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Essential roles of PI-3K/Akt/IKKβ/NFκB pathway in cyclin D1 induction by arsenite in JB6 Cl41 cells

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Skin is a major target of carcinogenic trivalent arsenic (arsenite, As^{3+}). It has been thought that cell proliferation is one of the central events involved in the carcinogenic effect of arsenite. Cyclin D1, a nuclear protein playing a pivotal role in cell proliferation and cell cycle transition from G_1 to S phases, has been reported to be induced in human fibroblast by arsenite via uncertain molecular mechanisms. In the present study, the potential roles of PI-3K/Akt/IKKβ/NFκB signal pathway in cyclin D1 induction by arsenite were addressed in mouse epidermal Cl41 cells. We found that exposure of Cl41 cells to arsenite was able to induce cell proliferation, activate PI-3K→Akt/$p70^{S6k}$ signal pathway and increase cyclin D1 expression at both transcription and protein levels. Pre-treatment of Cl41 cells with PI-3K inhibitor, wortmannin, significantly inhibited the phosphorylation of Akt and $p70^{S6k}$ and thereby dramatically impaired the cyclin D1 induction by arsenite, implicating the importance of the PI-3K signal pathway in the cyclin D1 induction by arsenite. Furthermore, inhibition of PI-3K/Akt by overexpression of Δp85 or DN-Akt blocked arsenite-induced IKK phosphorylation, IκBα degradation and cyclin D1 expression, indicating that IKK/NFκB is the downstream transducer of arsenite-triggered PI-3K/Akt cascade. Moreover, inhibition of IKKβ/NFκB signal pathway by overexpression of its dominant negative mutant, IKKβ-KM, also significantly blocked arsenite-induced cyclin D1 expression. Overall, arsenite exposure triggered PI-3K/Akt/IKKβ/NFκB signal cascade which in turn plays essential roles in inducing cyclin D1 expression.

Introduction

Arsenite is introduced into the environment during energy production based on coal, oil shale and geothermal sources (1). Once in the environment, arsenite represents a potential health hazard of unknown magnitude. Available epidemiological data have shown that exposure to arsenite is associated with increased risks of human cancer of the skin, respiratory tract, hematopoietic system and urinary bladder (1–4). Epidemiological investigations also indicated that long-term arsenic exposure results in promotion of carcinogenesis, especially in lung and skin via inhalation and ingestion (3). Many cases of skin cancer have been documented in people exposed to arsenite through medical or other occupational exposures (2,4). It has also been reported that high arsenic levels in drinking water (0.35–1.14 mg/l) increased the risks of cancer of skin, bladder, kidney, lung and colon (5). Based on these facts, the International Agency for Research on Cancer (IARC, 1980, 1987) and the US Environmental Protection Agency (EPA, 1988) classified inorganic arsenic as a known human carcinogen.

As a carcinogen, arsenite was demonstrated to induce cell proliferation both in cell culture model and animal model (6–13). In cell culture studies, arsenite increases cell proliferation in human keratinocytes (6–8) and enhances the mitogenic effect of suboptimal serum concentrations on quiescent C3H10T1/2 cells (9). In animal studies, low doses of arsenite cause hyperplasia in the urinary bladder epithelium and in skin (8,10–12), and mice exposed to arsenite in drinking water show an increased epidermal thickness and an increased fraction of epidermal cells expressing proliferating cell nuclear antigen (PCNA) as comparison with control mice (13). It is believed that alterations of gene expression that drive uncontrolled cell cycle progression are associated with the aberrant proliferation during tumorigenesis (14,15). Normal eukaryotic cells progress through a well-defined cell cycle consisting of four distinct stages, G_1, S, G_2 and M. The cell cycle progression is mainly controlled by several key checkpoints, including G_1/S checkpoint, S-phase DNA damage checkpoint and G_2/M spindle integrity checkpoint. Previous studies demonstrated that faulty G_1/S control caused by activation of many oncogenes or inactivation of tumor suppressor genes plays a critical role in tumorigenesis. Cyclin D1 is one of the key regulators of G_1/S transition (14–16). Previous studies revealed that antisense to cyclin D1 was able to inhibit the growth and tumorigenicity of human colon cancer cells and induce apoptosis in human squamous carcinomas (17,18). Increasing evidence indicated that the ability of arsenite to modulate the signaling pathways and genes expression responsible for cell growth may play a more important role in its carcinogenesis (19,20). Rossman *et al*. (21) reported that exposure of human fibroblasts to arsenic can induce cyclin D1 expression; however, it remains unknown whether arsenite exposure is able to cause the increase in cyclin D1 expression in skin cells which are the major target of arsenite; moreover, the molecular mechanisms which mediate this induction remain to be elucidated. Because cell proliferation is believed to be a central event of arsenite skin carcinogenic effect, investigation on the signal pathways leading to cyclin D1 induction may shed some light on the unraveling of the molecular mechanisms, and thereby provide some clues to prevent arsenite-induced carcinogenesis. So we

Abbreviations: APC, human adenomatous polyposis coli gene; CDKs, cyclin-dependent kinases; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GSK3β, glycogen synthase kinase-3β; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEM, Eagle's minimal essential medium; mTOR, mammalian target of rapamycin; NFκB, nuclear factor-κB; PI-3K, phosphotidylinositol-3 kinase; PKCα, protein kinase Cα.

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addressed these questions in the present study in mouse epidermal Cl41 cells.

