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PRINCIPAL INVESTIGATOR: Steven R. Schwarze, Ph.D.

CONTRACTING ORGANIZATION: University of Wisconsin-Madison
Madison, Wisconsin 53706-1490

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6. AUTHOR(S) :
Steven R. Schwarze, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Wisconsin-Madison
Madison, Wisconsin 53706-1490

E-Mail:schwarze@surgery.wisc.edu

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Cdc37 is a co-chaperone protein that recruits several immature client kinases to Hsp90 for proper folding. Cdc37 up-regulation is a common early event in localized prostate cancer as is p16 induction. While targeted overexpression in mice leads to prostate epithelial cell hyperplasia, the function of Cdc37 in human prostate cancer is unclear. Here I examine the role of Cdc37 in the growth regulation of prostate cells. Using laser capture microdissection, Cdc37 and p16 are both concomitantly induced in moderately differentiated prostate cancer tissue. However, Cdc37 overexpression was unable to overcome p16 induced growth arrest in prostate cancer cells. I demonstrate that Cdc37 overexpression drives proliferation in normal prostate epithelial cells, while loss of Cdc37 function arrests growth and leads to apoptosis. Molecular analysis of Cdc37 client pathways indicated enhanced Raf-1 activity, up-regulated Cdk4 levels and reduced p16 expression with Cdc37 overexpression. These findings suggest increased Raf-1 and/or Cdk4 activity might underlie the proliferative enhancement. Induced Raf-1 activation, however, slowed growth, while cyclin D1 overexpression was sufficient to promote proliferation. These data further suggest that Cdc37 may play an active role in the progression of prostate cancer through accelerating proliferation and preventing apoptosis.

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Introduction

Prostate cancer is the most common malignancy in men and is the second leading cause of male cancer related deaths. The multi-step molecular pathogenesis is not clearly defined. Our laboratory has been examining alterations in the interrelated Cdk4/p16 and pRB pathways. We, and others, have reported that the Cdk4 inhibitor p16 is commonly induced in primary prostate cancer. As p16 should be arresting growth it was a surprising finding that it was induced in prostate cancer epithelium. Another gene that is induced in early prostate cancer development is Cdc37. The 50 kDa product of the mammalian *cdc37* gene is a co-chaperone protein that preferentially recruits protein kinases and is responsible for their activity (1-3). Cdk4 is a well-characterized Cdc37 client kinase. We hypothesized that Cdc37 induction may play a positive growth role and counter p16 induction in prostate cancer. Mechanistically, Cdc37 may bind to and activate more Cdk4 to participate in active cyclin D:Cdk4 complexes, thus negating inactive p16:Cdk4 complexes.

To test this hypothesis and assess the role of Cdc37 in the progression of prostate cancer, I proposed three Specific Aims or Tasks. These Aims were designed to test the association of p16 and Cdc37 induction in prostate cancer tissues and, more directly, to assess the role each of these genes could play in normal and cancer prostate cells in vitro. I have outlined the ongoing experiments below point by point, noting progress and issues that remain to be addressed in the following year. Overall, the project has been very successful and has lead to both technological advancements and significant scientific findings. The progress thus far has culminated in a research paper that is currently in peer review and will serve as a basis for an NIH grant submission on June 1. In the coming year I look forward to carrying out the remaining proposed experiments.

Body

Task 1. To examine Cdc37 levels in focal regions of primary and metastatic prostate tumors and determine if they correlate with p16 expression.

Rationale: Both p16 and Cdc37 levels are elevated in primary prostate cancer (4-7). If Cdc37, through association with Cdk4, is preventing high levels of p16 from binding Cdk4, then Cdc37 should be upregulated in association with p16. Through analysis of serial sections, individual glands that contain high levels p16 should co-express Cdc37. This aim will determine whether or not Cdc37 levels are able to override the effects of p16 *in vivo* and examine the prognostic value of the Cdc37:p16 relationship in early-stage primary prostate cancer.

Previous goals:

a. *Obtain antibodies to Cdc37 and optimize immunohistochemistry protocols to allow for specific detection of Cdc37 (this has already been accomplished for p16).*

b. *Immunohistochemically stain serial sections of 50 primary prostate tumor and normal pairs of tissue for Cdc37 and p16.*

Progress: Two commercially available antibodies were obtained for Cdc37 detection from Santa Cruz Biotechnology (Santa Cruz, CA). These antibodies have not been verified for immunohistochemical (IHC) techniques. Therefore, through the University of Wisconsin IHC lab these antibodies were tried under various conditions and tissue types. However, no signal was obtained and we concluded that they were uninformative for IHC use. Recently, Santa Cruz Biotechnology has released an anti-Cdc37 antibody certified for IHC applications in paraffin-embedded tissue and this antibody is currently being optimized in prostate tissue.

As an alternative p16 and Cdc37 mRNA levels were assessed in laser capture microdissected tissues. Three patient matched tumor/normal tissue pairs were selected. Cryosections were cut, H&E stained, and Gleason score determined by a urologic pathologist. Only matched normal and tumor specimens were used that contained no or all cancer, respectively. Furthermore, only those specimens with moderately differentiated cancer were selected (Gleason score 5-7). To separate epithelium from stromal components, laser capture microscopy was used.

Specimens were cyrosections (10 μm), dehydrated through an ethanol series, and allowed to dry for 3 hours. Epithelium and stromal components were captured using the PixCell II (Acturus, Mountain View, CA) laser capture system. Total RNA was isolated with the RNeasy total RNA isolation kit (Qiagen, Valencia, CA). RNA was precipitated using ethanol and glycogen as a carrier. Each sample was converted into first strand cDNA with SuperScript reverse-transcriptase (Invitrogen, Carlsbad, CA). Real time Quantitative RT-PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye as described using a iCycler detection system (Bio-Rad)(8, 9). For comparison of transcript levels between samples, a standard curve of cycle thresholds for several serial dilutions of a cDNA sample was established. This value was then used to calculate the relative abundance of each gene. These values were then normalized to the relative amounts of 18S cDNA. Quantitative real-time RT-PCR (qRT-PCR) was performed on cDNA generated from each sample. After standardizing to 18S rRNA levels, tumor epithelium consistently expressed more *Cdc37* message than normal epithelium (2-9 fold; Table 1)

Table 1. *Cdc37* mRNA is upregulated in primary prostate cancer. Laser capture microscopy was performed on 3 tumor/normal pairs of tissue obtained from radical prostatectomy. Epithelium was captured and the amount of *Cdc37* and *p16* message was measured using real-time qRT-PCR.

Sample	Gleason score	T:N (fold increase)
0110	3+4=7	9±0.7
0112	3+4=7	2.3±0.3
0117	3+3=6	3±0.5

Year 2 plans: Although laser capture microdissection (LCM) is an alternative to co-localize p16 and Cdc37 expression it is not as informative as IHC as mRNA and not protein is being assayed, LCM does not clearly demonstrate expression levels in one individual cell, and LCM is not conducive to high throughput screening. Therefore, I will be aggressively pursuing the IHC studies from paraffin-embedded tissue using the newly acquired rabbit anti-Cdc37 antibody approved for this technique. If, however the IHC does not work for this specific aim, then western blotting for p16 and Cdc37 will be performed on tumor and normal prostate tissue extracts. Although this assay dose not demonstrate expression levels in one cell, it will alleviate concerns regarding looking at protein expression and is amenable to high throughput assays.

