Cyclin-Dependent Kinase 2 Functions in Normal DNA Repair and Is a Therapeutic Target in BRCA1-Deficient Cancers

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Abstract

Abnormal regulation of progression from G_1 to S phase of the cell cycle by altered activity of cyclin-dependent kinases (CDKs) is a hallmark of cancer. However, inhibition of CDKs, particularly CDK2, has not shown selective activity against most cancer cells because the kinase seems to be redundant in control of cell cycle progression. Here, we show a novel role in the DNA damage response and application of CDK inhibitors in checkpoint-deficient cells. CDK2$^{-/-}$ mouse fibroblasts and small interfering RNA–mediated or small-molecule–mediated CDK2 inhibition in MCF7 or U2OS cells lead to delayed damage signaling through Chk1, p53, and Rad51. This coincided with reduced DNA repair using the single-cell comet assay and defects observed in both homologous recombination and nonhomologous end-joining in cell-based assays. Furthermore, tumor cells lacking cancer predisposition genes *BRCA1* or *ATM* are 2- to 4-fold more sensitive to CDK inhibitors. These data suggest that inhibitors of CDK2 can be applied to selectively enhance responses of cancer cells to DNA-damaging agents, such as cytotoxic chemotherapy and radiotherapy. Moreover, inhibitors of CDKs may be useful therapeutics in cancers with defects in DNA repair, such as mutations in the familial breast cancer gene *BRCA1*. (Cancer Res 2006; 66(16): 8219-26)

Introduction

The DNA damage response in mammalian cells is an evolutionarily conserved pathway that protects cells from the lethal or tumor-promoting consequences of genotoxic insult. Mutation in DNA repair genes, such as *BRCA1, BRCA2,* and *ATM,* accounts for a significant proportion of the breast cancers attributed to a familial predisposition, highlighting the importance of this process in tumor protection. Cell cycle regulation is thought to be important in regulating the DNA repair process. For example, nonhomologous end-joining (NHEJ) and homologous recombination (HR) are differently regulated in cycling versus quiescent cells, and the overall rate of DNA repair is faster in cells that are rapidly cycling compared with those that are quiescent (1). We propose a candidate and therapeutic target in this temporal regulation, cyclin-dependent kinase 2 (CDK2).

CDKs are a family of serine/threonine kinases that control the progression of the cell cycle, including the onset of DNA synthesis and mitosis (2). However, recent knockout and small interfering RNA (siRNA) studies suggested that CDK2 is not essential for somatic cell division and that other kinases can substitute for its function in the normal cell cycle. As CDK2 is considered a potential therapeutic target in cancer and multiple CDK2 inhibitors have shown efficacy in preclinical models and clinical trials, this was an unexpected outcome. Given that CDK2 has several non-cell cycle–associated substrates in the DNA damage response pathway [e.g., familial breast cancer genes *BRCA1* (3) and *BRCA2* (4), the tumor suppressor p53 (5), and the NHEJ factor Ku70 (6)], we examined the rate of DNA repair in cells where CDK2 was abrogated.

Using several *in vitro* systems, we found that inhibition of CDK2 using small-molecule inhibitors leads to a decrease in DNA repair activity in mammalian cells. Induction of DNA damage following specific ablation of CDK2 activity using short hairpin RNA (shRNA) or in CDK2$^{-/-}$ mouse embryonic fibroblasts (MEF) leads to a similar reduction in either the repair of an exogenous double-strand break in end-joining or HR assays or endogenous DNA damage after ionizing radiation. We show that after CDK inhibition, multiple components of the DNA damage response are altered in their response to damage caused by ionizing radiation, including phosphorylation of H2A.X, p53, and Chk1, and increased expression of p21^{Cip1}. Based on this finding, we tested the efficacy of CDK inhibition on cells that are deficient in other components of DNA repair, BRCA1 or ATM. Interestingly, CDK inhibition showed up to 4-fold increased toxicity in cells deficient in these DNA repair components, which are often mutated in familial breast cancer families. These findings show that CDK2 activity plays a key role in the normal DNA damage response and that CDK inhibition may be a chemotherapeutic option for specifically targeting cancer cells with defects in DNA repair.

Materials and Methods

Cell culture and flow cytometry. Immortalized CDK2$^{-/-}$ MEFs and matched wild-type controls were kindly provided by Mariano Barbacid's laboratory (Spanish National Cancer Institute, Madrid, Spain). MCF7, U2OS, and MEF cells were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS). FTY-pEB and FTY-pEB-(Flag)ATM cells (7) were cultured in RPMI 1640 supplemented with 10% FBS. Bromodeoxyuridine (BrdUrd) flow cytometry was done as described (8) on an LSR flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Drug treatments. Roscovitine (LC Laboratories, Woburn, MA) and PHA533533 (9) were dissolved in DMSO at 1,000× final concentration. IC_{70} doses were determined after 72-hour drug exposure using the sulforhodamine B assay as described (10).

DNA damage response assays. Subconfluent MCF7 cells were treated with CDK2 inhibitors and then immediately exposed to cesium-137 in a

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Gammacell 400 at 1.5 min/Gy. Protein samples were then collected at the indicated time points.

Clonogenic survival assays. MCF7 or FTY cells were plated at 300 to 10,000 cells per 6-cm plate. Sixteen hours later, cells were treated with drugs at the specified doses and incubated in normal medium containing drug for 12 days, fixed in 90% methanol, and stained with 0.1% crystal violet. Colonies of >50 cells were counted in the surviving fraction.

Single-cell comet assay. The single-cell comet assay was done as described previously (11). The Olive tail moment was determined by analysis of at least 50 nuclei from each treatment group using MetaMorph software (Universal Imaging Corp., Downington, PA).