Materials and methods

Reagents and plasmids

Fetal bovine serum (FBS) was purchased from Life Technologies, Inc. (Gaithersburg, MD); MEM (minimal essential medium) was from Calbiochem (San Diego, CA); Arsenite (As^{3+}) was purchased from Aldrich (Milwaukee, WI). The luciferase assay substrate was purchased from Promega (Madison, WI). Wortmannin, PI-3K inhibitor and rapamycin, an mTOR/$p70^{S6k}$ pathway inhibitor, were purchased from Calbiochem (La Jolla, CA). Antibodies specific targeting phospho-Akt (Thr308), phospho-Akt (Ser473), Akt, phospho-$p70^{S6k}$ (Thr389), phospho-$p70^{S6k}$ (Thr421/Ser424), $p70^{S6k}$, phospho-IKKα/β, IκBα and cyclin D1 were purchased from Cell Signaling Technology (Berverly, MA). Antibody against GAPDH was from Abcam (Cambridge, MA). The cytomegalovirus-neo vector plasmid, the Akt mutant plasmid (SRα-Akt-T308A/S473A) and the dominant negative mutant PI-3K plasmid (Δp85), were as described previously (22–25). The dominant negative form of IKKβ (IKKβ-KM, K44A), which is a gift from Dr. Hiroyasu Nakano (Juntendo University, Japan). The cyclin D1 promoter-driven luciferase reporter (cyclin D1 Luc) construct was obtained by inserting a 1.23 kb EcoR I-Pvu II fragment of the cyclin D1 gene promoter, which contains the cyclin D1 promoter sequence from −1095 to +135 relative to the translation initiation site, into the pA3LUC vector as described previously (26,27).

Cell culture

Mouse epidermal JB6 Cl41 cells and their stable transfectants were cultured in monolayers at 37°C, 5% CO_2 using Eagle's MEM containing 5% FBS, 2 mM L-glutamine and 25 μg of gentamicin/ml. The cultures were detached with trypsin and transferred to new 75-cm^2 culture flasks (Fisher, Pittsburgh, PA) from once to thrice per week.

Cell proliferation assay

Confluent monolayers of Cl41 transfectants were trypsinized and 1×10^3 viable cells suspended in 100 μl MEM supplemented with 5% FBS were added to each well of 96-well plates. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. Twelve hours later, the cells were exposed to arsenite, and then lysed with 50 μl lysis buffer. The proliferation of the cells was measured using CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI) with a luminometer (Wallac 1420 Victor2 multipliable counter system). The results are expressed as luciferase activity relative to control medium (proliferation index).

Cell cycle assay

Cl41 cells (2×10^5) were seeded into each well of 6-well plates. After the confluence reached 70–80%, the cells were exposed to 5 μM of arsenite for 48 h, and then harvested and fixed with 3 ml of ice-cold ethanol 80% overnight. The fixed cells was then centrifuged (3000 rpm, 3 min), suspended in lysis buffer (100 mM sodium citrate and 0.1% Triton X-100) and incubated for 15 min at room temperature. The cells were incubated with RNAse A (10 mg/ml) (Sigma Chemical, St. Louis, MO) for 10 min at room temperature and DNA was stained with propidium iodide (50 μg/ml) for at least 1 h at 4°C. The cell proportion in sub-G_1 and S phases was determined by flow cytometry using a Epics XL FACS (Beckamn Coulter) and EXPO 32 software (27).

Stable transfection

Cl41 cells were transfected with cyclin D1 luciferase gene reporter, NFκB luciferase gene reporter, the dominant negative mutant of PI-3K regulatory subunit p85 (Δp85), the dominant negative mutant of Akt (DN-Akt), the dominant negative mutant of IKKβ (IKKβ-KM) and mock vector according to the manual of Lipofectamine 2000 reagent. Briefly, Cl41 cells were cultured in 6-well plates to 85–90% confluence. Five micrograms of plasmid DNA in combination with 1 μg of CMV-neo vector for co-transfection, were mixed with 10 μl of Lipofectamine 2000 reagent, and used to transfect each well in the absence of serum. After 6 h, the medium was replaced with 5% FBS MEM. Approximately 30–36 h after the beginning of the transfection, the cells were digested with 0.033% trypsin, and the cell suspensions were plated onto 75-ml culture flasks and cultured for 24–28 days with G418 selection (500 μg/ml). Stable transfectant was established and cultured in G418-free MEM for at least two passages before each experiment.

Gene reporter assay

Confluent monolayers of Cl41 transfectants were trypsinized, and 8×10^3 viable cells suspended in 100 μl MEM supplemented with 5% FBS were added to each well of 96-well plates. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. After the cell density reached 80–90%, the cell culture medium was replaced with an equal volume of MEM supplemented with 0.1% FBS and 2 mM L-glutamine. 12 h later, the cells were exposed to arsenite for cyclin D1 induction or NFκB activation. After 12-h culture, the cells were lysed with 50 μl lysis buffer, and luciferase activity was measured using Promega Luciferase assay reagent with a luminometer (Wallac 1420 Victor2 multipliable counter system). The results are expressed as cyclin D1 luciferase activity relative to control medium (relative cyclin D1 induction) or NFκB activity relative to control medium (relative NFκB activation) (22).

PI-3 kinase assay

PI-3 kinase activities were assayed as described in our previous reports (23,24). Cells were cultured in monolayers in 100 mm dishes using normal culture medium. The media were replaced with 0.1% FBS MEM containing 2 mM L-glutamine and 25 μg of gentamicin/ml after the cell density reached 70–80%. Forty-five hours later, the cells were incubated with fresh serum-free MEM for 3–4 h at 37°C. Arsenite was then added to cell cultures for PI-3K induction. The cells were washed once with ice-cold PBS and lysed in 400 μl of lysis buffer/plate [20 mM Tris (pH 8), 137 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1% NP-40, 1 mM DTT, 0.4 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride]. The lysates were centrifuged and the supernatants incubated at 4°C with 40 μl of agarose beads (conjugated previously with the monoclonal antiphosphotyrosine antibody Py20) overnight. Beads were washed twice with each of the following buffers: (i) PBS with 1% NP-40, 1 mM DTT; (ii) 0.1 M Tris (pH 7.6), 0.5 M LiCl, l mM DTT; and (iii) 10 mM Tris (pH 7.6), 0.1 M NaCl, 1 mM DTT. Beads were incubated for 5 min on ice in 20 μl of buffer 3 and then 20 μl of 0.5 mg/ml phosphatidylinositol [sonicated previously in 50 mM HEPES (pH 7.6), 1 mM EGTA, 1 mM NaH_2PO_4] were added. After 5 min at room temperature, 10 μl of the reaction buffer were added [50 mM $MgCl_2$, 100 mM HEPES (pH 7.6), 250 μM ATP containing 5 μCi of [γ-^{32}P]ATP], and beads were incubated for an additional 15 min. The reactions were stopped by the addition of 15 μl of 4 N HCl and 130 μl of chloroform–methanol (1 : 1). After vortexing for 30 s, the solutions, 30 μl from the phospholipid-containing chloroform phase, were spotted onto TLC plates coated with silica gel H containing 1.3% potassium oxalate and 2 mM EDTA applied in H_2O/methanol (3 : 2). The plates were heated at 110°C for at least 3 h before use. The plates were then placed in tanks containing chloroform–methanol–NH_4OH/H_2O (600 : 470 : 20 : 113) for 40–50 min until the solvent reached the top of the plates. The plates were dried at room temperature and autoradiographed.