Task 2. **To determine if Cdc37 is able to override p16 mediated growth arrest.**

Rationale: Our laboratory has identified that several prostate carcinoma cell lines are p16 negative due to promoter methylation (PPC-1, PC-3 and TSU-PR1) (10). p16 re-expression in these cell lines leads to G_1 arrest and acquisition of a senescent-like state (11). This model provides an excellent system to test this aim since bypassing one factor (p16) will allow the cells to continue to proliferate. In addition, the relative levels of Cdc37 and p16 can be controlled. The experiment will be carried out by determining a ratio of Cdk4:p16 that just restricts growth. Next, either Cdc37 or an empty expression vector will be introduced and the number of cells in S-phase determined. If Cdc37 overexpression is able to raise the number of cells in S-phase over the vector only, then we will conclude that Cdc37 is able to bypass p16-mediated growth arrest.

Previous goals:

a. *Develop a transfection plasmid for overexpressing Cdc37 (has already been accomplished for p16).*

b. *Transfect Cdc37 and p16 plasmids into prostate cancer cell lines and determine the cell cycle status and proliferation levels.*

Progress: Human Cdc37 cDNA was obtained (J. Wade Harper, Baylor College of Medicine). The open reading from was then cloned into an expression vector (pCDNA3.1). Human Cdk4 cDNA from our laboratory

was cloned into the pBABE puro expression vector. The Cdk inhibitor, p16, in the GFP expression vector, pLSG, was as described (12). The prostate cancer cell line PPC-1 was transfected using Effectene (Qiagen) with various combinations and ratios of these plasmids to determine if Cdc37 could override the growth arrest induced by p16. Cell cycle analysis of cells was accomplished by analyzing GFP positive cells pre-incubated with the fluorescent DNA dye Hoechst 33342 (Molecular Probes, Eugene, OR). Cell cycle position of GFP (transfected) cells was determined using MODFIT software (Becton Dickinson, San Jose, CA). Expression of all constructs was determined by western blotting.

The results of this experiment are demonstrated in Table 2. It was determined that a Cdk4:p16 ratio of 2:1 was still sufficient to arrest growth, while a ratio of 3:1 began to allow more cells in S-phase. At a Cdk4:p16 ratio of 2:1, Cdc37 or the empty vector (pBABE puro) were co-transfected. Cdc37, however, was not able to push more cells into S-phase. We therefore conclude that in the PPC-1 cell line Cdc37 cannot overcome p16 mediated cell cycle arrest.

	DNA transfected					
Sample	GFP	p16-GFP	Cdk4	Cdc37	pcDNA3.1	% in S-phase
GFP only	X					26.9%
Cdc37 only				X		25.9%
p16 only		X				1.6%
Cdk4 only	X		X			27.1%
Cdk4:p16 (1:2)		X	X			5.5%
Cdk4:p16 (1:1)		X	X			5.2%
Cdk4:p16 (2:1)		X	X			5.6%
Cdk4:p16 (3:1)		X	X			11.7%
Cdk4:p16 (2:1) +pCDNA3.1		X	X		X	5.4%
Cdk4:p16 (2.1) +Cdc37		X	X	X		5.9%

Table 2. Cdc37 cannot overcome p16-mediated cell cycle arrest in PPC-1 cells. PPC-1 cells were transfected with the indicated plasmids. At 72 hours post-transfection, cells were labeled with Hoechst 33342 and the DNA content of GFP positive (transfected) cells analyzed by FACS. Clearly, p16 was able to induced cell cycle arrest. Cdk4 was co-transfected to act as a reservoir to bind excess p16 and to form a potential large pool of immature Cdk4 for Cdc37 to fold. A ratio of 3:1 Cdk4:p16 was sufficient to partially alleviate the p16 growth arrest. Therefore a slightly lower Cdk4:p16 ratio of 2:1 was used to test the ability of Cdc37 to bypass the p16-mediated growth arrest. Either Cdc37 or the control pCDNA3 vector was co-transfected with Cdk4 and p16. However, Cdc37 was unable to enhance proliferation.

Year 2 plans: The experiments as described above will be repeated in two additional cell lines as proposed (PC-3 and TSU-PR1). There may be inherent cell-type specific factors that did not allow Cdc37 to bypass the p16-mediated cell cycle arrest that must be examined.

Task 3. To determine, using an in vitro approach, if Cdc37 overexpression is able to extend replicative lifespan in normal human prostate epithelial cells.

Rationale: Studies demonstrate that Cdc37 can be a positive growth regulator *in vivo*, causing prostate epithelium hyperplasia, and can act as an oncogene when combined with cyclin D1 or c-myc overexpression in mice (4, 13). As normal cells progress from proliferation to senescence they accumulate high levels of p16 (12). A necessary alteration for lifespan extension is bypassing p16 by inactivating pRb, mutating or deleting p16, or through gene silencing by p16 promoter methylation (14, 15). This aim will determine if Cdc37 exerts the observed hyperplastic effects and if Cdc37 can promote immortalization in normal HPECs.

Previous goals:

a. *Develop a retroviral vector for expressing Cdc37.*

b. *Obtain prostate explants from patients and culture normal human prostate epithelial cells.*

c. *Infect prostate epithelial cells with Cdc37 retrovirus and select for infected cells.*

d. *Compare cells infected with Cdc37 and vector only virus. Perform BrdU incorporation assays to address proliferative.*

Progress: The bulk of the significant finding thus far came from this Task. There has been a major accomplishment in the development of retroviral production methods, as well as, in the demonstration that Cdc37 can induce proliferation. Further molecular analyses derived from this finding have yielded a wealth of information regarding the mechanism of Cdc37 induced proliferation in HPECs. A more detailed analysis of this study can be found as a submitted manuscript in the appendix.

(a) *Develop a retroviral vector for expressing Cdc37.* Currently, transfection of HPECs is very inefficient (less than 0.5%) and therefore this method is not a viable method for gene delivery. Retroviral gene expression is, however, a method that allows stable incorporation of exogenous gene expression in normal cells. Standard methods for retrovirus generation rely on transfecting amphotropic cell lines with a linearized expression construct, followed by drug selection for stable cell lines. A number of stable cell lines are then expanded in culture and the viral titer assayed. Finally, large quantities of viral supernatant are harvested from the highest titer cell line and frozen away. The production of viral supernatant in this manner has several downfalls including:

1. Several months 2-3 are required just to make viral supernatant.
2. Stable cell lines cannot be generated from constructs that induce growth arrest or apoptosis.
3. Freeze-thawing viral supernatant reduced viral titer by 2-fold.
4. Many expressing cell lines have their titer reduced significantly if they are froze down and, therefore, the whole 2-3 month procedure needs to be performed if more supernatant is needed in the future.

As an alternative, Dr. Gary Nolan at Stanford University has devised a vastly improved method for retroviral production. This protocol has been adapted for use in HPECs. Briefly, To generate virus, the DNA vectors were transfected (Effectene, Qiagen) into Phoenix Ampho packaging cell lines (provided by Gary Nolan, Stanford University through ATCC, Manassas, VA) according to the Nolan lab protocol (http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html). At day 2 post-transfection, the supernatant w harvested, filtered, 4 μg/ml polybrene added and placed onto HPECs. After 8 hours, the viral containing supernatan was replaced by F12+. At two days post-infection, HPECs infected with the pBABE puro parent vector were treatec with 4 μg/ml puromycin. Drug treatment was carried out for three days, at which point no uninfected cells remainec viable. At day 5 post-infection, cells were re-plated to 50-70% confluency. Infection rates for all constructs varied between 5-22%.