DNA end-joining assay. The plasmid pZeoSV (Invitrogen, Carlsbad, CA) was completely digested with *Pvu*II and purified using JetStar DNA purification beads (Genomed, Lohne, Germany). MCF7 cells (1.5×10^5 per well) were plated in six-well plates. The following day, 0.75 μg digested plasmid was transfected per well using Fugene 6 reagent (Roche, Mannheim, Germany). Six hours later, CDK2 inhibitors or DMSO vehicle was added to cells. Following 12, 24, or 40 hours of incubation, DNA was extracted with proteinase K digestion overnight at 55°C followed by phenol/chloroform extraction and isopropanol precipitation. DNA (10 ng) was used as a PCR template. Quantitative real-time PCR was done using the SYBR Green detection method (Applied Biosystems, Warrington, United Kingdom) and primers specific for the rejoined cut site [5′-CGAGAATTCGAACGCGTGA-3′ (forward) and 5′-TGTGAAATTTGTGATGCTATTGCTT-3′ (reverse)] or an uncut region of the plasmid [pZeo, 5′-ATAATACGACTCACTATAGGAG-GGCC-3′ (forward) and pZeo, 5′-GAACTCGACCGCTCCGG-3′ (reverse)].

HR assay. A U2OS clone containing a single complete copy of the integrated HR reporter hprt-DR-GFP was generated as described previously (12, 13) U2OS-DR-GFP cells (1.5×10^5 per well) were seeded in six-well plates and, the following morning, transfected with 1 μg of pCBA-I-*Sce*I (12) plasmid using Fugene 6 reagent. Six hours later, medium was replaced with fresh medium containing drugs at the specified concentration. Cells were harvested after 2 days for flow cytometric analysis on a FACSCalibur cytometer (Becton Dickinson).

siRNA-mediated knockdown. Knockdown of BRCA1 using pSuper vector-mediated RNA interference (RNAi) has already been described (14). Stable knockdown of CDK2 was achieved after cotransfection of BS/U6/shCDK2 (15) and pBabe-hygro into MCF7 cells or the U2OS-DR-GFP line described above. Clones were selected in hygromycin and screened for CDK2 expression by Western blotting.

Western blotting and kinase assays. SDS-PAGE, Western blotting, and CDK kinase assays were done as described previously (14) using the following primary antibodies: p53 and BRCA1 (Oncogene Research, Darmstadt, Germany), p21, CDK2, and Chk1 (Santa Cruz Biotechnology, Santa Cruz, CA), γH2A.X (Upstate Biotechnology, Charlottesville, VA), $p53^{Ser15}$ (16G8) and $Chk1^{Ser317}$ (Cell Signaling Technology, Beverly, MA), ATM (Gentex, San Antonio, TX), and α-tubulin (Sigma, Castle Hill, NSW, Australia).

Immunofluorescence staining. The following antibodies were used: Rad51 (Ab1, 1:300; Oncogene Research), BRCA1 (Ab1, 1:100), 53BP1 (1:100; J. Chen, Mayo Clinic, Rochester, MN), and γH2A.X (1:1,000). Cells were grown in eight-well chamber slides (Medos, Mt Waverly, VIC, Australia), exposed to drug and irradiated with 5 Gy ionizing radiation. After 1 hour of incubation, cells were fixed in 3.7% paraformaldehyde for 20 minutes at room temperature and blocked for 1 hour in TNB [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% (w/v) bovine serum albumin] containing 0.2% (v/v) Triton X-100 followed by overnight incubation at 4°C with primary antibody in TNB. Secondary antibodies (anti-mouse or anti-rabbit Alexa Fluor 488/568, diluted 1:1,000) were from Molecular Probes (Eugene, OR). Representative images were taken on a Bio-Rad (Hercules, CA) MRC1000 confocal microscope at ×400 magnification.

Statistical analysis. Statistical analyses were done using the unpaired Student's two-tailed *t* test as indicated.

Results

Small-molecule CDK2 inhibitors arrest the cell cycle and interact with ATM/ATR target activation in MCF7 cells. To investigate the role of CDK inhibition in the mammary epithelial normal cell cycle, two small-molecule inhibitors of CDKs were used. The R-anantomer of roscovitine (also known as CYC202 or Seliciclib) is currently in phase II trials for the treatment of non–small cell lung cancers and B-cell malignancies and has similar activity against CDK2 and CDK1, whereas PHA533533 is a 3-aminopyrazole that is in preclinical development and is a more potent and selective inhibitor of CDK2 (9). The structure and chemical inhibitory doses of the drugs against a panel of purified kinases are shown in Fig. 1.

In cultured MCF7 mammary adenocarcinoma cells, doses of 3 μmol/L PHA533533 and 20 μmol/L roscovitine lead to complete