Western blot

Cl41 cells (3×10^4) were cultured in each well of 6-well plates to 70–80% confluence with normal culture medium. The cell culture medium was replaced with 0.1% FBS MEM with 2 mM L-glutamine and 25 μg of gentamicin and cultured for 33 h. The cells were incubated in serum-free MEM for 3–4 h at 37°C. After exposure to arsenite, the cells were washed once with ice-cold PBS and then extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with each of the antibodies against phosphospecific Akt (Thr308), phosphospecific Akt (Ser473), Akt, phosphospecific $p70^{S6k}$ (Thr389), phosphospecific $p70^{S6k}$ (Ser421/Ser424), $p70^{S6k}$, phosphospecific IKKα/β, and IκB. The protein bands specifically bound to the primary antibodies were detected using an anti-rabbit IgG alkaline phosphatase-linked secondary antibody and an ECF western blot system (Amersham, Piscataway, NJ) (28).

Cyclin D1 expression assay

Cl41 cells and their transfectants (3×10^4) were cultured in each well of 6-well plates to 90% confluence. After exposure to arsenite for different time points as indicated in the figure legends, the cells were washed once with ice-cold PBS and then extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with antibodies against cyclin D1 or GADPH. The protein bands specifically bound to the primary antibodies were detected as mentioned above.

Statistical analysis

The significance of the difference between treated and untreated groups were determined with the Student's *t*-test. The results are expressed as mean ± SEM.

Results

Arsenite exposure induces cell proliferation and cyclin D1 expression in mouse epidermal Cl41 cells

Arsenite was reported to be able to induce cell proliferation both in cell culture model and animal model (6–13). Here we

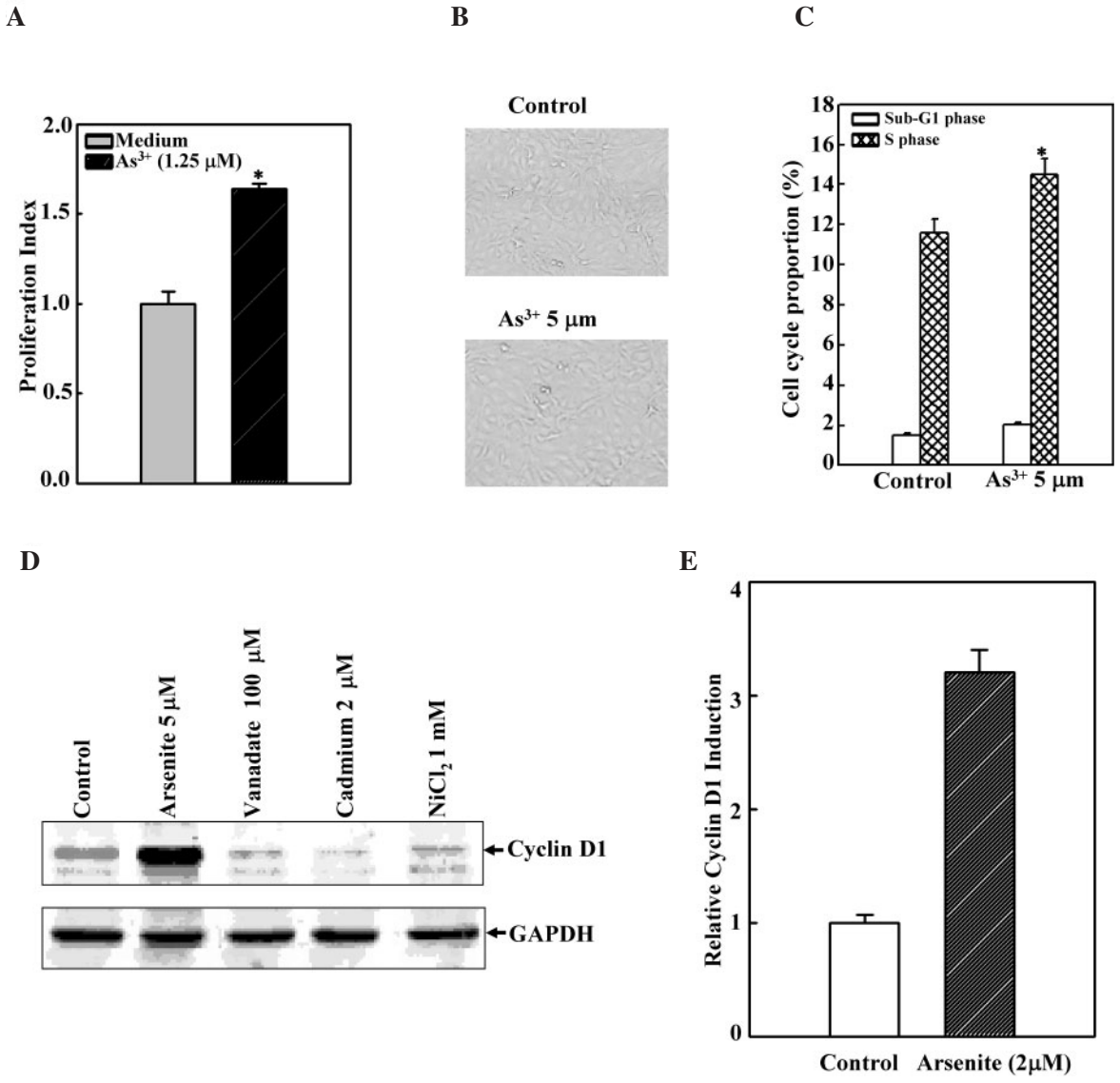
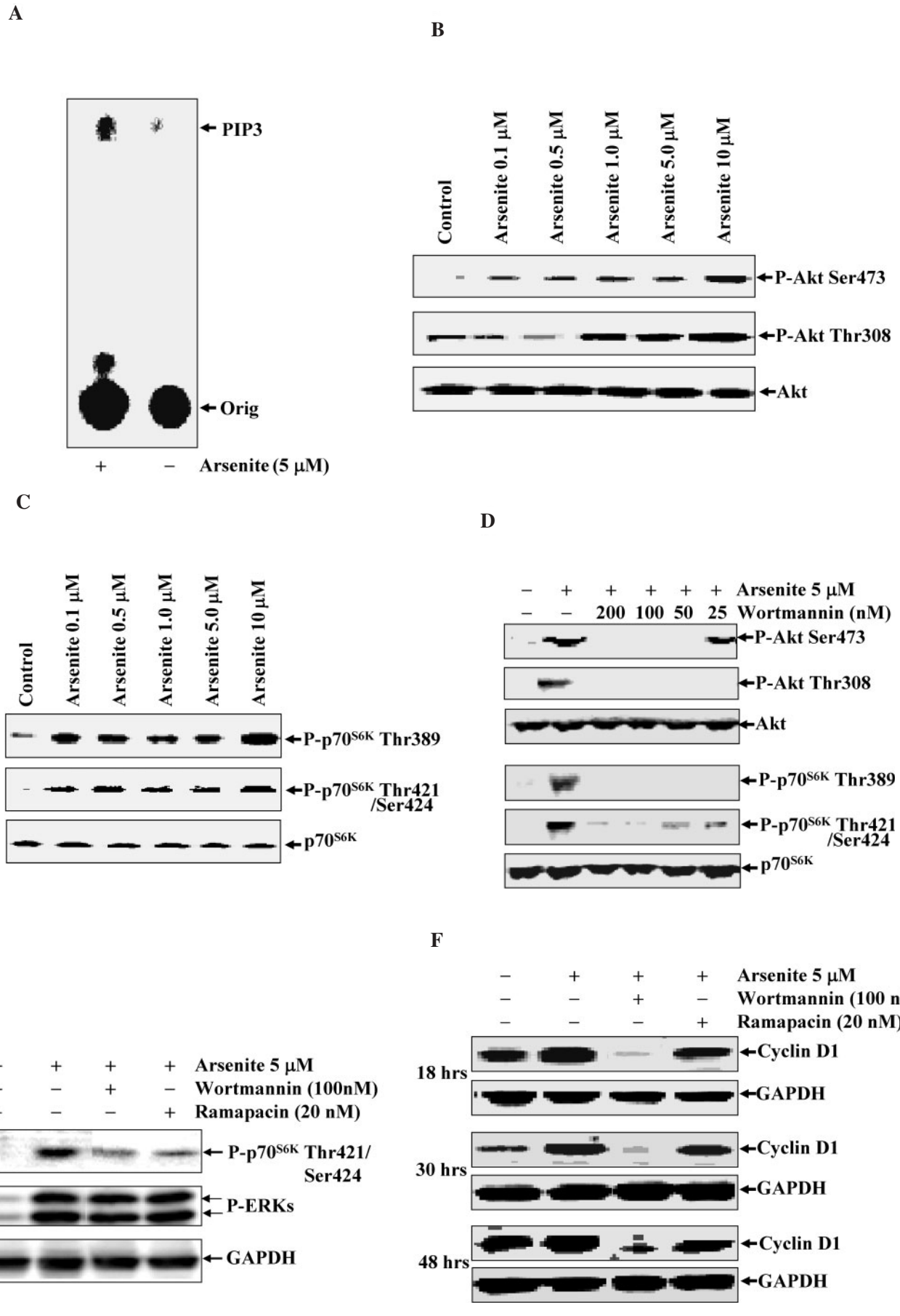