This improved retroviral production methodology has allowed vast improvements in:

1. Time: retrovirus is to be produced in 2 days instead of 2-3 months.
2. Retroviral production from all constructs: retrovirus has been made from the negative growth regulator, p16, and from the apoptosis-inducing construct, Cdc37ΔC, an impossible feat with standard technologies.
3. Reliable high viral titer: With transfection procedures optimized for the Phoenix Ampho cell line high viral titers are reliable made. Also, the timing of viral production can be synchronized to need, thus preventing loss of viral tite through freeze thawing procedures.
4. Convenience: No need to freeze down retroviral producing cell lines.

(b) *Obtain prostate explants from patients and culture normal human prostate epithelial cells.* With close collaboration with my sponsor, Dr. David Jarrard, the availability of human prostate tissue has been adequate. With our expertise, no problems have been encountered in culturing normal human prostate epithelial cells.

(c) *Infect prostate epithelial cells with Cdc37 retrovirus and select for infected cells.* As stated in (a), retroviral infections and expression in HPECs has been very good (5-22% infection rates). Further proof of the ability to infect HPECs is demonstrated in each of the figures in the appendix.

(d) *Compare cells infected with Cdc37 and vector only virus. Perform BrdU incorporation assays to address proliferative.* Cdc37 overexpression was able to drive proliferation compared to vector only infected HPECs (66-123%). Ensuing molecular analysis was performed on cellular extracts to gain insights as to mechanistic alterations that were potentially leading to cellular proliferation (Figure 1). Molecular analysis of Cdc37 client pathways indicated enhanced Raf-1 activity, up-regulated Cdk4 and cyclin D2 levels and lower p16 expression with Cdc37 overexpression. These findings suggest increased Raf-1 and/or Cdk4 activity might underlie the proliferative enhancement (Figure 1).

To address whether loss of Cdc37 function inhibits growth a dominant negative Cdc37 construct, Cdc37ΔC, was overexpressed in HPECs. This protein retains the ability to bind kinases and homodimerize, however it cannot bind to Hsp90 due to a C-terminal truncation. Thereby, maturation of Cdc37 client polypeptides is blocked (16). At 4 days post-infection, distinct morphological changes were readily apparent in HPECs expressing Cdc37ΔC. Cells lost their characteristic cuboidal epithelial morphology, instead becoming contracted, and highly light refractile (Figure 2A). At 5 days post infection, Hoechst 33342 stained cells were observed by fluorescent microscopy to assess nuclear DNA integrity. An abundance of fragmented, or pycnotic, nuclei suggested the cell death was apoptotic (Figure 2B). At the same time point, Cdc37ΔC and control pBABE puro infected cultures were assayed for proliferation by BrdU incorporation. Effective growth cessation (~10-fold) was caused by Cdc37ΔC expression with cells accumulating in G0/1 (Figure 2C). This data further demonstrates that Cdc37 is critical for proliferation and is necessary for survival.

To further address which of the observed molecular changes could lead to growth enhancement, the Raf-1 induction, Cdk4 and cyclin D1 overexpression were recapitulated. Constitutive Raf-1 activation however resulted in growth arrest (Figure 3). The mechanism behind this growth arrest is unclear, however overexpressed Raf-1 is unable to result in growth activation. Cdk4 was also overexpressed and was unable to generate a proliferative response. Interestingly, cyclin D1 overexpression was sufficient to promote proliferation. These findings are consistent with they hypothesis that Cdc37 mediates growth arrest through inducing Cdk4 activity.

One of the most surprising findings was that blocking Cdc37 activity resulted in apoptosis. This indicates that Cdc37 is necessary for survival. It would also be an important finding that Cdc37 also performs anti-apoptotic functions. Recently, it was demonstrated that Cdc37 is necessary for activity of the anti-apoptotic gene AKT (17). It is interesting to speculate that Cdc37 overexpression observed in human prostate tissue not only promotes proliferation, but also apoptosis resistance.

Key Accomplishments

- mRNA quantitation of genes using laser capture microdissection and quantitative real-time PCR
- Cloning of all desired cDNA constructs into expression vectors with confirmed gene expression
- Generation of a retrovirus using improved methodology for gene expression in normal human prostate epithelial cells (HPECs)
- Efficient infection and gene expression in HPECs

Reportable Outcomes

1. A submitted manuscript entitled: Cdc37 Enhances Proliferation and is Necessary for Normal Human Prostate Epithelial Cell Survival. The manuscript is attached in the appendix.

Conclusions

1. p16 and Cdc37 mRNA are co-induced in the prostate cancer compared to normal tissue.
2. Cdc37 is not able to overcome p16-mediated growth arrest in PPC-1 cells.
3. Cdc37 overexpression drives proliferation in HPECs.
4. Cdc37 overexpression results in molecular changes consistent with induction of Cdk4 activity.
5. The Cdc37 downstream Cdc37 target cyclin D1 is sufficient to drive proliferation alone.
6. Loss of Cdc37 function leads to growth arrest and apoptosis.

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Cdc37 Enhances Proliferation and is Necessary for Normal Human Prostate Epithelial Cell Survival

Steven R. Schwarze, Vivian, X. Fu and David F. Jarrard

Department of Surgery, Division of Urology, University of Wisconsin Medical School, Molecular and Environmental Toxicology and the University of Wisconsin Comprehensive Cancer Center, Madison, WI 53972

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†To whom correspondence should be addressed: David F. Jarrard, M.D. Department of Surgery, University of Wisconsin, 600 Highland Avenue, K6/530, Madison, WI 53792 Ph. 608-265-2225; Fax: 608-265-8133; E-mail: jarrard@surgery.wisc.edu

Abbreviations: HPEC, human prostate epithelial cell; FACS, fluorescence-activated cell sorter; 5-bromo-2-deoxy-uridine, BrdU; CDK, cyclin-dependent kinase

Abstract

Cdc37 is a co-chaperone protein that recruits several immature client kinases to Hsp90 for proper folding. Cdc37 up-regulation is a common early event in localized human prostate cancer. While targeted overexpression in mice leads to prostate epithelial cell hyperplasia, the effect of Cdc37 dysregulation in human prostate cells is unclear. In this study we examine the role of Cdc37 in the growth regulation of normal prostate epithelial cells utilizing a unique human model system. We demonstrate that Cdc37 overexpression drives proliferation, while loss of Cdc37 function arrests growth and leads to apoptosis. With increased Cdc37 expression, molecular analysis of Cdc37 client pathways demonstrates enhanced Raf-1 activity, upregulated Cdk4 levels, and reduced expression of the cyclin-dependent kinase inhibitor p16/*CDKN2*. To further investigate these downstream pathways, increased Raf-1 or Cdk4 activities were selectively induced in human prostate epithelial cells. Induction of Cdk4 activity using cyclin D1 overexpression was sufficient to promote proliferation. However, Raf-1 activation inhibited proliferation. These data indicate that Cdc37 induces proliferation and is critical for survival in human prostate epithelial cells. These alterations in cell division and survival may be important in the development and progression of early prostate cancer.