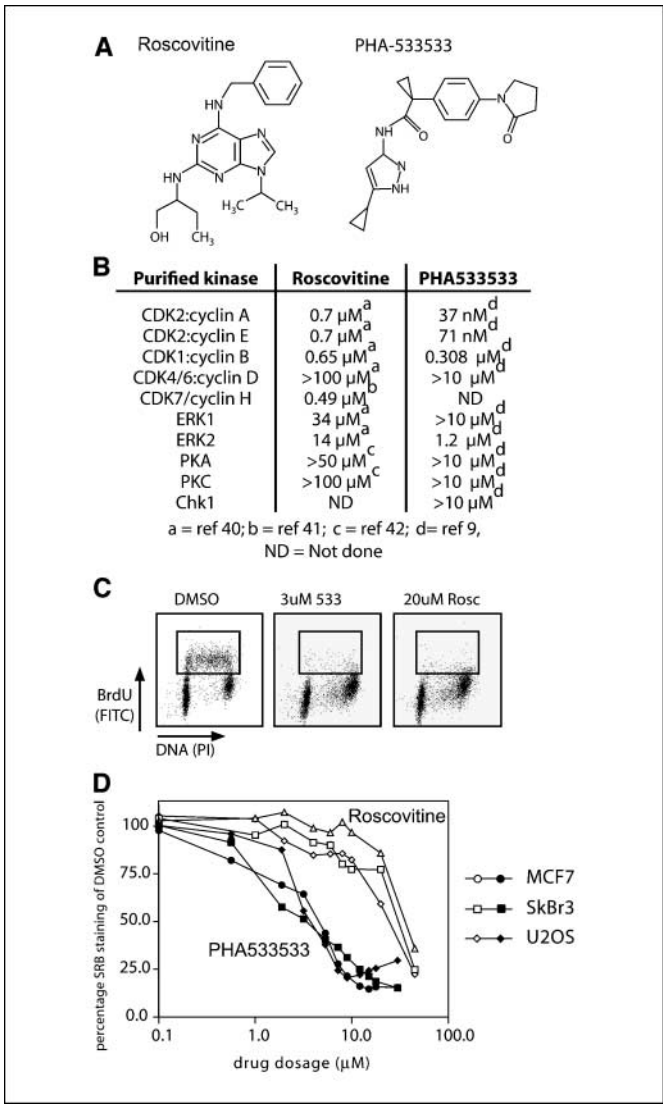

Figure 1. *A* and *B,* biochemical and antiproliferative properties of the CDK2 inhibitors used in this study: their chemical structure and chemical IC_{50} against selected purified kinases (*a,* ref. 40; *b,* ref. 41; *c,* ref. 42; *d,* ref. 9). *ERK,* extracellular signal-regulated kinase; *PKA,* protein kinase A; *PKC,* protein kinase C. *C,* synthetic CDK2 inhibitors arrest MCF7 cells at all phases of the cell cycle as measured by fluorescence-activated cell sorting analysis of propidium iodide (*PI*)–stained and BrdUrd-labeled MCF7 cells. For example, at 3 μmol/L PHA533533 (*533*) and 20 μmol/L roscovitine (*Rosc*), DNA synthesis is inhibited as can be noted by cells with a >2N<4N content that is not actively incorporating BrdUrd. *Boxed,* loss of cells in S phase, which are incorporating BrdUrd. *D,* several human cell lines have similar dose response curves to roscovitine and PHA533533 as measured by sulforhodamine B (*SRB*) staining of cells grown in the presence of various drug doses as per Materials and Methods.

