MicroRNA-29a regulates the benzo[a]pyrene dihydrodiol epoxide-induced DNA damage response through Cdc7 kinase in lung cancer cells

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INTRODUCTION
Mammalian cells are continuously exposed to a multitude of endogenous and exogenous forms of DNA damage. Unrepaired DNA damage can lead to mutations and therefore represents a major threat to genome stability. Cells have developed complex mechanisms that identify and repair damage, thereby reducing the harmful effects of DNA damage. Cell cycle checkpoints are regulatory pathways that respond to DNA damage by invoking transient delays in cell cycle progression. S-phase checkpoint mechanisms sense DNA damage or replication stress during S-phase and co-ordinates S-phase progression with DNA repair, thereby maintaining genetic stability. The presence of DNA damage hinders progression through S-phase, resulting in replication fork stalling and the S-phase checkpoint pathway prevents fork collapse and suppresses firing of late replication origins (reviewed in Nyberg et al.1). Considerable evidence indicates that the protein kinase ATR and its downstream effector Chk1 mediate the S-phase checkpoint.2

Cdc7 kinase is a key regulator of DNA replication and has an important role in the cellular DNA damage response by controlling checkpoint signaling and cell survival. Yet, how the activity of Cdc7 kinase is regulated is poorly understood. In silico analysis identified microRNA-29 (miR-29)-binding sites in the 3'-untranslated region (UTR) of both Cdc7 and its activating subunit Dbf4. We show that miR-29a binds to Cdc7 and Dbf4 3'-UTRs and regulates kinase levels. We find that in response to DNA damage, upregulation of Cdc7 kinase correlates with a downregulation in miR-29a. Enforced miR-29a expression prevents the accumulation of Cdc7 in response to the environmental genotoxin, benzo[a]pyrene dihydrodiol epoxide (BPDE) present in cigarette smoke, resulting in aberrant checkpoint signaling and increased cell lethality. As BPDE sensitivity was rescued by overexpression of miRNA-resistant Cdc7/Dbf4, we propose that Cdc7 kinase is an important target of miR-29a in determining cell survival from genotoxic stress caused by this environmental toxin.

Keywords: protein kinase; DNA damage; genotoxic stress; S-phase; microRNA
Figure 1. miR-29a targets Cdc7 and Dbf4 mRNA at specific 3′-UTR sites. (a) Putative miR-29a-binding sites in the 3′-UTR regions of Cdc7 and Dbf4. (b) Luciferase reporter assay constructs containing the full-length 3′-UTR of Cdc7 (wt-Cdc7 3′-UTR) or the full-length 3′-UTR of Dbf4 (wt-Dbf4 3′-UTR) were used to generate stable cell lines expressing wt-Cdc7 3′-UTR luciferase or wt-Dbf4 3′-UTR luciferase. Stable cell lines were transfected with miR-ve or miR-29a, and luciferase activity measured. miR-29a overexpression caused a significant decrease in luciferase activity in both wt-Cdc7 3′-UTR (n = 3, ***P = 0.015) and wt-Dbf4 3′-UTR (n = 3, ***P = 0.006) expressing cells compared with miR-ve. Values are presented as the mean ± s.e.m. (c) Mutant constructs (Cdc7 mut 29-1 and -Dbf4 mut 29) were used to generate stable cell lines, which were transfected with miR-29a or miR-ve and luciferase activity measured. miR-29a induced a significant decrease in luciferase activity in cells expressing wt-Cdc7 3′-UTR (n = 5, ***P = 5.7 × 10^{-5}) and wt-Dbf4 3′-UTR (n = 4, ***P = 0.0001), but caused no significant difference in luciferase activity in cells expressing the mutant 3′-UTRs of Cdc7 or Dbf4.

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MicroRNA-29a regulates DNA damage response in lung cancer cells. This study explores the role of miR-29a in regulating the DNA damage response pathway, and its potential implications in lung cancer. miR-29a is shown to be important for cell survival during chronic DNA damage, particularly in the context of tumor suppression. The well-characterized p53 responsive tumor suppressor represents a mechanism whereby BPDE-induced mutagenic lesions are regulated. miR-29a targets both Cdc7 and Dbf4, and its overexpression decreases luciferase activity in cells expressing wt-Cdc7 and wt-Dbf4.

