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MicroRNA-200b Suppresses Arsenic-transformed Cell Migration by Targeting Protein Kinase Cα and Wnt5b-Protein Kinase Cα Positive Feedback Loop and Inhibiting Rac1 Activation*

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Background: MiR-200b is able to inhibit tumor cell migration and metastasis, the underlying mechanism is not well understood.
Results: PKCα is a new direct target of miR-200b.
Conclusion: MiR-200b suppresses arsenic-transformed cell migration by targeting PKCα and Wnt5b-PKCα positive feedback loop and inhibiting Rac1 activation.
Significance: This study identifies a new target and mechanism for the inhibitory effect of miR-200b on tumor cell migration.

MicroRNA-200b (miR-200b) is a member of miR-200 family that has been found to inhibit cell migration and cancer metastasis; however, the underlying mechanism is not well understood. We previously reported that miR-200 expression is depleted in arsenic-transformed human bronchial epithelial cells with highly migratory and invasive characteristics, whereas stably re-expressing miR-200b strongly suppresses arsenic-transformed cell migration. This study was performed to investigate how miR-200b inhibits arsenic-transformed cell migration. We found that protein kinase Cα (PKCα) is significantly up-regulated in arsenic-transformed cells. Combining bioinformatics analysis with PKCα 3′-untranslated region vector luciferase reporter assays, we showed that PKCα is a direct target of miR-200b. Inhibiting PKCα activity or knocking down PKCα expression drastically reduced cell migration, phenocoping the inhibitory effect of overexpressing miR-200b. In contrast, forced expression of PKCα in miR-200b overexpressing cells impaired the inhibitory effect of miR-200b on cell migration. In addition, we also found a positive feedback loop between Wnt5b and PKCα in arsenic-transformed cells. Knocking down Wnt5b expression reduced phospho-PKC levels and cell migration; and knocking down PKCα expression decreased Wnt5b level and cell migration. Moreover, forced expression of PKCα increased Wnt5b and phospho-PKC levels and cell migration. Further mechanistic studies revealed that Rac1 is highly activated in arsenic-transformed cells and stably expressing miR-200b abolishes Rac1 activation changing actin cytoskeleton organization. Manipulating PKCα or Wnt5b expression levels significantly altered the level of active Rac1. Together, these findings indicate that miR-200b suppresses arsenic-transformed cell migration by targeting PKCα and Wnt5b-PKCα positive feedback loop and subsequently inhibiting Rac1 activation.

MicroRNAs (miRNAs)[2] are a large family of small non-coding RNAs (~22 nucleotide long) that negatively regulate protein-coding gene expression post-transcriptionally by interacting with 3′-untranslated region of messenger RNAs (mRNAs), causing mRNA degradation or translation inhibition (1, 2). The miRNA-200 (miR-200) family consists of 5 members divided into two groups locating in two different genomic regions: Group I contains miR-200b, −200a and −429 (miR-200b/200a/429) located on chromosome 1; and Group II consists of miR-200c and −141 (miR-200c/141) located on chromosome 12. Alternatively, miR-200 family can be categorized into two functional clusters based on the identities of their seed sequences: Cluster I of miR-200b/200c/429 and Cluster II of miR-200a/141. The miR-200 family members are among the first miRNAs reported to function as potent inhibitors of epithelial to mesenchymal transition (EMT) (3–6). EMT, an embryonic developmental program, is now believed to play crucial roles in cancer metastasis (7, 8). Reduced expression levels of miR-200 family have been observed in various types of cancer and are associated with increased cell motility and cancer metastasis (9–11). In contrast, ectopic expression of miR-200 family has been shown to inhibit cell migration and reduce cancer metastasis (12–14). However, the underlying mechanism has not been well understood and only a limited number of miR-200 family target genes that promote cancer cell migration and metastasis have currently been identified (9–11).

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[2] The abbreviations used are: miRNA, microRNA; 3′-UTR, 3′-untranslated region; EMT, epithelial-to-mesenchymal transition; p53[low]HBECs, human bronchial epithelial cells (HBECs) with p53 expression stably knocked down; PKCα, protein kinase Cα; miR-200b, microRNA 200b; siRNA, small interfering RNA; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

Cell migration is a multistep process requiring dynamic actin cytoskeleton reorganization, which is primarily regulated by the small Rho GTPases that belong to Ras GTPase super family (15, 16). Rac1 is one of the best characterized Rho GTPase family members, acting as a molecular switch cycling between an active GTP-bound state and an inactive GDP-bound state. The active form of Rac1 (Rac1-GTP) interacts with a variety of its effectors and increases cell migration mainly by promoting actin cytoskeleton reorganization resulting in membrane ruffling or lamellipodia formation, which produces physical forces, pushes the membrane forward and supports cell motility (17–19). While ectopic expression of miR-200 family has been shown to inhibit cell migration, whether miR-200 family has an effect on Rac1 activity is unknown.

Arsenic is a common environmental pollutant and long term exposure to arsenic is associated with increased risk of multiple types of cancer including lung cancer (20). Despite extensive studies, the mechanism of arsenic carcinogenicity has not been elucidated. Ours and other cell transformation studies showed that arsenic-induced cell malignant transformation is accompanied by drastic cellular morphological changes from epithelioid to fibroblast-like resembling EMT (21–24). Further characterization of arsenic-induced cell transformation revealed that arsenic-transformed cells are highly migratory and invasive, and inoculation of arsenic-transformed cells into nude mice produces invasive and metastatic xenograft tumors (24–26). However, the molecular mechanism by which arsenic-transformed cells exhibited enhanced migratory capability is not clear.

We recently reported miR-200 expression is depleted in arsenic-transformed human bronchial epithelial cells with highly migratory and invasive characteristics, whereas stably re-expressing miR-200b strongly suppresses arsenic-transformed cell migration (21, 26). In this study we found that PKCα is highly up-regulated in arsenic-transformed cells and identified PKCα as a direct target of miR-200b. We also found a positive feedback loop between Wnt5b and PKCα. Using siRNA knocking down gene expression and stable overexpression approaches we showed that miR-200b suppresses arsenic-transformed cell migration by targeting PKCα and Wnt5b-PKCα positive feedback loop and subsequently inhibiting Rac1 activation.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—Generation of human bronchial epithelial cells (HBECs) with p53 expression stably knocked down (defined as p53^{low}HBECs) and arsenic-transformed HBECs (defined as As-p53^{low}HBECs) were described in our recent studies (21, 26, 27). The green fluorescence protein (GFP) vector control and miRNA-200b (miR-200b) stably expressing As-p53^{low}HBECs were also generated in our previous study and named as As-p53^{low}HBEC-GFP and As-p53^{low}HBEC-GFP-200b, respectively (21). In current study, control p53^{low}HBECs, As-p53^{low}HBECs, As-p53^{low}HBEC-GFP, and As-p53^{low}HBEC-GFP-200b cells were cultured in chemically defined serum-free medium (K-SFM) (Invitrogen, Carlsbad, CA) in the absence of any arsenic further treatment as previously described (21, 26, 27). All cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere.

Generation of PKCα 3′-Untranslated Region (3′-UTR) Luciferase Reporter Wild Type and Mutant Type Vectors and Dual Luciferase Reporter Assays—Bioinformatics analysis with miRNA target predicting software TargetScan and DIANA-MICROT revealed that human PKCα 3′-UTR has a putative conserved binding site for miR-200b at nucleotide position 1319–1325. A fragment of human PKCα 3′-UTR containing nucleotide 1–1825 was synthesized by Blue Heron Biotech (Bothell, WA) and cloned into Origene cloning vector pMirTarget (OriGene Technologies), which served as the wild type PKCα 3′-UTR luciferase vector containing the miR-200b putative binding site. To generate the mutant type PKCα 3′-UTR luciferase vector, the same fragment human PKCα 3′-UTR containing nucleotide 1–1825 was synthesized except that the miR-200b putative binding site at nucleotide position 1319–1325 was completely mutated. The mutated PKCα 3′-UTR fragment was similarly cloned into pMirTarget, which served as the mutant type PKCα 3′-UTR luciferase vector. To perform dual luciferase reporter assays, cells were co-transfected with a wild type or mutant type PKCα 3′-UTR luciferase vector and a pRL-TK *Renilla* luciferase vector. 48 h after transfection the luciferase activities were measured using Promega Dual Luciferase Reporter Assay (Promega, Madison, WI). The relative luciferase reporter activity was calculated as the wild type or mutant type PKCα 3′-UTR firefly luciferase activity divided by the *Renilla* luciferase activity.

Ectopic Expression of PKCα in miR-200b Stably Expressing Cells—Human PKCα full-length cDNA was obtained from OriGene Technologies (Rockville, MD) and cloned into pLenti6.3/V5-DEST™ vector using Gateway® cloning technology (Invitrogen) following the manufacturer's instructions. Vector control (pLenti6.3) and PKCα expressing (pLenti6.3-PKCα) lentiviral particles were packaged using 293T cells following previously described protocols (21, 28). To establish the vector control and PKCα stably expressing cell lines, As-p53^{low}HBEC-GFP-200b cells were transduced with vector control (pLenti6.3) or PKCα-expressing (pLenti6.3-PKCα) lentiviral particles. 48 h after lentiviral particle transduction, cells were selected with Blasticidin. Ectopic expression of PKCα in As-p53^{low}HBEC-GFP-200b cells was confirmed by Western blot. Vector control and PKCα stably expressing cells were named as As-p53^{low}HBEC-GFP-200b-pLenti6.3 and As-p53^{low}HBEC-GFP-200b-pLenti6.3-PKCα, respectively. Both kinds of cells were cultured in chemically defined serum-free medium (K-SFM) in the absence of arsenic as described above.

