Cyclin D1 Integrates Estrogen-Mediated DNA Damage Repair Signaling

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Abstract

The cyclin D1 gene encodes the regulatory subunit of a holoenzyme that phosphorylates the retinoblastoma protein (pRb) and nuclear respiratory factor (NRF1) proteins. The abundance of cyclin D1 determines estrogen-dependent gene expression in the mammary gland of mice. Using estradiol (E2) and an E2-dendrimer conjugate that is excluded from the nucleus, we demonstrate that E2 delays the DNA damage response (DDR) via an extranuclear mechanism. The E2-induced DDR required extranuclear cyclin D1, which bound ERα at the cytoplasmic membrane and augmented AKT phosphorylation (Ser473) and γH2AX foci formation. In the nucleus, E2 inhibited, whereas cyclin D1 enhanced homology-directed DNA repair. Cyclin D1 was recruited to γH2AX foci by E2 and induced Rad51 expression. Cyclin D1 governs an essential role in the E2-dependent DNA damage response via a novel extranuclear function. The dissociable cytoplasmic function to delay the E2-mediated DDR together with the nuclear enhancement of DNA repair uncovers a novel extranuclear function of cyclin D1 that may contribute to the role of E2 in breast tumorigenesis. Cancer Res; 74(14); 3959–70. ©2014 AACR.

Introduction

Genomic integrity is monitored by cellular networks governing the DNA damage response (DDR). Defects in DNA damage signaling or repair contribute to degenerative diseases and cancer. The DDR involves a DNA damage signaling arm, which includes sensors, transducers (ATM and ATR), mediators, and effectors. The transducer kinases include ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR). The ATM signaling mediators (p53BP1, MDC, and BRCA1) and ATR mediators (TopBP1 and Claspin) in turn activate effectors, including CHK1 and CHK2 (1). ATR activity requires association with ATRIP and TopBP1 proteins. The downstream substrates of ATR and ATM include BRCA1, BRCA2, NBS1, and CHK2. ATR activation results from replication stress, whereas ATR and ATM both play a role in cellular response to double-stranded DNA breaks (DSB). The DDR induces assembly of nuclear repair foci. Phosphorylation of histone H2AX on serine 139 produces γH2AX, which recruits proteins that sense or signal the presence of DNA damage. DDR activation occurs early in human tumor cells in which activation of γH2AX occurs early, often in the preinvasive stages of human tumor correlating with the presence of senescence markers (2). Activated oncogenes, including c-Myc, Ras, Mos, CDC25, E2F1, cyclin D1, and cyclin E (3–6), induce DSBs and the DDR. DNA damage is a common feature of premalignant lesions, including breast ductal carcinoma in situ (7, 8).

In human breast cancer, estrogen receptor alpha (ERα) bound to its ligand estradiol (E2) contributes important survival and proliferative effects. The major adjuvant therapy for human breast cancer involves antagonists for the approximately 70% of ERα-expressing human tumors. E2 is known to delay the assembly and prolongs the resolution of γH2AX and Rad51 foci through inhibition of ATR kinase signaling (9). As E2/ERα increased chromosomal damage after irradiation, it has been suggested that E2-mediated restraint of ATR activation may be a novel estrogen action that promotes breast malignancy. Estrogen serves as a ligand for ERα inducing nuclear receptor activity and also participates in an acute cytoplasmic membrane-associated activity (reviewed in ref. 10). The ERα regulates nuclear gene expression via binding to both canonical DNA and noncanonical DNA sequences in the promoter of target genes. Extranuclear pools of ERα have been identified in the plasma membranes (11). The ability to distinguish nuclear from extranuclear ERα signaling has been enabled through the generation of 17β-estradiol dendrimer conjugates (EDC), which are localized to the extranuclear domain.

Supplementary data for this article are available at Cancer Research Online. (http://cancerres.aacrjournals.org/).
compartments (12, 13). The contribution of nuclear versus extranuclear effects of E2 is important in providing optimal patient treatment as, for example, the vascular protection and the protection of cortical bone mass are mediated at least in part via nongenomic E2 signaling (14, 15).

