## University of Louisville ThinkIR: The University of Louisville's Institutional Repository

Electronic Theses and Dissertations

8-2011

# Adenovirus-induced cyclin E activates CDK2 for virus replication.

Pei-Hsin Cheng 1983-University of Louisville

Follow this and additional works at: https://ir.library.louisville.edu/etd

#### **Recommended** Citation

Cheng, Pei-Hsin 1983-, "Adenovirus-induced cyclin E activates CDK2 for virus replication." (2011). *Electronic Theses and Dissertations*. Paper 239. https://doi.org/10.18297/etd/239

This Master's Thesis is brought to you for free and open access by ThinkIR: The University of Louisville's Institutional Repository. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of ThinkIR: The University of Louisville's Institutional Repository. This title appears here courtesy of the author, who has retained all other copyrights. For more information, please contact thinkir@louisville.edu.

## ADENOVIRUS-INDUCED CYCLIN E ACTIVATES CDK2 FOR VIRUS REPLICATION

By Pei-Hsin Cheng B.S., National Sun Yat-Sen University, 2005 M.S., National Sun Yat-Sen University, 2007

A Thesis Submitted to the Graduate Faculty of the University of Louisville School of Medicine in Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Pharmacology and Toxicology University of Louisville Louisville, Kentucky

August 2011

## ADENOVIRUS-INDUCED CYCLIN E ACTIVATES CDK2 FOR VIRUS REPLICATION

By Pei-Hsin Cheng B.S., National Sun Yat-Sen University, 2005 M.S., National Sun Yat-Sen University, 2007

A Thesis Approved on

February 2, 2011

by the following Thesis Committee

Kelly McMasters, M.D., Ph.D.

Heshan Zhou, Ph.D.

Donald Nerland, Ph.D.

David Powell, Ph.D.

Wolfgang Zacharias, Ph.D.

## DEDICATION

This thesis is dedicated to my beloved family and friends

for their continuous love and support.

#### ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Kelly McMasters, and my co-mentor, Dr. Heshan Zhou. Without their full support and guidance, it would not be possible to accomplish this work. I would also like to thank my graduate committee members, Drs. Donald Nerland, David Powell, and Wolfgang Zacharias, for their critical comments and valuable suggestions. My thanks are extended to the other members in the lab, Drs. Hongying Hao, Jorge Gutierrez, Lan Chen and X-Mei Rao, for their kind help with experiments. I especially appreciate my Louisville family, Dr. Rao and her family, for their moral support and fellowship. I would also like to express my thanks to Dr. Theresa Chen for her precious advice in my studies and life. In addition, I want to thank Colins Eno, Yu-Ting Chen, and Sharon Carpenter for their help in editing my thesis. Special thanks go to my beloved family and friends. Without all of you, I would not be able to pursue my dreams and achieve my goals.

#### ABSTRACT

## ADENOVIRUS-INDUCED CYCLIN E ACTIVATES CDK2 FOR VIRUS REPLICATION

**Pei-Hsin Cheng** 

#### February 2, 2010

Human adenoviruses (Ads) can infect and replicate in cells at different cell-cycle stages. Ads with the E1B55K deletion preferentially replicate in cancer cells and cause oncolysis. Our laboratory has previously shown that the Ad E1b gene is involved in induction of several cell-cycle regulative genes (Rao, X. et al. Virol. 350:418-28, 2006) and that cyclin E expression is required for efficient viral replication (Zheng, X. et al. J Virol 82:3415-27, 2008). In this study, we sought to investigate the interaction of cyclin E with its partner CDK2 in Ad-infected cells. We show four lines of evidence indicating the importance of CDK2 activation by cyclin E in Ad replication. First, the replication of E1B55K-deleted Ads was partially inhibited in the transgenic CHO cell line expressing a cyclin E mutant unable to bind CDK2, but not in those expressing wild-type cyclin E or a mutant unaffecting its CDK2 binding. Second, Ad-induced cyclin E protein formed complex with CDK2, correlating with the increased phosphorylation of CDK2 at T160 and pRb at S612. Third, the CDK2 chemical inhibitor, roscovitine, decreased viral DNA synthesis, protein production, and viral yield. Finally, a siRNA specifically inhibiting CDK2 repressed the viral replication with the decrease in pRb phosphorylation. Our findings indicate that Ad-induced cyclin E activates CDK2 to target the transcription repressor pRb for Ad productive replication.

## TABLE OF CONTENTS

DEDICATION iii
ACKNOWLEDGMENTSiv
ABSTRACT
LIST OF FIGURES
CHAPTER I. INTRODUCTION1
CHAPTER II. MATERIALS AND METHODS
CHAPTER III. RESULTS
CHAPTER IV. DISCUSSION
FIGURES
REFERENCES
APPENDIX
CURRICULUM VITAE

## LIST OF FIGURES

1. Transgenic cyclin E expression and the infectivity of AdGFP in CHO cells 23
2. Comparison of viral replication in normal and Myc-cycE transgenic CHO cells24
3. Cyclin E/CDK2 complex induced by viral infection in A549 cells25
4. Effects of viral replication on cellular proteins related to $G_1/S$ phase
5. Effects of viral replication on pRb and CDK inhibitors27
6. Effects of roscovitine on CPE and viral production
7. Effects of Ros on viral DNA synthesis, viral capsid proteins, virus-induced cyclin E and phospho-pRb S612
8. Effects of CDK2-specific siRNA on oncolytic viral replication
9. Proposed mechanism of cyclin E function in Ad replication

#### CHAPTER I

#### INTRODUCTION

The human adenoviruses (Ads) are double-stranded linear DNA viruses that can infect and replicate in cells at different cell-cycle stages (5). After infection, viral early proteins interact with cellular factors to create favorable environments for viral replication. The E1 region contains two sets of genes, *E1a* and *E1b*, that are dedicated to controlling cell cycle, inhibiting apoptosis, and regulating cellular and viral genes (69). Ads with *E1* modifications that preferentially replicate in cancer cells have been used for oncolytic virotherapy.

The viral *E1a* gene expresses immediately after infection. The primary role of E1A products is to regulate expression of multiple cellular and viral genes (5). Instead of directly binding to specific DNA sequences in transcriptional regulation elements, E1A proteins interact with several key regulators of cell proliferation (3, 20). The well-known cellular factors that E1A proteins bind with are products of the retinoblastoma (*Rb*) gene and its structurally related genes, p107 and p130 (41, 76). By sequestering the retinoblastoma protein (pRb), E1A activates transcriptional regulator E2F proteins. Studies have suggested that the pRb/E2F complex actively represses the transcription from target genes, mediates G<sub>1</sub> arrest triggered by p19 (ARF), p53, p16INK4a, TGF beta, and cell contact (38, 64, 83). Several groups have shown that expression of E1A triggers the accumulation

of p53 protein and p53-dependent apoptosis (16, 40, 44, 86) either by activating p53 transcription or preventing p53 from being degraded by the proteasome (11, 16, 40, 56).

E1B55K has been shown in some studies to counteract the E1A-induced stabilization of p53 (16, 58). E1B55K protein may inhibit the functions of p53 through at least two distinct mechanisms. E1B55K reportedly binds the amino terminus of p53 (35), and this binding may repress p53 transcriptional activation, as suggested in transcription assays (43) and transient transfection studies (79). E1B55K may also interfere with p53 function by cooperating with viral E4orf6 protein to cause proteolytic degradation of p53 protein (29, 51, 57, 77). Thereby, E1B55K blocks the expression of p53-regulated genes and, consequently, counteracts the p53-dependent apoptosis induced by E1A, allowing efficient viral replication (35, 43).