Quantitative PCR (Q-PCR) Analysis—Cellular total RNAs were extracted using Qiagen miRNeasy mini kit and used for Q-PCR analysis following manufacturers' instructions. Q-PCR analysis was carried out in ABI 7500 Fast Real Time PCR System using TaqMan gene expression assays for PKCα, Wnt5b, and miR-200b (Applied Biosystems, Inc., Foster City, CA). β-Actin or U6 snRNA was analyzed by TaqMan PCR assays and used as internal controls for normalizing relative PKCα, Wnt5b, and miR-200b expression levels, respectively, as previously described (21).

PKCα, Wnt5b, and Rac1 RNA Interference—Negative Control small interfering RNA (siRNA) and ON-TARGETplus SMARTpool siRNA for PKCα, Wnt5b, or Rac1 were obtained

from Thermo Scientific Dharmacon (Lafayette, CO). The second siRNA for PKCα with different targeting sequence (PKCα siRNA-2) was obtained from Invitrogen (Grand Island, NY) SiRNA duplexes (100 nM) were transfected into cells using Lipofectamine 2000 (Invitrogen) as described previously (21). 72 h after transfection cells were collected for Western blot analysis, Transwell cell migration assays, Rac1-GTP pull down assays or Rhodamine Phalloidin stainings as described below. Rescue experiments for Wnt5b siRNA were performed with recombinant human Wnt5b protein (Genemed, South San Francisco, CA).

Western Blot Analysis—Cells were lysed using Tris-sodium dodecyl sulfate (SDS) and subjected to SDS-polyacrylamide gel electrophoresis as described previously (21). The following primary antibodies were used: anti-Wnt5b, anti-PKCα, anti-phospho PKC (pan) (βII Ser660), anti-phospho-PKC (pan) (γ Thr514), anti-phospho-PKC (pan) (ζ Thr410) (Cell Signaling Technology, Inc. Danvers, MA); anti-PKCβI, anti-PKCβII, anti-ZEB1 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Rac1 (EMD Millipore, Billerica, MA); and anti-β-actin (Sigma). PKC isozyme sampling antibody kit was from BD Biosciences (San Jose, CA). The HRP conjugated secondary anti-mouse and anti-Rabbit IgGs were from Bio-Rad.

Transwell Cell Migration Assay—Cell migration was measured and quantified by Transwell cell migration assays using uncoated (8-μm pore size, Corning Costar, Cambridge, MA) filters in 24-well plates as previously described (26). Briefly, cells were trypsinized and seeded onto the upper chamber of the Transwells (5×10^4 cells/well) in supplements-free K-SFM. The lower chamber of the Transwells was filled with the K-SFM containing 100 ng/ml of EGF (R&D Systems). The chambers were incubated at 37 °C with 5% CO_2 for 6 h. At the end of incubation, cells on the upper surface of the filter were removed using a cotton swab. Cells migrating through the filter to the lower surface were fixed with 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet for 5 min. Migrated cells were viewed and photographed under a phase-contrast microscope and counted in five randomly chosen fields (magnification: ×100).

Rac1-GTP Pulldown Assay—Rac1-GTP pulldown assays were performed to determine the active level of Rac1 as previously described with modifications (29). Briefly, cells were cultured in chemically defined serum-free medium (K-SFM) in the absence of arsenic and 48 h later cells at 70–80% confluence were used for pulldown assays. Cells were washed once with ice cold phosphate-buffered saline (PBS), lysed, and scraped in a buffer containing 20 mMTris/HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 150 mM NaCl, 0.5% (v/v) Nonidet P40, 5 mM β-glycerophosphate, and the protease inhibitor mixture (Sigma). Cell lysates were centrifuged at 14,000 × *g* for 10 min at 4 °C. A small portion of the resulted supernatant was collected for Western blot analysis of total Rac1 levels. The rest of the supernatant was transferred to an Eppendorf tube and incubated with Rac/Cdc42 binding domain (PBD) of the human p21 activated kinase 1 protein (PAK) bound glutathione-agarose beads (Cytoskeleton Inc., Denver, CO) for 1 h at 4 °C. After extensive washing, the beads were boiled in sample buffer. The samples were then resolved on a SDS/polyacrylamide gel, transferred onto PVDF membranes and analyzed by Western blot using an anti-Rac1 antibody.

Rhodamine Phalloidin Staining—Cellular actin cytoskeleton organization was revealed by Rhodamine Phalloidin staining as previously described (30). Briefly, cells cultured on cover slides in chemically defined serum-free medium (K-SFM) for 48 h were washed twice with PBS, fixed in 4% paraformaldehyde in PBS for 10 min, and then permeabilized using 0.1% Triton X-100 in PBS for 3 min. Cells were stained with Rhodamine Phalloidin (Molecule Probes) in PBS containing 1% bovine serum albumin (BSA) for 20 min at room temperature and then counterstained with 4′,6′-diamidino-2-phenylindole (DAPI, 1 μg/ml) for 10 min at 4 °C. Cells were visualized and photographed with a Nikon Eclipse TE2000-U fluorescence microscope (Nikon, Inc., Melville, NY). The captured red fluorescent images (Rhodamine Phalloidin staining) were overlaid with the blue fluorescent images (nuclear DAPI staining) using MetaMorph software (Molecular Devices Corp., Downingtown, PA).

MTT Assay—The tetrazolium dye colorimetric test (MTT assay) was used to monitor cell growth indirectly as indicated by the conversion of the tetrazolium salt to the colored product, formazan, and its concentration can be quantified spectrophotometrically. Briefly, cells were cultured in 96-well plates (5×10^4 cells/well in 100 μl of chemically defined serum-free medium) for 24, 48, or 72 h, respectively. At the end of culture, 50 μl of the MTT reagent (5 mg/ml) was added to each well and incubated for 4 h. Then, 200 μl of dimethyl sulfoxide (DMSO) was added to each well and incubated for another hour. The plate was read using a microplate reader (SpectraMAX Plus, Molecular Devices, Sunnyvale, CA) at the wavelength of 570 nm.

Statistical Analysis—All experiments were repeated at least three times. The statistical analyses for the significance of differences in numerical data (means ± standard deviations) were carried out by testing different treatment effects by analysis of variance (ANOVA) using a general linear model (Statistical Analysis System (SAS) version 9.1, SAS Institute, Inc. Cary, NC). Differences between treatment groups were determined using two-tailed *t*-tests. A p value of <0.05 was considered statistically significant.

RESULTS

Stably Expressing miR-200b Reduces Wnt5b, Phospho-PKC (pan), and Total PKCα Levels in Arsenic-transformed Cells—Our recent studies showed that the mRNA level of a non-canonical Wnt ligand Wnt5b was significantly increased in arsenic-transformed highly migratory cells (As-p53^{low}HBECs) and stably expressing miR-200b reduced Wnt5b mRNA level to that of control cells (p53^{low}HBECs) and inhibited cell migration (26, 27). Since non-canonical Wnt signaling was shown to play a crucial role in cell migration (31, 32), we wanted to determine whether up-regulation of Wnt5b contributes significantly to arsenic-transformed cell migration; and whether down-regulation of Wnt5b by miR-200b plays an important role in its inhibitory effect on cell migration. All cells used in this study were cultured in the absence of any further arsenic treatment.

We first found that Wnt5b protein level is also remarkably increased in As-p53^{low}HBECs and stably expressing miR-200b

