meeting, Philadelphia, PA

Conclusions

In conclusion, the second year of funding proved to be quite fruitful with many insights into the mechanism by which cyclin E induces mitotic delays. Experiments documenting the cyclin E-induced mitotic delays were completed and several types of experiments were begun to investigate mechanistic aspects of this project. These included immunofluorescence and biochemical assays to explore mitotic protein accumulation. From these assays, it was found that substrates normally degraded in mitosis, such as cyclin B and Pds1/securin were accumulating to a greater extent during mitosis due to cyclin E deregulation. Hence, it appears that cyclin E is inhibiting the APC ubiquitin ligase complex. In addition, it was shown that by knocking out the spindle checkpoint, using BubR1 siRNA, and to a lesser degree Mad2 siRNA, one could alleviate the mitotic defects caused by cyclin E deregulation. Future experiments will be done to investigate APC inhibition directly with ubiquitination assays. In addition, the role of BubR1 in the observed mitotic delays will be explored.

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