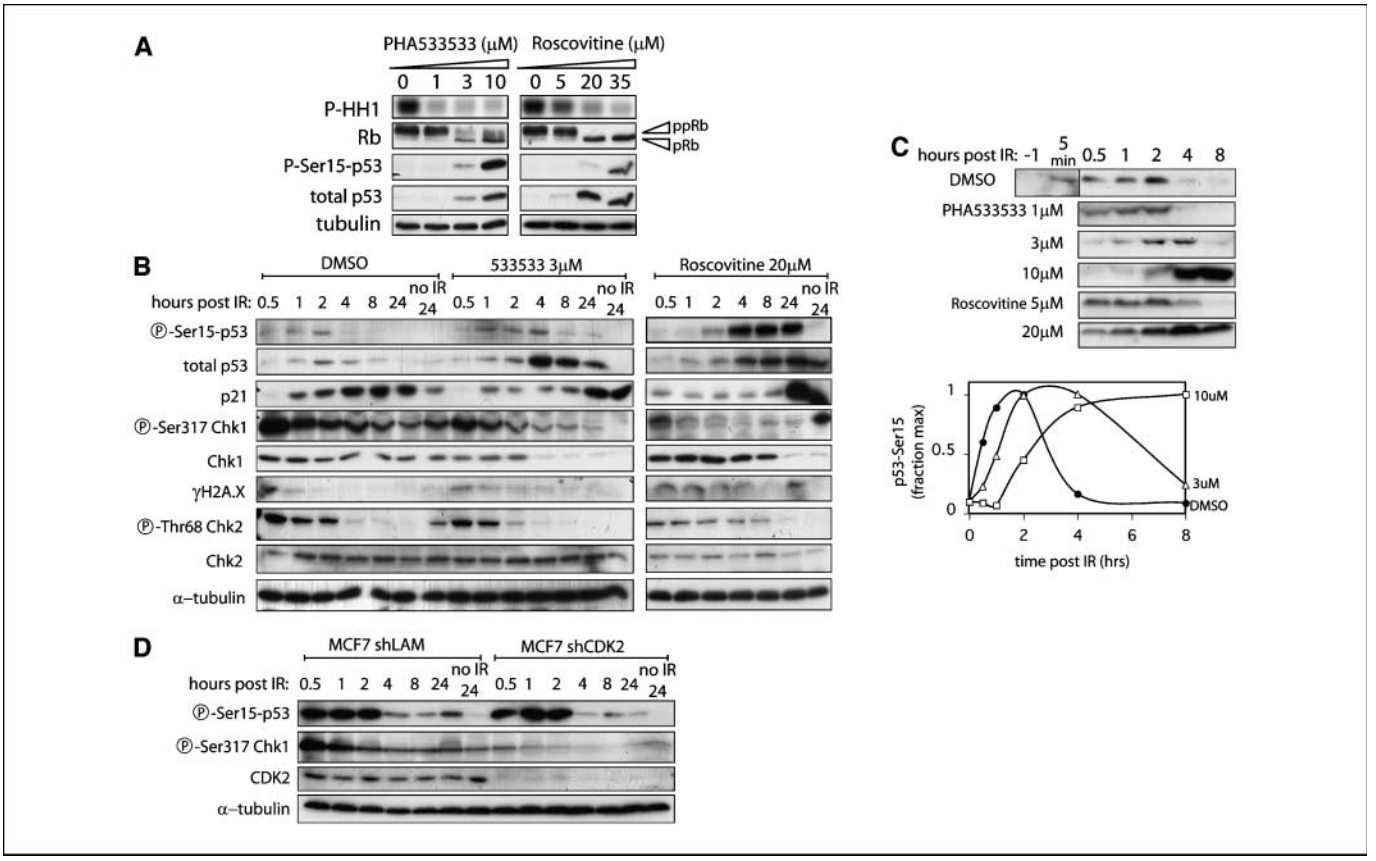

Figure 2. Synthetic CDK inhibitors interact with ATM/ATR target activation after DNA damage. *A,* CDK inhibitors inhibit MCF7 CDK2 kinase activity against recombinant histone H1 (*HH1*; measured by H1 kinase assay; see Materials and Methods) or the endogenous retinoblastoma (*Rb*) protein (band shift on Western blot). Both synthetic CDK inhibitors also induce p53 stabilization and phosphorylation on Ser^{15} after 24 hours of exposure. *B,* IC_{70} doses of PHA533533 and roscovitine alter the normal phosphorylation of p53, Chk1, and histone H2A.X, but not Chk2, after irradiation. MCF7 cells were refed with medium containing DMSO or drug immediately before 5 Gy γ-irradiation. Protein samples were harvested at the designated time points (hours). Unirradiated controls were exposed to the same dose of drugs for 24 hours without ionizing radiation (*IR*) treatment. Accumulation of total p53, Chk1, and p21 after irradiation. *C,* this effect is dose dependent, as increasing dose of PHA533533 or roscovitine enhances the effect of CDK2 inhibitors on Ser^{15} phosphorylation of p53. Loading controls are in Supplementary Fig. S1A. *D,* Chk1-Ser^{317} and p53-Ser^{15} phosphorylation after 5 Gy ionizing radiation in and MCF7-shCDK2 clone compared with control. Other Western results are in Supplementary Fig. S1B.

inhibition of endogenous CDK2 activity as measured by an *in vitro* kinase activity using immunoprecipitated CDK2 and a recombinant histone H1 substrate. Furthermore, drug treatment caused a shift in the SDS-PAGE mobility of the retinoblastoma protein, an endogenous target of CDK2 phosphorylation (Fig. 2*A*). The almost complete inhibition of DNA synthesis after 24 hours is measured by BrdUrd uptake (Fig. 1*C*) and >70% inhibition of proliferation over 72 hours as measured by the sulforhodamine B assay (Fig. 1*D*). Both drugs also induced phosphorylation at Ser^{15} and stabilization of p53 after 24 hours of exposure (Fig. 2*A*). p53-Ser^{15} phosphorylation is ATM/ATR dependent and a response to genotoxic insult (16). Others have reported that CDK inhibitors do not directly cause DNA damage, but given CDK inactivation is a downstream target of the pathway, we were interested to know if CDK inhibitors could modulate the DNA damage response after exogenous genotoxic insult. Surprisingly, after 5 Gy ionizing radiation, CDK inhibitor drugs lead to a delay in the activation of p53-Ser^{15} phosphorylation, the stabilization of p53 protein, and the up-regulation of the p53 target $p21^{Cip1}$ (Fig. 2*B*), although some p53-independent up-regulation of $p21^{Cip1}$ was also observed most likely through alteration of retinoblastoma activity as a downstream consequence of CDK2 inhibition (17). The effect on p53 phosphorylation was dose dependent, and increasing CDK inhibitor drug concentration further delayed the activation of p53 (Fig. 2*C*). The phosphorylation of another ATM target, Chk1-Ser^{317}, was also reduced at 30 minutes after ionizing radiation but continued to decrease to background levels by 2 hours after ionizing radiation. This coincides with the loss of total Chk1 expression after CDK inhibition as described previously (18). Interestingly, the phosphorylation of a third component of the DNA damage response, Chk2, was not altered in response to CDK inhibitors, suggesting some specificity in the effects to the repair pathways. Nonetheless, the clearance of phosphorylated H2A.X, a conserved marker of DNA double-strand breaks in eukaryotic cells (19), was significantly delayed in irradiated cells pretreated with either roscovitine or PHA533533 (Fig. 2*B*). Overall, these results provide evidence that CDK inhibition delays the normal activation of DNA damage signaling but prolongs the persistence of double-strand breaks.

While being relatively specific drugs, roscovitine and PHA533533 can have activity against other CDKs and extracellular signal-regulated kinases, although at higher doses than for CDK2 and Cdc2 (Fig. 1). Importantly, others have shown that, although 10 to 20 μmol/L roscovitine arrests $CDK2^{+/+}$ cells, no cell cycle– or cell

