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Arsenic Inhibits DNA Mismatch Repair by Promoting EGFR Expression and PCNA Phosphorylation

Arsenic Inhibits DNA Mismatch Repair by Promoting EGFR Expression and PCNA Phosphorylation*

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Background: Exposure to arsenic is linked to increased risk of human cancer.
Results: Arsenic-treated cells have higher levels of EGFR and tyrosine-phosphorylated PCNA and reduced mismatch repair activity.
Conclusion: The carcinogenic effect of arsenic may be mediated by its ability to promote EGFR-dependent PCNA phosphorylation, thereby inhibiting mismatch repair.
Significance: Arsenic-stimulated posttranslational modification of PCNA represents a novel mechanism of action for non-genotoxic carcinogens.

Both genotoxic and non-genotoxic chemicals can act as carcinogens. However, while genotoxic compounds lead directly to mutations that promote unregulated cell growth, the mechanism by which non-genotoxic carcinogens lead to cellular transformation is poorly understood. Using a model non-genotoxic carcinogen, arsenic, we show here that exposure to arsenic inhibits mismatch repair (MMR) in human cells, possibly through its ability to stimulate epidermal growth factor receptor (EGFR)-dependent tyrosine phosphorylation of proliferating cellular nuclear antigen (PCNA). HeLa cells exposed to exogenous arsenic demonstrate a dose- and time-dependent increase in the levels of EGFR and tyrosine 211-phosphorylated PCNA. Cell extracts derived from arsenic-treated HeLa cells are defective in MMR, and unphosphorylated recombinant PCNA restores normal MMR activity to these extracts. These results suggest a model in which arsenic induces expression of EGFR, which in turn phosphorylates PCNA, and phosphorylated PCNA then inhibits MMR, leading to increased susceptibility to carcinogenesis. This study suggests a putative novel mechanism of action for arsenic and other non-genotoxic carcinogens.

Arsenic is a widely distributed non-genotoxic environmental carcinogen. Exposure to arsenic is associated with increased risk of various types of human cancer, including bladder, kidney, lung, liver, and skin cancer, most notably in countries where arsenic in drinking water is not tightly regulated (1–8). In the United States, although the concentration of arsenic in drinking water is closely monitored and regulated by the US

Environment Protection Agency, one specific concern is the fact that arsenic exposure and tobacco use synergistically increase the risk of lung cancer (9, 10). However, the molecular basis by which exposure to arsenic increases susceptibility to cancer is not fully understood.

Although arsenic does not directly interact with DNA (reviewed in Ref. 11), it may generate reactive oxygen species (12–16) and inhibit DNA repair (17, 18). A subset of lung cancers, especially small cell lung cancers, display microsatellite instability (MSI)[3] (19–22), the hallmark of DNA mismatch repair (MMR) deficiency (23, 24). Here, we explore the hypothesis that arsenic contributes to risk of lung cancer by stimulating protein post-translational modifications that reduce the efficiency of MMR. This would be consistent with previous studies showing that tyrosine-phosphorylated PCNA inhibits MMR in human cells (25–27).

MMR is a critical genome-maintenance system that corrects potentially mutagenic DNA mispairs that arise during normal DNA replication and DNA repair synthesis (23, 24). MMR in human cells involves three stages: initiation, excision and resynthesis. The initiation stage involves mismatch recognition by MutSα (MSH2-MSH6) or MutSβ (MSH2-MSH3), followed by assembly of an MMR initiation complex, which includes MutSα (or MutSβ), MutLα and proliferating cell nuclear antigen (PCNA), and localization of the strand discrimination signal (a single strand nick) by this complex. The latter part of this process is incompletely understood. In the second stage of MMR, exonuclease 1 (Exo1) removes nascent DNA exonucleolytically from a nick distal to the mismatch, generating a single-strand DNA gap of variable length, in a reaction that requires MutSα (or MutSβ), MutLα, PCNA, and replication protein A (RPA). During the "resynthesis" phase of MMR, DNA polymerase δ fills in the single-strand DNA gap in a reaction that requires RFC, PCNA, and RPA; finally, DNA ligase I seals the