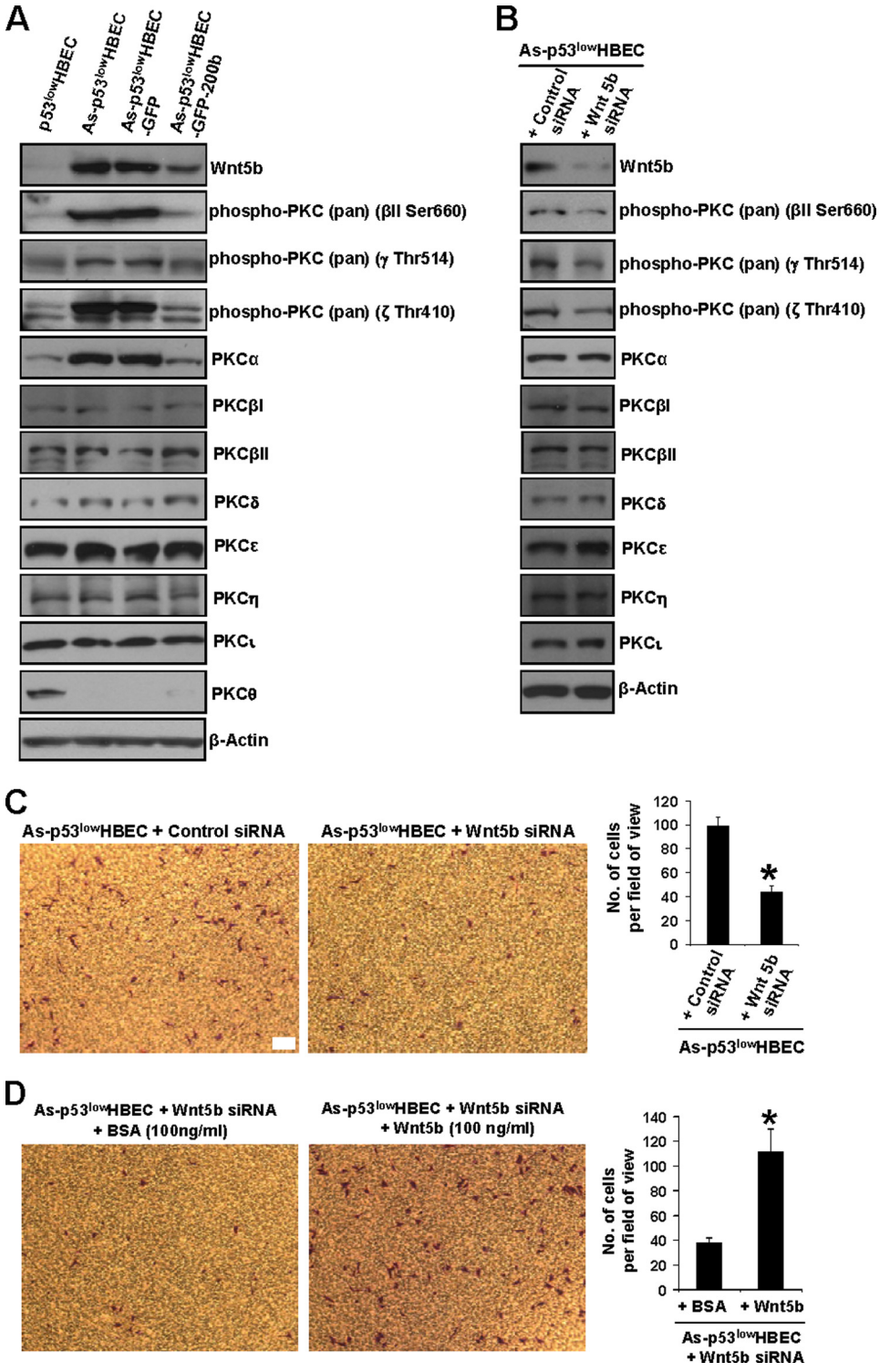

FIGURE 1. **Stably expressing miR-200b reduces Wnt5b, phospho-PKC (pan), and total PKCα levels, and siRNA knocking down Wnt5b decreases arsenic-transformed cell migration.** *A*, Western blot analysis of Wnt5b, phospho-PKC (pan), and individual PKC isozyme total levels in control, arsenic-transformed and miR-200b stably expressing cells. *B*, Western blot analysis of Wnt5b, phospho-PKC (pan), and individual PKC isozyme total levels in As-p53^{low}HBEC cells transfected with control siRNA or Wnt5b siRNA. *C*, representative images and quantification of Transwell cell migration assay. *D*, representative images and quantification of Transwell cell migration assay for cells transfected with Wnt5b siRNA and treated with bovine serum albumin (*BSA*) or recombinant human Wnt5b protein (100 ng/ml). Scale bar, 100 μm. The quantification of cell migration is presented as number of cells per field of view (means ± S.D., $n = 3$). * $p < 0.05$, compared with Control siRNA or BSA treatment group.

greatly reduces Wnt5b protein level (Fig. 1*A*), implying an important role of Wnt5b in arsenic-transformed cell migration. As non-canonical Wnt signaling can regulate cell migration through activating protein kinase C (PKC) pathway (31, 32), we next determined PKC activation status and expression levels in control and arsenic-transformed cells.

PKC activation status was analyzed by detecting overall phospho-PKC (pan) levels using three different anti-phospho-

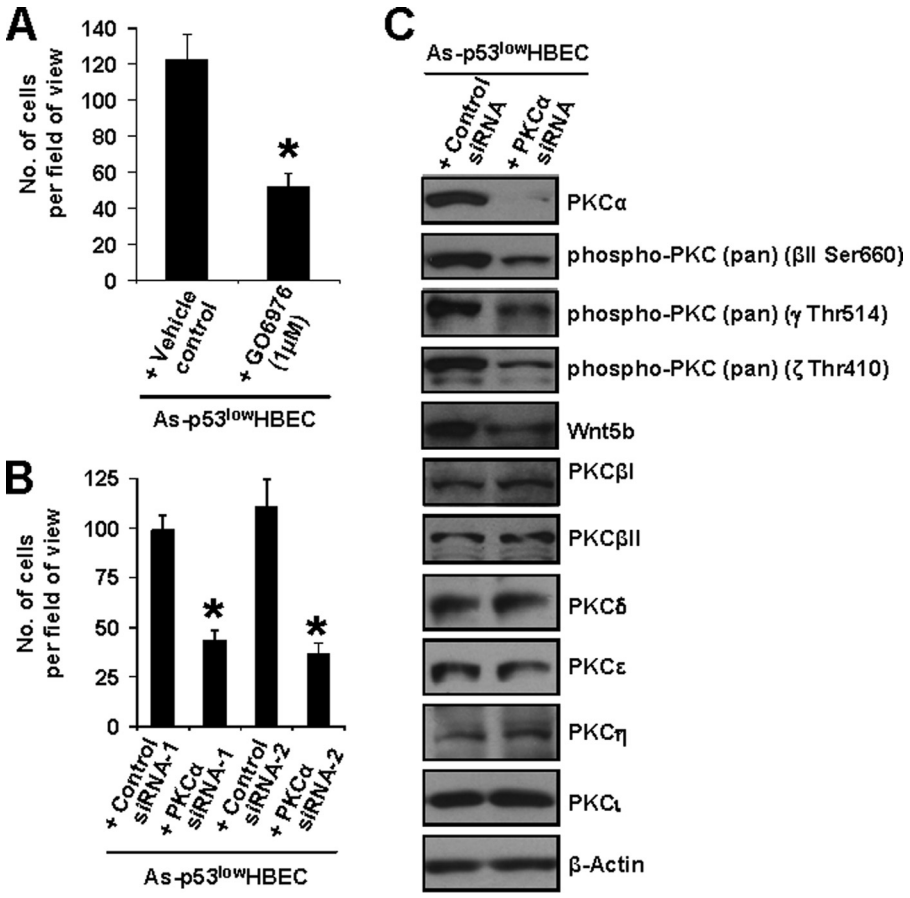

FIGURE 2. **Inhibiting PKCα activity or knocking down PKCα expression reduces arsenic-transformed cell migration.** *A and B*, quantification of Transwell migration of cells treated with vehicle control or GO6976 (*A*) or transfected with control siRNA or PKCα siRNA (*B*). The quantification of cell migration is presented as number of cells per field of view (means ± S.D., $n = 3$). *, $p < 0.05$, compared with vehicle control or control siRNA group. *C*, Western blot analysis of phospho-PKC (pan) and individual PKC isozyme total levels in As-p53^{low}HBEC cells transfected with control siRNA or PKCα siRNA.

PKC (pan) antibodies: (i) anti-phospho-PKC (pan) (βII Ser660): an antibody detects endogenous levels of PKCα, βI, βII, δ, ϵ, η, and θ isozymes only when phosphorylated at a C-terminal residue homologous to serine 660 of PKCβII; (ii) anti-phospho-PKC (pan) (γ Thr-514): an antibody detects endogenous levels of PKCα, βI, βII, γ, δ, ϵ, η, θ isozymes only when phosphorylated at a residue homologous to threonine 514 of PKCγ; and (iii) anti-phospho-PKC (pan) (ζ Thr410): an antibody detects endogenous levels of PKCα, βI, βII, γ, δ, ϵ, η, θ, and ι isozymes only when phosphorylated at a residue homologous to threonine 410 of PKCζ. Studies have shown that phosphorylations of PKCs are critical events in PKC activation and phosphorylated PKCs have higher specific activities than unphosphorylated PKCs (33, 34). As shown in Fig. 1*A*, the levels of phospho-PKCs detected by three phospho-PKC (pan) antibodies are significantly increased in As-p53^{low}HBECs and stable expression of miR-200b drastically reduces the phospho-PKC (pan) levels, which are well correlated with the protein levels of Wnt5b among these cells.

Up-regulation of phospho-PKC levels may also be due to increased PKC expression, we next compared the total protein levels of all PKC isozymes between control (p53^{low}HBECs) and arsenic-transformed cells (As-p53^{low}HBECs). Results showed that 8 PKC isozymes including PKCα, βI, βII, δ, ϵ, η, ι, and θ are expressed in p53^{low}HBECs (Fig. 1*A*), whereas PKCγ and PKCζ are not detectable by Western blot (data not shown). Interestingly, while the protein levels of PKCβI, βII, δ, ϵ, η, and ι are not changed, PKCα level is drastically increased and PKCθ level is depleted in As-p53^{low}HBECs compared with control p53^{low}HBECs (Fig. 1*A*). Strikingly, stably expressing miR-200b reduces the protein level of PKCα to that of non-transformed control cells but has no significant effect on the levels of the rest PKC isozymes (Fig. 1*A*).