The cyclin D1 gene is commonly overexpressed in human breast cancer correlating with chromosomal instability in the tumors (16, 17). The luminal B breast cancers, which overexpress cyclin D1, associated with chromosomal instability and poor prognosis, are uniformly ERα positive (17). Immunohistological staining for cyclin D1 in the breast cancer tissue sections showed that cyclin D1 associates with the ERα in the breast cancer tissue sections (18, 19). Cyclin D1 expression and promoter activity is induced by E2α and cyclin D1 associates with the ERα in the nucleus to enhance ligand-independent transcription (20). Genetic deletion studies of cyclin D1 in the mouse demonstrated a role for cyclin D1 in estrogen-dependent signaling in the mammary gland (21). In these studies, cyclin D1 was required for the induction of a gene module involved in E2-mediated gene expression in the mammary gland (21). Previous studies have implicated cyclin D1 in the DDR in response to ultraviolet (UV) and DNA damage signaling (21). Previous studies have implicated cyclin D1 in the DDR in response to ultraviolet (UV) and DNA damage signaling (21). Previous studies have implicated cyclin D1 in the DDR in response to ultraviolet (UV) and DNA damage signaling (21). Previous studies have implicated cyclin D1 in the DDR in response to ultraviolet (UV) and DNA damage signaling (21). Previous studies have implicated cyclin D1 in the DDR in response to ultraviolet (UV) and DNA damage signaling (21). Previous studies have implicated cyclin D1 in the DDR in response to ultraviolet (UV) and DNA damage signaling (21). Previous studies have implicated cyclin D1 in the DDR in response to ultraviolet (UV) and DNA damage signaling (21). Previous studies have implicated cyclin D1 in the DDR in response to ultraviolet (UV) and DNA damage signaling (21).

Materials and Methods

Plasmids

Tet-On TRIPZ inducible lentiviral human cyclin D1 shRNAs were purchased from Thermo Scientific. The retroviral vector encoding ERα was constructed as follows. The cDNA encoding human (ERα) was excised from HEGO vector with EcoRI digestion and subcloned into MSCV-IRES-GFP retroviral vector. The direction of cDNA and the protein expression was confirmed by restriction digestion and Western blotting, respectively. Rad51-luciferase plasmid (pGL3) was from Dr. Peter M. Glazer (Yale University School of Medicine, New Haven, CT; ref. 26). Cherry-lacR-NLS-ATM, Cherry-lacR-NLS-NBS1, Cherry-lacR-NLS-cyclin D1, or empty vector Cherry-lacR-NLS using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions and treated with 10 nmol/L E2. Twenty-four hours later, cells were processed for γH2AX immunostaining.

Luciferase reporter assays

Luciferase assays were performed as previously described (32). siRNA knockdown of endogenous cyclin D1 in MCF-7 cell

For suppression of endogenous cyclin D1 expression in MCF-7 breast cancer cell lines, three siRNAs (Hs_CCND_1, Hs_CCND_2, and Hs_CCND_3) that specifically target human cyclin D1 mRNA were purchased from Qiagen.

Western blot analysis

Western blot analyses were conducted as described previously (32).
Immunostaining

Immunostaining was conducted as described previously (6).

Homologous recombination–directed DNA repair assay

MCF-7 DR-GFP and U2OS DR-GFP cells harbor a chromosomally integrated copy of the DR-GFP reporter. The DR-GFP reporter contains an inactive expression cassette for GFP that is interrupted by a recognition site for the rare-cutting endonuclease I-SceI. When I-SceI is expressed in DR-GFP–expressing cells, it induces DSBs within the SceGFP fragment providing a signal for homologous recombination and leads to restoration of the functional GFP (Fig. 7A; ref. 31). To analyze the efficiency of HDR, MCF-7 DR-GFP and U2OS DR-GFP cells were transiently transfected with pCBA-Sce using the Nucleofector kit (Lonza) following the manufacturer’s recommendations. The cells were cultured for 3 days to allow completion of repair, then the percentage of GFP+ cells was analyzed by FACS analysis. The GFP expression plasmid in the same backbone, pCAGGS-NZEGFP, was used as a transfection efficiency control. The empty vector pCAGGS-BSKX was used as a negative control.

