Arsenite is a well-documented human carcinogen. Long-term exposure to inorganic arsenic from drinking water has been reported to induce various cancers (Centeno et al. 2002; Huang et al. 2004; Tseng et al. 1968; Yu et al. 2006). Chronic exposure to arsenite can lead to its accumulation in the skin and cause skin hyperpigmentation and hyperkeratosis (Centeno et al. 2002; Yu et al. 2006). This could in turn develop into skin cancers, including Bowen disease (carcinoma in situ), basal cell carcinoma (BCC), and squamous cell carcinoma (SCC) (Tseng et al. 1968; Yu et al. 2006).

Cancer development results from a synergism between genotoxic and nongenotoxic factors (Hecker 1987; Zoumpourlis et al. 2003). The former induces irreversible genetic alterations (tumor initiation), whereas the latter promotes tumor development by favoring the clone outgrowth of the genetically altered cells (tumor promotion) through activating cell survival and proliferation signal pathways and altering the machineries controlling cell proliferation and apoptosis. Previous studies have demonstrated that arsenite has a weak mutagen effect; therefore it is thought that its ability to activate signaling pathways leading to the alteration of gene expression responsible for cell growth may play an important role in its carcinogenic effect (Bernstam and Nriagu 2000). It has been demonstrated that signal pathways, including mitogen-activated protein kinases (MAPKs), activating factor 1 (AP-1) and nuclear factor kappa B (NF-kB), can be activated upon arsenite exposure and presumably contribute to arsenite-induced skin carcinogenic effect (Cooper et al. 2004; Huang et al. 2001, 2004). Phosphatidylinositol 3-kinase (PI-3K) comprises an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) and could be activated by multiple growth factors and cytokines (Cantley 2002; Vivanco and Sawyers 2002). Upon activation, PI-3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP3), a lipid second messenger essential for the activation of protein kinase B (Akt) (Alessi et al. 1997; Toker and Cantley 1997). Akt in turn regulates various cellular functions such as apoptosis and proliferation (Alessi et al. 1997; Franke et al. 2003). PI-3K/Akt has been demonstrated to be an important signaling pathway for cell survival and growth, and it also plays a pivotal role in cell transformation and tumorigenesis (Huang et al. 1999; Li et al. 2005; Nicholson and Anderson 2002; Ouyang et al. 2005a; Samuels and Ericson 2006). The elevated expression or high phosphorylation of Akt could be observed in many tumor cells (Asanuma et al. 2005; Bae et al. 2006; Misra et al. 2006). Most recently, He et al. (2006) reported that PI-3K/Akt is related to the malignant transformation associated with acquired apoptotic resistance in human HaCaT keratinocytes induced by chronic UVA irradiation. Souza et al. (2001) have reported that PI-3K is required for the induction of endothelial nitric oxide synthesis (eNOS) by arsenite in human keratinocytes. Our previous studies have also shown that arsenite exposure is able to activate the PI-3K/Akt pathway and induce cyclin D1 expression in mouse epidermal Cl41 cells (Ouyang et al. 2006). In HaCat cells, the PI-3K/Akt/cyclin D1 cascade activation contributed to arsenite-induced proliferation (Ouyang et al. 2007b). Although hyperproliferation is correlated with cellular transformation in some cases (Chen et al. 2001), our previous findings clearly demonstrated that in C41 cells, epidermal growth factor (EGF)-induced transformation was impaired by disrupting PI3K/p85 expression; however, cell proliferation was not affected (Huang et al. 1996), which indicates that the transformation ability is not always paralleled with the accelerated proliferation rate. Therefore, we performed the present studies to investigate whether the PI-3K/Akt signal pathway is indeed implicated in arsenite-induced cell transformation through the induction of cyclin D1.