Knocking Down Wnt5b Expression Reduces Phospho-PKC (pan) Levels and Arsenic-transformed Cell Migration—Next, we wanted to determine whether Wnt5b plays an important role in increased PKC phosphorylation and cell migration by knocking down Wnt5b expression using a pool of 4 Wnt5b siRNAs. Western blot analysis revealed that Wnt5b siRNA significantly decreases Wnt5b protein level (Fig. 1*B*). Knocking down Wnt5b expression significantly reduces the levels of phospho-PKCs but has not effect on total protein level of individual PKC isozyme (Fig. 1*B*), indicating that up-regulation of Wnt5b expression plays a significant role for increased levels of phospho-PKCs in arsenic-transformed cells. Moreover, Transwell cell migration assays revealed that knockdown of Wnt5b expression significantly reduces cell migration (Fig. 1*C*). Similarly, siRNA knocking down Wnt5b expression also significantly reduced the migration of a breast cancer cell line (SUM-159) that expresses barely detectable level of miR-200b but high

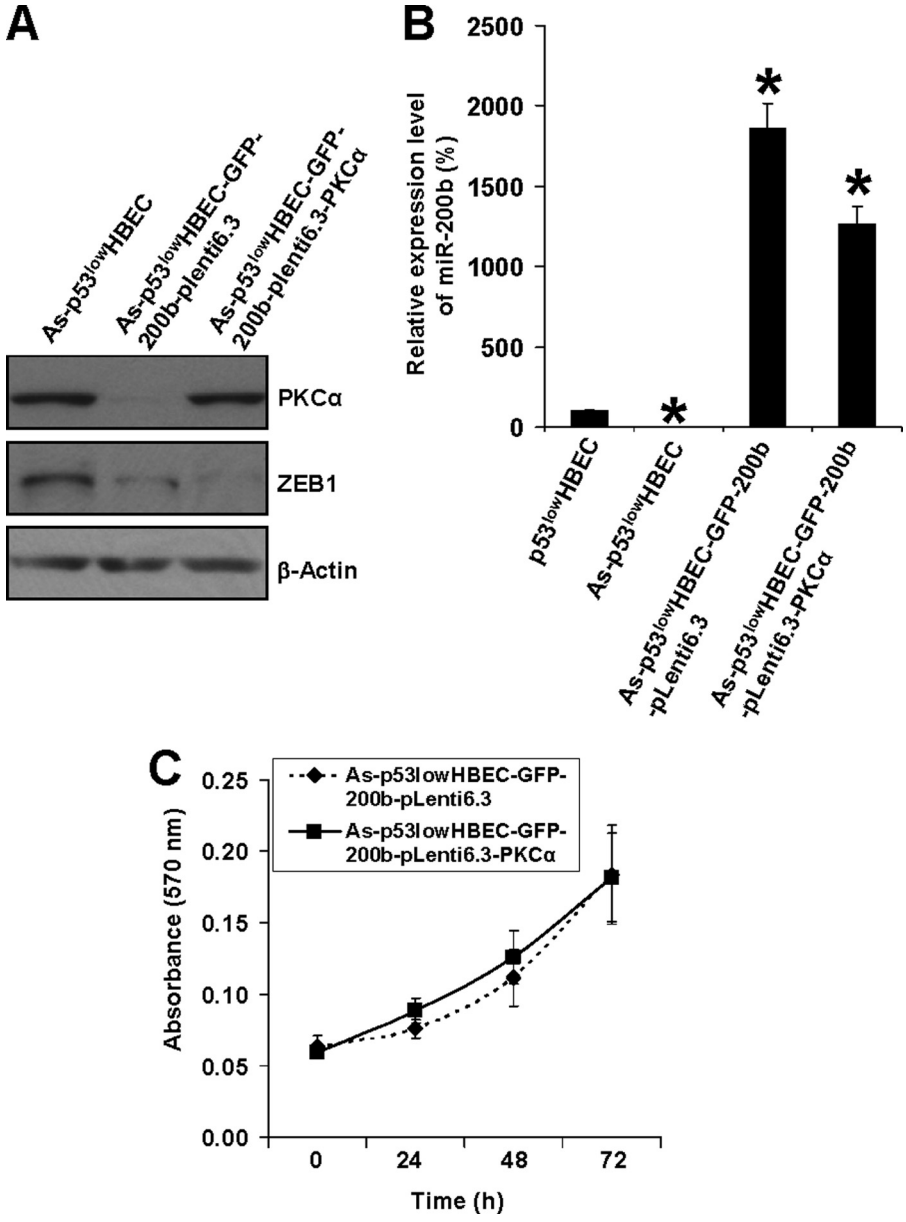

FIGURE 3. **Effect of forced expression of PKCα in miR-200b stably expressing cells on miR-200b and ZEB1 expression levels and cell proliferation.** *A*, Western blot analysis of PKCα and ZEB1 levels in As-p53^{low}HBEC, As-p53^{low}HBEC-GFP-200b-pLenti6.3, and As-p53^{low}HBEC-GFP-200b-pLenti6.3-PKCα cells. *B*, Q-PCR analysis of miR-200b level in non-transformed control (p53^{low}HBEC), arsenic-transformed (As-p53^{low}HBEC), miR-200b stably expressing vector control (As-p53^{low}HBEC-GFP-200b-pLenti6.3), and PKCα-miR-200b double stable expression (As-p53^{low}HBEC-GFP-200b-pLenti6.3-PKCα) cells. The expression levels of miR-200b are expressed relative to p53^{low}HBEC cells (means ± S.D., $n = 3$). *, $p < 0.05$, compared with p53^{low}HBEC group. *C*, cell proliferation determined by the MTT assay (means ± S.D., $n = 8$).

levels of Wnt5b and PKCα (data not shown). The inhibitory effect of Wnt5b siRNA on cell migration is not likely the off-target effect of Wnt5b siRNA, as treatment of cells with recombinant human Wnt5b protein is able to overcome the inhibitory effect of Wnt5b siRNA on cell migration (Fig. 1*D*), Together, these results suggest that up-regulation of Wnt5b plays an important role in arsenic-transformed cell migration and that Wnt5b promotes arsenic-transformed cell migration probably by increasing PKC activation.

Inhibiting PKCα Activity or Knocking Down PKCα Expression Reduces Wnt5b Level and Arsenic-transformed Cell Migration—Since stably expressing miR-200b was able to efficiently inhibit arsenic-transformed cell migration and reduce PKCα level, we next wanted to determine whether PKCα plays an essential role in arsenic-transformed cell migration. We first used a pharmacological inhibitor GO6976 that specifically inhibits PKCα and PKCβI activity. Transwell cell migration assays revealed that GO6976 treatment significantly reduces cell migration (Fig. 2*A*), suggesting that PKCα may play an important role in arsenic-transformed cell migration. This point is further determined by specifically knocking down PKCα expression using two PKCα siRNAs from different sources with different targeting sequences. As shown in Fig. 2*B*, transfection with PKCα siRNAs similarly and significantly decreases arsenic-transformed cell migration. Moreover, siRNA knocking down PKCα expression also significantly reduced the migration of SUM-159 breast cancer cells (data not shown). West-