A more detailed Materials and Methods are included in Supplementary Materials and Methods.

Results

Cyclin D1 is required for E2-mediated delay in the DDR

Previous studies had demonstrated a role for cyclin D1 in enhancing the DDR (6). To examine the functional interaction between endogenous cyclin D1 and estrogen in mediating γH2AX, MCF-7 cells were treated with either control siRNA or cyclin D1 siRNA and subsequently with E2 (10 nmol/L; Fig. 1A). Upon UV irradiation (100 J/m²), γH2AX was induced and the presence of foci was observed at 2 hours (Fig. 1B). Quantitation of the number of γH2AX foci was shown in Fig. 1C. E2 treatment delayed the number of irradiation-induced γH2AX foci for 2 hours. At 4 hours, E2–treated cells had significantly more γH2AX foci (Fig. 1C). Cyclin D1 siRNA abrogated the formation of γH2AX at 4

Figure 1. Endogenous cyclin D1 mediates E2–dependent DNA damage signaling in human breast cancer cell line MCF-7 cells. A, schematic representation of the experimental protocol. B, confocal microscopy of immunofluorescence for cyclin D1 (red) and γH2AX (green) and nuclear staining with DAPI in MCF-7 cells after UV (100 J/m²). The cyclin D1 siRNA–transduced cells are indicated. C, quantitation of γH2AX foci as number of foci per cell. Confocal microscopy of γH2AX immunofluorescence in MCF-7 cells after UV exposure either in the absence or presence of estradiol (10⁻⁷ mol/L) was used to determine data shown as mean ± SEM for N > 50 cells.
hours (Fig. 1B, arrows in column 3, row 6). The delayed induction of DDR by E₂, assessed by γH2AX foci number, was reduced by cyclin D1 siRNA (Fig. 1B and C). Collectively, these studies demonstrate that cyclin D1 is required for the E₂-mediated delayed induction of γH2AX foci.

**Extranuclear E₂-dependent DDR is mediated via cyclin D1**

The nongenomic extranuclear actions of the ERα are more rapid than the genomic actions and are unaffected by inhibitors of RNA or protein synthesis. Large abiotic nondegradable polyamidoamine (PAMAM) dendrimer macromolecules