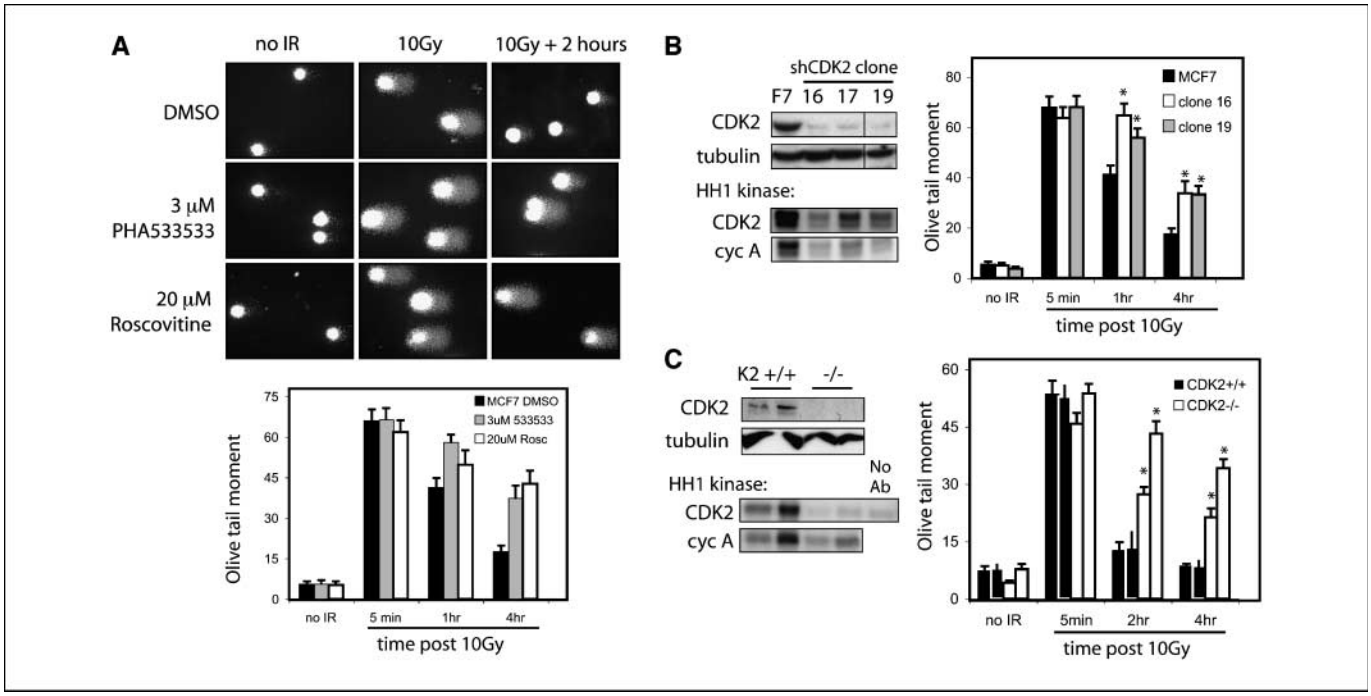

Figure 3. CDK2 inhibition delays repair as measured by the single-cell comet assay. *A,* MCF7 cells were treated with PHA533533 (3 μmol/L) or roscovitine (20 μmol/L) before exposure to 10 Gy ionizing radiation. Single-cell electrophoresis was done either immediately or 1, 2, or 4 hours after ionizing radiation under alkaline conditions. Example comets. The relative length and intensity of ethidium bromide–stained DNA tails to heads is proportional to the amount of DNA damage present in the individual nuclei and is measured by Olive tail moment [tail length × (tail fluorescence / (head + tail fluorescence))]. *B* and *C,* similar reductions in repair were seen in MCF7-shCDK2 cells and MEFs obtained from CDK2-deficient mice. *, $P > 0.01$. Knockdown in MCF7 cells (>90%) or knockout in MEFs of CDK2 protein levels by Western blotting, whereas *in vitro* kinase activity of immunoprecipitated CDK2 and cyclin A complexes against recombinant histone H1 compared with a no antibody (*No Ab*) control.

survival–associated effects are observed in CDK2$^{-/-}$ cells at up to 25 μmol/L (20), suggesting that CDK2 is the predominant target in mammalian cells. To further confirm a specific role for CDK2 in the DNA damage response, we used stable knockdown of CDK2 by shRNA. An MCF7-shCDK2 clone or MCF7-shLamin control was analyzed in a time course after DNA damage. In contrast to drug treatment, p53-Ser^{15} phosphorylation was normal in these cells at the 30-minute time point; however, Chk1-Ser^{317} phosphorylation was almost completely absent (Fig. 2*D*). Similar delays are also observed in two other knockdown clones (data not shown). This suggests that the residual CDK2 protein in these clones (Fig. 2*D*) is sufficient to assist with DNA damage signaling to p53 but not Chk1 or that other drug-sensitive kinases may partially compensate for CDK2 for some DNA damage response targets. It is likely that direct phosphorylation of p53 by CDK2 is not important, as a CDK phosphorylation site mutant of p53 (p53-S315A; ref. 5) was not impaired for Ser^{15} phosphorylation after ionizing radiation.[6]

Absence of CDK2 leads to persistence of unrepaired DNA damage in irradiated MCF7 cells. A direct role for CDKs in the DNA damage response was examined using the single-cell comet assay. MCF7 cells were exposed to 10 Gy ionizing radiation after treatment with 3 μmol/L PHA533533, 20 μmol/L roscovitine, or DMSO (vehicle control) and allowed to recover for up to 4 hours. Single cells were then electrophoresed following the comet protocol to separate damaged from intact DNA. Comet tails, representing a significant fraction of unrepaired genomic DNA damage, were still present in treated cells, whereas the majority of control cell DNA remained in the head (Fig. 3*A*), indicating that inhibition of CDKs inhibited DNA repair. Importantly, both CDK inhibitor drugs did not induce significant damage in the absence of ionizing radiation nor did they enhance the amount of tail DNA in association with ionizing radiation (Fig. 3*A*).

Although the small molecules potentially target several CDKs and show some off-target activity (Fig. 1), this result was replicated by specifically ablating CDK2. MCF7-shCDK2 clones were selected with >80% knockdown of CDK2 protein and associated kinase activity (Fig. 3*B*). These clones had significantly higher levels of unrepaired DNA than wild-type cells up to 4 hours after 10 Gy ionizing radiation ($P > 0.01$). Finally, two independently derived CDK2-deficient immortalized MEF lines retained a significant percentage of tail DNA at 2 to 4 hours when matched wild-type control lines had comet profiles similar to unirradiated samples. These three systems confirm that the absence of CDK activity results in delayed repair of endogenous DNA damage and that this activity can be substantially attributed to CDK2.

CDK inhibition directly slows the rate of DNA repair. A novel end-joining assay was used to assess the role of CDK2 in the repair of an exogenous double-strand break by NHEJ. Quantitative real-time PCR was used to follow the rejoining of a linearized transfected plasmid where only uncut template leads to production of a PCR product (Fig. 4*A*), whereas further primers outside the cut site were used as an input control. PHA533533 (3 μmol/L) or roscovitine (20 μmol/L) reduced the rejoining of the linearized plasmid up to 72 hours after

[6] A.J. Deans, unpublished observation.

transfection. Similarly, specific abrogation of CDK2-associated kinase activity using siRNA leads to a >2.5-fold reduction in cell-based end-joining activity. This was a comparable reduction in end-joining with what was seen using similar assays in p53- or BRCA1-deficient cells (21, 22) and shows that loss of CDK2 directly affects rates of NHEJ.