Materials and Methods

**Cell culture and reagents.** Spontaneously immortalized human keratinocytes, HaCat

**Address correspondence to C. Huang, Nelson Institute of Environmental Medicine, New York University School of Medicine, 57 Old Forge Rd., Tuxedo, NY 10987 USA. Telephone: (845) 731-3519. Fax: (845) 351-2320. E-mail: chuanshu@env.med.nyu.edu**

**These authors contributed equally to the work. This work was supported in part by grants from the National Institutes of Health (NIH)/National Cancer Institute R01 CA094964 (C.H.), R01 CA112557 (C.H.), R01 CA105380 (C.H.), R01 CA119028 (X.S.), NIH/National Institute of Environmental Health Sciences (NIEHS) R01 ES012451 (C.H.), and NIEHS Center grant ES00260. The authors declare they have no competing financial interests. Received 24 April 2007; accepted 4 October 2007.**
cell line, and their stable transfectants were cultured in monolayers at 37°C, 5% CO₂ using Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 µg gentamicin/mL. Normal human epidermal keratinocytes (NHEKs) were cultured in keratinocyte–SFM medium (Invitrogen Corp., Carlsbad, CA, USA) containing supplements (human epidermal growth factor, bovine pituitary extract; Invitrogen) and gentamycin (5 mg/mL; Sigma-Aldrich Corp., St. Louis, MO, USA). The cultures were detached with trypsin and transferred to new 75-cm² culture flasks (Fisher Scientific Co., Pittsburgh, PA, USA) twice a week. FBS was purchased from Life Technologies, Inc.; DMEM was from Calbiochem (San Diego, CA, USA); sodium arsenite was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). The dominant-negative mutants of Akt (DN-Akt) and PI-3K (∆p85) or vector control plasmids using Lipofectamine 2000 reagent (Invitrogen Corp.) according to manufacturer's instructions. Briefly, HaCat cells were cultured in a 6-well plate to 85–90% confluence. Five micrograms plasmid DNA, alone or in combination with pCMV-neo vector, were for co-transfection. DNA was mixed with 10 µL of Lipofectamine 2000 reagent and used to transfect each well in the absence of serum. After 6–8 hr, the medium was replaced with 10% FBS DMEM. Approximately 30–36 hr after the beginning of the transfection, the cells were detached with 0.033% trypsin, and cell suspensions were plated into 75-cm² culture flasks and cultured for 24–28 days with G418 selection (Ouyang et al. 1996; Li et al. 2004; Ouyang et al. 2006).

**Cyclin D1 small interference RNA construction.** The specific small interference RNA (siRNA)–targeted human cyclin D1 was described before (Ouyang et al. 2005b). The target sequence was inserted into the pSuppressor vector and verified by DNA sequencing. **Stable transfection.** We transfected HaCat cells with DN-Akt and ∆p85 or vector control plasmids using Lipofectamine 2000 reagent (Invitrogen Corp.) according to manufacturer's instructions. Briefly, HaCat cells were cultured in a 6-well plate to 85–90% confluence. Five micrograms plasmid DNA, alone or in combination with pCMV-neo vector, were for co-transfection. DNA was mixed with 10 µL of Lipofectamine 2000 reagent and used to transfect each well in the absence of serum. After 6–8 hr, the medium was replaced with 10% FBS DMEM. Approximately 30–36 hr after the beginning of the transfection, the cells were detached with 0.033% trypsin, and cell suspensions were plated into 75-cm² culture flasks and cultured for 24–28 days with G418 selection (Ouyang et al. 1996; Li et al. 2004; Ouyang et al. 2006).

**PI-3 kinase assay.** We conducted the PI-3 kinase activity assay as described in our previous studies (Brubaker et al. 2006; Jungwirth et al. 1997), where L is tumor length, W is width, and H is height (Ouyang et al. 2007a). Tumors were removed from mice, and fixed in 10% buffered formalin and embedded in paraffin; 5-µm sections were dehydrated and stained with hematoxylin and eosin (H&E).

**Statistical analysis.** The significant difference between the treated and untreated
groups was determined with the Student t-test. Results are expressed as mean ± SD.