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**Figure 2.** Endogenous cyclin D1 mediates the delayed DNA damage signaling mediated by extranuclear estrogen signaling. A, experimental protocol in which cells transduced with cyclin D1 siRNA or control siRNA were treated with the 17β-estradiol (E₂) dendrimer conjugate (EDC), which is localized to the cytoplasm and is incapable of stimulating nuclear actions of ERα. B, cells were then exposed to DNA damage by UV (100 J/m²) for time points as indicated. H2AX phosphorylation of Ser139 was detected by immunofluorescence. C, the percentage of γH2AX positive cells collated as mean ± SEM.
Figure 3. Endogenous cyclin D1 mediates the cytoplasmic membrane mediated E2-dependent DDR. A, Western blot analysis of MCF-7 cells treated with cyclin D1 siRNA and EDC (estrogen–dendrimer conjugate) at a dose equivalent to $10^{-9}$ mol/L E2. Western blot analysis was conducted using antibodies directed to $\gamma$H2AX, cyclin D1, or $\beta$-actin as a protein loading control. SE, short exposure; LE, long exposure. B, quantitation of relative $\gamma$H2AX protein abundance shown as mean ± SEM for $N > 3$ separate experiments. C, endogenous cyclin D1 enhances E2-induced Akt phosphorylation (Ser473) in MCF-7 cells exposed to UV. MCF-7 cells were transfected with cyclin D1 siRNAs or control siRNA. Forty-eight hours later, cells were treated with 10 nmol/L E2 for time points as indicated. Then, the cells were exposed to UV (100 J/m²). Specific antibodies to phospho-Serine 473 Akt1/2/3, total Akt1, and $\gamma$H2AX were used in Western blot analysis. D, membrane E domain of ER$\alpha$ binds to cyclin D1 in 293T cells. 293 T cells were cotransfected with FLAG-cyclin D1 (in CMV10 vector) and the E domain of ER$\alpha$ (in ECFP-Mem vector targeting the E domain to the plasma membrane). Forty-eight hours later, immunoprecipitation and Western blot analysis were conducted to detect membrane-ER$\alpha$ and FLAG-cyclin D1 interaction.
conjugated to estrogen remain outside the nucleus, providing optimal ligand access to ERα and conveying a binding affinity comparable with that of E2 (12, 33). EDC stimulates ERK, SHC, and Src phosphorylation, activating nongenomic activities at concentrations that do not alter the transcription of nuclear estrogen target genes (12, 13). EDC is therefore useful in studying nongenomic effects of estrogen action in target cells (15). To determine whether cyclin D1 contributes to nongenomic estrogen-mediated signaling, MCF-7 cells were treated with UV irradiation in the presence or absence of EDC, compared with the control dendrimer. Immunohistochemical analysis of γH2AX foci was conducted (Fig. 2). After UV-induced DDR, the number of γH2AX-positive cells was increased at 4 and 8 hours. Treatment with EDC resulted in the continued increase in γH2AX at 24 hours (Fig. 2C; right vs. left). Cyclin D1 siRNA reduced basal UV-induced γH2AX staining and reduced both the EDC and E2-induced γH2AX at 24 hours by approximately 50% (Fig. 2C and Supplementary Fig. S1). Thus, endogenous cyclin D1 participates in the persistence of EDC-dependent γH2AX.

To examine further the role of extranuclear E2 signaling to the DDR in breast cancer cells, Western blot analysis was conducted of MCF-7 cells transduced with cyclin D1 siRNA and treated with UV irradiation and either EDC or control FBS. Figure 4. Cyclin D1 is required for extranuclear E2-mediated expression and recruitment of Rad51 to nuclear foci in response to DNA damage. A, schematic depicting the experimental procedure. Immunofluorescence staining for cyclin D1 and Rad51, with the nucleus identified by DAPI staining. B, Western blot analysis of MCF-7 cells transduced with three distinct cyclin D1 siRNAs and subjected to treatment with E2 (10 nmol/L). C, quantitation of Rad51 mRNA abundance determined by qRT-PCR. Cells were treated with 10 nmol/L E2 for 24 hours.
Endogenous cyclin D1 is required for E2-induced Rad51 abundance

An important regulatory event that determines the type of DNA repair used in the cell is the process of double-stranded break repair. The Rad51 nucleofilament mediates homology search in the sister-chromatid followed by strand invasion. We, therefore, examined the role of EDC and cyclin D1 in the regulation of Rad51 abundance (Fig. 4). MCF-7 cells were treated with EDC or control. EDC treatment increased cyclin D1 and Rad51 abundance (Fig. 4A, 2 vs. 1). Cyclin D1 siRNA reduced cyclin D1 and Rad51 abundance was induced by EDC (Fig. 4A, 4 vs. 2). Three separate cyclin D1 siRNAs were used to reduce cyclin D1 abundance by Western blotting and each siRNA reduced E2 enhancement of Rad51 abundance (Fig. 4B). Rad51 mRNA induction by E2, as well as basal levels, were also reduced by cyclin D1 siRNA (Fig. 4C).