[3] The abbreviations used are: MSI, microsatellite instability; MMR, mismatch repair; PCNA, proliferating cellular nuclear antigen; RPA, replication protein A; EGFR, epidermal growth factor receptor.

ssDNA break restoring the integrity of the dsDNA genome. Defects in MMR correlate strongly with MSI and with susceptibility to hereditary non-polyposis colorectal cancer (HNPCC) and other cancers (23, 24).

This study explores the hypothesis that arsenic inhibits MMR by stimulating expression of epidermal growth factor receptor (EGFR) tyrosine kinase, which phosphorylates tyrosine 211 (Y211) of PCNA, which in turn inhibits initiation of MMR in human cells. The results of this study suggest a putative novel mechanism of action underlying the carcinogenicity of arsenic.

Experimental Procedures

Cell Culture, Nuclear Extract, and Whole Cell Extract—HeLa cells were grown in RPMI 1640 medium with 10% fetal bovine serum. A549 and Beas-2B cells were grown in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum in the presence or absence of virious concentrations (5–15 μM) of sodium *meta*-arsenite (NaAsO$_2$). All cells were cultured at 37 °C in a humidified atmosphere of 5% CO$_2$. Unless specified otherwise, nuclear extracts (NE) were prepared from 2×10^9 cells as previously described (28), and whole cell extracts (WCE) were prepared from 6×10^7 cells. For WCE preparation, cells were collected and treated with washing buffer (20 mM Hepes, pH 7.5, 5 mM KCl, 0.5 mM MgCl$_2$, 0.1% PMSF, 0.5 mM DTT, 1 μg/ml leupeptin, and 0.2 M sucrose). Cells were lysed in the same buffer without 0.2 M sucrose by passage through a 27-gauge needle. Proteins were precipitated by addition of ammonium sulfate to 65% saturation, collected by centrifugation, resuspended in lysis buffer, and dialyzed to equilibrium in buffer containing 20 mM Hepes (pH 7.5), 5 mM KCl, 0.1 mM EDTA, 0.1% PMSF, 0.5 mM DTT, 1 μg/ml leupeptin as described (28).

Western Blot and Immunoprecipitation—Antibodies to PCNA, EGFR, α-tubulin, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to Y211 phosphorylated PCNA was generated by Bethyl Laboratories, Inc. (Montgomery, TX). 50 μg of whole cell lysate or nuclear extract was fractionated by SDS-PAGE and the proteins electroblotted to nitrocellulose membrane for 1 h (29). The nitrocellulose membrane was blocked with 5% nonfat dry milk (NFDM) dissolved in TBST (10 mM Tris-HCl (pH 7.5), 0.8% NaCl, 0.1% (v/v) Tween 20) for 1 h at room temperature. Then the membrane was incubated overnight at 4 °C with primary antibody in 5% NFDM. The membrane was washed three times for 10 min in TBST. The membrane was then incubated with a secondary antibody (Sigma) at room temperature for 2 h. The membrane was washed three times for 10 min at room temperature in TBST. Proteins were detected by chemiluminescence (ECL, GE Healthcare) followed by autoradiography.

For immunoprecipitation, nuclear extract or whole cell extract (~1 mg) was incubated with 15 μl of protein G-agarose beads (Thermo) for 30 min at 4 °C with shaking. The reaction was centrifuged at 800 $\times$ *g* at 4 °C for 2 min, the supernatant was incubated overnight with antibody to Y211-phosphorylated PCNA (5 μg). The mixture was incubated with 15 μl of prewashed protein G-agarose for 2 h at 4 °C with shaking. The

beads were collected and washed three times with LS Buffer (PBS (pH 7.5), 10% (v/v) glycerol, 0.1% (v/v) Nonidet P-40, and 1$\times$ proteinase inhibitor mixture) for 10 min with shaking. The sample was resuspended in gel loading buffer and heated at 90 °C for 5 min prior to analysis by SDS-PAGE. The gel was transferred to nitrocellulose membrane and analyzed by Western blot using primary antibody to PCNA.