Ad *d*/1520 (ONYX-015) contains an 827-bp deletion and a point mutation generating a premature stop codon in the E1B55K coding sequence, preventing expression from the gene (4). It was originally proposed that the E1B55K-deleted Ads could only replicate in p53-deficient tumor cells because the E1B55K-mediated degradation of p53 protein might be not required in those cancer cells (8, 62). E1B55K-deleted oncolytic Ads have been applied in human clinical trials and are being marketed for cancer treatment in China (80). However, the original hypothesis was challenged by several studies showing E1B55K mutants are able to replicate in many cancer cell lines regardless of their p53

status (17, 26, 27, 63). Recent studies have shown that the accumulation of p53 protein in cells, after infection with Ads carrying mutated E1B55K genes that are unable to repress p53, can neither efficiently induce apoptosis nor transcriptionally activate expression of p53-responsive genes (32, 52). These studies have demonstrated that blocking of p53 activity by E1B55K protein is unlikely to be the major requirement for viral replication. Thus, the mechanism(s) of E1B55K-deleted viral replication of in cancer cells is still not established, even though the vectors have been applied in human cancer treatment (80).

Previously, our laboratory has shown that Ad E1B55K is involved in the induction of cell cycle-related genes, including cyclin E (60). E1B55K-mediated cyclin E induction normally plays a critical role in viral replication; however, E1B55K is not required for cyclin E induction and viral replication in cancer cells with deregulated cyclin E (85). We proposed that cyclin E deregulation in cancer cells may be an important molecular basis for oncolytic replication of E1B55K-deleted Ads (85).

Cyclin E regulates the DNA replication (14, 22), centrosome duplication (31, 45), and cell cycle progression in cells. In normal cell cycle, the level of cyclin E rises at late  $G_1$  phase and peaks at the  $G_1$ /S phase to promote the S-phase entry (37, 54). Deregulation of cyclin E is frequently detected in many types of cancers by gene amplification (42), overexpression of cyclin E mRNA or protein levels (18, 66), decrease of cyclin E turnover (73), and the presence of more active forms of cyclin E (2, 36, 78). Constitutive overexpression of cyclin E was shown to induce

chromosome instability and impair normal cell cycle progression (47, 72). The idea that abnormal cyclin E expression can trigger tumors has also been supported by several transgenic animal studies (10, 21, 39).

One of the functions of cyclin E in the cell cycle is to bind and activate cyclin-dependent kinase 2 (CDK2) (50). The cyclin E/CDK2 complex then phosphorylates substrates such as pRb and leads to transcriptional activation of downstream genes. Recent studies have indicated that cyclin E also has CDK2-independent functions (23, 24). In vivo animal studies revealed the variance between the phenotypes of cyclin E null (cyclin E1<sup>-/-</sup> E2<sup>-/-</sup>) mice and CDK2 null (CDK2<sup>-/-</sup>) mice. Mice lacking CDK2 are viable, showing no significant effects on normal development except defective germ cell development (6, 55). Yet double knockout of cyclin E1 and E2 genes in mice caused embryonic lethality owing to the deficiency in endoreplication of trophoblast giant cells and megakaryocytes (25). Matsumoto et al. (2004) identified a centrosomal localization signal (CLS) domain in cyclin E (46). This CLS domain allows cyclin E to target the centrosome and promote S phase entry in a CDK2-independent manner. Additionally, Geng et al. (2007) showed that a cyclin E kinase-deficient mutant (KD-E) is able to partially restore minichromosome maintenance protein (MCM) loading and S phase entry in cyclin E null cells (24). These results illustrate the CDK2-independent functions of cyclin E.

To further study the role Ad-induced cyclin E in the Ad infectious life cycle, we investigated whether the cyclin E function is dependent on activation of CDK2. This question may be especially important in the development of oncolytic

virotherapy strategies. We show that Ad-induced cyclin E binds with and activates CDK2 that targets transcription repressor pRb, which may in turn regulate expression of cellular and viral genes. The results suggest that the interaction between cyclin E and CDK2 generates a suitable environment for Ad productive replication.

#### CHAPTER II

#### MATERIALS AND METHODS

**Cell lines and culture conditions.** HEK 293 (ATCC no. CRL-1573), CHO (ATCC no. CCL-61) and human lung cancer A549 (ATCC no. CCL-185) cell lines were purchased from the American Type Culture Collection (Rockville, MD). The transgenic CHO cell lines were established and kindly provided to us by Dr. James L. Maller's laboratory (46). HEK 293 and A549 cell lines were cultured in MEM-Alpha. All CHO cell lines were cultured in F-12K medium. All media were supplemented with 10% FBS and penicillin/streptomycin (100 U/ml). Cells were cultured in a 5% CO<sub>2</sub> incubator at 37°C. All cell culture reagents were obtained from Gibco BRL (Bethesda, MD).

**Adenoviral vectors.** Wild-type adenovirus type 5 (Adwt, ATCC no. VR-5) was used as a replication-competent control. AdCMV/GFP, an Ad vector with E1 deletion, was used as a replication-defective control. Adhz63, an oncolytic Ad vector with the deletion of E1B55K region similar to *d*/1520, was constructed by our laboratory (59).

**Viral infection and titration.** Cells were seeded into 6-well plates at a density of  $2.5 \times 10^5$  (cells/well) and cultured under the indicated conditions. Subsequently, cells were mock-infected or infected with AdGFP, Adwt, or Adhz63 at an MOI of 5.

Cytopathic effect (CPE) caused by viral replication was observed at designed time points and photographed with an inverted microscope (Olympus CKX41). Total infected cells and cell supernatants were collected at 48 hr postinfection (p.i.) and lysed to release virus particles with three cycles of freezing and thawing. The viral titers were determined by the infective unit method as described previously (65, 84). Briefly, HEK 293 cells were seeded in 96-well plates at a density of 10<sup>3</sup> (cells/well) and then infected with 5-fold serially diluted viruses. CPE was recorded and scored after incubation for 7 days.

**Viral DNA synthesis assay.** After viral infection, A549 cells were collected at different time points. The viral DNA synthesis was determined with Southern blot; 1 µg of isolated genomic DNA was digested with the restriction enzyme *Pst*I and analyzed with 1% agarose gel, which was subsequently transblotted to a Hybond-N+ membrane (YA3609; Amersham Pharmacia Biotech, Arlington Heights, IL). The probe was prepared by digesting 0.5 µg pBHGE3 (7) with *Pst*I and labeled by following the protocol of Amersham AlkPhos Direct Labeling and Detection Systems (RPN 3690; GE Healthcare, Piscataway, NJ). The blot was prehybridized for 3 hrs at 60°C. The hybridization and stringency washes were performed at 60°C and followed by the chemiluminescent detection according to the manufacturer's protocol.

Western blot analysis. Infected A549 cells were harvested at indicated time points and lysed with CDK2 lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 5 mM

MgCl<sub>2</sub>, 0.5% Nonidet P-40, 0.1% Brij 35, 5 mM sodium glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol). The Western blot analyses were performed as described previously (86). Briefly, 80 µg of cell lysates were electrophoresed through 12% SDS-polyacrylamide gels and transferred onto an Immobilon-P Membrane (Millipore, Billerica, MA). The primary antibodies used in this study were rabbit anti-cyclin E (M-20), CDK4 (C-22), mouse anti-cyclin D1 (DCS-6), PCNA (PC10), p21 (F-5), pRb (IF8) (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-CDK2, p27 (BD Biosciences, San Jose, CA), pCDK2 T160 (Cell signaling Danvers, MA), rabbit anti-phospho-pRb S612, and actin (Sigma, St. Louis, MO), anti-phospho-pRb S795 (New England Biolabs, Beverly, MA), and anti-phospho-pRb T821 (Invitrogen, Carlsbad, CA). The membranes were then incubated with anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG peroxidase-linked species-specific whole antibody (GE Healthcare, Piscataway, NJ). Chemiluminescent detection was performed with ECL reagents according to the supplier's recommendations (GE Healthcare).