Introduction

Cdc37 is a co-chaperone protein that targets and activates multiple protein kinases. These interactions are important for a number of mitogenic signaling pathways. As a critical component of cell cycle control, the Cdc37dysregulation may be implicated in the development of a number of cancers. Recently, human prostate tissue specimens were surveyed for Cdc37 expression and increased immunoreactivity was found in all specimens analyzed when compared to normal tissues (1). Overexpression of Cdc37 was also found in the prostate cancer precursor lesion, prostatic intraepithelial neoplasia (PIN), indicating Cdc37 may be an important early step in prostate cancer development. To extend these findings, the targeted overexpression of Cdc37 in mice prostates was performed using a probasin promoter. These tissues displayed significant hyperplasia at 8 months of age (1) indicating Cdc37 can positively lead to loss of growth control in mice. In mice, Cdc37 has also been found to collaborate with *c-myc* in the development of tumors in multiple tissues suggesting it regulates a rate-limiting step in epithelial cell transformation (1). However, the effect of Cdc37 on the proliferation and survival of non-immortalized human epithelial cells, in which *in vitro* transformation is rare, is unknown.

The 50 kDa product of the mammalian *cdc37* gene is a co-chaperone that is absolutely required for Hsp90 substrate-specificity folding activity (2-5). The Hsp90 family members are molecular chaperones that provide maturation and folding to a number of client polypeptides in an ATP-dependent manner. Cdc37 binds immature protein kinases through interaction with its amino-terminal region (6) and links these to the Hsp90 carboxy-terminus (7). In additional to a physical role in targeting kinases to

Hsp90 for activation, it has been demonstrated in yeast that Cdc37p also exhibits protein chaperone activity (3). Functionally, Cdc37 is critical not only for kinase activation, but also protein stability. Blocking Cdc37 function in immortalized cell lines using either a dominant negative Cdc37 mutant (6) or inhibiting Hsp90 activity with ansamycins such as geldanamycin (8) results in both decreases in steady state protein levels and activity of client kinases such as Cdk4, Raf-1, v-Src, AKT and the androgen receptor (6, 9-12).

At least two Cdc37 client kinases, Cdk4 and Raf-1, have been implicated in the processes of proliferation. The decision to enter the cell division cycle is modulated by a series of signal transduction pathways acting on genes required for cell cycle progression. Cyclin D proteins (D1, D2 or D3) form an active kinase complex with Cdk4 or Cdk6 that phosphorylates pRB and functions in the G1 to S phase transition (13). Cdc37 binds to Cdk4 in high molecular weight complexes that also contain the molecular chaperone Hsp90. These positive proliferation signals are antagonized by p16, and other members of the cyclin-dependent inhibitor family INK4a, which compete with cyclin D for binding to Cdk4 and Cdk6, thereby preventing kinase activity [Weinberg, 1995 #10]. Overexpression of either cyclin D1 or replacement of wild-type Cdk4 with a Cdk4 mutant that cannot bind the CDK inhibitor p16, Cdk4 R24C, leads to elevated cyclinD:Cdk4 activity, induction of proliferation and elevated tumor incidence in mice (14, 15). Furthermore, fibroblasts derived from Cdk4 R24C homozygous mice or p16 knockout mice are immortal in culture (15). Thus, regulation of Cdk4 activity is critical in the proliferation of normal cells, and appears to play a role in cancer susceptibility at least in mouse models.

Another Cdc37 client kinase is Raf-1, a critical signaling molecule in the MAPK pathway that transmits information from the plasma membrane to the nucleus and cell cycle machinery. Upstream of Raf-1 is a membrane bound family of Ras GTPases (16, 17). Upon activation, Ras recruits Raf-1 to the membrane, a process that results in Raf-1 activation (18). Activated Raf-1 can then phosphorylate mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK1/2), which can then phosphorylate and activated the p42/p44 mitogen-activated protein kinases ERK1/2 (19). Raf-1 can positively regulate proliferation likely via the stimulation of cyclin D1 by MAPK signaling (20). Although this Raf-1 function is likely critical for proliferation, paradoxically Raf-1 can also lead to growth arrest and differentiation in non-immortalized cells (21, 22). It is unclear if this dual role is cell-type specific or dependent on the immortalization status.

In the present study, we examine the role of Cdc37 in the proliferation and survival of genetically intact, non-immortalized epithelial cells in a unique prostate epithelial cell model (23). Using molecular constructs, the mechanisms through which these signals are elicited are examined. Consistent with mouse transgenic studies, Cdc37 overexpression in human prostate epithelial cells is sufficient to enhance proliferation. The mechanism is consistent with an increase in Cdk4 activity. Furthermore, our studies open the possibility that the Cdc37-mediated hyperplastic response *in vivo* may also arise from resistance to apoptosis. This study supports the hypothesis that Cdc37 is a positive growth regulator, a survival gene, and may play an early role in the progression of human prostate cancer.

RESULTS

Cdc37 overexpression increases BrdU incorporation in HPECs. Using a retroviral system, Cdc37 or the empty pBABE puro vector was expressed in primary HPEC cultures. Infected cells were selected with puromycin until no viable cells remained in uninfected cultures (typically 72 hours). No morphological changes were apparent between Cdc37 overexpressing and control cells (data not shown). At 8 days post-infection, HPECs were pulsed with BrdU for one hour, labeled with an anti-BrdU antibody, and analyzed by FACS (Figure 1). Cells overexpressing Cdc37 showed a consistent marked increase in the number of BrdU positive cells compared to the pBABE vector infected control cells (66-123% increase; Figure 1A) and a decrease in the number of cells in G0/G1 (Figure 1B).

Cdc37 overexpression leads to alterations in the Cdk4/6 pathway. We next surveyed molecular changes that may lead to enhanced proliferation in Cdc37 overexpressing HPECs. Since Raf-1 and Cdk4 are known Cdc37 client kinases, we focused on genes involved in these pathways. Western blot analysis was performed on protein extracts isolated from HPECs overexpressing Cdc37 or the vector only (Figure 1C). Cdc37 protein was overexpressed at high levels compared to endogenous levels. Raf-1 steady-state levels were unchanged. However, Raf-1 activity, measured by the abundance of phosphorylated S217/221 MEK1/2, a direct target of Raf-1 (24), was consistently elevated in Cdc37 overexpressing cells. Significant increases in Cdk4 markedly reduced p16 expression were also consistently observed in Cdc37 overexpressing HPECs. No changes in cyclin D1 or Cdk6 expression were detected.

Cyclin D2 and D3 were not detectable in HPECs at the protein level. Thus, Cdc37 driven proliferation may be mediated through greater Cdk4 and/or Raf-1 activity.

Blocking Cdc37 function leads to growth arrest and reduced Raf-1 and Cdk4 expression. To address whether loss of Cdc37 function inhibits proliferation and progression through G1 in normal epithelial cells, a dominant negative Cdc37 construct, Cdc37ΔC, was employed. This protein retains the ability to bind protein kinases and homodimerize, however it cannot engage Hsp90 due to a C-terminal truncation (6) and, therefore, leads to a block in the maturation of Cdc37 client polypeptides. At 4 days post-infection, distinct morphological changes were readily apparent in HPECs expressing Cdc37ΔC when compared to control cells. The majority of mutant Cdc37 expressing cells lost their characteristic cuboidal epithelial morphology and became contracted and highly light refractile (Figure 2A). Hoechst 33342 stained cells were analyzed by fluorescent microscopy to assess nuclear DNA integrity. An abundance of fragmented, or pycnotic, nuclei indicated the mechanism of cell death was apoptotic (Figure 2B).