Fig. 1. Cell proliferation and cyclin D1 expression induced by arsenite in mouse epidermal Cl41 cells. (**A**) 1×10^3 of viable cells were seeded into each well of 96-well plates. After being cultured at 37°C in a humidified atmosphere of 5% CO_2 for 12 h, the cells were exposed to 1.25 μM of arsenite for 72 h. The proliferation of the cells was measured using CellTiter-Glo® Luminescent Cell Viability Assay kit. The results are expressed as luciferase activity relative to control medium (proliferation index). The symbol (∗) indicates a significant increase from medium control ($P < 0.01$). (**B** and **C**) 2×10^5 of Cl41 cyclin D1-Luc mass1 cells were seeded into each well of 6-well plates. After being cultured at 37°C overnight, the cells were treated for 24 h with 5 μM of arsenite. The photos for the cell morphology were taken under microscope (B); and the cell apoptosis (cell sub-G_1 phase) and S phase were analyzed by propidium iodide staining with Flow Cytometry (C). (**D**) 2×10^5 of Cl41 cyclin D1-Luc mass1 cells were seeded into each well of 6-well plates. After being cultured at 37°C overnight, the cells were treated for 24 h with 5 μM of arsenite, 100 μM of vanadate, 2 μM of cadmium and 1 mM of $NiCl_2$, respectively. The cells were then washed once with ice-cold PBS and extracted with SDS-sample buffer. Western blot was carried out as described in the Materials and methods section. GAPDH was used as a control for protein loading. (**E**) 8×10^3 of Cl41 cyclin D1-Luc mass1 cells were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were treated with various concentrations of arsenite as indicated for 12 h. The luciferase activity was then measured and the results are presented as cyclin D1 luciferase activity relative to control. Each bar indicates the mean ± SD of three replicate assay wells.

found that exposure of mouse epidermal Cl41 cells which are wildly used as a cell model for the study of tumor promotion, to arsenite also caused significant increase in cell proliferation (Figure 1A). The cell proliferation is strictly controlled by a set of proteins including cyclins. It is demonstrated that cyclin D1 plays a crucial role in controlling cell proliferation (29). Alteration in its expression is believed to associate with tumorigenesis (30). It has been reported that cyclin D1 can be induced by arsenite exposure in normal human fibroblasts (21). In this study, we examined whether cyclin D1 can also be induced by arsenite in Cl41 cells. The results showed that exposure of Cl41 cells to 5 μM of arsenite, a concentration showing no observed toxicity to the cells (Figure 1B and C), resulted in a marked cyclin D1 induction at protein level (Figure 1D) and significant elevation of cell proportion in S phase (Figure 1C). In contrast, other metals including vanadate, cadmium and nickel chloride showed no effect on cyclin D1 expression (Figure 1D). In cyclin D1-luciferase reporter assay, arsenite exposure also induced an increase in cyclin D1 transcription, with over 3-fold induction at 12 h after 2 μM arsenite exposure (Figure 1e). These results demonstrate that arsenite exposure is able to induce



cyclin D1 transcription and protein expression in mouse epidermal Cl41 cells.