**RESULTS**

miR-29a targets the 3′-UTR of Cdc7 and Dbf4 mRNA in lung cancer cells.

To identify miRNAs that could regulate human Cdc7 and Dbf4 expression, we used the web-based algorithms TargetScan and PITA. All these tools predict potential miR-29, miR-30a and miR-199-binding sites in the 3′-UTR of both Cdc7 and Dbf4. In particular, three miR-29-binding sites were predicted in the 3′-UTR of Cdc7 at positions 422–428, 428–434, and 681–687, and one in the 3′-UTR of Dbf4 at position 497–503 (Figure 1A). To experimentally verify these predictions, we constructed two reporter plasmids containing the full-length wild-type hCdc7 3′-UTR (wt-Cdc7) and hDbf4 3′-UTR (wt-Dbf4) fused to a firefly luciferase complementary DNA. Human H1299 lung carcinoma cells that express low levels of miR-29a (Supplementary Figure S1A) were used to generate stable wt-Cdc7-luciferase and wt-Dbf4-luciferase-expressing cell lines. These cells were then transfected with miR-29a or miR-ve, as a negative control, and luciferase activity was measured. miR-29a overexpression decreased luciferase activity driven by wt-Cdc7 and wt-Dbf4 3′-UTRs by 33% and 15% respectively, indicating that miR-29a regulates the 3′-UTR of Cdc7 and Dbf4 (Figure 1B). To determine if this effect is direct, the predicted miR-29-binding sites in the 3′-UTR of luciferase-Cdc7 and -Dbf4 plasmids were mutated generating luciferase-Cdc7 mut 29-1, -Cdc7 mut 29-2 and -Dbf4 mut 29 constructs (Figures 1C and D). Stable H1299 cell lines were again generated and reporter assays revealed that luciferase-Cdc7 mut 29-1 and -Cdc7 mut 29-2, as well as -Dbf4 mut 29 were all insensitive to miR-29a overexpression (Figures 1C and D). Taken together, these results suggest that miR-29a downregulation in lung cancer cells may represent a mechanism whereby BPDE-induced mutagenic lesions accumulate.

Cigarette smoke is one of the leading causes of lung cancer. The environmental carcinogen benzo[a]pyrene dihydrodiol epoxide (BPDE) present in cigarette smoke causes DNA adducts and is highly mutagenic. In vitro DNA damage caused by low doses of BPDE (for example, 100 nM), induces a Chk1-dependent response that transiently inhibits DNA synthesis, however, cells recover from the S-phase arrest and do not exhibit significant loss of viability. In contrast, higher doses of BPDE (for example, 600 nM) generate high levels of double-strand breaks (DSBs), ATM/Chk2 activation and phosphorylation of H2AX (γH2AX) causing a complete block to DNA synthesis and loss of viability.

Here, we show that miR-29a directly targets both Cdc7 and Dbf4 transcripts in lung cells. Furthermore, we show that overexpression of miR-29a leads to increased sensitivity of lung cancer cells towards BPDE and that this is mediated by Cdc7 kinase. These findings indicate that miR-29a, by targeting Cdc7, affects the cellular responses to BPDE-induced DNA damage, and suggest that miR-29a downregulation in lung cancer cells may represent a mechanism whereby BPDE-induced mutagenic lesions accumulate.
together these data indicate that miR-29a interacts with specific elements in the 3’-UTR of Cdc7 and Dbf4.

To investigate if miR-29a modulates endogenous Cdc7 and Dbf4 expression, we examined the effects of miR-29a overexpression on Cdc7 and Dbf4 protein levels in human primary lung MRC-5 and H1299 cell lines. Upon transfection miR-29a was overexpressed ~3 fold in MRC-5 cells and 15 fold in H1299 cells as determined by real-time quantitative PCR (RT–qPCR) (Figure 2a). This difference in miR-29a overexpression between the two cell lines could be attributed to dissimilar transfection efficiency or to higher endogenous levels of miR-29a in primary non-transformed MRC-5 cells compared with H1299 cancer cells (Supplementary Figure S1A). Nevertheless, miR-29a overexpression caused a reduction in Cdc7 and Dbf4 protein levels compared with miR-ve control in both cell lines (Figure 2b). In these experiments, we observed that partial downregulation of Cdc7/Dbf4 induced by miRNA-29a, similar to partial downregulation achieved by siRNA, was not sufficient to obviously affect bulk DNA synthesis as determined by 5-ethyl-2’-deoxyuridine incorporation experiments (Figure 2c).