By 8 days post-infection, ~10% of Cdc37ΔC expressing cells remained attached (data not shown). At this time Cdc37ΔC and control pBABE puro infected cultures were assayed for proliferation by BrdU incorporation 5 days after infection. Effective growth cessation (~10-fold) was caused by Cdc37ΔC expression with cells accumulating in G0/1 (Figure 2C). Two antibodies were unable detect the C-terminally truncated Cdc37 levels as their epitopes mapped to the C-terminus. Therefore, RT-PCR was used to amplify a segment of the N-terminus. Modest increases in the N-terminal were found in Cdc37ΔC infected cells. Presumably, higher cellular Cdc37ΔC levels led to apoptosis while

moderate to low levels resulted in growth arrest (Figure 2D). Western analysis demonstrated that the Cdc37 client kinases Raf-1 and Cdk4 expression levels were consistently reduced, indicating the Cdc37ΔC truncated protein was indeed preventing wild-type Cdc37 activity. Interestingly, wild-type Cdc37 expression was elevated with Cdc37ΔC expression (Figure 2D). This may be explained by a feedback mechanism. Another possibility is that overexpressed Cdc37ΔC binds to majority of the misfolded client kinases, sequestering them from wild-type Cdc37. Without misfolded kinases bound to endogenous Cdc37, it may be less likely targeted for ubiquitination. Thus, the Cdc37 dominant negative construct further demonstrates Cdc37 is critical for proliferation in HPECs and additionally plays a role in survival by preventing apoptosis.

Raf-1 activation causes growth arrest. Selective induction of specific kinase pathways activated by Cdc37 was then performed in HPECs. To test if overexpressing Raf-1 alone could generate a proliferative response in HPECs, an inducible activated Raf-1 estrogen receptor fusion construct (ΔRaf:ER) was stably incorporated into HPECs through retroviral means. Following infection, cells were drug selected and Raf-1 activity induced with ß-estradiol. After 6 days of Raf-1 induction, cells acquired a flattened, elongated morphology with many pseudopods (Figure 3A). BrdU labeling showed proliferation decreased and an accumulation of cells in G0/G1 with Raf-1 activation (Figure 3B). Western blot analysis shows the Raf-1 target, S217/221 MEK1/2 is highly phosphorylated, indicating Raf-l induction, compared to vehicle only treated ΔRaf:ER infected cells (Figure 3C). Further analysis of genes involved in G1:S transition shows Cdk4 to be repressed with ΔRaf:ER induction, while p16 and cyclin D1 protein expression remained unchanged. Thus, activation of Raf-1 alone leads to a non-apoptotic

differentiated response in HPECs indicating other pathways are critical in the proliferative induction with Cdc37.

Cdk4 is insufficient to foster proliferation, however cyclin D1 overexpression alone is sufficient generate a proliferative response. Molecular analysis of Cdc37 overexpressing HPECs suggested that the observed proliferative increases could be mediated through enhanced Cdk4 activity either by directly stabilizing Cdk4, or indirectly through p16 repression. To address the impact greater Cdk4 levels may have on proliferation, HPECs were infected with Cdk4 or the pBABE puro control vector and selected with puromycin. Cdk4 was unable to cause a greater BrdU proliferation index (Figure 4A), although consistently fewer cells accrued in G1 with a correspondingly higher G2/M DNA content (Figure 4B). Thus, it appears that increased Cdk4 levels are able to partially bypass the G1 block, however are not able to progress through the cell cycle.

To test whether greater Cdk4 activity is capable of increasing proliferation, we overexpressed cyclin D1 in HPECs. Cyclin D1 overexpression is well-established means of increasing Cdk4/6 activity (14, 25, 26). Following drug selection, infected HPECs were assayed for proliferation by BrdU incorporation. Cyclin D1 generated a marked proliferation induction (51-94% higher; Figure 5A) leading to both decreases in the G0/1 and G2/M DNA content (Figure 5B). Cellular extracts confirmed cyclin D1 overexpression in transfected cells (Figure 5C). These experiments indicate that the overexpression of cyclin D1 generates increases in proliferation similar to those found with Cdc37 and that Cdk4 activity is rate limiting.

DISCUSSION

It is generally accepted that most cancer cells arise from a combination of uncontrolled proliferative cues, as well as, inhibition of cell death pathways. Prostate cancer is no exception. As normal prostate cells transform into high-grade prostatic intraepithelial neoplastic (PIN) lesions, a putative prostate cancer precursor, there is an increase in the number of cells proliferating (27). Progression to localized prostate cancer involves no further proliferative increases when compared to normal epithelial cells, but a decrease in cell death (27). One gene induced in early PIN, that is also found to demonstrate high levels of expression in localized prostate cancer, is Cdc37. Cdc37 is critical in several cell growth and death pathways and may have a direct impact on both enhanced proliferation and evasion from apoptosis (6, 9, 11). Our laboratory has a longstanding interest in understanding how genes involved in the G1 cell cycle checkpoint regulate growth in human prostate cells in the progression to cancer. In this manuscript, we examined the role of Cdc37 in the growth of normal human primary prostate epithelial cells *in vitro*. Our primary finding is that Cdc37 overexpression leads to an increase in the proliferation rate. To our knowledge, the effect of Cdc37 on proliferation in human cells has not been reported. Likely this is due to the use of human normal prostate epithelial cells, as we were unable to demonstrate greater proliferation in prostate cancer cell lines overexpressing Cdc37. Prostate cancer cell lines have been selected for growth in vitro and, therefore exhibit a 2-5 fold higher proliferation rate. We would hypothesize that prostate cancer cells have already upregulated Cdc37 client pathways involved in proliferation. This example study illustrates nicely the utility our HPEC model when investigating early mechanisms of prostate cancer development.

Furthermore, this finding is consistent with the demonstration that targeted Cdc37 overexpression in mouse prostate (PB-Cdc37.1 line) tissues leads to >50% of the prostatic acini developing epithelial hyperplasia and dysplasia (1). Therefore, the overexpression of Cdc37 has a similar effect in human epithelial cells and is a candidate for the growth dysregulation seen in prostate cancer progression.

A second important observation was that the inhibition of Cdc37 function, utilizing a dominant negative construct, not only halted proliferation, but also efficiently induced apoptosis. Primary HPEC cultures demonstrate greater resistance to the effects of anti-cancer drugs when compared to established, immortalized prostate cancer cell lines (28, 29). It was remarkable that the degree of nuclear fragmentation seen with the Cdc37ΔC construct was significantly greater than that which we have observed with Docetaxel, thapsigargin or 5-fluoro-2-deoxy-uracil (data not shown). This finding suggests that not only does Cdc37 function in activating genes involved in proliferation, but also provides an anti-apoptotic role. Therefore it is possible that both higher proliferation rates and apoptosis resistance may lead to the hyperplasia seen in mouse prostate tissues overexpressing Cdc37 (PB-Cdc37.1) and in human prostate cancers (1). Support for Cdc37 overexpression leading to apoptosis resistance is also demonstrated by recent reports showing that Cdc37 binds to the anti-apoptotic AKT family members (11). Furthermore, Hsp90 inhibition leads to decreases in steady state AKT levels, as well as a reduction in kinase activity (11). Since AKT is part of an important survival pathway, reduced AKT activity may sensitize primary HPECs to cell death. Finally, we speculate that prostate cells may be especially responsive to loss of Cdc37 function since the androgen receptor binds to Cdc37 and this interaction is necessary for androgen receptor-

mediated transcriptional activity (12). Future studies will examine if Cdc37 overexpression can also confer apoptosis resistance.