As Rad51 abundance may be transcriptionally induced, we deployed the Rad51 promoter linked to a luciferase reporter gene (Fig. 3A). In transient expression studies, coexpression of a cyclin D1 expression vector enhanced Rad51 promoter activity in a dose-dependent manner (Fig. 5A). There was no effect of cyclin D1 coexpression on the luciferase reporter backbone (Fig. 5B). E2 is known to inhibit ATR signaling through rapid induction of PI3K/AKT activity (9) to thereby delay the assembly and prolong the resolution of γH2AX foci. As cyclin D1 was required for E2-mediated delay in γH2AX resolution, we determined a potential role for cyclin D1 in AKT activation by E2. MCF-7 cells transduced with cyclin D1 siRNA or scrambled siRNA control, demonstrated the E2-mediated induction of AKT phosphorylation (Ser473; Fig. 4A, 2 vs. 1). Cyclin D1 siRNA reduced cyclin D1 and Rad51 abundance was induced by EDC (Fig. 4A, lane 7 vs. 5 and lane 11 vs. 9). EDC enhanced UV irradiation-induced γH2AX. Cyclin D1 siRNA reduced cyclin D1 and Rad51 abundance (Fig. 4A, lane 8 vs. 6 and lane 12 vs. 10). Quantitation of multiplicate experiments confirmed the importance of cyclin D1 in EDC-mediated induction of γH2AX (Fig. 4B). E2 is known to inhibit ATR-mediated induction of AKT phosphorylation (Ser473; Fig. 3C). Cyclin D1 siRNA reduced cyclin D1 activity by pGL3-Luc vector

Normalized Rad51 luciferase activity by pGL3-Luc vector

Promoters

Signaling

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pGL3-luc (Fig. 5A). E2 induced the Rad51 promoter activity 1.5 fold. The addition of inhibitors for the PI3K, HDAC, and JNK signaling did not significantly reduce E2-mediated induction of the Rad51 promoter (Fig. 5B). In contrast, inhibitors of the ATM (KU55933) and ATM/ATR pathways (caffeine) abrogated E2-mediated induction of the Rad51 promoter (Fig. 5C). The DNA-PK inhibitor NU7026 and the CK2 inhibitor (TBB) did not significantly reduce the E2 induction of Rad51 (Fig. 5C). These studies suggest that E2 induces Rad51 transcription via ATM. To determine precisely the role of ATM and ATR in regulating Rad51 promoter activity, expression vectors encoding either ATM or ATR were introduced into cultured cells with the Rad51 promoter reporter gene. Coexpression of ATM was sufficient to induce Rad51 promoter activity in the presence of E2 (Fig. 5D). These studies suggest that cyclin D1 governs two apparently distinct functions in the DDR: first, to participate in an extranuclear signaling that augments AKT signaling, and delay the resolution of γH2AX foci; second, to augment expression of Rad51, a protein participating in DNA damage repair.

**E2 enhances the DDR induced by cyclin D1 tethered to chromatin**

To examine further the mechanism by which E2 regulated cyclin D1-dependent DNA damage signaling, we deployed a DNA-repair factor chromatin recruitment assay (27). The recruitment of DDR factors into chromatin can trigger and amplify the DDR signal via an ATM- and DNA-PK-dependent mechanism (27). Like the DDR factors, cyclin D1 is recruited to chromatin in these assays, requiring the cyclin D1 carboxyl terminus (6). The role of E2 in regulating the DDR induced by the DDR factor recruitment to chromatin is unknown.