Cdc7 and miR-29a expression following genotoxic stress
Owing to the role of Cdc7 in response to genotoxic insults, we explored the regulation of miR-29a and Cdc7 following various genotoxic stress stimuli. Firstly, A549 cells were treated with low-(100 nM) or high-dose (600 nM) BPDE (Figure 3a) and protein CSK-soluble, CSK-insoluble and RNA extracts were prepared. Treatment with low-dose BPDE resulted in Chk1 activation, monitored by its phosphorylation on serine 317, and accumulation of Cdc7 protein

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**Figure 2.** miR-29a inhibits Cdc7 and Dbf4 expression. (a) MRC-5 and H1299 cells were transfected with miR-29a and mir-29a levels were determined by real-time quantitative PCR (RT–qPCR). (b) Western blot analysis was performed to determine Cdc7 and Dbf4 protein levels. β-Actin served as loading control. (c) Cells were reverse transfected with mir-ve or miR-29a. Forty-eight hours after transfection, cells were incubated with 10 μM 5-ethyl-2’-deoxyuridine for 1 h. Levels of 5-ethyl-2’-deoxyuridine incorporation were determined by the click reaction and fluorescence-activated cell sorting analysis.
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Figure 3. miR-29a levels inversely correlate with Cdc7 and Dbf4 levels in response to BPDE. (a) A549 cells were treated with 100 or 600 nM BPDE for the indicated times and fractionated into CSK-soluble and CSK-insoluble fractions. Proteins were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting. (b) RNA was also prepared and miR-29a levels were determined by real-time quantitative PCR (RT–qPCR). (c) A549 cells were treated with 10J UV. RNA and protein extracts were prepared at the indicated times. miR-29a and Cdc7 levels were determined by RT–qPCR and western blot, respectively. (d) A549 cells were treated with 2 mM HU. RNA and protein extracts were prepared at the indicated times and miR-29a and Cdc7 levels were determined by RT–qPCR and western blot respectively.

miR-29a overexpression effects cellular response to BPDE

To understand the role of miR-29a in response to DNA damage we overexpressed miR-29a in A549 cells that were then treated with 100 nM BPDE. miR-29a overexpression in A549 cells did not result in obvious changes in the levels of DNA synthesis as determined by 5-ethynyl-2′-deoxyuridine analysis (Supplementary Figure S1B), however, unlike control cells miR-29a overexpressing cells failed to accumulate Cdc7 protein (Figure 4a and Supplementary Figure S2). Furthermore, in miR-29a-transfected cells the activation of Chk1 following BPDE treatment was markedly decreased (Figure 4a and Supplementary Figure S2) suggesting an altered cellular response to DNA damage. This data is consistent with previous reports that show depletion of Cdc7 by siRNA causes a defect in Chk1 activation. 

Deficient S-phase checkpoint signaling often results in enhanced sensitivity and decreased viability in situations of genotoxic or replication stress. Hence, we determined the effects of different doses of BPDE on clonogenic survival of miR-ve and miR-29a-expressing cells. Treatment with 50 nM BPDE did not affect the viability of miR-ve expressing cells but elicited a 33% decrease in clonogenic survival of miR-29a-expressing cells. 100 nM BPDE caused a 16% and 38% reduction in clonogenic survival of miR-ve and miR-29a-expressing cells, respectively. miR-29a-induced BPDE sensitization was no longer observed at higher doses of genotoxin treatment (200 and 600 nM) that generate DSBs (Figure 4b). These data demonstrate that miR-29a is important for cell survival after low-dose BPDE treatment.