Our analysis of Cdc37 overexpressing HPECs demonstrated the activation of several putative growth control pathways, in conjunction with an increase in proliferation, including Raf-1. Raf-1 activity, measured by phosphorylation of its downstream target MEK1/2, was induced in Cdc37 overexpressing cells. Overexpression of activated Raf-1 can induce a differential effect in various cell types. In a number of human cell lines Raf-1 overexpression can stimulate a malignant progression (Samuels, 1993). In contrast, activated Raf-1 can induce growth arrest in primary cells, as well as some cell lines (22, 30, 31). The mechanism behind Raf-1 induced growth arrest is unclear, however it does not required intact p53 or pRb pathways (21, 22). It is evident that the events during the conversion process from a finite lifespan to immortalization causes some cells lose the ability growth arrest. Consistent with previous studies in primary cells, Raf-1 overexpression and activation resulted in growth inhibition in HPECs. Our observations are strikingly similar to those performed in human primary breast epithelial cells and include similar morphological alterations, growth arrest, lack of senescence-associated ß-galactosidase staining (data not shown) and no change in levels of several cell cycle inhibitor proteins (22). In vivo, growth arrest in response to greater Ras or Raf-1 activity may play an important tumor suppressive role.

Through association with known, and undoubtably other unknown, client kinases, the manner in which Cdc37 promotes HPEC growth is likely multi-factorial. However, we did find that the induction of proliferation by Cdc37 is consistent with greater Cdk4 activity as Cdk4 levels were upregulated and the Cdk4 inhibitor p16 was downregulated.

Unfortunately, due to the low cell number following infection and selection we were unable to directly assay Cdk4 activity using kinase assays. As an alternative, we utilized a phospho-specific pRB antibody directed against the S780 site which cyclin D:Cdk4 phosphorylates during cell cycle progression (32). However, we found that phosphorylated S780 pRB was dependent only on total pRB expression and was insensitive in monitoring cell cycle progression in these cultures. Experimentally we have also shown that by overexpressing Cdk4 the G1 cell cycle checkpoint could be bypassed, although no change in proliferation was noted. Likely, Cdk4 levels are not rate limiting as has been observed in other primary cells (25). We overexpressed cyclin D1, a protein which functions as a Cdk4 binding partner needed for Cdk4 activity, and found higher proliferation could be attained in HPECs. Together, these data are consistent with the hypothesis that Cdc37 expression induces Cdk4 activity, and greater Cdk4 activity can drive proliferation in normal human prostate epithelial cells.

Since the Hsp90/Cdc37 association is important for both proliferation and survival it is a potential target in cancer therapy (33). The drug 17-allylamino, 17-demethoxy-geldanamycin (17-A-GA) indirectly destabilizes kinases that cells need for survival by inhibiting Hsp90 and related family members (8). Phase I clinical trials for the use of 17-A-GA are currently ongoing are ongoing (34). However, selectivity between cancer cells and normal cells may be difficult since Hsp90 provides critical functions for a number of normal cellular processes. A more direct and selective target may be Cdc37 itself. Blocking Cdc37 co-chaperone activity would inhibit growth in overexpressing cells, such as prostate tumors, yet still allow Hsp90 to associate with other biologically important co-chaperones, such as p23, HOP and Hsp70. Through this

increased specificity, this strategy may result in reduced cytotoxicity compared to anti-Hsp90 drugs.

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MATERIALS AND METHODS

Primary Cell Culture and Retroviral Infection—Prostate tissue was obtained under an approved IRB protocol from men (ages 44-66) undergoing cystoprostatectomy for bladder cancer at the University of Wisconsin Hospitals and Clinics. Histology confirmed that no bladder or prostate cancer was present in the tissues harvested for our studies. Prostate epithelial cultures were established as described previously (35). Prostate tissues were minced with a scalpel and digested in a solution containing collagenase (500 U/ml) (Sigma, St. Louis, MO) and plated on collagen-coated plates. Cells were maintained in Ham's F-12 media (Invitrogen, Carlsbad, CA) supplemented with regular insulin, 0.25 units/ml; hydrocortisone, 1 μg/ml; human transferrin, 5 μg/ml; dextrose, 2.7 mg/ml; non-essential amino acids, 0.1 mM; penicillin, 100 units/ml; streptomycin, 100 μg/ml; L-glutamine, 2 mM; cholera toxin, 10 ng/ml; bovine pituitary extract, 25 μg/ml and 1% FBS (36). Cells were passaged using trypsin-EDTA.

To generate virus, the DNA vectors were transfected using Effectene (Qiagen, Valencia, CA) into Phoenix Ampho packaging cell lines (provided by Gary Nolan, Stanford University through American Type Culture Collection, Manassas, VA) according to the Nolan lab protocol

(http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html). At day 2 post-transfection, the supernatant was harvested, filtered, 4 μg/ml polybrene (Sigma) added and placed onto HPECs. After 8 hours, the viral containing supernatant was replaced by F12+. At two days post-infection, HPECs infected with the pBABE puro parent vector were treated with 4 μg/ml puromycin (Invitrogen). Cells infected with the ΔRaf:ER vector were treated with 300 μg/ml of Geneticin (Invitrogen) with or without 1 μM ß-estradiol (Sigma). Drug treatment was carried out for three days, at which point no uninfected cells remained viable. At day 5 post-infection cells were re-plated to 50-70% confluency. Infection rates for all constructs routinely vary between 5-22%.

Generation of cDNA clones and retroviral stocks—Human Cdc37 cDNA (provided by J. Wade Harper, Baylor College of Medicine) was cloned into the retroviral expression vector pBABE puro. The Cdc37ΔC dominant negative construct was designed to encompass AA1-174 as previously described (6). The amino terminal fragment was PCR amplified using Expand proofreading DNA polymerase (Roche Biochemicals, Indianapolis, IN), cloned into pBABE puro, and sequenced. The ΔRaf:ER vector (provided by Herb Chen through Martin McMahon) consists of the human Raf-1 gene lacking the N-terminal regulatory domain fused to the human estrogen receptor hormone binding region. The construct has neomycin as a resistance marker and has been previously described (37). Human Cdk4 and cyclin D1 were PCR amplified from HPEC DNA with Expand proofreading DNA polymerase, sequenced and cloned into pBABE puro.

BrdU labeling and cell cycle analysis—HPECs were labeled with 5-Bromo-2-deoxy-uridine (BrdU) (Sigma) for one hour, harvested, and processed using an anti-BrdU monoclonal primary antibody followed by a goat anti-mouse FITC conjugated secondary according to the

manufacturers directions (Becton-Dickinson Immunocytometry Systems (B-D), San Jose, CA). Cells were analyzed with a FACScan (B-D) and the percentage of BrdU positive cells (10,000 gated events) determined using CellQuest software (B-D). Cell cycle phase was determined using MODFIT software (B-D).