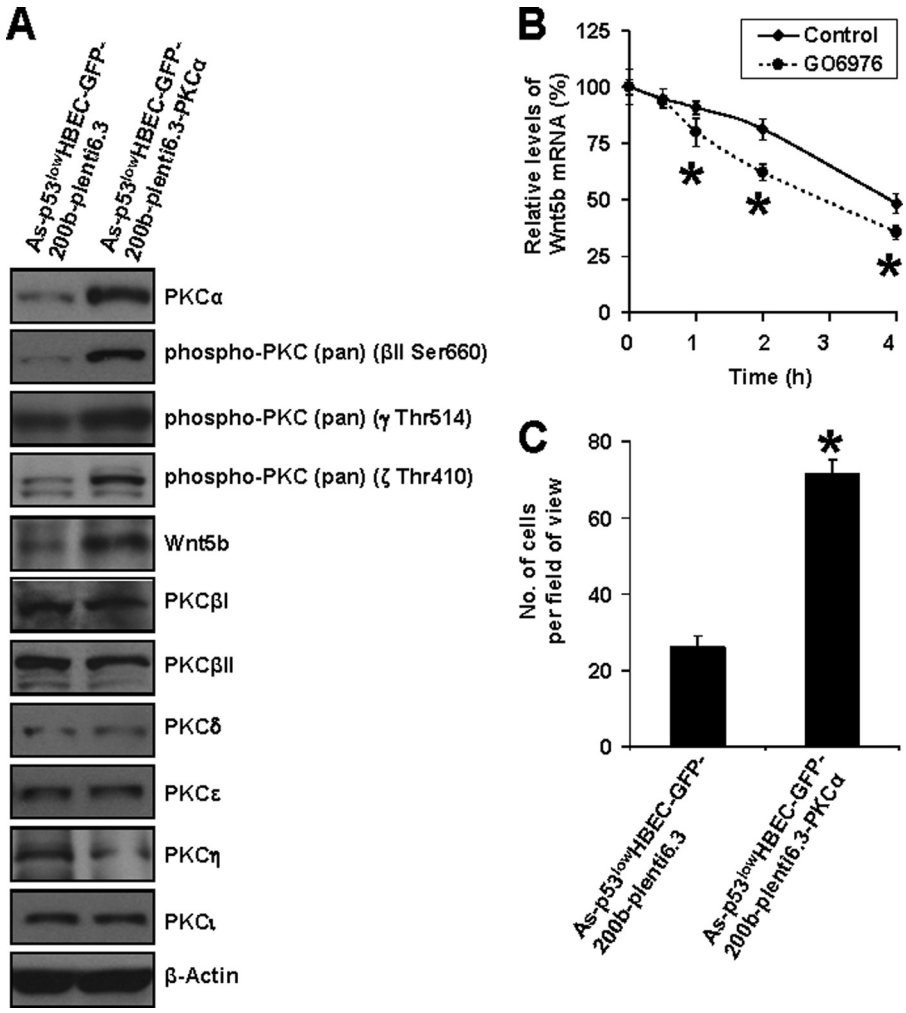

FIGURE 4. **Forced expression of PKCα in miR-200b stably expressing cells increases Wnt5b level and impairs the inhibitory effect of miR-200b on cell migration.** *A*, Western blot analysis of phospho-PKC (pan), individual PKC isozyme, and Wnt5b levels in As-p53^{low}HBEC-GFP-200b-pLenti6.3 and As-p53^{low}HBEC-GFP-200b-pLenti6.3-PKCα cells. *B*, time course of Wnt5b mRNA degradation. As-p53^{low}HBEC-GFP-200b-pLenti6.3-PKCα cells were cultured in K-SFM for 48 h and then treated with vehicle control (DMSO) or GO6976 (1 μM) for 45 min. At the end of treatment, all cells were treated with actinomycin D (2.5 μg/ml) for 0, 0.5, 1, 2, or 4 h and collected for Q-PCR analysis of Wnt5b mRNA. The levels of Wnt5b mRNA are expressed relative to 0 h Actinomycin D treatment (means ± S.D., $n = 3$). *, $p < 0.05$, compared with vehicle control-treated cells. *C*, quantification of Transwell cell migration. The quantification of cell migration is presented as number of cells per field of view (means ± S.D., $n = 3$). *, $p < 0.05$, compared with vector control cells.

ern blot analysis showed that PKCα siRNA efficiently knocks down PKCα level but has no obvious effect on the levels of PKCβI and other PKC isozymes (Fig. 2*C*). Strikingly, knocking down PKCα expression drastically reduces the levels of PKC phosphorylation detected by three phospho-PKC (pan) antibodies (Fig. 2*C*). Moreover, knocking down PKCα expression also greatly reduces Wnt5b protein level (Fig. 2*C*). This is not likely due to the off-target effect of PKCα siRNA. These findings suggest that: (i) up-regulation of PKCα expression plays a key role in arsenic-transformed cell migration; (ii) PKCα may regulate the level of Wnt5b; and (iii) down-regulation of PKCα by miR-200b may play a crucial role in its inhibitory effect on cell migration.

Forced Expression of PKCα in miR-200b Stably Expressing Cells Increases Wnt5b Level and Impairs the Inhibitory Effect of miR-200b on Cell Migration—To further demonstrate the critical role of PKCα down-regulation in the inhibitory effect of miR-200b on cell migration, we next stably expressed PKCα in miR-200b-stable expression cells and generated PKCα-miR-200b double stable expression cells. The PKCα cDNA used for generating the double stable expression cells lacks PKCα 3′-UTR, so the PKCα expression is not subject to the regulation by a miRNA. The protein level of PKCα in PKCα-miR-200b double stable expression cells is comparable to that of arsenic-transformed cells (As-p53^{low}HBEC) as detected by Western blot shown in Fig. 3*A*. Q-PCR analysis showed that miR-200b level is slightly reduced in PKCα-miR-200b double stable expression cells compared with miR-200b stably expressing vector control cells (Fig. 3*B*). However, the miR-200b level in PKCα-miR-200b double stable expression cells is still much significantly higher than that of control p53^{low}HBECs and As-p53^{low}HBECs (Fig. 3*B*). Moreover, the expression level of ZEB1, a well-established target of miR-200b, is not changed in PKCα-miR-200b double stable expression cells compared with miR-200b stably expressing vector control cells (Fig. 3*A*). Analysis of cell proliferation using MTT assays showed that forced expression of PKCα does not significantly affect cell proliferation, as no significant differences of cell growth are detected between vector control and PKCα stably expressing cells within 72 h (Fig. 3*C*).

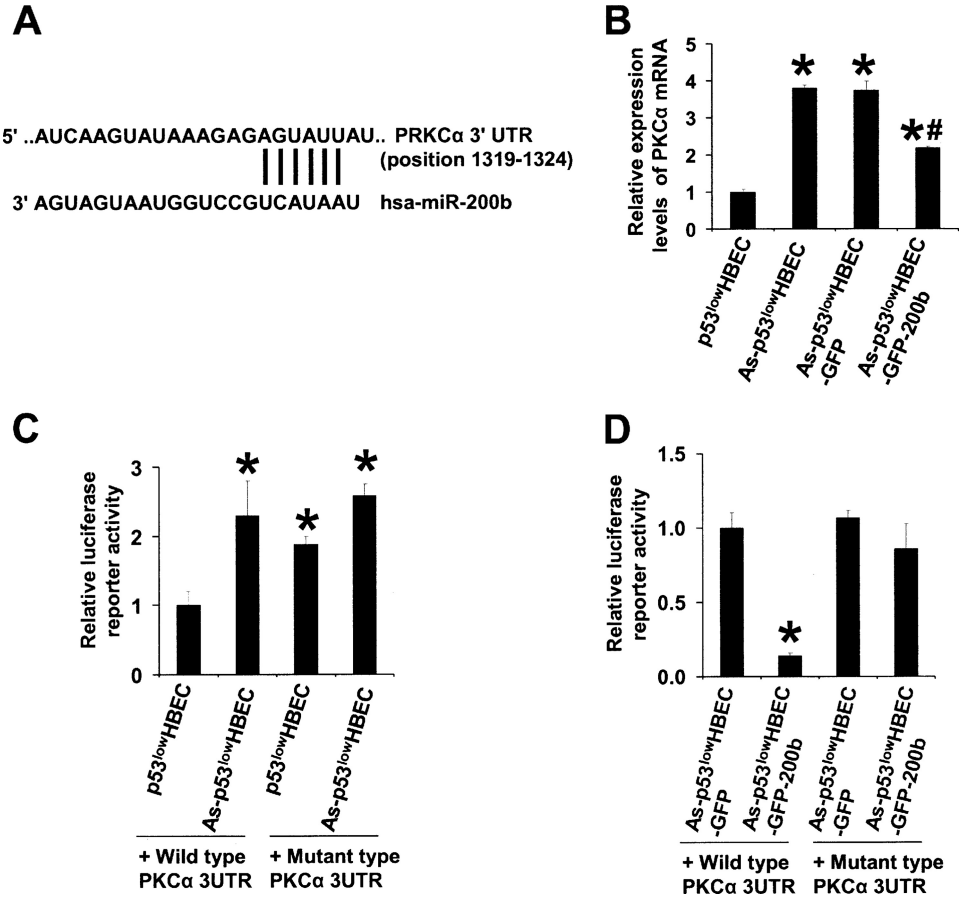

FIGURE 5. **PKCα is a direct target of miR-200b.** *A*, schematic description of predicted interaction between miR-200b seed region and the 3′-UTR of human PKCα. *B*, Q-PCR analysis of PKCα mRNA levels in control, arsenic-transformed and miR-200b stably expressing cells. The mRNA levels of PKCα are expressed relative to p53^{low}HBEC cells. *C* and *D*, quantification of PKCα 3′-UTR wild type and mutant type vector luciferase reporter activity. The luciferase reporter activity is expressed relative to p53^{low}HBEC (*C*) or As-p53^{low}HBEC (*D*) cells. All quantitative results are expressed as means ± S.D. ($n = 3$). *, $p < 0.05$, compared with p53^{low}HBEC (*B* and *C*) or As-p53^{low}HBEC (*D*) group; #, $p < 0.05$, compared with As-p53^{low}HBEC-GFP group.

Western blot analysis showed that forced expression of PKCα has no effect on the expression levels of other PKC isozymes (Fig. 4*A*). However, forced expression of PKCα drastically increases the levels of PKC phosphorylation detected by three phospho-PKC (pan) antibodies (Fig. 4*A*). Interestingly, Wnt5b protein level is also significantly elevated in PKCα and miR-200b double stable expression cells, again indicating that PKCα can regulate Wnt5b level and Wnt5b is not a direct target of miR-200b. To explore the potential mechanism by which PKCα regulates Wnt5b level, we treated PKCα stably expressing cells with or without GO6976 plus a transcription inhibitor actinomycin D and detected the rate of Wnt5b mRNA degradation. We found that Wnt5b mRNA degradation is significantly faster when PKCα activity is inhibited (Fig. 4*B*), suggesting that PKCα activity could increase Wnt5b mRNA stability. Transwell cell migration assays revealed that forced expression of PKCα significantly increases the migration of miR-200b stably expressing cells (Fig. 4*C*), impairing the inhibitory effect of miR-200b on cell migration. Similarly, forced expression of PKCα in control cells not treated with arsenic (p53^{low}HBECs) also significantly increased cell migration (data not shown). Together, these results suggest that: (i) down-regulation of PKCα plays a crucial role in the inhibitory effect of miR-200b on arsenic-transformed cell migration; (ii) while stably expressing miR-200b significantly reduces Wnt5b level, Wnt5b is not a direct target of miR-200b; and (iii) PKCα is capable of regulating Wnt5b level at least by increasing Wnt5b mRNA stability.