Cdc7/Dbf4 prevents miR-29a-induced BPDE-sensitization

miRNAs have multiple cellular targets, thus miR-29a-induced sensitization to low-dose BPDE may be dependent or independent of Cdc7 kinase function. To discriminate between these possibilities A549 cells were transiently co-transfected with Cdc7 and Dbf4 expression vectors lacking 3′-UTRs and therefore miR-29a regulatory elements, or empty plasmid control vector followed by a second transfection with miR-ve or miR-29a (Figure 5a). Cells were then treated with 100 nM BPDE and cell viability assessed. Under these slightly different experimental conditions, whereby cells undergo a double transfection before genotoxin treatment, BPDE had little effect on viability of cells transfected with empty vector and miR-ve, but similarly caused a 34% viability loss in cells transfected with empty vector and miR29a. Importantly, miR-29a-induced sensitization to BPDE was...
reversed by coexpression of miRNA-resistant Cdc7/Dbf4 constructs. Taken together, these data indicate that Cdc7/Dbf4 levels are a critical parameter controlled by miR-29a during the response to genotoxic stress.

**DISCUSSION**

The cell cycle kinase Cdc7 is essential for initiation of DNA synthesis and has a role in the DNA damage response, however, the mechanisms involved in regulating Cdc7 kinase are poorly understood. In this study, we have investigated the role of miRNAs in Cdc7 kinase regulation. Using bioinformatic target prediction algorithms, we identified multiple miR-29-binding sites in the 3′-UTR of Cdc7 and one in the 3′-UTR of Dbf4. We demonstrate that miR-29a directly binds and controls Cdc7 kinase levels. Although it is likely that other miRNAs target Cdc7 and Dbf4, to the best of our knowledge this is the first finding that a single miRNA targets both the catalytic and regulatory subunit of the kinase complex.

Cdc7 levels increase in response to DNA damage caused by the cigarette smoke carcinogen BPDE in lung cancer cells. The BPDE-induced increase in Cdc7 inversely correlates with a down-regulation in miR-29a at non-cytotoxic concentrations of BPDE that induce an S-phase cell cycle checkpoint.29 We demonstrate that miR-29a directly binds and controls Cdc7 kinase levels. Although it is likely that other miRNAs target Cdc7 and Dbf4, to the best of our knowledge this is the first finding that a single miRNA targets both the catalytic and regulatory subunit of the kinase complex.

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miR-29a sensitization is restricted to low doses of BPDE. We suggest the reason for this is due to the different types of DNA damage generated by low- and high-dose BPDE, that is, single-strand versus double-strand DNA breaks, respectively, as well as the diverse mechanisms used by cells to respond to these various genotoxic concentrations.29 Importantly, Cdc7 has only been implicated in regulating the cellular responses to replication fork stalling, a primary event caused by bulky adducts and single-strand DNA breaks by phosphorylating Claspin and promoting Chk1 activation.12,31 On the contrary, the involvement of Cdc7 in response to DSBs has not yet been documented. Thus, Cdc7 regulation by miR-29a may only be relevant at low doses of BPDE, whereas at higher doses miR-29a- and Cdc7-independent events likely determine cellular sensitivity to BPDE.

The importance of the miR-29 family in regulating cellular responses to DNA damage is further supported by recent findings that miR-29 family members also target Ppm1d/Wip1 phosphatase,25 a key regulator of the DNA damage checkpoint pathway that can dephosphorylate both p53 and Chk1.32 Thus, Cdc7 and miR-29a have been reported independently to contribute to the cellular responses and survival following DNA damage.11,24,25,33,34 Our work indicates that, at least for BPDE-induced lesions, these two essential players work concurrently in the same pathway.

miR-29 family members exert their tumor suppressive functions by targeting genes involved in cell proliferation and cell cycle (for example, Cdk6), differentiation (osteonectin), apoptosis (Mcl-115,36), cell senescence (Ppm1d phosphatase17 and B-Myb) and metastasis (TGF-β-Smad) in different cancer types (reviewed in Wang et al.35). However, in certain cancers (for example, breast...
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Materials and methods

Cell culture
A549, H1299 and MRC-5 cells were obtained from American Type Culture Collections (Rockville, MD, USA). All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C and 5% CO2.