Western Blot Analysis—Western blots were performed as described [Reznikoff, 1996 #5] from three independent infections. Cells were harvested by trypsin-EDTA and washed in 1X PBS. Protein was extracted by freeze thawing 3 times in ECB buffer (50 mM Tris pH 8.0, 125 mM NaCl, 100 mM NaF, 0.5% NP-40, 200 μM Na_3VO_4, 1 μg/ml Aprotinin, 1 μg/ml leupeptin, and 50 μg/ml PMSF). Protein extracts were quantified using the Bradford assay. Twenty-five μg of whole cell extract was separated on polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and blocked in 5% non-fat dry milk in PBS plus 0.1% Tween 20 (PBST). Polyclonal antibodies to Cdk4 (C-22), Cdk6 (C-21), and Raf-1 (C-20), in addition to, monoclonal antibodies to cyclin D1 (A-12) and Cdc37 (N-18) were obtained from Santa Cruz (Santa Cruz, CA). The monoclonal antibody to $p16^{INK4A}$ (AB-1) was obtained from Calbiochem (La Jolla, CA). The monoclonal antibody to α-tubulin (AB-1) was obtained from Oncogene (Cambridge, MA). The monoclonal antibody to pRB (14001A) was purchased from PharMingen (San Diego, CA). Monoclonal antibodies to phospho S780 pRB, phospho S217/221 MEK1/2 and MEK1/2 were obtained from Cell Signaling Technologies (Beverly, MA). Secondary goat anti-mouse IgG (HRP conjugate) and goat anti-rabbit IgG (HRP conjugate) antibodies were obtained from Pierce (Rockford, IL). Antibodies were applied in 2.5% non-fat dry milk in PBST for one hour and washed 3 times for 10 minutes in PBST. Bound antibody was detected using chemiluminescence (Pierce).

Reverse Transcriptase PCR—Total RNA was isolated from HPECs infected with either Cdc37ΔC or the pBABE puro backbone virus 5 days post-infection with the RNeasy total RNA isolation kit (Qiagen). First strand cDNA was generated with SuperScript reverse transcriptase (Invitrogen) from 1 μg of total RNA. The primers sequences used to amplify a segment of the Cdc37 amino-terminus were, 5'-AAGGAAAGATGGTGGACTACAGC-3' and 5'-TCACATGCCAAAGTGCTTGA. Following 30 PCR cycles, reactions were electrophoresed on an agarose gel, stained with Sybr Green (Molecular Probes) and visualized with the Storm imaging system (Molecular Dynamics, Piscataway, NJ).

Figure 1. Cdc37 overexpression increases proliferation and alters expression of cell cycle related genes. (A) Primary HPECs from individual patients were retrovirally infected with a Cdc37 construct or a vector only control, selected with puromycin and analyzed for proliferation through BrdU incorporation at 8-days post-infection. The values reflect the percentage of cells staining positively with an anti-BrdU antibody indicating DNA synthesis. (B) Cell cycle analysis of the infected HPEC cultures demonstrates a consistent decrease in the G0/1 DNA content in cells overexpressing Cdc37 compared to the pBABE puro vector only cells. (C) Cellular extracts were immunoblotted for known Cdc37 targets and genes involved in the Cdk4/6 pathway. Greater p-MEK1/2, and Cdk4 levels were consistently found in Cdc37 overexpressing protein extracts, as well as, decreases in p16 expression. The p-MEK1/2 antibody detects phosphorylated S217 and S221 residues.

Figure 2. Blocking Cdc37 function inhibits proliferation and leads to apoptotic death. (A) The Cdc37 dominant negative construct, Cdc37ΔC, or a vector only control, pBABE puro, were retrovirally expressed in HPECs. Phase-contrast microscopy demonstrates that Cdc37ΔC infected cells lose their cuboidal morphology characteristic of HPECs in contrast to cells infected with control virus at 4 days post-infection. Magnification is 100X. (B) HPECs expressing Cdc37ΔC or pBABE puro were harvested, fixed and stained with Hoechst 33342 and analyzed under fluorescent microscopy. Fragmented nuclei, indicative of apoptosis, were abundant in the Cdc37ΔC expressing cells and rare in vector only cells. The percentages of fragmented nuclei are shown ± the standard deviation from three separate infections. (C) BrdU analysis and propidium iodide staining demonstrates a marked proliferation reduction and an increase in the number of

cells in G0/G1 in HPECs infected with Cdc37ΔC compared to pBABE puro only controls at 5 days post-infection. (D) Reverse-transcriptase PCR directed at the N-terminus was used to demonstrate that the Cdc37ΔC was expressed. Our antibody did not react with the C-terminal truncated protein. Immunoblotting for the known Cdc37 target genes Raf-1 and Cdk4 indicates that the Cdc37ΔC is functional as the steady-state levels of these target genes are reduced. No alteration in the level of p16 was detected.

Figure 4. Cdk4 overexpression is insufficient to induce proliferation. (A) HPECs retrovirally overexpressing Cdk4 or the vector only were assayed at 8-days post-infection by BrdU analysis. Cdk4 overexpression did not result in proliferative increases. (B) The cell cycle profile determined by propidium iodide staining. Note consistently fewer cells in G0/1 in Cdk4 infected cells compared to the patient matched control pBABE puro infected cells (C) Immunoblotting of the cellular extracts shows Cdk4 to be overexpressed.

Figure 5. Cyclin D1 overexpression is sufficient to induce proliferation. (A) HPECs retrovirally overexpressing cyclin D1 or the vector only were assayed at 8-days post-infection by BrdU analysis. (B) The cell cycle profile determined by propidium iodide staining. Note fewer cells in both the G0/1 and G2 phases in cells overexpressing cyclin D1. (C) Immunoblotting analysis demonstrating cyclin D1 overexpression.

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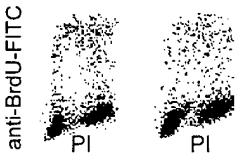
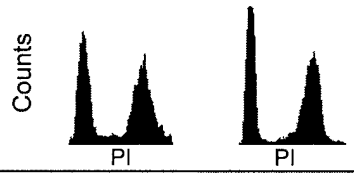
Figure 1 Schwarze et al.

A



Sample	pBABE-cdc37	pBABE
NP222	15.0%	9.2%
NP227	9.4%	4.2%
NP232	16.9%	9.6%

B



	pBABE-cdc37		pBABE	
Sample	G0/G1	G2/M	G0/G1	G2/M
NP222	34.3%	49.0%	36.1%	47.1%
NP227	47.6%	41.7%	53.7%	38.0%
NP232	39.2%	47.6%	53.8%	36.2%

C

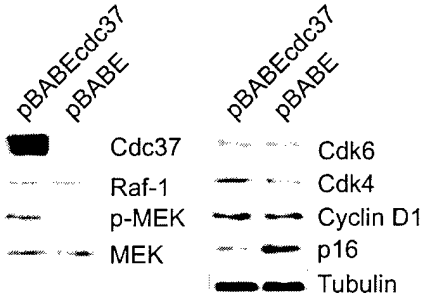


Figure 2 Schwarze et al.