Heteroduplex Preparation and MMR Assay—DNA substrates used in this study are circular heteroduplex DNA containing a unique G-T mismatch and a strand break 3′ to the mismatch (Fig. 1). The substrate was prepared from M13mp18-UKY phase series as described previously (30). MMR assays using nuclear extracts were performed essentially as described (28, 30). Briefly, 100 ng of heteroduplex DNA was incubated with 75 μg of nuclear extract or whole cell extract at 37 °C for 15 min in a 20-μl reaction containing 10 mM Tris-HCl (pH 7.5), 5 mM MgCl$_2$, 1.5 mM ATP, 0.1 mM dNTPs, 1 mM glutathione, and 110 mM KCl. Reactions were terminated by addition of Proteinase K. DNA samples were extracted with phenol and recovered by ethanol precipitation. For reactions with nuclear extract, DNA was digested with restriction enzymes NsiI (repair-scoring enzyme) and BseRI, and the reaction products were fractionated by 1% agarose gel electrophoresis. For reactions with whole cell extract (WCE), repair products were digested with PstI, NsiI (repair-scoring enzyme) and BglI, fractionated by polyacrylamide gel electrophoresis, and detected by Southern blot with a ^{32}P-labeled probe. DNA products were visualized by phosphorimager.

Analysis of MMR Incision/Excision Intermediates—Mismatch-provoked-excision was evaluated in the same manner as the MMR assay with 3′-nicked heteroduplex DNA, except the reaction buffer lacked dNTPs. Reaction products were digested with PstI and BglI, and fractionated by 6% denaturing polyacrylamide gel electrophoresis, followed by Southern blot using a ^{32}P-labeled oligonucleotide probe that hybridized near the BglI site in the nicked strand. Reaction products were detected by phosphorimager.

Gap-filling DNA Synthesis Assay—DNA resynthesis/gap-filling was performed using a circular M13mp18 dsDNA containing a 283-nt ssDNA gap in the same buffer, and reactions conditions as in the MMR assay. Reaction products were digested with HindIII and BseRI, fractionated through 1% agarose gel electrophoresis, and detected by UV light after ethidium bromide.

Protein Expression and Purification—Recombinant human RPA, PCNA, and GST-p21c were overexpressed in *Escherichia coli* BL21 (DE3) from plasmids provided by Marc Wold (University of Iowa), Jerald Hurwitz (Memorial Sloan-Kettering Cancer Center, New York, NY), and Anindya Dutta (University of Virginia, Charlottesville, VA), respectively. The recombinant proteins were purified as described previously (31–34). Recombinant human MutSα, MutLα, and Exo1 were expressed in insect cells using the Bac-to-Bac expression system (Invitrogen) as previously described (34–36). Purified proteins were stored in liquid nitrogen at -80 °C in 10 to 25-μl aliquots in buffer containing 1 mg/ml bovine serum albumin (BSA).
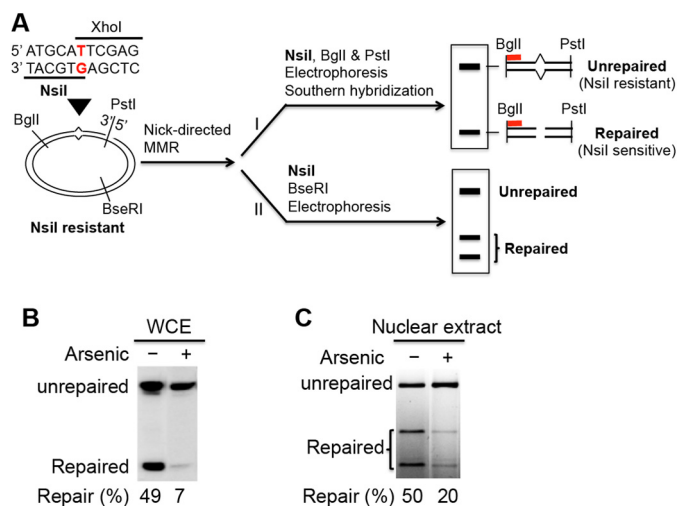
FIGURE 1. **Reduced MMR activity in arsenic-treated HeLa cells.** *A*, DNA substrate and MMR assay principles. MMR activity of arsenic-treated and control HeLa cells was determined by incubating cell extracts with a circular DNA heteroduplex containing a G-T mismatch and a strand break 172-bp 3′ to the mismatch as described previously (30). Several restriction enzymes used for scoring incision/excision or repair are shown. Assay I (*top panel*) shows a newly developed MMR assay, which employs whole cell extract (WCE) and Southern blot analysis. The *red bar* represents ^{32}P-labeled oligonucleotide probe complementary to the nicked strand at the indicated location. Assay II (*bottom panel*) shows the traditional MMR assay, which utilizes nuclear extract as previously described (28). *B*, MMR assay showing reduced MMR activity in WCE of arsenic-treated HeLa cells. *C*, MMR assay showing reduced MMR activity in nuclear extract of arsenic-treated HeLa cells.