DNA repair factors or cyclin D1 fused to the *Escherichia coli* lac-repressor (LacR) were tagged with the cherry-red fluorescent protein. An NIH 3T3 cell line that contains 256 repeats of the LacO site, stably integrated into chromosome 3 (NH2/4; ref. 30) was transduced with retroviral vectors encoding either ERα or control vector. The NH2/4-ERα cells were used to analyze the role of E2 in recruitment of cyclin D1 and DDR fusion proteins to DNA (Fig. 6B). The presence of ERα was readily detectable by Western blotting (Fig. 6B). The fusion protein accumulated at the LacO multimer site as distinct nuclear foci (Fig. 6D). NBS1 recruitment to the LacO site was sufficient to induce the DDR and activate γH2AX and form foci at the LacO site [Fig. 6D and E, LacR ERα (vehicle) vs. NBS1 ERα (vehicle)]. In the absence of E2, cyclin D1 and ATM were not sufficient to induce γH2AX foci at the LacO site [Fig. 6E, LacR ERα (vehicle) vs. cyclin D1 ERα (vehicle) and LacR ERα (vehicle) vs. ATM ERα (vehicle)]. E2 treatment in NH2/4-ERα cells enhanced NBS1 and ATM recruitment- mediated γH2AX foci [Fig. 6E, NBS1 ERα (vehicle) vs. NBS1 ERα (E2) and ATM ERα (vehicle) vs. ATM ERα (E2)]. The presence of ERα and E2 enhanced cyclin D1-mediated γH2AX foci formation at the LacO site [Fig. 6E, cyclin D1 ERα (vehicle) vs. cyclin D1 ERα (E2)]. Thus, E2 enhances cyclin D1 recruitment to γH2AX foci. Importantly, these studies demonstrate that cyclin D1 binds to the cytoplasmic membrane-associated ERα, which in turn governs γH2AX, a function distinct from the previously defined nuclear ERα/cyclin D1 interactions. Third, cyclin D1 was required for both basal and E2-induced expression of one of the key components of the DDR, Rad51. Finally, cyclin D1 siRNA reduced HDR. Collectively, these observations demonstrate that cyclin D1 mediates UV- and estrogen-induced DNA damage repair signaling via a novel mechanism in breast cancer epithelial cells.

**Endogenous cyclin D1 facilitates and E2 inhibits homology-directed DNA damage repair**

Double-stranded breaks generated by ionizing radiation are repaired by either homologous or nonhomologous end joining, DSB and repair can be simulated in mammalian cells by using the homing endonuclease I-SceI. Recombination repair can be monitored through the ligation-mediated formation of GFP protein from a reporter plasmid (DR-GFP; Fig. 7A). As a form of positive control, we deployed a previously described homology-directed repair (HDR) U2OS cell line, and assessed the role of endogenous cyclin D1 in the repair process by transducing the cells with three separate cyclin D1 siRNA under control of a tetracycline-inducible promoter (Fig. 7B). Cyclin D1 siRNA reduced the abundance of cyclin D1 by Western blotting (Fig. 7B) and reduced the I-SceI–mediated DNA damage repair in U2OS cells (Fig. 7C). In MCF-7 cells, the addition of E2 reduced the rate of HDR by approximately 30% (Fig. 7D). Thus, endogenous cyclin D1 facilitates homologous DNA repair in MCF-7 cells. To determine whether the DNA damage signaling (the formation of γH2AX foci), and the DNA damage repair process were both regulated by extranuclear E2 signaling, we compared the role of E2 and EDC on the assays of homologous DNA repair. In contrast with the DNA damage signaling, the repair of damaged DNA repair was regulated by E2 and not by extranuclear ERα, assessed by addition of the E2 dendrimer (EDC; Fig. 7E). Furthermore, the use of ATM versus ATM/ATR inhibitors demonstrated that the DNA repair process was dependent upon both ATM and ATR (Fig. 7F). Together, these data are consistent with a model in which cyclin D1 conducts distinct functions. The membrane-associated ERα bound cyclin D1 is associated with the augmentation of E2-mediated induction of AKT phosphorylation (Ser473; Fig. 7G). In contrast with the DNA damage signaling, cyclin D1 facilitates homologous DNA repair, being recruited to γH2AX foci by E2 and inducing Rad51 abundance.