**Immunoprecipitation.** A549 cells were seeded in 150 mm dishes at a cell density of 5  $\times 10^{6}$  (cells/dish) and then mock-infected or infected with AdGFP, Adwt, or Adhz63 at an MOI of 5. At 48 hr p.i., cells were collected and lysed with CDK2 lysis buffer according to the method described in previous publications (12, 85). 500 µg cell lysates were immunoprecipitated with cyclin E (HE111), the mouse monoclonal antibody, (Santa Cruz) or anti-CDK2 antibody (BD Transduction Laboratories) at 4°C for 4 hr, followed by adding protein A Sepharose CL-4B

(82506; Sigma) and incubating overnight. Immunocomplexes were analyzed by Western blot with anti-cyclin E and CDK2 antibodies.

**siRNA transfection.** The siRNA oligonucleotides were synthesized by Eurogentec (Fremont, CA). Three different siRNA duplexes were designed to target CDK2 on nucleotides 399 to 419 (#1), 619 to 639 (#2), and 691 to 711 (#3) according to Genbank accession NM001798.2 (National Center for Biotechnology Information GenBank). A negative control siRNA duplex containing two strands of 19 complementary RNA bases with 3'dTdT overhangs was obtained from Eurogentec (SR-CL000-005). The sequences have no significant homology to any known mouse, rat, or human gene sequences. A549 cells were seeded into a 6-well plate at a density of 10<sup>5</sup> (cells/well) and then transfected with 200 nM CDK2 siRNA duplexes or a negative control siRNA duplex with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Cells were harvested at 48 hr after transfection. Eighty μg of cell lysates were analyzed by Western blot with CDK2 and actin antibodies.

#### CHAPTER III

#### RESULTS

## Cyclin E mutant unable to bind CDK2 inhibits replication of E1B55K-deleted virus.

Our laboratory has previously established the link between cyclin E and replication of E1B55K-deleted viruses (85). Cyclin E can promote S phase entry DNA replication via CDK2-dependent (50) and participate in and CDK2-independent pathways (46). To investigate the connection of CDK2 and cyclin E in Ad replication, we first applied transgenic CHO cell lines expressing Myc-tagged wild-type rat cyclin E (cycE-wt), a cyclin E mutant that is still able to bind CDK2 (cycE-SWNQ-A), or a cyclin E mutant unable to bind CDK2 (cycE-S180D) (46) to evaluate whether cyclin E binding with CDK2 plays a role in viral replication. Expression of the knock-in cyclin E genes is under the control of the tetracycline-on (Tet-on) system. We first examined the protein levels of the Myc-tagged cyclin E proteins with or without tetracycline as an expression inducer. The results showed that the expression of knock-in rat cyclin E genes is not significantly repressed in the absence of Tet (Fig. 1A). Thus, the CHO cell line without any transgenic cyclin E was used as a control for the CHO cell lines with different knock-in cyclin E genes.

To verify the infectivity of human Ad on the hamster cells, the normal CHO cells and the three transgenic CHO cells were infected with AdGFP at an MOI of

10. AdGFP is a replication-defective Ad with deletion of *E1a* and *E1b* genes and carrying green fluorescent protein (GFP) as a reporter gene. Equal expression of the green fluorescence protein was observed in all four cell lines at 48 hr p.i., indicating that all the CHO cells can be efficiently and equally infected by Ads (Fig. 1B).

We then determined the CPE caused by viral replication in the cells infected with wild-type Ad5 (Adwt) or E1B55K-deleted Adhz63 at an MOI of 10. The repeated experiments showed that there was no difference among the Adwt-caused CPE in the normal CHO and the three transgenic cell lines at 96 hr p.i., showing cells rounded up and detached from the cell monolayer (Fig. 2, a-h). However, CPE caused by E1B55K-deleted Adhz63 was repressed in the CHO (Fig. 2, i and j) and in the CHO-cycE-S180D cells (Fig. 2, o and p). The CHO cells do not express any transgenic cyclin E, while the transgenic cycE-S180D is unable to bind CDK2. More CPE was observed in Adhz63-infected CHO cells expressing cycE-wt (Fig. 2, k and I) or cycE-SWNQ-A (Fig. 2, m and n), both of which are able to bind with CDK2.

It should be noted that all the CHO cells carry their endogenous cyclin E gene. It appeared that the endogenous cyclin E was enough to support Adwt replication in CHO cells, but overexpression of transgenic cyclin E that can bind with CDK2 improved replication of Adhz63 with deletion of E1B55K. This result agrees with our previous report that oncolytic replication of E1B55K-deleted viruses is dependent on overexpression or deregulation of cyclin E in cells (85). The results presented in Figure 2 suggest that cyclin E binding to CDK2 is required for viral

replication.

#### Cyclin E/CDK2 complex is induced by replication-competent viruses.

Our laboratory has reported that A549 cells constitutively express cyclin E protein, and that Ad infection mainly induces the large form of cyclin E protein (cyclin EL) (85). Cyclin E and cyclin EL are generated from splicing and using different start ATG codons in exons 2 and 3 (54). The N terminus of cyclin EL is 15 amino acids longer than that of cyclin E. To investigate the physical interaction between CDK2 and cyclin E, we used anti-cyclin E antibody to immunoprecipitate cyclin E protein and analyzed the immunocomplexes with Western blot. A549 cells were mock-infected or infected with AdGFP, Adwt, or Adhz63. At 48 hr p.i., cells were collected and lysed. The data show that cyclin E precipitated from cells mock-treated or treated with replication-defective AdGFP (as controls) did not exhibit significant association with CDK2 protein (Fig. 3A, lanes 1 and 2). However, immunocomplexes from Adwt- and Adhz63-infected A549 cells contained both cyclin E and cyclin EL with an increase of CDK2 binding (Fig. 3A, lanes 3 and 4). Thus, the cyclin EL induced by viral replication is associated with CDK2 in the precipitated complex. To verify this cyclin E/CDK2 interaction, we also used anti-CDK2 antibody to pull down the protein complex and examined the level of cyclin E. The immunoprecipitated CDK2 protein was increased in Adwt and Adhz63-infected cells with a concomitant precipitation of cyclin EL, especially for Adwt-infected cells (Fig. 3B, lanes 3 and 4). The results indicate that replication-competent viruses (Adwt and Adhz63) induce cyclin EL expression

and increase the formation of cyclin E/CDK2 complex.

#### Adenoviruses activate cyclin E/CDK2 but not cyclin D/CDK4.

Consistent with the previous published study in our laboratory (85), we detected that the faster-migrating band, cyclin E, was constitutively produced by A549 cells and that the slower-migrating band, cyclin EL, was markedly induced by the replicative Adwt and Adhz63 (Fig. 4A, lanes 3 and 4, 7 and 8). Previous studies showed that the CDK2 is activated by the phosphorylation on the T160 site and that this phosphorylation increases its electrophoretic mobility, resulting in faster-migrating bands (28). Analysis of the cell lysates with Western blot demonstrated that the cyclin EL induction led to an increase of the faster-migrating CDK2, consistent with pCDK2 T160 (the active form of CDK2), which is significantly increased at 48 hr p.i. (Fig. 4A, lanes 7 and 8). We verified that the phosphorylated pCDK2 T160 was increased by Adwt and Adhz63 with the antibody that specifically targets this phosphorylated CDK2 protein (Fig. 4B).

We also examined the level of cyclin D since both cyclin D and cyclin E are involved in the transition of the G<sub>1</sub>/S phase. Interestingly, the cyclin D level was decreased after viral infection (Fig. 4C, lanes 3 and 4, and 7 and 8). Meanwhile, the level of CDK4 and the proliferating cell nuclear antigen (PCNA) did not significantly change in any of the groups. CDK4 is regulated and activated by cyclin D to process the G<sub>1</sub>-S transition (34, 70). PCNA is known to regulate DNA replication and DNA repair, and is also associated with multiple cyclin/CDK complexes in the cell-cycle progression (48, 82). Our results show that Ads

specifically activate cyclin E and CDK2, but not cyclin D and CDK4, suggesting a critical role for cyclin E and CDK2 in Ad replication.