Results
Repeated arsenite exposure led to transformation of HaCat cells. Human skin is a major target of environmental carcinogen arsenite. To elucidate the mechanism implicated in arsenite-induced human skin carcinogenic effect in vitro, we first evaluated the cytotoxicity of arsenite to HaCat cells with CellTiter-Glo Luminescent Cell Viability Assay kit. We found that exposure of HaCat cell to 0.625 µM arsenite caused a significant increase in cell proliferation (Figure 1A) and no inhibition of cell proliferation at doses lower than 2.5 µM arsenite (Figure 1A). Thus, we used 2.5 µM arsenite to treat human keratinocyte HaCat cells to establish a cell transformation model. HaCat cells were exposed repeatedly to 2.5 µM arsenite twice a week for 8 weeks, and the anchorage-independent growth capability of arsenite-treated HaCat cells was evaluated. Compared with the medium control, repeated arsenite exposure resulted in increased the anchorage-independent growth capacity of HaCat cells (Figure 1B, C). Those results indicate that arsenite-exposed HaCat cells obtain the ability of anchorage-independent growth for colony formation in soft agar. The tumor characteristic of the transformed cells was further confirmed in nude mice. As shown in Figure 1D, injection of arsenite long-term exposed HaCat cells into nude mouse caused observable tumor formation (tumor volumes 786 ± 126, n = 6) compared with that of long-term culture HaCat cells (0 ± 0, n = 6). H&E staining also revealed a tumor formation in the arsenite long-term exposed HaCat cells (Figure 1E). On the basis of these results, we anticipate that repeated exposure of HaCat cells to arsenite could cause malignant transformation.

The PI-3K/Akt pathway is required for arsenite-induced transformation of HaCat cells. Our previous studies have shown that PI-3K is essential for Cl41 cells obtaining anchorage-independent growth capacity in TPA (12-O-tetradecanoylphorbol-13-acetate) and EGF treatments (Huang et al. 1999; Ouyang et al. 2005b). In addition, our published studies have shown that arsenite exposure is able to activate PI-3K in mouse epidermal Cl41 cells (Ouyang et al. 2006). To determine the potential involvement of the PI-3K pathway in arsenite-induced HaCat cell transformation, we tested the PI-3K activity in arsenite-exposed HaCat cells. The results showed that the arsenite exposure did increase PI-3K activation in HaCat cells compared with the medium control (Figure 2A, B). We also further confirmed this finding in NHEKs (Figure 2C, D). The aforementioned data demonstrate that PI-3K is implicated in human keratinocyte response to arsenite exposure.

Upon activation, PI-3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP3), a lipid second messenger essential for the translocation of Akt to the plasma membrane where it is phosphorylated and activated by phosphoinositide-dependent kinase-1 (PDK-1) (Alessi et al. 1997; Toker and Cantley 1997). Subsequently, Akt phosphorylates and regulates the function of many downstream cellular proteins involved in the processes of apoptosis, proliferation, and transformation (Alessi et al. 1997; Franke et al. 2003). To test possible Akt activation by arsenite in human keratinocytes, we determined Akt activation in both HaCat and NHEKs by evaluating its phosphorylation at Thr308 and Ser473. The results indicated...
that arsenite exposure was able to activate Akt in both cells (Figure 3A, B), which was consistent with PI-3K activation. To elucidate the PI-3K/Akt pathway and its role in human keratinocyte response to arsenite response, we established the stable HaCat Δp85 and DN-Akt transfectants. Ectopic expression of Δp85 and DN-Akt dramatically reduced arsenite-induced Akt activation (Figure 3A), and consequently blocked cell transformation upon chronic arsenite exposure in HaCat cells (Figure 3C, D). These results demonstrate the critical role of the PI-3K/Akt pathway in arsenite-induced HaCat transformation.