The alternate DNA repair pathway HR was also measured using U2OS osteosarcoma cells containing an integrated HR reporter, hprt-DR-GFP. DR-GFP is composed of two differentially mutated *GFP* genes oriented as direct repeats, and expression of I-*Sce*I in these cells leads to a unique double-strand break in the reporter. A gene conversion event within this substrate results in the expression of intact green fluorescent protein (GFP; Fig. 5*A*; ref. 12), which can be assayed by flow cytometry. The incidence of HR in U2OS-DR-GFP cells transfected with I-*Sce*I (transfection rate, 50-60%; data not shown) was dramatically decreased in cells treated with increasing concentrations of CDK inhibitor drugs, including at doses that do not show significant effects on cell cycle progression or cell survival (Fig. 5*A*).

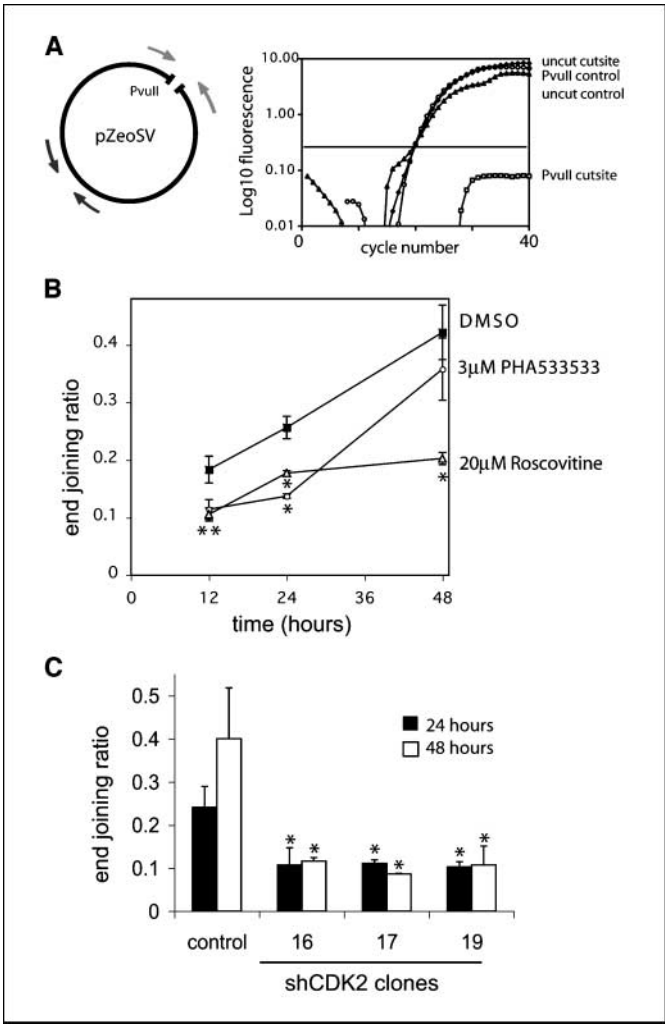

Figure 4. Repair of an exogenous double-strand break is delayed by CDK inhibition. *A,* primer sets used to measure end-joining assay. The cut site primer set (*gray*) generates a product only when pZeoSV is uncut, whereas the control primer set (*black*) generates a real-time PCR product with identical kinetics when the plasmid is cut or uncut. *B,* pZeoSV/*Pvu*II was transfected into MCF7 followed by addition of CDK inhibitors 6 hours later. DNA was extracted and subject to quantitative real-time PCR as per Materials and Methods. Cycle threshold (CT) scores were converted to absolute quantities via the equation 1/2^CT. The amount of cut site product was normalized to the control product. The ratio is a unitless relative measurement of the rejoining of the plasmid in the cell lines. *C,* similar reduction in end-joining was seen in three independent MCF7-shCDK2 clones (same clones used in Fig. 3*B*) compared with the MCF7 parental cell line. *Columns,* mean; *bars,* SD. *, $P < 0.05$.

To determine the specific contribution of CDK2 inhibition to this observation, three independently derived lines with stable knockdown of CDK2 were generated, which show >80% knockdown of total CDK2 protein yet normal cell cycle regulation (Fig. 5*B*). Despite having some residual CDK2 protein expression, HR is reduced by up to 55% in these clones compared with parental and matched control siRNA cell lines. Finally, as shown in Fig. 5*D*, dominant-negative (kinase dead) CDK2 can also decrease HR by 4-fold in this assay, whereas cyclin E, a positive regulator of CDK2, can increase HR by 60% (Fig. 5*C*).

At sites of HR, several proteins accumulate in discrete foci, including H2A.X, BRCA1, Rad51, and 53BP1 (19). As HR was greatly reduced in the absence of CDK2 activity in U2OS cells, we measured the accumulation of these proteins in foci after ionizing radiation. Whereas CDK inhibitor–treated cells displayed normal H2A.X, BRCA1, and 53BP1 accumulation at dsDNA breaks after ionizing radiation (Fig. 5*D*; Supplementary Fig. S2), Rad51 foci were absent 1 hour after ionizing radiation in U2OS-shCDK2 and CDK inhibitor–treated cells compared with vehicle only or control siRNA U2OS cells. These results suggest that CDK2 can regulate both HR and NHEJ independently of cell cycle–related events.

Inhibiting CDKs can selectively target BRCA1- and ATM-deficient cells. Several approaches have been taken to selectively target the DNA repair defects found in cancers that lack the familial breast cancer genes *BRCA1* or *ATM* (23). As we showed a repair failure after loss of CDK2 activity, we sought to determine if CDK inhibitors could also interact with ATM or BRCA1 loss to reduce clonogenic survival in tumor cell lines. Small hairpin–mediated knockdown of BRCA1 in MCF7 cells leads to a 2-fold and >4-fold increased sensitivity compared with a control shRNA vector with PHA533533 and roscovitine treatment, respectively (Fig. 6*A* and *B*). A >2-fold decrease in sensitivity to PHA533533 was also observed in the ATM-deficient FTY cell line when full-length wild-type ATM was exogenously expressed (Fig. 6*D*). We confirmed that CDK2 knockdown led to reduced viability in the absence of BRCA1 by transfecting shBRCA1 into MCF7-shCDK2 clones (knockdown shown in Fig. 3*C*). Colony-forming ability was reduced by 80% in these clones compared with parental cells (Fig. 6*C*). These data further establish a role for CDKs in the DNA repair process.