Results

Cells Exposed to Arsenic Exhibit Reduced Mismatch Repair Activity—Nuclear extracts derived from HeLa cells are proficient in MMR *in vitro* (28, 37). To determine the impact of arsenic on MMR, whole cell extracts of arsenic-treated or untreated HeLa cells were prepared and assayed for their ability to repair a circular DNA heteroduplex containing a G-T mismatch, using a newly developed MMR assay (Fig. 1*A*, *top panel*), which is more sensitive than the traditional MMR assay (Fig. 1*A*, *bottom*). It is worth mentioning that the traditional assay uses nuclear extract from $\geq 2 \times 10^9$ cells (28, 38), but the assay used here is performed with whole cell extract from 6×10^7 cells. The results reveal that HeLa cells treated with arsenic are ~2-fold less active than untreated HeLa cells (Fig. 1, *B* and *C*), which is consistent with the hypothesis that arsenic directly or indirectly inhibits MMR.

Arsenic-induced Inhibition of MMR Is Rescued by Exogenous PCNA—To identify which MMR component is sensitive to arsenic, purified key MMR components MutSα, MutLα, and PCNA were added in excess to both the traditional MMR assay (Fig. 2*A*) and the new MMR assay (Fig. 2*B*) containing extracts derived from arsenic-treated HeLa cells. The results show that exogenous PCNA stimulated MMR activity in treated HeLa cell extracts (Fig. 2*A*, *lane 5*; *B*, *lane 5*), but not in untreated HeLa extracts (Fig. 2*A*, *lanes 6* and *7*); addition of other MMR components did not restore MMR in this experimental system (Fig. 2*A*, *lanes 3* and *4*; *B*, *lanes 3* and *4*). These results suggest that arsenic could inhibit MMR through a mechanism involving functional alteration of PCNA.
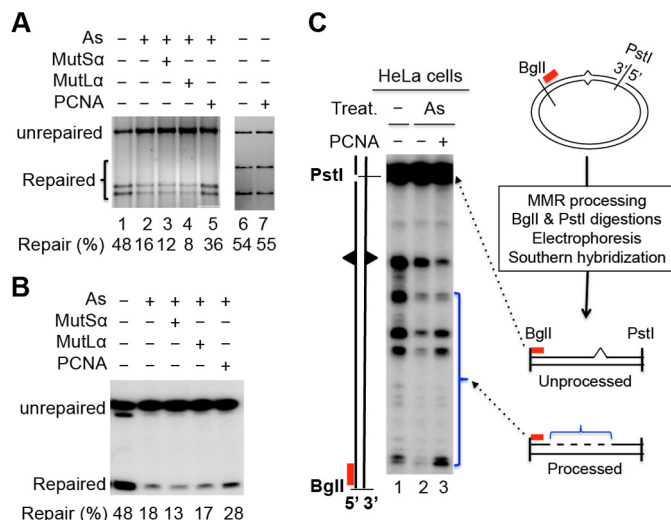