**Discussion**

The current studies provide new evidence for cyclin D1 to enhance both the activity of the DNA damage signaling pathway and HDR. First, UV-induced γH2AX was reduced by cyclin D1 siRNA. Second, the estradiol-mediated DDR was attenuated by cyclin D1 siRNA. A reduction in cyclin D1 reduced γH2AX foci. Importantly, these studies demonstrate that cyclin D1 binds to the cytoplasmic membrane-associated ERα, which in turn governs γH2AX, a function distinct from the previously defined nuclear ERα/cyclin D1 interactions. Third, cyclin D1 was required for both basal and E2-induced expression of one of the key components of the DDR, Rad51. Finally, cyclin D1 siRNA reduced HDR. Collectively, these observations demonstrate that cyclin D1 mediates UV- and estrogen-induced DNA damage repair signaling via a novel mechanism in breast cancer epithelial cells.

Cyclin D1 participates in a nongenomic ERα function. In the current studies, E2 induced a sustained DDR. Exposure to estrogen or its catechol metabolites results in oxidative DNA damage and DSBs. However, catechol metabolites function independently of ERα and E2 induces only low levels of...
Figure 6. Estradiol enhances the DDR of repair factor recruitment to DNA. A, schematic representation of the chimeric fusion proteins in which the DNA damage signaling repair factors, or cyclin D1, were linked to the cherry-lacR-NLS (red). B, Western blot NIH 2/4 cells and NIH 2/4 cells transduced with an ERα expression plasmid. Immunoblotting was conducted with antibodies as indicated. C, schematic representation of the cell treatment protocol. D, confocal immunofluorescence microscopy of NIH 2/4 cells transiently transfected with the DNA damage repair factor or cyclin D1 fused to cherry-lacR-NLS (red). Phosphorylation of γH2AX indicates activation of the DDR. E, the percentage of cells with γH2AX signal was determined after 24 hours of treatment (E2 10^{-8} mol/L). The data are mean ± SEM for N > 50 cells and N > 3 separate experiments. F, models of E2 on cyclin D1, ATM, and NBS1 chromatin recruitment-mediated γH2AX foci.
Figure 7. Estradiol inhibits HDR. A, schematic representation of the HDR reporter system in which I-SceI-induced DNA breaks are repaired by HDR, resulting in GFP fusion protein production. B, U2OS DR-GFP stable cell lines were transduced with three distinct doxycycline-inducible cyclin D1 shRNA and Western blotting was conducted after 72 hours of doxycycline treatment, with the antibodies as indicated. C, HDR rate of U2OS DR-GFP cells expressing doxycycline-inducible cyclin D1 shRNA shown as mean ± SEM for N > 3 separate experiments in which DSBs were introduced by I-SceI. D-F, HDR in MCF-7 cells expressing the Sce-GFP repair reporter plasmid shown in A. Cells were transduced with an I-SceI expression plasmid and the repair rate was assessed after 72 hours of E2 (10^{-8} mol/L) treatment (D), E2 (10^{-8} mol/L), and EDC (E), and specific ATM inhibitor (10 mmol/L KU55933), ATM/ATR inhibitor (5 mmol/L caffeine), or vehicle DMSO control (F). Data are mean ± SEM for three separate transfections. G, schematic representation of model by which cyclin D1 augments AKT signaling associated with ERα in the cytoplasmic membrane thereby participating in E2-dependent signaling; cyclin D1 augments HDR, in part through binding to DNA at γH2AX foci and through inducing Rad51 transcription and abundance.
oxidative stress (34–36). ERα-mediated transcription requires the decatenanting activity of topoisomerase IIβ, in which transient DSBs are generated (37, 38). E2 has been shown to induce DSBs and γH2AX foci (39), with careful kinetic analysis showing that E2 delays the resolution of the DDR essentially extending the response (9). In the current studies, the induction of the DDR by E2 was abrogated by cyclin D1 siRNA, demonstrating the importance of cyclin D1 in maintaining the DDR. E2 functions by genomic and nongenomic signaling (10). Extranuclear signaling by the estrogen receptor can be dissected using estrogen–dendrimer conjugates (EDC). The large abiotic nondegradable poly(amido)amine molecule is conjugated to estrogen via a stable covalent bond and has been shown to activate only extranuclear signaling (12, 13). Herein, cyclin D1 was required for EDC-induced γH2AX. Furthermore, cyclin D1 binds to ERα at the cytoplasmic membrane. Thus, cyclin D1 participates in an extranuclear ERα signaling pathway. As prior studies have demonstrated, cyclin D1 functions within the nuclear estrogen signaling pathway (24). Together, these studies suggest that cyclin D1 functions in both extranuclear and nuclear estrogen signaling pathways (23). Consistent with the finding that endogenous cyclin D1 mediates UV-induced γH2AX, the relative abundance of cyclin D1 also governed the abundance of genes involved in the DDR in vivo (21). Using cyclin D1−/− mammary gland or cyclin D1−/− liver cells, gene expression analysis, determined through microarray, identified a subset of genes involved in the DDR.