#### Adenoviruses increase pRb phosphorylation and repress CDK inhibitors.

CDK2 activated by cyclin E is known to control the G<sub>1</sub>-S transition by phosphorylation of the downstream substrates. Considering that pRb is one of the well-known targets for pCDK2, we examined whether the increase of active pCDK2 alters the phosphorylation of pRb on S612, which is a CDK2-preferred phosphorylation residue (67, 81). We found that phospho-pRb S612 was strongly increased in cells infected with replication-competent Adwt and Adhz63, even though the protein level of unphophorylated pRb is decreased approximately two fold (Fig. 5A, lanes 3 and 4). We could not detect any significant changes of pRb phosphorylation at the CDK2-prefferred phosphorylation residue T821 (33, 81) and the CDK4-prefferred S795 (13) (Fig. 5A), suggesting the specific selection of pRb phosphorylation at S612 by CDK2. We also observed that the protein levels of both p21 and p27 are decreased in the virus-infected cells, especially for p21 which is strongly inhibited (Fig 5B, lanes 3 and 4, and 7 and 8). p21 and p27 are the well-known CDK inhibitors, which negatively regulate the activity of cyclin/CDK complexes to prevent the cell-cycle progression (71). Our results suggest that Ads activate the CDK2 by inducing cyclin E and repressing p21 and p27, and that the cyclin E-activated CDK2 in turn increases pRb phosphorylation at S612 to promote viral production.

#### CDK2 chemical inhibitor reduces adenoviral replication.

To further investigate the role of CDK2 in viral replication, we used the chemical CDK2 inhibitor, roscovitine (Ros; CYC202), to interrupt cyclin E and CDK2 interaction. Ros is a purine derivative that inhibits the activity of CDK2 by binding to its active site (15). Ros reduces phosphorylation on CDK2 (61) and blocks the androstenedione-induced increase in active phosphorylated CDK2 (1). If CDK2 is required for viral replication, blocking CDK2 activity should reduce replication. Figure 6A, representing one of the three repeated experiments, shows that with increased Ros, CPE caused by infection with Adwt and Adhz63 was partially inhibited. Figure 6B shows that 5 µM of Ros led to a 2-fold decrease of Adwt titer (P = 2.17E-04) and a 3.5-fold decrease of Adhz63 titer (P = 0.034) when compared with the control group treated with virus and DMSO. Figure 6 B also shows that 10 µM of Ros led to even more decreases of viral titers, a 5-fold decrease for Adwt (P = 1.01E-05) and a 7.5-fold decrease for Adhz63 (P = 0.012). The repressed viral yields are consistent with the CPE phenomenon in Figure 6A. Thus, inhibiting CDK2 by Ros decreases viral production.

We further examined the levels of viral DNA and proteins produced in cells affected by Ros treatment. The viral DNA synthesis was determined by Southern blot probed with the Ad genome (Fig. 7A). The viral DNA synthesis of Adwt and Adhz63 at 24 hr p.i. was strongly inhibited in the presence of 10  $\mu$ M Ros. Consistently, the viral capsid proteins were significantly inhibited in the presence of 10  $\mu$ M Ros (Fig. 7B). We also examined the change of phospho-pRb S612 with Ros treatment. We found that inhibition of CDK2 activity by Ros repressed the

phosphorylation of pRb at S612 site induced by Ad infection (Fig. 7C, lanes 4 and 6). Interestingly, Ros treatment markedly repressed cyclin E protein production induced by Adwt and Adhz63 (Fig. 7C, lanes 4 and 6). To sum up, our data suggest inhibition of CDK2 with the chemical reagent roscovitine reduced viral DNA synthesis, capsid proteins expression, phospho-pRb S612 and cyclin E induction, inhibiting viral replication.

#### siRNA inhibiting CDK2 represses adenoviral replication by targeting pRb.

Since the chemical inhibitor Ros may also influence other CDKs and cellular kinases, we applied RNA interference to specifically silenced CDK2 expression in further experiments. We tested three different pairs of siRNA duplexes targeting CDK2 on the coding region and showed that all CDK2 siRNAs dramatically inhibited CDK2 expression in A549 cells (Fig. 8A). The #1 siRNA appears to have the most sizeable inhibiting effects. To evaluate the effects of CDK2 on the cellular protein production in response to viral infection, A549 cells were infected with Adhz63 after the cells treated with CDK2 siRNA duplex (#1) or a non-specific control siRNA for 48 hrs. We focused on the E1B55K-deleted Adhz63 because it is more sensitive to the inhibition of CDK2 activity as shown in figures 6 and 7. As expected, the CDK2 siRNA specifically repressed production of CDK2 protein and decreased pCDK2 T160 (Fig. 8B). Repression of CDK2 resulted in reduced CDK2-specific phosphorylation on pRb, but did not affect pRb protein level (Fig. 8B). We also observed that repression of CDK2 specifically decreased Ad-induced cyclin EL, but not cyclin E. Blockage of CDK2 expression with siRNA

notably caused a 3-fold decrease of viral titer (P = 0.03, Fig. 8C). These findings show that specifically inhibiting CDK2 with siRNA significantly represses viral production, correlating with the decreased CDK2 activation.

#### CHAPTER IV

#### DISCUSSION

Previously our laboratory has shown that induction of cyclin E is required for Ad replication (60, 85). In this report, we demonstrate that CDK2 activation by cyclin E is a critical molecular step in Ad replication. Four lines of evidence support the importance of activation of CDK2 by cyclin E in Ad replication. First, the replication of E1B55K-deleted virus was partially inhibited in the transgenic CHO cell expressing a cyclin E mutant unable to bind CDK2. Second, Ad-induced cyclin E directly interacted with CDK2 and formed the cyclin E/CDK2 complex, leading to increased phosphorylation of CDK2 and pRb. Third, the CDK2 chemical inhibitor, roscovitine, decreased viral replication. Finally, a siRNA specifically inhibiting CDK2 repressed the viral replication with the decrease in pRb phosphorylation. These four lines of evidence support the hypothesis that Ad-induced cyclin E activates CDK2, which targets the transcription suppressor pRb, resulting in controlling cellular and viral gene expression for productive viral replication (Fig. 9).

Considering that CDK2-independent functions of cyclin E are related to participation in DNA replication licensing (24) and oncogenic transformation (23), we first used the CHO cell line expressing transgenic wild-type or mutated cyclin E genes to investigate whether the ability of cyclin E to bind to CDK2 plays an important role in the viral replication. Expression of transgenic cyclin E genes did

not affect Adwt replication. Adwt, containing the intact E1B55K region, can elicit endogenous cyclin E expression (60, 85), and, thus, its replication is not dependent on expression of the transgenic cyclin E genes. However, replication of the E1B55K-deleted virus is enhanced in CHO cells expressing transgenic cyclin E proteins that can bind with CDK2, but not in CHO cells without transgenic cyclin E or expressing cycE-S180D mutant that is unable to bind CDK2 (Fig. 2). Consistent with the results of using transgenic CHO cell lines, the CDK2 chemical inhibitor Ros decreased viral DNA synthesis and protein production (Figs. 6 and 7). Ros inhibits the activity of CDK2 by binding to its active site (15). Our results suggest that cyclin E binding with and activating CDK2 is an important step in Ad replication.