PKCα Is a Direct Target of miR-200b—Given that down-regulation of PKCα by miR-200b plays a key role in its inhibitory effect on cell migration as shown above, we next wanted to investigate the mechanism by which miR-200b down-regulates the level of PKCα. Using two computer programs (TargetScan and DIANA-LAB) for miRNA target prediction, we found a potential binding site for miR-200b in the 3′-UTR of human PKCα as shown in Fig. 5*A*. Q-PCR analysis showed that the mRNA level of PKCα is significantly increased in arsenic-transformed cells and stably expressing miR-200b significantly reduces its level (Fig. 5*B*). To determine whether PKCα is a direct target of miR-200b, PKCα 3′-UTR luciferase reporter vectors containing intact (Wild type) or mutated (Mutant type) miR-200b binding site were generated and dual luciferase reporter assays were performed. In line with our previous observation that miR-200b expression is significantly lower in As-p53^{low}HBECs than control p53^{low}HBECs (21), a significantly higher luciferase reporter activity of the wild type PKCα 3′-UTR vector is detected in As-p53^{low}HBECs compared with control p53^{low}HBECs (Fig. 5*C*). Moreover, significantly higher luciferase reporter activities of the mutant type PKCα 3′-UTR

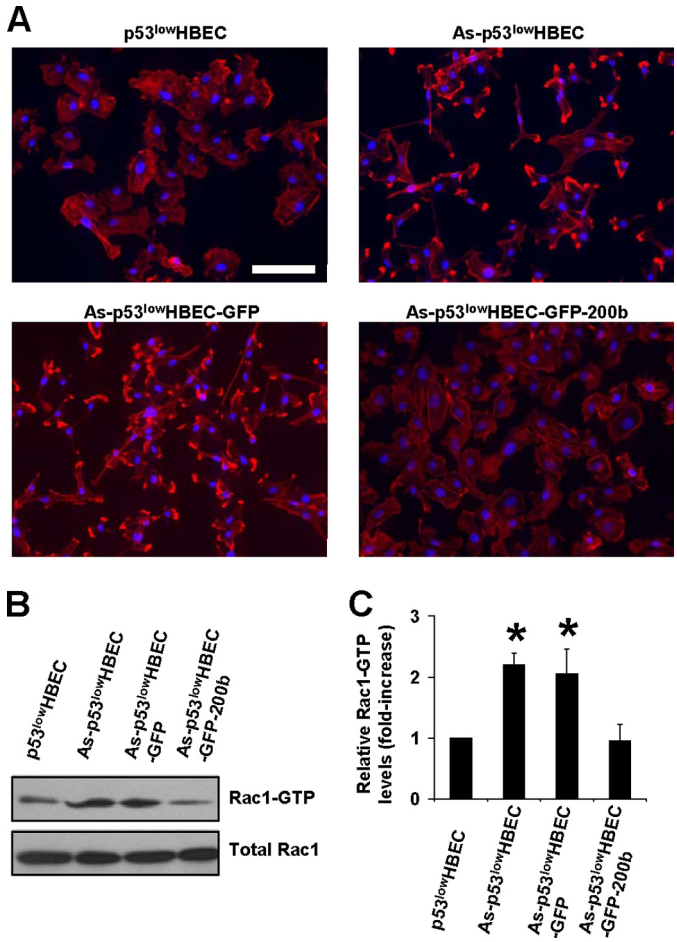

FIGURE 6. **Actin cytoskeleton is reorganized and Rho GTPase Rac1 is highly activated in arsenic-transformed cells and stably expressing miR-200b inhibits Rac1 activation and actin cytoskeleton reorganization.** *A*, representative overlaid images of actin cytoskeleton staining with Rhodamine Phalloidin (*red*) and nuclear staining with DAPI (*blue*). Scale bar, 100 μm. *B* and *C*, Rac1-GTP levels determined by Rac-GTP pulldown assays. Rac1-GTP levels were quantified using ImageJ software, and the quantifications are presented as the Relative Rac1-GTP levels (Rac1-GTP levels divided by the corresponding total Rac1 levels) (means ± S.D., $n = 3$). *, $p < 0.05$, compared with control p53^{low}HBEC group.

vector are observed in both As-p53^{low}HBECs and control p53^{low}HBECs (Fig. 5*C*). Together, these results suggest that PKCα is a direct target of miR-200b. This view is further supported by the observations that ectopic expression of miR-200b significantly reduces luciferase reporter activity of the wild type PKCα 3′-UTR vector but has no significant effect on luciferase reporter activities of the mutant type PKCα 3′-UTR vector (Fig. 5*D*).

Actin Cytoskeleton Is Reorganized and Rho GTPase Rac1 Is Highly Activated in Arsenic-transformed Cells—Next, we wanted to further investigate the mechanism by which Wnt5b and PKCα down-regulation by miR-200b inhibits cell migration. Because actin cytoskeleton reorganization is a key event in cell migration, we first compared actin cytoskeleton organization patterns between control p53^{low}HBECs and arsenic-transformed highly migratory cells (As-p53^{low}HBECs) by staining cells with Rhodamine Phalloidin. No specific pattern of actin organization is observed in the majority of control p53^{low}HBECs (Fig. 6*A*). In sharp contrast, almost all of As-p53^{low}HBECs show significant actin clustering at the edges of cells appearing to be membrane ruffling or lamellipodia formation (Fig. 6*A*), which is a distinct pattern of actin cytoskeleton reorganization mediated by Rho GTPase Rac activation and an indication of increased cell motility (17–19). Strikingly, stably expressing miR-200b abolishes membrane ruffling or lamellipodia formation and cells exhibit a similar actin staining pattern to that of control cells (Fig. 6*A*). Consistent with the actin cytoskeleton organization patterns, Rac1 GTPase pulldown assays revealed that Rac1 is highly activated in As-p53^{low}HBECs, but stably expressing miR-200b reduces the level of active Rac1 to that of control cells (Fig. 6, *B* and *C*). Moreover, siRNA knocking down Rac1 expression significantly reduced arsenic-transformed cell migration (data not shown). No significant changes of the active levels of other Rho GTPases such as Cdc42 and Rho A were detected in arsenic-transformed cells (data not shown).

siRNA Knocking Down Wnt5b or PKCα Inhibits Rac1 Activation and Actin Cytoskeleton Reorganization—To determine the role of Wnt5b or PKCα in Rac1 activation in arsenic-transformed cells, As-p53^{low}HBECs were transfected with control, Wnt5b or PKCα siRNA. We found that siRNA knocking down either Wnt5b or PKCα expression all significantly reduces Rac1 activation (Fig. 7, *A* and *B*). As a result, siRNA knocking down Wnt5b or PKCα expression also abolishes membrane ruffling or lamellipodia formation as revealed by Rhodamine Phalloidin staining (Fig. 7*C*). These results suggest that Wnt5b and PKCα play key roles in Rac1 activation and subsequent actin cytoskeleton reorganization in arsenic-transformed cells, and miR-200b inhibits Rac1 activation by down-regulating the expression of PKCα and Wnt5b. Similarly, siRNA knocking down Wnt5b expression also significantly reduced the activation of Rac1 in SUM-159 breast cancer cells (data not shown). Although we cannot completely rule out the possibility of an off-target effect of PKCα siRNA, a similar conclusion achieved by using multiple approaches to modulate PKCα expression or activity suggests that the effects observed from PKCα siRNA experiments are unlikely the off-target effects of PKCα siRNA but rather the effects of PKCα down-regulation.

Forced Expression of PKCα in miR-200b Stably Expressing Cells Impairs the Inhibitory Effect of miR-200b on Rac1 Activation—To further demonstrate the important role of PKCα and Wnt5b in Rac1 activation and actin cytoskeleton reorganization, we next analyzed Rac1 activation and actin cytoskeleton organization pattern in vector control and PKCα-miR-200b double stable expression cells. It was found that active Rac1 level is significantly higher in PKCα-miR-200b double stable expression cells (As-p53^{low}HBEC-GFP-200b-plenti6.3-PKCα) than that of vector control cells (As-p53^{low}HBEC-GFP-200b-plenti6.3) (Fig. 8, *A* and *B*). Moreover, Rhodamine Phalloidin staining reveals significant membrane ruffling or lamellipodia formation in PKCα-miR-200b double stable expression cells (Fig. 7*C*). These results indicate that forced expression of PKCα impairs the inhibitory effect of miR-200b on Rac1 activation and actin cytoskeleton reorganization.