Discussion

Small-molecule CDK2 inhibitors are under clinical trial for use in the treatment of cancers as well as a variety of other human diseases (reviewed in ref. 24). We provide evidence that these drugs may be particularly useful in treating cancers that are deficient in familial breast cancer genes *BRCA1* and *ATM* based on a novel role for CDK2 in the DNA damage response. Although CDK2 was originally thought to play an essential role in the initiation of DNA synthesis in S phase, CDK2 siRNA in human cancer cells or targeted deletion in the mouse is nondeleterious and CDK2$^{-/-}$ somatic cells continue to cycle

normally (25, 26). Yet, activation of CDKs can promote progression of the mammalian cell cycle, and pathways regulating CDKs are activated in the majority of human cancers (27). Unlike its role in G_1-S progression (28), this study shows a role for CDK2 that cannot be substituted by other redundant kinases in the normal cell cycle. Other reports have recently shown that cyclin A1 is induced early after γ-irradiation and that purified cyclin A1/CDK2 could promote DNA repair in an *in vitro* assay (29). Furthermore, although CDK2-deficient mice display a normal mitotic cell cycle, meiosis fails at prophase I, a phenotype analogous to that seen in mice lacking other components of DNA repair machinery (30, 31). CDK2 is found along chromosome synapses during prophase, at a time when DNA repair pathways are recruited to participate in genetic exchange between sister chromatids (32). Further evidence is found in *Saccharomyces cerevisiae*, where HR and activation of the ATM homologue Mec1 require the single CDK homologue Cdc28 for double-strand break processing to 3′-tails (33). These studies support our finding that *CDK2* is an essential gene in the DNA repair process both during HR and NHEJ.

The reduced HR observed in CDK-inhibited cells is potentially due to the absence of Rad51 in DNA damage foci. Furthermore, CDK inhibition leads to delayed p53 phosphorylation and activation of its target p21 after ionizing radiation as well as reduced Chk1 phosphorylation. An absence of Chk1 phosphorylation also leads to a reduction in ionizing radiation–induced Rad51 foci formation (34), so we hypothesize that CDK2 acts upstream of this kinase in the DNA damage response and potentially in multiple components of the pathway given the large number of CDK targets found in protein complexes involved in the DNA damage response. Such targets include both BRCA1 and its binding partner BARD1 (5, 35). Moreover, the "synergy" observed in our experiments (Fig. 6) and in human cancers between loss of CDK2 and BRCA1 may hint at an epistatic interaction between them. BRCA1- and BRCA2-mutant cancers seem to specifically select for activation of CDK2 activity, with loss of p27 (a CDK2 inhibitor protein) and overexpression of cyclin E (an activating cyclin) being two of several characteristics that have come to define familial breast cancers as a distinct subtype (14, 36). If BRCA-associated cancers have become dependent on CDK activity for their survival, this provides further support for the use of CDK inhibitor drugs in their selective cell killing.

This study provides a mechanism to examine the combined scheduling of CDK inhibitors with DNA-damaging agents. Already, flavopiridol has been investigated in combination with ionizing radiation in stage III clinical trials (24), whereas roscovitine can synergize with both ionizing radiation and the ATM/ATR inhibitor caffeine to induce cell killing in tumor cell lines (37, 38). We now provide evidence that cell types with defects in DNA repair pathways show increased sensitivity to CDK2 inhibitors in long-term clonogenic survival assays. ATM inhibitors and other DNA repair-inhibiting enzymes, which may be good candidates for combination with CDK inhibitors, are currently in development (23, 39), but based on our findings in cell culture models, BRCA1- and ATM-deficient