PI-3K-AKT/p70S6K pathway is involved in cyclin D1 induction by arsenite exposure

Numerous studies have demonstrated that PI-3K signal pathway may play a crucial role in eukaryotic cells by activating a set of transcription factors, including CREB and NFκB, and in turn modulating the expression of genes involved in cell proliferation, survival and malignant transformation (31,32). Here we found that exposure of Cl41 cells to arsenite led to PI-3K activation (Figure 2A), and increase in phosphorylation of Akt (Figure 2B) and $p70^{S6K}$ in dose-dependent manner (Figure 2C). Pretreatment of cells with wortmannin, a chemical inhibitor of PI-3K, resulted in a dramatic inhibition of the phosphorylation of Akt and $p70^{S6K}$ (Figure 2D). Furthermore, inhibition of PI-3K by pre-treatment of Cl41 cells with wortmannin resulted in a significant reduction in arsenite-induced cyclin D1 expression, whereas inhibition of $p70^{S6K}$ pathway by pre-treatment of cells with rapamycin, a chemical inhibitor of mTOR-$p70^{S6K}$ pathway, did not show any inhibitory effect on cyclin D1 induction by arsenite (Figure 2E and F). These results demonstrate that activation of PI-3K/Akt pathway may be implicated in cyclin D1 induction by arsenite in Cl41 cells.

PI-3K/AKT mediates arsenite-induced cyclin D1 expression via IKKβ/NFκB in Cl41 cells

The downstream of PI-3K may include Akt (33,34), mTOR/$p70^{S6K}$ (35), IKK/NFκB (36), MEK/ERKs/AP-1 (37) and GSK3β/β-catanin (38) pathways. The above result with utilization of rapamycin indicated that mTOR/$p70^{S6K}$ did not contribute to PI-3K-mediated cyclin D1 induction by arsenite, even though its activation is mediated by PI-3K. The above results using wortmannin also suggested that arsenite-induced Akt activation may be mediated by PI-3K activation. To test whether Akt is the downstream target of PI-3K, we determined the effect of dominant negative PI-3K mutant (**Δ**p85) on Akt phosphorylation induced by arsenite. As shown in Figure 3A, **Δ**p85 transfection led to impairment of arsenite-induced Akt phosphorylation, suggesting that Akt activation is mediated by PI-3K. To further unravel the downstream kinase of PI-3K/Akt responsible for cyclin D1 induction by arsenite, we addressed IKKβ/NFκB pathway which has been reported to be transactivated by arsenite and is believed to associate with tumor promotion effect of arsenite (39). NFκB is expressed in an inactive form in most cells, composed of NFκB p50 and Rel A p65 subunits and bound to an inhibitory protein, IκBα (40). In response to stress, cytokine, or growth factor, IκBα is phosphorylated by IKK, then ubiquitinated and degraded by the proteasome, enabling nuclear localization and binding of p50/p65 to the promoter region of target genes (40). Cyclin D1 promoter region includes NFκB binding sites which have been indicated to regulate cyclin D1 expression in Swiss 3T3 cells (41). To test the hypothesis that IKK/NFκB is the downstream of PI-3K/Akt cascade, we established Cl41-DN-Akt stable transfectants, and analyzed IKK phosphorylation and IκBα degradation after exposure of the transfectants to arsenite. The results demonstrated that arsenite exposure was able to induce phosphorylation of IKK and degradation of IκBα in Cl41 cells, which were blocked by **Δ**p85 and DN-Akt transfections (Figure 3A), indicating that IKK/NFκB does act as the downstream transducer of arsenite-triggered PI-3K/Akt cascade. Moreover, arsenite-induced cyclin D1 expression was also blocked in Cl41-**Δ**p85 and Cl41-DN-Akt stable transfectants (Figure 3B). It may be notable that the transfection of **Δ**p85 blocked arsenite-induced cyclin D1 expression more effectively than the transfection of DN-Akt, which may be explained by that endogenous PI-3K kinase can be more effectively inhibited by its dominant negative mutant as compared with Akt kinase, or that there are some signal pathways other than Akt can mediate the signal cascade from PI-3K to cyclin D1 expression. To further confirm the involvement of IKK/NFκB in arsenite-induced cyclin D1 expression, we examined the effect of arsenite on NFκB transactivation in Cl41 cells by gene reporter assay. The result indicated that exposure of Cl41 cells to arsenite markedly induced NFκB activation which was dramatically inhibited by the co-transfection of IKKβ-KM, the dominant negative mutant of IKKβ (Figure 4A and B). Moreover, the overexpression of IKKβ-KM inhibited arsenite-induced cyclin D1 expression dramatically at both transcription and protein levels (Figure 4C–E). It may be noted that cyclin D1 induction decreased when the dosage of arsenite is higher than 4 μM in the luciferase reporter assay (Figure 4D), whereas 5 μM of arsenite seems to cause the maximal cyclin D1 induction in western blot assay (Figure 4E). This inconsistent induction between transcription and protein levels may be due to that high dosage of arsenite may also affect the degradation of cyclin D1 which facilitates cyclin D1 accumulation. Overall, our data strongly demonstrate that induction of cyclin D1 expression by arsenite in Cl41 cells requires PI-3K/Akt/IKKβ/NFκB pathway.