FIGURE 2. **PCNA restores MMR to arsenic-treated HeLa cells.** *A* and *B*, *in vitro* MMR assays showing that nuclear extracts (*A*) or whole cell extracts (*B*) of arsenic-treated HeLa cells exhibit reduced MMR activity, but addition of exogenous PCNA, not other key MMR proteins, stimulates *in vitro* MMR. *B*, Southern blot analysis of mismatch-provoked incision/excision in extracts of arsenic-treated and control HeLa cells. The *red bar* represents ^{32}P-labeled oligonucleotide probe that anneals to the nicked DNA strand near the BglI site, and *blue bracket* includes incision/excision intermediates allowing mismatch removal by a 5′-3′ exonuclease such as exonuclease 1.

Exposure to Arsenic Blocks Mismatch-provoked Incision/Excision—PCNA is essential for mismatch-provoked incision/excision, especially in 3′ nick-directed MMR (30, 39). The incision/excision products can be visualized on a Southern blot, by carrying out the MMR reaction in the absence of deoxyribonucleotide triphosphates (dNTPs), which blocks DNA resynthesis (30, 40). This approach was used to examine whether arsenic inhibits mismatch-provoked incision/excision in HeLa cell extracts, and whether such inhibition involves PCNA.

As shown in Fig. 2*C*, extracts derived from untreated HeLa cells generated expected incision/excision products (*lane 1*, DNA bands within bracket). Arsenic treatment greatly reduced the production of the incision/excision intermediates (Fig. 2*C*, *lane 2*), however, addition of purified recombinant PCNA to the reaction nearly restored the incision/excision products to the normal level (compare lane 3 with lane 1). These results suggest that arsenic inhibits MMR at the initiation stage, and that this inhibition can be rescued by exogenous PCNA. Thus, we postulate that endogenous PCNA in arsenic-treated cells plays a role in inhibiting mismatch-provoked incision/excision.