Cyclin D1 is a key PI-3K/Akt downstream protein responsible for arsenite-induced transformation of HaCat cells. It has been thought that the contribution of the PI-3K/Akt pathway to tumorigenesis could be associated with either its regulation of cell apoptosis or cell growth. Our previous studies have shown that arsenite exposure is able to up-regulate cyclin D1 protein expression in HaCat cells, which further mediates cell cycle alternation in HaCat cells (Ouyang et al. 2005b). Thus, it is important to determine whether there is a link between arsenite-induced PI-3K/Akt activation and cyclin D1 protein expression. Arsenite treatment resulted in a marked increase in cyclin D1 protein expression in both HaCat cells (Figure 4A) and NHEKs (Figure 4B), and this cyclin D1 induction was dramatically impaired in Δp85 or DN-Akt stable transfectants (Figure 4C), indicating that the PI-3K/Akt pathway is critical for cyclin D1 protein induction by arsenite. It might be noted that overexpression of DN-Akt was able to block Akt activation, whereas Δp85 only showed a partial inhibition of Akt activation induced upon arsenite treatment (Figure 3A). This differential inhibition of Akt phosphorylation by DN-Akt and Δp85 could be due to the protein expression levels of those two exogenous dominant negative mutants, or alternate pathways may be involved in the Akt activation. It might also be noted that Δp85 is able to block arsenite-induced cyclin D1 expression completely, whereas it shows only partial inhibition on Akt phosphorylation. The explanation for this may be that Akt is only one of p85 downstream kinases, and the other p85 downstream kinases such as protein kinase C, serum glucocorticoid-inducible kinase, and Rac/CDC42 may also play some role in cyclin D1 protein expression in arsenite responses. In addition, cyclin D1 induction might need PI-3K activation to a certain level, so when Akt activation was relatively low, it was not able to cause cyclin D1 induction. The basal level of Akt phosphorylation in DN-Akt transfectants was higher than that of the vector control (Mock) transfectants. The explanation was that, due to the importance of Akt in normal cell function, the phosphorylation of the endogenous Akt in DN-Akt stable transfectant was elevated to overcome the biological effects caused by overexpression of exogenous DN-Akt. However, the arsenite-induced phosphorylation will be greatly inhibited, as shown in Figure 3A.

To evaluate the contribution of cyclin D1 protein expression to arsenite-induced HaCat cell transformation, we used HaCat cells stably transfected with cyclin D1 siRNA (Ouyang et al. 2005b). As shown in Figure 4D, introduction of cyclin D1 siRNA dramatically reduced the basal level of the cyclin D1 protein expression, whereas it did not affect the basal level of the cyclin D2 protein expression, verifying the specificity of cyclin D1 siRNA. Knockdown of cyclin D1 expression by its siRNA abrogated the HaCat cell transformation induced by arsenite (Figure 4D, E). Collectively, these results indicate that cyclin D1 is not only induced by arsenite exposure through the PI-3K/Akt-dependent pathway but it is also at least one of the key events responsible for arsenite-induced human keratinocyte transformation.

Discussion

Arsenite is a well-defined human carcinogen, with skin as its primary target organ (Centeno et al. 2002; Huang et al. 2004; Tseng et al. 1968; Yu et al. 2006). Because arsenite has only a weak mutagenic effect, it is thought that its ability to activate some signaling pathways and gene expression responsible for cell growth may play an important role in mediating its carcinogenetic effect (Bernstam and Nriagu 2000). In the present study, we demonstrated that repeated exposure of human keratinocytes to low doses of arsenite resulted in cell transformation with the characteristic of cell anchorage-independent growth in soft agar. The dose we used to repeatedly treat cells did not cause obvious cell death. On the contrary, it promoted cell proliferation as we reported in our recent publication (Ouyang et al. 2007b). The treatment of cells with arsenite also caused the activation of PI-3K/Akt, which thereby plays a critical role in arsenite-induced cell transformation through induction of cyclin D1 expression.