Cyclin E and the large form cyclin EL are generated from alternative splicing. The translation of cyclin EL is initiated at an ATG codon located in exon 2 and cyclin E is from the ATG codon in exon 3 (54). Previously our laboratory constructed a plasmid, pTet-cycE, containing cyclin E cDNA that produces these two forms of cyclin E proteins (85). The A549 cell line constitutively expresses the regular cyclin E protein (cyclin E), and Ad infection mainly induces the expression of large form cyclin E protein (cyclin EL) (85). In this study our finding indicates that Ad-induced cyclin EL preferentially associates with CDK2 protein (Fig. 3). We cannot exclude that cyclin E in A549 may also interact with CDK2. However, we only observed detectable levels of CDK2 along with cyclin EL after Ad infection with replication competent Adwt and Adhz63, suggesting cyclin EL highly interacts with CDK2. Consistently, Harwell *et al.* reported that the addition of exogenous

cyclin EL increases the activity and phosphorylation of CDK2 *in vitro* (30). They showed that cyclin EL can bind to endogenous CDK2 in complex with cyclin E, cyclin A, or unbound CDK2, and the cyclin-activating kinase is involved in the phosphorylation of CDK2 in this event. It is still unclear why Ad infection mainly induces cyclin EL that is highly associated with and activates CDK2.

We identified that Ad-induced cyclin EL correlates with the increase of phosphorylated CDK2 at T160 and phosphorylated pRb at S612 (Figs. 4A and 5A). Three phosphorylation sites have been identified in CDK2 (28). T160 phosphorylation is essential for CDK2 activity while T14 and Y15 phosphorylation cause an inhibitory effect. The retinoblastoma tumor suppressor pRb is inactivated by CDK's phosphorylation and, presumably, enables E2F transcription factor to be released from the pRb/E2F complex to carry out the downstream gene regulation (75). Phosphopeptide analysis of pRb showed that S612 is one of the CDK2-preferred phosphorylation sites (81). The presence of pCDK2 T160 and phospho-pRb S612 provides the evidence to indicate the increased CDK2 activity caused by Ad-induced cyclin EL. We also examined the level of pRb with phosphorylation of T821 (CDK2-preffered) and S795 (CDK4-preffered); however, we did not detect any significant change on these two sites (Fig. 5A). Inhibition of CDK2 expression with the CDK2 siRNA repressed phosphorylation on CDK2 and pRb (Fig. 8B) and decreased viral replication (Fig. 8C). Our results indicate that Ad-induced cyclin EL activates CDK2, which specifically introduces pRb phosphorylation on S612 site.

We detected a notable decrease of CDK inhibitors p21 and p27 in the

Ad-infected cells. The decrease may be the result of the highly induced cyclin E/CDK2 complex. p21 and p27 inhibit the activity of cyclin/CDK complexes to prevent the cell-cycle progression, and their protein stability is also regulated by cyclin/CDK complexes (68, 71, 87). Phosphorylation of p27 by cyclin E/CDK2 causes its degradation (68, 74). Montagnoli *et al.* showed that cyclin E/CDK2-dependent phosphorylation of p27 on threonine 187 facilitates the formation of a trimeric complex with cyclin E/CDK2 and leads to p27 ubiquitination (49). In agreement with our findings, recent studies also suggest that CDK may promote p21 degradation (9, 87). Thus, the activated cyclin E and CDK2 may decrease the CDK inhibitors p21 and p27 to benefit viral replication. Whether cyclin E/CDK2 complex directly or indirectly governs the degradation of p21 and p27 has yet to be clarified.

The pRb phosphorylation by cyclin E/CDK2 may lead to regulation of cellular and viral genes for Ad replication. Interestingly, Ad-induced cyclin EL expression is inhibited by a CDK2 chemical inhibitor and CDK2 siRNA (Figs. 7C and 8B). It seems that inhibition of CDK2 interferes in the cyclin E induction via a loopback regulation (Fig. 9). Previous studies have reported that cyclin E gene is the downstream target of E2F (19, 53). In our previous work, we showed that the cyclin E promoter is more active in cancer cells and that the promoter activity is further enhanced after viral replication (85). We suggest that cyclin E activates the cyclin E-CDK2-pRb/E2F pathway and cyclin E itself is also one of the targets of the pathway.

In summary, these results demonstrate that Ad-induced cyclin E plays a

critical role in Ad replication by activation of CDK2. Our results suggest that cyclin EL binds to and activates CDK2; subsequently, the active cyclin E/CDK2 phosphorylates the transcription suppressor pRb, which can regulate expression of multiple cellular and viral genes, including the looping back further increasing cyclin E production. Therefore, CDK2 activation by cyclin E appears to generate a suitable environment for viral replication (Fig. 9). Our results not only help to further reveal the mechanism of Ad replication, but also provide the basis for the development of future oncolytic vectors. The experiments regarding the novel oncolytic viruses are ongoing.





Fig. 1. Transgenic cyclin E expression and the infectivity of AdGFP in CHO cells. (A) 5  $\times 10^5$  of CHO cells expressing Myc-tagged wild-type rat cyclin E (cycE-wt), Myc-tagged cyclin E mutant able to bind CDK2 (cycE-SWNQ-A), or Myc-tagged cyclin E mutant unable to bind CDK2 (cycE-S180D) were cultured in the presence or absence of 10 µg/mL tetracycline for 96 hrs and cell lysates were analyzed by Western blot. (B) Wild-type (CHO) and transgenic CHO cells were infected with AdGFP at an MOI of 10. All fluorescent microscopy is at taken at 48 hr p.i. with an Olympus IX50 microscope (original magnification of x100).



Fig. 2. Comparison of viral replication in normal and Myc-cycE transgenic CHO cells.  $5 \times 10^5$  of CHO cells were mocked-infected or infected with AdGFP, Adwt or Adhz63 at an MOI of 10. CPE was observed at 96 hr p.i. Photographs are presented in duplicate (original magnification of x100).



Fig. 3. Cyclin E/CDK2 complex induced by viral infection in A549 cells. (A) A549 cell lysates were immunoprecipitated with anti-cyclin E antibody (1:50 dilution). Immunocomplexes were analyzed by Western blot with cyclin E and CDK2 antibodies. (B) The cell lysates were immunoprecipitated with anti-CDK2 antibody and immunoblotted for CDK2 and cyclin E.



Fig. 4. Effects of viral replication on cellular proteins related to G<sub>1</sub>/S phase. A549 cells were mock-infected or infected with AdGFP (GFP), Adwt (wt), or Adhz63 (63) at an MOI of 5. Cells were collected at 24 hr or 48hr p.i., and then analyzed by Western blot. Cell lysates were immunoblotted for (A) cyclin E, CDK2, and actin; (B) pCDK2 T160 and actin; and (C) cyclin D, CDK4, PCNA and actin. Actin was used as the loading control.



Fig. 5. Effects of viral replication on pRb and CDK inhibitors. A549 cells were mock-infected or infected with AdGFP (GFP), Adwt (wt), or Adhz63 (63) and collected at 24 hr or 48 hr p.i., followed by Western blot analysis. Cell lysates were immunoblotted for (A) phospho-pRb (p-pRb) at S612, T821, S795 and actin; or (B) p21, p27 and actin. Actin was used as the loading control.



Fig. 6. Effects of roscovitine on CPE and viral production. (A) Cells were treated with 0  $\mu$ M, 5  $\mu$ M or 10  $\mu$ M of roscovitine (Calbiochem, Darmstadt, Germany), and mock-infected or infected with AdGFP, Adwt or Adhz63 at an MOI of 5. All microscopy is originally at a magnification of x100 taken at 48 hr p.i. (B) Viral titers were determined at 48 hr p.i. with infection unit method. The values represent the means $\pm$  S.D. of independent quadruplicate. \* P<0.05 compared with the DMSO-control group, Student's *t*-test.