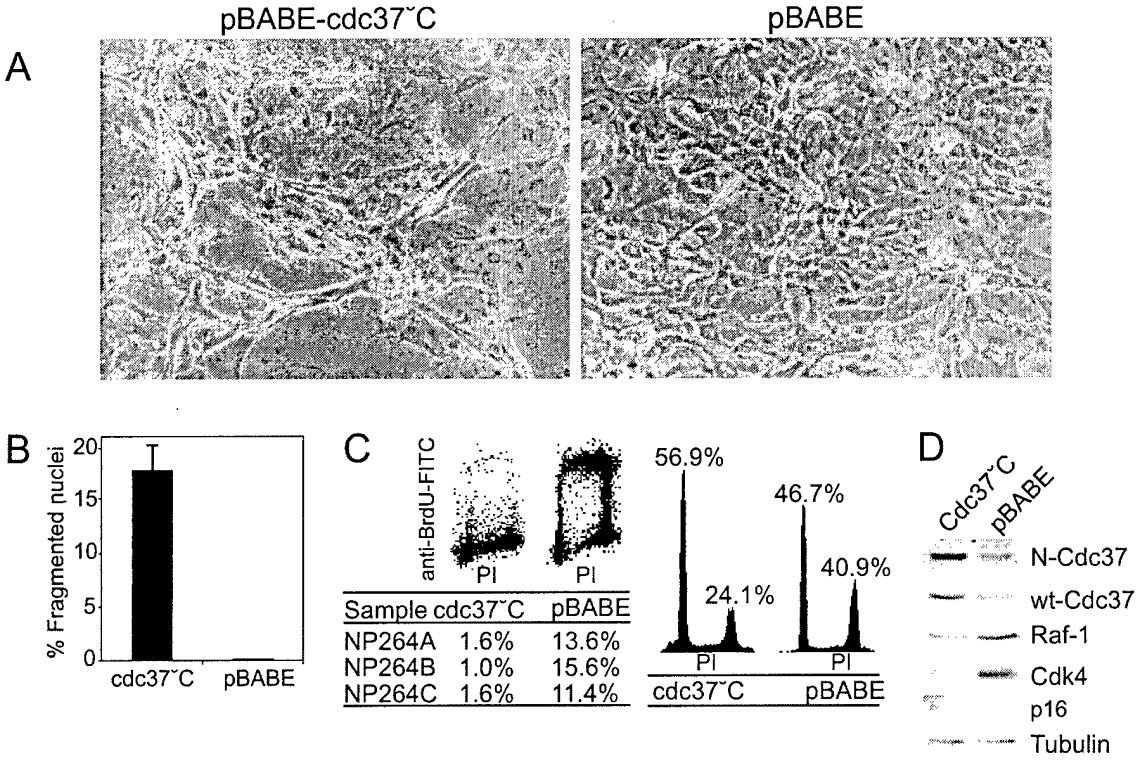


Figure 3 Schwarze et al.

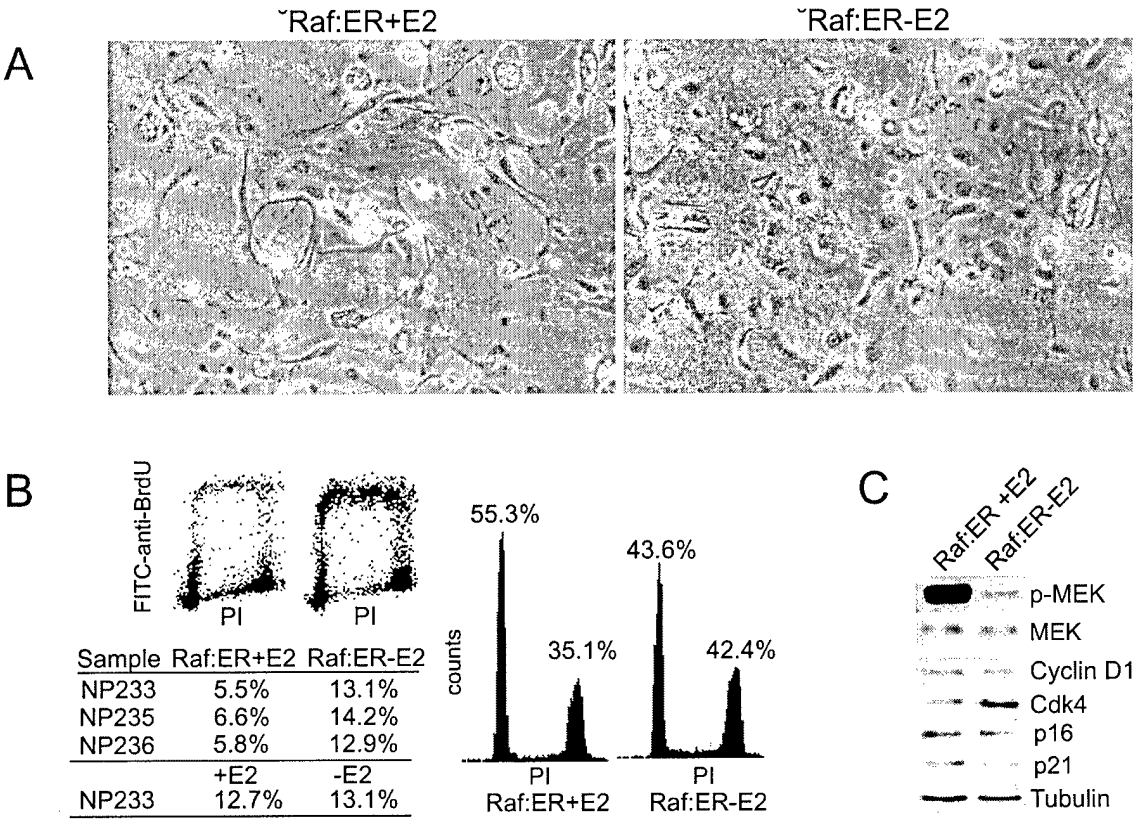
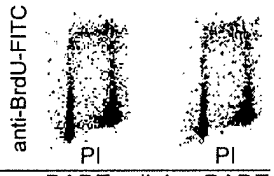


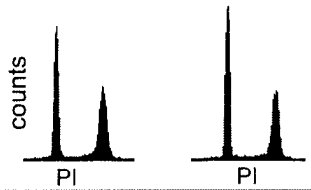
Figure 4 Schwarze et al.

A



	pBABE-cdk4	pBABE
NP233	8.7%	13.9%
NP235	12.4%	14.1%
NP248	7.8%	6.8%

B



	pBABE-cdk4		pBABE	
	G0/G1	G2/M	G0/G1	G2/M
NP233	34.2%	55.7%	44.9%	51.7%
NP235	27.7%	58.4%	31.5%	55.5%
NP248	43.2%	49.7%	49.8%	43.9%

C

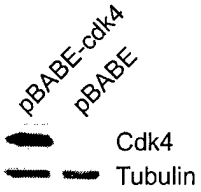
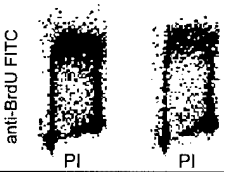


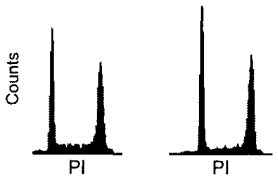
Figure 5 Schwarze et al.

A



Sample	pBABE-D1	pBABE
NP250	25.3%	16.8%
NP254	26.1%	16.3%
NP264	20.8%	10.7%

B



Sample	G0/G1	G2/M	G0/G1	G2/M
NP254	36.8%	39.6%	38.4%	45.2%
NP250	31.8%	43.8%	37.8%	45.8%
NP264	38.4%	44.0%	39.3%	47.8%

C