Effect of Arsenic on Phosphorylation Status of PCNA—Previous studies suggest that exposure to arsenic stimulates expression of epidermal growth factor receptor (EGFR) in human cells, potentially activating EGFR and its downstream targets and increasing risk of lung cancer (41). Data also show that the activated EGFR tyrosine kinase phosphorylates PCNA at tyrosine 211 (Y211) (42), and that Y211-phophorylated PCNA (PCNA-p) inhibits initiation of MMR *in vitro* (25). Therefore, it is reasonable to propose that arsenic-dependent carcinogenesis is at least in part mediated by EGFR-phosphorylated PCNA. To test this hypothesis, EGFR expression, PCNA phosphorylation status, and MMR capacity were evaluated in arsenic-treated and control HeLa cells. The results show a time- and dose-de-
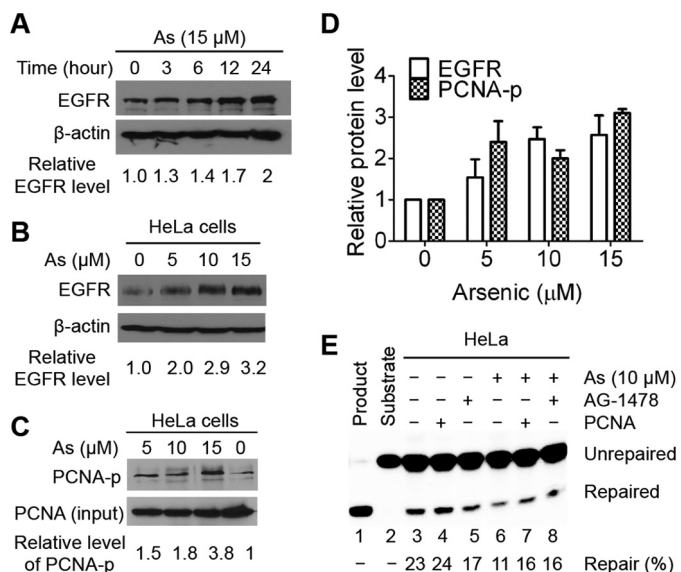
FIGURE 3. **Arsenic stimulates EGFR expression and PCNA phosphorylation.** *A,* Western blot quantification of EGFR in HeLa cells treated with 15 μM arsenic for the indicated times. *B,* Western blot of EGFR in HeLa cells treated with the indicated concentrations of arsenic for 24 h. *C,* immunoprecipitation followed by Western blot of Y211-phosphorylated PCNA (PCNA-p) in HeLa cells treated with arsenic at the indicated concentration for 24 h. Cell extracts (1 mg) were immunoprecipitated using a PCNA-Y211 phosphorylation-specific antibody, and probed using antibody to PCNA. Relative protein levels were determined using the levels of EGFR (*A* and *B*) or PCNA-p (*C*) in untreated cells as a reference. *D,* correlation between EGFR expression and PCNA phosphorylation. Error bars represent S.D., which were determined from variations in three independent experiments. *E, in vitro* MMR activity in arsenic-treated or untreated HeLa cells in the presence or absence of 10 μM AG-1478.

pendent correlation between arsenic exposure and expression of EGFR in HeLa cells (Fig. 3, *A* and *B*). PCNA phosphorylation was quantified in immunoprecipitates associated with a phosphorylation-specific antibody to Y211-PCNA, followed by Western analysis (Fig. 3*C*). The results reveal that increased levels of PCNA-p correlate with increased levels of EGFR in arsenic-treated HeLa cells (Fig. 3, *B*, *C*, and *D*). To determine that arsenic-mediated MMR inhibition is indeed related to the EGFR tyrosine kinase activity, HeLa cells were treated with arsenic in the presence or absence of an EGFR tyrosine kinase inhibitor, AG-1478, and WCEs derived from these cells were analyzed for MMR activity using the newly developed MMR assay (Fig. 1*A, top panel*). As shown in Fig. 3*E*, although AG-1478 slightly inhibits MMR activity (compare lane 5 with lane 3), it blocks arsenic-mediated inhibitory effect on MMR (compare lane 5 with lane 8). These data support the notion that arsenic stimulates expression/activation of EGFR, which in turn phosphorylates PCNA, leading to inhibition of MMR.

To test the idea that the effect of arsenic on PCNA and MMR increases susceptibility to lung carcinogenesis, we treated normal human lung bronchial cells, Beas-2B, and human non-small lung cancer cells, A549, with arsenic (10 μM) for 24 h, and analyzed EGFR expression, PCNA phosphorylation and MMR activity. As shown in Fig. 4*A*, exposure to arsenic led to increased expression and activation of EGFR (increased abundance of phosphotyrosine proteins) in Beas-2B and A459 cells as well as to increased levels of PCNA-p (Fig. 4*A*). Whole cell extracts derived from arsenic-treated and untreated A549 and
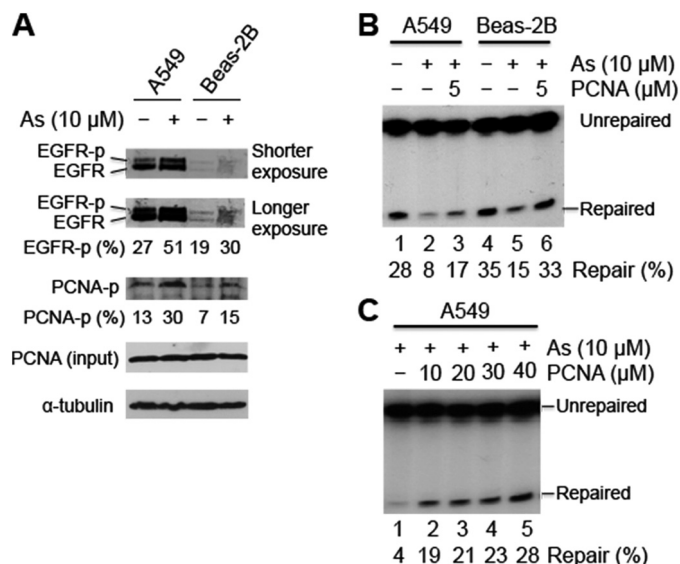