Finally, we wanted to determine whether Wnt5b plays an essential role by which forced expression of PKCα impairs the inhibitory effect of miR-200b on Rac1 activation, actin cytoskeleton reorganization and cell migration. PKCα-miR-200b double stable expression cells were transfected with

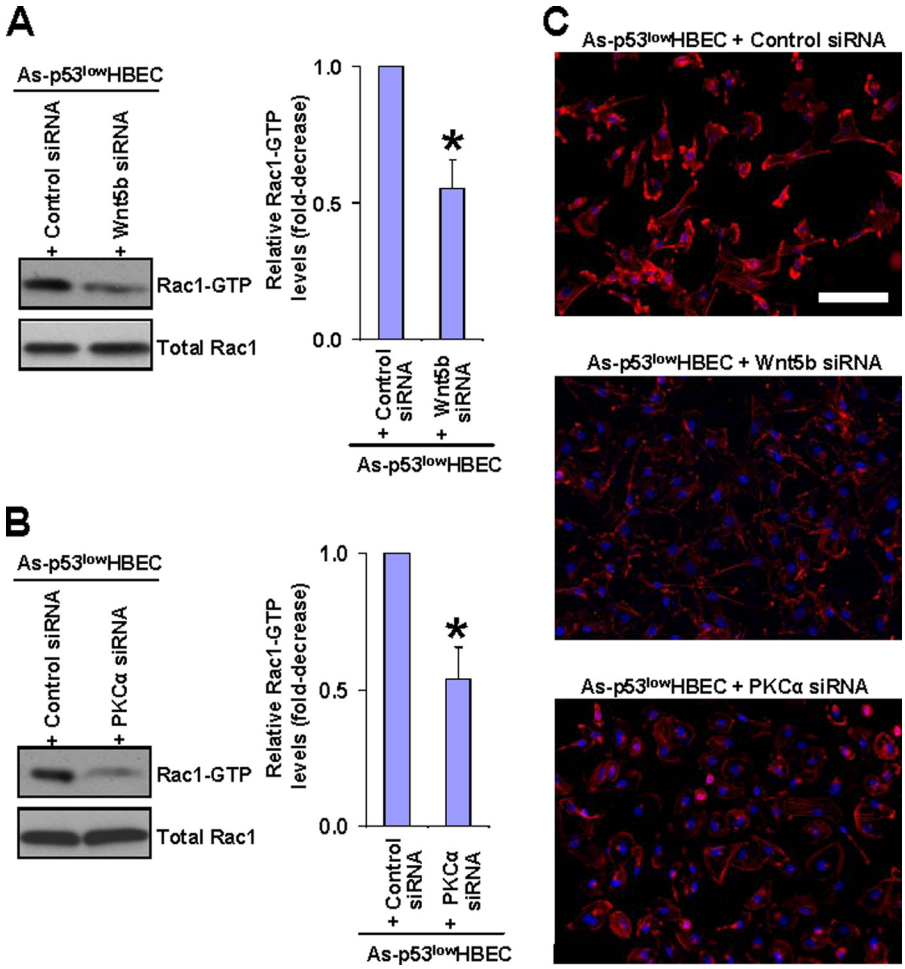

FIGURE 7. **siRNA knocking down Wnt5b or PKCα expression significantly reduces Rac1 activation and actin cytoskeleton reorganization in arsenic-transformed cells.** *A* and *B*, Rac1-GTP levels determined by Rac-GTP pulldown assays. Rac1-GTP levels were quantified using ImageJ software and the quantifications are presented as the Relative Rac1-GTP levels (Rac1-GTP levels divided by the corresponding total Rac1 levels) (means ± S.D., $n = 3$). *, $p < 0.05$, compared with Control siRNA group. *C*, representative overlaid images of actin cytoskeleton staining with Rhodamine Phalloidin (*red*) and nuclear staining with DAPI (*blue*). Scale bar, 100 μm.

control or Wnt5b siRNA. It was found that siRNA knocking down Wnt5b expression has no effect on total PKCα level but drastically reduces the levels of phospho-PKCs (Fig. 9*A*), again indicating a key role of Wnt5b in PKC phosphorylation. Moreover, knocking down Wnt5b expression greatly decreases Rac1 activation (Fig. 9*B*), abolishes actin cytoskeleton reorganization (Fig. 9*C*) and significantly reduces the migration of PKCα stably expressing cells (Fig. 9*D*). These results indicate that forced expression of PKCα impairs the inhibitory effect of miR-200b on cell migration is mediated by Wnt5b. Moreover, these results further support an important role of Wnt5b-PKCα-Rac1 pathway in promoting arsenic-transformed cell migration. A model that describes the main signaling pathway mediating arsenic-transformed cell migration and the inhibitory effect of miR-200b re-expression is presented in Fig. 9*E*.

DISCUSSION

The inhibitory effect of miR-200 family members on cell migration and cancer metastasis has been reported in a number of studies; however, the underlying mechanism has not been well understood. Only a limited number of miR-200 target genes that promote cancer cell migration and metastasis have been identified. The majority of studies showed that miR-200 family inhibits cell migration and cancer metastasis through targeting epithelial to mesenchymal transition (EMT)-inducing transcription factors zinc-finger E-box-binding homeobox factor 1 (ZEB1) and ZEB2 (9–11). Fewer studies indicated that miR-200 family may also be capable of inhibiting cell migration and cancer metastasis by targeting other genes such as actin cytoskeleton regulator WAVE3 (Wiskott-Aldrich syndrome protein family member 3), moesin and ROCK2 (Rho-kinase2), or extracellular matrix component fibronectin 1 (13, 35–37). As summarized in Fig. 9*E*, in this study we identified PKCα as a new direct target of miR-200b and showed that miR-200b inhibits arsenic-transformed cell migration by targeting PKCα and Wnt5b-PKCα positive feedback loop and thus reducing the activation of the Rho GTPase Rac1. These findings are not likely cell type-specific effects as our recent breast cancer research work showed that miR-200b also directly targets PKCα in two breast cancer cell lines (MDA-MB-231 and SUM-159) and suppresses breast cancer cell migration and tumor metastasis.[3]

[3] B. Humphries, Z. Wang, A. L. Oom, T. Fisher, D. Tan, Y. Cui, Y. Jiang, and C. Yang, manuscript under review.

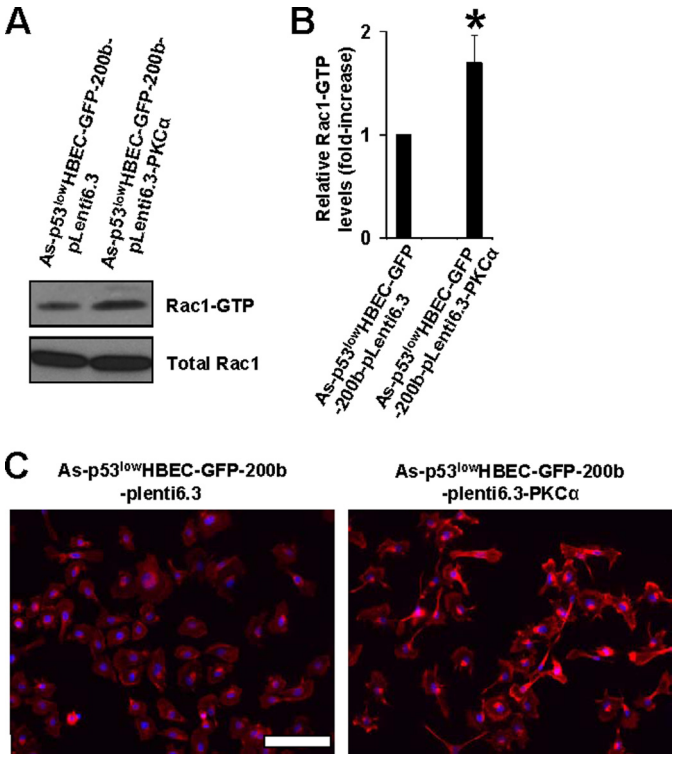

FIGURE 8. **Forced expression of PKCα significantly increases Rac1 activation and causes actin cytoskeleton reorganization in miR-200b stably expressing cells.** *A* and *B*, Rac1-GTP levels determined by Rac-GTP pulldown assays. Rac1-GTP levels were quantified using ImageJ software, and the quantifications are presented as the Relative Rac1-GTP levels (Rac1-GTP levels divided by the corresponding total Rac1 levels) (means ± S.D., $n = 3$). *, $p < 0.05$, compared with vector control cells (As-p53^{low}HBEC-GFP-200b-pLenti6.3). *C*, representative overlaid images of actin cytoskeleton staining with Rhodamine Phalloidin (*red*) and nuclear staining with DAPI (*blue*). Scale bar, 100 μm.

Wnt5b is a non-canonical Wnt ligand having about 80% total-amino acid identities with another non-canonical Wnt ligand Wnt5a (38). Non-canonical Wnt signaling has been shown to play important roles in development and various diseases including cancer. Our knowledge about the role of non-canonical Wnt signaling and its regulation come mainly from studies on Wnt5a, much less studies have been done on Wnt5b and other non-canonical Wnt ligands (32, 39). It has been shown that non-canonical Wnt can promote cell migration through activating protein kinase C (PKC)-mediated signaling pathway (31, 32). PKC is a family of serine/threonine kinases consisting of 10 isozymes and can be grouped into 3 subfamilies including the conventional/classical PKCs (PKCα, βI, βII, γ); the novel PKCs (PKCδ, ϵ, η, θ); and the atypical PKC (PKCι/λ and ζ). Many PKC isozymes have been shown to play crucial roles in cancer initiation, cell migration and cancer metastasis (40), it is thus important to identify new regulators that can down-regulate PKC expression thus inactivating PKC-mediated oncogenic signaling.