Fig. 7. Effects of Ros on viral DNA synthesis, viral capsid proteins, virus-induced cyclin E and phospho-pRb S612. (A) A549 cells were collected at 0 hr and 24 hr p.i. Viral DNA synthesis was determined by Southern blot. (B) At 24 hr p.i., cells were harvested and cell lysates were immunoblotted for adenovirus type 5 capsid proteins, (C) cyclin E (M-20), pRb S612, and actin. Actin was used as a loading control.



Fig. 8. Effects of CDK2-specific siRNA on oncolytic viral replication. (A) A549 cells were transfected with 200 nM siRNA duplexes targeting different coding regions of CDK2. "Mix" represents the mixture of three pairs of siRNA duplexes (#1 to #3). Cells were harvested at 48 hr after transfection and analyzed by Western blot. (B) At 48 hr after transfection with CDK2 siRNA duplex (#1) or a negative control siRNA, cells were infected with Adhz63 at an MOI of 5. Cells were harvested at 24 hr after infection and analyzed by Western blot. (C) The viral titers were determined at 48 hr p.i. with the infection unit method. The values are means  $\pm$ S.D. of independent triplicate. \* P<0.05 compared with the negative control group, Student's *t*-test.



Fig. 9. Proposed mechanism of cyclin E function in Ad replication. In Ad-infected cells, cyclin E binds to and activates CDK2. Subsequently, active pCDK2 phosphorylates the transcription repressor pRb, leading to the downstream cellular gene expression to provide a suitable environment for the viral replication.

#### REFERENCES

- Akli, S., T. Bui, H. Wingate, A. Biernacka, S. Moulder, S. L. Tucker, K. K. Hunt, and K. Keyomarsi. 2010. Low-molecular-weight cyclin E can bypass letrozole-induced G1 arrest in human breast cancer cells and tumors. Clin Cancer Res 16:1179-1190.
- Akli, S., P. J. Zheng, A. S. Multani, H. F. Wingate, S. Pathak, N. Zhang, S. L. Tucker, S. Chang, and K. Keyomarsi. 2004. Tumor-specific low molecular weight forms of cyclin E induce genomic instability and resistance to p21, p27, and antiestrogens in breast cancer. Cancer Res 64:3198-3208.
- 3. Avvakumov, N., M. Sahbegovic, Z. Zhang, M. Shuen, and J. S. Mymryk. 2002. Analysis of DNA binding by the adenovirus type 5 E1A oncoprotein. J Gen Virol 83:517-524.
- 4. Barker, D. D., and A. J. Berk. 1987. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. Virology 156:107-121.
- 5. Ben-Israel, H., and T. Kleinberger. 2002. Adenovirus and cell cycle control. Front Biosci 7:d1369-1395.
- 6. Berthet, C., E. Aleem, V. Coppola, L. Tessarollo, and P. Kaldis. 2003. Cdk2 knockout mice are viable. Curr Biol 13:1775-1785.
- 7. Bett, A. J., W. Haddara, L. Prevec, and F. L. Graham. 1994. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc Natl Acad Sci U S A 91:8802-8806.
- Bischoff, J. R., D. H. Kirn, A. Williams, C. Heise, S. Horn, M. Muna, L. Ng, J. A. Nye, A. Sampson-Johannes, A. Fattaey, and F. McCormick. 1996. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science 274:373-376.
- Bornstein, G., J. Bloom, D. Sitry-Shevah, K. Nakayama, M. Pagano, and A. Hershko. 2003. Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. J Biol Chem 278:25752-25757.
- 10. Bortner, D. M., and M. P. Rosenberg. 1997. Induction of mammary gland hyperplasia and carcinomas in transgenic mice expressing human cyclin E.

Mol Cell Biol 17:453-459

- Braithwaite, A., C. Nelson, A. Skulimowski, J. McGovern, D. Pigott, and J. Jenkins. 1990. Transactivation of the p53 oncogene by E1a gene products. Virology 177:595-605.
- Chou, M. M., J. M. Masuda-Robens, and M. L. Gupta. 2003. Cdc42 promotes G1 progression through p70 S6 kinase-mediated induction of cyclin E expression. J Biol Chem 278:35241-35247.
- 13. Connell-Crowley, L., J. W. Harper, and D. W. Goodrich. 1997. Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol Biol Cell 8:287-301.
- 14. Coverley, D., H. Laman, and R. A. Laskey. 2002. Distinct roles for cyclins E and A during DNA replication complex assembly and activation. Nat Cell Biol 4:523-528.
- De Azevedo, W. F., S. Leclerc, L. Meijer, L. Havlicek, M. Strnad, and S. H. Kim. 1997. Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine. Eur J Biochem 243:518-526.
- 16. Debbas, M., and E. White. 1993. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev 7:546-554.
- 17. Dix, B. R., S. J. Edwards, and A. W. Braithwaite. 2001. Does the antitumor adenovirus ONYX-015/dl1520 selectively target cells defective in the p53 pathway? J Virol 75:5443-5447.
- 18. Donnellan, R., and R. Chetty. 1999. Cyclin E in human cancers. FASEB J 13:773-780.
- 19. Duronio, R. J., and P. H. O'Farrell. 1995. Developmental control of the G1 to S transition in Drosophila: cyclin Eis a limiting downstream target of E2F. Genes Dev 9:1456-1468.
- 20. Dyson, N., and E. Harlow. 1992. Adenovirus E1A targets key regulators of cell proliferation. Cancer Surv 12:161-195.
- 21. Freemantle, S. J., and E. Dmitrovsky. 2010. Cyclin E transgenic mice: discovery tools for lung cancer biology, therapy, and prevention. Cancer Prev Res (Phila) 3:1513-1518.
- 22. Furstenthal, L., B. K. Kaiser, C. Swanson, and P. K. Jackson. 2001. Cyclin E uses Cdc6 as a chromatin-associated receptor required for DNA replication. J

Cell Biol 152:1267-1278.

- 23. Geisen, C., and T. Moroy. 2002. The oncogenic activity of cyclin E is not confined to Cdk2 activation alone but relies on several other, distinct functions of the protein. J Biol Chem 277:39909-39918.
- 24. Geng, Y., Y. M. Lee, M. Welcker, J. Swanger, A. Zagozdzon, J. D. Winer, J. M. Roberts, P. Kaldis, B. E. Clurman, and P. Sicinski. 2007. Kinase-independent function of cyclin E. Mol Cell 25:127-139.
- 25. Geng, Y., Q. Yu, E. Sicinska, M. Das, J. E. Schneider, S. Bhattacharya, W. M. Rideout, R. T. Bronson, H. Gardner, and P. Sicinski. 2003. Cyclin E ablation in the mouse. Cell 114:431-443.
- 26. Geoerger, B., J. Grill, P. Opolon, J. Morizet, G. Aubert, M. J. Terrier-Lacombe, B. Bressac De-Paillerets, M. Barrois, J. Feunteun, D. H. Kirn, and G. Vassal. 2002. Oncolytic activity of the E1B-55 kDa-deleted adenovirus ONYX-015 is independent of cellular p53 status in human malignant glioma xenografts. Cancer Res 62:764-772.
- 27. Goodrum, F. D., and D. A. Ornelles. 1998. p53 status does not determine outcome of E1B 55-kilodalton mutant adenovirus lytic infection. J Virol 72:9479-9490.
- 28. Gu, Y., J. Rosenblatt, and D. O. Morgan. 1992. Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. EMBO J 11:3995-4005.
- 29. Harada, J. N., A. Shevchenko, D. C. Pallas, and A. J. Berk. 2002. Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery. J Virol 76:9194-9206.
- 30. Harwell, R. M., B. B. Mull, D. C. Porter, and K. Keyomarsi. 2004. Activation of cyclin-dependent kinase 2 by full length and low molecular weight forms of cyclin E in breast cancer cells. J Biol Chem 279:12695-12705.
- 31. Hinchcliffe, E. H., C. Li, E. A. Thompson, J. L. Maller, and G. Sluder. 1999. Requirement of Cdk2-cyclin E activity for repeated centrosome reproduction in Xenopus egg extracts. Science 283:851-854.
- Hobom, U., and M. Dobbelstein. 2004. E1B-55-kilodalton protein is not required to block p53-induced transcription during adenovirus infection. J Virol 78:7685-7697.
- 33. Hsieh, W. S., R. Soo, B. K. Peh, T. Loh, D. Dong, D. Soh, L. S. Wong, S. Green, J. Chiao, C. Y. Cui, Y. F. Lai, S. C. Lee, B. Mow, R. Soong, M. Salto-Tellez, and B. C. Goh. 2009. Pharmacodynamic effects of seliciclib, an

orally administered cell cycle modulator, in undifferentiated nasopharyngeal cancer. Clin Cancer Res 15:1435-1442.