Discussion

Arsenite is a well-documented skin carcinogen (1,3). Numerous studies have demonstrated that exposure to arsenite can induce cell proliferation and cell transformation. It is believed that modulation on cell cycle machinery is associated with arsenite-induced carcinogenesis although the precise mechanisms remain to be fully elucidated. D type cyclins (particularly cyclin D1) play a crucial role in controlling the checkpoint of G_0/G_1 to S phase transition (16), and are implicated in cell proliferation and tumorigenesis (42). Cyclin D1 expression varies in abundance during the cell cycle, and its protein level is mainly controlled by the rate of cyclin D1 gene transcription, which is regulated by multiple transcription factors. The cyclin D1 promoter region contains multiple cis-elements, includes binding sites for NFκB (32,41,43–45), AP-1 (46,47),

Fig. 2. Activation of PI-3K signaling pathway and its role in cyclin D1 induction by arsenite. 2×10^5 of Cl41 cells were seeded into each well of 100 mm dishes (**A**) or 6-well plates (**B–F**) and cultured in 5% FBS MEM at 37°C. After the cell density reached 80–90% (**A**), the cells were exposed to 5 μM of arsenite for 20 min and then extracted. PI-3K activity was measured as described in the Materials and methods section; (B and C) the cells were exposed to various concentrations of arsenite as indicated for 1 h; (D) the cells were pretreated with various concentrations of wortmannin for 30 min and exposed to 5 μM of arsenite for 1 h; (E and F) the cells were pretreated with wortmannin (100 nM) or rapamycin (20 nM) for 30 min and then exposed to 5 μM of arsenite for 12 h (E) or different time period as indicated (F). The cells were washed once with ice-cold PBS and extracted with SDS-sample buffer. Western blot was carried out as described in the Materials and methods section. GAPDH was used as a control for protein loading.

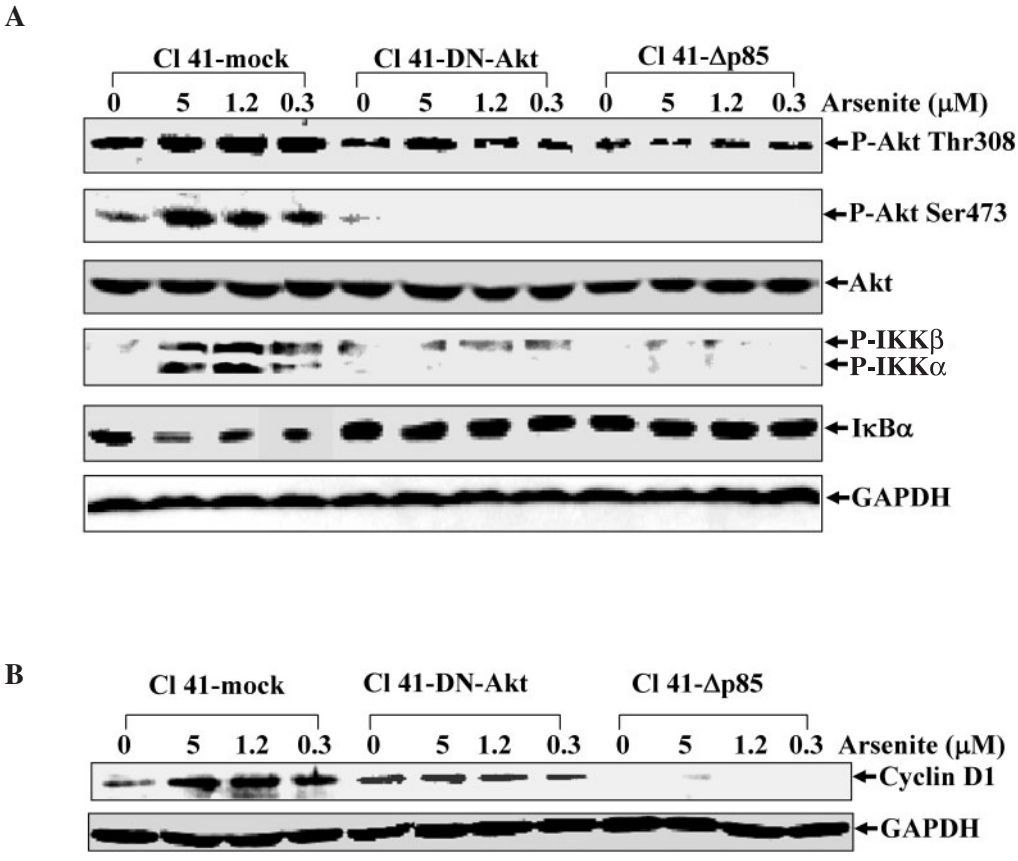


Fig. 3. The role of PI-3K/AKT in the activation of IKKβ/NFκB and cyclin D1 expression induced by arsenite. 2×10^5 of Cl41-mock vector, Cl41-Δp85 and Cl41-DN-Akt cells were seeded into each well of 6-well plates and cultured in 5% FBS MEM at 37°C. After the cell density reached 80–90%, the cells were exposed to various concentrations of arsenite as indicated for 1 h (**A**) or 24 h (**B**). The cells were then extracted with SDS-sample buffer. Western blot was carried out using specific antibodies as described in the Materials and methods section.

STAT (48), E2F/DP (49), ATF/CREB (47,50–52) and sp-1/sp-3 (52,53). These cis-elements are potentially important for transcriptional activation of the cyclin D1 gene. In fact, it was reported that NFκB binding sites in the cyclin promoter were implicated in transcriptional activation of the gene (32,41,43–45). Angiotensin II induced activation of the cyclin D1 promoter through the AP-1 binding site (46,47). It was also reported that STAT-binding sites were implicated in cytokine-induced transcriptional activation of the cyclin D1 gene in hematopoietic cells (48). E2F-1 mediated a Neu-signaling cascade to cyclin D1 induction (49). The ATF/CREB-binding site was also demonstrated to mediate the expression of the cyclin D1 gene (47,50–52). $pp60^{v\text{-}src}$ induced transcriptional activation of the cyclin D1 gene mediated by the ATF/CREB site (47). Moreover, it was found that estrogen-induced activation of the cyclin D1 gene depended on the ATF/CREB site in which ATF-2 and c-Jun formed heterodimers (51). Whereas in vascular endothelial cells, activation of the cyclin D1 promoter was largely mediated by the ATF/CREB and SP1 sites (52). These results suggest that the transcriptional activation of the cyclin D1 gene may occur in a cell type- and mitogen-specific manner.