RNA and protein analyses
Total RNA was prepared by the TRI Reagent extraction method (Life Technologies/Ambion, Carlsbad, CA, USA). miRNA and mRNA analyses were performed using TaqMan MicroRNA Assays and TaqMan Gene Expression Assays, respectively (Life Technologies/Applied Biosystems, Carlsbad, CA, USA). Relative quantification was performed using RNU24 as endogenous control for miRNAs and TATA-box binding protein for mRNAs. Western blot on CSK extracts were performed as described in Barkley et al.40

miRNA and anti-miRNA overexpression
miR-29a was overexpressed by reverse transfection of 100 nm Pre-miR miR-29a precursor molecule (miR-29a) (Life Technologies/Ambion) using siPORT NeoFX transfection reagent (Life Technologies/Ambion) as per the manufacturer’s instructions and compared with pre-miR negative control (miR-ve) (Life Technologies/Ambion). Inhibition of miR-29a was achieved using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). miR-29a-expressing cells were treated with different doses of BPDE or vehicle dimethyl sulfoxide treated. Twenty-four hours later, control and BPDE-treated cells were trypsinized, counted and replated at a density of 500 cells/10-cm dish. Ten days later, colonies resulting from the surviving cells were fixed, stained with crystal violet and counted. Colony formation assay
miR-ve and miR-29a-expressing cells were treated with different doses of BPDE or vehicle dimethyl sulfoxide treated. Twenty-four hours later, transfected cells were reverse transfected with miR-ve or miR-29a and treated with BPDE and colony formation performed as described above.

Flow cytometry
S-phase populations were determined by 5-ethynyl-2'-deoxyuridine incorporation and the click reaction. Briefly, cells were incubated for 1 h with 10 μM 5-ethyl-2'-deoxyuridine, permeabilized with 0.1% Triton X-100 in PBS for 10 min on ice and washed with PBS. To perform the click reaction, the following components were added sequentially, 10 μM (7)-sodium-l-ascorbate, 0.1 mM 6-carboxyfluorescein-triethylene glycol azide and 2 μM copper (II) sulfate, and cells were incubated in the dark for 30 min at room temperature followed by addition of 10 volumes 1% BSA, 0.5% Tween 20 in PBS and washed three times in PBS.

Statistical analysis
All data shown are representative of experiments that were repeated at least three times with similar results on each separate occasion. Data are shown as mean ± s.e.m. Data were compared by the paired Student’s t-test. A P-value of <0.05 was considered statistically significant.

Conflict of interest
The authors declare no conflict of interest.

Acknowledgements
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Removal of miR-29a by reverse transfection
miR-29a was removed by reverse transfection of 100 nm anti-miR-29a Inhibitor (anti-miR-29a) (Life Technologies/Ambion) using siPORT NeoFX transfection reagent and compared with anti-miR negative control (anti-miR-ve).

Luciferase constructs and assays
Full-length human Cdc7- and Dbf4- 3’-UTR sequences were amplified by PCR and cloned into the SpeI/HindIII restriction sites of the pMIR Report Luciferase plasmid (Life Technologies/Invitrogen, Carlsbad, CA, USA). The putative miR-29a target sites in the 3’-UTR of Cdc7 and Dbf4 were mutated using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). H1299 cells were transfected with 750 ng wild-type or mutant Cdc7 and Dbf4 3’-UTR luciferase reporter constructs and stable cell lines generated by puromycin selection. Stable cell lines were reverse transfected with 10 nm miR-29a or miR-ve. Cells were incubated for a further 24 h and luciferase activity was assessed using the Dual-Luciferase Assay System (Promega, Fitchburg, WI, USA).

Drug treatments
BPDE (National Cancer Institute) was added directly to the growth medium as a 1000 stock to give a final concentrations indicated. For hydroxurea treatment, hydroxurea was added directly to the growth medium as a 1000 stock to give a final concentration of 2 μM. For ultraviolet-C treatment, cells were transferred to an ultraviolet crosslinker (Stratagene) and irradiated.

Colony formation assay
miR-ve and miR-29a-expressing cells were treated with different doses of BPDE or vehicle dimethyl sulfoxide treated. Twenty-four hours later, control and BPDE-treated cells were trypsinized, counted and replated at a density of 500 cells/10-cm dish. Ten days later, colonies resulting from the surviving cells were fixed, stained with crystal violet and counted. Colony formation assay
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