- 34. Hunter, T., and J. Pines. 1994. Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. Cell 79:573-582.
- 35. Kao, C. C., P. R. Yew, and A. J. Berk. 1990. Domains required for in vitro association between the cellular p53 and the adenovirus 2 E1B 55K proteins. Virology 179:806-814.
- 36. Keyomarsi, K., S. L. Tucker, T. A. Buchholz, M. Callister, Y. Ding, G. N. Hortobagyi, I. Bedrosian, C. Knickerbocker, W. Toyofuku, M. Lowe, T. W. Herliczek, and S. S. Bacus. 2002. Cyclin E and survival in patients with breast cancer. N Engl J Med 347:1566-1575.
- 37. Le Cam, L., J. Polanowska, E. Fabbrizio, M. Olivier, A. Philips, E. Ng Eaton, M. Classon, Y. Geng, and C. Sardet. 1999. Timing of cyclin E gene expression depends on the regulated association of a bipartite repressor element with a novel E2F complex. EMBO J 18:1878-1890.
- 38. Liu, Y., M. E. Costantino, D. Montoya-Durango, Y. Higashi, D. S. Darling, and D. C. Dean. 2007. The zinc finger transcription factor ZFHX1A is linked to cell proliferation by Rb-E2F1. Biochem J 408:79-85.
- 39. Loeb, K. R., H. Kostner, E. Firpo, T. Norwood, D. T. K, B. E. Clurman, and J. M. Roberts. 2005. A mouse model for cyclin E-dependent genetic instability and tumorigenesis. Cancer Cell 8:35-47.
- 40. Lowe, S. W., and H. E. Ruley. 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev 7:535-545.
- 41. Ludlow, J. W., and G. R. Skuse. 1995. Viral oncoprotein binding to pRB, p107, p130, and p300. Virus Res 35:113-121.
- 42. Marone, M., G. Scambia, C. Giannitelli, G. Ferrandina, V. Masciullo, A. Bellacosa, P. Benedetti-Panici, and S. Mancuso. 1998. Analysis of cyclin E and CDK2 in ovarian cancer: gene amplification and RNA overexpression. Int J Cancer 75:34-39.
- 43. Martin, M. E., and A. J. Berk. 1998. Adenovirus E1B 55K represses p53 activation in vitro. J Virol 72:3146-3154.
- 44. Mathai, J. P., M. Germain, R. C. Marcellus, and G. C. Shore. 2002. Induction and endoplasmic reticulum location of BIK/NBK in response to apoptotic signaling by E1A and p53. Oncogene 21:2534-2544.

- 45. Matsumoto, Y., K. Hayashi, and E. Nishida. 1999. Cyclin-dependent kinase 2 (Cdk2) is required for centrosome duplication in mammalian cells. Curr Biol 9:429-432.
- 46. Matsumoto, Y., and J. L. Maller. 2004. A centrosomal localization signal in cyclin E required for Cdk2-independent S phase entry. Science 306:885-888.
- 47. Minella, A. C., J. Swanger, E. Bryant, M. Welcker, H. Hwang, and B. E. Clurman. 2002. p53 and p21 form an inducible barrier that protects cells against cyclin E-cdk2 deregulation. Curr Biol 12:1817-1827.
- 48. Moldovan, G. L., B. Pfander, and S. Jentsch. 2007. PCNA, the maestro of the replication fork. Cell 129:665-679.
- 49. Montagnoli, A., F. Fiore, E. Eytan, A. C. Carrano, G. F. Draetta, A. Hershko, and M. Pagano. 1999. Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev 13:1181-1189.
- 50. Moroy, T., and C. Geisen. 2004. Cyclin E. Int J Biochem Cell Biol 36:1424-1439.
- 51. Nevels, M., S. Rubenwolf, T. Spruss, H. Wolf, and T. Dobner. 1997. The adenovirus E4orf6 protein can promote E1A/E1B-induced focus formation by interfering with p53 tumor suppressor function. Proc Natl Acad Sci U S A 94:1206-1211.
- 52. O'Shea, C. C., L. Johnson, B. Bagus, S. Choi, C. Nicholas, A. Shen, L. Boyle, K. Pandey, C. Soria, J. Kunich, Y. Shen, G. Habets, D. Ginzinger, and F. McCormick. 2004. Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. Cancer Cell 6:611-623.
- 53. Ohtani, K., J. DeGregori, and J. R. Nevins. 1995. Regulation of the cyclin E gene by transcription factor E2F1. Proc Natl Acad Sci U S A 92:12146-12150.
- 54. Ohtsubo, M., A. M. Theodoras, J. Schumacher, J. M. Roberts, and M. Pagano. 1995. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol Cell Biol 15:2612-2624.
- Ortega, S., I. Prieto, J. Odajima, A. Martin, P. Dubus, R. Sotillo, J. L. Barbero, M. Malumbres, and M. Barbacid. 2003. Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. Nat Genet 35:25-31.
- 56. Pomerantz, J., N. Schreiber-Agus, N. J. Liegeois, A. Silverman, L. Alland, L. Chin, J. Potes, K. Chen, I. Orlow, H. W. Lee, C. Cordon-Cardo, and R. A. DePinho. 1998. The Ink4a tumor suppressor gene product, p19Arf, interacts

with MDM2 and neutralizes MDM2's inhibition of p53. Cell 92:713-723.

- 57. Querido, E., P. Blanchette, Q. Yan, T. Kamura, M. Morrison, D. Boivin, W. G. Kaelin, R. C. Conaway, J. W. Conaway, and P. E. Branton. 2001. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes Dev 15:3104-3117.
- 58. Querido, E., R. C. Marcellus, A. Lai, R. Charbonneau, J. G. Teodoro, G. Ketner, and P. E. Branton. 1997. Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. J Virol 71:3788-3798.
- 59. Rao, X. M., M. T. Tseng, X. Zheng, Y. Dong, A. Jamshidi-Parsian, T. C. Thompson, M. K. Brenner, K. M. McMasters, and H. S. Zhou. 2004. E1A-induced apoptosis does not prevent replication of adenoviruses with deletion of E1b in majority of infected cancer cells. Cancer Gene Ther 11:585-593.
- 60. Rao, X. M., X. Zheng, S. Waigel, W. Zacharias, K. M. McMasters, and H. S. Zhou. 2006. Gene expression profiles of normal human lung cells affected by adenoviral E1B. Virology 350:418-428.
- 61. Raynaud, F. I., S. R. Whittaker, P. M. Fischer, S. McClue, M. I. Walton, S. E. Barrie, M. D. Garrett, P. Rogers, S. J. Clarke, L. R. Kelland, M. Valenti, L. Brunton, S. Eccles, D. P. Lane, and P. Workman. 2005. In vitro and in vivo pharmacokinetic-pharmacodynamic relationships for the trisubstituted aminopurine cyclin-dependent kinase inhibitors olomoucine, bohemine and CYC202. Clin Cancer Res 11:4875-4887.
- 62. Rogulski, K. R., S. O. Freytag, K. Zhang, J. D. Gilbert, D. L. Paielli, J. H. Kim, C. C. Heise, and D. H. Kirn. 2000. In vivo antitumor activity of ONYX-015 is influenced by p53 status and is augmented by radiotherapy. Cancer Res 60:1193-1196.
- 63. Rothmann, T., A. Hengstermann, N. J. Whitaker, M. Scheffner, and H. zur Hausen. 1998. Replication of ONYX-015, a potential anticancer adenovirus, is independent of p53 status in tumor cells. J Virol 72:9470-9478.
- 64. Rowland, B. D., S. G. Denissov, S. Douma, H. G. Stunnenberg, R. Bernards, and D. S. Peeper. 2002. E2F transcriptional repressor complexes are critical downstream targets of p19(ARF)/p53-induced proliferative arrest. Cancer Cell 2:55-65.
- Sandig, V., R. Youil, A. J. Bett, L. L. Franlin, M. Oshima, D. Maione, F. Wang, M. L. Metzker, R. Savino, and C. T. Caskey. 2000. Optimization of the helper-dependent adenovirus system for production and potency in vivo. Proc

Natl Acad Sci U S A 97:1002-1007.