In the present study, we demonstrated that exposure of mouse epidermal Cl41 cells to arsenite led to activation of PI-3K/Akt which thereby induce cyclin D1 expression via its downstream IKK/NFκB signal pathway. Inhibitions of either PI-3K/Akt or IKKβ dramatically impaired cyclin D1 induction by arsenite. In our previous study, arsenite was shown to be able to induce cyclin D1 expression and thereby cause the progression of G_1/S transition in human keratinocytes (27), which also requires PI-3K/Akt activation demonstrated in our recent studies (Ouyang *et al*. unpublished data). Although most previous studies on NFκB activation through Akt/IKK signaling have focused their attention on the IKKα, and it has been shown that Akt directly phosphorylates the IKKα under stimulation by TNF, EGF, insulin-like growth factor-I and platelet-derived growth factor, there are some studies that most recently demonstrated that IKKβ is also a substrate of Akt in HTLV-1-transformed cells (54), and in breast cells when treated by FGF-2 (55). The results in this study suggest that IKKβ may also be the substrate of Akt in arsenite-exposed Cl41 cells. In addition, the effect of arsenite on NFκB activation has been indicated to be time-, dose- and cell type-dependent (56). Whereas high concentrations of arsenic (>10 μM) generally inhibit NFκB which was recently demonstrated to be caused by reaction with the critical cysteine in the activation loop of IKKs (56), low and non-cytotoxic concentrations of arsenic (1–10 μM) usually activate this transcription factor. In the current and our previous studies, arsenite was demonstrated to induce NFκB activation in mouse epidermal JB6 cell model and human keratinocytes (27).

However, it is interesting that the contributions of IKKs/NF-κB pathway to carcinogen-induced skin cancer remain controversial. IKKα has been demonstrated to be an inhibitory factor for the proliferation of skin epidermis (57–59); and overexpression of active p50 and p65 NFκB subunits in