Herein, E2-activated the γH2AX foci formation induced by recruitment of cyclin D1, ATM, or NBS1 to chromatin. E2 inhibited homologous DNA repair. The finding that E2 inhibits HDR is consistent with our prior studies that E2 and BRCA1 functions are mutually antagonistic (40). Herein, endogenous cyclin D1 enhanced the HDR. Our recent studies provided evidence for a direct physical interaction between cyclin D1 and the distal end of the DNA damage repair complex (24). The RAD50/MRE11/NBS1 complex functionally interacts with BRCA1 to regulate repair of DNA DSBs. Cyclin D1 bound to BRCA1 via the carboxyl terminal domain of BRCA1 (21). The carboxyl terminus of BRCA1 binds cyclin D1 (21). The BRCA1 gene product is important for DNA repair of both chromosomal DSBs by homologous recombination and transcription-coupled repair (41, 42). Cyclin D1 colocalizes with BRCA1 by immunohistochemistry in breast cancer epithelial cells and coassociated by immune precipitation (24). Chromatin immunoprecipitation (ChIP) assays have demonstrated dynamic interactions of ERα, cyclin D1, and BRCA1 at target estrogen response elements after E2 treatment. The current findings are consistent with prior observations. In ChIP assays, BRCA1 inhibited E2-induced ERα recruitment, consistent with functional antagonism between BRCA1 and ERα signaling. At an ERα binding site, the BRCA1/ERα complex is displaced by cyclin D1, to form a cyclin D1/ERα complex (24). Cyclin D1 is also recruited in ChIP assays to BRCA1 binding sites, such as the Areg gene (21). Importantly, and consistent with the current studies demonstrating a role for cyclin D1 in promoting HDR, cyclin D1 genetic knockout demonstrated that cyclin D1 functioned as a scaffold factor for assembly of BRCA1 complex (21). The distinct functions of cytoplasmic versus nuclear cyclin D1 in the DDR remain to be further explored in vivo.

E2 is known to delay the assembly and prolong the resolution of the DDR. E2 inhibition of DNA damage-induced check point activity might allow the passage of unrepaired mutations, which may in turn enhance mutation acquisition. The finding that cyclin D1 was required for the E2-induced delay in the assembly of the DDR is consistent with studies in which cyclin D1 was required for tumorigenesis by carcinogens and oncogenes. Oncogenes induce both DSB and the DDR (3–6). Cyclin D1 is a limiting step in cellular transformation by oncogenes (Ras, Notch, Stat3, and ErbB2; refs. 43, 18). DNA damage and cyclin D1 expression are both features of preinvasive breast cancer (7, 8). Cyclin D1 overexpression is associated with induction of expression of genes involved in DNA replication and DNA damage checkpoint control (MCM3, MCM4, and several H2A histone family members; ref. 44). Conversely, inducible mammary epithelial cell–targeted cyclin D1 antisense transgenic mice showed reduced abundance of DNA damage repair signaling proteins, including MCM2, CDC20, and Rad51 (45). Together, these studies increase the possibility that cyclin D1 may participate in the induction of gene expression governing the DDR in early tumorigenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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