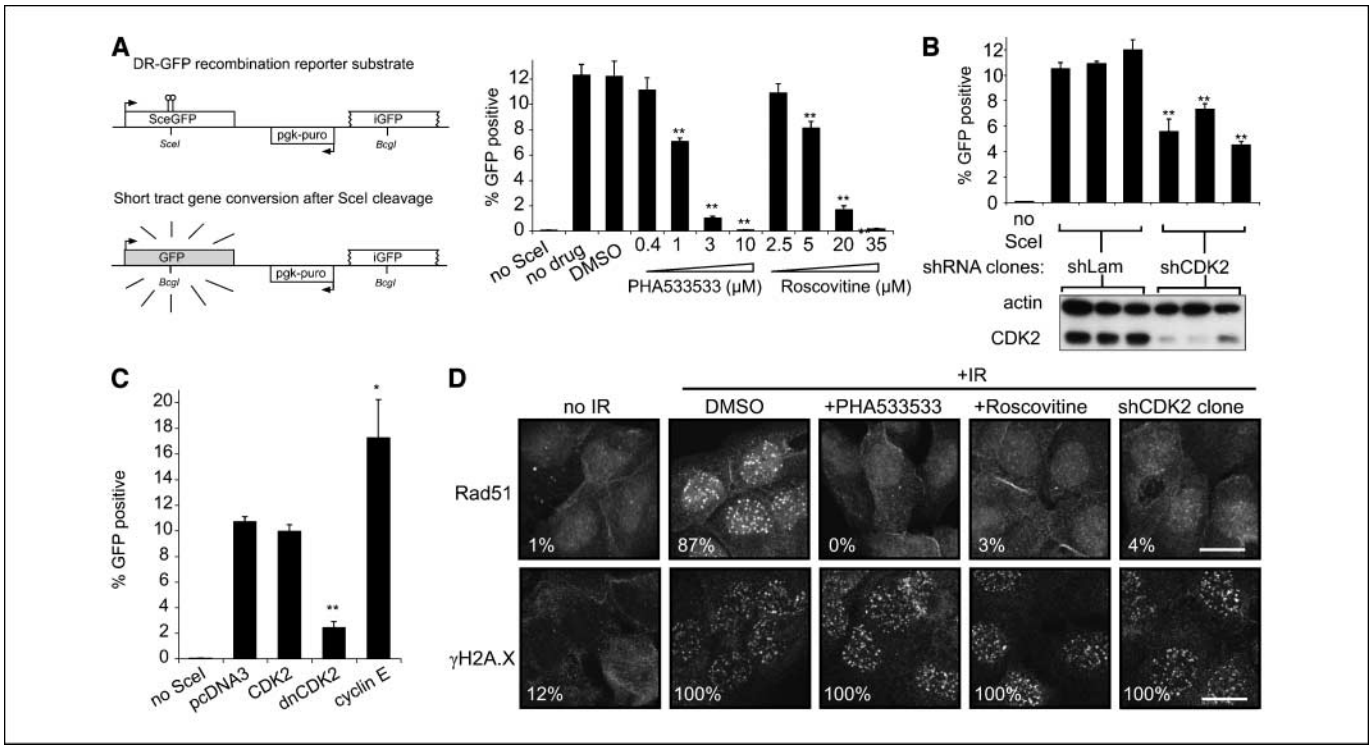

Figure 5. *A,* the HR reporter hprt-DR-GFP and GFP-expressing repair product. GFP expression increases after I-*Sce*I expression but is reduced with increasing doses of CDK inhibitors PHA533533 and roscovitine. Similar effects are seen with (*B*) stable CDK2 knockdown in three independently derived controls (CDK2 expression versus actin in the knockdown clones by Western blotting compared with three control clones) and (*C*) a dominant-negative kinase-dead CDK2 cotransfected at the same time as the *Sce*I plasmid. Cyclin E expression leads to increased HR in this assay. Percentage GFP-positive cells were determined by flow cytometry done 48 hours after transfection. *Columns,* mean; *bars,* SD. *, $P < 0.01$; **, $P < 0.001$. *D,* CDK inhibition inhibits Rad51 recruitment to DNA damage foci after ionizing radiation. U2OS cells or a U2OS-shCDK2 clone was treated with CDK inhibitors or DMSO and 5 Gy of ionizing radiation, and after 1-hour recovery, cells were fixed and stained with anti-Rad51 or anti-H2A.X antibody. *Inset,* percentage foci-positive cells for each sample. Bar, 10 μm.

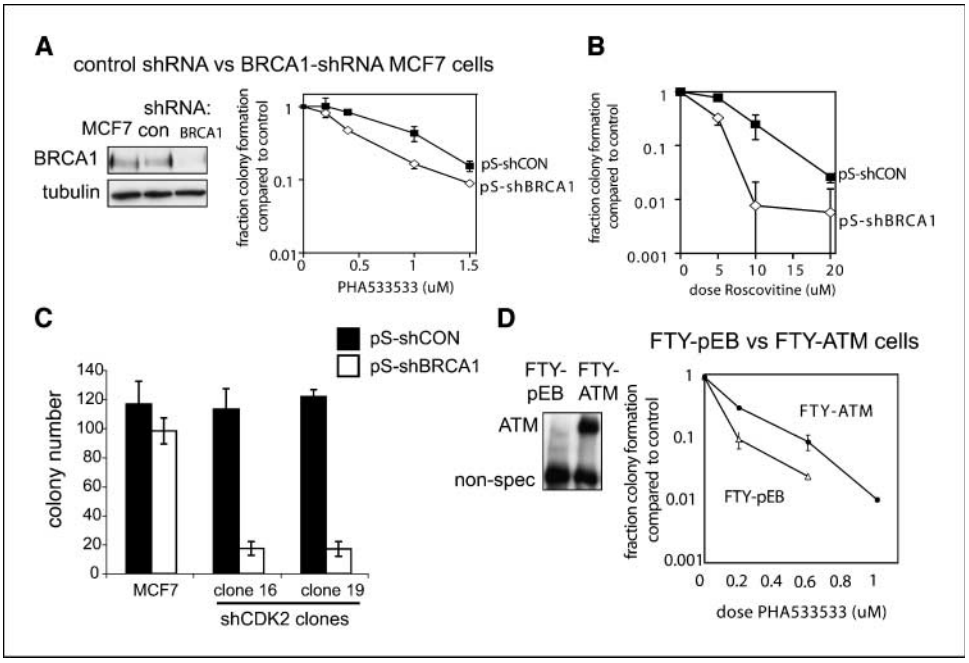

Figure 6. Reduced clonogenic survival of BRCA1- and ATM-deficient cells after CDK2 inhibition. *A* and *B,* clonogenic survival assays for MCF7 cells transfected with pEGFP-C1 and BRCA1-specific RNAi (pSuper-BRCA1i) or control RNAi (pSuper-CONi) and collected by flow cytometry based on green fluorescence. Cells were grown in the presence of varying doses of PHA533533 or roscovitine for 12 days and fixed, and colonies of >50 cells were counted. *Points,* mean; *bars,* SD. *C,* colony formation assays of MCF7-shCDK2 clones (same as those shown in Fig. 3*C*) after transfection with BRCA1-specific RNAi, and 800 cells were plated per 6-cm plate and cultured for 12 days. The number of colonies formed was counted. *Columns,* mean; *bars,* SD. *D,* clonogenic survival assays for ATM-mutant FTY cells stably expressing Flag-ATM or a vector only (pEB) grown in the presence of increasing doses of PHA533533 for 12 days. BRCA1 knockdown and Flag-ATM expression were confirmed by Western blotting using an anti-BRCA and anti-ATM antibody.

cancers are an excellent candidate for evaluation of CDK inhibitors in early phase clinical trials.

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