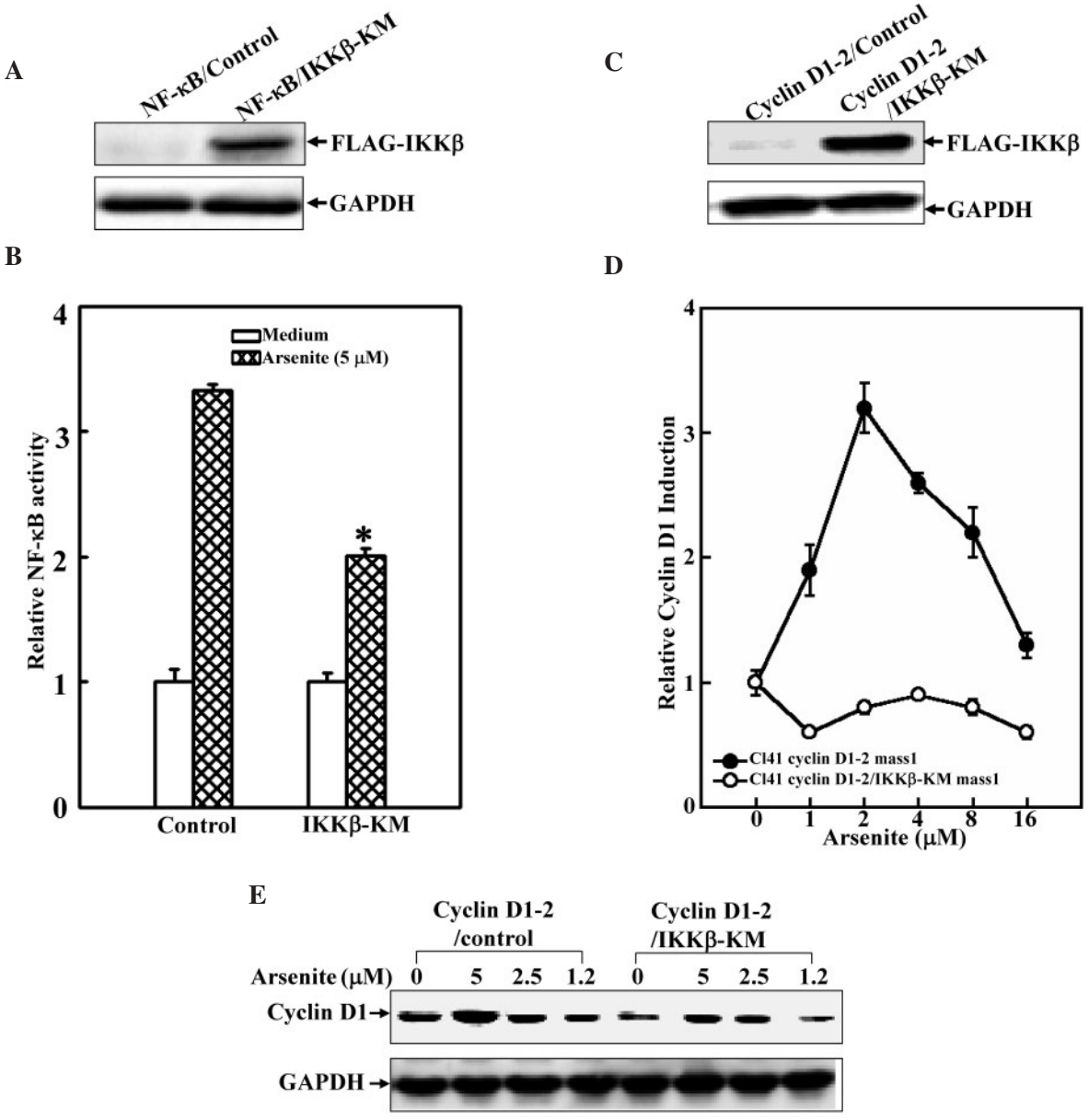


Fig. 4. Involvement of IKKβ/NFκB in arsenite-induced cyclin D1 expression. (**A**) Cl41 NFκB/vector control mass1 cells and stable co-transfectant of Cl41 NFκB/IKKβ-KM mass1 cells were extracted with SDS-sample buffer. The cell extracts were analyzed by western blot with specific anti-FLAG antibody (M2) or GAPDH antibody. (**B**) 8×10^3 of Cl41 NFκB/control mass1 cells and stable co-transfectant of Cl41 NFκB/IKKβ-KM mass1 cells were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were treated with 5 μM of arsenite for 12 h. The luciferase activity was then measured and the results are presented as NFκB luciferase activity relative to control. Each bar indicates the mean and SD of three replicate assay wells. The symbol (∗) indicates a significant difference between IKKβ-KM- and control vector-transfected cells ($P < 0.01$). (**C**) Cl41 cyclin D1-2/control mass1 cells and stable co-transfectant of Cl41 cyclin D1-2/IKKβ-KM mass1 cells were extracted with SDS-sample buffer. The cell extracts were analyzed by western blot with specific anti-FLAG antibody (M2). (**D**) 8×10^3 of Cl41 cyclin D1-2/control mass1 cells and stable co-transfectant of Cl41 cyclin D1-2/IKKβ-KM mass1 were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were treated with various concentrations of arsenite as indicated for 12 h. The luciferase activity was then measured and the results are presented as cyclin D1 luciferase activity relative to control. Each bar indicates the mean and SD of three replicate assay wells. (**E**) 2×10^5 of Cl41 cyclin D1-2/control mass1 and Cl41 cyclin D1–2/IKKβ-KM mass1 cells were seeded into each well of 6-well plates and cultured in 5% FBS MEM at 37°C. After the cell density reached 80–90%, the cells were exposed to various concentrations of arsenite as indicated for 24 h. The cells were then extracted with SDS-sample buffer. Western blot was carried out as described in the Materials and methods section. GAPDH was used as protein loading control.

transgenic epithelium produced hypoplasia and growth inhibition (60). But it has been reported that the deletion of IKKβ did not affect the proliferation of skin epidermis (61); IκBα deficiency results in a sustained NFκB response and severe widespread dermatitis characterized by epidermal hyperplasia in mice (62); and NFκB is a key mediator of integrin β4-induced epidermal growth (63). Although it is claimed that some carcinogens may exert their carcinogenic effect by inhibiting IKKα activation, and thereby promote the proliferation of skin epidermis, while a growing body of evidence indicates NFκB might be involved in keratinocyte transformation and skin carcinogenesis (64,65). It has been demonstrated that epidermal inflammation and hyperplasia play a critical role in skin tumor promotion and NF-κB is one of the well-known mediators of these effects (64). Substances like phorbol ester and okadaic acid, which are promoters of

skin cancer, are also strong inducers of an NF-κB response in keratinocytes (64). Furthermore, a Ha-ras oncogene point mutation is found in over 90% of mouse skin tumor, in which NF-κB activation has been observed (64). In the Ha-ras point mutation mouse model, it has also been shown that while in normal epidermis NF-κB was expressed in the cytoplasm of basal cells, p50 and p52 are elevated in squamous cell carcinoma as well as in skin papillomas (65).

In addition to IKK/NFκB (36), mTOR (35), MEK/ERKs/AP-1 (37) and GSK3β/β-catanin (38) pathways are also the downstreams of PI-3K/Akt cascade. In this study, inhibition of mTOR by rapamycin did not show any inhibitory effect on the cyclin D1 induction by arsenite, suggesting that mTOR may not be the downstream transducer of PI-3K/Akt cascade in arsenite-induced cyclin D1 expression. GSK3β has been reported to play a very important role in the negative-regulation of cyclin D1 level at both transcription and protein levels (38,50,66). It has been demonstrated that GSK3β can phospholate cyclin D1 and thereby facilitate its degradation (38,66). In addition, GSK3β is able to phosphorylate β-catanin and retain it in cytoplasm with APC (38). Phosphorylation of GSK3β by Akt leads to its inactivation, and in turn dephosphorylation of β-catanin, which enables its translocation into nuclear and activation of its target genes expression including cyclin D1 (38,50,67). Actually, we have observed that β-catanin can be activated after arsenite exposure. Its role in cyclin D1 induction by arsenite is currently under investigation. In addition, we found that exposure of Cl41 cells to arsenite can also induce the activation of other transcription factors, such as AP-1 (68). It remains to be explored if these transcription factors are also involved in cyclin D1 induction by arsenite.

It has been well-accepted that alterations in the cell cycle machinery at checkpoints are associated with cell proliferation, tumor development (29,30,69). The re-entry of cell cycle of the quiescent cells from G_0 to G_1 phase and the progression of G_1/S transition in actively proliferating cells are regulated by a set of cyclins (particularly cyclin D) via regulating the activity of the cyclin-dependent kinases (CDK4 and CDK6) (16,42,70,71). Aberrant cyclin D1 expression has been observed early in carcinogenesis (72–74), and overexpression of cyclin D1 was reported in several human cancers, including uterine cervix (75), ovary (76), breast (77), urinary bladder (78) and endometrium cancers (79). Moreover, antisense to cyclin D1 was reported to inhibit the growth and tumorigenicity of human colon and human lung cancer cells (17,18). Here, we demonstrated that exposure of mouse epidermal Cl41 cells to arsenite was able to induce cyclin D1 expression through PI-3K/Akt/IKK/NFκB pathway, which may play a key role in arsenite-induced cell proliferation. In fact, we found that cyclin D1 induction is critical for arsenite-induced cell cycle progression from G_0/G_1 phase to S phase in human keratinocyte (27). The exact role of the increased cyclin D1 in arsenite-induced proliferation and cell transformation is currently also under investigation in our laboratory.

In summary, our studies demonstrated that exposure of mouse epidermal Cl41 cells to arsenite leads to activation of PI-3K/Akt/IKK/NFκB signal pathway which thereby induce cyclin D1 expression. Because of the important role of cyclin D1 in controlling cell cycle progression, its induction may associate arsenite-induced cell proliferation and transformation. Thus, our present studies provide novel information for understanding the molecular mechanisms involved in cyclin D1 expression and carcinogenic effect by arsenite in its major target tissue of skin.

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