FIGURE 4. **Correlation between EGFR expression, PCNA phosphorylation, and MMR activity in normal human lung and human lung cancer cells.** *A,* lung cancer cell line A549 and normal lung cell line Beas-2B were treated with 10 μM arsenic for 24 h, cell extracts prepared and analyzed by Western blot with antibody to EGFR or PCNA-p by IP-Western, as described in Fig. 3. *B* and *C,* MMR activity in arsenic-treated A549 and Beas-2B cells with or without exogenous PCNA.

Beas-2B cells were examined for MMR activity (Fig. 1*A, top panel*). As shown in Fig. 4*B*, MMR capacity is lower in extracts from arsenic-treated A549 and Beas-2B cells than those from untreated control cells (Fig. 4*B, lanes 1* and *2, 4* and *5,* respectively). Addition of 5 μM of non-phosphorylated recombinant PCNA completely restored MMR activity in extracts from arsenic-treated Beas-2B, but only partially restored MMR activity in A549 cells. This might indicate a higher level of PCNA-p in A549 cells than in Beas-2B cells. Indeed, MMR activity was fully restored when more exogenous PCNA was added to WCE of arsenic-treated A549 cells (Fig. 4*C, lane 5*). In summary, these data are consistent with the hypothesis that arsenic stimulates EGFR-dependent phosphorylation of PCNA.

Arsenic Exposure Delays Mismatch-provoked DNA Resynthesis—PCNA is an essential cofactor for efficient DNA resynthesis during MMR (43). Therefore, the efficiency of DNA resynthesis in nuclear extracts from arsenic-treated and control cells was examined using a DNA gap-filling assay (see Fig. 5*A*). After the DNA resynthesis reaction, reaction products were cleaved by restriction enzymes HindIII and BseRI, which cleave ssDNA (gapped DNA substrate) with very low efficiency. In reactions carried out for 20 min at 37 °C, the efficiency of DNA resynthesis appeared similar in extracts from arsenic-treated HeLa cells and control cells in the presence or absence of exogenous PCNA (Fig. 5*B*). However, when reactions were carried out for 1, 3, 5, or 10 min, the efficiency of DNA resynthesis was slightly lower in reactions containing extracts from arsenic-treated HeLa cells (Fig. 5*C, lanes 6–9*) than in NE from control cells (Fig. 5*C, lanes 2–5*). This effect was reversed by addition of exogenous PCNA (Fig. 5*C, lanes 10–13*), consistent with the hypothesis that the effect of arsenic on MMR is mediated by endogenous PCNA.
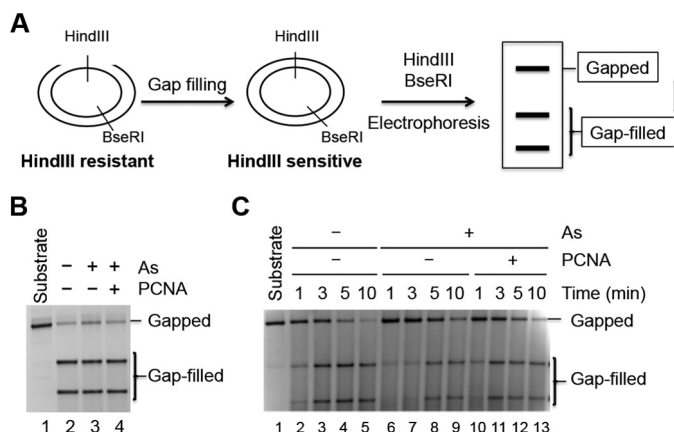
FIGURE 5. **Effect of arsenic on the kinetics of DNA resynthesis during MMR.** *A*, schematic representation of gap-filling DNA synthesis assay. The DNA substrate contains a 283-bp gap, and is resistant to HindIII digestion, while the product of gap-filling DNA synthesis extending past the HindIII site is cleaved by HindIII. *B* and *C*, gap-filling assay using nuclear extracts from arsenic-treated HeLa or control HeLa cells, as indicated. Reactions were incubated for 20 min (*panel B*), or for the indicated length of time (*panel C*).