- 66. Sawasaki, T., K. Shigemasa, Y. Shiroyama, T. Kusuda, T. Fujii, T. H. Parmley, T. J. O'Brien, and K. Ohama. 2001. Cyclin E mRNA overexpression in epithelial ovarian cancers: inverse correlation with p53 protein accumulation. J Soc Gynecol Investig 8:179-185.
- 67. Schmitz, N. M., K. Leibundgut, and A. Hirt. 2005. CDK2 catalytic activity and loss of nuclear tethering of retinoblastoma protein in childhood acute lymphoblastic leukemia. Leukemia 19:1783-1787.
- 68. Sheaff, R. J., M. Groudine, M. Gordon, J. M. Roberts, and B. E. Clurman. 1997. Cyclin E-CDK2 is a regulator of p27Kip1. Genes Dev 11:1464-1478.
- 69. Shenk, T. 2001. Adenoviridae: the viruses and their replication, p. 2265-2300. *In* D. M. Knipe and P. M. Howley (ed.), Fields Virology, 4 ed, vol. 2. Lippincott Williams & Wilkins, Philadelphia, PA.
- 70. Sherr, C. J. 1995. D-type cyclins. Trends Biochem Sci 20:187-190.
- 71. Sherr, C. J., and J. M. Roberts. 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 9:1149-1163.
- 72. Spruck, C. H., K. A. Won, and S. I. Reed. 1999. Deregulated cyclin E induces chromosome instability. Nature 401:297-300.
- 73. Strohmaier, H., C. H. Spruck, P. Kaiser, K. A. Won, O. Sangfelt, and S. I. Reed. 2001. Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line. Nature 413:316-322.
- 74. Vlach, J., S. Hennecke, and B. Amati. 1997. Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. EMBO J 16:5334-5344.
- 75. Weinberg, R. A. 1995. The retinoblastoma protein and cell cycle control. Cell 81:323-330.
- 76. Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow. 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature 334:124-129.
- 77. Wienzek, S., J. Roth, and M. Dobbelstein. 2000. E1B 55-kilodalton oncoproteins of adenovirus types 5 and 12 inactivate and relocalize p53, but not p51 or p73, and cooperate with E4orf6 proteins to destabilize p53. J Virol 74:193-202.

- 78. Wingate, H., A. Puskas, M. Duong, T. Bui, D. Richardson, Y. Liu, S. L. Tucker, C. Van Pelt, L. Meijer, K. Hunt, and K. Keyomarsi. 2009. Low molecular weight cyclin E is specific in breast cancer and is associated with mechanisms of tumor progression. Cell Cycle 8:1062-1068.
- 79. Yew, P. R., and A. J. Berk. 1992. Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. Nature 357:82-85.
- 80. Yu, W., and H. Fang. 2007. Clinical trials with oncolytic adenovirus in China. Curr Cancer Drug Targets 7:141-148.
- Zarkowska, T., and S. Mittnacht. 1997. Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J Biol Chem 272:12738-12746.
- Zhang, H., Y. Xiong, and D. Beach. 1993. Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes. Mol Biol Cell 4:897-906.
- 83. Zhang, H. S., A. A. Postigo, and D. C. Dean. 1999. Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition. Cell 97:53-61.
- 84. Zhao, T., X. M. Rao, X. Xie, L. Li, T. C. Thompson, K. M. McMasters, and H. S. Zhou. 2003. Adenovirus with insertion-mutated E1A selectively propagates in liver cancer cells and destroys tumors in vivo. Cancer Res 63:3073-3078.
- 85. Zheng, X., X. M. Rao, J. G. Gomez-Gutierrez, H. Hao, K. M. McMasters, and H. S. Zhou. 2008. Adenovirus E1B55K region is required to enhance cyclin E expression for efficient viral DNA replication. J Virol 82:3415-3427.
- 86. Zheng, X., X. M. Rao, C. L. Snodgrass, K. M. McMasters, and H. S. Zhou. 2006. Selective replication of E1B55K-deleted adenoviruses depends on enhanced E1A expression in cancer cells. Cancer Gene Ther 13:572-583.
- 87. Zhu, H., L. Nie, and C. G. Maki. 2005. Cdk2-dependent Inhibition of p21 stability via a C-terminal cyclin-binding motif. J Biol Chem 280:29282-29288.

## APPENDIX

## List of Abbreviations

Ad	adenovirus
CPE	cytopathic effect
CDK2	cyclin-dependent kinase 2
СНО	Chinese hamster ovary
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
GFP	green fluorescent protein
IP	immunoprecipitation
ΜΟΙ	multiplicity of infection
MEM-Alpha	minimal essential medium Alpha
PBS	phosphate buffered saline
Rb	retinoblastoma gene
pRb	retinoblastoma protein
phospho-pRb	phosphorylated pRb
Ros	roscovitine
siRNA	small interfering RNA
Tet	tetracycline
wt	wild-type

#### CURRICULUM VITAE

- NAME: Pei-Hsin Cheng
- ADDRESS: 627 S. Preston St. Apt. 5K Louisville, KY 40202
- DOB: June 19 1983; Kaohsiung City, Taiwan

#### EDUCATION AND TRAINING:

B.S., Biological Sciences National Sun Yat-Sen University, Taiwan 2001-2005

M.S., Biological Sciences National Sun Yat-Sen University, Taiwan 2005-2007

Ph.D., Pharmacology and Toxicology University of Louisville, KY, USA 2008-present

HONORS AND AWARDS:

2006	Graduate Teaching Assistantship, Department of Biological Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan
2008-2010	K. C. Huang Scholarship, Department of Pharmacology and Toxicology University of Louisville, KY, USA
2008-present	Doctoral Fellowship, Integrated Program in Biomedical Science (IPIBS), University of Louisville, KY, USA

PROFESSIONAL SOCIETIES:

2006-present Chinese Society of Cell and Molecular Biology

2009-present Golden Key International Honors Society

PRESENTATION AND PUBLICATIONS:

Pei-Hsin Cheng, Xiao-Mei Rao, Kelly M McMasters, and H. Sam Zhou. "Oncolytic Adenovirus Replication Depends on Cyclin E Activating CDK2" Research Louisville (2010)

Pei-Hsin Cheng, Xiao-Mei Rao, Kelly M McMasters, and H. Sam Zhou. "Oncolytic Adenovirus Replication Depends on Cyclin E Activating CDK2" Research Louisville (2009)

Pei-Hsin Cheng. Expression and characterization of truncated HGF in human breast cancer cell. (2007)

Pei-Hsin Cheng, Ya-Ling Lin, and Shiping He. "Expression and characterization of truncated HGF in human breast cancer cell" 22th Joint Annual Conference of Biomedical Sciences, Taipei, Taiwan (2007)