As an important signal pathway for cell survival and growth, PI-3K/Akt has been demonstrated to be associated with tumorigenesis (Nicholson and Anderson 2002; Samuels and Ericson 2006). More than 30% of various solid tumor were found recently to contain mutations in PIK3CA, the catalytic subunit of PI-3K (Samuels and Ericson 2006). The mutation in p85, a regulatory subunit of PI-3K, has also been reported in previous studies (Jimenez et al. 1998; Philp et al. 2001). Recent studies also indicate that Akt is frequently constitutively activated in many types of human cancer (Nicholson and Anderson 2002). Although the mechanisms

![Figure 3. Requirement of the PI-3K/Akt pathway activation for HaCat cell transformation upon arsenite exposure. (A) HaCat cells stably transduced with dominant negative mutants of Akt (DN-Akt) or p85 (Δp85) or vector control (Mock) were treated with arsenite in different doses as indicated for 180 min. The number was the relative wells density of phosphorylated Akt compared with total Akt. (B) NHEKs were treated with 2.5 μM arsenite at different time points and the phosphorylation of Akt was detected with specific antibodies. (C,D) The anchorage-independent growth was evaluated among the HaCat cells stably transduced with vector control, DN-Akt, and Δp85 after repeated exposure to arsenite for 8 weeks. Each bar indicates the mean and SE of triPLICATE assay wells.

*Significant decrease compared with that from HaCat cells transfected with vector (Mock) (p < 0.05).
have not yet been fully characterized, constitutive PI-3K/Akt signaling is believed to promote proliferation and increase cell survival, which is an indispensable event during the process of cancer development (Samuels and Ericson 2006). Current studies demonstrated that arsenite exposure was able to activate PI-3K and Akt, and inhibition of either PI-3K or Akt by their dominant mutants impaired arsenite-induced cell transformation in human skin keratinocytes HaCat, suggesting that the PI-3K/Akt pathway may contribute to arsenite human skin carcinogenic effects.

Reactive oxygen species (ROS) at low concentration may function as a signaling intermediately of cellular responses (Sullivan et al. 1994). The production of ROS in response to arsenite treatment has been observed in various cancer cells (Duyndam et al. 2001; Ozaki et al. 2000), suggesting that arsenite may act early in the growth factor signaling pathway. Jung et al. (2005) have clearly demonstrated that the predominant product by arsenite appeared to be hydrogen peroxide (H$_2$O$_2$) because the arsenite-induced increase in dichlorofluorescein (DCF) fluorescence was completely abolished by pretreatment with catalase but not with heat-inactivated catalase. By eliminating H$_2$O$_2$ with catalase or N-acetylcysteine, they further found that H$_2$O$_2$ might act as an upstream molecule of PI-3K as well as ERK1/2 (Jung et al. 2003). So we propose that the generation of ROS by arsenite may be associated with various cellular processes, such as PI-3K/Akt pathway activation.

Cyclin D1 could be induced by growth factors and stress, then regulate cell cycle and proliferation (Cook et al. 2000; Perry et al. 1998; Winston and Pledger 1993). Ablation of growth factor signaling pathway by its dominant-negative mutants, which also suggests that the PI-3K/Akt/cyclin D1 pathway might be a target for chemoprevention of arsenite-induced skin cancer.

**Figure 4.** A key role of cyclin D1 in arsenite-induced HaCat cell transformation. HaCat cells (A) and NHEKs (B) were treated with 5 µM arsenite for the indicated time period (A) or for 24 hr (B), and the cells were extracted with sample lysis buffer for Western blot analysis to determine cyclin D1 expression. (C) HaCat cells stable transfected with vector, DN-Akt, or Δp85, were treated with arsenite at concentrations indicated, and cyclin D1 protein expression levels were evaluated with Western blot analysis. (D) Specific knockdown of cyclin D1 in HaCat cells was identified with Western blot analysis compared with normal expression of cyclin D2 expression. (E,F) The capability of anchorage-independent growth activities was compared between cyclin D1 siRNA transfectant and nonspecific control siRNA transfectant after repeatedly treated with arsenite for 8 weeks. Each bar indicates the mean and SE of triplicate assay wells.

*Significant decrease compared with that from HaCat cells transfected with control siRNA (Scramble).
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