Wnt5a has been shown to be able to activate PKCs, and interestingly, activated PKCs are capable of stabilizing Wnt5a mRNA forming a positive feedback loop between Wnt5a and PKC, which plays an important role in cancer cell migration and metastasis (41, 42). Although Wnt5b has high total-amino acid identities with Wnt5a, whether a similar positive feedback loop between Wnt5b and PKC exists is not clear. In this study we found both Wnt5b and PKCα levels are significantly up-regulated in arsenic-transformed cells. SiRNA knocking down Wnt5b expression did not affect total PKCα level but significantly reduced the levels of phospho-PKCs and cell migration, suggesting that Wnt5b plays an important role in PKC activation and cell migration. Moreover, siRNA knocking down PKCα expression greatly decreased Wnt5b level, phospho-PKC level and cell migration, and forced expression of PKCα significantly increased Wnt5b mRNA stability and its protein level, phospho-PKC level and cell migration. Collectively, these results indicate that a similar positive feedback loop between Wnt5b and PKC also exists, which plays an important role in promoting cell migration.

Our findings that PKCα is a direct target of miR-200b and stably expressing miR-200b is able to down-regulate both PKCα and Wnt5b levels are novel and important. Although very little is known about the role of Wnt5b in cancer, studies have shown that Wnt5a expression is critically involved in increased invasiveness of various kinds of cancer (39). Moreover, up-regulation of PKCα expression and activation has also been reported in many types of cancer and plays important roles in cancer cell migration and metastasis (40, 43, 44). Therefore, Wnt5a/b-PKCα positive feedback loop could play crucial roles in cancer progression, and Wnt5a, Wnt5b, and PKCα could be a useful target for inhibiting cancer metastasis. Because of the ubiquitous expression nature of the majority of PKC isozymes and the complicated biological functions performed by different PKC isozymes, it is essential to target the specifically deregulated PKC isozymes to achieve the desired therapeutic effect. However, because of the high homology of kinase domains among different PKC isozymes, efficiently targeting a specific PKC isozyme has remained to be a big challenge despite extensive studies (45). Our finding that miR-200b efficiently and specifically reduces PKCα expression with no significant effect on the levels of other PKC isozymes provides a novel alternative strategy for specifically targeting PKCα.

Arsenic is a well-recognized human carcinogen; however, the mechanism of its carcinogenicity has not been elucidated. Previous studies showed that short term arsenic exposure causes Rac1 activation in endothelial cells and Rac1 activity is required for arsenic-stimulated endothelial cell remodeling and angiogenesis (46, 47). However, whether Rac1 is activated in cells malignantly transformed by long term arsenic exposure and whether Rac1 plays a role in arsenic carcinogenesis are not clear. Interestingly, one previous study reported that total Rac1 protein level is significantly higher in mouse skin tumors induced by fetal arsenic exposure plus topical 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) treatment than in skin tumors induced by TPA treatment alone (48). In current study we found while total Rac1 protein level is not changed but Rac1 is highly activated in arsenic-transformed cells, and Rac1 activity is closely associated with actin cytoskeleton reorganization and enhanced migratory behavior of arsenic-transformed cells. Moreover, we also showed that Rac1 activation in arsenic-transformed cells depends on PKCα- and Wnt5b-PKCα-mediated signaling pathway. Given the critical role of Rac1 activity in cell malignant transformation, cancer cell survival, prolifera-

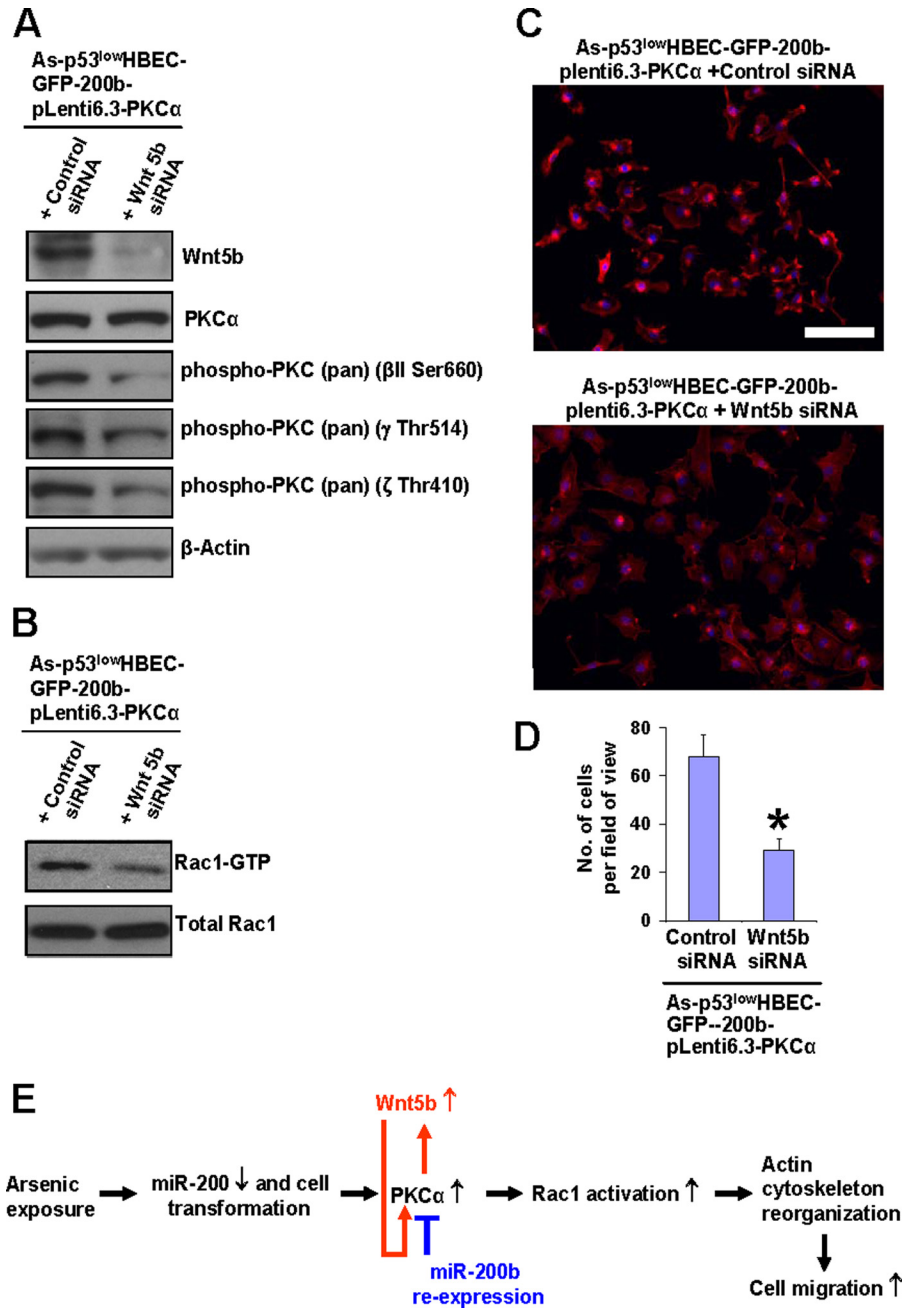

FIGURE 9. **SiRNA knocking down Wnt5b expression in PKCα-miR-200b double stable expression cells drastically reduces the levels of phospho-PKC (pan) and Rac1 activation and inhibits actin cytoskeleton reorganization and cell migration.** *A*, Western blot analysis of Wnt5b, PKCα, and phospho-PKC (pan) levels. *B*, representative Western blot analysis of Rac1-GTP level determined by the Rac-GTP pulldown assay. *C*, representative overlaid images of actin cytoskeleton staining with Rhodamine Phalloidin (*red*) and nuclear staining with DAPI (*blue*). Scale bar, 100 μm. *D*, quantification of Transwell cell migration. The quantification of cell migration is presented as number of cells per field of view (means ± S.D., $n = 3$). *, $p < 0.05$, compared with Control siRNA group. *E*, model describing the main signaling pathway that promotes arsenic-transformed cell migration and the inhibitory effect of miR-200b re-expression. →, promote; ⊥ inhibit; ↑, up-regulate or activate; and ↓ down-regulate. *Red color* shows the Wnt5b-PKCα positive feedback loop. *Blue color* indicates miR-200b directly targets PKCα.

tion, and migration (15, 49), the findings from current and previous studies suggest that deregulation of Rac1 expression and activity may play important roles in arsenic carcinogenesis.

In summary, although miR-200 family members have been shown to be able to inhibit cell migration and cancer metastasis, the underlying mechanisms have not been well understood. In this study we identified PKCα as a new direct target of miR-200b and showed that miR-200b suppresses arsenic-transformed cell migration by targeting PKCα and Wnt5b-PKCα positive feedback loop and subsequently inhibiting the Rho GTPase Rac1 activation and abolishing actin cytoskeleton reorganization.

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