Discussion

Genotoxic carcinogens or their metabolic products directly or indirectly damage nucleic acids including genomic DNA. In contrast, the mechanism of many non-genotoxic carcinogens, while poorly understood, is likely to involve an impact on signal transduction pathways that regulate or mitigate genetic instability and/or cell growth. The carcinogenicity of arsenic in humans is incompletely understood, but it may include genotoxic effects mediated by ROS-induced DNA damage (44) or a non-genotoxic mechanism mediated by induction of EGFR expression/activity (41) or both.

We show here that arsenic produces similar effects in HeLa, A549, and BEAS-2B cells, including: 1) inhibition of MMR by a mechanism mediated by endogenous PCNA (Figs. 1, 2, and 4); 2) induction of EGFR expression/activity (Fig. 3); and 3) increased abundance of Y211-phosphorylated PCNA. These data support a model in which arsenic induces EGFR expression and/or activates the EGFR tyrosine kinase, which in turn phosphorylates PCNA at Y211, stimulating cell proliferation (42) while inhibiting MMR and indirectly increasing susceptibility to carcinogenesis. Our recent studies show that Y211-phosphorylated PCNA promotes misincorporation during DNA synthesis (25), which could undermine mechanisms that preserve replication fidelity while promoting mechanisms that lead to genome instability and cellular transformation (23, 45–47).

It is well accepted that cancer expresses a mutator phenotype (48). Based on this theory, acquisition of a large number of mutations in genes regulating cell growth is a prerequisite for tumorigenesis. Environmental carcinogens, particularly the genotoxic carcinogens, can directly or indirectly modify DNA to form carcinogen-DNA adducts, thereby inducing gene mutations. However, a cell possesses an array of DNA damage response and repair pathways to efficiently remove all types of DNA lesions, including those caused by environmental carcinogens (49, 50). Because of quality and fidelity controls by these DNA repair systems, it is not easy for a carcinogen-DNA adduct

to be converted into a mutation. In other words, mutations may only occur when a genome maintenance systems is not functional. How can environmental carcinogens impair DNA repair systems to induce mutations? The results presented here provide a clue for the long-standing question: arsenic inhibits MMR by inducing expression/activation of EGFR, which phosphorylates PCNA, a critical component of MMR as well as DNA replication and DNA repair.

Here, we postulate that post-translational modification of PCNA impairs MMR in human cells (25), leading to genome instability and cancer susceptibility. However, many questions about arsenic-induced carcinogenesis are still not resolved, and additional studies are needed. Critical unresolved questions include the mechanism by which arsenic stimulates expression/activation of EGFR and the translocation of EGFR into the nucleus, which is presumably a prerequisite to EGFR-dependent phosphorylation of PCNA. It is also not yet understood how or why tyrosine-phosphorylation of PCNA reduces the efficiency of MMR.

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Arsenic Inhibits DNA Mismatch Repair by Promoting EGFR Expression and PCNA Phosphorylation

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